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Expression of neurofibromin, the neurofibromatosis 1 gene product: studies in human neuroblastoma cells and rat brain

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Neurofibromatosis type 1 (NF1) is one of the most frequent Mendelian disorders in man and one of the most common autosomal dominant disorders affecting the nervous system. The NF1 gene has recently been cloned, and shows homology to the GTPase activating protein. In order to characterize the NF1 gene product, now called neurofibromin, we raised polyclonal antibodies against C- and N-terminal regions of neurofibromin and analyzed the protein by Western blot analysis and immunohistochemical studies of rat brain.

Von Recklinghausen neurofibromatosis (NF1) is one of the most common Mendelian diseases affecting the development and growth control of a variety of tissues. Among the common manifestations are benign tumors of peripheral nerves (neurofibromas) and pigment changes of the skin (cafe-au-lait spots). More than 40% of NF1 patients have learning disabilities and a small percentage have frank mental retardation indicating that a mutation in the NF1 gene may disturb cognitive development and function (reviewed in ref. 8). Although not exclusively, it mainly affects tissues that are derived from the neural crest.

Using positional cloning the NF1 gene was recently identified. Sequence analysis of the cDNA predicted a protein of 2818 amino acids [1, 2, 12, 13, 15]. This protein has recently been named neurofibromin. Protein sequence analysis revealed that neurofibromin is a hydrophilic protein with a GTPase activating protein (GAP)-like domain at its center. The catalytic domain shows 22% homology to mammalian GTPase-activating protein (GAP) and 26% homology to yeast IRA proteins [1, 15]. Fusion proteins of the GAP-like domain (GAP-LD) have been shown to interact with ras-proteins and inhibit their GTPase functions [4, 6, 7, 14]. Recently, antibodies (ABs) have been raised to fusion proteins and synthetic peptides of neurofibromin [3, 5]. These studies indicated that the molecular weight of rodent neurofibromin is approximately 250 kDa, and that the NF1 gene is expressed ubiquitously in tissues other than neural crest derivatives [5]. We are now providing the first anatomical studies on neurofibromin in rodent CNS.

We raised antibodies against two synthetic peptides predicted from the cDNA sequence [15]. Peptide NF1C (KAPKRQEMESGITT, amino acids (aa) 2198–2211, numbered according to [15]) is located 1200 aa-residues C-terminally from GAP-LD while peptide NF1D (KFKEKPTDLETRSY, aa 142–155) is located 650 aa-residues N-terminally from the GAP-LD (Fig. 1). After coupling to keyhole limpet hemocyanin peptides were injected into two rabbits each. The antisera were affinity-purified using peptide bound to activated Sepharose-4B as described previously [9].

To determine the molecular weight of neurofibromin and to test the specificity of the ABs, we performed Western blot analyses of protein extracts of a human neuroblastoma cell line (HTB10). Methods were adapted from Towbin et al. [11] with the following modifications: whole cell lysates were extracted with lysis buffer (100 mM Tris, pH 0.8, 150 mM CaCl, 1% Triton X-100 phenyl methanesulfonyl fluoride (PMSF), 2 U/ml aprotinin). The proteins were then resolved on a 4–20% SDS–polyacrylamide gradient gel (BioRad). Molecular weight markers were purchased from BioRad. The fractionated proteins were blotted essentially as previously described [11], and immunostained as described in the BioRad Immuno-Blot Assay kit for alkaline phosphatase-conju-

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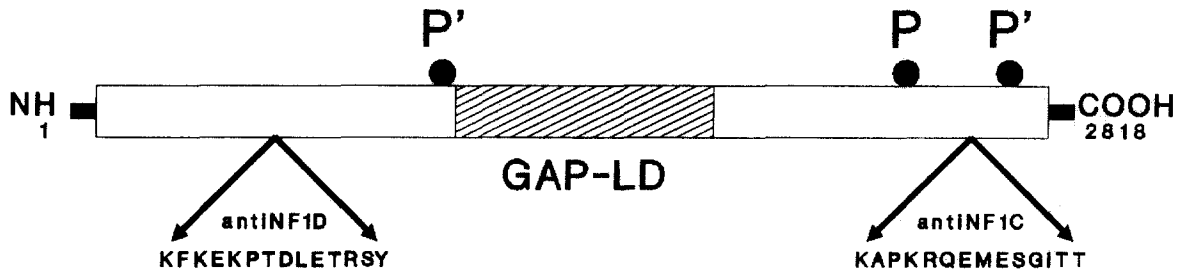

Fig. 1. Map of neurofibromin showing the positions of the GTPase activating protein-like domain (GAP-LD), the potential phosphorylation sites (P), and location of the two synthetic peptides.

gated anti-rabbit IgG, except that the primary antibodies were incubated at 4°C overnight.

Both ABs detected two similar sized proteins of 290 kDa and 70 kDa. In addition, the anti-NF1C AB detected two more proteins of 60 kDa and 38 kDa, while the other detected a 30 kDa antigen (Fig. 2, lanes 1 and 2). However, the lower molecular weight bands were inconsistently observed indicating that some of the bands were likely products of nonspecific degradation. All of these bands were absorbed out by addition of 10 μM of the respective peptide to the antibody solution (Fig. 2, lanes 3 and 4).

