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# Extracellular Matrix-Associated Serine Protease Inhibitors ($M_r$ 33,000, 31,000, and 27,000) Are Single-Gene Products with Differential Glycosylation: cDNA Cloning of the 33-kDa Inhibitor Reveals Its Identity to Tissue Factor Pathway Inhibitor-2[1]

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**Recently, we reported the identification and partial characterization of three serine protease inhibitors ($M_r$ 33,000, 31,000, and 27,000) from the extracellular matrix (ECM) of human umbilical vein endothelial cells and skin cells. Here, we report that a full-length cDNA clone for the 33-kDa inhibitor from SV-40 transformed human skin fibroblasts (t12FB) is identical to a recombinant trypsin/tissue factor pathway inhibitor called TFPI-2 from placenta. By immunoblotting, the three inhibitors from ECM and cell lysates demonstrated cross-reactivity with an antiTFPI-2 IgG. To further elucidate how these inhibitors are related, pulse-chase labeling of t12FB with [$^{35}$S]methionine followed by immunoprecipitation with antiTFPI-2 IgG was performed on ECM and cytosolic proteins. A precursor–product relationship did not exist between the three inhibitors from ECM. In contrast, the various species of inhibitors from cytosolic fractions demonstrated a precursor–product relationship. Within the cytosolic fraction, 26-, 29-, and 30-kDa inhibitors were detected in the early chases (0 and 15 min) but they form precursors to the synthesis of the 33-kDa inhibitor which accumulated in the later chases (30 min to 1 h). When pulse-chase experiments were performed in the presence of tunicamycin, synthesis as well as sequestration of the three inhibitors into ECM was completely inhibited. In the presence of tunicamycin, the cells synthesized and sequestered a single 25.5-kDa inhibitor into ECM. Peak quantities of the 25.5-kDa inhibitor appeared in the ECM after 6 h chase while they were 1 h for the 27- and 31-kDa inhibitors and 3 h for the 33-kDa inhibitor. To further support that the three inhibitors are related but only differ in the extent of glycosylation, the 33-kDa inhibitor from the t12FB ECM was deglycosylated with *N*-glycosidase F and the products were identified by immunoblotting with anti-TFPI-2 IgG. The enzyme released the 31-, 27-, and 25.5-kDa inhibitors from the 33-kDa inhibitor. Collectively, these results demonstrate that the ECM-associated 33-, 31-, and 27-kDa inhibitors are biosynthetic products of a single gene with differential glycosylation. The 25.5-kDa inhibitor is unglycosylated, whereas 27- and 30- to 31-kDa inhibitors are partially glycosylated and the 33-kDa inhibitor is fully glycosylated. Inhibition of glycosylation significantly retarded the rate of secretion of the inhibitor but did not prevent its association with ECM. Quantitation of the inhibitors with cell-conditioned medium and ECM fractions reveals that 70–75%**

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**were ECM-associated and 25–30% cell-associated. None or very little of the inhibitors (0–2%) remained in a conditioned medium. Because they are primarily associated with ECM, the inhibitors may play a major role in ECM remodeling and turnover.** 

***Key Words:*** **extracellular matrix; serine protease inhibitors; fibroblasts; glycosylation; tissue factor pathway inhibitor-2.**

Several studies have demonstrated that the extracellular matrix (ECM)[3] functions as a reservoir of a variety of growth factors (1–4), growth factor binding proteins (5, 6), cytokines (7), proteases (8, 9), and protease inhibitors (10–14). It has been suggested that the sequestration of these growth regulatory molecules within the ECM protects them from rapid inactivation (11, 15). For example, plasminogen activator inhibitor-1 (PAI-1), which is rapidly inactivated in solution, remained active for more than 6 h after binding to ECM (15). Similarly, ECM-bound basic fibroblast growth factor and transforming growth factor-$\beta$ were stored in the matrix as active forms and released with full biological activity upon proteolysis of the matrix by plasmin and related serine proteases (16). Thus, cultured cells deposit matrices that are composed of not only structural macromolecules but also growth regulatory molecules which play important roles in matrix remodeling.

Previously, we reported the partial characterization of three novel serine protease inhibitors, related to the superfamily of Kunitz-type serine protease inhibitors, from the ECM of human skin cells (17, 18) and human umbilical vein endothelial cells (HUVEC) (19). These inhibitors have molecular sizes of 33, 31, and 27 kDa; inhibited the gelatinolysis and caseinolysis by trypsin, plasmin, $\alpha$-chymotrypsin, and pancreatic elastase but not by thrombin, thermolysin, and papain; and required acid for partial extraction from ECM. The synthesis of the three inhibitors was increased several-fold in cells after treatment with phorbol esters (17–19). The enzyme inhibitory profiles, molecular size, and the Kunitz classification of the inhibitors closely resembled those of a recombinant 32-kDa trypsin/tissue factor pathway inhibitor, designated TFPI-2 (20). Recently, a 29-kDa trypsin inhibitor was isolated from the conditioned medium of the human glioblastoma cell line T98G and cDNA cloning revealed that it is identical to TFPI-2 (21) and to a 30- to 36-kDa placental inhibitor designated placental protein 5 or PP5 (22).

This study was designed to investigate (i) if one of the ECM-associated inhibitors is TFPI-2 and (ii) if the three ECM-associated inhibitors are structurally related. A full-length cDNA clone for the 33-kDa inhibitor was characterized and found it to be identical to TFPI-2. The results suggest that the three inhibitors are biosynthetic products of a single gene with different levels of glycosylation. We also report that inhibition of glycosylation did not prevent the inhibitor from binding to ECM but significantly delayed its secretion and accumulation in the ECM.

## MATERIALS AND METHODS

*Reagents.* Phorbol 12-myristate 13-acetate (PMA) and tunicamycin were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Glycosidase F (PNGase F) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Protein A-Sepharose and heparin-Sepharose beads were purchased from LKB/Pharmacia Biotechnology Inc. (Uppsala, Sweden). RPMI, glutamine, fetal bovine serum (FBS), and trypsin-EDTA were purchased from Northwestern University Cancer Center, Tissue Culture Facility. 3′ RACE (rapid amplification of cDNA ends) kit was obtained from Life Technologies Inc. (Gaithersburg, MD). TA-cloning kit was purchased from In-Vitrogen (San Diego, CA). RNAZol was purchased from TEL-TEST Inc. (Friendswood, TX). ECL reagent system was a product of Amersham Life Sciences (England). [$^{35}$S]Methionine was purchased from ICN Radiochemicals (Irvine, CA). Recombinant TFPI-2 (rTFPI-2) was purified from baby hamster kidney cell culture medium as described (20). Antibodies against rTFPI-2 were generated in rabbits (23) and the IgG fraction was purified by protein A column chromatography.

