Cloning of a brain protein identified by autoantibodies from a patient with paraneoplastic cerebellar degeneration* 
(paraneoplastic syndrome/cDNA expression libraries/autoimmunity)

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ABSTRACT Autoantibodies directed against neuronal proteins have been identified in some patients with paraneoplastic cerebellar degeneration. To identify the molecular targets for these autoantibodies, we constructed a Agt11 cDNA expression library from human cerebellum and screened the library with IgG from a patient with paraneoplastic cerebellar degeneration. A single clone, pCDR2, produced a fusion protein that reacted strongly with the patient's IgG. The isolated pCDR2 clone was used to identify six overlapping cDNA clones. Sequencing of the pCDR2 clones revealed a distinctive pattern consisting of a unit of 18 nucleotides (6 amino acids) repeated in tandem along the entire cDNA sequence. This sequence is unlike any previously described eukaryotic gene. Southern blot analysis was consistent with single-copy representation of the CDR (cerebellar degeneration-related) gene in the human and mouse genome. RNA blotting studies with normal tissues showed expression of the CDR gene to be largely restricted to brain. Expression of the CDR message was also noted in cell lines derived from cancers of neuroectodermal, kidney, and lung origin.

Several distinctive neurologic disorders of unknown etiology occur as “paraneoplastic syndromes” or “remote effects” of systemic cancer (1). The syndrome of paraneoplastic cerebellar degeneration (PCD) is among the most common of these disorders and generally occurs in patients with neoplasms of the lung (especially small cell carcinoma), breast, ovary, or with Hodgkin disease (1, 2). Neuropathologic features of PCD include extensive loss of Purkinje cells, degenerative changes in the remaining Purkinje cells, variable loss of granule and basket neurons, proliferation of Bergmann glial cells, and a variable degree of perivascular and meningeal infiltration by lymphocytes and other mononuclear cells. The presence of anti-Purkinje cell antibodies in some PCD patients suggests an autoimmune etiology (3–6). Immunofluorescent staining of Purkinje cell cytoplasm has been reported in cases of PCD associated with Hodgkin disease (3), small cell lung cancer (4), breast cancer (5), and ovarian cancer (5, 6). Several investigators have attempted to characterize the putative neuronal antigens reacting with anti-Purkinje cell antibodies. Immunoblots with sera from PCD patients have identified several brain proteins of various molecular weights (7–10), but the identity of these “target” proteins and their role in the pathogenesis of PCD remain unknown. Direct efforts at purifying the target proteins have been hampered by their restricted cellular distribution and relatively low abundance. We have used the autoantibodies from a patient with PCD to identify and clone a gene from a human cerebellum cDNA library. The gene is expressed predominantly in neuroectodermal tissues and encodes a protein with a distinctive tandemly repeating structure.

MATERIALS AND METHODS

Human and Mouse Tissue. Normal human cerebellar cortex, cerebral cortex, and other tissues were obtained at autopsy. BALB/c mice were from our breeding colony. Tumor cell lines were from the Sloan–Kettering Institute collection.

DNA and RNA Preparations. High molecular weight cellular DNA and total cellular RNA were prepared as described (11). Poly(A)+ RNA was isolated by oligo(dT)-cellulose column chromatography (12).

Construction of Agt11 cDNA Library. A Agt11 cDNA library was constructed from human cerebellar poly(A)+ RNA according to Huynh et al. (13). cDNA species >300 base pairs (bp) were size-selected by 5% polyacrylamide gel electrophoresis and ligated to EcoRI-digested dephosphorylated Agt11 arms in a molar ratio of 2:1 (13, 14). The ligated DNA was packaged using a high-efficiency in vitro packaging extract (Vector Cloning Systems, San Diego, CA). Approximately 2.3 × 105 plaques were obtained, with 70% being recombinants. An amplified stock of the library was prepared in Escherichia coli Y1088 (12) and used for antibody screening.

