Expression and Purification of Human Stromelysin 1 and 3 from Baculovirus-Infected Insect Cells

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INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of endopeptidases that may play a major role in matrix remodeling. Their proteolytic activity is tightly controlled by the secretion of the enzymes as latent zymogens and by the action of tissue inhibitors of metalloproteinases (TIMPs) (1, 2). An imbalance in the equilibrium between proteases and inhibitors leads to the net extracellular matrix degradation required for angiogenesis and metastasis (3–5). Within the MMP family, stromelysins and gelatinases have been specially incriminated in basement membrane degradation of the tumor–host interface, where key events in invasion occur (6). Stromelysins, which are characterized by their relatively broad substrate specificity, have four protein domains: (i) a leader sequence or predomain; (ii) a prodomain that is removed when the enzymes are activated; (iii) a catalytic domain, containing the zinc-binding site, and (iv) a carboxyl domain with sequence similarity to hemopexin (7).

Although particular attention has been focused on the stromelysin 1 (ST1) role in cancer invasion and metastasis, ST1 is not widely expressed normally, but can be readily induced by a number of factors, including cytokines, tumor promoters, and growth factors (8, 9). A high level of ST1 mRNA transcripts has been correlated with increased local invasiveness in head and 

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Abbreviations used: α2-M, α2-macroglobulin; FCS, fetal calf serum; hpi, hours postinfection; MMP, matrix metalloproteinase; m.o.i., multiplicity of infection; pfu, plaque-forming unit; ST, stromelysin; TIMP, tissue inhibitor of metalloproteinase.
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...expression of ST1 has been described for gastric carcinoma tissues (11) and its presence has been implicated in the invasive phenotype of pancreatic and ampullary cancer (12). ST1 is produced mainly by the fibroblasts of the tumor stroma adjacent to areas of basement membrane disruption (13, 14). The fact that transgenic mice expressing an active form of this enzyme developed mammary tumors has led to the suggestion that ST1 might act as an oncogene (15).

Stromelysin 3 (ST3) is an interesting member of the MMP family, which may play a role in the progression of human carcinomas. It was first cloned by differential screening of a cDNA library prepared from a human breast carcinoma subtracted with a breast fibroadenoma (16). Its deduced amino acid sequence has the characteristic structure of the matrix metalloproteinases, a leader peptide, prodomain, and catalytic and hemopexin-like domains. By in situ hybridization it was found that ST3 RNA is expressed in fibroblastic cells immediately surrounding the neoplastic cells, both in invasive and in situ breast carcinomas and in metastatic lymph nodes (16). The ST3 gene, which is generally not expressed at significant levels in benign breast tumors, has been found to be expressed in all invasive breast carcinomas tested so far (16–18). The increased expression of ST3 was observed in other types of human carcinomas, head and neck squamous cell carcinoma (18, 19) and skin and colon cancer (18, 20), and the highest ST3 RNA levels are observed in tumors that exhibit high local invasiveness. ST3 expression in stromal fibroblasts may be under the control of factors produced by cancer cells during carcinoma progression and may thus represent a stroma-derived factor necessary for the advance of epithelial malignancies. In fact, recent studies in nude mice showed that ST3 promotes tumor incidence (21).

Understanding the mechanisms by which MMP family members are activated and degrade substrates will enhance the pharmacological intervention of cancer. The development of methods allowing an effective production of active MMPs could be a valuable source of proteins to undertake further functional studies. The baculovirus expression system is an attractive alternative for high-level expression of recombinant proteins in a complex eukaryotic host system (22). This system has been previously employed by us to express matrilysin, a member of the MMP family, and the activity of the purified recombinant protein was shown to be analogous to that of the native enzyme (23). In the present paper, the usefulness of the baculovirus expression system to express active forms of ST1 and ST3 is discussed.

MATERIALS AND METHODS

Cell culture. Spodoptera frugiperda (Sf9) insect cells were obtained from Invitrogen (San Diego, CA). Sf9 cells were grown at 28°C as monolayer or suspension cultures in TNM-FH medium (JRH Biosciences, Sussex, UK), supplemented with 10% FCS (JRH Biosciences) as described (24).

