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A Role for RAS in V-CRK Transformation

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A ROLE FOR RAS IN V-CRK TRANSFORMATION

A thesis submitted to the faculty of
The Rockefeller University
in partial fulfillment of the requirements
of the degree of Doctor of Philosophy

by

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ABSTRACT

The v-Crk oncogene product, while possessing no intrinsic tyrosine kinase activity, causes an elevation in levels of cellular phosphotyrosine as it transforms chicken embryo fibroblasts (CEF). Because of this similarity to v-Src transformation and EGF receptor (EGFR) stimulation, signalling pathways that might be activated in v-Crk transformed cells were investigated by analogy to pathways utilized by v-Src and the activated EGFR, and a search for serine/threonine kinases activated in v-Crk transformed CEF was conducted.

Mapk was identified as one kinase, the activity of which is constitutively elevated in v-Crk transformed CEF. Mek activity was also somewhat elevated. However, the Shc proteins, which probably mediate activation of the Ras pathway in v-Src transformed and EGF-stimulated cells, were not tyrosine phosphorylated, indicating that the activation of Mapk was probably mediated by a different mechanism.

v-Crk transformed NIH-3T3 cell lines were established in order to examine the effects of blocking the Ras pathway in v-Crk transformed cells. In contrast to previous studies done in mammalian cells, v-Crk caused morphological transformation and promoted anchorage-independent growth of the transfected NIH-3T3 cells. In addition, elevated levels of tyrosine phosphorylation were observed on the characteristic p70 and p130.

Expression of dominant negative Ras in the v-Crk NIH-3T3 caused morphological reversion of these cells, as well as an inhibition of colony formation in soft agar, indicating a requirement for Ras function in v-Crk transformation. However, no decrease in tyrosine phosphorylation of cellular

proteins was observed upon dominant negative Ras expression.

A delay in serum stimulation of Mapk activity in oncogene transformed cells was also observed. This delay was shown to correlate with increase in metabolic rate, rather than degree of morphological transformation. A possible mechanism involving a delay in Shc phosphorylation is proposed.

INTRODUCTION

Cell growth is tightly controlled by a series of regulatory proteins, responsible for transducing signals to the DNA replication machinery in the nucleus. The genes encoding these essential regulatory proteins are capable of causing oncogenic transformation if they are mutated in such a way that increases the activity of a growth-promoting protein, or abolishes the activity of a growth-suppressing protein.

Growth-promoting genes include those encoding growth factors, their receptors, the signal transduction proteins necessary to transmit growth information to the nucleus, and the nuclear proteins (including transcription factors) that receive growth signals and initiate DNA replication. Signal transduction pathways have been substantially explored for several growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, and colony-stimulating factor-1 (CSF-1). The EGF signal transduction pathway will be described as a pathway typical of these growth factors (reviewed in Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992). This pathway is schematically diagrammed in Figure 1 for reference.

Activation of the EGF Receptor

EGF, a polypeptide hormone comprised of 53 amino acids, was first isolated from murine submaxillary glands (Savage et al., 1972) as an activity promoting epithelial cell growth. Human EGF, or urogastrone, has been detected in salivary glands, but it is not known if this is a site of synthesis (Carpenter and Cohen, 1979).

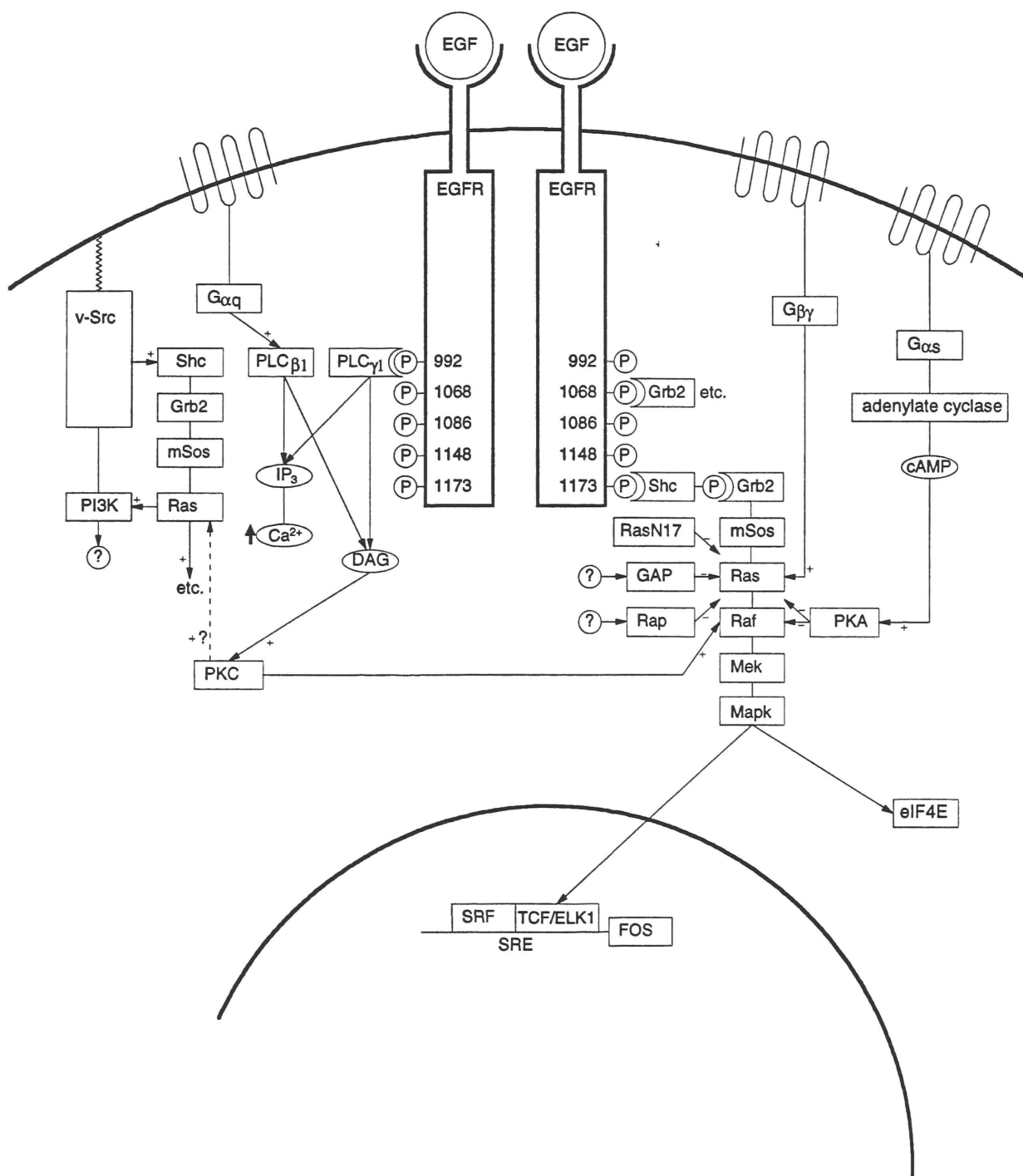


Figure 1. Signal transduction from the EGFR and v-Src, and input from participating G proteins. References in text; also Taylor et al., 1991. Ras farnesylation omitted for clarity.

The epidermal growth factor receptor (EGFR) is a transmembrane protein tyrosine kinase. It consists of an extracellular EGF binding domain, a transmembrane region, an intracellular kinase domain, and a carboxy-terminal tail with several autophosphorylation sites (Ullrich et al., 1984). Ligand binding induces receptor dimerization (Cochet et al., 1988; Chantry, 1995) and activates EGFR tyrosine kinase activity. Receptor dimerization is an essential function of EGF: Biochemically isolated dimers have an elevated kinase activity relative to the monomers (Sorokin et al., 1994), even in the absence of EGF (Boni-Schnetzler and Pilch, 1987), and expression of a kinase-negative EGFR acts as a dominant negative, suppressing stimulation of the endogenous EGFR via receptor heterodimerization (Kashles et al., 1991; Redemann et al., 1992).

Activated EGFR dimers undergo intermolecular autophosphorylation (Honegger et al., 1990), resulting in receptor phosphorylation at five sites on the carboxyterminal tail: tyrosines 992, 1068, 1086, 1148, and 1173. Several signal transduction proteins bind to these autophosphorylated tyrosines via Src-homology 2 (SH2) domains, named for a region in the amino-terminal regulatory portion of the Src tyrosine kinase. SH2 domains are found in a wide variety of signal transduction proteins, and have been shown to bind phosphotyrosine moieties in specific sequence contexts (Matsuda et al., 1991). The crystal structure of the Src SH2 domain revealed a fairly shallow peptide binding surface containing a deep pocket into which the phosphorylated tyrosine residue is inserted. Several positively charged residues interact with the phosphate group, as well as aromatic ring of the tyrosine. Phosphoserine and phosphothreonine are clearly too short to make contact with the positive amino acids at the bottom of the phosphotyrosine binding pocket (Waksman et al., 1992). Variable sequences exposed on the peptide binding surface

probably account for SH2 specificity. Although the first three residues following the phosphorylated tyrosine are the most important specificity determinants, a contribution from the +4 and +5 amino acids has also been documented (Larose et al., 1995). The preferences of several SH2 domains were determined by measuring binding to a degenerate library of synthetic phosphopeptides (Songyang et al., 1993).

A second binding module often found in SH2-containing signalling proteins is the Src-homology 3 (SH3) domain. SH3 domains bind to specific proline-rich sequences which form local left-handed type II polyproline helices. The minimal consensus sequence for SH3 binding peptides is Pro-X-X-Pro, in which the two prolines directly contact the flat, mostly hydrophobic surface of the SH3 domain. Additional basic residues surrounding these four amino acids help to confer specificity for the SH3 of a particular protein (Ren et al., 1993; Yu et al., 1994; Wu et al., 1995).

Different receptor tyrosine kinases bind different subsets of SH2/SH3 containing proteins when activated, and the phosphotyrosine docking sites of these receptors are not necessarily in the carboxy-terminal tails. For example, the platelet-derived growth factor receptor (PDGFR) can bind SH2-containing proteins via tyrosines in an “insert” region of the catalytic domain; and the insulin receptor does not directly bind all of its effector molecules, but rather phosphorylates the docking protein IRS-1 (insulin receptor substrate 1), to which specific SH2 containing proteins can then bind (reviewed in Pawson and Schlessinger, 1993).

Signalling Proteins Recruited to the Activated EGFR

SH2-containing proteins which bind the EGFR tail can be divided into

two groups: those which contain a known catalytic activity, and those which consist primarily of SH2 and/or SH3 domains. Phospholipase C γ (PLC γ) falls into the first category. PLC γ is capable of hydrolyzing phosphatidylinositols to produce 1,2-diacylglycerol and a series of inositol phosphates (reviewed in Majerus et al., 1990). Diacylglycerol, in turn, activates protein kinase C (PKC), a serine/threonine kinase involved in mitogenic signalling, while inositol phosphates cause release of intracellular stores of calcium. The accumulation of inositol phosphates in EGF-stimulated cells is well-documented (Wahl and Carpenter, 1988; Hepler et al., 1987). Tyrosine 992 appears to be the major PLC γ binding site on the EGFR carboxyterminal tail, with lesser affinities for tyrosines 1068 and 1173 (Vega et al., 1992; Rotin et al., 1992). It is likely that binding of PLC γ to phosphorylated tyrosine 992 is accompanied by tyrosine phosphorylation and activation of the enzyme by the EGFR (Nishibe et al., 1990).

The second group of growth factor receptor binding proteins, those that consist primarily of SH2 and SH3 domains, are termed “adaptor proteins” because their function appears to be linking tyrosine phosphorylated proteins bound to the SH2 domain with proline-rich signalling proteins bound to the SH3 domain. This group is exemplified by the Grb2 protein, which consists of domains SH3-SH2-SH3. Grb2 was isolated by screening an expression library with the tyrosine-phosphorylated EGFR carboxy-terminal tail (Lowenstein et al., 1992), and binds the phosphorylated EGFR at tyrosines 1068 and 1086 (Batzer et al., 1994; Okutani et al., 1994). In addition, Grb2 can bind Shc, another SH2-containing adaptor protein that is tyrosine phosphorylated upon EGF stimulation (Pelicci et al., 1992). Shc binds the phosphorylated EGFR at tyrosines 1173 and 1148, creating two additional, indirect binding sites for Grb2 (Okabayashi et

al., 1994; Batzer et al., 1994; Okutani et al., 1994). Recent evidence suggests that most of the binding of Grb2 to the EGFR is in fact mediated by Shc (Sasaoka et al., 1994).

This simple picture is complicated by studies involving EGFR mutants with all five autophosphorylation sites mutated to phenylalanine. Unexpectedly, these mutant receptors still permitted EGF-induced mitogenesis when expressed in a strain of NIH-3T3 cells void of endogenous EGFR. Although PLC_γ was not activated in response to EGF in these cells, Shc was still phosphorylated and complexed with Grb2, and signals were sent to effector molecules downstream of Grb2 (Decker, 1993; Gotoh et al., 1994; Li et al., 1994). Tyrosine phosphorylated Shc may be able to bypass binding to the EGFR by binding to a recently-identified 145 kDa protein which is also tyrosine phosphorylated in response to EGF stimulation. Shc binds the phosphotyrosine moiety of p145 with its PTB domain, a newly-described phosphotyrosine binding domain with little homology to SH2 sequences (Kavanaugh and Williams, 1994).

Activation of Ras

Under normal circumstances, Grb2 thus binds the tyrosine-phosphorylated EGFR via Shc or with its own SH2 domain. The Grb2 SH3 domains bind proline rich motifs of the mSos protein, a guanine nucleotide exchange factor (GEF) for the Ras low molecular weight GTP binding protein (Buday and Downward, 1993a; Egan et al., 1993; Chardin et al., 1993; Rozakis-Adcock et al., 1993; Li et al., 1993). Ras cycles between GTP-bound “on” (growth signal transmitting) and GDP-bound “off” states. Ras activity is modified by two sets of proteins: GTPase activating proteins, which stimulate the intrinsic

GTP hydrolysis activity of Ras, thereby switching Ras off; and GEFs, which release bound GDP from Ras, allowing free GTP to bind and activate Ras (reviewed in Satoh et al., 1992). EGF stimulation increases the GTP:GDP ratio of Ras by upregulating the rate of guanine nucleotide exchange (Satoh et al., 1990; Medema et al., 1993; Buday and Downward, 1993b; Gale et al., 1993).

Several Ras GEFs have been isolated to date, including a brain specific Ras-GRF, the widely expressed mSos, and the *S. cerevisiae* Cdc25 and Scd25 (reviewed in Boguski and McCormick, 1993). Cdc25 was the first GEF identified. Otherwise lethal Cdc25 mutations can be complemented by Scd25, the GEF domain of which can also function to exchange bound nucleotide on mammalian Ras (Broek et al., 1987; Crechet et al., 1990). The exchange factor participating in EGF signalling is mSos. mSos is constitutively bound to Grb2 in most cell types examined, and its exchange activity is upregulated when translocated to the membrane by the binding of Grb2 to the EGFR. In fact, membrane targeting of mSos by farnesylation or myristylation is sufficient to activate Ras, independent of growth factor stimulation (Quilliam et al., 1994; Aronheim et al., 1994). Overexpression of mSos with a deleted GEF domain can prevent growth-factor induced activation of Ras (Sakaue et al., 1995).

The first Ras GAP (p120 GAP) was identified as an activity that stimulated the intrinsic GTPase activity of Ras (Trahey and McCormick, 1987). GAP is phosphorylated on tyrosine in response to EGF stimulation, as are two associated proteins (p62 and p190), but initial reports of EGFR binding by GAP remain unsubstantiated despite the presence of two SH2 domains and one SH3 domain (Ellis et al., 1990; Liu and Pawson, 1991; Margolis et al., 1990). No change in GAP activity *in vitro* after phosphorylation has been reported, and the role of this protein in growth factor-induced Ras activation remains unclear.

There are three low molecular weight Ras proteins that function in mitogenesis: H-Ras, K-Ras, and N-Ras. The relative contributions of each to EGF-stimulated growth induction has not been elucidated, except for the lack of a phenotype in N-Ras null transgenic mice (Umanoff et al., 1995). H-Ras has been shown to function as a substrate for the mSos GEF (Chardin et al., 1993), and is generally used as a prototypical Ras protein. Ras proteins are posttranslationally farnesylated on a cysteine appearing in a carboxy-terminal CAAX motif, in which the A is an aliphatic residue and the X is any amino acid. In addition, the hypervariable region just preceding the CAAX motif is palmitoylated; or in the case of K-Ras B, a polybasic domain exists that cooperates with the CAAX farnesyl. Both of these modifications are required for Ras localization to the membrane (Hancock et al., 1990), which is required for mSos-promoted nucleotide exchange on Ras and Ras-mediated cell growth (Porfiri et al., 1994; Cox et al., 1992).

The role of Ras in mitogenesis has been underlined by studies with a dominant-negative form of the protein, Ras N17. Substitution of Ser 17, a residue near the GTP binding site, with Asn created a protein with a decreased affinity for GTP, favoring the GDP bound state (Feig and Cooper, 1988; Milburn et al., 1990). The resulting protein is thought to sequester nucleotide exchange factors, preventing them from acting on endogenous Ras after growth factor stimulation. Ras N17 suppresses endogenous Ras activation, but not signalling by constitutively-activated Ras (Feig and Cooper, 1988; Cai et al., 1990; Vries-Smits et al., 1992). A second recently-identified dominant negative Ras, in which Asp 57 was replaced with Tyr, is thought to function in a manner similar to Ras N17 (Jung et al., 1994).

Activation of Raf

It is only recently that a downstream effector of Ras has been identified. Evidence including genetic studies in *Drosophila* (to be later discussed) and similarities of phenotypes induced by activated forms of these signal transduction proteins implicated the Raf-1 protein serine/threonine kinase. The widely-expressed Raf-1 is a 70-75 kDa phosphoprotein that consists of an amino-terminal regulatory region and a carboxy-terminal kinase domain (reviewed in Daum et al., 1994). The tissue-specific Raf family genes *A-raf* and *B-raf* have a similar structure, but have not been extensively studied.

N-terminal truncation causes constitutive activation of Raf protein kinase activity (Rapp et al., 1988). In addition, phosphorylation of Raf on serines 259 and 499 by PKC or tyrosines 340 and 341 by activated tyrosine kinases has also been shown to stimulate Raf enzymatic activity (Kolch et al., 1993; Fabian et al., 1993). None of the major growth factor-induced serine phosphorylation sites (Ser 43, 259, and 621) appear to be autophosphorylated; Thr 268 is the major autophosphorylation site. Ser 621 -> Ala mutants were nonactivatable in the baculovirus system, indicating a role for phosphorylation in Raf-1 kinase activation (Morrison et al., 1993).

A dominant negative Raf with a deleted kinase domain presumably functions by binding and sequestering its upstream regulator without activating downstream signal transduction proteins. Dominant negative Raf has been shown to block EGF-induced Ras activation of these downstream proteins in COS cells, thus establishing a position for Raf somewhere downstream of Ras (Schaap et al., 1993). Results from two hybrid system screening and direct co-immunoprecipitation experiments indicate that activated, GTP-bound Ras binds to the amino-terminal region of Raf-1 (Moodie et al., 1993; Aelst et al., 1993;

Zhang et al., 1993; Warne et al., 1993; Vojtek et al., 1993; Koide et al., 1993).

Raf-1 also binds a constitutively activated form of Ras. However, Raf-1 does not complex with GDP-bound Ras or inactive Ras “effector domain” mutants. While no Ras-Raf complexes were detected in intact cells without mitogenic stimulation, 3% of total cellular Raf was found to associate with Ras after EGF addition (Hallberg et al., 1994).

The region of Raf responsible for binding Ras was mapped to amino acids 51 to 131 (Chuang et al., 1994). A Raf [Arg 89 -> Leu] mutation abolishes binding to Ras and inhibits Ras-mediated activation of Raf kinase activity. The same mutation in the truncated dominant negative Raf interferes with its ability to block Ras-mediated growth stimulation (Fabian et al., 1994). However, Raf activity stimulated by the nonreceptor-type tyrosine kinase v-Src is unaffected, indicating a possible Ras-independent mode of Raf activation (Pumiglia et al., 1995).

It is possible that the sole purpose of the binding of activated Ras to Raf is translocation of Raf from the cytosol to the plasma membrane, where additional factors would carry out activation of the Raf kinase. Several observations support this hypothesis. First, Ras-GTP cannot stimulate Raf activity *in vitro* (Zhang et al., 1993). Second, Ras farnesylation is necessary for activation of Raf mediated by Ras binding (Kikuchi and Williams, 1994). Finally, membrane targeting of Raf by addition of the Ras CAAX motif obviates the need for Ras: membrane-bound Raf is constitutively activated, and this activity is not affected by activated Ras. EGF stimulation, however, can further increase the level of Raf activity, even in the presence of a dominant negative Ras, again indicating a Ras-independent pathway of Raf activation (Leevers et al., 1994; Stokoe et al., 1994).

The 14-3-3 protein family, implicated in yeast cell cycle control (Ford et al., 1994), has been suggested to enhance Raf kinase activity. Members of this family, identified by two-hybrid screening, binding assays, and yeast genetic experiments, can bind Raf-1 and activate its kinase activity in yeast or *Xenopus* expression systems (reviewed in Morrison, 1994). However, 14-3-3 complexes with Raf *in vivo* regardless of Raf subcellular localization, and attempts to activate Raf *in vitro* with 14-3-3 are less than convincing (Freed et al., 1994; Irie et al., 1994; Fu et al., 1994; Fantl et al., 1994; Li et al., 1995). 14-3-3 is therefore probably not the direct activator of Raf at the membrane, although it may play a structural or recruitment role. Interestingly, 14-3-3 has also been found in complex with growth-promoting proteins bcr-abl and polyomavirus middle T antigen (Reuther et al., 1994; Pallas et al., 1994).

The amino-terminal half of Raf was also found to interact with the Rap1b protein in a two-hybrid assay (reviewed in Avruch et al., 1994). Rap1a and Rap1b are Ras-related low molecular weight GTP binding proteins, 95% identical to each other and 50% identical to Ras. However, these proteins, also known as K-rev and Smg, were identified in a screen for factors that inhibit cell growth and transformation otherwise promoted by constitutively activated Ras (Kitayama et al., 1989; Kitayama et al., 1990). The Rap proteins are widely expressed at levels similar to Ras in most tissues examined, and no differences in Rap expression were found in neoplastic versus normal human tissues (Takayama et al., 1991). The role of Rap in growth control is still unclear, but a possible mechanism for antagonism of Ras activity may involve competition with GTP-bound Ras for the N-terminal domain of Raf.

One further level of modulation of Raf activation involves the observation that 8-bromo-cyclic AMP, an activator of the cAMP-regulated protein kinase A

(PKA), inhibits EGF stimulation of DNA synthesis (Burgering et al., 1989). EGFR activation, Grb2 and Shc association, and Ras activation are not affected by cAMP, while Raf activation is inhibited (reviewed in Marx, 1993). cAMP-induced PKA phosphorylation of Raf appears to accomplish this by two concomitant mechanisms: reduction of affinity for Ras (Wu et al., 1993a), and downregulation of Raf kinase activity (Hafner et al., 1994).

The Kinase Cascade Initiated by Raf: Mek and Mapk

In the chain of signal transduction events from the EGFR, Raf is responsible for initiating a kinase cascade that ultimately results in phosphorylation of several transcription factors. The immediate substrate of Raf is Mek, also known as MAP kinase-kinase, and Mek is responsible for activating MAP kinase (Mapk), also known as Erk (Extracellular signal-Regulated Kinase).

Mapk was originally identified as an activity in insulin-stimulated 3T3-L1 cells capable of phosphorylating microtubule-associated protein 2 on serine and threonine *in vitro* (Ray and Sturgill, 1987). A similar activity was observed in many other cell types in rapid response to various stimuli, such as the injection of sea star oocytes with maturation-promoting factor (Sanghera et al., 1991) and EGF stimulation of quiescent Swiss 3T3 cells (Ahn et al., 1991). Three Mapk proteins were subsequently cloned: p44 Erk1, p42 Erk2, and p65 Erk3 (Boulton et al., 1990; Boulton et al., 1991a). Erk1 and 2 share 90% amino acid identity, while the more distantly related Erk3 is 50% identical to Erk1 and 2. Erk1 contains a seven amino acid amino-terminal extension relative to Erk2, while Erk3 has an additional 180 carboxy-terminal residues compared with Erk1 and 2. Messages for the three Erks were detected in all adult tissues examined, but relative levels of each varied. However, many cell lines and

primary cell types have been identified that express only Erk1 or Erk2. Low-stringency Southern hybridization has indicated the possible existence of more Erk family members, and cloning of a 97 kDa human homolog of Erk 3 has recently been reported (Zhu et al., 1994).

Mapk requires two phosphorylation events for activation: phosphorylation of Thr 183 and Tyr 185 (Anderson et al., 1990; Crews et al., 1991; Payne et al., 1991). Treatment of Mapk with either a tyrosine-specific phosphatase or a serine/threonine specific phosphatase inactivates the enzyme. In fact, MKP-1, a dual specificity tyrosine/threonine phosphatase with a greater activity toward Mapk than any other substrate tested, can dephosphorylate and inactivate Mapk *in vitro*. Transcription of MKP-1 is induced by serum with kinetics similar to the eventual dephosphorylation of Mapk after the peak of Mapk activation, raising the possibility that MKP-1 is a physiological regulator of Mapk (Charles et al., 1993; Sun et al., 1993).

Mapk is a dual-specificity enzyme itself, and is capable of autophosphorylating the two residues sufficient for activation, but the kinetics of *in vivo* activation imply the existence of an activator kinase (Seger et al., 1991). The identification of this activator kinase became the focus of a large number of labs.

Several groups isolated an activity capable of phosphorylating kinase-negative Mapk, or a bacterially produced, nonphosphorylated Mapk (Alessandrini et al., 1992; Rossomando et al., 1992; Nakielnny et al., 1992; Kyriakis et al., 1992; Matsuda et al., 1993b). The first Mapk kinase cloned, *MEK1*, encodes a widely expressed 393 amino acid, 43.5 kDa dual-specificity kinase (Crews et al., 1992a; Wu et al., 1993b; Ashworth et al., 1992). *MEK2*, cloned soon thereafter, encodes a 400 amino acid, 44.5 kDa dual specificity

kinase displaying 80% amino acid identity with Mek1 (Zheng and Guan, 1993a; Brott et al., 1993; Wu et al., 1993c). Mek1 and 2 are both activated by EGF-stimulated Swiss 3T3 cell lysate and are capable of phosphorylating and activating p42 and p44 Mapk *in vitro*, but with different apparent activities (Zheng and Guan, 1993b). MEK1 and 2 also exhibit different patterns of expression, presaging a complex network of regulatory pathways.

Although Mek1 is able to autophosphorylate on serine, threonine, and tyrosine residues, physiological activation seems to involve only serine phosphorylation, specifically serines 218 and 222 (Kosako et al., 1992; Ahn et al., 1993; Zheng and Guan, 1994; Alessi et al., 1994). Activation of Mek1 in response to EGF is abolished by mutation of either of the serines to alanine. Replacement of serine 218 with aspartate, mimicking constitutively phosphorylated Mek, was shown to increase Mek1 activity greater than 100-fold without further activation by substitution of serine 222 (Huang and Erikson, 1994). However, there are conflicting reports of similar experiments which render the relative contributions of the two serines to Mek activation unclear (Seeger et al., 1994; Alessi et al., 1994; Cowley et al., 1994; Mansour et al., 1994).

As indicated above, activated Raf-1 is responsible for phosphorylating and activating Mek in response to EGF stimulation. It was first shown that Mek and Mapk are upregulated in cells expressing a constitutively active Raf, and that active Raf-1 was capable of activating dephosphorylated, inactive Mek *in vitro* (Kyriakis et al., 1992; Dent et al., 1992; Howe et al., 1992). Raf-1 can stably bind Mek1, and can even act as a bridge in a ternary complex of Ras-GTP, Raf, and Mek (Huang et al., 1993; Moodie et al., 1993; Aelst et al., 1993; Vaillancourt et al., 1994). A linear pathway showing chain-activation of Raf-1, Mek, and p42

Mapk was created in baculovirus-infected Sf9 cells expressing constitutively activated Ras, indicating a kinase cascade of direct substrates (Macdonald et al., 1993).

This sequence of signalling events, growth factor -> receptor tyrosine kinase -> Shc/Grb2 -> GEF -> Ras -> Raf -> Mek -> Mapk, has been further substantiated by genetic studies in *C. elegans*, *Drosophila*, and yeast. *C. elegans* vulval induction is the process by which an existing gonadal cell transmits a signal to vulval precursor cells, resulting in the differentiation of three of these cells along the vulval pathway (reviewed in Pawson, 1992). Mutation of the Let-23 receptor tyrosine kinase, Sem-5 adaptor protein (a homolog of Grb2), or Let-60 Ras homolog prevent vulval development; genetic studies place Sem-5 between Let-23 and Let-60.

The Ras Pathway In *Drosophila* Photoreceptor Development

Even more information has been gathered from the *Drosophila* photoreceptor development system (reviewed in Rubin, 1991). The *Drosophila* compound eye contains about 200 ommatidia, each containing eight photoreceptors and twelve support cells. The R7 photoreceptor, the last of the eight photoreceptors to be recruited in the developing ommatidium, must receive a signal from the neighboring R8 photoreceptor for induction of its neuronal fate; lack of a signal allows the precursor to develop in the default mode of a nonneuronal cone cell.

Genetic studies indicate a signal initiated by the transmembrane ligand "bride of sevenless" (boss) expressed on the surface of the R8 photoreceptor binding to the sevenless (sev) tyrosine kinase receptor expressed on the R7

photoreceptor (Tomlinson and Ready, 1986; Reinke and Zipursky, 1988). Other cells, including R3 and R4 (which contact R8), express *sevenless* as well, but are presumably committed to their respective lineages before *boss* is expressed. The development of R3 and R4 is unaffected by mutations in *sevenless* or *boss*. Interestingly, the entire transmembrane ligand is internalized by R7 (Cagan et al., 1992).

Genes functioning downstream of *sevenless* were identified in several screens that identified dominant suppressors, mutations in which increase efficiency of signal transduction from a weakened temperature-sensitive *sevenless* mutant, and dominant enhancers, mutations in which abrogate signal transduction from a weakened *sevenless* mutant. The assay in both cases was the presence of an R7 cell.

Suppressors of *sevenless* were identified to be mutations in the *Drosophila Ras-1* gene; *drk*, the *Drosophila* homolog of *Grb2*; and *Sos*, a *Drosophila* GEF, “son of *sevenless*” (Simon et al., 1991; Simon et al., 1993; Olivier et al., 1993; Bonfini et al., 1992). A loss-of-function mutation in *Ras1* or *Sos* blocks *sevenless* function (Simon et al., 1991), while constitutively activated *Ras1* induces formation of supernumerary R7 cells, even in the absence of a functional *sevenless* receptor (Fortini et al., 1992). Enhancers of *sevenless* were identified to be mutations in the *Drosophila* homologs of either *Rap* or *GAP*, encoding two previously described proteins that interfere with *Ras* function. These mutations also result in supernumerary R7 cells without requiring a functional *sevenless* receptor (Hariharan et al., 1991; Gaul et al., 1992).

Drosophila *Raf*, *Mek*, and *Mapk* also behave in a manner similar to that in EGF signalling. *Drosophila* *Raf* (*Draf*) was cloned with a human *Raf* probe

(Nishida et al., 1988); subsequent genetic studies placed Draf downstream of Ras in sevenless signalling. A constitutively activated Draf also induced R7 development in the absence of sevenless (Dickson et al., 1992). *Drosophila* Mek, Dsor1, was isolated in a screen for gain-of-function mutations that rescue R7 development in flies with a weakened Draf (Tsuda et al., 1993). *Drosophila* Mapk (ERK-A), encoded by *rolled*, was identified in a screen for mutations which blocked signalling from a constitutively activated Draf (Biggs et al., 1994). A gain-of-function ERK-A mutant was also identified in a screen for sevenless pathway function in the absence of boss (Brunner et al., 1994a). The gain-of-function mutation was mapped to substitution of Asn for kinase domain residue Asp 334, conserved in every Mapk homolog known. The mechanism of activation by this substitution is still unknown.

The *Drosophila* *Ellipse* gene encodes the receptor tyrosine kinase most closely related to the mammalian EGFR. *Ellipse* null mutants are lethal, and gain-of-function mutants have decreased numbers of (intact) ommatidia (Baker and Rubin, 1989). Inactivating mutations in *drk*, *Sos*, and *Ras-1* also suppress *Ellipse* gain-of-function mutants, indicating a role for the Ras pathway in signal transduction from multiple tyrosine kinase receptors (Simon et al., 1991).

Downstream effectors of Mapk in the sevenless system are poorly understood. One candidate is *seven in absentia* (*sina*), a nuclear protein of unknown function, the mutation of which abrogates sevenless signalling (Carthew and Rubin, 1990). In addition, two Ets family transcription factors, *pointed* and *yan*, have been identified as potential Mapk substrates with opposing effects on R7 development (O'Neill et al., 1994; Brunner et al., 1994b). Loss of function mutations in *phyllopod* were also identified in a screen for suppressors of constitutively activated Ras; this gene functions downstream of

yan and upstream of *sina* (Chang et al., 1995; Dickson et al., 1995). Finally, expression of a dominant negative form of the nuclear transcription factor Jun blocked photoreceptor development (Bohmann et al., 1994).

The Ras Pathway in Yeast

Saccharomyces cerevisiae contains two Ras proteins, RAS1 and RAS2. Cells remain viable with deletion of either one, but not both. Unlike in mammalian cells, yeast RAS controls activation of adenylate cyclase (Toda et al., 1985). Human H-Ras, although only 189 amino acids long compared to 322 for yeast RAS2, is 62% homologous to RAS2 in the amino-terminal 172 amino acids, and can rescue RAS null mutants, indicating conserved biochemical properties (Kataoka et al., 1985; Temeles et al., 1985). Several genes encoding regulators of RAS in *S. cerevisiae* have been cloned, including a GEF, *CDC25*; and two GAPs, *IRA1* and *IRA2* (Broek et al., 1987; Tanaka et al., 1989; Tanaka et al., 1990). Disruption of *IRA1* increased cellular levels of cyclic AMP and rescued *CDC25* mutants, but not *RAS1/RAS2* mutants (Tanaka et al., 1989).

The picture downstream of RAS, outlined in Table 1, quickly becomes more complicated in yeast. *S. cerevisiae* appear to have at least three pathways dependent on Mapk homologs, none of which require RAS (reviewed in Blumer and Johnson, 1994). For example, initiation of mating requires binding of a pheromone to a pheromone receptor, activation of a trimeric G protein, and initiation of a kinase cascade that results in activation of STE11, which functions in a manner analogous to mammalian Raf. STE11 activates Mek homolog STE7, which activates Mapk homolog FUS3/KSS1, which ultimately results in mating response. The STE11-STE7-FUS3 pathway is duplicated in the initiation of cell wall biosynthesis, which utilizes analogous

[-----S.cerevisiae-----]		[S.pombe]		[Dros.]		[-----mammalian-----]	
<u>mating</u>	<u>cell wall biosynth.</u>	<u>osmotic stress</u>	<u>mating</u>	<u>photo-reception</u>	<u>growth stim.</u>	<u>UV stress</u>	<u>osmotic stress</u>
STE11	BCK1	?	byr2	Draf	Raf	Mekk	?
STE7	MKK1/2	PBS2	byr1	Dsor	Mek	Sek	Mkk3/4
FUS3	MPK1	HOG1	spk1	Mapk	Mapk	Jnk	p38/RK

Table 1. Summary of genes involved in conserved kinase cascades in yeast and higher eukaryotes. Bulk of table from Blumer and Johnson (1994); other references in text. More recently elucidated details of yeast pathways reviewed in Herskowitz (1995).

proteins BCK1-MKK1/MKK2-MPK1, and glycerol accumulation in response to hyperosmotic media, which utilizes an unknown STE11 family protein to activate Mek homolog PBS2 and Mapk homolog HOG1. These pathways all function independently of one another; disruption of a gene in one pathway does not affect the functions of the other two pathways.

The mating response in *S. pombe* is yet again a different story: RAS1 does play a role in mating. Genetic evidence indicates that the pombe mating pheromone binds the pheromone receptor, causing activation of a trimeric G protein, which sends a signal through RAS1 to the pombe equivalents of the STE11-STE7-FUS3 pathway, *byr2-byr1-spk1*. Complex formation between RAS1 and *byr2* has even been detected using the two-hybrid system (Aelst et al., 1993).

Complexities of Ras Pathway Signalling in Mammalian Systems

Evidence contradicting a single, linear physiological pathway is now accumulating for mammalian cells as well. While the MEKs are similar in sequence to the cerevisiae STE7 and pombe *byr1*, Raf shows no homology to STE11 or *byr2*. Using PCR primers derived from regions of identity between *STE11* and *byr2*, a cDNA was identified in NIH-3T3 cells with similarity to these to genes (Lange-Carter et al., 1993). This sequence was used as a probe to clone Mek kinase (Mekk) from a mouse brain library. The 78-80 kDa protein exhibits a serine/threonine rich amino-terminus and a carboxy-terminal kinase domain that is 75% homologous to the *byr2* kinase domain.

Expression of Mekk in COS cells caused a 4-5 fold activation of MAP kinase activity, which was only slightly increased by addition of EGF. Mekk purified from these cells by column chromatography or immunoprecipitation

was able to phosphorylate a catalytically inactive Mek, as well as autophosphorylate. However, overexpression of Raf in this system did not phosphorylate or activate Mek unless EGF was added, indicating that Raf and Mekk are two independent Mek regulators, responding to different physiological stimuli (Lange-Carter et al., 1993). Raf-1 and Mekk also have different specificities for the Mek regulatory serines 218 and 222: Mekk preferentially phosphorylates Ser 218 with less activity towards Ser 222, while Raf-1 phosphorylates each residue to similar extents (Yan and Templeton, 1994a).

Recent studies indicate the existence of additional Mek activators expressed in different cell types and responding to different stimuli. B-Raf was purified as the major Mek activator in bovine brain; Raf-1 was expressed in this tissue, but did not copurify with Mek activator activity in this system (Catling et al., 1994). Similar results were obtained from NIH-3T3 cells (Reuter et al., 1995), in which an unidentified 40-50 kDa MEK activator was also observed. In addition, an activity was purified from insulin-stimulated adipocytes that separated into two distinct peaks, both of about 56 kDa. This activity did not co-elute with Raf-1 or Mekk and its predicted molecular weight precluded B-Raf, so it was named I-Mekk, representing a novel protein or proteins (Haystead et al., 1994). The phorbol ester PMA activated Mek in these cells without affecting I-Mekk, intimating the presence of another activator specific for the protein kinase C pathway in adipocytes. To date, a total of four Mekk isoforms with different amino-termini have been cloned (G. Johnson, personal communication).

There is substantial evidence, already discussed, that Raf-1 is responsible for activating Mek and Mapk in response to EGF stimulation. More specifically, *in vitro* experiments indicate that Raf-1 can bind Mek1 but not Mek2 in NIH-3T3 fibroblasts (Jelinek et al., 1994). However, some reports indicate

that several exceptions to this rule may exist. It is likely that more exceptions will surface as this pathway is studied in more cell types and with more stimuli.

One group used dominant negative Raf to show that Raf is not required for stimulation of Mapk activity by EGF or phorbol ester in Balb/c 3T3 cells, while it is necessary for stimulation of Mapk by insulin-like growth factor I (IGF-I). Ras was activated in all cases (Chao et al., 1994). A second group examined Raf function in insulin stimulated 3T3-L1 adipocytes (Porrás et al., 1994). A dominant negative Raf blocked differentiation of 3T3-L1 cells in response to insulin without blocking activation of Mapk after insulin stimulation, indicating an alternate effector pathway for Raf in this system. Finally, two groups used a dominant negative Ras to show that Ras is necessary for Mapk activation stimulated by insulin in NIH-3T3 cells and by PDGF in rat-1 cells, but is dispensable for phorbol ester and EGF stimulation of Mapk in rat-1 cells (Vries-Smiths et al., 1992; Burgering et al., 1993).

Of course, before one makes any generalizations, it is important to note that some reports indicate that Ras is required for phorbol ester stimulation of Mapk in native NIH-3T3 cells, as well as phorbol ester superstimulation of Mapk in NIH-3T3 cells transformed with v-Raf, indicating a pathway from PKC through Ras (Nori et al., 1992; Mitra et al., 1993). There are also conflicting reports concerning the role of Ras and Raf in stimulation of MAP kinase via heterotrimeric G-protein coupled receptors, although evidence seems to be accumulating for involvement of these signal transduction proteins in at least some G-protein coupled pathways that activate Mapk (Nori et al., 1992; Corven et al., 1993; Winitz et al., 1993; Alblas et al., 1993; Howe and Marshall, 1993). It is unclear if PKC activation is obligatory in these systems, but PKC and Ras may cooperate in full activation of Raf under some conditions. Further complexities

of interaction between heterotrimeric G protein signalling, PKC signalling, and Ras pathway signalling are reviewed in (Burgering and Bos, 1995).

The opposite specificity problem occurs in the rat pheochromocytoma cell line PC12 (reviewed in Chao, 1992). PC12 cells differentiate to a sympathetic neuronal phenotype upon stimulation by nerve growth factor (NGF), while EGF induces PC12 cell proliferation. NGF and EGF initiate seemingly identical signal transduction pathways in these cells, including activation of Ras, Raf, and Mapk, while eliciting two very different responses (Thomas et al., 1992; Wood et al., 1992; Ohmichi et al., 1992). The specificity of the signal in transit over the Ras pathway may lie in kinetics of Mapk activation (Marshall, 1995), or there may be additional stimulus-specific signals that have not yet been identified.

One example of such a stimulus-specific pathway in fibroblasts may be the emerging Jak/STAT pathway (reviewed in Briscoe et al., 1994), originally characterized as an interferon-stimulated pathway (Darnell et al., 1994). Many cytokines and growth factors activate a specific Jak family cytosolic tyrosine kinase. The activated Jak family kinase then phosphorylates a specific SH2-containing STAT family transcription factor, causing SH2-dependent homo- or hetero-dimerization and translocation to nucleus. The nuclear dimer can bind specific sequence elements, dependent on the nature of the STAT family proteins in the complex, and initiate transcription of genes involved in the response to the initiating stimulus.

