Identification, characterization, and tissue distribution of apolipoprotein D in the rat

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Abstract We recently described an unknown apolipoprotein that is present on the lipoprotein particles isolated from regenerating rat sciatic nerves. In the regenerating nerve, the concentration of this apolipoprotein rises 500-fold over its concentration in the normal nerve. In this report we have identified the apolipoprotein by partial amino acid sequence analysis as apolipoprotein (apo) D. Characterization of rat apoD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed it to be composed of a series of molecular weight isoforms of between 27 kDa and 31 kDa that increase 2 kDa in apparent molecular mass upon reduction. Rat apoD has multiple isoelectric points between pH 4.05 and 4.37, apparently resulting from *N*-linked glycosylation. In the rat, unlike the human, little apoD is found in plasma. However, immunocytochemical localization of apoD in 12 tissues (liver, kidney, bladder, adrenal, cerebrum, duodenum, testis, lung, spleen, pancreas, heart, and skin) showed that a variety of cells contained substantial levels of apolipoprotein. The broad distribution of apoD suggests that it may play a general role in cellular metabolism. Moreover, many of the same cell types varied dramatically in their content of apoD in different tissues, suggesting that the uptake or secretion of apoD by cells is regulated. —**Boyles, J. K., L. M. Notterpek, M. R. Wardell, and S. C. Rall, Jr.** Identification, characterization, and tissue distribution of apolipoprotein D in the rat. *J. Lipid Res.* 1990. **31:** 2243–2256.

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In the regenerating peripheral nerve, massive membrane degradation and lipid release is rapidly followed by massive membrane biogenesis and lipid utilization. Peripheral nerve regeneration, therefore, serves as an excellent model in which to establish the mechanism of lipid transport between cells. After a denervating injury, as the nerve undergoes Wallerian degeneration, large quantities of phospholipids and cholesterol are released by the breakdown of myelin (1–5). Much of this lipid remains in the nerve, stored in macrophages, neurolemmal cells, and Schwann cells (2, 6–8). Large quantities of lipid are in turn required for the assembly of new axonal and myelin membranes. Both stored lipids and lipids produced locally or imported from the plasma are utilized in this process (2, 6–9). During nerve regeneration, lipids and lipid-binding proteins accumulate in lipoprotein particles within the interstitial matrix of the nerve (8, 10). Presumably, it is these lipoprotein particles that provide the nerve with a lipid transport system.

It has been our goal to understand this lipid transport system. We and others have already identified three of the apolipoproteins that are components of the lipoprotein particles accumulating in the regenerating peripheral nerve of the rat: apolipoprotein (apo) E (8, 10), apoA-I (8), and apoA-IV (11). Among the several unidentified proteins is one that is produced by neurolemmal cells and increases in concentration over 500-fold during regeneration of the rat sciatic nerve (11). On the first day after injury, this protein increases severalfold in concentration, and it reaches its peak concentration at 3–4 weeks after injury. Thus, both during axon regeneration (1 day to 2 weeks after injury) (8) and active myelination (2–6 weeks after injury) (8), this protein is present in greatly increased concentrations.

We have now isolated, characterized, and identified this protein. It is apoD. Apolipoprotein D had previously been identified only in humans and the baboon, where it is a minor component of plasma lipoprotein particles (12–16). The human apoD protein has been characterized (12–16), and its mRNA (17) and gene (18) sequences have been determined. In the human, apoD mRNA has been identified in several tissues, including the brain (17, 19). Rat apoD, as shown by our study, is very similar to human apoD. Unlike the human protein, the rat protein is not

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; LCAT, lecithin:cholesterol acyltransferase.

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present to a significant extent in the plasma or on plasma lipoproteins. The expression of apoD by tissues may, however, be similar in both species. Using an affinity-purified antibody and immunocytochemistry, we have identified apoD in a wide variety of cells from a dozen rat tissues.

MATERIALS AND METHODS

Animals, nerve crush, and lipoprotein isolation

Male and female Sprague-Dawley rats (300–450 g) were used for all experiments. The procedures used for nerve crush, nerve collection, and lipoprotein isolation were identical to those described in an earlier study of peripheral nerve regeneration in the rat (8). Briefly, while the animal was under deep anesthesia, the sciatic nerves of both legs were exposed through a small incision and crushed with jeweler's forceps just below the sciatic notch. The wound was closed, and regeneration was allowed to proceed for 3–4 weeks. Prior to lipoprotein isolation, the animals were anesthetized and perfused to remove the blood and the lipoproteins it contains. The segment of the sciatic nerve distal to the crush injury and the nerve's major branches were then harvested. The lipoproteins were allowed to diffuse out of the coarsely chopped nerve segments into a buffer and were isolated by density centrifugation; KBr was used to adjust the density of the nerve extract to 1.21 g/ml or 1.24 g/ml. Similarly, rat plasma lipoproteins were isolated by density centrifugation. Human plasma high density lipoproteins (HDL) were isolated between d 1.063 and 1.21 g/ml.

Electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of all samples was performed according to the procedure of Wyckoff, Rodbard, and Chrambach (20). Ten percent acrylamide gels were used with or without the reducing agent β-mercaptoethanol (2%). Silver staining was done according to the procedure described by Morrissey (21). Immunoblots were produced as previously described (8, 22). For immunoblotting, an antibody to the 27-kDa isoform of apoD isolated from nonreduced SDS-PAGE gels of nerve lipoproteins was used at a dilution of 1:1000. The preparation and characterization of this antibody has been described (11). The relative concentration of apoD in various fractions was determined by densitometry scans of immunoblots and was corrected for the quantity of total protein applied to each lane and present in a fraction. The method of Lowry et al. (23) was used for protein determinations.

Protein isolation

The major 27-kDa isoform of apoD was isolated by electroelution from nonreduced preparative SDS-PAGE gels of nerve lipoproteins. Electroelution of the protein was performed according to the procedure of Hunkapiller et al. (24), with modifications to reduce the concentration of SDS. The presoaking step was omitted. The gel pieces were rinsed in water, loaded into the wells of the elution chamber, soaked for 3–5 h in elution buffer, and the protein was electroeluted overnight. An additional 4 days of electrophoresis, using frequent changes of 10 mM NH_4HCO_3, was included in the protocol to remove residual SDS from the isolated protein. Approximately 40 μg of the 27-kDa isoform of apoD was isolated from each group of 20 rats (40 nerves). For amino acid and amino sugar analysis, the 29-kDa to 31-kDa isoforms of apoD from nonreduced gels were also isolated.

Isoelectric focusing

The procedure for isoelectric focusing of the proteins of nerve lipoproteins was modified from Menzel and Utermann (25). After delipidation with $CHCl_3$–CH_3OH 2:1 (v/v), 10 μg of the apolipoprotein was dissolved in 0.01 M Tris-HCl, pH 8.0, containing 1% sodium decyl sulfate, 1% NP-40 (Pierce, Rockford, IL), and 20% sucrose, and applied to a polyacrylamide focusing gel. The gel contained pH 3.5–5 ampholines (Pharmacia LKB, Biotechnology Inc., Piscataway, NJ), 8 M urea, and 0.5% NP-40. Electrophoresis was performed for 2 h at 200 V, 3 h at 400 V, and 2 h at 800 V. The isoelectric focusing gels were then either silver-stained, used to determine the pH gradient, or applied to the top of a 10% SDS-PAGE gel for electrophoresis without reduction in the second dimension. The pH gradient within the gel was determined by dividing one lane from the middle of each gel into 1/4-inch segments. These sections were then soaked in boiled distilled water, and the pH was determined. A plot of pH versus position in the gel was used to extrapolate the isoelectric points of the protein bands.

