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PRINCIPAL INVESTIGATOR: Robert B. Darnell, M.D., Ph.D.

CONTRACTING ORGANIZATION: Rockefeller University
New York, New York 10021-6399

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Robert B. Darnell, M.D., Ph.D.

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Paraneoplastic neurologic disease (PND) antigens are proteins normally expressed specifically in neurons that are expressed ectopically in tumors. It is believed that when expressed in tumors outside of the immunologically privileged nervous system, PND antigens stimulate the development of anti-tumor immunity, but also stimulate an autoimmune neuronal degeneration. We are studying PND's associated with breast cancer and immunity to the Nova and cdr2 antigens, assaying PND expression in clinical breast tumor specimens, and establishing animal models for the disorders. A prerequisite to studying the abnormal expression of PND antigens is an understanding of their normal expression. We have found that the Nova gene and antigen is normally restricted in expression to neurons. Strikingly, we have discovered that the cdr2 gene is widely expressed, but that its mRNA is under tight translational control, so that the cdr2 antigen is normally made only in neurons and (immune privileged) testes. This observation provides a novel insight to the biology of breast tumors, suggesting that they may dysregulate translational control to express abnormal proteins, and alters our approach to the analysis of cdr2 expression *in vivo*. We have also made significant progress in generating gene constructs to generate mouse models of PND.

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INTRODUCTION:

Paraneoplastic neurologic disease (PND) antigens are proteins normally expressed specifically in the central nervous system (CNS) that are expressed ectopically in tumors, most commonly breast, ovarian and lung cancers (Posner and Furneaux, 1990), and we have termed such proteins onconeural antigens (Darnell et al., 1991; Darnell, 1994; Darnell, 1996). It is believed that their expression in tumors outside of the immunologically privileged CNS allows for their recognition by the immune system, and consequently a clinically effective anti-tumor immune response (Posner and Furneaux, 1990; Darnell, 1996). Tumor suppression is a significant factor in the majority of PND patients (Darnell and DeAngelis, 1993; Darnell, 1994; Darnell, 1996).

Studies of the expression and function of onconeural antigens in tumors and brain are lacking, as are animal models of their role in tumor biology and immunology. The purpose of the present work is to expand our understanding of the expression of onconeural antigens, and to develop an animal model for these syndromes. These studies are necessary to test and refine our model of the pathogenesis of these disorders. PND's provide examples of *bona fide* anti-breast tumor immunity in humans, and our hope is that the studies underway in this project will enable a detailed analysis of the mechanisms of successful anti-tumor immunity.

The methods of study in this project depend on the use of cloned genes (Sakai et al., 1990; Fathallah-Shaykh et al., 1991; Buckanovich et al., 1993) encoding the PND breast tumor antigens Nova and cdr2. The first specific aim is to assay clinical tumor specimens for the expression of PND genes and the presence of PND antigens. In some cases, it will also be appropriate to assay for co-factors that might be associated with the immune recognition of PND antigen (i.e. if we cannot establish a direct relationship between PND antigen expression and anti-tumor immunity). The second and third aims are complementary approaches aimed at establishing animal models for the breast tumor associated PND's, using breast tumor cell lines transfected with PND genes and transgenic mice making breast tumors expressing PND genes, respectively.

BODY:

Characterization of the Expression of PND Genes:

One test of the model presented for the pathogenesis of the PND's is that the expression of onconeural antigens is normally restricted to neurons, allowing them to be recognized by the immune system as "foreign" antigens when ectopically expressed in breast tumors. We have focused on the "Yo" antigen, which is expressed in breast and ovarian tumors. This antigen is normally expressed in Purkinje neurons and is targeted in patients with paraneoplastic cerebellar degeneration (PCD). Recently an additional neuronal gene termed cdr3, highly related to the cdr2 gene thought to encode the Yo tumor antigen, has been cloned (Posner et al., personal communication, unpublished data). To establish which cdr gene, if either, is expressed and thereby able to encode the 52kD Yo breast and ovarian tumor antigen, we obtained three clinical tumor specimens from patients with PCD. From one patient, we were also able to obtain a section of cerebellum (courtesy of Dr. J. Dalmau) to confirm that the patient had typical PCD. We performed immunohistochemical staining of a section of brain from patient 3 and a normal control using PCD antiserum. There is no cellular neuronal staining in the section of brain from the PCD, and all of the Purkinje neurons are absent (presumably destroyed), confirming that this patient suffered from typical immune-mediated PCD Corradi et al., 1997. RNA was extracted from tumor samples from this and two other patients and the expression of cdr2 and cdr3 was evaluated by RT-PCR analysis. We found that each tumor expressed readily detectable cdr2 mRNA, but no detectable cdr3 mRNA Corradi, et al., 1997. As a control, brain RNA assayed for expression of cdr2 and cdr3 mRNA was found to express readily detectable levels of both.

