PG-M/Versican-like Proteoglycans Are Components of Large Disulfide-stabilized Complexes in the Axolotl Embryo*

(Received for publication, June 5, 1996, and in revised form, October 11, 1996)

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Large disulfide-stabilized proteoglycan complexes were previously shown to be synthesized by the epidermis of axolotl embryos during stages crucial to subepidermal migration of neural crest cells. We now show that the complexes contain PG-M/versican-like monomers in addition to some other component with low buoyant density. Metabolically $^{35}$S-labeled proteoglycans were extracted from epidermal explants and separated by size exclusion chromatography and density equilibrium gradient centrifugation. The complexes, which elute in the void volume on Sepharose CL-2B, were recovered at buoyant density 1.42 g/ml in CsCl gradients, whereas the monomer proteoglycans, which could only be liberated from the complexes by reduction, had a higher buoyant density (1.48 g/ml). The native complexes did not aggregate with hyaluronan. The purified complexes reacted with antibodies against a portion of a cloned PG-M/versican-like axolotl proteoglycan. These antibodies were found to stain the subepidermal matrix of axolotl embryos, suggesting that the proteoglycan complexes are encountered by neural crest cells during subepidermal migration. From Western blot analysis, the core protein of the PG-M/versican-like monomers was found to be of similar size (~500 kDa) as those of PG-M/versican variants of other species. Another chondroitin sulfate proteoglycan that was present in small amounts in the epidermal extracts was found to be distinctly different from the similarly sized PG-M/versican-like monomers.

The role of proteoglycans (PGs)

1 in the modulation of cell matrix interaction and cell migration depends on their structure and localization (1–3). Interstitial PGs that carry chondroitin/dermatan sulfate (CS/DS) chains have been implicated to have such modulatory roles by several studies (4–18). In this regard, since both the core protein (7, 18–19) and the glycosaminoglycan moiety (15, 18, 20) appear to be of functional importance, the structure of the intact PG may be crucial (18). In addition to the inherent potential for heterogeneity in the carbohydrate component (21), the core proteins of PGs in the PG-M/versican family, for instance, may exist as several splice variants (22–25) with potentially diverse expression patterns and functions during various stages of the life cycle of an animal.

The importance of the extracellular matrix in the regulation of subepidermal neural crest cell migration in the axolotl embryo was demonstrated by transplantation experiments in which epidermal grafts or microcarriers with matrix adsorbed in situ from developmentally more advanced embryos were found to trigger the onset of premature migration (26). Ultrastructural studies of the matrix after fixation in the presence of cetylpyridinium chloride (27) or ruthenium red (28–30) revealed an abundance of granular proteoglycan precipitates along collagen fibrils. Interestingly, in embryos of the mutant axolotl, in which subepidermal migration of the neural crest-derived pigment cells is defective (28–29, 31), the subepidermal matrix was significantly less granulated (27, 29–30) suggesting a potential role of the PGs in the matrix.

In an attempt to isolate and characterize the PGs that might be encountered by migrating neural crest cells in the axolotl embryo, a previous study (32) took advantage of the possibility to utilize epidermal explants for the metabolic radiolabeling of PGs with $^{35}$S sulfate. Dorsal trunk epidermis was explanted at a premigratory stage and was maintained in tissue culture together with radiolabel for a time period corresponding to the transition from a nonpermissive matrix to one that permits subepidermal migration of neural crest cells. It was found that the major PGs synthesized were large disulfide-stabilized PG complexes with monomer PGs in the size range of aggrecan or the major PGs synthesized were large disulfide-stabilized PG complexes with monomer PGs in the size range of aggrecan or PG-M/versican but with unusually large ($M_r \approx 150,000$) chondroitin/dermatan sulfate (CS/DS) side chains. In the present work these complexes are further studied, and their PG component is purified, characterized, and identified. We report that the complexes contain PG-M/versican-like PG monomers in addition to some other component of low buoyant density. Furthermore, the PG-M/versican-like monomers of the complex are shown to be distinctly different from another CSPG that is present in small amounts in the epidermal extracts.