Amino acid, amino sugar, and carbohydrate analysis

For amino acid analysis, samples were hydrolyzed for 20 h at 110°C with 6 N HCl in sealed, evacuated tubes. For amino sugar analysis, hydrolysis was performed for 4 h at 100°C in 4 N HCl in sealed, evacuated tubes. Amino acids and amino sugars were quantified on a Beckman 121MB Amino Acid Analyzer (Beckman Instruments, Fullerton, CA) equipped with a model 126 data system. For sugar analysis, a modification of the standard three-buffer sodium citrate system was used (26): only the second buffer (0.35 N sodium citrate, pH 3.9, with 2% isopropanol) and the high temperature setting (65°C) were used. The two amino sugars (glucosamine and galactosamine) are well resolved by this buffer and elute between phenylalanine and the basic amino acids.

N-glycanase (N-Glycosidase F) (Genzyme, Boston, MA) digestion was carried out according to the supplier's recommended procedure. Nerve lipoproteins were digested without prior delipidation.

Amino acid sequencing

Amino acid sequencing was done on an Applied Biosystems 477A pulsed-liquid sequencer (Applied Biosystems, Foster City, CA) equipped with an on-line 120A analyzer. The protein was fragmented by protease digestion and CNBr cleavage. *Staphylococcus aureus* V-8 protease (ICN Biomedicals, Costa Mesa, CA) digestion of the isolated protein was carried out in two stages. Apolipoprotein D (25 μg) in 300 μl of 10 mM NH_4HCO_3 was digested with V-8 protease (10 μg) for 16 h at 37°C; a second addition of V-8 protease (10 μg) was made, and the digestion was allowed to continue for an additional 16 h. The peptides generated by V-8 protease were separated by reverse-phase high-performance liquid chromatography on a Beckman Model 334 using a Waters Delta Pak C-4 column (15 cm × 3.9 mm, Waters Chromatography Division, Millipore Corp., Milford, MA) maintained at 36°C. A gradient of two buffers was used: buffer A, 0.1% trifluoracetic acid; and buffer B, 80% acetonitrile with trifluoracetic acid added so that the absorbance of buffer B at 214 nm was identical to that of buffer A. The digest was injected onto the column after equilibration with 90% buffer A and 10% buffer B. Buffer B was then increased linearly to 100% in 90 min at a flow rate of 0.5 ml/min. The absorbance was monitored at 214 nm, and the peptides were collected manually. The major peptides were subjected to sequence analysis.

CNBr cleavage of 10 μg of apoD was carried out directly on the sequencer glass fiber filter after three cycles of Edman degradation. Twenty microliters of CNBr (50 mg/ml in 70% formic acid) was applied to the filter, and digestion was carried out overnight at room temperature in an argon atmosphere saturated with the same solution. The filter was oven-dried at 45°C for 30 min, and sequencing was reinitiated on the digested sample.

Immunocytochemisty

Immunocytochemical detection of apoD was performed on cryostat sections using procedures identical to those used previously to detect apoE (8, 22), apoA-I (8, 22), and apoD (11) in neural tissue. The rabbit anti-rat apoD was used on frozen cryostat sections, and the bound antibodies were detected using a peroxidase ABC kit (Vector Laboratories, Burlingame, CA). An affinity-purified apoD antibody was used for all immunocytochemistry. This antibody was isolated using 30 μg of the 27-kDa isoform of apoD electroeluted from a nonreduced SDS-PAGE gel and bound to nitrocellulose. After the residual protein-binding sites were quenched with 5% Carnation nonfat milk in phosphate-buffered saline (PBS) for 5 min, the nitrocellulose was washed in PBS and incubated in 0.5 ml of anti-apoD serum overnight. The nitrocellulose was then washed in PBS and the bound antibodies were released with 5 M NaCl and 0.15 M glycine, pH 2.8. The antibody solution was neutralized with an equal volume of 0.5 M Tris-HCl, pH 8.0, and then dialyzed against PBS to which 0.02% sodium azide had been added.

Two controls for nonspecific antibody binding were done. First, the sections were incubated without a primary antibody in order to detect endogenous peroxidase and the nonspecific sticking of the secondary reagents. Second, as a control for the nonspecific sticking of rabbit IgG, the sections were incubated in preimmune sera. Neither control resulted in a reaction product, i.e., they were negative.

RESULTS

Characterization of new apolipoprotein in the regenerating peripheral nerve

To identify the apolipoproteins present within the regenerating nerve, lipoprotein particles were isolated from the distal segment of rat sciatic nerves at the time of peak accumulation of the apolipoproteins (3–4 weeks after a crush injury) (11). All the blood within the nerve was removed to ensure that the isolated nerve lipoproteins were not contaminated with plasma lipoproteins (8). The nerve lipoproteins were then allowed to leach out of chopped regenerating nerve segments into a buffer for collection by density centrifugation. As shown in **Fig. 1**, only a limited number of proteins were present in the nerve lipoproteins, as assessed by both reduced and nonreduced SDS-PAGE and silver staining. Many of these proteins have already been identified, apoA-I (M_r ~26,000), apoE (M_r ~34,000 to 36,000), apoA-IV (M_r ~45,000), and albumin (M_r ~66,000 reduced and M_r ~59,000 nonreduced) (8, 10, 11, 26, 27). Several proteins above M_r ~120,000 that have not yet been isolated or identified were also found in the nerve lipoproteins. In addition, a series of lower molecular weight bands was seen in gels of the nerve lipoproteins. These had molecular weights between 27,000 and 31,000 when no reducing agent was used. A similar series was seen between 29,000 and 33,000 when a reducing agent was added (Fig. 1). Although readily identified in silver-stained gels of nerve lipoprotein, this lower molecular weight series of bands was poorly stained by Coomassie Blue.

An antibody raised against the most prominent of these bands isolated from nonreduced preparative SDS-PAGE gels (M_r ~27,000) recognized the series of lower molecular weight bands in both reduced and nonreduced gels. This suggested that these bands were isoforms of a single protein. The increase in apparent molecular weight of these isoforms upon reduction further suggested the presence of one or more internal disulfide bonds in the pro-