In addition to confirming that cdr2 is likely to be the relevant gene encoding the *bona fide* breast and ovarian "Yo" tumor antigen, we have pursued our studies of cdr2 mRNA expression. Surprisingly, we have found that cdr2 mRNA is abundant in all tissues, as assessed by Northern blot and RT-PCR analysis Corradi, et al., 1997. Moreover, sequencing of full length cDNA clones from brain and spleen revealed no changes in the sequence of the mRNA in either tissue. We also have shown that antibody to cdr2 only recognizes protein in brain and testis, as assessed by Western blot analysis (Figure 3A in 1995 Report).

To confirm and extend these results, we have now completed immunohistochemical and *in situ* hybridization analysis of cdr2 protein and mRNA expression Corradi, et al., 1997. These studies demonstrate that the cdr2 antigen is indeed expressed in a tissue-specific manner. Within the nervous system, both cdr2 protein and mRNA are found within discrete sets of neurons (primarily Purkinje cerebellar neurons, a few brainstem nuclei, and dorsal root ganglia). Outside of the nervous system cdr2 mRNA is widely expressed, although, surprisingly, with a high degree of tissue specificity within some tissues (e.g. splenic cortex, testicular spermatogoni). However, immunohistochemical analysis of Yo antigen expression revealed no expression outside of the nervous system except within the spermatogonia of the testis Corradi, et al., 1997. Notably, normal breast tissue or ovarian tissue did not express cdr2 antigen, while tumor tissue does (Darnell, 1996; Corradi, et al., 1997). We also note in this context that testis is normally the major immunologically isolated site outside of the nervous system.

The observation that cdr2 antigen expression is tissue specific is consistent with the current model of PND pathogenesis, and is particularly significant for the current project. It indicates that the study of cdr2 mRNA in breast and ovarian tumor tissues, as originally proposed, is not the appropriate level of analysis. Rather, an analysis of cdr2 antigen expression, as assayed by Western blot and immunohistochemistry, will be the relevant focus of study for this antigen. We have now pursued this observation by examining expression of the cdr2 antigen from non-PND human tumor samples. We have found that ~60% of all human ovarian tumors appear to express the cdr2 antigen by Western blot analysis (N=16; Figure 1). To determine whether the immunoreactive band co-migrating on one dimensional SDS-PAGE in tumor and brain is

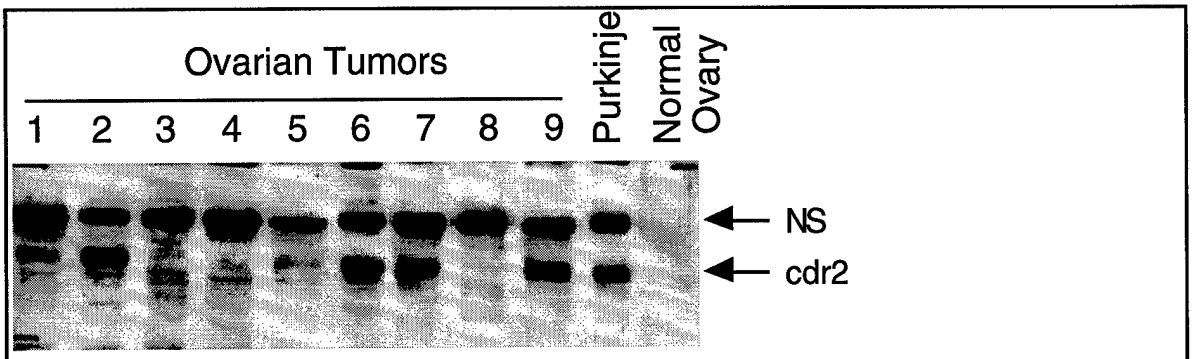

Figure 1. Western blot analysis of cdr2 expression in human ovarian tumors. Protein extracts from 9 human ovarian tumors were run on Western blots and probed with biotinylated affinity purified PCD antibody. Strong cdr2 reactivity was evident in tumors 1, 2, 6, 7 and 9, as well as in extracts of human Purkinje neurons; there was no reactivity with a protein extract of normal human ovary. Reprobing the blot with an anti-tubulin antibody showed equal immunoreactive protein in each lane (including normal ovary; data not shown). A non-specific (NS) band was present in Purkinje extracts and ovarian tumors that reacted with streptavidin-peroxidase secondary alone (data not shown).