*Quantitation of inhibitors in serum-free conditioned medium, cell lysate, and ECM of dermal fibroblasts.* SV-40 transformed human skin fibroblasts (t12FB) were cultured in RPMI containing 10% FBS, 50 $\mu$g penicillin, and 50 $\mu$g/ml streptomycin (24). Cells were grown to subconfluence in 100-mm tissue culture dishes or six-well Costar culture plates and the medium was replaced with serum-free medium supplemented with PMA, at a concentration of 100 ng/ml. After culturing the cells overnight, serum-free conditioned medium (CM) was collected and cells were lysed by incubation with lysis buffer containing PBS, 0.5% Triton X-100 for 20 min at 23°C. Inhibitors from CM and cell lysate proteins were concentrated by heparin-Sepharose chromatography (18). Heparin-Sepharose-bound proteins were extracted directly into 1.5× SDS–PAGE sample buffer. The ECM was prepared as described before (17, 19). Briefly, cell monolayers were washed three times with PBS and removed from culture dishes by incubation with lysis buffer as described above. The remaining ECM was washed three times with 20 mM Tris–HCl, pH 7.40, 100 mM NaCl, 0.1% (v/v) Tween 80 and five times with PBS. The ECM-bound inhibitors were extracted by incubation of the ECM with 1.5× SDS–PAGE sample buffer without dithiothreitol for 30 min at 23°C. In some experiments, PMA was added to cells already in incubation with tunicamycin for 8–14 h. After adding PMA, the cells were cultured overnight and CM, cell lysate proteins, and ECM-bound inhibitors were collected as described above. The inhibitors from CM, cell lysate, and ECM fractions were identified by immunoblotting with antiTFPI-2 IgG and quantitated by scanning the inhibitor bands with an imaging densitometric scanner (Model GS-670, Bio-Rad Laboratories, Richmond, CA).

*Immunoblotting.* CM, cell lysate, and ECM samples were boiled for 3 min and proteins were separated by SDS–PAGE using 12 or

[3] Abbreviations used: ECM, extracellular matrix; t12FB, SV-40 transformed human foreskin fibroblasts; PMA, phorbol-12-myristate 13-acetate; FBS, fetal bovine serum; CM, serum-free conditioned medium; TFPI-2, tissue factor pathway inhibitor-2; u-PA, urokinase type plasminogen activator; PP5, placental protein-5; PNGase F, *N*-glycosidase F; RACE, rapid amplification of cDNA ends; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; MMP, matrix metalloprotease.

15% polyacrylamide gels (25). After electrophoresis, proteins were electroblotted onto nitrocellulose membranes as described (26) and blocked with 4% nonfat dry milk in 10 mM Tris–HCl, 150 mM NaCl, pH 7.4, containing 0.1% Tween 20 (TTBS) for 2 h at 23°C. Then, the membranes were incubated for 2 h at 23°C or overnight at 4°C with normal rabbit serum or antiTFPI-2 IgG, diluted 1:3000 in TTBS containing 1% BSA. After several washes, the membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody, diluted 1:3000 in TTBS, 1% BSA. The immunoreactive proteins were identified using the enhanced chemiluminescence (ECL) reagent system, following the manufacturer's instructions.

*Pulse-chase metabolic labeling and immunoprecipitations.* t12FB were cultured to subconfluence in 100-mm tissue culture dishes. For these experiments, cell monolayers were incubated for 3 h with 100 ng/ml PMA in growth medium. The cells were rinsed three times with PBS and once with RPMI without glutamine/methionine/cysteine. In some experiments, PMA was added to cells already in incubation with 1 $\mu$g/ml tunicamycin for 12 h. Cell cultures were then labeled with 50 $\mu$Ci/ml [$^{35}$S]methionine (1153 Ci/mmol) for 20 min in RPMI without glutamine/methionine/cysteine. The cells were then chased for different time periods, as indicated in the figure legends, by culturing the cells in 10% FBS containing growth medium. After the chase, the medium was discarded and the cell layers were washed four times with PBS. Then, the cultures were lysed in 3 ml of lysis buffer containing PBS, 0.5% Triton X-100 for 20 min at 23°C. Triton X-100-soluble proteins were collected by centrifugation (20 min at 22,000*g* and 4°C). ECM-bound inhibitors were extracted as described previously (17, 18). Briefly, ECM-bound inhibitors were collected after incubation for 30 min at 23°C with 3 ml of a buffer containing 0.1 M glycine–HCl, pH 3.0. The extract was immediately neutralized with 1.5 M Tris–HCl, pH 8.8, and diluted with an equal volume of 30 mM Tris–HCl, pH 7.4, containing 1% Triton X-100. Both cell lysate and ECM-bound proteins were subjected to immunoprecipitation with normal rabbit serum or antiTFPI-2 IgG. Briefly, 1-ml aliquots of the radiolabeled cell lysate and ECM-associated proteins were incubated with 1 $\mu$l of antiTFPI-2 IgG or normal rabbit serum overnight at 4°C. The antigen–antibody complexes were captured onto protein A-Sepharose beads by end-over-end rotation of the reaction mixtures with beads for 1 h at 23°C. The beads were pelleted by centrifugation and washed three times with cold 15 mM Tris–HCl, pH 7.40, 0.5% Triton X-100, 0.15 M NaCl (buffer A) and two times with buffer A containing 10% KCl. Finally, protein A-Sepharose beads were washed twice with 15 mM Tris–HCl, pH 7.4, and resuspended in 70 $\mu$l of 1.5× SDS–PAGE sample buffer. The immunoprecipitates were boiled for 3 min, and proteins from aliquots of 30 $\mu$l were separated by SDS–PAGE using 12% polyacrylamide gels and identified by autoradiography. AntiTFPI-2 immunoreactive proteins were quantitated by scanning the autoradiograms with a densitometric scanner.

*Treatment of ECM-bound proteins and rTFPI-2 with N-glycosidase F.* rTFPI-2 (2 $\mu$g) or t12FB ECM-associated proteins (7 $\mu$g) were heat denatured in the presence of 0.2% SDS in 100 mM phosphate buffer, pH 7.0, 10 mM EDTA. The reaction mixtures were diluted with an appropriate volume of 1% Triton X-100 to make the concentration of this detergent fivefold higher than that of SDS. Then, 2 units of PNGase F was added to each reaction mixture to a final reaction mixture volume of 300 $\mu$l. The reaction mixtures were incubated at 37°C and aliquots (35 $\mu$l) were removed at time points indicated in the figure legends. The samples were made to 1× by diluting the samples with 5× SDS–PAGE sample buffer. Aliquots (20 $\mu$l) were separated on 12% polyacrylamide gels and products were identified by immunoblotting with antiTFPI-2 IgG.