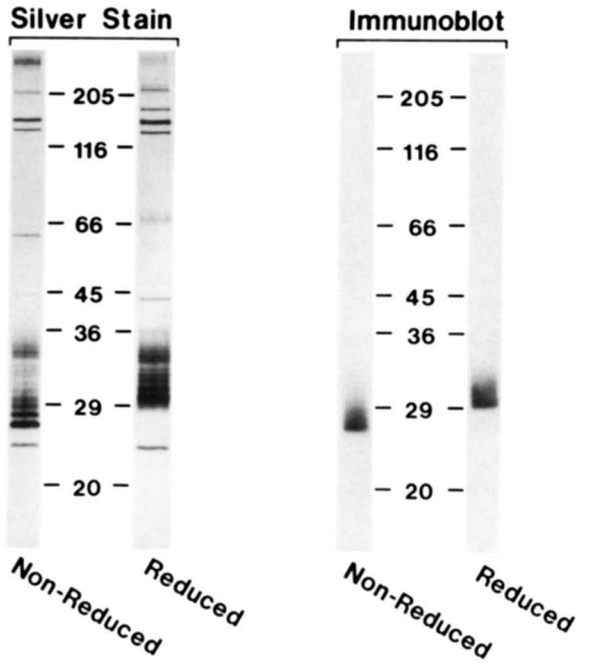


Fig. 1. SDS-PAGE and immunoblot of lipoprotein particles (d < 1.21 g/ml) isolated from the regenerating rat sciatic nerve 4 weeks after a crush injury. Left: Reduced and nonreduced nerve lipoproteins after SDS-PAGE and silver staining. Right: Immunoblot of reduced and nonreduced nerve lipoproteins separated by SDS-PAGE and probed with an antibody raised against the major 27-kDa protein isolated from a nonreduced gel of rat sciatic nerve apolipoproteins.

tein. As indicated by the lack of high molecular weight bands in the immunoblot of the nonreduced nerve lipoproteins, this protein did not form disulfide-linked complexes with itself or with other proteins of the nerve lipoprotein particles.

The distribution of this protein between lipoprotein (d < 1.21 g/ml) and lipoprotein-deficient (d > 1.21 g/ml) fractions of the regenerating nerve extract and plasma was determined using immunoblots and densitometry scans of the resultant autoradiograms (**Fig. 2**). Significant quantities of this protein were identified by immunoblots in the d > 1.21 g/ml fraction of nerve extracts; 13 to 15% of the total was in this fraction. After the density was raised to 1.24 g/ml, the same amount of the protein remained in the d > 1.24 lipoprotein-deficient fraction, suggesting that the protein in this fraction is substantially free of lipid and not part of a very high density lipoprotein. No significant amount of this unknown lipid-binding protein, or apolipoprotein, was found in the plasma of the rat. It could not be detected on immunoblots of either whole rat plasma or the lipoprotein-deficient fraction of rat plasma. It was detected, however, in the lipoprotein fraction of rat plasma (Fig. 2), but at a concentration significantly less than 0.1% of its concentration in the nerve lipoprotein particles.

The nerve apolipoprotein was further characterized by isoelectric focusing. As seen in **Fig. 3A**, it focused as a series of six bands having isoelectric points between pH 4.05 and 4.37. When two-dimensional electrophoresis was performed, each of these differently charged isoforms separated into a series of molecular weight isoforms (Fig. 3B), resulting in a complex and distinctive pattern of isoforms. The more acidic isoforms also tended to be the higher molecular weight isoforms of the apolipoprotein.

The amino sugar compositions of the 27-kDa and the combined 29- to 30-kDa isoforms of the rat nerve apolipoprotein isolated from nonreduced SDS-PAGE gels were determined. Amino sugar analysis of both of these fractions demonstrated only glucosamine, but no galactosamine, thus demonstrating the presence of *N*-linked sugars, but not *O*-linked sugars. The presence of *N*-linked carbohydrate residues on the rat nerve apolipoprotein was confirmed by *N*-glycanase digestion. As shown in **Fig. 4**, after *N*-glycanase digestion and analysis by reduced SDS-PAGE and immunoblotting, most of the rat nerve apolipoprotein present in a sample of nerve lipoproteins was reduced in molecular weight to a single band of 22 kDa.

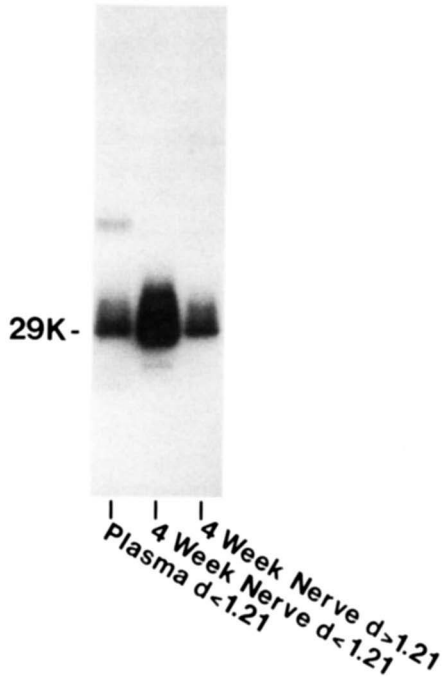


Fig. 2. Immunoblot for the detection of the nerve apolipoprotein in the lipoprotein (d < 1.21 g/ml) fraction of rat plasma and in the lipoprotein (d < 1.21 g/ml) and lipoprotein-deficient (d > 1.21) fractions of regenerating nerve extracts obtained 4 weeks after injury. Twenty micrograms of plasma lipoproteins, 0.1 μg of nerve lipoproteins, and 4 μg of the lipoprotein-deficient fraction of the regenerating nerve extract were electrophoresed under reduced conditions. The blot was probed with an antibody raised against the 27-kDa isoform of the rat nerve apolipoprotein isolated from a nonreduced gel.

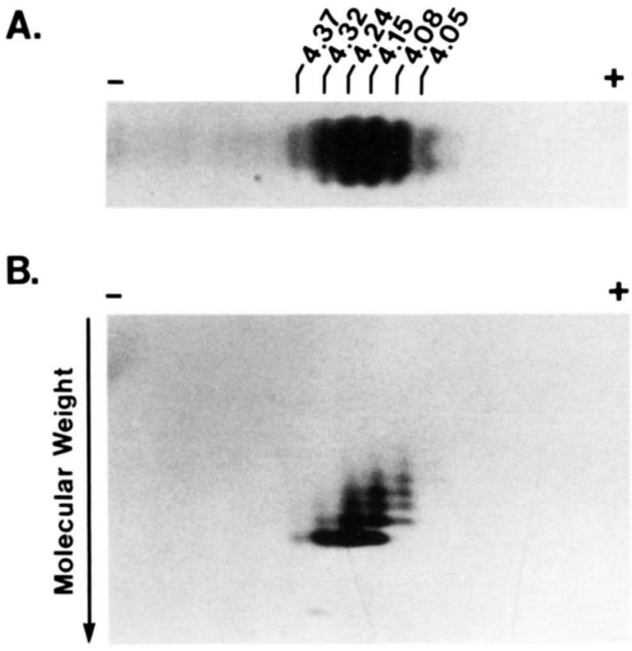


Fig. 3. Isoelectric focusing gels of silver-stained nerve apolipoproteins. A. One-dimensional isoelectric focusing. B. Two-dimensional electrophoresis using isoelectric focusing in a pH 3.5-5 gradient in the first dimension and nonreduced SDS-PAGE in the second dimension. The arrow points in the direction of decreasing molecular weight.

Intermediate digestion products formed a series of bands from 23.5 kDa to 29 kDa, and a residue of undigested apolipoprotein remained as a series of bands above 29 kDa. These results suggest that the many molecular weight isoforms of this apolipoprotein may result from differences in glycosylation, and that this glycosylation is *N*-linked and occurs at two or more sites.