Parallel Pathways: Response to Cellular Stress in Higher Eukaryotes

Some specificity of Mekk versus Raf is now being examined in systems analogous to the parallel response pathways in yeast. A protein kinase

cascade induced by cellular stress in response to ultraviolet (UV) light phosphorylates the amino terminus of the nuclear protein c-Jun, resulting in increased transcriptional activity by AP-1 (to be later discussed), of which Jun is a component (Devary et al., 1992). This phosphorylation event was originally thought to be dependent on the Raf-Mek-Mapk pathway, but any phosphorylation of Jun by Mapk appears to be in the carboxy-terminal region, which does not contribute to activation of Jun transcriptional activity (Minden et al., 1994a). However, an activity was identified that could both bind and phosphorylate the amino terminus of c-Jun (Hibi et al., 1993).

JNK1 (Jun kinase) was cloned by homology as a member of the MAP kinase family of proteins (Derijard et al., 1994), and encodes a 46 kDa protein that is about 40% identical to the MAP kinases Erk1 and Erk2, as well as HOG1, FUS3, and MPK1 (see Table 1). The identical *Sapk* (stress-activated protein kinase) was simultaneously purified and cloned by a laboratory investigating cellular stress induced by tumor necrosis factor α (TNF α) (Kyriakis et al., 1994). Jnk1 was able to efficiently phosphorylate the activating amino-terminal serines 63 and 73 of c-Jun in response to ultraviolet light and activated Ras (Derijard et al., 1994), although the TNF α pathway was Ras-independent (Minden et al., 1994b).

Jnk1 contains a regulatory threonine and tyrosine, analogous to the phosphorylation sites of other family members, which are required for UV induced activation of kinase activity (Derijard et al., 1994). The *Mek*-related gene *Sek*, cloned by homology, encodes a protein capable of phosphorylating these sites and activating Jnk1 (Sanchez et al., 1994). Since activated Raf causes a strong, immediate activation of Mapk upon induction, but only a weak, delayed activation of Jnk1; and activated Mekk causes a strong, immediate

activation of Jnk1, but a only weak activation of Mapk, it appears that Mekk and Raf control two different pathways. Growth factor stimulation activates Raf, Mek, and Mapk, while cellular stress activates Mekk, Sek, and Jnk1 (Minden et al., 1994b; Yan et al., 1994b). Overexpression of activated Mekk in Swiss 3T3 cells causes massive apoptosis, as opposed to the DNA synthesis induced by activated Raf (Gary Johnson, personal communication), seemingly appropriate responses for the two very different stimuli from which Mekk and Raf transduce signals.

The gene encoding a 55 KDa Jnk2, exhibiting 83% identity to Jnk1, has recently been cloned (Kallunki et al., 1994). It seems to be regulated in a similar fashion, but binds Jun approximately 25 times more efficiently than Jnk1. The specificity and relative contributions of these two genes to stress response remains to be characterized.

One group reported activation of Jnk1 in response to osmotic shock (Galcheva-Gargova et al., 1994), but it now seems likely that yet another MAP kinase family member also plays a role in this pathway. A 38 kDa protein was purified from LPS-treated B cells and cloned from a murine liver cDNA library (Han et al., 1994). This protein is most closely related to the cerevisiae HOG1 (52% identity). Both share the phosphorylation site sequence TGY, as opposed to TPY for the Jnks and TEY for the Mapks. Like HOG1, p38 is activated in response to hyperosmotic stress, although Mapk activation is also observed in these experiments (Han et al., 1994). p38 is probably the rat homolog of a reactivating kinase (RK) recently cloned for MAPKAP Kinase-2, a substrate of Mapk under conditions in which Mapk is activated (Stokoe et al., 1992). However, MAPKAP Kinase-2 can also be activated by several types of cellular stress, conditions under which Mapk is not activated, but p38/RK is (Rouse et

al., 1994). Two kinases capable of activating p38/RK were subsequently cloned: MKK3 and MKK4 (Derijard et al., 1995). Neither MKK3 nor MKK4 can activate the Erk family kinases, but MKK4, the human homolog of Sek, is able to activate Jnk.

Only one substrate of Jnk besides c-Jun has been described so far: Activating Transcription Factor 2 (ATF2) (Gupta et al., 1995). Both Jnk1 and Jnk2 were able to phosphorylate threonines 69 and 71 of ATF2 in response to UV irradiation, increasing ATF2 transcriptional activity in a luciferase reporter assay. Mutation of these two threonines to alanine decreased reporter gene expression, and a catalytically inactive Jnk1 inhibited serum stimulation of ATF2 activity. Interestingly, ATF2 can also heterodimerize with c-Jun to bind the ATF2 target “CRE-like” elements.

Downstream Effectors of Mapk

There is also some information concerning the downstream targets of the Raf-Mek-Mapk pathway. All Mapk substrates contain the phosphorylation site consensus sequence Pro-X-Ser/Thr-Pro, in which the Ser or Thr is phosphorylated by Mapk (reviewed in Crews et al., 1992b). Three cytoplasmic targets of Mapk are the previously mentioned MAPKAP kinase-2, cytosolic phospholipase A₂ (cPLA₂), and p90 Rsk (reviewed in Blenis, 1993).

MAPKAP kinase 2 is a serine/threonine kinase, originally purified as a NaF-stabilized activity capable of phosphorylating glycogen synthase. The two related isoforms, p53 and p60, are active in conditions under which Mapk is also active (Stokoe et al., 1992). A possible function for MAPKAP Kinase-2 is phosphorylation of the small heat shock proteins, Hsp 25/27, but the role this plays in mitogenesis is unknown (Rouse et al., 1994).

Cytosolic PLA₂ is activated by stimuli which elevate cellular levels of calcium, including growth factors and agonists of G-protein coupled receptors, to contribute to arachidonic acid production. Mapk phosphorylation of cPLA₂ activates its phospholipase activity, and mutation of the phosphorylation site Ser 505 impairs the cPLA₂-mediated release of arachidonic acid in response to phorbol esters or thrombin (Lin et al., 1993).

Rsk was originally purified as a mitogenically stimulated kinase capable of phosphorylating ribosomal protein S6 *in vitro*. However, it now appears that this function is physiologically accomplished by the 70 kDa S6 kinase, which is also mitogenically stimulated, although not phosphorylated by Mapk (Ballou et al., 1991; Blenis et al., 1991). The physiological substrates of Rsk are not known. There also may be another kinase responsible for phosphorylating Rsk in response to growth factor stimulation, and there exists evidence that Mapk can only partially activate Rsk *in vitro*, making the precise role of Mapk in Rsk activation unclear (Chen et al., 1992; Sturgill et al., 1988; Chung et al., 1991).

The above cytoplasmic substrates of Mapk therefore do not provide too many clues about the function of Mapk in growth factor stimulated cells, since a mechanism for cell growth must eventually link up to induction of transcription and translation of proteins necessary to induce DNA synthesis and cell division. A fourth recently described cytoplasmic substrate may be more informative. PHAS-I, a protein which normally binds to and sequesters translation initiation factor eIF-4E, is phosphorylated on serine 64 by Mapk in response to insulin or EGF (Lin et al., 1994). eIF-4E is then released from its complex with PHAS-I, and becomes available to participate in translation initiation. Overexpression of eIF-4E is mitogenic in serum-starved NIH-3T3 cells, but this phenotype is paradoxically abrogated by expression of a dominant negative Ras (Lazaris-

Karatzas et al., 1992).

There is also evidence that Mapk can function in the nucleus. Serum or mitogenic growth factor stimulation induces translocation of Mapk, but not Mek, into the nucleus (Gonzales et al., 1993; Lenormand et al., 1993), raising the possibility of physiologically relevant nuclear targets. Rsk has been detected in the nucleus as well (Chen et al., 1992). The most viable candidate for a nuclear Mapk effector is the transcription factor Elk-1, which is involved in the transcriptional activation of the nuclear gene *c-fos* (Hipskind et al., 1991).

Fos is a component of the serum-induced transcription complex AP-1, of which the previously-mentioned c-Jun is also a component (reviewed in Curran and Franza, 1988). The Fos-Jun AP-1 complex recognizes a conserved nine base pair motif, called the TPA-responsive element (TRE), which is sufficient to confer phorbol ester or serum responsiveness to a promoter containing this element. Genes containing such elements include those encoding collagenase and human metallothionein IIA (Angel et al., 1987; Lee et al., 1987), but the role of these genes in initiation of cell division is not known.

Fos is considered an early response gene, in that its transcription is induced within minutes of serum or growth factor stimulation (Greenberg and Ziff, 1984; Kruijer et al., 1984). Serum stimulated transcription of *Fos* is dependent on the formation of a ternary complex consisting of the cis serum response element (SRE) in the *fos* promoter, the trans serum response factor (SRF), and a trans ternary complex factor (TCF) family member, such as Elk-1 (Norman et al., 1988; Shaw et al., 1989; Treisman, 1994). Elk-1, which contains an Ets domain similar to Pointed and Yan of the *Drosophila* sevenless pathway, is phosphorylated at many sites in its carboxy-terminus by Mapk in response to mitogenic stimulation. This phosphorylation event potentiates the

transcriptional transactivation function of Elk-1 (Marais et al., 1993). There is also evidence that phosphorylation of TCFs by Mapk enhances ternary complex formation between the SRE, SRF, and the TCFs (Gille et al., 1992), and that the extent and kinetics of Mapk activation, TCF phosphorylation, and Fos promoter activation all correlate (Hipskind et al., 1994). Phosphorylation of the Fos protein itself may involve a MAP kinase family member other than Erk 1 or 2, contradicting previous reports (Chen et al., 1993; Deng and Karin, 1994).

The Ultimate Target: The Cell Cycle

The relationship between mitogen-induced transcription factors and initiation of DNA synthesis is still a black box. Growth factor stimulation can prompt a cell to enter S phase from G1, or from G0 if the cell is in a quiescent state (reviewed in Scherr, 1993; Scherr, 1994). This is accomplished by the sequential accumulation of specific cyclin proteins, which are produced and degraded in characteristic timeframes spanning various phases of the cell cycle. Cyclins are able to associate with and activate specific cyclin-dependent kinases (CDK), the expression levels of which generally do not vary with cell cycle. The cyclins responsible for transit across G1 include D1, D2, and D3; cyclin E functions specifically at the G1/S boundary (Lew et al., 1991; Dulic et al., 1992). Once a cell passes the cell cycle restriction point in late G1, around the time of Rb phosphorylation, the cell is committed to divide, and removal of the growth factor has no effect on further progression of the cell cycle.

The D cyclins are likely targets of growth factors. Synthesis of the D cyclins begins in the G0/G1 transition after serum stimulation, while degradation begins in S phase (Baldin et al., 1993; Won et al., 1992). In addition, they are rapidly degraded when mitogens are removed. This degradation can occur at

any point in the cell cycle, but only prevents progression to S phase if the restriction point has not been passed (Matsushime et al., 1991). There is evidence that the cyclin D2-Cdk4 complex can directly phosphorylate Rb, releasing Rb-sequestered transcription factor E2F (Ewen et al., 1993; Dowdy et al., 1993; Chellappan et al., 1991). Unfettered E2F may participate in transcription of genes involved in growth response; E2F binding sites are found in the promoters of early response gene *c-myc*, M-phase cyclin dependent kinase *cdc2*, and DNA polymerase α (reviewed in Nevins, 1992).

Preliminary results indicate a possible link between the Ras pathway and cell cycle progression: Raf is able to phosphorylate and activate the phosphatase responsible for activating Cdc2 (David Beach, unpublished data). In addition, it appears that Myc, which is transcribed in early response to serum with kinetics preceding that of cyclin D1, is capable of stimulating cyclin D1 transcription (Daksis et al., 1994). The relationship between the transcription of Myc in immediate response to serum and the implied cyclin D-dependent transcription of Myc in delayed response to Rb phosphorylation has not been elucidated.

Cell Transformation

Each of the Ras pathway signal transduction components involved in mitogenic signalling from the EGFR is also associated with cell transformation. Cell transformation results from the deregulation of cell growth, with phenotypes including altered cell morphology, increased rate of DNA synthesis and cell proliferation, growth to higher saturation densities, lack of contact inhibition, anchorage-independent growth, and tumor formation in an immunocompromised animal. The transforming potential of a normal cellular

gene, known as a proto-oncogene, can be unmasked by mutation, overexpression, or co-expression with another gene involved in signal transduction. A single transforming gene, or oncogene, is often enough to elicit many hallmarks of transformation in established cell lines, which have already undergone the initiating process of immortalization. However, primary cells often require the cooperating functions of two or more oncogenes before transformation occurs (Ruley, 1983; Land et al., 1983; Sager et al., 1983). As implied by many examples given above, oncogenic activation of one signal transduction protein can often cause constitutive activation of downstream signalling molecules, and transformed phenotypes can often be inhibited by a dominant inactivating mutation in a downstream signalling molecule.

Both deletion of the amino-terminal ligand binding domain and mutation of carboxy-terminal sequences can confer transforming potential to the EGFR when expressed in NIH-3T3 fibroblasts (Massaglia et al., 1990).

Overexpression of Shc in NIH-3T3 cells is sufficient for transformation, while Grb2 must be overexpressed with H-Ras to stimulate DNA synthesis in a rat embryo fibroblast cell line (Pelicci et al., 1992; Lowenstein et al., 1992).

Overexpression of *Drosophila* Sos in Rat1 cells was also sufficient to confer many of the transformed phenotypes (Egan et al., 1993). Several transforming Ras mutations have been identified and exhaustively studied, but the most popular mutation is the substitution of Gly 12 with Val, or one of several other amino acids that confer oncogenicity in NIH-3T3 cells when present at position 12 (Fasano et al., 1984).

Amino-terminal truncation of Raf is sufficient for unmasking of transforming potential in cell lines, while mutations in Mek that substitute Asp or Glu for Serines 218 and 222 (mimicking constitutive phosphorylation) enable

Mek to transform NIH-3T3 cells (Stanton et al., 1989; Cowley et al., 1994; Mansour et al., 1994; Huang and Erikson, 1994). An oncogenic Mapk has not yet been described, but the activating mutation identified for *Drosophila* Mapk should prove to be a useful reagent.

Downstream of Mapk, oncogenic activity has been described for overexpressed eIF-4E in NIH-3T3 cells (Lazaris-Karatzas et al., 1992). Overexpressed c-Fos is transforming in both cell lines and primary chicken embryo fibroblasts without structural alteration (Iba et al., 1988). Overexpression of c-Jun is sufficient for transformation of cell lines, but amino-terminal truncation of Jun is necessary for efficient transformation of primary cells (Schutte et al., 1989; Bos et al., 1990). Expression of cyclin D1 can transform primary rat kidney cells under very narrow conditions: Coexpression of activated Ras and a mutant adenovirus E1A protein that does not interact with Rb is not transforming, while the additional overexpression of cyclin D1 pushes the cells over the transforming precipice (Hinds et al., 1994).

The involvement of these signal transduction proteins in human cancer is quite another matter. Of the proteins with transforming potential described above, only Ras and possibly cyclin D1 have been identified as agents in human neoplasia. Mutationally activated Ras has been observed in many different tumors, including bladder carcinoma (Tabin et al., 1982) and colorectal carcinoma (reviewed in Fearon and Vogelstein, 1990). Evidence implicating cyclin D1 originates from a common parathyroid tumor chromosomal translocation. This translocation causes juxtaposition of the 5' regulatory region of the parathyroid hormone gene with the coding sequence of the human cyclin D1 gene, known as *PRAD1* before sequence identification, causing D1 overexpression (Motokura et al., 1991). Human cancer has been more

frequently associated with inactivation of growth suppressing genes, such as p53 and Rb, than activation of growth promoting genes (Baker et al., 1990; Huang et al., 1988).

Oncogenes are also found in transforming retroviruses, which can incorporate cellular sequences, often accompanied by mutation, while replicating. The mechanism of transduction of cellular sequences is still unclear, but several hypotheses based on sequence information from existing oncogenic viruses have been proposed (reviewed in Bishop and Varmus, 1985). The most likely hypothesis involves capture of cellular RNA in a nascent virion, as all viral oncogenes analyzed to date have already undergone splicing events.

The v-src Oncogene

One of the best studied viral oncogenes is *v-src*, isolated from the Rous sarcoma virus. *v-src* encodes a 60 kDa cytosolic protein tyrosine kinase which associates with the plasma membrane due to a posttranslational myristylation event (Brugge and Erikson, 1977; Collett et al., 1980; Schultz et al., 1985; Buss and Sefton, 1985). The cellular counterpart of *v-src*, *c-src*, encodes a highly homologous protein with extra carboxy-terminal sequences not encoded by *v-src*. In fact, it was *src* with which the cellular origin of viral oncogenes was proposed (Stehelin, et al., 1976; Hanafusa et al., 1977; Spector et al., 1978; Takeya and Hanafusa, 1983).

The Src protein, illustrated in Figure 2, consists of unique amino-terminal sequences (divergent within the Src family of tyrosine kinases), an SH3 domain, an SH2 domain, and a kinase domain. Kinase activity is integral to v-

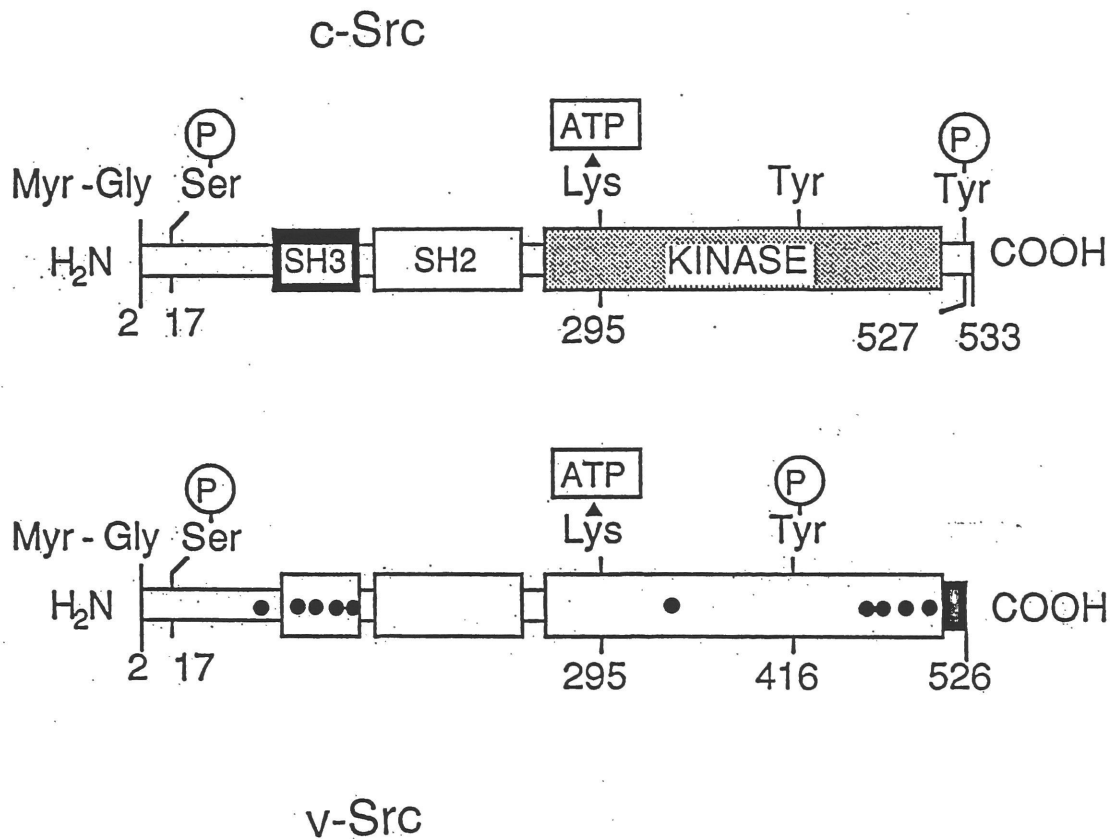


Figure 2. A comparison of the c-Src and v-Src proteins. Dots indicate positions of point mutations in v-Src relative to c-Src. Tyrosine 527 is preferentially phosphorylated in c-Src, while tyrosine 416 is preferentially phosphorylated in v-Src, which is truncated just before tyrosine 527. Both proteins are myristylated at their amino termini.

Src transforming ability, and v-Src transformed cells exhibit elevation of phosphotyrosine on many cellular proteins (Hamaguchi et al., 1988; Kanner et al., 1990). A negative regulatory phosphorylation site, Tyr 527, resides in the c-Src carboxy-terminus, but is deleted by the v-Src carboxy-terminal truncation. Phosphorylation of this site represses the kinase activity of c-Src, and mutation of this residue to Phe is sufficient to activate the kinase activity of c-Src to levels comparable to that of v-Src, accompanied by activation of c-Src transforming potential (Kmiecik and Shalloway, 1987; Piwnicka-Worms et al., 1987; Cartwright et al., 1987). Csk (c-Src kinase) was identified as a kinase capable of phosphorylating Tyr 527 (Okada and Nakagawa, 1989; Nada et al., 1991), and it is thought that c-Src is maintained in the inactive state by intramolecular binding of the SH2 domain to phosphorylated Tyr 527 (Roussel et al., 1991).

The role of c-Src in growth control *in vivo* is not yet completely understood. c-Src can bind PDGFR Tyr 857 with its SH2 after PDGF stimulation, resulting in phosphorylation of c-Src and activation of c-Src kinase activity (Kypta et al., 1990; Gould and Hunter, 1988; Courtneidge et al., 1991; Twamley et al., 1992). It is difficult to reconcile this with data showing translocation of c-Src to the cytosol from the plasma membrane under the same conditions (Walker et al., 1993; Weernink and Rijksen, 1995), although the kinetics of the release of Src from the membrane may be somewhat delayed compared to association with the PDGFR, so they may be sequential events. However, microinjection of a catalytically inactive c-Src can block PDGF- and EGF-mediated induction of DNA synthesis, but not G-protein-mediated induction of DNA synthesis, defining a likely role for c-Src in signal transduction initiated by growth factor receptor tyrosine kinases (Twamley-Stein et al., 1993; Roche et al., 1995).

c-Src may play an additional role in regulation of M phase: the amino-terminus of c-Src is phosphorylated in mitotic cells on serine and threonine by cdc2, the M phase cyclin-dependent kinase, accompanied by an increase in c-Src kinase activity (Chackalaparampil and Shalloway, 1988; Shenoy et al., 1989; Morgan et al., 1989). The c-Src SH2 domain is also more accessible in mitosis (Bagrodia et al., 1994).

v-Src transformation is not completely understood, either.

Phosphorylation of cellular substrates is obviously essential, but how these phosphorylated proteins connect with signal transduction pathways leading to DNA synthesis is still unclear. However, activation of some signalling pathways has been described.

Transforming variants of v-Src, but not c-Src or kinase-inactive v-Src, associate with phosphatidylinositol-3-kinase (PI3K). This interaction, mediated by the Src SH3, is accompanied by phosphorylation and activation of PI3K and elevation of cellular levels of phosphoinositides phosphorylated at the D3 position (Fukui and Hanafusa, 1989; Liu et al., 1993; Pleiman et al., 1994). PI3K is comprised of an 85 kDa regulatory subunit containing one SH3 and two SH2 domains, and a 110 kDa catalytic subunit, which associates with a region of p85 located between the two SH2 domains (Escobedo et al., 1991; Skolnik et al., 1991; Otsu et al., 1991; Hiles et al., 1992; Klippel et al., 1993). Multiple isoforms of these two subunits have been identified, but examination of functional distinctions is just beginning (Baltensperger et al., 1994; reviewed in Parker and Waterfield, 1992).

PI3K can also associate with the autophosphorylated PDGFR upon growth factor stimulation; this interaction is mediated by the PI3K p85 SH2 domains (McGlade et al., 1992a; Klippel et al., 1992). PI3K probably does not

bind to the EGFR, but EGF does elevate cellular levels of phosphatidylinositol-3-phosphates. The EGFR may be able to activate PI3K indirectly, since activated Ras has been shown to bind and activate PI3K (Rodriguez-Viciano et al., 1994). Downstream effectors of the products of activated PI3K have not been identified.

The other major signalling pathway known to be activated by v-Src is the Ras pathway. Shc is tyrosine phosphorylated and associates with Grb2 in v-Src transformed cells (McGlade et al., 1992b). v-Src transformed cells contain elevated levels of GTP-bound Ras (Sato et al., 1990), and v-Src can cooperate with c-Ras to activate Raf in the baculovirus system (Williams et al., 1992). Furthermore, overexpression of GAP suppresses v-Src transformation, and v-Src fails to rescue cell growth blocked by dominant negative Ras N17 (Nori et al., 1991; DeClue et al., 1991; Feig and Cooper, 1988). There is no evidence for participation of c-Src in activation of Ras in response to growth factor stimulation.

The v-*crk* Oncogene

Another viral oncogene, v-*crk*, was identified as the transforming gene of the CT10 avian sarcoma virus (Mayer et al., 1988). It encodes a 47 kDa protein consisting of viral gag sequences fused to an SH2 and SH3 domain. Chicken embryo fibroblasts (CEF) transformed by v-Crk exhibit morphological transformation, grow to higher cell densities, and form colonies in soft agar. v-Crk transformed CEF also contain elevated levels of phosphotyrosine on three proteins (p130, p110, and p70), despite the conspicuous absence of a kinase domain in the transforming protein. In addition, transformation and increased phosphotyrosine levels correlate in cells expressing a panel of v-Crk mutants

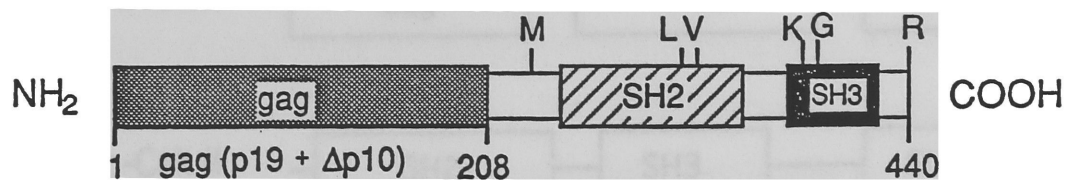
(Mayer and Hanafusa, 1990b). Both the SH2 and SH3 domains of v-Crk are required for transformation (Mayer and Hanafusa, 1990b).

The cellular counterpart of the *v-crk* oncogene, *c-crk*, was cloned from a chicken embryonic library and shown to consist of one SH2 domain, followed by two SH3 domains (Reichman et al., 1992). A comparison of v- and c-Crk is shown in Figure 3. The widely expressed 35 kDa c-Crk protein is almost identical to v-Crk in the colinear sequences; the carboxy-terminus of v-Crk occurs between the two SH3 domains of c-Crk. A shorter, alternatively-spliced form of c-Crk, termed Crk-I, has been identified in CEF and cloned from a human embryonic lung library (Reichman et al., 1992; Matsuda et al., 1992b). Overexpression of the Crk-I protein can cause cell transformation similar to that caused by v-Crk (Matsuda et al., 1992b). A second human *c-crk*-like gene, *CRKL*, encodes a 36 kDa protein consisting of one SH2 and two SH3 domains. *CRKL* is most highly homologous (76% identity) in the SH2 and SH3 domains, with little similarity in the spacer regions (ten Hoeve et al., 1993).

v- and c-Crk clearly belong to the SH2/SH3 adaptor protein family, for which v-Crk served as prototype. Representative members of the adaptor protein family are illustrated in Figure 4. The v-Crk SH2, which recognizes the consensus motif YXXP (Songyang et al., 1993), was shown to bind tyrosine-phosphorylated epidermal growth factor receptor *in vitro*, concomitantly protecting the receptor from phosphatase activity (Birge et al., 1992). c-Crk could only bind the phosphorylated receptor if an N-terminal extension was added, such as GST fusion, or 31 amino acids provided by an upstream CUG translation initiation site (Fajardo et al., 1993).

The v-Crk SH2 domain can also bind tyrosine phosphorylated p130 and p70 from CT10-transformed CEF (Birge et al., 1993). p70 has been identified

gag-crkr



c-crkr

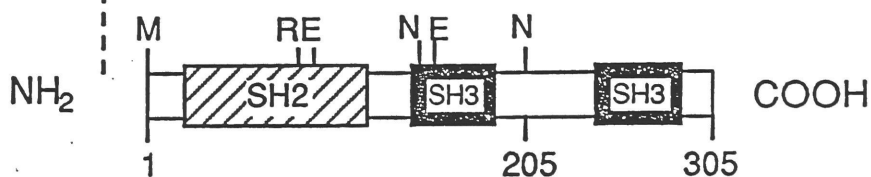


Figure 3. A comparison of the v-Crk ("gag-crkr") and c-Crk proteins. Amino acid differences are indicated. v-Crk is synthesized as a fusion of viral gag sequences with cellular Crk, but is truncated after the first SH3 domain.

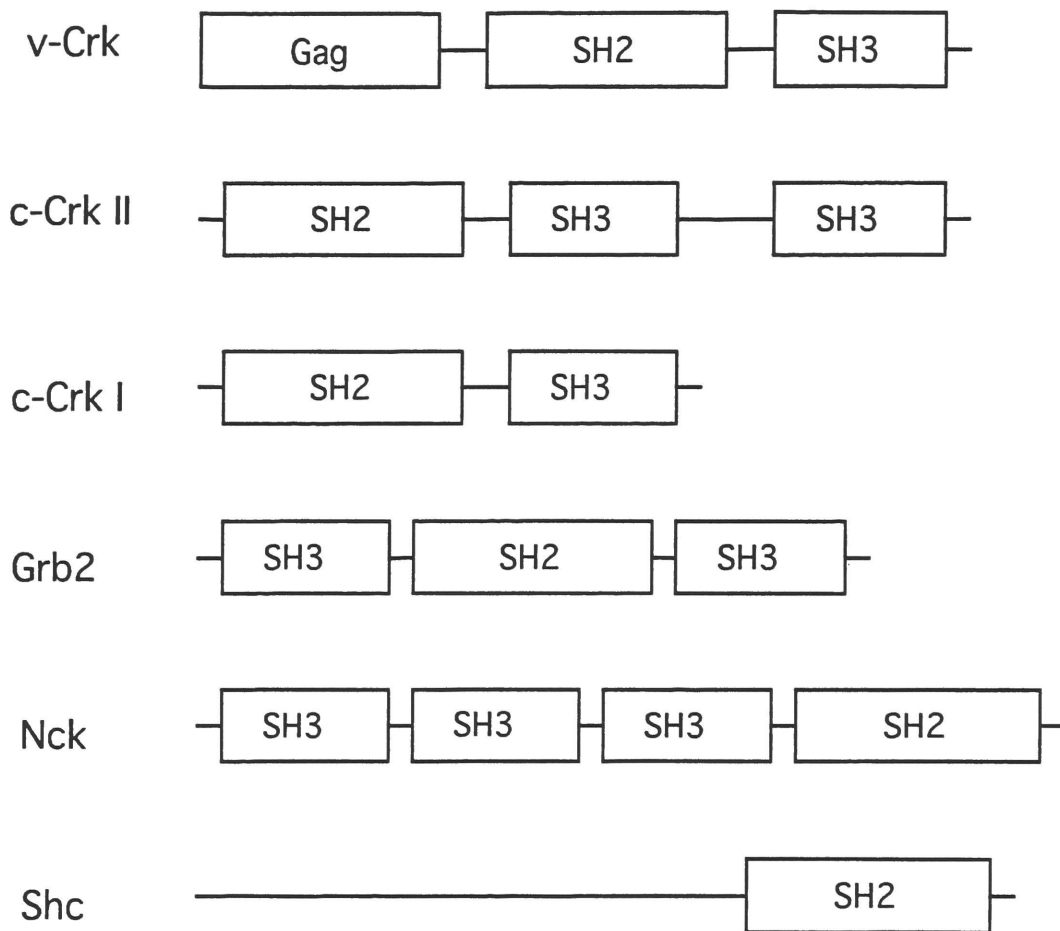


Figure 4. Representative adaptor proteins

as the focal adhesion protein paxillin (Birge et al., 1993; Turner and Miller, 1994), suggesting that v-Crk may be localized to sites of cell-to-substratum attachment. The gene encoding Crk-associated p130 has recently been cloned (Sakai et al., 1994); p130 consists of an amino-terminal SH3 domain, a cluster of tyrosines forming putative SH2 binding sites, and a carboxy-terminal sequence of unknown function. This protein, termed Cas, is also tyrosine phosphorylated in v-Src transformed cells, and is able to bind the v-Src SH2. In addition, a 116 kDa protein antigenically related to Cas is tyrosine phosphorylated in T cells upon stimulation, enabling it to bind to the c-Crk SH2 (Sawasdikosol et al., 1995).

Crk SH3 binding proteins have been identified as well. Solution binding assays were used to demonstrate binding of the first c-Crk SH3 to a series of high molecular weight proteins. One was identified as mSos, the Ras GEF. A second was shown to be c-Abl, a cytosolic tyrosine kinase that can specifically phosphorylate c-Crk Tyr 222, forming a high-affinity binding site for the Crk SH2. This raises the possibility that c-Crk *in vivo* binding activity is inhibited by an intramolecular binding event, much like that hypothesized for c-Src. Furthermore, Y222 is deleted in the v-Crk carboxy-terminal truncation, indicating a possible role of Y221 phosphorylation in regulation of Crk growth-signalling activity (Feller et al., 1994). A third Crk SH3 binding protein, C3G, was independently cloned by two groups (Tanaka et al., 1994; Knudsen et al., 1994). C3G is a 145-150 kDa protein consisting of a central domain containing four proline-rich Crk SH3 binding sites and a carboxy-terminal domain with homology to Ras-family GEFs. Sos and C3G thus provide a possible link to the Ras pathway. These three proteins also bind the v-Crk SH3 domain.

The effects of v-Crk on the PC12 system have also been examined.

Overexpression of a v-Crk cDNA potentiated NGF-induced neurite outgrowth, and switched the EGF stimulus from a growth-promoting to a differentiation-promoting signal (Hempstead et al., 1994). The v-Crk SH2 can associate with tyrosine-phosphorylated EGFR or TrkA, the NGF receptor, and v-Crk itself is tyrosine phosphorylated upon EGF or NGF stimulation. On the other hand, microinjection of v-Crk protein can induce neurite outgrowth in PC12 cells in a Ras-dependent manner without any additional stimuli (Tanaka et al., 1993). Both the SH2 and SH3 are required for this phenotype, and mutations in these domains cause v-Crk overexpression to inhibit NGF-induced activation of Ras, indicating an intersection between v-Crk signalling and Ras activation in these systems (Matsuda et al., 1994).

Although v-Crk can single-handedly transform CEF, it is not sufficient for transformation of 3Y1 cells when expressed at medium levels without additional cellular mutations. However, v-Crk and c-Src can cooperate to efficiently transform 3Y1 cells. This transformation is accompanied by elevation of c-Src kinase activity, and is suppressed by Csk, which concomitantly decreases the specific activity of c-Src (Sabe et al., 1992). A possible mechanism for this cooperation involves SH2-mediated binding of c-Src and Csk to paxillin, maintaining c-Src Tyr 527 phosphorylation. *In vitro*, Crk is capable of binding the same sites on paxillin as Csk, so overexpression of v-Crk could replace paxillin-bound Csk with v-Crk, allowing dephosphorylation of c-Src Tyr 527 and activation of c-Src activity (H. Sabe, unpublished data).

Although little is known about physiological upstream activators of c-Crk, the identification and characterization of Crk binding proteins provide some clues to downstream signalling events which v-Crk may initiate. However, the

connection of v-Crk to signal transduction pathways leading to induction of DNA synthesis in the nucleus has not been studied. It was therefore undertaken to examine signalling pathways activated in v-Crk transformed cells, and to identify pathways necessary for v-Crk transformation. Because v-Crk transformation results in an increase in tyrosine phosphorylation of specific cellular proteins, the pathways utilized by v-Crk will be studied in comparison to those utilized by v-Src and activated growth factor receptors.

MATERIALS AND METHODS

DNA construction

Plasmid M17 (Feig and Cooper, 1988) carrying dominant negative Ras N17 (a gift from G. Cooper) was digested with BamHI and PvuII, and a 1.2 kb insert containing the 489 bp coding sequence surrounded by 173 5' untranslated bp and 539 3' untranslated bp was isolated. Both ends of the 1.2 kb insert were blunted, and the fragment was placed into the SnaBI site of the puromycin resistance vector pBabe puro.

Plasmid pAAU-HRY57 (Jung et al., 1994) carrying dominant negative Ras Y57 (a gift from M. Wigler) was digested with BamHI and EcoRI and the 700 bp insert was blunted and placed into the SnaBI site of the puromycin resistance vector pBabe puro. Both orientations were isolated.

Plasmid pUC101a (Trahey et al., 1988) carrying the human GAP gene (a gift from F. McCormick) was digested with EcoRI and the 3.2 kb insert was placed into the EcoRI site of the puromycin resistance vector pBabe puro.

Plasmid pVC3G3 (Tanaka et al., 1994) carrying C3G coding sequences (a gift from M. Matsuda) was digested with EcoRI and BamHI, and the 2.1 kb fragment E2.1 was isolated. E2.1, containing three of the Crk binding sites and all downstream sequences, was blunted and inserted into vector pET 21c+ digested with BamHI and blunted. This resulted in the simultaneous provision of an in-frame ATG start codon (frame confirmed by sequencing) and N-terminal fusion to an eleven amino acid T7 tag. The T7E2.1 insert was cut back out of pET with NdeI and SalI and blunted. Blunted T7E2.1 was inserted into the SnaBI site of puromycin vector pBabe puro. The T7E2.1 fragment was also

digested with *Apal* to create the 800 bp T7E.8A, containing the last three Crk binding sites but no GEF sequences. T7E.8A was blunted and inserted into the *SnaBI* site of puromycin vector pBabe puro.

pCEP4-MKP-1, a hygromycin resistance vector carrying the MKP-1 (Sun et al., 1993) coding sequences attached to a myc tag (a gift from N. Tonks) was transfected without further manipulation.

Orientations were confirmed by restriction analysis. Large-scale plasmid preps for transfection were done with Qiagen columns, and DNA was sterilized by ethanol precipitation prior to transfection. All sequencing was done with the Sequenase sequencing kit.

Cell Culture

Chicken embryo fibroblasts (CEF) prepared from eleven day old embryos as previously described (Hanafusa, 1969) were cultured in F-10 medium supplemented with 10% tryptose phosphate broth (TPB), 5% calf serum, and 1% chicken serum. Secondary CEF were plated at 8×10^5 cells per 6 cm plate, and infected with virus 4-6 hours later, facilitated by addition of 5 μ g/ml DEAE-dextran. After infection, cells were overlaid with Scherer's soft agar (Scherer's medium containing 0.375% agar) and fed periodically with Scherer's medium supplemented with 10% TPB and 5% calf serum.

NIH-3T3 cells obtained from ATCC were maintained in a subconfluent state in DEM supplemented with 10% calf serum. v-Raf 3T3 (Yu et al., 1993) were a gift from R. Jove. 3Y1 cells were grown in DEM supplemented with 5% calf serum. Cos cells were grown in DEM containing 10% fetal calf serum. All cell culture media contained 100 U/ml penicillin, 50 μ g/ml streptomycin, and 1

µg/ml fungizone. Cells were incubated at 37 °C.

CEF and NIH-3T3 were placed in low-serum medium containing 0.5% calf serum 16-24 hours prior to serum stimulation with medium containing 10% calf serum. Cells were similarly incubated in low-serum medium prior to stimulation with 100 ng/ml TPA or 10 ng/ml TGFα. After stimulation, cells were placed immediately on ice and lysed.

B581 (Garcia et al., 1993) was obtained from the Eisai Research Institute, made up as a 100x stock in DMSO, and used at a final concentration of 0.1 mM.

Transfection

5x10⁶ Cos cells were plated on 6 cm plates 24 hours prior to transient transfection with 2 µg DNA and 1 µg/ml DEAE-dextran. Cells were incubated with the transfection mix for 30 min, treated with 50 µM chloroquine for four hours, then fed with complete medium.

NIH-3T3 cells were stably transfected by the calcium phosphate method, using a 5 Prime -> 3 Prime transfection kit. Briefly, 1x10⁵ cells were plated on 10 cm plates 16-20 hours prior to transfection with 0.5-2 µg construct DNA and 40 µg salmon sperm carrier DNA. Cells were incubated 4 hours with the transfection mix, then glycerol shocked for 5 min and fed with complete medium. Two days after transfection, cells were placed under drug selection (400 µg/ml G418 or 2 µg/ml puromycin) and cultured for approximately 2 weeks until individual drug-resistant colonies could be identified. Colonies were trypsinized with the aid of cloning cylinders and replated in separate plates for further analysis. Pooled transfectants were grown to confluence, then split into

2-3 plates for further analysis.

Colony formation assays

Infected CEF were assayed for colony formation in soft agar by suspending approximately 10^6 cells in Minimal Essential Medium (MEM) supplemented with 10% TPB, 10% calf serum, 1% chicken serum, 1% DMSO, antibiotics, and 0.4% agar. The suspension was plated on a layer of bottom agar (MEM with the same supplements and 0.7% agar) in 10 cm plates. CEF colony plates were incubated at 40 °C for 2-4 weeks.

Transfected NIH-3T3 were assayed for colony formation by suspending 10^4 - 10^6 cells in DEM containing 10% fetal calf serum, antibiotics, and 0.4% noble agar. The suspension was plated on a layer of bottom agar (DEM containing 10% calf serum, antibiotics, and 0.5% bacto-agar) in 10 cm plates. NIH-3T3 colony plates were incubated at 37 °C for 3-5 weeks. Anchorage-independent NIH-3T3 colonies were picked from the soft agar for further analysis by removal directly into a syringe containing 0.15% trypsin in phosphate-buffered saline (PBS).

Thymidine incorporation

Cells were analyzed for thymidine incorporation by labelling with medium containing 20 $\mu\text{Ci/ml}$ ^3H thymidine for 30 min. Unincorporated isotope was washed away in PBS, and the cells were treated with cold 5% TCA, washed with ethanol, and lysed in 0.1 M NaOH. The base was neutralized with HCl and aliquots were counted in the scintillation counter. Counts were normalized for protein content.

Cell lysis

For most experiments, cells were placed on ice, washed 2x with cold PBS (Tris-Glu for CEF), and lysed directly on the plate in a buffer containing 10 mM Tris (pH 7.6), 1% Triton, 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 1 mM EGTA, 1 mM sodium vanadate, 1 mM PMSF, 1% trasylol, and 0.1 mM sodium molybdate. Lysates were scraped off the plates and clarified by spinning in microfuge tubes for 5 min at 4 °C. Bradford protein assays were performed using the Bio-Rad protein protein assay reagent.

