Modulation of matrix metalloproteinase activity by a syndecan-like proteoglycan in the lactating mammary gland

4.1 Introduction

As indicated before, ECM plays a crucial role in maintaining the form and function of the lactating mammary gland. Remodeling of the mammary gland is associated with the degradation of ECM macromolecules. Degradation is caused by enzymes, the principal group being the matrix metalloproteinases (MMPs) [Alexander and Werb, 1991; Birkedel–Hansen et al., 1993; Mignatti et al., 1996], which are regulated either transcriptionally or by their specific endogenous inhibitors called the tissue inhibitors of metalloproteinases (TIMPs) [Matrisian, 1992]. At least three types of gelatinases are involved in rat mammary gland involution viz., 130K which is seen during the early involuting phase, 72/68K which is seen throughout all the stages of development and a 60K gelatinase which appears on the 5th day of involution [Talhouk et al., 1991; Ambili et al., 1997].

It was reported that the mammary epithelial function during involution is regulated by the coordinated expression of ECM–degrading proteinases and their inhibitors. Inhibitors of MMPs play a role in minimizing ECM remodeling and modulate cell morphology and function [Talhouk et al., 1992b]. Even though the 72/68K gelatinase is present in the mammary gland throughout the development, ECM remains intact and the glandular structure is maintained during the lactating stage suggesting the occurrence of possible mechanisms that control action of MMPs on ECM. Recent reports from our laboratory, indicated that in addition to the regulation by TIMPs, these gelatinases are inhibited by glycosaminoglycans (GAGs), particularly chondroitin sulfates (CS) [Ambili and Sudhakaran, 1999]. Although these in vitro studies do not indicate whether the GAGs modulate the
activity of these MMPs in intact mammary gland, investigations have shown reciprocal changes in GAGs and MMPs in mammary gland during development [Sudhakaran et al., 1999]. The results of experiments included in this chapter relate to the isolation and characterization of a proteoglycan from lactating mammary gland that inhibits MMP activity.

4.2 Materials and Methods

Proteoglycans were isolated from both involuting and lactating goat mammary glands. The effect of these proteoglycans on the activity of MMPs involved in mammary gland involution was assessed by multiwell zymography. The PG exhibiting maximum inhibitory effect was further purified by gel filtration over sepharose 4B – CL column and characterized by SDS – PAGE and autoradiography. The GAG chains of the PG were released by subjecting the PG to β - elimination and were separated by gel filtration over sepharose 4B – CL column. The GAGs were characterized by chondroitinase ABC/AC digestion and nitrous acid degradation. The core protein of the PG was separated by gel filtration over sephacryl S – 300 and characterized by SDS – PAGE, followed by autoradiography and HPLC. The binding characteristics of the PG were studied by gel filtration and dot blot assay. Antibody against the purified PG was raised in male albino rabbit and the distribution of the PG in intact tissue was studied by immunocytochemistry and the cellular source was determined by cell culture experiments. The changes in the level of the PG during mammary gland ontogeny were studied by ELISA and immunoblot. The details of these procedures have been given in chapter 2.

4.3 Results

4.3.1 Isolation of proteoglycans from goat mammary gland

To isolate proteoglycans, goat mammary glands from both involuting and lactating stages were extracted with 4M guanidine hydrochloride in presence of protease inhibitors and fractionated over a DEAE-cellulose column, using a gradient of 0.5M to 2.5M NaCl. Coelution of protein and uronic acid was observed in a single peak in the case of involuting mammary gland (Fig. 4.1A) and three different peaks viz., A, B and C (Fig. 4.1B) in the case of lactating tissue. While the fraction
Proteoglycans isolated from both lactating and involuting mammary glands, as described in the methods were subjected to ion-exchange chromatography over DEAE—cellulose column (28 x 2.8 cm), using a gradient of 0.5 M - 2.5 M NaCl in 6M urea / 50 mM Tris / HCl, pH 7.4. Coelution of proteins and uronic acid indicated the presence of a single peak in the case of involuting tissue (A); while three such uronic acid containing peaks (A, B, C) were obtained for the lactating goat mammary gland (B).

eluted at low salt concentrations (fraction A) was present in both lactating and involuting tissues, fractions eluted with high salt (fractions B and C) were observed only in lactating tissue. The uronic acid content in the extracts of lactating tissue was found to be greater compared to the involuting gland. The uronic acid...
containing fractions C, from the lactating mammary gland was found to represent nearly 15% of the total uronic acid containing fractions from that stage. The fractions which contain uronic acid from the involuting tissue and those from the lactating tissue were collected separately, dialysed, concentrated and tested for their ability to affect the activity of MMPs involved in mammary gland involution.