*cDNA cloning and protein expression in Escherichia coli.* cDNA clones for the 33-kDa inhibitor were identified by 3′ RACE as described previously (27). Briefly, monolayers of t12FB were incubated with PMA for 6 h and total cellular RNA was isolated using guanidine thiocyanate (RNAZol) reagent (28). Adaptor primer (Life Technologies Inc.) was annealed to 1 $\mu$g of total cellular RNA and then reverse transcribed with SuperScript reverse transcriptase. After the reverse transcription reaction, RNA was degraded by treatment of the reaction mixture with RNase H and the remaining cDNA was mixed with a 100-$\mu$l PCR mixture containing 100 to 1000 nM 3′- and 5′-primers (Life Technologies Inc.). The 5′-primers consisted of either a degenerate oligonucleotide [5′-CAA(G)TTT(C)ATT(CA)ATT(CA)AAT(C)C(T)T-3′; amino acid sequence AEQEP is common to 33- and 31-kDa inhibitors, shared homology to TFPI-2] or a single oligonucleotide sequence [5′-CGATGCTGCTCAGCCACAC-3′; nt 104–123, amino acid sequence AAQEP of TFPI-2 shared homology to the 33- and 31-kDa inhibitors]. After incubation of the reaction mixture for 5 min at 94°C, a 30-cycle PCR was carried out with denaturing, annealing, and extension temperatures set at 92°C, 1 min; 52°C,1 min; and 72°C, 3 min, respectively. The amplified cDNA products were separated by electrophoresis on 1% agarose gels. cDNA products of approximately 1.2 kb were purified and subcloned into TA-vector. The recombinants were isolated, and plasmid DNA was purified and sequenced using an Applied Biosystems 373 A sequencer (29), as adopted by Applied Biosystems Inc. for fluorescent DNA sequencing. Cycle sequencing using the M13 universal primer in the presence of dye-conjugated dideoxyterminators was carried out according to the manufacturer's protocol.

A *Nde*I restriction endonuclease recognition sequence 5′-CATATG-3′ at the initiation site and a *Bam*HI recognition sequence 5′-GGATTC-3′ after the termination codon of the inhibitor gene was introduced by PCR. The PCR product was digested with *Nde*I and *Bam*HI restriction endonucleases and the inhibitor gene was cloned into the respective sites of the pRE expression vector (30). A recombinant was isolated in *E. coli* C600 lambda lysogen. *E. coli* strain MZ1 carrying the pRE1 recombinant plasmid containing the inhibitor gene was grown in 50 ml of LB medium containing ampicillin (50 $\mu$g/ml) at 32°C to an $A_{650}$ of 0.40. At this temperature, the synthesis of the recombinant inhibitor in the bacterial culture was uninduced. A 30-ml aliquot of this culture was saved for estimating the background synthesis of the recombinant inhibitor. The synthesis of the recombinant inhibitor was induced in the remainder of the culture by diluting with an equal volume of fresh LB, previously equilibrated to 50°C, and then immediately placing the culture in a 40°C waterbath shaker for 2 h. Cells were collected by centrifugation at 10,000*g* for 15 min and washed with 25 mM Tris–HCl buffer, pH 7.50 (buffer A). The cells were suspended in buffer A and broken by passage through a French pressure cell at 10,000 psi. The cell lysates were examined by SDS–PAGE to evaluate the level of expression of the 33-kDa inhibitor gene product. The extract from the heat-induced but not from the uninduced bacteria contained the 25-kDa recombinant protein. The cell lysate extract containing the recombinant inhibitor protein was centrifuged at 10,000*g* for 15 min and the pellet was washed three times with cold 15 mM Tris–HCl, pH 7.4, buffer and extracted with 15 mM Tris–HCl, pH 7.4, 6 M urea for 2 h at 23°C. The extract was centrifuged at 22,000*g* for 15 min and the supernatant was used as the source of the recombinant inhibitor protein.

## RESULTS

### *cDNA Cloning and Identification of the 33-kDa Inhibitor as TFPI-2*

Microsequencing analysis of the 33- and 31-kDa inhibitors revealed that the amino-terminal amino acid sequence of the 33-kDa inhibitor shared 70–90% homology to that of the mature TFPI-2 (18). The first five residues [AA(E)QEP] between the two matrix-associated inhibitors are identical and found in TFPI-2 at matching positions. To isolate cDNA clones for both

TAAACATTCTTAATATGTCATCTTGTTTGTCTTTATGGCTTATTTGCCTTTATG
GTTGTATCTGAAGAATAATATGACAGCATGAGGAAACAAATCATTGGTGATT
TATTCACCAGTTTTTATTAATACAAGTCACTTTTTCAAAAATTTGGATTTTTT
TATATATAACTAGCTGCTATTCAAATGTGAGTCTACCATTTTTAATTTATGGT
TCAACTGTTTGTGAGACTGAATTCTTGCAATGCATAAGATATAAAAGCAAAT
ATGACTCACTCAAAAAAAAAAAAAAAAA

**FIG. 1.** Nucleotide sequence (nucleotides 898 to 1189) of the complete 3′ untranslated region of the 33-kDa inhibitor cDNA. The new sequence that was not determined by Sprecher *et al.* (20) for TFPI-2 is underlined.

inhibitors, 3′ RACE was performed using a single oligonucleotide primer (to the sequence AAQEP, nt 104–123 of TFPI-2 cDNA) or a degenerate oligonucleotide primer (to the sequence AEQEP, sequence common to both inhibitors). Sixteen different cDNAs were selected and their 5′ nucleotide sequences were determined. The nucleotide sequences of all the cDNAs were identical with TFPI-2. The two strands of 1 of the 16 cDNA clones designated ECM-33SPI-2 which has a length of 941 nucleotides were completely sequenced. The nucleotide and deduced amino acid sequence of the ECM-33SPI-2 were identical to TFPI-2. Sprecher *et al.* (20) isolated a partial cDNA clone for the placental TFPI-2. The TFPI-2 cDNA was missing about 58 nucleotides at the 3′ end of the TFPI-2 mRNA. It was possible to determine the entire nucleotide sequence of the 3′ untranslated region extending into the poly(A) tail because of the application of 3′ RACE. The entire nucleotide sequence of the 3′ untranslated end of the TFPI-2/33-kDa mRNA was shown in Fig. 1.

### *33-, 31-, and 27-kDa Serine Protease Inhibitors Are Related to TFPI-2: A Majority of the Inhibitors Are Associated with ECM*

cDNA cloning studies revealed that the 33-kDa inhibitor is identical to TFPI-2 from placenta. To further understand how the three ECM-associated inhibitors are related to one another, immunoblotting analysis of the three inhibitors from t12FB was performed with a polyclonal antibody to TFPI-2. The experiment was also designed to determine the distribution of anti-TFPI-2 cross-reactive inhibitors in CM, cell, and ECM compartments. These antibodies do not cross-react with TFPI (W. Kisiel, unpublished data). As shown in Fig. 2B, antiTFPI-2 IgG intensely cross-reacts with the three inhibitors from cell lysate (lanes 2 and 6) and ECM (lanes 3 and 7) of both control (lanes 2 and 3) and PMA-treated (lanes 6 and 7) t12FB. The 33-kDa inhibitor was detected from the CM of PMA-treated t12FB (lane 5) but not in the control cells (lane 1). These three inhibitors from human foreskin fibroblasts, neonatal keratinocytes, and transformed dermal microvascular endothelial cells also cross-reacted with antiTFPI-2 IgG (Rao, unpublished data). Normal rabbit serum did not recognize the inhibitors from the ECM of control and PMA-treated t12FB (Fig. 2A, lanes 3 and 7, respectively). However, normal rabbit IgG faintly cross-reacted with one or two proteins between the molecular sizes 30 and 33 kDa in cytosolic proteins of control (Fig. 2A, lane 2) and PMA-treated t12FB (Fig. 2A, lane 6). At present, it is not clear if these proteins are the novel 33- and 31-kDa serine protease inhibitors or irrelevant proteins which might nonspecifically reacts with IgG because of overloading of these two lanes with cytosolic proteins. Serum-free growth medium did not contain any antiTFPI-2 IgG-reactive proteins (Fig. 2B, lane 4).