Subcellular fractionation

Plates were washed 2x with cold PBS and cells were scraped off the plates in a hypotonic lysis buffer containing 5 mM Tris (pH 7.4), 2.5 mM KCl, 1 mM MgCl₂, 1 mM DTT, 1 mM PMSF, and 1% trasylol. The lysate was dounce homogenized in a 15 ml small clearance homogenizer and spun in a microfuge tube for 2 min at 3500 RPM (1000 x g). The supernatant was separated into an S100 and P100 by spinning 30 min at 36,000 RPM (10⁵ x g) in an ultracentrifuge. The pellet (P100) was solubilized in RIPA buffer containing 10 mM Tris (pH 7.4), 5 mM EDTA, 150 mM NaCl, 1% Triton, 1% Na deoxycholate, 0.1% SDS, 1 mM PMSF, and 1% trasylol.

Antibodies

Unless otherwise indicated, the following antibodies were used for all immunoprecipitations and Western blots. Mapk kinase was immunoprecipitated with polyclonal anti-Mapk 7727 (prepared in the lab), and Western blotted with monoclonal Z033 from Zymed. Anti-Crk polyclonal RF51 and anti-Gag monoclonal 3C2 were prepared in the lab. Polyclonal anti-Shc 06-203 was

obtained from UBI, as was monoclonal anti-phosphotyrosine 4G10. GAP was immunoprecipitated with the polyclonal antibody 16-153 from UBI, and Western blotted with monoclonal antibody Z030 from Zymed. The monoclonal antibody specific for the GAP-associated p190 was a gift from J. Settleman. Mouse monoclonal anti-pan-Ras Ab-3 from Oncogene Science was used to recognize endogenous and dominant negative Ras. Monoclonal anti-Src 2-17 was prepared in the lab, and polyclonal anti-Raf-1 was obtained from Santa Cruz Biotechnology. Monoclonal anti-Mek1 3D9 was obtained from Zymed, monoclonal anti-T7-Tag from Novagen, and monoclonal anti-c-Myc 9E10 from Oncogene Science.

Immunoprecipitation

Immunoprecipitation was generally done with 50-200 μ g lysate in a final volume of 300 μ l lysis buffer. Diluted lysates were incubated with the appropriate antibody at 4 °C for 1.5 hr on a nutator. Polyclonal antibody complexes were precipitated with protein A sepharose, and monoclonal antibody complexes with protein G sepharose, at 4 °C for 30 min on a nutator. The beads were washed 2x with lysis buffer prior to kinase assay or electrophoresis. In order to preserve the complex of GAP and its associated proteins, GAP immunoprecipitates (IPs) were washed in a less stringent buffer containing 20 mM Hepes (pH 7.5), 10% glycerol, 0.1% Triton, 150 mM NaCl, and 1 mM sodium vanadate.

p42 Erk2 was immunoprecipitated by diluting 250-500 μ g lysate to 200 μ l with lysis buffer, adding 10 μ l 10% SDS (final conc. 0.5%), heating at 90 °C for 3

min, and diluting with 800 µl lysis buffer (SDS final conc. 0.1%) before adding antibody (Ward et al., 1994). Denatured IPs were precipitated with protein A sepharose as described above.

Gel Electrophoresis

Proteins were separated by standard SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples were boiled 5 min in 2x sample buffer, clarified 5 min, and loaded onto a 4% stacking gel. Resolving gels were generally 8-12% polyacrylamide. Some gels were stained in 0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid for 20 min, then destained in 50% methanol and 10% acetic acid for 3-6 hours to visualize high abundance proteins.

Western Blotting

Proteins separated by SDS-PAGE were electrophoretically transferred to PVDF membranes in transfer buffer containing 20 mM Tris base, 150 mM glycine, and 20% methanol. Filters were rinsed in TBST (20 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween) and blocked for one hour in TBST containing 5% BSA. Filters were then incubated in primary antibody solution (dilutions generally of 1:4000-1:10,000 in 0.5% TBST) for 1.5 hours. Filters were washed in TBST and incubated in TBST containing 0.5% BSA and secondary antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse, or protein A (dilutions generally of 1:4000-1:10,000) for one hour. Finally, the filters were washed again in TBST and visualized by Enhanced Chemiluminescence (ECL; Amersham or NEN).

In order to visualize immunoprecipitated bands with a migration similar to

that of the immunoglobulin heavy chain, a rabbit polyclonal was usually used for one step (i.e. IP or Western) and a mouse monoclonal for the other. In cases in which this was not possible, a low dilution of primary antibody was used in the Western blot, followed by a high dilution of secondary. For example, Shc was immunoprecipitated with a rabbit polyclonal, then Western blotted with the same primary antibody diluted 1:3000, followed by a secondary HRP-anti-rabbit antibody diluted 1:20,000.

Resolution of hyperphosphorylated Mapk was achieved by running a large 10% polyacrylamide gel until the 30 kDa marker had run off.

Kinase Assays

Solution kinase assays were performed by incubating whole cell lysate or fractions with 10 μ Ci γ^{32} P-ATP and 5 μ g substrate per sample with 20 μ M cold ATP in a final volume of 20 μ l kinase buffer (30 mM Hepes pH 7.1, 10 mM MgCl_2 , 1 mM DTT) at 30 °C for 10 min. Reactions were terminated by adding 2x sample buffer containing 50 mM EDTA and boiling for 5 min. The reactions were clarified and separated by SDS-PAGE (15% polyacrylamide for most low molecular weight substrates), the radioactive dye front was cut off, and the gel was washed for one hour in fixer (10% methanol, 7% acetic acid, 10% glycerol), then dried and exposed to film. Alternatively, substrate could be visualized by Coomassie staining and cut out and counted in a scintillation counter without drying.

IPs to be analyzed by immunocomplex kinase assay were washed 2x in kinase buffer (after the initial 2 washes in lysis buffer). p44 Erk1 IPs were incubated in 20 μ l of kinase buffer (30 mM Hepes pH 7.1, 10 mM MgCl_2 , 1 mM

DTT) containing 10 μCi $\gamma^{32}\text{P}$ -ATP, 1 μl 500 μM cold ATP, 1 μl 2 μM staurosporine, and 10 μg myelin basic protein (MBP) per sample for 30 min at 30 $^{\circ}\text{C}$. Mek IPs were incubated in 30 μl kinase buffer (50 mM Tris pH 8.0, 5 mM DTT, 0.1 mg/ml ovalbumin, 3 mM Mg acetate, 10 mM NaF, 1 mM EGTA, 1 mM Na vanadate) containing 10 μCi $\gamma^{32}\text{P}$ -ATP, 2 μg GST-Erk1 K63M (Huang and Erikson, 1994; a gift from R. Erikson), and 1 μl 500 μM cold ATP per sample for 15 min at 30 $^{\circ}\text{C}$. Reactions were terminated by the addition of 2x sample buffer containing 50 mM EDTA and were boiled for 5 min. The reactions were clarified and separated by 10% SDS-PAGE as described above.

In Gel Kinase Assay (IGKA) analysis (Kameshita and Fujisawa, 1989) was performed on IPs, whole cell lysate, and fractions. Samples were boiled for 5 min in 2x sample buffer and clarified, then loaded in duplicate onto 10% mini polyacrylamide gels with or without 0.5 mg/ml MBP cast into the gel. SDS was washed out of the gel in 2 30-min washes with 20% isopropanol in 50 mM Tris (pH 8.0). The gels were equilibrated for one hour in buffer A (50 mM Tris pH 8.0, 5 mM 2-mercaptoethanol), then denatured in 2 30-min washes of 6 M guanidine hydrochloride. The gels were slowly renatured with several washes of buffer A containing 0.04% Tween-20 over a period of 16-24 hours at 4 $^{\circ}\text{C}$. The gels were then equilibrated for 30 min in kinase buffer (30 mM Hepes pH 7.4, 10 mM MgCl_2 , 10 mM MnCl_2 , 2 mM DTT, 0.5 mM Na vanadate, 5 mM β -glycerophosphate), and incubated for 1 hour at room temperature in kinase buffer containing 10 μM cold ATP and 100 μCi $\gamma^{32}\text{P}$ -ATP. The unincorporated isotope was removed by several washes at room temperature in 5% TCA/1% Na pyrophosphate over a period of 16-24 hours, and the gels were dried and

exposed to film.

Ion exchange column chromatography

Overlaid CEF, or NIH-3T3 placed in media containing 0.5% calf serum for 16-24 hours prior to lysis, were lysed in a low-salt buffer containing 20 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton, 10% glycerol, 1 mM Na vanadate, 0.1 mM Na molybdate, 5 mM β -glycerophosphate, 25 mM NaF, 20 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF, 1 mM DTT, and 25 mM NaCl.

Lysates were separated by FPLC over an anion-exchange Mono-Q column (Pharmacia). In general, 2 mg lysate diluted to 2 ml in low-salt lysis buffer was injected onto the column and eluted in 0.5 ml fractions. In the experiment shown in Figure 6, the proteins were eluted in a salt gradient from 25 mM to 610 mM over 60 fractions, with the gradient beginning at fraction 20. In subsequent experiments, proteins were eluted in a salt gradient from 25 mM to 512 mM over 64 fractions, with the gradient beginning at fraction 24.

Gel filtration column chromatography

200 μ l of CT10 Mono-Q fraction 34 was applied to a Superose 12 gel filtration column in a buffer similar to that described above for ion exchange column chromatography, containing 150 mM NaCl. Proteins were eluted in the same buffer, and 80 fractions of 250 μ l each were collected. Marker proteins were subsequently run. Blue dextran (2×10^6 kDa) eluted in fractions 27-29, BSA (66 kDa) in 36-38, ovalbumin (45 kDa) in 41-43, and cytochrome C (14 kDa) in 48-50.

Phosphoamino acid analysis

Solution kinase assays were performed on the designated fractions as described above. Coomassie-stained MBP bands were cut out of the dried gel and rehydrated in 10% methanol, washed 5x15 min in 10% methanol, and washed 10 min in 50 mM ammonium bicarbonate. The gel slices were cut into small pieces and lyophilized, then rehydrated in 50 mM ammonium bicarbonate and treated with TPCK-treated trypsin overnight at 37 °C. The digested samples were spun in a microfuge, and the supernatant was lyophilized and washed 3x in dH₂O.

Washed, lyophilized pellets were dissolved in 50 µl 6 M HCl and sealed in 100 µl capillary tubes, then boiled for 90 min in a water bath. The hydrolysates were ejected into microfuge tubes and washed 3x in dH₂O. Pellets were resuspended in 10 µl dH₂O containing 0.5 mg/ml each phospho- serine, threonine, and tyrosine. Samples and a marker dye mix, containing 0.01% xylene cyanol FF and acid fuchsin red, were spotted onto a cellulose thin layer chromatography plate (Merck #5716) and electrophoresed for 90 min at 1000 V in acetic acid containing 8% pyridine and 8% dH₂O. The plate was dried, sprayed with ninhydrin solution, baked for 5 min at 110 °C, and exposed to film.

Immunofluorescence

Cells were fixed with 3% formaldehyde in PBS for 30 min at room temperature, then washed 2x with PBS. Cells were permeabilized in 0.2% Triton for 5 min, washed 2x with PBS, and incubated with 50 mM glycine in PBS for 10 min. The cells were then washed 2x with PBS containing 0.2% gelatin (PBS-gel). Cells were incubated with primary antibody diluted in PBS-gel for

30-60 min, then washed 4x5 min with PBS-gel. Cells were incubated with secondary antibody conjugated to fluorescein isothiocyanate (Jackson Immunologicals) diluted in PBS-gel for 30-60 min, then washed 4x with PBS-gel and embedded in the polyvinyl alcohol Mowiol/PPD. F-actin was stained with phalloidin conjugated to tetra-rhodamine-isothiocyanate (Sigma).

RESULTS

Activation of Mapk in CT10-transformed CEF

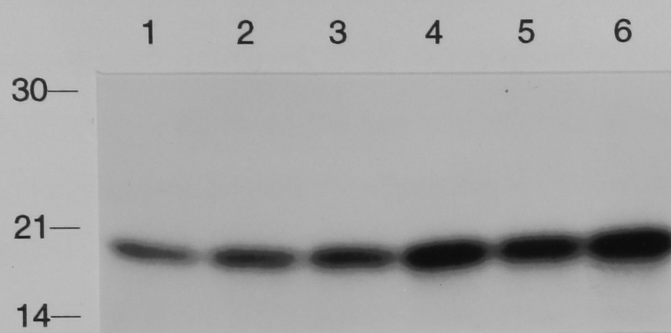
Because serine/threonine kinase cascades play such a prominent role in signal transduction from the activated EGFR and from v-Src, the first approach to examining signalling pathways activated by v-Crk was to search for kinases that may be activated in CT10-transformed CEF. CEF were infected with the CT10 virus and grown under a soft agar overlay for about ten days, at which time the cells displayed the characteristic transformed morphology and high saturation density. The overlay selects for infected, transformed cells, probably by effectively lowering the concentration of available serum. Cells infected with the nontransforming avian retrovirus UR2AV served as a control.

Lysates prepared from UR2AV- and CT10-infected CEF were normalized for protein and incubated with γ - ^{32}P ATP and myelin basic protein (MBP) or casein, two common serine/threonine kinase substrates. Three different amounts of each lysate were used to verify linearity. The reaction products were separated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and the gels were washed to remove unincorporated isotope and exposed to film. As shown in Figure 5, an approximately twofold increase in phosphorylation of both substrates by the CT10 lysate was observed. The MBP kinase activity was chosen for further characterization.

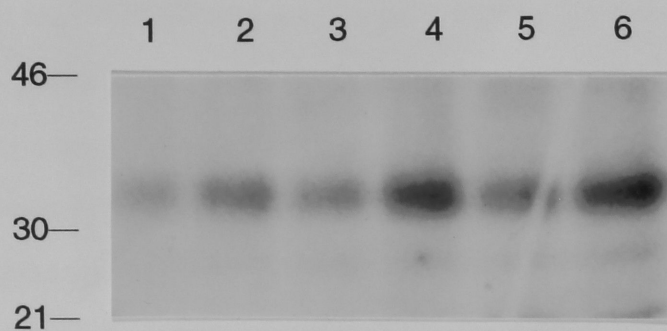
It was possible that the increase in MBP kinase activity was partially masked by a constitutively highly active kinase present in both lysates, or by the presence of an inhibitor. UR2AV and CT10 lysates were therefore fractionated over a Mono-Q ion exchange column. The MBP kinase activity in each fraction

Figure 5. Increase in MBP (A) and casein (B) kinase activity in CT10-transformed CEF. Solution-phase kinase assays with 5 μ g substrate, 10 minute reaction at 30 °C. In both A and B, 0.25 μ g lysate were used in lanes 1 and 2, 0.5 μ g in lanes 3 and 4, and 1 μ g in lanes 5 and 6. UR2AV lysate was used in lanes 1,3, and 5; CT10 in lanes 2, 4, and 6.

A



B



was assayed by solution kinase assays similar to those performed on the whole cell lysates. The results are shown in the upper panel of Figure 6. Several CT10 fractions contained elevated MBP kinase activity, the increase in which was greater than that seen in the whole cell lysate kinase assays. However, corresponding fractions in the two lysates may not completely correlate, as the excess of negative charge on tyrosine phosphorylated proteins, as well as general changes in transcription and translation patterns in the CT10 lysate could affect the relative elution patterns of all proteins in this lysate.

The largest difference between the two lysates was in the region of fractions 34-35. CT10 fraction 34 was therefore run on a Superose 12 gel filtration column to establish the approximate molecular weight of the kinase or kinases in the fraction. Solution MBP kinase assays were performed on the Superose 12 fractions, and the peak of kinase activity eluted in fractions 41 and 42, while the 45 kDa ovalbumin eluted in fractions 41-43 (data not shown). Peak fraction 41 was used to assay substrates in yet another solution kinase assay. As shown in Figure 7A, both MBP and Histone 2B (H2B) were efficiently phosphorylated by this fraction, while Histone 1 (H1) was phosphorylated to a lesser extent, and Histones 2A and 4 were only slightly phosphorylated. Casein was not phosphorylated at all by the peak Superose 12 fraction, indicating that the kinases responsible for the increase seen in Figure 4B are not identical to the kinases responsible for the MBP phosphorylation by this fraction.

MBP phosphorylated by fractions 34 and 40 of both lysates was subjected to phosphoamino acid analysis, shown in Figure 7B. Not enough activity was present in UR2AV fraction 34 for analysis, but CT10 fraction 34 clearly phosphorylated MBP on both serine and threonine. No tyrosine phosphorylation was observed. On the other hand, fraction 40 of both lysates

Figure 6. Activated Mapk is found in CT10 fractions containing elevated MBP kinase activity. Upper panel, solution-phase kinase assay on 1 μ l each Mono-Q fraction with 5 μ g MBP, 10 min reaction at 30 °C. Lower panel, Western blot of UR2AV and CT10 Mono-Q fractions (30 μ l each) probed with anti-Mapk.

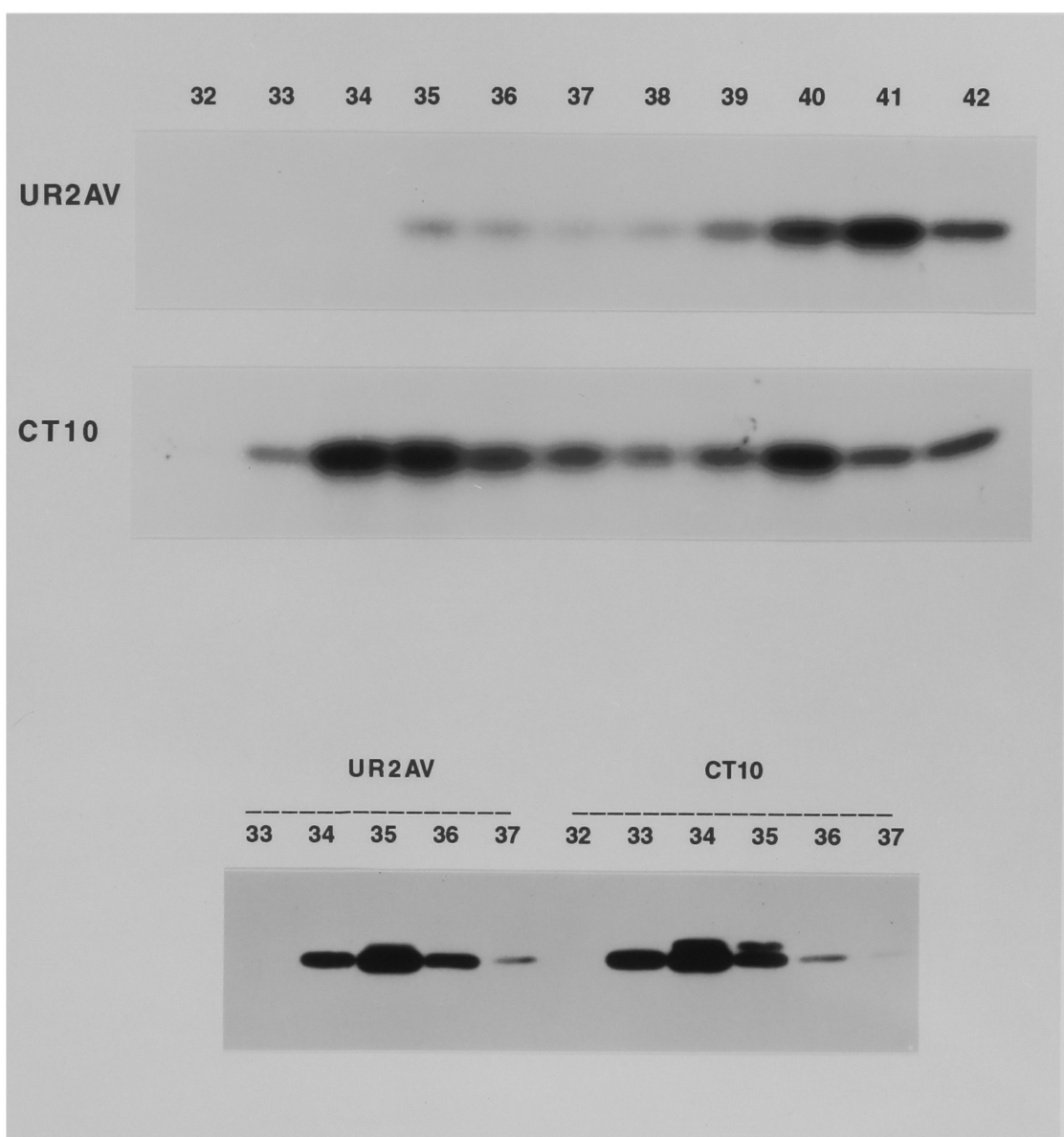
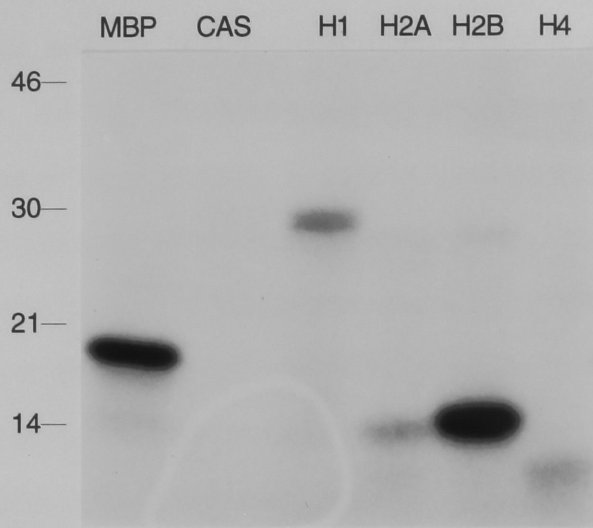
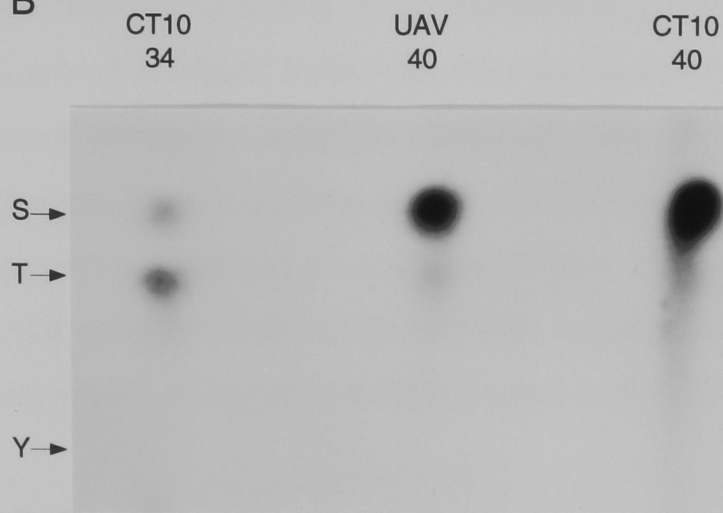


Figure 7. The v-Crk-activated MBP kinase activity preferentially phosphorylates MBP and H2B. A, solution-phase kinase assay of Superose 12 fraction 41 on 5 μ g of various substrates, 10 minute reaction at 30 °C. MBP, casein, and histones H1, H2A, H2B, and H4. B, phosphoamino acid analysis on 5 μ g MBP phosphorylated by 1 μ l each Mono-Q fraction, 10 min reaction at 30 °C.

A



B



phosphorylated MBP predominantly on serine, with only a trace of activity towards threonine, and no activity towards tyrosine.

Information concerning the molecular weight, substrate specificity, and amino acid specificity of the kinases in CT10 Mono-Q fraction 34 suggested the involvement of Mapk, a 42-44 kDa serine/threonine kinase with a preference for MBP (Boulton et al., 1991a). The fractions were therefore analyzed by Western blot for the presence of Mapk protein. Fractions were separated by SDS-PAGE, Western blotted, and probed with a monoclonal antibody specific for Mapk. CEF express only one isoform of Mapk, immunologically related to p42 Erk2, but which migrates at 43 kDa on SDS-PAGE (Sanghera et al., 1992 and data not shown). When activated, the protein becomes hyperphosphorylated on threonine and tyrosine, and its migration is further slowed.

As shown in the bottom panel of Figure 6, Mapk protein is found in fractions 34-37 of UR2AV, and fractions 33-37 of CT10. Furthermore, hyperphosphorylated Mapk elutes in CT10 fractions 34 and 35, which correlate with the peak of MBP kinase activity. A tiny fraction of Mapk may be hyperphosphorylated in UR2AV fraction 35; this may account for the subtle increase in kinase activity of this fraction compared to the neighboring fractions. It must be noted that Mapk is probably not the only active kinase in these fractions, because the kinases in the Superose 12 peak fraction, derived from CT10 fraction 34, were able to phosphorylate H2B, a substrate which Mapk does not phosphorylate to any great extent (Boulton et al., 1991b and data not shown).

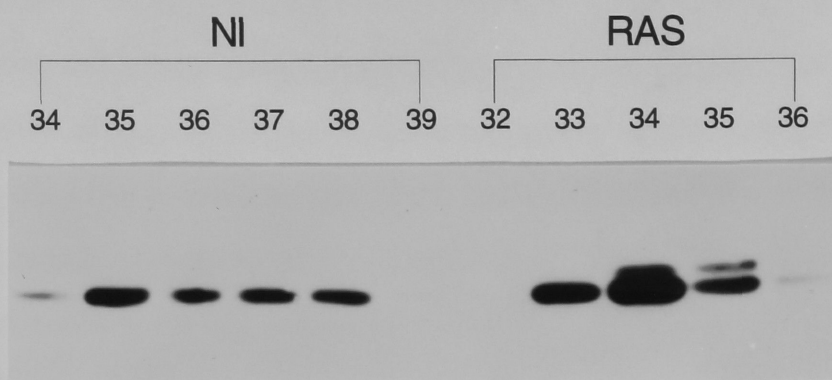
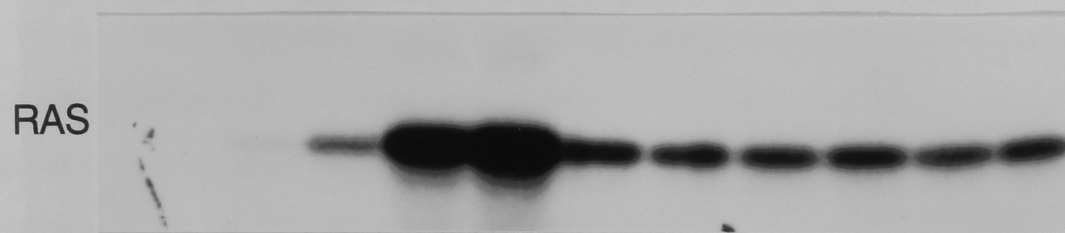
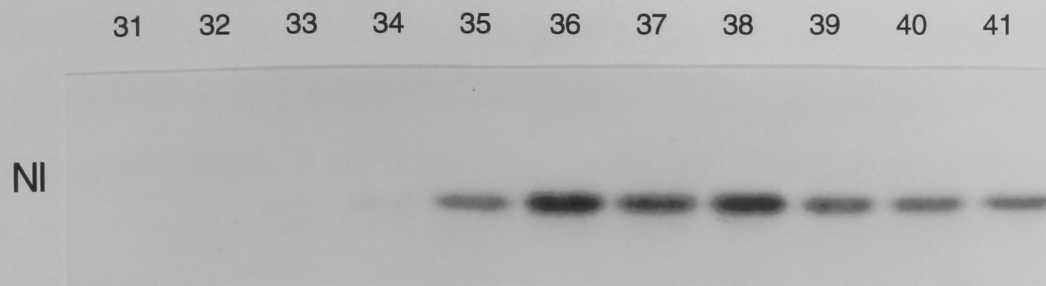
It can also be inferred from this figure that the elution profile of specific proteins may peak one fraction later in UR2AV lysates than in CT10 lysates, possibly due to the reasons outlined above. Further analysis of the MBP kinase

activity peaks in UR2AV fraction 41 and CT10 fraction 40 was therefore not attempted, due to the high probability that these activities represented the same kinases, with no significant activation in CT10-transformed lysates.

Because Mapk was implicated in the fractionation experiments, the behavior of MBP kinase activity in CEF lysates transformed by v-Ras, an upstream activator of Mapk, was also examined. Lysates from uninfected ("NI") or v-Ras infected CEF were separated over the Mono-Q column, and solution MBP kinase assays were performed on the resulting fractions. The data are shown in the top panel of Figure 8. Similar to the results obtained from Crk-transformed CEF, elevated MBP kinase activity can be seen in fractions 34 and 35 of the Ras lysates, although these fractions phosphorylated the MBP to a greater extent than the CT10 lysates. This corresponds to the presence of hyperphosphorylated Mapk, as assayed by Western blot analysis, shown in the bottom panel of Figure 8. Mapk is expected to be activated in these lysates, since Ras most likely sends many of its growth promoting signals through Mapk (Macdonald et al., 1993). A similar experiment was performed on serum-stimulated lysates, and again the increase in MBP phosphorylation, even greater than that observed for Ras-transformed cells, correlated with the presence of hyperphosphorylated Mapk (data not shown).

A correlation between Mapk activation and elevated MBP kinase activity in CT10-transformed CEF was therefore established. It was next undertaken to definitively demonstrate activation of Mapk in CT10-transformed CEF, most easily accomplished by precipitating Mapk with a specific antibody prior to assaying kinase activity. However, no antibody was available to precipitate p42 Erk2 in its native form. An assay was therefore devised utilizing an antibody that could precipitate p42 under denaturing conditions, similar to that used in

Figure 8. Activated Mapk is also found in v-Ras fractions with elevated MBP kinase activity. Upper panel, solution-phase MBP kinase assay on Mono-Q fractions (NI, uninfected CEF) with 5 μ g MBP, 10 min reactions at 30 °C. To compensate for an error in normalization of protein run over the column, 2.5 μ l each NI fraction and 1.8 μ l each v-Ras fraction were assayed. Lower panel, Western blot of NI and v-Ras Mono-Q fractions (30 μ l and 22 μ l, respectively) probed with anti-Mapk.



(Ward et al., 1994).

Figure 9A shows a Western blot of Mapk immunoprecipitated from native and denatured lysates. The lysates run in lanes 2 and 4 were pretreated with 0.5% SDS, which was then diluted to 0.1% before the precipitating antibody was added. Lanes 1 and 3 were not denatured. The immunoprecipitates (IPs) were separated by SDS-PAGE, Western blotted, and probed with a monoclonal antibody against Mapk. A Mapk band comigrating with that in whole cell lysate (lane 5) was seen only in the IPs from the denatured lysates (lanes 2 and 4).

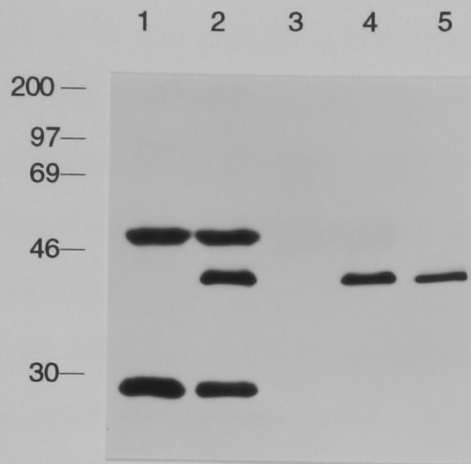
Because a kinase assay cannot be performed with denatured protein, a method for assaying renatured proteins in the gel was used. After precipitation of Mapk from denatured lysates, the IPs are separated by SDS-PAGE on a gel containing MBP. The entire gel is then denatured in guanidine hydrochloride, and slowly renatured in a Tween-containing buffer. An In Gel Kinase Assay (IGKA) is performed on the renatured gel, in which the MBP cast into the gel is phosphorylated by any renatured, activated kinases, visualized as bands comigrating with the responsible kinases.

Several oncogene-transformed lysates were thus subjected to Mapk immunoprecipitation followed by IGKA. As shown in Figure 9B, lysates from v-Ras and v-Src transformed cells contained the expected high levels of activated Mapk, while the level of Mapk activity precipitated from v-Crk transformed cells was slightly lower, but still elevated compared to uninfected CEF or CEF infected with either of the nontransforming retroviruses (PNR200 and UR2AV). The phosphorylated bands were cut out of the gel and counted in a scintillation counter. Mapk activity was elevated about eight-fold in the v-Ras and v-Src lysates, and three-fold in the v-Crk lysates.

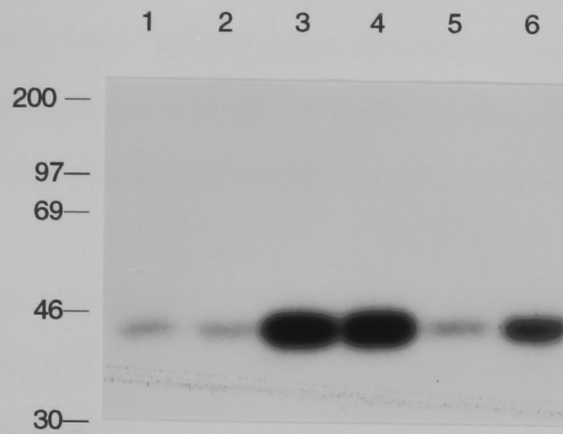
A comparison of the activation of Mapk in CT10 transformed CEF and

Figure 9. Mapk activity is elevated in CT10-transformed CEF. A, anti-Mapk Western blot of Mapk immunoprecipitates from 500 μ g uninfected CEF lysate. Lanes 1 and 2, IP with monoclonal anti-Mapk Z033. Lanes 3 and 4, IP with polyclonal anti-Mapk 7727. Lane 5, 50 μ g whole cell lysate. Lanes 2 and 4 were denatured in 0.5% SDS. The IP immunoglobulin heavy and light chains can be seen in lanes 1 and 2 because a mouse monoclonal antibody was used for both IP and Western. B, in gel MBP kinase assay. 140 μ g of each lysate was denatured in 0.5% SDS and immunoprecipitated with anti-Mapk 7727. Lane 1: NI, 2: PNR200, 3: v-Ras, 4: v-Src, 5: UR2AV, 6: CT10. C, in gel MBP kinase assay. 50 μ g each lysate was denatured in 0.5% SDS and immunoprecipitated with anti-Mapk 7727. Lanes 1 and 2: UR2AV, 3-5: CT10. Lanes 1 and 3: no stimulation, 2 and 4: 10% serum stimulation for 5 minutes, 5: 10% serum stimulation for 20 min.

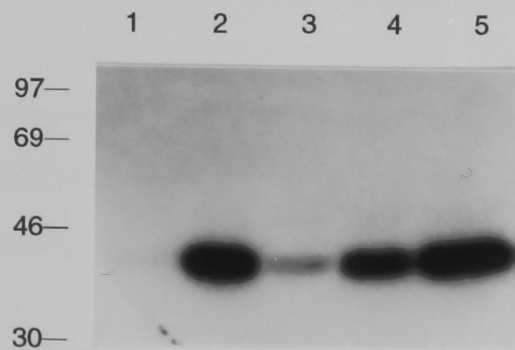
A



B



C

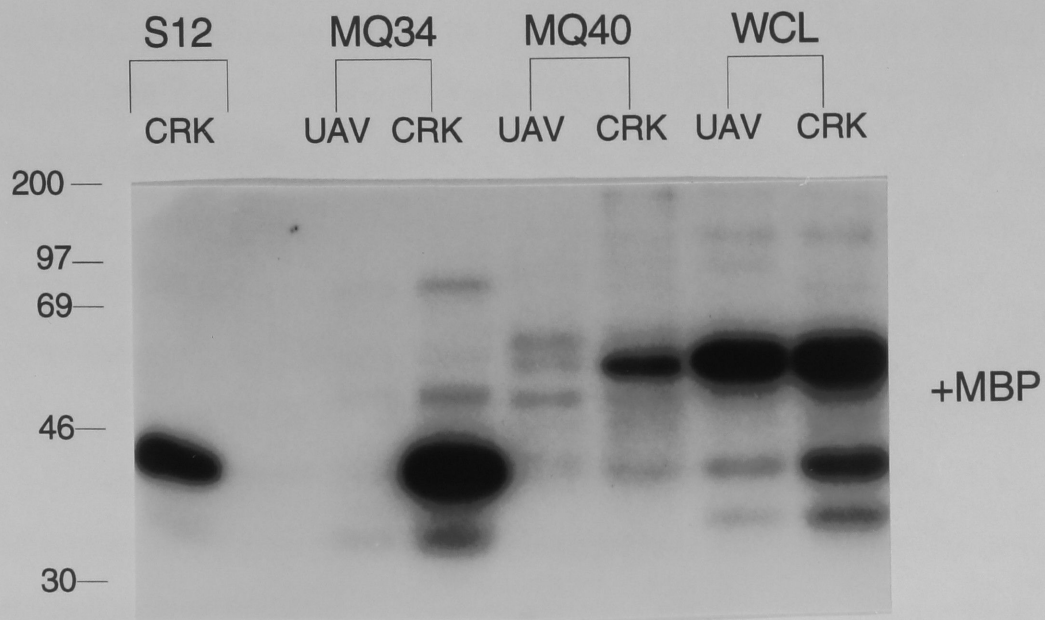
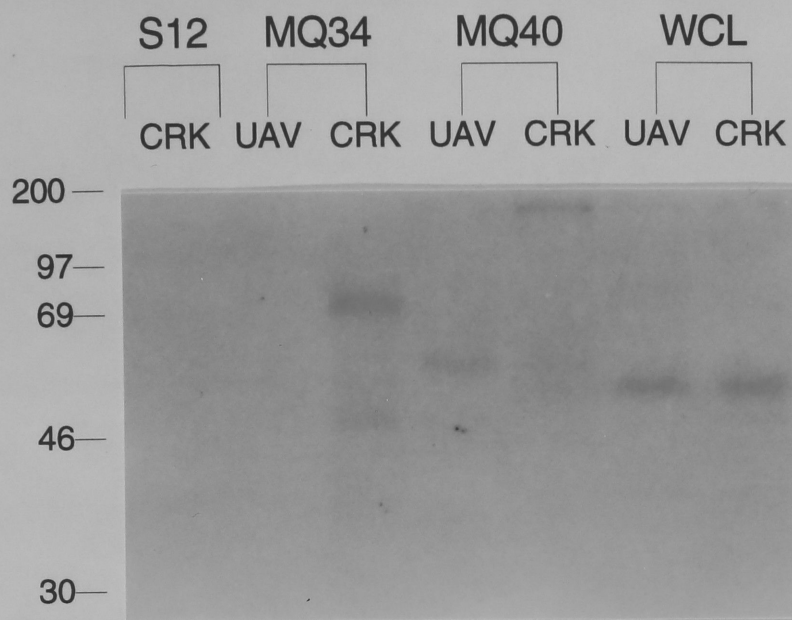


serum stimulated CEF was then done. Mapk was immunoprecipitated from lysates from UR2AV- and CT10- infected cells that had been placed in medium containing 0.5% calf serum for one day, then stimulated with 10% serum for 0, 5, or 20 minutes. An IGKA was performed on the precipitates. As shown in Figure 9C, serum stimulation elevates Mapk activity to a far greater extent than v-Crk transformation, indicative of an immediate response, as opposed to a situation of chronic growth stimulation.

The extent of Mapk activation in CT10-transformed CEF depends on the serum conditions under which the cells are grown. A difference in Mapk activity is observed in cells maintained under soft agar overlay and cells transferred to medium containing only 0.5% calf serum one day before lysis. However, cells maintained in standard growth medium containing 5% calf serum do not exhibit as great a difference in Mapk activity when transformed by v-Crk; the continuous presence of high concentrations of serum seems to suppress the constitutive elevation of Mapk observed in CEF under low serum conditions (data not shown).

IGKA analysis was also used to examine proteins present in the Mono-Q fractions from UR2AV and CT10 transformed lysates. Figure 10 shows IGKA analysis of whole cell lysates (WCL), Mono-Q fractions (MQ34 and MQ40), and Superose 12 fraction 41 (S12), derived from CT10 Mono-Q fraction 34. The upper gel contains no MBP, and therefore visualizes only autophosphorylated bands, while the lower gel contains MBP and indicates the presence of kinases capable of phosphorylating this exogenous substrate. No bands were significantly autophosphorylated under these conditions, but many MBP kinases were present in the whole cell lysates of UR2AV and CT10 (last two lanes). Bands with elevated activity of about 43 kDa and 35 kDa were observed

Figure 10. Many MBP kinases are present in the UR2AV and CT10 (v-Crk) Mono-Q fractions. Upper panel, autophosphorylation (no MBP in gel). Lower panel, MBP added to gel. In both gels, S12: Superose 12 fraction 41, MQ34: UR2AV or CT10 Mono-Q fraction 34, MQ40: UR2AV or CT10 Mono-Q fraction 40, WCL: UR2AV or CT10 whole cell lysate. Assay was performed on 50 μ l each fraction and 5 μ g whole cell lysate.



