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Interaction domains of neurofilament light chain and brain spectrin

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We have previously demonstrated that brain spectrin binds to the low-molecular-mass subunit of neurofilaments (NF-L) [Frappier, Regnouf & Pradel (1987) Eur. J. Biochem. **169**, 651–657]. In the present study, we seek to locate their respective binding domains. In the first part we demonstrate that brain spectrin binds to a 20 kDa domain of NF-L.This domain is part of the rod domain of neurofilaments and plays a role in the polymerization process. However, the polymerization state does not seem to have any influence on the interaction. In the second part, we provide evidence that NF-L binds to the β-subunit of not only brain spectrin but also human and avian erythrocyte spectrins. The microtubule-associated protein, MAP2, which has also been shown to bind to microfilaments and neurofilaments, binds to the same domain of NF-L as spectrin does. Finally, among the tryptic peptides of brain spectrin, we show that some peptides of low molecular mass (35, 25, 20 and 18 kDa) co-sediment with either NF-L or F-actin.

INTRODUCTION

Brain spectrin appears to be a particularly important cross-linking component of the cytoplasmic matrix of the neuron. In studies of axonal transport, it has been shown that spectrin has unusual transport kinetics. It is the only protein that moves down the axon in association with membrane organelles during fast axonal transport, and mainly with cytoskeletal structures, i.e. microfilaments, microtubules and neurofilaments, during slow axonal transport. This idea is supported by quantitative analysis of the distribution of spectrin, which indicates that most of the axonal spectrin (80%) is present in the central axoplasm (Lasek *et al.*, 1984). The remainder is located in the cortex, where it is known to form a meshwork with microfilaments (Levine & Willard, 1981; Hirokawa, 1982). Experiments *in vitro* with purified proteins have demonstrated this interaction (Glenney *et al.*, 1982*a*), but brain spectrin is also able to bind to microtubules (Fach *et al.*, 1985) and to neurofilaments (Frappier *et al.*, 1987). We have demonstrated that, *in vitro*, brain spectrin binds specifically to the light subunit of neurofilaments (NF-L), under physiological conditions of pH and ionic strength. We have also demonstrated that the Ca^{2+}–calmodulin complex, known to bind to brain spectrin (Glenney *et al.*, 1982*b*), may increase the association at physiological Ca^{2+} concentrations.

Interestingly, microtubule-associated protein, MAP2, has been shown to bind to the same subunit of neurofilaments (Heimann *et al.*, 1985), with the same affinity, as for spectrin. NF-L binds to the 30 kDa domain of MAP2, which is also the binding domain of microtubules and F-actin (Sattilaro, 1986).

In the present study, we examine, on the one hand, the NF-L subdomain organization and identify the brain spectrin- and MAP2-binding fragment. On the other hand we identify the β-subunit of spectrin as the one that binds to NF-L, and provide evidence that some spectrin tryptic peptides co-sediment with NF-L and F-actin.

MATERIALS AND METHODS

Preparation of neurofilaments

Crude pure neurofilaments and NF-L subunit of neurofilament proteins were isolated from frozen pig spinal cord according to modifications of the methods previously described (Delacourte *et al.*, 1980; Minami & Sakai, 1983; Frappier *et al.*, 1987), except that pure neurofilaments and NF-L were reassembled by dialysis overnight at 37 °C against 50 mM-Mes/175 mM-NaCl/1 mM-dithiothreitol (DTT), pH 6.25 (Aebi *et al.*, 1988).

Preparation of MAP2

MAP2 was prepared by the procedure of Vallee (1986), from pig brain.

Purification of human erythrocyte spectrin

Erythrocytes, erythrocyte ghosts and spectrin were purified from freshly drawn anticoagulated human blood (Fondation Nationale de Transfusion Sanguine, Paris, France) as described by Bennett (1983), except that for the erythrocyte preparation complete removal of leucocytes was achieved by passage through a Micropore L filter (Baxter S.A.) as described by Wong *et al.* (1985).

Purification of avian erythrocyte spectrin

Chicken blood was collected on citrate in a slaughterhouse. Erythrocytes and ghosts were obtained as reported by Granger *et al.* (1982). The ghosts were mechanically enucleated in a home-built hydrodynamic system (Cassoly *et al.*, 1989). Synemin and vimentin were extracted as described by Granger *et al.* (1982), and spectrin was extracted in 2 mM-EDTA, pH 8, at 37 °C.

Purification of brain spectrin

All steps were performed at 4 °C. Frozen pig brain (500 g) was homogenized for 3 × 10 s at high speed in a Waring blender with 3 vol. of 0.14 M-NaCl/5 mM-EDTA/1 mM-EGTA/1.5 mM-NaN_3, pH 7, solution containing 1 mM-di-isopropyl phosphorofluoridate (DIFP), 0.25 mM-phenylmethanesulphonyl fluoride (PMSF), 0.20 mM-benzamidine and 5 μg each of leupeptin and pepstatin/ml. The homogenate was centrifuged in a JA10 Beckman rotor at 9500 rev./min for 30 min. Pellets were homogenized with 2 vol. of 20 mM-Tris/0.9 M-KCl/15 mM-$Na_4P_2O_7$/3 mM-DTT/1.5 mM-NaN_3, pH 8.2, containing the same inhibitors, gently stirred for 25 min at 4 °C and centrifuged

Abbreviations used: NF-L, light subunit of neurofilaments; DTT, dithiothreitol; DIFP, di-isopropyl phosphorofluoridate; PMSF, phenylmethanesulphonyl fluoride; TLCK, *N*-tosyl-L-lysylchloromethane; TPCK, *N*-tosyl-L-phenylalanylchloromethane; BNPS-skatole, 3′-bromo-2-(2′-nitrophenylsulphenyl)-3-methylindolene; MAP, microtubule-associated protein.

