Presence of a Protein Immunologically Related to Lamin B in the Postsynaptic Membrane of *Torpedo marmorata* Electrocyte

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Abstract. The *Torpedo* electrocyte is a flattened syncytium derived from skeletal muscle, characterized by two functionally distinct plasma membrane domains. The electrocyte is filled up with a transversal network of intermediate filaments (IF) of desmin which contact in an end-on fashion both sides of the cell. In this work, we show that polyclonal antibodies specific for lamin B recognizes a component of the plasma membrane of *Torpedo* electrocyte. This protein which thus shares epitopes with lamin B has a relative molecular mass of 54 kD, an acidic IP of 5.4. It is localized exclusively on the cytoplasmic side of the innervated membrane of the electrocyte at sites of IF–membrane contacts.

Since our previous work showed that the noninnervated membrane contains ankyrin (Kordeli, E., J. Cartaud, H. O. Nghiêm, L. A. Pradel, C. Dubreuil, D. Paulin, and J.-P. Changeux. 1986. *J. Cell Biol.* 102:748–761), the present results suggest that desmin filaments may be anchored via the 54-kD protein to the innervated membrane and via ankyrin to the noninnervated membrane. These findings would represent an extension of the model proposed by Georgatos and Blobel (Georgatos, S. D., and G. Blobel. 1987*a*. *J. Cell Biol.* 105:105–115) in which type III intermediate size filaments are vectorially inserted to plasma and nuclear membranes by ankyrin and lamin B, respectively.

THE interaction of intermediate sized filaments (IF)[1] with membranes was until now poorly documented. Recently, various reports have indicated that type III IF (vimentin–desmin) are nucleated on lamin B of the nuclear envelope (Georgatos and Blobel, 1987*a*,*b*; Georgatos et al., 1987), then vectorially assembled in the cytoplasm and finally capped at the plasma membrane via ankyrin (Georgatos and Marchesi, 1985; Georgatos et al., 1985). These observations may have important implications for the understanding of IF–membrane interactions in other systems. The peculiar cellular architecture of *Torpedo* electrocyte offers a unique model for the investigation of such interactions. Moreover, the innervated plasma membrane domain is of special interest, since it constitutes a useful model system for the deciphering of the molecular events involved in the stabilization of the synaptic nicotinic acetylcholine receptor (AchR) (see Changeux, 1981; Kordeli et al., 1987*a*).

The electrocyte is a flattened syncytium embryologically derived from skeletal muscle, and it receives a dense cholinergic innervation on one of its surfaces. This results in a highly asymmetric structure characterized by two functionally distinct plasma membrane domains: the innervated, rich in AchR; and the noninnervated, rich in Na^+, K^+ ATPase. The cytoplasm of the cell is filled with a dense transversal network of desmin filaments which contact in an end-on fashion both membrane domains of the cell (Kordeli et al., 1986; Sealock et al., 1989; Walker et al., 1985).

To each plasma membrane domain corresponds a specialized cortical skeleton. Peripheral proteins such as the relative molecular mass 43 kD, also called ν_1-protein (Gysin et al., 1981), which is directly engaged in AchR immobilization (Barrantes et al., 1980; Lo et al., 1980; Cartaud et al., 1981; Rousselet et al., 1982) and a quantitatively minor component of relative molecular mass 58 kD of unknown function (Froehner, 1984; Froehner et al., 1987) are found beneath the innervated membranes. The noninnervated membrane is associated with a membrane skeleton which includes actin polymers, ankyrin, and fodrin (Kordeli et al., 1986, 1987*a*,*b*).

In this work, we have investigated the possible implication of capping proteins, in the insertion of desmin filaments in the two opposite membrane domains of the electrocyte. Using a serum from a patient with systemic lupus erythematosus, which contains polyclonal antibodies directed against lamin B (Guilly et al., 1987), we have identified an immunoreactive component with a relative molecular mass of ~54

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; IEF, isoelectrofocusing; IF, intermediate filaments; IP, isoelectric point.

kD and isoelectric point (IP) ~5.4, present exclusively on the cytoplasmic side of the innervated membrane in particular at sites of IF–membrane contacts. We suggest that some of the epitopes common to the 54-kD protein and lamin B are of functional significance and involved in the anchoring of desmin filaments to the innervated membrane.

Materials and Methods

Chemicals

Ampholines were purchased from LKB Instruments Inc. (Bromma, Sweden); SDS-PAGE molecular mass standards and isoelectrofocusing (IEF) standards from Bio-Rad Laboratories (Cambridge, MA); ultrapure urea from Schwarz/Mann (Spring Valley, NY). Nitrocellulose paper (BA 85, membrane filters: 0.45 μm) was from Schleicher & Schuell, Inc. (Keene, NH). Phosphatase alkaline-conjugated goat anti-human was from Promega Biotec (Madison, WI). 5-Bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate system for electrophoretic blots and rhodamine-conjugated goat anti-human IgG were from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD); fluorescein-labeled α-bungarotoxin was from Sigma Chemical Co. (St. Louis, MO), colloidal gold (10 nm) conjugated to IgG was from Janssen Pharmaceutica (Beerse, Belgium).