The immunoblotting technique using the ECL reagent system readily detected 0.1 to 0.3 ng of recombinant TFPI-2 (data not shown). Intensities of the 33-, 31-, and 27-kDa inhibitor bands were scanned in an imaging densitometric scanner, and their total (in terms of peak areas) in each compartment was determined and adjusted to the total volume of the sample. It was found that in both control and PMA-treated cells, 70–75% of the inhibitors were associated with ECM, 25–30% with cells, and only 0–2% with CM. These data confirm our previous observations which indicated that the inhibitors are primarily associated with ECM (17). By reverse zymography (17) we could not detect the three inhibitors from the cytosolic fraction of both control and PMA-treated t12FB. However, these cytosolic inhibitors were readily detected by anti-TFPI-2 antibody, suggesting that the identification of

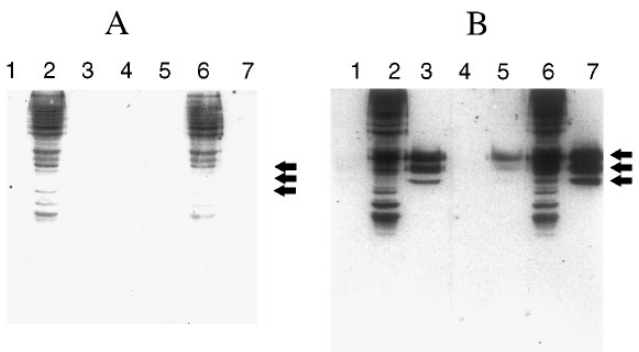


**FIG. 2.** Cross-reactivity of 33-, 31-, and 27-kDa inhibitors with anti-TFPI-2 IgG. CM, cell lysate, and ECM samples from control and PMA-treated cells were prepared as described under Materials and Methods and subjected to SDS–PAGE using 15% acrylamide slab gels. The proteins were electroblotted onto nitrocellulose membranes and subjected to immunoblotting with normal rabbit serum (A) and antiTFPI-2 IgG (B). In A and B, the samples are as follows: lanes 1, 2, and 3 are CM, cell lysate, and ECM, respectively, from control t12FB; lanes 5, 6, and 7 are CM, cell lysate, and ECM, respectively, from PMA-treated t12FB. In lane 4, serum-free medium was included as control. The 33-, 31-, and 27-kDa inhibitors are marked by arrowheads to the left of lane 7 in B.

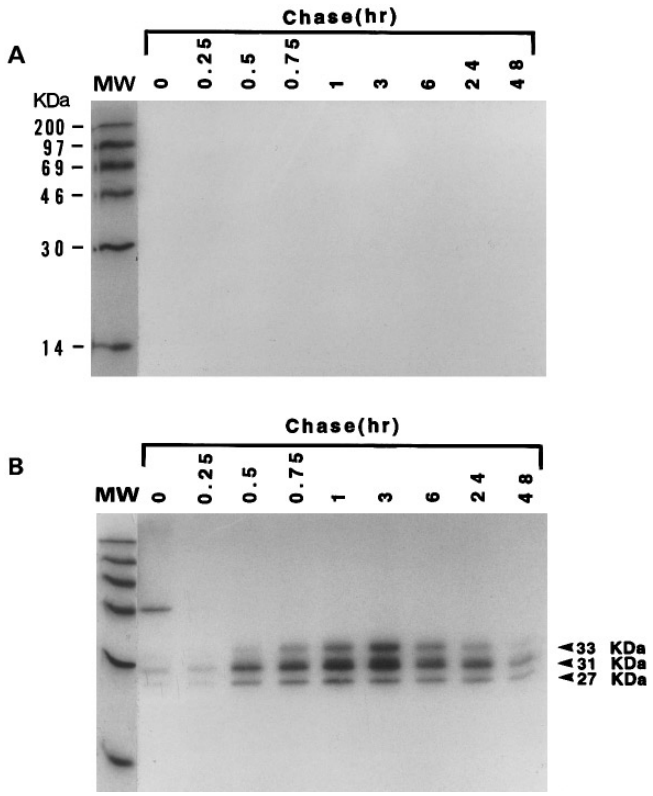


**FIG. 3.** Pulse-chase labeling of the inhibitors from the extracellular matrix (ECM) of PMA-treated t12 fibroblasts. Cells ($4 \times 10^6$ cells in 100-mm dishes) were pulsed for 20 min with [$^{35}$S]methionine and chased for intervals ranging from 0 to 48 h. The inhibitors from the ECM were immunoprecipitated with antiTFPI-2 IgG (B) or normal rabbit serum (A). In A and B, lane MW contains molecular weight standards and their sizes were represented in A only. The 33-, 30- to 31-, and 27-kDa inhibitors are marked to the right of lane 48 in B.

the inhibitors by immunoblotting is more sensitive than the function-based reverse zymography assay. Compared to untreated cells, cells treated with PMA contained threefold higher levels of the inhibitors, in both the cytosolic and ECM fractions (Fig. 2B, lanes 6 and 7 compared to lanes 2 and 3).

### *Within ECM, 33-, 31-, and 27-kDa Inhibitors Do Not Display a Precursor–Product Relationship*

As antiTFPI-2 IgG strongly cross-reacted with all three inhibitors, the antibody was used to investigate whether the smaller species of inhibitors are precursors to the synthesis of the larger 33-kDa inhibitor or degradation products from the 33-kDa inhibitor. PMA-treated t12FB were labeled for 20 min with radioactive methionine and chased with unlabeled amino acid for 0 to 48 h. ECM-bound proteins were extracted and subjected to immunoprecipitation with normal rabbit serum (Fig. 3A) or antiTFPI-2 IgG (Fig. 3B). The inhibitors were identified by autoradiography. The three inhibitors gradually accumulated in the ECM of cells subjected to chases from 0 to 3 h. The 31-kDa inhibitor appears to be a doublet of 30 and 31 kDa. Peak quantities of the 33-kDa inhibitor were observed in the ECM of cells subjected to 3 h chase. The highest levels of the 27-kDa inhibitor appeared in the ECM of cells subjected to 1 h chase, whereas the 30- to 31-kDa inhibitors were equally maximal after 1- and 3-h chases. After 3 h chase all three inhibitors gradually declined, indicating that they were degraded. Thus, the steady-state accumulation of the three inhibitors in ECM indicates that the smaller species of inhibitors are neither degradation products from the 33-kDa inhibitor nor precursors to the synthesis of the 33-kDa inhibitor. The inhibitors also displayed different half-lives, approximately 20–22 h for the 30- to 31-kDa doublet, 5 h for the 27-kDa inhibitor, and 3 h for the 33-kDa inhibitor.