Antibody Screening of Agt11 Library. Serum used for screening was obtained from a patient with adenocarcinoma of unknown origin, PCD, and high-titer anti-Purkinje cell antibodies. IgG was isolated from the serum by ammonium sulfate precipitation and DEAE-Sephadex chromatography (15). Recombinant phage were screened at a density of 8000 plaque-forming units per 85-mm plate of E. coli Y1090. The plates were incubated at 42°C for 4 hr, overlaid with nitrocellulose filters (Millipore) saturated with isopropyl β-d-thiogalactopyranoside (IPTG), and incubated 3 hr at 37°C. Filters were washed in Tris/HCl/NaCl/Tween 20 and incubated at 4°C overnight with the primary antibody solution containing 2% bovine serum albumin and preabsorbed with a lysate of E. coli. Incubation at room temperature for 4 hr with horseradish peroxidase-conjugated goat anti-human IgG (Bio-Rad) was followed by staining with chloronaphthol (Bio-Rad). Clones yielding positive signals were purified by several rounds of antibody screening until 100% of the plaques gave positive signals.

Preparation and Immunoblotting of Agt11 Fusion Proteins. Lysogens of recombinant phage were prepared in E. coli Y1089 (13) at a multiplicity of infection of 5. Cultures were grown at 32°C to an OD560 of 0.5, followed by induction of lytic growth at 44°C for 30 min, addition of IPTG to a concentration of 10 mM, and incubation at 37°C for 2 hr.

Abbreviations: PCD, paraneoplastic cerebellar degeneration; IPTG, isopropyl β-d-thiogalactopyranoside; CDR, cerebellar degeneration-related.

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Bacterial pellets were lysed in cold Tris-HCl/EDTA/lysozyme buffer (16), sonicated, and centrifuged. The supernatants were diluted in NaDodSO4/polyacrylamide gel sample buffer containing 5% 2-mercaptoethanol, electrophoresed in 7% NaDodSO4/polyacrylamide gel (17), and electrophoretically transferred to nitrocellulose (18). Blots were analyzed with the patient’s serum as described above for antibody screening.

**Plaque Hybridization.** Nitrocellulose filters were overlaid on 85-mm phage plates for 4 min, washed at room temperature for 4 min each in 1 M NaCl/0.5 M NaOH, 2 M NaCl/1 M Tris-HCl, pH 6.8, and 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate), then vacuum-dried at 80°C for 2 hr. Filters were hybridized and washed as described for Southern blot analysis (see below), except that dextran sulfate was omitted from the hybridization solution.

**DNA Sequencing and Analysis.** cDNA clones were subcloned into M13mp18 and M13mp19, sequenced by the dideoxy chain-termination method (19), and analyzed with computer programs from BIONET.

**RNA Blot and Cytoplasmic Dot Blot.** RNA blot and RNA dot blot analyses were done as described (20) using 32P-labeled nick-translated probes. For cytoplasmic dot blot analysis, cultured tumor cells were washed with phosphate-buffered saline, lysed in Tris-HCl/EDTA/0.5% Nonidet P-40 containing 0.1 mM vanadyl ribonucleoside complex (Bethesda Research Laboratories), and centrifuged (21). The supernatants were transferred to an equal volume of 12× SSC and 15% formaldehyde, bound to nitrocellulose paper in a Minifold apparatus (Schleicher & Schuell), and analyzed with nick-translated probes.

**Southern Blot Analysis.** High molecular weight genomic DNA (15 μg) was digested with restriction endonucleases, electrophoresed in 0.7% agarose gels, and transferred to nitrocellulose (22). Filters were prehybridized and hybridized to nick-translated probes at 65°C for 15 hr. Hybridization was performed in 5x SSC/4x Denhardt’s solution (1x Denhardt’s solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/50 mM phosphate buffer/50 μg of salmon sperm DNA per ml/10% (vol/vol) dextran sulfate. Filters were then washed and autoradiographed.

**RESULTS**

**Isolation of Positive Clones.** Initial antibody screening of 110,000 λgt11 recombinants from the human cerebellar cDNA library by the PCD patient’s IgG yielded a single reproducibly positive clone designated pCDR2. To confirm reactivity with antigenic determinants encoded by the cDNA fusion protein, pCDR2 and wild-type λgt11 were used to infect lysogenic strain Y1089. Bacterial lysates were prepared after induction of the phage β-galactosidase gene by IPTG, and analyzed by NaDodSO4/PAGE (Fig. 1). The 116-kDa β-galactosidase present in the wild-type culture was replaced by a 140-kDa fusion protein in the pCDR2 culture. Immunoblotting revealed that this fusion protein reacted specifically with the patient’s IgG. No reaction was seen with IgG from normal individuals.