4.3.2 Effect of the PGs from goat mammary gland on MMPs involved in mammary gland remodeling

In order to test the effect of PGs isolated from goat mammary gland on the activity of MMP – 2 (72/68 K gelatinase) and on 60K gelatinase, which are involved in mammary gland involution, the activity of these enzymes in presence of PGs was assessed by multiwell zymography. The enzymes were preincubated with the PG – containing fractions, and multiwell zymography was done. Enzyme activity appeared as white zones in the gel; while the inhibition of enzyme activity appeared as increase in intensity of blue colour. This is because the enzyme is inhibited from degrading gelatin, which is copolymerised with the gel. Changes in the intensity of the multiwell zymograms were measured using a laser densitometer, and the results are given in Figs. 4.2 and 4.3.

Both the 68K and 60K gelatinases were found to be inhibited by the fractions containing proteoglycans from lactating and involuting stages. But, the proteoglycan eluted as peak C, from the lactating tissue, between 1.25 – 1.5M salt concentrations was found to inhibit the enzyme activity greater than the other fractions and hence this specific PG fraction was selected for further studies.

4.3.3 Purification of the MMP inhibitory proteoglycan from lactating goat mammary gland

Since the PG from the lactating mammary gland caused maximum inhibition of MMP – 2 activity, this was further purified by gel filtration over sepharose 4B – CL column and the elution profile is given in Fig. 4.4. The uronic acid containing material was coeluted with protein as a major peak; the peak fractions were pooled together, concentrated and subjected to SDS – PAGE analysis. On SDS – PAGE, this proteoglycan fraction appeared as a single diffuse band with an average molecular size of 190 kDa, as presented in the figure 4.5. The purity of this PG was further confirmed by autoradiography. For this, the PG isolated by gel filtration
Fig. 4.2  Effect of PGs isolated from goat mammary gland on 68K gelatinase

Equal amounts of (10µg uronic acid equivalent) of the three different peak fractions (A, B & C) isolated from the goat mammary gland were incubated with 30 µg of 68 K gelatinase and was subjected to zymography in multiwell plates as described in the methods. A representative multiwell zymogram corresponding to each sample is given as inset.

Fig. 4.3  Effect of PGs isolated from goat mammary gland on 60 K gelatinase

Equal amounts (10 µg uronic acid equivalent) of the three different peak fractions (A, B and C) isolated from the goat mammary gland were incubated with 30 µg of 60 K gelatinase and was subjected to zymography in multiwell plates as described in the methods. A representative multiwell zymogram corresponding to each sample is given as inset.
The proteoglycan isolated from the lactating mammary gland which caused maximum inhibition of gelatinase (C), was subjected to gel filtration over Sepharose 4 B – CL column (50 x 1.3 cm) in 0.05 M phosphate buffer / 0.15 M NaCl / pH 7.5. 2 ml fractions were collected and the amount of protein was measured at 280 nm and uronic acid at 530 nm. \( V_0 \) and \( V_t \) represent the elution volumes of dextran blue and potassium dichromate respectively.

The purified proteoglycan from goat mammary gland was subjected to SDS – PAGE on 7.5% gel and the bands were visualized by coomassie staining (A). \(^{125}\)I-labeled proteoglycan was also used for SDS – PAGE followed by autoradiography (B). Myosin (205 kDa), \( \beta \)-galactosidase (116 kDa) and bovine albumin (66 kDa) were used as markers.
Fig. 4.6  Effect of purified PG on the activity of 68 K gelatinase - concentration dependence

Different concentrations of the PG (0 to 12.5 µg uronic acid equivalent) were incubated with 30 µg of 68 K gelatinase and was subjected to zymography in multiwell plates as described in the methods. A representative multiwell zymogram corresponding to each sample is given as inset. Values given are the average of three experiments (±SD).

Fig. 4.7  Effect of purified PG on the activity of 60 K gelatinase - concentration dependence

Different concentrations of the PG (0 to 12.5 µg uronic acid equivalent) were incubated with 30 µg of 60 K gelatinase and was subjected to zymography in multiwell plates as described in the methods. A representative multiwell zymogram corresponding to each sample is given as inset. Values given are the average of three experiments (±SD).
was radioiodinated, electrophoresed and subjected to autoradiography. As is seen from Fig. 4.5 autoradiogram showed a single band indicating the absence of any contaminant proteins.

The inhibitory effect of the purified PG on MMPs was also studied by zymography in multiwell plates. The results are shown in Figs. 4.6 and 4.7. A progressive decrease in the activity of both 68K gelatinase (Fig. 4.6) and 60K gelatinase (Fig. 4.7), with increase in concentration of this PG was observed.