The amino acid compositions of the 27-kDa and combined 29- to 30-kDa isoforms of the rat nerve apolipoprotein were also determined. The amino acid composition of the 27-kDa isoform is shown in **Table 1**. (The 29- to 30-kDa isoforms gave essentially identical results in both amino sugar and amino acid analysis; data not shown.) Also shown for comparison in Table 1 is the amino acid composition of human apoD, a plasma apolipoprotein that is similar in molecular weight (12–17) and glycosylation (17).

Identification of the new apolipoprotein of the regenerating peripheral nerve

The properties of the rat nerve apolipoprotein suggested that it might be rat apoD. To confirm this tentative identification, the M_r ~27,000 isoform was isolated from nonreduced SDS-PAGE gels for amino acid sequence analysis. The amino terminus was found to be blocked (as in human apoD (17)); therefore, internal sequences were sought. Because of the possible presence of residual SDS in our preparations, *S. aureus* V-8 protease digestion, which is resistant to inhibition by SDS, was chosen. The 27-kDa protein proved extremely resistant, however, to hydrolysis by this protease, and CNBr cleavage was therefore used to generate additional sequence information. The results of this sequence analysis identified the rat apolipoprotein as apoD. In **Fig. 5**, the CNBr- and V-8 protease-derived amino acid sequences of rat apoD have been aligned with the corresponding sequences of human apoD, as determined by Drayna and his collaborators (17) from the nucleotide sequence of human apoD cDNA.

The first rat V-8 peptide corresponded to amino acid residues 61 through 68 of the human apoD sequence; the sequences were identical at 6 of the 8 amino acids. The second of the rat V-8 peptides corresponded to amino acid residues 87 through 98 of the human sequence; the sequences were identical at 7 of the 12 amino acids. The third rat V-8 peptide corresponded to the human sequence beginning at amino acid residue 159 and included the apparent carboxy-terminal residue of the protein, amino acid 169; the sequences were identical at 5 of the 10 amino acids.

CNBr cleavage of the rat nerve apolipoprotein was used to generate additional sequence information. Assuming that the three methionine residues of the human protein were conserved in the rat protein, we expected three se-

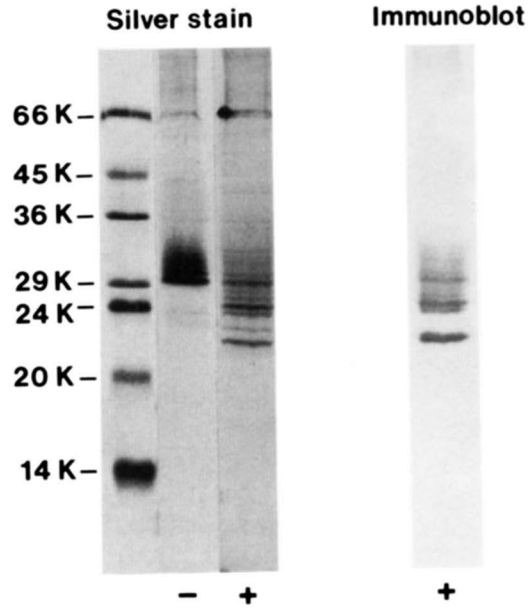


Fig. 4. SDS-PAGE gel and immunoblot of nerve lipoprotein particles that have (+) or have not (-) been digested by *N*-glycanase. The reduced SDS-PAGE gel is stained by silver. The immunoblot is also from a reduced gel. The blot was incubated with an antibody raised to the major 27-kDa isoform of the rat nerve apolipoprotein isolated from a nonreduced gel.

TABLE 1. Comparison of the amino acid composition of regenerating rat sciatic nerve apolipoprotein and human apolipoprotein D

Amino Acid	Amino Acid Analysis 27-kDa Rat Apolipoprotein	Amino Acid Composition[a] Human Apolipoprotein D	
	mole %	*mole %*	*residues*
Lysine	6.4	6.5	11
Histidine	1.7	1.2	2
Arginine	2.7	2.4	4
Aspartic Acid	11.7	13.6	23
Threonine	5.4	6.5	11
Serine	7.0	4.1	7
Glutamic Acid	13.1	11.2	19
Proline	6.1	7.1	12
Glycine	8.8	3.6	6
Alanine	7.2	5.9	10
Cysteine	ND[b]	3.0	5
Valine	5.7	7.1	12
Methionine	1.0	1.8	3
Isoleucine	5.4	6.5	11
Leucine	9.8	8.3	14
Tyrosine	4.1	4.1	7
Phenylalanine	3.9	4.7	8
Tryptophan	ND	2.4	4

[a]From Drayna et al. (17).
[b]ND, Not determined.

quences; the fourth possible fragment was expected to be blocked at the amino terminus. Upon sequencing, two prominent amino acids were detected at each cycle, and a third, less prominent amino acid sequence was identified at most cycles. The two prominent amino acids at each cycle were present in sufficiently different yields to allow for reasonably confident sequence assignment. These two prominent sequences corresponded to amino acid sequences in human apoD beginning after methionine residues 49 and 93. The third, weaker sequence corresponded to the human sequence beginning with residue 80. Because residue 79 of the human se-

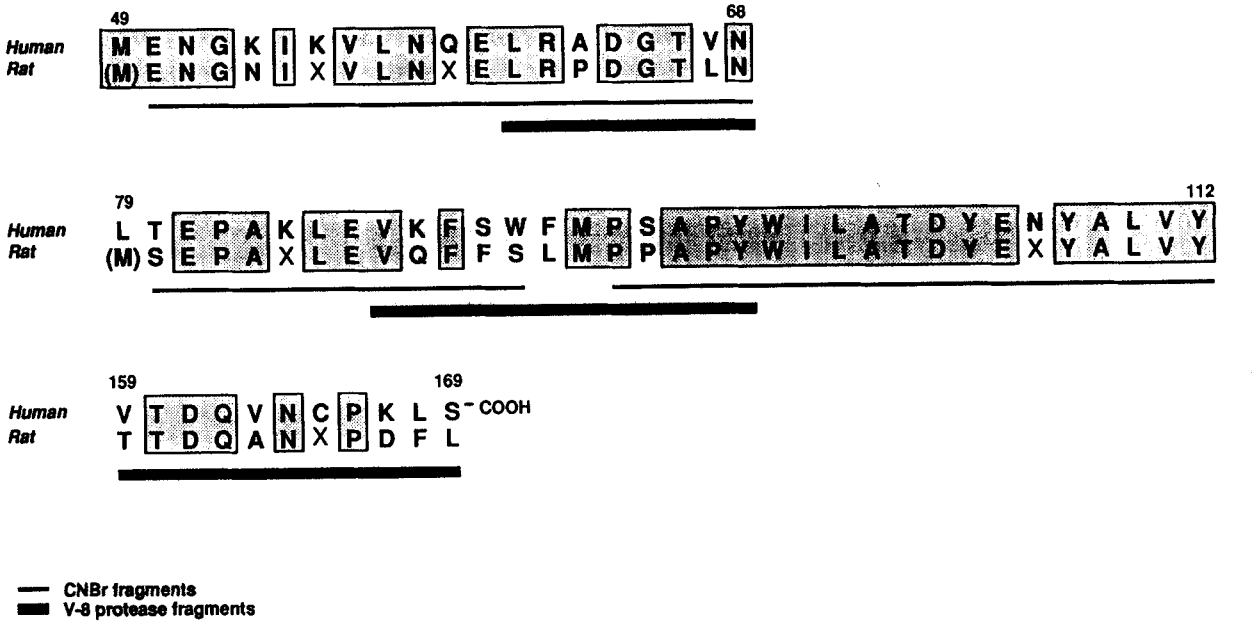


Fig. 5. Comparison of the sequences of several rat nerve apolipoprotein peptides with the corresponding sequences of human apoD. Identical residues in both species are enclosed in shaded boxes. At positions 49 and 79, the two methionine residues in parentheses are inferred because they precede CNBr sequences. The human sequence was derived from that of Drayna et al. (17).

