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Expression of the integrin $\alpha_6\beta_4$ in peripheral nerves: localization in Schwann and perineural cells and different variants of the β_4 subunit

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SUMMARY

Integrin $\alpha_6\beta_4$ is expressed in human peripheral nerves, but not in the central nervous system. This integrin heterodimer has previously been found in perineural fibroblast-like cells and in Schwann cells (SCs), which both assemble a basement membrane but do not form hemidesmosomes. We show here that in SCs, which had formed a myelin sheath, $\alpha_6\beta_4$ was enriched in the proximity of the nucleus, at Ranvier paranodal areas and at Schmitt-Lanterman clefts; $\alpha_6\beta_4$ was also found at the grooved interface between small axons and non-myelinating SCs. Immunoprecipitation of human peripheral nerves, in combination with Western blotting showed that β_4 is associated with the α_{6A} subunit. Northern blot analysis of human peripheral nerves showed a single β_4 transcript of 6 kb. Using the reverse transcriptase polymerase chain reaction, we detected two mRNA species, one for the most common (−70, −53) form of β_4 and the other encoding the (+53) variant of β_4. Cultured SCs were devoid of $\alpha_6\beta_4$ but expressed $\alpha_6\beta_1$, indicating that SCs lose β_4 expression when contact with neurons is lost. Thus, resting SCs in contact with axons express α_{6A} in combination with β_4, irrespective of myelin formation. We suggest that $\alpha_6\beta_4$ expressed in SCs plays a role in peripheral neurogenesis.

Key words: integrin $\alpha_6\beta_4$, peripheral nerve, Schwann cell, perineural fibroblast, β_4 variant

INTRODUCTION

The integrin family of adhesion receptors mediates cell-cell and cell-extracellular matrix contact. Integrins consist of an α- and a β-subunit that are non-covalently bound to form a heterodimeric receptor. Thus far, 14 α chains and 8 β chains have been identified, which, together, give rise to at least 20 integrin complexes (Hynes, 1992).

The integrin α_6 subunit can associate with either the β_1 or the β_4 subunit. Two variants of α_6 have been described, α_{6A} and α_{6B} (Hogervorst et al., 1991; Cooper et al., 1991), which have identical extracellular and transmembrane domains but differ completely in a large part of their cytoplasmic domain. The $\alpha_6\beta_1$ integrin is widely expressed and binds to the extracellular matrix molecule laminin (Sonnenberg et al., 1988). Laminin has also been identified as a ligand for the $\alpha_6\beta_4$ integrin expressed on colon carcinoma cells (Lee et al., 1992). The ligand of $\alpha_6\beta_4$ in other cell types has not yet been identified (Sonnenberg et al., 1990b; De Luca et al., 1990); it may be laminin, one of its isoforms or a conformational determinant of the multimolecular basement membrane region.

The tissue distribution of $\alpha_6\beta_4$ is more restricted than that of $\alpha_6\beta_1$. It is expressed in almost all epithelial tissues, certain types of endothelial cells and peripheral nerves (Sonnenberg et al., 1990a; Kennel et al., 1992; Natali et al., 1992). Compared to the other β subunits, the β_4 subunit has an unusually large cytoplasmic domain of more than 1,000 amino acids (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). So far, this domain shares no homology with any other known protein sequence except for the presence of four fibronectin type III repeats, which reside in two pairs. Such type III repeats have also been found in certain cytoskeletal proteins, like titin and twitchin (Benian et al., 1989; Labeit et al., 1990), and this suggests a possible functional similarity between β_4 and these cytoskeletal proteins.

In addition to the most common form of β_4, two variants have been identified, each containing insertions of 70 or 53 amino acids, respectively (Hogervorst et al., 1990; Tamura et al., 1990). In both the β_4(+70) and the β_4(+53) variants the insertions are located between the two sets of fibronectin type III repeats and probably arise by alternative splicing. At present nothing is known about the possible difference in function between the different variants of the β_4 subunit.

In basal epithelial cells of skin, which express the most common (−70,−53) form of the β_4 subunit, $\alpha_6\beta_4$ integrin is localized in hemidesmosomes (Sonnenberg et al., 1991; Stepp et al., 1990; Jones et al., 1991). Hemidesmosomes are specialized structures of keratinocytes and other epithelial cells. They mediate firm adhesion to the underlying basement membrane by anchoring fibrils, and represent organizing sites for the

intermediate filament system (Garrod, 1993). In fact, one function of the large cytoplasmic domain of β_4 might be the interaction with keratin filaments, either directly or via other proteins localized in hemidesmosomes. There are no such indications for cytoskeletal association of $\alpha_6\beta_4$ in cell types that do not form hemidesmosomes.

As reported in previous papers (Sonnenberg et al., 1990a; Hogervorst et al., 1993; Jaakkola et al., 1993) $\alpha_6\beta_4$ is expressed in peripheral nerves where it is found in perineural fibroblasts and SCs. The latter are the major class of glial cells in the peripheral nervous system and consist of non-myelinating and myelinating subtypes. Non-myelinating SCs accommodate small axons in membrane-limited grooves, whereas myelinating SCs are larger and wrap around individual axon segments on a one-cell-to-one-cell basis, forming a myelin sheath of variable thickness (for a review see Jessen and Mirsky, 1991). Both SC types are supported by a basement membrane, containing several laminin isoforms, nidogen and collagen type IV (Leivo and Engvall, 1988; Hunter et al., 1989; Sanes et al., 1990; Engvall et al., 1990; Jaakkola et al., 1993). Myelin-forming SCs express the intermediate filaments vimentin and neurofilament-M (NF-M) (Kelly et al., 1992) whereas the non-myelinating SCs express vimentin and glial fibrillary acidic protein (GFAP) (Jessen et al., 1990). The function of $\alpha_6\beta_4$ expressed on SCs is unknown but it may play a role in the myelination process. Upon peripheral nerve injury, SCs are released from the degenerating distal tract of the nerve, remove debris and are involved in supporting the regeneration of the distal nerve stump that is eventually remyelinated. This complex phenomenon restores peripheral nerve function; it does not occur in the central nervous system where myelination is supported by oligodendrocytes, which do not express $\alpha_6\beta_4$ integrin.

