Domain Structure of the Basement Membrane Heparan Sulfate Proteoglycan

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ABSTRACT: We have used proteolytic digestions and immunological reactivity to map regional domains of the 400-kilodalton (kDa) core protein of the heparan sulfate containing basement membrane proteoglycan from the Englebreth–Holm–Swarm tumor. Digestion with V8 protease caused the rapid release of numerous large peptides ranging in size from 80 to 200 kDa and a 44-kDa peptide. The 44-kDa peptide (P44) was stable to further digestion, but the larger peptides were eventually degraded to a 46-kDa peptide (P46). Both the P44 and P46 fragments migrate slower in the presence of a reducing agent, indicating intrachain disulfide bonding, and do not have heparan sulfate side chains. Antisera to the P46 fragment, however, did not react with P44 fragment, and the amino acid compositions of P46 and P44 fragments were different. This suggests that these two fragments are unrelated. Trypsin digestion of the proteoglycan immediately released a 200-kDa peptide (P200) that also lacked heparan sulfate side chains. Digestion of the P200 fragment with V8 protease produced the P44 and P46 fragments in the same temporal sequence seen with V8 protease digestion of the proteoglycan. Antisera to the P200 fragment reacted strongly with the P44 and P46 fragments. These results show that the P44 and P46 domains are contained within the P200 domain. The rapid release of the P44 domain indicates that it is located at one end of the core protein. The large size of these proteolytic fragments suggests the core protein contains considerable conformational structure, and the absence of heparan sulfate on the P200 domain indicates that the side chains are asymmetrically located on the core.

Basement membranes are thin sheets of extracellular matrix that separate epithelial, endothelial, muscle, nerve, and fat cells from adjacent matrix. They contain type IV collagen (Kefalides, 1971; Orkin et al., 1977; Timpl et al., 1978; Kleinman et al., 1982), laminin (Timpl et al., 1979; Laurie et al., 1982), entactin/nidogen (Carlin et al., 1981; Hogan et al., 1982; Timpl et al., 1983; Paulsson et al., 1985), fibronectin (Mayer et al., 1981; Thesleff et al., 1981), and a basement membrane specific proteoglycan with heparan sulfate side chains (Kanwar & Farquhar, 1979; Hassell et al., 1980; Kanwar et al., 1981). The proteoglycan, due to the presence of sulfate esters on the glycosaminoglycan side chains, is the most highly charged component in basement membranes and has been shown to be responsible for the ionic filtration accomplished by the glomerular basement membrane (Rennke et al., 1975; Kanwar et al., 1980). Other studies indicate that it is also involved in salivary gland (Thompson & Spooner, 1982) and mammary gland (Smith & Bernfield, 1982) morphogenesis as well as in the initial processes of cell attachment but not in maintenance of attachment (Gill et al., 1986). This proteoglycan may also act as a mitogen for Schwann cells (Ratner et al., 1985).

Heparan sulfate containing basement membrane proteoglycans isolated from various sources differ in size, ranging from large (M_r 750 000) to small (M_r 130 000) [see Hassell et al. (1986a) for a review]. Immunological studies, however, indicate that these proteoglycans have determinants in common despite size differences. For examples, antibodies against basement membrane proteoglycans isolated from a variety of sources including the Englebreth–Holms–Swarm (EHS) tumor (Hassell et al., 1980), muscle cells (Anderson & Fambrough, 1983), yolk sac tumor (Fenger et al., 1984), kidney glomerulus (Stow et al., 1985a), and Schwann cells (Eldridge et al., 1986) localize to all basement membranes as well as recognize the same size (M_r 400 000) precursor protein (Ledbetter et al., 1985; Wever et al., 1985; Eldridge et al., 1986; Hassell et al., 1986a). Furthermore, studies of the EHS proteoglycans show the small proteoglycan to have immunological determinants in common with the large proteoglycan (Hassell et al., 1985). Metabolic studies with both EHS (Ledbetter et al., 1985) and endothelial cells (Kinsella & Wight, 1985) in culture indicate that the majority of the small proteoglycans is derived from the large proteoglycan. Thus, we have proposed that the large proteoglycan is the initial biosynthetic product and the small proteoglycan is probably a proteolytic fragment derived from the large proteoglycan (Ledbetter et al., 1985). These immunologically and metabolically related matrix proteoglycans are immunologically distinct from cell surface heparan sulfate proteoglycans (Stow et al., 1985b; Jalkanen et al., 1985).

Biochemical and electron microscopic studies have provided evidence for the basic structure of the large basement membrane proteoglycan. Heparitinase removes the glycosaminoglycan side chains, leaving a large (M_r 400 000) core protein (Hassell et al., 1985). Each proteoglycan molecule contains three or four heparan sulfate side chains (Hassell et al., 1985) located at one end of the core protein (Laurie et al., 1984; Paulsson et al., 1986). In the present study, we have mapped proteolytic fragments and immunological determinants within the proteoglycan to construct a structural model confirming the asymmetrical location of heparan sulfate side chains and revealing the presence of a large (M_r 200 000) trypsin-resistant domain free of heparan sulfate.

Materials and Methods

Tumor Preparation and Proteoglycan Extraction. The EHS tumor was maintained by serial passage in C57BL mice and harvested as previously described (Orkin et al., 1977). Radiolabeled tumor was prepared by injecting mice bearing

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the tumor with sodium [^{35}S]sulfate (1–1.5 mCi/mouse) 18 h before harvesting the tumor (Hassell et al., 1980). Harvested tumor was quickly dissected in small pieces (2–4 mm^3) and frozen in liquid nitrogen. Frozen tumor harvested from mice not injected with radiolabel was combined with labeled tumor to provide enough material to yield sufficient amounts of purified proteoglycan.

The large, low-density basement membrane proteoglycan was extracted and purified by using modifications of methods described previously (Hassell et al., 1985). Weighed amounts of frozen tumor were thawed in 6 volumes (w/v) of 3.4 M NaCl containing 0.1 M 6-aminohexanoic acid, 0.04 M ethylenediaminetetraacetic acid (EDTA), 0.008 M *N*-ethylmaleimide (NEM), 0.002 M phenylmethanesulfonyl fluoride (PMSF), and 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 6.8, with vigorous stirring for 15–20 min. The tumor was then homogenized with a Polytron for 30 s and then stirred at 4 °C for 1 h. Insoluble material was collected by centrifugation (12000*g* for 10 min), and the supernatant fraction was discarded. The insoluble residue was reextracted with 2 volumes (original tissue weight) of the 3.4 M NaCl solution for 30 min with stirring at 4 °C. Insoluble material was again collected by centrifugation, and the supernatant fraction was discarded. These discarded supernatants contained the small, high-density heparan sulfate proteoglycan described previously (Hassell et al., 1985) as well as some small proteins. The insoluble residue was then suspended in 6 volumes of 6 M urea containing 0.1 M 6 aminohexanoic acid, 0.04 M EDTA, 0.002 M PMSF, and 0.05 M Tris-HCl, pH 6.8, homogenized with a Polytron for 30 s, and stirred for 2 h at 4 °C. The mixture was homogenized again with a Polytron for 1 min and centrifuged to remove insoluble material. The supernatant fraction was saved, and the insoluble material was reextracted with 2 volumes of the 6 M urea solution. The insoluble material was removed by centrifugation, and the supernatant fluids were combined and saved for proteoglycan isolation.