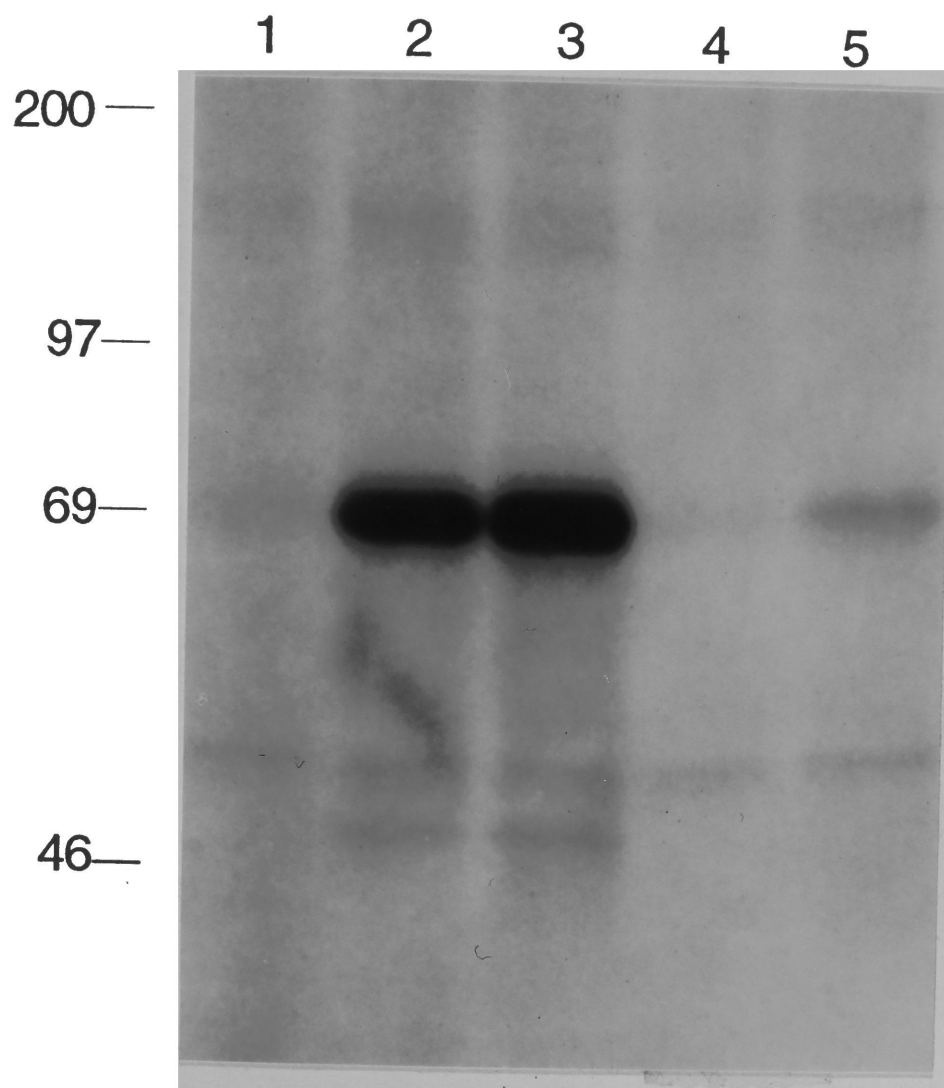
in the CT10 whole cell lysate lane. The 43 kDa band is a good candidate for Mapk, but the identity of the 35 kDa band is not known. A 65 kDa band appeared with equally high intensity in both lysates, potentially capable of masking the increases in the lower molecular weight bands from linear detection in whole cell lysate kinase assays, such as that shown in Figure 5A.

Mono-Q fractionation enriched the activity of this 43 kDa putative Mapk in CT10 fraction 34 relative to other activities remaining in the fraction (Figure 10, CRK MQ34), and Superose 12 fractionation of this fraction eliminated still more of the contaminating kinase activity. However, low levels of the 35 kDa kinase remained in the peak Superose 12 fraction, as well as CT10 Mono-Q fraction 34, possibly contributing to any observed elevation of MBP kinase activity in these fractions. Some activity comigrating with the whole cell lysate 65 kDa kinase was present in UR2AV and CT10 Mono-Q fraction 40; this may account for the serine kinase activity shown in Figure 7B for this fraction.

The activity of the upstream activator of Mapk, Mek, was then examined. Mek1 was immunoprecipitated from lysates of cells transformed with various oncogenes and maintained under overlay, and an immune-complex kinase assay was carried out on the Mek IPs, using the kinase-inactive GST-Erk1 K63M (a gift from R. Erikson) as an exogenous substrate (Huang and Erikson, 1994). The results, shown in Figure 11, indicate that while v-Ras and v-Src transformed CEF contain highly elevated levels of Mek activity, v-Crk transformation only slightly elevates Mek activity (about two-fold), as visualized by phosphorylation of the 70 kDa GST-Erk1 K63M.

The Ras pathway is therefore activated to some extent in v-Crk transformed CEF. Since Ras pathway activation is accompanied by tyrosine phosphorylation of Shc and the GAP-associated p190 in v-Src transformed

Figure 11. Mek immune-complex kinase assay. 75 µg each lysate was immunoprecipitated with anti-Mek-1 and assayed 15 minutes at 30 °C using 3 µg of the 70 kDa GST-Erk1-K63M as an exogenous substrate. Lane 1: PNR200, 2: v-Ras, 3: v-Src, 4: UR2AV, 5: CT10.



cells and serum stimulated cells (Pelicci et al., 1992; Ellis et al., 1990; McGlade et al., 1992b), the phosphorylation states of these proteins in v-Crk transformed CEF were examined next.

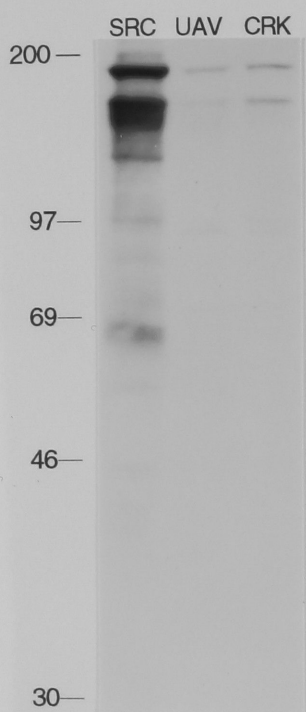
Ras GAP was immunoprecipitated from v-Src, UR2AV, and v-Crk CEF lysates. These IPs were run on SDS-PAGE and Western blotted with anti-phosphotyrosine (Figure 12A). A p190 band, as well as a broad band running around 150 kDa, were heavily phosphorylated on tyrosine in v-Src transformed CEF. However, the comigrating bands in the UR2AV and CT10 lysates only faintly reacted with the antiphosphotyrosine antibody, indicating that phosphorylation of the GAP-associated p190 does not accompany v-Crk transformation. The tyrosine-phosphorylated band migrating around 65 kDa in the v-Src lane may be the GAP-associated p62.

When the filter was stripped and reprobed with anti-GAP, three bands of equal intensity appeared at 120 kDa in all three lanes, shown in Figure 12B, indicating that the immunoprecipitation was equally efficient for all three lysates. The filter was stripped once more and reprobed with anti-p190 (a gift from J. Settleman), which showed that only two-fold more p190 was precipitated with GAP in the v-Src lysates (see Figure 12C), indicating that the level of phosphorylation of p190 is much more than two-fold greater in v-Src transformed CEF. Figure 12C also provides evidence that the broad tyrosine-phosphorylated band around 150 kDa in the v-Src transformed lysate is either a breakdown product of p190, or an antigenically-related protein.

A similar analysis was performed for Shc. Shc was immunoprecipitated from v-Src, UR2AV, and CT10 transformed lysates, and the IPs were separated by SDS-PAGE and assayed for the presence of tyrosine-phosphorylated proteins by Western blot analysis. Figure 13A shows that Shc isoforms p46,

Figure 12. Ras GAP and its associated p190 are not tyrosine phosphorylated in v-Crk transformed CEF. Western blots of GAP immunoprecipitated with 25 μ l polyclonal anti-GAP from 1 mg lysate derived from v-Src transformed, UR2AV infected, or v-Crk transformed CEF. The same filter was probed with A, anti-phosphotyrosine; B, monoclonal anti-GAP; or C, anti-p190.

A



B



C

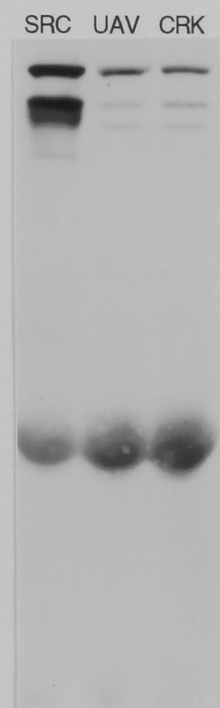
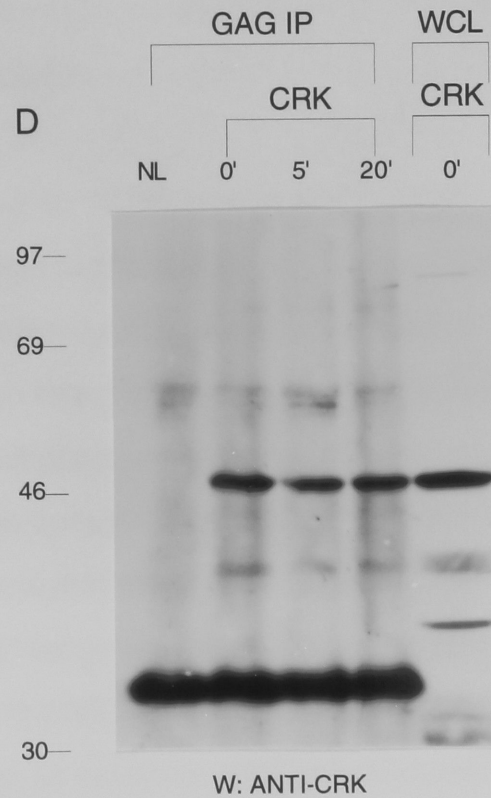
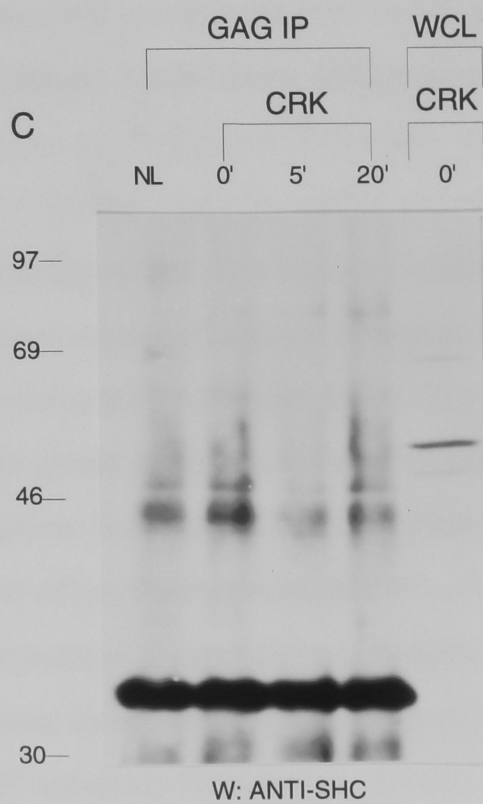
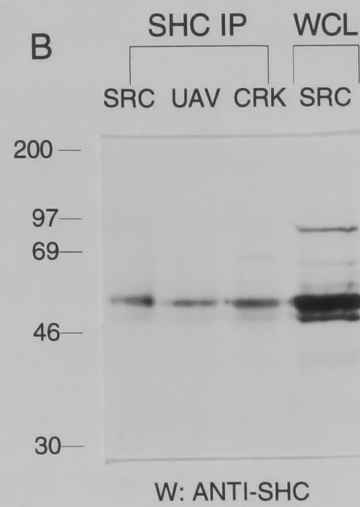
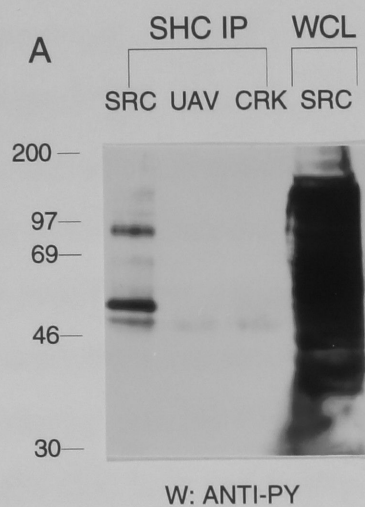


Figure 13. Shc is not tyrosine phosphorylated in v-Crk transformed CEF, nor does it associate with v-Crk after serum stimulation. A, anti-phosphotyrosine Western of Shc immunoprecipitated from 200 μ g each v-Src, UR2AV, and CT10 (v-Crk) lysate. v-Src whole cell lysate was run in the last lane. B, filter was reprobed with anti-Shc. C, anti-Shc Western blot of anti-Gag immunoprecipitates from 80 μ g lysate derived from v-Crk transformed CEF 10% serum stimulated for 0 min, 5 min, or 20 min. NL: no lysate, WCL: v-Crk whole cell lysate. D, filter was reprobed with anti-Crk.



p52, and what may be p66 are tyrosine phosphorylated in v-Src, but not UR2AV or v-Crk lysates. The Shc proteins from chicken lysates migrate somewhat more slowly than those from mammalian lysates (data not shown). Reprobing of the Western with anti-Shc indicated similar levels of Shc in all three lysates, shown in Figure 13B. The anti-Shc antibody seems to react most strongly with p52. The mechanism of activation of the Ras pathway in v-Crk transformed CEF is therefore different than the Shc-dependent mechanism utilized by v-Src.

The possible association of Shc with v-Crk after serum stimulation was then examined, because serum-induced tyrosine phosphorylation of Shc may allow it to interact with the SH2 domain of v-Crk. Anti-gag IPs (precipitating v-Crk, but not c-Crk) from serum-stimulated CT10-transformed CEF were Western blotted with anti-Shc (Figure 13C) or anti-Crk (Figure 13D). Although v-Crk was efficiently precipitated from serum-stimulated cells, no association of Shc was observed either before or after serum stimulation.

The next step was to examine if Ras function is necessary for v-Crk transformation. The easiest method by which to accomplish this is use of the dominant negative Ras. However, it is technically difficult to express two proteins (i.e. v-Crk and Ras N17) in chicken cells because the viruses carrying the genes of interest must be of different subgroups, binding to different cell surface receptors, so that infection with one does not interfere with infection of the other. Furthermore, it is difficult to ensure that every cell in a given population is expressing both genes. A "B" subgroup CT10 virus (CT10B) was made by transfecting CEF with cloned CT10 DNA, then infecting with the RAV2 "B" subgroup helper virus. CT10B was able to transform CEF and increase cellular phosphotyrosine on p70 and p130 (data not shown). Preliminary

experiments were done with CT10B and dominant negative Ras cloned into an “A” subgroup vector, but the results of these experiments were ambiguous due to apparent interference between these two viruses.

The system of study was therefore switched from CEF to the murine cell line NIH-3T3. Unlike CEF, NIH-3T3 have already undergone the process of immortalization, and can be passaged indefinitely. These cells can be transfected with two genes in vectors carrying different drug resistances, selected with these drugs to substantially increase the percentage of cells expressing both proteins, and cloned to obtain homogenous populations of cells expressing quantifiable levels of the proteins of interest.

Characterization of v-Crk NIH-3T3

v-Crk expressing cell lines were first obtained and characterized. NIH-3T3 cells were transfected with the vector pMEXneo or pMEXneo-v-Crk. Three stable cell lines transfected with the vector alone and ten stable cell lines transfected with v-Crk were obtained by G418 selection. Several of the G418-resistant v-Crk clones exhibited morphological alteration, including being more refractile and growing to higher saturation densities. The level of v-Crk expression in each cell line was quantitated by Western blot analysis, and was found to correlate with degree of transformation (data not shown).

NIH-3T3 cells are relatively genetically unstable, able to spontaneously undergo transformation when cultured for extended periods of time at high densities (Yao and Rubin, 1994). Several of the v-Crk transformed lines did appear to accumulate mutations during passaging, becoming more transformed. The vector transfected cells and a line expressing very low levels of v-Crk, “V8” (see Figure 15A for expression levels) did not undergo

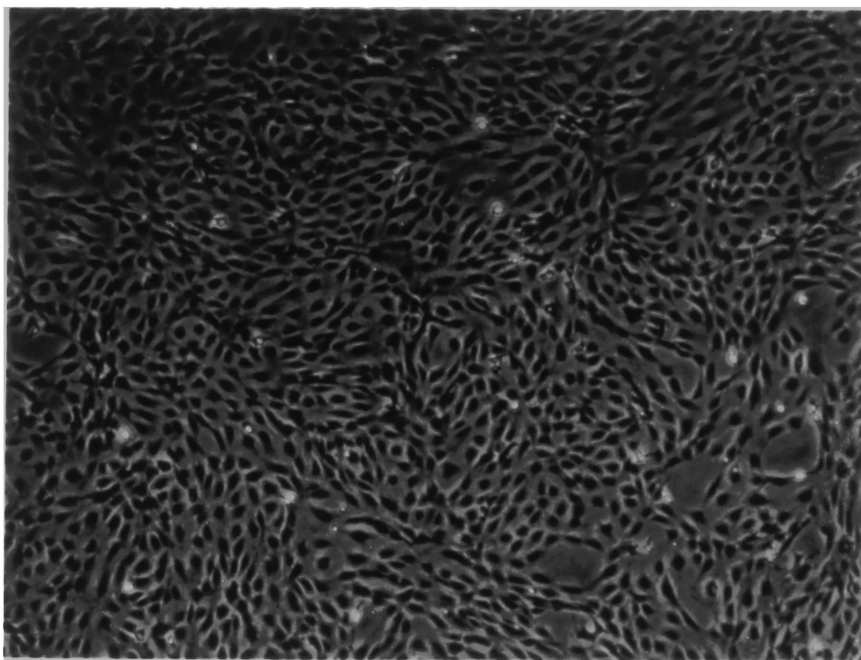
observable morphological alteration, indicating that even this extreme morphology was v-Crk dependent (data not shown). However, to minimize the effects of random mutation, a high-expressing v-Crk cell line that was very transformed without extensive passaging was chosen for further characterization. This cell line, "V4", was maintained at low densities for short periods of time (less than one month), after which a new, early passage isolate was thawed for use. The control cell line transfected with vector alone, "M2", was maintained under identical conditions.

The morphologies of M2 and V4 are shown in Figure 14. The M2, in panel A, are flat and contact inhibited. The V4, in panel B, are refractile and tend to pile up at high densities. The levels of p47 v-Crk expression in some representative clones are shown in figure 15A, a whole cell lysate Western blot with anti-Crk. The levels of v-Crk expression relative to endogenous c-Crk, marked with an arrow, are difficult to quantitate because of the variability of detection of c-Crk with this anti-Crk SH2 antibody in Western blot analysis. However, somewhat arbitrarily designating line V8 as overexpressing v-Crk 1.5-fold over endogenous c-Crk allows quantitation of v-Crk levels. Densitometry of the gel shown in figure 15A indicates 2.5-fold overexpression of v-Crk in V1, 11-fold in V3, 20-fold in V4, 16-fold in V6, and 18-fold in V7. Immunofluorescence with anti-gag confirmed the homogenous expression of v-Crk in V4 and V7 cells, and indicated partial localization to structures that may represent focal adhesions, consistent with the binding of v-Crk to tyrosine phosphorylated paxillin (Birge et al., 1993). This is shown in Figure 16 (experiment done by P. van Bergen en Henegouwen).

The v-Crk expressing cell lines also contained elevated levels of phosphotyrosine on the characteristic p70 (paxillin) and p130 (Cas), shown for

Figure 14. v-Crk can cause morphological transformation in NIH-3T3 cells. A, M2. B, V4.

A



B

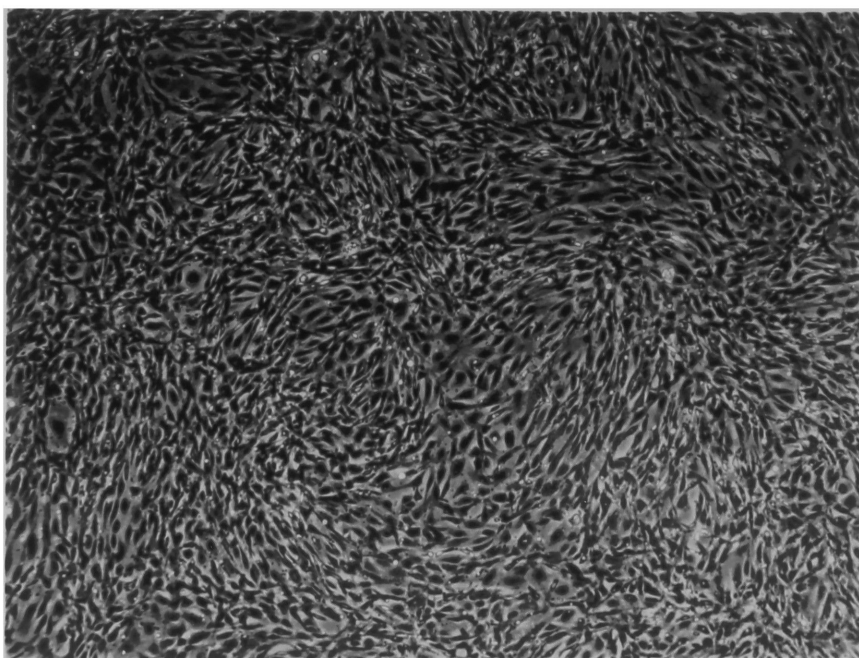
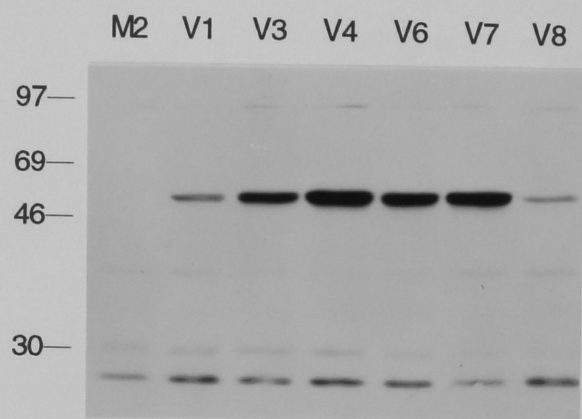


Figure 15. Overexpression of v-Crk causes an elevation of cellular phosphotyrosine in NIH-3T3 cells. A, whole cell lysate anti-Crk Western showing expression levels of p47 gag-v-Crk in various stable cell lines. c-Crk is marked with an arrow. B, whole cell lysate anti-phosphotyrosine Western.

A



B

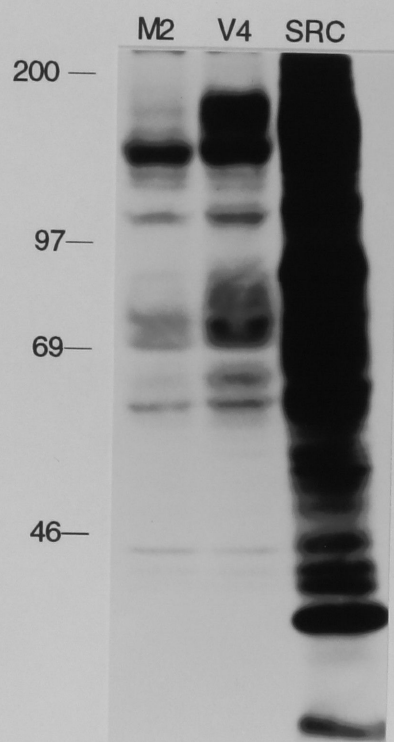
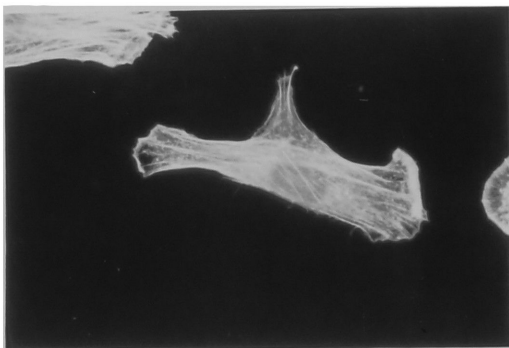


Figure 16. Immunofluorescence experiments. Top left: M2 stained with anti-actin. Top right: M2 stained with anti-Gag. Bottom left: V4 stained with anti-actin. Bottom right: V4 stained with anti-Gag. Cells were maintained in 10% calf serum prior to formaldehyde fixation.

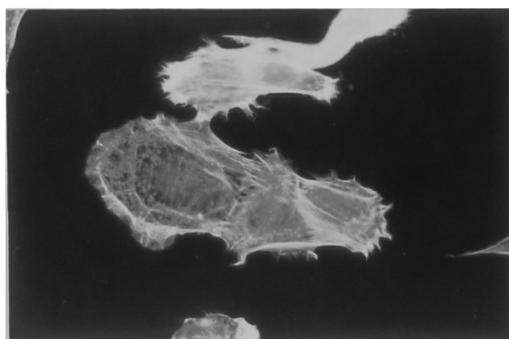
M2, anti-actin



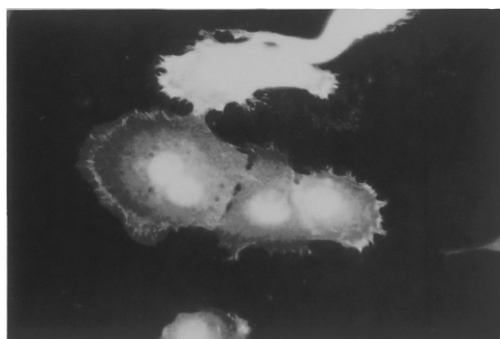
M2, anti-gag



V4, anti-actin



V4, anti-gag



V4 in Figure 15B, an anti-phosphotyrosine Western blot of whole cell lysates. Some tyrosine phosphorylated proteins are present in M2, indicative of the relatively high background level of cellular phosphotyrosine in NIH-3T3.

The effects of v-Crk on the growth properties of the NIH-3T3 were next examined. The rate of incorporation of ^3H -thymidine at confluence in regular medium containing 5% calf serum was about two-fold faster in the V4 than in the M2 (average of three experiments), indicating a faster rate of DNA synthesis. The increase was not as great at subconfluence, averaging 1.35-fold.

The v-Crk transformed NIH-3T3 also grew in an anchorage-independent manner: they formed colonies when suspended in soft agar. The number and size of the colonies formed were roughly proportional to the level of v-Crk expressed in a given line of low passage number, although long-term passage at high density increased the average size of the colonies (data not shown). However, additional, undefined mutations may be necessary for colony formation by v-Crk expressing cells for two reasons. First, a relatively low percentage of cells seeded grew colonies in soft agar. For example, in one experiment in which 10^4 cells were seeded, 58 v-Src transformed cells grew colonies larger than 1 mm in diameter and hundreds more grew colonies between 0.5 and 1 mm in diameter, while early passage V4 cells grew only one colony larger than 1 mm in diameter and 9 colonies between 0.5 and 1 mm in diameter. No colonies were detected in plates seeded with M2 or the low-expressing V8.

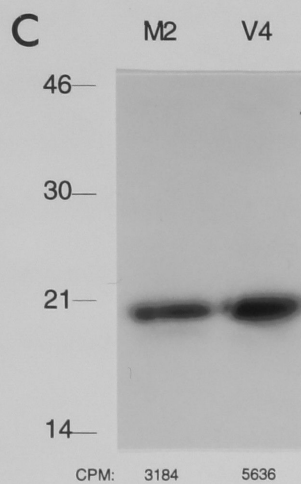
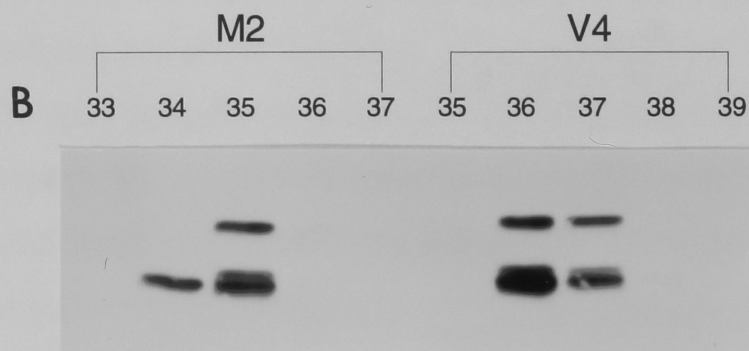
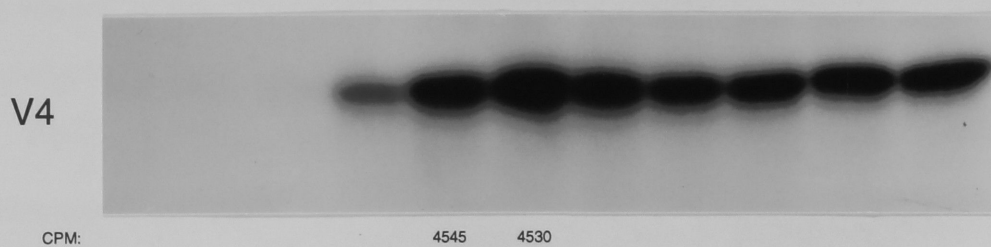
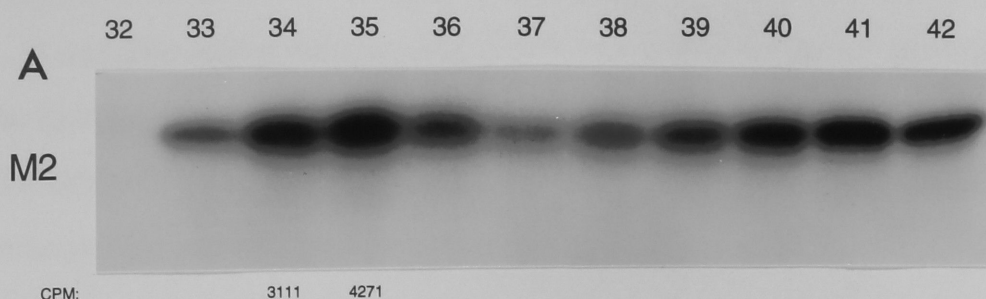
The second reason why additional mutations may be necessary for colony formation is that cells derived from V4 soft agar colonies, picked and expanded in liquid culture, are more transformed than the parental cells with which the colonies were originally seeded. The colony-derived cells are

morphologically more refractile, and themselves form more colonies in soft agar than the parental V4, approaching the uncountable “hundreds” observed for v-Src transformed cells (data not shown). No increase in v-Crk expression over levels present in the parental V4 cells was observed in the colony-derived cells (data not shown).

The activation state of Mapk in the v-Crk NIH-3T3, which express both p42 Erk2 and p44 Erk1, was the next question at hand. M2 and V4 cells were placed in medium containing 0.5% calf serum for one day, then lysed and fractionated over a Mono-Q column. The fractions were assayed for MBP kinase activity by solution kinase assay, as done previously with the CEF. The results are shown in figure 17A. A much higher background level of MBP kinase activity was observed with these lysates, but distinct peaks of activity were evident in M2 fractions 34-36, and V4 fractions 36-38. Again, activated, hyperphosphorylated Mapk was detected in these fractions by anti-Mapk Western blot (see Figure 17B), but the correlation was not as tight as in the CEFs, indicating the presence of other MBP kinases in these fractions. When the phosphorylated MBP bands from the fractions containing peak Mapk protein were cut out and counted in a scintillation counter (counts listed under each fraction in Figure 17A), a 23% increase was detected in the V4 lysate.

An immune-complex kinase assay was also performed on lysates from M2 and V4 grown for 1 day in media containing 0.5% calf serum. In this experiment, the Mapk immunoprecipitation was performed on native, nondenatured lysate, so that only p44 contributed to the observed phosphorylation of MBP, shown in figure 17C. As with the fractionation results, the increase in incorporated ^{32}P for V4 was small, a 77% increase in this experiment. The high basal levels of Mapk activation in NIH-3T3, not observed

Figure 17. Activated Mapk is found in v-Crk 3T3 fractions containing elevated MBP kinase activity. A, solution-phase MBP kinase assay on 1 μ l each Mono-Q fraction with 5 μ g MBP, 10 min at 30 °C. CPM normalized for background are listed under bands counted in the scintillation counter. B, anti-Mapk Western blot of 25 μ l each Mono-Q fraction. C, Mapk immune-complex kinase assay. Mapk was immunoprecipitated with anti-Mapk 7727 from 50 μ g M2 and V4 lysates from cells cultured 1 day in media containing 0.5% calf serum prior to lysis. IPs assayed on 10 μ g MBP 30 min at 30°C. CPM normalized for background are listed under each lane.



in CT10-transformed CEF, may partially mask a greater increase in an unidentified subpopulation of Mapk.

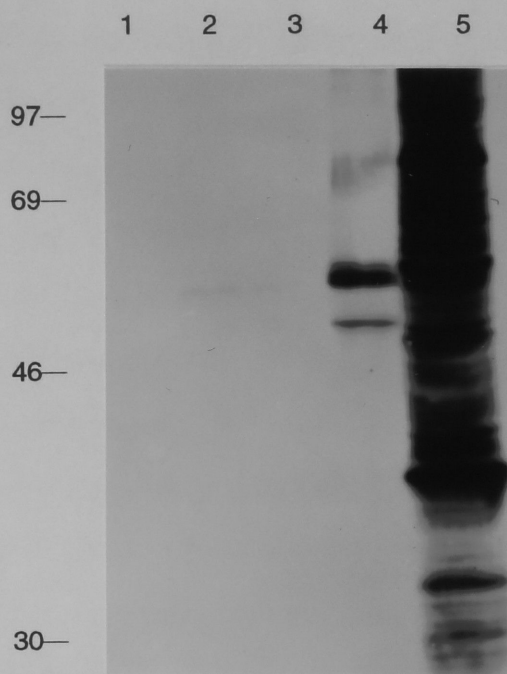
It was also asked whether Shc is phosphorylated in v-Crk NIH-3T3. Similar to the experiment done with the CEF, Shc was immunoprecipitated from M2, V4, and v-Src transformed NIH-3T3, the IPs were separated by SDS-PAGE, and Western blotted with anti-phosphotyrosine. As shown in Figure 18A, the three Shc isoforms were tyrosine phosphorylated in v-Src transformed cells, but not in the M2 or V4. Reprobing the Western with anti-Shc indicated that similar amounts of Shc were precipitated from all three lysates, shown in Figure 18B.

Finally, the phosphorylation states of p120 Ras GAP and its associated proteins were examined. GAP was immunoprecipitated from 250 μ g M2, V4, and v-Src NIH-3T3 lysates, and the IPs were separated by SDS-PAGE and Western blotted with anti-phosphotyrosine. While a putative p120 GAP and the GAP-associated p62 were clearly tyrosine phosphorylated in v-Src 3T3 lysates, no tyrosine phosphorylation of these bands was observed in M2 or V4 lysates. There was some basal tyrosine phosphorylation on GAP-associated p190 in the M2 lysate, but no increase in phosphorylation in the V4 lysate was observed, while p190 exhibited about a three fold increase in tyrosine phosphorylation in the v-Src lysate (data not shown). Phosphorylation of GAP and its associated proteins probably does not play a role in v-Crk transformation.

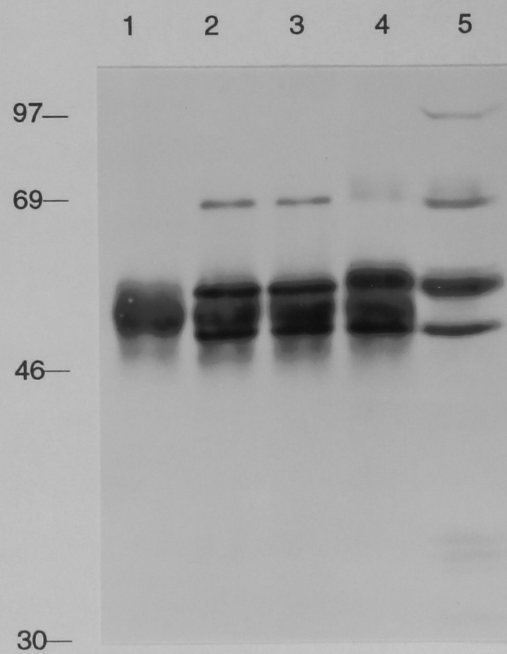
The v-Crk transformed NIH-3T3 therefore exhibit similar transformed phenotypes, tyrosine phosphorylated proteins, activation of Mapk, and lack of Shc phosphorylation, as observed in CT10-transformed CEF. These cells constitute a suitable system for studying the effects of dominant negative Ras on Crk transformation.

Figure 18. Shc is not tyrosine phosphorylated in v-Crk transformed NIH-3T3. Shc was immunoprecipitated from 150 μ g each lysate. Lane 1: no lysate, 2: M2, 3: V4, 4: v-Src 3T3. Lane 5: v-Src 3T3 whole cell lysate. A, anti-phosphotyrosine Western. B, same filter reprobed with anti-Shc.

A



B



Requirement of Ras function for v-Crk transformation

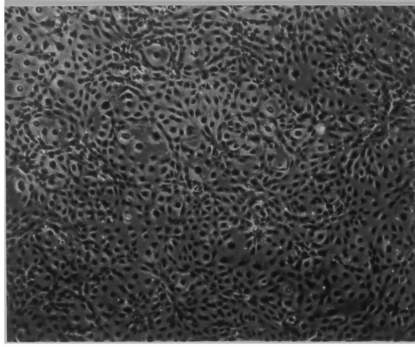
The two dominant negative Ras mutants used in this study, N17 (a gift from G. Cooper) and Y57 (a gift from M. Wigler), are point mutants of c-H-Ras isolated by random mutagenesis (Feig and Cooper, 1988; Jung et al., 1994). Both of these mutants have a decreased affinity for GTP, suggesting that they may function by sequestering upstream GEFs without exchanging bound nucleotide. The sequences encoding these two dominant negative Ras proteins were inserted into the puromycin resistance vector pBabe puro (pBp) by blunt-end ligation. Expression of Ras N17 was confirmed by transient transfection into Cos7 cells (data not shown).

The first experiment was designed as a pooled transfectant assay. M2, V4, V7, negative control v-Raf, and positive control v-Src were transfected with vector alone, pBp N17, pBp Y57, or a Y57 antisense construct that should not produce any protein. The cells were placed under drug selection two days after transfection, and split 1:3 when confluent. One of the three resulting plates was used to photograph morphology, one was used for a colony formation assay, and one was lysed to check expression of the overexpressed proteins. This approach allows quick phenotype identification without giving the cells time to accumulate mutations that alter growth properties. However, not all drug resistant cells will express the same (or any) level of the intended protein, yielding a heterogeneous population of cells.

The morphologies of the resulting transfectants are shown in Figure 19. The morphology of the M2 was unaffected by Ras N17 expression, although their growth rate was decreased. Transfection of the V4 with pBp or antisense Y57 had no effect on cell morphology, while transfection with Y57 partially reverted Crk transformation and N17 fully reverted the transformed morphology.

Figure 19. Morphologies of pooled revertant cells transfected with dominant negative Ras. Cells were transfected with puromycin vector pBp, dominant negative Ras Y57, an antisense construct for dominant negative Ras Y57, and dominant negative Ras N17.

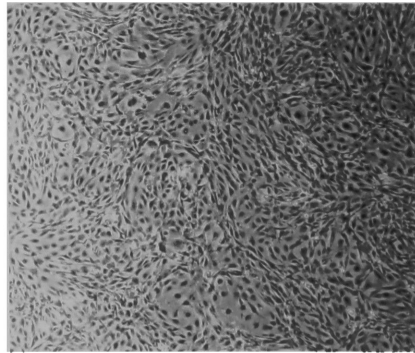
**M2
pBp**



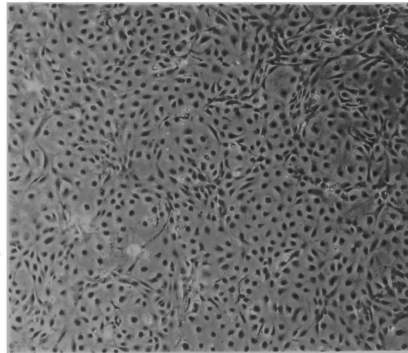
**M2
N17**



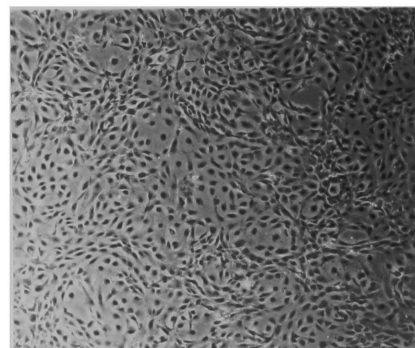
**V4
pBp**



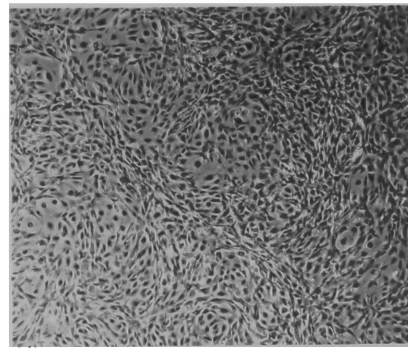
**V4
N17**



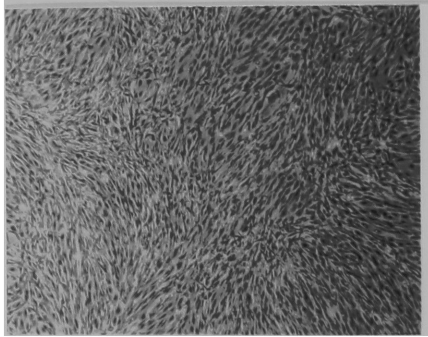
**V4
Y57**



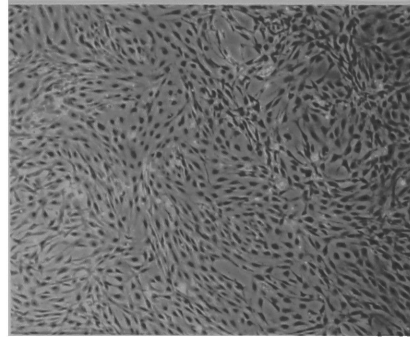
**V4
Y57
AS**



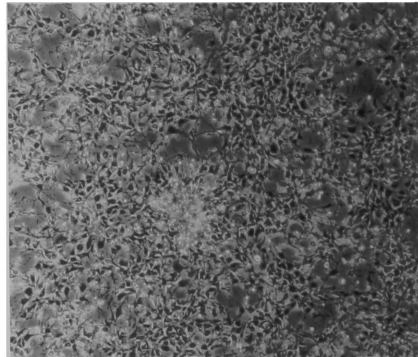
**V7
pBp**



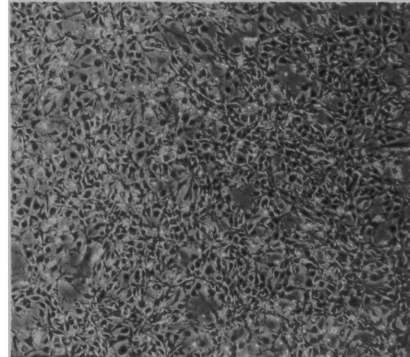
**V7
N17**



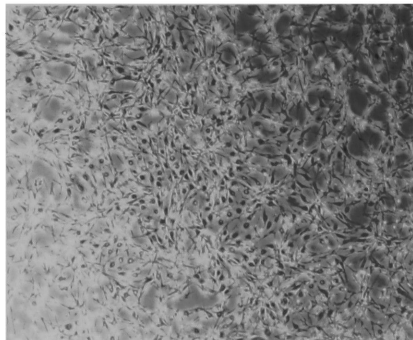
**v-Src
pBp**



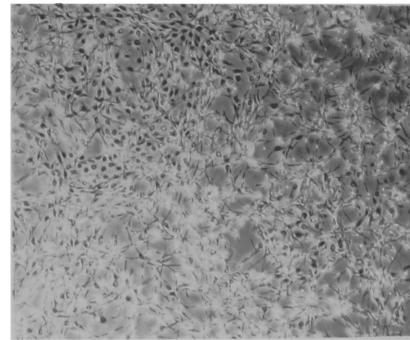
**v-Src
N17**



**v-Raf
pBp**



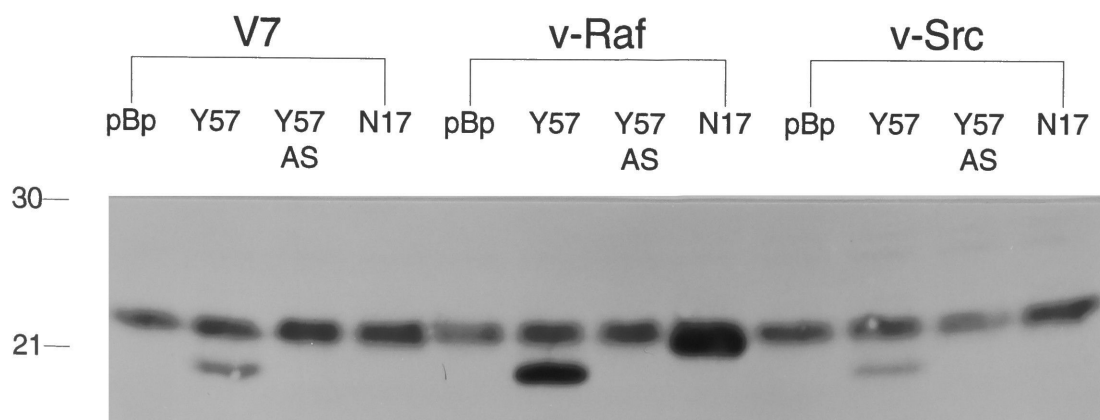
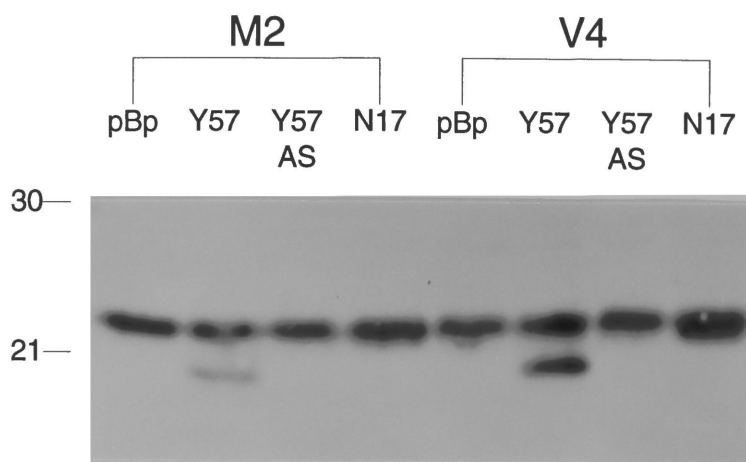
**v-Raf
N17**



	pBp			Y57			N17		
	1	2	3	1	2	3	1	2	3
M2	0	0	1	0	0	1	0	0	0
V4	0	26	NC	0	10	NC	0	1	5
V7	0	12	NC	0	0	3	0	1	0
v-Src	4	136	NC	13	194	NC	0	37	NC

Table 2. Suppression of v-Crk induced soft agar colonies by dominant negative Ras Y57 and N17. Column 1: colonies 1 mm in diameter or larger. Column 2: colonies 0.5 mm in diameter to 0.9 mm in diameter. Column 3: colonies less than 0.5 mm in diameter. NC: too many colonies to accurately count.

