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Domain Structure of Neurofilament Subunits as Revealed by Monoclonal Antibodies

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Monoclonal antibodies have been prepared against purified neurofilament (NF) subunits (NF68, NF150, and NF200). From 25 fusions, several hundred strongly positive antibodies have been obtained. Among them are antibodies against the specific subunits as well as antibodies recognizing common antigenic determinants. These have all been characterized according to the following properties: ELISA (enzyme-linked immunosorbant assay) testing against each subunit, immunoblots against enriched neurofilament preparation, immunoblots of cyanogen bromide or chymotrypsin-treated neurofilaments, immunofluorescence with PC12 cells, and immunohistochemistry of cerebellum. Whereas the antibodies against the NF68 and NF150 appear to react with single cyanogen bromide fragments, the antibodies against the NF200 react with multiple cyanogen bromide fragments. These data are consistent with the hypothesis that the NF200 is partially composed of several repeated structural determinants. Furthermore, all of the antibodies that react with the NF200 recognize the solubilized "sidearm" domain from limited chymotryptic digestions. The locations of the common and variable domains of the three subunits are discussed in light of these results.

Key words: neurofilaments, monoclonal antibodies, intermediate filaments

Neurofilaments are intermediate filaments of neurons and comprise the major cytoskeletal structure of large neuritic processes [1,2]. They are but one of several subclasses of intermediate filaments based on the size and differing immunoreactivities of protein subunits [3,4]. Most intermediate filaments are composed of a single major protein, which can be enriched by isolation and purification of the parent intermediate filament [5]. Neurofilaments, however, are unique in that they contain three major proteins with molecular weights of approximately 68,000 (NF68), 150,000 (NF150), and 200,000 (NF200) [6–8]. Although it has been suggested that these "triplet proteins" are structurally related, the evidence has been primarily indirect. In fact, peptide maps comparing the three polypeptides show no apparent similarities [9–12].

In contrast, immunological evidence has suggested that the three neurofilament polypeptides are related [13,14]. One monoclonal antibody has been developed that

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appears to detect a determinant common to all intermediate filaments [15]. Antibodies have recently been obtained that have reactivity against both subunit-specific epitopes or epitopes common to two or three of the subunits [16]. In the present manuscript, we report the analysis of the sets of neurofilament monoclonal antibodies that have been derived in our laboratory. In particular, it has been found that the NF200 appears to possess multiple repeated antigenic determinants. Furthermore, the distribution of common and unique epitopes within the polypeptide can be described.

MATERIALS AND METHODS

Bovine neurofilament proteins were prepared from spinal cord with some modification of the method previously described [17]. Monoclonal antibodies were prepared using electrophoretically purified neurofilament subunits as immunogens [17]. The hybrid clones were obtained from spleen cells of immunized Lewis rats fused with mouse myeloma cells. Positive clones were subcloned in agarose to obtain pure and stable monoclonal antibody-producing cells. Cyanogen bromide fragments (CNBr) were produced by incubation of purified neurofilament subunits with excess CNBr in 70% formic acid [18]. Soluble chymotrypsin fragments were obtained after limited digestion of enriched neurofilaments with chymotrypsin [5,19–21]. After digestion, the neurofilament preparation was centrifuged removing the undigested filaments and the low-molecular-weight "core" material from the reaction mixture. Analyses of intact and cleaved neurofilament samples were performed in sodium dodecyl sulfate on 6% or 7.5% polyacrylamide slab gels (0.75 mm) according to Laemmli [22]. The protein was transferred to nitrocellulose paper with an adaptation of the method of Towbin et al [23] utilizing a horizontal assembly. The electrophoretic blots were blocked for 1 hr in 1% normal rabbit serum and 1% bovine serum albumin in phosphate-buffered saline. Strips were then incubated overnight with the monoclonal antibodies diluted in 1% bovine serum albumin. After washing, the blots were developed with 4-chloro-1-naphthol.

RESULTS

Specificity of Monoclonal Antibodies

The hybridoma supernatants were tested for subunit specificity first by the ELISA test and then by immunoblotting enriched neurofilament fractions. Several thousand clones obtained from 25 fusions were screened, and only the strongest were maintained in culture. Typical results using the immunoblotting technique are seen in Figure 1. We obtained nine antibodies specific for the NF200, 25 specific for the NF150, and seven antibodies reactive only with the NF68. In addition, antibody-secreting clones were obtained that reacted with common determinants in two or more subunits. The largest of the cross reacting categories, 95 clones, was that identifying epitopes common to both NF150 and NF200. These included antibodies very strong or very weak against both polypeptides and antibodies that reacted strongly with one antigen and more weakly against the other. A single antibody was obtained that had specificity towards both NF68 and NF150; seven hybrids secreted antibodies reacting with NF68 alone. A single antibody was found that reacted with both NF68 and vimentin.