In the present study we investigated the fine localization of the β_4 subunit in peripheral nerves. We confirm the expression of $\alpha_{6A}\beta_4$ integrin in SCs and the perineurium and present new structural details. Using immunohistochemistry and immunoelectron microscopy analysis, we localized $\alpha_6\beta_4$ integrin within individual myelin segments, suggesting that this integrin plays a role in myelin sheath formation. Both SCs as well as perineural fibroblasts are cell types that do not express the molecular components required for hemidesmosome assembly. We wondered therefore whether peripheral nerves express a variant of the β_4 subunit. Using reverse transcriptase polymerase chain reaction (RT-PCR) analysis, we observed expression of β_4 of the (−70,−53) form but also of the (+53) variant. The (+70) variant could not be identified in peripheral nerves. The (+53) variant has already been identified in placenta (Tamura et al., 1991) and recently in mouse spleen (Kennel et al., 1993). Thus, it is possible that expression of an alternative form of β_4 is associated with a different function of $\alpha_6\beta_4$ integrin in distinct cell types.

MATERIALS AND METHODS

Cell lines and antibodies

SCs were prepared from sciatic nerves of 3.5 day neonatal rats according to the method of Brockes et al. (1979). Briefly, sciatic nerves were dissected out under sterile conditions, rinsed in serum-free F-10 medium (Sigma Chemical Co., St Louis, MO) and then digested in 0.25% trypsin and 0.1% collagenase (Sigma Chemical Co.) in HBSS (Hanks' Balanced Salt Solution) for 60-90 minutes at 37˚C; digestion was stopped by the addition of F-10 medium supplemented with 10% FCS, and followed by mechanical dissociation with vigorous pipetting. After centrifugation, cells were recovered in F-10, filtered through a siever-mesh 100 and seeded on plastic 10 cm dishes that had been coated with 10 mg/ml poly-L-lysine (Sigma Chemical Co.). After seeding, cells were treated for 5 days with 10^{-5} M arabinoside-cytosine (Sigma Chemical Co.) to remove contaminating fibroblasts, and then supplemented with pituitary extract (500 mg/ml) and cholera toxin (0.5 mg/ml) (Sigma Chemical Co.) to enhance SC proliferation until confluence. Schwann cells were routinely cultured in F12 medium supplemented with 10% FCS, 1% glutamine, streptomycin and penicillin, and passaged at confluence for three or four times.

HBL100, a normal human mammary epithelial cell line, and UMSCC-22B, a squamous cell carcinoma cell line (kindly provided by Dr T.E. Carey, University of Michigan, Ann Arbor, MI) were cultured in DMEM supplemented with 10% FCS, streptomycin and penicillin. Dr S. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN) provided the mouse mAbs 450-9D, 450-10D, 450-11A and the rat mAb 439-9B, all directed against the β_4 subunit, the anti-α_6 mouse mAbs 450-33D and 450-30A1 and the rat mAb 135-13C (Kennel et al., 1989, 1990). The mouse mAb 4.3E1 (Hessle et al., 1984), directed against the β_4 subunit, was kindly provided by Dr E. Engvall (La Jolla Cancer Foundation, La Jolla, CA). Rabbit anti-β_1 serum was prepared against a synthetic peptide of the carboxy terminus of β_1 (Marcantonio and Hynes, 1988) and was a gift from Dr R. Hynes (MIT, Boston, MA). The rat mAb GoH3, specific for the α_6 subunit, the mouse mAb 1A10, directed against the α_{6A} variant, and the mouse mAb 6B4, against the α_{6B} variant, have been described previously (Sonnenberg et al., 1986; Hogervorst et al., 1993). Rabbit anti-β_4 serum was generated against a bacterial 6xHis fusion protein containing the entire cytoplasmic domain of the β_4 (+53) subunit.

Immunoprecipitation and electrophoresis

SCs and perineural fibroblasts were surface-labelled with ^{125}I by the lactoperoxidase method (Sonnenberg et al., 1988). After washing, the cells were lysed with 1% (v/v) Nonidet P-40 in 20 mM Tris-HCl (pH 7.5), 4 mM EDTA and 100 mM NaCl; 1 mM phenylmethanesulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor and 10 μg/ml leupeptin were added as protease inhibitors. Peripheral nerve tissue was homogenized in 20 mM Tris-HCl (pH 7.5) using a Polytron mincer and extracted with 1% NP-40. The lysate was clarified at 10,000 ***g*** and precleared by incubation with Protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Precleared lysates were then added to Protein A-Sepharose, previously incubated with rabbit anti-mouse IgG and monoclonal antibody. After incubation for 2 hours at 10,000 ***g*** 4˚C beads were washed and sample buffer was added. The precipitated samples were analyzed by SDS-PAGE according to Laemmli (1970).

Immunoblot analysis

Proteins were separated on a 5% polyacrylamide gel under non-reducing and reducing conditions and blotted onto nitrocellulose at 100 V for 1 hour. Filters were blocked in TBST (20 mM Tris-HCl, pH 8, 150 mM NaCl and 0.05% Tween-20) containing 1% (w/v) BSA for 30 minutes at room temperature, incubated with the primary antibody (diluted 1:500 in TBST) for 1 hour at room temperature, washed and then incubated with the appropriate secondary antibody (anti-mouse or anti-rabbit IgG/Fc alkaline phosphatase conjugate; Promega Corp., Madison, WI). After washing the filters again, they were stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$ for 15-30 minutes at room temperature. The colour reaction was stopped by rinsing the filters in deionized water.

Northern blot analysis

Total RNA was isolated as described by Chomczynsky and Sacchi (1987), electrophoresed on a 1% agarose gel containing 6% formaldehyde and subsequently blotted onto nitrocellulose using standard procedures. The filters were hybridized at 63˚C with a ^{32}P-labeled probe in Hybmix containing 5× Denhardt's, 3× SSC, 10% dextran sulphate and 50 μg/ml herring sperm DNA. Filters were washed with 2× SSC/0.1% SDS for 30 minutes at 64˚C, dried and exposed to KODAK-X-OMAT AR film using an intensifying screen at –70˚C.

Probes were labelled with [α-^{32}P]dATP using the random primer labelling kit from Gibco-BRL (Gaithersburg, MD). A 1 kb *Hin*dIII cDNA fragment coding for the N terminus of β_4, a 2.4 kb *Hin*dIII fragment of β_1 cDNA, a *Pst*I fragment of N-CAM cDNA (a kind gift from Dr R. Michalides, The Netherlands Cancer Institute, Amsterdam, The Netherlands) and a 700 bp *Bam*HI fragment of actin cDNA were used as probes in the northern blot analysis.