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for 30 min in a JA10 Beckman rotor at 9500 rev./min. The supernatant was centrifuged for 30 min at 44 000 rev./min in a 45Ti Beckman rotor. To this supernatant 3 vol. of 2 mM-$MgCl_2$ was added and the pH was adjusted to 6.6. The extract was incubated for 10 min in a cold-room before being centrifuged in a JA10 Beckman rotor at 9500 rev./min for $1\frac{1}{2}$ h. The supernatant was pelleted with $(NH_4)_2SO_4$ at 35% saturation and centrifuged at 9500 rev./min for 30 min. Pellets were resuspended in 20 mM-Tris/5 mM-EGTA/0.6 M-KI/15 mM-2-mercaptoethanol/1.5 mM-NaN_3, pH 8.2, containing 10% sucrose, and centrifuged overnight at 32500 rev./min in a 70Ti Beckman rotor. The supernatant was applied to a Sepharose CL4B column (5 cm × 90 cm), equilibrated with 20 mM-Tris/5 mM-EGTA/0.6 M-KCl/15 mM-2-mercaptoethanol/1.5 mM-NaN_3, pH 8.2, at a flow rate of 50 ml/h. Enriched fractions were concentrated by dialysis against Aquacide II, then dialysis residue was allowed to precipitate for 30 min with $(NH_4)_2SO_4$ at 30% saturation, and centrifuged for 15 min at 11000 rev./min in a JA17 Beckman rotor. Pellets were suspended in 10 mM-Tris/50 mM-KCl/1 mM-$MgCl_2$/0.1 mM-$CaCl_2$/1.5 mM-NaN_3/0.1 mM-DTT, pH 8.2, and dialysed against the same buffer. Non-diffusible material was centrifuged for 2 h at 46000 rev./min in a Beckman SW55 rotor; EGTA was added to the supernatant to 1 mM, and concentrated by dialysis against 1 mM-NaH_2PO_4/0.1 mM-DTT/1.5 mM-NaN_3, pH 6.8, containing glycerol and stored at −20 °C.

Preparation of total pig brain extract

Total brain extract was prepared by the procedure of Davis & Bennett (1984).

Separation of brain spectrin subunits

The brain spectrin subunits α and β were separated on a calmodulin–Sepharose column as described by Glenney & Weber (1985).

Antibody preparation

Antibodies directed against the α- and β-subunits of brain spectrin were prepared as described by Glenney & Glenney (1984) from 2.5 mg of each subunit of brain spectrin. Antibodies were further purified by affinity on nitrocellulose sheets (Fowler & Bennett, 1983).

Iodination

Brain spectrin, MAP2 and NF-L were iodinated by the procedure of Bolton & Hunter (1973). After iodination and before chromatography on a column (0.7 cm × 25 cm) of Sephadex G-25 (fine grade), NF-L was solubilized in 6 M-urea.

Immunoblot experiments

After SDS/PAGE, proteins were transferred to nitrocellulose as described by Towbin *et al.* (1979). Nitrocellulose sheets were then washed for 30 min in TBST (10 mM-Tris/150 mM-NaCl, pH 8, containing 0.05% Tween 20), and for 30 min in TBST containing 5% non-fat dry milk. Incubation with antibodies was at 37 °C for 1 h. Sheets were then washed for 4 × 15 min at room temperature in TBST. The detection system employed was anti-(rabbit IgG–alkaline phosphatase conjugate) allowed to react with the 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium substrate system.

Binding to transblotted proteins

After migration in a polyacrylamide gel, proteins were electrotransferred on to nitrocellulose sheets. Strips were washed in PBS (10 mM-NaH_2PO_4/150 mM-NaCl, pH 7.5), containing 2.5% Triton X-100 (PBST) and saturated in PBST containing 40 mg of BSA/ml. Binding was carried out by overlaying with radioactive proteins in PBST/BSA overnight at 4 °C. Then after six washes in PBS sheets were exposed to Hyperfilm MP (Amersham) for 48 h at −70 °C using an intensifying screen.

Protein hydrolysis

BNPS-skatole peptides of NF-L were prepared by the method of Mahboub *et al.* (1986). NF-L 30 kDa fragment was electro-eluted from SDS/PAGE and then dialysed against 10 mM-NaH_2PO_4/6 M-urea/1 mM-EGTA/1 mM-2-mercaptoethanol/1.5 mM-NaN_3, pH 7.5. NF-L 30 kDa fragment (25 μM; 75 μg in 100 μl) was hydrolysed with 0.5 μg of Lys-C endoproteinase (1 μl at 30 units/mg) (Boehringer Mannheim) for 1 h at room temperature. Reaction was stopped with 4 μM-aprotinin (1.5 μg at 10 mg/ml) (Geisler *et al.*, 1983). Hydrolysates were processed as described above for autoradiography.