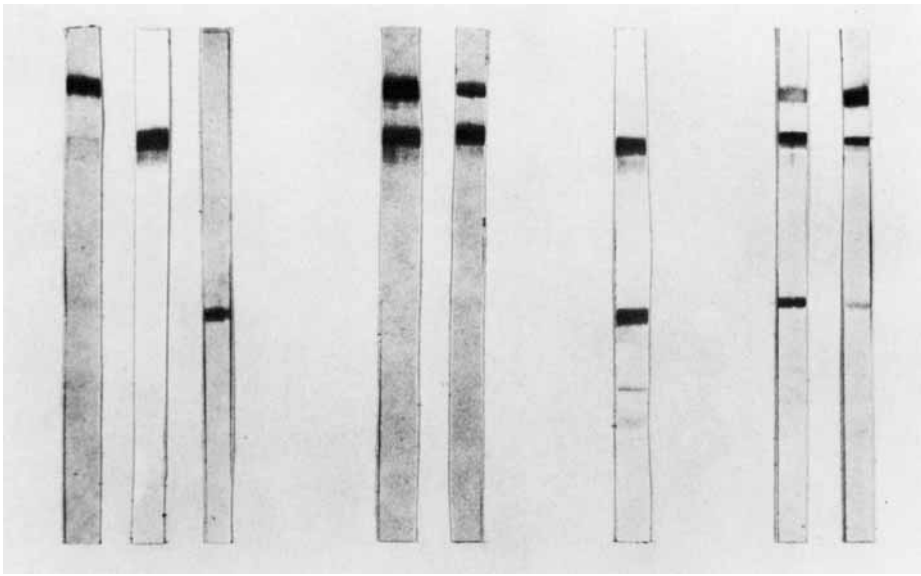

Fig. 1. Immunoblots of neurofilament preparation with selected monoclonal antibodies. Lane 1, anti-NF200; lane 2, anti-NF150; lane 3, anti-NF68; lanes 4 and 5, anti-NF150/200; lane 6, anti-NF68/150; lanes 7 and 8, anti-NF68/150/200.

Immunopeptide Mapping of Monoclonal Antibodies

In order to ascertain which portion of the individual neurofilament subunits reacted with specific antibodies, purified polypeptides were subjected to cyanogen bromide fragmentation, immunoblotted, and reacted with the hybridoma supernatants. Under these conditions, polyclonal antibodies usually react with multiple peptides, whereas monoclonal antibodies will react with unique peptides. In the case of the NF68 and NF150 immunopeptide maps, the latter case was most common (Fig. 2). However, using digests of the NF200, antibodies detected multiple peptide bands (Fig. 2). All antibodies that reacted with NF200 of whatever specificity (NF200, NF200/NF150, NF200/NF150/NF68) detected antigenic reactivity in the largest 125 kD fragment as well as in two smaller fragments. Subclones of these hybrid cells produced the same reactivity, indicating that true monoclonal antibodies were being produced.

The data obtained above for the NF200 indicate that this protein is formed in part from identical or closely related structural domains repeated throughout the polypeptide chain. Furthermore, the identity of this pattern of reactivity for NF200-reactive antibodies of all specificities indicates that several determinants are repeated in this manner.

Immunological Analysis of Chymotryptic Fragments

From work performed in several laboratories, it is known that short-term incubation of enriched neurofilament preparations with chymotrypsin produces sev-

