Schwann Cells Synthesize Type V Collagen That Contains a Novel Alpha 4 Chain:
Molecular Cloning, Biochemical Characterization,
and High Affinity Heparin Binding of α4(V) Collagen*

Michael A. Chernousov, Katrina Rothblum, William A. Tyler,
Richard C. Stahl, and David J. Carey‡

Sigfried and Janet Weis Center for Research
Penn State College of Medicine
100 Academy Avenue
Danville, PA 17822
Tel: (570) 271-6679
FAX: (570) 271-6701
E-mail: djcarey@psghs.edu

*This work was supported by National Institutes of Health Grant NS21925.
‡To whom correspondence should be addressed.

Copyright 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
SUMMARY

Previously, we reported the isolation of a heparan sulfate-binding collagenous protein, p200, that is expressed by Schwann cells in developing peripheral nerves (J. Biol. Chem. 271:13844-13853, 1996; J. Neurosci. Res. 56:284-294, 1999). Here we report the cloning of p200 cDNA from a Schwann cell cDNA library. The deduced amino acid sequence identifies p200 as a novel member of the collagen type V gene family. This polypeptide, which we have named α4 type V collagen, contains an uninterrupted Gly-X-X collagen domain of 1011 amino acids that shows 82% sequence identity to human α3(V) collagen and 71% identity to rat α1(V) collagen. α4(V) is secreted by Schwann cells as a collagen heterotrimer that also contains α1(V) chains. α4(V)-containing collagen molecules synthesized by Schwann cells retain their N-terminal non-collagenous domains. α4(V) mRNA was detected by reverse transcriptase-linked PCR amplification in neonatal and adult brain and neonatal peripheral nerve. α4(V) mRNA and protein were not detected in most other tissues, including placenta and heart, which are known to contain α3(V). This pattern of α4(V) expression contrasted with that of α1(V) mRNA and protein, which were ubiquitously expressed. The isolated α4(V) chain demonstrated an unusually high affinity for heparin. The restricted expression and unusual properties of α4(V)-containing collagen type V molecules suggest a unique and important role for these molecules in peripheral nerve development.
INTRODUCTION

Interactions of cells of the developing peripheral nervous system with the extracellular matrix (ECM)\(^1\) are critical for nervous system development. Cell-ECM interactions strongly influence cell shape and cell migration, which play major roles in determining tissue structure and cellular organization, as well as cellular terminal differentiation. Studies carried out with primary cultures of embryonic peripheral nerve cells have revealed that Schwann cells are major producers of the peripheral nerve ECM (1,2).

Previously, we identified an ECM protein that is secreted by Schwann cells that binds with high affinity to heparin and heparan sulfate proteoglycans. This 200 kDa protein, which we called p200, is expressed in developing peripheral nerves and appears in the late embryonic period before the onset of myelination (3). p200 is not expressed in adult nerves, but its expression is induced by axotomy. This pattern of expression is distinct from that exhibited by proteins of the Schwann cell basal lamina, such as laminin and collagen type IV, which are major components of the mature endoneurial ECM. Expression of p200 is restricted. The protein is readily detected by immunoblot analysis in extracts of developing peripheral nerve tissue or Schwann cells, but not in most other neonatal or adult tissues (4).

p200 was originally isolated from conditioned medium of cultured Schwann cells on the basis of its high affinity binding to the transmembrane heparan sulfate proteoglycan, syndecan-3 (4). This interaction is of sufficient affinity to mediate adhesion of cultured Schwann cells to immobilized p200, by a mechanism that is inhibited by soluble heparin. Biochemical and partial amino acid sequence analysis revealed p200 to be a collagen-like protein. Schwann cells cultured in medium containing ascorbate secrete p200 as pepsin-resistant, non-covalent trimers. p200 is incorporated into fibrillar ECM produced by Schwann cells, where it co-localizes with
collagen type I (3; R. Stahl, M. Chernousov, and D. Carey, unpublished observations). In addition to a large collagen domain, p200 contains a glycosylated non-collagen domain with an apparent Mr ~ 80,000. This domain appears to contain the main heparin-binding site of p200 (4).

We now report the molecular cloning from a neonatal rat Schwann cell library of cDNA that encodes p200. The cDNA sequence reveals p200 to be a collagen polypeptide with a high degree of sequence homology to type V/type XI collagen alpha chains. Ascorbate-treated Schwann cells secrete p200 as a non-covalent heterotrimer that also contains α1(V) collagen.

p200 appears to be the rat homologue of a mouse cDNA that was cloned recently (5). The authors of this report concluded that the mouse cDNA encoded the α3(V) collagen chain. Our analysis of the available data leads us to conclude, however, that p200 and the mouse cDNA actually encode a novel member of the type V/type XI collagen family, which we propose to name α4 type V collagen. The rat and mouse α4(V) collagen chains show significant homology to human α3(V). The abundant and transient expression of p200 by Schwann cells and its unique biochemical properties suggest that p200 containing collagen molecules play an important role in the development of the peripheral nervous system.
MATERIALS AND METHODS

Cell culture – Schwann cells were cultured from neonatal rat sciatic nerves as described previously (6). Human embryonic kidney cells (293 cells) were obtained from American Type Culture Collection and cultured in DME-10% fetal bovine serum. 293 cells were transfected using Lipofectamine-Plus (GIBCO-BRL).

