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# Characterization of the Paraneoplastic Cerebellar Degeneration Antigen Cdr2

John Peter Corradi

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# **Characterization of the Paraneoplastic Cerebellar Degeneration Antigen cdr2**

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

by

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April 1997

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## Abbreviations

AChR	acetylcholine receptor
BBB	blood-brain barrier
bHLHzip	basic helix-loop-helix leucine zipper
BTEB	basic transcription element binding protein
CAR	cancer-associated retinopathy
CAT	chloramphenicol acetyltransferase
cc-zip	coiled-coil leucine zipper
cdr	cerebellar degeneration related
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
DRG	dorsal root ganglia
GAD	glutamic acid decarboxylase
glu	glucose
gal	galactose
GST	glutathione S-transferase
HSP	heat shock protein
IEG	immediate early gene
MHC	major histocompatibility class
( $\beta$ )NAP	neuronal adaptin protein
NGF	nerve growth factor
NMJ	neuromuscular junction
PCD	paraneoplastic cerebellar degeneration
PND	paraneoplastic neurologic disorder
Tag	SV40 large T antigen
UTR	untranslated region

## Abstract

Paraneoplastic cerebellar degeneration (PCD) is a remote effect of breast and ovarian cancer on the nervous system. It is believed to arise when these tumors ectopically express a neuron-specific protein, thereby initiating an anti-tumor immune response that subsequently develops into a neuronal degeneration. Three cerebellar degeneration related (cdr) genes encoding putative antigens were previously identified using PCD antisera. We have demonstrated that *cdr2*, which encodes a cytoplasmic coiled-coil leucine zipper protein, is the only *cdr* gene expressed in PCD-associated tumors, and is therefore the true PCD tumor antigen. We have isolated the mouse *cdr2* homologue and examined expression of the mRNA and protein in adult tissues. The *cdr2* antigen is detected only in the brain and testis, both considered to be immune-privileged sites. Within the nervous system, *cdr2* is expressed predominantly in the cerebellum and brainstem, the regions most affected in PCD. These findings are therefore consistent with the proposed role of *cdr2* in the autoimmune hypothesis for PCD. While the *cdr2* protein is detected only in brain and testis, the mRNA is expressed in almost all tissues, suggesting that *cdr2* is regulated at a post-transcriptional level. In order to elucidate the function of the *cdr2* gene product, we have performed protein-protein interaction studies. Using the coiled-coil leucine zipper domain of *cdr2* in a yeast two-hybrid screen, we have identified c-Myc as a *cdr2*-binding protein. Immunohistochemical analysis of cerebellar cortex revealed that *cdr2* and c-Myc colocalize exclusively to the cytoplasm of Purkinje neurons. We have found that the full length *cdr2* and c-Myc proteins interact *in vitro*, and that this binding requires the basic helix-loop-helix leucine zipper region of c-Myc. In addition, *cdr2* represses c-Myc transcriptional activity in a transfected cell line. We conclude from these findings that the *cdr2* antigen is a regulator of the oncoprotein c-Myc in cerebellar Purkinje neurons, and possibly in tumors.

## Chapter 1 - Introduction

### Paraneoplastic Neurologic Disorders

Paraneoplastic neurologic disorders (PND's) are remote effects of systemic malignancies that are not attributable to metastasis (for reviews, see Posner, 1995b; Darnell, 1996). While such effects may be the result of opportunistic infection, vascular disorders, or metabolic and nutritional complications, the term PND is used to refer to a group of syndromes that defy such categorization. Several distinct PND's have been identified and classified according to the specific regions of the central and/or peripheral nervous system in which the primary dysfunction occurs. These syndromes include subacute cerebellar degeneration, opsoclonus-myoclonus (an eye and muscle movement disorder), subacute sensory neuronopathy/encephalomyelitis, retinal degeneration, and Lambert-Eaton Myasthenic Syndrome (LEMS), a disorder of the neuromuscular junction. The occurrence of these PND's often suggests the presence of a specific underlying cancer; for example, paraneoplastic sensory neuronopathy/encephalomyelitis is found in association with small cell lung cancer (SCLC) exclusively.

In addition to their affiliation with particular neoplasms, PND's share several other characteristics that distinguish them from other neurologic complications of cancer. Presentation of the neurological symptoms most often precedes detection of the tumor. When the tumor is identified, it is usually indolent and nonmetastatic, though these tumors often grow aggressively in neurologically normal patients. The disorders are profoundly debilitating and the symptomatology and corresponding pathological damage are restricted to the nervous system. Finally, with very few exceptions, paraneoplastic syndromes are irreversible and refractory to treatment of the cancer.

There are several potential mechanisms that could account for the remote effects of cancer on the nervous system observed in PND's. The

tumor may secrete “toxins” that interfere with normal CNS function, as has been demonstrated for certain peptide hormones and cytokines. It is also possible for the tumor to competitively deprive the nervous system of important nutrients required for active neuronal metabolism. Immunosuppression related to the cancer can sometimes allow for viral infections that affect the CNS. However, there is no evidence to date that supports any of these etiologies for the PND's. The usually small, nonmetastatic tumors associated with paraneoplastic disease are not likely to deplete the body of any essential substrates for nervous system function. Patients with paraneoplastic syndromes are not overtly immunosuppressed, as evidenced by the absence of common opportunistic infections associated with cancer and the presence of high titers of antibodies in their serum (to be discussed below).

Although the pathogenesis of PND's remains unknown, the cumulative clinical evidence suggests an autoimmune mechanism. PND patients harbor high titers of characteristic autoantibodies in both their serum and cerebrospinal fluid (CSF) that are selectively reactive with both the regions of the nervous system affected in the disorder and the tumor tissue. There is a strong correlation between the presence of the antibodies and neurologic dysfunction. PND antibodies are rarely found at detectable levels in healthy individuals, and only infrequently found in cancer patients that are free of neurological disease. In addition, the fact that the tumors found in PND patients are often occult suggests that there may be active anti-tumor immunity. There are several reports of spontaneous regression of tumors associated with paraneoplastic disease (Darnell and DeAngelis, 1993; Zaheer et al., 1993), and one study found a correlation between the serum titer of the Hu antibody associated with paraneoplastic sensory neuronopathy and the degree to which the small cell lung cancer progresses (Dalmau et al., 1990). The presence of inflammatory lymphocytic infiltrates in both the nervous system and tumor tissue of some PND patients also provides evidence of immune system involvement.

Taken together, the clinical indications of effective anti-tumor immunity with concomitant anti-neuronal immunity in PND suggest the following model for their pathogenesis. First, an immune response is elicited when a systemic tumor ectopically expresses a neuronal protein (termed an "onconeural" antigen). An unidentified secondary event allows the immune response to become competent to recognize the PND antigens in their normal cellular context, resulting in subsequent neuronal dysfunction or degeneration. This autoimmune model of PND is predicated on the assumption that the ectopic expression of these neuronal proteins is necessary for their immunogenicity. While direct evidence for this is lacking, there are several observations concerning the immune privilege of the nervous system which support the autoimmune hypothesis. Limited surveillance of the CNS by immune cells and the absence of detectable antigen presentation complexes on neurons may render the immune system naive to proteins that are restricted in their expression throughout development to the nervous system (Streilein, 1993; Streilein, 1995). In fact, the PND antigens for which a rigorous analysis of expression patterns has been carried out all adhere to this requirement. The Hu and Nova onconeural antigens are RNA binding proteins expressed exclusively in neurons both early in embryogenesis and in adults (Szabo et al., 1991; Dalmau et al., 1992; Buckanovich et al., 1993; Buckanovich et al., 1995; Okano and Darnell, 1996). Similarly, the autoimmune cerebellar degeneration antigen  $\beta$ -NAP is a neuron-specific vesicle coat protein (Newman et al., 1995), and the stiff-man syndrome antigens GAD (Solimena et al., 1988) and amphiphysin (DeCamilli et al., 1993) are nerve terminal vesicle-associated proteins.

With the exception of the neuromuscular junction (NMJ) antigens, all of the PND antigens identified are intracellular proteins with varied localization. It remains unclear how the immune response becomes competent to recognize the neuronal antigens or exactly how the pathologic damage is mediated. One possibility is that the antigens are merely markers

of an independently initiated tumor degeneration, rather than being direct targets of the immune response. It has been proposed that some common intracellular antigens (e.g. hnRNP's) may become accessible to the immune system via apoptotic blebbing of dying cells (Tan, 1994). This appears to be an overly simplistic mechanism for antitumor immunity in PND, however, since the PND antibodies are not typically found in combination with other autoantibodies to intracellular proteins.

Clinical findings of localized lymphocytic infiltrates and high titer serum and CSF antibodies suggest a role for both cellular and humoral immune responses, though the relative contributions of either of these mechanisms to the antitumor and antineuronal immunity in PND remains unknown. While it is likely that cellular immunity may account for the pathologic damage to the nervous system, there is also the possibility that the neuronal dysfunction may be antibody mediated. The relative concentration of specific to total IgG in the CSF of PND patients is higher than in the serum, indicating that there is active antibody synthesis within the CNS (Graus et al., 1988; Furneaux et al., 1990b). Targeted disruption of PND antigen function could only occur if the antibody has access to the intracellular proteins. In fact, several studies have indicated that there is selective uptake of large macromolecules such as immunoglobulins (Borges et al., 1985; Fabian and Petroff, 1987), and specifically PND antibodies (Graus et al., 1991; Greenlee et al., 1993; Hormigo and Lieberman, 1994), in some neurons. The observation that many autoantibodies target functional domains of proteins (Tan, 1991) has been shown to be true for the PND antibodies. PND epitopes have been mapped to the leucine zipper domain of the cerebellar degeneration antigen (Sakai et al., 1993), and to RNA binding motifs found in the Hu and Nova family members (Buckanovich, et al., 1995; Manley et al., 1995). Interestingly, antibodies from paraneoplastic opsoclonus/myoclonus (POMA) patients' sera can inhibit RNA binding of the Nova-1 protein *in vitro* (Buckanovich, et al., 1995). It should be noted, however, that efforts to produce an animal model of PND's by either passive transfer of autoantibodies or immunization with

the PND antigens have so far failed to reconstitute the syndromes (Graus, et al., 1991; Sakai et al., 1995).

Recently, Darnell (Darnell, 1996) proposed a reclassification of the PND's according to the nature of the target antigens. In this approach it is believed that examination of the expression and function of these proteins may lead to an understanding of their potential involvement in the pathogenesis of the syndromes. Identification of common characteristics may lend insight into the immunogenicity of the antigens, and perhaps the consequences of disrupted functionality of these proteins in neurons. These newly defined categories include nerve terminal or vesicle-associated proteins (e.g.  $\beta$ -NAP, amphiphysin, GAD), neuron-specific RNA binding proteins (e.g. Hu and NOVA family members), NMJ proteins (e.g.  $\text{Ca}^{2+}$   $\beta$ -subunit, AChR  $\alpha$ -subunit), and putative neuronal signaling proteins. PND antigens of the last category include recoverin, associated with paraneoplastic blindness, and the major paraneoplastic cerebellar degeneration antigen cdr2, which is the focus of this thesis.

It is estimated that the various PND's identified affect only 0.1-1.0% of cancer patients (Posner, 1995b). Despite the rare occurrence of these disorders, they are of importance to the clinician in providing evidence of a malignancy that may otherwise have gone undetected. In addition, the identification of autoantibodies in PND patients has provided clues to the etiology of the syndromes, and offers the researcher opportunities to investigate the roles of the disease antigens in neurobiology, tumor biology and mechanisms of autoimmunity.

### **Antibody-Associated Paraneoplastic Cerebellar Degeneration**

The best characterized of the paraneoplastic syndromes that affect the CNS is paraneoplastic cerebellar degeneration (PCD) (for review, see Posner, 1993). PCD most often complicates gynecological tumors of the ovary or uterus, breast cancer, lung (particularly small-cell) cancer, and lymphomas

(particularly Hodgkin's disease). First described clinically in 1919 (Brouwer, 1919), its association with cancer was not fully documented until 1951 (Brain et al., 1951), and the finding of characteristic autoantibodies in patients with ovarian and breast cancer was not made until the 1980's (Greenlee and Brashear, 1983; Jaeckle et al., 1983). It is this last observation that distinguishes gynecological tumor-associated PCD from other occurrences of the syndrome.

As is the case for most of the PND's, the neurologic symptoms of PCD usually precede the detection of an underlying malignancy by a few months to a year. The predominant clinical signs are of pancerebellar dysfunction, and display a subacute onset of weeks to months. Within that time symptoms may rapidly progress from slight incoordination in walking to the inability to walk, sit, or eat independently. PCD patients also commonly have dysarthric speech and oscillopsia (rapid involuntary eye movements). The symptoms usually stabilize at their peak, and treatments directed toward the neurologic dysfunction, such as immunosuppression, or the tumor usually have no effect on the disease. In addition to the cerebellum, a study of 55 antibody positive PCD patients revealed that there was involvement of numerous regions of the nervous system, particularly the brain stem (Peterson et al., 1992).

The most striking pathologic feature of PCD is the extensive loss of Purkinje neurons in the cerebellar cortex (Verschuuren et al., 1996). These large cells essentially form a monolayer that receive multiple afferent inputs and constitute the sole output of the cerebellar cortex to the deep cerebellar nuclei. Other, perhaps secondary, findings include the thinning of the molecular and granule cell layers of the cerebellar cortex and Bergmann gliosis (proliferation of Bergmann astrocytes). Occasionally lymphocytic infiltrates are present in the cerebellum, but rarely in the Purkinje cell layer of the cortex. This may be due to the often long time lapse between onset of the degeneration and autopsy, since the neurologic disease itself is not fatal. Most PCD-associated malignancies are localized and show an indolent course after discovery, but are not histologically distinct from non-PCD tumors arising in

the same tissues. However, several studies have documented a greater percentage of PCD tumors with lymphocytic infiltrates than in those of patients without PCD (Hetzel et al., 1990; Verschuuren, et al., 1996).

Although many cases of PCD may be clinically and pathologically indistinguishable, the presence of a characteristic autoantibody in some patients directs the search for the tumor to ovary or breast, and may indicate a unique pathogenesis for this antibody-associated syndrome. The antibody, dubbed "anti-Yo" by Posner and Furneaux (Posner and Furneaux, 1990), is a polyclonal IgG found at high titers in both the serum and CSF of PCD patients. By immunohistochemical analysis anti-Yo is reactive with several discrete populations of neurons and the PCD tumor. Reactivity is found at limiting dilutions in the cytoplasm of Purkinje neurons (Jaekle, et al., 1983), though at higher antibody concentrations neurons in the deep cerebellar nuclei, brainstem, retina, and dorsal root ganglia have also been reported to be immunoreactive (Altermatt et al., 1991; Tomimoto et al., 1993). On Western blots of Purkinje cell extract anti-Yo identifies a major species reported as either 62 kDa or 52 kDa and a minor species of 34 kDa. In addition to immunohistochemical reactivity, Furneaux et al. (Furneaux et al., 1990a) found that the anti-Yo antibody recognized the 62 kDa species on Western blots of PCD patients' tumors, but not on those of neurologically normal patients. Verschuuren et al. (Verschuuren, et al., 1996) found evidence of IgG deposits in a PCD tumor sample, although the identity of the antibody as anti-Yo could not be made definitively. In a clinical study describing 55 PCD patients with the anti-Yo antibody, all had either ovarian or breast cancer (Peterson, et al., 1992). With the exception of one report that describes two neurologically normal ovarian cancer patients with Purkinje cell reactive antibodies (Brashear et al., 1989), anti-Yo antibody is not found in healthy individuals or those with cancer and no PCD (Posner, 1995b).

Attempts to further define the intracellular localization of the PCD antigen have yielded somewhat different results. Immunoelectron microscopy was used by two groups to examine the staining of Purkinje

neurons by PCD antisera. Ultrastructural examination revealed staining of discrete cytoplasmic organelles in both reports, but the distribution varied. Rodriguez et al. (Rodriguez et al., 1988) found that staining was primarily confined to the perinuclear rough endoplasmic reticulum (ER), vesicles on the *trans* face of the Golgi complex, and rough ER in dendrites. However, a subsequent study found that immunoperoxidase staining was associated with both smooth and rough ER and free polyribosomes in the cell soma, but only smooth ER in the Purkinje cell dendrites; no Golgi staining was observed (Tomimoto, et al., 1993). These inconsistent findings have not been clarified, but it should be noted that only the latter study characterized the antibody by Western blot analysis.

A critical advance in the study of PND's was the use of patients' sera to screen cDNA expression libraries to identify the genes encoding potential PND antigens. In the case of PCD this approach has led to the isolation of three cerebellar degeneration related genes, referred to here as cdr1, 2, and 3. The first cDNA characterized (cdr1) encodes the 34 kDa protein recognized by PCD patients' sera (Dropcho et al., 1987). Encoded by a single exon, the predicted amino acid sequence reveals an unusual structure composed of nearly identical hexapeptide repeats over 91% of the protein. RNA dot blot analysis indicated that cdr1 is expressed predominantly in the brain, with expression five- to tenfold greater in the cerebellum than the cerebral cortex. The cdr1 mRNA was also detected in several tumor cell lines of neuroectodermal, kidney and lung origin, but not in breast or gynecological tumor cell lines. Although the authors reported the detection of cdr1 mRNA in two PCD-associated tumors, neither the method of detection nor the percentage of tumors examined that this result represented were described.

Rabbit antisera was generated against a synthetic 16 amino acid peptide corresponding to cdr1 sequence and used for both immunohistochemical and Western blot analysis (Furneaux et al., 1989). This polyclonal antibody specifically identified the 34 kDa protein from Purkinje cells and one PCD-associated breast tumor. Immunoperoxidase staining of human brain

sections was entirely restricted to the cytoplasm of Purkinje neurons. However, in another study the 34 kDa species was not detectable in PCD and non-PCD tumor extracts using native PCD patients' sera (Furneaux, et al., 1990a), suggesting that the relative titer of cdr1-specific antibody in disease antisera is very low. The function of the cdr1 gene product cannot be inferred from its primary amino acid sequence, and its relevance to the cerebellar syndrome is still unclear.

The second cDNA (cdr2) was isolated by two groups independently from both a cerebellar (Fathallah-Shaykh et al., 1991) and a HeLa cell (Sakai et al., 1991) library, and is the major focus of this study. A highly related protein, cdr3, was also isolated from a HeLa cDNA library by Fathallah-Shaykh and Posner (Genbank accession L02687). Aside from the noted 45% amino acid homology with cdr2, this protein has not been characterized further.

### **The PCD Antigen cdr2**

Sakai et al. (Sakai, et al., 1991) first isolated a cdr2 cDNA from a human cerebellum library by screening with previously characterized sera from a PCD patient with a uterine carcinoma. The cdr2 amino acid sequence shares no significant homology with cdr1 nor with other proteins in the databases. They found that the cdr2 open reading frame predicts a protein of molecular weight 52 kDa, and this full length protein expressed in bacteria comigrates with the major cerebellar species identified by PCD antisera. Expression of the cdr2 gene in nine different tissues was examined by RT-PCR analysis. The cdr2 mRNA was detected in cerebellum, brainstem, and intestine; but not in heart, lung, liver, spleen, ovary, or uterus. However, there are several important weaknesses in the methodology used: first, an appropriate control for the integrity of the RNA in the negative samples was omitted; second, the reverse transcription reaction was randomly primed, whereas a poly (dA)<sub>12-18</sub> oligonucleotide primer was used in the PCR amplification; lastly, the primers corresponding to the human cdr2 nucleotide sequence were used with rat

RNA as a starting template without knowledge of the degree of sequence homology between species. Therefore, an accurate expression profile for *cdr2* is not provided by this study.

A HeLa cell *cdr2* cDNA was also isolated and found to encode a 510 amino acid protein of predicted MW 54 kDa (Fathallah-Shaykh, et al., 1991), though the 5' end of the coding region was not identified. With the exception of the 5'-extended open reading frame, this sequence is essentially identical to that isolated by Sakai et al. Hydropathy predictions based on the algorithm by Kyte and Doolittle indicated that the protein is highly hydrophilic over the length of the sequence. There is a weak homology with many coiled-coil forming proteins of the myosin class based on the spacing of certain amino acids, and Fathallah-Shaykh et al. (Fathallah-Shaykh, et al., 1991) identified what they termed a "super leucine zipper" domain present in the N-terminal region of the protein. Allowing for substitution of one serine residue and one histidine residue, the heptad leucine repeat is found over a 70 amino acid region. The authors also reported the identification of a zinc finger motif in the *cdr2* protein, but the criteria for forming this DNA binding domain are not met by the sequence upon closer inspection (personal observation). Another salient feature of the primary sequence is a highly acidic region of 28 amino acids near the N-terminus with a predicted isoelectric point (pI) of 3.4, reminiscent of the acidic activation domains present in some transcription factors.

Importantly, PCD antisera affinity purified from a *cdr2* fusion protein was found to yield the same immunohistochemical and Western blot patterns as the native sera (Fathallah-Shaykh, et al., 1991). This observation increases the likelihood that *cdr2* encodes the major PCD antigen or at least harbors a highly related epitope recognized by the anti-Yo antibody. Inspection of the overlapping original clones identified by antibody screening in addition to the presence of a highly homologous leucine zipper encoded by another HeLa cDNA (*cdr3*) suggested that the epitope may include this most conspicuous structural feature (Fathallah-Shaykh, et al., 1991). In fact Sakai et

al. further dissected the cdr2 cDNA to define the region encoding the anti-Yo epitope (Sakai, et al., 1993). A series of nested deletions were made in the cdr2 cDNA using restriction endonucleases, and these deleted constructs were used to express fusion proteins in bacteria. Immunoblots using sera from five different PCD patients revealed that the common epitope lies within a 37 amino acid region that encompasses the leucine zipper motif. The full length fusion protein was not recognized by the sera of several ovarian cancer patients who were neurologically normal.

Further support for the identity of the major PCD antigen as cdr2 comes from a more recent study by Sakai et al. (Sakai, et al., 1995), in which mice were immunized with the full length recombinant cdr2 protein. A robust immune response was elicited in five different strains of mice, resulting in the production of anti-cdr2 antibody at or above the titers typically found in PCD patients. More importantly, the antibodies recognized a protein in human cerebellum that comigrated with the recombinant protein by Western blot. Immunohistochemical analysis also revealed a parallel pattern of reactivity with PCD patients' sera, specifically staining the Purkinje cell cytoplasm as well as many large and small neurons in the brainstem. The authors reported that the immunoreactivity of the mouse derived anti-cdr2 antibody was more widespread than that seen with the PCD antisera in their study. However, several other studies, including the one presented here, have revealed that the reactivity of the anti-Yo antibody is not restricted exclusively to cerebellar Purkinje neurons (Altermatt, et al., 1991; Tomimoto, et al., 1993).

## **Specific Aims of This Study**

### *Expression of cdr2 in tumor and normal tissue*

Tumors of PCD patients have previously been shown to be immunoreactive with Yo antisera, and only the major reactive species is consistently detectable on immunoblots of tumor extracts (Furneaux, et al., 1990a). Identification of

the PCD tumor antigen as cdr2 cannot be made definitively based on this evidence, however, because there are two known genes (cdr2 and cdr3) which may encode proteins with the anti-Yo epitope of the corresponding size. It is essential to analyze expression of all three cdr genes at the level of the mRNA in order to distinguish which of these genes encodes the *in vivo* PCD tumor antigen.

Given the autoimmune hypothesis for PCD, which is strongly supported by the available clinical evidence, it is important to clearly define the expression pattern of the putative disease antigen(s) in normal tissues. The reasons for this are twofold: first, the distribution of the antigen is expected to generally correspond with the localization of the symptomatology and degenerative pathology; second, a prerequisite for the autoimmune pathogenesis is the restricted expression of the antigen to immune privileged tissues. In the case of PCD, symptoms are confined primarily to the CNS and predominantly to the cerebellum and brainstem (Peterson, et al., 1992), and the pathological findings are usually limited to the tumor, cerebellum and brainstem (Verschuuren, et al., 1996). There have been numerous reports of the distribution of the cdr2 antigen and/or corresponding transcript, but the studies have been either inadequate in their analysis, incomplete in their scope or inconsistent with independent studies. Observations of either the cdr2 mRNA or PCD antisera immunoreactivity outside of the nervous system (Sakai, et al., 1991; Tomimoto, et al., 1993) suggested that the antigen may not be restricted to immune privileged tissues. However, weaknesses in the analyses used (as discussed above) have left the precise expression pattern of cdr2 in normal tissues an unresolved issue.

A major aim of this study was to define the possible role of cdr2 in the pathogenesis of PCD by determining the expression of the mRNA and protein in both PCD tumors and normal tissues. The data presented here indicate that the expression of cdr2 is indeed consistent with its proposed role as an onconeural antigen. Specifically, the *in vivo* tumor antigen is encoded by the cdr2 gene, and the normal expression of cdr2 is restricted to immune

privileged tissues. Moreover, the distribution of cdr2 in the nervous system is confined predominantly to the cerebellum and brainstem, and provides a correlation with the symptoms and pathology of the disease.

#### *The function of the cdr2 antigen*

PND's in general, and PCD in particular, offer an interesting opportunity to investigate mechanisms of neurobiology, tumor biology, autoimmunity and neurodegeneration. The PND antigens are generally neuron-specific proteins that may be targets of a neurodegenerative process. In addition, the proteins serve as *bona fide* tumor antigens, in that their expression elicits an effective anti-tumor immune response. Ectopic expression of the antigens in subsets of specific types of malignancies suggests that they may also provide a selected function in tumors. For these reasons the biology of the PND antigens is inherently interesting to examine, and may provide insight into the complex etiology of these syndromes.