Brain spectrin (2 mg at 1.42 mg/ml) was digested with *N*-tosyl-L-phenylalanylchloromethane (TPCK)-treated trypsin (32 μg at 80 μg/ml) at 4 °C for 4 h in 20 mM-Tris/25 mM-NaCl/0.5 mM-EDTA/1 mM-2-mercaptoethanol, pH 8, as described by Harris & Morrow (1988) using an enzyme/substrate molar ratio of 1:1.56 (final volume 1.8 ml). The reaction was stopped by adding a 10-fold molar excess of DIFP and a 10-fold molar excess of *N*-tosyl-L-lysylchloromethane (TLCK). After hydrolysis, the pH of the solution was adjusted to 6.8 to allow interaction experiments. Hydrolysate was first sedimented in a Beckman Airfuge at 190 kPa for 20 min, then the supernatant was incubated with either F-actin or NF-L. For F-actin interaction studies, final concentrations were adjusted to 50 mM-KCl/2 mM-$MgCl_2$/1 mM-EGTA/17 mM-Tris, pH 6.8, whereas, for neurofilament interaction studies, concentrations were adjusted to 50 mM-KCl/1 mM-EGTA/2 mM-$MgCl_2$/0.1 M-Mes, pH 6.8, to provide optimal conditions for binding. Hydrolysate (150 μg) was incubated at room temperature for 1 h with 22.5 μg of either NF-L or F-actin. Then they were centrifuged for 20 min in a TLA 100.2 ($r_{av.}$ 31.8 mm) Beckman rotor at 55000 rev./min. Pelleted peptides were separated by SDS/PAGE (5–17.5% linear gradient of acrylamide), electro-transferred to nitrocellulose, and then processed as described in immunoblot experiments with anti-(brain α-spectrin) and anti-(brain β-spectrin) as antibodies. In the same conditions, hydrolysates were incubated without NF-L and F-actin. A second control experiment was done in which NF-L and F-actin were incubated alone under the same conditions as described above.

Low-angle rotatory-shadowing experiments

Brain spectrin (0.37 mg/ml) and neurofilaments (0.37 mg/ml) were incubated in 0.1 M-Mes (pH 6.8)/1 mM-EGTA/0.5 mM-$MgCl_2$/50 mM-NaCl for 30 min at room temperature. Then, after being fixed with glutaraldehyde for 30 min (Glenney *et al.*, 1982*a*), the mixtures were diluted in 155 mM-ammonium acetate (pH 7.4)/100 μM-EDTA/10 μM-DTT/70% glycerol buffer and sprayed on freshly cleaved mica by the method of Shotton *et al.* (1979). The mica pieces were transferred to a rotatory-shadowing 'Balzers' apparatus to be shadowed with platinum/carbon, and finally they were coated with carbon film. The replicas so formed were floated on distilled water, and picked up on 300-mesh carbon-coated collodion grids. Specimens were examined and photographed in a Phillips EM 300 electron microscope at 80 kV.

Miscellaneous

Protein concentrations were determined by the methods of either Bradford (1976) or Lowry *et al.* (1951), with BSA as a standard. SDS/PAGE was carried out as described by Laemmli (1970).

RESULTS

Characterization of the NF-L domain that interacts with brain spectrin

BNPS-skatole is useful for cleaving tryptophan residues. When NF-L was cleaved by BNPS-skatole at Trp-278, two fragments were produced, one a 30 kDa *N*-terminal fragment and the other a 40 kDa *C*-terminal fragment (Geisler *et al.*, 1983; Mahboub *et al.*, 1986). Fig. 1 shows an autoradiogram of an overlay experiment with ^{125}I-labelled brain spectrin on BNPS-skatole hydrolysate products. Brain spectrin binds to the 30 kDa fragment of NF-L. Contrary to our earlier results (Frappier *et al.*, 1987), no binding is observed to the 70 kDa NF-L, since more than 80% of NF-L is cleaved in this experiment.

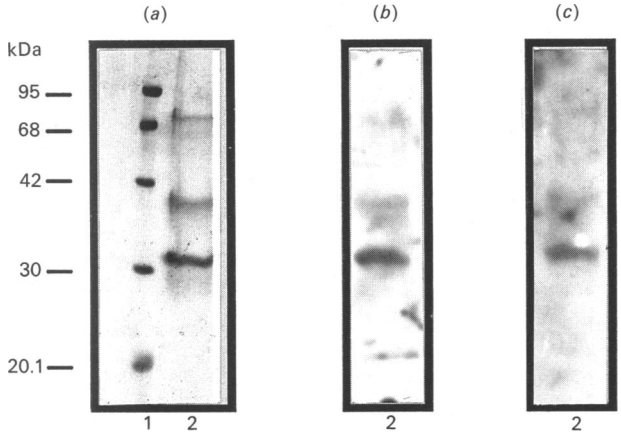

Fig. 1. Binding of ^{125}I-labelled brain spectrin and ^{125}I-labelled MAP2 to NF-L BNPS-skatole hydrolysate

Proteins were separated on SDS/5–15%-PAGE gels and stained with Coomassie Blue (*a*) or electrophoretically transferred to a nitrocellulose sheet that was then incubated with ^{125}I-labelled brain spectrin (*b*) or ^{125}I-labelled MAP2 (*c*) (specific radioactivity 0.1 μCi/μg). Lane 1, molecular-mass markers: phosphorylase *a*, 95 kDa; BSA, 68 kDa; actin, 42 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa. Lanes 2, BNPS-skatole hydrolysis products (15 μg): 70 kDa, 40 kDa and 30 kDa.

