Biosynthesis of Type IV Collagen by Cultured Rat Schwann Cells

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ABSTRACT We have obtained evidence that rat Schwann cells synthesize and secrete type IV procollagen. Metabolic labeling of primary cultures of Schwann cells plus neurons and analysis by SDS PAGE revealed the presence of a closely spaced pair of polypeptides in the medium of these cultures that (*a*) were susceptible to digestion by purified bacterial collagenase, (*b*) co-migrated with type IV procollagen secreted by rat parietal endoderm cells, and (*c*) were specifically immunoprecipitated by antibodies against mouse type IV collagen. Limited pepsin digestion of metabolically labeled medium or cell layers produced a pepsin-resistant fragment characteristic of pro-α1(IV) chains. Removal of neuronal cell bodies from the cultures immediately before labeling did not reduce the amount of type IV procollagen detected in the medium. This indicated that Schwann cells, not neurons, were responsible for synthesis of type IV procollagen. We believe type IV procollagen is a major constituent of the Schwann-cell extracellular matrix based upon (*a*) its presence in a detergent-insoluble matrix preparation, (*b*) its presence in the cell layer of the cultures in a state in which it can be removed by brief treatment with bacterial collagenase or trypsin, and (*c*) positive immunofluorescence of Schwann cell-neuron cultures with anti-type-IV collagen antibodies. Secretion of type IV procollagen was substantially reduced when Schwann cells were maintained in the absence of neurons. This observation may account for the previously reported finding that Schwann cells assemble a basal lamina only when co-cultured with neurons (Bunge, M. B., A. K. Williams, and P. M. Wood, 1982, *Dev. Biol.*, 92:449).

For the past several years we have been interested in the extracellular matrix (ECM)[1] of the peripheral nervous system. Histologically, this ECM consists of a well-defined basal lamina and a less well-defined endoneurial sheath surrounding each Schwann cell, whether related to unmyelinated or myelinated axons (32). These axon-Schwann cell basal lamina units are, in turn, embedded within a matrix of collagen fibrils. Together, the basal lamina, the endoneurial sheath, and the surrounding collagen fibrils comprise the endoneurium (25). In most of our studies of peripheral nerve ECM, we have utilized primary cultures of developing rat dorsal root ganglion neurons and Schwann cells. These cells, when grown together in culture, assemble a basal lamina with associated small collagen fibrils which is morphologically similar to that produced in vivo. In addition, the cultured Schwann cells provide unmyelinated ensheathment and myelination for the axons (6).

With this culture system we have been able to demonstrate that Schwann cells synthesize and secrete certain components of the ECM, including collagen (6) and laminin (13). Matrix assembly appears to be an essential step in Schwann cell development, based upon the observation that growth of Schwann cells and neurons under conditions that prevent normal ECM assembly leads to abnormal development. These conditions include growth in the presence of the proline

[1] *Abbreviation used in this paper:* ECM, extracellular matrix.

THE JOURNAL OF CELL BIOLOGY · VOLUME 97 AUGUST 1983 473-479

analogue *cis*-hydroxyproline (11) and growth in a serum-free defined medium in which collagen production by Schwann cells is reduced (9, 22; Carey and Bunge, unpublished observations).

Another finding from the studies of these cells in tissue culture is that neurons appear to regulate ECM assembly by Schwann cells. This is based upon the observation that Schwann cells do not deposit a basal lamina and collagen fibrils unless they are co-cultured with neurons (7). Our initial observations indicated, however, that neurons neither significantly alter the secretory activity of Schwann cells nor contribute to the secretion of extracellular proteins (9). Thus, until recently, the mechanism underlying the requirement for neurons in the production of ECM by Schwann cells could not be explained.

Type IV collagen is a specific and ubiquitous component of basal laminae as demonstrated in many biochemical (17, 33) and immunoelectron microscopic (15, 19, 27, 31) studies. The protein is assembled into a regular network connected by covalent cross-links (34), thus providing these matrices with mechanical stability. Type IV collagen is synthesized as a soluble precursor, type IV procollagen, that is only slightly larger than the final matrix product (4, 23). Because of the essential role that the Schwann cell basal lamina seems to play in normal development, we have investigated type IV collagen production by cultured Schwann cells. Here, we present evidence that: (*a*) Schwann cells synthesize and secrete type IV procollagen; (*b*) at least a portion of the secreted collagen is incorporated into an insoluble ECM; and (*c*) neurons modulate the secretion of a limited number of polypeptides by Schwann cells, including type IV collagen. The latter observation may account for the observed effect of neurons on basal lamina formation by Schwann cells (7).