Despite considerable interest in the PCD antigen, there have been no studies reported seeking to elucidate the possible function of the cdr2 protein. Fathallah-Shaykh et al. (Fathallah-Shaykh, et al., 1991) identified several structural motifs predicted by the amino acid sequence (the leucine zipper, acidic domain, and zinc finger) to infer a function for cdr2. Taken together, the authors argue, these structural features suggest a possible role for the cdr2 protein in the regulation of gene expression. To support this speculation they cited their unpublished observation that the cdr2 protein present in HeLa cell cytoplasmic extracts can bind with high affinity to native DNA cellulose (Fathallah-Shaykh, et al., 1991). The rigor of such an approach to identify a true sequence-specific DNA binding protein is questionable. In addition, the lack of a clearly identifiable DNA binding domain and the entirely cytoplasmic localization of the immunoreactive protein (by both light and electron microscopy) suggest that cdr2 is not likely to function as an orthodox transcriptional activator/repressor. However, this does not preclude the

possibility that cdr2 may play a more indirect role in the regulation of neuronal gene expression.

Another major aim of this study was to determine the possible function of the cdr2 protein. Leucine zipper structural motifs mediate specific protein-protein interactions that serve to regulate the biological activity of a complex. The presence of this oligomerization domain in cdr2 suggested a reasonable approach to investigate the function of the protein. Using the leucine zipper domain of cdr2 in a yeast two-hybrid screen, we sought to identify specific cdr2-interacting proteins. In this way, the product of the proto-oncogene c-myc was identified as a cdr2 binding partner. Evidence of colocalization, direct binding *in vitro*, and an effect on c-Myc transcriptional activity suggest that this interaction is physiologically relevant. Taken together, the data support a role for cdr2 in the regulation of c-Myc, and have important implications for neurobiology, tumor biology and the pathogenesis of PCD.

## Chapter 2- Materials and Methods

### Expression Studies

#### *Tumor RNA Extraction and RT-PCR*

Frozen samples of ovarian tumors removed from patients with Yo-positive PCD were obtained from the Memorial Sloan Kettering Cancer Center. 100mg fragments were used for purification of either total or poly A+ RNA. Total RNA was prepared by the method of Chomcynski and Sacchi (Chomcynski and Sacchi, 1987) , and the samples were subsequently treated with RQ1 RNase-free DNase (Promega, Madison, WI) before RT-PCR analysis. For the purification of polyA+ RNA, the section was homogenized by sonication in 400µl extraction buffer (4M guanidinium thiocyanate, 0.1M Tris-HCl pH8.0, 1% dithiothreitol, 0.5% lauryl sarcosinate), 800µl of binding buffer (0.1M Tris-HCl pH8.0, 0.4M LiCl, 20mM EDTA) was added and the lysate was centrifuged at 18,000 x g for five minutes. Magnetic Dynabeads Oligo (dT)<sub>25</sub> (Dyna, Inc., Great Neck, NY) were prepared by washing 300µl of beads once with 200µl Binding Buffer. The lysate supernatant was added to the Dynabeads, mixed, and incubated at room temperature for 3-5 minutes. A magnet was used to immobilize the beads, the supernatant was removed and the beads were washed three times with 0.5ml wash buffer (10mM Tris-HCl pH 8.0, 0.15M LiCl, 1mM EDTA). PolyA+ RNA was then eluted in 20µl 2mM EDTA pH 8.0 at 65°C for two minutes.

For RT-PCR reactions 2µl tumor polyA+ RNA, 1-2µg total tumor RNA or 20ng polyA+ RNA from normal tissues (CLONTECH Laboratories, Inc., Palo Alto, CA) were denatured at 70°C for ten minutes and placed on ice. The RNA was reverse transcribed using random hexanucleotide primers (Boehringer Mannheim, Indianapolis, IN) and Superscript reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 42°C for 50 minutes and the

reaction stopped by incubation at 95°C for 5 minutes. One-tenth of the first strand cDNA sample was used as template for a PCR reaction using Amplitaq polymerase (Perkin Elmer Corp., Norwalk, CT) and the following forward and reverse oligonucleotide primers corresponding to cdr2: 5'-TGAATGGAGTTGAGAAGCTGGTG-3' and 5'-GAGATGCCCCTCTGTTTCACAG-3'; and cdr3: 5'-CATTGAGCGCCTCCAGGCT-3' and 5'-AGCTCCTTGAGGCAGGGGAA-3'. The products were amplified for 35 cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute). PCR products were labeled by addition of trace amounts of  $\alpha^{32}\text{P}$ -dCTP (Amersham Life Science Inc., Arlington Heights, IL) to the reaction mixtures and were loaded on a 10% non-denaturing acrylamide gel and visualized by autoradiography.

#### *cDNA library screening and sequencing*

Adult mouse brain and spleen cDNA libraries (Stratagene, La Jolla, CA) were plated at a density of  $1 \times 10^5$  pfu per 135mm dish. Plaques were lifted onto nitrocellulose filters for hybridization with  $^{32}\text{P}$ -labeled probes (Sambrook et al., 1989).  $1.2 \times 10^6$  pfu of the mouse brain library were screened at low stringency with a probe corresponding to bp 1-872 of the human cdr2 cDNA (Fathallah-Shaykh, et al., 1991). This screen resulted in the isolation of a single 2.4kb clone.  $6 \times 10^5$  pfu of the mouse spleen library were screened with a cDNA probe corresponding to bp 153-720 of the mouse brain cdr2 cDNA (see Figure 4). This screen resulted in the isolation of three overlapping clones, one comprising the full coding and UTR regions. The cDNA clones were sequenced by the dideoxy method of Sanger et al. (Sanger et al., 1977) using Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH), and sequence data were analyzed using the MacVector software package (International Biotechnologies, Inc., New Haven, CT).

### *Northern blot and RT-PCR analysis of mouse tissues*

Adult ICR mouse (Charles River) organs were dissected and total RNA was prepared using the TRIZOL Reagent (Gibco BRL) and the protocol recommended by the manufacturer. 30µg of total RNA was resolved on an agarose/50% glyoxal gel (Sambrook, et al., 1989) and transferred to Biotrans B nylon membrane (Pall Corp., Glen Cove, NY). The RNA was UV cross-linked to the membrane and the blot was prehybridized in 6X SSC, 0.5% SDS, 5X Denhardt's solution, 0.05% sodium pyrophosphate, 100µg/ml denatured herring sperm DNA for 30 minutes at room temperature. cDNA's corresponding to a 580 base pair fragment of the mouse cdr2 3'-UTR (bp 1468-2048 in Figure 4) and the full length GAPDH coding region were labeled with <sup>32</sup>P-dCTP using the Prime-It Kit (Stratagene, La Jolla, CA). The RNA blot was hybridized with 1x10<sup>6</sup> cpm/ml labeled probe in 6X SSC, 1X Denhardt's solution, 0.05% sodium pyrophosphate, 100µg/ml yeast tRNA for 16 hours at 65°C. The membrane was then washed twice in 2X SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 65°C, and twice in 0.1X SSC, 0.1% SDS, 0.1% sodium pyrophosphate at room temperature, and hybridization was visualized by autoradiography.

The RT-PCR analysis was carried out as described above. First strand cDNA was synthesized using random hexanucleotide primers from total or polyA<sup>+</sup> RNA from adult mouse cerebellum, spleen, heart, and testes. cdr2 primers flanking the leucine zipper coding region (bp 153-720 in Figure 4) were used to amplify a 567 bp product by PCR. The cdr2 primer sequences were 5'-CATGCTGGCAGATAACC-3' and 5'-AGGGCTCTGTTGGCTCTG-3'.

### *Antibody affinity purification and Western blot analysis*

The region coding for amino acids 16-192 of human cdr2 was fused in frame to glutathione-S-transferase (GST) in the GSTag vector (gift of David Ron, New York University, NY). Bacteria transformed with the GSTag-cdr2 plasmid were grown to an OD<sub>600</sub> of 0.5, and 1mM IPTG added for an

additional three hours. Cells were harvested and resuspended in ice cold PBS, lysed by sonication and the lysate cleared by centrifugation. The cleared lysate was incubated with glutathione Sepharose (Pharmacia Biotech Inc., Piscataway, NJ), washed in PBS, and fusion protein eluted with 10mM reduced glutathione. Purity and immunoreactivity of the GST-cdr2 fusion protein were verified by SDS-PAGE, Coomassie blue staining, and Western blot analysis.

All human sera were obtained from either PCD patients at Memorial Sloan-Kettering Cancer Center and The Rockefeller University Hospital, or from neurologically normal individuals. Prior to experimental use, PCD sera were assayed for characteristic Purkinje cell Western blot and immunohistochemistry, as well as for specific reactivity with a cdr2 fusion protein.

For affinity purification of PCD antisera, Immobilon membrane (Millipore Corp., Bedford, MA) was wetted with methanol and rinsed well with ddH<sub>2</sub>O. 20-30µg of GST-cdr2 fusion protein was spotted on a 0.5x3cm strip of membrane and blocked for 60 minutes in 25mM Tris-HCl pH 8.0, 20mM NaN<sub>3</sub>, 150mM NaCl, 5% non-fat dry milk. The strip was washed with PBS/0.02% sodium azide and incubated with 1ml of PCD antiserum for two hours at 4°C. The strip was then washed four times with 25mM Tris-HCl pH 8.0, 20mM NaN<sub>3</sub>, 150mM NaCl, 0.1% Triton-X-100, two times with 25mM Tris-HCl pH 8.0, 20mM NaN<sub>3</sub>, 150mM NaCl, 2mM EDTA, and antibody was eluted with 1ml 0.2M glycine pH 3.0. The elution was repeated and the pooled eluates were neutralized with 0.5N NaOH to a final pH of 7.5-8.0. Ultrafiltration in a Centricon-10 unit (Amicon, Inc., Beverly, MA) was used to remove the glycine from the affinity purified antibody.

For Western blot analysis the indicated tissues were dissected from adult ICR mice, homogenized in PBS, 2X SDS sample buffer was added, and the samples were boiled. Frozen sections of PCD ovarian tumors were pulverized with a mortar and pestle under liquid nitrogen, and homogenized

in lysis buffer (10mM Tris-HCl pH 7.4, 50mM NaH<sub>2</sub>PO<sub>4</sub>, 50mM KF, 1% NP-40, 5mM EDTA). 45µg of total protein from each tissue extract was resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated with affinity purified PCD antibody diluted 1:50 or native PCD antisera diluted 1:400, washed and incubated with anti-human IgG conjugated to horseradish peroxidase (Amersham) at a 1:5000 dilution. Reactive proteins were detected using the ECL kit (Amersham) according to the manufacturer's instructions, and stripped of antibody according to the ECL protocol.

### *Two-dimensional gel electrophoresis*

Cerebellum and testis from Sm/Ckc mice were homogenized in 2D lysis buffer (9.5M urea, 2% NP-40, 5% β-mercaptoethanol, 2% Biolyte ampholytes (BioRad Labs, Hercules, CA) consisting of 75% 3/5 range and 25% 3/10 range Biolytes). The lysate was clarified by centrifugation at 2100 x g for five minutes, and protein concentrations were adjusted with 2D lysis buffer. Isoelectric focussing (IEF) gels were performed essentially by the method of O'Farrell (O'Farrell, 1975). 0.75mm IEF slab gels (9.2M urea, 4% acrylamide (ReadySol IEF, Pharmacia Biotech), 2% NP-40, and 5% Biolyte ampholytes) were loaded with 40µg of total protein per lane, and the samples were covered with sample overlay buffer (7% urea, 2.5% ampholytes, 5% β-mercaptoethanol). The gels were run using 0.01M H<sub>3</sub>PO<sub>4</sub> and 0.02M NaOH buffers as described by O'Farrell (O'Farrell, 1975) at 4W constant power. Voltage was limited to 700V and gels were run for 1800V-hr. Lanes containing the samples were cut from the gel, equilibrated with 1X SDS sample buffer for five minutes, and loaded horizontally onto a 1mm 9% SDS-PAGE gel with a 3% stacking gel. A single well was loaded with 40µg protein extract in SDS sample buffer for one-dimensional analysis. Gels were transferred to nitrocellulose and probed with PCD antisera.

### *In situ hybridization*

The protocol used was essentially the same as that described by Newman et al. (Newman, et al., 1995). Adult ICR mouse tissues were embedded and frozen in O.C.T. Compound (Miles Inc., Elkhart, IN). 10µm sections were cut using a cryostat and applied to Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA). Tissue sections were fixed in 4% paraformaldehyde, rinsed in PBS, and dehydrated in incremental ethanol baths. The sections were then treated in 0.1M triethanolamine pH 8.0 / 0.25% acetic anhydride, rinsed in 0.2X SSC buffer, dehydrated in ethanol again, and dried in a dessicated chamber overnight. Tissue sections were overlaid with 40µl of prehybridization solution (10mM Tris pH 7.4, 0.6M NaCl, 50% formamide, 2.5X Denhardt's reagent, 0.05% SDS, 1mM EDTA, 150µg/ml denatured herring sperm DNA, 50µg/ml yeast RNA) and incubated at room temperature for 2 hours in a humidified chamber. A plasmid containing the same region of the 3'-UTR of the mouse *cdr2* gene as that used for the Northern blot analysis (in both orientations) was linearized and both sense and antisense riboprobes were transcribed using T7 RNA polymerase (Stratagene) and <sup>33</sup>P-UTP (Dupont/NEN). The labeled probe was purified on a Sepharose G50 column. Labeled riboprobe was added to hybridization solution (prehybridization solution + 10mM DTT, 10% dextran sulfate), denatured at 95°C, and aggregates were removed by centrifugation. The prehybridization solution was removed from the tissue sections, replaced with 40µl of hybridization solution (~5x10<sup>5</sup> - 1x10<sup>6</sup> cpm labeled probe per section) and the sections were overlaid with parafilm to prevent evaporation. Sections were incubated at 50°C for 36-48 hours in a humidified chamber. The sections were rinsed in 1X SSC buffer at room temperature, washed in 50% formamide, 1X SSC, 10mM DTT at 49°C for 30 minutes, then washed in 0.5X SSC at room temperature for 30 minutes. The slides were then incubated in 20µg/ml RNase A in RNase

buffer (10mM Tris pH 8.0, 0.5M NaCl, 1mM EDTA) at 37°C for 30 minutes, and washed in RNase buffer alone at 37°C for 30 minutes. Finally, the sections were washed in 0.2X SSC at 50°C for 1 hour, 0.2X SSC + 7mM DTT at 50°C for 2 hours, and dehydrated in incremental ethanol baths containing 0.3M ammonium acetate. The dried sections were then dipped in photographic emulsion (Kodak) and exposed at 4°C for 7-10 days.

### *Immunohistochemistry*

For the human tissues, IgG from PCD and normal human sera was isolated and biotinylated as described previously (Furneaux, et al., 1990a). Paraffin embedded sections of ovarian tumor and cerebellum from PCD patients were reacted with the biotinylated antibodies as described by Verschuuren et al. (Verschuuren, et al., 1996).

For the mouse tissues, whole organs were dissected from adult ICR mice and tissues were embedded and frozen in O.C.T. Compound. 10µm sections were fixed in methanol/0.3% H<sub>2</sub>O<sub>2</sub> at room temperature for 30-60 minutes to quench endogenous peroxidase activity, washed in PBS, and blocked with PBS/2% normal goat serum (NGS) at room temperature for one hour. Sections were incubated with primary antibody diluted in PBS/2% NGS at 4°C overnight, washed in PBS and incubated with biotinylated anti-human IgG (Vector Laboratories Inc., Burlingame, CA) diluted 1:5,000 in PBS/2% NGS at room temperature for 1-2 hours. The signal was enhanced by addition of an avidin-biotin complex (Vectastain Elite Kit, Vector Laboratories Inc.) and visualized with diaminobenzidine in the presence of H<sub>2</sub>O<sub>2</sub>.

### **Protein Interaction Studies**

#### *Prediction of cd2 secondary structure*

The human cdr2 amino acid sequence was analyzed using the Paircoil algorithm described by Berger et al. (Berger et al., 1995). This algorithm uses

pairwise residue correlations from a database of known amino acid sequences that form coiled coil structures. The cdr2 sequence was submitted for analysis via the internet at <http://ostrich.lcs.mit.edu/cgi-bin/score> and the output is represented in Figure 9. A helical wheel representation of the human cdr2 leucine zipper domain was generated using the Protean program included in the Lasergene software package (DNASTar Inc., Madison, WI).

### *Yeast Two-Hybrid Screen*

The Interaction Trap assay, including all plasmids, strains, and the HeLa cDNA library, was kindly provided by Dr. Roger Brent and colleagues (Massachusetts General Hospital, Boston). Three fragments of the human cdr2 cDNA encoding amino acids 1-419, 1-173, and 65-140 were generated by PCR and were cloned in frame into the pEG202 vector to express LexA-cdr2 fusion proteins (the “baits”). The host strain for all assays was EGY48 (MATa trp1 ura3 his3 LEU2::pLexAop6-LEU2), in which the endogenous LEU2 gene has been replaced by a LEU2 reporter with six LexA binding sites.

All bait constructs were tested for their ability to activate the LEU2 reporter gene independently, and their ability to enter the nucleus and bind to the LexA operon was examined using a repression assay. For the activation assay, EGY48 was transformed with the bait plasmids and the pSH18-34 lacZ reporter to simulate the library screening conditions. The pSH17-4 bait plasmid (containing the yeast Gal4 activation domain) and the pRFHM1 bait plasmid (containing a transcriptionally inert fragment of the *Drosophila* bicoid protein) were used as positive and negative controls, respectively. Transformants were grown in non-selective liquid media at 30°C overnight, and serial dilutions of  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  were spotted on selective media that did or did not contain leucine. Baits that activated the LEU2 reporter gene allowed for growth in the absence of leucine (leu-). For the repression assay, EGY48 was transformed with the cdr2 baits, pRFHM1, and with pJK101, a modified lacZ reporter with a constitutive promoter interrupted by the LexA

operon. Binding of the bait fusion protein to the LexA site represses transcription of the lacZ reporter, and the yeast do not turn blue in the presence of the X-gal substrate.

For the cDNA library screen, the EGY48 strain was transformed sequentially with the pSH18-34 plasmid (a lacZ reporter under the control of a modified Gal1 promoter containing eight LexA binding sites), then with the cdr2<sup>65-140</sup> bait plasmid, and finally with the pJG4-5-based HeLa cDNA library (encoding B42 acidic activation domain fusion proteins under the control of the conditional Gal1 promoter). Approximately  $4 \times 10^5$  transformants were first grown on non-selective media containing glucose to prevent expression of potentially toxic library fusion proteins. Colonies were then replica plated to leu- and X-gal selective media in the presence of either glucose or galactose. Transformants that grew on leu- media and turned blue on X-gal plates only in the presence of galactose were picked for further analysis. Library plasmids from these colonies were isolated and the host strain was co-transformed with either the bait or a control and the purified library plasmid to test for the specificity of the interaction. True positives were sequenced using pJG4-5 vector primers to determine identity and confirm correct reading frame. pJG4-5 plasmids encoding Max and Mxi1 activation domain fusion proteins were kindly provided by Dr. Erica Golemis (Fox Chase Cancer Center, Philadelphia).

### *Immunohistochemistry*

An adult Sprague-Dawley rat was perfused with 4% paraformaldehyde/PBS and the brain dissected out. The tissue was post-fixed in 4% paraformaldehyde/PBS at 4°C for 4 hours and stored in 10% sucrose/PBS at 4°C overnight. 30µm sections of cerebellum were cut on a freezing sliding stage microtome and placed in PBS. Floating sections were blocked for 1 hour at room temperature in PBS/0.05% tritonX-100/2% normal horse serum (NHS). c-Myc monoclonal antibody C-33 (Santa Cruz Biotechnology) was

used at 1 $\mu$ g/ml and 2 $\mu$ g/ml and Yo antisera was used at a 1:200 dilution. Primary antibody was diluted in PBS/0.05% tritonX-100/1% NHS, and sections were incubated at 4°C for 36-48 hours. After washing in PBS/0.05% tritonX-100, sections were incubated in appropriate secondary antibody for 2 hours. The c-Myc sections were incubated in 1:100 biotinylated, rat adsorbed anti mouse IgG (Vector Labs); the Yo sections were incubated in 1:1000 biotinylated anti human IgG (Vector Labs). After washing in PBS/triton, signal was enhanced with the Vectorstain ABC Elite Kit (Vector Labs). The substrate diaminobenzidine (DAB) was added in the presence of peroxide. Staining was visualized by light microscopy.

### *Recombinant Proteins*

Plasmids encoding GST fusions with Max, USF, c-Myc amino acids 250-353 and c-Myc amino acids 250-439 and a plasmid containing the full length coding region of mouse c-myc were kindly provided by Dr. Katherine Calame and colleagues (Columbia University, New York). All fusion proteins were expressed in bacteria and affinity purified with glutathione sepharose. The GST-cdr2 fusion protein contains amino acids 16-192 of human cdr2 (described above). To increase the yield of mostly insoluble fusion protein, it was purified from bacterial inclusion bodies by dissolving the bacterial pellet in 8M urea and then removing the urea by dialysis against PBS (Sambrook, et al., 1989). Full length mouse c-Myc and mouse cdr2 were transcribed *in vitro* using T7 RNA polymerase (Stratagene), and the RNA's were then translated *in vitro* using a rabbit reticulocyte lysate system (Promega) and labeled by incorporation of <sup>35</sup>S-methionine (Dupont NEN).

### *In Vitro Binding Assay*

The assay was performed essentially as described in Harper et.al. (Harper et al., 1993) with some modifications. Programmed reticulocyte lysate (containing either the *in vitro* translated cdr2 or c-Myc) was pre-cleared by incubation

with glutathione sepharose at 4°C for 30 minutes. GST fusion proteins immobilized on glutathione sepharose were washed in binding buffer (50mM Tris pH7.5, 120mM NaCl, 2mM EDTA, 0.1% Nonidet P-40, 1mM NaF, 2µg/ml aprotinin, 100µg/ml PMSF). 15µl of fusion protein sepharose was incubated with 10µl of programmed reticulocyte lysate diluted in 150µl binding buffer, rotating at 4°C for 30 minutes. The beads were washed three times with 1 ml of binding buffer, 25µl of 2x SDS sample buffer was added, the samples were boiled and resolved by 12% SDS-PAGE. The gel was treated with the Amplify fluor (Amersham) and bound proteins were detected by autoradiography. 1-5µg of each GST fusion protein was used in the assay (as determined by Coomassie blue stain), and the bead volume was normalized by addition of glutathione sepharose.

#### *Plasmids and transfection of NIH3T3 cells*

To generate a cdr2 eukaryotic expression construct, the full length coding region of mouse cdr2 was first cloned into the pET21B plasmid (Novagen) in frame with the T7 epitope tag in that vector. The N-terminal T7-tagged full length mouse cdr2 was then subcloned into the pcDNA3 expression vector with the constitutive CMV promoter (Pharmacia).

The SpMyc and (+/-)M4minCAT plasmids were kindly provided by Dr. Robert Eisenman (FHCRC, Seattle, WA). SpMyc contains the full length coding region of human c-myc driven by a constitutive SV40 promoter. The (+/-)M4minCAT vectors contain the chloramphenicol acetyltransferase (CAT) gene in front of the basal HSV thymidine kinase promoter with or without a fourfold repeat of the E-box Myc binding site, CACGTG. These plasmids were described previously (Kretzner et al., 1992a). The pSV-GFP plasmid was kindly provided by Davorka Bilicic. It is a modification of the pEGFP-1 plasmid (CLONTECH) that encodes a red-shifted variant of wild-type green fluorescent protein, into which the SV40 early promoter has been

inserted. The pKS-CMV- $\beta$ gal plasmid was a kindly provided by Kiki Broccoli, and contains the lacZ gene and the CMV promoter inserted into the pBluescript II KS vector (Stratagene).

For transient transfections, NIH3T3 cells were plated at a density of  $3\text{--}4 \times 10^5$  cells per 35mm dish and maintained in Dulbecco's Modified Eagles Media (DMEM) plus 10% fetal calf serum (FCS), 2mM L-glutamine, and 5 $\mu$ g/ml gentamicin (Gibco BRL) at 37°C and 5% CO<sub>2</sub>. The following day the cells (~80% confluent) were transfected using the lipofection method and the Lipofectamine reagent (Gibco BRL). All transfections were performed in triplicate. The appropriate plasmids were added to 100 $\mu$ l of serum-free DMEM per 35mm dish. Transfections included 0.5 $\mu$ g (+/-)M4minCAT, 0.25 $\mu$ g of either pKS-CMV- $\beta$ gal or pSV-GFP, 0.5 $\mu$ g SpMyc, and varying amounts of pcDNA3-cdr2. Total DNA was kept constant at 2 $\mu$ g by supplementing with salmon sperm DNA when necessary - transfections using more than 2 $\mu$ g of DNA were found to be generally toxic. For each 35mm dish, 6 $\mu$ l of Lipofectamine reagent was added to 100 $\mu$ l serum-free DMEM. The DNA and the Lipofectamine reagent were then mixed and allowed to complex at room temperature for 45 minutes. Cells were washed twice with serum-free DMEM and then overlaid with 800 $\mu$ l of DMEM. 200 $\mu$ l DNA-Lipofectamine complexes were added, and the cells were maintained serum-free at 37°C for 5-6 hours. FCS and L-glutamine were then added to final concentrations of 10% and 2mM, respectively. No antibiotic was added up to this point. After 24 hours, the media was replaced with whole media, including gentamicin. Cells were harvested between 40-48 hours after the transfection.

#### *CAT assays*

Transfected NIH3T3 cells were washed twice with PBS, 500 $\mu$ l TEN (40mM Tris-HCl pH 7.5, 1mM EDTA, 150mM NaCl) was added, and the cells were

placed on ice for 5 minutes. The cells were then scraped and transferred to microfuge tubes on ice. Cells were pelleted by spinning at 10,000xg for no longer than 1 minute, the TEN was removed and the cells were resuspended in 35 $\mu$ l of ice cold 0.25M Tris-HCl pH 7.5. Three successive freeze-thaw cycles of 5 minutes in ethanol/dry ice bath followed by 5 minutes in a 37°C water bath were used to gently lyse the cells. Cell debris was pelleted by a 5 minute spin and the cytoplasmic extracts were either used directly or stored at -20°C.