Figure 20. Overexpression of dominant-negative Ras in pooled transfected cells. Whole cell lysate anti-Ras Western. Cells were transfected with puromycin vector pBp, dominant negative Ras Y57, an antisense (AS) construct for dominant negative Ras Y57, and dominant negative Ras N17.



The N17-overexpressing V4 were flattened and grew to a lower saturation density, comparable to the that of the M2. The v-Crk overexpressing cell line V7, suspected of accumulating additional alterations, was partially reverted by N17. v-Src transformed NIH-3T3 were also partially reverted by Ras N17, as evidenced by the increase of adherent cells. This is consistent with the results of GAP overexpression experiments (Nori et al., 1991; DeClue et al., 1991). However, the morphology of v-Raf transformed NIH-3T3 (a gift from R. Jove) was not affected, in agreement with the placement of Raf downstream from Ras (Schaap et al., 1993). GAP overexpression also partially reverted v-Crk transformed NIH-3T3 (data not shown).

The results of the colony assay are shown in Table 2. Ras N17 effectively reduced the number of colonies formed by both v-Crk and v-Src transformed NIH-3T3, although some anchorage-independent cells remained, probably due to the heterogeneity of the Ras N17 expression levels. The Y57 mutant was less effective, and did not affect v-Src colony formation at all, for unknown reasons.

The levels of mutant Ras protein expression were examined by whole cell lysate anti-Ras Western blot, shown in Figure 20. The expression levels were relatively low, equal to or less than the level of endogenous Ras in every case except for v-Raf 3T3, which are not dependent on Ras function for growth. Ras Y57 is clearly visible under the endogenous Ras band, while N17 migrates just slightly ahead of endogenous Ras, and can be observed as a broadening of the endogenous Ras band into the lower molecular weight range. No overexpression of any protein was observed in the Y57 antisense-overexpressing cells.

The level of transforming protein was then checked for each cell type to

ensure that reversion was not simply due to loss of the oncogene. A whole cell lysate anti-Crk Western blot is shown in Figure 21A. The comparable levels of v-Crk in the V4 and V7 cells were unaffected by expression of either of the dominant negative Ras proteins. The same is true for v-Raf (Figure 21B) and v-Src (Figure 21C), also assayed by whole cell lysate Western blot.

The effects of dominant negative Ras on levels of cellular phosphotyrosine was examined next, by whole cell lysate anti-phosphotyrosine Western blot, shown in the upper panel of Figure 22. The levels of cellular phosphotyrosine in the v-Crk NIH-3T3, most evident on p130, are directly proportional to the levels of v-Crk expression (compare with Figure 15A). Neither v-Crk 3T3 nor v-Src 3T3 show a decrease in cellular tyrosine phosphorylation in response to Ras N17 expression. Paxillin phosphorylation is also essentially unchanged, so it is not surprising that v-Crk is still localized to the focal adhesion-like structures in V4-N17 cells, shown in the lower panel of Figure 22 (immunofluorescence done by P. van Bergen en Henegouwen).

Posttranslational modification is a requirement for Ras function (Cox et al., 1992). Ras farnesylation inhibitors have thus been the focus of much inquiry (Hara et al., 1993; Gibbs et al., 1993; Garcia et al., 1993). A competitive tetrapeptide farnesylation inhibitor modelled after the Ras CAAX motif (Garcia et al., 1993), B581, was obtained as a gift from the Eisai Research Institute. v-Crk and v-Src NIH-3T3 were also reverted by treatment with the B581 farnesylation inhibitor, the resulting morphologies shown in Figure 23A, while B581 merely slowed the growth of the nontransformed M2 cells (data not shown). Neither the v-Crk nor the v-Src protein levels were affected, as assayed by whole cell lysate Western blot analysis (Figures 23B and 23C).

The activation state of Mapk in the pooled Ras N17 transfectants was

Figure 21. Oncogene expression is not affected by coexpression of dominant negative Ras. Whole cell lysate Western blot probed with: A, anti-Crk; B, anti-Raf-1; C, anti-src. Positions of p75 gag-v-Raf (B) and p60 v-Src (C) are marked with arrows. p47 gag-v-Crk runs above the endogenous p35 c-Crk (A). Cells were transfected with puromycin vector pBp, dominant negative Ras Y57, an antisense (AS) construct for dominant negative Ras Y57, and dominant negative Ras N17.

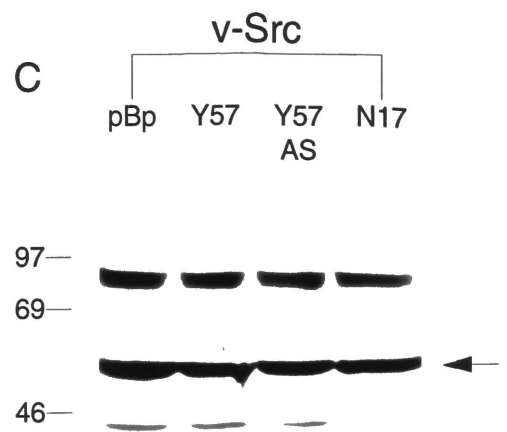
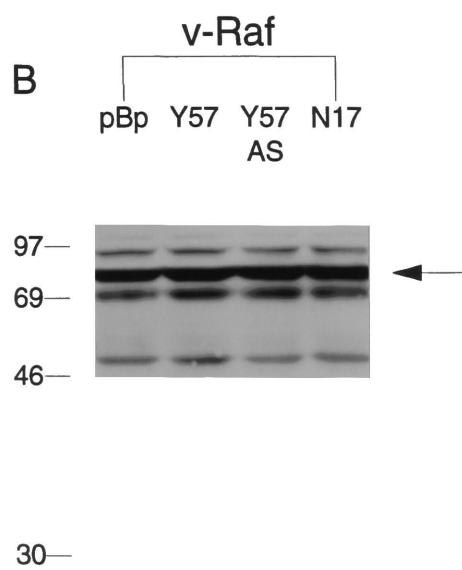
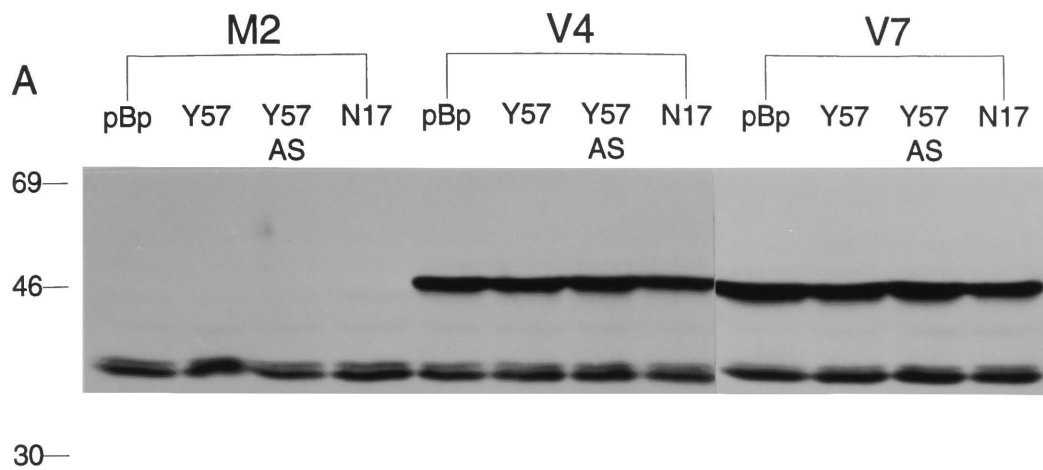
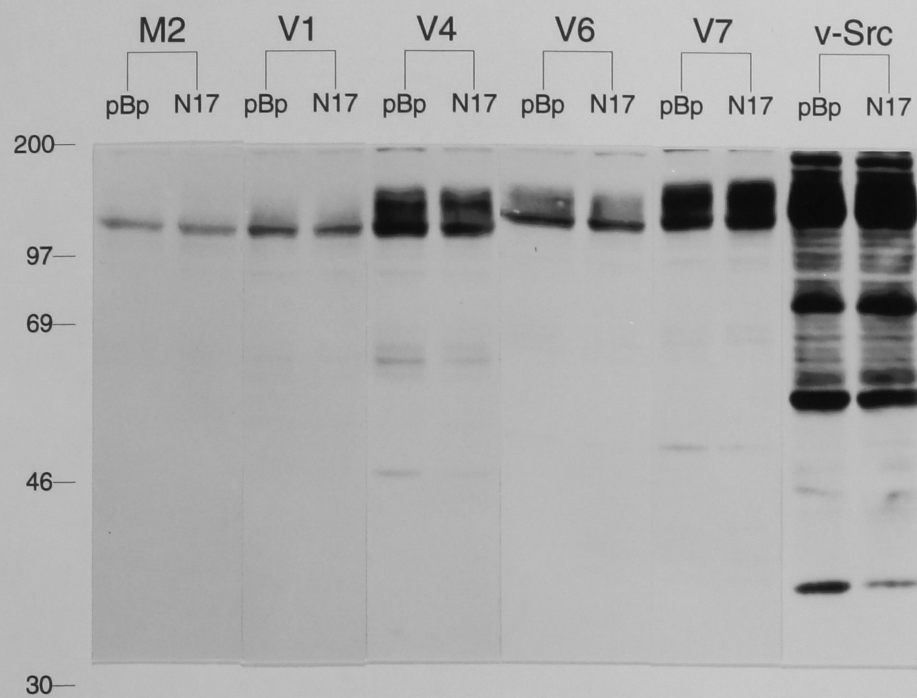
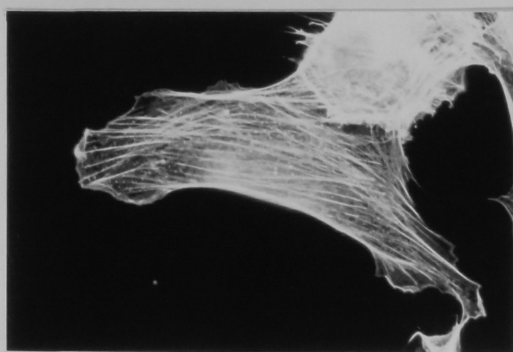


Figure 22. Tyrosine phosphorylation is not affected by dominant negative Ras. Upper panel, whole cell lysate anti-phosphotyrosine Western blot. Lower panel, immunofluorescence showing continued localization of v-Crk to putative focal adhesions. Left: V4N17 stained with anti-actin. Right: V4N17 stained with anti-gag.



V4 N17, anti-actin



V4 N17, anti-gag

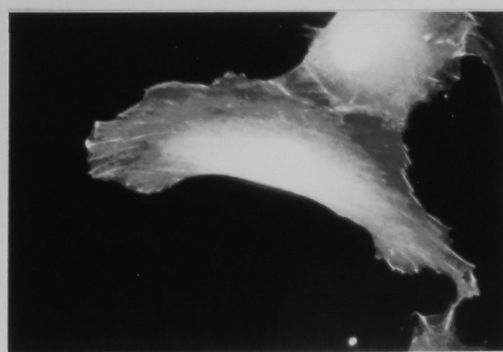
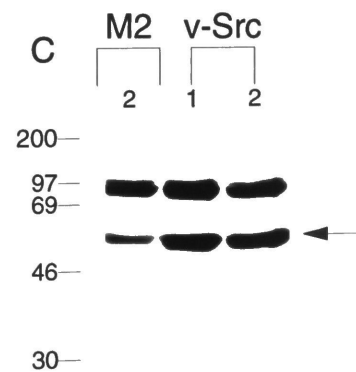
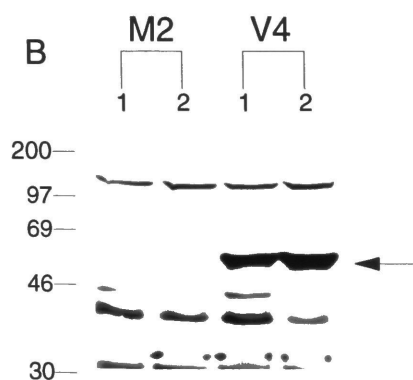
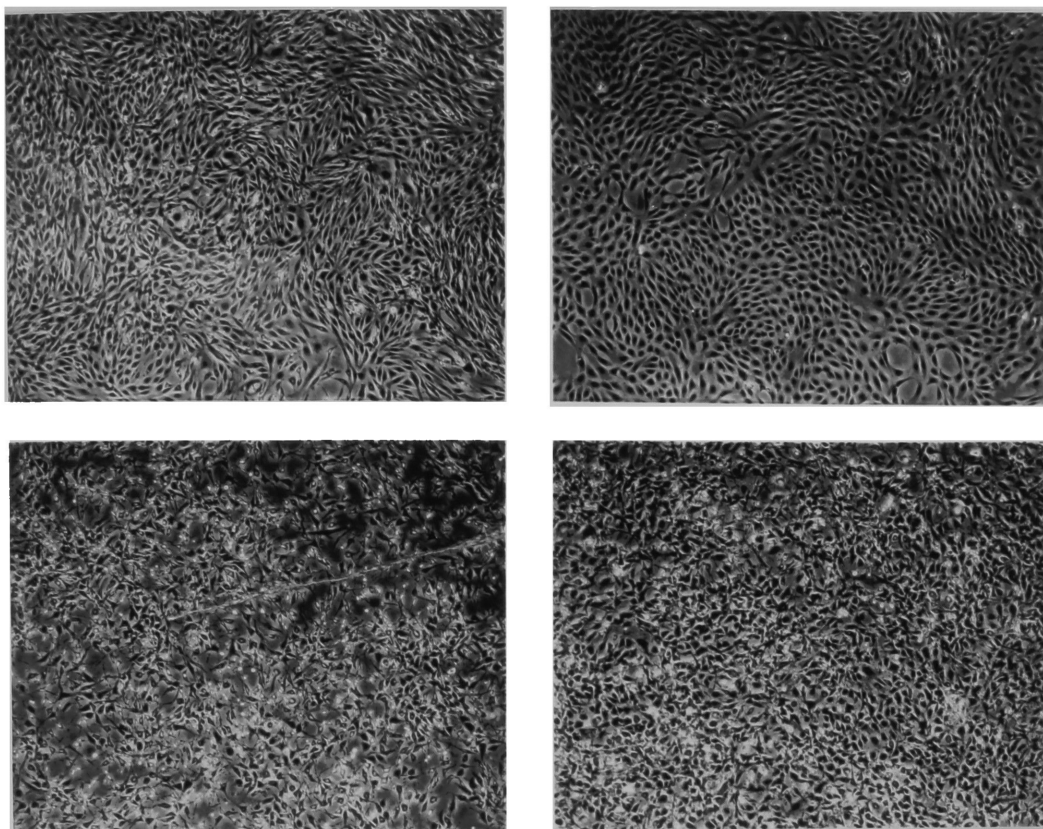


Figure 23. Ras farnesylation inhibitor B581 reverts v-Crk and v-Src transformed NIH-3T3. 100 μ M B581 in DMSO added once; reversion observable in 2-4 days. A, morphologies of cells treated with B581. Top left: V4 + DMSO; top right: V4 + B581; bottom left: v-Src + DMSO; bottom right: v-Src + B581. B, whole cell lysate Western blot probed with anti-Crk. C, whole cell lysate Western blot probed with anti-Src. In B and C, lane 1: +DMSO, 2: +B581. Arrows mark positions of p47 gag-v-Crk (B) and p60 v-Src (C).

A



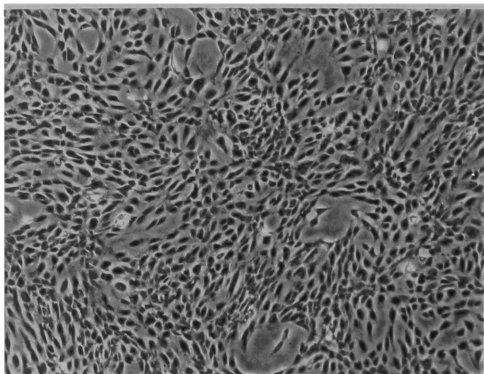
examined both by Western blot and immunocomplex kinase assay. Surprisingly, no suppression of Mapk activity was observed in the cells expressing dominant negative Ras (data not shown). Furthermore, dominant negative Ras did not suppress serum stimulation of Mapk activity either (data not shown). However, these observations could be accounted for by the heterogeneity of the pooled drug-resistant cells. Clonal cell lines stably expressing Ras N17 were therefore made.

M2 and V4 cells were plated at low density and transfected with vector pBabe puro (pBp) or dominant negative Ras (pBpN17). The cells were placed under drug selection two days after transfection and grown for about a week in puromycin. During this time, the cells which did not stably integrate one or more copies of pBp or pBpN17 died, while cells with successful integration events were resistant to the puromycin and grew into small colonies. Several colonies from each transfection event were isolated and replated into separate dishes. These drug resistant colonies were grown and assayed for Ras N17 expression by whole cell lysate Western blot analysis. The two highest expressors in the M2 and V4 backgrounds were chosen for further analysis.

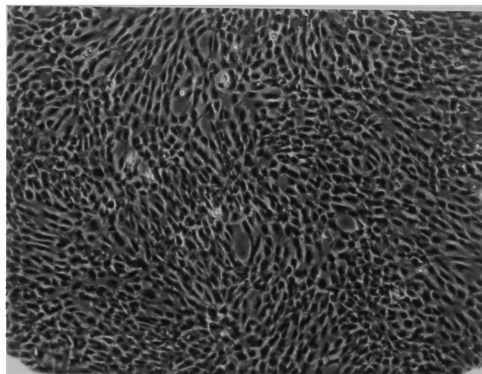
The morphologies of the representative stable transfectants are shown in Figure 24. Expression of dominant negative Ras did not significantly alter the morphology of the M2 cells (compare B and C with A), but did revert the transformed morphology of the V4 cells (compare E and F with D). During the six week selection and growth process, the V4pBp-2 cell line presumably accumulated transformation-promoting mutations, because its morphology became more extreme than the parental V4 cells. The revertants (E and F) would therefore more accurately be compared to the parental V4 line shown in Figure 14B, which is still significantly more transformed than either of the V4N17

Figure 24. Morphologies of clonal cell lines expressing dominant negative Ras.
A, M2pBp-1; B, M2N17-3; C, M2N17-4; D, V4pBp-2; E, V4N17-4; F, V4N17-8.

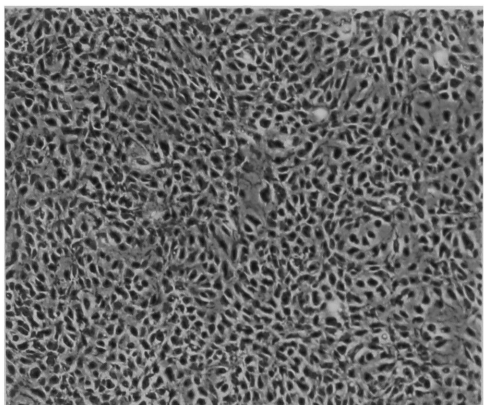
A



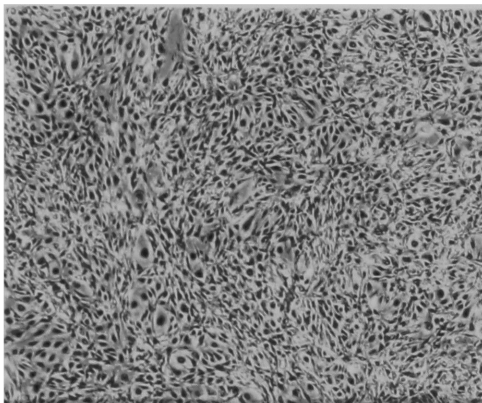
B



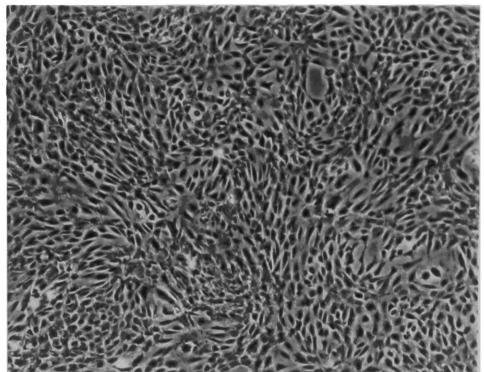
C



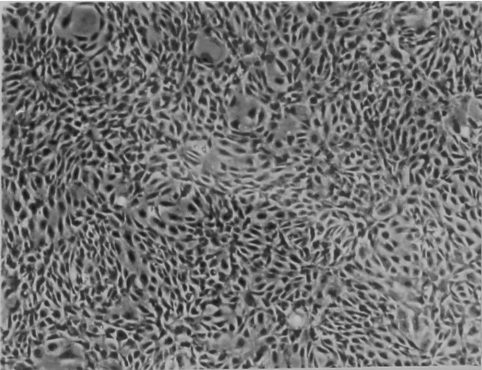
D



E



F



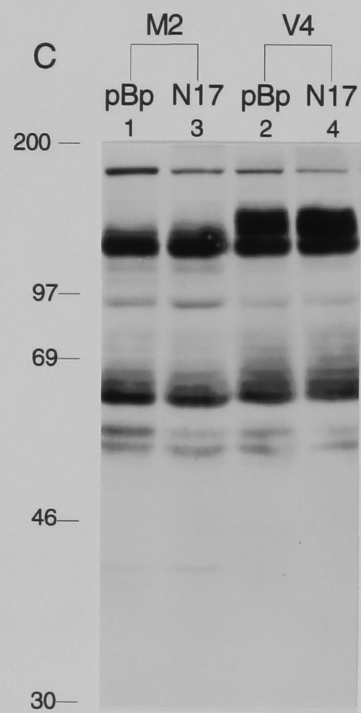
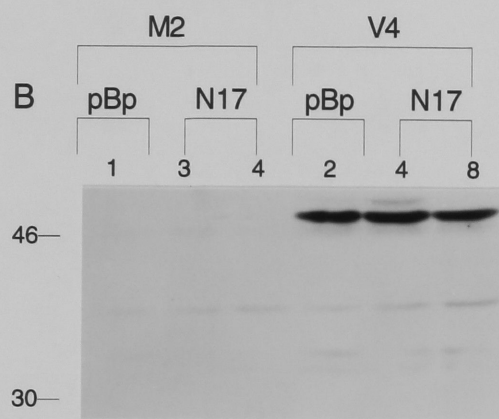
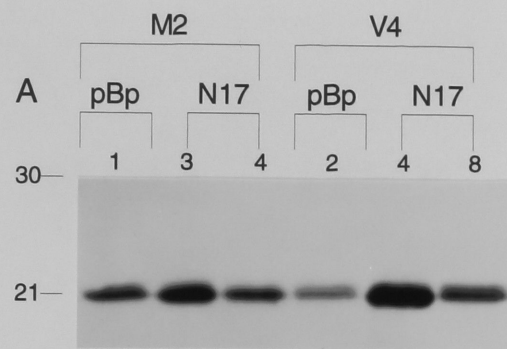
lines.

The levels of expression of Ras N17 are shown in Figure 25A, a whole cell lysate anti-Ras Western blot. Lines M2N17-3 and M2N17-4 exhibited about three-fold overexpression of Ras N17, compared to the vector transfected M2pBp-1 line. V4N17-4 overexpressed Ras N17 about ten-fold, and V4N17-8 about two-fold, compared to the vector transfected V4pBp-2 line. V4N17-4 grew extremely slowly, and the level of Ras N17 expression was downregulated to about two-fold after ten weeks of passage. The levels of v-Crk in the reverted V4N17 lines were not decreased compared to V4pBp-2 (Figure 25B), shown by reprobing the Western from Figure 25A with anti-Crk. The levels of phosphotyrosine on paxillin and p130 were also unchanged, as assayed by whole cell lysate anti-phosphotyrosine Western blot analysis (Figure 25C).

The stably transfected cells were then assayed for anchorage independent growth by colony formation in soft agar. The results are shown in Table 3. None of the M2 lines formed any colonies in soft agar, but V4pBp-2 was clearly capable of anchorage-independent growth. Expression of Ras N17 almost completely abolished the ability of V4 cells to form colonies in soft agar.

Finally, the effects of dominant negative Ras expression on Mapk activity were examined. An anti-p44 immunocomplex MBP kinase assay is shown in Figure 26A. Expression of Ras N17 in both M2 and V4 partially inhibited the ability of Mapk to phosphorylate MBP. A greater suppression was observed in V4N17-4, which expressed higher levels of dominant negative Ras than M2N17-3. The ability of overexpressed dominant negative Ras to block serum stimulation of Mapk was also investigated. M2 and V4 lines stably expressing pBp or Ras N17 were placed in media containing 0.5% serum for one day, then serum stimulated in 10% serum for the indicated amount of time and lysed. An

Figure 25. Levels of v-Crk and phosphotyrosine are not affected by Ras N17 expression. A, whole cell lysate Western blot probed with anti-Ras. B, same filter reprobed with anti-Crk. C, whole cell lysate Western blot probed with anti-phosphotyrosine.

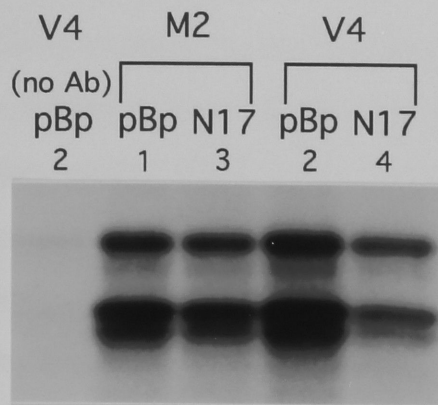


		<u>≥ 1 mm</u>	<u>≥ 0.5 mm</u>	<u>< 0.5 mm</u>
A	M2pBp-1	0	0	0
	M2N17-3	0	0	0
	M2N17-4	0	0	0
	V4pBp-2	2	7	21
	V4N17-4	0	0	3
	V4N17-8	0	0	1
B	V4pBp-2	3	12	61
	V4N17-8	0	0	4

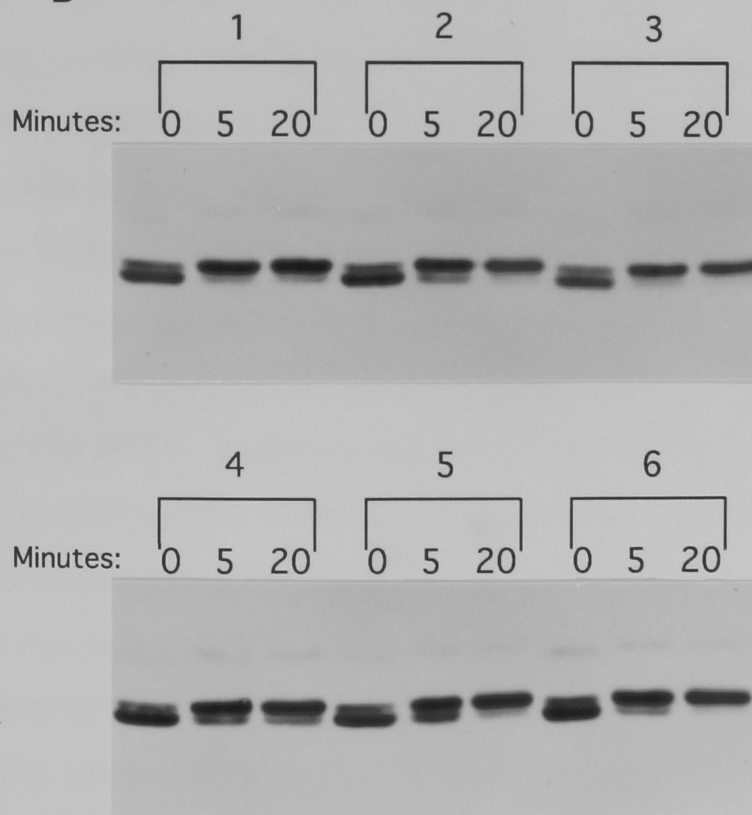
Table 3. Suppression of v-Crk induced soft agar colonies by dominant negative Ras N17 in stable cell lines. A, 2×10^5 cells; B, 4×10^5 cells.

Figure 26. Dominant negative Ras inhibits Mapk activity. A, Mapk immunocomplex kinase assay on lysates placed in low serum for 24 hours prior to lysis. IPs with anti-Mapk 7727 from 50 μ g each lysate assayed on 10 μ g MBP, 30 min at 30 °C. Lane 1, mock IP with protein A sepharose only to indicate background level of nonspecific kinase activity adhering to beads. B, whole cell lysate Western blot probed with anti-Mapk. Cells serum stimulated in media containing 10% calf serum for the number of minutes indicated on top of each lane. 1: M2pBp-1, 2: M2N17-3, 3: M2N17-4, 4: V4pBp-2, 5: V4N17-4, 6: V4N17-8.

A



B



anti-Mapk Western blot was performed on these lysates, shown in Figure 26B. While the expression of low levels of Ras N17 in these lines does not inhibit serum stimulation of Mapk hyperphosphorylation, the high-expressing V4N17-4 line does exhibit a slight delay of Mapk hyperphosphorylation in response to serum stimulation. This is evident in the presence of a larger percentage of faster-migrating hypophosphorylated Mapk in the V4N17-4 line serum stimulated for 5 min, as compared to the V4pBp-2 line serum stimulated for 5 min. However, serum-inducible, Ras-independent pathways to Mapk may also exist in NIH-3T3 cells, complicating interpretation of these results. The activation of Ras-independent pathways is not unlikely, since serum may stimulate several growth factor receptors and their cognate signal transduction pathways at the same time (Burgering et al., 1993).

In order to examine the role of Mapk activation in the v-Crk NIH-3T3 cells more precisely, the Mapk-specific, serum-inducible phosphatase MKP-1 (Sun et al., 1993) carrying a Myc epitope tag in the hygromycin resistance vector pCEP4 (a gift from N. Tonks) was introduced into M2, V4, and v-Src 3T3 cells. The cells were analyzed both by pooled transfectant assay and the attempted establishment of clonal stable cell lines.

Preliminary results indicate that Mapk activity, or another unknown activity inhibited by MKP-1, is not only required for transformation, but also for growth. While a small number of MKP-1 transfected drug resistant cells survived in the pooled assay, they were enlarged, flattened, and quiescent, as evidenced by their lack of division during the selection period (data not shown). No such morphological alterations were observed in the vector (pCEP4) transfected cells, which eventually grew to confluence (data not shown). Unfortunately, the small number of nondividing drug resistant MKP-1

transfectants precluded analysis of MKP-1 protein levels or Mapk activity.

The establishment of clonal MKP-1 cell lines was also attempted. Many more drug resistant colonies resulted from pCEP4 vector transfection than from MKP-1 transfection. The few MKP-1-transfected drug resistant colonies were picked, expanded, and analyzed for MKP-1 expression by anti-Myc Western blotting (data not shown). No expression was detected in any of the MKP-1 transfectants analyzed, indicating a strong selection against inhibition of Mapk activity.

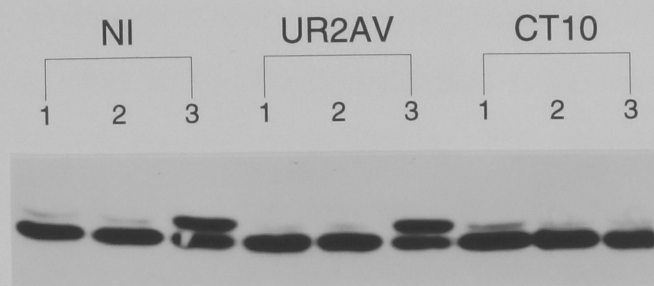
Delay of serum stimulation of Mapk by oncogene transformation

During the studies of Mapk in CT10-transformed CEF, it was fortuitously observed that hyperphosphorylation of Mapk in response to serum stimulation was markedly inhibited in v-Crk transformed CEF. For serum stimulation, CEF were infected with helper virus UR2AV or transforming virus CT10 and maintained under soft agar overlay for about ten days, at which time the cells were placed into liquid medium containing 0.5% calf serum for 24 hours. The following day, the medium was removed and replaced with medium containing 10% calf serum for 5 min, and the cells were then immediately placed on ice and lysed. Mapk hyperphosphorylation was visualized as slowed migration by probing a long-run whole cell lysate Western blot with anti-Mapk.

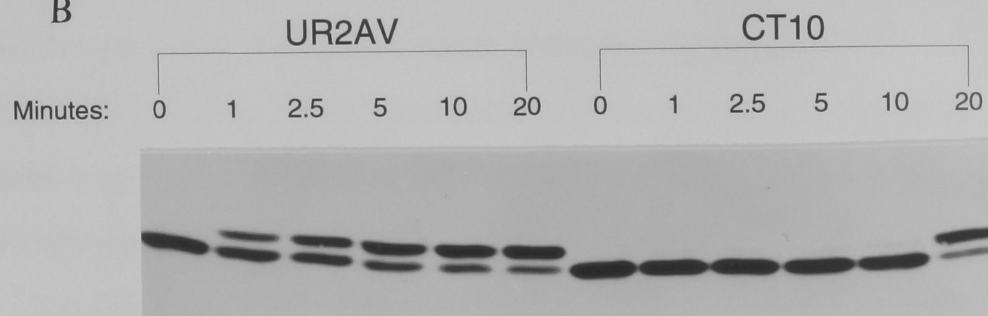
A typical observation is shown in Figure 27A. Little or no hyperphosphorylation of Mapk was observed in unstimulated CEF that were maintained in standard growth media containing 5% calf serum (lane 1) or low-serum media containing 0.5% serum (lane 2); basal conditions varied somewhat from experiment to experiment. When cells were serum stimulated for 5 min (lane 3), an increase in the slower-migrating hyperphosphorylated

Figure 27. v-Crk transformation inhibits serum stimulation of Mapk. A, Whole cell lysate anti-Mapk Western blot. Lane 1: cells maintained in 5% calf serum, lane 2: cells maintained in 0.5% calf serum, lane 3: cells incubated 24 hours in 0.5% calf serum, then stimulated with 10% serum for 5 min. NI = uninfected cells. B, Whole cell lysate anti-Mapk Western blot. Cells serum stimulated for the number of minutes indicated at the top of each lane.

A



B



Mapk isoform was observed in uninfected ("NI") and UR2AV-infected cells. However, no hyperphosphorylation of Mapk was detected after a 5-min serum stimulation of CT10-transformed CEF.

Further study revealed that v-Crk transformation did not abolish, but rather delayed the serum stimulation of Mapk. As revealed by the time course shown in Figure 27B, Mapk responded to serum stimulation in UR2AV-infected cells within one minute, while CT10-infected cells did not significantly respond until between 10 and 20 min after serum stimulation. The degree of the delay varied from experiment to experiment, correlating with the degree of transformation of the culture. In Figure 28A, UR2AV- and CT10-infected cells were maintained under soft agar for 6 (I), 8 (II), 10 (III), and 12 (IV) days, placed in low serum for 24 hours, then serum stimulated for the indicated number of minutes. A significant delay in serum stimulation of Mapk was only seen in the cells maintained 10 or more days post infection under soft agar. The whole cell lysate anti-Crk Western in Figure 28B indicates greater levels of v-Crk expression in the longer-term cultures, but cannot distinguish between greater expression per cell or a greater number of expressing cells in these heterogenous cultures. Other conditions, such as greater morphological alteration and greater acidity due to faster metabolic rate, also characterize the longer-term cultures.

The direct correlation between hyperphosphorylation and activation state of Mapk was examined next. First, to demonstrate that the hyperphosphorylated bands seen in the whole cell lysate Western blots contained the requisite tyrosine phosphorylation, Mapk was immunoprecipitated from denatured lysates of UR2AV- and CT10-infected cells serum stimulated for the indicated amount of time (Figure 29). The IPs were separated by SDS-PAGE, Western

Figure 28. A greater degree of transformation correlates with a greater delay in serum stimulation of Mapk hyperphosphorylation. Cells were maintained under soft agar for 6 (I), 8 (II), 10 (III), or 12 (IV) days, placed in medium containing 0.5% calf serum for 24 hours, then stimulated with 10% serum for the indicated number of minutes and lysed. A, whole cell lysate anti-Mapk Western blot. B, Western was reprobed with anti-Crk.