4.3.4 Characterization of the proteoglycan which inhibits MMPs

In order to characterize the purified PG, it was digested with chondroitinase ABC and AC and the digests were separated by paper chromatography. The absorption of unsaturated disaccharides liberated was measured at 232nm. The chromatographic profile is given as Fig. 4.8. The results of this experiment is given in Table 4.1. 50% of the uronic acid containing material was found to be susceptible to degradation by chondroitinase ABC and AC indicating the presence of chondroitin sulfates A and C in the sample. The PG was also subjected to nitrous acid degradation and the results are shown in Table 4.1. About 50% of the material was susceptible to nitrous acid degradation suggesting the presence of heparan sulfate.

<table>
<thead>
<tr>
<th>Mode of digestion</th>
<th>% digested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitinase ABC digestion</td>
<td>58.6</td>
</tr>
<tr>
<td>Chondroitinase AC digestion</td>
<td>55</td>
</tr>
<tr>
<td>Nitrous acid degradation</td>
<td>51</td>
</tr>
</tbody>
</table>

In order to further characterize the GAGs, the PG was subjected to β - elimination by treating with 0.15M NaOH and fractionated over sepharose 4B - CL and the results are shown in Fig. 4.9. Two uronic acid containing peaks of average molecular size 50 kDa and 12 kDa were found. Uronic acid containing fractions of these peaks were pooled separately, concentrated and subjected to further characterization by chondroitinase ABC/AC digestion and nitrous acid degradation. More than 80% of the large GAG fraction (50 kDa) was susceptible to chondroitinase ABC/AC digestion (Fig. 4.10 and Table 4.2). But the material
Fig. 4.8 Digestion of PG with Chondroitinase ABC/AC - paper chromatography

The purified PG (100 µg in 50 µL 0.25 M tris buffer) was incubated with 0.1 unit of chondroitinase ABC and 0.3 unit of chondroitinase AC for 30 min. at 37°C, paper chromatography was done as described in the methods. Chromatogram containing the disaccharides and the corresponding regions of the blank were cut into small fragments and dissolved in 0.01 N HCl. Centrifuged and O.D was measured at 232 nm.

Fig. 4.9 Separation of GAG chains by gel filtration over sepharose 4B-CL

Purified PG was subjected to β - elimination and the GAG chains were separated by gel filtration over sepharose 4B-CL column (50 x 1.3 cm) as described in the methods. 2 ml fractions were collected and uronic acid in each fraction was estimated. The proteoglycan before (O-O) and after (●-●) β - elimination. $V_0$, $V_{66}$, $V_{12}$ and $V_4$ represent the elution volume of dextran blue, bovine albumin, cytochrome C (MW - 12.4 kD) and potassium dichromate, respectively.
Fig. 4.10  Digestion of 50 kDa fraction with Chondroitinase ABC / AC - paper chromatography

The purified 50 kDa fraction (100 µg in 50 µl 0.25 M tris buffer) was incubated with 0.1 unit of chondroitinase ABC and 0.3 unit of chondroitinase AC for 30 min. at 37°C, paper chromatography was done as described in the methods. Chromatogram containing the disaccharides and the corresponding regions of the blank were cut into small fragments and dissolved in 0.01 N HCl. Centrifuged and O. D was measured at 232 nm.

Table 4.2
Digestion of the GAG chains by chondroitinase ABC / AC and nitrous acid

<table>
<thead>
<tr>
<th>Fraction digested</th>
<th>Mode of Digestion</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Chondroitinase ABC</td>
</tr>
<tr>
<td>21st</td>
<td>86.5%</td>
</tr>
<tr>
<td>25th</td>
<td>------</td>
</tr>
</tbody>
</table>

was resistant to nitrous acid degradation. More than 95% of the other peak was completely susceptible to nitrous acid degradation and resistant to chondroitinase digestion, indicating that the material was heparan sulfate. The results of these experiments are summarized in Table 4.2.

For the characterization of the core protein, iodinated proteoglycan was subjected to digestion with chondroitinase ABC and nitrous acid and gel filtration
over sephacryl S – 300. The protein was found to be eluted as a single peak (Fig. 4.11) SDS – PAGE and autoradiography of the protein showed the presence of a single band appearing at around 65 kDa as shown in Fig. 4.11. The purity of the core protein was also tested by HPLC and the results are given in Fig. 4.12. The HPLC profile of the core protein also indicated the presence of a single protein having a molecular size of around 65 kDa. As no low molecular weight peptides were detected in HPLC, it is unlikely that the core protein has been affected by nitrous acid. These results together indicate that a proteoglycan having an average molecular size of 190 kDa with a core protein of 65 kDa, possessing two or three HS (MW-12 kDa) and two CS chains (MW-50 kDa) is present in the lactating goat mammary gland.