### *Within the Cytoplasm 27- and 30- to 31-kDa Inhibitors Are Precursors to the Synthesis of the 33-kDa Inhibitor*

Radiolabeled cytosolic proteins were also immunoprecipitated with normal rabbit serum (Fig. 4A) or anti-TFPI-2 IgG (Fig. 4B). As shown in Fig. 4B, three species of inhibitors, $M_r$ 26,000, 29,000, and 31,000, were detected in immunoprecipitates from cells after 0 h chase. Compared to the cells from 0 h chase, the cells from 0.25 h chase contained 25–40% less of these inhibitors. In addition, this sample contains new higher molecular size species at $M_r$ 33 kDa as a faint band. When the chase was increased from 0.25 to 0.5 h, the 26-, 29-, and 31-kDa inhibitors were undetected or weakly detected. In these cells, the 33-kDa inhibitor was readily detectable. By 0.75 h chase, the cells contain 33-kDa species as the prominent inhibitor immunoprecipitable with antiTFPI-2 IgG. The 29- and 31-kDa inhibitors do not appear as clear bands but are detected as a faint broad smear. In cells from chases of 1, 3, and 6 h, the 33-kDa inhibitor was the major species immunoprecipitable with antiTFPI-2 IgG. Indeed, the immunoprecipitates from cells after 3 and 6 h chase contain only the 33-kDa inhibitor. The half-life of the cytosolic 33-kDa inhibitor was approximately 6 h. In addition to the four TFPI-2-related inhibitors, two proteins of $M_r$ 77,000 and 80,000 were detected in immunoprecipitates from 0 to 3 h chase (Fig. 4B). The 77- and 80-kDa proteins are also detected in immunoprecipitates with normal rabbit serum (Fig. 4A) and therefore represent nonspecific contaminants. These data clearly suggest that the smaller 26-, 29-, and 31-kDa inhibitors are acting as precursors to the synthesis of the 33-kDa inhibitor within the cytoplasm.

### *Tunicamycin Blocked the Synthesis of 27-, 30- to 31-, and 33-kDa Inhibitors*

Pulse-chase experiments with cell lysate inhibitors demonstrated a precursor–product relationship be-

tween the smaller 26-, 29-, and 31-kDa inhibitors and the mature 33-kDa inhibitor. To assess if N-linked glycosylation is involved in the formation of the 33-kDa inhibitor, pulse-chase labeling of cells was performed in the presence of tunicamycin, and antiTFPI-2 IgG-immunoprecipitable cytosolic inhibitors were identified by autoradiography. As shown in Fig. 5B, antiTFPI-2 IgG immunoprecipitated bands of 26-, 29-, and 31-kDa inhibitors from the cytosolic proteins of cells subjected to 0 h chase (lane 0). In contrast, cells in the presence of tunicamycin contained only a 25.5-kDa inhibitor (lane marked 0 + T). The 25.5-kDa inhibitor is the only species detected with antiTFPI-2 IgG from tunicamycin-treated cells subjected to 0.25, 0.5, 0.75, 1, and 3 h chase. It was barely detected in cells after 6 h chase (lane 6 + T) and not detected from cells after 24 h chase (lane 24 + T). Several proteins between the molecular sizes of 45,000 and 90,000 were also detected in all the immunoprecipitates with antiTFPI-2 IgG. These proteins were also detected with normal rabbit serum (Fig. 5A), indicating that they are nonspecific contaminants. These results suggest that tunicamycin blocked the synthesis of 27-, 29-, 30-, 31-, and 33-kDa inhibitors in PMA-treated t12FB.

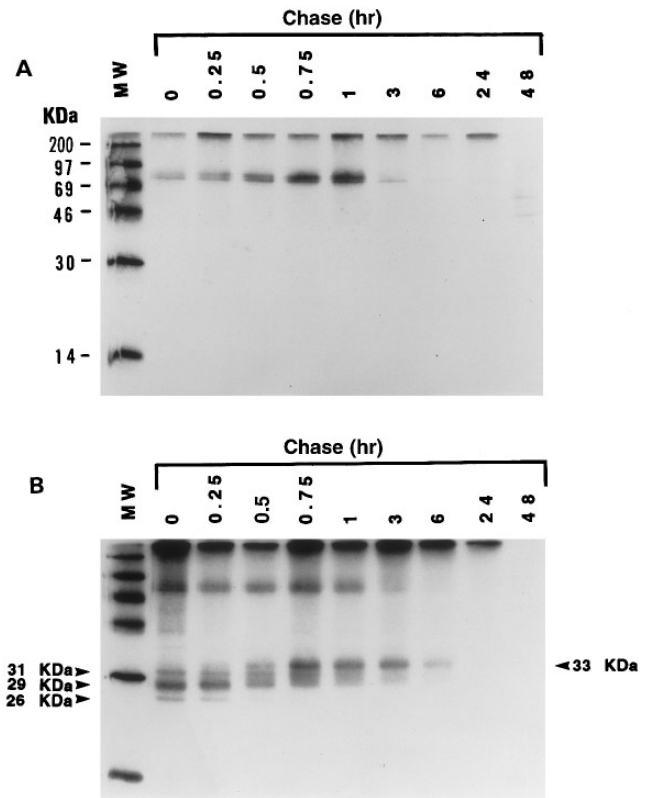


**FIG. 4.** Pulse-chase labeling of cytosolic inhibitors from PMA-treated t12 fibroblasts. Cells were pulsed for 20 min with [$^{35}$S]-methionine and chased for intervals ranging from 0 to 48 h. The inhibitors were immunoprecipitated with antiTFPI-2 IgG (B) or normal rabbit serum (A). In A and B, lane MW contains molecular weight standards and their sizes were represented in A only. The 26-, 29-, and 31-kDa inhibitors are marked to the left of lane MW in B. The location of the 33-kDa inhibitor was marked to the right of lane 48 in B.

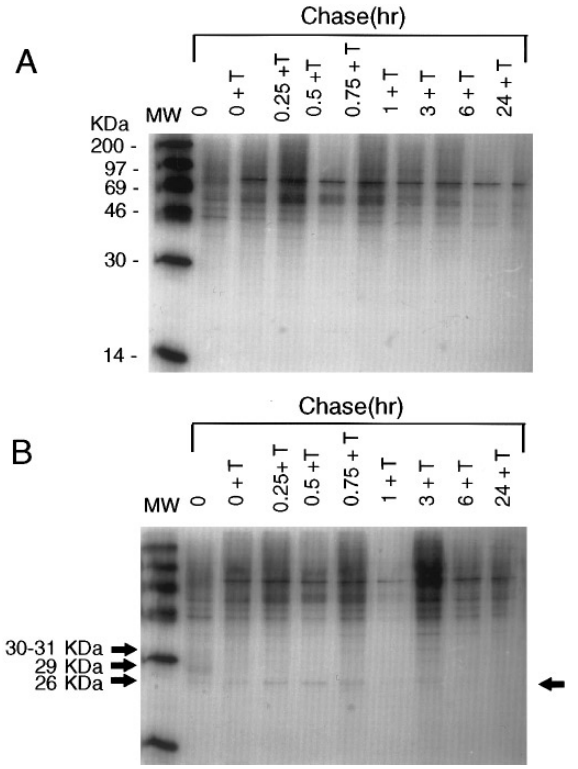


**FIG. 5.** Effect of tunicamycin on the pulse-chase labeling of cytosolic inhibitors from PMA-treated t12 fibroblasts. Prior to incubation of cells with PMA, the cells were incubated with 1 $\mu$g/ml of tunicamycin for 12 h. Cells were pulsed for 20 min with [$^{35}$S]methionine and chased for intervals ranging from 0 to 24 h. The cytosolic inhibitors were immunoprecipitated with antiTFPI-2 IgG (B) or normal rabbit serum (A). In A and B, the lane MW contains molecular weight standards and their sizes were represented in A only. Lane 0 in A and B contained immunoprecipitates from PMA-treated cells only. The samples from cells treated with tunicamycin were represented by the addition + T. The location of the 25.5-kDa inhibitor was marked to the right of lane 24 + T in B. The location of the 26- and 29 kDa inhibitors in lane 0 was also marked to the left of lane MW in B.