For each sample in the CAT assay, the following mixture was prepared: 4 $\mu$ l (0.2 $\mu$ Ci)  $^{14}$ C-chloramphenicol (Dupont NEN), 5 $\mu$ l 5mg/ml n-butyryl CoA (Sigma), 16.6 $\mu$ l Tris-HCl pH 8.0, and ddH<sub>2</sub>O to a volume of 70 $\mu$ l. 30 $\mu$ l of the transfected cell cytoplasmic extracts was added to this mix and incubated at 37°C for 90 minutes. 1U of purified CAT enzyme (Sigma) was added to one tube as a positive control. To specifically extract the acetylated  $^{14}$ C-chloramphenicol, 200 $\mu$ l of a 2:1 mixture of tetramethyl pentadecane (TMPD)/xylenes was added and the tubes were vortexed. After a 2 minute spin, the top (organic) phase was transferred to 5ml Ready Safe liquid scintillation fluid (Beckman), and measured in a scintillation counter.

To measure  $\beta$ -galactosidase activity, 10 $\mu$ l of NIH3T3 cytoplasmic extract was added to 40 $\mu$ l ddH<sub>2</sub>O, 450 $\mu$ l Buffer A (100mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.5, 10mM KCl, 1mM MgSO<sub>4</sub>, 50mM  $\beta$ -mercaptoethanol) and 100 $\mu$ l ONPG substrate (4mg/ml O-nitrophenyl  $\beta$ -D galactopyranoside (Sigma) in 100mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.5). The tubes were incubated at 37°C for at least 30 minutes, the reactions were stopped by adding 250 $\mu$ l 1M Na<sub>2</sub>CO<sub>3</sub>, and the A<sub>420nm</sub> was measured.

If the pSV-GFP plasmid was cotransfected as an independent measure of transfection efficiency, the GFP positive cells were visualized just prior to harvesting using an inverted microscope with an ultraviolet lamp. Two randomly chosen fields of cells were scored for each triplicate sample, and the average number of GFP-expressing cells was used.

For the CAT assay data analysis, the transfection efficiency was determined by either  $\beta$ -galactosidase activity or GFP expression in all experimental samples relative to the transfection with the CAT reporter alone. The relative CAT activity was then normalized to the transfection efficiency. Data was analyzed and plotted using the Cricket Graph III software package (Computer Associates).

## Chapter 3 - Expression of cdr2

### Introduction

An autoimmune model has been proposed for the pathogenesis of PND's (Anderson et al., 1987). In this model, a systemic tumor ectopically expressing a neuron-specific protein initiates an appropriate immune response that subsequently develops into autoimmune neurologic disease. Evidence for this mechanism includes limited tumor growth and the presence of high titer autoantibodies in patients' serum that react with both the tumor and neurons in affected regions of the nervous system. For example, in paraneoplastic sensory neuronopathy/encephalomyelitis, patients with small cell lung cancers expressing the neuronal protein Hu develop an immune response to their tumors and a neurologic syndrome affecting multiple regions of the CNS and PNS (Dalmau et al., 1991; Posner, 1995a). Patients with cancer-associated retinopathy (CAR) harbor antibodies against the recoverin protein that is expressed only in photoreceptor cells and the peripheral tumor (Polans et al., 1995). It is the coordinate expression of the paraneoplastic antigens in both discrete populations of neurons and specific tumors that led to the term "onconeural antigens" to describe these proteins (Darnell et al., 1991).

Central to the autoimmune hypothesis is the traditional belief that the brain is a site of immune privilege (Medawar, 1948). Physical and molecular barriers protect neurons from normal immune surveillance by lymphocytes (Pollack and Lund, 1990; Streilein, 1993). Therefore, the ectopic expression of onconeural antigens in tumors outside of the nervous system may be able to elicit effective anti-tumor immunity. A necessary condition for this autoimmune mechanism is the restricted expression of onconeural antigens to immune privileged tissues. This holds true for the known paraneoplastic antigens Nova-1, Hu, and recoverin, all of which have been observed to be neuron-specific proteins (Dizhoor et al., 1991; Szabo, et al., 1991; Dalmau, et

al., 1992; Buckanovich, et al., 1993; Buckanovich, et al., 1995; Okano and Darnell, 1996).

It has been suggested that *cdr2* may be widely expressed in normal tissues, which is problematic for its proposed role in the pathogenesis of PCD. Widespread expression of *cdr2* would also be inconsistent with the clinical features of PCD, which are restricted to evidence of anti-tumor immunity and neuronal (primarily cerebellar) dysfunction (Peterson, et al., 1992). Early studies using PCD antisera found immunoreactivity with cerebellar Purkinje cells when the antibody was used at limiting dilutions (Jaekle et al., 1985). Subsequent reports have found Purkinje cell immunoreactivity at limiting antibody dilutions, but more widespread reactivity both within and outside the nervous system using high concentrations of antibody (Altermatt, et al., 1991; Tomimoto, et al., 1993). However, the antibodies used in those studies were not completely characterized by Western blot analysis with both tissue extract and *cdr2* fusion protein. Furthermore, Sakai et al. (Sakai, et al., 1991) detected the *cdr2* mRNA in cerebellum, brainstem and intestine by RT-PCR; additional samples were negative but were not controlled for integrity of the RNA.

The present study was undertaken to clarify the expression pattern of the PCD antigen. We have examined three tumors obtained from PCD patients for the expression of *cdr* genes, and found that the *cdr2* gene encodes the PCD tumor antigen in each. We then defined the tissue distribution of the *cdr2* mRNA and immunoreactive protein in the adult mouse, and have found that expression of the PCD antigen is restricted to the brain and testis, tissues that are known to be sites of immunologic privilege. These results demonstrate that the expression pattern of the PCD antigen is consistent with the proposed autoimmune model of PCD. Interestingly, the *cdr2* mRNA displays a wider distribution than the protein, indicating that expression of the *cdr2* antigen is regulated at a post-transcriptional level.

## Results

### *Detection of cdr2 message in PCD-associated ovarian tumors*

Previous studies demonstrated that PCD antisera recognized a protein of ~62kD in all PCD tumor samples examined, but detected the 34kD species (cdr1) in only one (Furneau, et al., 1990a), suggesting that cdr2 might encode the PCD tumor antigen. However, the antigenic epitope in cdr2 localizes to the protein's leucine zipper domain (Sakai, et al., 1993), which is nearly identical in sequence to the leucine zipper of cdr3, and both cDNAs were cloned from expression cDNA libraries using PCD antisera. To address which of these two genes encodes the *in vivo* PCD tumor antigen, PCD tumors were assayed for the presence of cdr gene transcripts by the reverse transcription-polymerase chain reaction (RT-PCR). Single strand cDNA was synthesized from polyA<sup>+</sup> or total RNA isolated from three ovarian tumors obtained from PCD patients and amplified using gene-specific primers corresponding to cdr2 or cdr3 (Figure 1). While both cdr transcripts could be detected in the cerebellum, only cdr2 mRNA was found in the PCD ovarian tumors. In addition, cdr1 mRNA was detected in cerebellum, but could not be detected in the PCD tumors (data not shown).

To confirm that the cdr2 positive PCD tumors we assayed came from typical PCD patients, tumor tissues were examined for immunoreactivity with PCD antisera in collaboration with Dr. Josep Dalmau at Memorial Sloan Kettering Cancer Center. Figure 2 demonstrates that tumor tissue from one patient (tumor 2) was immunoreactive with PCD antisera (Fig. 2B) but not control antisera (Figure 2A); similar results were found with tumors 1 and 2 (data not shown). In addition, tissue from tumor 3 was assayed for PCD reactivity by Western blot analysis. Figure 2C demonstrates that PCD antisera recognize a protein in tumor extracts that co-migrates with the PCD antigen in human Purkinje cell extracts. Finally, cerebellar tissue obtained from the autopsy of a patient (tumor 3) was examined. Immunohistochemical analysis of cerebellar tissue using PCD antisera revealed the complete absence of

immunoreactivity and Purkinje neurons in PCD cerebellum (Figure 3A), but showed characteristic staining pattern in Purkinje neurons of control cerebellum (Figure 3B). We conclude from the RT-PCR and protein studies that the PCD tumor antigen is the *cdr2* gene product.

In order to facilitate the study of *cdr2* expression, a human *cdr2* cDNA clone (Fathallah-Shaykh, et al., 1991) was used to isolate a 2.4kb cDNA encoding *cdr2* from an adult mouse brain library (Figure 4). Since the initiating methionine of the human cDNA has not been defined (Fathallah-Shaykh, et al., 1991; Sakai, et al., 1991), we compared the degree of nucleic acid homology between the murine and human *cdr2* sequences. The sequence homology decreases dramatically immediately upstream of a methionine codon at position 154 and there is an in frame stop codon at position 88, indicating that the ATG at position 154 is the initiation codon. The full length murine *cdr2* open reading frame encodes a protein of predicted molecular weight 52kD and its amino acid sequence is 87% identical to its human homologue (Figure 4). Within the region of the PCD antigenic epitope (the leucine zipper domain) the mouse and human proteins are identical.

*cdr2 mRNA is expressed widely in adult mouse tissues, but the protein is restricted to the brain and testis*

To determine whether *cdr2* gene expression was limited to the nervous system, Northern blot analysis was performed using a 580 bp *cdr2* cDNA probe corresponding to the 3' untranslated region (UTR) (Figure 5A). A single *cdr2* transcript of 2.8kb was detected in 8 of 9 tissues examined; it was most abundant in testis and spleen, and was not detectable in liver (Figure 5B). This result was confirmed by hybridization with a 224 bp probe corresponding to the *cdr2* coding region (Figure 5A), and by RT-PCR analysis of mouse cerebellum, spleen, heart and testis RNA (Figure 5C, and data not shown). The *cdr2* primer pair used for PCR amplification flanks the region

encoding the PCD epitope (Figure 5A), suggesting that this region of the *cdr2* transcript is the same in each tissue.

To investigate the expression pattern of the PCD antigen, Western blot analysis using affinity purified PCD antisera was performed on the same battery of adult mouse tissues used for Northern blot analysis. Interestingly, a single band of  $M_r = 56\text{kD}$  was detected in cerebellum and testis but not in other tissues (Figure 6A). On a longer exposure a faint band was also detected in the cerebral cortex (not shown). The blot was stripped of antibody and reprobed with a monoclonal antibody to  $\beta$ -tubulin (Figure 6A), demonstrating that equivalent amounts of protein were loaded in each lane. Native sera from several PCD patients revealed the same distribution of the 56kD reactive species (not shown). To confirm the identity of the immunoreactive species in brain and testis, two-dimensional (2-D) gel electrophoretic analysis was performed using mouse cerebellar and testis extract. Figure 6B shows that the protein recognized by PCD antisera in cerebellum (top panel) and testis (bottom panel) exactly co-migrate by both their molecular weights and isoelectric points (pI). The protein(s) run as a broad band with a pI of 6.1-6.4, consistent with previous 2-D gel analysis of human Purkinje cell lysate (Cunningham et al., 1986). This migration pattern in the dimension of isoelectric focusing could be due to post-translational or chemical modifications, and may also explain the difference between the observed pI and the predicted pI of 4.76.

There are several potential explanations for the discrepancy between the tissue distribution of the *cdr2* message and the protein detected by the PCD antisera. In tissues other than brain and testis the PCD antigen may not be recognized by the Yo antibody, either as a result of alternative processing of the primary transcript, differential post-translational modification of the protein, or regulation at the level of translation. To test for the first of these possibilities, *cdr2* cDNA's were cloned from spleen. Mouse spleen was chosen because it represents a tissue in which the *cdr2* mRNA was abundant, while

the protein was undetectable. Two overlapping clones, one of near full length, were isolated and found to be identical to the brain cDNA sequence throughout the coding region and UTR. These results suggest that the apparent differences in detectable cdr2 protein in brain and spleen cannot be entirely accounted for by alternatively spliced cdr2 mRNAs.

To address the possibility that the detection of cdr2 may be affected by tissue-specific post-translational modifications or protein stability, a non-neuronal cell line was transfected with an expression vector containing the full cdr2 open reading frame without UTR sequences. The transfected cdr2 plasmid yielded abundant immunoreactive protein in NIH3T3 cells, suggesting that the protein was stable and the epitope was not masked in this fibroblast cell line (Figure 6C).

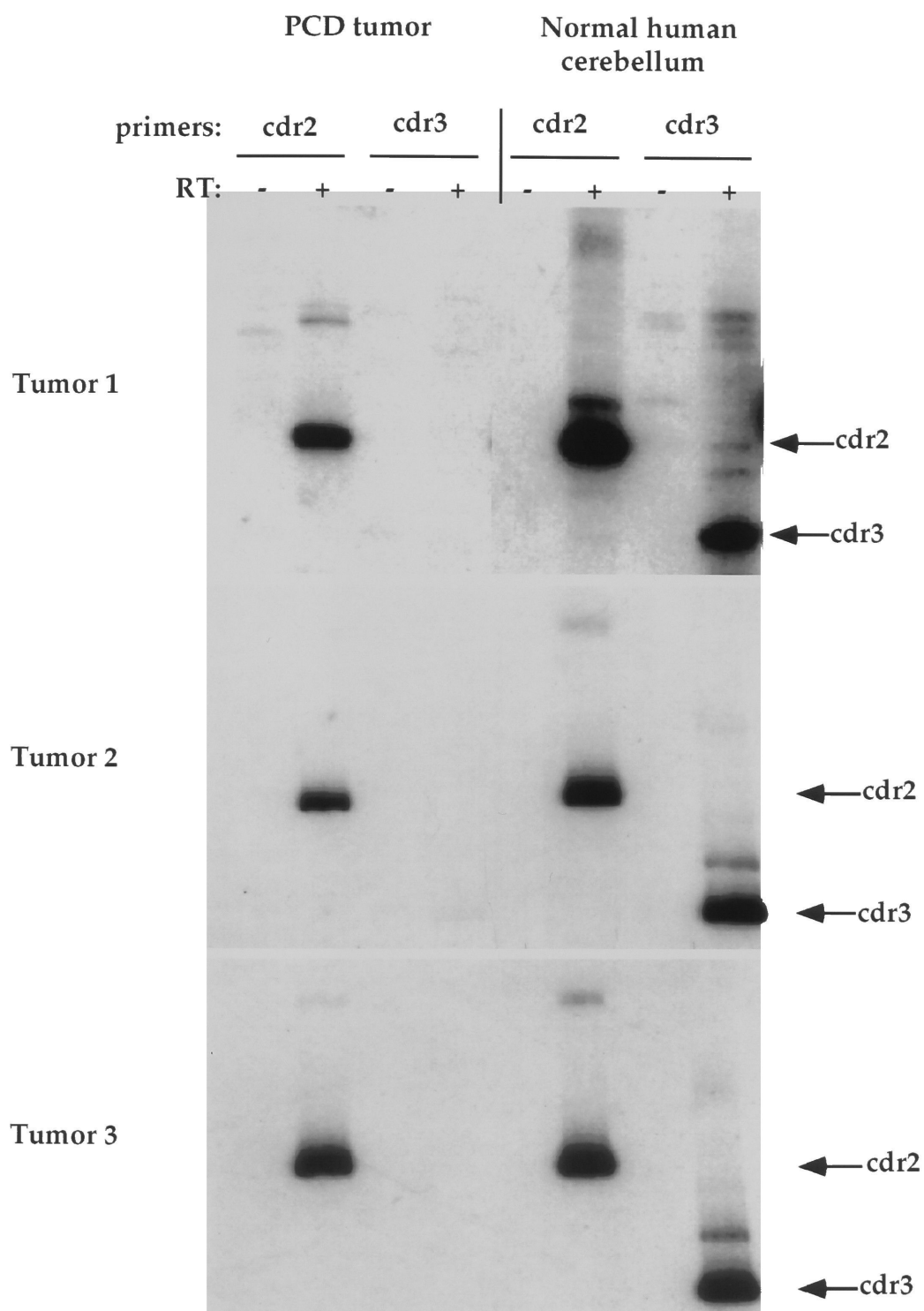
*cdr2 expression is also regulated at the level of transcription*

To extend the cdr2 expression data, the tissue distribution of cdr2 mRNA and protein was compared by *in situ* hybridization and immunohistochemistry. A specific *in situ* hybridization probe was generated from the mouse cdr2 3'-UTR (Figure 5A), and the expression in mouse tissues was compared with the pattern of immunoreactivity seen with affinity purified or native PCD serum. Both sagittal and coronal sections of adult mouse brain showed a pattern of cdr2 mRNA expression that corresponded closely to the pattern of immunoreactivity. In the cerebellum, abundant cdr2 mRNA and PCD antigen were detected specifically in the cortical Purkinje neurons and neurons of the deep cerebellar nuclei, whereas no expression was detected in the dense granule cell layer (Fig. 7A, E, and F). The most prominent cdr2 expression found outside of the cerebellum was in the brainstem, where many neurons were cdr2-positive, including those in the medial vestibular, pontine, inferior olivary, and red nuclei (Figure 7C and D). cdr2 mRNA and protein were absent from hippocampus, basal ganglia, and neocortex, with the exception of scattered cerebral cortical neurons (Figure 7B, G, and H).

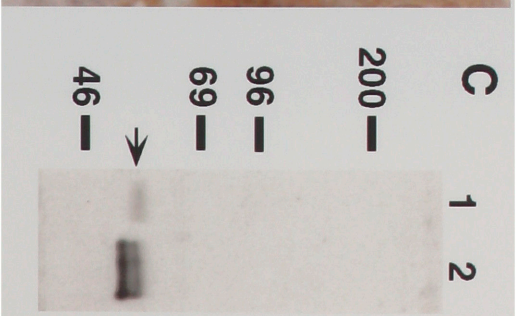
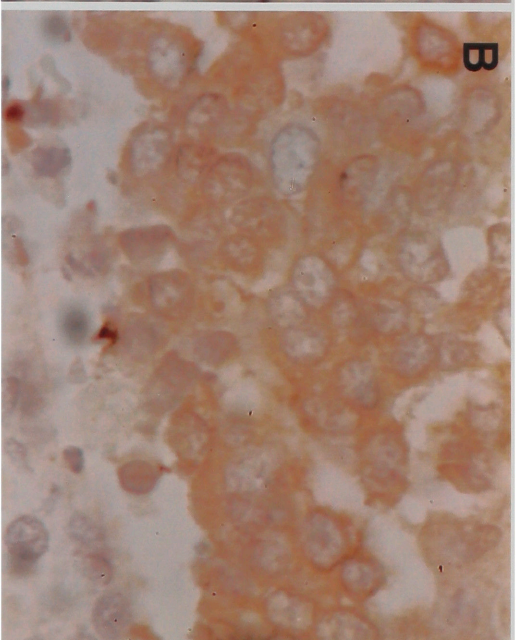
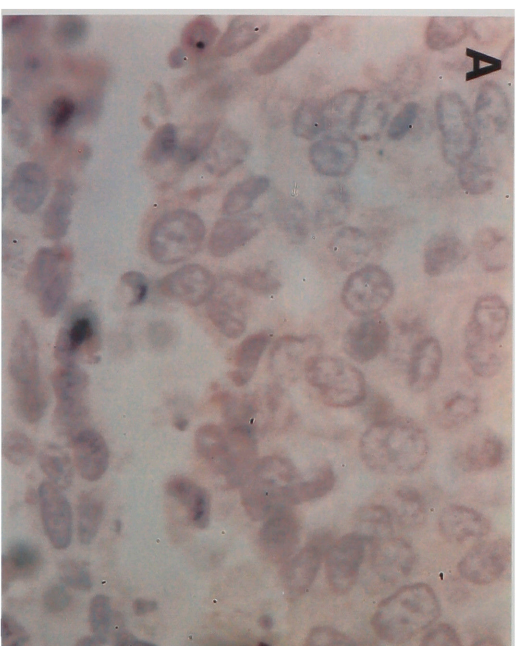
Another region to which the *cdr2* riboprobe hybridized was the mitral cell layer of the olfactory bulb (Figure 7B). However, *cdr2* immunoreactivity in this region could not be clearly demonstrated.

Outside of the nervous system, there was no correlation between *cdr2 in situ* hybridization and immunohistochemistry except in testis. In sections of adult spleen the *cdr2* mRNA was readily detected, where it was found to be restricted to the splenic cortex (an area rich in lymphocytes) but absent from the red pulp (Figure 8A). In contrast, there was no detectable *cdr2* immunoreactivity in either the splenic cortex or pulp (Figure 8B). Similarly, no immunoreactivity could be detected in any other non-neuronal tissue examined except testis. Immunohistochemical staining of testis revealed that *cdr2* cytoplasmic reactivity was restricted to the outermost cell layer of the seminiferous tubules (Figure 8D). By their relative position in the tubules and by morphological criteria, these cells appear to be spermatogonia, the least differentiated type in the germ cell lineage. *In situ* hybridization of testis revealed that *cdr2* mRNA is abundantly expressed in spermatogonia, and could be detected to a lesser degree in early differentiating spermatocytes (Figure 8C). Taken together, these data demonstrate an uncoupling of *cdr2* mRNA and protein expression, suggesting that a post-transcriptional mechanism restricts *cdr2* protein expression to spermatogonia and a subset of neurons.

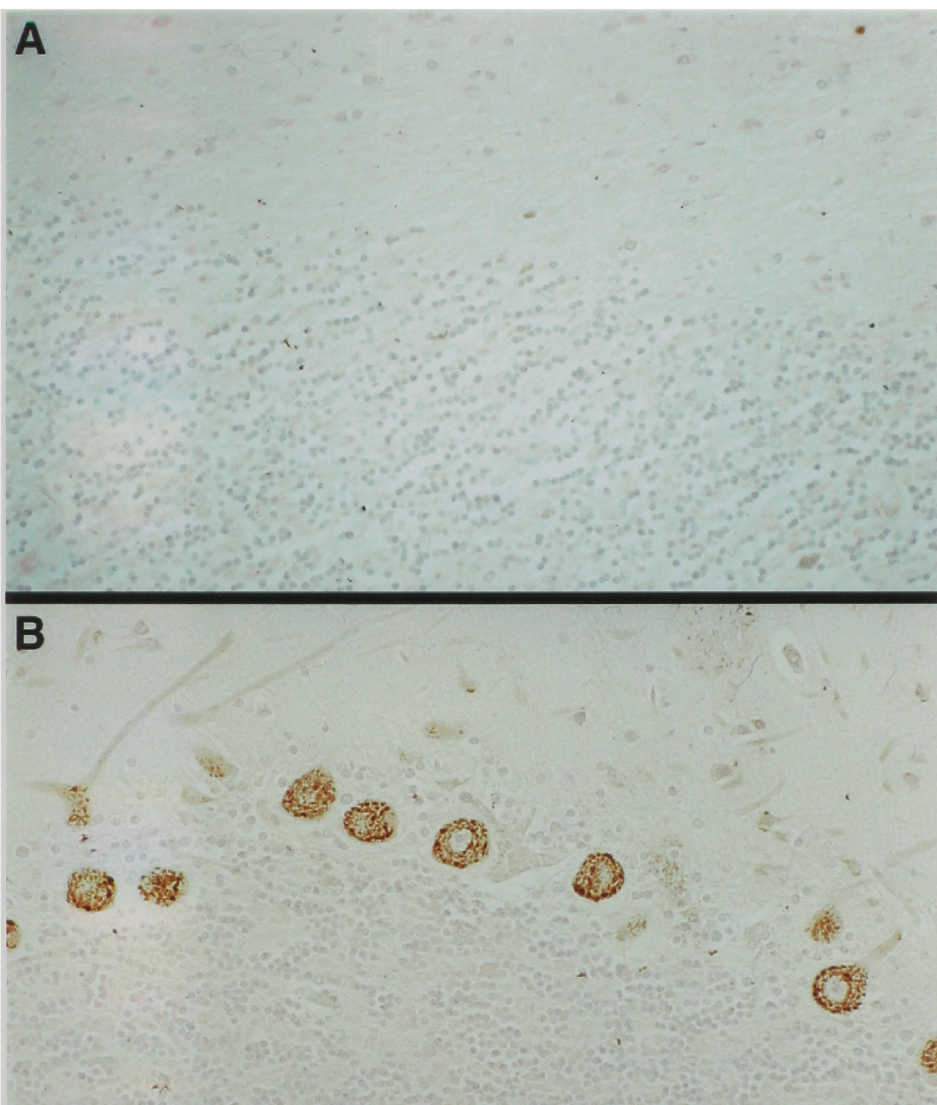
**Figure 1.** RT-PCR analysis of PCD-associated ovarian tumors. Total or polyA<sup>+</sup> RNA purified from tumor tissue or normal human cerebellar polyA<sup>+</sup> RNA were used as templates for the reverse transcription. Gene-specific primers corresponding to the coding region of either cdr2 or cdr3 were used for PCR amplification of the first strand cDNA. Reactions were carried out both in the presence and absence of reverse transcriptase (RT) to control for DNA contamination. A  $\beta$ -actin primer pair was also used as a control for RNA integrity (not shown). While the transcripts of the expected size for both cdr2 and cdr3 were detectable in human cerebellum, only the cdr2 transcript was detected in the PCD tumors.



**Figure 2.** Immunoreactivity of PCD ovarian tumors with PCD antisera. Serial sections of a paraffin embedded PCD ovarian tumor (tumor 2 from Fig. 1) stained with either biotinylated normal human serum (**A**) or biotinylated PCD antisera (**B**). PCD antisera displays a characteristic cytoplasmic reactivity in the tumor tissue, and not in the surrounding connective tissue seen in the bottom of the photomicrograph. **C**, Detection of the PCD ovarian tumor antigen by Western blot. PCD antisera was immunoreactive with a protein of  $M_r$  56kD (arrowhead) s present in both human Purkinje (lane 1) and PCD tumor (lane 2) protein extracts. The lower reactive band in the tumor extract is IgG, determined by probing the same blot with the anti-human IgG secondary antibody alone (data not shown).



**Figure 3.** Immunohistochemical analysis of paraffin sections of human cerebellum using the anti-Yo antibody. **A**, Cerebellum of a Yo-positive PCD patient (corresponding to tumor 3 in Fig. 1) showing complete lack of reactivity with biotinylated anti-Yo and the absence of Purkinje neurons. **B**, Cerebellum of a neurologically normal patient demonstrating anti-Yo reactivity with Purkinje cell cytoplasm.