Anti-Lamin Antibodies

Human auto antibodies directed against lamin B used in this study have been previously characterized (Guilly et al., 1987). In some experiments another human autoantibody to lamin A and C was also used (Mc Keon et al., 1983).

Preparation of Pore Complex/Lamina Fractions

Pore complex/lamina fractions from rat liver and *Torpedo* liver were prepared according to Gerace et al. (1984), with slight modifications (Guilly et al., 1987). Briefly minced rat and *Torpedo* liver were homogenized in 2.2 M sucrose in 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5 (TKM) containing 1 mM PMSF, 10 mM *N*-ethyl-maleimide with a Chauveau homogenizer (10 strokes). Nuclei were collected by sedimentation through a 30% sucrose cushion in the same buffer. These were then digested with DNase I (20 μg/ml) and RNase A (20 μg/ml) for 1 h at 4°C. Nuclei envelopes were collected similarly. Purified nuclear lamina were obtained by extraction of nuclear envelopes with 0.6 M NaCl and 4% Triton X-100 in TKM and stored at −70°C in 90% glycerol until further analysis.

Preabsorption of Serum F

Serum F (1:200 dilution) was incubated overnight at 4°C with a pellet of purified rat liver nuclear lamina (see above). After centrifugation (12,000 *g*, 30 min) the supernatant was used for immunoblotting and immunocytochemical experiments.

Alternatively, preabsorption of serum F (1:200 dilution) with partially renatured lamin B (the 68-kD band) purified by SDS-PAGE from rat liver was achieved.

Preparation of Torpedo AchR-rich Membranes

AchR-rich membranes were purified from fresh adult electric tissue according to Saitoh and Changeux (1980) and Nghiêm et al., (1983).

SDS-PAGE and IEF

AchR-rich membranes or pore complex/lamina fractions were separated on one-dimensional 10% SDS-PAGE according to Laemmli (1970) using a slab cell (Miniprotean II; Bio-Rad Laboratories) operating at 200 V for 45 min. For pore complex/lamina fractions, proteins were allowed to migrate a further 15 min at 200 V after the dye front reached the bottom of the gel to obtain good separation of the three lamins.

Two-dimensional gel electrophoresis was performed according to O'Farrell (1975) with the following modifications: 40 μl of AchR-rich membranes (~50-μg proteins) were dissolved in 1% Triton X-100, 0.02% SDS for 10 min before the addition of first dimension sample buffer lacking SDS. IEF gels were made in 2% ampholine 3,5–10, 2% ampholine 5–7, and 2% Ampholine 7–9 for 4 h at 400 V and then 30 min at 800 V. Pore complex/lamina fractions (20–40-μg proteins) were dissolved in 1% Triton X-100, 0.02% SDS for 10 min before the addition of the first dimension sample buffer lacking SDS. IEF experiments were made in 1% ampholine 3.5–10, 4% ampholine 5–7, and 1% ampholine 7–9 for 5 h at 450 V and 30 min at 800 V. Electrophoresis was carried out in 10% polyacrylamide gels containing 0.15% SDS.

Immunoblots

Proteins separated by one- or two-dimensional SDS-PAGE were electrophoretically transferred to nitrocellulose paper (Towbin et al., 1979), stained with Ponceau red (Coudrier et al., 1983), then incubated with serum F (1:200 to 1:1,000 dilution). Alkaline phosphatase–conjugated goat anti-human IgG were used as a second antibody and the color reaction developed with the 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium reaction.

Immunofluorescence Microscopy

Preparation of cryostat sections of *Torpedo* electric organ has been previously described (Kordeli et al., 1986). Serum F (1:200 and 1:500 dilution) was used as first antibody and was revealed by the addition of rhodamine-conjugated goat anti-human IgG. Fluorescein-conjugated α-bungarotoxin was used to identify the postsynaptic membrane of the electrocyte. The neural region of 3% paraformaldehyde fixed rat diaphragm muscle was excised using underneath lighting. Small strips were then infiltrated with sucrose, and subsequently, rapidly frozen and sectioned (mean thickness ~4 μm). Double immunofluorescence labeling was performed as above. Stained sections mounted using cityfluor as antibleach (City University, London, UK) were observed with a Leitz Aristoplan microscope equipped with epifluorescence illumination (filters I_3 and N_2 for fluorescein and rhodamine) and with 63×, Na 1.4 or 100×, Na 1.32 immersion optics and were photographed with Kodak Tri-X or Ektachrome 400 films (exposure index 400 ASA).

Immunogold Labeling

The immunogold labeling of the electric tissue was carried out on cryostat sections, as previously described (Nakane, 1982; Kordeli et al., 1986).