Our results are in agreement with two previous reports showing that neurofibromin has a molecular weight of 250–280 kDa in tissues of rats and mice [3, 5]. Using polyclonal antibodies directed against peptides of different regions of the predicted NF1 protein, Gutmann et al. [5] observed an antigen at 250 kDa in mouse tissues and human HeLa cells, while DeClue et al. [5] found a 280 kDa protein in mouse NIH-3T3 and human HeLa cells in agreement with our MW estimate of 290 kDa. In recent experiments, using a 4–15% gradient PAGE Western blots, we determined the size of human neurofibromin to be 350 kDa, whereas the rat neurofibromin had a distinctively smaller apparent M.W. of 250 kDa (data not shown). This observation may indicate differential transcription or post-translational modifications of the NF1 products in human and rat.

In order to study the localization of the NF1 product, we incubated sections of frozen, paraformaldehyde-fixed rat brain and spinal cord with both ABs. Rat brain and spinal cord tissues were prepared as follows: 150–200 g anesthetized male Sprague–Dawley rats were perfused via cardiac puncture with 200 ml of 100 mM PBS, pH 7.4, followed by 200 ml of 100 mM PBS-buffered 2% paraformaldehyde (PFA). Brains and spinal cords were fixed with 2% PFA for another 24 h followed by cryoprotection with 30% sucrose in 100 mM PBS for 48 h. The samples were rapidly frozen in crushed dry-ice, and stored at −20°C. Thirty μm sections were prepared and incubated in 4% PFA overnight. For immunocytochemistry, the procedure described in the Vector ABC elite Peroxidase kit was modified as follows: the fixed sections were washed extensively and briefly incubated with Auto/Zyme (Biomeda), followed by Peroxidase blocker (Biomeda) and Avidin/Biotin blocker (Vector). The tissue sections were incubated with primary ABs at 4°C overnight, washed four times with 100 mM PBS, pH 7.4, then followed with the second AB incubation for 1 h at

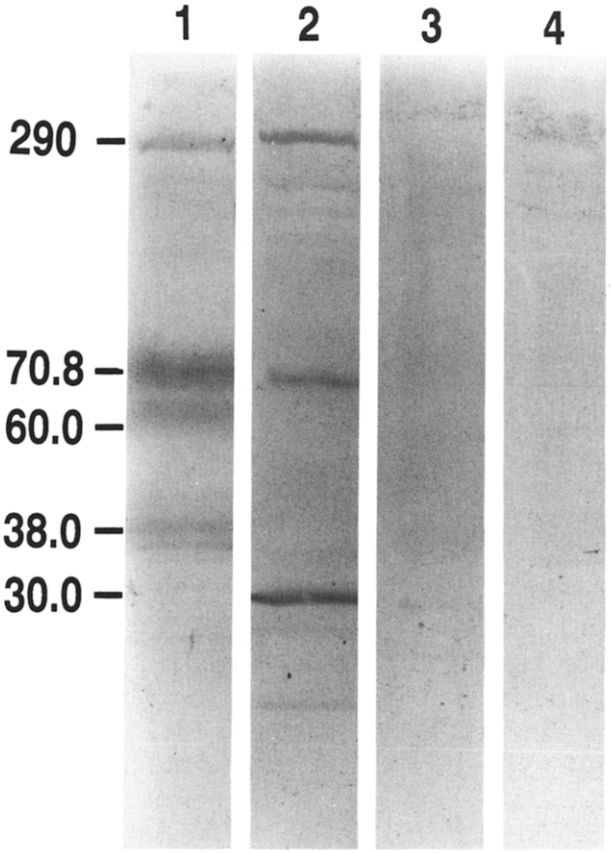

Fig. 2. Western blot analysis of extracts from a human neuroblastoma cell line (HTB10). Lanes 1 and 2 were incubated with anti-NF1C AB and anti-NF1D AB, respectively. Lanes 3 and 4 were incubated with anti-NF1C AB and anti-NF1D AB preabsorbed with 10 μM of the respective peptides. Both antibodies detected a common 290 kDa antigen, and other low molecular weight species at 70 kDa, 60 kDa, 38 kDa, and 30 kDa.

room temperature. Prior to DAB substrate incubation (Biomeda), the sections were briefly exposed to Peroxidase Enhancer (Biomeda).

Both anti-NF1C and anti-NF1D ABs had a similar staining pattern in the spinal cord (Fig. 3A,C) and brain detecting intense neurofibromin-like immunoreactivity. Staining of both ABs was absorbed out by the addition of 100 μM of the respective peptide, but not by the addition of 100 μM of other peptides, indicating the specificity of these anti-neurofibromin antibodies (Fig. 3B,D).