**Fig. 4.11 Isolation and characterization of the proteoglycan core protein**

The purified proteoglycan was iodinated using $^{125}\text{I}$ - Nal and subjected to gel filtration over sephacryl S-300 column (54 x 1.3 cm) before (●●) and after (○○) treatment with chondroitinase ABC and nitrous acid. 2 ml fractions were collected and the radioactivity of each of the fractions was measured as cpm / ml. The SDS - PAGE of the purified core protein (A) (silver staining) and the autoradiogram (B) are given as inset. $V_0$, $V_{66}$, $V_{12}$ and $V_i$ represent the elution volume of dextran blue, bovine albumin, cytochrome C (MW - 12.4 kD) and potassium dichromate, respectively.
The purity of the core protein was checked by HPLC (SPD 10 A) analysis, using DIOL 150 (shimpack) column at a flow rate of 1 ml / min. O. D was measured at 280 nm. The peak obtained was compared with authentic standards.

4.3.5 Effect of the core protein and the GAG chains of the PG on MMP activity

To test whether the inhibitory effect of the PG is due to the presence of CS or HS side chains, zymographic assay of MMP – 2 (68 K gelatinase) and 60K gelatinase was done in the presence of the CS and HS chains separately and in combination and also in the presence of the core protein and the results are shown in Fig. 4.13 and 4.14. It was found that both CS and HS inhibit both MMP – 2 and 60K gelatinase of the mammary gland. It appears that HS can inhibit MMP activity more strongly than CS. The core protein did not inhibit the activity of MMPs. When both the CS and HS chains were simultaneously present in assay system, the enzyme activity was inhibited more strongly.
Fig. 4.13 Effect of the GAG chains and core protein of the PG on the activity of 68 K gelatinase

The GAG chains and core protein were incubated with 30 μg of 68 K gelatinase and subjected to zymography in multiwell plates. Enzyme activity in the absence of PG (1), in the presence of 5 μg uronic acid equivalent of CS (2), in the presence of 5 μg uronic acid equivalent of HS (3), equal amounts of both CS and HS (4) and 20 μg of the core protein (5) are given. A representative multiwell zymogram corresponding to each of the sample is given as inset. Values given are the average of three experiments (± SD).

Fig. 4.14 Effect of the GAG chains and core protein of the PG on the activity of 60 K gelatinase

The GAG chains and core protein were incubated with 30 μg of 60 K gelatinase and subjected to zymography in multiwell plates. Enzyme activity in the absence of PG (1), in the presence of 5 μg uronic acid equivalent of CS (2), in the presence of 5 μg uronic acid equivalent of HS (3), equal amounts of both CS and HS (4) and 20 μg of the core protein (5) are given. A representative multiwell zymogram corresponding to each of the sample is given as inset. Values given are the average of three experiments (± SD).
The effect of different concentrations of CS and HS chains on the activity of these MMPs was also studied and the results are given in Figs. 4.15 and 4.16. Increase in the extent of inhibition was observed with increase in concentration of GAG. 50% of inhibition of 68 K gelatinase was caused by 6 µg uronic acid equivalent of the HS chain and 8.5 µg uronic acid equivalent of CS. Also 50% inhibition of 60 K gelatinase was caused by 8.5 µg uronic acid equivalent of HS and 10 µg uronic acid equivalent of CS. Thus it is evident that in both cases HS inhibits MMP activity more strongly than CS.

Fig. 4.15 Inhibition of 68 K gelatinase activity by CS and HS chains - concentration dependence

The effect of different concentrations of CS (●) and HS (○) chains on the activity of 68 K gelatinase was studied by multiwell zymography as discussed in the methods. 0 to 12.5 µg uronic acid equivalent of CS and HS chains were pre incubated with 30 µg of the enzyme and carried out zymography. Values given are the average of three experiments (± SD).

4.3.6 Binding of PG, core protein and GAG chains to the 68K gelatinase

The binding of the PG, core protein and GAG chains separately and in combination to the gelatinases was studied by gel filtration of the MMP in the presence of PG, GAGs and core protein and the results are shown in Figs. 4.17 and 4.18. The MMP – 2 which was used for the experiment was collected as a peak fraction at the elution volume of the standard bovine albumin. But after incubation with PG, the MMP was eluted as a high molecular weight fraction, suggesting the binding of the PG with the enzyme. But no such shift in elution profile of the enzyme was observed on incubation with core protein, indicating that the binding of this PG to 68K gelatinase
The effect of different concentrations of CS and HS chains on the activity of 60 K gelatinase was studied by multiwell zymography as discussed in the methods. 0 to 12.5 µg uronic acid equivalent of CS and HS chains were preincubated with 30 µg of the enzyme and carried out zymography. Values given are the average of three experiments (± SD).

required the GAG chains. Results are shown in Fig. 4.17. This was further examined by incubation with both CS and HS chains and subjected to gel filtration. The results are shown in Fig. 4.18. There was a shift in the elution profile of the enzyme, on incubation with GAG chains, when given separately or in combination. These results suggest the binding of the PG to the gelatinase. These results further suggest that the PG is capable of binding to 68K gelatinase through the GAG side chains.