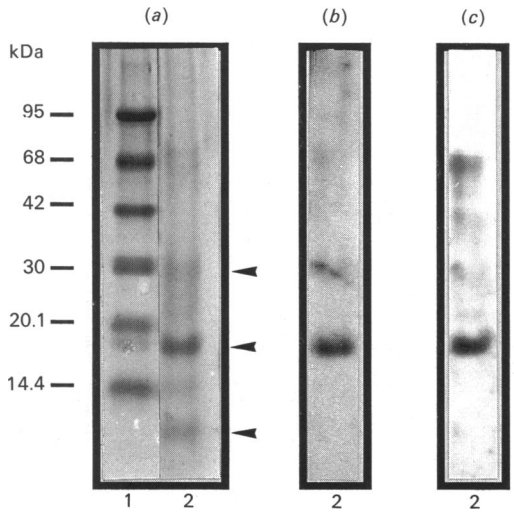

Fig. 2. Binding of ^{125}I-labelled brain spectrin and ^{125}I-labelled MAP2 to 30 kDa Lys-C endoproteinase hydrolysis products

Proteins separated by SDS/PAGE (5–20%) were stained with Coomassie Blue (*a*) or transblotted electrophoretically to nitrocellulose and incubated with either ^{125}I-labelled brain spectrin (*b*) or ^{125}I-labelled MAP2 (*c*) (specific radioactivity 0.11 μCi/μg). Lane 1, molecular-mass markers as in Fig. 1 but with the addition of α-lactalbumin, 14.4 kDa. Lanes 2, 30 kDa Lys-C endoproteinase hydrolysis products: 30 kDa, 20 kDa and 9 kDa (arrowheads).

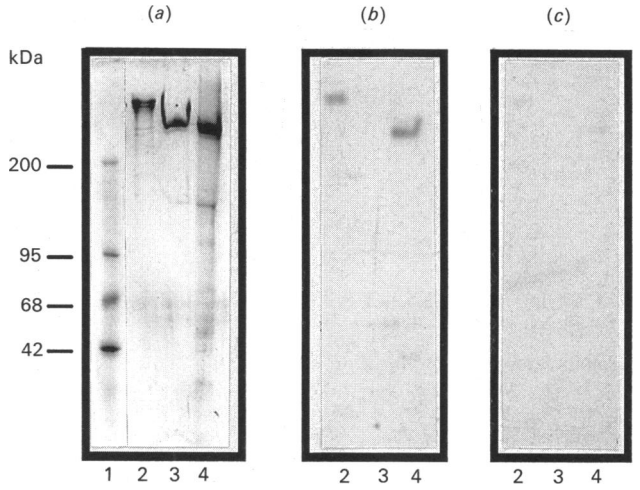

Fig. 3. Binding of polymerized or depolymerized ^{125}I-labelled NF-L to MAP2 and brain α- and β-spectrin

^{125}I-labelled NF-L was depolymerized by dialysis against 20 mM-Tris (pH 8.5)/1 mM-$MgCl_2$/1 mM-EDTA/1.5 mM-NaN_3 for 70 h at 4 °C. Non-diffusible material was centrifuged for 1 h at 100000 *g* in a 70.1 Ti Beckman rotor. MAP2 and brain spectrin subunits, separated by SDS/PAGE (4–10%), were stained with Coomassie Blue (*a*), or overlayed with 10 μg of either polymerized (*b*) or depolymerized (*c*) ^{125}I-labelled NF-L (specific radioactivity 0.33 μCi/μg). Lane 1, molecular-mass markers: myosin, 200 kDa; phosphorylase *a*, 95 kDa: BSA, 68 kDa; actin, 42 kDa. Lanes 2, MAP2 (2 μg). Lanes 3, Brain α-spectrin (1 μg). Lanes 4, Brain β-spectrin (1 μg).

This 30 kDa fragment electroeluted from the gel and hydrolysed by the Lys-C endoproteinase gives 9 kDa and 20 kDa hydrolysis products. Fig. 2 shows an autoradiogram of an overlay experiment in which ^{125}I-labelled brain spectrin is incubated with these hydrolysis fragments. Brain spectrin binds to the 20 kDa fragment.

The same experiments were done with ^{125}I-labelled MAP2. Fig. 1 shows that MAP2 interacts with NF-L on the 30 kDa domain, and Fig. 2 shows that it binds to the 20 kDa domain. NF-L is not present in this preparation (see the Materials and methods section), so the binding observed at the top of the autoradiogram is an artifact. Thus brain spectrin and MAP2 bind to the same domain of NF-L.

Determination of the brain spectrin subunit that binds to NF-L

The α- and β-subunits of brain spectrin were separated on a calmodulin–Sepharose column and electrophoresed for overlay experiments with either polymerized or depolymerized ^{125}I-labelled NF-L overnight at 4 °C. In these experiments MAP2 is used as a control. As shown in Fig. 3, the β-subunit is the only subunit of brain spectrin that binds to NF-L, as for MAP2. This binding of NF-L to the β-subunit of brain spectrin and MAP2 occurs strongly when NF-L subunits are polymerized, but for the depolymerized form only faint spots were observed, probably due to the binding of small NF-L protomers.

Determination of the subunit from erythrocyte spectrin that interacts with NF-L

Using the same experimental procedure, the behaviour of spectrins purified from human and avian erythrocytes was studied. Fig. 4 shows that for both spectrins strong binding is

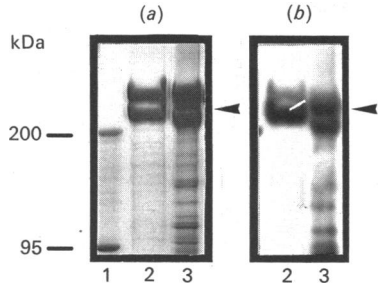

Fig. 4. Binding of ^{125}I-labelled NF-L to human and avian erythrocyte spectrins

Human and avian erythrocyte spectrins (10 μg) were separated on SDS/PAGE (4–10%), stained with Coomassie Blue (*a*), or transblotted to nitrocellulose and overlayed with 3.5 μg of polymerized ^{125}I-labelled NF-L/ml (specific radioactivity 0.15 μCi/μg). (*b*) Lane 1, Molecular-mass markers (see Fig. 3). Lanes 2, human erythrocyte spectrin. Lanes 3, avian erythrocyte spectrin. Arrows show synemin or β^1-spectrin.