MATERIALS AND METHODS

Cell Culture Methods: The Schwann cell and neuron culture methods have been described previously (6, 8, 9, 39). Briefly, lumbar dorsal root ganglia from late embryonic rats were cultured as explants or after dissociation on a substratum of rat tail collagen in 2-cm diam dishes molded from Aclar plastic. During the first 7–14 d of culture, the cells were fed a medium containing 10^{-5} M 5-fluorodeoxyuridine (to kill fibroblasts), and subsequently fed a medium lacking the drug but containing human placental serum and chick embryo extract. In this medium (and in the presence of neurons) Schwann cells proliferate, assemble an ECM, and eventually ensheath and myelinate axons.

Parietal endoderm cells were obtained by dissecting Reichert's membranes and attached cells from 14–16-d-old rat embryos. The trophoblast cells were stripped off with the aid of a dissecting microscope. The parietal endoderm cells (attached to the Reichert's membrane) were radiolabeled as described below, and the medium was used as a source of type IV procollagen (10).

Radiolabeling and Electrophoresis: Cultures were labeled with [^{3}H]leucine or [^{3}H]proline as described previously (9). SDS PAGE was performed in 6% slab gels according to Laemmli (18). Runs were calibrated by using globular proteins as standards. In some experiments rat endodermal type IV procollagen and rat tail tendon (type I) collagen were used as standards. In all cases where molecular weights are indicated in the text, they were determined from calibration curves derived from the mobilities of globular protein standards. This has resulted in slightly higher estimates of molecular weights for type IV procollagen chains than reported by others. For analysis of disulfide bonding, samples were electrophoresed first in 6% tube gels in the absence of 2-mercaptoethanol, and then electrophoresed after equilibration in gel sample buffer containing 5% 2-mercaptoethanol in the second dimension in 6% slab gels. For experiments in which the cell layers were analyzed, the cultures were rinsed three times with PBS containing 2.5 mM phenylmethylsulfonylfluoride, 10 mM EDTA, 10 mM *N*-ethylmaleimide, scraped into a Dounce homogenizer, and homogenized in 0.5–1.0 ml of the same buffer.

Collagen Analysis: Type IV collagen was immunoprecipitated from radiolabeled culture medium with affinity-purified rabbit anti-mouse type IV collagen antibodies (33). The antigen-antibody complexes were collected by adsorption to fixed and killed *Staphylococcus aureus* cells (Pansorbin, Calbiochem-Behring Co., San Diego, CA), washed with 7.5 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 1% Tween-80, 50 μg/ml ovalbumin, dissolved in electrophoresis sample buffer, and analyzed by SDS PAGE.

For collagenase digestion of radiolabeled culture medium, samples of medium were dialyzed into 7.5 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, and then incubated for 1 h at 37°C with purified bacterial collagenase (Advanced Biofactures) at a concentration of 50 U/ml. The reaction was stopped by addition of an equal volume of two-times concentrated electrophoresis sample buffer. Controls were treated identically except that the incubations were done in the absence of enzyme.

For pepsin digestion, samples of radiolabeled medium or cells were dialyzed into 0.5 M acetic acid and incubated at 4°C for 18 h with 100 μg/ml pepsin. The reaction was stopped by freezing in dry ice/acetone and lyophilization.

The relative amounts of collagen released by Schwann cells in the presence and absence of neurons were quantitated in cultures either still containing neuronal explants or two weeks after removal of neuronal explants. The cultures were radiolabeled as described above and medium samples containing equal amounts of total acid-precipitable radioactivity were electrophoresed under reducing conditions. The areas of the bands of pro-α(IV) chains were then determined by densitometric scanning of the fluorograms.