Purification of p200-containing collagen trimer – Native p200-containing trimer was purified from conditioned medium of ascorbate-treated Schwann cells, as described previously (3), with several modifications. Briefly, the conditioned medium was supplemented with protease inhibitors and acidified with acetic acid to pH 3.0. p200-containing collagen was precipitated by addition of crystalline NaCl to a final concentration of 1.2 M. The precipitate was recovered by centrifugation, resuspended in 0.5 M acetic acid and dialyzed against 2 M urea, 100 mM NaCl, 50mM Tris-HCl, pH 7.8, plus protease inhibitors. The dialyzed material was loaded onto a Q-Sepharose column and eluted with a linear gradient of 0.1 M to 1 M NaCl in 2 M urea, 50 mM Tris-HCl, pH 7.8. Elution of p200 was monitored by immunoblot analysis. p200-containing fractions (eluted at approximately 0.55-0.75 M NaCl) were pooled, diluted with 50 mM Tris-HCl, pH 7.8 to a urea concentration of 0.7 M, and applied to a heparin-agarose column. The column was washed with 0.2 M NaCl, 50 mM Tris-HCl, pH 7.8 and bound material was eluted with a linear gradient of 0.2-1 M NaCl in 50 mM Tris-HCl, pH 7.8. Aliquots of column fractions were analyzed on 4-15% SDS-polyacrylamide gradient gels followed by silver staining and immunoblotting with anti-p200 or anti-α1(V) collagen antibodies. Aliquots were also analyzed by immunoblotting with antibodies against the heparan sulfate proteoglycans syndecan-3 (7) and glypican-1 (8). Anti-proteoglycan immunoreactivity was not detected in the purified collagen preparation (data not shown).
cDNA cloning – PolyA-containing RNA was isolated from cultured Schwann cells (Micro-FastTrack, Invitrogen) and used to synthesize cDNA using Superscript II reverse transcriptase (Superscript Choice System, Life Technologies) and a mixture of oligo dT and random hexamers as primers. The cDNA products were ligated to EcoRI-Not I-Sal I adapters, size fractionated, and ligated to dephosphorylated λGT11 arms (Promega). Library DNA was packaged into phage using Gigapack III Gold packaging extracts (Stratagene) and amplified in E.coli Y1090r. 86,000 recombinant phage were screened with affinity purified anti-p200 antibodies (1:500 dilution). Bound antibodies were detected by incubation with anti-rabbit IgG-alkaline phosphatase conjugate (1:7500 dilution) in 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05 % Tween-20 with 2% BSA. Positive plaques were visualized colorimetrically using 0.33 mg/ml NBT and 0.165 mg/ml BCIP as substrates.

Phage cDNA inserts from positive plaques were obtained by PCR amplification using λforward (CCT GGA GCC CGT CAG TAT CG) and λreverse (CAA CTG GTA ATG GTA GCG AC) primers and rTth DNA polymerase XL (PE-Applied Biosystems). The PCR products were subcloned into pCR3.1 (Invitrogen) and sequenced using Big Dye™ terminators and a 377 Automated Sequencer (PE-Applied Biosystems).

Full length rat α1(V) collagen cDNA was cloned by reverse transcriptase-linked PCR using oligonucleotide primers based on the hamster α1(V) sequence (9). The deduced amino acid sequence of rat α1(V) was 98% identical to the mouse and hamster sequences, and 94% identical to human α1(V). The non-collagenous domain of rat α1(XI) collagen cDNA was cloned by a similar method using oligonucleotide primers based on the partial rat sequence (10). Schwann cells expressed the “acidic” form of α1(XI) mRNA.
**Antibody preparation** – Preparation and characterization of polyclonal anti-p200 antibodies have been described previously (4). For library screening the antibodies were affinity purified as follows. p200 monomer partially purified from Schwann cell conditioned medium was subjected to SDS-gel electrophoresis and transferred to Immobilon. The p200 containing portion of the membrane was excised and incubated with anti-p200 antibodies. The membrane was rinsed with phosphate buffered saline, and the bound antibodies were eluted with 0.1 M triethanolamine, pH 11.5, neutralized with HCL, and dialyzed against phosphate buffered saline.

Polyclonal anti-α1(V) antibodies were prepared by immunizing rabbits with the recombinant N-terminal non-collagenous domain expressed as a GST fusion protein. BL21-DE3-pLysS transformants were induced with IPTG and lysed by sonication. The GST-fusion protein was purified by chromatography on glutatione-Sepharose and heparin-agarose. The purified protein was mixed with RIBI adjuvant and used to immunize rabbits as described previously (7).

Polyclonal anti-α1(XI) antibodies were prepared by a similar method, except the insoluble GST fusion protein was solubilized in 8 M urea and purified by preparative SDS gel electrophoresis. The region of the gel containing the GST-fusion protein was excised and emulsified by passing through a 25 gauge syringe needle before being used for immunization.

Preparation and characterization of affinity purified anti-syndecan-3 and anti-glypican-1 antibodies have been described previously (7).

Specificity and titer of antibodies were determined by immunoblot analysis using purified recombinant proteins and Schwann cell conditioned medium and cell lysates.
Biochemical characterization of collagen – Immunoblot analysis was carried out as described previously (11). Bound antibodies were detected by enhanced chemiluminescence. Signals were detected and quantitated with a Lumi-Imager (Boehringer-Roche Biochemicals).

Schwann cells were labeled with 100 µCi/ml of $^{35}$S-methionine (1175 Ci/m mole, NEN Life Science Products, Inc.) for 24 hours in low-methionine medium. The medium was supplemented with protease inhibitor cocktail, pre-cleared with protein A-Sepharose, and mixed with an equal volume of 1% NP-40, 120 mM NaCl, 50 mM Tris-HCl, pH 7.4. Anti-p200 antibody was added and the samples were incubated for 1 hr on a rotator. Immune complexes were collected with protein A-Sepharose, rinsed twice with 0.5% NP-40, 120 mM NaCl, 50 mM Tris-HCl, pH 7.4, and dissolved in SDS gel sample buffer with β-mercaptoethanol. The samples were resolved on 5% polyacrylamide gels, transferred to Immobilon-P and subjected to immunoblotting with anti-collagen antibodies. For these experiments bound antibodies were detected colorimetrically using alkaline phosphatase conjugated secondary antibodies. The membrane was then air dried and exposed to Kodak X-ray film to detect $^{35}$S-labeled polypeptides.