**Figure 4.** Nucleotide and predicted amino acid sequence of the adult mouse brain cdr2 cDNA. Amino acids are numbered on the left and the nucleotides on the right. Leucine residues forming the core leucine zipper are indicated in bold. The in-frame stop codon upstream of the presumptive initiating methionine and a polyadenylation signal are underlined. The translational stop codon is indicated with an asterisk. The 455 amino acid protein of predicted MW = 52kD is 87% identical to the human sequence. The amino acids that are not conserved are underlined.

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 214 K R V S V E A E Y K V V L K E N S E L E Q Q L G  
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**Figure 5.** Expression of the *cdr2* mRNA in adult mouse tissues. **A**, Schematic representation of the full length mouse *cdr2* cDNA and the regions used in the generation of labeled probes and PCR primers. The shaded grey box represents the *cdr2* coding region and the lines represent the UTR's. The white box contains the region encoding the leucine zipper epitope. Nucleotide sequences of the probes and primers are described in more detail in Chapter 2. **B**, A Northern blot of total RNA prepared from the indicated tissues was hybridized with a  $^{32}\text{P}$ -labeled cDNA probe made from the 3' UTR of mouse *cdr2* (represented by the longer bar shown in **A**). The bottom panel shows hybridization of a GAPDH probe to the same blot as a control for loading of RNA. The mouse tissues used in the analysis were: cerebellum (Cb), cerebral cortex (Cx), heart (Ht), lung (Lu), liver (Li), kidney (Kd), spleen (Sp), ovary (Ov), and testis (Ts). The relative positions of 28S (5.1kb) and 18S (2.0kb) rRNA are shown. A single *cdr2* transcript of 2.8kb was detected in all tissues tested, with the exception of the liver. This result was confirmed by using another *cdr2* cDNA probe corresponding the coding region (represented by the shorter bar shown in **A**). **C**, RT-PCR analysis of *cdr2* expression in cerebellum versus spleen RNA was performed as described in Figure 1, using primers flanking the PCD epitope (represented by the arrow heads shown in **A**) or  $\beta$ -actin primers. The *cdr2* transcript was also detected in heart and testis by this assay (data not shown).

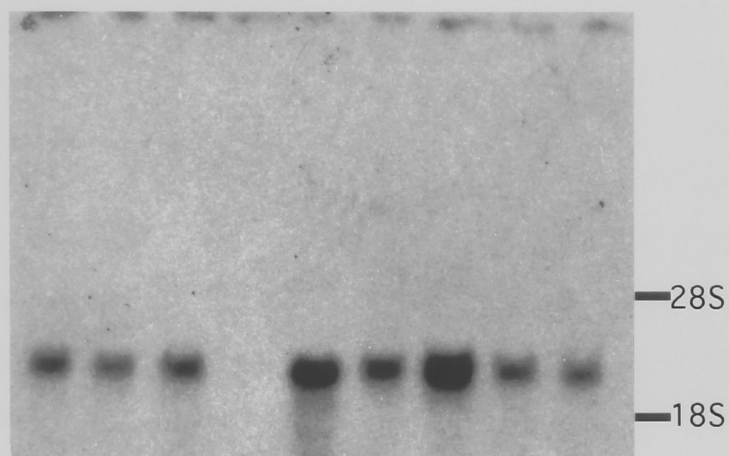
**A.**

Mouse *cdr2* cDNA

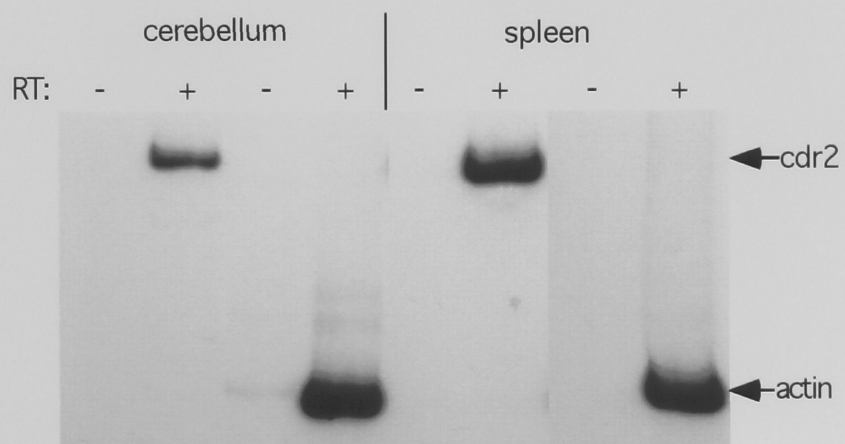


**B.**

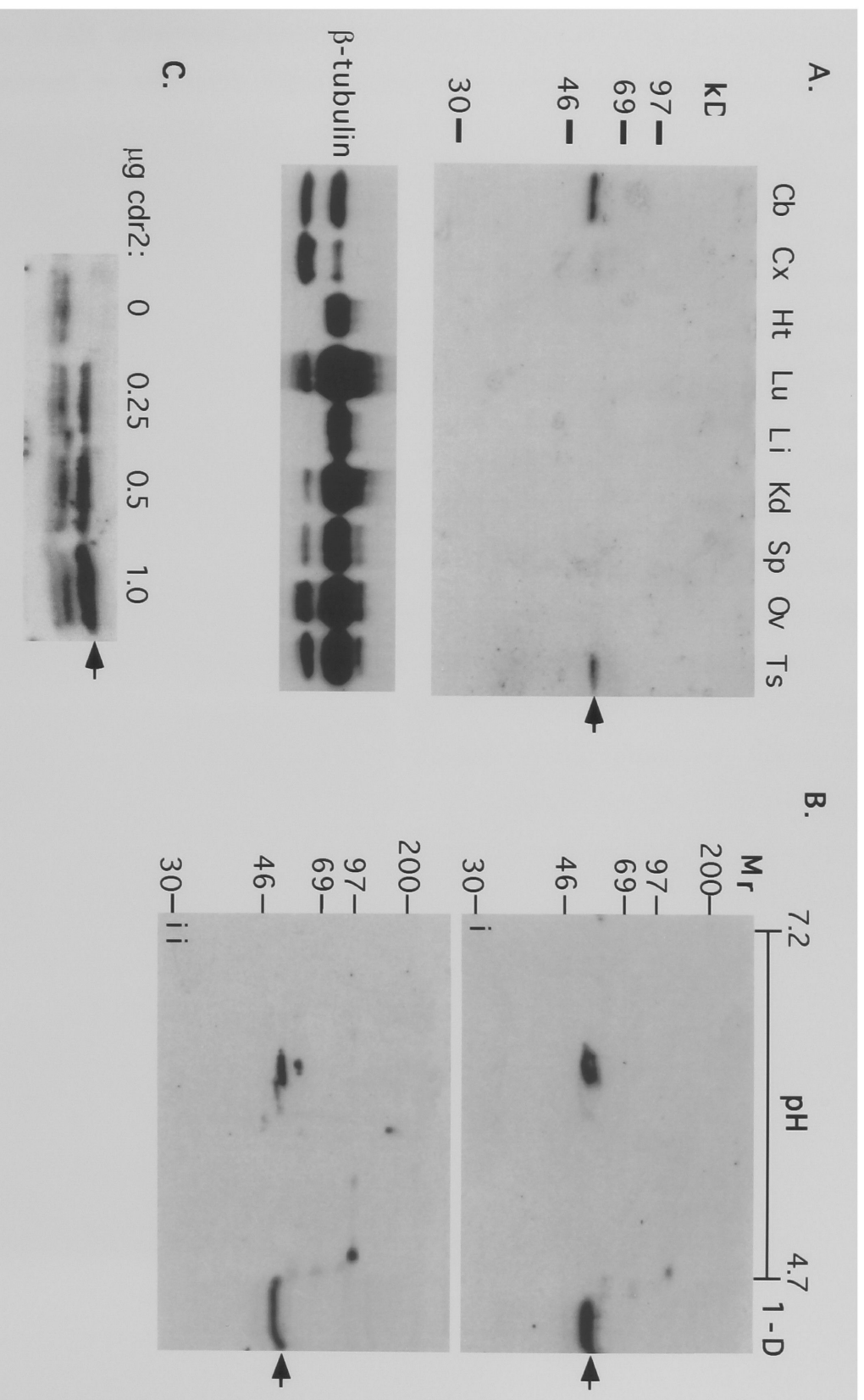
Cb Cx Lu Li Sp Kd Ts Ov Ht



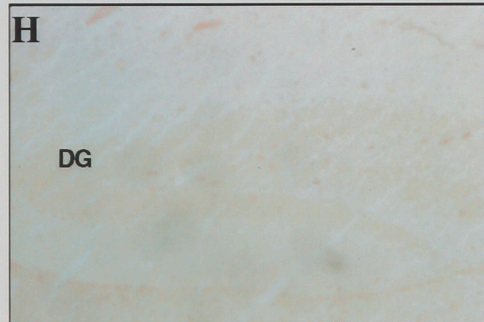
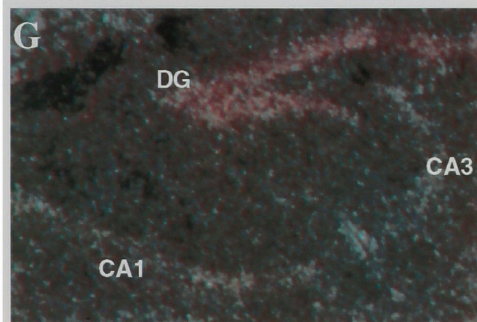
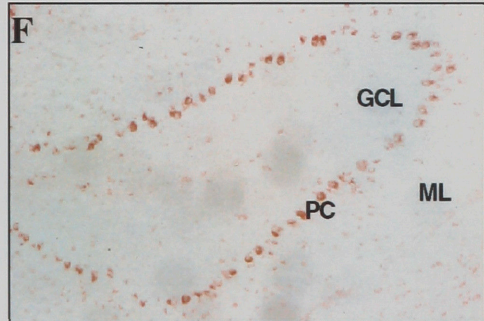
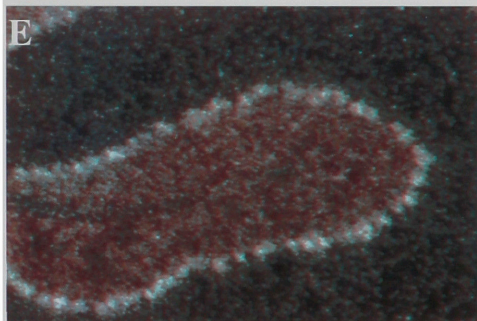
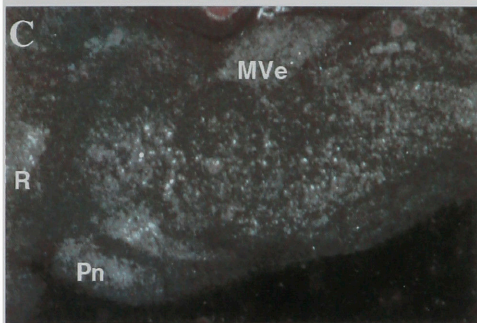
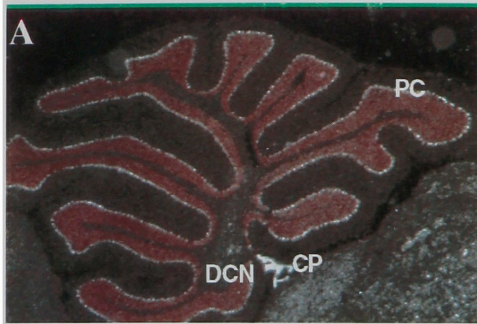
**C.**



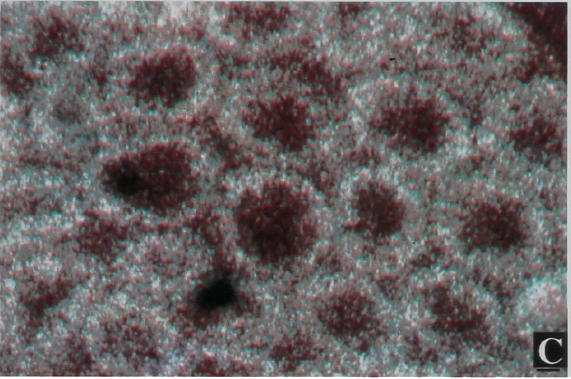
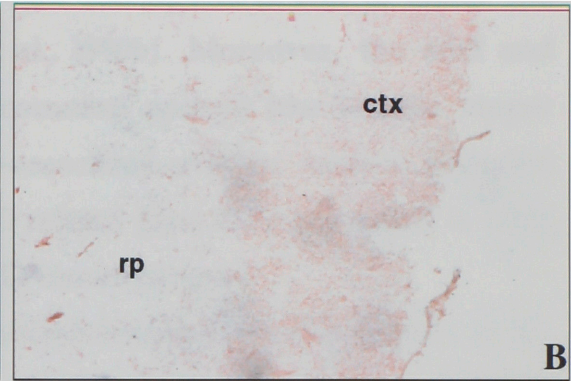
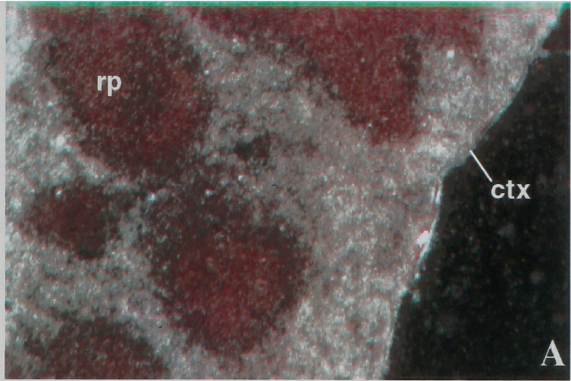
**Figure 6.** Detection of the PCD antigen in adult mouse tissues and a transfected non-neural cell line. **A**, Affinity purified PCD antisera was used to probe a Western blot of the indicated protein extracts (top panel, abbreviated as in Figure 3). The blot was stripped of antibody and reprobed with a monoclonal antibody to  $\beta$ -tubulin as a protein loading and transfer control (bottom panel); the lower band is mouse Ig heavy chain. The cdr2 affinity purified antibody recognizes a 56kD antigen only in brain and testis. **B**, Two dimensional gel electrophoresis of protein extracts from cerebellum (i) and testis (ii). Proteins were resolved by their isoelectric points in the horizontal direction (the direction and end points of the pH gradient are shown above) and by their molecular weights in the vertical direction. The major species detected by PCD antisera in cerebellum and testis comigrate in both dimensions. **C**, Western blot analysis of NIH3T3 cells transfected with increasing amounts of the pcDNA3-cdr2 expression plasmid, containing just the cdr2 coding region (see Chapter 2 for details). Whole cell lysates were separated by SDS-PAGE and probed with native PCD antisera. The cdr2 protein is readily detectable with the autoantibody in this non-neural cell line.



**Figure 7.** Analysis of *cdr2* expression in adult mouse brain by *in situ* hybridization (**A**, **B**, **C**, **E**, and **G**) and immunohistochemistry (**D**, **F**, and **H**). Sections were hybridized with a <sup>33</sup>P labeled *cdr2* riboprobe or reacted with either affinity purified or native PCD antisera. Dark field photomicrographs reveal that the *cdr2* mRNA is detected in cerebellar Purkinje neurons and deep cerebellar nuclei neurons (**A** and **E**). No signal was detected in the dense granule cell layer or in the molecular layer of the cerebellar cortex (**E**). *cdr2*-positive cells were detected throughout the brainstem, with prominent hybridization in particular nuclei (**C**). Immunoreactivity with PCD antisera was found in the same brainstem and cerebellar neurons in which *cdr2* mRNA was detected, illustrated by the examples shown in **D** and **F**. Expression of *cdr2* was scarce in the cerebrum, where only scattered cortical neurons were detectable (**B**, and not shown). Higher magnification of the hippocampus shows the complete absence of *cdr2* mRNA and protein (**G** and **H**). No hybridization or immunoreactivity was observed with *cdr2* sense probes or with control antisera in any of the tissues analyzed (not shown). *PC*, Purkinje cell layer; *DCN*, deep cerebellar nuclei; *CP*, choroid plexus; *Mi*, mitral cell layer; *R*, red nucleus; *Pn*, pontine nuclei; *MVe*, medial vestibular nucleus; *IO*, inferior olive; *GCL*, granule cell layer; *ML*, molecular layer; *DG*, dentate gyrus.



**Figure 8.** Analysis of *cdr2* expression in adult mouse spleen and testis by *in situ* hybridization (**A** and **C**) and immunohistochemistry (**B** and **D**) as in Figure 7. A dark field photomicrograph shows hybridization of the *cdr2* probe specifically to the splenic cortical cells and not to cells in the red pulp (**A**). In contrast, immunoreactivity with PCD antisera was completely absent in spleen (**B**) when compared to a normal human serum control (not shown). *cdr2* mRNA (**C**) and protein (**D**) were detected specifically in spermatogonia, cells of the outermost layer of the seminiferous tubules in the testis. The mature germ cells, or spermatocytes, showed no detectable *cdr2* expression. *rp*, red pulp; *ctx*, cortex; *spg*, spermatogonia; *spc*, spermatocytes.



## Discussion

### *The onconeural antigen cdr2*

Three genes encoding putative PCD antigens have been identified by expression library screening with PCD antisera (Dropcho, et al., 1987; Fathallah-Shaykh, et al., 1991; Sakai, et al., 1991), but which of these encode proteins that might be relevant to PCD *in vivo* has been uncertain. Western blot analysis of PCD tumor tissue probed with PCD antisera previously identified immunoreactive species that might correspond in size to either the cdr2 or cdr3 gene products (Furneaux, et al., 1990b). Moreover, the cdr2 and cdr3 genes encode proteins that share a common epitope (the leucine zipper domain). RT-PCR analysis of three PCD-associated ovarian tumors revealed that in each of these tumors, the only PCD related gene to be expressed is cdr2, indicating that the cdr2 protein is the PCD tumor antigen.

Based on RNA analysis and immunohistochemical studies (Sakai, et al., 1991; Tomimoto, et al., 1993), it had previously been thought that the PCD antigen cdr2 might be expressed outside of the nervous system, generating uncertainty regarding its role as an onconeural antigen. We have demonstrated that the expression of the PCD antigen is normally restricted to neurons and testis, sites exhibiting the characteristics of immune privilege. Two dimensional gel analysis confirms that the immunoreactive species evident on Western blot analysis is the same protein in both tissues. This expression pattern is consistent with the proposed role of cdr2 as an onconeural antigen.

The expression of previously characterized onconeural antigens and some autoimmune antigens has been found to be very tightly restricted to neurons, including the Nova and Hu RNA binding proteins (Szabo, et al., 1991; Dalmau, et al., 1992; Buckanovich, et al., 1993; Buckanovich, et al., 1995), the autoimmune cerebellar degeneration antigen  $\beta$ -NAP (Newman, et al., 1995), and the stiff-man syndrome antigens GAD and amphiphysin (Solimena, et al., 1988; DeCamilli, et al., 1993). Our findings are thus concordant with the

strict regulation of onconeural antigen expression, but indicate that their expression may extend to immune privileged cells outside of the nervous system.

The phenomenon of immune privilege, traditionally described as the prolonged survival of allogeneic or xenogeneic grafts, has been studied most extensively in the brain, eye, and testis (Streilein, 1993). Immune privilege in the nervous system has both a physical and a molecular component, defined by the blood-brain barrier and the lack of detectable MHC class I or II antigens, respectively (Bradbury, 1984; Pollack and Lund, 1990). There are also active mechanisms by which immune privileged tissues evade immune surveillance. For example, cells in the anterior chamber of the eye and the testis express fas ligand as a means of inducing apoptosis of autoreactive immune cells (Bellgrau et al., 1995; Griffith et al., 1995). While these studies have examined the immune response to foreign antigens expressed within immune privileged tissues, they imply that proteins whose expression is normally restricted to such sites may be highly immunogenic when ectopically expressed.

In PND it is believed that sequestration of onconeural antigens from immune surveillance in the brain results in lack of immune tolerance to these proteins when they are ectopically expressed in tumor cells. *cdr2* is the only PCD-associated gene expressed in ovarian tumors from PCD patients, and thus appears to be the inciting onconeural antigen. Ectopic expression of *cdr2* is associated with a robust immune response to the antigen. The presence of a specific high-titer autoantibody and limited tumor growth in PCD patients provide clinical evidence for an active anti-tumor immune response (Anderson et al., 1988; Peterson, et al., 1992), and suggests that the *cdr2* protein may act as a *bona fide* tumor antigen.

It remains uncertain how a systemic immune response to ectopically expressed *cdr2* protein becomes competent to recognize the antigen within the brain. However, it does appear that the autoimmune response within the nervous system in PCD is likely to be directed against *cdr2*. Pathologic

examination of PCD brains reveals degeneration of the same neurons in which *cdr2* is expressed, most prominently Purkinje neurons of the cerebellum (Fig. 3 and Peterson, et al., 1992; Verschuuren, et al., 1996). Taken together, these observations suggest that autoimmunity to *cdr2* in PCD proceeds in two steps. First, a naive immune system is naturally competent to recognize *cdr2* in PCD tumors. A second unidentified event, perhaps involving cytokines or a change in the nature of the cellular immune response, allows the immune privilege of the brain to be breached, culminating in autoimmune neurologic disease.

#### *Regulation of the cdr2 antigen at a post-transcriptional level*

The nature of paraneoplastic cerebellar degeneration has been clarified by definitively identifying *cdr2* as the neuronal gene that is ectopically expressed in PCD tumors. Given the significance of such onconeural genes to tumor biology and neurobiology (for review see Darnell, 1996) , this observation focuses attention on studies of the regulation of *cdr2* expression. We have defined the tissue-specific expression pattern of the *cdr2* antigen, and found that it is regulated at a post-transcriptional level. A single *cdr2* transcript is detected in nearly all tissues, while the PCD antigen is expressed specifically in brain and testis. Sequence analysis of both brain and spleen *cdr2* cDNAs reveals that these mRNAs are identical, indicating that there is a tissue-specific regulatory mechanism responsible for restricting expression of the *cdr2* protein that operates after mRNA processing.

The discrepancy between the distribution of *cdr2* message and protein suggests several possible underlying mechanisms. Perhaps the most likely is that translational control regulates the expression of *cdr2*. Such a mechanism might relate either to an induction of translation specifically in brain and testis, or repression of translation in other tissues. There are several examples of tissue or cell type-specific regulation of translation, including the testis proenkephalin mRNA, S-adenosylmethionine decarboxylase

(AdoMetDC) and the transcription factor, BTEB (Hill and Morris, 1992; Rao and Howells, 1993; Imataka et al., 1994). Interestingly, the expression of BTEB closely resembles that of *cdr2*, in that the mRNA is detected in many tissues, while the protein is found only in brain and testis.

Most cases of translational regulation involve sequence elements in the 5' or 3'-UTRs of the mRNA. These elements may form stable secondary structures that either directly impede the translation initiation complex or may act as binding sites for trans-acting regulatory factors (Meleforts and Hentze, 1993). In addition to complex secondary structure, many tightly regulated genes contain multiple upstream AUG codons (uAUGs), often present in long 5'-UTRs, that serve to decrease translational efficiency (Kozak, 1989; Kozak, 1991a; Kozak, 1991b; Geballe and Morris, 1994). Both AdoMetDC and BTEB require the presence of such uAUGs in their 5'-UTRs for inhibition of translation. The 5'-UTR of human and mouse *cdr2* cDNAs have ~80% G+C content in the 135 base pairs immediately upstream of the initiating methionine, predicting stable secondary structure. In addition, there are specific sequence elements within the *cdr2* 5'-UTR that are conserved across species.

Several alternate explanations for the discrepancy between the expression of *cdr2* mRNA and protein may be considered. The *cdr2* protein could be translated constitutively, but selectively unstable due to a tissue-specific degradation mechanism. While there are examples of proteins targeted for degradation in response to specific signals, there is little precedence for such a mechanism regionally restricting protein expression. Moreover, the observation that the *cdr2* protein is able to be expressed at high levels when transfected into non-neuronal cells (data not shown) suggests that the stability of the protein is not likely to be dependent on tissue-specific factors.

It is also possible that these results reflect tissue-specific differences in post-translational modifications affecting the PCD epitope, such that the protein is only immunoreactive in brain and testis. Notably, the *cdr2* leucine zipper harbors several potential phosphorylation sites. However, bacterially

expressed cdr2 fusion protein, full length cdr2 translated in reticulocyte lysate, or cdr2 protein expressed in a transfected fibroblast cell line are all readily detected by PCD antisera. It is therefore unlikely that a neuron-specific post-translational modification, or lack thereof, is a significant factor in recognition of the cdr2 epitope. A more direct approach to address this question would be to generate antibodies against other epitopes of the cdr2 protein to examine expression of the antigen.

## Chapter 4 - cdr2 Functional Studies

### Introduction

The PCD antigen cdr2 is normally expressed in specific neuronal populations and ectopically expressed in a subset of breast and ovarian tumors. Clinical evidence of immune pathology, neuronal degeneration and antitumor immunity indicate that the cdr2 antigen is at the center of a complex disease pathogenesis (for review, see Darnell, 1996). High titers of autoantibodies in PCD patients combined with evidence of limited tumor growth suggest that cdr2 serves as a *bona fide* tumor antigen. cdr2 protein is expressed in neurons that are targeted in the degenerative disease, implicating cdr2 in the neuropathology as well. In addition, selective expression of cdr2 in particular types of neoplasms suggests a possible role for the onconeural antigen in tumor biology.