Detergent-insoluble Extracellular Matrix: We used a modification of the method of Meezan et al. (21) for purification of basement membrane from tissues to prepare a detergent-insoluble ECM from Schwann cell-neuron cultures. Briefly, the procedure consisted of sequential extractions of radiolabeled cultures with Triton X-100 (3%), deoxyribonuclease I (0.1 mg/ml in 1 M NaCl), and deoxycholate (4%). Extractions were carried out for 30 min at 4°C except for the deoxyribonuclease extraction, which was done at room temperature. The material remaining on the culture dishes after these extractions was scraped into a Dounce homogenizer, homogenized in a small volume of PBS containing 2.5 M phenylmethylsulfonylfluoride, 10 mM EDTA, 10 mM *N*-ethylmaleimide, and subjected to SDS PAGE. In some cases samples were treated with either bacterial collagenase or pepsin as described above prior to SDS PAGE analysis.

Immunocytochemistry of Cell Cultures: Live cultures were incubated with the primary antibody directed against type IV collagen (30 μg/ml) in a HEPES-buffered Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum (Gibco Laboratories, Grand Island, NY) for 1 h at 37°C. Binding was visualized by indirect methods, using a fluorescein-conjugated goat anti-rabbit secondary antibody (Cappel Laboratories Inc., Cochranville, PA.). Cultures were fixed in 3% paraformaldehyde/PBS and photographed in a Zeiss Universal microscope equipped for fluorescence with epi-illumination.

RESULTS

Schwann Cells Secrete Type IV Procollagen

Bacterial collagenase digestion of [^{3}H]leucine-labeled medium from cultures of Schwann cells and neurons resulted in the disappearance of at least three high-molecular-weight polypeptides when analyzed by SDS PAGE (Fig. 1). The largest of these collagenase-sensitive polypeptides, which is actually a closely-spaced pair of bands (M_r 190,000), co-migrated with type IV procollagen secreted by rat parietal endoderm cells (Fig. 1). Both bands were, as expected, specifically immunoprecipitated by antibodies to mouse type IV collagen (Fig. 2). These two bands very likely are the pro-α1(IV) and pro-α2(IV) chains of basement membrane procollagen (3, 16, 36). The other two collagenase-sensitive bands were not identified but showed a mobility similar to that of the chains of interstitial procollagens.

Pepsin digestion of the medium or the cell layer produced five to six major polypeptides (Fig. 3). The largest polypeptide migrated slightly faster than intact pro-α(IV) chains, indicating that it is derived from the pro-α1(IV) chain (3, 16). This polypeptide co-migrated with pepsinized type IV procollagen of parietal endoderm cells and contained interchain disulfide bonds as demonstrated by two-dimensional electrophoresis (data not shown). Some of the other pepsin-resistant chains observed were derived from interstitial procollagen types I, III

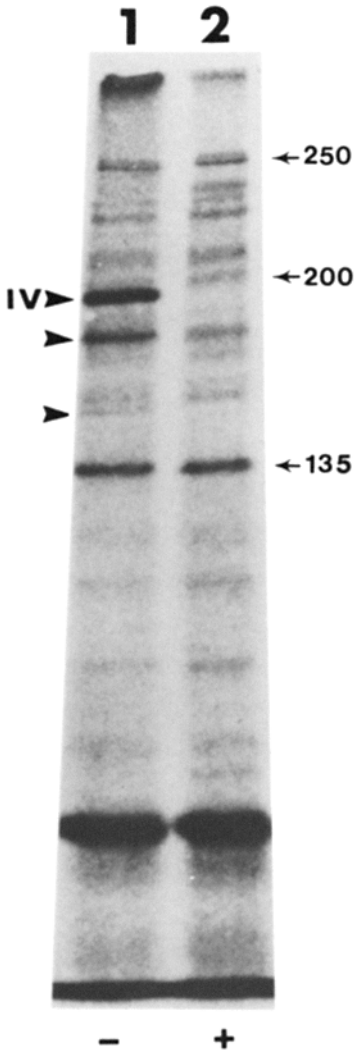

FIGURE 1 Bacterial collagenase digestion of medium obtained from radiolabeled Schwann cell-neuron cultures. Fluorogram of a 6% SDS polyacrylamide gel of [^{3}H]leucine-labeled medium incubated without (lane *1*) or with (lane *2*) bacterial collagenase. Collagenase-sensitive bands are indicated by arrowheads; arrows and numbers to the right indicate molecular weights ($M_r \times 10^{-3}$). The position of migration of parietal endoderm type IV procollagen is indicated to the left (*IV*).