Internal peptides derived from p200 monomer were obtained by in-gel digestion of the protein as described previously (4), except Lysyl Endopeptidase (Wako BioProducts) was used instead of trypsin, and the digestion was carried out in 0.1 M Tris-HCl, pH 9.1. Amino-terminal sequencing of p200 was carried out as described previously (12), after transfer of the protein from an SDS polyacrylamide gel onto Immobilon P.

Immunofluorescence – Hind limbs from 18 d gestation rat embryos were mounted in Tissue-Tek and frozen immediately. Ten micrometer thick cryosections were applied to clean glass slides and fixed for 30 minutes in 3% paraformaldehyde in phosphate buffered saline.
sections were rinsed with phosphate buffered saline and incubated with primary antibodies (diluted in 5% non-fat milk, 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5) for 1 hour at room temperature. The slides were rinsed and incubated with fluorescent secondary antibodies for 30 minutes. The sections were rinsed, overlayed with cover slips and viewed with a fluorescence microscope (6). Goat anti-rabbit IgG conjugated with Texas Red-X was obtained from Molecular Probes (Eugene, OR). Mouse monoclonal anti-neurofilament antibody and goat anti-mouse IgG coupled to fluorescein isothiocyanate were obtained from Sigma Chemical Co. (St. Louis, MO).

**RT-PCR** – Reverse transcriptase-linked PCR amplification was carried out as follows. One µg of total RNA isolated from the tissues indicated in Results was used as template for synthesis of cDNA using SuperScript™ II RNase´Reverse Transcriptase (Life Technologies). Oligonucleotide primer pairs based on the rat p200 and α1(V) sequences were used in PCR amplification reactions. Sequences of the primers were: p200 (sense: 5’-CCC GGG AAG ACC AGT CCA CAT C- 3’; antisense: 5’-GAG AGC AGC CTG AGG GTC TGG- 3’); α1(V) (sense: 5’-GGA TGT TGC CTA CCG AGT CTC TAA- 3’; antisense: 5’-TCA TAG GCA GCT CGG TGG TC’). One hundred pmoles of each primer was used per reaction with Taq DNA polymerase (FisherBiotech). The predicted sizes of the p200 and alpha-1 type V PCR products were 700 bp and 493 bp respectively.

**Pepsin digestion** – Rat tissues were minced and homogenized in 0.5M acetic acid on ice. Pepsin (Worthington) was added to a final concentration of 0.5 mg/ml, and digestion was carried out for 6 hrs at 4°C. Insoluble material was removed by centrifugation.
RESULTS

Cloning of rat α4(V) collagen – Affinity purified anti-p200 antibodies were used to screen a neonatal rat Schwann cell cDNA expression library. Nine antibody positive clones were sequenced and found to contain overlapping cDNA inserts that encoded a collagen-like protein with a high degree of homology to collagen type V. Two overlapping clones yielded the full length sequence of 6111 nucleotides, not including a terminal poly A stretch. A consensus polyadenylation and splice recognition motif (AATAAA) is present 13 nucleotides upstream of the poly A sequence.

The deduced amino acid sequence is shown in Figure 1. The amino acid sequence begins at the 5'-most ATG of the cDNA, which initiates an open reading frame of 1373 amino acids, producing a polypeptide with a predicted molecular weight of 171,574.

The p200 amino acid sequence is homologous to rat α1(V) collagen and α1(XI) collagen, as well as to mouse and human cDNAs that were recently cloned (5) and reported as α3(V) collagen. The p200 amino acid sequence is 95% identical to the putative mouse α3(V) sequence, but only 82% identical to the human α3(V) sequence (Figure 2). Comparison of the mouse and human amino acid sequences also revealed a sequence identity of 82%. This is significantly lower than the cross-species sequence conservation typically observed for homologous collagen alpha chains. Comparison of the full length human α1(V) sequence with the rat, mouse and hamster sequences, for example, revealed 94% amino acid identity (Figure 2). Collagen domain sequences are even more highly conserved. The collagen domains of rat and human α1(V) collagen show greater than 98% amino acid sequence identity. In contrast to this high degree of sequence homology, the collagen domains of p200 and human α3(V) show only 88% sequence identity. Similarly, comparison of the deduced p200 sequence with sequences of peptides
derived from the collagen domain of human placental \( \alpha 3(V) \) collagen revealed an overall sequence identity of 86% in 363 amino acids from 8 non-contiguous peptides. In contrast, the human \( \alpha 3(V) \) amino acid sequence deduced from the cDNA sequence was 97% identical to the peptide sequences.

The sequence data, plus information on the expression and biochemical properties of p200 presented below, lead us to conclude that p200 is a novel member of the collagen type V/type XI family. The putative mouse \( \alpha 3(V) \) reported recently (5) appears to be the mouse homologue of p200. We propose the name \( \alpha 4 \) type V for this collagen chain.