EXPERIMENTAL PROCEDURES

Embryos—Embryos of the Mexican axolotl (Ambystoma mexicanum, Urodela, Amphibia) were obtained from the Indiana University Axolotl Colony, Bloomington, IN, and from the Department of Zoology, Uppsala University. Before manual decapsulation, eggs were sterilized in 70% ethanol and rinsed in modified Steinberg’s solution (MS), composed of 58 mM NaCl, 0.67 mM KCl, 0.34 mM CaCl$_2$, 0.83 mM MgSO$_4$, 4.6 mM HEPES, pH 7.4 (33), supplemented with 0.05% penicillin/streptomycin. All storage, handling, and manipulations of embryos were done in sterile MS. Embryos were staged according to Bordzilovskaya et al. (34).

Metabolic Radiolabeling with $^{35}$S Sulfate and Extraction of PGs—Using sharp tungsten needles, the epidermis was carefully removed from the dorsal trunk (the area between the levels of the gills and anus.
overlying the somites and neural tube/crest of stage 30 embryos (around the onset of neural crest cell migration). The epidermal explants were incubated in MS containing 2.5 mM CaCl₂ and 0.65 mM Na₂SO₄ (carrier-free; Du Pont NEN). After incubation for 20 h at 20°C, corresponding to the time between stages 30 to 35 (as determined by the parallel increase in size of the fibronection fibres), the tissue was dissolved in extraction buffer (about 400 µl per 50 explants) containing 4 mM guanidine HCl, 50 mM sodium acetate, pH 5.8, and 0.2% Triton X-100, together with 1 mM CsCl gradients with and without added aggrecan. The 3H radioactivity of another 1 h.

glycosaminoglycan side chains was achieved by adding 1 volume of 0.1 M NaOH, 0.6 M NaBH₄ followed by incubation at room temperature for 24 h, after which the samples were acidified, neutralized, and dialyzed against water. The chain length of the glycosaminoglycans was assayed by chromatography on a calibrated Sepharose CL-4B (Pharmacia) ion exchange column, equilibrated with a buffer containing the same ingredients to the time between stages 30 to 35 (as determined by the parallel increase in size of the fibronection fibres), the tissue was dissolved in extraction buffer (about 400 µl per 50 explants) containing 4 mM guanidine HCl, 50 mM sodium acetate, pH 5.8, and 0.2% Triton X-100, together with 1 mM CsCl gradients with and without added aggrecan. The 3H radioactivity of another 1 h.

Additional Treatments to Dissociate the HMPG Complexes—Acid treatment was done by titrating the sample with acetic acid to pH 3.8, after which the samples were incubated for 1.5 h at +4°C. For digestion with collagenase (form III; Advanced Biofuicides, Lynnbrook, NY), samples were dialyzed into a digestion buffer of 50 mM Tris-HCl, pH 7.5, 0.36 mM CaCl₂, and the mixture of protease inhibitors excluding EDTA. To test the effectiveness and specificity of the collagenase treatment, collagenase (type I) was added to the digested buffer containing aggrecan (52), was incubated with 150 mM NaCl, 0.1 M NaAc, pH 5.8, 0.1% Triton X-100, with the mixture of protease inhibitors. As a positive control of enzyme activity, aggrecan was digested under identical conditions and analyzed by chromatography on a Sepresso 6 HPLC column.

Chondroitinase ABC Digestion—Before digestion with chondroitinase ABC (protease-free; Seikagaku Co., Japan), the purified PG samples were dialyzed into the appropriate buffer. Digestion with chondroitinase ABC (0.1 units/ml) was done at 37°C for 3 h in 50 mM Tris, pH 8, 30 mM NaAc, 0.1% Triton X-100, with the mixture of protease inhibitors. As a positive control of enzyme activity, aggrecan was digested under identical conditions and analyzed by SDS-PAGE and immunoblotting.