Proteoglycan Purification. The urea extract was dialyzed overnight against 5 volumes of 6 M urea containing 0.15 M NaCl and 0.05 M Tris-HCl, pH 6.8, at 4 °C. The extract was adjusted to contain 0.5% Triton X-100 (RPI), centrifuged to remove insoluble material, and chromatographed on a column of DEAE-Sephacel in 6.0 M urea containing 0.5% Triton using a 0.15–1.15 M NaCl gradient as previously described (Hassell et al., 1985). The extract from 240 g of tumor was chromatographed on a DEAE-Sephacel column (9.0 × 10.0 cm). Fractions were sampled for radioactivity, and those containing the $^{35}SO_4$ label that eluted at >0.3 M NaCl were pooled. The macromolecular material in the pooled fractions was precipitated by the addition of 4 volumes of absolute methanol at 4 °C for 2 h. The precipitate was collected by centrifugation, solubilized in 4.0 M guanidine hydrochloride containing 0.02 M Tris-HCl, pH 7.0, and 0.5% Triton X-100, dialyzed against 6 M urea containing 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.0, and rechromatographed on DEAE-Sephacel as before but on a smaller column (5.0 × 9.0 cm). The proteoglycan eluting in the salt gradient was collected by methanol precipitation and solubilized as described above. Solid CsCl was added (0.5 g/g), and this solution was centrifuged in a 50.2 Ti rotor (Beckman) at 33000 rpm for 68 h. The gradient was fractionated into 10 equal parts, and each fraction was sampled for radioactivity. Selected fractions were pooled and dialyzed against 0.2 M NaCl, and their macromolecular components were collected by methanol precipitation as described above. The precipitate was solubilized in 4.0 M guanidine hydrochloride containing 0.02 M Tris-HCl, pH 7.0, and 1.0% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and chromatographed on a column (1.5 × 150 cm) of Sepharose CL-4B in 4.0 M guanidine hydrochloride containing 0.1% CHAPS and 0.02 M Tris-HCl, pH 7.0. Fractions were sampled for radioactivity by liquid scintillation counting, and those containing proteoglycan were pooled, dialyzed against distilled water, and lyophilized.

Enzymatic Digestions and Electrophoresis. Weighed amounts of lyophilized proteoglycan and fragments of proteoglycan were dissolved at 1 or 10 mg/mL in 4.0 M guanidine hydrochloride containing 0.02 M Tris-HCl, pH 7.0, and 0.01% CHAPS and then dialyzed against either distilled water or 0.02 M Tris-HCl, pH 8.0, buffer. Water-dialyzed samples were digested with heparitinase (Miles) as previously described (Hassell et al., 1985) while Tris buffer dialyzed samples were digested with either V8 protease (Miles) or trypsin (Sigma) using 10 μg of enzyme/mg of substrate. Digestion was monitored by electrophoresis in sodium dodecyl sulfate (SDS)–polyacrylamide gels as used previously (Hassell et al., 1985). Protein and peptide bands were visualized by Coomassie Blue staining. All running gels were 7.5% acrylamide except where otherwise stated. Samples were adjusted to 0.02 M phosphate buffer, pH 6.8, and to 2% SDS and were boiled 3 min before electrophoresis. Protein molecular weight standards of M_r 200000, 116000, 92000, 68000, 45000, and 31000 were obtained from Bio-Rad. The A chain of laminin was used as an M_r 400000 marker. In some instances, samples were reduced with 0.04 M dithiothreitol (DTT) prior to boiling.

Purification of Peptide Fragments. Enzymatic digests were chromatographed on a column (1.5 × 90 cm) of Sepharose CL-6B in 0.2 M NaCl containing 0.02 M Tris-HCl, pH 8.0. The eluant was monitored at 280 nm. Fractions were sampled for radioactivity by liquid scintillation counting to detect the $^{35}SO_4$-labeled glycosaminoglycans and for peptide content by SDS–polyacrylamide gel electrophoresis (PAGE) as described above. Selected fractions were pooled, dialyzed against distilled water, and lyophilized. Glycosaminoglycan-containing fractions were dissolved in 4.0 M guanidine hydrochloride containing 0.02 M Tris-HCl, pH 7.0, and 0.1% CHAPS, adjusted to contain 0.5 g of CsCl/g, and further purified by centrifugation in a 50 Ti rotor (Beckman) at 40000 rpm for 68 h. The tubes were sliced into five equal fractions and sampled for radioactivity. Selected fractions were pooled, dialyzed against distilled water, and lyophilized. Peptide-containing fractions from the Sepharose CL-6B chromatography were dissolved in 0.05 M ammonium acetate and were further purified by high-performance liquid chromatography (HPLC) on a column of DEAE 5PW (Waters) using a 0–1.0 M NaCl gradient in 0.05 M ammonium acetate. The eluent was monitored at 280 nm, and fractions were sampled for peptide content by SDS–PAGE. Selected fractions were pooled, dialyzed against distilled water, and lyophilized. The amino acid composition of the purified peptides was determined on a Dionex D-500 analyzer after gas-phase hydrolysis with 6 N HCl containing 1% phenol at 110 °C for 22 h.

Antibody Preparation. Rabbits were initially injected subdermally with 0.5 mL of a saline/Freund's complete adjuvant emulsion containing 500 μg of the proteoglycan or proteolytic fragment. The animals were boosted 4 and 6 weeks later with 0.5 mL of a saline/Freund's incomplete adjuvant containing 300 μg of either the proteoglycan or the proteolytic fragment. Rabbits were bled every 2–4 weeks, and the titer of the antisera was measured by the enzyme-linked immu-