*Glycosylation Is Required for the Rapid Secretion of the Inhibitors But Is Not Required for the Binding of the Inhibitors to ECM*

The rate of secretion and the binding of the 25.5-kDa unglycosylated inhibitor to ECM were assessed using two different approaches. In the first approach, which determined the rate of secretion of the unglycosylated inhibitor, 0.1 M glycine–HCl, pH 3.0, extracts of radiolabeled ECM from pulse-chase experiment described above (Fig. 5) were subjected to immunoprecipitation with antiTFPI-2 IgG. The results are shown in Fig. 6. A single inhibitor species of 25.5 kDa was detected in

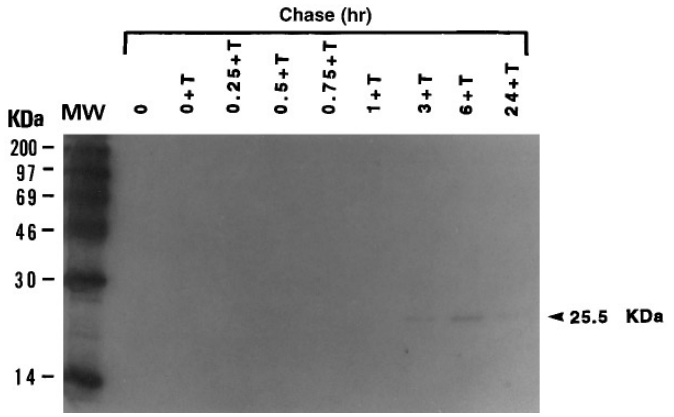


**FIG. 6.** Effect of tunicamycin on the pulse-chase labeling of inhibitors from the extracellular matrix (ECM) of PMA-treated t12 fibroblasts. Prior to incubation of cells with PMA, the cells were incubated with 1 $\mu$g/ml of tunicamycin for 12 h. Cells were pulsed for 20 min with [$^{35}$S]methionine and chased for intervals ranging from 0 to 24 h. The inhibitors from the ECM were immunoprecipitated with anti-TFPI-2 IgG or normal rabbit serum (not shown). Lane MW contains molecular weight standards. Lane 0 contained an immunoprecipitate from PMA-treated cells. The samples from cells treated with tunicamycin were represented by the addition + T. The 25.5-kDa inhibitor was marked to the right of lane 24 + T.

the ECM from tunicamycin-treated cells subjected to chases for 3 h (lane 3 + T), 6 h (lane 6 + T), and 24 h (lane 24 + T). The ECM of the cells from 6 h chase (lane 6 + T) contained the peak amounts of the 25.5-kDa inhibitor. In contrast, the peak amount for the 27- and 30- to 31-kDa inhibitors was at 1 h and for the 33-kDa inhibitor at 3 h (Fig. 3B). Thus, the rate of secretion of nonglycosylated inhibitor was one-sixth that of the 27- and 31-kDa inhibitors.

The second approach was the use of immunoblotting analysis to quantitate the nonglycosylated inhibitor sequestered in the ECM by t12FB in the presence of tunicamycin during an overnight culture. For this experiment, cells were plated in six-well culture plates, exposed to 0.5–3 $\mu$g/ml of tunicamycin for 8 h, and then treated with PMA for 16 h. ECM was extracted into 250 $\mu$l of 1.5× SDS–PAGE sample buffer and 30-$\mu$l aliquots were subjected to immunoblotting with anti-TFPI-2 IgG. The results are shown in Fig. 7. As shown before (Fig. 2B, lane 6), the ECM from control t12FB contains the 33-, 30- to 31-, and 27-kDa inhibitors (lane 2). In contrast, the ECM of cells treated with tunicamycin 0.5 $\mu$g/ml (lane 3), 1 $\mu$g/ml (lane 4), 2 $\mu$g/ml (lane 5), and 3 $\mu$g/ml (lane 6) contains a single inhibitor of approximately $M_r$ 25,500. The molecular size of the 25.5 inhibitor species was compared to that of a recombinant inhibitor from *E. coli* which lacked glycosylation (lane 1). The inhibitor from cells treated with 3 $\mu$g/ml tunicamycin comigrated with the *E. coli* recombinant inhibitor (lane 1). The *E. coli* recombinant inhibitor contained an extra glycine at its amino-terminal end for protein stability (31). Quantitation of the 25- to 26-kDa inhibitor species (lanes 3 to 6) from tunicamycin-treated cells reveals that it constitutes 40 to 50% of all the four inhibitors of the control cells (lane 2). The 25.5-kDa inhibitor was not detected in the CM or cell lysates from tunicamycin-treated cells. In comparison, the 33-, 31-, and 27-kDa inhibitors were detected in the cytosol and CM of PMA-treated cells (data not shown). When combining the 25.5-kDa inhibitor in the cytosolic, CM, and ECM fractions from tunicamycin-treated cells, it represented 25 to 30% of the three glycosylated inhibitors from control cells.

### *The 33-kDa Inhibitor Is Deglycosylated to Produce the Unglycosylated 25.5-kDa Inhibitor and Partially Glycosylated 27- and 31-kDa Inhibitors*

The experiments described above with and without tunicamycin revealed that the 33-kDa inhibitor is the final product of the addition of N-linked oligosaccharides to an unglycosylated inhibitor protein of approximately 25.5 kDa. The intermediates in this conversion process appears to be 27- and 30- to 31-kDa inhibitors. To investigate if the 33-kDa inhibitor is the N-glycosylation product of the lower molecular size 25.5-, 27-, and 31-kDa inhibitors, the 33-kDa inhibitors from PMA-treated t12FB ECM and rTFPI-2 were deglycosylated with *N*-glycosidase F and the products were identified by immunoblotting with antiTFPI-2 IgG. The results are shown in Fig. 8. rTFPI-2 also contains a minor but detectable 31-kDa inhibitor as the contaminant (Fig. 8A, lane 1). Nonetheless, degradation of rTFPI-2 with

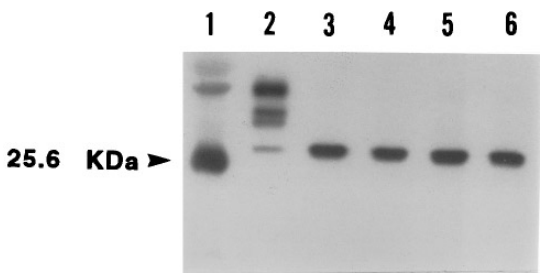


**FIG. 7.** Extracellular matrix (ECM) association of the unglycosylated inhibitor. Cells ($0.2 \times 10^6$) were plated in six-well Costar plates. Prior to the addition of PMA to the confluent cultures, the cells were treated with the following concentrations of tunicamycin for 8 h in growth medium: lane 2, 0 $\mu$g/ml; lane 3, 0.5 $\mu$g/ml; lane 4, 1 $\mu$g/ml; lane 5, 2 $\mu$g/ml; and lane 6, 3 $\mu$g/ml. Tunicamycin was kept during the subsequent incubation of the cells with PMA for an additional 16 h. The inhibitors from CM, cell lysates, and ECM were prepared as described under Materials and Methods. An equal amount of the sample from each treatment was separated by SDS–PAGE using 12% polyacrylamide gels and immunoblotted with antiTFPI-2 IgG. Immunoblots for the inhibitors from CM and cell lysates were not shown. An immunoblot of the inhibitors from ECM fraction was shown. Lane 1 contains recombinant inhibitor protein from *E. coli* (25.6 kDa) as a molecular size marker for the unglycosylated inhibitor.