actually the same protein, we have also examined cdr2 expression by two-dimensional IEF/SDS-PAGE analysis. We find that brain, testis, and ovarian tumors and HeLa cells all express identically migrating PCD antigen reactive with affinity purified Yo antisera (Figure 2). These data indicate that the majority of human ovarian tumors express the cdr2 tumor antigen. We have also begun to look at human non-PND breast tumors, and find in a small sample that 25% of these tumors express cdr2 (N=4; data not shown).

Preliminary work indicated that the expression of the Nova antigen was normally restricted to neurons using PND antisera to assay protein expression (Buckanovich, et al., 1993) However, since we have found evidence for a second Nova gene family member, Nova-2 (Darnell et al., unpublished data), we have pursued this observation at the level of expression of the Nova-1 gene, using a gene specific probe for *in situ* hybridization. These studies (Buckanovich et al., 1996) confirm that the Nova-1 gene and antigen are restricted to neurons at all points during the normal development of mice, and support the model for the pathogenesis of the PNDs outlined in the introduction. Expression of the Nova-2 gene also seems restricted to neurons, with the possible exception of an unidentified population of cells within the lung.

To determine the specific expression pattern of Nova-2, and to determine which of the Nova genes encodes the paraneoplastic opsoclonus-myoclonus ataxia (POMA) breast tumor antigen, we have used the Nova-2 cDNA to predict a unique peptide fragment. Antibodies generated to this peptide have been made, and these discriminate completely between Nova-1 and Nova-2 fusion proteins. This antisera also works to recognize the neuronal Nova-2 antigen. We are currently using this antisera to probe a tumor block obtained from a POMA patient and to probe mouse tissues.