Within the spinal cord, staining appeared the strongest in the dorsal root and the dorsal root entry zone (Fig. 3A,C, arrows). Immunoreactivity was detected in sensory as well as motor neurons. Although most of the staining was localized to neurons, glial cells, presumably oligodendroglia, in spinal cord white matter stained as well (Fig. 3E). Within neurons, staining was seen in the cytoplasm as well as in the nucleus (Fig. 3F).

The relatively broad distribution of neurofibromin-like immunoreactivity in the spinal cord was also seen in a survey of rat brain (data not shown). Staining was most pronounced in layers II and V of the cerebral cortex. Among the strongest staining neuronal cell groups were Purkinje cells in the cerebellum, pyramidal cells in the CA1-CA4 regions of the hippocampus, the supraoptic nuclei, and neurons in the gigantocellular nucleus of the medulla oblongata.

Neurofibromin appears to be widely distributed in different mouse tissues as shown by Western blot analysis [3]. Similarly, our studies indicate a broad distribution of neurofibromin within the nervous system. Neurons in different anatomical regions and functional systems showed neurofibromin-like immunoreactivity. However, the neuronal populations showing strong staining did not appear to conform to known anatomical or functional groups. The detection of nuclear as well as cytoplasmic staining raises the possibility that neurofibromin may encode other functions distinct from cytoplasmic interaction with the GTP-ras complex. It is interesting to note that a recently identified second transcript from the NF1 gene [10] encodes a potential nuclear localization signal.

In summary, we have raised two polyclonal ABs directed against C-terminal and N-terminal regions of neurofibromin. These ABs recognized proteins on Western blots with a molecular weight close to that predicted from the NF1 cDNA sequence. Both ABs stained CNS tissue specifically with a nearly identical staining pattern. Staining was detected in neurons and glial cells. The widespread distribution of neurofibromin in the nervous system is consistent with the clinical observation that a mutation in the NF1 gene may lead to structural as well as functional CNS abnormalities.

Dedicated to the memory of Dr. Hiltrud Pulst. Supported by the Steven and Lottie Walker Foundation, the Carmen and Louis Warschaw endowment fund and grant NS01428-01A1 from the National Institutes of Health.

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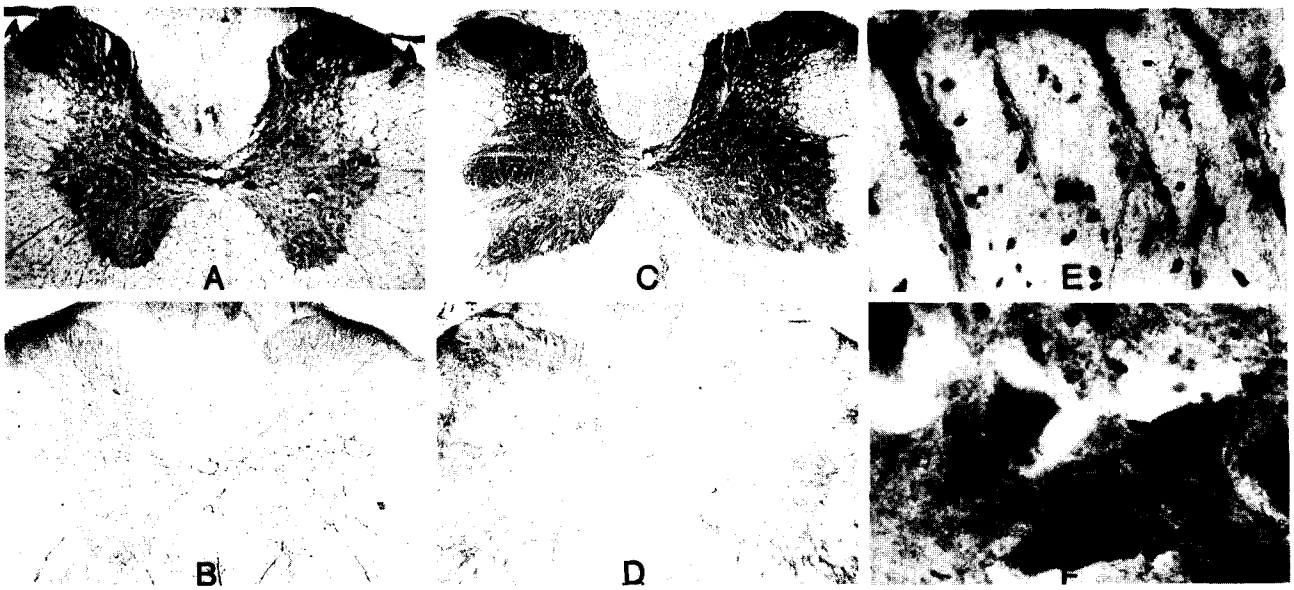

Fig. 3. Detection of neurofibromin-like immunoreactivity in the rat nervous system. A–D: transerve sections of rat cervical spinal cord. The sections were stained with anti-NF1C AB (A), anti-NF1C AB preabsorbed with 100 μM peptide C (B), anti-NF1D AB (C), and anti-NF1D AB preabsorbed with 100 μM peptide D (D). E,F: higher magnification view of oligodendrocytes in spinal cord white matter (E) and ventral horn motor neurons (F).

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