quence is leucine, we presume that in the rat a methionine substitutes for the leucine at this position. Amino acid 157 is a methionine in the human sequence, but in the rat a different amino acid probably substitutes for this methionine because we detected no corresponding sequence in the rat protein after CNBr cleavage. The final assignment of amino acids was made to the three CNBr peptide sequences so as to maximize homology between the human and rat sequences. On this basis, and assuming that the residues in the rat sequence corresponding to human residues 49 and 79 in the human sequence are methionines, the identity between this portion of the human and rat sequences is 45 of 65 residues (69%), with five residues (8%) unassigned.

Immunocytochemical localization of apolipoprotein D in tissues

Immunocytochemical demonstration of apoD in several rat tissues was accomplished using an affinity-purified antibody raised to the 27-kDa isoform of rat apoD, a biotinylated second antibody, and an avidin-biotin complex coupled to horseradish peroxidase. Those tissues previously shown in the human to contain apoD mRNA (liver, kidney, adrenal, brain, duodenum, spleen, and pancreas (17, 19)), as well as several additional tissues (bladder, testis, lung, heart, and skin), were examined. Those cells of each tissue reacting with the apoD antibody are listed in **Table 2**, along with an estimation of their relative reactivity. The details of a 12-tissue survey for apoD are given below.

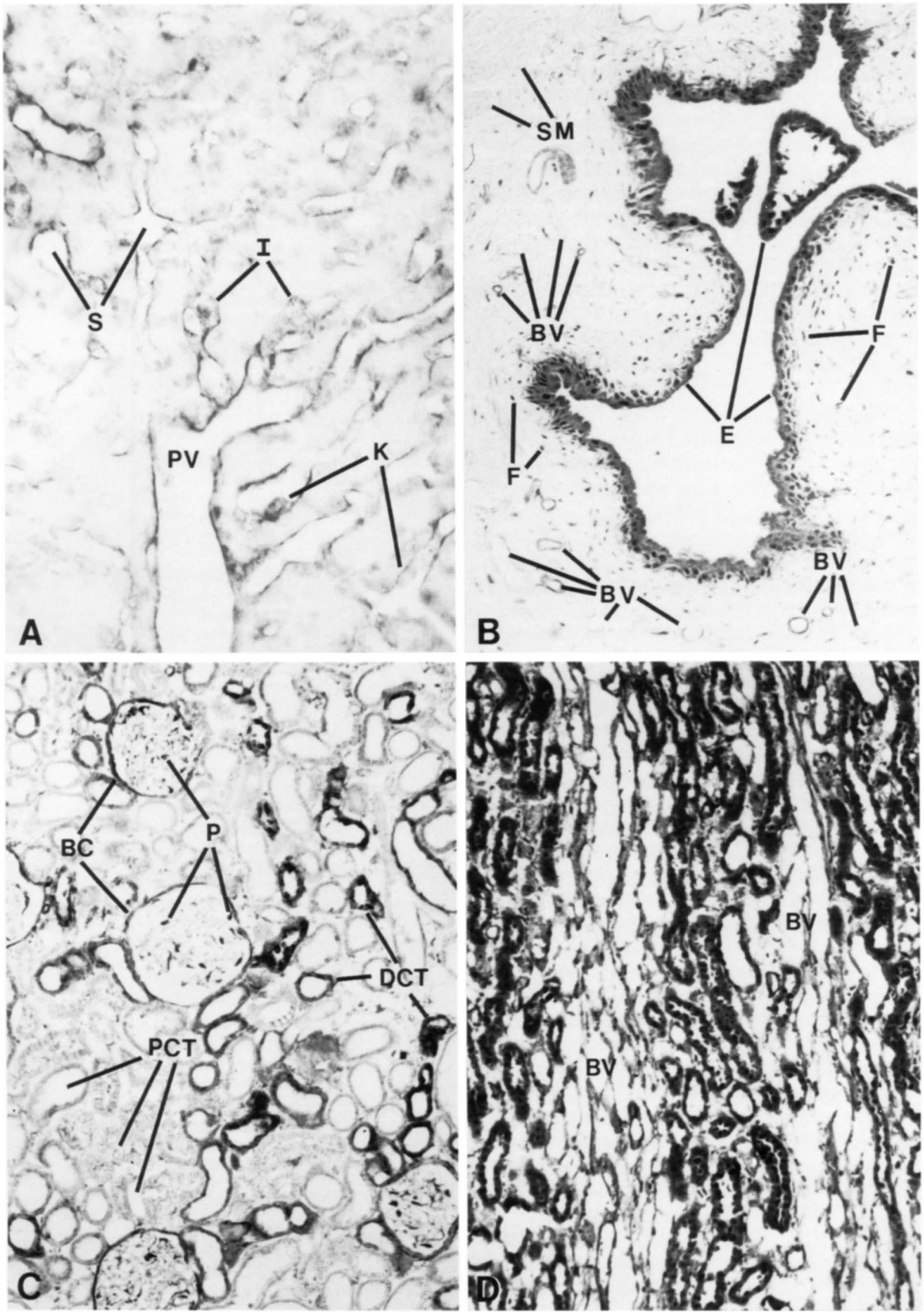
Liver **(Fig. 6A)**. In the liver, a reaction for apoD was found in the sinusoidal endothelial cells, particularly on the portal and arterial sides of the lobule. The endothelia and smooth muscle of the larger blood vessels were reactive, as were the fibroblasts of the triads (not shown) and many Ito cells and Kupffer cells. A weak reaction for apoD was also seen in the parenchymal cells on the portal side of the lobule.

Bladder and kidney (Figs. 6B–D). Investigation of the urinary bladder demonstrated reaction for apoD in the transitional epithelial cells of this tissue as well in the mesothelial cells covering this organ (not shown) and in the sympathetic ganglia and nerve fibers of the tissue (not shown). No reaction was found in the smooth muscle cells, but reaction was seen in most of the vasculature and some fibroblasts. In the kidney, a strong reaction for apoD was seen in the podocytes of the glomerulus and in the epithelial cells of Bowman's capsule. A strong reaction was also found in all parts of the kidney tubule except the proximal convoluted tubule. In the cortex of the kidney, weak reaction for apoD was also seen in many interstitial cells, but was not seen in the blood vessels until below the level of the medullary rays. Within the medulla of the