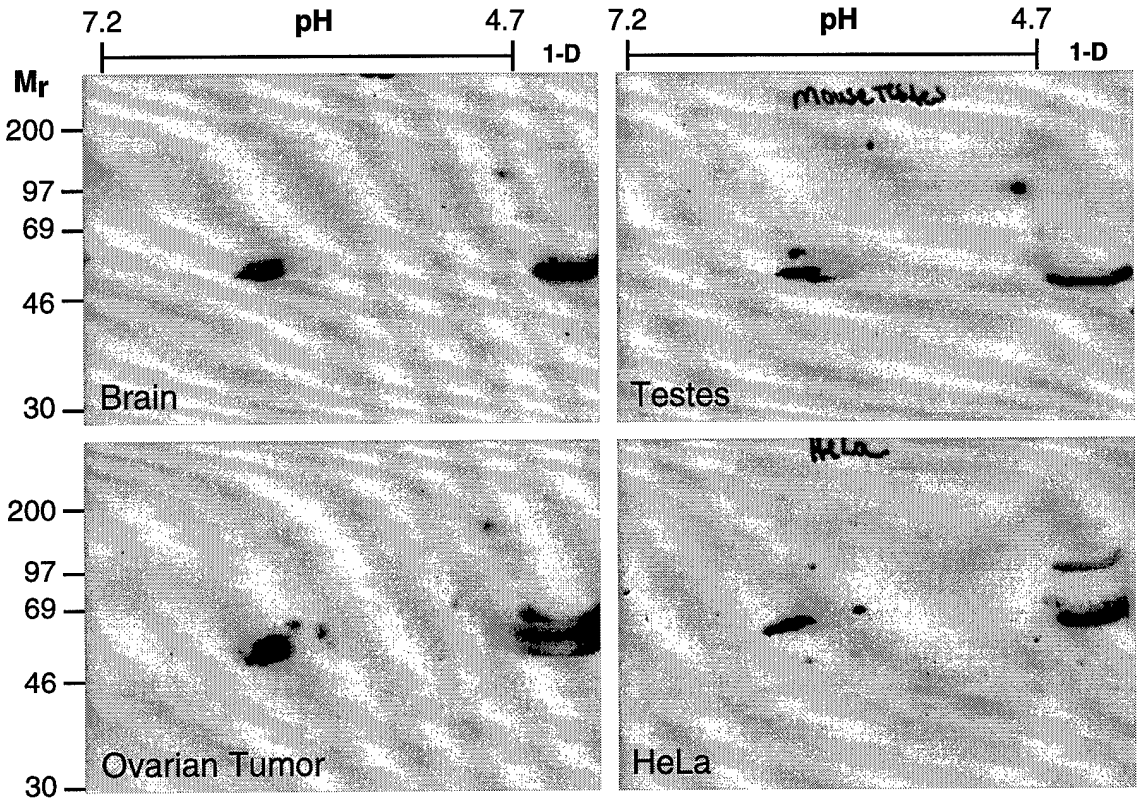

Figure 2. IEF/SDS-PAGE analysis of cdr2 expression. An immunoreacti band of identical Mr and pI is present in each indicated tissue. Extraneous cross reactive ban seen in standard SDS gels (1-D, as indicated) using HeLa or ovarian tumor extracts do n resolve on IEF. Identical results were obtained with human Purkinje cell extracts and affini purified cdr2 antibody (data not shown).

Animal Models of Breast Tumor Immunity

We have proposed to establish a model for anti-breast tumor immunity in mice using a breast tumor cell line (RAC) transfected with the PND breast tumor antigens Nova and cdr2. For reasons that we do not understand, we have not been able to maintain stable RAC cell lines expressing cdr2. In several independent experiments, cells transfected with cdr2 expression constructs and selectable markers (pcDNA3 or pMSG) were selected for 3-4 weeks in the presence of either G418 or gpt selection medium. In every case, drug resistant clones were obtained, but in no case did they express cdr2 protein (assayed by Western blot analysis). This contrasts with our background data (presented in Figure 5, 1995 Report) demonstrating that these constructs do express high levels of cdr2 in transient transfection assays. We are currently pursuing these observations by analyzing whether cdr2 expression might cause functional changes in these cells that interfere with cell growth. One such function would be impingement on cell cycle progression or viability.

A new approach to overcome the problem with RAC cells is to use EL4 (B-cell lymphoma) cells and SVB6 transformed cells, which are mouse cell lines of H-2D^b and H-2D^d haplotype, respectively. These cells generate tumors when injected into C57B/6 or Balb/c mice, respectively. Thus if we are able to generate stable cdr2 expressing cell lines from either or both of these tissue culture cells, we will have a viable alternative approach to establishing an animal model.

We have also proposed using currently available breast tumor transgenic mice (MMTV-wnt, neu and myc) to test the role of PND antigens in anti-tumor immunity. To adequately control these studies, we planned to adoptively transfer transgenic breast tumors that either do or do not express PND antigens into naive syngeneic mice. The background work to establish such experiments includes an analysis of transgenic breast tumors for endogenous PND antigen expression, followed by construction of MMTV-driven PND expression vectors. To date, we have obtained MMTV-myc and MMTV-wnt breast tumor tissue (from Charles River and H. Varmus, respectively) for analysis of PND expression. We have analyzed these tumors for Nova antigen expression, and find no evidence that they express this antigen (data not shown). Initial studies indicated that each tumor type expressed cdr2 mRNA (data not shown); however, given our results indicating that cdr2 is under tight translational control, we also evaluated cdr2 antigen expression in these tumors by Western blot analysis. We found that the myc, and possibly wnt tumors, appear to express cdr2 antigen by one-dimensional SDS-PAGE analysis. While this may be consistent with our observation that up to 60% of non-PND tumors express cdr2, it would also complicate our animal model studies. We are thus currently re-analyzing the putative cdr2 expression in these tumors using the 2D-SDS-PAGE analysis we have established.

Studies in the immunology laboratory of Dr. Ralph Steinman at the Rockefeller University have recently indicated that cellular apoptosis may lead to the effective presentation of intracellular antigen to dendritic cells (unpublished data). We now plan to pursue this observation in our animal models by taking stable cdr2 expressing cell lines, apoptosing them (by UV irradiation), and using them as adjuvants to stimulate T cell immunity in animals. We will then assay for anti-tumor immunity by implanting tumor producing cdr2-expressing cell lines at distant sites as planned.