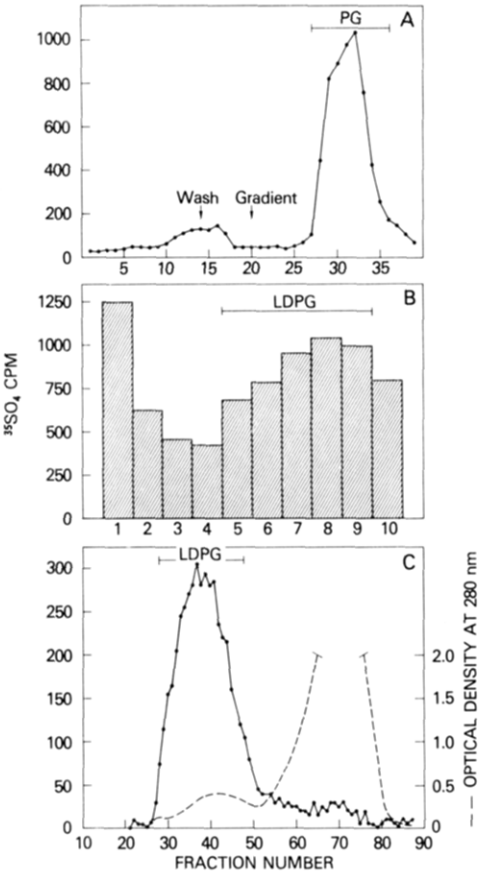

FIGURE 1: Purification of low-density heparan sulfate proteoglycan from extracts of $^{35}SO_4$-labeled EHS tumor. (A) Chromatography of the urea extract on a column of DEAE-Sephacel. The proteoglycans (PG) elute during the salt gradient at approximately 0.30 M NaCl. (B) CsCl density gradient centrifugation of the proteoglycan fraction obtained by DEAE-Sephacel chromatography. Fraction 1 is from the bottom of the tube and 10 from the top of the tube. The low-density proteoglycan (LDPG) localized to the upper part of the tube (densities 1.38–1.30 g/mL). (C) Chromatography of the LDPG fraction obtained by CsCl density gradient centrifugation on a column of Sepharose CL-4B. V_0 = fraction 28, V_t = fraction 85. The LDPG elutes at a K_{av} of 0.15, just after the void volume of the column.

nosorbent assay (ELISA) in microtiter wells (Rennard et al., 1980) using a goat anti-rabbit IgG second antibody coupled to horseradish peroxidase. The reaction of the various antisera with the different proteolytic fragments was also determined by ELISA (Rennard et al., 1980), as described in the legend to Figure 7.

Results

The tumor tissue was extracted first with 3.4 M NaCl. This solvent removed weakly associated material such as the small, high-density proteoglycan described previously (Hassell et al., 1985), free glycosaminoglycans, and small proteins (data not shown). Subsequent extraction with 6.0 M urea removed the strongly associated, large, low-density proteoglycan (Hassell et al., 1985). The proteoglycan in the urea extract was separated from glycoproteins in the extract by chromatography on a column of DEAE-Sephacel (Figure 1A). The glycoproteins did not bind to the column, and the proteoglycan, detected by its incorporated $^{35}SO_4$, eluted in the salt gradient. The fractions containing the proteoglycan were pooled as indicated by the brackets in Figure 1A and rechromatographed on a column of DEAE-Sephacel (not shown). The proteoglycan fraction was then subjected to CsCl density gradient centrifugation (Figure 1B). Most of the proteoglycan localized to the low-density (top portion of the centrifuge tube) region

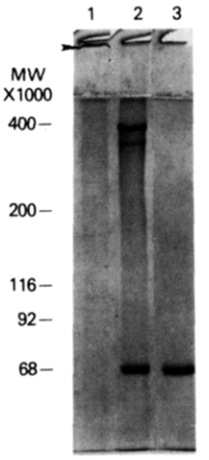

FIGURE 2: SDS–polyacrylamide gel electrophoresis of low-density proteoglycan before and after heparitinase digestion. 5 μg of proteoglycan was electrophoresed in lanes 1 and 2. The same amount of heparitinase used to digest the proteoglycan in lane 2 was electrophoresed alone in lane 3. Samples were reduced before electrophoresis, and gels were stained with Coomassie Blue. The running gel was 5% acrylamide. (Lane 1) Interact proteoglycan migrates just into the top of the stacking gel (arrow). (Lane 2) Heparitinase digestion of proteoglycan before electrophoresis eliminates the band in the stacking gel and produces a major band at M_r 400 000. The band at M_r 68 000 is in the heparitinase preparation. (Lane 3) Electrophoresis of heparitinase alone yields a band at M_r 68 000.

of the gradient. The fractions containing the low-density proteoglycan were pooled as indicated by the brackets in Figure 1B (densities 1.38–1.30 g/mL) and chromatographed on a column of Sepharose CL-4B (Figure 1C). The proteoglycan eluted just after the void volume at a K_{av} of 0.15.

The purity of the proteoglycan fraction obtained after molecular sieve chromatography was determined by SDS–PAGE under reducing conditions followed by Coomassie Blue staining (Figure 2). Electrophoresis of the intact proteoglycan yielded a single major band that migrated just into the top of the stacking gel (arrow, Figure 2, lane 1). Heparitinase digestion of the proteoglycan before electrophoresis abolished the band in the stacking gel and produced a new major band (M_r 400 000), the core protein of the proteoglycan (Figure 2, lane 2). The less prominent band at M_r 350 000 varies in amount with different proteoglycan preparations and is thought to be proteolytically derived from the M_r 400 000 band (Hassell et al., 1986b; Paulsson et al., 1986). The band at M_r 68 000 is a protein present in the heparitinase preparation (Figure 2, compare lanes 2 and 3). Low-density proteoglycan prepared previously (Hassell et al., 1985) contained small amounts of contaminating laminin that could only be removed after disulfide bond reduction by an additional chromatography step. The low-density proteoglycan prepared in the present report did not contain disulfide-bonded material [compare Figure 2, lane 1, in this report to Figure 4, lane 3, in Hassell et al. (1985)], and reducing conditions were not needed for purification. The methods in this and in the previous study (Hassell et al., 1985) are similar with the exception of the use of a high-salt (3.4 M NaCl) preextraction instead of a low-salt (0.15 M NaCl) preextraction. The high-salt preextraction used in this study yielded proteoglycan of greater purity and, because reduction was not used, in a more native state than the low-salt preextraction methods used in the previous (Hassell et al., 1985) study.