(which migrates just below α1(I) and is most evident in the medium) and V (which migrates as a doublet between types I and IV). Other polypeptides may in part be fragments of the pro-α2(IV) chain, which is less resistant to pepsin digestion than the pro-α1(IV) chain (17, 35).

Excision of neuronal cell bodies from Schwann cell-neuron explant cultures immediately prior to radiolabeling does not alter the pattern of labeled proteins released into the culture medium, indicating that Schwann cells synthesize most of the labeled-medium polypeptides detected by electrophoresis (9). The axons that are left behind are devoid of ribosomes and thus incapable of protein synthesis. In the present study we observed that the same treatment did not reduce the amount of labeled type IV procollagen detected in the culture medium (data not shown). Thus Schwann cells, and not neurons, were producing the type IV procollagen. Further support for this conclusion came from the fact that we were unable to detect type IV procollagen by electrophoresis in the medium of radiolabeled neuron cultures, and that neurons cultured in the absence of Schwann cells did not stain for type IV collagen by immunofluorescence (data not shown).

Type IV Procollagen is Part of an Insoluble Extracellular Matrix

To determine whether type IV procollagen is present in the Schwann cell ECM, we extracted radiolabeled Schwann cell-neuron cultures with Triton X-100, deoxyribonuclease I, and deoxycholate, as described in Materials and Methods. PAGE of the radiolabeled material resistant to this extraction revealed four major and several minor polypeptides (Fig. 4). The most prominent polypeptide, with M_r 190,000, was identical to the one shown above to be type IV procollagen. In addition, we observed a band that co-migrated with pepsinized type IV procollagen after SDS PAGE analysis of pepsinized ECM (data not shown). These results indicated that type IV procollagen is a major constituent of the Schwann cell ECM. The polypeptides in the ECM preparation appearing at M_r 250,000 and 200,000 are immunoprecipitated by antilaminin antibodies (12).

We obtained additional evidence that the Schwann cell ECM contains type IV collagen from the observation that radiolabeled pepsin-resistant type IV collagen could be detected in the cell layer of Schwann cell-neuron cultures (Fig. 3). This collagen was removed from the cultures by brief treatment with bacterial collagenase under conditions in which the cells remained intact (data not shown). When radiolabeled cultures were subjected to brief trypsin digestion, the cell-associated type IV collagen was selectively removed (data not shown). Trypsin has been shown to remove the

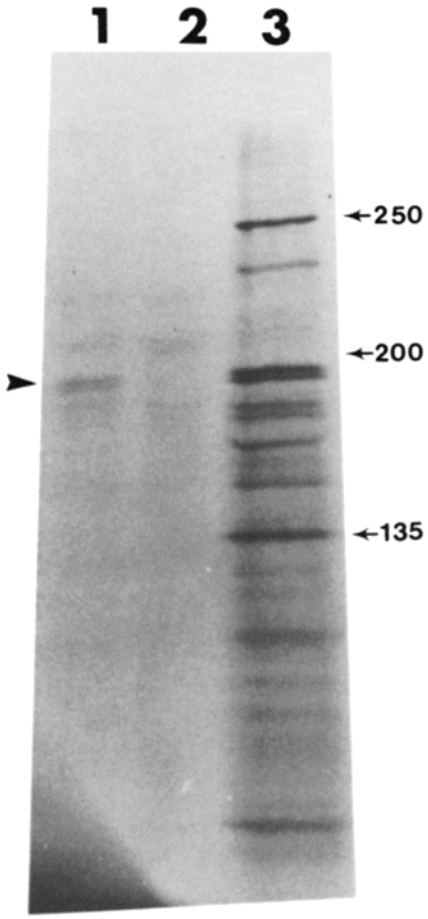

FIGURE 2 Anti-type-IV collagen immunoprecipitation of medium obtained from radiolabeled Schwann cell-neuron cultures. Fluorogram of a 6% SDS polyacrylamide gel of medium polypeptides from [^{3}H]leucine-labeled cultures; lane *1*: anti-type-IV IgG immunoprecipitate; lane *2*: nonimmune IgG immunoprecipitate; lane *3*: total medium. The arrow to the left indicates the position of migration of type IV procollagen. The arrows and numbers to the right indicate molecular weight ($M_r \times 10^{-3}$).