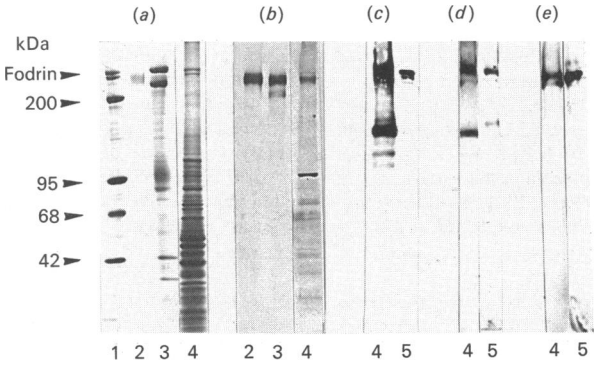

Fig. 5. Specificity of the antibodies used

(*a*) SDS/PAGE (4–12%) and Coomassie Blue staining. Immunoblot with anti-ankyrin (*b*), anti-(brain spectrin) (*c*), anti-(brain α-spectrin) (*d*) and anti-(brain β-spectrin) (*e*) antibodies. Lane 1, molecular-mass markers (see Fig. 3) and fodrin. Lanes 2, ankyrin. Lanes 3, human erythrocyte ghosts. Lanes 4, brain total extract. Lanes 5, fodrin.

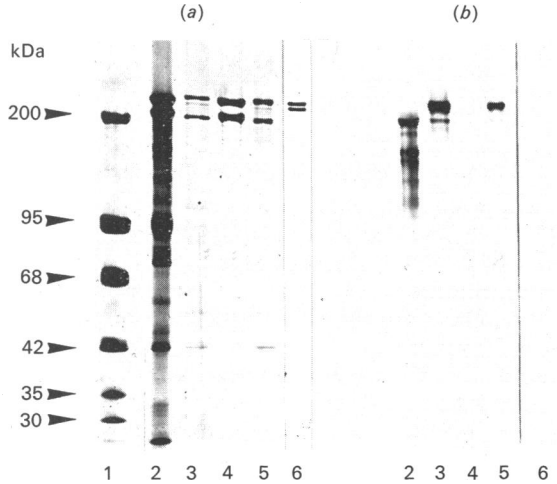

Fig. 6. Determination of the presence of ankyrin in spectrin preparations

(*a*) SDS/PAGE (4–12%) and Coomassie Blue staining. (*b*) Anti-ankyrin immunoblot. Lane 1, Molecular-mass markers (see previous figures). Lanes 2, human erythrocyte ghosts. Lanes 3, avian erythrocyte ghosts. Lanes 4, human erythrocyte spectrin. Lanes 5, avian erythrocyte spectrin. Lanes 6, fodrin.

observed for the β-subunit and weak binding for the α-subunit. These results are in good agreement with those obtained for brain spectrin.

To verify that our protein preparations were devoid of contaminating proteins, anti-(erythrocyte ankyrin), anti-(brain spectrin) or anti-(brain α- and β-spectrin) antibodies were used. As shown in Fig. 5, these antibodies are specific and react exclusively with the protein against which they were raised. The anti-(erythrocyte ankyrin) also reacted with two degradation products of ankyrin. One, of molecular mass 200 kDa, was observed in the erythrocyte ghost preparation (Fig. 5*b*, track 3), and the other of molecular mass 72 kDa, observed in total pig brain extract (Fig. 5*b*, track 4), has been shown to be the fragment that contains the spectrin-binding site (Davis & Bennett, 1984). We verified that our spectrin preparation was not contaminated with ankyrin, because this protein binds to desmin and vimentin intermediate filament proteins. As shown in Fig. 6, the brain spectrin and human erythrocyte spectrin were not contaminated by ankyrin; only the avian erythrocyte spectrin preparation contained small amounts of ankyrin. In the case of avian spectrin, a strong additional band was observed between the α- and β-subunit, probably corresponding to β^1-spectrin (Nelson & Lazarides, 1983; Moon & Lazarides, 1984) or to synemin (Granger *et al.*, 1982), a 230 kDa protein known to interact with intermediate filaments (Granger & Lazarides, 1982). Because of its high molecular mass (250 kDa) it would seem that this additional band is not due to goblin (ankyrin).

Electron microscopy of shadowed neurofilament samples

Neurofilaments were shown to have a rough surface with a periodicity of about 20 nm, and in addition some protomeric structures were seen (Fig. 7*a*). Isolated brain spectrin appears to have different morphology; it is found as a long and flexible molecule of about 200 nm (Fig. 7*b*). When spectrin is incubated with neurofilaments, it appears to bind mainly along the filament (Fig. 7*c*). Nevertheless a possible cross-link at the end of the filament is also observed (Fig. 7*d*). Fig. 7(*e*) shows associations between brain spectrin and neurofilament protomers. Fig. 7(*f*) shows isolated protomers.