TABLE 2. Identification of cells reacting with an apolipoprotein D antibody

Tissue	Cell Type	Level of Reactivity[a]
Liver	Sinusoidal and large-vessel endothelium	+ +
	Fibroblasts	+ +
	Ito cells	+
	Kupffer cells	+
	Parenchymal cells	+
Urinary bladder	Mesothelium	+ +
	Transitional epithelium	+ +
	Vascular endothelium and smooth muscle	+ and −
	Fibroblasts	+ and −
	Neurons and glial cells	+ +
Kidney	Bowman's capsule endothelium	+ + +
	Podocytes	+ + +
	Cortical interstitial cells	+
	Ascending and descending loop of Henle	+ + +
	Distal convoluted tubule	+ + +
	All cells of the medulla	+ + +
Adrenal	Chromaffin cells	+ + +
	Capsule fibroblasts	+
	Large-vessel endothelium	+
	Pericytes, fibroblasts, or macrophages of cortex	+ + +
	Steroid-secreting cells	+ and −
Brain	Astrocytes, protoplasmic	+
	Astrocytes, fibrous	+ + +
	Oligodendrocytes	+
	Neurons	+ and −
	Pial cells	+ + +
	Perivascular cells	+ and −
	Vascular endothelial cells	+ and −
Testis	Leydig cells	+ +
	Fibroblasts	+ +
	Macrophages	+ +
	Vascular smooth muscle	+ +
	Vascular endothelium	+ + +
	Sertoli cells	+ +
Duodenum	Enterocytes	+ + +
	Smooth muscle cells of the villus	+ + +
	Fibroblasts of the villus	+ + +
	Enteric ganglia	+ + +
	Enteric glia or fibroblasts	+ + +
	Cells of the crypts	+
	Smooth muscle cells of the muscularis	+ and −
Lung	Alveolar epithelial cells	+
	Bronchiolar epithelium	+
Spleen	Stellate cells of white pulp	+
	Cells of the marginal zone	+
	Macrophages of red pulp	+
Pancreas	Islets of Langerhans	+ + +
	Ductule cells	+
	Interstitial fibroblasts and macrophages	+
	Exocrine cells	+ and −
Heart	Fibroblasts	+
Skin	Neurolemmal cells	+ + +

[a]Where both reactive and negative cells of the same cell type were identified, "+ and −" has been used to denote this fact.



kidney, however, all cells, including those of the vasculature, were found to be strongly reactive for apoD.

Adrenal (**Fig. 7A**). In the adrenal gland, reaction for apoD was strong within the neural cells of the medulla, but was not found in the vascular cells of the medulla. In the cortex, the fibroblasts of the capsule, the endothelial cells of the large vessels, and some steroid-producing cells were reactive, in particular the cells of the glomerulus. Scattered cells (which appear to be the pericytes of the sinusoids and macrophages or fibroblasts) contained the greatest reaction in the cortex.

Nervous tissue (Fig. 7B). In the cerebrum, we found the tissue generally reactive. Various levels of reaction for apoD were present in the glial cells (astrocytes and oligodendrocytes), but reaction was particularly strong in the cells of the pia and arachnoid. In addition, occasional endothelial cells and perivascular cells (which may be macrophages or pericytes) were reactive (not shown). Many neurons showed reactivity as well, particularly in the larger processes of these cells. Previously, we demonstrated the detection of apoD in the spinal cord, dorsal root ganglia, and sciatic nerve (11). The enteric ganglia of the duodenum and the sympathetic ganglia of the bladder are discussed in the sections describing these tissues.

Testis (Fig. 7C). In the testis, reaction for apoD was found in all the cells of the interstitium (fibroblasts, Leydig cells, and macrophages) and in the Sertoli cells of the seminiferous tubules, but the highest level of reactivity was found in the endothelial cells of the vasculature. The smooth muscle cells of the vasculature were also reactive.

Duodenum (Fig. 7D). In the duodenum, the enterocytes were strongly reactive for apoD. The cells of the villus core were reactive as well, in particular the smooth muscle cells and fibroblasts. The circular and longitudinal smooth muscle cells of the muscularis, in contrast, contained little reactivity. The mesothelium covering the duodenum showed no reaction, unlike the bladder, where the mesothelium was reactive (not shown). The enteric ganglia and the glial cells (or fibroblasts) that accompany this neuronal network were intensely reactive.

Lung and spleen (**Figs. 8A** and 8B). In the lung and spleen, a low level of reaction for apoD was seen in virtually every cell, but not in the vasculature. In the lung, reaction was most concentrated in the various cells of the alveolus, particularly the large pneumocytes and in the bronchiolar epithelium. In the spleen, reaction was most concentrated in the cells of the marginal zone and in the macrophages of the red pulp. Some reactivity was also seen in occasional cells of the white pulp.

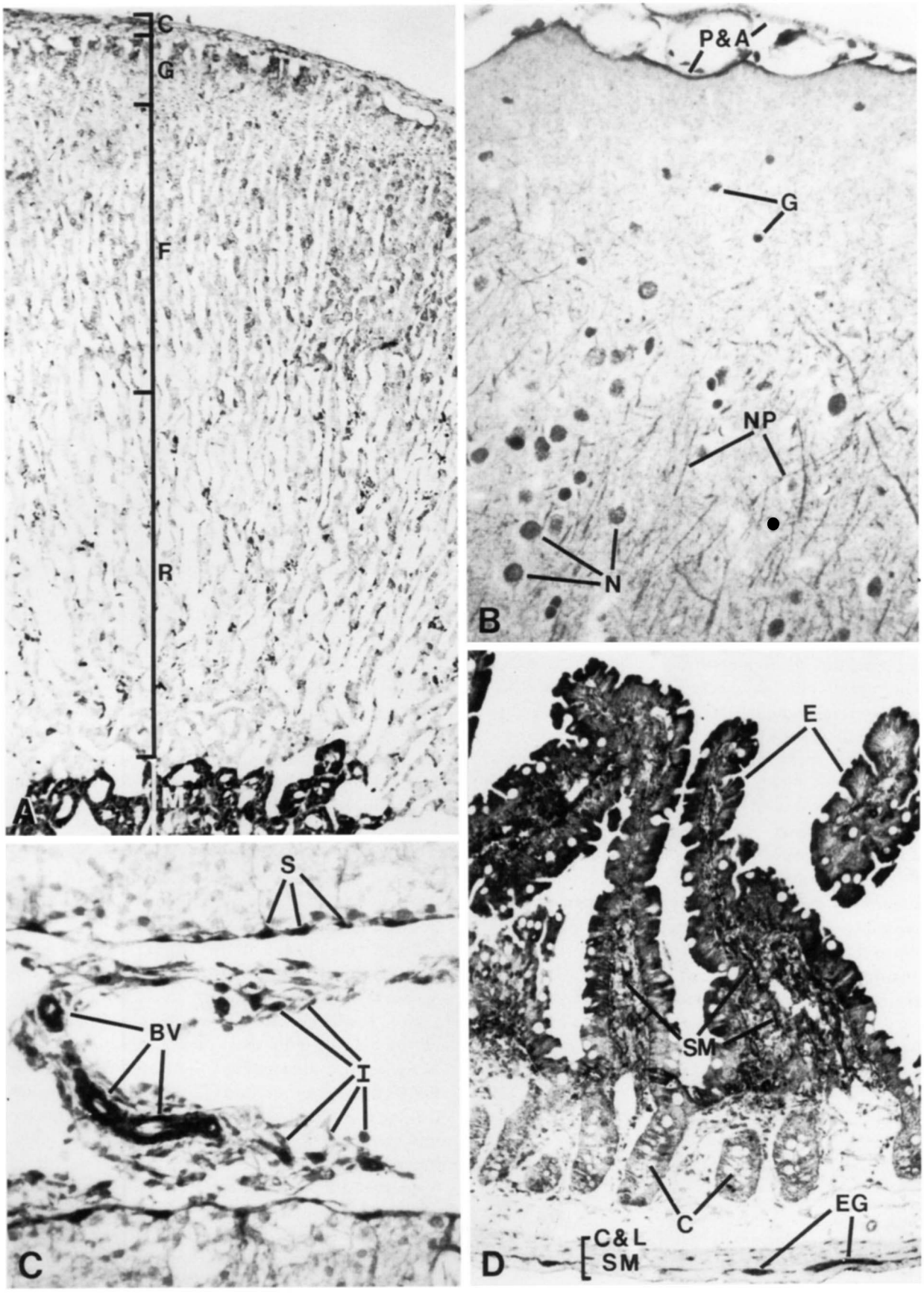
Pancreas (Fig 8C). In the pancreas, a strong reactivity for apoD was identified in the cells of the islets of Langerhans. Weaker reaction was present in the interstitial cells and in occasional ductule cells. Reactivity was also seen occasionally at the brush borders of the acinar cells and in the contents of the ducts. Very weak reactivity was sometimes seen in the exocrine cells.