4.3.7 Binding of the PG to the ECM components, Col I and Col IV

In order to test whether the proteoglycan can bind directly to Col I and Col IV, dot blot assay and gel filtration were done. The binding of PG to Col I, an important constituent of the strona and Col IV, the major constituent of the BM was assessed. Different concentrations of $^{125}$[I] - PG were dotted on nitrocellulose discs precoated with Col I and Col IV. Gelatin was taken as the control. The results are given in Fig. 4.19. It is seen that, the binding of PG to Col I and Col IV increased with increase in concentration of the PG. Also, the binding of PG was more to Col I than to Col IV.
The binding of the PG and its core protein to 68 K gelatinase was checked by gel filtration over sephacryl S - 300 column (54 x 1.3 cm). The 68 K gelatinase was eluted from the column alone and in presence of PG and core protein. 2 ml fractions were collected and OD was measured at 280 nm. \( V_0 \) indicates the elution volume of bovine albumin (66 kDa). \( V_0 \) and \( V_1 \) represent the elution volumes of dextran blue and potassium dichromate respectively.
The binding of the CS and HS chains separately and in combination to 68 K gelatinase was checked by gel filtration over sephacryl S - 300 column (54 x 1.3 cm). The 68 K gelatinase was eluted from the column alone and in presence of CS and HS. 2 ml fractions were collected and OD was measured at 280 nm. \( V_0 \) and \( V_1 \) represent the elution volumes of dextran blue and potassium dichromate respectively.
Nitrocellulose discs were coated with 50 µg/ml each of Col I, Col IV and gelatin and different concentrations of the $^{125}$I- PG was added and incubated at 37°C for 1 hr. The discs were washed with TBS thrice and the radioactivity bound to the membrane was measured. Values given are the average of three experiments (± SD).

The binding of PG to collagen was further confirmed by gel filtration experiments. The gel filtration profile of Col I is found to be shifted towards the high molecular weight side indicating that the PG binds directly to Col I. But no such shift in pattern was observed in the case of Col IV and gelatin. The results of these experiments are given in Fig. 4.20.

4.3.8 The distribution of the proteoglycan in the mammary gland

Antibody against the PG appearing in lactating goat mammary gland was raised in male albino rabbit. The cross-reaction of the antibody with the PG was tested by immunoblot and the results are shown in Fig. 4.21. A single band was seen on immunoblotting of the total tissue homogenate, which is corresponding to that obtained for purified PG suggesting that the antobody was specific.
Fig. 4.20 Binding of $^{125}$I PG to Col I, Col IV and gelatin -
Gel filtration over sepharose 4B - CL

$^{125}$I Radioactivity (cpm / ml)

- Col I
- Col IV
- Gelatin

Fraction numbers

$^{125}$I PG was subjected to gel filtration over sepharose 4B - CL column (50 x 1.3 cm) in 0.05 M phosphate buffer / 0.15 M NaCl / pH 7.5. 2ml fractions were collected and the radioactivity of each fraction was measured as cpm / ml. Elution of PG alone (●●) and in combination (○○).
Antibody against PG, which was raised in male albino rabbit was tested by immunoblot. Tissue extract from goat lactating mammary gland was subjected to SDS-PAGE, transferred to nitrocellulose membrane by Western blotting and then probed with the antibody against PG followed by IgG-HRP as described in the methods. Myosin (205 kDa), β-galactosidase (116 kDa) and bovine albumin (66 kDa) were used as markers.

4.3.9 Immunocytochemical localization of PG in mammary gland

The localization of the PG in mammary gland during different stages of development was studied by immunocytochemistry and the results are shown in Fig. 4.22. The PG was found to be localized on the cell surface of epithelial cells in the acini and also in the extracellular matrix of the lactating mammary gland. But no positive staining was seen in the mammary glands from early and later stages of involution.

4.3.10 Production of $^{35}$[S] - PGs by mammary epithelial cells in culture

In order to identify the cellular source of the PG, mammary epithelial cells were isolated and maintained in culture in medium supplemented with $^{35}$[S] - Na$_2$SO$_4$ and the PGs synthesized and secreted into the medium and those appearing on the cell surface were quantitated by measuring the radioactivity and the results are given in Fig. 4.23. The cell layer was treated with trypsin to isolate PGs appearing on the cell surface. The amount of PGs appearing both at the cell surface and in the medium were found to increase with time in culture.
Legends to figure

Fig. 4.22 Immunocytochemical localization of the PG in the mammary gland

Tissue sections from lactating (B), early involuting (C) and late involuting (D) stages of goat mammary gland were stained with antibody against the PG and counterstained with Mayer's hematoxylin as described in the methods. Section of lactating gland untreated with primary antibody served as control (A). Straight arrows indicate positive staining on the acinar cells while curved arrows represent extracellular staining. Bar = 100 µm.
Rat mammary epithelial cells from the lactating stage were metabolically labeled with $^{35}$[S] - Na$_2$SO$_4$ (20 µCi / ml) for 24 hrs, the medium was separated (□) and the cell layer was digested with trypsin (■). Radioactivity (cpm / mg protein) associated with PGs was measured. Values given are the average of three experiments (± SD).