Determination of the domain of the β-subunit of brain spectrin that interacts with NF-L

The peptides obtained by proteolysis of brain spectrin with TPCK-treated trypsin were incubated with NF-L and sedimented. The origin of the peptides sedimented with NF-L were determined with antibodies to the α- and β-subunits of brain spectrin. Three peptides of average molecular mass 78, 43 and 42 kDa were found to be hydrolysis fragments of the β-subunit, whereas one major band of 92 kDa, with two minor bands of 120 and 116 kDa, which also co-sedimented with NF-L, reacted positively towards anti-(α-spectrin) antibodies. There were also other polypeptides of lower molecular mass (35, 25, 20 and 18 kDa) that co-sediment with NF-L, but we could not determine their subunit origins by the method used. The same profile of peptides co-sedimenting with F-actin was observed (Fig. 8). Some of these peptides sedimented in the control experiment without NF-L or F-actin. For this reason, we quantified the sedimenting peptides by analysing the gel with a microdensitometer. The pelleted fraction in Fig. 9 is given as a percentage of the control experiment. We observed that the low-molecular-mass peptides sedimented alone, but it appears that the pelleted fraction is larger in the presence of NF-L or F-actin than without. Because molecular masses are close to those of NF-L and F-actin, we were unable to quantify the ratio of the 78 kDa, 43 kDa and 42 kDa peptides in the assays. This preparation was totally free of ankyrin (Fig. 10).

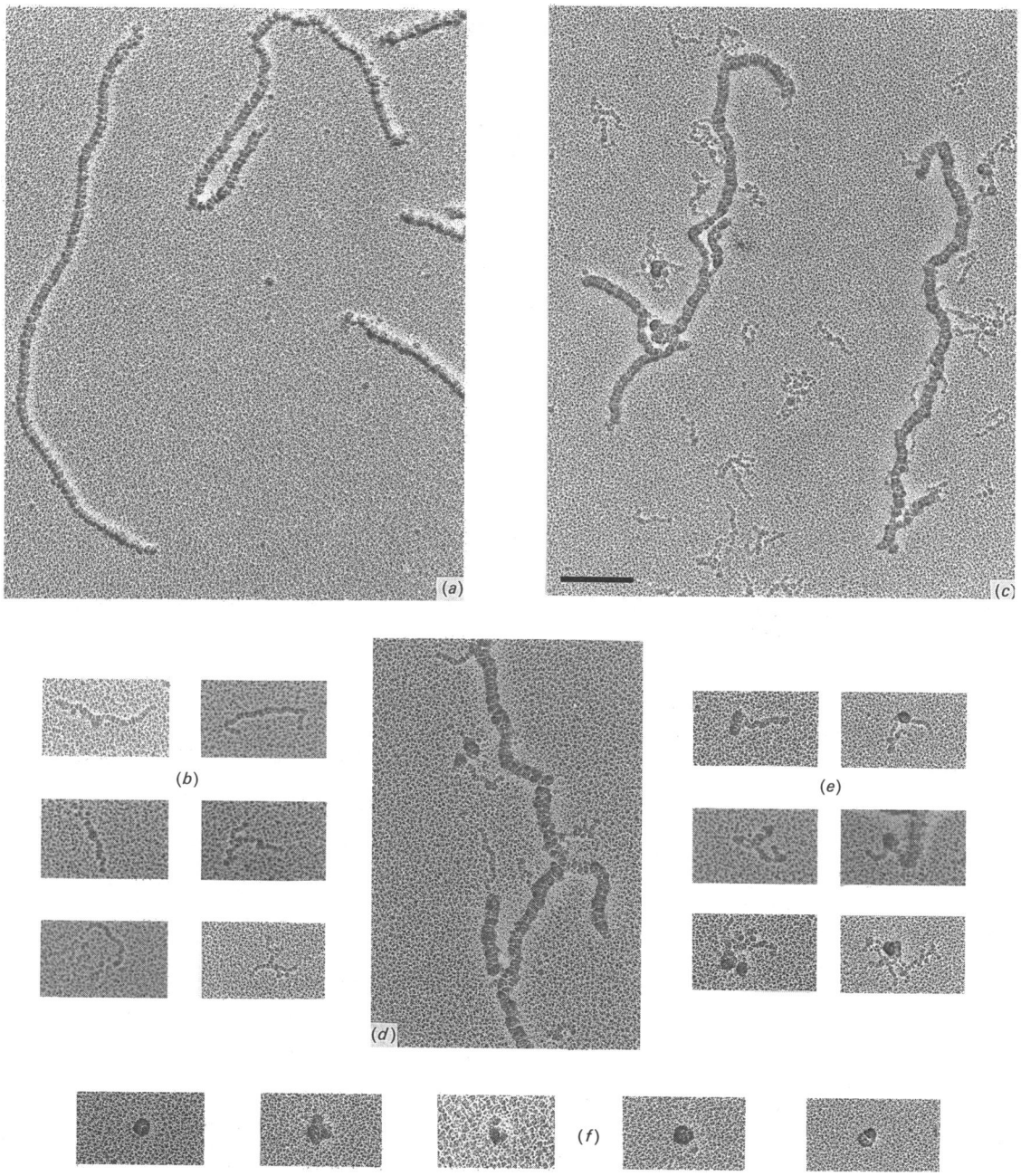

Fig. 7. Electron micrographs of brain spectrin and neurofilaments

(*a*) Neurofilaments. (*b*) Brain spectrin. Cross-links between brain spectrin and neurofilaments: along the filaments (*c*), at the end of the filaments (*d*) and to small neurofilament protomers (*e*). (*f*) Isolated neurofilament protomers. Magnification × 52000; bar = 200 nm.

DISCUSSION

The purpose of our research was to locate the binding domains for the interaction of NF-L with brain spectrin. This study was developed in two steps; the first was the determination of the binding domain on the NF-L subunit protein and the second was the determination of the binding site on the brain spectrin.