Skin and heart (not shown). Reactivity for apoD in hair-covered skin was confined exclusively to small nerve fibers and their sheaths. These contained extremely high levels of reactivity for apoD. In the heart, weak reactivity was found in the fibroblasts, but generally the tissue showed little reaction.

Fig. 6. Immunocytochemical detection of apoD in the (A) liver, (B) bladder, and (C and D) kidney. Dark peroxidase reaction product identifies antibodies specific for apoD. A: Portal region of the liver lobule. PV, portal vein; S, sinuses; I, Ito cells; K, Kupffer cells. B: Epithelial surface of the bladder. BV, blood vessels; F, fibroblasts; SM, smooth muscle cells; E, epithelium. C: Cortex of the kidney. P, podocytes; DCT, distal convoluted tubule; PCT, proximal convoluted tubule; BC, Bowman's capsule. D: Medullary rays of the kidney. BV, blood vessels. A, × 340. B-D, × 140.

DISCUSSION

We recently described an unknown apolipoprotein that is a component of the lipoprotein particles isolated from regenerating rat sciatic nerves and made by neurolemmal cells (11). During the response of the nerve to a denervating injury and subsequent regeneration and myelination, this protein increases over 500-fold (11). In this report, we have identified this apolipoprotein by partial amino acid sequence analysis as apoD (Fig. 5). Characterization of rat apoD isolated from regenerating sciatic nerves demonstrated that it is very similar to human apoD in molecular weight (Fig. 1), isoelectric point (Fig. 3), glycosylation pattern (Fig. 4), and amino acid composition (Table 1). Rat apoD in SDS-PAGE analysis occurs as a series of molecular weight isoforms (Figs. 1 and 3), which in nonreduced gels run between 27 and 31 kDa and in reduced gels between 29 and 33 kDa (Fig. 1). Rat apoD is a very acidic protein with multiple-charge isoforms whose isoelectric points range between pH 4.05 and 4.37 (Fig. 3). Like human apoD (17), it is apparently *N*-glycosylated (Fig. 5). Unlike the human protein, however, its concentration in plasma and on plasma lipoprotein particles is low (Fig. 2). By immunocytochemistry, we can identify numerous types of cells that contain apoD in various rat tissues (Figs. 6–8 and Table 2). This wide tissue distribution of apoD in the rat resembles the wide tissue distribution of apoD mRNA previously shown in the human (17, 19). From tissue to tissue, or even in different parts of the same tissue of the rat, the reaction for apoD in a given cell type is often variable. For example, in some tissues many fibroblasts contain apoD, as do endothelial cells and smooth muscle cells, whereas in other rat tissues, these same cell types lack apoD. This result suggests that the uptake or secretion of apoD may be regulated.



Rat apoD has marked sequence identity with the human protein. In those portions of the rat sequence we have analyzed, 45 of the 65 amino acids identified in rat apoD are identical to those of the human sequence (69%). For 5 of the remaining 20 amino acids, no identification could be made, and these may or may not be conserved. Six of the other 15 known amino acid changes are conservative. Most of the amino acid changes (i.e., 12) can result from single base substitutions, whereas the remaining 3 require two-base-pair substitutions. The degree of conservation between the human apoD sequence and our partial rat apoD sequence is similar to the degree of conservation between human and rat sequences of several other apolipoproteins. For example, the percentage for protein sequence identity of human and rat apoE (27, 28) is 71%; for human and rat apoA-I (29, 30), 41%, with the rat having numerous deletions and changes not seen in other species; for human and rat apoA-II (31–35), 53%; and for human and rat apoA-IV (36–38), 62%.

Apolipoprotein D is part of a large family of proteins, the α_{2u}-microglobulin superfamily (18, 39–41). These are all relatively small proteins, which, where their functions have been established, have been found to transport small hydrophobic molecules. The members of the family include bilin-binding protein, retinol-binding protein, insecticyanin, the γ component of C8, β-lactoglobulin, odorant-binding protein, the androgen-dependent epididymal secretory protein, purpurin, α-1-microglobulin, α_1-acid glycoprotein, BG protein, heterogeneous charge protein, and α_{2u}-microglobulin. Where structural information is available, it has also been found that members of this family share a common structural motif. In retinol-binding protein (42), β-lactoglobulin (43–45), insecticyanin (46), and bilin-binding protein (47, 48), the ligand-binding pocket is formed by two orthogonal β-sheets composed of eight antiparallel β-strands and an α-helix. The structure of apoD, based on its sequence and on molecular modeling, appears to be similar (17, 39). Some members of this family have little ligand specificity; for example, the odorant-binding protein presents various hydrophobic molecules to odor-sensing neurons (49). Others, such as retinol-binding protein, appear to be very specific. Still others have yet to have their ligand or ligands identified. Apolipoprotein D is one of these. It is clear from the association of apoD with lipoproteins in the plasma and nerve, as well as the ability of purified apoD to form lipoproteins when added to phospholipids and cholesterol (50), that it binds one or more types of lipid. In addition, apoD could bind other hydrophobic small molecules. Recent work by Peitsch and Boguski (39) showed that apoD has a hydrophobic surface that surrounds the ligand-binding pocket of the protein. This could explain its binding of lipids. In addition, the hydrophobic ligand pocket formed by the orthogonal β-sheets of the protein appears to bind bilirubin (39). They have speculated that apoD may be part of the antioxidant defense system in which the conversion of heme to biliverdin and then to bilirubin serves to protect cells and tissues from oxidant damage. If apoD does carry an antioxidant, it may also help to protect lipoproteins from oxidant damage. The binding of heme or compounds derived from heme suggests that apoD might serve as a cellular heme transport protein.