The peptide fragments generated by proteolytic digestion of the proteoglycan with trypsin and V8 protease were evaluated by SDS–PAGE under nonreducing conditions. Trypsin digestion for only 15 min produced a major band (M_r 200 000)

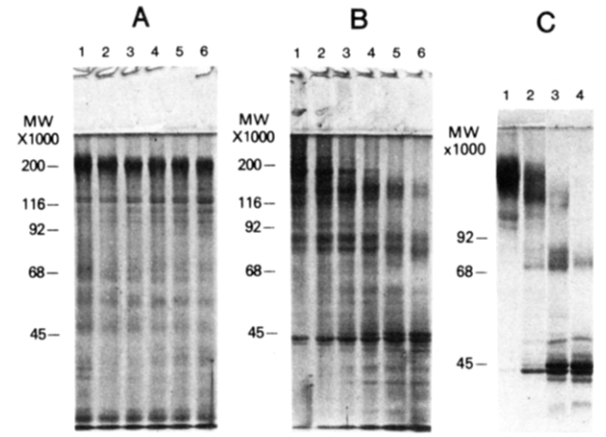

FIGURE 3: SDS–polyacrylamide gel electrophoresis of proteolytically digested proteoglycan. Samples were not reduced before electrophoresis, and the gel was stained for protein with Coomassie Blue. (A) Electrophoresis of 10 μg of trypsin-digested proteoglycan in each lane: (lane 1) 15-min digestion; (lane 2) 30-min digestion; (lane 3) 1-h digestion; (lane 4) 2-h digestion; (lane 5) 4-h digestion; (lane 6) 8-h digestion. (B) Electrophoresis of 20 μg of V8 protease digested proteoglycan in each lane: (lane 1) 15-min digestion; (lane 2) 30-min digestion; (lane 3) 1-h digestion; (lane 4) 2-h digestion; (lane 5) 4-h digestion; (lane 6) 8-h digestion. (C) Electrophoresis of 20 μg of V8-digested P200 fragment in each lane: (lane 1) 5-min digestion; (lane 2) 1-h digestion; (lane 3) 4-h digestion; (lane 4) 20-h digestion.

plus several smaller minor components (Figure 3A, lane 1). The M_r 200 000 peptide resisted further trypsin digestion and was still the major peptide after 2 h (Figure 3A, lane 4), 8 h (Figure 3A, lane 6), and 20 h (not shown) of digestion, although slightly diminished at the latter time. In contrast, digestion with V8 protease for 15 min (Figure 3B, lane 1) to 30 min (Figure 3B, lane 2) produced numerous peptides ranging in size from M_r 200 000 to M_r 80 000 as well as a major peptide of M_r 44 000. Further digestion for the time periods between 1 h (Figure 3B, lane 3) and 8 h (Figure 3B, lane 6) produced a simultaneous loss of the M_r 200 000–80 000 peptides and the appearance of an M_r 46 000 peptide but did not reduce the amount of M_r 44 000 peptide. The amounts of M_r 44 000 and 46 000 peptide were unchanged even after 20 h of digestion (not shown). The almost quantitative production of the M_r 44 000 peptide by only short digestion times (30 min) suggests that it is located in a region of the core that is readily accessible to enzymatic attack, perhaps from a terminal domain on the core. The more gradual appearance of the M_r 46 000 peptide, on the other hand, suggests that it may be derived from a more internal region of the core. Electrophoresis of either enzyme alone at the concentration used for digestion produced no bands (not shown).

The M_r 200 000 peptide (P200), the M_r 46 000 peptide (P46), and the M_r 44 000 peptide (P44) were selected for isolation and characterization. These peptides, as well as glycosaminoglycans, were initially purified by fractionation on a column of Sepharose CL-6B. The elution position of the glycosaminoglycans was determined by the presence of $^{35}SO_4$ label and the elution position of the peptide fragments by SDS–PAGE. Fractionation of trypsin-digested proteoglycan on Sepharose CL-6B produced glycosaminoglycans that eluted just after the void volume and the later eluting P200 fragment (Figure 4A). V8 protease digestion of the proteoglycan (Figure 4B) yielded glycosaminoglycans that eluted slightly later than the trypsin-released glycosaminoglycans, and P44 and P46 fragments that eluted substantially later than the P200 fragment. The glycosaminoglycans released by trypsin and V8 protease digestion were pooled separately as indicated by the brackets on Figure 4A,B and further purified by CsCl

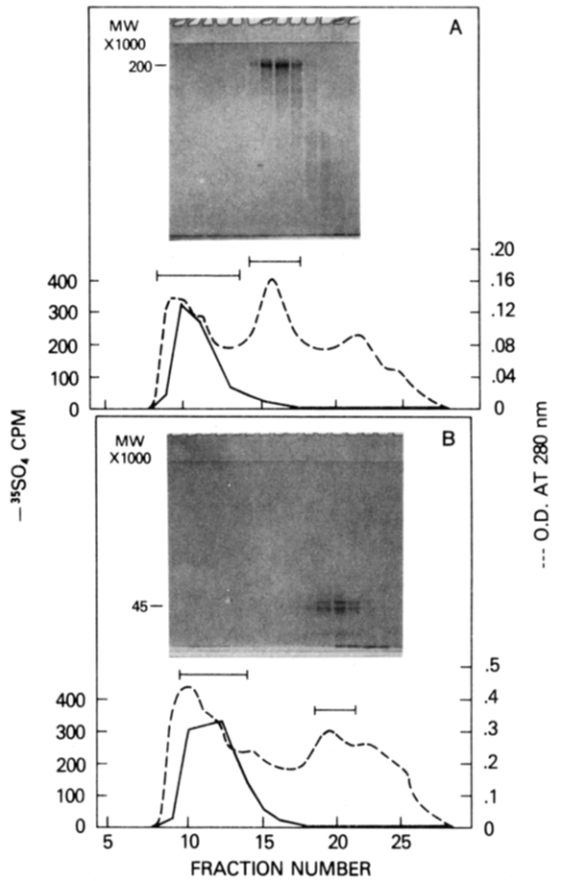

FIGURE 4: Chromatography of proteolytically digested proteoglycan on a Sepharose CL-6B column. The absorbance of the column eluant was measured at 280 nm, and aliquots of the column fractions were measured for radioactivity. Aliquots of the column fraction were also assessed for peptide fragments by SDS–PAGE followed by Coomassie Blue staining. Fractions containing the glycosaminoglycans as determined by incorporated $^{35}SO_4$, and the peptide fragments, as determined by SDS–PAGE, were pooled separately as indicated by the bars. (A) Chromatography of 15 mg of proteoglycan digested for 40 min with trypsin. (B) Chromatography of 35 mg of proteoglycan digested for 20 h with V8 protease.

density gradient centrifugation. Between 86% and 88% of the $^{35}SO_4$ in the glycosaminoglycans obtained from both digests localized to the bottom two-fifths of the gradient (not shown), indicating that most of the protein core had been removed by the enzymatic treatment. The trypsin-derived and V8 protease derived glycosaminoglycans, which had been isolated from the bottom two-fifths of the gradient, were termed T-GAG and V-GAG, respectively, and used in subsequent experiments. Heparitinase digestion of V-GAG and T-GAG followed by SDS–10% PAGE and Coomassie Blue staining did not reveal a "core" protein (not shown). This indicates that the size of the remaining core protein on these glycosaminoglycan fragments either is very heterogeneous or is less than M_r 20 000.