Although the PCD antigen has been the focus of many studies since its isolation in 1991, little progress has been made in elucidating the function of the protein. The only clues to the biology of the cdr2 gene product are structural motifs found in its predicted amino acid sequence. Specifically, the presence of a typical leucine zipper at the C-terminal end of a long  $\alpha$ -helical region forms an extended amphipathic helix reminiscent of structural proteins and some transcription factors. It was this putative protein interaction domain along with an acidic region at the N-terminus (and the erroneous identification of a zinc finger motif) that led Fathallah-Shaykh et al. (Fathallah-Shaykh, et al., 1991) to suggest that cdr2 might be involved in the regulation of gene expression. However, detection of the antigen exclusively in the cytoplasm is inconsistent with a function as a DNA binding protein (Rodriguez, et al., 1988). The reported ultrastructural localization of cdr2 to free and membrane-bound ribosomes (Rodriguez, et al., 1988; Tomimoto, et al., 1993) suggests that the antigen may functionally interact with the translational machinery. Although it is tempting to speculate that

cdr2 (itself translationally controlled) may be involved in the regulation of translation, there is currently no functional data to support such a role for cdr2. Identification of cdr2-interacting proteins represents one approach to determining the function of cdr2, which may provide critical insights into mechanisms of neurobiology, tumor biology, neurodegeneration, and anti-tumor immunity.

Several strategies for the direct cloning of genes encoding interacting proteins have been developed (for review, see Guarente, 1993). One is an *in vitro* screening approach in which the protein of interest is labeled and subsequently used as a probe to screen a cDNA expression library. This method has been used successfully to identify partners of leucine zipper proteins, such as the Myc binding protein Max (Blackwood and Eisenman, 1991). However, this experimental approach may not be ideal for several reasons. The conditions of this assay do not accurately reflect the *in vivo* binding environment. Since the protein of interest is usually expressed as a fusion protein in bacteria, it is not post-translationally modified nor likely to be in its native conformation. In addition, the system may not be sensitive enough to detect interactions that are transient or unstable.

An *in vivo* screening method using *Saccharomyces cerevisiae* was first described in 1989 by Fields and Song (Fields and Song, 1989). This system, often referred to as the "yeast two-hybrid" or "interaction trap," exploits the modular nature of many transcriptional activators to detect protein-protein interactions. Although there are several variations on the assay, usually the protein of interest is fused to the DNA binding domain of a transcription factor, and a random pool of cDNAs encode fusion proteins containing the activation domain of the same or different factor. Also present in the yeast strain are reporter genes with the corresponding cognate binding sites. If the protein of interest interacts with a fusion protein from the library, the complex will bring together the DNA binding and activation domains to transcribe the reporter genes. Increased sensitivity, detecting even transient interactions, is only one of the advantages of this method. The

interactions also occur "*in vivo*" in a eukaryotic cell. Any fusion protein to be used in a two-hybrid screen as the "bait" must not activate transcription of the reporter genes independently to be useful in the system, and this can only be determined empirically. A major disadvantage of the dramatic increase in sensitivity of this method is that often many false positives must be retested and discarded before finding true specific binding partners. Several controls have been incorporated into the system to aid in determining the validity of interactions (Bartel et al., 1993; Gyuris et al., 1993). Finally, the relevance of any putative interactors identified in a two-hybrid screen must be verified using independent assays.

Through the use of the yeast two-hybrid system, we have identified an interaction between the PCD antigen cdr2 and c-Myc. Additional evidence for their colocalization in Purkinje cell cytoplasm, direct binding of the proteins via their helix-leucine zipper domains, and an effect of cdr2 on c-Myc activity support the conclusion that cdr2 and c-Myc form a functional complex *in vivo*. Thus, cdr2 represents a cell type-specific factor that may regulate the function of c-Myc in post-mitotic neurons as well as some tumors.

## Results

### *Identification of c-Myc as a cdr2-interacting protein*

Although the *cdr2* gene product exhibits no extensive homology to known proteins, there are distinguishing structural features that may provide clues to the protein's function. Database searches reveal a significant homology to myosin and many myosin-related proteins that form coiled-coil oligomeric complexes. Consistent with the homology data, an analysis of the predicted amino acid sequence of *cdr2* using the Paircoil algorithm (Berger, et al., 1995) indicates that the N-terminal domain containing the leucine zipper motif has a high probability of forming a coiled-coil (Figure 9, left). The final 28 amino acids of this  $\alpha$ -helical region represented on a helical wheel demonstrate the characteristics of a classical leucine zipper (Figure 9, right). Hydrophobic residues in positions *a* and *d* of the heptads provide the interface for protein binding on the amphipathic helix (Alber, 1992). Therefore, *cdr2* contains a coiled-coil leucine zipper domain that is likely to mediate specific protein-protein interactions.

HeLa cell lines express endogenous *cdr2* protein, as detected by Western blot and immunoprecipitation with PCD sera (data not shown). To identify proteins that may form functional complexes with *cdr2* we used the yeast two-hybrid system, or interaction trap (Zervos et al., 1993), to screen a HeLa cell cDNA library. This is a pJG4-5 based library that conditionally expresses activation domain fusion proteins in the presence of galactose (Gyuris, et al., 1993). All plasmids were transformed into the yeast strain EGY48, containing both *Leu2* and *lacZ* reporter genes under the control of the LexA operon (see Figure 10). Three "bait" constructs, in which full length human *cdr2* or fragments including the dimerization domain of *cdr2* were fused in frame with the LexA DNA binding protein, were tested for their ability to activate transcription in the absence of library plasmids. Only the *cdr2*<sup>65-140</sup> bait construct, encoding the 75 amino acids of *cdr2* that comprise the predicted coiled-coil leucine zipper domain, was found to be transcriptionally inert

(Figure 11A). The  $\text{cdr2}^{65-140}$  LexA fusion protein bait was also able to enter the nucleus and bind to the LexA operon, as determined by a transcriptional repression assay (Figure 11B).

Approximately  $4 \times 10^5$  primary library transformants were screened, and 41 single  $\text{leu}^+ \text{lacZ}^+$  colonies were isolated for further characterization. An additional criterion used for identifying potential  $\text{cdr2}$ -interacting proteins would be the presence of a similar dimerization motif (i.e. a leucine zipper) in the cDNA library fusion proteins. From this initial pool of positive clones 34 library plasmids were purified and analyzed by restriction digest and partial sequencing, representing 12 different cDNAs. The clones with open reading frames that corresponded to known sequences or showed partial homology to known sequences were transformed again into the EGY48 strain with either the  $\text{cdr2}$  bait plasmid or an irrelevant bait (in this case a transcriptionally inert fragment of the *Drosophila* bicoid protein). Results of the interaction trap and specificity assays are summarized in Table 1.

A large number of the cDNAs identified in the two-hybrid screen encoded either the N-terminal or C-terminal halves of the heat shock protein hsp90, and one clone encoded for another chaperonin family member, hsp60. Although the N-terminal hsp90 clone showed some degree of specificity in its interaction with  $\text{cdr2}$  in this assay, the interaction with the C-terminal half of HSP90 was clearly non-specific. While we have not pursued the *in vivo* relevance of this interaction, and it should be noted that heat shock proteins are the most commonly reported artifact of this screening method (Erica Golemis, personal communication).

Only one clone (represented by a single cDNA) showed a strong interaction with  $\text{cdr2}$  by the intensity of staining for  $\beta$ -galactosidase activity, and showed no reactivity with a control bait fusion protein (Figure 12A). This clone encoded the C-terminal region of c-Myc, a member of the bHLHzip family of proteins. The bHLHzip DNA binding and protein dimerization motif, which c-Myc requires for its activity, is encompassed by the fragment

encoded by the HeLa library cDNA. To further assess the specificity of the cdr2/c-Myc interaction in yeast, plasmids encoding two other bHLHzip proteins, Max and Mxi1, were also transformed into yeast with the cdr2 bait. Interestingly, cdr2 interacted only weakly with Mxi1 and not at all with Max using the sensitive lacZ reporter (Figure 12B). Therefore the interaction with c-Myc in the yeast two-hybrid system is highly specific, even among proteins with homologous dimerization domains.

*cdr2 and c-Myc immunoreactivity colocalize to Purkinje cell cytoplasm*

c-Myc is a nuclear phosphoprotein that is expressed abundantly in proliferating cells (Evan and Littlewood, 1993), whereas the cdr2 protein appears to be exclusively cytoplasmic, and is expressed abundantly in post-mitotic neurons. Therefore, a significant concern regarding the *in vivo* relevance of the cdr2/Myc interaction is the apparent difference in the regional and subcellular localization of the two proteins. To determine whether cdr2 and c-Myc are colocalized in adult brain, sections of rat cerebellum were analyzed by immunohistochemistry. Interestingly, immunoreactivity with PCD sera and an anti-c-Myc monoclonal antibody was confined to the cytoplasm of Purkinje neurons in the cerebellar cortex (Figure 13A, B). Higher magnification inspection of the Purkinje staining revealed a somewhat punctate pattern in the cytoplasm and little or no c-Myc reactivity was detected in the nucleus (Figure 13B, see inset). Anti-c-Myc staining of neurons was not uniform throughout the Purkinje cell layer, but rather found in clusters of cells. Although other neurons in the deep cerebellar nuclei and the brainstem were positively stained with the PCD sera, no other c-Myc positive cells were observed. These results are consistent with a previous study in which c-myc mRNA was detected only in some Purkinje cells in the adult cerebellum (Ruppert et al., 1986). Since the apparent subcellular localization of some antigens may be altered as a result of specific tissue fixation conditions, serial sections of rat cerebellum were stained with

other antibodies to known nuclear antigens. Figure 13C shows the predominantly nuclear stain of Purkinje and other cerebellar cortical neurons obtained with the paraneoplastic anti-Ri antibody (recognizing a family of primarily nuclear RNA binding proteins). This was also verified with the paraneoplastic anti-Hu antibody that recognizes another family of nuclear RNA binding proteins (data not shown). These results indicate that the immunoreactivity of nuclear proteins was not generally affected by our fixation conditions, and we conclude that c-Myc colocalizes with cdr2 specifically in the cytoplasm of adult cerebellar Purkinje neurons.

*Full length cdr2 and c-Myc proteins bind in vitro and require the bHLHzip domain of c-Myc*

Interactions detected in the yeast two-hybrid system may be mediated by bridging proteins or nucleic acids, rather than reflecting direct protein-protein binding. In addition, small fragments of bait proteins are often used in the screen that may not identify true binding partners of the full length proteins (Fields and Sternglanz, 1994). To determine whether full length cdr2 and c-Myc proteins can interact, we analyzed their ability to form a complex *in vitro*. Various GST fusion proteins were immobilized on glutathione sepharose and used as binding substrates for full length *in vitro* translated, <sup>35</sup>S-methionine labeled cdr2 or c-Myc proteins. Schematics of the GST-cdr2 and two different GST-c-Myc fusion proteins are shown in Figure 14A, and a Coomassie blue stain of all the purified fusion proteins is shown in Figure 14B. All of the fusion proteins contained the respective protein dimerization domains. The results of the *in vitro* binding assay indicated that cdr2 and c-Myc can interact directly. c-Myc formed a complex with cdr2 and Max, its known dimerization partner, but not with GST alone or with another bHLHzip protein, USF (Figure 14C). Full length cdr2 bound to c-Myc, but not to GST alone or to Max (Figure 14D), in agreement with the yeast two-hybrid data. Interestingly, the *in vitro* translated cdr2 formed a complex with the

GST-cdr2 fusion protein, suggesting that cdr2 may form homodimers *in vivo*. Deletion of the bHLHzip domain of c-Myc abrogated binding to full length cdr2 (Figure 14D), indicating that this 86 amino acid region of c-Myc is necessary for the interaction.

#### *cdr2 represses c-Myc transcriptional activity*

c-Myc is necessary for cell cycle entry and progression, can induce neoplastic transformation, and can initiate apoptotic cell death. All of these processes are dependent on c-Myc's activity as a transcriptional regulatory protein. The cytoplasmic localization of cdr2 suggests that it may have a significant effect on the ability of c-Myc to activate transcription in the nucleus. To assess the functional consequences of a cdr2:c-Myc complex *in vivo*, the transcriptional activity of c-Myc was assayed in NIH3T3 cells that were transiently transfected with a cdr2 expression plasmid. A plasmid (M4minCAT) containing a chloramphenicol acetyl transferase (CAT) reporter gene linked to a minimal SV40 promoter with a fourfold repeat of the Myc:Max recognition sequence CACGTG ("E-box") was used to measure Myc transcriptional activity, and an identical construct without the E-box sequences (minCAT) was used as a negative control (Figure 15, Kretzner et al., 1992b). All transfections included either a CMV promoter driven  $\beta$ -gal reporter or an SV40 promoter driven green fluorescent protein (GFP) reporter as an independent measure of transfection efficiency. Relative CAT activity for all experimental conditions was normalized to the transfection efficiency.

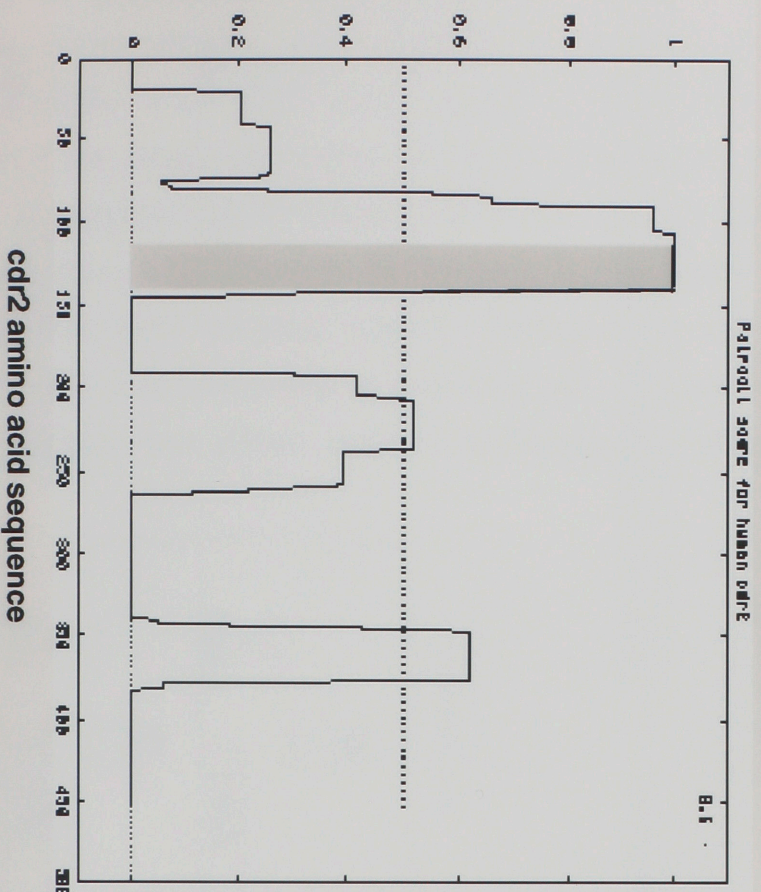
Transfection of the cells with M4minCAT alone resulted in a significant level of endogenous activity when compared to the basal activity of the minCAT reporter. Cotransfection of increasing amounts of the cdr2 expression plasmid with M4minCAT resulted in a corresponding decrease in endogenous E-box dependent CAT activity (Figure 16). The levels of specific repression ranged from two- to fourfold. When c-myc was transfected alone, a modest two- to threefold increase in CAT activity resulted, in agreement

with previous studies using both the CAT and luciferase reporter genes (Kretzner, et al., 1992a; Hurlin et al., 1997). Cotransfection of c-myc with increasing amounts of cdr2 expression plasmid also resulted in a titratable repressive effect on Myc-mediated transcription (Figure 17). The average maximal repression was more than twofold, resulting in levels of CAT activity at or below the endogenous level. Parallel experiments were carried out with the minimal promoter CAT reporter to demonstrate the E-box sequence dependence of the observed activation and repression. Higher titrations of cdr2 were not possible due to the cellular toxicity of increased total DNA used in those transfections. These results demonstrate that cdr2 has an inhibitory effect on the endogenous activity of E-box binding transcription factors, such as the Myc:Max complex, as well as the transcriptional activity of exogenous c-Myc.

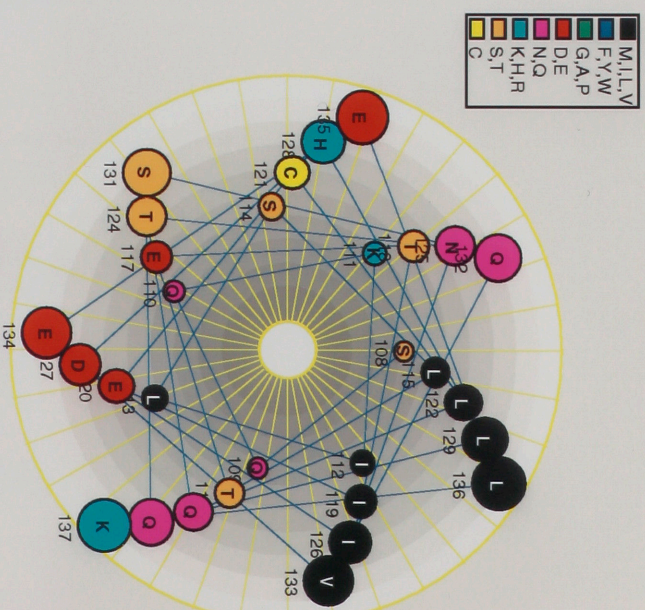
**Figure 9.** Schematic representation of the predicted secondary structure of cdr2. **Left**, probability (y-axis) that the amino acid sequence of cdr2 (x-axis) forms a coiled-coil, as determined by the Paircoil algorithm. The authors have set the minimum level of significance at a probability of 0.5 (dotted line). A 75 amino acid region near the N-terminus displays a high probability of forming a coiled-coil structure. **Right**, a helical wheel representation of the region shaded in grey on the Paircoil graph. This domain shows all the features of a typical leucine zipper: it is an amphipathic helix with hydrophobic residues (in black) at positions *a* and *d* of the heptad repeat.

# **cd<sub>r</sub>2 Amino Acid Sequence Predicts Coiled-Coil / Leucine Zipper Domain**

**Probability of forming coiled-coil**  
Paircoil algorithm (Berger et al., PNAS 92:8259)

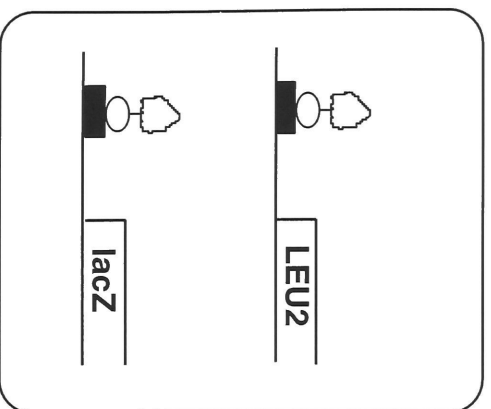


**Helical wheel**

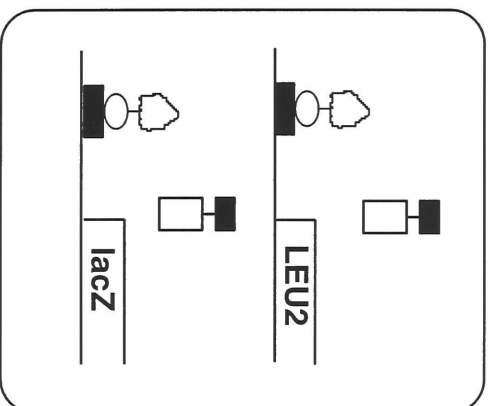


**Figure 10.** The yeast interaction trap used to screen a HeLa cDNA library for cdr2-interacting proteins. Several fragments of cdr2, all encompassing the coiled-coil leucine zipper domain, were fused in frame with the entire coding region of the *E. coli* DNA binding protein, LexA. This “bait” plasmid was cotransformed with the HeLa cDNA library, in which the inserts are fused to the B42 acidic activation domain under the control of the conditional Gal1 promoter. The yeast strain EGY48 contains LEU2 and lacZ reporter genes under the control of the LexA operon. Fusion proteins from the library cDNA’s are expressed only in the presence of galactose, and bait-library interactions are detected by both growth on leucine<sup>-</sup> media and staining with the X-gal substrate.

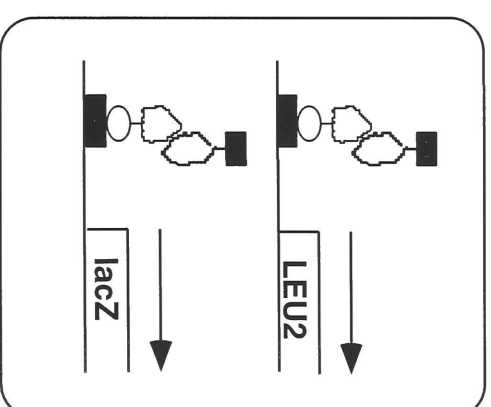
# Yeast Interaction Trap (R. Brent Lab, MGH)



+ galactose

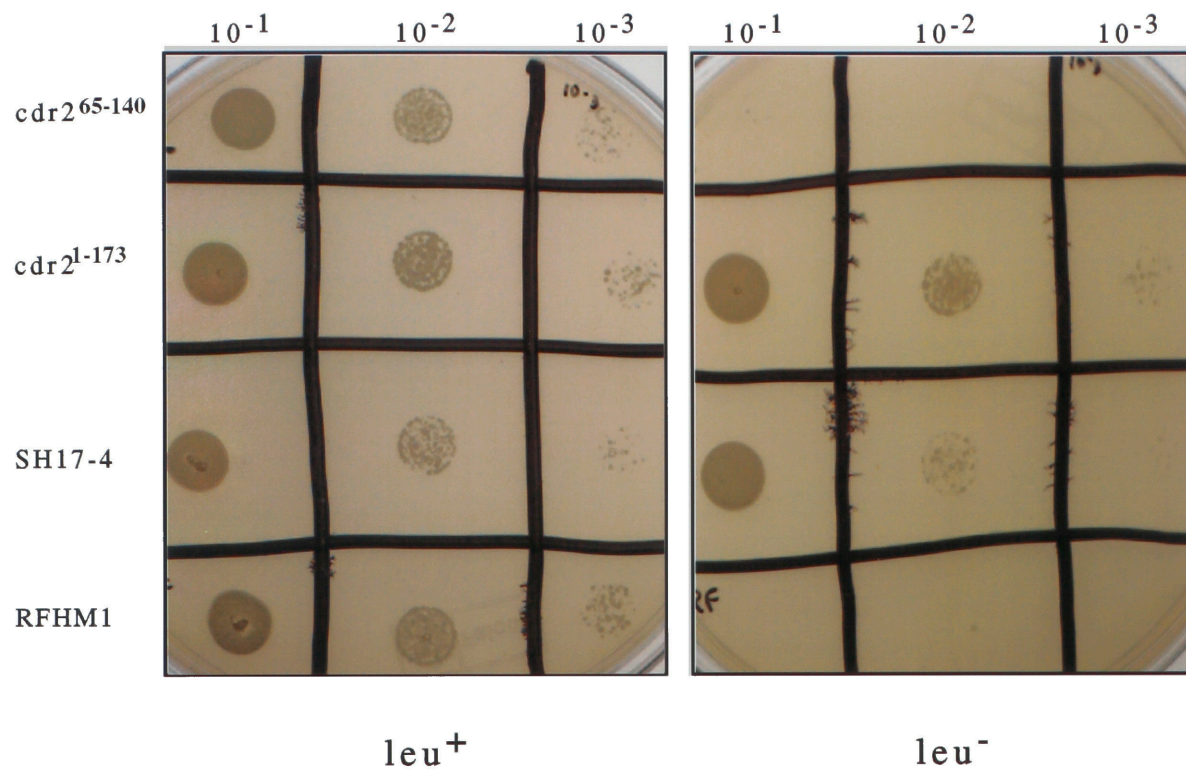


- 1) growth on leu<sup>-</sup> media
- 2) blue in presence of X-gal

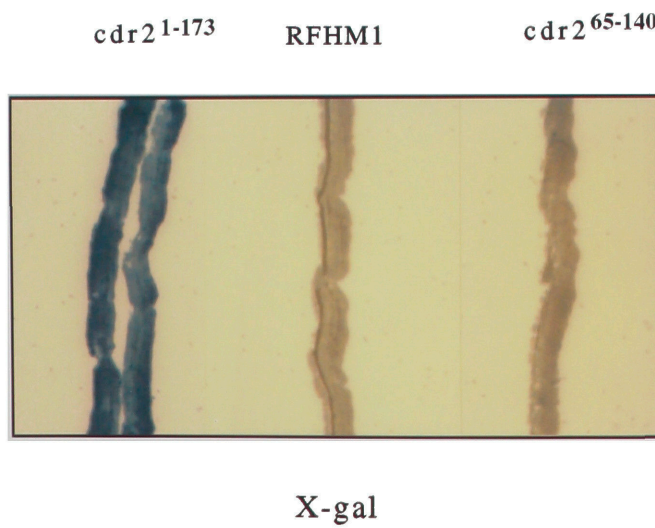


**Figure 11.** Yeast interaction trap bait fusion protein tests. **A**, Test to determine if the *cdr2* bait fusion proteins activate transcription of the *LEU2* reporter gene independent of the cDNA library. Here two different *cdr2* baits (numbers indicate amino acids) are tested along with a positive control encoding the yeast Gal4 activation domain (SH17-4) and a negative control encoding a transcriptionally inert fragment of the *Drosophila* bicoid protein (RFHM1). Serial dilutions of liquid cultures were spotted on media with or without leucine. Both the *cdr2*<sup>1-173</sup> and the positive control clearly activate transcription of the *LEU2* gene, as evidenced by the growth in the absence of leucine. In contrast, yeast transformed with either the *cdr2*<sup>65-140</sup> bait or the negative control did not grow in the absence of leucine at the highest concentration, indicating that these fusion proteins are not transcriptionally active. **B**, Repression assay to determine if the bait fusion proteins can enter the nucleus and bind to the LexA operon. Bait plasmids are transformed with the modified reporter, pJK101, which contains the *lacZ* gene under a constitutive promoter with a LexA operon insert. Binding of transcriptionally inert fusion proteins to the LexA site represses transcription of the *lacZ* gene. Both the *cdr2*<sup>65-140</sup> and the bicoid baits repress  $\beta$ -gal expression, as detected by X-gal staining. The *cdr2*<sup>1-173</sup> bait, which has been shown to activate transcription, is unable to repress the reporter gene expression.