Transfer vectors construction. The complete ST1 cDNA was amplified in three overlapping fragments from ASJ-4 human fibroblasts kindly provided by Dov Zipori (Rehovot, Israel) and cloned into the BamHI site of pBacPAK1. The complete ST3 cDNA was obtained from the plasmid pREP9-ST3 (25) kindly provided by Dr. S. Weiss (Michigan). After digestion with SflI and KpnI, it was cloned into the SmaI site of plasmid pBacPAK9 (Clontech, Palo Alto, CA) to obtain the transfer vector pBacproST3. The cDNA corresponding to the 45-kDa form of ST3 (mST3) was PCR amplified from the complete ST3 cDNA and the sequences of the primers used were deduced from the published sequence of human ST3 cDNA (16). EcoRI and XhoI sites were included respectively at the 5′ and 3′ ends of the oligonucleotide sequences to allow further cloning into the EcoRI and XhoI sites of pBacPAK9 and to obtain the transfer vector pBacmST3.

Recombinant baculovirus production. Sf9 cells were transfected with a mixture of 500 ng of transfer vector DNA and 100 ng of Bsu36I-digested BacPAK6 DNA (Clontech), according to the manufacturer's instructions. After 3–4 days the medium, containing the viruses generated during transfection, was harvested and stored at 4°C. The transfection supernatant was plated on Sf9 monolayers according to standard techniques (26). Plaques were visible by staining with 0.033% neutral red in PBS; they were resuspended in 0.1 plaque-forming units (pfu) per cell. The medium was harvested when signs of infection appeared, usually at the third or fourth day postinfection. This passage one virus stock, 2 × 10^6 cells were infected with 100–200 μl of the viral stock showing the best expression of the protein and the medium was harvested when signs of infection appeared, usually at the third or fourth day postinfection. This passage one virus stock was used to generate larger working stocks of virus for protein production. Stocks of recombinant viruses were obtained by infection of monolayer cultures of Sf9 cells at a multiplicity of infection (m.o.i.) of 0.1 plaque-forming units (pfu) per cell. The medium was harvested when signs of infection appeared, usually at 3–4 days postinfection, with titers of about 10^8 pfu per milliliter.

Purification of ST1 and mST3. A m.o.i. of 10 pfu per cell was used in infections for both protein production. Infected cells (5 × 10^6 cells in 500 ml of culture medium with FCS) were harvested at 72 h postinfection (hpi). Cells were washed with 50 mM Tris–HCl, 1 mM EDTA, pH 8.5 (buffer A), containing 150 mM...
NaCl, resuspended in the same buffer (10 ml for 10^8 cells), and lysed by sonication (Labsonic U. B. Braun, 4 x 15 s, 200 W). After centrifugation (33,700g, 30 min, 4°C), the pellet was dissolved (to a protein concentration <1 mg per milliliter) in 8 M urea, 100 mM 2-mercaptoethanol in buffer A containing 150 mM NaCl. After overnight stirring at 4°C, insoluble material was removed by centrifugation (33,700g, 15 min, 4°C) and discarded. The resulting supernatant was diluted 1:10 in buffer A and allowed to stand for 16 h at 4°C. After centrifugation (22,000g, 90 min, 4°C) the supernatant was applied to a Q-Sepharose Fast Flow (1.75 cm^2 x 5 cm) column equilibrated with 0.8 M urea, 10 mM 2-mercaptoethanol in buffer A. Unbound proteins were washed out with buffer A until the absorption (280 nm) reached baseline. Bound proteins were eluted with a gradient (60 ml) from 0 to 1 M NaCl. Bound proteins were washed out with the same buffer until no absorption was detected. Bound proteins were eluted with a 1 M NaCl, 10 mM CaCl_2, and 0.1% Brij 35 (assay buffer) with a 2-M (500 μg/ml, Calbiochem-Novabiochem, La Jolla, CA) for 18 h at 37°C in the absence or presence of 20 mM EDTA, 10 μM BB-94 (kindly provided by Dr. F. Colotta, Pharmacia & Upjohn, Milan, Italy), and 4 μg/ml aprotinin (Sigma). In the case of ST1 a casein zymography (12.5% polyacrylamide gel) was performed to analyze the results. ST3 samples were resolved by SDS-PAGE on a 10% resolving gel under nonreducing conditions, electroblotted, and probed with the ST3 polyclonal antiserum. A horseradish peroxidase-conjugated, goat anti-rabbit IgG (Dako A/S, Glostrup, Denmark) was used as the secondary antibody and Western blots were developed by chemiluminescence with ECL (Amersham, Arlington Heights, IL).