The pCDR2 clone was found to contain a 320-bp cDNA insert, which was then used as a probe to rescreen the cerebellar cDNA library. Six positive overlapping clones were obtained (designated pCDR5, -6, -8, -12, -13, and -18) with inserts ranging in size from 500 to 1200 bp. None of the six clones identified by plaque hybridization produced a fusion protein that reacted with the patient’s IgG. The pCDR13 clone was studied in detail, as it was the largest clone and contained overlapping sequences of the other six clones.

**Sequence Analysis and Repetitive Features of Cerebellar Degeneration-Related (CDR) Clones.** Fig. 2 shows the restriction map of pCDR13 and the six other clones. Fig. 3 shows the nucleotide and derived amino acid sequences of pCDR13. A continuous open reading frame commences with an ATG codon at position 55. This is the only long open reading frame present in the sequence, and it is in register with the junctional lacZ sequence of λgt11. The 669-nucleotide open reading frame is followed by a 3’ untranslated sequence. The protein encoded by the sequence from nucleotide positions 55–724 is composed of 223 amino acids and has a calculated molecular mass of 27,038 Da.

The most striking feature of the sequence is a pattern of tandem repeats consisting of units of 18 nucleotides throughout the entire cDNA sequence. The derived amino acid sequence contains 34 inexact tandem repeats of six amino acids that compose 91% of the predicted peptide (Figs. 3 and 4). The hexapeptide repeating unit features a nearly invariant core of Glu-Asp (codons GAA GAC), flanked on the amino side by hydrophobic residues (generally leucine and phenyl-
The repeating units are underlined.

A hydrophathy profile (23) of the predicted amino acid sequence (Fig. 5) reveals an initial hydrophobic segment, followed by cycles of alternating very hydrophilic and slightly hydrophobic regions with a periodicity of \( \approx 40 \) amino acid residues, and ending with a slightly hydrophilic nonrepetitive segment. The predicted secondary structure of the sequence according to the method of Chou and Fasman (24) is an \( \alpha \)-helix, with a \( \beta \)-sheet conformation extending along 30 amino acids at the carboxyl end.

In comparison to the pCDR13 sequence, pCDR6 and pCDR8 both lack a 204-bp segment that includes the translational termination codon of pCDR13. This indicates a possible alternative splicing of mRNA, as supported by the presence of multiple mRNA species in RNA blots (see below). Because of the deletion, the predicted amino acid sequences of pCDR6 and pCDR8 are slightly different from pCDR13 in the carboxyl end of the molecule, with the main repetitive feature remaining unchanged.

**Southern Blot Analysis of the CDR Gene in Human and Mouse.** Southern blot analysis showed hybridization of the 1.2-kilobase (kb) pCDR13 probe to single fragments in digests of human genomic DNA by BamHI, EcoRI, HindIII, Pst I, and Pvu II (Fig. 6). These findings are consistent with single-copy representation of the CDR gene. The observation that different restriction enzymes generated single fragments also suggests that the entire pCDR13 cDNA clone was derived from one exon or from exons lacking restriction sites in the intervening sequences. Southern analysis of restriction digests of mouse genomic DNA also showed strong hybridization.

**Fig. 4.** Tandem repeats in the CDR sequence. The sequence of the 34 repeats is summarized to illustrate the consensus 18-nucleotide/hexapeptide unit.

Nucleotide: TTG GAA GAC GTA CCT TGG GTA GAC ATT AGG CTG GAA GAC GTA CCT TGG GTA GAC AGC GAT TTC CTG GTA GAC GTG CAT Arg Leu Glu Asp Ile Ser Leu Leu Glu Asp Pro Leu Leu Glu Asp Val Ser

Amino acid: Leu Phe Leu Glu Asp Met Val Asp

No. appeared: 26 26 34 32 18 21

**Fig. 5.** Hydrophathy plot of the predicted amino acid sequence of the CDR protein based on the method of Kyte and Doolittle (23). Hydrophathy values for a span of seven amino acid residues were averaged, assigned to the middle residue of the span, and plotted (y axis) according to the position of the middle residue along the sequences (x axis).
not expressed in leukemia/lymphoma cell lines or in lines derived from colon or breast carcinoma.