Results

Characterization of the Anti-Lamin B Antibodies

Serum F previously characterized in mammals (Guilly et al., 1987) was checked on *Torpedo marmorata* liver nuclear envelopes. A major immunoreactive component with a relative molecular mass of ~68 kD close to that of rat liver lamin B (Fig. 1 *a*, lanes *1* and *2*) was detected in *Torpedo* (Fig. 1 *a*, lanes *3* and *4*). Further characterization of the 68-kD protein was achieved by immunoblotting of equilibrium pH gradient electrophoresis followed by SDS-PAGE. The ~68-kD immunoreactive component disclosed an acidic IP of ~5.8 ± 0.2 (Fig. 1 *b*). Direct comparison with rat liver lamin B was achieved by immunoblotting of the two lamina fractions separated on the same gel (Fig. 1, *c* and *d*). *Torpedo* lamin B clearly exhibits a slightly lower molecular weight and a less acidic IP compared to rat lamin B.

Subcellular Localization of Anti-Lamin B Immunoreactivity in Torpedo Electric Tissue

Serum F was used in double fluorescence experiments carried out on cryostat sections of electric organ tissue. Transversal sections stained with a fluorescent derivative of α-bungarotoxin allowed unambiguous identification of the innervated membrane surface of the electrocytes (Fig. 2, *a* and *d*). Double fluorescence experiments with serum F (1:200 dilution) showed that the two fluorescences coincided at the level of the innervated membranes (Fig. 2, *b* and *e*).

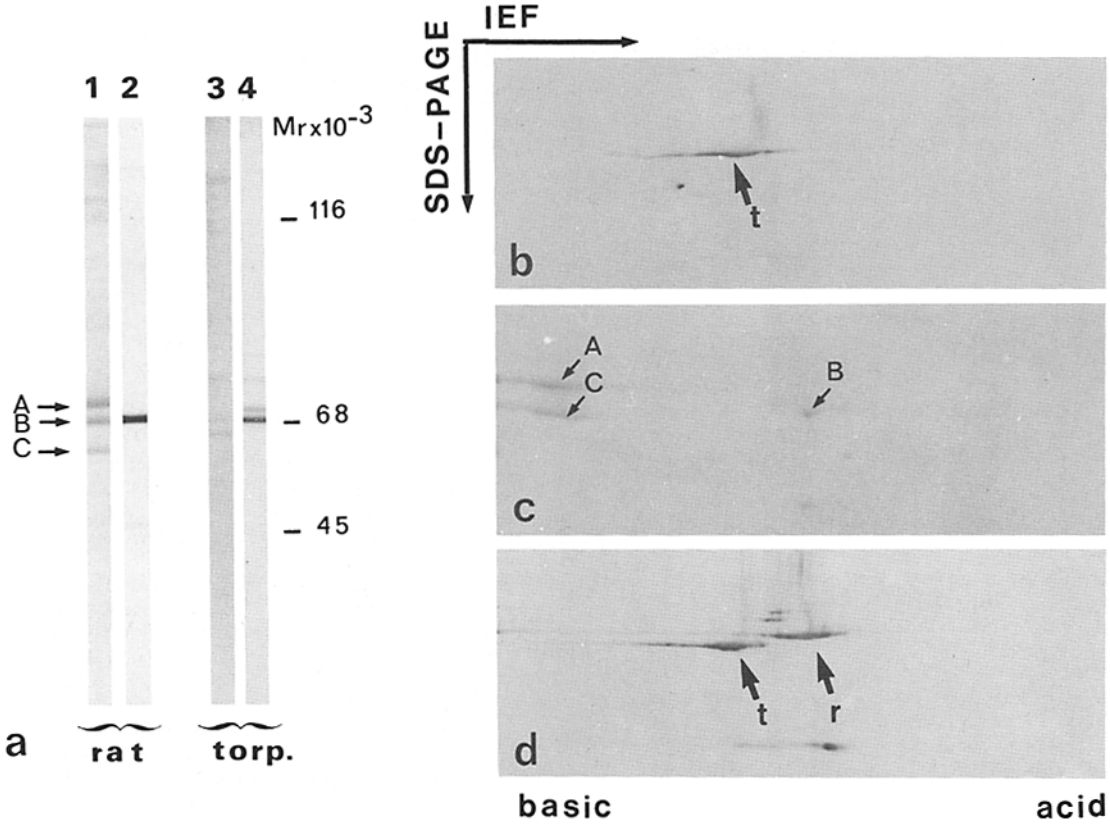

Figure 1. Characterization of serum F on rat and *Torpedo* nuclear lamina fractions. (*a*) Immunoreplicas of one-dimensional SDS-PAGE of lamina/pore complex fractions from rat liver (lane *1*, Ponceau red staining; lane *2*, serum F, dilution 1:250) and *Torpedo* liver (lane *3*, Ponceau red staining; lane *4*, serum F 1:250 dilution). The ~68-kD component (type B lamin) in each fraction was recognized with serum F. (*b*) Immunoblot experiment of an IEF/SDS-PAGE from *Torpedo* pore/lamina complex showing that the immunoreactive component (*arrow, t*) possessed an acidic IP (~5.8) (serum F 1:1,000 dilution). (*c*) Two-dimensional Coomassie blue staining of a rat pore/lamina complex in which lamins A, B, and C are shown (*arrows*). (*d*) Two-dimensional immunoblot experiment carried out on a mixture of pore/lamina complexes from rat (*r*) and *Torpedo* (*t*). Rat and *Torpedo* lamins of type B (*arrows*) are revealed with serum F (1:1,000 dilution). The 46-kD degradation peptides are also observed.