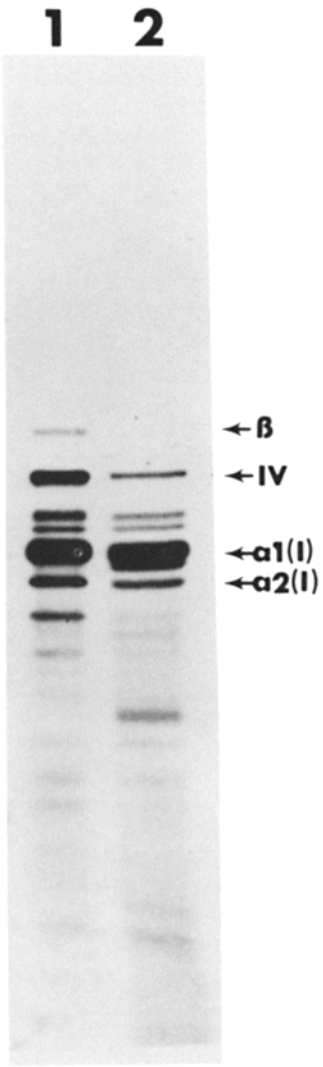

FIGURE 3 Pepsin-resistant polypeptides from medium or cell layer of radiolabeled Schwann cell-neuron cultures. Fluorogram of 6% SDS polyacrylamide gel of [^{3}H]leucine-labeled cell layer (lane *1*) or medium (lane *2*) after limited pepsin digestion. The symbols to the right indicate the position of migration of pepsinized collagen standards; pepsinized type IV collagen was obtained from rat parietal endoderm cells; pepsinized β-chains (i.e., cross-linked dimers of collagen α-chains) and α1(I)- and α2(I)-chains are from rat tail tendon collagen.

basal lamina, but not the small-diameter fibrils, from Schwann cell-neuron cultures (7) and to degrade type IV collagen of other species (33). Thus, this result is consistent with the presence of type IV collagen in the basal lamina.

Indirect immunofluorescence with anti-type-IV collagen antibodies produced bright and specific staining of Schwann cell-neuron cultures. The staining associated with myelinated fibers suggested the presence of type IV collagen in the Schwann cell basal lamina (Fig. 5).

Neurons Alter Schwann Cell Collagen Secretion

Schwann cells deposit a basal lamina and collagen fibrils only when co-cultured with neurons (7). Because earlier observations had shown that neurons do not exert a significant effect on the overall amount or pattern of polypeptides released by Schwann cells (9), we next examined whether neurons exert a specific effect on collagen secretion by Schwann cells. In these experiments we used Schwann cells that had been devoid of axonal contact for 2 wk. The results indicated that in the presence of neurons, Schwann cells release into the medium significantly more procollagen than in the absence of neurons (Fig. 6). The increase in type IV procollagen in the medium of neuron-containing cultures was 6–9-fold relative to the amount of the M_r 250,000 and 150,000 polypeptides, as determined by densitometry (range of three experiments). The same result was obtained when pepsinized culture medium was analyzed (data not shown). The cell layers of neuron-containing cultures also contained more type IV collagen than cultures without neurons as determined by pepsin digestion and SDS PAGE analysis. Thus, the increase in type IV procollagen in the medium of neuron-containing cultures cannot be accounted for by a decreased deposition of type IV procollagen into the matrix.

In addition to type IV procollagen, a polypeptide with M_r 135,000 appeared to be present in increased amounts in the medium of neuron-containing cultures. This polypeptide was also present in the detergent-insoluble ECM of Schwann cell-neuron cultures but was not identified (Fig. 4). The amount of several other Schwann cell-released polypeptides was not increased by co-culture with neurons. These included some ECM constituents, e.g., the M_r 250,000 polypeptide of laminin (Fig. 6; see also Fig. 4), and type I collagen (data not shown).

We also observed that the release of some polypeptides by Schwann cells was enhanced in the absence of neurons (Fig. 6). The identity of these polypeptides is unknown.