Enzymatic activity—For slot blotting, the chondroitinase ABC-treated samples were diluted to <0.05% Triton X-100 in 4 mM guanidine-HCl/TBS (10 mM Tris, pH 8, and 150 mM NaCl), serially diluted, and immobilized on a nitrocellulose membrane (Schleicher & Schuell) using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad). For Western blotting, samples were precipitated with 10% trichloroacetic acid, rinsed in ethanol:diethyl ether (1:1), briefly dried, and dissolved in sample buffer. Reduced and alkylated samples were subjected to SDS-PAGE in 7.5% homogeneous PhastGels for 130 V·h followed by semi-dry electrotransfer for 30 min onto nitrocellulose using a Pharmacia PhastSystem. Prestained SDS-PAGE Standards (high range; Bio-Rad) and laminin (detected by Coo massie staining of parallel gels) were used as molecular weight markers. Blots were blocked with 5% non-fat milk in Tris-buffered saline, 0.1% Tween (TBS-T), washed with primary antibodies (see below) in TBS-T for 1 h at room temperature or overnight at +4°C, washed, incubated with horseradish peroxidase-linked anti-mouse IgG or anti-rabbit Ig (Amersham Corp.; secondary antibodies diluted 1:5000) for 30 min at room temperature, washed, reacted with enhanced chemiluminescence reagent (Amersham Corp.) according to the manufacturer's instructions, and detected by exposure to Fuji RX X-ray film.

Expression of a Portion of a Cloned Axolotl PG-M/Versican Homologue as a Fusion Protein—A portion of the CS attachment domain of a partially cloned axolotl PG-M/versican homologue was selected for cloning (M. Stigson and L. Kjellen, work in progress). Briefly, a cDNA encoding corresponding to a portion of the conserved C-terminal domain of chicken PG-M (22) prepared from a partial cDNA clone generously provided by Drs. K. Kimata and T. Shinomura was used for the screening of an oligo(dT)′-primed embryonic axolotl cDNA library. A 4.67-kilobase pair AxPG partial cDNA clone (the full transcript was estimated to be around 12 kilobases) was sequenced and found to consist of 3422 of coding and 1245 base pairs of 3′-untranslated sequence. The deduced amino acid sequence comprises the C-Terminal 1140 amino acid residues of the core protein including 830 amino acid residues of the CS attachment domain and the complete conserved C-terminal end with two epidermal growth factor domains, a lectin-like domain, and a complement regulatory protein-like domain. The C-terminal domain of AxPG shows an extensive amino acid sequence identity to that of the published chicken (22), mouse (24), and human (51) PG-M/versican, i.e. 83% identity to the avian and 78% to the mammalian species. Furthermore, the 830 C-terminal residues of AxPGs is significantly lower, e.g. 58% identity to chicken aggrecan (52) and 56% to rat neuronin (53). However, the CS attachment region apparently shows limited sequence conservation between the species and was chosen for expression as a fusion protein and generation of AxPG-specific antibodies (cf. 54).
transfection of *Escherichia coli* JM83, a recombinant clone with the correct insert was selected after sequencing. The recombinant plasmid was cultured in *E. coli* UT5600, and after induction with isopropyl β-D-thiogalactopyranoside, the bacteria were further cultured and lysed, after which the axolotl PG-glutathione S-transferase (AxPG-GST) fusion protein was purified by affinity chromatography on a glutathione-Sepharose (Pharmacia) column.

### Preparation of Antibodies Against the AxPG-GST Fusion Protein

Rabbits were immunized intramuscularly with the purified AxPG fusion protein using standard procedures, and the production of antibodies was monitored by enzyme-linked immunosorbent assay. Anti-AxPG-GST antibodies (0.3 mg/ml) were obtained by affinity chromatography on AxPG-GST-coupled Sepharose 4B. The antibodies were used in immunoblotting (30 µg/ml in TBS-T) and immunofluorescence (as described below). As a control, similarly prepared affinity-purified antibodies against GST were used under the same conditions as the anti-AxPG-GST antibodies.