ST1 and mST3 proteolytic activities. ST1 (4.2 μg/ml) was incubated with α-casein (250 μg/ml, Merck, Darmstadt, Germany), β-casein (250 μg/ml, Sigma), fibronectin (250 μg/ml, Sigma), laminin (250 μg/ml, Collaborative Biomedical Products, Bedford, MA), type I collagen (250 μg/ml, Collaborative Biomedical Products), and α1-antitrypsin (6.5 μg/ml, Calbiochem-Novabiochem) for 18 h at 37°C in assay buffer. rhTIMP-2 (10 μg/ml) expressed in Escherichia coli in our laboratory was included in the reaction when indicated. mST3 (100 and 50 μg/ml) was incubated with β-casein (25 μg/ml) for 18 h at 37°C in assay buffer. Digestion products were analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue R-250.

ProST3 processing by proteases. ProST3 (35 μg/ml) was incubated in assay buffer alone or in the presence of ST1 (4 μg/ml), plasmin (0.5 μg/ml, Sigma), and urokinase (1.3 U/ml, Sigma) for different periods of time. Reactions were stopped by boiling for 3 min in Laemmli sample buffer and samples were separated by SDS-PAGE on 10% polyacrylamide gels under reducing conditions, electroblotted, and probed with the ST3 polyclonal antibody as previously described.

RESULTS AND DISCUSSION

The present paper reports for the first time the production of human ST1 and two forms of ST3 using the baculovirus expression system. Plasmids pBacST1, pBacproST3, and pBacmST3 were constructed by cloning the corresponding cDNAs into the transfer vectors pBacPAK1 and pBacPAK9. These cDNAs were inserted into the baculovirus genome under the control of the polyhedrin promoter, thereby directing a high-level expression of ST1 and ST3 in baculovirus-infected insect cells. Recombi-
nant baculoviruses were named BacST1, BacproST3, and BacmST3, respectively.

Monolayer cultures of Sf9 insect cells were infected with the recombinant baculovirus BacST1 and an over-expressed band of an approximate molecular weight of 60 kDa, corresponding to that expected for proST1, was only detected in cellular extracts and neither in conditioned medium nor in uninfected cell extracts (Fig. 1A). A similar level of proST1 expression was obtained from 24 to 72 hpi. The baculovirus expression system has been employed successfully for the expression of several secreted proteins (22). However, when this system was used to express ST1, most of the expressed protein (99%) accumulated in the intracellular space. This intracellular accumulation could be due to an inefficient recognition of heterologous signal peptides by the insect cell secretion machinery or to a saturation of the secretion pathway. The same result was obtained when matrilysin was expressed in the baculovirus system (23).

The pellet coming from $5 \times 10^8$ cells after 72 hpi with BacST1 was washed to remove residues of the FCS used in the cell culture and lysed by sonication. After centrifugation most of the ST1, as judged by SDS–PAGE and casein zymography, remained insoluble in the sonication pellet and a negligible amount was discarded in the lysis supernatant (Fig. 1B, lanes 1–3). The pellet was subjected to an unfolding (8 M urea, 100 mM 2-mercaptoethanol) and refolding process by dilution, and the solubilized protein was applied to a Q-Sepharose Fast Flow column (Fig. 1C, lane 1). Elution of bound protein was achieved with a gradient from 0 to 1 M NaCl (Fig. 1C, lanes 3–5). The Q-Sepharose eluate was a mixture of the latent form (60 kDa) and several active intermediates. ST1 complete activation was achieved by an overnight incubation at 55°C, yielding a band of 30 kDa with caseinolytic activity (Fig. 1C, lanes 6A and 6B). This result agrees with the molecular weight that has been previously described for the fully activated ST1 (28). After a further chromatographic step through the Superose S-12 column, an average of 0.3 mg of ST1 of about 90% homogeneity per $5 \times 10^8$ infected cells was obtained.