A



B

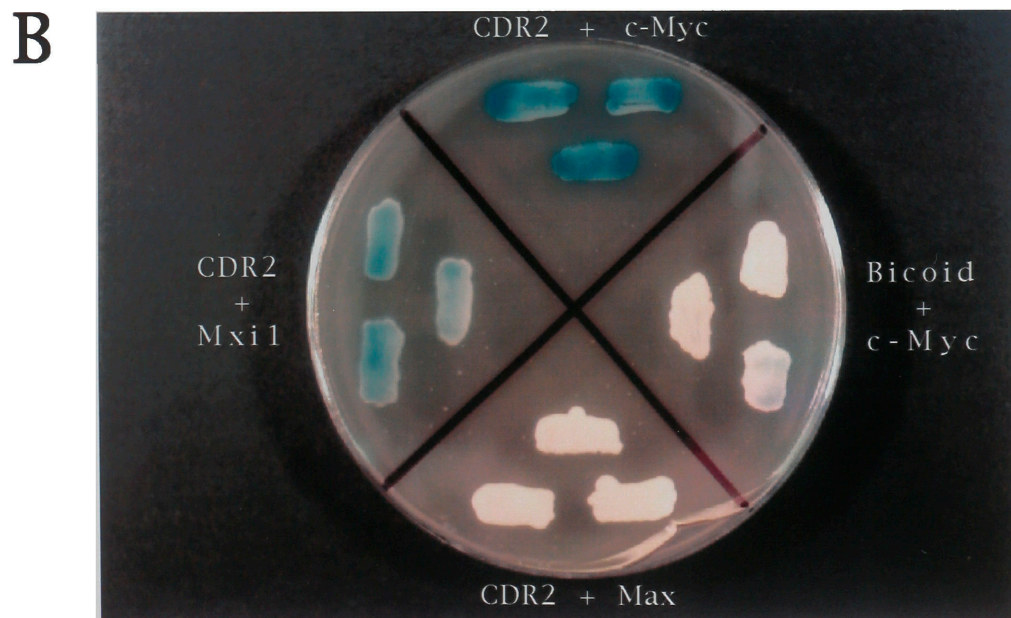
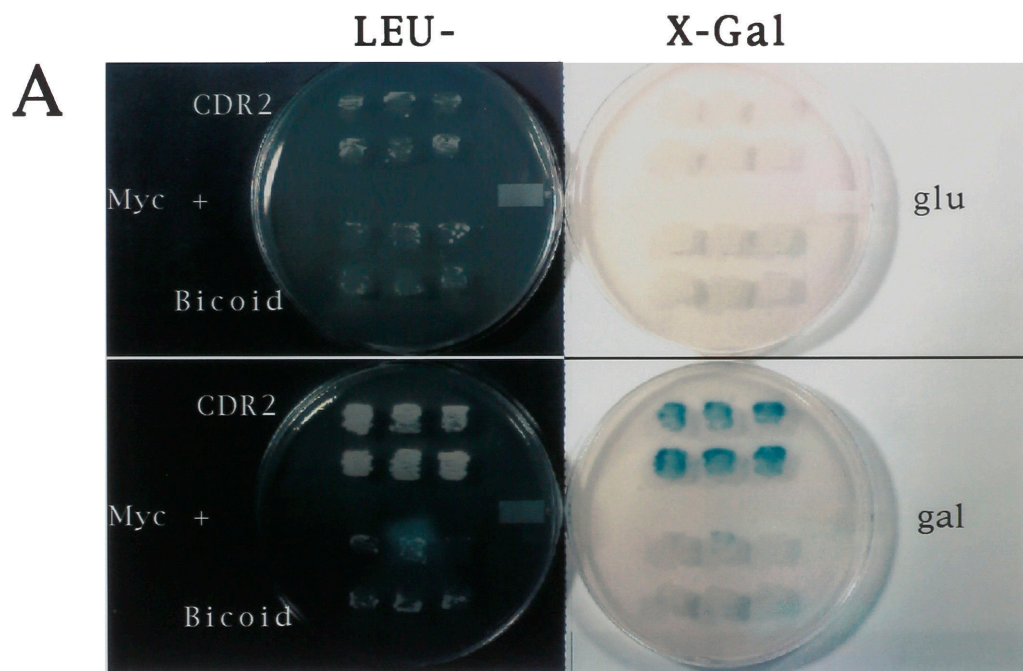


**Table 1.** Summary of results of the yeast interaction trap screen of a HeLa cell cDNA library. Library plasmids from colonies that grew in the absence of leucine and turned blue in the presence of X-gal (only on galactose containing media) were isolated and partially sequenced. The number of times each cDNA was isolated in the screen is indicated in column 2. Some of these purified library plasmids were transformed again with either the bicoid or the *cdr2*<sup>65-140</sup> bait plasmid to test for the specificity of the interaction. Shown here are the relative intensities of X-gal staining with each bait. Similar results were obtained when the LEU2 reporter gene expression was examined (not shown). nt = not tested

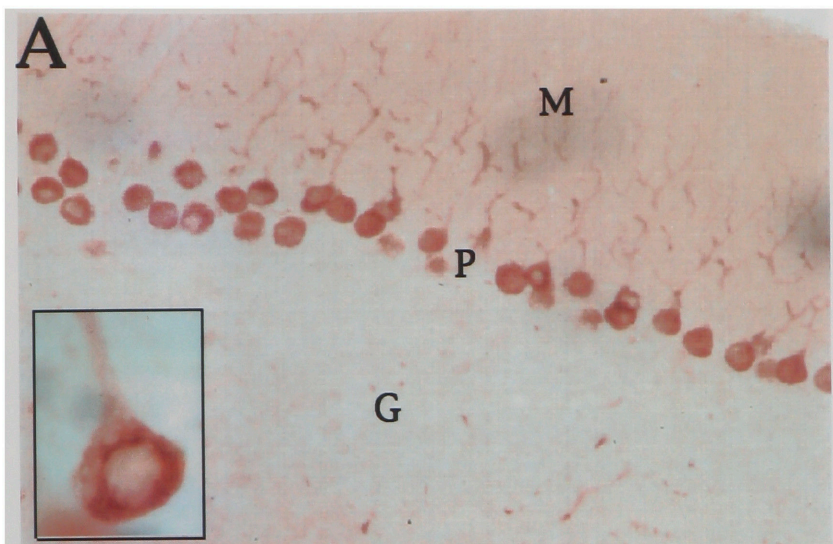
## Interaction Trap Results

cDNA	# clones	lacZ w/ cdr2 bait	lacZ w/ bicoid bait
HSP90 (N-terminal)	18	+++	+
HSP90 (C-terminal)	6	+++	+++
Fetal brain EST .	1	++	++
NADH isocitrate dehydrogenase	1	+	-
unknown	2	++	+
c-myc (C-terminal)	1	+++	-
26S protease subunit	1	n t	n t
EST	1	n t	n t
HSP60	1	n t	n t
unknown	1	n t	n t
EST	1	n t	n t

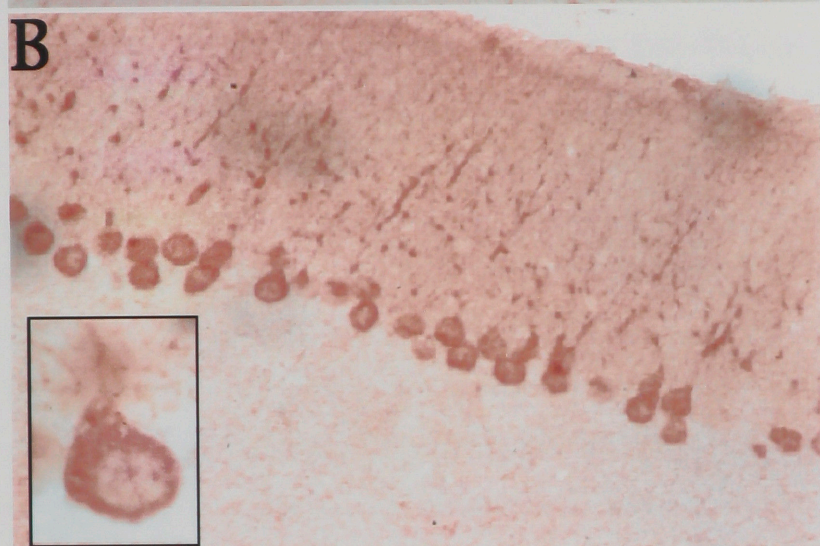
**Figure 12.** Specificity of the cdr2/c-Myc interaction in yeast. **A**, The activation domain/c-Myc fusion protein identified in the HeLa cDNA library screen interacts specifically with the LexA/cdr2<sup>65-140</sup> bait protein. Activation of both the LEU2 (allowing growth on media lacking leucine) and lacZ (producing blue color in the presence of X-gal) reporter genes is dependent on the presence of galactose. There is no interaction of c-Myc with the control *Drosophila* bicoid bait protein. **B**, The interaction of cdr2<sup>65-140</sup> with proteins of the bHLHzip family in the yeast two-hybrid assay. As determined by activation of the lacZ reporter gene, the interaction between cdr2<sup>65-140</sup> and c-Myc is strong and specific. There is no detectable interaction of cdr2<sup>65-140</sup> with Max, and a relatively weak interaction between cdr2<sup>65-140</sup> and Mxi1. Again, the bicoid control bait does not interact with c-Myc.



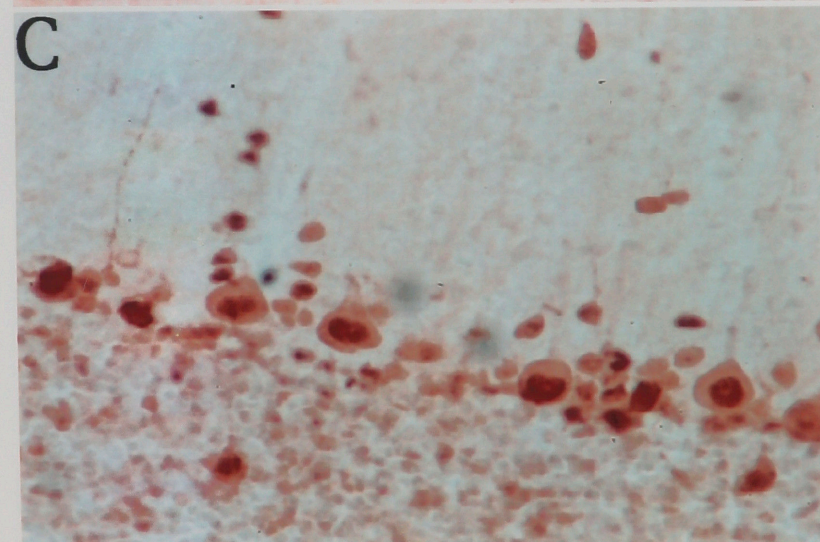
**Figure 13.** **A**, Immunohistochemical colocalization of cdr2 and c-Myc in the cytoplasm of Purkinje neurons. A section of adult rat cerebellar cortex stained with a PCD patient antisera ("Yo") reveals cdr2 immunoreactivity in the cytoplasm of Purkinje cells. **B**, A serial section of adult rat cerebellar cortex stained with a monoclonal antibody against c-Myc shows the same localization. Insets show cytoplasmic staining, sparing the nucleus. **C**, Another paraneoplastic disease antisera ("Ri"), that recognizes a family of neuronal RNA binding proteins, was used to stain serial sections (shown at slightly higher magnification) to demonstrate that nuclear staining was not generally affected by the tissue fixation conditions. Serial sections processed without primary antibody showed no cellular staining (data not shown). The three cellular layers of the cerebellar cortex are: the molecular layer *M*, the Purkinje cell layer *P*, and the granule cell layer *G*.



anti-Yo

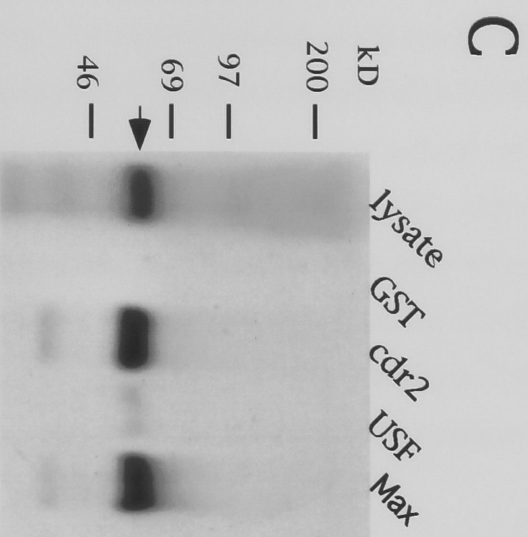
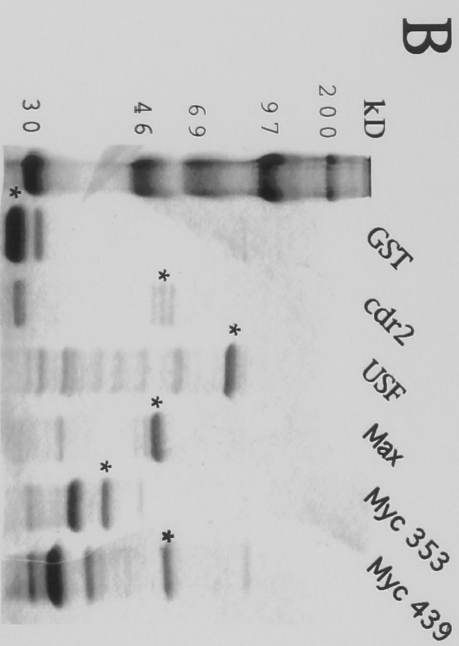
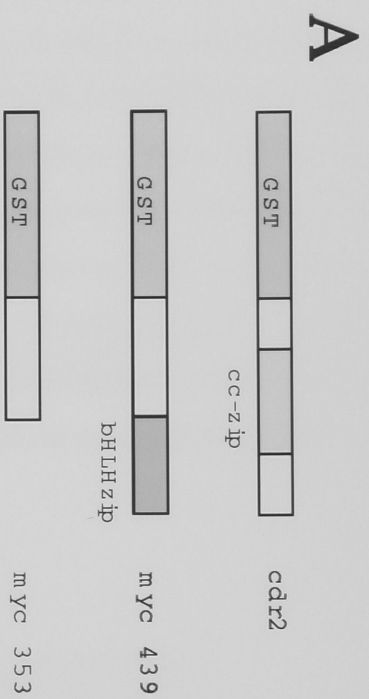


anti-  
c-Myc

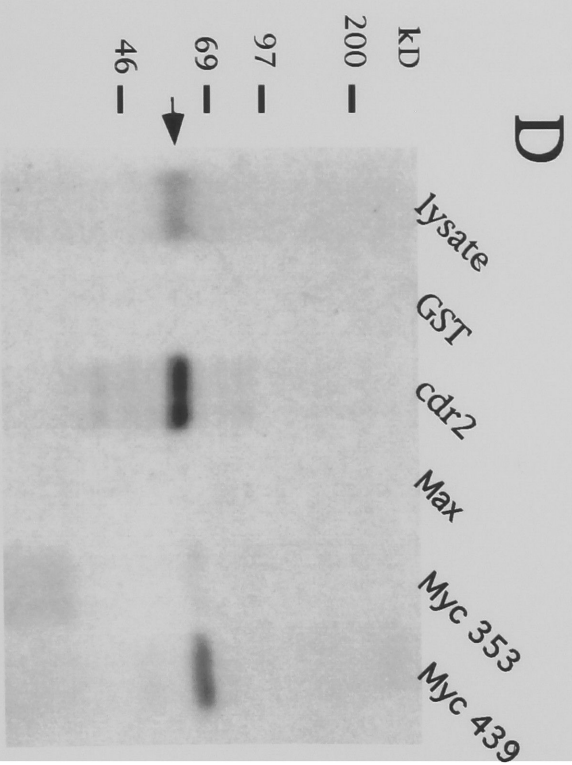


anti-Ri

**Figure 14.** *cdr2* and c-Myc interact *in vitro*. A GST pull-down assay was used to examine the *in vitro* binding of *cdr2* and c-Myc. Various immobilized GST fusion proteins were used as binding substrates for full length *in vitro* translated *cdr2* and c-Myc. **A**, Schematic representation of some of the GST fusion proteins used in the assay. The *cdr2* fusion protein contains the coiled-coil leucine zipper domain. Two different fragments of the c-myc gene were fused in frame with GST, yielding either a 189 amino acid domain including the bHLHzip motif or a 103 amino acid domain with the bHLHzip motif deleted. **B**, All the GST fusion proteins were partially purified on glutathione sepharose and resolved by SDS-PAGE. Shown is a coomassie blue stain of the fusion proteins; the full length proteins are indicated by an asterisk. **C**, Full length <sup>35</sup>S-c-Myc binds to GST-*cdr2* *in vitro*. Myc did not bind to GST alone and only weakly to GST-USF, another b-HLH-zip protein. GST-Max was a positive control for the assay. **D**, Full length <sup>35</sup>S-*cdr2* interacted with a truncated Myc fusion protein that includes the b-HLH-zip domain (Myc 439), but not to a fusion protein lacking this region (Myc 353). *cdr2* interacted with itself, indicating that it may potentially form homodimers *in vivo*. GST-Max did not bind *cdr2*, in agreement with the yeast two-hybrid data, nor did GST alone. The arrows indicate the size of the full length labeled proteins. The protein in the lanes marked "lysate" represents 10% of the protein used in each binding assay.



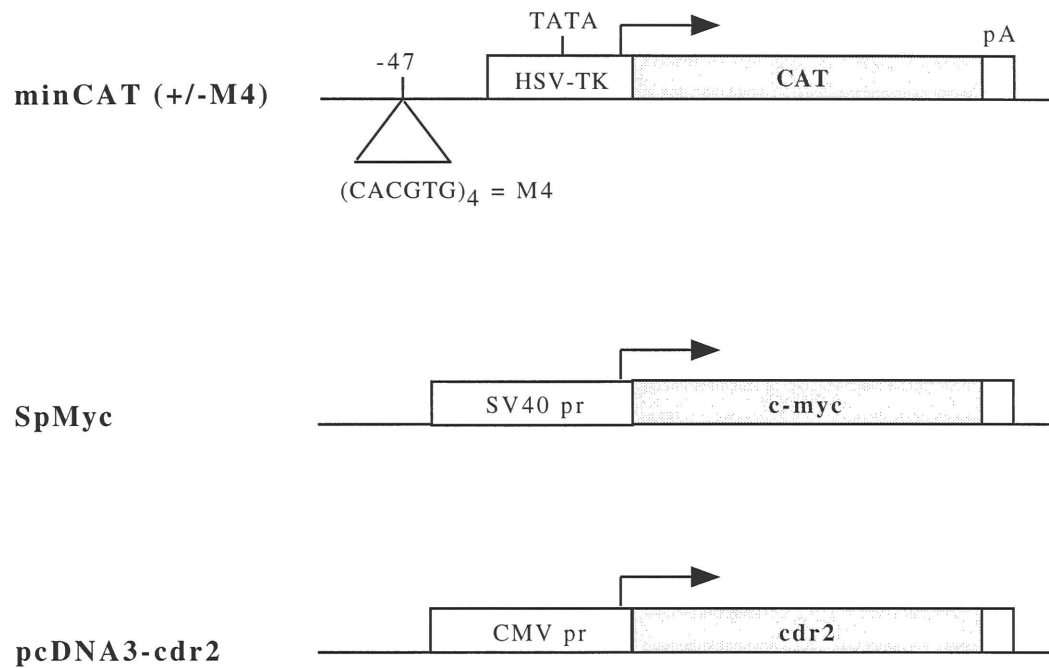
$[^{35}\text{S}]$  c-Myc



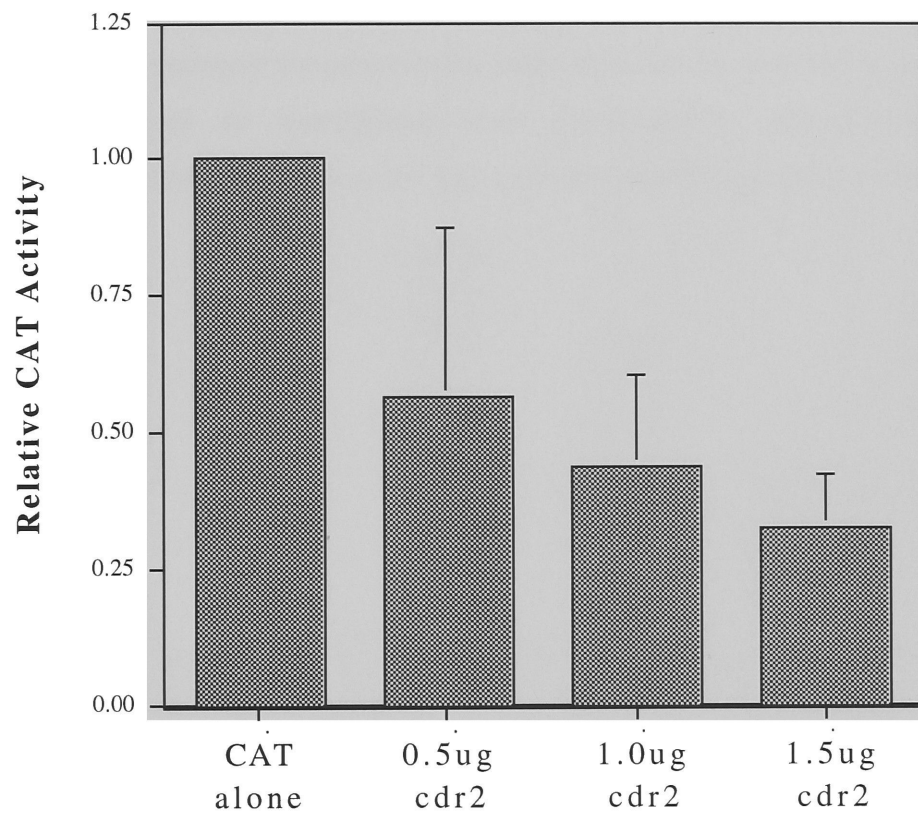
$[^{35}\text{S}]$  CDR2

**Figure 15.** Schematic representation of the plasmids used in the E-box reporter assay. NIH3T3 cells were transiently transfected with the following plasmids: minCAT (+/-M4) is a chloramphenicol acetyltransferase (CAT) reporter gene in front of a minimal HSV thymidine kinase promoter, with or without a fourfold repeat of the CACGTG E-box sequence; SpMyc contains the full length c-myc coding region driven by the constitutive SV40 promoter; and pcDNA3-cdr2 contains the full length cdr2 coding region driven by the constitutive CMV promoter. CAT activity was determined by measuring the amount of acetylated <sup>14</sup>C-chloramphenicol in each cell lysate. An SV40-GFP (green fluorescent protein) or CMV-lacZ reporter construct was cotransfected in all experiments as an independent measure of transfection efficiency.

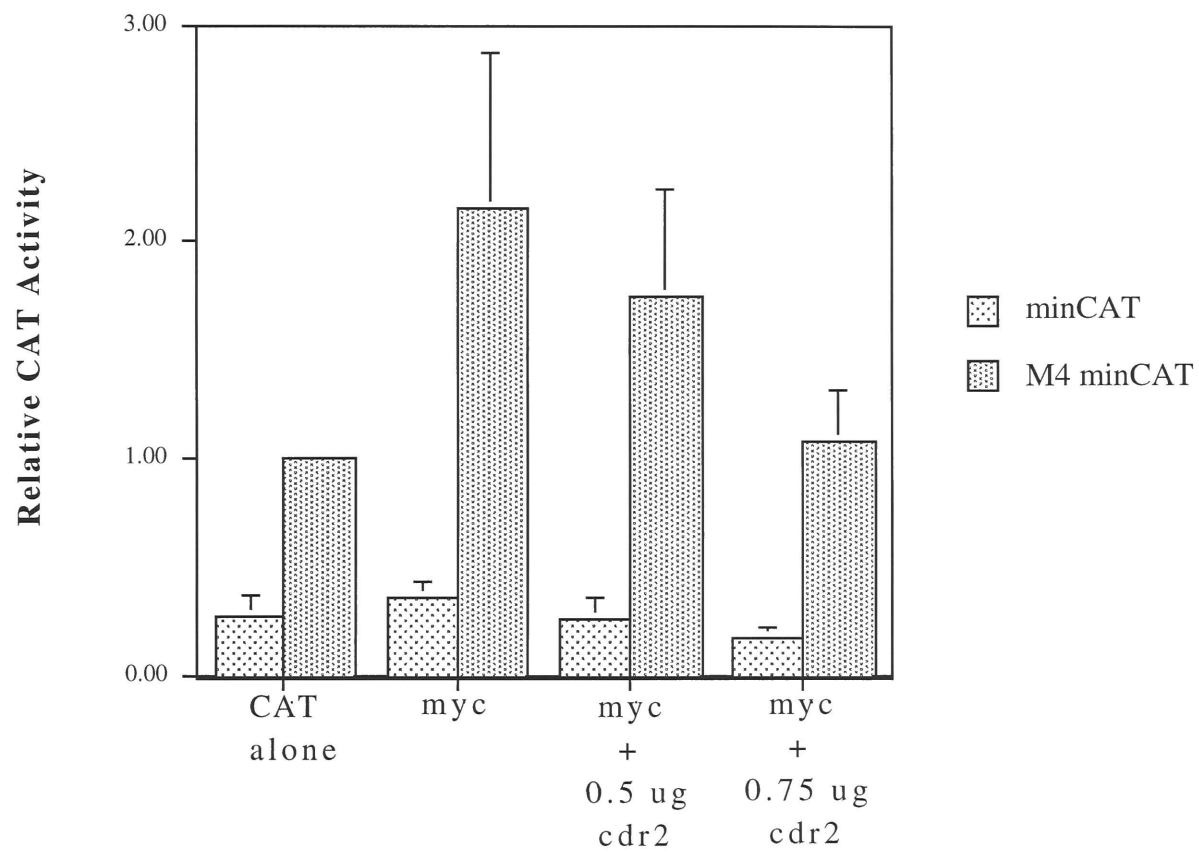
## "E-box" reporter assay - NIH3T3 cells



**Figure 16.** *cdr2* represses endogenous E-box activity. NIH3T3 cells were transiently transfected with the M4minCAT reporter gene and increasing amounts of the pcDNA3-*cdr2* plasmid. Exogenous *cdr2* repressed E-box driven CAT activity in a titratable manner, with the average levels of repression ranging from 44-68%. Results shown here are the average of four independent experiments, and in each experiment all samples were carried out in triplicate (n=12). Relative CAT activity was normalized to the transfection efficiency. CAT activity from the minimal promoter was typically near 10% of that obtained from the E-box promoter (not shown).