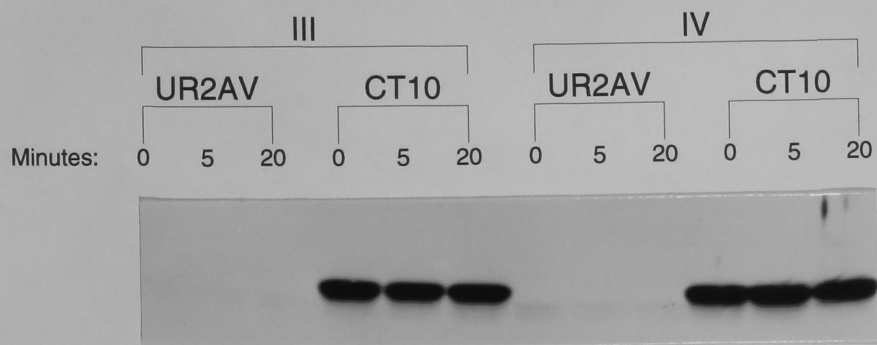
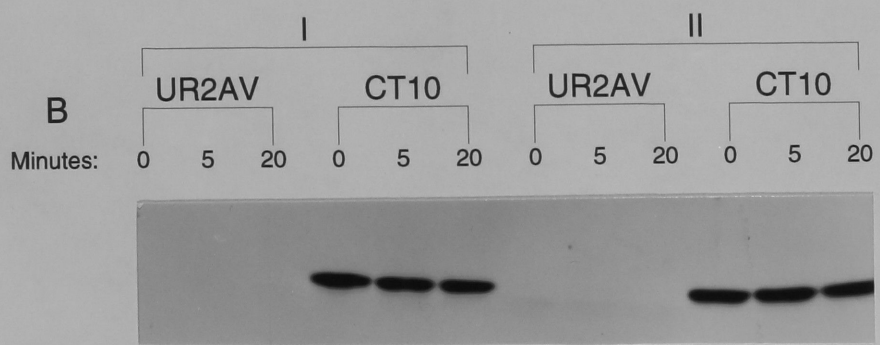
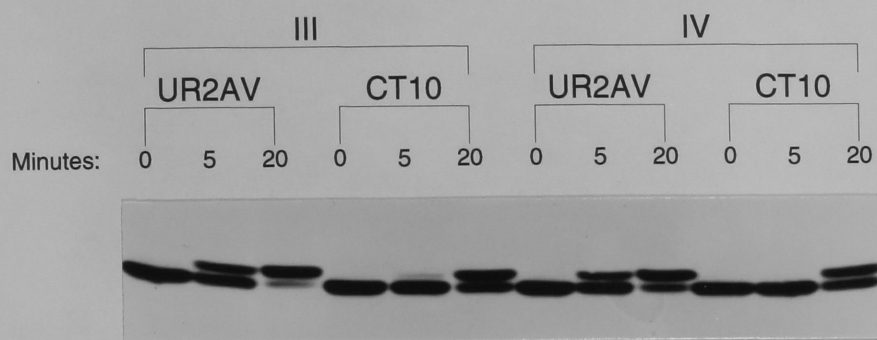
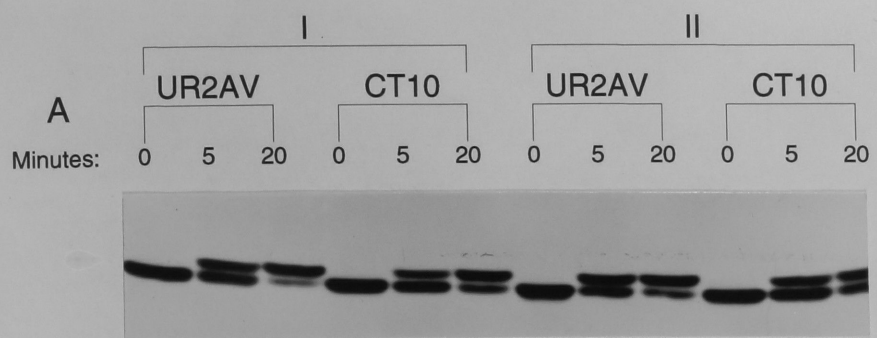
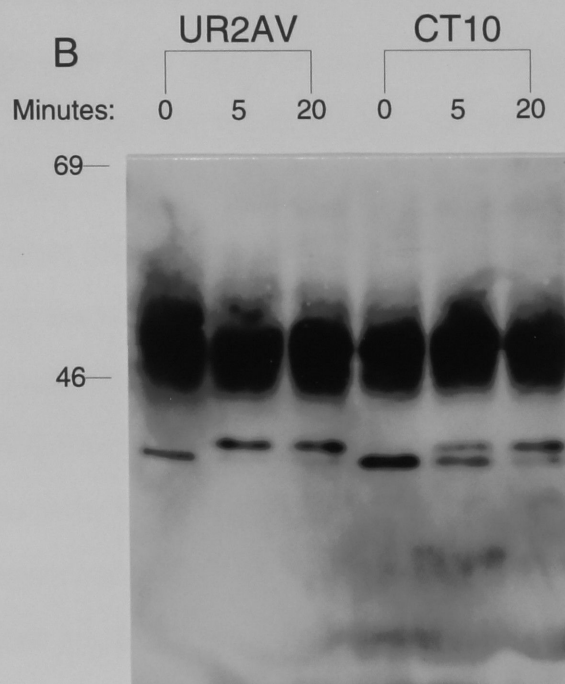
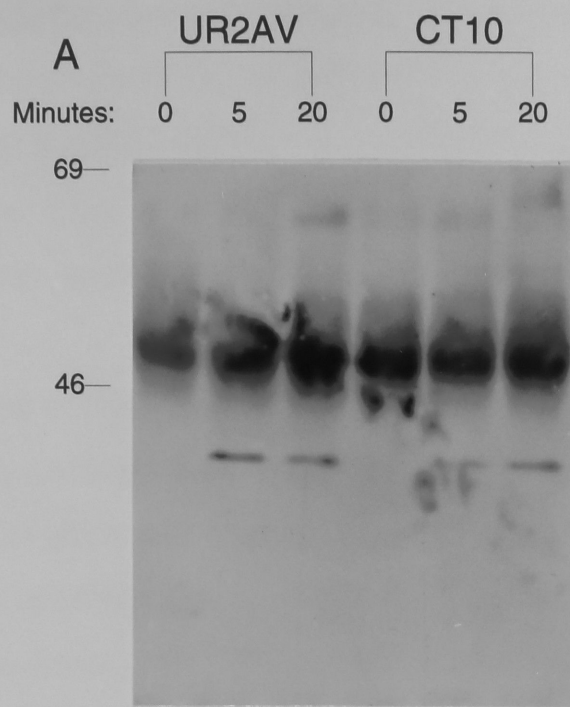


Figure 29. Tyrosine phosphorylation of Mapk is also delayed in v-Crk transformed CEF. Mapk was immunoprecipitated from 80 μ g of lysate denatured in 0.5% SDS, diluted to 0.1% before antibody (anti-Mapk 7727) addition. A, IPs were separated by SDS-PAGE, Western blotted, and probed with anti-phosphotyrosine. B, the Western was reprobed with anti-Mapk.



blotted, and probed with anti-phosphotyrosine (A), then stripped and reprobed with anti-Mapk (B). The upper, hyperphosphorylated bands in B exactly comigrate with the tyrosine-phosphorylated bands seen in A. Furthermore, the level of tyrosine phosphorylation on Mapk in CT10-infected cells serum stimulated for 5 min is clearly less than that exhibited by UR2AV-infected cells serum stimulated for 5 min. Finally, a delay in full activation of kinase activity is observed by Mapk in gel kinase assays, such as the experiment shown in Figure 9C.

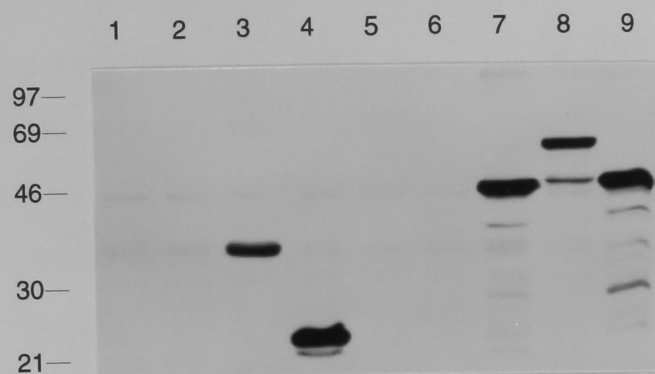
The response of Mapk to serum stimulation was then examined in CEF transformed by other oncogenes. v-Ras, v-Src, and v-Yes all caused a delay in serum stimulation of Mapk, with the degree of delay being dependent on the degree of transformation (data not shown). The response of Mapk to other stimuli in oncogene-transformed cells was also examined. TGF α , which binds and stimulates the chicken EGFR, was examined to investigate if stimulation by a single growth factor was affected in the same manner as stimulation by heterogenous serum. TGF α stimulation of Mapk hyperphosphorylation was delayed to the same extent as serum stimulation in CT10-transformed cells (data not shown). The response to phorbol myristate acetate (PMA), which directly stimulates PKC activity, indirectly activating Mapk, was also delayed in v-Crk transformed cells (data not shown). Normal cellular responses to several mitogenic stimuli are therefore inhibited by oncogene transformation.

Serum stimulation of Mapk was then examined in CEF expressing Crk constructs of differing transforming potentials (C. Reichman, graduate dissertation). These v-/c- chimeric Crk constructs, diagrammed in the legend to Figure 30, were expressed in CEF. Proteins of the expected size were detected by anti-Crk whole cell lysate Western blot (Figure 30A), except for the gag-

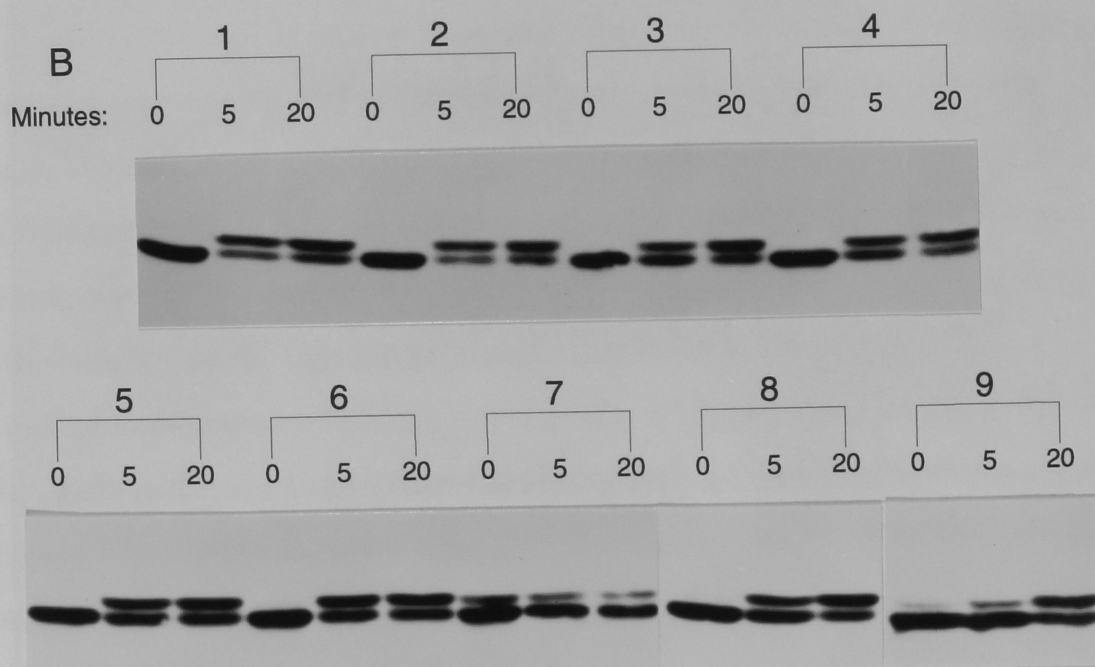
Figure 30. Delay in serum stimulation of Mapk in CEF infected with Crk constructs of different transforming potentials. A, Whole cell lysate anti-Crk Western blot. B, Whole cell lysate anti-Mapk Western blot of cells incubated in medium containing 0.5% calf serum for 24 hours, then 10% serum stimulated for the indicated number of minutes. The constructs are diagrammed below (adapted from C. Reichman, graduate dissertation). Lane 1, no infection; 2, PNR200; 3, c-Crk (CC); 4, SHB; 5, UR2AV; 6, GCC (GCL); 7, GC (GCS); 8, GVC; 9, CT10.

		Morpho- logical Changes	Anchorage Indepen- dence	Tumor Formation
CT10 (GV)		+++	+++	+++
GC		++	++	++
GVC		++	++	++
GCC		+	+	-
SHB (V)		++	+	+
c-Crk (CC)		+	+	-

A



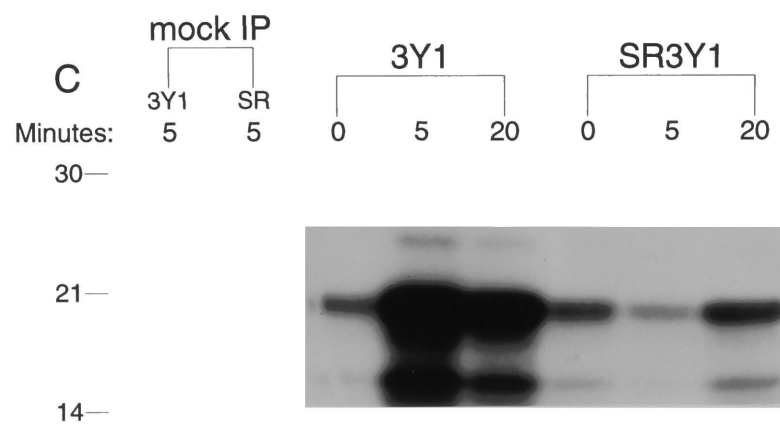
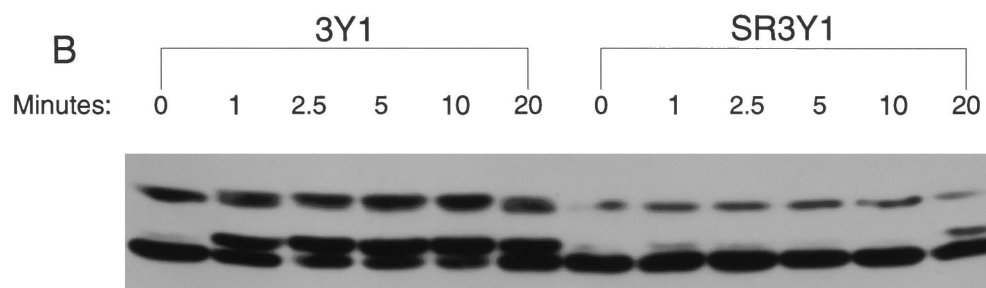
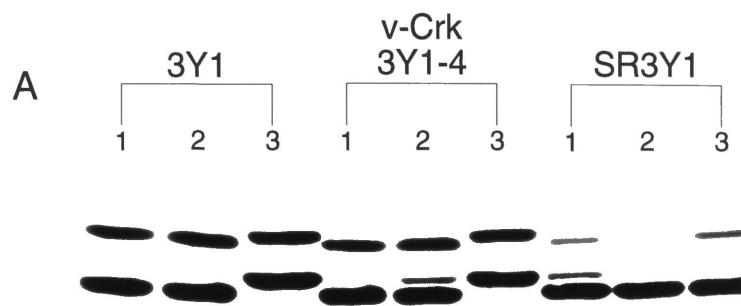
B



fusion c-Crk construct, GCL, for which no expression was detected. After extended culture under soft agar, cells were placed in liquid medium containing 0.5% calf serum for 24 hours, then serum stimulated for the number of minutes indicated in Figure 30B, and lysed. Interestingly, delay of serum stimulation seemed more tightly correlated with metabolic rate than transformation. For example, CT10 is more efficient at promoting colony growth in soft agar than “gag-c-short” (GCS), a gag fusion c-Crk construct with truncation of the second SH3 domain and Tyr 222, while GCS-infected cells metabolized faster than CT10-infected cells, as evidenced by acidity of the culture media. Yet GCS was even more efficient than CT10 in inhibition of serum stimulation of Mapk. Also, while c-Crk has almost no ability to promote anchorage-independent growth, it did cause cells to grow and metabolize faster than helper virus, and did cause a subtle delay in serum stimulation of Mapk.

The effects of oncogene transformation on serum stimulation of Mapk were also examined in clonal cell lines. As shown in Figure 31A, 5 minute serum stimulation of Mapk hyperphosphorylation in the very transformed (and fast-metabolizing) Schmidt-Rupin 3Y1 cells (SR3Y1) was inhibited, while the low-expressing, non-transformed v-Crk 3Y1-4 line exhibited a response similar to the parental 3Y1s. Like the v-Crk transformed CEF, the SR3Y1 Mapk response to serum was delayed, illustrated by the time course shown in Figure 31B. Both p42 and p44 Mapk are present in these cell lines, and the response of these two proteins to serum seems to be uniform. The V4 v-Crk NIH-3T3 cell line was also tested for kinetics of Mapk response to serum, but only an infinitesimal delay was observed, probably because these cells do not metabolize at the same elevated rate as the SR3Y1. However, a long-term passage isolate of the V7 v-Crk NIH-3T3 that was very transformed and

Figure 31. Delay of serum stimulation of Mapk hyperphosphorylation and kinase activity in Schmidt-Rupin 3Y1 cells. A, Whole cell lysate anti-Mapk Western blot of 3Y1, nontransformed v-Crk 3Y1, and transformed v-Src 3Y1 (SR3Y1). Lane 1: cells maintained in medium containing 5% calf serum, 2: cells maintained in medium containing 0.5% calf serum, 3: cells incubated 48 hours in medium containing 0.5% calf serum, then stimulated with medium containing 10% calf serum for 5 min. B, Whole cell lysate anti-Mapk Western blot of cells incubated 48 hours in medium containing 0.5% calf serum, then 10% serum stimulated for the indicated number of minutes. C, Mapk immune-complex kinase assay. Mapk was immunoprecipitated from 30 μ g lysate of cells stimulated with 10% serum for the indicated number of minutes with anti-Mapk C-1 (same lysates as in B). The first two lanes are mock IPs with only protein A sepharose added. IPs were assayed on 10 μ g MBP for 30 min at 30 °C.

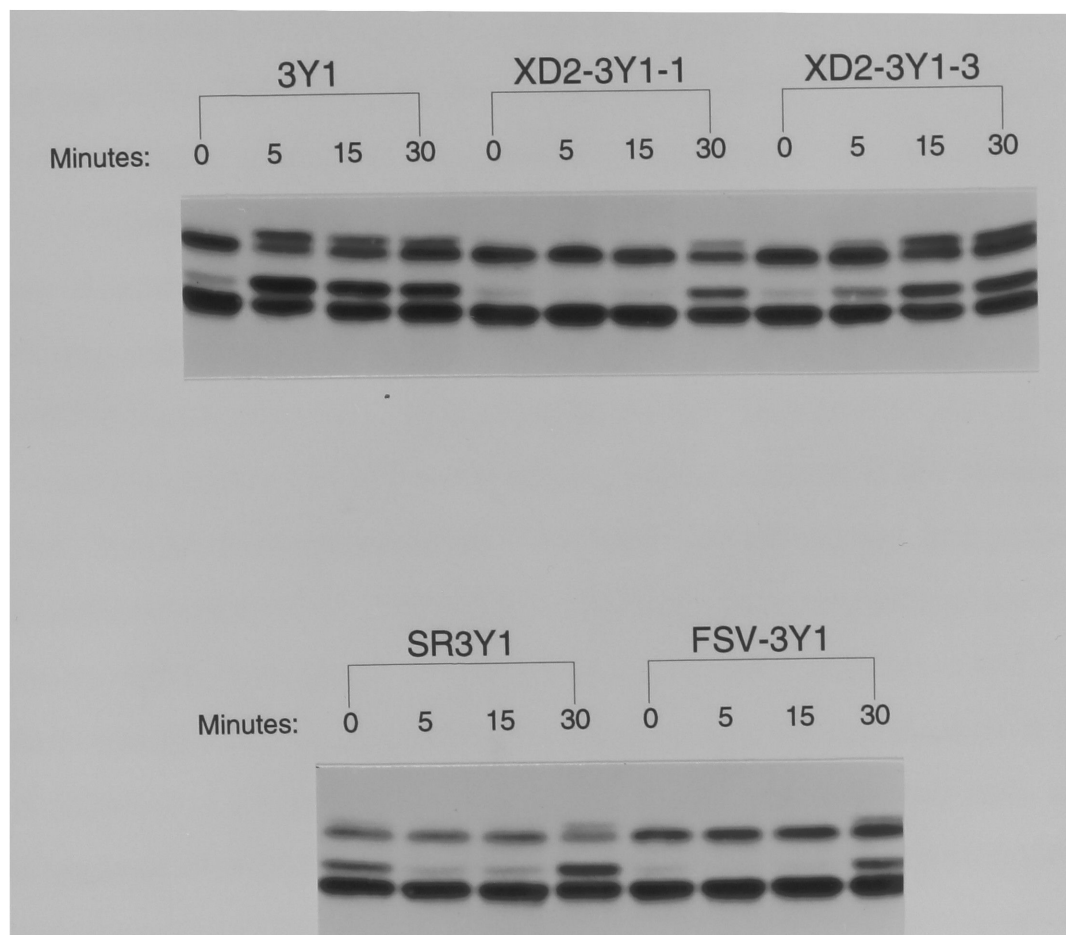


metabolized at a high rate did exhibit a delay in serum stimulation of Mapk hyperphosphorylation (data not shown).

The hyperphosphorylation results were mirrored by MBP immune complex kinase assay results, shown in Figure 31C. Because the lysates are not denatured in this assay, only p44 activity is reflected in the levels of MBP phosphorylation. The first two lanes are mock IPs, with protein A sepharose but not anti-Mapk added to the indicated lysates, to assay the activity of nonspecific MBP kinases that bind at low levels to protein A beads. The contribution of these kinases to the observed MBP phosphorylation is negligible at this exposure. The remaining lanes represent Mapk IPs from 3Y1 and SR3Y1 cells serum stimulated for the indicated number of minutes. Interestingly, serum stimulation seems to temporarily decrease basal levels of Mapk activity in SR3Y1 cells, before the activity eventually begins to rise after 20 min. This phenomenon is also observed as a decrease in basal levels of Mapk hyperphosphorylation in the whole cell lysate Westerns in Figures 31A and 31B.

The degree of inhibition of Mapk serum response in other cell lines expressing various levels of v-Src was next examined. Fast-metabolizing lines SR3Y1 and XD2-3Y1-1, and slower-metabolizing XD2-3Y1-3 (expressing lower levels of v-Src), as well as the v-Fps transformed line FSV-3Y1, were serum stimulated for the number of minutes indicated in Figure 32. As shown in the whole cell lysate anti-Mapk Western blot, the inhibition of Mapk hyperphosphorylation in XD2-3Y1-1 was comparable to that observed for SR3Y1, while the XD2-3Y1-3 displayed less inhibition. A delay in serum stimulation of Mapk hyperphosphorylation was also observed in the FSV-3Y1 cells.

Figure 32. Delay in Mapk hyperphosphorylation in 3Y1 cell lines expressing different levels of v-Src. Whole cell lysate anti-Mapk Western blot. Cells were incubated 24 hours in medium containing 0.5% calf serum, then stimulated with 10% serum for the indicated number of minutes.

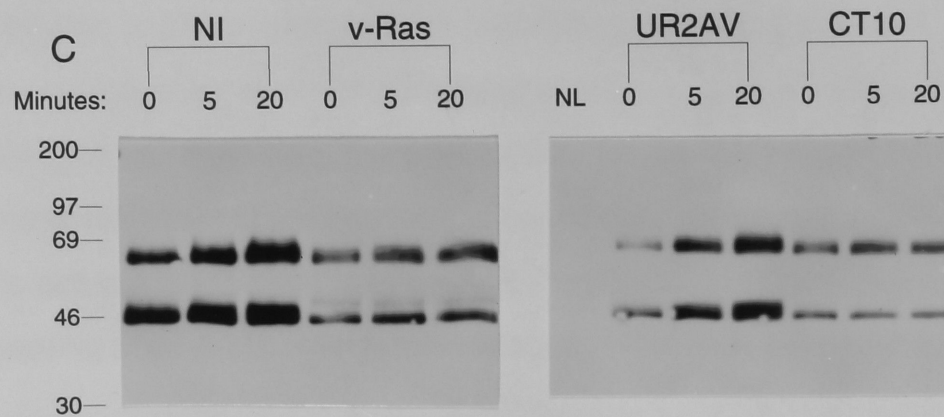
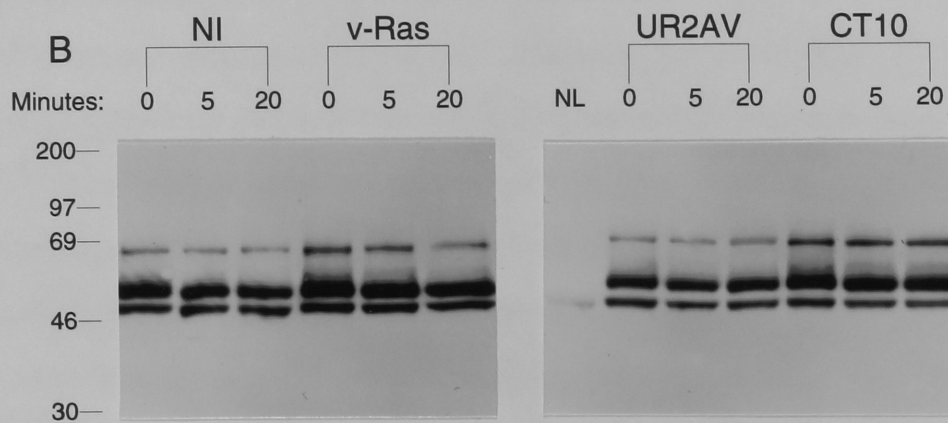
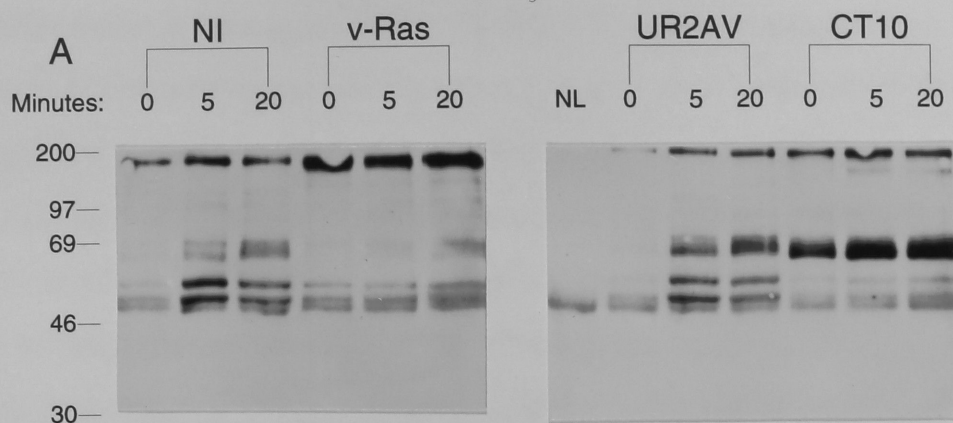


Possible mechanisms for the transformation-induced inhibition of serum stimulation were examined. The possible contribution of media acidity to the delay was studied by experiments in which media on transformed and nontransformed cells were switched 24 hours after initial incubation with 0.5% calf serum, as well as experiments in which the low-serum media on the transformed cells was changed twice after the initial 24 hour incubation to avoid extreme acidity. These medium manipulations had no effect on the delay of serum stimulation of Mapk in oncogene-transformed cells (data not shown).

Preliminary studies implicated the Shc protein as a participant in the delay of serum stimulation of Mapk in oncogene-transformed cells. Shc was immunoprecipitated from uninfected ("NI"), v-Ras transformed, UR2AV-infected, and CT10-transformed CEF. In each panel, the "NL" lane is an IP with no lysate to reveal the contribution of the precipitating immunoglobulin to the observed signal. The IPs were separated on SDS-PAGE, Western blotted, and probed with anti-phosphotyrosine (Figure 33A). Tyrosine phosphorylation of the three isoforms of Shc upon serum stimulation was observed in uninfected and UR2AV-infected CEF by 5 min. However, little tyrosine phosphorylation of Shc was observed in the serum stimulated v-Ras and v-Crk transformed cells, and this low level of phosphorylation exhibited delayed kinetics. Reprobing the same blot with anti-Shc (Figure 33B) indicated the presence of similar amounts of the three Shc isoforms in all IPs.

The anti-Shc Western in Figure 33B also revealed a discrepancy in the "thickness" of the p66 Shc band and the tyrosine phosphorylated band observed around 66 kDa in Figure 33A: another tyrosine phosphorylated protein of similar migration had apparently been coprecipitated with Shc. The suggestive molecular weight, as well as the presence of this tyrosine

Figure 33. Delay in serum stimulation of Shc phosphorylation in oncogene-transformed CEF. Cells were incubated in medium containing 0.5% serum, stimulated in 10% serum for the indicated number of minutes, then lysed. 200 μ g each lysate was immunoprecipitated with anti-Shc, separated by SDS-PAGE, and Western blotted. Westerns were probed with monoclonal anti-phosphotyrosine (A), and reprobbed with polyclonal anti-Shc (B), and monoclonal anti-paxillin (C). NL = no lysate.



phosphorylated protein in Shc IPs from v-Crk transformed cells in the absence of serum stimulation provided the impetus to reprobe the Western with anti-paxillin, shown in Figure 33C.

p70 paxillin is clearly present in all of the Shc immunoprecipitates, and the amount of coprecipitating paxillin increased with serum stimulation in the uninfected and UR2AV infected cells. Furthermore, the additional coprecipitating paxillin migrates more slowly than the paxillin present in the cells prior to serum stimulation, suggesting that paxillin is tyrosine phosphorylated upon serum stimulation, although the isoforms created migrate differently than tyrosine phosphorylated paxillin in v-Crk transformed cells. Binding could be mediated by the Shc SH2 domain, but this would not explain the presence of paxillin in IPs from unstimulated NI and UR2AV cells that is not observed in the anti-phosphotyrosine blot. It is more likely that Shc and paxillin associate constitutively in a phosphotyrosine-independent manner, and that growth factor-induced tyrosine phosphorylation of paxillin allows a greater percentage to associate with Shc, possibly in a phosphotyrosine-dependent manner. Only a small percentage of cellular paxillin precipitated with Shc in these experiments (data not shown). The protein of about 45 kDa that reacts with anti-paxillin could be a breakdown product. However, it too does not react with anti-phosphotyrosine.

Inhibition of Shc phosphorylation therefore represents a potential mechanism of the delay of serum stimulation of Mapk in oncogene-transformed cells. However, this most likely does not account for the delay observed in phorbol ester treatment of oncogene-transformed cells, as phorbol esters are thought to activate the Ras pathway by phosphorylation of Raf by activated PKC, thus bypassing Shc. A separate mechanism may function in the phorbol ester

system, or there may exist a more universal mechanism. In either case, further study is required.

DISCUSSION

The data presented here constitute an attempt to investigate the nature of signalling pathways utilized by the v-Crk oncogene in the process of transformation. Like v-Src transformation and EGFR activation, v-Crk transformation is accompanied by an elevation of tyrosine phosphorylation on specific proteins. The first approach therefore consisted of a search for serine/threonine kinases that might be activated in v-Crk transformed cells, by analogy to the kinase cascades initiated by v-Src and the activated EGFR. The results of this search implicated the Ras pathway in v-Crk transformation, and the specific role of c-Ras function in v-Crk transformation was analyzed.

Mapk is activated in CT10-transformed CEF

Mapk is implicated as a component of the signalling machinery utilized by v-Crk in the process of CEF transformation. After an increase in total MBP kinase activity was demonstrated in whole cell lysate from CT10 transformed cells (Figure 5), the increase in MBP kinase activity was demonstrated to be localized to specific Mono-Q fractions (Figure 6). While the presence of activated Mapk correlates with the elevated MBP kinase activity of the fractions in question, there may be other MBP kinases in these fractions. In fact, it is likely that another kinase of similar Mono-Q elution profile and similar molecular weight is present, because the Superose 12 peak fraction was able to phosphorylate H2B (Figure 7), while Mapk has only a low level of activity towards H2B (Boulton et al., 1991b and data not shown). Other MAP kinase family members, previously described or novel, are possible candidates.

An activation of Mapk in CT10 transformed CEF has been demonstrated by immunoprecipitation of denatured protein, followed by IGKA analysis (Figure 9B). However, this three-fold elevation of kinase activity is significantly lower than the eight-fold constitutive elevation observed in v-Ras and v-Src transformed cells. It is therefore likely that other, possibly novel, signalling pathways are also involved in v-Crk transformation in these cells.

The possible existence of other kinases with properties similar to those of Mapk in the Mono-Q and Superose 12 fractions, mentioned above, would help explain the discrepancy between the three-fold activation of Mapk observed by IP and IGKA, and the approximately ten-fold activation of MBP kinase activity in the Mono-Q fractions in which Mapk elutes (Figure 6). The possibility of nonlinear IGKA conditions also exists, as the kinetics of this assay are crude; for example, time must be allowed for diffusion of the isotope into the gel. However, different amounts of the same lysate analyzed by IGKA generated signals roughly proportional to the quantity of protein in the sample, indicating that no reagents are limiting (data not shown).

Consistent with the IGKA data (Figures 9B and 9C), Mono-Q fractionation of lysates from v-Ras transformed cells revealed an even greater elevation of MBP kinase activity in v-Ras fractions containing hyperphosphorylated Mapk protein (Figure 8). Furthermore, serum stimulation, the most potent Mapk activating stimulus of those described here, caused the greatest increase in MBP phosphorylation with Mono-Q fractions containing activated Mapk. These data suggest that increases in MBP kinase activity in fractions co-eluting with hyperphosphorylated Mapk can at least partially be attributed to Mapk.

IGKA analysis revealed a complex combination of MBP kinases in the whole cell lysates and various fractions (Figure 10). The MBP kinase of about

60 kDa present in both fractions could be responsible for masking the large elevation of MBP kinase activity, seen in the Mono-Q fractions (Figure 6), from observation in the whole cell lysate (Figure 5). The 60 kDa kinase is almost completely absent from UR2AV fraction 34 and CT10 fraction 34. The MBP kinase of approximately 35 kDa potentially represents another kinase activated by v-Crk transformation, or may even be the H2B kinase (Figure 7). However, it is unlikely that it plays a major role in MBP phosphorylation by Mono-Q fraction 34, since it is not particularly enriched in this fraction in CT10 cells.

One limitation of IGKA analysis is that not all kinases are capable of renaturing under these conditions. The presence of MBP kinases of other molecular weights potentially activated in CT10 cells therefore cannot be excluded from CT10 Mono-Q fraction 34, although kinases of this nature were not detected in the Superose 12 fractions. It is unfortunately impossible to preclear p42 Erk2 from nondenatured lysate (due to lack of an antibody) to allow examination of nonrenaturable kinases that may exist in the Mono-Q and Superose 12 fractions.

A Mek immunocomplex kinase assay confirmed upregulation of Mek kinase activity in v-Crk transformed chicken embryo fibroblasts (Figure 11). However, Mek activity was elevated only about two-fold. It is likely that this is sufficient for the three-fold upregulation of Mapk activity, although Mek2 may also contribute to the activation of Mapk in CT10 transformed CEF (not tested).

Tyrosine phosphorylation of Shc mediates activation of the Ras pathway in both v-Src transformation and EGFR activation (McGlade et al., 1992b). However, Shc is not tyrosine phosphorylated in v-Crk transformed CEF, nor is there any evidence that it can associate with v-Crk after serum stimulation of these cells (Figure 13). The GAP-associated p190, which is tyrosine

phosphorylated in v-Src transformed and EGF-stimulated cells (Ellis et al., 1990), is also not tyrosine phosphorylated in v-Crk transformed CEF (Figure 12). Taken together, these data indicate that putative activation of the Ras pathway in v-Crk transformed CEF, as manifested by Mek and Mapk activation, is not mediated by the same mechanism as utilized by v-Src and the activated EGFR. Alternatively, the formal possibility remains that Mapk is activated by v-Crk through a Ras-independent pathway.

v-Crk transforms NIH-3T3 cells

v-Crk had been previously reported as only “marginally transforming” when overexpressed in the rat 3Y1 fibroblast cell line (Sabe et al., 1992), raising the question of whether v-Crk is capable of transforming mammalian cell lines. Although the data presented here provide evidence that v-Crk can transform the murine NIH-3T3 cell line, a contribution from spontaneous mutations in culture cannot be ruled out. The inability of v-Crk to transform 3Y1 cells may therefore be accounted for by the lower expression levels of v-Crk attained in these cells (data not shown), a genetic background of these cells requiring more signals than those sent by v-Crk for full transformation, or a greater resistance of these cells to spontaneous mutation than the NIH-3T3.

The morphological alteration (Figure 14), colony formation in soft agar (data not shown), and elevation of tyrosine phosphorylation on p70 and p130 (Figure 15), as well as the dependence of the extent of these phenotypes on levels of v-Crk expression, all indicate that v-Crk transforms NIH-3T3 cells in a manner similar to CEF. In addition, Shc and the GAP-associated p190 are not phosphorylated in the v-Crk 3T3, similar to the situation in the CT10 transformed CEF (Figure 18 and data not shown). The only discrepancy is the lack of

significant elevation of MBP kinase activity in Mono-Q fractions (Figure 17A and B), and the lesser activation of Mapk (Figure 17C), compared with that observed in CEF. One confounding factor is the level of expression of v-Crk. While the V4 3T3 overexpress v-Crk about 20-fold, the levels of v-Crk expressed by fully transformed CT10-infected CEF are more than two-fold higher (data not shown). In fact, MDCK cells that express levels of v-Crk about four-fold higher than found in the V4 NIH-3T3 exhibit constitutive elevation of Mapk activity (data not shown). The possibility that a greater constitutive activation of Mapk would occur in NIH-3T3 expressing a higher level of v-Crk remains.

The lack of apparent increase of MBP kinase activity in the V4 3T3 fractions containing Mapk protein (Figure 17A and 17B) can be accounted for by analysis of the fractions by IGKA (data not shown). Although a 42-44 MBP kinase activity is present specifically in the V4 fractions, an MBP kinase of approximately 35 kDa is present in M2 fractions 34 and 35, and is even more active in IGKA than the 42-44 kDa MBP kinase. However, the 42-44 MBP kinase activity (or activities) does not exactly correlate with the presence of hyperphosphorylated Mapk in the V4 fractions, implicating other activated MBP kinases in these cells. These results suggest that the classical Ras-Raf-Mek-Mapk pathway is not the only signalling pathway activated in v-Crk transformation of NIH-3T3 cells.

Dominant negative Ras reverts v-Crk transformation

Expression of dominant negative Ras (Figure 19), expression of Ras GAP (data not shown), and addition of the Ras farnesylation inhibitor B581 (Figure 23) all caused morphological reversion of v-Crk transformed 3T3 cells. Inhibition of colony formation in soft agar was also observed (Table 2). This

indicates that a Ras-dependent signal transduction pathway is necessary for v-Crk transformation, although, for reasons described above, this Ras-dependent pathway is probably not the classical Ras-Raf-Mek-Mapk pathway.

Dominant negative Ras (N17 and Y57) was expressed at low levels in the pooled transfectants, consistent with its growth-inhibitory properties (Figure 20). However, higher levels were observed in the v-Raf 3T3, in which growth and transformation are driven by a constitutively activated Raf kinase, which lies downstream of Ras on the classical Ras-Raf-Mek-Mapk pathway. The expression of Ras N17 in the clonal stable cell lines indicates either that higher levels of dominant negative Ras are required for full growth inhibition than for reversion of transformation, or that Ras-independent mechanisms of growth stimulation exist in these cells. The latter is not unlikely, since several growth-promoting pathways, such as those initiated by PLC and PI3K, are activated by growth factor stimulation (Valius and Kazlauskas, 1993). In addition, ligands for the G-protein-coupled receptors capable of activating PKC may also upregulate Raf activity in a Ras-independent manner by PKC-mediated phosphorylation of Raf (Kolch et al., 1993). The stable N17 lines did, however, lose Ras expression with time in passage (data not shown), indicating the existence of selective pressure against high levels of dominant negative Ras.

Unaltered levels of v-Crk expression in the dominant negative Ras-expressing and B581-treated v-Crk 3T3 cells confirmed that reversion of transformation in these cells was not due to a loss of v-Crk protein (Figures 21 and 23). However, recently published data indicate a potential caveat of the farnesylation inhibitor experiments. At least in one system, the fast kinetics of reversion (less than 18 hours) did not correlate with inhibition of Ras processing (Prendergast et al., 1994). Although the examination of the state of Ras

processing yielded ambiguous results in the B-581-treated V4 cells (data not shown), the reversion in this experiment did require several days of treatment, more consistent with the expected kinetics of farnesylation inhibitor action.

The isolation of stable clonal cell lines expressing dominant negative Ras N17 confirmed the results of the pooled transfectant assay. The morphological reversion was more uniform in these lines than in the heterogenous transfectants (compare Figure 24 with Figure 19), and the V4N17-4 line, which initially exhibited about ten-fold overexpression of dominant negative Ras (Figure 25A), grew markedly slower than the other lines examined. V4 colony formation was also inhibited in the N17 lines (Table 3). Levels of v-Crk protein expression were not decreased in the V4N17 lines (Figure 25B). However, the length of time necessary for selection, growth, and analysis of clonal cell lines was sufficient for the apparent accumulation of transformation-promoting mutations in the V4pBp cells.

Expression of dominant negative Ras in the clonal cell lines was not sufficient to block serum stimulation of Mapk hyperphosphorylation (Figure 26), although a partial delay was observed in the V4N17-4 cell line that overexpressed Ras N17 about ten-fold. However, this can easily be accounted for by the presence of Ras-independent pathways leading to Mapk activation, such as the previously-mentioned G protein-dependent pathways resulting in PKC activation (Kolch et al., 1993). EGF stimulation of Mapk activity in Rat-1 cells was shown to be independent of Ras function (Burgering et al., 1993), but these phenomena are clearly cell-type specific. Insulin stimulation of Mapk might provide a more appropriate system to study, since insulin stimulation of Mapk in NIH-3T3 cells was shown to be Ras-dependent (Vries-Smits et al., 1992).

v-Crk sends multiple signals

Expression of dominant negative Ras in either pooled transfectants or clonal cell lines did not decrease levels of tyrosine phosphorylation of p130 or p70 in the v-Crk transformed NIH-3T3 cells (Figures 22A and 25C), indicating both that Ras function is not necessary for v-Crk-induced tyrosine phosphorylation of cellular proteins, and that tyrosine phosphorylation of cellular proteins is not sufficient for v-Crk transformation. While the dominant negative Ras experiments indicate that Ras function is necessary for v-Crk transformation, it is not yet known if tyrosine phosphorylation of cellular proteins is necessary. The only information available is the correlation of v-Crk mutant transforming potential with ability to induce tyrosine phosphorylation of cellular proteins (Mayer and Hanafusa, 1990b). This could potentially be further examined by use of a general tyrosine kinase inhibitor, such as genistein.

These data suggest that v-Crk sends multiple, cooperating signals in the process of cellular transformation, as is the case for both v-Src transformation and EGFR stimulation. v-Src is capable of stimulating PI3K activity (Fukui and Hanafusa, 1989) and sends a myriad of other as yet undefined tyrosine phosphorylation-dependent signals, as well as stimulating the Ras pathway (Satoh et al., 1990). Similarly, the EGFR activates the pathways headed by PLC γ activation (Nishibe et al., 1990), and STAT phosphorylation (Ruff-Jamison et al., 1993; Silvennoinen et al., 1993; Fu and Zhang, 1993), as well as Ras activation (Satoh et al., 1990).

The Jak-STAT pathway involves direct tyrosine phosphorylation of a specific subset of the STAT family of transcription factors in response to stimuli such as interferons (IFNs), cytokines, and growth factors (reviewed in Darnell et al., 1994). This phosphorylation event is most likely mediated by one or more of

the Jak family kinases, a specific subset of which is also activated by specific stimuli. The phosphorylated STATs then dimerize in an SH2- and phosphotyrosine- dependent manner, and translocate to the nucleus, where they can bind specific response elements in gene promoters and activate transcription. However, a role for these STAT proteins in cell transformation has not been described. Preliminary experiments showed that while STAT3 is tyrosine phosphorylated in v-Src transformed 3T3 cells, neither STAT1 nor STAT3 are tyrosine phosphorylated in v-Crk transformed cells (data not shown). There is thus no evidence to date for the participation of the Jak-STAT pathway in v-Crk transformation. However, this does not rule out the possible contribution of other members of the growing family of STAT proteins.