Reverse transcriptase and PCR

Total RNA was isolated using the urea/lithium chloride method. cDNA was synthesized from 5 μg RNA using a cDNA kit (Promega Corp.) and a poly(A)$^+$ primer as template. PCR reactions were performed as described before (Hogervorst et al., 1991) using the following reaction temperatures: denaturation for 1 minute at 94˚C, annealing for 1.5 minutes at 50˚C and extension for 5 minutes at 72˚C. A final extension of 5 minutes was at 72˚C after 35 cycles. Samples were analyzed on a 1% (w/v) agarose gel. Primers used in PCR were:

(1) GGTGTCCTTTGAGCAGCCTGAGTTCTC, sense, position 2982-3008

(2) CCCATCATCCCTGACATCCCTATC, sense, position 3988-4011

(3) CTCAGAACACTCACACTCGACCACACT, sense, position 4289-4316

(4) CAGTGTGGTCGAGTGTGAGTGTTCTGAG, antisense, position 4289-4317

(5) CGGAATTCCATGGGCCTCCCTCCCATCTGGGAACAC, sense, position 4357-4380

(6) CCAAGCTTGGTCATGGGCCCCAGGAGGGCGCTGCTGG, antisense, position 4492-4515

(7) CTCACAAACTCCTGGGTCACATGC, antisense, position 5340-5363.

Positions are according to Hogervorst et al. (1990).

Immunoperoxidase staining

Cryostat sections of human peripheral nerves were immunoperoxidase-stained by an indirect procedure using horseradish peroxidase-conjugated second antibody (Sonnenberg et al., 1986). Reactivity was visualized using hydrogen peroxide and 3′3′-diaminobenzidine as substrate. The sections were counterstained with haematoxylin and mounted in Depex (British Drug House Chemicals, Poole, UK). Control reactions were performed with Sp2/0 culture medium. Immunostained slides were inspected in Zeiss Axiophot microscope using a ×63/1.4 NA Planapochromatic lens. Pictures were taken with Kodak T-Max 100 films using a Kodak Wratten 44 blue-green correction filter.

Electron microscopy

The procedure was that reported by Tokuyasu (1980, 1984) and by Keller et al. (1984) with a few modifications. Briefly, specimens of human nerves (mostly vagus nerve removed during major gastric surgery) were immediately dissected in 1 mm blocks or less and fixed in 3% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 hour at room temperature. Blocks were then infused with 2.3 M sucrose in PBS and frozen in Freon-12 cooled in liquid nitrogen. Ultrathin cryosections were obtained using a Reichert Ultracut E ultramicrotome (C. Reichert AG, Wien, Austria) with the cryoattachment FC-4D (C. Reichert AG) at −90 to −110˚C. Only the sections with a reflected colour from yellow to green, were transferred to carbon/Formvar-coated grids, using a droplet of 2.5 M sucrose. The supporting film was ionized by UV irradiation immediately before transferring the sections. The specimens were quenched with 50 mM glycine in PBS (pH 7.4) and then incubated on droplets of PBS containing 0.5% BSA and 0.2% gelatin (PBG) to avoid non-specific protein-protein interactions; PBG was also used for all the washings until the post-fixation procedure. The primary antibodies (a gift from Dr V. Quaranta, Scripps Research Institute, La Jolla, CA) were rabbit antisera to α_6 (R6844 and R6842; see Tamura et al., 1990, 1991) and β_4 integrin subunits (R5710; Kajiji et al., 1989), and rabbit IgG to laminin (Sigma Chemical Co.); the secondary antibodies were goat anti-rabbit IgG conjugated to 5 nm gold particles (BioCell Research Laboratories, Cardiff, UK). After labelling, the sections were post-fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) followed by 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.3) for 10 minutes. The specimens were stained with a saturated solution of uranyl acetate for 10 minutes, dehydrated in ethanol and embedded in LR-White resin (London Resins, London, UK) (Keller et al., 1984). Grids were examined in a Philips 410 electron microscope operating at 80 kV accelerating voltage.

RESULTS

Immunohistochemistry

Peripheral nerve fibers have previously been shown to express $\alpha_6\beta_4$ and this has been attributed to SCs and perineural fibroblasts (Sonnenberg et al., 1990a; Hogervorst et al., 1993). In this study, we have examined the distribution of $\alpha_6\beta_4$ in human peripheral nerves in more detail using immunoperoxidase staining of frozen sections and a panel of monoclonal antibodies directed against several epitopes of the α_6 and β_4 subunits. For the α_6 subunit the following mAbs were used: GoH3, J8H, 135-13C, 450-30A1, 450-33D, which are all ectodomain-specific, and 1A10 and 6B4, which are specific for the cytoplasmic domain of α_{6A} or α_{6B}, respectively. The following mAbs were used for the β_4 subunit: 4.3E1 and 450-9D, which are both directed against the ectodomain, and 450-10D and 450-11A, which are specific for the cytoplasmic domain.

Two populations of peripheral nerve cells expressed the α_6 and β_4 subunits i.e. SCs and the perineural fibroblast-like cells (Fig. 1a). The latter reacted with all antibodies except mAb 6B4, directed against the α_{6B} cytodomain. Therefore, perineural cells express and assemble the heterodimer $\alpha_{6A}\beta_4$, in which the β_4 subunit seems to be full length as the cytoplasmic domain contains both the very carboxyl terminus recognized by 450-11A and the cytoplasmic epitope recognized by mAb 450-10D (not shown) (Kennel et al., 1990).

SCs reacted strongly with all antibodies directed against the ectodomain of both α_6 and β_4 chains. Reaction was also observed with mAb 1A10 (Fig. 1g), directed against the α_{6A} cytodomain but not with the anti-α_{6B} mAb 6B4 (Fig. 1h), indicating that SCs, like perineural cells, express the heterodimer $\alpha_{6A}\beta_4$. The reactivity with mAbs specific for the cytodomain of β_4 was clearly different: staining was only observed with mAb 450-10D, which recognizes an epitope close to the transmembrane domain, whereas no reaction was seen with mAb 450-11A. In the same sections, the perineurium was stained by both mAbs. These findings suggest that the carboxyl terminus of β_4 in SCs is either absent or not accessible to antibodies, due to masking of the 450-11A defined epitope.