**Domain structure of \( \alpha 4(V) \)** – Inspection of the p200 protein sequence reveals several distinct structural domains. The collagen domain is the largest, and is made up of 1011 amino acids of uninterrupted Gly-X-X collagen repeats. This region is 71% homologous to the collagen domain of rat collagen \( \alpha 1(V) \) and 88% identical to the corresponding domain of human \( \alpha 3(V) \) (Figure 2). The non-collagenous amino terminal domain of 369 amino acids can be divided into several subdomains (Figures 1 and 2). There is an amino terminal signal sequence of 29 amino acids, followed by a region of 196 amino acids that shows 76% and 40% identity to the corresponding regions of collagens \( \alpha 3(V) \) and \( \alpha 1(V) \), respectively (Figure 2). Following this is a region of 144 amino acids in which the sequence identities drop to 50% and 14%. This unique region contains a highly basic segment in which 16/23 residues are either arginine or lysine, that is flanked by proline-rich sequences. The basic segment contains 4 nearly perfect contatameric repeats of the consensus heparin-binding motif XBBXB (where B is a basic amino acid). Thus, this region might account for the high affinity binding of the polypeptide to heparan sulfate (4). Separating the amino terminal non-collagenous domain and collagen domain is a region of 106 amino acids that contains Gly-X-X repeats interrupted by non-collagenous
sequences of 10 and 36 amino acids. The deduced sequence contains 5 consensus sites for N-linked glycosylation, 3 of which are located in the unique non-collagenous region. The conserved region of the non-collagenous domain contains 4 cysteine residues that are also present in similar positions in collagens α1(V) and α1(XI).

Residues 30-44 of the deduced sequence match the N-terminal amino acid sequence determined for p200 (Figure 1), identifying the alanine at position 30 as the N-terminus of the mature polypeptide. The deduced sequence is also identical to the amino acid sequences of 6 internal peptides derived from the collagen domain of p200. These results confirm the identification of the cDNA as p200.

Transfection of 293 cells with an expression plasmid that contained the putative coding sequence resulted in the secretion of a polypeptide that reacted strongly with anti-p200 antibodies but not α1(V) antibodies and co-migrated on SDS polyacrylamide gels with p200 obtained from Schwann cell conditioned medium (Figure 3). These results confirm that the cDNA contains the full length coding sequence of p200.

*Schwann cells secrete p200 as heterotrimers that contain collagen α1(V) chains* – Ascorbate treated Schwann cells secrete p200 as non-covalently linked pepsin-resistant trimers (3). SDS gel electrophoresis and silver staining of p200 purified from the medium of ascorbate-treated Schwann cells revealed the presence of 3 polypeptide chains of 205 kDa, 200 kDa and 190 kDa. Of these polypeptides only the 200 kDa chain reacted on immunoblots with antibodies made against p200 (Figure 4A). All three polypeptides were immunoprecipitated from conditioned medium of ascorbate-treated Schwann cells with anti-p200 antibodies, however, demonstrating their tight association (Figure 4B). In contrast, only the 200 kDa chain was immunoprecipitated with anti-p200 antibodies when medium from Schwann cells not treated
with ascorbate was used (not shown). These results are consistent with ascorbate-dependent assembly of p200 into a collagen heterotrimer.

It has been shown that other members of the type V/type XI family form heteromeric collagen trimers. We hypothesized that p200 containing collagen heterotrimers were produced by co-assembly of p200 with other type V/XI collagen alpha chains. As shown in 4B, anti-\(\alpha_1(V)\) antibodies stained on immunoblots the 205 kDa polypeptide that was co-immunoprecipitated with anti-p200 antibodies. The anti-\(\alpha_1(V)\) antibodies also stained the 205 kDa band in the purified p200-containing heterotrimer (Figure 4C). When conditioned medium from ascorbate-treated Schwann cells was fractionated on an anion exchange column the \(\alpha_1(V)\) and p200 polypeptides bound to the column and co-eluted in precisely the same fractions (Figure 5). The 2 polypeptides were not co-eluted, however, when medium from Schwann cells that were not treated with ascorbate was used. Together, these findings demonstrate the ascorbate-dependent co-assembly of \(\alpha_1(V)\) and p200 into collagen heterotrimers in Schwann cells.

In contrast to these results, anti-\(\alpha_1(XI)\) antibodies failed to stain any bands in the purified p200-containing collagen preparation. The anti-\(\alpha_1(XI)\) antibodies did react strongly with an approximately 200 kDa band in detergent extracts of Schwann cells (Figure 4C). These results demonstrate that despite the fact that Schwann cells synthesize \(\alpha_1(XI)\), this alpha chain does not co-assemble with \(\alpha_1(V)\) and p200 chains in collagen trimers.

Expression of \(\alpha_1(V)\) and p200 collagen chains – p200 was identified originally on the basis of its high affinity binding to the heparan sulfate proteoglycan syndecan-3 (4). Syndecan-3 and p200 also exhibit similar patterns of expression during embryonic and postnatal development (3,4,7,11). As shown in Figure 6B and C, immunofluorescent staining of hind limbs of embryonic day 18 rats revealed a co-localization of syndecan-3 and p200. The regions of intense
syndecan-3/p200 staining corresponded to bundles of peripheral nerve fibers that course through the tissue, as revealed by staining with anti-neurofilament antibodies (Figure 6A). These nerve fiber bundles were also stained with anti-α1(V) antibodies (Figure 6D), consistent with the presence of α1(V)/p200 containing collagen trimers in the nerves. However, in contrast to the syndecan-3 and p200 staining, the α1(V) staining was not restricted to the nerve.

Expression of α1(V) collagen is fairly widespread, while our previous biochemical analyses indicated a more restricted pattern of p200 expression. α3(V) has also been reported to be widely expressed, but at low levels. Expression of α1(V) and p200 mRNAs was analyzed in a panel of neonatal and adult rat tissues by reverse transcriptase-linked PCR amplification. As shown in Figure 7, an amplified product derived from α1(V) mRNA was detected in every sample tested. In contrast, p200 mRNA-derived product was detected at high levels only in neonatal brain and peripheral nerve, cultured Schwann cells, and adult brain. Lesser but detectable amounts were seen in neonatal heart and skeletal muscle and adult heart. p200 mRNA-derived product was not detected in neonatal lung, spleen, or kidney or placenta.