The peptide fragments obtained from chromatography on Sepharose CL-6B were further fractionated by HPLC on a column of DEAE 5PW using a NaCl gradient. The elution positions of peptides were monitored by the absorbance of the eluate at 280 nm as well as by SDS–PAGE of selected fractions followed by Coomassie Blue staining. Chromatography on DEAE 5PW essentially purified the P200 trypsin-produced fragment (Figure 5A). This chromatographic procedure also separated the V8 protease derived P46 and P44 fragments from each other, but both were slightly contaminated with minor amounts of an M_r 30 000 peptide (Figure 5B). The fractions containing the P200 fragment, the P46 fragment, and

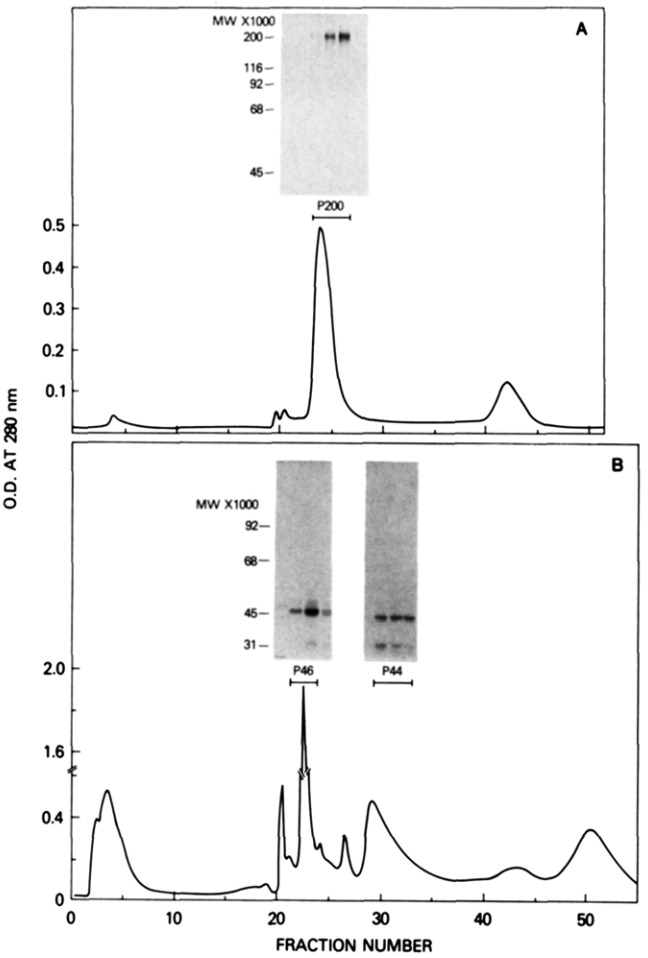

FIGURE 5: HPLC on DEAE-5PW of peptides initially isolated after proteolytic digestion and chromatography on a Sepharose CL-6B column (Figure 4). The column eluant was measured for the absorbance at 280 nm, and selected fractions were assessed for peptide fragment by SDS–PAGE. Fractions were pooled as indicated by the brackets. (A) Chromatography of the fraction containing trypsin-released P200 fragment. (B) Chromatography of the fraction containing the V8 protease released P44 and P46 fragments.

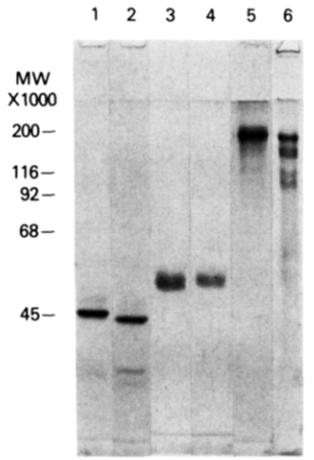

FIGURE 6: SDS–polyacrylamide gel electrophoresis of isolated core protein fragments under reducing and nonreducing conditions. The gel was stained for protein with Coomassie Blue. (Lane 1) P46 fragment, nonreduced; (lane 2) P44 fragment, nonreduced; (lane 3) P46 fragment, reduced; (lane 4) P44 fragment, reduced; (lane 5) P200 fragment, nonreduced; (lane 6) P200 fragment, reduced.

Table I: Amino Acid Composition of Proteolytic Fragments from Basement Membrane Proteoglycan[a]

	P200	P46	P44
Asp	89	82	89
Thr	61	53	59
Ser	80	103	74
Glu	137	152	151
Pro	98	96	97
Gly	120	120	128
Ala	72	70	82
Val	44	38	34
Met	11	9	10
Ile	23	14	22
Leu	75	64	77
Tyr	38	37	48
Phe	39	33	38
His	34	45	31
Lys	26	19	16
Arg	54	64	43

[a] Data are expressed in residues per 1000.

the P44 fragment were pooled as indicated by the brackets in Figure 5A,B and used in subsequent experiments.

The effect of disulfide bond reduction on the apparent molecular weight of the fragments was evaluated by SDS–PAGE (Figure 6). The apparent size of both the P46 fragment (Figure 6, lane 1) and the P44 fragment (Figure 6, lane 2) increased to M_r 50 000 (Figure 6, lanes 3 and 4) upon reduction. The apparent size of a proportion of the P200 fragment (Figure 6, lane 5) did not change upon reduction (Figure 6, lane 6), but several peptides in the range of M_r 150 000–100 000 also appeared. This suggests that the trypsin made some internal cleavages in some of the P200 fragments, thereby generating peptides that were still held together by disulfide bonds.

The amino acid compositions of the purified peptide fragments are shown in Table I. All three fragments contain similar high levels of glutamic acid and glycine. The P44 and P46 fragments differ in their serine, histidine, isoleucine, and arginine content.

The levels of reaction of the purified core protein peptide fragments with antisera raised against the proteoglycan as well as antisera against the P200 and P46 fragments were measured by ELISA (Figure 7). Antiserum raised against the intact proteoglycan reacted more strongly with the P44 and P46 fragments than with the proteoglycan and even more strongly with the P200 fragment (Figure 7A). This antiserum reacted weakly with the T-GAG and showed even less reaction with the V-GAG. Antiserum to the P200 fragment reacted strongly with the P200 fragment, as expected (Figure 7B), but also reacted strongly with the P44 and P46 fragments. This antiserum showed a substantially weaker reaction with the proteoglycan and did not react at all with the T- and V-GAG. These results indicate that the P44 fragment and the P46 fragment have immunological domains in common with the P200 fragment. Antiserum to the P46 fragment reacted most strongly with the P200 fragment, showed slightly less reaction with the P46 fragment and with the proteoglycan, and showed only a minor reaction with the P44 fragment (Figure 7C). These results indicate that the P44 and P46 fragments may have some determinants in common but, for the most part, contain immunologically distinct domains. The antiserum to the P46 fragment did not react with the V- or T-GAG.