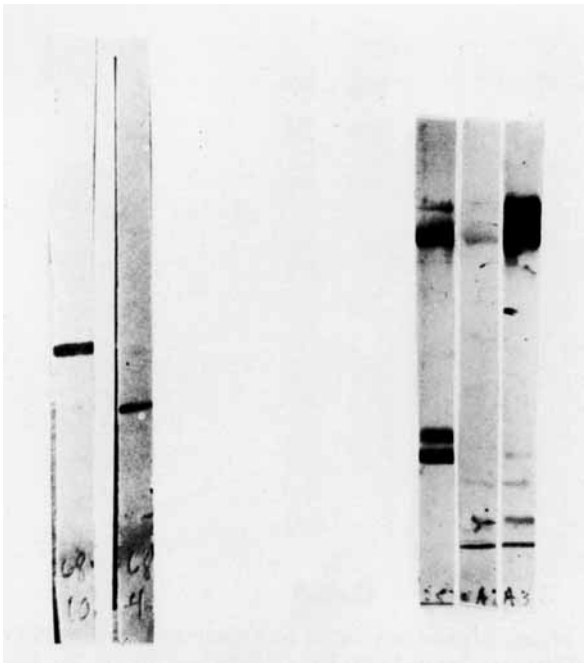

Fig. 2. Immunopeptide maps of neurofilament subunits. The individual neurofilament polypeptide chains were treated with cyanogen bromide and the resultant fragments separated on 7.5% (NF150) or 4–20% (NF68) sodium dodecyl sulfate polyacrylamide gels. After electrophoretic transfer, the nitrocellulose strips were incubated with monoclonal antibodies for the detection of immunoreactive fragments. On the left are two representative strips from anti-NF68 antibodies. On the right are three representative strips from anti-NF200 reactive antibodies whether subunit-specific or common determinants.

eral specific polypeptide fragments that are water soluble [19–21]. At the termination of the reaction, the filaments are centrifuged, removing unreacted neurofilaments plus a "core," α-helical region of approximately 40,000 daltons, presumably common to all neurofilaments. Indeed, it is this portion of the structure that is believed to be shared among all intermediate filaments [5,19–21]. The resultant supernatant contains four soluble polypeptides. A 160,000 dalton fragment is derived from the NF200, and a triplet of fragments at 130,000, 125,000, and 110,000 daltons is derived from the NF150 (Fig. 3). These soluble fragments, the carboxyterminal regions of the neurofilaments, are believed to represent "sidearms" from the main filamentous structure that extend exposed in the cytoplasm [24]. The structural differences between the neurofilament subunits are thought to reside in this region of the molecule (see model, Fig. 3). Our library of monoclonal antibodies was tested against chymotryptic digests of both assembled neurofilaments and purified subunits in order to determine where the unique and common epitopes lie.

Figure 4 shows examples of the typical reactivity of our antibodies. All antibodies that react with NF200, of whatever specificity, react with the solubilized 160 kD fragment. In turn, all of the NF150-specific antibodies react with the lower triplet polypeptides. In contrast, of the other antibodies that cross react with NF150, including antibodies that react with NF150/NF200 or NF68/150/200, only approximately one-third detect these solubilized fragments. The remainder also do not react

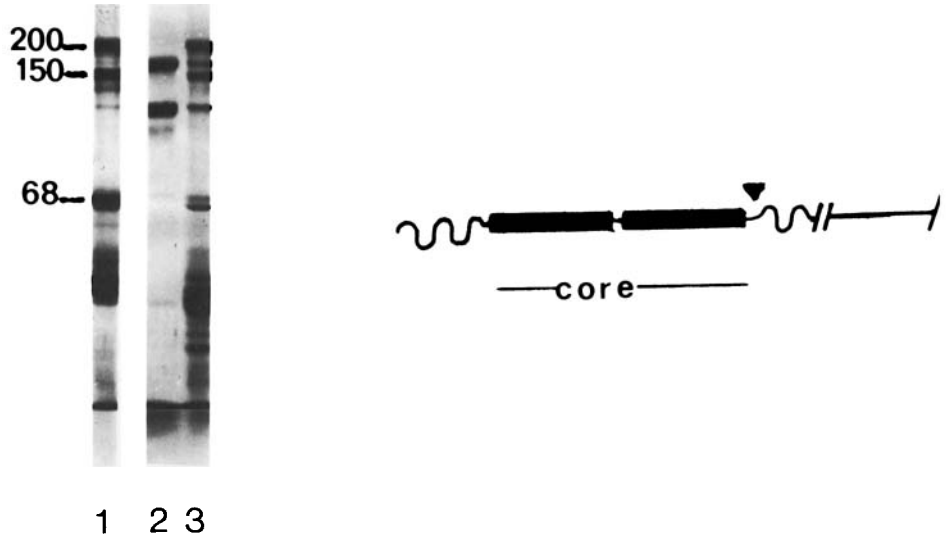

Fig. 3. Chymotrypsin digestion of intact neurofilaments. Left, sodium dodecyl sulfate electrophoretograms. Lane 1, enriched S1 neurofilament preparation; lane 2, supernatant from a 15′ digestion of S1 neurofilaments with chymotrypsin; lane 3, pellet from a 15′ digestion of S1 neurofilaments with chymotrypsin. Right, model of an individual neurofilament. A short amino terminal region is followed by an α-helical (two helices), or "core," region of ca 40,000 daltons. The carboxy-terminal region released by chymotrypsin (at arrow) is the location of the fragments released (lane 2 above). The "core" is thought to be a common element of all intermediate filaments [5,19–21].