The presence of the particular PG in the medium and the trypsin digest was detected by immunoprecipitation. The immunoprecipitated sample was then subjected to SDS – PAGE along with the purified PG as marker. The gels were silver stained and destained. Each lane of the gel was cut into 1 cm pieces and the radioactivity was measured and the result is given in Fig. 4.24. The presence of a high molecular weight PG was observed both in the medium and in the trypsin digest.

4.3.11 Changes in the level of the PG during different stages of mammary gland development

In order to assess the changes in the level of this specific PG during mammary gland ontogeny, immunoblotting using specific antibody was done and the results are shown in Fig. 4.25. The virgin, lactating and involuting stages of rat and also lactating and involuting stages of the goat mammary gland were used for these experiments. The level of the PG was maximum in the lactating stage and only slightly detectable in the involuting stage of the goat tissue. The PG was also detected in the virgin and lactating rat mammary gland. The level of the PG was highest in the lactating stage and only faintly detectable in the involuting stage.
Fig. 4.24  Synthesis of the PGs by mammary epithelial cells in culture - 
Immunoprecipitation using specific antibody for PG

The medium (●●) and trypsin digest (○○) from the primary culture of mammary 
epithelial cells after 16 hrs were used for immunoprecipitation as described in the methods 
using specific antibody against the PG. The immunoprecipitated samples were then 
subjected to SDS - PAGE along with the purified PG as marker. The gels were cut into 1 
cm pieces, dissolved in $\text{H}_2\text{O}_2$ and the radioactivity was measured. 205, 116 and 66 represent 
the molecular weight of myosin, $\beta$ - galactosidase and bovine albumin respectively.

Fig. 4.25  Immunoblot of PG from different stages of goat 
and rat mammary gland

Plasma membrane extracts prepared from different stages of goat and rat mammary glands 
were subjected to SDS - PAGE and then transferred to nitrocellulose membrane by Western 
blotting. The PG was located using specific antibody. V - Virgin, L - Lactating and 
I - Involuting stages. Myosin (205 kDa) was selected as marker.
This was further confirmed by quantitating the PG in different stages of mammary gland development by ELISA, and the results are given in Fig. 4.26. The level of PG was maximum in the lactating stage, both in the case of goat and rat tissues. The level of PG in the involuting stage was nearly one third of that of the lactating tissue in both the rat and goat tissues.

**Fig. 4.26 Changes in the level of the PG during different developmental stages of the mammary gland**

Equal amounts of plasma membrane proteins (50 µg/ml) from different stages of goat (□) and rat (■) mammary gland were coated on multiwell plates and analysed by ELISA. OD units/mg protein was calculated. Values given are the average of 4 experiments (± SD).

4.3.12 Immunoblotting of the PG from the lactating goat mammary gland using antibody specific for syndecan – 1

Since the molecular size and nature of the proteoglycan, the GAG chains and core protein as well as the distribution pattern resemble the syndecan family of cell surface proteoglycans, it was further examined using antibodies against syndecan – 1. The total tissue homogenate of goat lactating mammary gland was immunoprecipitated using the antibody produced against the PG and was subjected to SDS – PAGE and transferred to a nitrocellulose membrane. It was then overlaid
with primary antibody against syndecan-1 and located by HRP conjugated secondary antibody. A single band was obtained as given in Fig. 4.27. This suggests that the particular proteoglycan appearing in lactating mammary gland is related to the syndecan family of cell surface proteoglycans.

**Fig. 4.27 Immunoblot of the PG from lactating goat mammary gland with anti syndecan -1 antibody**

![Immunoblot image]

The plasma membrane extract of mammary gland was immunoprecipitated using antibody specific for PG and after SDS-PAGE, the purified PG was transferred to nitrocellulose membrane by Western blotting and then located with anti syndecan -1 antibody. Myosin (205 kDa), β-galactosidase (116 kDa) and bovine albumin (66 kDa) were used as markers.