In addition, the periphery of the nuclei was also labeled with serum F. The cytoplasm, the noninnervated membrane and the network of IF were not labeled. To demonstrate that anti-lamin B antibodies of the serum were specifically involved in the immunostaining of the innervated membrane, inhibition experiments were carried out with serum F (1:200 dilution) absorbed on purified rat nuclear lamina. Under these conditions, we have verified (Fig. 1 *a*, lane *2* and Fig. 4, lanes *2* and *3*) that the only component recognized by serum F was a protein with a relative molecular mass of ~68 kD corresponding to lamin B. Preabsorption resulted in a complete extinction of the fluorescence associated with the innervated membranes and nuclear periphery (Fig. 2 *c*). Alternatively, preabsorption of serum F with lamin B purified by SDS-PAGE resulted in incomplete inhibition as observed by immunoblot and immunofluorescence experiments (not shown).

Immunogold staining further disclosed that the labeling was associated with the cytoplasmic surface of the innervated membrane (Fig. 3 *a*). At high magnification, the labeling occurred at regularly spaced patches (Fig. 3, *a–c*). The dotted distribution of this staining contrasted sharply with the almost continuous labeling previously described for two major components of the innervated membrane, the nicotinic AchR and the 43-kD protein (Nghiêm et al., 1983; Kordeli et al., 1986, 1987*a*; Sealock et al., 1984). Furthermore, close examination of the innervated membrane revealed that the labeling was frequently present at sites where IF contact the membrane (Fig. 3, *b* and *c*). As predicted by immunofluorescence experiments, the noninnervated membrane and the network of desmin-filaments were not labeled (Fig. 3 *d*). Labeling of the innervated membrane was not observed with antibodies to lamin A and C (not shown).

Biochemical Identification of the Immunoreactive Component of the Innervated Membrane

Immunoblotting experiments (1 and 2D) were carried out on membrane fractions highly enriched in the innervated membrane (~80%). Contaminants mostly derive from the noninnervated membrane but not from nuclear envelope, as shown by electron microscopy (Cartaud et al., 1978).

In one-dimensional analysis, serum F revealed a component of ~54 kD (Fig. 4 *a*, lane *4*). Preabsorption of serum F with purified rat liver nuclear pore/lamina fraction prevented the immunodetection of lamin B in rat liver nuclear lamina (Fig. 4 *a*, lanes *1–3*) and abolished the immunodetection of the 54-kD component in *Torpedo* mem-