The experiments for localization of the NF-L binding site led us to conclude that brain spectrin and MAP2 bind to a 20 kDa domain on NF-L that corresponds to the proximal part of the rod, i.e. from *N*- to *C*-termini, helix 1a, linker 1, helix 1b, linker L12 and finally 23 amino acid residues of helix 2 (Geisler *et al.*, 1983). It may be noted that a postulated polymerization process occurs via the interaction of intermediate filament chains by their rod domains to form first a dimer and then a tetramer. The tetrameric form is the smallest unit observed when intermediate filaments are depolymerized *in vitro* at low ionic strength and slightly alkaline pH (for a review see Steinert & Roop, 1988). Part of this domain alone, helix 1b, was shown, in avian desmin, to be able to form dimeric, trimeric and tetrameric structures (Saeed & Ip, 1989). When brain spectrin subunits, or MAP2, are overlayed with either polymerized or depolymerized NF-L, a stronger band is observed with the polymeric structure. A possible explanation for this is that when ^{125}I-labelled NF-L tetramer binds to brain β-spectrin or MAP2 it blocks the binding of other tetramers to the same β-subunit or MAP2, whereas binding of ^{125}I-labelled NF-L filaments to β-subunit or MAP2 leads to the binding of many more protomers, thereby increasing the labelling

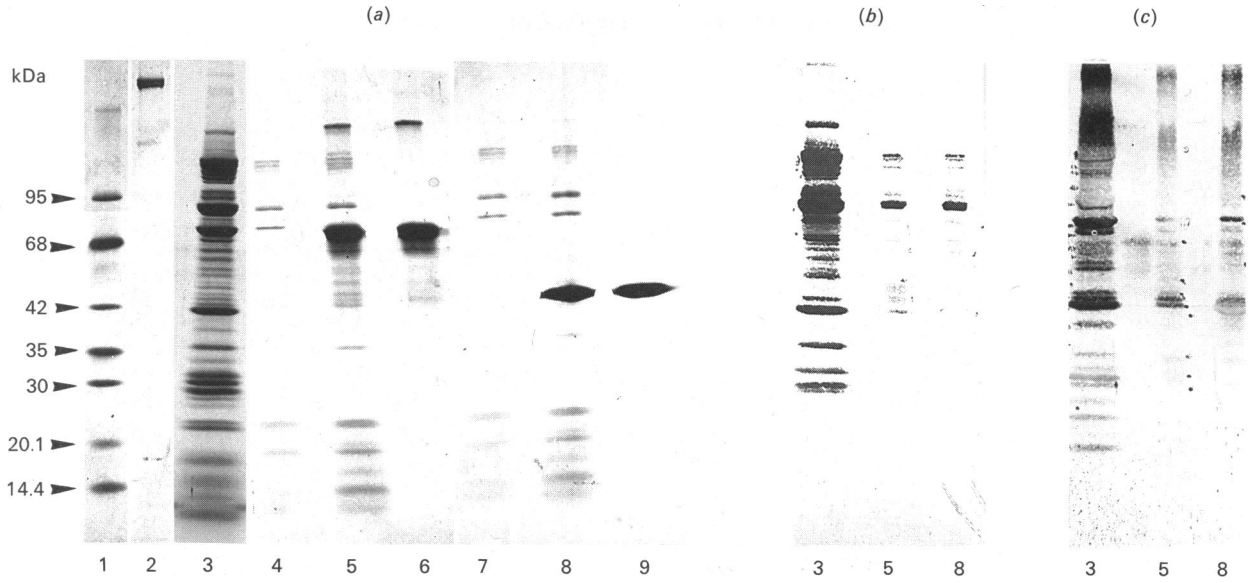

Fig. 8. Determination of the subunit origin of the tryptic peptides that co-sedimented with either microfilaments or neurofilaments

Antibodies were used at a 1 μg/ml dilution. (*a*) Coomassie Blue staining. Lane 1, low-molecular-mass markers. Lane 2, brain spectrin (5 μg). Lanes 3, total tryptic hydrolysate (100 μg). Lane 4, pelleted spectrin peptides from 100 μg of hydrolysate in NF-L condition control. Lanes 5, pelleted peptides with NF-L from 100 μg of hydrolysate. Lane 6, pelleted NF-L in control experiment. Lane 7, pelleted spectrin peptides from 100 μg of hydrolysate in F-actin control experiment. Lanes 8, pelleted peptides with F-actin from 100 μg of hydrolysate. Lane 9, pelleted F-actin in control experiment. (*b*) Anti-(brain α-spectrin) immunoblot. (*c*) Anti-(brain β-spectrin) immunoblot.

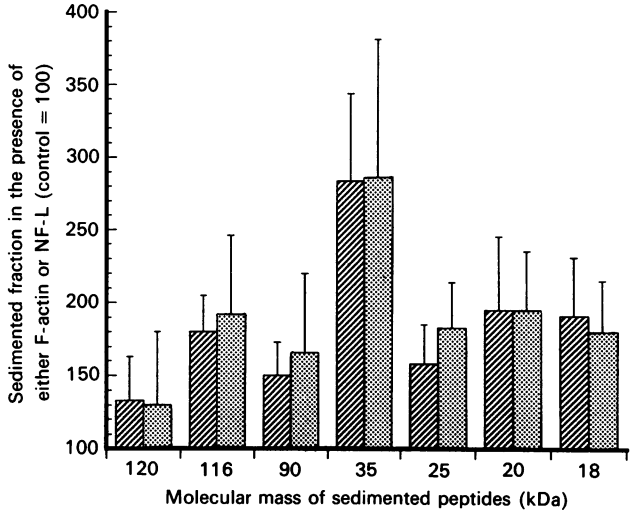

Fig. 9. Determination of the percentage of pelleted peptides over the control experiment

The control experiments are referred to 100. The percentage of sedimented peptides was determined by microdensitometric analysis of the SDS/PAGE gels ($n = 6$). ▨, F-actin, ⊡, NF-L.