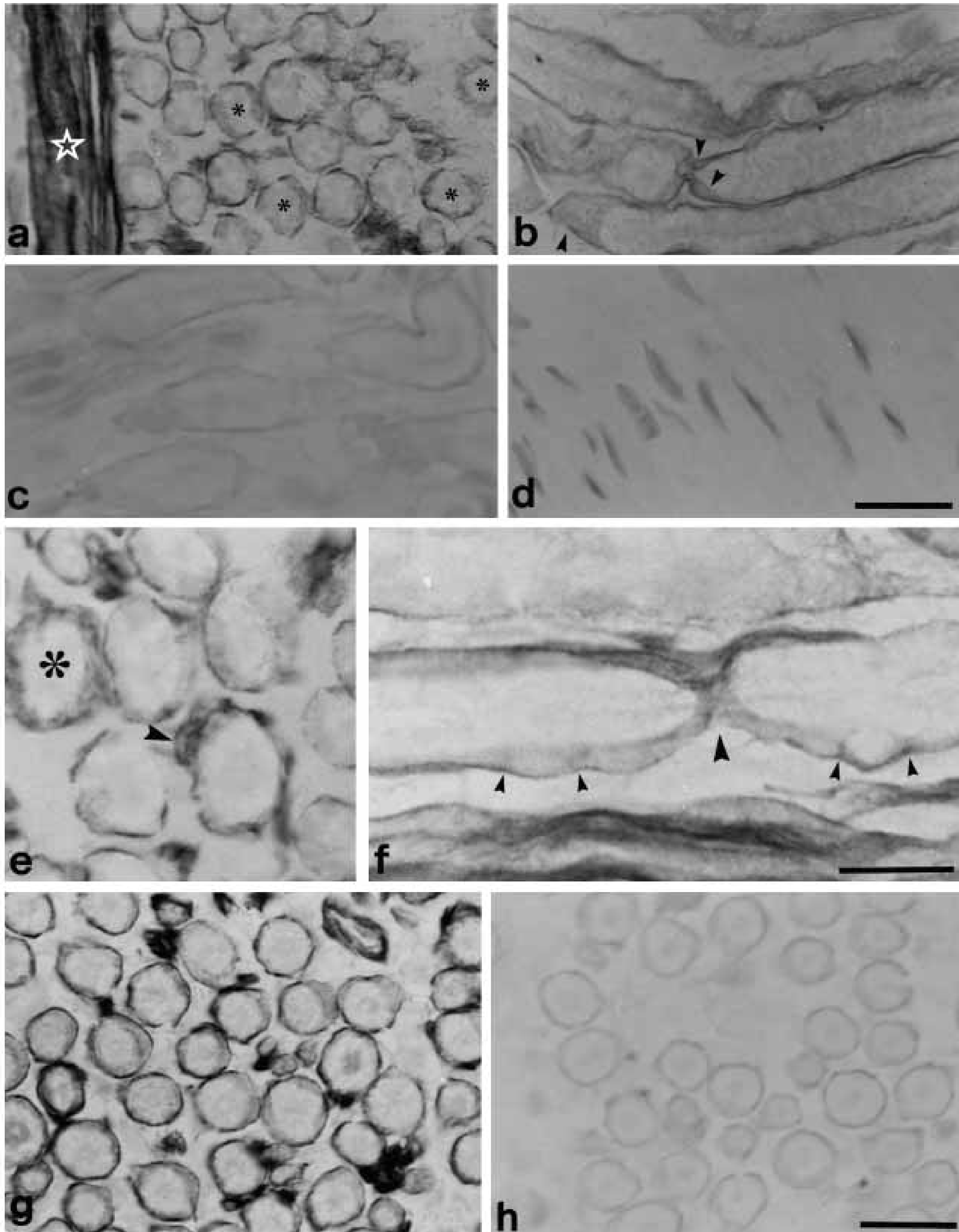

Fig. 1

The topography of $\alpha_{6A}\beta_4$ in human peripheral nerve fibers was quite interesting. In cross-sections of myelin segments $\alpha_{6A}\beta_4$ was found in a thin rim at the outer boundary and staining was somewhat more intense around the SC nucleus (Fig. 1a and e, e.g. indicated by arrowhead). Also a more intense reaction was seen at the level of the Ranvier nodes (Fig. 1b, e and f): in fact, in longitudinal as well as in oblique sections a rather strong signal was observed in the nucleus-con-

Fig. 1. Immunoperoxidase staining of frozen sections of human peripheral nerve. (a, e and f) Immunostaining with mAb 4.3E1 to the ectodomain of β_4; (b) with mAb 450-10D; (c) with mAb 450-11A to the carboxyl terminus of β_4; (g) with mAb 1A10 to α_{6A}; (h) with mAb 6B4 to α_{6B} and (d) to a control irrelevant mAb. The transverse section in (a) also shows a tract of the strongly positive perineurium (indicated by a white star) and a bundle of cross-sections of myelinated nerve fibres. The latter have in general a sharply positive outline but some of them (e.g. those marked by asterisks) show a sort of 'cloudy' outline that may reflect sectioning in the proximity of a Ranvier node (see also (e) at asterisk). The longitudinal section in (b) indicates that more intense staining indeed occurs close to Ranvier nodes (marked by small arrowheads). This is also seen at higher magnification in (f), which also shows the presumed position of two Schmitt-Lanterman clefts (in between the small arrowheads). The arrowhead in (e) indicates the position of a Schwann cell nucleus and the positive reaction in the nuclear area. The positive reaction to mAb 1A10 raised against the variant chain α_{6A} is shown in (g) while no reaction was found with mAb 6B4 to α_{6B} (h). Bars: 5 μm (a-d,g,h) and 2.5 μm (e-f).

taining hillock and at the Ranvier nodes. In some of the sections of myelinated nerve fibers, a weak reaction was seen along individual myelin segments, usually not far from and in between two consecutive Ranvier nodes (Fig. 1f, at smaller arrowheads). Given their position and their slightly bulging appearance, we think that these weak signals may be due to $\alpha_6\beta_4$ located at Schmitt-Lanterman clefts. The topography of $\alpha_{6A}\beta_4$ indicates that this integrin is enriched in areas where thin patches of cytoplasm prevent myelin membranes from making contact with each other. All β_4 positive structures also reacted with anti-α_6 mAbs. No reaction was detected in nerve sections incubated with irrelevant mAbs (Fig. 1d).

In addition to these sites, a positive reaction for α_6 and β_4 was detected in non-myelinating SCs interspersed among myelinated fibres (Fig. 2a, some are indicated by arrowheads). The staining was restricted to the contact area between thin axons engulfed in SC grooves, indicating that $\alpha_{6A}\beta_4$ can apparently also be present at the surface of SCs in direct contact with individual axons (Fig. 2b) and not only at the surface involved in myelin wrapping. Therefore, the presence of $\alpha_6\beta_4$ appears to be a constitutive feature of SCs directly contacting axons, whether or not they are involved in myelin sheath formation. Finally, no $\alpha_6\beta_4$ could be detected in neurons or glial cells in various selected areas of adult human brain and in cross-sections of human optic nerve (not shown).