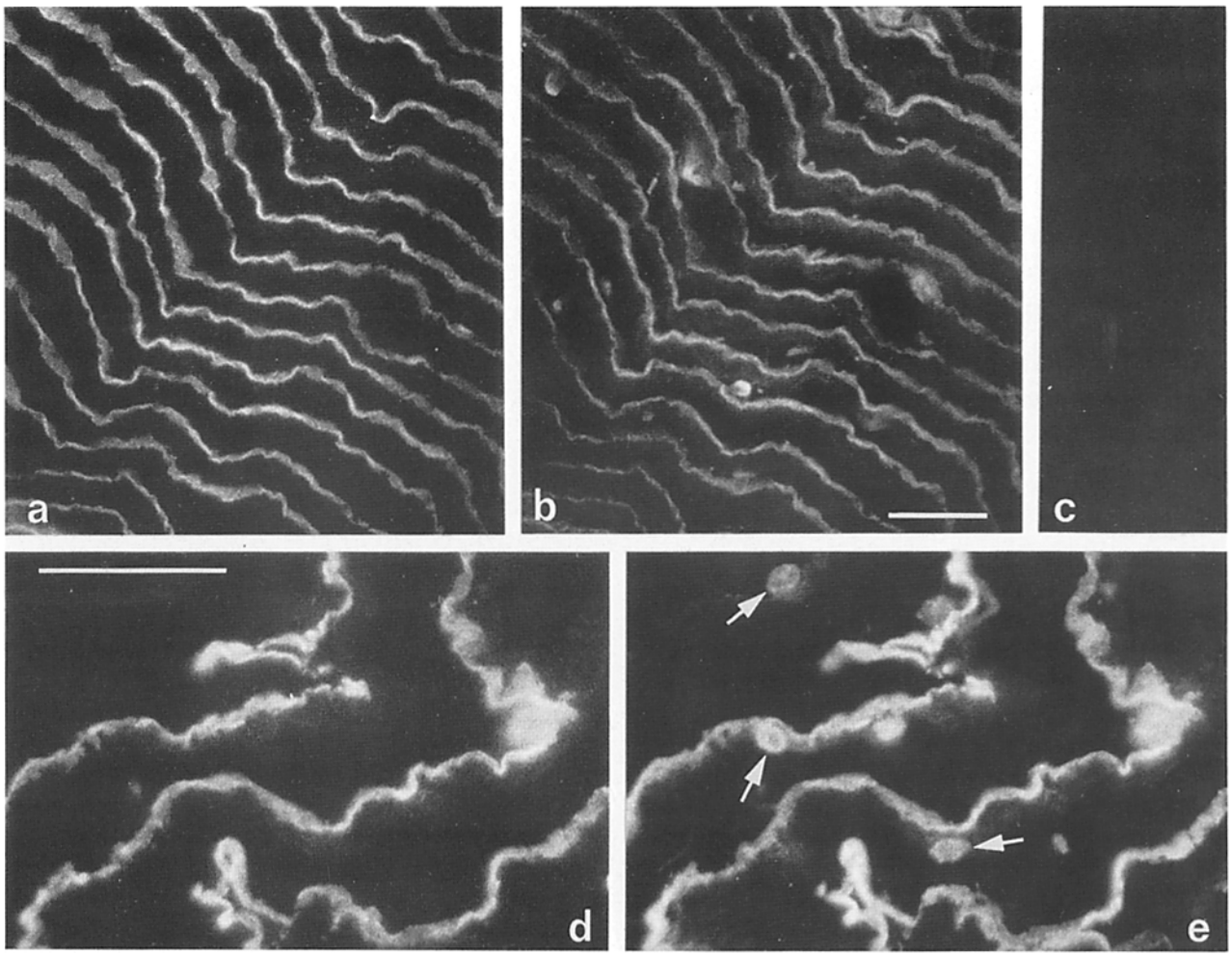

Figure 2. Immunofluorescence localization of lamin B immunoreactivity in membranes of *Torpedo* electric tissue. Double fluorescence experiments were performed on cryostat sections of *Torpedo* electric tissue. Note the codistribution of the α-bungarotoxin (*a* and *d*) and anti-lamin B (*b* and *e*) stainings at the innervated face of the electrocytes. Nuclei were also labeled in *b* and *e* (*arrows*). Control experiment (*c*) was performed using anti-lamin B antibodies absorbed with purified rat nuclear lamina (see also Fig. 4 for corresponding immunoblotting experiments). Bars, 20 μm.

brane fractions (Fig. 4 *a*, lanes *4* and *5*). Since the only component of the pore/lamina complex recognized by serum F is lamin B (Fig. 1 *a*, lane *2* and Fig. 4, lanes *2* and *3*) this inhibition experiment demonstrated that lamin B and the 54-kD protein of the innervated membrane share common epitopes. As expected from the immunofluorescence experiments antibodies to lamin A and C did not label this 54-kD or any other proteins in immunoblot experiments (not shown).

Further characterization of the 54-kD protein was achieved by immunoblotting of two-dimensional SDS-PAGE/IEF gel (Fig. 4, *b* and *c*). In these experiments, serum F revealed a spot with an IP of ~5.4. Quantitatively, this 54-kD protein represents a minor component of the innervated membrane. It was not detectable by Coomassie blue staining.

Immunofluorescence Localization of Anti-Lamin B Immunoreactivity in Rat Muscle

To check if the presence of a protein immunologically related to lamin B was not merely a singularity of the innervated membrane of the electric tissue, we looked for lamin B immunoreactivity at rat neuromuscular junctions where the postsynaptic membrane is also a potential site of IF insertion.

Double fluorescence experiments conducted on cryostat sections of adult rat diaphragm muscle showed that anti-lamin B reactivity was, in addition to the nuclei, associated with motor endplates (identified by α-bungarotoxin labeling). On transversal sections (Fig. 5) the labeling coincided with α-bungarotoxin at each gutter. No significant labeling was observed on the sarcolemma outside the synaptic area.