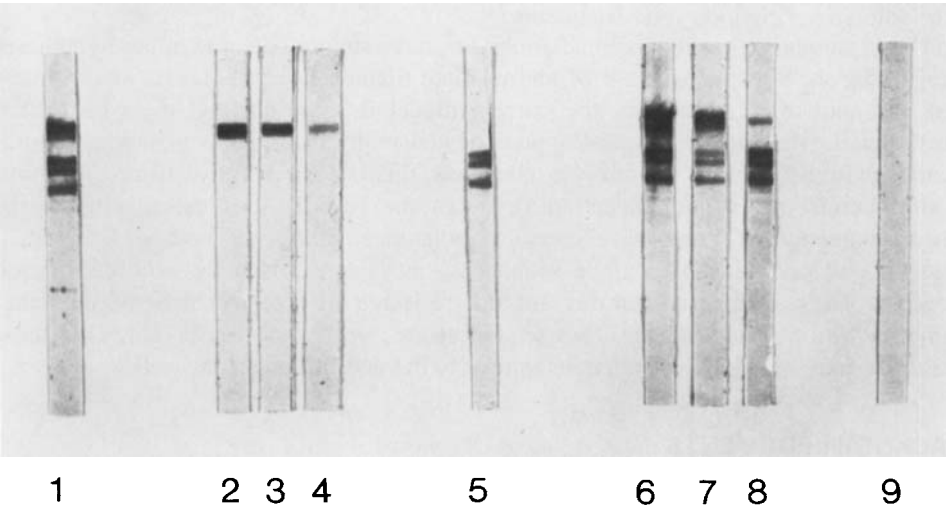

Fig. 4. Immunoblots of soluble chymotryptic fragments. Lane 1, polyclonal antiserum; lanes 2–4, typical reactions of antibodies that react with NF200; lane 5, reaction of antibodies specific to NF150; lanes 6–8, reaction of monoclonal antibodies that reveal both the fragments derived from NF200 and from NF150; lane 9, controls or negatives (see text for details).

with the "core," or common, region. Thus, it seems likely that the epitopes for these antibodies lie within the region of the polypeptide chain in which chymotrypsin cleaves. In addition, three of the NF68/NF150/NF200 antibodies react with the fragment derived from NF200, and a fourth antibody reacts with all of the solubilized fragments. None of these antibodies react with the "core" or presumably common region. Indeed, only five antibodies were found that did react with this insoluble "core" region. Three of these possessed reactivity unique to the NF68, one was specific to NF150, and the last cross reacted with both NF68 and NF150.

DISCUSSION

From these straightforward immunological peptide mapping experiments of neurofilaments we have been able to draw some straightforward conclusions. First, NF200 appears to have a portion of its structure composed of repeated domains. However, these epitopes lie wholly within the portion of the polypeptide that can be solubilized with chymotrypsin. Second, there are common antigenic determinants that lie within the fragments of both the NF150 and NF200 solubilized by chymotrypsin from intact neurofilaments. These regions of the molecules had previously been presumed to contain primarily variable determinants specific to the individual neurofilament subunits [5,20,21]. It should be mentioned that we do find subunit-specific determinants to be present in these regions as well. In addition, four of the antibodies that we have found to react with the "core" or putative common region instead react with determinants unique to specific subunits. Thus, the region of the molecule can contain variable epitopes as well as common ones. Finally, our antibodies that recognize all three neurofilament polypeptides are distinct from the monoclonal antibody previously found to react with all three as well as with all other intermediate filaments [15]. That antibody reacts with the "core" region; our antibodies react with the solubilized chymotryptic fragments.

In summary, our monoclonal antibodies have supported some of the hypotheses regarding the domain structure of intermediate filaments [5,20,21]. However, many of our antibodies reveal that the current model is too rigid and must be further elaborated. Molecular biological approaches currently in progress in several laboratories will be able to answer the questions raised. Specifically, from the above observations one would expect to find that the carboxy-terminal regions of the neurofilaments (sidearms) have several regions identical to one another. These identical sequences, plus the unique sequences, may have arisen by gene duplication events. These structural features are not reflected in the structures of the other intermediate filaments whose DNA sequences are already known [25–27]. Thus these features may be related to functions unique to the neurofilaments as well.

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