The peptide fragments generated by digestion of the P200 fragment by V8 protease were also evaluated by SDS–PAGE under nonreducing conditions (Figure 3C). V8 protease digestion for 5 min had little effect on the P200 fragment (Figure 3C, lane 1), but digestion for 1 h (Figure 3C, lane 2) produced a major band at M_r 44 000 which increased in amount at 4 h (Figure 3C, lane 3) and remained at a constant level even after 20 h of digestion (Figure 3C, lane 4). An additional band

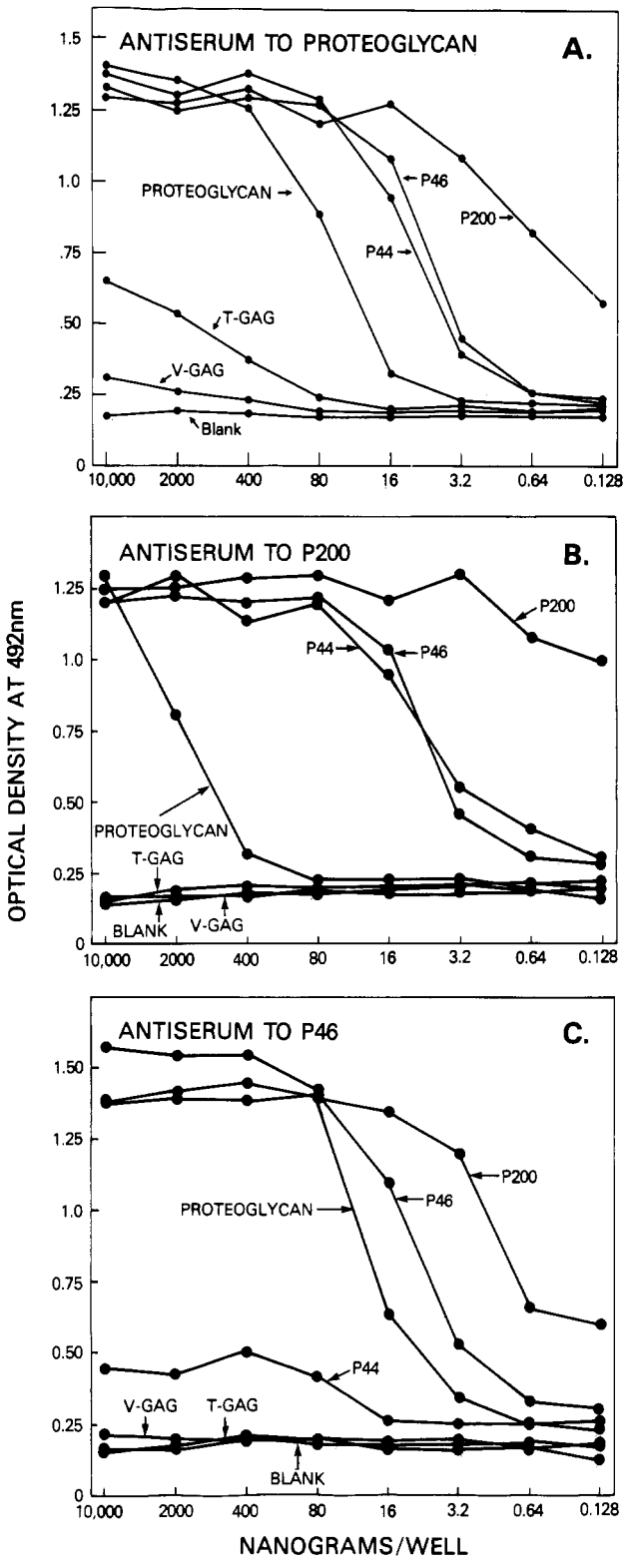

FIGURE 7: Immunoreactivity of antisera as determined by ELISA. Wells were coated serially with 10 μg to 0.128 ng of each of the purified proteoglycan fragments as well as the intact proteoglycan. The proteoglycan fragments used were the M_r 200 000 fragment (P200), the M_r 46 000 fragment (P46), the M_r 44 000 fragment (P44), and the glycosaminoglycans obtained from both trypsin digestion (T-GAG) and V8 protease digestion (V-GAG). A 1:100 dilution of the rabbit antisera to the proteoglycan or to the fragment was allowed to react with each well, and the amount of antibody bound was detected with a horseradish peroxidase conjugated goat anti-rabbit IgG second antibody. The blank is uncoated wells. Preimmune serum at a 1:100 dilution did not react with the proteoglycan or the fragments (not shown). (A) Antiserum to the proteoglycan. (B) Antiserum to the P200 fragment. (C) Antiserum to the P46 fragment.

at M_r 46 000 appeared after 1 h of digestion (Figure 3C, lane 2), and this band increased to maximum amount at 20 h of digestion (Figure 3C, lane 4). These two bands were similar in size to the P44 and P46 fragments produced by digestion of the proteoglycan with V8 protease (Figure 3B). Western blots of lane 6 in Figure 3B and lane 4 in Figure 3C showed that antiserum to the P200 fragment reacted with both the M_r 44 000 and M_r 46 000 fragments but antiserum to the P46 fragment reacted with only the M_r 46 000 fragments (not shown).

Discussion

The results of these studies show the core protein of the large, low-density basement membrane proteoglycan contains an extensive trypsin-resistant domain equal to half the size of the core that is devoid of glycosaminoglycan side chains plus a trypsin-sensitive region which contains the side chains. The separate elution of the glycosaminoglycans and the trypsin-resistant domain on Sepharose CL-6B shows these components are from separate regions of the core. The M_r 200 000 trypsin-resistant domain (P200) was released quantitatively from the proteoglycan after only 15 min of digestion, indicating the presence of a trypsin-sensitive site or region between itself and the glycosaminoglycan binding domain. Trypsin produced some internal clipping in a proportion of the P200 fragments, but these peptides were held together by disulfide bonds. This suggests that the P200 fragment has considerable secondary structure. Prolonged digestion of the proteoglycan with V8 protease produced M_r 44 000 (P44) and 46 000 (P46) fragments, and there are several lines of evidence that show these fragments to be contained within the P200 fragment. First, immunoassays showed the antiserum to the P200 trypsin-produced fragment reacted strongly with both the P44 and P46 V8 protease produced fragments. Second, prolonged digestion of the P200 fragment with V8 protease produced fragments of the same size (M_r 44 000 and 46 000) and immunoreactivity as that produced by V8 protease digestion of the proteoglycan. Third, in timed digestions of both the proteoglycan and the P200 fragment, the P44 fragment appeared first. Thus, we find an M_r 200 000 domain of the M_r 400 000 core protein of the basement membrane proteoglycan to be devoid of glycosaminoglycan and contain the M_r 44 000 and 46 000 V8 protease resistant domains.