Identification of $\alpha_6\beta_4$ in peripheral nerves by immunoblotting

The expression of both α_6 and β_4 subunits by peripheral nerves indicated the presence of the $\alpha_6\beta_4$ complex. To demonstrate this, integrins were purified from peripheral nerves by immunoprecipitation with the mAbs 450-9D (anti-β_4), 450-33D (anti-α_6) or AIIB2 (anti-β_1), separated by SDS-PAGE under reducing and non-reducing conditions, and examined by immunoblotting, using a polyclonal antiserum to β_4. As shown in Fig. 3, the anti-β_4 antiserum recognized a band of 180 kDa (non-reduced) or 200 kDa (reduced), corresponding to intact β_4, in the samples precipitated by mAbs 450-9D (lane 2) and 450-33D (lane 3), but not by mAb AIIB2 (lane 4). The 150 kDa band seen in all three precipitates under non-reducing conditions is the IgG used for immunoprecipitation of integrins. The anti-β_4 antiserum also recognized the non-reduced 180 kDa protein in total lysates of peripheral nerves (Fig. 3, lane 1). These findings confirm the immunohistochemical observations, showing that the $\alpha_{6A}\beta_4$ integrin complex is present in peripheral nerves.

Electron microscopic analysis of the β_4 subunit in peripheral nerves

To fine-localize the $\alpha_6\beta_4$ integrin in SCs, we performed electron microscopic analysis of ultrathin frozen sections of human peripheral nerves that had been incubated with rabbit sera against α_6, β_4 and laminin, and visualized with secondary antibodies coupled to 5 nm colloidal gold particles.

The β_4 subunit was found between individual laminae of the myelin sheath (Fig. 4a). Labelling was found at discrete sites as indicated by clusters of gold particles. The pattern obtained with antibodies specific for α_6 and laminin, respectively, was very similar, indicating that the $\alpha_6\beta_4$ and a potential ligand are sequestered in minute amounts within the intact myelin sheath.

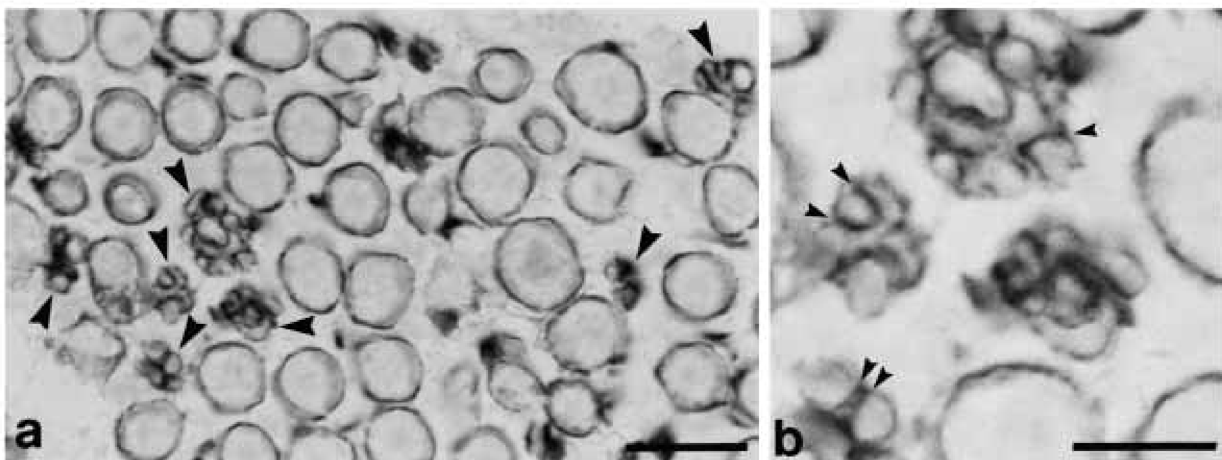

Fig. 2. Immunoperoxidase staining of frozen sections of human peripheral nerve with mAb 4.3E1 to the ectodomain of β_4 showing bundles of small axons (indicated by arrowheads in (a)) associated with non-myelinating SCs scattered among large myelinated axons. (b) A detail of (a) where the positive interfaces between SCs and individual small axons are indicated by arrowheads. Bar, 2.5 μm.

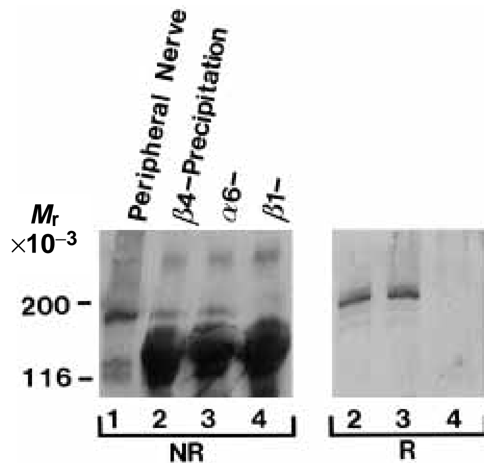

Fig. 3. Immunoblot analysis of immunoprecipitated proteins, using mAbs 450-9D (anti-β_4), 450-33D (anti-α_6) or AIIB2 (anti-β_1) from peripheral nerves. Proteins were separated on a non-reducing and reducing SDS-polyacrylamide (5%) gel and immunoblotted using a polyclonal antiserum to β_4. Lane 1, total lysate of peripheral nerves; lane 2, β_4 precipitation; lane 3, α_6 precipitation; and lane 4, β_1 precipitation.

This result further shows that, although the myelin sheath is formed by tightly apposed membranes in close reciprocal contact, accommodation of the cytoplasmic domain of the β_4 subunit is possible. This may occur at sites where thin cytoplasmic inclusions are present, like Ranvier nodes and Schmidt-Lanterman clefts (Berthold, 1982; Price et al., 1988) as suggested by immunohistochemical data.

RNA expression of β_4 variants in peripheral nerves

We performed northern blot analysis to determine the size of the mRNA of β_4 in peripheral nerves. RNAs from HBL100 and UMSCC-22B were used as positive controls. Transcripts of 6 kb were detected in human peripheral nerves, UMSCC-22B and HBL100 cell lines (Fig. 5).

Neither SCs nor perineural fibroblasts are capable of hemidesmosome formation although these cells express the $\alpha_6\beta_4$ integrin. These cells lack most components required for hemidesmosome formation including the BP230 protein and do not express keratin components, but other types of intermediate filaments. These facts point to a different role for $\alpha_6\beta_4$ in peripheral nerves other than its involvement in hemidesmosome organization. Therefore, it was of interest to examine whether peripheral nerves express a variant β_4 subunit.