**Figure 17.** *cdr2* represses c-Myc transcriptional activity. NIH3T3 cells were transiently transfected with the (+/-M4)minCAT reporter plasmids, 0.5 $\mu$ g of the SpMyc plasmid and increasing amounts of the pcDNA-*cdr2* plasmid. Transfection with c-myc alone resulted in an average 2.2-fold induction of CAT activity, consistent with previous findings. Cotransfection with *cdr2* inhibited the c-Myc induced CAT activity in a titratable manner, resulting in levels near the endogenous activity. In contrast, the activity of the minimal promoter was relatively unaffected. The results shown represent the average of four independent experiments, each performed in triplicate (n=12). Relative CAT activity was normalized to the transfection efficiency.



## Discussion

The use of the cdr2 coiled-coil leucine zipper protein interaction domain in a yeast two-hybrid screen has led to the identification of c-Myc as a cdr2-interacting protein. Colocalization of the two proteins in the cytoplasm of Purkinje neurons in the cerebellar cortex, as well as the demonstration of direct binding of cdr2 and c-Myc *in vitro*, suggest that this interaction may be physiologically relevant. Transient transfection of NIH3T3 cells with either cdr2 alone or cotransfection with c-myc showed that the overexpression of the cdr2 protein has a significant and titratable repressive effect on c-Myc transcriptional activity. Taken together, these data provide both *in vitro* and *in vivo* evidence for a functional interaction between cdr2 and c-Myc.

The c-myc proto-oncogene encodes a member of the basic helix-loop-helix leucine zipper (bHLHzip) family of transcription factors (for reviews, see Evan and Littlewood, 1993; Bernards, 1995). Sequence specific DNA binding to a CACGTG element ("E-box") is mediated by the basic region, and the HLHzip domain is required for efficient protein oligomerization. Two conserved motifs in the amino terminus are involved in either transcriptional activation (Myc box I) or repression (Myc box II, Li et al., 1994). Expression of c-myc is rapidly induced upon mitogenic stimulation of quiescent cells, and studies in which c-myc expression is inhibited have shown that it is required for cell cycle entry. In addition to its role in normal cellular proliferation, c-myc has been implicated in the processes of apoptosis and neoplastic transformation. Overexpression of c-myc under conditions that do not favor proliferation (e.g. low serum) can cause p53-dependent programmed cell death (Hermeking and Eick, 1994). c-myc cooperates with the ras proto-oncogene to transform primary fibroblasts (Land et al., 1983), and mice carrying a c-myc transgene develop mammary tumors (Stewart et al., 1984). Several members of the myc family of genes, including c-myc, have been found to be deregulated in a variety of malignancies.

Multiple levels of regulation affect the activity of the c-myc proto-oncogene. Gene expression is induced in response to mitogenic signals, and phosphorylation of the activation domain regulates c-Myc transcriptional activity. However, the primary level of control comes from a network of protein-protein interactions that either promote or inhibit the function of c-Myc. Heterodimerization of c-Myc with a related bHLHzip protein, Max, is required for sequence specific DNA binding and transcriptional activation (Blackwood and Eisenman, 1991). In fact, the functions of c-Myc in cell proliferation, transformation and apoptosis all require dimerization with Max (Amati et al., 1993a; Amati et al., 1993b). Formation of the Myc:Max heterodimer is strongly favored over either Myc:Myc or Max:Max homodimers, the latter of which acts as a transcriptional repressor (Kretzner, et al., 1992a). Several other recently discovered bHLHzip proteins interact specifically with Max, and serve to form repressor complexes that inhibit Myc function by competing for both Max and the CACGTG binding site (Ayer et al., 1993; Zervos, et al., 1993). Examples of proteins that may negatively regulate Myc by direct interaction are the pRb-related p107 and the zinc finger transcription factor YY-1 (Shrivastava et al., 1993; Gu et al., 1994).

Although many studies have indicated that c-myc expression is required for cell cycle entry, others have provided evidence for a role in differentiation of some cell types. The expression of c-myc in the developing nervous system is not as tightly linked to proliferation as observed in other tissues. In the developing cerebellum, peaks of c-myc expression are associated not only with well defined periods of proliferation, but also with specific stages of differentiation (Ruppert, et al., 1986). This is illustrated by expression in terminally differentiating Purkinje neurons long after these cerebellar cortical cells have become post-mitotic. Induction of the embryonic carcinoma cell line P19 to differentiate along a neuronal lineage leads to two transient increases in c-myc expression, also suggesting a direct role in neuroectodermal differentiation (St-Arnaud et al., 1988). The function and

regulation of the c-Myc protein during neural differentiation events as well as in mature neurons is not understood.

#### *The cdr2:c-Myc interaction*

Domain mapping data are consistent with the finding that cdr2 and c-Myc form a complex. The yeast two-hybrid data indicate that the 75 amino acid coiled-coil leucine zipper domain of cdr2 is sufficient for binding to c-Myc. Similarly, *in vitro* translated full length cdr2 bound to a GST-c-Myc fusion protein that included the bHLHzip domain, but not to a fusion protein with a truncation of this 86 amino acid region. These results demonstrate that the coiled-coil domains of both cdr2 and c-Myc are necessary and sufficient for their interaction.

Preferential formation of heterodimers of c-Myc and Max is also mediated through the HLHzip domain, and several studies have contributed to an understanding of the structural basis for this interaction. Domain swapping experiments demonstrated that both the second helix in the HLH motif and the contiguous leucine zipper were required for efficient and specific dimerization (Davis and Halazonetis, 1993; Marchetti et al., 1995). This represents a critical difference between the bHLHzip family of proteins and the related basic leucine zipper (bZip) proteins, for which the leucine zipper is the only determinant of dimerization. Structural studies support this finding and extend the observations to include characterization of residues contributing to complex stability. Ferré-D'Amaré et al. (Ferre et al., 1993) determined the structure of Max:Max homodimers bound to DNA by X-ray crystallography at a 2.9Å resolution, and found that the dimer forms a four-helix bundle. The first  $\alpha$ -helix of each monomer is composed of the basic region and helix1 of the HLH and is involved in binding DNA; the second  $\alpha$ -helix is the helix2-leucine zipper region and mediates protein-protein interactions. The predicted coiled-coil domain of cdr2 is analogous to the helix2-leucine zipper domain of c-Myc and related proteins. All form an

extended amphipathic  $\alpha$ -helix with heptad repeats of hydrophobic residues continuing in register with the leucine zipper. A study using synthetic peptides corresponding to the c-Myc and Max leucine zippers identified specific interhelical electrostatic interactions that act as stability determinants (Lavigne et al., 1995). Most important among these is a potential interaction between a charged histidine side-chain in the Max leucine zipper and two glutamic acid side-chains in the c-Myc leucine zipper. Interestingly, cdr2 has a single histidine in register with three leucine heptad repeats in the coiled-coil domain in the same configuration as seen in the Max leucine zipper. Whether this specific region of cdr2 is involved in the interaction with c-Myc remains to be determined.

#### *c-Myc colocalizes with cdr2 in the cytoplasm of Purkinje neurons*

The regional and subcellular localization of cdr2 and c-Myc is critical to the physiological relevance of their interaction. Early studies on the tissue distribution of c-myc expression identified expression in populations of proliferating cells in development, but no expression in the adult brain by Northern blot analysis (Zimmerman et al., 1986). However, studies examining c-myc expression in the nervous system using more sensitive techniques such as RNase protection and *in situ* hybridization found that the proto-oncogene is expressed in development and into adulthood, but only in restricted regions of the CNS. Ruppert et al. (Ruppert, et al., 1986) generated such an expression profile for c-myc mRNA in the developing mouse cerebellum. The authors demonstrated that peaks of c-myc expression correspond both to critical proliferation and differentiation events in the cerebellum. Interestingly, a peak in expression between postnatal days 7-10 occurs at a time when Purkinje neurons are undergoing terminal differentiation, and c-myc mRNA was detected only in Purkinje neurons in the adult cerebellum. No hybridization was seen with a sense strand c-myc riboprobe or after ribonuclease treatment of the probes. These findings were

corroborated by two other reports, in which c-myc expression persisted in the adult cerebellum (McCormack et al., 1992), and specifically in Purkinje neurons (Takahashi et al., 1993).

The results presented here are the first report of the immunohistochemical localization of c-Myc protein in post-mitotic Purkinje neurons. Colocalization of cdr2 and c-Myc in the cytoplasm of these large neurons in the cerebellar cortex suggests that the cdr2:Myc interaction detected in yeast may reflect an *in vivo* association. Although the detection of c-Myc in the Purkinje cytoplasm was unexpected, this observed subcellular distribution of the protein is not without precedent. Cytoplasmic c-Myc has been detected in adult neurons of the enteric nervous system and dorsal root ganglia (Parr et al., 1994). Takahashi et al. (Takahashi, et al., 1993) examined the expression of c-Myc protein in developing and adult rat cerebellum by immunoblotting, and noted that most of the detectable protein was found in the cytosolic fraction. Studies using various cell lines have revealed that the subcellular localization of c-Myc can be altered by conditions that either halt proliferation or induce differentiation. When the myeloid cell line ML-1 differentiates in response to treatment with a phorbol ester, c-Myc translocates to the cytoplasm, displaying a perinuclear staining pattern (Craig et al., 1993). Metabolic labeling indicated that new protein continued to be synthesized, but transport into the nucleus had been slowed. Similarly, when a fibroblast cell line (NIH3T3) was serum starved, the cells became quiescent and c-Myc localization shifted to the cytoplasm (Vriz et al., 1992). Both studies included internal controls and used both histological and biochemical analysis, ruling out the possibility of artifactual staining due to fixation techniques. c-Myc is also sequestered in the cytoplasm of oocytes, and upon fertilization undergoes a rapid translocation to the nucleus (Lemaitre et al., 1995). In all of these circumstances, the mechanism(s) by which c-Myc protein is excluded from the nucleus have yet to be identified.

### *Regulation of c-Myc activity by cdr2*

The results presented here demonstrate that *cdr2* can inhibit the transcriptional activity of c-Myc. In transiently transfected NIH3T3 cells, *cdr2* repressed transcription of an E-box promoter driven reporter gene. The levels of repression were similar to those found by Kretzner et al. (Kretzner, et al., 1992a) when the Myc-binding protein Max was overexpressed alone in NIH3T3 cells with the M4minCAT reporter. Much of the activity from this promoter can be attributed to endogenous c-Myc and related factors. In addition, the induction of reporter gene transcription resulting from overexpression of c-Myc can be blocked by *cdr2*. Therefore, the cytoplasmic *cdr2* protein is able to inhibit the nuclear activity of c-Myc.

Control of the subcellular localization of proteins has been observed in the heat shock response, early embryonic development, ribosomal component shuttling, and targeting of nuclear hormone:receptor complexes. Cytoplasmic sequestration is a common mechanism regulating the activity of proteins, including transcription factors such as NF $\kappa$ B, c-Fos, and c-Myc (Baeuerle and Baltimore, 1988; Roux et al., 1990; Craig, et al., 1993). In the case of NF $\kappa$ B, a specific cytoplasmic inhibitor (named I $\kappa$ B) of the transcription factor has been identified. I $\kappa$ B serves as an anchor, maintaining the majority of NF $\kappa$ B in the cytoplasmic pool, inactive but readily available (Baeuerle and Baltimore, 1988). Upon stimulation I $\kappa$ B is phosphorylated, releasing it from the cytoplasmic complex and freeing NF $\kappa$ B for transport into the nucleus (Rice and Ernst, 1993). Similar to the studies on c-Myc, the subcellular distribution of c-Fos has been observed to change from nuclear to predominantly cytoplasmic upon the withdrawal of serum factors from cultured cells (Roux, et al., 1990). Evidence from time course experiments with cycloheximide treatment in the same study supports the role of a labile protein in the retention of c-Fos in the cytoplasm, and demonstrated that this mode of regulation could be overcome by addition of cyclic AMP.

In one study, Craig et al. (Craig, et al., 1993) found that in differentiating ML-1 myeloid cells the nuclear translocation of newly synthesized c-Myc was inhibited. If an I $\kappa$ B-like mechanism is involved in the regulation of c-Myc activity, cytoplasmic binding proteins might account for the altered distribution of c-Myc. The cytoplasmic colocalization of c-Myc and the cdr2 antigen in post-mitotic Purkinje neurons suggests that cdr2 could play such a role in Myc biology in the nervous system. An immunofluorescence study of the related N-Myc protein in the developing mouse and chick nervous system revealed that the localization of N-Myc shifts from nuclear in proliferating neuroblasts to cytoplasmic in certain post-mitotic neuronal populations (Wakamatsu et al., 1993). Interestingly, there appears to be a correlation between those neurons displaying altered N-Myc localization and PCD antisera immunoreactivity; namely, cerebellar Purkinje neurons, retinal ganglion neurons, and neurons in the dorsal root ganglia (Tomimoto, et al., 1993). Though we also found N-Myc immunoreactivity in adult Purkinje neurons, the staining was predominantly nuclear (data not shown). The ability of cdr2 to interact with the N-Myc protein *in vitro* has not yet been determined.

Also consistent with this model of cytoplasmic sequestration is the ability of exogenous cdr2 to repress c-Myc transcriptional activity in proliferating NIH3T3 cells. cdr2 was found to negatively effect both endogenous activity from an E-box promoter as well as the induction of transcription due to overexpressed c-myc. The activity of this artificial promoter cannot be attributed completely to Myc:Max complexes because other ubiquitous factors (e.g. USF) are able to bind this same sequence. However, the effect observed in the cdr2 and c-myc cotransfection experiments, as well as the specificity of the cdr2:c-Myc interaction, support the role of cdr2 as an inhibitor of c-Myc stimulated transcription. Although the precise mechanism of the observed transcriptional repression awaits further study, preliminary data indicates that the overexpressed cdr2 protein

is exclusively cytoplasmic (data not shown). The possibility that *cdr2* expression has an indirect effect on c-Myc activity cannot be ruled out, but the protein binding data strongly suggests that the *cdr2*-mediated repression occurs via direct interaction with c-Myc. Co-immunoprecipitation of *cdr2* and c-Myc would provide direct evidence of an *in vivo* interaction, and immunofluorescence analysis and/or subcellular fractionation of cotransfected cells may help to support or discount cytoplasmic sequestration as the particular mode of regulation involved.

Gene disruption studies showed that *c-myc* is required for nervous system development (Davis et al., 1993), an observation that can be attributed to the essential role of the proto-oncogene in regulating proliferation. However, *c-myc* expression in the developing nervous system is clearly uncoupled from the cell cycle and often persists in post-mitotic neurons (Ruppert, et al., 1986). Observed peaks of expression suggest that *c-myc* may also be required for events associated with neuronal differentiation, such as process outgrowth and synaptogenesis. Restricted expression of c-Myc in the adult brain indicates that the protein may have specialized functions in mature neurons that have yet to be defined. The interaction between c-Myc and the *cdr2* onconeural antigen may represent a novel cell type-specific regulatory mechanism characteristic of post-mitotic Purkinje neurons.

## Chapter 5 - Discussion

The results presented in this thesis represent a careful characterization of the PCD onconeural antigen cdr2, and serve to illustrate the significance of this protein to disease pathogenesis as well as to neurobiology and tumor biology. Identification of cdr2 as the *in vivo* PCD antigen and evidence supporting its role in the neurodegeneration associated with PCD have come from studies examining the expression of cdr2 mRNA and protein in tumor and normal tissue. In addition, protein interaction studies have revealed a functional interaction between cdr2 and the oncoprotein c-Myc, a finding with broad implications for the role of the cdr2 antigen in neurons, tumors, and the etiology of PCD.

### Expression of the cdr2 onconeural antigen

*The cdr2 protein is expressed in immune privileged tissues*

Western blot and immunohistochemical analyses demonstrated that the cdr2 antigen is restricted in its expression to specific cell types in the brain and testis. Specifically, deep cerebellar nuclei and Purkinje neurons, many brainstem nuclei neurons, and scattered cerebral cortical neurons are cdr2 immunoreactive. In the testis, only the spermatogonia express the onconeural antigen. All cells stained with the anti-Yo antibody display cytoplasmic reactivity and a sparing of the nucleus.

Within the cerebellum there is a clear developmental and functional relationship between the cdr2-positive cell types. Purkinje neurons and neurons in the deep cerebellar nuclei originate from the ventricular germinal zone of the rhombic lip during the same period (E10-E13 in the mouse) of development (Jacobson, 1991). These two neuronal populations are also functionally linked in the cerebellar circuit as the deep cerebellar nuclei receive afferent input from the Purkinje axonal projections. Whether the cdr2 protein is required for the development and/or survival of these

neurons is unknown. Targeted disruption of the *cdr2* gene or a transgenic mouse expressing a dominant negative *cdr2* (discussed below) may serve to answer such questions.

The spermatogonia of the testis are the least differentiated cell type of the germ cell lineage, and are therefore mitotically active. Thus the functional significance of the shared *cdr2* expression with post-mitotic neurons is unclear. One important common feature of both tissues is their immune privileged status. Transplanted allogeneic or xenogeneic grafts experience prolonged survival in brain and testis relative to other tissues. However, the mechanistic basis of this privilege is quite different for each tissue. In the testis there is excellent lymphatic drainage and immune surveillance by B and T lymphocytes is unimpeded. The ability to survive rejection when transplanted themselves, and the expression of the apoptosis-inducing fas ligand suggested that cells in the testis use an active mechanism to achieve their immune privilege. Such a mechanism was demonstrated in a study by Belgrau et al. (Bellgrau, et al., 1995) in which the survival of testicular grafts was dependent on both the expression of the fas ligand by the testis and the expression of the CD95 fas receptor on the surface of T lymphocytes. By inducing the apoptotic death of reactive lymphocytes, cells in the testis (and in the anterior chamber of the eye, Griffith, et al., 1995) escape immune-mediated destruction.

Immune privilege in the brain is more directly relevant to paraneoplastic disease, and can be accounted for by physical and molecular barriers to systemic immune surveillance. Tight junctions between vascular endothelial cells in the brain serve to selectively exclude large macromolecules and lymphocytes from the brain parenchyma. In addition, there is a paucity of cells in the CNS that express either of the major histocompatibility class (MHC) antigens I or II. Survival of xenogeneic grafts in the brain was found to be dependent on the integrity of the blood-brain-barrier (BBB) (Pollack and Lund, 1990). The authors demonstrated that a chemically induced breach in the BBB not only allowed for infiltration by

circulating lymphocytes, but also increased the number of cells with significant MHC expression (presumably astrocytes and microglia) in the region of the transplant. These findings have important implications for the pathogenesis of PCD in two respects: first, the restricted expression of cdr2 to immune privileged tissues is consistent with the autoimmune hypothesis of PCD as discussed in Chapter 3; and secondly, the factors contributing to the immune privilege of the brain must be altered in PCD patients for the anti-tumor response to become targeted to the nervous system. Secondary events contributing to the onset of cerebellar degeneration in PCD have not been identified, but could include infection, injury or a cytokine-mediated breach of the BBB.

*The cdr2 antigen is ectopically expressed in ovarian tumors*

Since several immunoreactive antigens have been identified with PCD antisera, it is important for understanding the pathogenesis of the tumor and brain immunity to determine the true PCD antigen. RT-PCR analysis was used in combination with Western blotting and immunohistochemistry to definitively identify cdr2 as the PCD tumor antigen. That cdr2 serves as a *bona fide* tumor antigen is evidenced by the robust immune response to cdr2 coupled with the limited tumor growth seen in PCD patients. The cdr2 protein is not expressed in normal ovary, and therefore the antigen is ectopically expressed in this tumor.

The ectopic expression of PND antigens in specific neoplasms suggests that these neuronal proteins could play functional roles in tumorigenesis. Although the exact function of the sensory neuronopathy/encephalomyelitis antigen HuD is unknown, its expression in all small cell lung cancers (Dalmau, et al., 1992) suggests that it may have a function in this tumor. It is possible that the onconeural antigens provide the tumor with a selective growth advantage or contribute to the survival of transformed cells. In cancer-associated retinopathy (CAR) the paraneoplastic antigen is recoverin, a

cytoplasmic calcium binding protein that regulates the activity of guanylate cyclase in photoreceptor cells (Dizhoor, et al., 1991; Polans, et al., 1995). Given its role in the regulation of signal transduction, ectopic expression of recoverin in the small cell lung cancers of CAR patients suggests that the protein could contribute to tumorigenesis. It is now clear that *cdr2* is expressed in a significant number of ovarian tumors, approximately 60% of those assayed to date (J.C. Darnell and M. Albert, unpublished observation). Deregulated expression of *cdr2* in ovarian cancer may represent a general mechanism characteristic of this tumor type, and underscores the relevance of this protein in tumor biology. Further support for this hypothesis comes from the finding that *cdr2* interacts with the oncoprotein c-Myc, which is implicated in both ovarian and breast tumorigenesis.

Taken together, the characteristic and widespread expression of the onconeural antigens in certain tumors suggests that their ectopic expression is necessary, but not sufficient, for the initiation of the anti-tumor immune response seen in PND patients. One possible distinguishing feature of the tumors of PND patients might be the co-expression of MHC I and/or costimulators such as B1. Interestingly, there is evidence for a correlation between tumor MHC I expression and anti-Hu seropositive patients with neuroblastoma and small cell lung cancer (Dalmau et al., 1995). Alternatively, the PND antigens may be presented after release by apoptotic tumor cells in a manner analogous to the model of lupus autoimmunity proposed by Casciola-Rosen et al. (Casciola-Rosen et al., 1994), and the development of tumor immunity may then be dependent on specific features of the host immune system (e.g. MHC haplotype). Another mechanism of antigen presentation involving members of the heat shock protein family (HSP90, HSP70, and gp96) was proposed recently and has been supported by experimental evidence (for review, see Srivastava and Udono, 1994). Cellular peptides complexed with HSP's can be taken up by phagocytic cells and the peptides transferred to MHC I for presentation, thus inducing a cytotoxic lymphocyte (CTL) response (Suto and Srivastava, 1995). This presentation

pathway is intriguing in light of the yeast two-hybrid interaction between *cdr2* and HSP90. Regardless of the mechanism involved, it is clear from the expression of *cdr2* that it is the primary target of the autoimmune response in both the PCD tumor and in the CNS.

#### *Post-transcriptional regulation of cdr2*

The differential distribution of the *cdr2* mRNA and protein in adult tissues indicates that the expression of the onconeural antigen is regulated at a post-transcriptional level. Based on the results of this study, the most likely mechanism responsible for the tissue-specific distribution of the protein is translational control of the *cdr2* mRNA. Several regions of mRNA have been shown to be involved in the regulation of translational efficiency (for review, see Mathews et al., 1996). *Cis*-acting elements present in the untranslated regions (UTR) of the transcript are the most common examples. Since the initiation of translation usually occurs near the 5' m7G cap, the 5' UTR is often the site of these regulatory regions. Analysis of many eukaryotic mRNA's has revealed that a disproportionate number of genes involved in growth control contain regions of stable secondary structure (G-C rich) and the presence of multiple upstream (u)AUG codons in the 5' UTR (Kozak, 1991a). The prototypic mechanism of mRNA-specific translational control is the iron response element (IRE) in the 5' UTR of the ferritin message (for review, see Rouault et al., 1996). Under conditions of low iron, the iron regulatory protein (IRP) binds the IRE stem-loop structure and prevents the association of the 43S ribosomal complex with the cap structure, thereby blocking initiation of translation.

While ferritin protein is produced in response to metabolic signals, some mRNA's are translated in a cell- or tissue-specific manner, suggesting a role for *trans*-acting factors in their regulation. Interaction with such RNA binding proteins may serve to either enhance or repress the active translation of the mRNA. One specific uAUG out of seven in the 5' UTR of the

transcription factor BTEB inhibits translation of the mRNA in HeLa cells, but not in a neuroblastoma cell line (Imataka, et al., 1994). Interestingly, the distribution of BTEB mRNA and protein matches that of *cdr2* in adult tissues. The mRNA is ubiquitously transcribed while the BTEB protein is found only in brain and testis. Other examples of the role of uAUG's in cell- or tissue-specific translational control include the retinoic acid receptor  $\beta_2$  and S-adenosylmethionine decarboxylase mRNA's (for review, see Geballe, 1996). Although the identification of trans-acting factors have not been made for these specific examples, there is indirect evidence for the existence of tissue-specific translational regulatory factors. Translation of a testis-specific Cu/Zn superoxide dismutase (SOD-1) and protamine 1 (Prm-1) are regulated by testis proteins that bind to the 5' UTR and 3' UTR of the mRNA's, respectively (Gu and Hecht, 1996; Lee et al., 1996). A study by Han et al. (Han et al., 1995) identified a brain/testis-specific protein implicated in translational control. These findings indicate that there is precedent for shared mechanisms in the regulation of translation of specific mRNA's in the brain and testis.

Alternative explanations for the discrepancy between the *cdr2* mRNA and protein distributions were considered in Chapter 3. Analysis of the polysome distribution in brain/testis versus non-brain tissue would provide more definitive biochemical evidence of translational control of the *cdr2* mRNA. Estimates based on the *cdr2* Northern blot (Figure 5B) and primer extension analysis (Chingwen Yang, unpublished observation) indicate that approximately 400 bp of 5' UTR sequence of the mouse *cdr2* mRNA remain to be characterized. With the complete transcript available for analysis, *cis*-acting regulatory elements may be identified by using reporter gene constructs in cell lines that either do or do not express endogenous *cdr2* protein.