We also compared the steady state levels of p200 and α1(V) polypeptide chains in a panel of newborn rat tissues by immunoblot analysis. As shown in Figure 8, p200 was detected in extracts of newborn rat peripheral nerve and Schwann cells. Expression of p200 mRNA (Figure 7) and protein (Figure 8) in Schwann cells strongly suggests that these cells are the major producers of this protein in peripheral nerves. p200 was detected at a low level in skeletal muscle, but was undetectable in extracts of the other neonatal tissues examined. These results are consistent with our previously reported findings on p200 expression (3,4).

In contrast to this result, expression of α1(V) collagen was detected in all of the tissue extracts examined, albeit at varying levels. Two major forms of the collagen chain were
detected, which displayed different apparent molecular weights on SDS polyacrylamide gels. The larger form, which appeared as a closely spaced doublet of approximately 205 kDa, was present at the highest levels in extracts of Schwann cells and newborn rat peripheral nerve, where it was the predominant α1(V) form detected. Immunoblot analysis of p200 containing collagen trimers purified from Schwann cell cultures also revealed only the high molecular weight form of the α1(V) chain (see Figure 4). Variable but lesser amounts of the high molecular weight α1(V) chain were detected in other tissues. In tissues other than peripheral nerve, however, a smaller immunoreactive polypeptide, probably representing a processed form of α1(V) collagen, was predominant.

Because the α3(V) collagen chain has been reported to be expressed in placenta and heart, we investigated expression of p200 in those tissues in more detail. Neonatal peripheral nerve, placenta, and adult heart were extracted with saline buffer, or digested with pepsin to release collagenous polypeptides. These samples were then analyzed by immunoblotting with anti-p200 antibodies. As shown in Figure 9, p200 was readily identified in saline extracts of nerve. An approximately 80 kDa immunoreactive band was also detected. Pepsin digested material released from the nerve contained an approximately 140 kDa immunoreactive product, corresponding to the collagen domain of p200. In contrast to these results, neither saline extracts nor pepsin released material from placenta or heart contained detectable amounts of polypeptides that were immunoreactive with anti-p200 antibodies. Anti-p200 immunoreactive material was also not detected in detergent extracts of placenta, or in tissue sections of placenta examined by immunohistochemical methods (data not shown).

*Heparin-binding activities of collagens α1(V) and α4(V)* – Previous studies have demonstrated that collagen type V binds heparin and heparan sulfate. Our earlier biochemical
characterization of Schwann cell p200 revealed a high affinity for heparin and heparan sulfate proteoglycans. This contrasts with reports that suggested a low affinity of \( \alpha_3(V) \) chains for heparin. We were interested in comparing the heparin binding activities of \( \alpha_1(V) \) and p200. Conditioned medium from Schwann cells was applied to a heparin-agarose column, which was eluted with a linear gradient of NaCl. Since the Schwann cells used for these experiments were not treated with ascorbate, the \( \alpha_1(V) \) and p200 chains were secreted as monomers. Elution of the collagen chains was monitored by immunoblot analysis. As shown in Figure 10, both collagen chains bound to the heparin-affinity column and were eluted by the salt gradient. p200 was eluted significantly later than \( \alpha_1(V) \), however, demonstrating a markedly higher affinity of p200 for heparin.
DISCUSSION

Previously, we reported the purification and characterization of a 200 kDa protein, p200, that is secreted by Schwann cells and binds with high affinity to the heparan sulfate proteoglycan syndecan-3 (4). In this paper we report the cloning of p200 cDNA and additional information on its structure and expression. The cDNA and deduced amino acid sequences identify p200 as a novel member of the family of collagen genes that includes type V and type XI collagens. As the fourth member of the type V collagen family, we propose the name \( \alpha 4(V) \) collagen for this polypeptide. Confirmation that the cloned cDNA corresponds to p200 comes from several lines of evidence. The protein encoded by the cloned cDNA is recognized by specific anti-p200 antibodies that do not recognize other collagens, including the closely related \( \alpha 1(V) \) and \( \alpha 1(XI) \) collagen chains. The protein structure predicted from the cDNA sequence matches the known properties of the native polypeptide (3,4). Finally, sequences that are identical to those of peptides derived from purified p200 are found in the deduced amino acid sequence. These include the N-terminal peptide, which provided information on the apparent site of signal peptide cleavage, and internal peptides derived from the collagen domain.

The sequence homology of rat \( \alpha 4(V) \) collagen to a recently reported mouse type V collagen (5) is sufficiently high to conclude that these are homologous polypeptides. The rat \( \alpha 4(V) \) (p200) sequence displays significantly lower homology to a human type V collagen polypeptide described in the same report (5). The authors identified these polypeptides as the mouse and human homologues of \( \alpha 3(V) \) collagen. The human sequence shows a very high degree of sequence identity with the available, but fragmentary, peptide sequence data from human \( \alpha 3(V) \) collagen (13), establishing, in our opinion, the human cDNA as \( \alpha 3(V) \) collagen. The rat p200 sequence and putative mouse \( \alpha 3(V) \) sequence, on the other hand, show a very high
degree of sequence identity to each other, but significantly lower homology to the human α3(V) sequence. The degree of sequence homology between the rat and mouse sequences (94% identity) is consistent with cross-species sequence variation typically observed for homologous members of this highly conserved gene family. In contrast, the homology between the rat and mouse sequences and the human α3(V) sequence (82%) is significantly lower than expected for homologous genes. We conclude, therefore, that p200 and the mouse cDNA represent a previously unrecognized member of the type V collagen family. Furthermore, the failure to detect using highly sensitive and specific methods p200 expression in tissues that have been shown to contain α3(V) chains provides additional evidence that p200 represents a novel collagen alpha chain.