Discussion

In most cells, the IF network appears to be connected to both plasma membrane and perinuclear envelope (Lazarides, 1980; Geiger, 1987). Recently, the interactions of two type III-IF proteins (vimentin and desmin) with the plasma membrane and the nuclear lamina have been analyzed in vitro by reconstitution experiments. The site of attachment of vimentin to the avian erythrocyte plasma membrane resides in the 55-60-kD domain of ankyrin; a major constituent of the submembrane skeleton (Georgatos and Marchesi, 1985). This

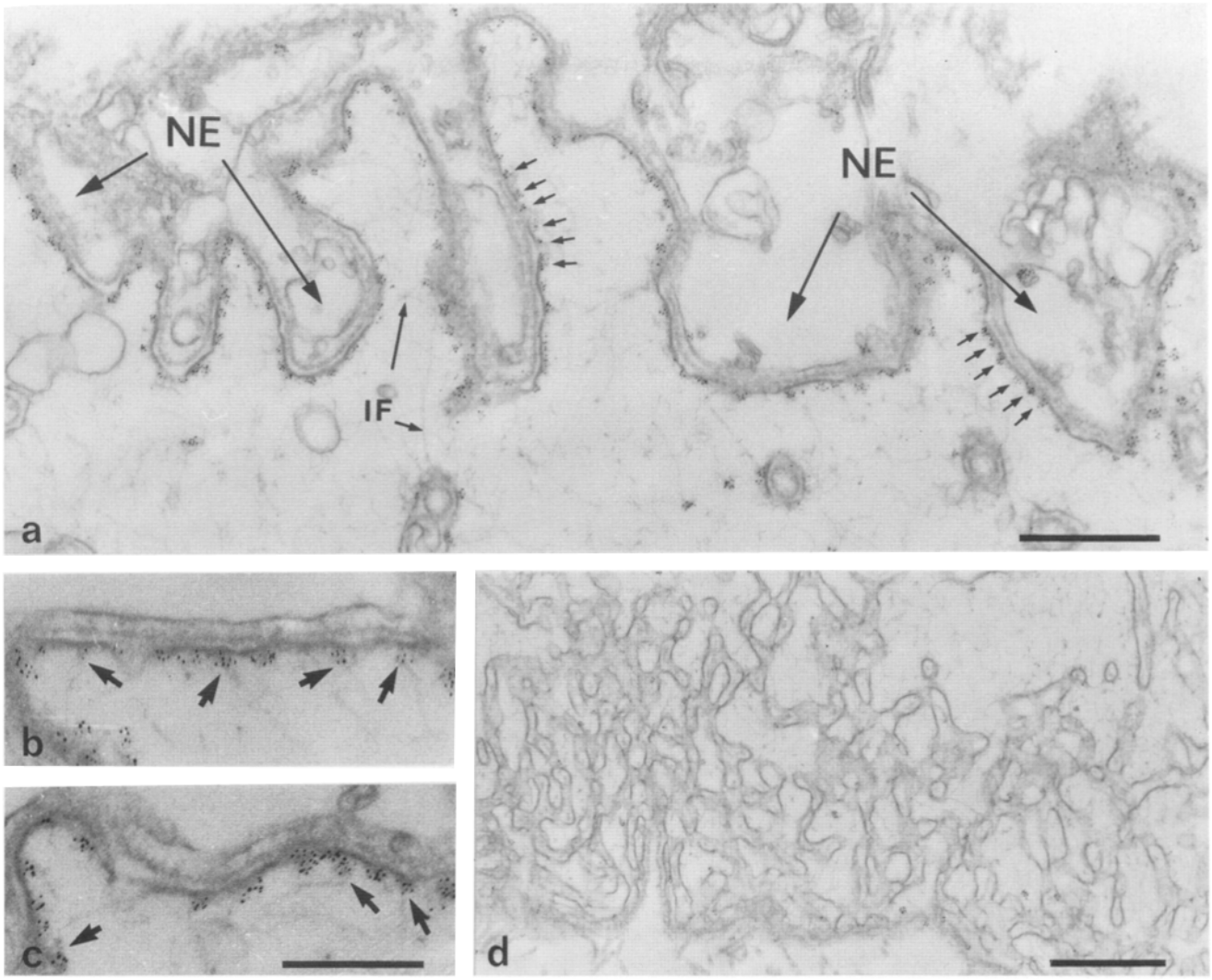

Figure 3. Immunogold localization of lamin B immunoreactivity in *Torpedo* electric tissue. A discontinuous labeling of the cytoplasmic surface of the innervated membrane domain of the cell (*a*) was observed (*IF*, intermediate filaments; *NE*, nerve endings). Spots of gold granules regularly spaced on the membrane surface often corresponded to the attachment sites of desmin-containing IF with the membrane (*arrows*, *b* and *c*). The convoluted noninnervated membrane (*d*) was labeled at background level. Bars: (*a* and *d*) 1 μm; (*b* and *c*) 0.5 μm.

interaction involves the amino terminal head domain of vimentin or desmin (Georgatos et al., 1985; Georgatos et al., 1987). Being saturable and noncooperative, it essentially provides attachment (capping) sites for IF. Conversely, a functionally distinct set of interactions takes place at the nuclear envelope where lamin B appears to be the receptor for the carboxy termini of vimentin or desmin (Georgatos and Blobel, 1987*a*,*b*; Georgatos et al., 1987). This latter interaction being cooperative and nonsaturable, lamin B likely represents a nucleation site from which IF could elongate. According to this model, the structurally apolar IF may acquire vectorial properties via their differential attachment sites.