As shown in Fig. 6A, RT-PCR analysis of RNA from human peripheral nerves with primers flanking the sequence in β_4 mRNA encoding the 53 amino acid insertion (Fig. 6C), yielded three PCR products (Fig. 6A, lane 2). The upper DNA fragment co-migrated with the PCR product of the cDNA in plasmid 903 (lane 4) encoding the (+53) variant of β_4, whereas the lower band co-migrated with the PCR product of HBL-100 cDNA (lane 3), which encodes the (−53) common form. The band observed in between these two products is a heteroduplex, formed between the DNA strands of the upper and lower bands. To further confirm the presence of mRNA for the (+53) variant of β_4, RT-PCR analysis was also performed with two primer sets both consisting of one primer in the domain encoding for the 53 amino acid insertion and another primer 5′ or 3′ outside this domain (Fig. 6C). In both cases, a PCR product of the expected length (Fig. 6B, lanes 6 and 7) was obtained. Only one PCR product was found with primers for the DNA around the area encoding the 70 amino acid insertion (Fig. 6A, lane 1), which was of the same length as the product of plasmid 903 encoding the (−70) variant of β_4 (not shown). Thus, peripheral nerves express two variants of the β_4 subunit, the common (−53,−70) form and the (+53) variant, while the (+70) variant is not expressed in these cells.

Expression of $\alpha_6\beta_4$ in cultured SCs

SCs isolated from myelin-forming sciatic nerves from newborn rats and cultured in vitro without neurons lose their ability to form myelin sheaths and become cells of the nonmyelinating type within 24 hours (Jessen et al., 1990). This can be determined by several markers specific for the two stages of SC differentiation (Jessen and Mirsky, 1991). We wished to determine whether SCs cultured without neurons still express $\alpha_6\beta_4$ integrin by immmunoprecipitation of ^{125}I surface labelled rat SCs. Cell lysates were immunoprecipitated with a polyclonal anti-peptide antiserum to β_1 and mAbs 1A10 (anti-α_{6A}) and 6B4 (anti-α_{6B}) and the precipitated proteins were analyzed by SDS-PAGE under non-reducing and reducing conditions. The anti-α_{6A} mAb 1A10 precipitated the α_6 subunit together with the β_1 subunit but not with β_4 (Fig. 7, lane 1). Integrin $\alpha_{6A}\beta_1$ was also immunoprecipitated by the polyclonal antiserum to β_1. No α_6-containing integrins were detected in immunoprecipitations by mAb 6B4 from cultured SCs. We therefore conclude that cultured SCs express $\alpha_{6A}\beta_1$, but not $\alpha_{6A}\beta_4$. Immunoprecipitation of proteins from cultured murine perineural fibroblasts with the anti-α_6 mAb GoH3 yielded α_6, β_1 as well as the β_4 subunit (result not shown), indicating that perineural fibroblasts do not lose their β_4 surface expression when cultured in vitro.

As expected, northern blot analysis revealed the presence of β_1 (3.4 and 3.8 kb) and the absence of β_4 mRNA in cultured SCs (Fig. 8). The absence of β_4 mRNA was further confirmed by RT-PCR, which yielded no β_4 cDNA products, using several primer sets. Since N-CAM is a known marker for nonmyelinating SCs (Jessen and Mirsky, 1991), we used a N-CAM probe, as a control in the northern blot analysis. As expected, N-CAM mRNA (2.9 and 6.7 kb) was found to be present in cultured SCs (Fig. 8).

DISCUSSION

Peripheral nerves consist of axons individually wrapped by SCs and collected in bundles surrounded by the perineurium. In this paper we show that the receptor $\alpha_{6A}\beta_4$ is expressed in peripheral nerves and is located both in SCs and in the fibroblast-like cells that form the perineurium.

There are several unique features related to this finding. First, this location of $\alpha_{6A}\beta_4$ is not related to hemidesmosomes, since these junctions are missing from peripheral nerves. There is no evidence for the presence of keratins in either SCs or in perineural fibroblasts and this rules out the formation of hemidesmosomes. Indeed other molecular components of hemidesmosomes like BP180 and BP230, the latter of which is one of the possible candidates for interaction with the cyto-