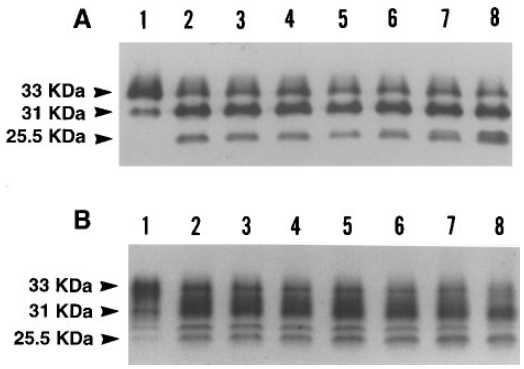


**FIG. 8.** Deglycosylation of rTFPI-2 and the 33-kDa inhibitor by *N*-glycosidase F. Two micrograms of rTFPI-2 (A) and 7 $\mu$g of a crude mixture of t12FB ECM proteins (B) were treated with *N*-glycosidase F for intervals ranging from 0 min to 16 h. The reaction was stopped by the addition of SDS–PAGE sample buffer and stored at −20°C until analysis. Samples were separated by SDS–PAGE using 12% polyacrylamide gels under nonreducing conditions and subjected to immunoblotting with antiTFPI-2 IgG. In A and B, the lanes represent the time of incubation of the proteins with the enzyme: lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; lane 4, 45 min; lane 5, 1 h; lane 6, 3 h; lane 7, 6 h; and lane 8, 16 h.

*N*-glycosidase F produced 31-kDa and in particular 25.5-kDa inhibitors which accumulated as the deglycosylation of the inhibitor progressed from 15 min through 16 h (Fig. 8A, lanes 2–8). Similarly, *N*-glycosidase F converted the 33-kDa inhibitor from ECM into three major species of $M_r$ 31, 000, 27, 000, and 25, 500 (Fig. 8B, lanes 2–8). In the sample incubated for 16 h, 95% of the 33-kDa inhibitor is converted into 31- and 25.5-kDa inhibitors (lane 8). These data suggest that the removal of N-linked sugars from the 33-kDa inhibitor produces 31-, 27-, and 25.5-kDa inhibitors, suggesting that the three inhibitors are related but only differ in the extent of their glycosylation.

## DISCUSSION

We report that the 33-, 30- to 31-, and 27-kDa ECM-associated serine protease inhibitors are biosynthetic products of a single gene with different degrees of glycosylation. All three inhibitors are glycosylated products of a nascent inhibitor protein of approximately 25.5 kDa. Significantly, the different glycosylation forms of this inhibitor are readily sequestered into ECM and function as independent inhibitors. We also report that blocking the inhibitor from glycosylation significantly delayed its secretion but did not prevent the inhibitor from binding to ECM. cDNA cloning revealed that the 33-kDa inhibitor is identical to TFPI-2.

Three lines of evidence support that the 33-, 30- to 31-, and 27-kDa inhibitors are products of a single gene with different degrees of glycosylation. The first line of evidence comes from pulse-chase kinetic analysis of the inhibitors within the cytosolic compartment. Within the cytosol, but not in the ECM, the lower molecular size inhibitors are synthesized first and they form precursors to the fully mature 33-kDa inhibitor. The second line of evidence comes from pulse-chase kinetic experiments in the presence of tunicamycin. Tunicamycin effectively inhibits N-linked glycosylation of membrane and secreted glycoproteins by blocking the transfer of *N*-acetylglucosamine to the dolichyl phosphate (32). In the presence of tunicamycin, a single 25.5-kDa inhibitor is synthesized and incorporated into ECM. The higher molecular size inhibitors were neither synthesized nor sequestered into ECM. This observation clearly demonstrate that the 27-, 30- to 31-, and 33-kDa inhibitors are biosynthetic products involving the addition of N-linked glycosylation of an unglycosylated 25.5-kDa inhibitor protein. The third line of evidence is obtained from experiments using *N*-glycosidase F to convert rTFPI-2 or the 33-kDa inhibitor from t12FB ECM into smaller inhibitors. The enzyme cleaves all types of asparagine-bound N-glycans when the amino group, as well as the carboxyl group, is present in a peptide linkage (33). *N*-Glycosidase F generated 31- and 25.5-kDa inhibitors from both rTFPI-2 and the 33-kDa inhibitor from t12FB ECM.

Based on these three lines of evidence, we hypothesize the following sequential events leading to the biosynthesis of the newly identified ECM-associated 33-, 30- to 31-, and 27-kDa serine protease inhibitors (Fig. 9). A nascent 25.5-kDa inhibitor undergoes posttranslational modifications involving N-linked glycosylation to synthesize the partially glycosylated 27- and 30- to 31-kDa inhibitors. Glycosylation of the nascent inhibitor occurs in the intracellular component, but most likely in the endoplasmic reticulum as usual. A certain proportion of these partially glycosylated inhibitor proteins are secreted and sequestered into ECM. The remaining pool of the intracellular 30- to 31-kDa inhibitors undergoes further glycosylation to produce the fully glycosylated 33-kDa inhibitor which is also sequestered into ECM. Certain batches of the t12FB ECM, in addition to the partially and fully glycosylated inhibitor species, also contain a 25- to 26-kDa inhibitor protein (Fig. 8, lane 1). Thus, a minor proportion of the unglycosylated inhibitor is also secreted and sequestered into ECM.