DISCUSSION

The results presented here demonstrate that cultured rat Schwann cells synthesized, secreted, and incorporated type IV procollagen into an insoluble extracellular matrix. Type IV collagen is an abundant component of basal laminae, and has been shown to be synthesized in culture by many cell

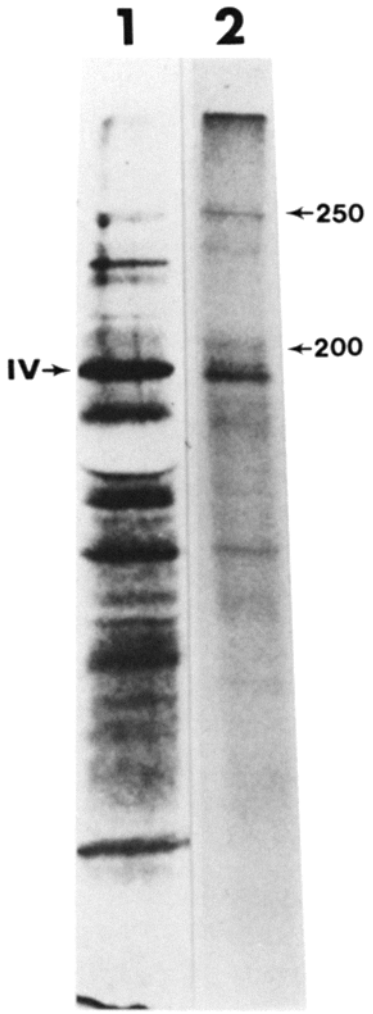

FIGURE 4 Polypeptides of the detergent-insoluble extracellular matrix from radiolabeled Schwann cell-neuron cultures. Fluorogram of 6% polyacrylamide gels of [^{3}H]leucine-labeled cultures; lane *1*: unfractionated medium; lane *2*: detergent-insoluble ECM, prepared as described in Materials and Methods. The arrows and numbers to the right indicate molecular weight ($M_r \times 10^{-3}$). The position of migration of type IV procollagen is indicated to the left.