Imamura et al. examined expression of human collagen α3(V) mRNA in fetal and adult human tissues (5). Among the human fetal tissues examined the heart and lung showed the highest levels of mRNA expression. Northern blot analysis of adult human tissues revealed the heart, placenta, uterus and brain to be the major sites of collagen α3(V) mRNA expression (although the transcript detected in brain was smaller than what was seen in other tissues). Expression of α3(V) polypeptide has also been demonstrated by biochemical methods in placenta (14) and uterus (15). In situ hybridization analysis of 15.5 dpc mouse embryos, on the other hand, failed to detect putative collagen α3(V) mRNA expression in heart or lung. The highest levels of mRNA expression were observed in the epimysium, a connective tissue sheath surrounding skeletal muscle fibers, the epineurium, a connective tissue sheath surround peripheral nerve fiber bundles, and sites of ligament attachment to developing bone (5). Although we have not examined the same developmental ages, our analyses of p200 (collagen α4 type V) expression in embryonic and postnatal rats are consistent with the reported
observations on mouse, but not human, α3(V) mRNA expression. Specifically, p200 was detected in skeletal muscle and peripheral nerve of early post-natal rats, but not in lung or heart. We were unable to detect p200 in adult heart or placenta. Northern blot analysis has shown that expression of the putative mouse collagen α3(V) mRNA in whole mouse embryos increases between embryonic days 15 and 17 (5). We have shown previously that expression of p200 in developing peripheral nerves increases dramatically on embryonic day 16 in the rat (3).

α4(V) is the sixth known member of a family of related genes that make up the type V and type XI collagens (16). (Due to their sequence homology and co-assembly into collagen heterotrimers it has been suggested that the type V and type XI collagens should be considered a single group). The sequence homology between α4(V) and α3(V) is greater than that between these collagens and the closely related α1(V) and α1(XI) chains (see Figure 2). Thus, α3(V) and α4(V) appear to make up a distinct subfamily within the type V/type XI collagen family.

Consistent with our earlier findings, expression of the α4(V) collagen chain is highly regulated, with high level expression restricted mainly to cells of neural origin. This is in contrast to α1(V), which is expressed in many tissues. The mechanisms that regulate the tissue specific expression of these collagens are not known. The human α1(V) collagen gene promoter has been described (17). The sequence reveals features that are unusual for a collagen gene promoter, but that are characteristic of “housekeeping” genes. This is consistent with the ubiquitous expression of α1(V) collagen. Interestingly, we observed expression of p200 mRNA in neonatal and adult brain, but could not detect the protein in those tissues by immunoblot analysis. The latter result is consistent with our earlier findings on p200 expression. This suggests that regulation of α4(V) expression occurs at both transcriptional and post-transcriptional levels.
Our results also demonstrate that peripheral glial cells produce an unusual form of type V collagen, characterized by the presence of the $\alpha 4(V)$ chain. Schwann cells secrete collagen heterotrimeric that contain both $\alpha 1(V)$ and $\alpha 4(V)$ chains. $\alpha 1(V)$ and $\alpha 4(V)$ chains are co-localized in regions surrounding developing peripheral nerves.

The $\alpha 4(V)$-containing collagen molecules synthesized by Schwann cells and present in developing peripheral nerves retain their N-terminal non-collagen domains. This conclusion is based on apparent molecular weights, collagenase digestion (3,4), and immunoreactivity with specific antibodies. This property has been described previously for members of the type V collagen family (18,19), but appears to occur to an unusually high degree in peripheral nerve. Thus, in Schwann cell cultures and newborn rat peripheral nerves the $\alpha 1(V)$ and p200 chains appear to undergo only limited proteolytic processing. Limited processing of the $\alpha 4(V)$ chains appears to be a cell type-dependent phenomenon. This is based on the observation that treatment of $\alpha 4(V)$-transfected human 293 cells with ascorbate results in the accumulation in the medium of an anti-p200 immunoreactive polypeptide of approximately 140 kDa, consistent with more extensive proteolytic processing that what is seen in Schwann cells (K. Rothblum and D. Carey, unpublished observations). Cell type-dependent limited processing of collagen alpha chains is also evident from immunoblot analysis of tissue extracts. In peripheral nerve extracts and Schwann cell cultures, where $\alpha 4(V)$ is expressed and displays limited processing, the $\alpha 1(V)$ also shows limited processing. In tissues that do not express $\alpha 4(V)$, the $\alpha 1(V)$ chain appears to be processed normally.

Our results also suggest that the N-terminal domains of these chains are retained in collagen molecules that are incorporated into the ECM. Antibodies made against the N-terminal domains of $\alpha 1(V)$ and $\alpha 4(V)$ stain extracellular fibrils in Schwann cell cultures (Chernousov,
Stahl, and Carey, unpublished observations) as well as ECM in rat embryo tissue sections. This is likely to have important consequences for the biological activity of the collagen. The N-terminal domain of \( \alpha 4(V) \) binds heparan sulfate chains with high affinity (4). This binding is of sufficient strength to mediate heparan sulfate-dependent adhesion of Schwann cells. Thus, collagen molecules that contain the N-terminal domain of \( \alpha 4(V) \) would possess unique ligand binding activities. The repeating basic motif in the \( \alpha 4(V) \) N-terminal domain is likely to be involved in mediating heparin binding. Recent work by San Antonio and colleagues using model peptides has shown that concatameric repeats of the basic heparin binding motif BBXB bind heparin with significantly higher affinity than single motifs (20).

Association of \( \alpha 1(V) \) and \( \alpha 1(XI) \) chains into collagen heterotrimers has been described, and is consistent with the high degree of structural similarity between these collagens. It is interesting to note that although Schwann cells synthesize both \( \alpha 1(V) \) and \( \alpha 1(XI) \), these alpha chains do not appear to co-assemble into collagen trimers produced by Schwann cells. Neither the mechanisms that regulate collagen chain assembly, nor the fate of the \( \alpha 1(XI) \) chains in Schwann cells are known.