CONCLUSIONS:

The completed research demonstrates that the Nova and cdr2 PND antigens are both normally expressed in immune-privileged sites Buckanovich, et al., 1996; Corradi, et al., 1997. This supports our general model for the pathogenesis of the breast tumor immunity seen in PND patients. That is, the immune system appears to recognize these antigens in the context of breast tumor cells as foreign antigens; their normal restriction to neurons (and testis for cdr2) sequesters them from immune surveillance and prevents their recognition as self antigens. Strikingly, we have found that the mechanisms for sequestering Nova and cdr2 antigens to neurons are very different. Nova mRNA and protein are found to be sequestered to neurons at all developmental times. In contrast, cdr2 mRNA is widely expressed in various tissues; within the nervous system its expression is restricted to Purkinje cerebellar neurons, brainstem neurons and dorsal root ganglia, but outside the nervous system many tissues express the gene. Tight restriction of cdr2 antigen expression to neurons and testis is demonstrated by Western blot and immunohistochemical analysis, leading to the conclusion that post-transcriptional (likely translational) regulatory mechanisms restrict the expression of cdr2 to immune-privileged sites.

These studies suggest that the cdr2 gene is under strict regulatory control. Our findings are reminiscent of observations of neuron-specific gene transcription mediated by transcriptional silencers outside of the nervous system (Chong et al., 1995). Moreover, a direct precedent for our findings can be found in studies of the expression of the BTEB transcription factor. BTEB mRNA is made in many tissues but, due to 5'-UTR translational control elements, the protein is made only in brain and testis (Imataka et al., 1994).

Our observations have important implications for the role of cdr2 in breast and ovarian tumor biology, suggesting for example that tumors that ectopically express cdr2 antigen have dysregulated translational control mechanisms. Such dysregulation has not been previously observed in breast tumors, and suggests a possible target for therapeutic intervention. Moreover, these observations suggest a means for identifying this control mechanism. We

have begun a systematic analysis of cdr2 translational control, work that should bear on the mechanism of its induction in breast tumors.

By substantiating the observation that the cdr2 antigen is expressed *de novo* in a significant number of ovarian and perhaps breast tumors, we have accomplished several of the goals of this work. First, we have established the identification of these antigens as bona fide tumor markers. Second, by expanding their utility from markers in rare patients with PND to markers in many breast and ovarian tumor patients, we will have greatly broadened the practical significance of this observation.

Most importantly, we are now interested in exploring the significance of this result to anti-tumor immunity. Specifically, it is known that large numbers (perhaps all) small cell lung cancers (SCLCa) express the Hu onconeural antigen, and that up to 15% of SCLCa harbor low titer antibodies to the Hu antigen. Remarkably, this subset of SCLCa patients also have limited disease (Dalmau et al., 1990), as well as an associated increased complete response to therapy and improved survival (Graus et al., in press). By analogy, then, our observation that large numbers of ovarian and perhaps breast tumor patients express the cdr2 and perhaps Nova onconeural antigens predicts a simple way to identify patients with inherent anti-tumor immunity without neurologic disease. We hypothesize that some of the patients (perhaps 15%) whose tumors express these onconeural antigens will have evidence of active anti-tumor immunity--limited disease, complete response to therapy, and/or improved survival. To establish support for this hypothesis, however, we will need to generate a sensitive assay for anti-cdr2 or anti-Nova low level immunity. This would be established using the recombinant onconeural fusion proteins. One complication that would need to be addressed in setting up such a study is the insolubility of the cdr2 fusion protein. One straightforward solution to this problem would be to express the protein in baculovirus, a project currently underway.

METHODS

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 sections 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 poly A+ RNA, the section was homogenized by sonication in 400µl extraction buffer (4M guanidinium thiocyanate, 0.1M Tris-HCl pH 8.0, 1% dithiothreitol, 0.5% lauryl sarcosinate), 800µl of binding buffer (0.1M Tris-HCl pH 8.0, 0.4M LiCl, 20mM EDTA) was added and the lysate was centrifuged at 18,000 x g for five minutes. Magnetic Dynabeads Oligo $(dT)_{25}$ (Dynal, 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 0.5 ml 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., Norfolk, CT) and the following forward and reverse oligonucleotide primers corresponding to cdr2: 5'-TGAATGGAGTTGAGA AGCTGGTG-3' and 5'-GAGATGCCCCTCTGTTTCAC AG-3';

and cdr3: 5'-CATTGAGCGCCTCCAGGCT-3' and 5'-AGCTCCTTGAGG CAGGGGAA-3'. The product was 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 α^{32}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.

Two-dimensional gel electrophoresis. Cerebella and testes 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 focusing (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_3PO_4 and 0.02M NaOH buffers as described (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.

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