The rapid and quantitative appearance of the P44 fragment suggests that it is located in an accessible region of the core protein (and P200 fragment), possibly from one end of the core. In contrast, the slower emergence of the P46 fragment indicates that it could be located more internally. Although these two fragments are similar in size and in disulfide bonds, the immunological studies indicated they have only a minor number of epitopes in common and they also differ in amino acid composition. These studies indicate that the P44 and P46 fragments are distinct from one another and are not derived from the same region of the core protein. The increase in apparent molecular weight of the P44 and P46 fragments after reduction indicates that they have a compact structure that is maintained by disulfide bonds.

Several lines of evidence suggest that the trypsin-released glycosaminoglycans (T-GAG) have more peptide associated with them than the V8 protease-released glycosaminoglycans (V-GAG). The slightly earlier elution of T-GAG compared to V-GAG on Sepharose CL-6B indicates that the T-GAG are larger in size. In addition, the T-GAG reacted more strongly in immunoassay with the antiserum to the proteoglycan than the V-GAG. Since the size of heparan sulfate on both the T- and V-GAG would be identical, the increased

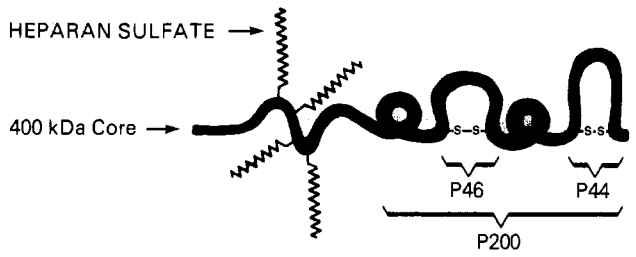

FIGURE 8: Structural map showing the possible location of the P44, P46, and P200 fragments within the M_r 400 000 core of the proteoglycan. The S–S symbols indicate the presence of disulfide bonds.

size and immunoreactivity of the T-GAG could possibly be due to the presence of additional peptide on the T-GAG not found on the V-GAG.

A proposed model of the proteoglycan, depicting the location of the glycosaminoglycan attachment region and the P200 fragment containing the P44 fragment and P46 fragments, is shown in Figure 8. The P200 fragment and the glycosaminoglycan binding region are located at opposite regions of the core. Both the P44 and P46 fragments are located within the P200 fragment, but because of its rapid release during proteolysis, the P44 fragment is placed at the end of the core protein. The presence of residual protein on the T-GAG indicates there may be conformational structure of the core in the glycosaminoglycan attachment region that provides some trypsin resistance. The P44 and P46 fragments have internal disulfide bonds as indicated by their change in apparent molecular weight upon reduction.

Although basement membrane proteoglycans from other sources have not been similarly analyzed, the core proteins of most characterized proteoglycans show regional domains. The structure of the rat yolk sac proteoglycan, deduced from genetic clones, contains 3 structural domains: a 14 amino acid N-terminal region followed by a 49 amino acid serine-glycine repeat region and a 41 amino acid COOH-terminal region (Bourdon et al., 1985). It is thought that the serine residues in the repeat region are conjugated with chondroitin sulfate side chains. The small proteoglycan in skin is considered to have its glycosaminoglycan side chain on the serine located four residues from the N-terminal end of its 45 000 molecular weight core protein (Chopra et al., 1985). Proteoglycans related to this one are also found in bone, tendon, cornea, and articular cartilage and constitute a family known as PG II [see Hassell et al. (1986a) for a review]. Recent studies with bone and tendon PG II also show the glycosaminoglycan side chains to be located near one end (Vogel & Fisher, 1986). The heparan sulfate containing proteoglycan from fibroblasts, that may be closely related to the transferrin receptor, has separate domains for heparan sulfate attachment, oligosaccharide attachment, transferrin binding, and disulfide bonding (Fransson et al., 1985). The cartilage proteoglycan [see Hassell et al. (1986a), Hardingham (1984), and Heinegard & Paulsson (1984) for recent reviews] has a glycosaminoglycan free globular region, maintained by disulfide bonds, at the N-terminal end of the core that binds hyaluronic acid and link protein with an adjacent keratan sulfate rich attachment region. The chondroitin sulfate attachment region starts in the keratan sulfate rich region and extends nearly to the C-terminal end of the core. Recently obtained cDNA clones to this proteoglycan indicate that there is a disulfide-bonded globular domain at the C-terminal end of the core as well (Doege et al., 1986). The studies presented in this report show that the basement membrane proteoglycan contains a trypsin-resistant region maintained by disulfide bonds that extend for half the length of the core protein. Although no functional activity is yet known for this domain, it should be noted that the proteoglycan is only extracted from the matrix by denaturing solvents (Hassell et al., 1985). It is possible that the region may be involved in interactions with other basement membrane components in the matrix.

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REFERENCES

Anderson, M. J., & Fambrough, D. M. (1983) *J. Cell Biol. 97*, 1396–1411.

Bourdon, M. A., Oldberg, A., Pierschbacher, M., & Ruoslahti, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1321–1325.

Carlin, B., Jaffe, R., Bender, B., & Chung, A. E. (1981) *J. Biol. Chem. 256*, 5209–5214.

Chopra, R. K., Pearson, C. H., Pringle, G. A., Fackre, D. S., & Scott, P. G. (1985) *Biochem. J. 232*, 277–279.

Doege, K., Fernandez, P., Hassell, J. R., Sasaki, M., & Yamada, Y. (1986) *J. Biol. Chem. 261*, 8108–8111.

Eldridge, C. F., Sanes, J. R., Chiu, A. Y., Bunge, R. P., & Cornbrooks, C. J. (1986) *J. Neurocytol. 15*, 37–51.

Fenger, M., Wewer, U., & Albrechten, R. (1984) *FEBS Lett. 173*, 75–79.

Fransson, L., Cöster, L., Carlstedt, I., & Malmström, A. (1985) *Biochem. J. 231*, 683–687.

Gill, P. J., Silbert, C. K., & Silbert, J. E. (1986) *Biochemistry 25*, 405–410.

Hardingham, T. E. (1984) in *Molecular Biophysics of the Extracellular Matrix* (Arnott, S., Rees, D., & Morris, E., Eds.) pp 1–19, Humana Press, Clifton, NJ.

Hassell, J. R., Robey, P. G., Barrack, H. J., Wilczek, J., Rennard, S. I., & Martin, G. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4494–4498.

Hassell, J. R., Leyshon, W. C., Ledbetter, S. R., Tyree, B., Suzuki, S., Kato, M., Kimata, K., & Kleinman, H. K. (1985) *J. Biol. Chem. 260*, 8098–8105.

Hassell, J. R., Kimura, J. H., & Hascall, V. C. (1986a) *Annu. Rev. Biochem. 55*, 539–567.

Hassell, J. R., Noonan, D. M., Ledbetter, S. R., & Laurie, G. W. (1986b) in *The Functions of Proteoglycans* (Whelan, J., Ed.) pp 204–222, Wiley, New York.