RNA blots of CDR mRNA-positive human cell lines demonstrated three closely migrating mRNA species (from 1.3 kb to 1.5 kb), best illustrated by the neuroblastoma line SMS-KAN (Fig. 8). This mRNA heterogeneity may be due to alternative splicing, as suggested by structural studies of cDNA clones pCDR6 and pCDR8 (Fig. 3). RNA blot analysis of rabbit and mouse brain confirmed the greater expression of the CDR gene in cerebellum than in cerebrum (Fig. 8). Rabbit CDR messages were similar in size to the human transcripts, except for the absence of the 1.3-kb species. In contrast, the blot of mouse brain RNA showed only a single 3.5-kb message.

**DISCUSSION**

To identify the molecular targets for anti-brain antibodies in PCD, we have used the serum from a patient with PCD to isolate a cDNA clone from a human cerebellum λgt11 expression library. The nucleotide sequence of the CDR cDNA contains a pattern of tandem repeats with a structure that is unlike any known eukaryotic gene. In addition, the

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**Table 1. Detection of CDR mRNA in human tumor cell lines by cytoplasmic dot blot analysis**

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<thead>
<tr>
<th>Tumor cell lines</th>
<th>No. positive/</th>
<th>% positive</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>no. tested</td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
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<td>67</td>
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<tr>
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</tr>
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<td>21</td>
</tr>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Other carcinomas*</td>
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<td>0</td>
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*Two ovarian, one cervical, one bladder, one pancreatic, one choriocarcinoma.
pattern of expression of the CDR gene does not resemble any previously described neuroectodermal gene. CDR gene transcripts are found much more abundantly in normal adult brain, especially cerebellum, than in non-neural tissues. The low level of CDR expression in human lung, kidney, and heart muscle could be due to expression in the small subpopulation of cells of neuroectodermal origin in these tissues.

Tandemly repeated sequences are known to characterize a number of surface proteins of malarial parasites. For example, the circumsporozoite protein of Plasmodium falciparum consists almost entirely of tandem repeats of 4 amino acids (25), while that of Plasmodium knowlesi contains 12 repeats of a 12-amino acid unit (26). These Plasmodium circumsporozoite proteins show a high degree of antigenicity and are able to serve as the targets for neutralizing antibodies. Most eukaryotic proteins with known tandem repeats are structural proteins, such as the intermediate filaments (27), nuclear lamins (28), myosin (29), and α-tropomyosin (30). These proteins form coiled coils and share a common structural pattern of tandemly repeating units of 7 amino acids, with hydrophobic residues occupying the first and fourth positions. However, there are otherwise no restrictions on the amino acids in the repeating units of these proteins, in contrast to the CDR molecule. Two structural proteins show certain similarities to the CDR gene product. Involucrin, a keratinocyte envelope component, contains a central segment of 39 repeats of 10 amino acids and appears to be a substrate for cross-linking by transglutaminase (31). The PrP prion protein, thought to polymerize into fibrils, contains well-conserved tandem repeats of 8 amino acids extending over 13% of its sequence (32, 33). To our knowledge, the only nonstructural eukaryotic protein with tandem repeats is RNA polymerase II, with a highly conserved unit of 7 amino acids repeated 26 times in the COOH-terminal sequence (34, 35). Knowledge of the tandem repeat composition of the CDR protein itself, therefore, does not allow insight into possible cellular functions.

Is the protein product of the CDR gene an important target in the pathogenesis of PCD? The fact that this molecule is recognized by the PCD patient’s serum and that transcripts for it are expressed predominantly in neural tissues (especially cerebellum) is consistent with a significant disease association. The absence of the CDR mRNA in breast and ovarian cancer cell lines and its presence in primary tumors from two PCD patients (one breast adenocarcinoma and one ovarian carcinoma; unpublished data) add to possible association. It is also likely that a molecule with such a tandemly repeated structures might be highly immunogenic and thus the target of an autoimmune response. However, recognition of this molecule by the immune system may be secondary to cell destruction in PCD and not causally related to the pathogenesis of PCD. The observation that the CDR gene is highly conserved in mouse and rabbit may provide valuable animal models for further studies of this molecule.

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