### Immunofluorescence

Axolotl embryos of stage 32 or epidermal explants of stage 30 embryos after 20 h incubation were fixed overnight (embryos) or 15 min (epidermal explants) in 3% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4. For whole embryos, fixative penetration was facilitated by cutting off the head and tail. The fixed embryos and epidermal explants were rinsed in phosphate-buffered saline (PBS), dehydrated, embedded in Paraplast, and sectioned transversely. Sections were dewaxed, rehydrated, washed in PBS, and subjected to chondroitinase ABC digestion (1 unit/ml) in 50 mM Tris, pH 8, 30 mM NaAc for 30 min at 37 °C. The incubation with primary antibodies (10 µg/ml in PBS containing 0.1% bovine serum albumin) was performed overnight at 4 °C. After washing in PBS, the sections were incubated with fluorescein isothiocyanate-conjugated secondary anti-rabbit IgG antibodies (diluted 1:200 in PBS, 0.1% bovine serum albumin; Sigma) for 1 h at room temperature, after which the immunofluorescence was visualized in a fluorescence microscope. As control, anti-AxPG antibody binding was competitively blocked by the addition of a 5-fold molar excess of AxPG fusion protein to the primary antibody. Anti-GST antibodies were used as negative control.

### RESULTS

To further characterize the large PG complexes previously shown to be synthesized by the epidermis of the axolotl embryo (32), explants of dorsal trunk epidermis from axolotl embryos were maintained in tissue culture for a time period corresponding to stages 30 to 35 in the presence of [35S]sulfate. After dissociative extraction and dialysis to recover macromolecular radiolabel, the [35S]-labeled PGs were separated by size exclusion chromatography on Sepharose CL-2B under dissociative conditions (Fig. 1A). The high molecular weight PGs (HMPGs) eluting in the void volume fractions and PGs of intermediate molecular weight (IMPGs) eluting with Kav 0.2–0.3 were separately pooled and subjected to density equilibrium centrifugation in self-forming gradients of CsCl under dissociative conditions (ρ = 1.42 g/ml). The HMPGs were mostly found in mid-gradients, fractions with ρ > 1.42 g/ml (Fig. 1B) which were pooled as indicated in the figure and used for further characterization. In contrast, the majority of IMPGs were recovered in fractions of significantly higher density (ρ > 1.55 g/ml; Fig. 1C). Evidently, the rather prominent second peak that appears at ρ < 1.42 g/ml in Fig. 1C represents contaminating HMPG which by its relative abundance is present in the IMPG pool after separation by Sepharose CL-2B chromatography. The IMPGs, pooled as indicated in the figure, were further purified from contaminating HMPG by ion exchange chromatography

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3 Introducing ion exchange chromatography on DEAE-Sepharose as a first step before Sepharose CL-2B chromatography or CsCl gradient centrifugation was found to result in a significantly reduced recovery of HMPGs compared with IMPGs (M. Stigson, unpublished data). For unknown reasons, HMPGs appear to bind irreversibly to the ion exchange resin and are only partly eluted by increasing the salt concentration. This property of the HMPG was taken advantage of for purification of IMPGs, which were enriched after ion exchange chromatography of HMPG-contaminated IMPG preparations.
The HMPG fraction has previously been shown to contain PG complexes stabilized by intermolecular disulfide bonds (32). After reduction, the monomers were found to elute at a $K_{av}$ of 0.2 when analyzed by Sepharose CL-2B chromatography (32). To investigate if the HMPG monomers resemble the IMPGs, the HMPG fraction obtained after chromatography on Sepharose CL-2B was subjected to CsCl gradient centrifugation before and after reduction and alkylation (Fig. 2). The reduced PGs were found at a higher buoyant density (1.48 g/ml; Fig. 2B) than the unreduced HMPGs (1.42 g/ml; Fig. 2A), indicating that the complex in addition to the HMPG monomers contain other components with lower buoyant density. Since the significantly higher buoyant density of the IMPGs (1.55 g/ml; Fig. 1C) indicated that the HMPG monomers and the IMPGs are not identical, it was decided to further compare the two PG preparations.

The HMPG Monomers Are Distinctly Different from the IMPGs

Glycosaminoglycan Chain Length—After alkaline liberation from the core protein, the glycosaminoglycan side chains of HMPG eluted significantly earlier ($K_{av}$ = 0.3) than those of IMPG ($K_{av}$ = 0.4) when chromatographed on a Sepharose CL-4B column previously calibrated with hyaluronan fragments (32). The arrow shows the elution position of $^3$H-labeled hyaluronan fragments of $M_r$ = 40,000, which were used as internal standard.