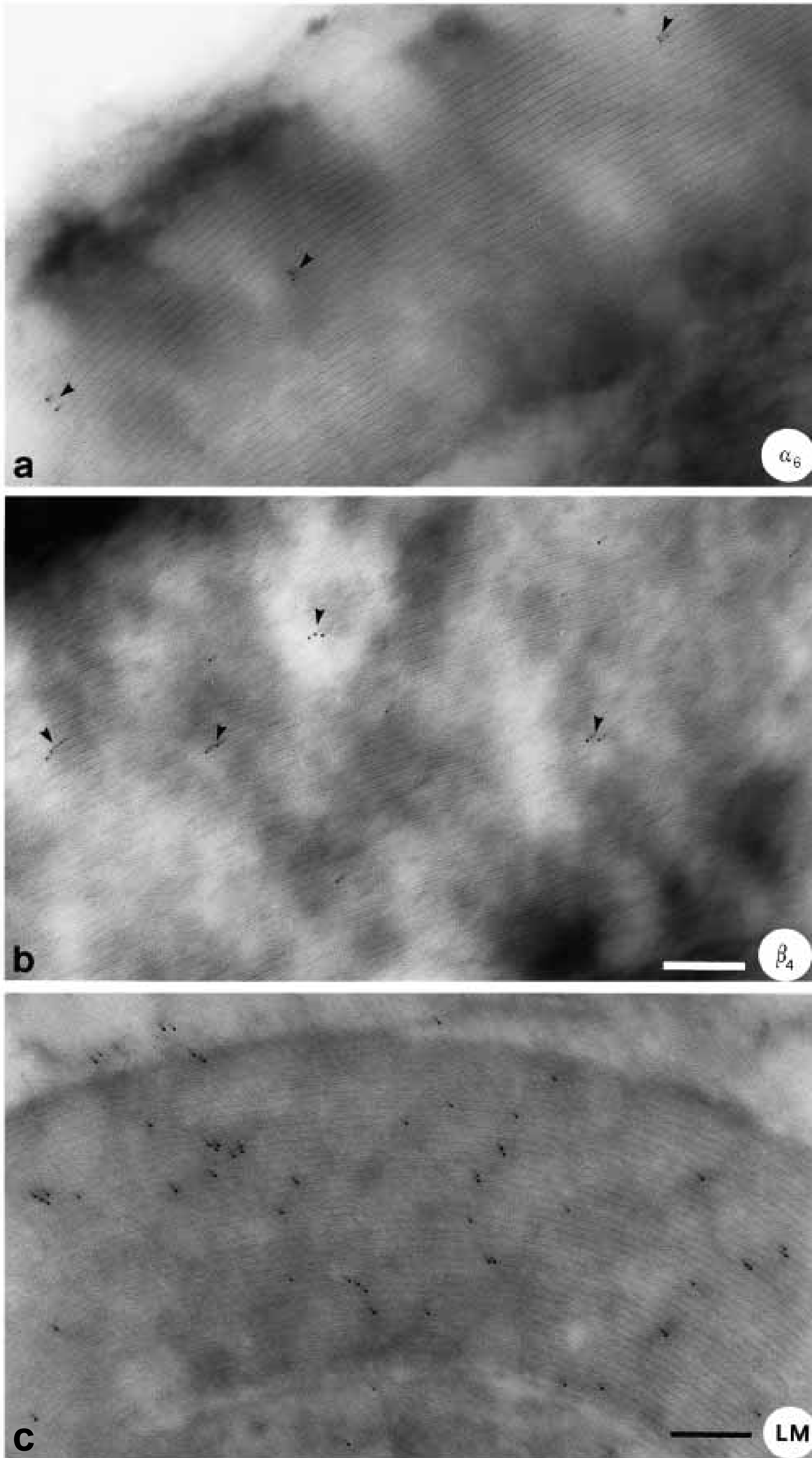

Fig. 4. Ultrastructural localization of α_6 (a) and β_4 (b) integrin subunits and laminin in human peripheral nerve (vagus nerve) using polyclonal sera. Ultrathin cryosections were first incubated with the rabbit antiserum R6844 against α_6 (a), R5710 against β_4 (b) or rabbit anti-laminin (c) and subsequently with gold-labeled goat anti-rabbit IgG. Gold particles of 5 nm are present in clusters (arrowheads) inside the myelin sheath. In view of the limited conservation of the structure obtained by this procedure the precise intramyelin position of the label cannot be defined; however, the gold particle clusters, particularly those indicated by arrowheads in (b), are found in areas where the myelin structure is looser and may be closer to Schmitt-Lanterman clefts. Bar, 0.1 µm

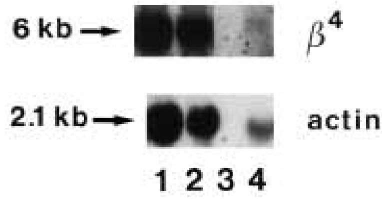

Fig. 5. Northern blot analysis of total RNA from human peripheral nerves and UMSCC-22B and HBL-100 cell lines. Total cellular RNA (10 µg/lane) was separated on a 1% agarose/formaldehyde gel and subsequently blotted to nitrocellulose. The filter was hybridized with ^{32}P-labeled probes encoding the N-terminal domain of β_4 and actin. Lane 1, HBL-100; lane 2, UMSCC-22B; lane 3; no sample loaded; and lane 4, peripheral nerves.

plasmic domain of β_4, are absent in peripheral nerves. Another candidate for such an interaction is HD1, a 500 kDa cytoplasmic protein, which is not hemidesmosome-specific, since it was shown to be present in peripheral nerves as well (Hogervorst et al., 1993). It is possible that $\alpha_6\beta_4$ interacts with other cytoskeletal proteins than keratin, and that this interaction is mediated by the HD1 protein.

A second characteristic is the location of $\alpha_6\beta_4$ within the myelin sheath, presumably at Ranvier nodes and Schmitt-Lanterman clefts where thin cytoplasmic inclusions prevent contact of apposing myelin membranes. These are probably the only structures that can accommodate the large cytoplasmic domain of the β_4 subunit. The quantity of $\alpha_{6A}\beta_4$ in between the myelinating sheaths is low and hardly visible by light microscope immunohistochemistry. It may represent a minor fraction of $\alpha_{6A}\beta_4$ that got trapped in resting SC membrane during myelin wrapping.

The β_4 subunit in peripheral nerves was found to be encoded by two different mRNA species. One mRNA coding for the most common (−70,−53) form and the other encoding the (+53) variant, whereas the (+70) variant could not be detected by RT-PCR. The (+53) variant has previously been described to be present in placenta (Tamura et al., 1990), murine spleen and to a much lesser extent in murine skin, uterus and thymus (Kennel et al., 1993). At present we do not know whether the two variants are expressed in both of the β_4-positive cell types that are found in peripheral nerves or that the (+53) variant is restricted to one cell type. Both variants were associated with the α_{6A} subunit.

In the immunohistochemical assays, all mAbs reacted with perineural fibroblasts and SCs except for mAb 450-11A, which did not react with SCs. This difference in staining with mAb 450-11A cannot be explained by a lack of reactivity of this antibody with one of the variants of β_4, because both the common form and the +53 variant of β_4 were recognized (unpublished observations). There is evidence that truncated β_4 proteins can arise from post-translational proteolytic processing (Giancotti et al., 1992). Also, in other studies, smaller products of the β_4 subunit have been detected (Hemler et al., 1989; Kajiji et al., 1989; Kennel et al., 1990). However, by immunoblotting of immunoprecipitated proteins from peripheral nerves, we observed only a major band of 180 kDa under non-reducing conditions and one of 200 kDa under reducing conditions, corresponding to intact β_4. It seems therefore unlikely that SCs express a truncated β_4 protein that lacks the epitope recognized by 450-11A. We suggest that the epitope for mAb 450-11A is masked in SCs.

SCs as well as perineural fibroblasts produce and assemble a basement membrane consisting of several laminin isoforms, collagen type IV and nidogen. This fits with other cell types where $\alpha_6\beta_4$ is associated with the basement membrane. The most obvious ligand for $\alpha_6\beta_4$ on SCs is laminin, which we also found to be trapped within the myelin sheath. Laminin has been shown to be the ligand for this integrin in colon carcinoma cells (Lee et al., 1992). The laminin isoform kalinin is co-localized with $\alpha_6\beta_4$ in skin (Rouselle et al., 1991) and in cultured keratinocytes (Marchisio et al., 1993), and was recently found to be a ligand for this integrin (Sonnenberg et al., 1993; Niessen et al., unpublished data). However, kalinin cannot be a ligand for $\alpha_6\beta_4$ in peripheral nerves, since kalinin is only found in basement membranes of squamous epithelia (Rouselle et al., 1991) and of certain endothelia (Jaspars et al., 1993). We cannot, however, exclude the existence of other laminin isoforms in the basement membrane of peripheral nerves that could bind to $\alpha_6\beta_4$. For example, a laminin variant apparently