Immunofluorescence studies indicated that v-Crk was at least partially localized to putative focal adhesions (Figure 16). This is consistent with both the likely gag-mediated membrane association of v-Crk and the ability of v-Crk to bind tyrosine-phosphorylated paxillin (Birge et al., 1993). High levels of v-Crk expression were necessary for focal adhesion localization (data not shown), suggesting that the level of tyrosine phosphorylation on paxillin is a determining factor. The role of the v-Crk SH2 domain in focal adhesion localization is now being examined by expression of v-Crk mutants (P. van Bergen en Henegouwen, personal communication).

Dominant negative Ras expression did not affect localization of v-Crk to the putative focal adhesions in the V4 3T3 cells (Figure 22). This is consistent with the lack of an effect of Ras N17 expression on tyrosine phosphorylation of paxillin in v-Crk transformed 3T3 cells. However, the physical mechanism of morphological reversion of v-Crk cells expressing dominant negative Ras remains a black box.

A model for v-Crk activation of Ras-dependent pathways

A model for v-Crk activation of Ras-dependent pathways is therefore proposed (illustrated in Figure 34). v-Crk associates with cellular membrane fractions (Mayer and Hanafusa, 1990b; data not shown), and could function similarly to the Shc/Grb2/Sos complex by binding Sos with its SH3 domain, bringing Sos into proximity with Ras by virtue of its gag-mediated membrane association. This would promote nucleotide exchange on Ras, thereby activating Ras. v-Crk may similarly bind and relocate C3G, another Ras family guanine nucleotide exchange factor, but the consequences of this event are less clear. In CEF, Ras activation results in upregulation of the classical Ras-Raf-Mek-Mapk pathway, or a Ras-independent pathway leading to Mapk activation, as evidenced by constitutive elevation of Mek and Mapk activities. However, this pathway may not be the driving force for transformation in the v-Crk NIH-3T3 cells. Instead, Ras may activate a second, undefined Ras-dependent pathway.

There is ample evidence that such Ras-dependent, Raf-independent pathways exist in specific contexts. The best-described example is the Jnk family of stress-activated dual specificity kinases, related to the Mapks. These kinases phosphorylate the nuclear protein c-Jun in response to cellular stress, such as exposure to ultraviolet light, in a Ras-dependent (Hibi et al., 1993; Derijard et al., 1994), Raf-independent (Minden et al., 1994b; Yan et al., 1994b) manner. The activity of Jnk in v-Crk transformed cells is currently being studied. In addition, other isolated examples of Ras-dependent, Raf-independent signal transduction pathways are emerging, such as thyrotropin stimulation of DNA synthesis in thyroid cells (Al-Alawi et al., 1995).

The Nck adaptor protein, which consists of three SH3 domains and one

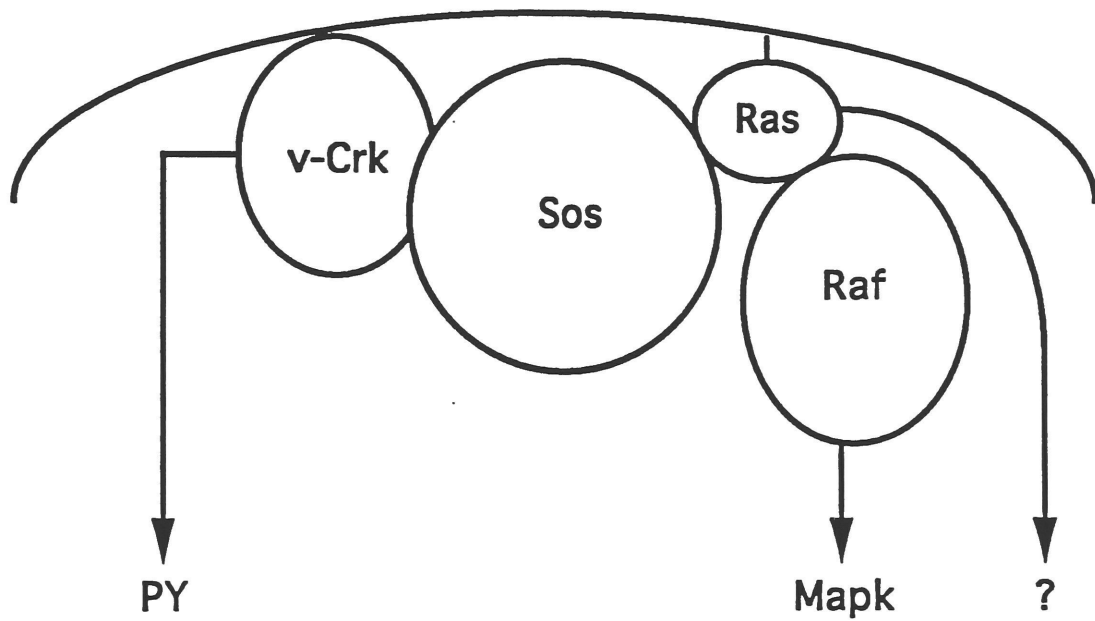


Figure 34. Model for the activation of a Ras-dependent pathway by v-Crk

SH2 domain, has also been shown to function in a manner analogous to Shc/Grb2. While SH2-mediated binding of Nck to activated growth factor receptors had already been established (Park and Rhee, 1992; Li et al., 1992), it has only recently been reported that Nck is capable of SH3-mediated association with Sos, and that co-expression of the Nck cDNA and a fos reporter construct in NIH-3T3 cells activates the *fos* gene promoter in a Ras-dependent manner (Hu et al., 1995). Examination of fos reporter construct activation in CT10-transformed CEF yielded ambiguous results (data not shown).

The model for v-Crk activation of Ras is currently being examined by overexpression of a C3G-derived protein fragment containing three of the four proline-rich Crk binding sites in C3G (but not the C3G GEF domain) in the M2, V4, and v-Src NIH-3T3 cells. This “dominant negative C3G” should bind the v-Crk SH3 domain and block binding of both Sos and C3G (as well as c-Abl) to v-Crk. Cell morphology, the binding of Sos and v-Crk, and Ras pathway activation will be examined in these transfectants.

In addition to the Ras-dependent signal, v-Crk sends a second, Ras-independent signal by tyrosine phosphorylation of cellular proteins p130 (Cas) and p70 (paxillin). This signal results in the binding of v-Crk to both Cas and paxillin (Birge et al., 1992; Birge et al., 1993) and concomitant localization of at least some v-Crk to focal adhesions. In addition, tyrosine-phosphorylated paxillin and Cas may serve as “docking proteins” for additional signalling molecules. Paxillin contains a proline rich motif recognized by the c-Src SH3 (Weng et al., 1993), and several tyrosines that may bind additional SH2 domains when phosphorylated (Turner and Miller, 1994). Cas contains one SH3 domain and as many as 15 YXXP motifs, all of which form potential Crk

SH2 binding sites when phosphorylated (Sakai et al., 1994; Songyang et al., 1993). A full analysis of proteins capable of binding paxillin and Cas has not yet been completed. However, paxillin and Cas may function in a manner analogous to the Insulin Receptor Substrate 1 (IRS-1), which is phosphorylated at multiple tyrosines upon insulin stimulation, creating binding sites for several SH2-containing proteins, including PI3K, Grb2, the protein tyrosine phosphatase Syp, and the Nck adaptor protein (reviewed in White and Kahn, 1994).

Because c-Src kinase activity was found to be upregulated in 3Y1 cells overexpressing both c-Src and v-Crk, v-Crk was hypothesized to transform primarily by activating the c-Src kinase (Sabe et al., 1992), possibly by displacing Csk from paxillin. This may represent yet another parallel signalling pathway activated by v-Crk, and is being further investigated by the overexpression of Csk in v-Crk transformed NIH-3T3 cells.

Oncogene transformation delays the serum stimulation of Mapk activity

A delay in the serum stimulation of Mapk activity in oncogene-transformed cells has been observed (Figures 27 and 31). While the exact kinetics and extent of activation vary from experiment to experiment, a delay is always observed in the transformed cells relative to the nontransformed cells in the same assay. In addition, long timecourses have revealed that final level of peak activation in oncogene transformed cells is often reduced in magnitude as well, also with some variation from experiment to experiment (data not shown).

The degree of inhibition of Mapk activation in response to serum in CT10 transformed CEF increased with time in culture (Figure 28). The total levels of v-Crk expressed in these cultures also increased during the period of

examination, but it cannot be distinguished whether this is a result of greater expression levels of v-Crk in individual cells or if this reflects a greater number of infected cells. This question could be further analyzed using immunofluorescence. In addition to increased levels of oncogene expression, the cells exhibiting a greater delay in Mapk activation metabolized faster, as evidenced by the greater acidity of the media.

Metabolic rate may in fact be the crucial determinant of this phenotype. Transforming potential of various v-Crk mutants was not as tightly correlated with the delay in serum stimulation of Mapk as medium acidity (Figure 30). The delay of serum stimulation of Mapk also correlated with metabolic rate in a series of v-Src 3Y1 cell lines (Figure 32), despite a lack of drastic differences in oncogene expression (data not shown). However, medium acidity per se does not seem to be the cause of this phenotype (data not shown).

The most intriguing observation that may reflect the mechanism of this phenotype is a similar delay in serum stimulation of Shc tyrosine phosphorylation (Figure 33). The delay in serum stimulation of Shc phosphorylation was observed in both v-Crk transformed and v-Ras transformed CEF. However, while this could account for the delay in serum stimulation of Mapk, it probably cannot account for the delay in phorbol ester stimulation of Mapk, which is most likely not mediated by Shc. Furthermore, it cannot be distinguished at this time whether Shc phosphorylation is an integral part of the mechanism of the delay in serum stimulation of Mapk, or just another manifestation of a more universal phenomenon.

Oncogene transformation affects signal transduction at many levels

Others have made observations that may may be related to the delay in

serum stimulation of Mapk in oncogene transformed cells. Transformation of 3Y1 cells by v-Src, v-Sis, v-Ras, and v-Raf, but not SV40, attenuated serum stimulation of immediate early gene transcription, including that of *c-fos*, *c-jun*, *junB*, and *egr-1* (Yu et al., 1993). The authors hypothesized that transcriptional upregulation of these nuclear genes was inhibited by chronic stimulation of signaling molecules in cells transformed by these oncogenes, all of which activate the Ras pathway at some level. Similar results were reported by a group studying PDGF stimulation of *c-myc* and *c-fos* transcription (Zullo and Faller, 1988). However, this study found that the phenotype may be specific for PDGF; no suppression of *c-myc* transcription was observed in FGF treated v-Ras transformed Balb/c-3T3 cells. A third group observed inhibition of serum stimulation of *c-fos*, *c-jun*, and *c-myc* transcription in v-Src transformed cells (Welham et al., 1990).

Effects of oncogene transformation on Ras pathway elements upstream of immediate early gene transcription have also been examined (Samuels and McMahon, 1994). Expression of Δ Raf-1:ER, a fusion of the hormone binding domain of the estrogen receptor to an oncogenic form of Raf, caused morphological transformation in C2-3T3 cells upon β -estradiol stimulation, but no induction of DNA synthesis was observed. Furthermore, activation of Δ Raf-1:ER inhibited PDGF and EGF stimulation of Raf and Mapk activity, as well as DNA synthesis. However, no inhibition of serum or phorbol ester stimulation of Raf or Mapk activity occurred. In all cases, Mek activity was maintained at a relatively high level in β -estradiol-stimulated cells, and was not much further increased by additional growth stimuli. Treatment of the Δ Raf-1:ER cells with sodium orthovanadate alleviated the inhibition of PDGF stimulation of Mapk

activity, implicating a phosphatase, such as MKP-1, in the mechanism of inhibition.

This group proposed that two levels of regulation are uncoupled by Raf transformation. The first involves uncoupling of PDGFR stimulation and Raf activation (but not p70 S6 kinase activation, which occurs as in nontransformed cells). There is no evidence for involvement of PKA in this uncoupling, as no increase in intracellular cAMP was detected, and the ability of Raf to physically interact with Ras-GTP in these cells was not affected. The authors hypothesized that an element upstream of Ras may be involved, because Sos was constitutively phosphorylated at high levels in the Δ Raf-1:ER cells. It has recently been shown that only unphosphorylated Sos is found in complexes with Shc (Vries-Smiths et al., 1995), suggesting that phosphorylated Sos is not capable of transducing growth signals.

The abrogation of Raf activation in this system is probably not the direct cause of inhibition of Mapk activation. Instead, evidence points to the existence of a second level of regulation uncoupled by v-Raf transformation: a phosphatase capable of preventing Mapk activation in the Δ Raf-1:ER cells. A search for the vanadate sensitive inhibitor of Mapk in these cells is being attempted by the authors, who suggest that the phenomena they observe are due to constitutive activation of feedback inhibition loops normally activated after growth factor stimulation.

This group's results support the possibility that several levels of signal transduction may be affected by oncogene transformation. While a delay in Shc phosphorylation may contribute to the delay in Mapk activation by serum stimulation, other signalling molecules that function in the pathway from PKC to

Mapk may be simultaneously affected, causing a delay in phorbol ester stimulation of Mapk. It is unclear why the response of Mapk to serum and phorbol ester stimulation described by Samuels and McMahon (1994) showed no inhibition in the v-Raf transformed cells. However, it would be interesting to examine the effects of vanadate treatment on the delay of serum stimulation of Mapk in the oncogene transformed cells described here.

Several potential mechanisms of the delay of serum stimulation of Mapk in oncogene transformed cells can thus be envisioned. The driving force may be global cellular changes, such as radical alteration of cellular architecture, as manifested by morphological transformation, or increased activity of metabolic pathways, which may affect the machinery responsible for transmission of growth signals in some undefined manner. Alternatively, the mechanism may be more subtle, involving specific inhibition of individual proteins functioning in signal transduction pathways stimulated by growth factors. This could be accomplished by gradual desensitization of key Ras pathway signalling molecules due to chronic stimulation, or by the constitutive activation of feedback inhibition loops already in place to ensure a normal transient response to growth factor stimulation. The downregulation of the Ras pathway also implies that oncogenes can signal via other as yet undefined pathways to maintain a cell in a growing, transformed state.

BIBLIOGRAPHY

Aelst, L Van, M Barr, S Marcus, A Polverino, and M Wigler (1993). Complex formation between Ras and Raf and other protein kinases. *Proc Natl Acad Sci USA* 90: 6213-6217.

Ahn, NG, R Seger, RL Bratlien, CD Diltz, NK Tonks, and EG Krebs (1991). Multiple components in an epidermal growth factor-stimulated protein kinase cascade. *J Biol Chem* 266: 4220-4227.

Ahn, NG, JS Campbell, R Seger, AL Jensen, LM Graves, and EG Krebs (1993). Metabolic labeling of mitogen-activated protein kinase kinase in A431 cells demonstrates phosphorylation on serine and threonine residues. *Proc Natl Acad Sci USA* 90: 5143-5147.

Al-Alawi, N, DW Rose, C Buckmaster, N Ahn, U Rapp, J Meinkoth, and JR Feramisco (1995). Thyrotropin-induced mitogenesis is Ras dependent but appears to bypass the Raf-dependent cytoplasmic kinase cascade. *Mol Cell Biol* 15: 1162-1168.

Alblas, J, EJ van Corven, PL Hordijk, G Milligan, and WH Moolenaar (1993). G_i-mediated activation of the p21^{ras}-mitogen-activated protein kinase pathway by α_2 -adrenergic receptors expressed in fibroblasts. *J Biol Chem* 268: 22235-22238.

Alessandrini, A, CM Crews, and RL Erikson (1992). Phorbol ester stimulates a protein-tyrosine/threonine kinase that phosphorylates and activates the *Erk-1* gene product. Proc Natl Acad Sci USA 89: 8200-8204.

Alessi, DR, Y Saito, DG Campbell, P Cohen, G Sithanandam, U Rapp, A Ashworth, CJ Marshall, and S Cowley (1994). Identification of the sites in MAP kinase kinase-1 phosphorylated by p74^{raf-1}. EMBO 13: 1610-1619.

Anderson, NG, JL Maller, NK Tonks, and TW Sturgill (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature 343: 651-653.

Angel, P, M Imagawa, R Chiu, B Stein, RJ Imbra, HJ Rahmsdorf, C Jonat, P Herrlich, and M Karin (1987). Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell 49: 729-739.

Aronheim, A, D Engelberg, N Li, N Al-Alawi, J Schlessinger, and M Karin (1994). Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. Cell 78: 949-961.

Ashworth, A, S Nakielnny, P Cohen, and C Marshall (1992). The amino acid sequence of a mammalian MAP kinase kinase. Oncogene 7: 2555-2556.

Avruch, J, X Zhang, and J Kyriakis (1994). Raf meets Ras: completing the framework of a signal transduction pathway. TIBS 19: 279-283.

Bagrodia, S, AP Laudano, and D Shalloway (1994). Accessibility of the c-Src SH2-domain for binding is increased during mitosis. J Biol Chem 269: 10247-10251.

Baker, NE and GM Rubin (1989). Effect on eye development of dominant mutations in *Drosophila* homologue of the EGF receptor. Nature 340: 150-153.

Baker, SJ, S Markowitz, ER Fearon, JKV Willson, and B Vogelstein (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249: 912-915.

Baldin, V, J Lukas, MJ Marcote, M Pagano, and G Draetta (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G₁. Genes & Dev 7: 812-821.

Ballou, LM, H Luther, and G Thomas (1991). MAP2 kinase and 70K S6 kinase lie on distinct signalling pathways. Nature 349: 348-350.

Baltensperger, K, LM Kozma, SR Jaspers, and MP Czech (1994). Regulation by insulin of phosphatidylinositol 3'-kinase bound to α - and β -isoforms of p85 regulatory subunit. J Biol Chem 269: 28937-28946.

Batzer, AG, D Rotin, JM Urena, EY Skolnik, and J Schlessinger (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol Cell Biol 14: 5192-5201.

Biggs, WH, KH Zavitz, B Dickson, A van der Straten, D Brunner, E Hafen, and SL Zipursky (1994). The *Drosophila rolled* locus encodes a MAP kinase required in the sevenless signal transduction pathway. EMBO 13: 1628-1635.

Birge, RB, JE Fajardo, BJ Mayer, and H Hanafusa (1992). Tyrosine-phosphorylated epidermal growth factor receptor and cellular p130 provide high affinity binding substrates to analyze Crk-phosphotyrosine-dependent interactions *in vitro*. J Biol Chem 267: 10588-10595.

Birge, RB, JE Fajardo, C Reichman, SE Shoelson, Z Songyang, LC Cantley, and H Hanafusa (1993). Identification and characterization of a high-affinity interaction between v-Crk and tyrosine-phosphorylated paxillin in CT10-transformed fibroblasts. Mol Cell Biol 13: 4648-4656.

Bishop, JM and H Varmus (1985). Functions and origins of retroviral transforming genes. RNA Tumor Viruses 2: 249-356.

Blenis, J, J Chung, E Erikson, DA Alcorta, and RL Erikson (1991). Distinct mechanisms for the activation of the Rsk kinases/MAP2 kinase/pp90^{rsk} and pp70-S6 kinase signaling systems are indicated by inhibition of protein synthesis. Cell Growth & Differentiation 2: 279-285.

Blenis, J (1993). Signal transduction via the MAP kinases: Proceed at your own RSK. Proc Natl Acad Sci USA 90: 5889-5892.

Blumer, KJ and GL Johnson (1994). Diversity in function and regulation of MAP

kinase pathways. TIBS 19: 236-240.

Boguski, MS and F McCormick (1993). Proteins regulating Ras and its relatives. Nature 366: 643-654.

Bohmann, D, MC Ellis, LM Staszewski, and M Mlodzik (1994). Drosophila Jun mediates Ras-dependent photoreceptor determination. Cell 78: 973-986.

Bonfini, L, CA Karlovich, C Dasgupta, and U Banerjee (1992). The *Son of sevenless* gene product: a putative activator of Ras. Science 255: 603-606.

Boni-Schnetzler, M and PF Pilch (1987). Mechanism of epidermal growth factor receptor autophosphorylation and high-affinity binding. Proc Natl Acad Sci USA 84: 7832-7836.

Bos, TJ, FS Montecarlo, F Mitsunobu, AR Ball, CHW Chang, T Nishimura, and PK Vogt (1990). Efficient transformation of chicken embryo fibroblasts by c-Jun requires structural modification in coding and noncoding sequences. Genes & Dev 4: 1677-1687.

Boulton, TG, GD Yancopoulos, JS Gregory, C Slaughter, C Moomaw, J Hsu, and MH Cobb (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 249: 64-67.

Boulton, TG, SH Nye, DJ Robbins, NY Ip, E Radziejewska, SD Morgenbesser, RA DePinho, N Panayotatos, MH Cobb, and GD Yancopoulos (1991a). ERKS: A

family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65: 663-675.

Boulton, TG, JS Gregory, and MH Cobb (1991b). Purification and properties of extracellular signal-regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase. *Biochemistry* 30: 278-286.

Briscoe, J, D Guschin, and M Muller (1994). Just another signalling pathway. *Current Biology* 4: 1033-1035.

Broek, D, T Toda, T Michaeli, L Levin, C Birchmeier, M Zoller, S Powers, and M Wigler (1987). The *S. cerevisiae* Cdc25 gene product regulates the *RAS*/adenylate cyclase pathway. *Cell* 48: 789-799.

Brott, B, A Alessandrini, DA Largaespada, NG Copeland, NA Jenkins, CM Crews, and RL Erikson (1993). MEK2 is a kinase related to MEK1 and is differentially expressed in murine tissues. *Cell Growth & Differentiation* 4: 921-929.

Brugge, JS and RL Erikson (1977). Identification of a transformation-specific antigen induced by an avian sarcoma virus. *Nature* 269: 346-347.

Brunner, D, N Oellers, J Szabad, WH Biggs, SL Zipursky, and E Hafen (1994a). A gain-of-function mutation in drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. *Cell* 76: 875-888.

Brunner, D, K Ducker, N Oellers, E Hafen, H Scholz, and C Klambt (1994b). The Ets domain protein Pointed-P2 is a target of MAP kinase in the Sevenless signal transduction pathway. *Nature* 370: 386-388.

Buday, L and J Downward (1993a). Epidermal growth factor regulates p21^{ras} through the formation of a complex of receptor, Grb2 adaptor protein, and Sos nucleotide exchange factor. *Cell* 73: 611-620.

Buday, L and J Downward (1993b). Epidermal growth factor regulates the exchange rate of guanine nucleotides on p21^{ras} in fibroblasts. *Mol Cell Biol* 13: 1903-1910.

Burgering, BMT, AJ Snijders, JA Maassen, AJ van der Eb, and JL Bos (1989). Possible involvement of normal p21 H-*ras* in the insulin/insulin-like growth factor 1 signal transduction pathway. *Mol Cell Biol* 9: 4312-4322.

Burgering, BMT, AMM de Vries-Smits, RH Medema, PC van Weeren, LGJ Tertoolen, and JL Bos (1993). Epidermal growth factor induces phosphorylation of extracellular signal-regulated kinase 2 via multiple pathways. *Mol Cell Biol* 13: 7248-7256.

Burgering, BMT and JL Bos (1995). Regulation of Ras-mediated signalling: more than one way to skin a cat. *TIBS* 20: 18-22.

Buss, JE and BM Sefton (1985). Myristic acid, a rare fatty acid, is the lipid

attached to the transforming protein of Rous sarcoma virus and its cellular homolog. J Virol 53: 7-12.

Cagan, RL, H Kramer, AC Hart, and SL Zipursky (1992). The bride of sevenless and sevenless interaction: internalization of a transmembrane ligand. Cell 69: 393-399.

Cai, H, J Szeberenyi, and GM Cooper (1990). Effect of a dominant inhibitory Ha-*ras* mutation on mitogenic signal transduction in NIH 3T3 cells. Mol Cell Biol 10: 5314-5323.

Carpenter, G and S Cohen (1979). Epidermal growth factor. Ann Rev Biochem 48: 193-216.

Carthew, RW and GM Rubin (1990). *seven in absentia*, a gene required for specification of R7 cell fate in the Drosophila eye. Cell 63: 561-577.

Cartwright, CA, W Eckhart, S Simon, and PL Kaplan (1987). Cell transformation by pp60^{c-src} mutated in the carboxy-terminal regulatory domain. Cell 49: 83-91.

Catling, AD, CWM Reuter, ME Cox, SJ Parsons, and MJ Weber (1994). Partial purification of a mitogen-activated protein kinase kinase from bovine brain. J Biol Chem 269: 30014-30021.

Chackalaparampil, I and D Shalloway (1988). Altered phosphorylation and

activation of pp60^{c-src} during fibroblast mitosis. Cell 52: 801-810.

Chang, HC, NM Solomon, DA Wassarman, FD Karim, M Therrien, GM Rubin, and T Wolff (1995). *phyllopod* functions in the fate determination of a subset of photoreceptors in *Drosophila*. Cell 80: 463-472.

Chantry, A (1995). The kinase domain and membrane localization determine intracellular interactions between epidermal growth factor receptors. J Biol Chem 270: 3068-3073.

Chao, MV (1992). Growth factor signaling: where is the specificity? Cell 68: 995-997.

Chao, TSO, DA Foster, UR Rapp, and MR Rosner (1994). Differential Raf requirement for activation of mitogen-activated protein kinase by growth factors, phorbol esters, and calcium. J Biol Chem 269: 7337-7341.

Chardin, P, JH Camonis, NW Gale, L Van Aelst, J Schlessinger, MH Wigler, and D Bar-Sagi (1993). Human Sos-1: A guanine nucleotide exchange factor for Ras that binds to Grb2. Science 260: 1338-1343.

Charles, CH, H Sun, LF Lau, and NK Tonks (1993). The growth factor-inducible immediate-early gene 3CH134 encodes a protein-tyrosine-phosphatase. Proc Natl Acad Sci USA 90: 5292-5296.

Chellappan, SP, S Hiebert, M Mudryj, JM Horowitz, and JR Nevins (1991). The

E2F transcription factor is a cellular target for the Rb protein. Cell 65: 1053-1061.

Chen, R, C Sarnecki, and J Blenis (1992). Nuclear localization and regulation of *erk*- and *rsk*- encoded protein kinases. Mol Cell Biol 12: 915-927.

Chen, R, C Abate, and J Blenis (1993). Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. Proc Natl Acad Sci USA 90: 10952-10956.

Chuang, E, D Barnard, L Hettich, X Zhang, J Avruch, and MS Marshall (1994). Critical binding and regulatory interactions between Ras and Raf occur through a small, stable N-terminal domain of Raf and specific Ras effector residues. Mol Cell Biol 14: 5318-5325.

Chung, J, SL Pelech, and J Blenis (1991). Mitogen-activated Swiss mouse 3T3 Rsk kinases I and II are related to pp44^{mpk} from sea star oocytes and participate in the regulation of pp90^{rsk} activity. Proc Natl Acad Sci USA 88: 4981-4985.

Cochet, C, O Kashles, EM Chambaz, I Borrello, CR King, and J Schlessinger (1988). Demonstration of epidermal growth factor-induced receptor dimerization in living cells using a chemical covalent cross-linking agent. J Biol Chem 263: 3290-3295.

Collett, MS, AF Purchio, and RL Erikson (1980). Avian sarcoma virus-

transforming protein, pp60^{src} shows protein kinase activity specific for tyrosine. Nature 285: 167-169.

Corven, EJ van, PL Hordijk, RH Medema, JL Bos, and WH Moolenaar (1993). Pertussis toxin-sensitive activation of p21^{ras} by G protein-coupled receptor agonists in fibroblasts. Proc Natl Acad Sci 90: 1257-1261.

Courtneidge, SA, RM Kypta, JA Cooper, and A Kazlauskas (1991). Platelet-derived growth factor receptor sequences important for binding of *src* family tyrosine kinases. Cell Growth & Differentiation 2: 483-486.

Cowley, S, H Paterson, P Kemp, and CJ Marshall (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77: 841-852.

Cox, AD, MM Hisaka, JE Buss, and CJ Der (1992). Specific isoprenoid modification is required for function of normal, but not oncogenic, Ras protein. Mol Cell Biol 12: 2606-2615.

Crechet, JB, P Poulet, MY Mistou, A Parmeggiani, J Camonis, E Boy-Marcotte, F Damak, and M Jacquet (1990). Enhancement of the GDP-GTP exchange of RAS proteins by the carboxyl-terminal domain of Scd25. Science 248: 866-868.

Crews, CM, AA Alessandrini, and RL Erikson (1991). Mouse *Erk-1* gene product is a serine/threonine protein kinase that has the potential to phosphorylate

tyrosine. Proc Natl Acad Sci USA 88: 8845-8849.

Crews, CM, A Alessandrini, and RL Erikson (1992a). The primary structure of Mek, a protein kinase that phosphorylates the *Erk* gene product. Science 258: 478-480.

Crews, CM, A Alessandrini, and RL Erikson (1992b). Erks: their fifteen minutes has arrived. Cell Growth & Differentiation 3: 135-142.

Cross, FR, EA Garber, D Pellman, and H Hanafusa (1984). A short sequence in the p60^{src} N terminus is required for p60^{src} myristylation and membrane association and for cell transformation. Mol Cell Biol 4: 1834-1842.

Curran, T and BR Franza (1988). Fos and Jun: the AP-1 connection. Cell 55: 395-397.

Daksis, JI, RY Lu, LM Facchini, WW Marhin, and LJZ Penn (1994). Myc induces cyclin D1 expression in the absence of *de novo* protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. Oncogene 9: 3635-3645.

Darnell, JE, IM Kerr, and GR Stark (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264: 1415-1421.

Daum, G, I Eisenmann-Tappe, HW Fries, J Troppmair, and UR Rapp (1994). The

ins and outs of Raf kinases. TIBS 19: 474-480.

Decker, SJ (1993). Transmembrane signaling by epidermal growth factor receptors lacking autophosphorylation sites. J Biol Chem 268: 9176-9179.

DeClue, JE, K Zhang, P Redford, WC Vass, and DR Lowy (1991). Suppression of *src* transformation by overexpression of full-length GTPase-activating protein (GAP) or of the GAP C terminus. Mol Cell Biol 11: 2819-2825.

Deng, T and M Karin (1994). c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from Jnk and Erk. Nature 371: 171-175.

Dent, P, W Haser, TAJ Haystead, LA Vincent, TM Roberts, and TW Sturgill (1992). Activation of mitogen-activated protein kinase kinase by v-Raf in NIH-3T3 cells and in vitro. Science 257: 1404-1407.

Derijard, B, M Hibi, I Wu, T Barrett, B Su, T Deng, M Karin, and RJ Davis (1994). Jnk1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 76: 1025-1037.

Derijard, B, J Raingeaud, T Barrett, IH Wu, J Han, RJ Ulevitch, and RJ Davis (1995). Independent human MAP kinase signal transduction pathways defined by Mek and Mkk isoforms. Science 267: 682-685.

Devary, Y, RA Gottlieb, T Smeal, and M Karin (1992). The mammalian ultraviolet response is triggered by activation of the Src tyrosine kinases. Cell 71: 1081-

1091.

Dickson, B, F Sprenger, D. Morrison, and E Hafen (1992). Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. *Nature* 360: 600-602.

Dickson, BJ, M Dominguez, A van der Straten, and E Hafen (1995). Control of drosophila photoreceptor cell fates by Phyllopod, a novel nuclear protein acting downstream of the Raf kinase. *Cell* 80: 453-462.

Dowdy, SF, PW Hinds, K Louie, SI Reed, A Arnold, and RA Weinberg (1993). Physical interaction of the retinoblastoma protein with human D cyclins. *Cell* 73: 499-511.

Dulic, V, E Lees, and S Reed (1992). Association of human cyclin E with a periodic G₁-S phase protein kinase. *Science* 257: 1958-1961.

Egan, SE, BW Giddings, MW Brooks, L Buday, AM Sizeland, and RA Weinberg (1993). Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* 363: 45-51.

Ellis, C, M Moran, F McCormick, and T Pawson (1990). Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. *Nature* 343: 377-381.

Escobedo, JA, S Navankasattusas, WM Kavanaugh, D Milfay, VA Fried, and LT

Williams (1991). cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β -receptor. *Cell* 65: 75-82.

Ewen, ME, HK Sluss, CJ Scherr, H Matsushime, J Kato, and DM Livingston (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* 73: 487-497.

Fabian, JR, IO Daar, and DK Morrison (1993). Critical tyrosine residues regulate the enzymatic and biological activity of the Raf-1 kinase. *Mol Cell Biol* 13: 7170-7179.

Fabian, JR, AB Vojtek, JA Cooper, and DK Morrison (1994). A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. *Proc Natl Acad Sci USA* 91: 5982-5986.

Fajardo, JE, RB Birge, and H Hanafusa (1993). A 31-amino-acid N-terminal extension regulates c-Crk binding to tyrosine-phosphorylated proteins. *Mol Cell Biol* 13: 7295-7302.

Fantl, WJ, AJ Muslin, A Kikuchi, JA Martin, AM MacNicol, RW Gross, and LT Williams (1994). Activation of Raf-1 by 14-3-3 proteins. *Nature* 371: 612-614.

Fasano, O, T Aldrich, F Tamanoi, E Taparowsky, M Furth, and M Wigler (1984). Analysis of the transforming potential of the human H-*ras* gene by random mutagenesis. *Proc Natl Acad Sci USA* 81: 4008-4012.

Fearon, ER and B Vogelstein (1990). A genetic model for colorectal tumorigenesis. *Cell* 61: 759.

Feig, LA and GM Cooper (1988). Inhibition of NIH-3T3 cell proliferation by a mutant *ras* protein with preferential affinity for GDP. *Mol Cell Biol* 8: 3235-3243.

Feller, SM, B Knudsen, and H Hanafusa (1994). c-Abl kinase regulates the protein binding activity of c-Crk. *EMBO* 13: 2341-2351.

Ford, JC, F Al-Khodairy, E Fotou, KS Sheldrick, DJF Griffiths, and AM Carr (1994). 14-3-3 protein homologs required for the DNA damage checkpoint in fission yeast. *Science* 265: 533-535.

Fortini, ME, MA Simon, and GM Rubin (1992). Signalling by the *sevenless* protein tyrosine kinase is mimicked by Ras1 activation. *Nature* 355: 559-561.

Freed, E, M Symons, SG Macdonald, F McCormick, and R Ruggieri (1994). Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science* 265: 1713-1716.

Fu, X and J Zhang (1993). Transcription factor p91 interacts with the epidermal growth factor receptor and mediates activation of the *c-fos* gene promoter. *Cell* 74: 1135-1145.

Fu, H, K Xia, DC Pallas, C Cui, K Conroy, RP Narsimhan, H Mamon, RJ Collier, and TM Roberts (1994). Interaction of the protein kinase Raf-1 with 14-3-3

proteins. Science 266: 126-129.

Fukui, Y and H Hanafusa (1989). Phosphatidylinositol kinase activity associates with viral p60^{src} protein. Mol Cell Biol 9: 1651-1658.

Galcheva-Gargova, Z, B Derijard, I Wu, and RJ Davis (1994). An osmosensing signal transduction pathway in mammalian cells. Science 265: 806-808.

Gale, NW, S Kaplan, EJ Lowenstein, J Schlessinger, and D Bar-Sagi (1993). Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature 363: 88-92.

Garcia, AM, C Rowell, K Ackermann, JJ Kowalczyk, and MD Lewis (1993). Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. J Biol Chem 268: 18415-18418.

Gaul, U, G Mardon, and GM Rubin (1992). A putative Ras GTPase activating protein acts as a negative regulator of signaling by the sevenless receptor tyrosine kinase. Cell 68: 1007-1019.

Gibbs, JB, DL Pompliano, SD Mosser, E Rands, RB Lingham, SB Singh, EM Scolnick, NE Kohl, and A Oliff (1993). Selective inhibition of farnesyl-protein transferase blocks Ras processing *in vivo*. J Biol Chem 268: 7617-7620.

Gille, H, AD Sharrocks, and PE Shaw (1992). Phosphorylation of transcription

factor p62^{TCF} by Map kinase stimulates ternary complex formation at *c-fos* promoter. Nature 358: 414-417.

Gonzales, FA, A Seth, DL Raden, DS Bowman, FS Fay, and RJ Davis (1993). Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. J Cell Biol 122: 1089-1101.

Gotoh, N, A Tojo, K Muroya, Y Hashimoto, S Hattori, S Nakamura, T Takenawa, Y Yazaki, and M Shibuya (1994). Epidermal growth factor-receptor mutant lacking the autophosphorylation sites induces phosphorylation of Shc protein and Shc-Grb2/Ash association and retains mitogenic activity. Proc Natl Acad Sci USA 91: 167-171.

Gould, KL and T Hunter (1988). Platelet-derived growth factor induces multisite phosphorylation of pp60^{c-src} and increases its protein-tyrosine kinase activity. Mol Cell Biol 8: 3345-3356.

Greenberg, M and EB Ziff (1984). Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. Nature 311: 433-438.

Gupta, S, D Campbell, B Derijard, and RJ Davis (1995). Transcription factor ATF2 regulation by the Jnk signal transduction pathway. Science 267: 389-393.

Hafner, S, HS Adler, H Mischak, P Janosch, G Heidecker, A Wolfman, S Pippig, M Lohse, M Ueffing, and W Kolch (1994). Mechanism of inhibition of Raf-1 by

protein kinase A. Mol Cell Biol 14: 6696-6703.

Hallberg, B, SI Rayter, and J Downward (1994). Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. J Biol Chem 269: 3913-3916.

Hamaguchi, M, C Grandori, and H Hanafusa (1988). Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis by use of anti-phosphotyrosine antibodies. Mol Cell Biol 8: 3035-3042.

Han, J, JD Lee, L Bibbs, and RJ Ulevitch (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265: 808-811.

Hanafusa, H (1969). Rapid transformation of cells by Rous sarcoma virus. Proc Natl Acad Sci USA 63: 318-325.

Hanafusa, H, CC Halpern, DL Buchhagen, S Kawai (1977). Recovery of avian sarcoma virus from tumors induced by transformation-defective mutants. J Exp Med 146: 1735-1747.

Hancock, JF, H Paterson, and CJ Marshall (1990). A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21^{ras} to the plasma membrane. Cell 63: 133-139.

Hara, M, K Akasaka, S Akinaga, M Okabe, H Nakano, R Gomez, D Wood, M Uh, and F Tamanoi (1993). Identification of Ras farnesyltransferase inhibitors by

microbial screening. Proc Natl Acad Sci USA 90: 2281-2285.

Hariharan, IK, RW Carthew, and GM Rubin (1991). The *Drosophila* *roughened* mutation: activation of a *rap* homolog disrupts eye development and interferes with cell determination. Cell 67: 717-722.

Haystead, CMM, P Gregory, A Shirazi, P Fadden, C Mosse, P Dent, and TAJ Haystead (1994). Insulin activates a novel adipocyte mitogen-activated protein kinase kinase kinase that shows rapid phasic kinetics and is distinct from c-Raf. J Biol Chem 269: 12804-12808.

Hempstead, BL, RB Birge, JE Fajardo, R Glassman, D Mahadeo, R Kraemer, and H Hanafusa (1994). Expression of the v-*crk* oncogene product in PC12 cells results in rapid differentiation by both nerve growth factor- and epidermal growth factor-dependent pathways. Mol Cell Biol 14: 1964-1971.

Hepler, JR, N Nakahata, TW Lovenberg, J DiGuseppi, B Herman, HS Earp, and TK Harden (1987). Epidermal growth factor stimulates the rapid accumulation of inositol (1,4,5)-trisphosphate and a rise in cytosolic calcium mobilized from intracellular stores in A431 cells. J Biol Chem 262: 2951-2956.

Herskowitz, I (1995). MAP kinase pathways in yeast: for mating and more. Cell 80: 187-197.

Hibi, M, A Lin, A Minden, and M Karin (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun

activation domain. *Genes & Dev* 7: 2135-2148.

Hiles, ID, M Otsu, S Volinia, MJ Fry, I Gout, R Dhand, G Panayotou, F Ruiz-Larrea, A Thompson, NF Totty, JJ Hsuan, SA Courtneidge, PJ Parker, and MD Waterfield (1992). Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell* 70: 419-429.

Hinds, PW, SF Dowdy, EN Eaton, A Arnold, and RA Weinberg (1994). Function of a human cyclin gene as an oncogene. *Proc Natl Acad Sci USA* 91: 709-713.

Hipskind, RA, VN Rao, CGF Mueller, ESP Reddy, and A Nordheim (1991). Ets-related protein Elk-1 is homologous to the *c-fos* regulatory factor p62^{TCF}. *Nature* 354: 531-534.

Hipskind, RA, M Baccarini, and A Nordheim (1994). Transient activation of Raf-1, Mek, and Erk2 coincides kinetically with ternary complex factor phosphorylation and immediate-early gene promoter activity in vivo. *Mol Cell Biol* 14: 6219-6231.

Honegger, AM, A Schmidt, A Ullrich, and J Schlessinger (1990). Evidence for epidermal growth factor (EGF)-induced intermolecular autophosphorylation of the EGF receptors in living cells. *Mol Cell Biol* 10: 4035-4044.

Howe, LR, SJ Leever, N Gomez, S Nakielnny, P Cohen, and CJ Marshall (1992). Activation of the MAP kinase pathway by the protein kinase Raf. *Cell* 71: 335-342.

Howe, LR and CJ Marshall (1993). Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring p21^{ras} and p74^{raf-1}. J Biol Chem 268: 20717-20720.

Hu, Q, D Milfay, and LT Williams (1995). Binding of Nck to Sos and activation of *ras*-dependent gene expression. Mol Cell Biol 15: 1169-1174.

Huang, HS, J Yee, J Shew, P Chen, R Bookstein, T Friedmann, EYHP Lee, and W Lee (1988). Suppression of the neoplastic phenotype by replacement of the Rb gene in human cancer cells. Science 242: 1563-1566.

Huang, W, A Alessandrini, CM Crews, and RL Erikson (1993). Raf-1 forms a stable complex with Mek1 and activates Mek1 by serine phosphorylation. Proc Natl Acad Sci USA 90: 10947-10951.

Huang, W and RL Erikson (1994). Constitutive activation of Mek1 by mutation of serine phosphorylation sites. Proc Natl Acad Sci USA 91: 8960-8963.

Iba, H, Y Shindo, H Nishina, and T Yoshida (1988). Transforming potential and growth stimulating activity of the *v-fos* and *c-fos* genes carried by avian retrovirus vectors. Oncogene Res 2: 121-133.

Irie, K, Y Gotoh, BM Yashar, B Errede, E Nishida, and K Matsumoto (1994). Stimulatory effects of yeast and mammalian 14-3-3 proteins on the Raf protein kinase. Science 265: 1716-1719.