In *Torpedo* electrocyte the IF network, instead of being organized radially around the nuclei as in most animal cells, appears basically anchored to the two opposite sides of the cell. The two domains of the plasma membrane as well as the cortical skeleton related to the two sides of the cell have no protein in common. Consequently, ankyrin, a major component of the cortical skeleton, is associated only with the noninnervated membrane (Kordeli et al., 1986). This particular asymmetrical architecture of the electrocyte was thus evocative of a vectorial insertion of the IF network. In the present work, we show that a human serum containing antibodies to lamin B of mammals (Guilly et al., 1987), amphibians (Krohne et al., 1987), as well as *Torpedo* (this work) reacts with a 54-kD acidic protein (IP ~5.4) of the innervated membrane domain. The reaction being abolished by absorption of the serum with purified rat liver nuclear lamina in which the only immunoreactive component is lamin B, we concluded that the 54-kD protein of the plasma membrane and lamin B share common antigenic determinant(s). This cross-reactivity between peripheral proteins of nuclear and plasma membranes is striking. A likely explanation would be that the epitopes common to both proteins would correspond to a conserved functional domain engaged in the interaction with IF. Along this line, it is noteworthy that Georgatos and Blobel (1987*b*) have shown that antibodies directed

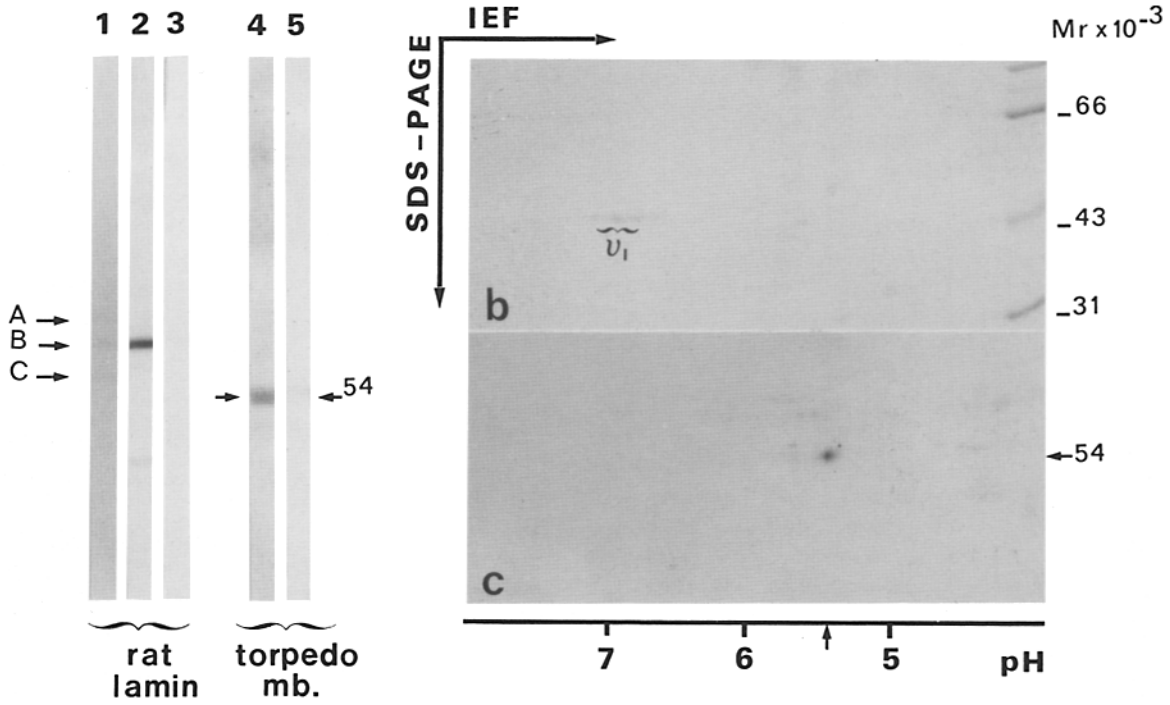

Figure 4. Identification of the component of the *Torpedo* postsynaptic membrane which cross-reacts with antibodies to lamin B. Proteins of rat liver lamina (*a*, lanes *1–3*) and purified *Torpedo* innervated membranes (*a*, lanes *4*, *5*, and *b* and *c*) were separated by one- (*a*) and two-dimensional gel electrophoresis (*b* and *c*); blotted, then revealed by either Ponceau red staining (*a*, lane *1*, and *b*) or serum F at 1:200 dilution (*a*, lanes *2* and *4*, and *c*) or serum F absorbed with purified rat liver nuclear lamina at 1:200 dilution (*a*, lanes *3* and *5*). A protein with a relative molecular mass of ~54 kD and IP ~5.4 was recognized by serum F (*a*, lane *4*, and *c*) but not by the absorbed serum (*a*, lane *5*). Note that the major extrinsic components of the membrane, the 43-kD (ν_1), isoforms (*b*) were not labeled.