A most noticeable effect of tunicamycin on the biosynthesis of the ECM-associated inhibitors is that the drug significantly delayed the rate of secretion of the unglycosylated inhibitor into ECM. Unlike the partially glycosylated 27- and 30- to 31-kDa inhibitors which accumulated in the ECM in peak amounts 1 h after their synthesis, the unglycosylated 25.5-kDa inhibitor was detected in peak amounts after 6 h of synthesis. Thus, the rate of secretion of the unglycosylated inhibitor is one-sixth that of the partially glycosylated

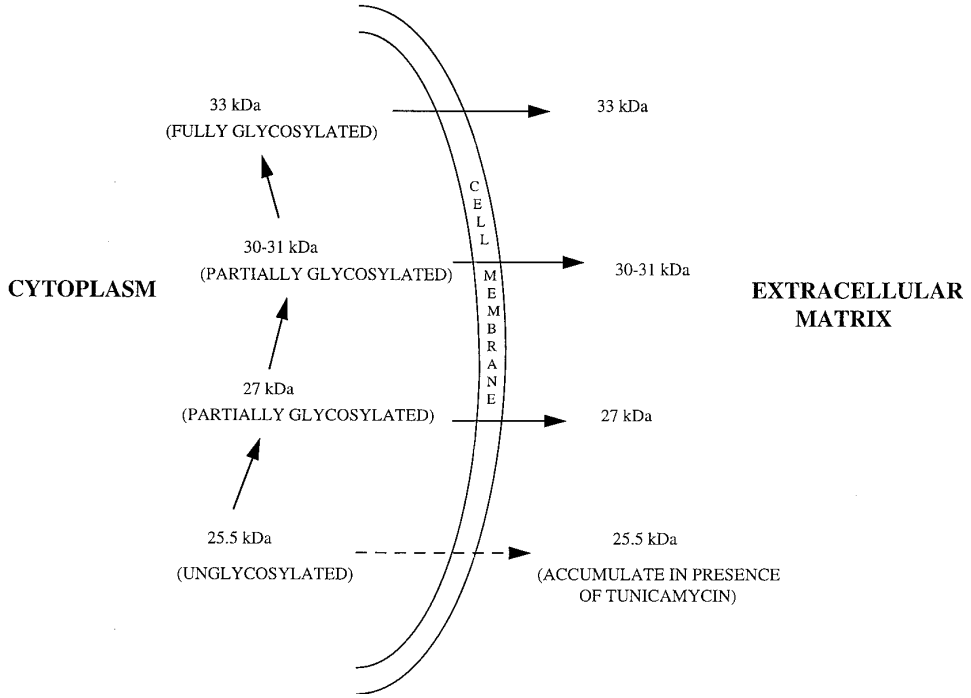


**FIG. 9.** Schematic representation of the biosynthesis of ECM-associated 33-, 30- to 31-, and 27-kDa serine protease inhibitors.

inhibitors. A similar inhibition in the secretion of several ECM proteins was also observed. In murine teratocarcinoma-derived parietal yolk sac cells, tunicamycin markedly delayed the secretion of laminin (34). In human fibroblast cultures, the cells treated with tunicamycin secreted procollagenase and fibronectin (35) at a rate fivefold less than that of control cells. In addition, the number of oligosaccharide moieties per heavy chain has been shown to influence the rate of immunoglobulin secretion (36). Thus, glycosylation appears to be required for the rapid secretion of the 33-, 31-, and 27-kDa inhibitors also. Apart from this role, glycosylation does not appear to be necessary for the binding of the inhibitors to ECM (components) and in their antiprotease activity. By reverse zymography, we observed that 31- and 27-kDa inhibitors derived from the 33-kDa inhibitor retained antiprotease activity (Rao, unpublished data). Nevertheless, reverse zymography is a qualitative assay and, therefore, subtle differences might still exist in the apparent inhibition constants of the unglycosylated, partially glycosylated, and fully glycosylated inhibitor species. Similarly, the various glycosylation forms of inhibitors might be binding to ECM (components) with different affinities.

The nucleotide and the deduced amino acid sequence of the 33-kDa inhibitor cDNA from t12FB were identical to those of TFPI-2 from placenta. The mature protein (rTFPI-2/33-, 31-, and 27-kDa matrix-associated inhibitors) contains 213 amino acids with 18 cysteine residues and two consensus N-linked glycosylation sites (Ref. 20 and this investigation). Although 16 independent cDNA clones were sequenced, a cDNA clone different from TFPI-2 was not found. These observations and those derived from pulse-chase kinetic studies with and without tunicamycin and *N*-glycosidase F treatment experiments clearly suggest that the smaller 30- to 31- and 27-kDa matrix-associated inhibitors are underglycosylated forms of the mature 33-kDa inhibitor. Interestingly, the various species of inhibitors are found in the ECM and all are functional by reverse zymography (16–18).

Distribution studies suggest that the ECM from various cell types including normal human keratinocytes, foreskin fibroblasts, and dermal microvascular endothelial cells contained more than 70% of the 33-, 31-, and 27-kDa inhibitors. The inhibitors were not found in their conditioned media (Rao, unpublished data). The specific association of the three novel serine protease inhibitors with ECM is of significant physiological importance. ECM remodeling is critical to a number of physiological and pathological processes which include fertilization, embryonic development, cell migration, angiogenesis, wound healing, and tumor invasion and metastasis (37). Recently, Petersen *et al.* measured the inhibitory properties of the human rTFPI-2/PP5/ECM-associated 33-kDa serine protease inhibitor (from this investigation) and found that it is a potent inhibitor of factor V11a-tissue factor, plasmin, trypsin, chymotrypsin, cathepsin G, plasma kallikrein, and factor XIa. The inhibitor was not active against the amidolytic activi-

ties of glandular kallikrein, urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator, leukocyte elastase, and factor Xa (38). By reverse zymography, we reported that the ECM-associated inhibitors inhibited the gelatinolytic and caseinolytic activities of plasmin, trypsin, chymotrypsin, and pancreatic elastase (17, 19). The inhibition of plasmin by the rTFPI-2/33-kDa inhibitor suggests a major role for this inhibitor in u-PA-mediated ECM degradation and turnover. A number of studies suggest that plasminogen-dependent degradation of ECM by tumor cells is responsible to a large extent for their successful invasion through human amniotic and matrigel membranes (39–42) and the formation of *in vivo* metastasis (43).

When plasmin is generated on ECM and on cell-surfaces, it is resistant to inhibition by serum plasmin inhibitors, namely $\alpha$2-antiplasmin and $\alpha$2-macroglobulin (44–48). We have found that the rTFPI-2/33 kDa ECM-associated inhibitor effectively inhibits the amidolytic activity of plasmin (u-PA + plasminogen) on ECM and fibrosarcoma HT-1080 cells in nanomolar concentrations (Rao *et al.*, unpublished data).

Plasmin is a broad-spectrum serine protease known to degrade glycoprotein constituents of basement membranes and connective tissue stroma (49). In addition to degrading ECM components and fibrin, plasmin has been suggested to be the physiological activator of promatrix metalloprotease (MMP)-1 (50), proMMP-3 (51), and latent transforming growth factor-$\beta$ (52). Thus, plasmin commands a central role in the degradation and turnover of ECM. We also found that the rTFPI-2/33 kDa inhibitor effectively inhibited the activation of proMMP-1 by soluble plasmin (uPA + plasminogen) as well as by plasmin bound to the cell surface of HT-1080 fibrosarcoma cells and to a fibroblast derived ECM-surface (Rao *et al.*, unpublished data). As the newly characterized rTFPI-2/33-kDa inhibitor is a potent inhibitor of cell- and ECM-bound plasmin, it is likely that this family of inhibitors consisting of the 31-, 30-, and 27-kDa ECM-associated serine protease inhibitors plays a major role in ECM degradation during tumor cell invasion and metastasis, wound healing, and angiogenesis.

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