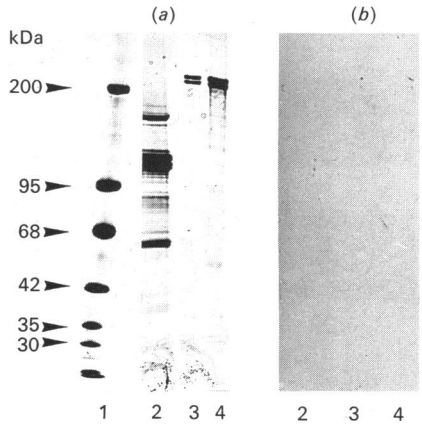

Fig. 10. Demonstration of the lack of ankyrin in the tryptic spectrin hydrolysate

(*a*) SDS/PAGE (4–12%) with Coomassie Blue staining. (*b*) Anti-ankyrin immunoblot. Lane 1, molecular-mass markers (see previous Figures). Lanes 2, tryptic hydrolysate (100 μg). Lanes 3, fodrin (1 μg). Lanes 4, fodrin (2 μg).

observed on the autoradiogram. Furthermore when ^{125}I-labelled spectrin or MAP2 overlay the NF-L 20 kDa domain, a strong band was observed. Finally, direct observation of the binding of brain spectrin to neurofilaments is provided by electron-microscopy images. Electron micrographs show brain spectrin protruding from neurofilaments apparently bound via one of its two ends. In some cases, binding of brain spectrin occurs at the end of the neurofilaments, and interestingly, according to our overlay experiments, to neurofilament protomers.

It is well known that intermediate filaments emanate from a perinuclear network and extend throughout the cytoplasm, terminating at the plasma membrane. Binding of desmin and vimentin to the nuclear membrane, by lamin B, was described by Georgatos *et al.* (1987). This binding occurs via the *C*-terminus of desmin and vimentin. On the other hand, binding of vimentin and desmin to the plasma membrane occurs via ankyrin (Georgatos & Marchesi, 1985; Georgatos *et al.*, 1987). These authors described an interaction via the basic *N*-terminal domains of these intermediate filament proteins. They showed that ankyrin binds to small polymers (tetramer) and prevents further assembly into filaments.

We describe here a possible link between the plasma membrane and the neurofilaments. Ankyrin and brain spectrin bind to intermediate filaments in two different ways. Ankyrin binds to tetramers of vimentin and desmin via their *N*-termini, whereas brain spectrin binds to filaments, as well as to protomers, via

their rod domains. This difference in binding domain may be important physiologically. This indirect binding to membrane via spectrin gives an easily deformable structure, as for the association of F-actin with spectrin, because of the highly flexible structure of spectrins.

The fact that NF-M and NF-H do not bind to brain spectrin can be explained by the fact that neither of them can form homopolymers *in vitro* (Moon *et al.*, 1981; Geisler & Weber, 1981; Liem & Hutchinson, 1982; Zackroff *et al.*, 1982).

The second part of our study was the determination of the binding domain on brain spectrin. The first finding was that the β-subunit is the binding subunit, not only for brain spectrin, but also for human and avian erythrocyte spectrins. During this study, the behaviour of MAP2 was compared with that of spectrin, because MAP2 has been shown to bind to F-actin and NF-L in the same way as spectrin.

MAP2 is a protein with an L shape that gives, on tryptic or chymotryptic proteolysis, a 30 kDa *C*-terminal domain and a 240 kDa *N*-terminal domain (Vallee & Borisy, 1977; Kim *et al.*, 1979). The 30 kDa domain contains the binding domain(s) for microtubules (Joly *et al.*, 1989), F-actin (Sattilaro, 1986) and neurofilaments (Flynn *et al.*, 1987). If MAP2 possesses a common binding site for F-actin and neurofilaments, it can be suggested that spectrin, which also binds to F-actin and to neurofilaments, possesses, like MAP2, a common binding site for the two cytoskeletal structures.

There is at present little information about the binding site of brain spectrin to F-actin. From electron-microscopy experiments and biochemical data, spectrin has been shown to bind to microfilaments by the end of the tetramer (Glenney *et al.*, 1982*a*), which seems to be composed of the *C*-terminus of the α-subunit and the *N*-terminus of the β-subunit (Speicher & Marchesi, 1984; Byers *et al.*, 1989; Dubreuil *et al.*, 1989).

These data suggest that, like F-actin, NF-L binds to the *N*-terminus of the β-subunit, since overlay experiments have provided evidence of it binding to the β-subunit of brain spectrin, and also human and avian erythrocyte β-spectrins.

Because of the high sensitivity of isolated brain β-spectrin to proteinases, we carried out a mild tryptic hydrolysis on native brain spectrin (Harris & Morrow, 1988); for this reason, NF-L or F-actin was incubated and sedimented with heterodimeric fragments of brain spectrin, and both α- and β-subunit fragments were found in the pellets. Antibodies to brain α- and β-spectrin allowed us to determine the origin of the tryptic peptide subunits. We have identified some peptides that co-sediment with NF-L. The same peptides co-sediment with microfilaments. The peptides of lower molecular mass are not recognized by the antibodies used. Nevertheless, these preliminary results strongly suggest that NF-L binds to the same domain of brain spectrin as F-actin. The purification of the lower-molecular-mass peptides that co-sedimented with NF-L and F-actin might allow us to localize the peptides to one of the brain spectrin subunits.

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