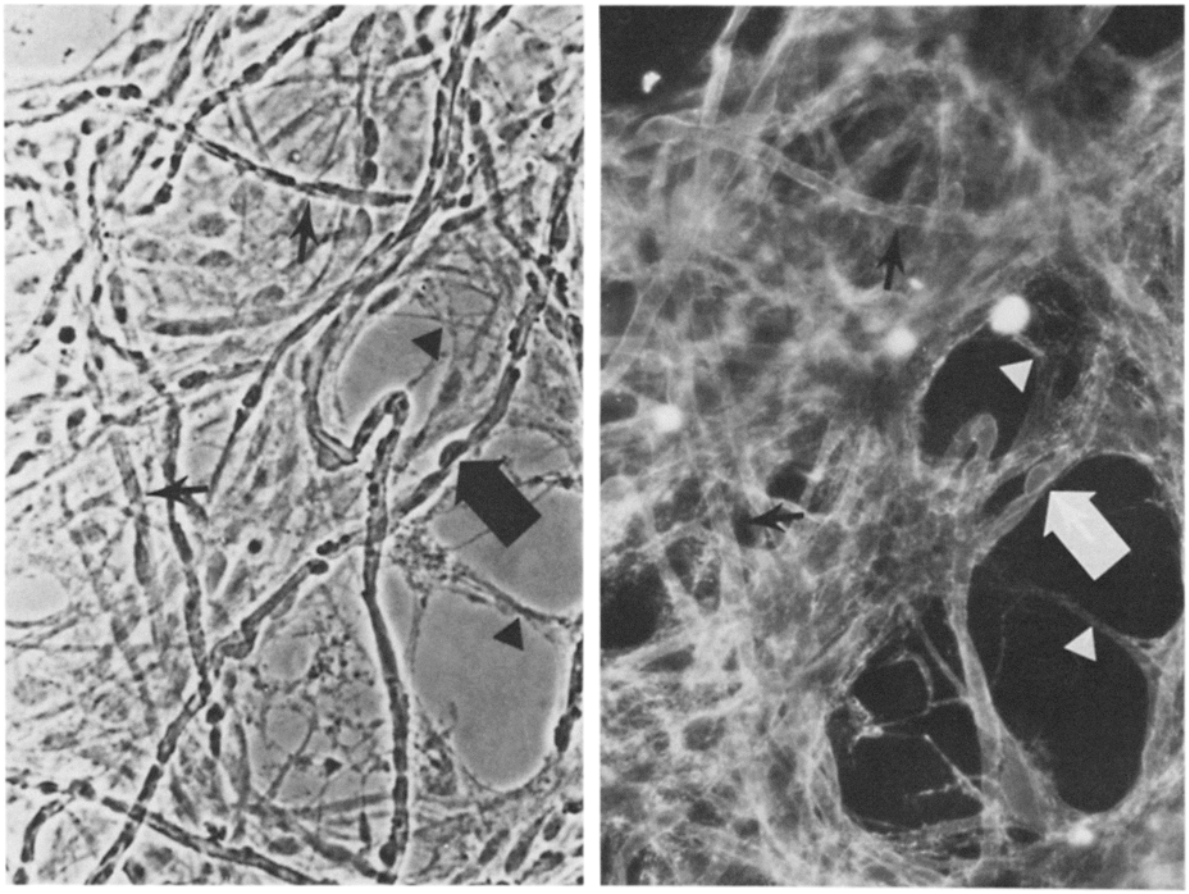

FIGURE 5 Immunofluorescence staining for type IV collagen of Schwann cell-neuron culture. This culture was maintained in enriched medium for 7 wk and contained myelinated as well as unmyelinated neurites, and is shown in phase contrast (*left*) and after immune staining (*right*). Antibodies directed against collagen type IV decorated the myelinated fibers (arrows) and included the myelinated Schwann cell soma (large arrow) within the linear staining pattern. A majority of the unmyelinated fibers (triangles) were also decorated in this culture. These probably represent unmyelinated neurites, which are ensheathed by Schwann cells. × 315.

types, including mammary epithelium (20) and parietal endoderm cells (10). To our knowledge, this is the first documentation of the synthesis of type IV procollagen by neural cells not derived from a tumor. Our data also indicate that while neurons themselves did not contribute directly to type IV collagen synthesis in a significant manner in our cultures, their presence was required for maintaining a high-level production of the protein by Schwann cells. A neuroblastoma cell line has been found to synthesize type IV procollagen and other basal lamina proteins (2). This difference could be due to altered phenotypic expression of transformed cells or could indicate that different types of neurons vary in their capacity to produce basal lamina proteins.

The neuronal regulation of ECM production by Schwann cells presents an intriguing and possibly unique example of intercellular regulation. From the results presented here it appears that neurons may control the assembly of the Schwann cell ECM by specifically stimulating the secretion of certain ECM constituents, notably type IV procollagen, while not affecting others. Elsewhere, we have reported that in the absence of neurons, Schwann cells produce and maintain the basal lamina glycoprotein laminin on their surface (12), even though these cells do not deposit a visible basal lamina (7). This indicates a lack of close coordination in the production of type IV procollagen and laminin, as was also found in studies with carcinoembryonic cells (26).

The mechanism of the regulation of collagen secretion by neurons is unknown. For example, the experiments reported here cannot distinguish between an increase in the rate of synthesis and a decrease in the turnover rate of the regulated polypeptides. It also remains to be determined whether neurons exert this effect by direct contact with Schwann cells, or by a diffusible factor. The regulation by neurons of Schwann cell ECM assembly is only one of several instances in which these cells appear to communicate. Growing axons stimulate Schwann cell proliferation (40; for review see 28), and the mitogen is believed to be an axonal membrane protein. Experiments with culture systems similar to those employed in the present study have shown that stimulation of Schwann cell proliferation by axons requires direct contact between Schwann cells and axons and may be mediated by particulate fractions prepared from axons (29, 30). Also, the signal that directs Schwann cells to provide either myelinated or unmyelinated ensheathment for axons comes from the axon itself (1). The mechanism of this process is unknown.