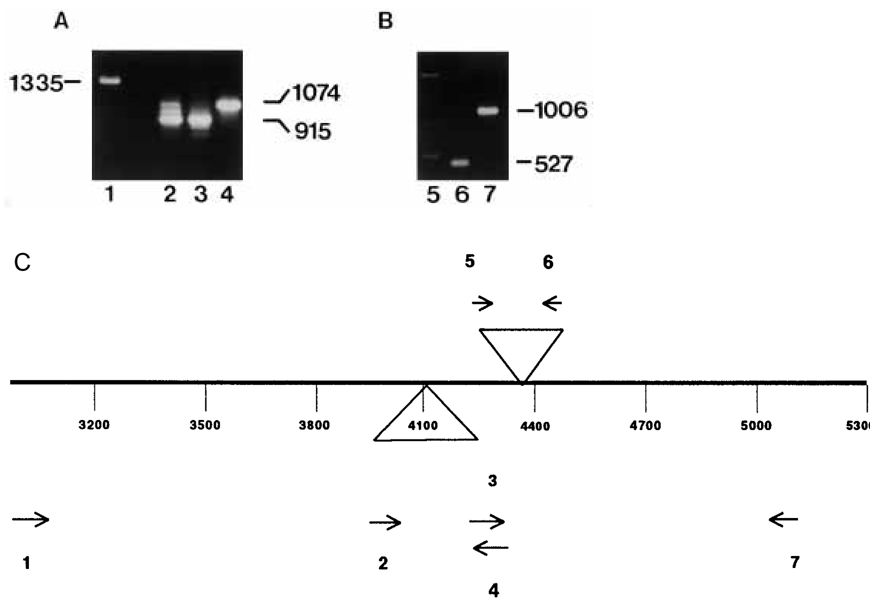

Fig. 6. Detection of variant β_4 mRNAs by reverse transcriptase-PCR amplification. PCR amplifications of cDNAs from peripheral nerves, human HBL-100 cells and a human α_6 cDNA clone (plasmid 903) were performed with primers in and around the domains coding for the (+70) (lane 1) and (+53) (lanes 2-4, 6 and 7) insertions. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. (A) Lane 1, peripheral nerves, primers 1 and 4; lane 2, peripheral nerves, primers 3 and 7; lane 3, HBL-100 cells, primers 3 and 7; lane 4, plasmid 903, primers 3 and 7. (B) Detection of transcripts coding for the (+53) variant. Lane 5, 100 bp molecular size marker; lane 6, peripheral nerves, primers 2 and 6; and lane 7, peripheral nerves, primers 5 and 7. (C) Diagram showing the alternatively spliced regions on the β_4 cDNA and the positions of the primers used for the RT-PCR reactions.

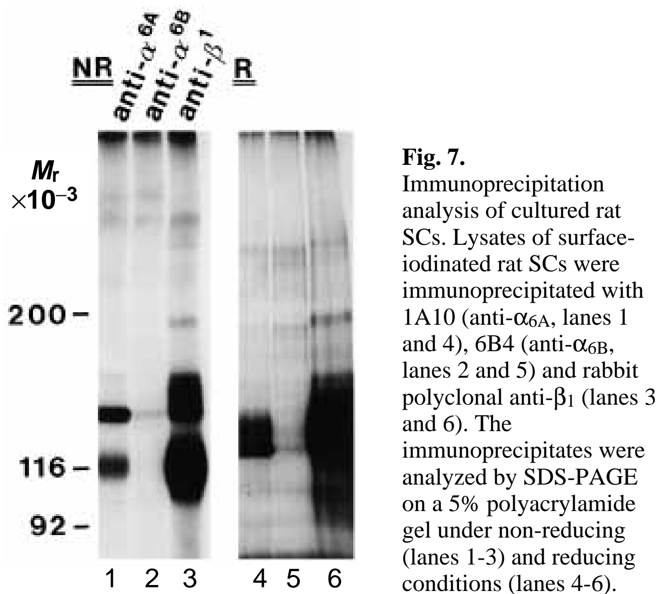

Fig. 7. Immunoprecipitation analysis of cultured rat SCs. Lysates of surface-iodinated rat SCs were immunoprecipitated with 1A10 (anti-α_{6A}, lanes 1 and 4), 6B4 (anti-α_{6B}, lanes 2 and 5) and rabbit polyclonal anti-β_1 (lanes 3 and 6). The immunoprecipitates were analyzed by SDS-PAGE on a 5% polyacrylamide gel under non-reducing (lanes 1-3) and reducing conditions (lanes 4-6).

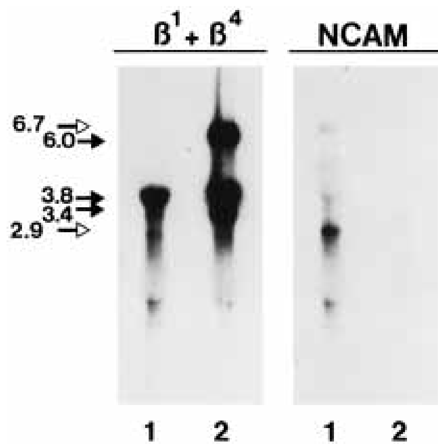

Fig. 8. Northern blot analysis of total RNA from cultured rat Schwann cells. Total RNA was separated on a 1% agarose/formaldehyde gel and subsequently blotted onto nitrocellulose. The filter was hybridized with either a combination of ^{32}P-labeled β_1 and β_4 probes or with a N-CAM cDNA probe. Lane 1, cultured SCs; lane 2, HBL-100 cells. SCs show transcripts of 3.4 and 3.8 kb for β_1 and of 2.9 and 6.7 kb for N-CAM. HBL-100 cells express β_1 and β_4 (6 kb transcript) but no N-CAM.

lacking an A-chain has been described to be produced by the rat Schwannoma cell line RN22 (Davis et al., 1985; Edgar et al., 1988), although it is not known whether this variant is also expressed in vivo.

In conclusion, peripheral nerves express $\alpha_{6A}\beta_4$ in two cell populations: perineural cells and SCs. Perineural cells represent a subpopulation of mesenchyme-derived cells whose cytoskeleton is based on microfilaments containing the α-smooth muscle actin isoform (C. Chaponnier et al., unpublished data) and in fact can be considered as a sub-population of vascular smooth muscle cells (O. Cremona et al., unpublished data). SCs derive from the neuroepithelium and have entirely different properties. First, they are specialized to engulf individual axons with their membrane and may wrap around neurite segments to form the myelin sheath, a vital structure involved in peripheral nerve conduction. The peculiar expression of $\alpha_{6A}\beta_4$ only in those cells that are in contact with axons, and its absence in cultured cells, indicate that this integrin is specifically up-regulated in the process of axon recognition and myelin formation. We suggest that resting SCs, even when embodied in myelin segments, express $\alpha_{6A}\beta_4$ integrin, which can be used for adhesion to the basement membrane when cells are called to support nerve repair. Therefore, we propose that this unique adhesion receptor may eventually prove to be involved in nerve regeneration supported by SCs (Richardson et al., 1980). The absence of $\alpha_6\beta_4$ from oligodendrocytes, in analogy with the protein P0 (reviewed by Colman, 1991), may represent a further crucial difference distinguishing the unique regenerative properties of peripheral versus central nerve fibres (reviewed by Carbonetto, 1991).

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