Jelinek, T, AD Catling, CWM Reuter, SA Moodie, A Wolfman, and MJ Weber (1994). Ras and Raf-1 form a signalling complex with Mek-1 but not Mek-2. *Mol Cell Biol* 14: 8212-8218.

Jung, V, W Wei, R Ballester, J Camonis, S Mi, L van Aelst, M Wigler, and D Broek (1994). Two types of RAS mutants that dominantly interfere with activators of RAS. *Mol Cell Biol* 14: 3707-3718.

Kallunki, T, B Su, I Tsigelny, HK Sluss, B Derijard, G Moore, R Davis, and M Karin (1994). Jnk2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes & Dev* 8: 2996-3007.

Kameshita, I and H Fujisawa (1989). A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. *Analytical Biochemistry* 183: 139-143.

Kanner, SB, AB Reynolds, RR Vines, and JT Parsons (1990). Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc Natl Acad Sci USA* 87: 3328-3332.

Kashles, O, Y Yarden, R Fischer, A Ullrich, and J Schlessinger (1991). A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization. *Mol Cell Biol* 11: 1454-1463.

Kataoka, T, S Powers, S Cameron, O Fasano, M Goldfarb, J Broach, and M Wigler (1985). Functional homology of mammalian and yeast *RAS* genes. *Cell*

40: 19-26.

Kavanaugh, WM and LT Williams (1994). An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science* 266: 1862-1865.

Kikuchi, A and LT Williams (1994). The post-translational modification of *ras* p21 is important for Raf-1 activation. *J Biol Chem* 269: 20054-20059.

Kitayama, H, Y Sugimoto, T Matsuzaki, Y Ikawa, and M Noda (1989). A *ras*-related gene with transformation suppressor activity. *Cell* 56: 77-84.

Kitayama, H, T Matsuzaki, Y Ikawa, and M Noda (1990). Genetic analysis of the Kirsten-*ras*-revertant 1 gene: Potentiation of its tumor suppressor activity by specific point mutations. *Proc Natl Acad Sci USA* 87: 4284-4288.

Klippel, A, JA Escobedo, WJ Fantl, and LT Williams (1992). The c-terminal SH2 domain of p85 accounts for the high affinity and specificity of the binding of phosphatidylinositol 3-kinase to phosphorylated platelet-derived growth factor β receptor. *Mol Cell Biol* 12: 1451-1459.

Klippel, A, JA Escobedo, Q Hu, and LT Williams (1993). A region of the 85-kilodalton (kDa) subunit of phosphatidylinositol 3-kinase binds the 110-kDa catalytic subunit in vivo. *Mol Cell Biol* 13: 5560-5566.

Kmiecik, TE and D Shalloway (1987). Activation and suppression of pp60^{c-src} transforming ability by mutation of its primary sites of tyrosine phosphorylation.

Cell 49: 65-73.

Knudsen, BS, SM Feller, and H Hanafusa (1994). Four proline-rich sequences of the guanine-nucleotide exchange factor C3G bind with unique specificity to the first Src homology 3 domain of Crk. J Biol Chem 269: 32781-32787.

Koide, H, T Satoh, M Nakafuku, and Y Kaziro (1993). GTP-dependent association of Raf-1 with Ha-Ras: Identification of Raf as a target downstream of Ras in mammalian cells. Proc Natl Acad Sci USA 90: 8683-8686.

Kolch, W, G Heidecker, G Kochs, R Hummel, H Vahidi, H Mischak, G Finkenzeller, D Marme, and UR Rapp (1993). Protein kinase C α activates Raf-1 by direct phosphorylation. Nature 364: 249-252.

Kosako, H, Y Gotoh, S Matsuda, M Ishikawa, and E Nishida (1992). *Xenopus* MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. EMBO 11: 2903-2908.

Kruijer, W, JA Cooper, T Hunter, and IM Verma (1984). Platelet-derived growth factor induces rapid but transient expression of the *c-fos* gene and protein. Nature 312: 711-716.

Kypta, RM, Y Goldberg, ET Ulug, and SA Courtneidge (1990). Association between the PDGF receptor and members of the *src* family of tyrosine kinases. Cell 62: 481-492.

Kyriakis, JM, H App, X Zhang, P Banerjee, DL Brautigan, UR Rapp, and J Avruch (1992). Raf-1 activates MAP kinase kinase. *Nature* 358: 417-421.

Kyriakis, JM, P Banerjee, E Nikolakaki, T Dai, EA Rubie, MF Ahmad, J Avruch, and JR Woodgett (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369: 156-160.

Land, H, LF Parada, and RA Weinberg (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 304: 596-602.

Lange-Carter, CA, CM Pleiman, AM Gardner, KJ Blumer, and GL Johnson (1993). A divergence in the MAP kinase regulatory network defined by Mek kinase and Raf. *Science* 260: 315-319.

Larose, L, G Gish, and T Pawson (1995). Construction of an SH2 domain-binding site with mixed specificity. *J Biol Chem* 270: 3858-3862.

Lazaris-Karatzas, A, MR Smith, RM Frederickson, ML Jaramillo, Y Liu, H Kung, and N Sonenberg (1992). Ras mediates translation initiation factor 4E-induced malignant transformation. *Genes & Dev* 6: 1631-1642.

Lee, W, P Mitchell, and R Tjian (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 49: 741-752.

Leevers, SJ, HF Paterson, and CJ Marshall (1994). Requirement for Ras in Raf

activation is overcome by targeting Raf to the plasma membrane. *Nature* 369: 411-414.

Lenormand, P, C Sardet, G Pages, G L'Allemain, A Brunet, and J Pouyssegur (1993). Growth factors induce nuclear translocation of MAP kinases (p42^{mapk} and p44^{mapk}) but not of their activator Map kinase kinase (p45^{mapkk}) in fibroblasts. *J Cell Biol* 122: 1079-1088.

Lew, DJ, V Dulic, and SI Reed (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell* 66: 1197-1206.

Li, P, K Wood, H Mamon, W Haser, and T Roberts (1991). Raf-1: A kinase currently without a cause but not lacking in effects. *Cell* 64: 479-482.

Li, W, P Hu, EY Skolnik, A Ullrich, and J Schlessinger (1992). The SH2 and SH3 domain-containing Nck protein is oncogenic and a common target for phosphorylation by different surface receptors. *Mol Cell Biol* 12: 5824-5833.

Li, N, A Batzer, R Daly, V Yajnik, E Skolnik, P Chardin, D Bar-Sagi, B Margolis, and J Schlessinger (1993). Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* 363: 84-88.

Li, N, J Schlessinger, and B Margolis (1994). Autophosphorylation mutants of the EGF-receptor signal through auxiliary mechanisms involving SH2 domain proteins. *Oncogene* 9: 3457-3465.

Li, S, P Janosch, M Tanji, GC Rosenfeld, JC Waymire, H Mischak, W Kolch, and JM Sedivy (1995). Regulation of Raf-1 kinase activity by the 14-3-3 family of proteins. *EMBO* 14: 685-696.

Lin, L, M Wartmann, AY Lin, JL Knopf, A Seth, and RJ Davis (1993). cPLA2 is phosphorylated and activated by MAP kinase. *Cell* 72: 269-278.

Lin, T, X Kong, TAJ Haystead, A Pause, G Belsham, N Sonenberg, and JC Lawrence (1994). PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science* 266: 653-656.

Liu, X and T Pawson (1991). The epidermal growth factor receptor phosphorylates GTPase-activating protein (GAP) at tyr-460, adjacent to the GAP SH2 domains. *Mol Cell Biol* 11: 2511-2516.

Liu, X, LEM Marengere, CA Koch, and T Pawson (1993). The v-Src SH3 domain binds phosphatidylinositol 3'-kinase. *Mol Cell Biol* 13: 5225-5232.

Lowenstein, EJ, RJ Daly, AG Batzer, W Li, B Margolis, R Lammers, A Ullrich, EY Skolnik, D Bar-Sagi, and J Schlessinger (1992). The SH2 and SH3 domain-containing protein Grb2 links receptor tyrosine kinases to ras signaling. *Cell* 70: 431-442.

Macdonald, SG, CM Crews, L Wu, J Driller, R Clark, RL Erikson, and F McCormick (1993). Reconstitution of the Raf-1-Mek-Erk signal transduction pathway in vitro. *Mol Cell Biol* 13: 6615-6620.

Majerus, PW, TS Ross, TW Cunningham, KK Caldwell, AB Jefferson, and VS Bansal (1990). Recent insights in phosphatidylinositol signaling. *Cell* 63: 459-465.

Mansour, SJ, WT Matten, AS Hermann, JM Candia, S Rong, K Fukasawa, GF Vande Woude, and NG Ahn (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* 265: 966-970.

Marais, R, J Wynne, and R Treisman (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional transactivation domain. *Cell* 73: 381-393.

Margolis, B, N Li, M Mohammadi, DR Hurwitz, A Zilberstein, A Ullrich, T Pawson, and J Schlessinger (1990). The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC- γ . *EMBO* 9: 4375-4380.

Marshall, CJ (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* 80: 179-185.

Marx, J (1993). Two major signal pathways linked. *Science* 262: 988-989.

Massaglia, S, A Gray, TJ Dull, S Munemitsu, H Kung, J Schlessinger, and A Ullrich (1990). Epidermal growth factor receptor cytoplasmic domain mutations trigger ligand-independent transformation. *Mol Cell Biol* 10: 3048-3055.

Matsuda, M, BJ Mayer, and H Hanafusa (1991). Identification of domains of the v-*crk* oncogene product sufficient for association with phosphotyrosine-containing proteins. *Mol Cell Biol* 11: 1607-1613.

Matsuda, M, CT Reichman, and H Hanafusa (1992a). Biological and biochemical activity of v-Crk chimeras containing the SH2/SH3 regions of phosphatidylinositol-specific phospholipase C and Src. *J Virol* 66: 115-121.

Matsuda, M, S Tanaka, S Nagata, A Kojima, T Kurata, and M Shibuya (1992b). Two species of human *CRK* cDNA encode proteins with distinct biological activities. *Mol Cell Biol* 12: 3482-3489.

Matsuda, M, S Nagata, S Tanaka, K Nagashima, and T Kurata (1993a). Structural requirements of *CRK* SH2 region for binding to phosphotyrosine-containing proteins. *J Biol Chem* 268: 4441-4446.

Matsuda, S, Y Gotoh, and E Nishida (1993b). Phosphorylation of *Xenopus* mitogen-activated protein (MAP) kinase kinase by MAP kinase kinase kinase and MAP kinase. *J Biol Chem* 268: 3277-3281.

Matsuda, M, Y Hashimoto, K Muroya, H Hasegawa, T Kurata, S Tanaka, S Nakamura, and S Hattori (1994). Crk protein binds to two guanine nucleotide-releasing proteins for the Ras family and modulates nerve growth factor-induced activation of Ras in PC12 cells. *Mol Cell Biol* 14: 5495-5500.

Matsushime, H, MF Roussel, RA Ashmun, and CJ Scherr (1991). Colony-

stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65: 701-713.

Mayer, BJ, M Hamaguchi, and H Hanafusa (1988). A novel viral oncogene with structural similarity to phospholipase C. Nature 332: 272-275.

Mayer, BJ and H Hanafusa (1990a). Association of the *v-crk* oncogene product with phosphotyrosine-containing proteins and protein kinase activity. Proc Natl Acad Sci USA 87: 2638-2642.

Mayer, BJ and H Hanafusa (1990b). Mutagenic analysis of the *v-crk* oncogene: requirement for SH2 and SH3 domains and correlation between increased cellular phosphotyrosine and transformation. J Virol 64: 3581-3589.

McGlade, CJ, C Ellis, M Reedijk, D Anderson, G Mbamalu, AD Reith, G Panayotou, P End, A Bernstein, A Kazlauskas, MD Waterfield, and T Pawson (1992a). SH2 domains of the p85 α subunit of phosphatidylinositol 3-kinase regulate binding to growth factor receptors. Mol Cell Biol 12: 991-997.

McGlade, J, A Cheng, G Pelicci, PG Pelicci, and T Pawson (1992b). Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. Proc Natl Acad Sci USA 89: 8869-8873.

Medema, RH, AMM deVries-Smits, GCM van der Zon, JA Maassen, and JL Bos (1993). Ras activation by insulin and epidermal growth factor through enhanced exchange of guanine nucleotides on p21^{ras}. Mol Cell Biol 13: 155-162.

Milburn, MV, L Tong, AM DeVos, A Brunger, Z Yamaizumi, S Nishimura, and S Kim (1990). Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic *ras* proteins. *Science* 247: 939-945.

Minden, A, A Lin, T Smeal, B Derijard, M Cobb, R Davis, and M Karin (1994a). c-Jun N-terminal phosphorylation correlates with activation of the Jnk subgroup but not the Erk subgroup of mitogen-activated protein kinases. *Mol Cell Biol* 14: 6683-6688.

Minden, A, A Lin, M McMahon, C Lange-Carter, B Derijard, RJ Davis, GL Johnson, and M Karin (1994b). Differential activation of Erk and Jnk mitogen-activated protein kinases by Raf-1 and Mekk. *Science* 266: 1719-1723.

Mitra, G, M Weber, and D Stacey (1993). Multiple pathways for activation of MAP kinases. *Cell and Mol Biol Res* 39: 517-523.

Moodie, SA, BM Willumsen, MJ Weber, and A Wolfman (1993). Complexes of Ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* 260: 1658-1661.

Morgan, DO, JM Kaplan, JM Bishop, and HE Varmus (1989). Mitosis-specific phosphorylation of p60^{c-src} by p34^{cdc2}-associated protein kinase. *Cell* 57: 775-786.

Morrison, DK, G Heidecker, UR Rapp, and TD Copeland (1993). Identification of

the major phosphorylation sites of the Raf-1 kinase. J Biol Chem 268: 17309-17316.

Morrison, D (1994). 14-3-3: Modulators of signaling proteins? Science 266: 56-57.

Motokura, T, T Bloom, HG Kim, H Juppner, JV Ruderman, HM Kronenberg, and A Arnold (1991). A novel cyclin encoded by a *bc1*-linked candidate oncogene. Nature 350: 512-515.

Nada, S, M Okada, A MacAuley, JA Cooper, and H Nakagawa (1991). Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60^{src}. Nature 351: 69-72.

Nakielnny, S, P Cohen, J Wu, and T Sturgill (1992). MAP kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. EMBO 11: 2123-2129.

Nevins, JR (1992). E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. Science 258: 424-429.

Nishibe, S, MI Wahl, SM Teresa Hernandez-Sotomayor, NK Tonks, SG Rhee, and G Carpenter (1990). Increase of the catalytic activity of phospholipase C- γ 1 by tyrosine phosphorylation. Science 250: 1253-1255.

Nishida, Y, M Hata, T Ayaki, H Ryo, M Yamagata, K Shimizu, and Y Nishizuka

(1988). Proliferation of both somatic and germ cells is affected in the *Drosophila* mutants of *raf* proto-oncogene. EMBO 7: 775-781.

Nori, M, US Vogel, JB Gibbs, and MJ Weber (1991). Inhibition of v-*src*-induced transformation by a GTPase-activating protein. Mol Cell Biol 11: 2812-2818.

Nori, M, G L'Allemain, and MJ Weber (1992). Regulation of tetradecanoyl phorbolacetate-induced responses in NIH-3T3 cells by GAP, the GTPase activating protein associated with p21^{c-ras}. Mol Cell Biol 12: 936-945.

Norman, C, M Runswick, R Pollock, and R Treisman (1988). Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-*fos* serum response element. Cell 55: 989-1003.

O'Neill, EM, I Rebay, R Tjian, and GM Rubin (1994). The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. Cell 78: 137-147.

Oda, T, C Heaney, JR Hagopian, K Okuda, JD Griffin, and BJ Druker (1994). Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. J Biol Chem 269: 22925-22928.

Ogawa, S, H Toyoshima, H Kozutsumi, K Hagiwara, R Sakai, T Tanaka, N Hirano, H Mano, Y Yazaki, and H Hirai (1994). The C-terminal SH3 domain of the mouse c-Crk protein negatively regulates tyrosine-phosphorylation of Crk associated p130 in rat 3Y1 cells. Oncogene 9: 1669-1678.

Ohmichi, M, L Pang, SJ Decker, and AR Saltiel (1992). Nerve growth factor stimulates the activities of the *raf*-1 and the mitogen-activated protein kinases via the *trk* proto-oncogene. J Biol Chem 267: 14604-14610.

Ohtsubo, M and JM Roberts (1993). Cyclin-dependent regulation of G₁ in mammalian fibroblasts. Science 259: 1908-1912.

Okabayashi, Y, Y Kido, T Okutani, Y Sugimoto, K Sakaguchi, and M Kasuga (1994). Tyrosines 1148 and 1173 of activated human epidermal growth factor receptors are binding sites of Shc in intact cells. J Biol Chem 269: 18674-18678.

Okada, M and H Nakagawa (1989). A protein tyrosine kinase involved in regulation of pp60^{c-src} function. J Biol Chem 264: 20886-20893.

Okutani, T, Y Okabayashi, Y Kido, Y Sugimoto, K Sakaguchi, K Matuoka, T Takenawa, and M Kasuga (1994). Grb2/Ash binds directly to tyrosines 1068 and 1086 and indirectly to tyrosine 1148 of activated human epidermal growth factor receptors in intact cells. J Biol Chem 269: 31310-31314.

Olivier, JP, T Raabe, M Henkemeyer, B Dickson, G Mbamalu, B Margolis, J Schlessinger, E Hafen, and T Pawson (1993). A drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. Cell 73: 179-191.

Otsu, M, I Hiles, I Gout, MJ Fry, F Ruiz-Larrea, G Panayotou, A Thompson, R

Dhand, J Hsuan, N Totty, AD Smith, SJ Morgan, SA Courtneidge, PJ Parker, and MD Waterfield (1991). Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60^{c-src} complexes, and PI3-kinase. Cell 65: 91-104.

Pallas, DC, H Fu, LC Haehnel, W Weller, RJ Collier, and TM Roberts (1994). Association of polyomavirus middle tumor antigen with 14-3-3 proteins. Science 265: 535-537.

Park, D and SG Rhee (1992). Phosphorylation of Nck in response to a variety of receptors, phorbol myristate acetate, and cyclic AMP. Mol Cell Biol 12: 5816-5823.

Parker, PJ and MD Waterfield (1992). Phosphatidylinositol 3-kinase: a novel effector. Cell Growth & Differentiation 3: 747-752.

Pawson, T (1992). Conviction by genetics. Nature 356: 285-286.

Pawson, T and J Schlessinger (1993). SH2 and SH3 domains. Current Biology 3: 434-442.

Payne, DM, AJ Rossomando, P Martino, AK Erickson, J Her, J Shabanowitz, DF Hunt, MJ Weber, and TW Sturgill (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO 10: 885-892.

Pelicci, G, L Lanfrancone, F Grignani, J McGlade, F Cavallo, G Forni, I Nicoletti, F Grignani, T Pawson, and PG Pelicci (1992). A novel transforming protein (Shc) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* 70: 93-104.

Piwnicka-Worms, H, KB Saunders, TM Roberts, AE Smith, and SH Cheng (1987). Tyrosine phosphorylation regulates the biochemical and biological properties of pp60^{c-src}. *Cell* 49: 75-82.

Pleiman, CM, WM Hertz, and JC Cambier (1994). Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. *Science* 263: 1609-.

Porfiri, E, T Evans, Pierre Chardin, and JF Hancock (1994). Prenylation of Ras proteins is required for efficient hSos1-promoted guanine nucleotide exchange. *J Biol Chem* 269: 22672-22677.

Porras, A, K Muszynski, UR Rapp, and E Santos (1994). Dissociation between activation of Raf-1 kinase and the 42-kDa mitogen-activated protein kinase/90 kDa S6 kinase (Mapk/Rsk) cascade in the insulin/Ras pathway of adipocytic differentiation of 3T3 L1 cells. *J Biol Chem* 269: 12741-12748.

Prendergast, GC, JP Davide, SJ deSolms, EA Giuliani, SL Graham, JB Gibbs, A Oliff, and NE Kohl (1994). Farnesyltransferase inhibition causes morphological reversion of *ras*-transformed cells by a complex mechanism that involves regulation of the actin cytoskeleton. *Mol Cell Biol* 14: 4193-4202.

Pumiglia, K, Y Chow, J Fabian, D Morrison, S Decker, and R Jove (1995). Raf-1 N-terminal sequences necessary for Ras-Raf interaction and signal transduction. *Mol Cell Biol* 15: 398-406.

Quilliam, LA, SY Huff, KM Rabun, W Wei, W Park, D Broek, and CJ Der (1994). Membrane-targeting potentiates guanine nucleotide exchange factor Cdc25 and Sos1 activation of Ras transforming activity. *Proc Natl Acad Sci USA* 91: 8512-8516.

Rapp, UR, G Heidecker, M Huleihel, JL Cleveland, WC Choi, T Pawson, JN Ihle, and WB Anderson (1988). *raf* family serine/threonine protein kinases in mitogen signal transduction. *Cold Spring Harbor Symp Quant Biol* 53: 173-184.

Ray, LB and TW Sturgill (1987). Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 *in vitro*. *Proc Natl Acad Sci USA* 84: 1502-1506.

Redemann, N, B Holzmann, T Ruden, EF Wagner, J Schlessinger, and A Ullrich (1992). Anti-oncogenic activity of signalling-defective epidermal growth factor receptor mutants. *Mol Cell Biol* 12: 491-498.

Reichman, CT, BJ Mayer, S Keshav, and H Hanafusa (1992). The product of the cellular *crk* gene consists primarily of SH2 and SH3 regions. *Cell Growth and Differentiation* 3: 451-460.

Reinke, R and SL Zipursky (1988). Cell-cell interaction in the *Drosophila* retina:

The *bride of sevenless* gene is required in photoreceptor cell R8 for R7 cell development. Cell 1988: 321-330.

Ren, R, BJ Mayer, P Cicchetti, and D Baltimore (1993). Identification of a ten-amino acid proline-rich SH3 binding site. Science 259: 1157-1161.

Reuther, GW, H Fu, LD Cripe, RJ Collier, and AM Pendergast (1994). Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. Science 266: 129-133.

Roche, S, M Koegl, MV Barone, MF Roussel, and SA Courtneidge (1995). DNA synthesis induced by some but not all growth factors requires Src family protein tyrosine kinases. Mol Cell Biol 15: 1102-1109.

Rodriguez-Viciano, P, PH Warne, R Dhand, B Vanhaesebroeck, I Gout, MJ Fry, MD Waterfield, and J Downward (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370: 527-532.

Rossomando, A, J Wu, MJ Weber, and TW Sturgill (1992). The phorbol ester-dependent activator of the mitogen-activated protein kinase p42^{mapk} is a kinase with specificity for the threonine and tyrosine regulatory sites. Proc Natl Acad Sci USA 89: 5221-5225.

Rotin, D, B Margolis, M Mohammadi, RJ Daly, G Daum, N Li, EH Fischer, WH Burgess, A Ullrich, and J Schlessinger (1992). SH2 domains prevent tyrosine dephosphorylation of the EGF receptor: identification of Tyr 992 as the high-

affinity binding site for SH2 domains of phospholipase C. EMBO 11: 559-567.

Rouse, J, P Cohen, S Trigon, M Morange, A Alonso-Llamazares, D Zamanillo, T Hunt, and AR Nebreda (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP Kinase-2 and phosphorylation of the small heat shock proteins. Cell 78: 1027-1037.

Roussel, RR, SR Brodeur, D Shalloway, and AP Laudano (1991). Selective binding of activated pp60^{c-src} by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60^{c-src}. Proc Natl Acad Sci USA 88: 10696-10700.

Rozakis-Adcock, M, R Fernley, J Wade, T Pawson, and D Bowtell (1993). The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 363: 83-85.

Rubin, G (1991). Signal transduction and the fate of the R7 photoreceptor in *Drosophila*. TIG 7: 372-377.

Ruff-Jamison, S, K Chen, and S Cohen (1993). Induction by EGF and interferon- γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. Science 261: 1733-1736.

Ruley, HE (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304: 602-606.

Sabe, H, M Okada, H Nakagawa, and H Hanafusa (1992). Activation of c-Src in cells bearing v-Crk and its suppression by Csk. *Mol Cell Biol* 12: 4706-4713.

Sager, R, K Tanaka, CC Lau, Y Ebina, and A Anisowicz (1983). Resistance of human cells to tumorigenesis induced by cloned transforming genes. *Proc Natl Acad Sci USA* 80: 7601-7605.

Sakai, R, A Iwamatsu, N Hirano, S Ogawa, T Tanaka, H Mano, Y Yazaki, and H Hirai (1994). A novel signaling molecule, p130, forms stable complexes *in vivo* with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO* 13: 3748-3756.

Sakaue, M, D Bowtell, and M Kasuga (1995). A dominant-negative mutant of mSOS1 inhibits insulin-induced Ras activation and reveals Ras-dependent and -independent insulin signaling pathways. *Mol Cell Biol* 15: 379-388.

Samuels, ML and M McMahon (1994). Inhibition of platelet-derived growth factor- and epidermal growth factor-mediated mitogenesis and signaling in 3T3 cells expressing Δ Raf-1:ER, an estradiol-regulated form of Raf-1. *Mol Cell Biol* 14: 7855-7866.

Sanchez, I, RT Hughes, BJ Mayer, K Yee, JR Woodgett, J Avruch, JM Kyriakis, and LI Zon (1994). Role of Sapk/Erk kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature* 372: 794-798.

Sanghera, JS, HB Paddon, and SL Pelech (1991). Role of protein

phosphorylation in the maturation-induced activation of a myelin basic protein kinase from sea star oocytes. J Biol Chem 266: 6700-6707.

Sanghera, JS, M Peter, EA Nigg, and SL Pelech (1992). Immunological characterization of avian MAP kinases: evidence for nuclear localization. Mol Biol Cell 3: 775-787.

Sasaoka, T, WJ Langlois, JW Leitner, B Draznin, and JM Olefsky (1994). The signaling pathway coupling epidermal growth factor receptors to activation of p21^{ras}. J Biol Chem 269: 32621-32625.

Satoh, T, M Endo, M Nakafuku, T Akiyama, T Yamamoto, and Y Kaziro (1990). Accumulation of p21^{ras}-GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. Proc Natl Acad Sci 87: 7926-7929.

Satoh, T, M Nakafuku, and Y Kaziro (1992). Function of Ras as a molecular switch in signal transduction. J Biol Chem 267: 24149-24152.

Savage, CR, T Inagami, and S Cohen (1972). The primary structure of epidermal growth factor. J Biol Chem 247: 7612-7621.

Sawasdikosol, S, KS Ravichandran, KK Lee, JH Chang, and SJ Burakoff (1995). Crk interacts with tyrosine-phosphorylated p116 upon T cell activation. J Biol Chem 270: 2893-2896.

Schaap, D, J van der Wal, LR Howe, CJ Marshall, and WJ van Blitterswijk (1993). A dominant-negative mutant of *raf* blocks mitogen-activated protein kinase activation by growth factors and oncogenic p21^{ras}. J Biol Chem 268: 20232-20236.

Scherr, CJ (1993). Mammalian G1 cyclins. Cell 1059-1065.

Scherr, CJ (1994). G1 phase progression: cycling on cue. Cell 79: 551-555.

Schlessinger, J and A Ullrich (1992). Growth factor signaling by receptor tyrosine kinases. Neuron 9: 383-391.

Schultz, AM, LE Henderson, S Oroszlan, EA Garber, and H Hanafusa (1985). Amino terminal myristylation of the protein kinase p60 *src*, a retroviral transforming protein. Science 227: 427-429.

Schutte, J, JD Minna, and MJ Birrer (1989). Deregulated expression of human *c-jun* transforms primary rat embryo cells in cooperation with an activated *c-Ha-ras* gene and transforms rat-1a cells as a single gene. Proc Natl Acad Sci USA 86: 2257-2261.

Seger, R, NG Ahn, TG Boulton, GD Yancopoulos, N Panayotatos, E Radziejewska, L Ericsson, RL Bratlien, MH Cobb, and EG Krebs (1991). Microtubule-associated protein 2 kinases, ERK1 and ERK2, undergo autophosphorylation on both tyrosine and threonine residues: implications for their mechanism of activation. Proc Natl Acad Sci USA 88: 6142-6146.

Seger, R, D Seger, AA Reszka, ES Munar, H Eldar-Finkelman, G Dobrowolska, AM Jensen, JS Campbell, EH Fischer, and EG Krebs (1994). Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. J Biol Chem 269: 25699-25709.

Shaw, PE, H Schroter, and A Nordheim (1989). The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human *c-fos* promoter. Cell 56: 563-572.

Shenoy, S, J Choi, S Bagrodia, TD Copeland, JL Maller, and D Shalloway (1989). Purified maturation promoting factor phosphorylates pp60^{c-src} at the sites phosphorylated during mitosis. Cell 57: 763-774.

Silvennoinen, O, C Schindler, J Schlessinger, and DE Levy (1993). Ras-independent growth factor signaling by transcription factor tyrosine phosphorylation. Science 261: 1736-1739.

Simon, MA, DDL Bowtell, GS Dodson, TR Lavery, and GM Rubin (1991). Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell 67: 701-716.

Simon, MA, GS Dodson, and GM Rubin (1993). An SH2-SH3-SH2 protein is required for p21^{Ras1} activation and binds to sevenless and Sos proteins in vitro. Cell 73: 169-177.

Skolnik, EY, B Margolis, M Mohammadi, E Lowenstein, R Fischer, A Drepps, A Ullrich, and J Schlessinger (1991). Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* 65: 83-90.

Songyang, Z, SE Schoelson, M Chaudhuri, G Gish, T Pawson, WG Haser, F King, T Roberts, S Ratnofsky, RJ Lechleider, BG Neel, RB Birge, JE Fajardo, MM Chou, H Hanafusa, B Schaffhausen, and LC Cantley (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* 72: 767-778.

Sorokin, A, MA Lemmon, A Ullrich, and J Schlessinger (1994). Stabilization of an active dimeric form of the epidermal growth factor receptor by introduction of an inter-receptor disulfide bond. *J Biol Chem* 269: 9752-9759.

Spector, DH, HE Varmus, and JM Bishop (1978). Nucleotide sequences related to the transforming gene of avian sarcoma virus are present in DNA of uninfected vertebrates. *Proc Natl Acad Sci USA* 75: 4102-4106.

Stanton, VP, DW Nichols, AP Laudano, and GM Cooper (1989). Definition of the human *raf* amino-terminal regulatory region by deletion mutagenesis. *Mol Cell Biol* 9: 639-647.

Stehelin, D, HE Varmus, JM Bishop, PK Vogt (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* 260: 170-173.

Stokoe, D, DG Campbell, S Nakielnny, H Hidaka, SL Leever, C Marshall, and P Cohen (1992). MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. EMBO 11: 3985-3994.

Stokoe, D, SG Macdonald, K Cadwallader, M Symons, and JF Hancock (1994). Activation of Raf as a result of recruitment to the plasma membrane. Science 264: 1463-1467.

Sturgill, TW, LB Ray, E Erikson, and JL Maller (1988). Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature 334: 715-718.

Sun, H, CH Charles, LF Lau, and NK Tonks (1993). MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell 75: 487-493.

Tabin, CJ, SM Bradley, CI Bargmann, RA Weinberg, AG Papageorge, EM Scolnick, R Dhar, DR Lowy, and EH Chang (1982). Mechanism of activation of a human oncogene. Nature 300: 143-149.

Takayama, K, K Furukawa, K Abe, Y Kawase, A Mizoguchi, A Kikuchi, Y Takai, Y Matsui, Y Teranishi, E Nakayama, and H Shiku (1991). Similarity of expression of low molecular weight G proteins *smg p21A* and *ras p21* in normal and malignant human tumors. Cancer Res 51: 2223-2228.

Takeya, T and H Hanafusa (1983). Structure and sequence of the cellular gene

homologous to the RSV *src* gene and the mechanism for generating the transforming virus. Cell 32: 881-890.

Tanaka, K, K Matsumoto, and A Toh-e (1989). *IRA-1*, an inhibitory regulator of the *RAS*-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol Cell Biol 9: 757-768.

Tanaka, K, M Nakafuku, T Satoh, MS Marshall, JB Gibbs, K Matsumoto, Y Kaziro, and A Toh-e (1990). *S. cerevisiae* genes *IRA1* and *IRA 2* encode proteins that may be functionally equivalent to mammalian *ras* GTPase activating protein. Cell 60: 803-807.

Tanaka, S, S Hattori, T Kurata, K Nagashima, Y Fukui, S Nakamura, and M Matsuda (1993). Both the SH2 and SH3 domains of human Crk protein are required for neuronal differentiation of PC12 cells. Mol Cell Biol 13: 4409-4415.

Tanaka, S, T Morishita, Y Hashimoto, S Hattori, S Nakamura, M Shibuya, K Matuoka, T Takenawa, T Kurata, K Nagashima, and M Matsuda (1994). C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of Crk and Grb2/Ash proteins. Proc Natl Acad Sci USA 91: 3443-3447.

Taylor, SJ, HZ Chae, SG Rhee, and JH Exton (1991). Activation of the $\beta 1$ isozyme of phospholipase C by α subunits of the G_q class of G proteins. Nature 350: 516-518.

Temeles, GL, JB Gibbs, JS D'Alonzo, IS Sigal, and EM Skolnick (1985). Yeast and mammalian *ras* proteins have conserved biochemical properties. *Nature* 313: 700-703.

ten Hoeve, J, C Morris, N Heisterkamp, and John Groffen (1993). Isolation and chromosomal localization of *CRKL*, a human *crk*-like gene. *Oncogene* 8: 2469-2474.

Thomas, S, M DeMarco, G D'Arcangelo, S Halegoua, and JS Brugge (1992). Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* 68: 1031-1040.

Toda, T, I Uno, T Ishikawa, S Powers, T Kataoka, D Broek, S Cameron, J Broach, K Matsumoto, and M Wigler (1985). In yeast, RAS proteins are controlling elements of adenylate cyclase. *Cell* 40: 27-36.

Tomlinson, A and DF Ready (1986). *Sevenless*: a cell-specific homeotic mutation of the *Drosophila* eye. *Science* 231: 400-402.

Trahey, M and F McCormick (1987). A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. *Science* 238: 542-545.

Trahey, M, G Wong, R Halenbeck, B Rubinfeld, GA Martin, M Ladner, CM Long, WJ Crosier, K Watt, K Kohts, and F McCormick (1988). Molecular cloning of two types of GAP complementary DNA from human placenta. *Science* 242: 1697-1700.

Treisman, R (1994). Ternary complex factors: growth factor regulated transcriptional activators. *Curr Op Gen Dev* 4: 96-101.

Tsuda, L, YH Inoue, M Yoo, M Mizuno, M Hata, Y Lim, T Adachi-Yamada, H Ryo, Y Masamune, and Y Nishida (1993). A protein kinase similar to MAP kinase activator acts downstream of the Raf kinase in drosophila. *Cell* 72: 407-414.

Turner, CE and JT Miller (1994). Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and pp125^{Fak}-binding region. *J Cell Sci* 107: 1583-1591.

Twamley, GM, RM Kypta, B Hall, and SA Courtneidge (1992). Association of Fyn with the activated platelet-derived growth factor receptor: requirements for binding and phosphorylation. *Oncogene* 7: 1893-1901.

Twamley-Stein, GM, R Pepperkok, W Ansorge, and SA Courtneidge (1993). The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. *Proc Natl Acad Sci USA* 90: 7696-7700.

Ullrich, A, L Coussens, JS Hayflick, TJ Dull, A Gray, AW Tam, J Lee, Y Yarden, TA Libermann, J Schlessinger, J Downward, ELV Mayes, N Whittle, MD Waterfield, and PH Seeburg (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309: 418-425.

Ullrich, A and J Schlessinger (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* 61: 203-212.

Umanoff, H, W Edelmann, A Pellicer, and R Kucherlapati (1995). The murine *N-ras* gene is not essential for growth and development. *Proc Natl Acad Sci USA* 92: 1709-1713.

Vaillancourt, RR, AM Gardner, and GL Johnson (1994). B-Raf-dependent regulation of the Mek-1/mitogen-activated protein kinase pathway in PC12 cells and regulation by cyclic AMP. *Mol Cell Biol* 14: 6522-6530.

Valius, M and A Kazlauskas (1993). Phospholipase C- γ 1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell* 73: 321-334.

Vega, QC, C Cochet, O Filhol, C Chang, SG Rhee, and GN Gill (1992). A site of tyrosine phosphorylation in the C terminus of the epidermal growth factor receptor is required to activate phospholipase C. *Mol Cell Biol* 12: 128-135.

Vojtek, AB, SM Hollenberg, and JA Cooper (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 74: 205-214.

Vries-Smits, AMM de, BM Burgering, SJ Leervers, CJ Marshall, and JL Bos (1992). Involvement of p21^{ras} in activation of extracellular signal-related kinase 2. *Nature* 357: 602-604.

Vries-Smits, AMM de, GJ Pronk, JP Medema, BMT Burgering, and JL Bos (1995). Shc associates with an unphosphorylated form of the p21 *ras* guanine nucleotide exchange factor mSos. *Oncogene* 10: 919-925.

Wahl, M and G Carpenter (1988). Regulation of epidermal growth factor-stimulated formation of inositol phosphates in A431 cells by calcium and protein kinase C. *J Biol Chem* 263: 7581-7590.

Waksman, G, D Kominos, SC Robertson, N Pant, D Baltimore, RB Birge, D Cowburn, H Hanafusa, BJ Mayer, M Overduin, MD Resh, CB Rios, L Silverman, and J Kuriyan (1992). Crystal structure of the phosphotyrosine recognition domain SH2 of *v-src* complexed with tyrosine-phosphorylated peptides. *Nature* 358: 646-653.

Walker, F, J deBlaquiere, and AW Burgess (1993). Translocation of pp60^{c-src} from the plasma membrane to the cytosol after stimulation by platelet-derived growth factor. *J Biol Chem* 268: 19552-19558.

Ward, Y, S Gupta, P Jensen, M Wartmann, RJ Davis, and K Kelly (1994). Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. *Nature* 367: 651-654.

Warne, PH, P Viciana, and J Downward (1993). Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. *Nature* 364: 352-355.

Weernink, PAO and G Rijksen (1995). Activation and translocation of c-Src to

the cytoskeleton by both platelet-derived growth factor and epidermal growth factor. J Biol Chem 270: 2264-2267.

Welham, MJ, JA Wyke, A Lang, and AW Wyke (1990). Mitogenesis induced by pp60^{v-src} is not accompanied by increased expression of immediate early response genes. Oncogene 5: 161-169.

Weng, Z, JA Taylor, CE Turner, JS Brugge, and C Seidel-Dugan (1993). Detection of Src homology 3-binding proteins, including paxillin, in normal and v-Src-transformed Balb/c 3T3 cells. J Biol Chem 268: 14956-14963.

White, MF and CR Kahn (1994). The insulin signaling system. J Biol Chem 269: 1-4.

Williams, NG, TM Roberts, and P Li (1992). Both p21^{ras} and p60^{v-src} are required, but neither alone is sufficient, to activate the Raf-1 kinase. Proc Natl Acad Sci USA 89: 2922-2926.

Winitz, S, M Russell, N Qian, and GL Johnson (1993). Involvement of Ras and Raf in the G_i-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. J Biol Chem 268: 19196-19199.

Won, K, Y Xiong, D Beach, and MZ Gilman (1992). Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc Natl Acad

Sci USA 89: 9910-9914.

Wood, KW, C Sarnecki, TM Roberts, and J Blenis (1992). *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and Rsk. Cell 68: 1041-1050.

Wu, J, P Dent, T Jelinek, A Wolfman, MJ Weber, and TW Sturgill (1993a). Inhibition of the EGF-activated Map kinase signaling pathway by adenosine 3',5'-monophosphate. Science 262: 1065-1069.

Wu, J, JK Harrison, LA Vincent, C Haystead, TAJ Haystead, H Michel, DF Hunt, KR Lynch, and TW Sturgill (1993b). Molecular structure of a protein-tyrosine/threonine kinase activating p42 mitogen-activated protein (MAP) kinase: MAP kinase kinase. Proc Natl Acad Sci USA 90: 173-177.

Wu, J, JK Harrison, P Dent, KR Lynch, MJ Weber, and TW Sturgill (1993c). Identification and characterization of a new mammalian mitogen-activated protein kinase kinase, MKK2. Mol Cell Biol 13: 4539-4548.

Wu, X, B Knudsen, SM Feller, J Zheng, A Sali, D Cowburn, H Hanafusa, and J Kuriyan (1995). Structural basis for the specific interaction of lysine-containing proline-rich peptides with the amino-terminal SH3 domain of c-Crk. Structure, submitted.

Yan, M and DJ Templeton (1994a). Identification of 2 serine residues of Mek-1 that are differentially phosphorylated during activation by *raf* and Mek kinase. J

Biol Chem 269: 19067-19073.

Yan, M, T Dai, JC Deak, JM Kyriakis, LI Zon, JR Woodgett, and DJ Templeton (1994b). Activation of stress-activated protein kinase by Mekk1 phosphorylation of its activator Sek1. Nature 372: 798-800.

Yao, A and H Rubin (1994). A critical test of the role of population density in producing transformation. Proc Natl Acad Sci USA 91: 7712-7716.

Yu, C, EV Prochownik, MJ Imperiale, and R Jove (1993). Attenuation of serum inducibility of immediate early genes by oncoproteins in tyrosine kinase signaling pathways. Mol Cell Biol 13: 2011-2019.

Yu, H, JK Chen, S Feng, DC Dalgarno, AW Brauer, and SL Schreiber (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell 76: 933-945.

Zhang, X, J Settleman, JM Kyriakis, E Takeuchi-Suzuki, SJ Elledge, MS Marshall, JT Bruder, UR Rapp, and J Avruch (1993). Normal and oncogenic p21^{ras} proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 364: 308-313.

Zheng, C and K Guan (1993a). Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. J Biol Chem 268: 11435-11439.

Zheng, C and K Guan (1993b). Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. J Biol Chem 268: 23933-23939.

Zheng, C and K Guan (1994). Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. EMBO 13: 1123-1131.

Zhu, AX, Y Zhao, DE Moller, and JS Flier (1994). Cloning and characterization of p97^{MAPK}, a novel human homolog of rat Erk-3. Mol Cell Biol 14: 8202-8211.

Zullo, JN and DV Faller (1988). p21 v-*ras* inhibits induction of c-*myc* and c-*fos* expression by platelet-derived growth factor. Mol Cell Biol 8: 5080-5085.

End