Previous results have suggested that the elaboration of the Schwann cell basal lamina is a necessary step in subsequent normal Schwann cell development, which includes ensheathment and myelination of axons by Schwann cells (8, 9, 11, 22). The time during peripheral nerve development at which the Schwann cell basal lamina is formed has not been precisely established. In the early stages of development, Schwann cells

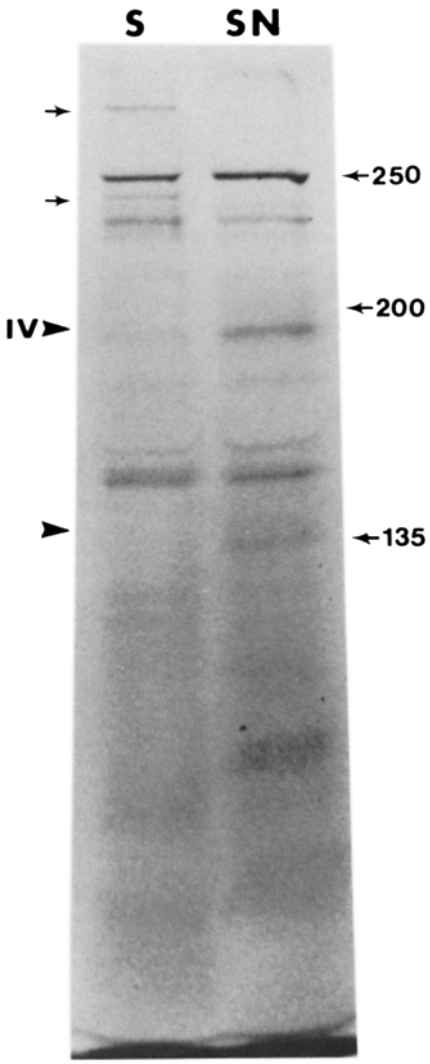

FIGURE 6 Radiolabeled medium proteins released by Schwann cells cultured in the presence or absence of neurons. Fluorogram of 6% polyacrylamide gel of [^{3}H]leucine-labeled polypeptides found in the medium of Schwann cells grown for 3 wk in the presence of neurons followed by 2 wk in the absence of neurons (*S*) and Schwann cells grown continuously for 5 wk in the presence of neurons (*SN*). Arrows and numbers to the right indicate molecular weight ($M_r \times 10^{-3}$). Arrowheads to the left indicate polypeptides whose numbers are decreased in cultures without neurons. The position of migration of type IV procollagen is indicated (*IV*). Arrows to the left indicate polypeptides whose numbers are increased in cultures without neurons.

exhibit a proliferative and migratory phase prior to the onset of ensheathment and myelination of axons (37). The Schwann cell basal lamina is not yet present at this time (5). A basal lamina appears, however, when Schwann cells begin to sort the axons and initiate myelin formation (37). Thus, the production of Schwann cell basal lamina may coincide with the onset of this stage of Schwann cell development, and may, as suggested by the culture studies cited above, be an obligatory condition for subsequent ensheathment and myelination of axons by Schwann cells.

These observations in vivo, along with the tissue culture studies that have shown that neurons control the assembly of Schwann cell basal lamina (7) and that axon-Schwann cell units interact with fibroblasts in the formation of the perineurium (38), place the axon in a central role in the regulation of peripheral nerve ECM production during development. The identification of proteins governed by neuronal regulation should facilitate further studies on the molecular mechanism. Of interest in this context are certain mutant strains of mice that possess ECM abnormalities in their peripheral nervous system. The use of culture preparations from the mutant strain dystrophic has made it possible to demonstrate that the basal lamina defect found in the peripheral nervous system of this mutant in vivo is expressed in organotypic culture (24), and that dystrophic Schwann cells express a basal lamina deficiency even though grown in relation to normal axons (14). These mutant strains could be useful in exploring the links between ensheathment deficiencies and defects in ECM production.

We thank Artree James for technical assistance and Susan Mantia and Carol Ann Ardoin for secretarial aid.

Supported by National Institutes of Health (NIH) grants NS 09923 and GM 28002 to R. P. Bunge and a grant from the Deutsche Forschungsgemeinschaft (Project Ti/955) to R. Timpl. Fellowship support to D. J. Carey was from the Muscular Dystrophy Association; training support to C. F. Eldridge was from NIH training grant NS 07071.

Received for publication 7 February 1983, and in revised form 19 April 1983.

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