### 4.4 Discussion

In vitro studies using cultures of mammary epithelial cells maintained on isolated matrix components have demonstrated the role of ECM in regulating tissue specific functions such as milk protein production. Degradation of the ECM during the involutary phase of the gland is brought about by matrix metalloproteinases. Three different MMPs have been found to be involved in the degradation of ECM in rat mammary gland [Talhouk et. al., 1991; Ambili et al., 1997]. The TIMPs
have been shown to regulate the activity of these MMPs. Results presented above indicate that apart from inhibition by TIMP, a proteoglycan present in lactating mammary gland inhibits these MMPs. The PG which was isolated from lactating goat mammary gland inhibited MMP – 2 and 60K gelatinase activity.

This proteoglycan was found to be made up of both chondroitin sulfate and heparan sulfate side chains. It has an average molecular size of 190 kDa as evidenced by SDS – PAGE analysis. Nature of the GAG chains was established by digestion with chondroitinase ABC/AC and by nitrous acid degradation. 50% of the material was found to be CS as evidenced by susceptibility to chondroitinase digestion and the rest of the material was HS as evidenced by nitrous acid degradation. CS was of an average molecular size of 50 kDa and HS was of molecular size of 12 kDa. The core protein of this PG appeared to have a molecular size of about 65 kDa on SDS – PAGE, gel filtration and HPLC. From the molecular size of the proteoglycan core protein and the GAG chains, and their relative amounts, it appears to be a hybrid PG, consisting of two or three HS chains and two CS chains attached to the core protein. A proposed model of the PG based on these observations, is given in the Fig. 4.28.

**Fig. 4.28 Proposed model for the proteoglycan**

![Proposed model for the proteoglycan](image)

The proteoglycan was found to possess a molecular size of 190 kDa with a core protein of molecular size 65 kDa. It also carries two CS (●●●) (mol. wt. 50 kDa) and two HS (○○○) (mol. wt. 12 kDa) side chains.
The two major groups of cell surface PGs that contain heparan sulfate chains are members of the syndecan and glypican families. Most cells and tissues express at least one member of the syndecan family, most express multiple syndecans with characteristic expression patterns in individual cell types and tissues. Syndecan – 1 is expressed mainly by epithelial cells and the major cell surface proteoglycan of mammary epithelial cells have been identified as syndecan – 1. It was identified on mouse mammary epithelia [Saunders et al., 1989] and also on human mammary epithelia [Mali et al., 1990]. Syndecan – 1 localizes to basolateral surfaces on simple epithelial cells and surrounds stratified epithelial cells. It possesses two heparan sulfate and two chondroitin sulfate chains and the electrophoretic profile is similar to that of the proteoglycan mentioned here. The molecular size and nature of the side chains resemble the syndecan family of the proteoglycans. The cross reaction of the PG with the antibody for syndecan is also a strong evidence for its resemblance to syndecan 1. Similarity of the nature of the PG, that we discuss in this chapter to syndecan suggests the presence of a syndecan – like proteoglycan in the lactating stage of the mammary gland and its localization in the epithelial and myoepithelial cell lining of the acini. It is also found to be present on the extracellular space as well.

Cell culture experiments provide further evidence that the proteoglycan is synthesized by mammary epithelial cells and it appears on the cell surface and is also secreted into the medium. Thus it is evident that the PG is present both on the cell surface and in the extracellular space. The cell surface PGs, especially HSPGs which include the syndecan family also, bind to extracellular proteins and form signaling complexes with receptors. Also HSPGs immobilize proteins at the cell surface and mediate protein internalization. Binding of cell surface HSPGs to insoluble ligands, such as cells and ECM components, immobilizes the PG, interact with the actin cytoskeleton and form a variety of cell – cell and cell - matrix adhesions [Bernfield et al., 1999]. The cell surface HSPGs are coreceptors in most of these interactions. Syndecans play a role as matrix receptors, involved in the formation of focal adhesions and also in cell spreading.

Earlier reports indicated that the basement membrane remodeling activity in vivo is lowest during lactation, when the mammary gland is fully differentiated and highest during involution, when the gland ceases to produce milk [Talhouk et al., 1991]. It is also reported that in the mammary gland, a critical and co-ordinated balance between ECM – degrading proteinases and their inhibitors regulate tissue
development and function in vivo [Talhouk et al., 1992b]. It appears that the process of mammary gland involution slows down when the level of the inhibitors of MMP, especially TIMPs exceeded the level of MMPs involved in involution. As indicated before, the functional role of MMPs in the loss of tissue specific function in mammary gland was studied by maintaining high concentrations of TIMP in a normally involuting gland and the results indicated that TIMP causes delay in alveolar regression and maintains high level of $\beta$-casein. Thus it appears that the balance of ECM degrading proteinases and their inhibitors regulates the organization of the basement membrane and the tissue specific function of the mammary gland. During involution, an excess of active ECM – degrading proteinases and/or an absence of inhibitors disrupts cell – ECM interaction leading to the loss of a lactational phenotype [Talhouk et al., 1992b].