against lamin B are able to inhibit the binding of vimentin to nuclear lamina. Another common feature to lamin B and the 54-kD protein is their acidic character with an IP close to 5.4 (Gerace and Blobel, 1980; Shelton et al., 1980). However, the apparent molecular weight of the innervated membrane protein is lower than that of the authentic nuclear lamin of type B (~68 kD) from *Torpedo* liver and that reported for type B lamins from other vertebrates (reviewed in Krohne and Benavente, 1986). It does not correspond also to the ~46-kD proteolytic product of lamins (Burke et al., 1983). The 54-kD protein is also different from the two other major extrinsic proteins associated with the innervated membrane of the electrocyte: the 43-kD (ν_1) isoforms (Saitoh and Changeux, 1980; Gysin et al., 1983; Froehner, 1984) and the 58-kD protein (Froehner, 1984; Froehner et al., 1987) which exhibits IP of ~7.0 and 6.4, respectively. Moreover, the 54-kD protein is different from desmin. Notwithstanding their close physico-chemical properties (relative molecular mass is ~52 kD, IP ~5.5; Lazarides and Hubbard, 1976; Granger and Lazarides, 1979; Gard et al., 1979), no cross-immunoreactivity between the two proteins was observed neither by immunoblotting nor by immunocytochemistry. In particular, these later experiments disclosed clearly a differential localization for desmin which is exclusively associated with the network of filaments across the cell (Kordeli et al., 1986; Sealock et al., 1989) while anti-lamin B labeling was strictly associated with the nuclear periphery and the innervated plasma membrane.

Immunogold labeling experiments have illustrated a discontinuous distribution of the 54-kD protein at the cytoplasmic surface of the innervated membrane. This contrasts with the distribution of the 43-kD and 58-kD proteins which forms a continuous coating of the AchR-rich membrane domain of the membrane (Nghiêm et al., 1983; Sealock et al., 1984; Froehner et al., 1987). The examination of the cytoplasmic surface of the innervated membrane labeled with anti-lamin antibodies revealed the occurrence of gold granules at/or near membrane sites at which IF terminate. This localization strengthens our hypothesis that the 54-kD protein could be engaged in IF–plasma membrane interaction.

We have previously reported (Kordeli et al., 1986, 1987*b*) that the noninnervated membrane domain of the electrocyte contains ankyrin. Using the immunogold staining method, we have also observed for ankyrin a nonuniform distribution compatible with the anchoring of IF at discrete sites on the noninnervated membrane. Taken together, these and the present data offer a structural basis for the anchorage of the IF network in a vectorial manner to the two opposite plasma membrane domains via a 54-kD protein and ankyrin. This configuration is basically that proposed by Georgatos and Blobel (1987*a*), and may constitute a general scheme of insertion of IF into specialized membrane domains. The latter hypothesis is further supported by our finding that anti-lamin B antibodies are able to label the mammalian neuromuscular junction. Though still nonidentified, the cross-reacting protein in the neuromuscular junction is likely to participate to the anchorage of the subneural network of IF (Hirokawa and Heuser, 1982; Sealock et al., 1989).

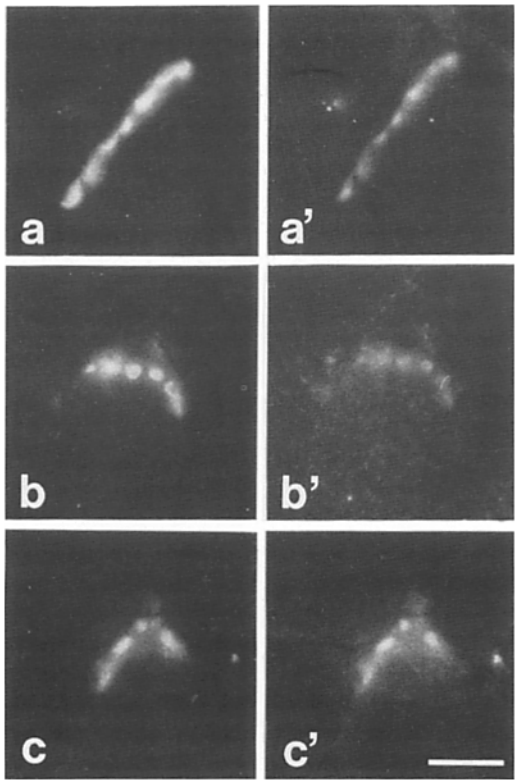

Figure 5. Immunofluorescence localization of lamin B immunoreactivity in rat muscle (diaphragma). Cryostat sections from the neural region of rat diaphragm muscle were doubly stained with fluorescein-conjugated α-bungarotoxin (*a*, *b*, and *c*) and serum F at 1:200 dilution (rhodamine fluorescence) (*a'*, *b'*, and *c'*). Note that each motor endplate gutter identified by α-bungarotoxin staining was also stained with the anti-lamin B antibody. Bar, 10 μm.

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