Since the *cdr2* mRNA is expressed in normal ovary, the ectopic expression of the protein in many ovarian tumors suggests that it is the translational control of *cdr2* that is deregulated in these tumors. Many components of the translational machinery have been shown to be important

in the control of cell growth. Overexpression or deregulation of the mRNA 5' cap binding protein eIF4E results in malignant transformation of rodent fibroblasts (Lazaris-Karatzas et al., 1990; Lazaris-Karatzas and Sonenberg, 1992). However, such factors are involved in the general control of the initiation and rate of translation. The ectopic expression of *cdr2* in tumors may be the first evidence of subverted (for an inhibitory mechanism) or co-opted (for a stimulatory mechanism) mRNA-specific translational control associated with tumorigenesis. In addition to the function of the *cdr2* protein, the regulation of its expression may also be important in tumor biology, perhaps affecting the expression of other genes that provide selected functions in the growth and/or survival of tumors. Given that 60% of ovarian tumors ectopically express *cdr2*, such a regulatory mechanism could be clinically significant as a target of intervention in these and perhaps other tumors.

### **The function of the *cdr2* onconeural antigen**

#### *cdr2 interacts with c-Myc*

Using a genetic approach, the yeast interaction trap, c-Myc was identified as a *cdr2* binding protein. Although this finding is consistent with the similar structural features found in both proteins, their interaction was unexpected for several reasons. First, the expression of the c-myc proto-oncogene is most often associated with actively proliferating cells and is barely detectable in adult brain (Zimmerman, et al., 1986). As a transcription factor, the subcellular localization of c-Myc protein is predominantly nuclear, corresponding with its *in vivo* targets. In seeming contrast to the temporal and spatial expression pattern of c-myc, the *cdr2* protein is expressed in the cytoplasm of post-mitotic neurons. However, the immunohistochemical colocalization of the proteins in the cytoplasm of cerebellar Purkinje neurons, the demonstration of specific binding *in vitro*, and the significant effect of *cdr2* on c-Myc activity support the conclusion that the *cdr2*:c-Myc interaction is relevant *in vivo*.

What is the function of the cdr2:c-Myc complex in Purkinje neurons? It is possible that there is an as yet undefined function for c-Myc in the cytoplasm that requires interaction with cytoplasmic factors such as cdr2. Although this hypothesis cannot be discounted, there is no evidence to date that supports such a role for c-Myc. A more likely scenario is one in which cdr2 may serve to regulate Myc activity by affecting its subcellular distribution. In a manner analogous to I $\kappa$ B and NF $\kappa$ B, association with cdr2 may prevent the translocation of c-Myc into the nucleus. Complex formation with cdr2 may mask the nuclear localization signal (NLS) of c-Myc or serve to anchor c-Myc to distinct cytoplasmic structures. Given the ultrastructural localization of cdr2 immunoreactivity determined by EM studies (Rodriguez, et al., 1988; Tomimoto, et al., 1993), the latter mechanism seems plausible. In addition to the EM findings, both peroxidase labeling and immunofluorescence with anti-Yo antibodies have yielded punctate cytoplasmic staining at the light microscopic level (Rodriguez, et al., 1988). Interestingly, the anti-c-Myc monoclonal antibody used in this study for immunohistochemical analysis yielded a similar punctate pattern of staining in the Purkinje cell cytoplasm (Figure 13), suggesting that the protein may be associated with structural components.

Sequestration of c-Myc in the cytoplasm would be expected to result in the downregulation of its activity as a transcriptional activator/repressor. The observation that exogenous cdr2 can repress c-Myc transcriptional activity in this study supports such a role for cdr2 in the regulation of c-Myc. Several alternative explanations for the repressive effect of cdr2 may be considered. cdr2 may form a stable complex with c-Myc that is unable to bind to DNA or activate transcription. The absence of an identifiable DNA binding domain in the cdr2 amino acid sequence suggests that a cdr2:c-Myc complex may not bind DNA. This can be examined *in vitro* by using an electrophoretic mobility shift assay (EMSA) with a labeled oligonucleotide containing the Myc binding site CACGTG. It is also possible that the interaction of c-Myc with cdr2 may

preclude the formation of Myc:Max heterodimers, a requirement for c-Myc activity. Though the relative affinities of cdr2 and Max for c-Myc appear to be comparable in the *in vitro* binding assay used in this study, a quantitative measure of their respective binding affinities is required to determine if cdr2 could competitively inhibit Myc:Max heterodimerization. Despite the fact that these mechanisms cannot be definitively ruled out, the subcellular localization of cdr2 indicates that it is not likely to regulate c-Myc activity within the nucleus.

#### *c-myc in the nervous system*

Cytoplasmic interaction of c-Myc with cdr2 may represent a Purkinje neuron-specific regulatory mechanism that serves to inhibit the nuclear activity of c-Myc in these post-mitotic cells. One aspect of such down-regulation of c-myc dependent gene transcription could be to provide a protective mechanism against inappropriate mitogenic signaling, and therefore tight control of c-Myc activity may be required in mature neurons. At the same time, by keeping a cytoplasmic pool of c-Myc protein available in the cell, cdr2 might allow for a rapid c-Myc response to external stimuli, similar to the NF $\kappa$ B:I $\kappa$ B signaling mechanism. This raises the question as to whether there is a distinct function for c-myc in post-mitotic neurons.

Studies on the temporal and spatial expression profile of c-myc in the developing nervous system demonstrate that it is clearly uncoupled from cell division in neurons. Hirvonen et al. (Hirvonen et al., 1990) analyzed the expression of myc family member mRNA's in the human fetal brain by Northern blot, RNase protection and *in situ* hybridization. c-, N-, and L-myc were all detected in the periventricular, intermediate, and cortical layers of the developing cerebrum, indicating that expression was maintained through stages of proliferation and mitotic arrest. Similar findings were reported by Ruppert et al. (Ruppert, et al., 1986) concerning the expression of c-myc mRNA in the developing mouse cerebellum. The cerebellum has a highly

regular laminar structure defined by three cellular layers and only five principal neuronal types, and developmental stages corresponding to the proliferation and differentiation of cerebellar neurons have been well characterized. Low levels of c-myc mRNA were detected throughout cerebellar development into adulthood, but there were transient increases in expression associated with the proliferation of the dense granule neuron layer at embryonic (E) day 17 and with the terminal differentiation of the Purkinje neurons at postnatal (P) days 7-10 (Ruppert, et al., 1986). Purkinje neurons are postmitotic by E13, and the postnatal period P7-10 is a time of extensive synaptogenesis for these large neurons with massive dendritic arborizations. Taken together these data suggest that c-myc functions not only in the replicative phase of neuroblasts, but also in the process of neuronal differentiation.

Further support for the role of c-myc in post-mitotic neurons comes from studies using differentiated neural cell lines. Expression of immediate early genes, including c-myc, is induced upon the neuronal differentiation of PC12 pheochromocytoma cells by exposure to nerve growth factor (NGF) (Greenberg et al., 1985; Milbrandt, 1986). The embryonic carcinoma cell line P19 can be differentiated specifically along a neuroectodermal lineage by treatment with retinoic acid. St-Arnaud et al. (St-Arnaud, et al., 1988) found that there were two transient increases in c-myc mRNA and protein corresponding to stages of early commitment and neuronal differentiation in this paradigm. These increases in c-myc expression were found to be specific to neuroectoderm since mesodermal and endodermal differentiation using dimethyl sulfoxide (DMSO) did not result in a similar effect.

Immediate early gene (IEG) expression in both neuronal cell lines and the intact nervous system can be induced by growth factors, electrical stimulation and neurotransmitters (for review, see Sheng and Greenberg, 1990). Early studies that focused primarily on the c-fos proto-oncogene demonstrated a strong activity-dependent induction of expression in neurons. In NGF treated PC12 cells, benzodiazepines and the acetylcholine

receptor agonist nicotine rapidly induce IEG expression (Curran and Morgan, 1985; Greenberg et al., 1986), demonstrating that these genes play an important role in the response of nondividing neuronal cells to external signals. Similarly, c-fos expression can be modulated in the dorsal horn of the spinal cord by sensory stimulation (Hunt et al., 1987), and electrical stimulation of the sensory/motor cortex led to induction of c-fos immunoreactivity in various brain regions involved in sensory and motor signal processing (Sagar et al., 1988). Finally, c-fos expression is upregulated in granule neurons of the dentate gyrus after chemically induced seizures (Morgan et al., 1987). The "kindling" model is a paradigm of neuronal plasticity in which the induction of seizures leads to changes in the brain that result in increased sensitivity to further seizure activity. Persistent increased c-fos expression observed in kindling was an early indication of the functional importance of IEG expression in the plasticity of mature neurons (Dragunow and Robertson, 1987).

Given its observed expression in differentiating neural cell lines and terminally differentiating cerebellar Purkinje cells in vivo, c-myc is likely to have a comparable function to c-fos in post-mitotic neurons. A Purkinje neuron receives thousands of synaptic contacts from its two main sources of afferent inputs, the climbing fibers from the inferior olivary nucleus and the parallel fibers of the cerebellar granule neurons. Axonal projections from Purkinje neurons represent the sole efferent output from the cerebellar cortex, and project to the deep cerebellar nuclei. The involvement of the cerebellum in motor learning and the electrophysiological phenomenon of long term depression exhibited by Purkinje neurons indicates that there is significant plasticity in the cerebellar cortex. Persistent c-myc expression in some adult Purkinje cells (Ruppert, et al., 1986; Takahashi, et al., 1993) suggests that the proto-oncogene may also be important for the normal activity and/or plasticity of these neurons.

An intriguing finding of the immunohistochemical analysis performed in this study is the nonuniform expression of c-Myc in the Purkinje cell layer.

This distribution is consistent with the detection of c-myc mRNA by Ruppert et al. (Ruppert, et al., 1986), in which c-myc expression was found in isolated patches of Purkinje neurons. Although morphologically identical, individual Purkinje neurons display both biochemical and electrophysiological heterogeneity (Wassef et al., 1992). Several known genes display differential expression patterns in the Purkinje cell layer; some define characteristic parasagittal stripes (e.g. zebrin I & II), some are expressed in broad parasagittal bands (e.g. glutamic acid decarboxylase), and others have a more "patchy" pattern of expression (e.g. acetylcholinesterase) (reviewed by Hawkes et al., 1992). Molecular dissection of the pcp-2/L7 Purkinje-specific promoter in lacZ transgenic mice also revealed a parasagittal banding pattern that changed in a developmental manner (Smeyne et al., 1991; Vandaele et al., 1991). The parasagittal bands often correspond to the organization of afferent projections from the inferior olive, but their formation is not dependent on this input (Hawkes, et al., 1992).

Examination of the expression of neurotransmitter synthesizing enzymes and electrophysiological studies have subdivided the cerebellar cortex into microzones of 0.5 to 1mm in length (Bower et al., 1981; Chan-Palay et al., 1981; Chan-Palay et al., 1982; Bower and Woolston, 1983 ). In the study by Sagar et al. (Sagar, et al., 1988), in which c-fos induction was analyzed in the brain after electrical stimulation, nuclear immunoreactivity in the granule cell layer of the cerebellar cortex was clustered in a pattern reminiscent of this microzonal organization. However, reactivity to a fos-related antigen was found in the cytoplasm of Purkinje neurons, in a patchy pattern reminiscent of that found for c-Myc in this study (Sagar, et al., 1988). The cytoplasmic immunoreactivity was only found in the stimulated animals and was abolished upon addition of a c-fos peptide corresponding to the antigenic epitope. While a correlation between c-myc expression and known Purkinje compartments cannot be made, these observations are consistent with the suggestion that c-myc expression in the adult cerebellar cortex is activity-dependent. Cytoplasmic localization of IEG's in Purkinje neurons seems

somewhat paradoxical if the nuclear activity of the proteins is required for response to stimuli, and suggests that there is something unique about the regulation of IEG products in Purkinje neurons.

#### *Regulation of c-Myc in Purkinje neurons*

Why would cerebellar Purkinje neurons require a cell type-specific regulatory mechanism to govern c-Myc activity? That question may best be answered by considering the possible consequences of deregulated Myc activity in post-mitotic neurons. In addition to its well characterized roles in the control of normal cell proliferation, transformation and tumorigenesis, c-myc has been shown to be involved in the process of apoptosis. Under conditions which block normal proliferation in cell culture (e.g. serum starvation), constitutively expressed c-myc is a potent inducer of programmed cell death (Evan et al., 1992). Further studies have demonstrated that this Myc-mediated apoptosis requires dimerization with Max (Amati, et al., 1993b), requires expression of wildtype p53 (Wagner et al., 1994), and can be blocked by overexpression of bcl-2 (Wagner et al., 1993). The seemingly contradictory functions of c-myc in both proliferation and cell death can be reconciled when the expression of c-myc is considered in the context of cell growth conditions. In the presence of other mitogenic signals, c-myc expression induces proliferation and prevents growth arrest. However, when cellular proliferation is blocked or cells have been growth arrested, c-myc overexpression induces programmed cell death (Evan, et al., 1992).

Mature neurons represent a somewhat unique post-mitotic cell type. Unlike quiescent cells in other tissues that can be stimulated to reenter the cell cycle (e.g. liver), fully differentiated neurons appear to be completely refractory to cell cycle reentry (for review, see Heintz, 1993). It follows that these post-mitotic cells would also be unable to be transformed, an hypothesis supported by both clinical and experimental evidence. There are no reports of tumors arising from mature neurons in the medical literature. In a study

aimed at testing this hypothesis directly, Baetscher et al. (Baetscher et al., 1991) used the calcitonin-CGRP promoter to drive oncogene expression in discrete populations of neurons and non-neuronal cells in transgenic mice. Overexpression of the SV40 large T antigen (Tag) caused tumors to arise in the thyroid (containing calcitonin expressing C cells), but not in neurons of the dorsal root ganglia (DRG). The authors also overexpressed c-myc, but did not observe transformation in either tissue. Although this clearly demonstrated a difference in the propensity for transformation of neurons, the authors did not carefully examine tissue for evidence of apoptosis.

Another report of forced oncogene expression in post-mitotic neurons provides insight into Purkinje cell biology specifically. Using the Purkinje-specific L7 promoter, Feddersen et al. (Feddersen et al., 1992) expressed the SV40 Tag in these post-mitotic neurons in the mouse cerebellum. Interestingly, the SV40 Tag expressing Purkinje cells selectively degenerated, and the extent of disrupted cerebellar cortical development was directly related to the transgene copy number. In a follow-up study it was demonstrated that the expression of SV40 Tag led to the induction of DNA synthesis in post-mitotic Purkinje cells, and the observed cell-specific death was characterized by DNA fragmentation, a typical feature of apoptosis (Feddersen et al., 1995). A critical mechanism by which SV40 Tag promotes oncogenesis is by association with and inactivation of the retinoblastoma gene product pRb (DeCaprio et al., 1988), and the pRb binding domain of SV40 Tag was shown to be essential for the Purkinje cell ablation in the transgenic mice (Feddersen, et al., 1995). These results suggest a direct link between cell cycle regulation and neurodegeneration.

A more careful consideration of the SV40 Tag/pRb signaling mechanism implicates c-myc as an important component in the Purkinje cell death pathway. pRb and related proteins complex with E2F, a ubiquitous protein that transactivates genes required for DNA synthesis (Qin et al., 1992). Normal inactivation of pRb by hyperphosphorylation or viral inactivation by binding to SV40 Tag results in free E2F. An E2F binding site in the c-myc

promoter is critical for its efficient expression in response to mitogenic signals (Moberg et al., 1992), and repression of c-myc transcription by pRb is dependent on this E2F site (Hamel et al., 1992). The importance of this signaling pathway in neurons was illustrated by analysis of pRb mutant mice. The targeted disruption of the Rb gene in mice leads to embryonic lethality with increased free E2F, aberrant S phase entry and p53-dependent apoptosis in the CNS (Jacks et al., 1992; Macleod et al., 1996). In addition to effecting the transcriptional control of the c-myc gene, it is believed that the pRb-related p107 may directly interact with the c-Myc protein and negatively regulate its activity (Beijersbergen et al., 1994). Therefore, the S phase initiation and subsequent apoptotic death of post-mitotic Purkinje neurons in the SV40 Tag mice is likely to involve modulated c-myc activity.

In conclusion, c-myc is normally expressed in some post-mitotic Purkinje neurons, and there is an apparent need for tight regulation of Myc activity in these cells. Taken together, the results of this study suggest that the onconeural antigen cdr2 may function in such a regulatory capacity. cdr2 and c-Myc interact both in the yeast two-hybrid system and *in vitro*, and the proteins are colocalized in the Purkinje neuron cytoplasm. In addition, cdr2 down-regulates the transcriptional activity of c-Myc from an E-box promoter. Moreover, disruption of the cdr2:c-Myc cytoplasmic complex *in vivo* could result in deregulated Myc activity, leading to inappropriate cell cycle entry and apoptosis (Figure 18). One approach to testing this hypothesis is to generate a dominant negative form of cdr2, perhaps the isolated coiled-coil leucine zipper domain. In the absence of other functionally important domains, the truncated cdr2 could compete with the full length protein for interactions with c-Myc (and possibly the endogenous cdr2 itself). In this way the sequestration of c-Myc in the cytoplasm could be blocked, allowing the protein to translocate to the nucleus and activate its target genes. Such a dominant negative expression construct could first be assayed in the transient transfection E-box assay for an effect on the full length cdr2-mediated repression. If it is effective in blocking the functional interaction of cdr2 and

c-Myc in cell lines, then the truncated cdr2 could be overexpressed in post-mitotic Purkinje neurons in transgenic mice using the pcp-2/L7 promoter.

### *Implications for disease*

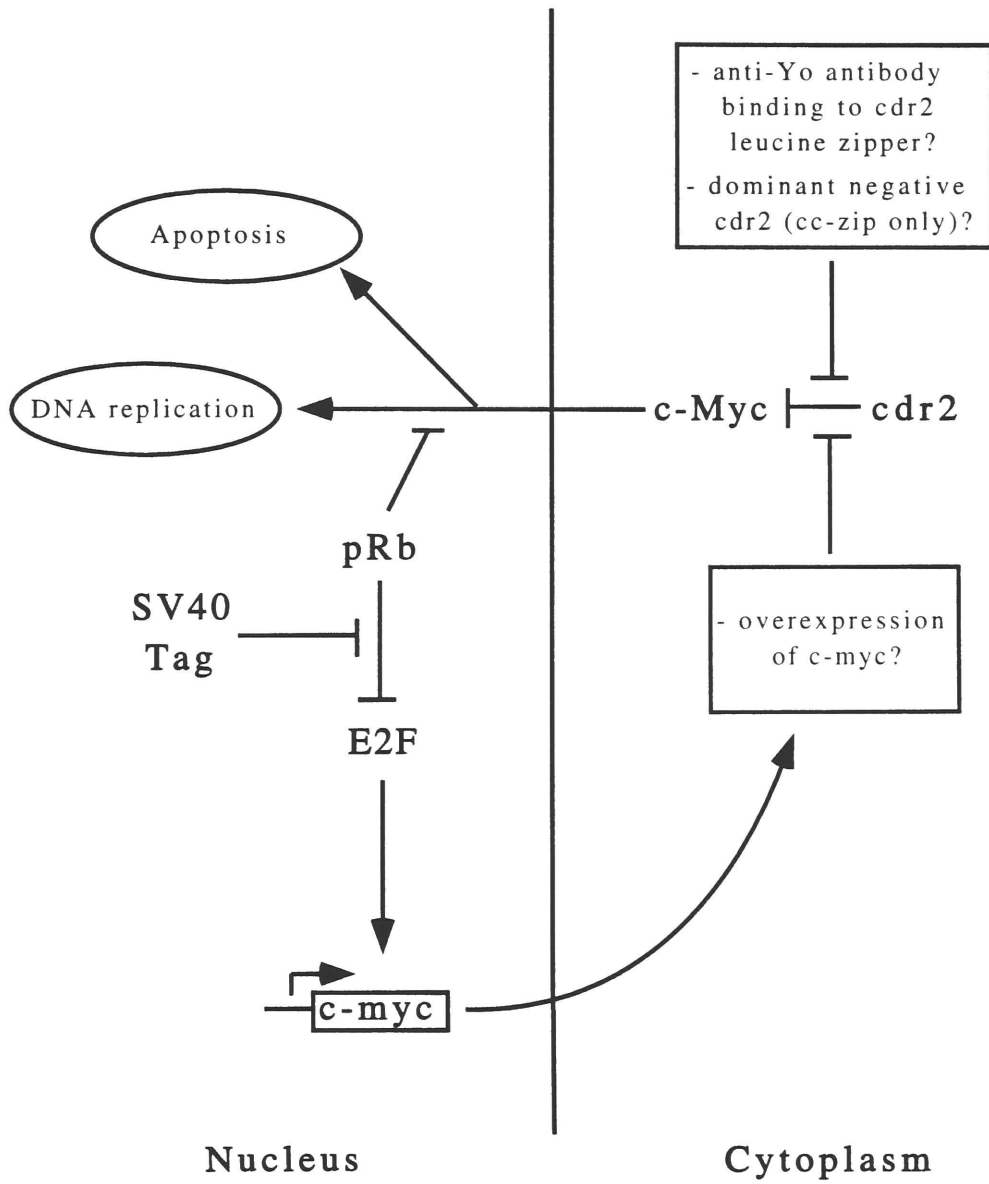
The precise nature of the autoimmune neurodegeneration in PCD is not understood. However, the cdr2 expression data presented in this thesis suggest that the PCD tumor antigen is also the target of the immune-mediated pathology in the nervous system. As is characteristic of many autoantibodies (Tan, 1991), the anti-Yo antibody recognizes the leucine zipper region of cdr2, which represents an important functional domain of cdr2 that may normally be buried within protein complexes. Paraneoplastic autoantibodies typically target functional regions of their corresponding antigens. The anti-Ri antibody, which recognizes the RNA-binding KH domains of the Nova-1 protein, effectively inhibits the binding of Nova-1 to RNA *in vitro* (Buckanovich, et al., 1995), suggesting that the antibody might disrupt functional complexes *in vivo*. Since the epitope of the anti-Yo antibody is, it is tempting to consider that the damage to Purkinje neurons in PCD may be antibody-mediated. Deregulation of c-Myc activity in Purkinje neurons may occur if the anti-Yo autoantibody is able to disrupt the formation of cdr2:c-Myc dimers, allowing c-Myc to enter the nucleus (Figure 18). The ability of the antibody to competitively inhibit the cdr2:c-Myc interaction can be assessed *in vitro* using the GST pull-down assay (as described in Chapter 4) and affinity purified anti-Yo antibody.

Although traditional immunological dogma leaves little room for such an antibody-mediated mechanism being responsible for autoimmune degeneration, there is accumulating evidence that autoantibodies can and do penetrate living cells (Alarcon-Segovia et al., 1996). In fact, paraneoplastic autoantibodies have been shown to penetrate cells both in culture and in the intact nervous system. When added to culture media, the paraneoplastic anti-Hu antibody was detected in the nucleus of cell lines expressing the Hu

nuclear antigen (Hormigo and Lieberman, 1994). In attempts to reproduce PCD in mice, animals were either passively injected with the anti-Yo antibody or actively immunized against a recombinant cdr2 protein (Tanaka et al., 1994; Sakai, et al., 1995). Both cdr2 studies found deposition of IgG in Purkinje neurons only in experimental animals, although the integrity of these results has been questioned (Smitt et al., 1995). However, neither the anti-Hu nor the anti-Yo studies revealed any deleterious effects of autoantibody uptake. An animal model that more closely reflects the complexity of the human syndrome is warranted to gain further insight into the pathogenesis of PCD. For example, the cdr2 antigen could be ectopically expressed in mammary tumors of transgenic mice carrying MMTV-driven oncogenes (Stewart, et al., 1984; Muller et al., 1988).

PCD is associated almost exclusively with breast and ovarian cancers, tumors that often display deregulated c-myc expression. Although tumors from PCD patients have not been analyzed for c-myc expression, the expression of cdr2 in ovarian tumors from neurologically normal patients suggests that its interaction with c-Myc may be generally relevant to tumor biology. Given the cytoplasmic localization of the cdr2 protein and the functional data presented in this thesis, expression of cdr2 might be expected to have an anti-tumorigenic effect. However, current hypotheses on the multistep process of tumorigenesis hold that the growth promoting effects of oncogenes (e.g. c-myc) must be carefully balanced with the suppression of oncogene-induced apoptosis (Canman and Kastan, 1995). In fact, several reports of cytoplasmic c-Myc immunoreactivity in ovary, breast, and uterine carcinomas (Ambros, 1992; Pavelic et al., 1992; Sasano et al., 1992) indicate that this subcellular distribution is found in malignant tissues. Whether the cdr2 and c-Myc proteins colocalize in tumor cells remains to be determined, but the interaction of these proteins is intriguing in light of their deregulated expression in similar tumors.

**Figure 18.** Model for the regulation of c-Myc in postmitotic Purkinje neurons. Constitutive expression of the retinoblastoma gene product, pRb, serves to inhibit the activity of the E2F transcription factor by direct association. If the complex is dissociated by phosphorylation of pRb or by binding of the SV40 large T antigen (Tag), E2F is free to activate many growth promoting genes, including c-myc. In the cytoplasm, c-Myc protein may be prevented from nuclear translocation by association with cdr2, thus downregulating c-Myc transcriptional activity. Disruption of the cdr2:c-Myc complex, perhaps by binding of the anti-Yo antibody to the cdr2 leucine zipper domain, could result in translocation of c-Myc into the nucleus and deregulated c-Myc activity. The consequences of this deregulation are predicted by studies in which pRb has been inactivated in Purkinje neurons. Specifically, Purkinje neurons undergo inappropriate entry into S phase and subsequent programmed cell death (Feddersen, et al., 1995). The cdr2:c-Myc complex could also be competitively inhibited by overexpression of c-myc or by a dominant negative cdr2 protein, containing only the coiled coil leucine zipper dimerization domain.



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