In summary, we have identified what appears to be a novel member of the collagen type V gene family that is expressed by Schwann cells in developing peripheral nerves. The unusual structure and biochemical properties of the Schwann cell type V collagen and its restricted pattern of expression strongly suggest that this collagen plays a critical role during peripheral nerve development.
ACKNOWLEDGEMENTS

We thank Ms. Barbara Rabold for help with peptide sequence determinations.
REFERENCES

1. Bunge, M. B., Williams, A. K., Wood, P. M., Uitto, U., and Jeffrey, J. J. (1980) *J. Cell Biol.* 84, 184-202

2. Chernousov, M. A., Stahl, R. C., and Carey, D. J. (1998) *J. Cell Sci.* 111, 2763-2777

3. Chernousov, M. A., Scherer, S. S., Stahl, R. C., and Carey, D. J. (1999) *J. Neurosci. Res.* 56, 284-294

4. Chernousov, M. A., Stahl, R. C., and Carey, D. J. (1996) *J. Biol. Chem.* 271, 13844-13853

5. Imamura, Y., Scott, I. C., and Greenspan, D. S. (2000) *J. Biol. Chem.* 275, 8749-8759

6. Carey, D. J. and Stahl, R. C. (1990) *J. Cell Biol.* 111, 2053-2062

7. Carey, D. J., Evans, D. M., Stahl, R. C., Asundi, V. K., Conner, K. J., Garbes, P., and Cizmeci-Smith, G. (1992) *J. Cell Biol.* 117, 191-201

8. Asundi, V. K., Keister, B. F., Stahl, R. C., and Carey, D. J. (1997) *Exp. Cell Res.* 230, 145-153

9. Greenspan, D. S., Cheng, W., and Hoffman, G. C. (1991) *J. Biol. Chem.* 266, 24727-24733

10. Oxford, J. T., Doege, K. J., and Morris, N. P. (1995) *J. Biol. Chem.* 270, 9478-9485

11. Carey, D. J., Conner, K., Asundi, V. K., O'Mahony, D. J., Stahl, R. C., Showalter, L. J., Cizmeci-Smith, G., Hartman, J., and Rothblum, L. I. (1997) *J. Biol. Chem.* 272, 2873-2879

12. Matsudaira, P. (1987) *J. Biol. Chem.* 262, 10035-10038

13. Mann, K. (1992) *Biol. Chem. Hoppe-Seyler* 373, 69-75.

14. Niyibizi, C., Fietzek, P.P. and van der Rest, M. (1984) *J. Biol. Chem.* 259, 14170-14174

15. Abedin, M.Z., Ayad, S. and Weiss, J.B. (1982) *Biosci. Rep.* 2, 493-502
16. Prockop, D. J. and Kivirikko, K. I. (1995) *Ann. Rev. Biochem.* **64**, 403-434

17. Lee, S. and Greenspan, D. S. (1995) *Biochem. J.* **310**, 15-22

18. Moradi-Ameli, M., Rousseau, J.-C., Kleman, J.-P., Champliaud, M.-F., Boutillon, M.-M., Bernillon, J., Wallach, J., and Van der Rest, M. (1994) *Eur. J. Biochem.* **221**, 987-995

19. Linsenmayer, T. F., Gibney, E., Igoe, F., Gordon, M.K., Fitch, J. M., Fessler, L. I., and Birk, D. E. (1993) *J. Cell Biol.* **121**, 1181-1189

20. Verrecchio, A., Germann, M. W., Schick, B. P., Kung, B., Twardowski, T., and San Antonio, J. (2000) *J. Biol. Chem.* **275**, 7701-7707
FOOTNOTES

¹The abbreviations used are: ECM, extracellular matrix
FIGURE LEGENDS

Figure 1. Amino acid sequence of α4(V) collagen (p200). The deduced amino acid sequence of p200 is shown. Sequences that match those of internal peptides derived from purified p200 are underlined. The amino-terminal sequence determined for purified p200 is double-underlined. The highly basic region in the non-collagenous domain is enclosed by a dashed box. The 4 conserved cysteines in the N-terminal domain are in bold font. Consensus sites for N-linked glycosylation are indicated by dashed underlines. The uninterrupted collagen domain is boxed. Sequence data reported in this paper is available from GenBank/EMBL under accession numbers AF272661 and AF272662.

Figure 2. Amino acid sequence homology and structural similarities among members of the type V collagen family. The upper part of the figure shows the predicted domain structure of the α4(V) polypeptide. The tables in the lower left part of the figure show amino acid sequence identities between rat (R) and mouse (Mu) α4(V) and human (Hu) α3(V) collagens (upper table) and rat, mouse, hamster (Ha) or human α1(V) collagen (lower table). Rat α4(V) is p200. Mouse α4(V) sequence was taken from the putative α3(V) sequence (5). Human α3(V) was from the published cDNA sequence (5). The lower right part of the figure shows amino acid sequence homologies between corresponding domains of rat α4(V), α1(V) and α1(XI) and human α3(V) collagen chains. The numbers indicate the percent amino acid sequence identity between the indicated domains. α4(V) shows greatest homology with α3(V) collagen. α4(V) shows less sequence homology to α1(V) or α1(XI) than the latter chains show.
to each other. The full length rat $\alpha_1$(XI) sequence is not available. Sequence homologies were determined by the Clustal method.

**Figure 3. Expression of recombinant p200.** Medium from Schwann cells (lane 1), $\alpha_4$(V) transfected 293 cells (lane 2) or control-transfected 293 cells (lane 3) was subjected to immunoblot analysis with anti-p200 antibodies. The position of the 200 kDa marker is shown (arrow).