Aggregation With Hyaluronan—A characteristic feature of several large PGs such as aggrecan (38), PG-M/versican (39, 40), and neurocan (41) is their ability to specifically bind to hyaluronan (i.e. noncovalent protein-carbohydrate binding) to form large supramolecular complexes. To investigate whether the HMPG complexes can bind to hyaluronan, samples of $^{35}$S-labeled HMPG without (Fig. 4A) or with (Fig. 4B) the addition of unlabeled hyaluronan and aggrecan were dialyzed into dissociative buffer and analyzed by associative CsCl gradient centrifugation. As shown in Fig. 4, the position of the $^{35}$S-labeled HMPG in the gradient was the same in the presence and absence of the aggrecan-hyaluronan complexes (see “Experimental Procedures”). In contrast, $^{35}$S-labeled IMPG, which was run on an associative Sepharose CL-2B column...
under the same conditions, was found to elute in the void volume after addition of aggrecan and hyaluronan (Fig. 5). Apparently, the IMPG can form aggregates with hyaluronan, whereas the HMPG complexes lack this property. Any hyaluronan binding activity of native HMPG monomers could not be tested since such activity is destroyed by reduction. Attempts to obtain native HMPG monomers by treatment with acid (pH 4), collagenase, or hyaluronidase (see “Experimental Procedures”) were unsuccessful as neither of these treatments altered the size or buoyant density of the HMPG complexes (data not shown).

Keratan Sulfate Content—To investigate if the two PG preparations contained keratan sulfate, equal amounts of $^{35}$S radioactivity of purified HMPG and IMPG were digested with chondroitinase ABC and analyzed by slot blotting, using monoclonal anti-keratan sulfate antibodies 5D4 (Fig. 6A). The antibodies reacted well with IMPG, whereas no immunoreactivity was found for HMPG. To exclude that the observed difference was due to lower amounts of HMPGs analyzed with acid (pH <4), collagenase, or hyaluronidase (see “Experimental Procedures”) were unsuccessful as neither of these treatments altered the size or buoyant density of the HMPG complexes (data not shown).

The HMPG Monomers Are Related to PG-M/Versican

Immunoreactivity—To investigate if the monomer PGs of the HMPG complex are related to PG-M/versican, HMPG and IMPG were purified from a large number of epidermal explants and analyzed by slot blotting (Fig. 7) using affinity-purified polyclonal antibodies (anti-AxPG-GST) raised against a fusion protein (AxPG-GST) of glutathione S-transferase and an 80-amino acid segment of the CS attachment domain of a partially cloned axolotl PG (AxPG; see “Experimental Procedures”) with homology to PG-M/versican.2 Chondroitinase ABC-digested HMPG was clearly immunoreactive to these antibodies, whereas IMPG and bovine nasal cartilage aggrecan were unreactive. The immunopositive signal of HMPG increased after the membrane was stripped in a reducing buffer and reprobed, suggesting that reduction of the complex further exposes the core protein epitopes (data not shown).
Core Protein Size—To estimate the size of the core protein of the HMPG monomers, the PGs purified from a large number of epidermal explants were analyzed by SDS-PAGE and immunoblotting after chondroitinase ABC treatment and reduction. Detection of the core protein by using the anti-AxPG-GST antibodies resulted in a weak band with a size (~500 kDa) in the same range as the core proteins of PG-M/versicans (data not shown). A stronger signal was obtained when using a mixture of the monoclonal antibodies 3B3, 2B6, and 1B5 (37), directed against the unsaturated stubs generated by digestion with chondroitinase ABC, which recognized the same band (Fig. 8). Since only CS/DS is present in the HMPG preparation (32), and only one band (~500 kDa) was detected with anti-CS-stub antibodies, the detected core protein must be derived from the major PG in the preparation. It thus appears unlikely that any significant amount of contaminating PGs would be present in the HMPG preparation. The core protein of IMPG could not be detected by immunoblotting using these conditions.