In human plasma, apoD is found in association with LCAT (lecithin:cholesterol acyltransferase) and apoA-I (51, 52). This has led to the suggestion that apoD plays a role in cholesterol esterification or in cholesterol or cholesteryl ester transfer between lipoprotein particles (51, 52). Recent work has also shown that apoD is a potent LCAT activator (50). Moreover, apoD seems to stabilize LCAT during cholesterol esterification, unlike two other well-known activators, apoC-I and apoA-I, which allow rapid inactivation of the enzyme (50). The activation of LCAT could be an important function of apoD in neural tissue. Lecithin:cholesterol acyltransferase activity has been identified in human cerebrospinal fluid (53). The lipoprotein particles of human cerebrospinal fluid contain significant amounts of esterified cholesterol (54), as expected if appreciable esterifying activity is present. This supports the suggestion that apoD could play a role in cholesterol metabolism in neural tissue. Such a role need not, however, exclude a role in heme metabolism or oxidant defense; multiple functions have been ascribed to several other apolipoproteins.

The identification of apoD as a possible transport protein carrying one or more small hydrophobic molecules (including lipids) would be consistent with its presence in many tissues, as suggested by immunocytochemistry using an affinity-purified apoD antibody. Expression of apoD by the enterocytes of the intestine and the distal portions of the kidney tubule and bladder epithelium is in keeping with a role for apoD in the transport of small hydrophobic molecules derived either from the diety or from the filtration of plasma. Evidence of apoD uptake by neurons and macrophages (11) is also in keeping with the delivery of small hydrophobic molecules to cells. The pre-

Fig. 7. Immunocytochemical detection of apoD in the (A) adrenal, (B) cerebrum, (C) testis, and (D) duodenum. Dark peroxidase reaction product identifies antibodies specific for apoD. A: Adrenal section that includes tissue from the capsule to the medulla. C, capsule; G, glomerulus; F, fasciculata; R, reticulata; M, medulla. B: Section from the surface of the cerebrum. N, nuclei of neurons; NP, neuronal processes; G, glia; P & A, pia and arachnoid. C: Area between two seminiferous tubules of the testis. S, Sertoli cells; I interstitial cells (macrophages, fibroblasts, and Leydig cells); BV, blood vessel. D: Cross-section of the duodenum. E, enterocytes; C, crypt cells; SM, smooth muscle cells of the villus; C & L SM, circular and longitudinal smooth muscle; EG, enteric ganglia; M, mesothelium. A, × 110. B and C, × 340. D, × 140.

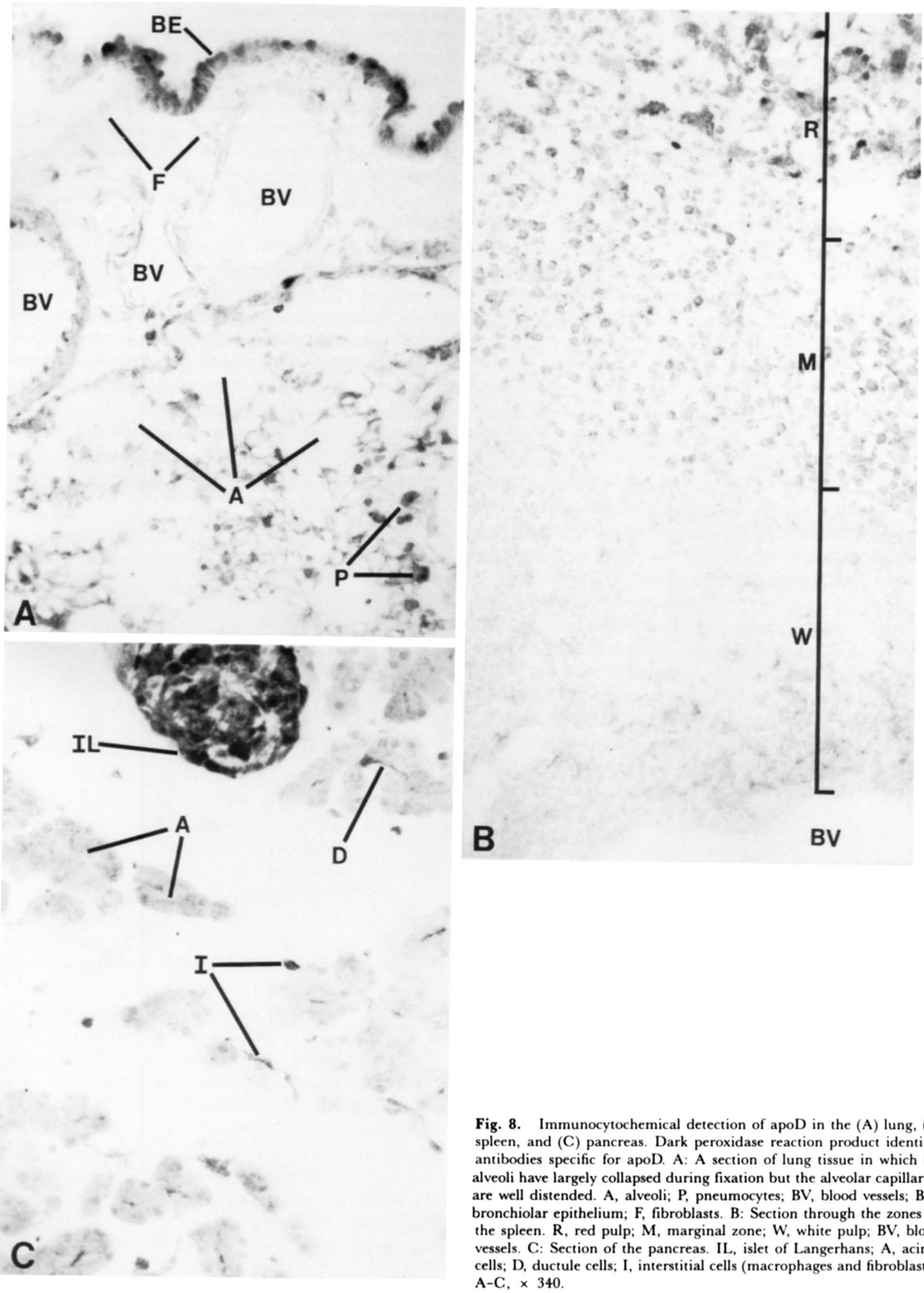


Fig. 8. Immunocytochemical detection of apoD in the (A) lung, (B) spleen, and (C) pancreas. Dark peroxidase reaction product identifies antibodies specific for apoD. A: A section of lung tissue in which the alveoli have largely collapsed during fixation but the alveolar capillaries are well distended. A, alveoli; P, pneumocytes; BV, blood vessels; BE, bronchiolar epithelium; F, fibroblasts. B: Section through the zones of the spleen. R, red pulp; M, marginal zone; W, white pulp; BV, blood vessels. C: Section of the pancreas. IL, islet of Langerhans; A, acinar cells; D, ductule cells; I, interstitial cells (macrophages and fibroblasts). A-C, × 340.

sence of apoD in a wide variety of cell types in many tissues, whether due to secretion or uptake, further suggests that the role of apoD is a general one of cellular "housekeeping" or metabolism. Moreover, the increase in apoD observed in the regenerating nerve and the variable expression of apoD by endothelial cells, mesothelial cells, fibroblasts, and smooth muscle cells suggest that the uptake or secretion of apoD may be regulated.

In summary, we have identified apoD as one of several proteins that accumulate in the regenerating rat sciatic nerve during regeneration. The expression of apoD is not unique to neural tissue, but is seen in numerous cells of various tissues, where its expression or uptake may be regulated.

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