The activity of this purified activated ST1 was analyzed by the $\alpha_2$-M entrapment assay (29). Both proteins were incubated at 37°C for 18 h and the casein zymography analysis showed the disappearance of most of the caseinolytic ST1-associated activity (Fig. 2A). This result indicates that an endoprotease activity is present in our ST1 preparation that is in accordance with previously published data (30). Moreover, the proteolytic activity of ST1 was shown on several of its known substrates such as fibronectin, casein, and $\alpha_1$-antitrypsin (30–32) (Fig. 2B). Cleavage activity on laminin was barely detected as a much lower activity of ST1 for this substrate has been reported (33). Type I collagen was included as a negative control of the activity. In all cases the proteolytic activity of ST1 was inhibited by TIMP-2, a physiologic inhibitor of matrix metalloproteinases (34).

The expression peak of proST3 was obtained in the cellular extract of Sf9-infected cells after 72–96 hpi as a band of an approximate molecular weight of 55 kDa (Fig. 3A). ProST3 does not show activity either on gelatin or on casein zymograms (25) so this purification was analyzed by Western blot by using a polyclonal antibody raised against proST3. The cellular pellet was lysed by sonication and after centrifugation the supernatant, containing most of the proST3 (Fig. 3B, lane 1), was applied onto a Q-Sepharose Fast Flow column. A negligible amount of proST3 was lost in the
flowthrough and washing of the chromatography (Fig. 3B, lane 2). Elution was performed with a gradient from 0 to 1 M NaCl and most of the desired protein eluted at 250–350 mM NaCl (Fig. 3B, lane 3). At this point of the purification process, a mean of 10 mg of total protein per $5 \times 10^8$ cells was obtained in a mixture whose percentage in proST3 is estimated at ca. 80%. A further purification step through Mono Q PC 1.6/5 produced a 95% homogeneity of proST3 preparation, with a final yield of 2.5 mg protein per $5 \times 10^8$ cells (Fig. 3B, lane 4).

It has been previously reported that the proST3 produced in other eukaryotic expression systems is processed along the purification procedures (25, 33). A number of discrete bands are generated whose apparent sizes range from 45 to 30 kDa. The 45-kDa form has been assigned to a mature form of ST3 that has lost the prodomain. The 30- to 20-kDa forms are supposed to correspond to mature forms that only contain the catalytic domain. The fact that a complete form of proST3 (55 kDa) can be purified from baculovirus-infected insect cells could be probably explained by the lack of the furin system in insect cells. This system is present in certain mammalian cells and it has been described to be responsible for the intracellular activation of proST3 (35, 36).

The expression and purification of an unprocessed complete form of proST3 may be a valuable resource for the characterization of the mechanisms of proST3 activation. ProST3 is autocatalytically processed neither in the presence of 4-aminophenylmercuric acetate (25, 36) nor by heating at 55°C (data not shown). To study some possible processing pathways of proST3, the ability to cleave the purified recombinant proST3 by several proteases so far desribed to process other MMPs (37, 38) was tested. As shown in Fig. 4A, the incubation of proST3 with ST1 produced the disappearance of the 55-kDa form and several bands of 40 and 35–30 kDa appeared. Incubation of proST3 with plasmin or urokinase, members of the serine protease family, gave rise to several bands ranging in size from 50 to 40 kDa concomitantly with the disappearance of the 55-kDa form (Figs. 4B and 4C). ProST3 showed a weak autoproteolytic activity since when incubated without proteases a 45-kDa band appeared. This result agrees with the autoproteolytic activity exhibited by other MMPs (39). It could be speculative to attribute any physiological significance to the processed bands ob-

![FIG. 2. Enzymatic activities associated with purified ST1. (A) α2-Macroglobulin entrapment assay. ST1 was incubated in the absence (lane 1) or presence (lane 2) of α2-macroglobulin for 18 h at 37°C and the activity was evaluated by casein zymography. (B) Digestion of different substrates by ST1. The indicated substrates were incubated in the absence (lane 1) or presence of ST1 (lanes 2 and 3). Lane 3 shows the result of TIMP-2 addition to the incubation mixture (lane 3). Digestions were carried out at 37°C for 18 h and analyzed by SDS-PAGE.](image-url)