Heinegard, D., & Paulsson, M. (1984) in *Extracellular Matrix Biochemistry* (Piez, K., & Reddi, H., Eds.) pp 277–328, Elsevier, New York.

Hogan, B. L. M., Taylor, A., Kurkinen, M., & Couchman (1982) *J. Cell Biol. 95*, 197–204.

Jalkanen, M., Rapraeger, A., Nguyen, H., Banerjee, S., Hassell, J., & Bernfield, M. (1985) *J. Cell Biol. 101*, 99a.

Kanwar, Y. S., & Farquhar, M. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4493–4497.

Kanwar, Y. S., Linker, A., & Farquhar, M. G. (1980) *J. Cell Biol. 86*, 688–693.

Kanwar, Y. S., Hascall, V. C., & Farquhar, M. G. (1981) *J. Cell Biol. 90*, 527–532.

Kefalides, N. A. (1971) *Biochem. Biophys. Res. Commun. 45*, 226–234.

Kinsella, M. G., & Wight, T. N. (1985) *J. Cell Biol. 101*, 338a.

Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., & Martin, G. R. (1982) *Biochemistry 21*, 6188–6193.

Laurie, G. W., Leblond, C. P., & Martin, G. R. (1982) *J. Cell Biol. 95*, 340–344.

Laurie, G. W., Hassell, J. R., Kleinman, H. K., & Martin, G. R. (1984) *J. Cell Biol. 99*, 78a.
Ledbetter, S. R., Tyree, B., Hassell, J. R., & Horigan, E. A. (1985) *J. Biol. Chem. 260*, 8106–8113.
Mayer, B. W., Hay, E. D., & Hynes, R. O. (1981) *Dev. Biol. 82*, 267–286.
Orkin, R. W., Gehron, P., McGoodwin, E. B., Martin, G. R., Valentine, T., & Swarm, R. (1977) *J. Exp. Med. 145*, 204–220.
Paulsson, M., Dziadek, M., Suchonek, C., Huttner, W. B., & Timpl, R. (1985) *Biochem. J. 321*, 571–579.
Paulsson, M., Fujiwara, S., Dziadek, M., Timpl, R., Pyler, G., Backstrom, G., Lindahl, U., & Engel, J. (1986) in *The Functions of Proteoglycans* (Whelan, J., Ed.) pp 189–203, Wiley, New York.
Ratner, N., Bunge, R. P., & Glaser, L. (1985) *J. Cell Biol. 101*, 744–754.
Rennard, S. I., Berg, R., Martin, G. R., Foidart, J. M., & Robey, P. G. (1980) *Anal. Biochem. 104*, 205–214.
Rennke, H. G., Cotran, R. S., & Venkatachalam, M. A. (1975) *J. Cell Biol. 67*, 638–646.
Smith, R. L., & Bernfield, M. (1982) *Dev. Biol. 94*, 378–390.
Stow, J. L., Sawada, H., & Farquhar, M. G. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3296–3300.
Stow, J. L., Kjeller, L., Unger, E., Hook, M., & Farquhar, M. G. (1985b) *J. Cell Biol. 100*, 975–980.
Thesleff, I., Barrach, H. J., Foidart, J. M., Vaheri, A., Pratt, R., & Martin, G. R. (1981) *Dev. Biol. 81*, 182–192.
Thompson, H. A., & Spooner, B. S. (1982) *Dev. Biol. 89*, 417–424.
Timpl, R., Martin, G. R., Bruckner, P., Wick, G., & Weidemann, H. (1978) *Eur. J. Biochem. 84*, 43–52.
Timpl, R., Rhode, H., Robey, P. G., Rennard, S. I., Foidart, J. M., & Martin, G. R. (1979) *J. Biol. Chem. 254*, 9933–9937.
Timpl, R., Dziadek, M., Fujiwara, S., Nowack, H., & Wick, G. (1983) *Eur. J. Biochem. 137*, 455–465.
Vogel, K. G., & Fisher, L. W. (1986) *J. Biol. Chem. 261*, 11334–11340.
Wewer, U., Albrechtsen, R., & Hassell, J. R. (1985) *Differentiation (Berlin) 30*, 61–67.

Chemical Modification and Cross-Linking of Neurophysin Tyrosine-49†

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ABSTRACT: Photoaffinity labeling of the single neurophysin tyrosine, Tyr-49, with Met-Tyr-azido-Phe amide has been reported to inhibit both neurophysin self-association and peptide binding. Accordingly, we investigated the functional consequences of modification, principally by tetranitromethane, of Tyr-49. Tetranitromethane-mediated tyrosine–tyrosine cross-linking permitted synthesis of covalent neurophysin "dimers" and of peptide–protein conjugates, the latter potentially analogous to the photoaffinity-labeled product. The self-association and binding properties of the covalent dimers were found to be similar or enhanced relative to those of the native protein. In contrast to the photoaffinity-labeled product, covalent conjugates of Tyr-49 with the ligand peptides Met-Phe-Tyr amide, Phe-Tyr amide, and Tyr-Phe amide also generally exhibited normal or increased binding affinity for exogenous peptide; a subfraction of the Phe-Tyr amide adducts showed evidence of reduced affinity. Diiodination of Tyr-49 had no significant effect on binding. However, among the products of tetranitromethane treatment in the absence of peptide was a novel inactive non-cross-linked product, representing modification only of Tyr-49 but containing no demonstrable nitrophenol. As evidenced by circular dichroism and nuclear magnetic resonance (NMR), this product was not significantly unfolded and retained the ability to self-associate. These latter results provide the strongest evidence thus far of a role for Tyr-49 in peptide–hormone binding. The disparate effects of different Tyr-49 modifications are collectively interpreted and reconciled with NMR data and the properties of the photoaffinity-labeled protein to suggest potential mechanisms of Tyr-49 participation in binding and the probable orientation of Tyr-49 relative to peptide residue 3 in neurophysin complexes.

The posterior pituitary protein neurophysin binds the peptide hormones oxytocin and vasopressin within the hypothalmo–neurohypophyseal tract and interacts similarly with di- and tripeptides resembling the amino-terminal region of the hormones (Breslow, 1979; Cohen et al., 1979). The binding site common to these peptides is largely unidentified.[1] A central question has been the relative contribution to binding of nonduplicated and internally duplicated regions of the protein [e.g., see Breslow (1979) and Cohen et al. (1979)]. In this context, the single neurophysin tyrosine, Tyr-49, of the evolutionarily conserved nonduplicated region has been extensively

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[1] The binding site investigated in this study is the principal hormone site, to which the smaller peptides also bind. A second site, which may also bind the hormones under some conditions, but which is not of thermodynamic significance for peptides, may also be present and close to Tyr-49 [e.g., see Cohen et al. (1979) and Breslow (1984)].

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