In addition to the regulation of MMPs in the lactating stage through TIMPs, our observations suggested the existence of another regulatory mechanism. GAGs particularly CS A and CS C inhibited the activity of MMPs indicating the possibility of the control of MMPs in vivo through proteoglycans [Ambili and Sudhakaran, 1999]. This is significant in the light of the developmental regulation of the GAGs in the mammary gland. The amount of CS and HS was found to be lowest during involution and highest during lactation [Sudhakaran et al., 1999] and this appears to be inversely related to the expression of MMPs. We now have asked whether the syndecan – like proteoglycan appearing in high concentrations in the lactating mammary gland exert an inhibitory effect on MMP activity. Data indicate that the PG and its side chains both CS and HS, inhibit the activity of 68K gelatinase and 60K gelatinase strongly. Reports from other laboratories have indicated the role of syndecan -1 in regulating MMP activity. Human myeloma cells which invade into type I collagen gels become non invasive when engineered to express syndecan – 1 in regulating the activity of 92K gelatinase (MMP – 9) [Kaushal et al., 1999]. The syndecans have also been shown to bind and modify the action of various growth factors, cytokines, proteases/antiproteases and ECM components [Subramanian et al., 1997]. Reciprocal relation between expression of MMP and syndecan – 1 was suggested by the observation that myeloma cells produce MMP – 9 and MMP – 2 and the accumulation of MMP – 9 protein is suppressed upon expression of syndecan – 1 [Kelly et al., 2000].
The inhibition of MMP by PG does not appear to be an artefact or due to the protein core of PG acting as a competitive substrate because (a) the concentration of the proteoglycan is several fold lower than that of the gelatin (2 mg/ml) which is used as the substrate. (b) The protein core of the PG in the absence of the GAG chains did not cause any inhibition whereas the GAG chains caused inhibition. These experiments do not clearly indicate how the PG inhibits MMP activity. It is possible that the PG may inhibit MMP activity by direct binding to the gelatinases as evidenced by the shift in the gel filtration pattern of MMP – 2 in presence of the PG. While the GAG chains inhibited the enzyme, the core protein was found to possess no inhibitory effect. Both the CS and HS chains exert their effect on the MMP, probably by binding to distinct sites as evidenced from a lack of any competitive effect and the gel filtration pattern. The other possibility is the binding of the PG to substrates thus blocking the enzyme cleavage sites. But the binding to Col IV, which is a major component of the basement membrane was very low.

The level of the PG during different stages of mammary gland ontogeny was found to be different indicating that its expression is developmentally regulated. Investigations from our laboratory showed quantitative and qualitative changes in the GAGs as the mammary gland undergoes developmental changes [Sudhakaran et al., 1999]. The level of GAGs was found to be highest in the lactating phase and lowest during the involutary phase, where the remodeling of the gland occurs. This observation correlates with the enhanced activity of MMPs during involution. But the enzyme activity was found to be inhibited during the lactating phase, where the gland is fully functional. Of the three different gelatinases involved in mammary gland involution, the 68K is found to be a constitutive enzyme which appears throughout development.

To maintain the cell - matrix ratio and the ECM in the mammary gland intact during lactation so that the expression of tissue specific phenotype is maintained, it is essential that the remodeling of ECM is limited during this stage. For this the activity of the constitutive MMP must be low or must be inhibited. The activity of TIMP is also minimum during this stage, suggesting the occurrence of other mechanisms in the regulation of the activity of the MMPs. The elevated level of this PG during the lactating phase and its inhibitory effect on MMP, contribute towards a novel regulatory mechanism (Fig. 4.29). It appears that proteoglycan, a major component of the matrix itself can regulate the degradation
1. During the lactating stage, the PGs are found to be present both on the cell surface and in the extracellular space. The PG may bind directly to the 68 K gelatinase which is present in the lactating tissue and prevent the enzyme from degrading the matrix and/or it may bind to collagen I which is the major component of the matrix thus protecting the matrix from the degradative enzymes.

2. During early involution as the level of the PG is decreased, the 68 K gelatinase becomes active and acts on the basement membrane. 130 K gelatinase also appears.

3. The late involution is characterised by the absence of inhibitory mechanisms and appearance of a 60 K gelatinase enzyme. The dissolution of basement membrane and stroma begins.
of the ECM by inhibiting the activity of MMPs, which are involved in ECM remodeling and therefore the regulation of the levels of PG in tissue can affect the molecular events involved in matrix remodeling. As this PG appears to resemble syndecan, this observation suggests a new role for syndecan family of PGs in the regulation of MMP activity and the control of cellular microenvironment whereby the cell - matrix interaction is influenced.