![FIG. 3. Expression and purification of proST3 from BacproST3-infected Sf9 cells. (A) Time-course expression of proST3 in Sf9 cells. Cellular extracts of BacproST3-infected Sf9 cells for different postinfection hours were analyzed after SDS-PAGE. Lane 1, noninfected cells; lane 2, 24 hpi; lane 3, 48 hpi; lane 4, 72 hpi; and lane 5, 96 hpi. (B) Western blot (left) and SDS-PAGE (right) analysis of different steps in the purification of proST3 from 72 h infected Sf9 cells. Lane 1, supernatant lysate; lane 2, Q-Sepharose flowthrough; lane 3, Q-Sepharose eluate (250–350 mM NaCl); and lane 4, Mono Q PC 1.6/5 eluate. The migration of molecular weight markers and proST3 is indicated.](image-url)
tained by incubation of proST3 with other proteases in vitro. However, it seems suggestive that some of these proteases play a major role in the protease cascade leading to tumor cell spreading. It is also interesting through this observation that urokinase and ST3 genes exhibit very similar patterns of expression in breast carcinoma (17).

To test the usefulness of the baculovirus expression system to produce the 45-kDa mature form of ST3, the corresponding cDNA was cloned from the complete coding sequence of ST3 and inserted in a baculovirus vector, and Sf9 cells were infected with the recombinant baculovirus. As observed in Fig. 5A, peak levels of expression of a 45-kDa band, corresponding to the predicted molecular weight of mST3, were observed in cellular extracts at 72–96 hpi. Unlike proST3, mST3 remained insoluble in the sonication pellet, so it had to be subjected to the unfolding process and subsequently refolded by dilution, as described above for recombinant ST1. The solubilized protein was purified through a Q-Sepharose column in a similar manner to that described for proST1 and proST3 purification (Fig. 5B).

The partially, 80% homogeneity, purified mST3 (2.5 mg per 5 × 10^8 cells) was active on the α2-M entrapment assay (Fig. 6A) as described (25), this activity being inhibited by the chelating agent EDTA and not by the serine protease inhibitor aprotinin (Fig. 6B) indicative of metalloproteinase activity. The degrading activity of mST3 on β-casein observed in Fig. 6C agrees with results presented previously (25), showing that the mature form of ST3 cleaved β-casein (30 kDa) at a single position, leading to species of about 25 kDa. This β-casein fragment is unable to diffuse out of the acrylamide gel, which explains why ST3 activity cannot be detected by casein zymography.

In summary, the baculovirus expression system has been successfully applied to produce and purify mature forms of the matrix metalloproteinases ST1 and ST3. The recombinant protein activities agree with those previously described for the mentioned proteases. On the other hand, the baculovirus system has allowed us to obtain a highly purified preparation of zymogen proST3 that may be specially useful to undertake further studies of the mechanisms of activation of this protease. All these results indicate the usefulness of the baculovirus expression system for the production of ST1 and ST3 preparations suitable for performing biochemical studies and/or screening of inhibitors.
FIG. 6. Proteolytic activities of mST3. (A) Purified mST3 (8.6 μg/ml) was incubated in the absence (lane 1) or presence of α2-M for 1, 2, 4, 8, and 24 h (lanes 2–6) at 37°C. Samples were electrophoresed under nonreducing conditions, blotted, and probed with the ST3 polyclonal antibody. (B) Purified mST3 (50 μg/ml) was incubated alone (lanes 1, 3, and 5) or with α2-M (lanes 2, 4, and 6) in the presence of EDTA (lanes 3 and 4) and aprotinin (lanes 5 and 6). (C) mST3 (100 and 50 μg/ml) was incubated alone or with β-casein (25 μg/ml) for 18 h at 37°C and the result was analyzed by SDS–PAGE. Lane 1, mST3 (100 μg/ml); lane 2, β-casein; lanes 3 and 4, β-casein and mST3 (100 and 50 μg/ml). The migration of molecular weight markers and mST3 is indicated.

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