Localization—The affinity-purified anti-AxPG-GST fusion protein antibodies stained the subepidermal extracellular matrix of axolotl embryos (Fig. 9A), as well as the matrix inside epidermal vesicles (Fig. 10). Immunofluorescence was found along fibrils in the matrix. This staining was inhibited by the addition of fusion protein (AxPG-GST), whereas weak unspecific fluorescence of the epididermal layer remained (Fig. 9B); antibodies against GST gave no staining (data not shown). From these results, it may be inferred that the major PGs encountered by neural crest cells in the subepidermal extracellular matrix of the axolotl embryo are PG-M/versican-like PGs that are components of large disulfide-stabilized complexes.

DISCUSSION

In the present study, antibodies against a portion of a partially cloned PG-M/versican-like axolotl PG (AxPG) expressed as a fusion protein (AxPG-GST) were demonstrated to recognize the large disulfide-stabilized PG complexes (HMPG; Fig. 7), previously found to be the major PGs synthesized by the epidermis of axolotl embryos during stages crucial to the subepidermal migration of neural crest cells (32). Another chondroitin sulfate PG (IMPG), which constitutes a minor fraction of the PGs present in epidermal extracts, was not recognized by these antibodies when equal amounts of 35S radioactivity of the purified PGs were analyzed by slot blotting (Fig. 7). Since the amount of core protein molecules present in the IMPG sample may be lower than in the HMPG sample, as indicated by the lower immunoreactivity to anti-CS stub antibodies (Fig. 6), it cannot be excluded that the IMPGs may have escaped detection with the anti-AxPG-GST antibodies (Fig. 7). It appears more likely, however, that the IMPGs which in contrast to the HMPGs were shown to contain KS (Fig. 6) may be related to the aggrecans. 4

Large PGs of the extracellular matrix have been suggested to interact with a rich variety of matrix components, both proteins and carbohydrates. Interactions may be mediated both by the glycosaminoglycan side chains and by the core protein itself. The classical case is the noncovalent binding of aggrecan to hyaluronan in cartilage (38). In PG-M/versican the N-terminal domain of the core protein exhibits hyaluronan-binding properties (39–41), whereas the C-terminal domain has lectin-like and heparin binding activity (19) and can bind to tenascin-R (42). The HMPG complexes, in addition to the PG-M/versican-like monomers, evidently contain some other component to account for their lower buoyant density (1.42 g/ml; Fig. 1) compared with that of the monomers (1.48 g/ml; Fig. 2). In the previous study (32), it was shown that the integrity of the large HMPG complexes is resistant to treatment with strong chaotropic agent (6 x guanidine) but susceptible to reduction and alkylation. This suggested that the HMPG complexes, unlike aggregates with hyaluronan, are covalently held together by intermolecular disulfide bonds. Due to the low pH of the extraction buffer (pH 5.8) and the presence of the alkylating agent NEM, it appears unlikely that the PG complexes could be artificially formed by disulfide exchange during extraction rather than being the natural molecular organization in the embryonic extracellular matrix. In further support of this conclusion, the HMPG fraction has been found to have a very low relative protein content. 5

4 Since the buoyant density of the HMPG monomers is lower, 1.48 g/ml (Fig. 2) versus 1.55 g/ml (Fig. 1) for IMPG, while their glycosaminoglycan side chains are longer, M, ~150,000 versus Mr ~ 75,000 for IMPG (Fig. 3), HMPG is likely to have fewer side chains per core protein molecule than IMPG. Nevertheless, the immunoreactivity of IMPGs to anti-CS-stub antibodies was found to be lower than for the HMPGs when equal amounts of 35S radioactivity of purified PGs were analyzed by slot blotting (Fig. 6). A restricted access of primary antibodies due to a relative epitope clustering could account for the lower immunoreactivity of IMPG.

5 When epidermal explants were simultaneously labeled with [3H]leucine and [35S]sulfate (M. Stigson, unpublished data) and analyzed by chromatography on Sepharose CL-2B, essentially all 3H-labeled macromolecules of the epidermal explants eluted with Kav ~ 0.8, whereas ~1% of the 3H radioactivity was recovered in the void volume. The low protein content of the HMPG fraction did not allow for further characterization of its protein component.
axolotl embryo, PGs appear to be localized along collagen fibrils, seemingly attached to them (27–30). Moreover, chicken PG-M has been shown to be able to bind to type I collagen (39). However, neither the size nor the buoyant density of HMPGs were altered by collagenase treatment, arguing against the possibility that the isolated HMPG complexes may owe their size (and buoyant density) to their association with collagen. Furthermore, since hyaluronidase treatment was also ineffective in dissociating the complexes, the present study rules out that any binding to hyaluronan could be involved in creating the large size of the complexes. Hyaluronan binding activity was demonstrated for the IMPGs (Fig. 5) but not for the HMPG complexes (Fig. 4). Although the intact complexes seem to be unable to bind to hyaluronan under the present experimental conditions, it cannot be precluded that the native monomers might have the capacity to do so. The protein-protein interactions leading to intermolecular disulfide bridges could, for instance, obscure the recognition sites for hyaluronan. Nevertheless, since the PG-M/versican-like PGs apparently form large supramolecular complexes by protein-protein interaction, the aggregation with hyaluronan may have been rendered redundant in the axolotl embryo. Whether the complex formation is compatible with a role for PG-M/versican in bridging cell surfaces to pericellular hyaluronan (40), or in cell attachment/ inhibitory binding to cell surface hyaluronan (7), remains to be investigated.

It is not known whether the ability to form intermolecular disulfide bonds may be unique to the axolotl variant of PG-M/versican or if it may be a general feature of some specific splice variant. When the structure of bovine PG-M/versican was investigated in the transmission electron microscope with the glycerol spraying/rotary shadowing technique (43), a pronounced tendency for these PGs to self-aggregate was noted. By that technique, however, it could not be judged whether covalent intermolecular bonds were involved in stabilizing the aggregates. Accordingly, it remains to be studied whether PG-M/versican-like PGs exist in the form of large disulfide-stabilized PG complexes in other systems. Intriguingly, PG-1000/TAP-1, a large PG component of disulfide-stabilized complexes associated with the electric organ basement membranes of electric rays (44–45), might be related to the PG-M/versicans. Very large CS/DS/SPG, which like the HMPGs of the axolotl embryo elute in the void volume in Sepharose CL-2B chromatography under dissociative conditions, have previously been isolated from sea urchin embryos (46–47). However, it remains unknown whether the sea urchin PGs owe their large size to complex formation or to the presence of exceedingly large CS/DS side chains (47). Although the molecular identity of the large sea urchin PGs has not yet been reported, the dependence on these molecules for the migration of primary mesenchyme cells during sea urchin development (47–49) is intriguing.

Previously, bovine aggrecan was found to inhibit axolotl neural crest cell migration in vitro (6–7), whereas aggrecans, when coated onto microcarriers and introduced into the migratory pathways of axolotl embryos (50), affected neural crest cells differently depending on what species the PGs were obtained. The choice of model PG for the study of neural crest cell migration in the axolotl embryo may thus be crucial. Since ample purification of PGs from axolotl embryos is not readily feasible, it is presently difficult to directly address the question whether the large PG-M/versican complexes affect migration in the embryo. Such studies may have to await the identification of all components in the complexes so that these may be assembled from components expressed and purified from other sources or the development of gene targeting techniques in the axolotl.

Acknowledgments—We express our gratitude to Drs. Koji Kimata and Tamayuki Shinomura for their generous gift of chicken PG-M cDNA and to Sandra Bloomfield at the Indiana University Axolotl Colony, Bloomington, IN, for patiently providing embryos. We are grateful to Dr. Staffan Johansson for oligonucleotide synthesis, helpful discussion, and critical reading of the manuscript. We thank Inger Eriksson, Brit-Marie Fogelfolm, and Charlotte Fällström for technical assistance.

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doi: 10.1074/jbc.272.6.3246

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