**Figure 4. Purification and characterization of p200-containing Schwann cell collagen.** Panel A: p200 was purified from conditioned medium of ascorbate-treated Schwann cells as described in Materials and Methods. The figure shows the p200-containing column fractions from the final step of the purification, heparin-agarose chromatography. Aliquots were subjected to SDS gel electrophoresis and analyzed by immunoblotting with anti-p200 antibodies (top) or silver staining. Arrows indicate position of migration of the 200 kDa size marker. Panel B: Schwann cell cultures were incubated in medium that contained ascorbate and $^{35}$S-methionine. Aliquots of the medium were immunoprecipitated with anti-p200 antibodies, subjected to SDS gel electrophoresis and analyzed by autoradiography to detect radiolabeled polypeptides ($^{35}$S) and immunoblot analysis with anti-p200 or anti-$\alpha_1$(V) collagen antibodies. The arrow indicates the position of migration of the 200 kDa size marker. Panel C: purified p200-containing collagen was subjected to SDS gel electrophoresis and analyzed by silver staining and immunoblot analysis with anti-$\alpha_1$(V) collagen, anti-p200, or anti-$\alpha_1$(XI) collagen antibodies, as indicated. An aliquot of a detergent lysate of ascorbate-treated Schwann cells (SC extract) was also subjected to immunoblot analysis with anti-$\alpha_1$(XI) collagen antibodies.
Figure 5. Co-assembly of $\alpha_1$V and $\alpha_4$V collagen chains in ascorbate-treated Schwann cells. Conditioned medium from Schwann cells that were incubated for 48 hours in the absence (A) or presence (B) of 50 $\mu$g/ml ascorbate was applied to a DEAE-5PW high pressure liquid chromatography column and eluted with a linear gradient of 0-1 M NaCl (dashed line). Aliquots of column fractions were subjected to SDS gel electrophoresis and immunoblot analysis with anti-$\alpha_1$V (upper panels) or anti-p200 antibodies (lower panels). Antibody staining was quantitated using a Lumi-Imager and is displayed in graphical form on the left.

Figure 6. Immunofluorescent staining of rat embryo hind limb tissue. Cryosections of the hind limb from an 18 day rat embryo were stained with anti-neurofilament (A), anti-syndecan-3 (B), anti-p200 (C), or anti-$\alpha_1$V collagen (D) antibodies. Adjacent sections are shown. The bar = 100 $\mu$m.

Figure 7. $\alpha_4$V and $\alpha_1$V mRNA expression in neonatal and adult rat tissues. Upper panel: RNA was isolated from tissues obtained from postnatal day 2 rats and neonatal Schwann cells. These RNAs were used as templates for reverse transcriptase-linked PCR amplification using primer pairs specific for $\alpha_1$V and p200. The expected sizes of the p200 and $\alpha_1$V-derived products are indicated. Lower panel: RNA isolated from tissues obtained from adult rats and from neonatal Schwann cells was used as templates as described above. Ethidium bromide stained gels are shown.

Figure 8. Immunoblot analysis of p200 and $\alpha_1$V steady state levels in neonatal rat tissues. Equivalent amounts (wet weight) of the indicated neonatal rat tissues were extracted
with buffered saline solution. Equivalent aliquots of the extracts were applied to SDS polyacrylamide gels and analyzed by immunoblotting with anti-p200 (upper panel) or anti-α1(V) (lower panel) antibodies. Schwann cell conditioned medium (SC) and purified p200-containing collagen isolated from Schwann cells (SC col V) were included on each gel. The arrows indicate the position of migration of the 200 kDa molecular weight marker.

**Figure 9. Expression of p200 in rat tissues.** Neonatal rat sciatic nerve (nerve), placenta and adult heart were extracted with buffered saline or digested with pepsin in acetic acid (see Materials and Methods). Equivalent aliquots of the saline extract (S) or pepsin digest (P) from each tissue were applied to SDS polyacrylamide gels and subjected to immunoblot analysis with anti-p200 antibodies. Numbers and arrows to the right indicate position of migration of molecular weight markers.

**Figure 10. Heparin affinity chromatography of Schwann cell α1(V) and p200 collagen chains.** Conditioned medium from Schwann cells that were cultured in medium without ascorbate was applied to a heparin-agarose column and eluted with a linear gradient of sodium chloride (dashed line). Aliquots of column fractions were subjected to immunoblot analysis with anti-α1(V) (upper left panel) or anti-p200 (lower left panel) antibodies. The immunoblots were quantitated with a Lumi-Imager and the data are shown graphically on the right.
Figure 1
Rat and Murine $\alpha_4(V)$
vs Human $\alpha_3(V)$

|       | Mu | Hu |
|-------|----|----|
| R     | 95 | 82 |
| Mu    | 82 |    |

$\alpha_1(V)$

|       | Mu | Ha | Hu |
|-------|----|----|----|
| R     | 98 | 98 | 94 |
| Mu    | 100| 94 |    |
| Ha    |    | 94 |    |

1a 1b b c d

1a conserved N-terminal domain region
1b unique N-terminal domain region
b interrupted Gly-X-X domain
c uninterrupted Gly-X-X domain
d C-terminal non-collagenous domain
Figure 4
Figure 5
Figure 7
Figure 8
Figure 9
Figure 10
Schwann Cells Synthesize Type V Collagen That Contains a Novel Alpha 4 Chain: Molecular Cloning, Biochemical Characterization and High Affinity Heparin Binding of Alpha4(V) Collagen
Michael A Chernousov, Katrina Rothblum, William A Tyler, Richard C Stahl and David J Carey

J. Biol. Chem. published online June 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M003922200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts