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# Detection and partial characterization of a chymostatin-sensitive endopeptidase in transformed fibroblasts

(protease inhibitor/membrane/plasminogen activator)

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**ABSTRACT** **A chymostatin-sensitive step in the release of plasminogen activator from transformed fibroblasts has been described recently. By using synthetic peptidyl substrates, we have detected and characterized a chymostatin-sensitive peptidase activity in chicken embryo fibroblasts transformed by Rous sarcoma virus. The activity represents a neutral endopeptidase that exhibits phenylalanine specificity and is inhibited by diisopropyl fluorophosphate. A detailed inhibitor profile of the enzyme activity shows that it is distinct from other chymotrypsin-like phenylalanine-preferring peptidases. The endopeptidase activity in transformed fibroblasts is increased over that of parallel cultures of normal fibroblasts. The mechanism of enzyme inhibition by chymostatin is indicated by these studies, and the possible role of the enzyme in modulating plasminogen activator secretion is discussed.**

The mechanism whereby specific polypeptides are processed and secreted from cells is well established (1–3). The secretory events in this pathway have been defined for a number of specific proteins. However, the mode of release of some proteins into the extracellular milieu is not entirely consistent with the features of this pathway. The properties of one such protein, the serine protease plasminogen activator (PA) that is released in enhanced amounts from a variety of activated and transformed cells (4), indicate that it may not be released via the classical secretory pathway. The cellular form of PA, in contrast to the soluble released form of the enzyme, has been shown to be firmly associated with smooth plasma membrane-like elements of the cell (5). The cell-associated form of PA does not appear to be located within membrane-enclosed vesicles because treatments that readily release vesicle-enclosed enzymes fail to release or solubilize PA (5). In addition, treatment of Rous sarcoma virus (RSV)-transformed chicken embryo fibroblasts (RSVCEF) with colchicine at concentrations that inhibit the secretion of other proteins and the movement of secretion granules (6–9) does not inhibit the release of PA (10). Finally, inhibition of protein synthesis for short periods of time, which has no effect on the secretion of established proteins (11), rapidly inhibits the release of PA from RSVCEF (10). Thus, PA appears to exist as a firmly bound membrane-associated enzyme that is actively released into the extracellular medium as a soluble enzyme by an as yet undefined mechanism. It has been suggested that PA, similar to some tumor-specific proteins, is "shed" from the surface of the malignant cell (12), but conclusive evidence for a PA-shedding phenomenon is still lacking. We have determined that in cultures of RSVCEF the appearance of PA in the extracellular medium is inhibited by the protease inhibitor chymostatin (13). This inhibition of PA release is accompanied by concomitant accumulation of the cell-associated form of PA. The effect is specific, is dose and time dependent, and appears to involve the aldehyde moiety of the chymostatin molecule (13). By using specific fluorescent substrates, we have found a chymostatin-sensitive enzyme associated with a membrane fraction isolated from RSVCEF.

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## MATERIALS AND METHODS

**Cell Culture.** Cultures of chicken embryo fibroblasts were prepared, maintained, and infected with RSV as described (5).

**Fluorescence Spectroscopy.** The release of 7-amino-4-methylcoumarin (AMC) from the fluorescent substrates Phe-AMC, succinyl (Suc)-Ala-Ala-Phe-AMC, Suc-Ala-Ala-Pro-Ala-AMC, and benzyloxycarbonyl (Cbz)-Gly-Gly-Arg-AMC was measured in a spectrofluorimeter at an excitation wave length of 380 nm and an emission wave length of 460 nm (14). The standard system used to measure hydrolysis of the various substrates consisted of 0.5 ml of a 0.4 mM solution of substrate in 5% dimethyl sulfoxide ($Me_2SO$) 0.05 M Hepes, pH 7.4, containing 10–100 μg of membrane protein. The linear release of AMC was recorded over 2–15 min and the amount (μmol) of AMC released was calculated against a standard solution of AMC.

**Membrane Isolation.** The preparation and fractionation of cellular homogenates was as described (5). A membrane fraction was isolated by high-speed centrifugation of a postnuclear supernatant; the resulting pellet is referred to as the total membrane fraction. Protein content (15), PA activity (5), and endopeptidase activity were determined for all fractions. The membrane fraction contained a majority of the cellular PA and endopeptidase activity and 25–30% of the cellular protein.

**Materials.** Suc-Ala-Ala-Phe-AMC and other peptidyl substrates were purchased from Bachem Fine Chemicals (Torrance, CA). Chymostatin, leupeptin, antipain, and elastatinal were gifts from Walter Troll (New York University School of Medicine). Benzamidine, ε-aminocaproic acid, nitrophenylguanidinobenzoate, tosylphenylalanine chloromethyl ketone (Tos-Phe$CH_2Cl$), dithiothreitol, diisopropyl fluorophosphate ($iPr_2P$-F), 1,10-phenanthroline, EDTA, EGTA, and iodoacetamine were purchased from Sigma. α-1-Antichymotrypsin was obtained from James Travis (University of Georgia, Athens). *N*-Benzoyl-1-phenylalaninal (Bz-Phe-al) was a gift of Richard Schultz (Loyola University Stritch School of Medicine, Maywood, IL). Cbz-Gly-Leu-Phe-$Ch_2Cl$ was provided by James Powers (Georgia Institute of Technology, Atlanta).

Abbreviations: RSV, Rous sarcoma virus; RSVCEF, Rous sarcoma virus-transformed chicken embryo fibroblasts; PA, plasminogen activator; $iPr_2P$-F, diisopropyl fluorophosphate; AMC, 7-amino-4-methylcoumarin; Suc, succinyl; Cbz, benzyloxycarbonyl; $Me_2SO$, dimethyl sulfoxide; Tos-Phe$CH_2Cl$, tosylphenylalanine chloromethyl ketone; Bz-Phe-al, *N*-benzoyl-1-phenylalaninal.

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## RESULTS

**Detection of a Chymostatin-Sensitive Activity in RSVCEF.** The release of PA from cultures of RSVCEF is inhibited by treatment of the cultures with the protease inhibitor chymostatin (13). A chymostatin-sensitive enzymatic activity has been detected in the total membrane fraction isolated from RSVCEF by using the fluorogenic peptide substrate Suc-Ala-Ala-Phe-AMC. Proteases or peptidases that have phenylalanine specificity will cleave on the COOH-terminal side of phenylalanine to release the fluorescent group AMC. The presence of the $NH_2$-terminal succinyl blocking group precludes the activity of amino peptidase-initiated sequential hydrolysis of the substrate. As shown in Fig. 1 *A* and *B*, a membrane preparation isolated from RSVCEF exhibits an activity capable of hydrolyzing this substrate that is both time and concentration dependent. Chymostatin inhibits the activity in a dose-dependent manner (Fig. 1*C*), yielding essentially total inhibition at a concentration of 100 μg/ml (0.17 mM). In separate experiments, it was shown that optimal enzyme activity occurred at pH 7.5–8.1, with little or no activity below pH 6 or above pH 9 (data not shown).

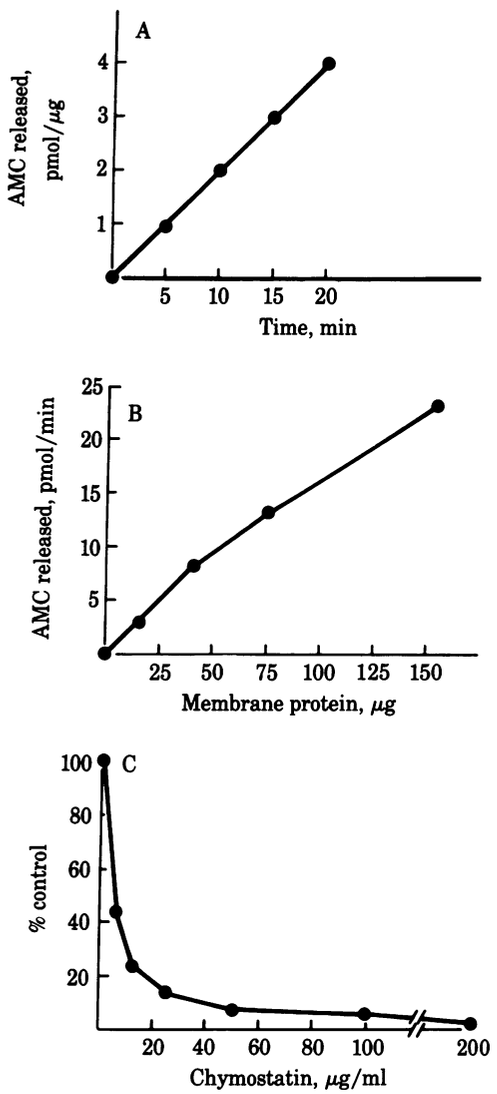


FIG. 1. Detection of a chymostatin-sensitive peptidase activity in a RSVCEF membrane fraction. A 0.5-ml assay mixture containing 0.4 mM Suc-Ala-Ala-Phe-AMC in 5% $Me_2SO$/0.05 M Hepes, pH 7.4, was used to detect peptidase activity. (*A*) Thirty-eight micrograms of total membrane fraction was added to the assay mixture and the release of AMC was measured at the indicated times. (*B*) Various amounts of membrane protein were added to the assay mixture, release of AMC was monitored for 10 min, and the amount of AMC released per minute was calculated. (*C*) Forty-six micrograms of total membrane fraction was added to the assay mixture in the presence of various concentrations of chymostatin, the rate of AMC release was measured, and the percent inhibition by chymostatin was calculated.

**Cleavage Site in Suc-Ala-Ala-Phe-AMC.** Release of AMC from Suc-Ala-Ala-Phe-AMC could be due to several enzymatic activities. Direct cleavage on the COOH-terminal side of phenylalanine by a specific endopeptidase would release AMC. However, an endopeptidase exhibiting alanine specificity could also cleave the substrate on the COOH-terminal side of either of the alanine residues, generating Ala-Phe-AMC, Phe-AMC, or both as products. These products, no longer having a blocked amino terminus, could be hydrolyzed by aminopeptidases, resulting in the release of free AMC. As shown in Table 1, the membrane preparation contains an aminopeptidase activity that hydrolyzes an unblocked Phe-AMC substrate. This activity, however, is inhibited by 1,10-phenanthroline and is relatively insensitive to chymostatin. In contrast, the activity measured with Suc-Ala-Ala-Phe-AMC is insensitive to 1,10-phenanthroline and highly sensitive to chymostatin (Table 1), indicating that exopeptidase activity is not involved in the membrane-catalyzed release of AMC. Further evidence that the release of AMC from Suc-Ala-Ala-Phe-AMC is not part of a multistep reaction is that the membrane preparation contains no detectable activity capable of cleaving at alanine residues when the substrate Suc-Ala-Ala-Pro-Ala-AMC is used (data not shown).

The total membrane preparation exhibits other peptidase activities. The fluorescent substrate Cbz-Gly-Gly-Arg-AMC also is hydrolyzed by the membrane preparation (Table 1). This activity, however, is insensitive to chymostatin but is strongly inhibited by the protease inhibitor antipain and thus distinct from the AMC-releasing activity detected when Suc-Ala-Ala-Phe-AMC is used. The chymostatin-sensitive activity in the membrane preparation, therefore, appears to be a specific endopeptidase that has phenylalanine specificity.

**$K_m$ and $K_i$ Values for the Endopeptidase Activity.** The effect of substrate concentration on the endopeptidase activity in the absence and the presence of various concentrations of chymostatin is shown in Fig. 2. A double reciprocal plot of the data (*B*) indicates a $K_m$ of 0.5 mM for Suc-Ala-Ala-Phe-AMC. A Dixon (16) plot (*C*) indicates a $K_i$ for chymostatin of 5.4 μg/ml ($9.2 \times 10^{-6}$ M). The various plots in Fig. 2 indicate that chymostatin is behaving as a competitive inhibitor, in that high substrate concentration affords protection from chymostatin inhibition. Additional experiments, however, suggest that the inhibition of the endopeptidase by chymostatin is more complex than that of a simple competitive inhibitor. If the membrane fraction is incubated with chymostatin at 20 μg/ml for 2 min, the endopeptidase activity is inhibited 70–80%. When this chymostatin/membrane mixture is diluted 1:20 with buffer and the diluted mixture is incubated for 15 min to allow for any reversal of chymostatin binding, the endopeptidase activity is inhibited only 28%, indicating significant reversibility. However, if the endopeptidase is incubated with chymostatin at 20 μg/ml for 30 min or more and then diluted 1:20 to 1:50 with buffer, little or no reversibility occurs and the enzyme remains 70% inhibited. These data are inconsistent with those for a classical competitive reversible inhibitor and suggest that binding of chymostatin to the enzyme during the short times (as used to generate the data in Fig. 2) can be inhibited by high concentrations of substrate but that, during prolonged incubation, the aldehyde moiety (phenylalaninal) in chymostatin reacts with the enzyme and irreversibly inhibits it. We have shown previously

Table 1. Membrane peptidase activities measured with various fluorescent substrates

| | Final conc., μg/ml | AMC released, (pmol/μg of protein)/hr | | | | | |
|---|---|---|---|---|---|---|---|
| | | Suc-Ala-Ala-Phe-AMC | | Phe-AMC | | Cbz-Gly-Gly-Arg-AMC | |
| | | Specific activity | % control | Specific activity | % control | Specific activity | % control |
| Control | | 4.81 | 100 | 9.48 | 100 | 2.87 | 100 |
| Chymostatin | 50 | 1.24 | 26 | 8.52 | 90 | 3.41 | 119 |
| | 200 | 0.39 | 8 | 7.20 | 76 | 2.56 | 89 |
| 1,10-Phenanthroline | 50 | 5.53 | 115 | 6.35 | 67 | NT | — |
| | 200 | 4.43 | 92 | 1.42 | 15 | NT | — |
| Antipain | 50 | 4.81 | 100 | NT | — | 0.77 | 27 |

Inhibitor solutions were prepared at various concentrations, and equal volumes were added to the assay mixture to establish the indicated inhibitor concentration (conc.). At the same concentration of membrane protein and the sensitivity used to measure AMC release from these substrates, no activity capable of hydrolyzing Suc-Ala-Ala-Pro-Ala-AMC was detected (data not shown). NT, not tested.

(13) that reduction of the aldehyde in chymostatin eliminates its antiproteolytic activity.

**Inhibitor Profile of RSVCEF Endopeptidase.** The effects of several protease inhibitors on the endopeptidase-catalyzed hydrolysis of Suc-Ala-Ala-Phe-AMC are summarized in Table 2. Effectors of thiol and metallo proteases, inhibitors of trypsin-like enzymes, and the microbial inhibitors elastatinal and bestatin had no effect on the endopeptidase activity. Each of these compounds also was incubated with the RSVCEF membranes for 30 min prior to the addition of Suc-Ala-Ala-Phe-AMC and still exhibited no inhibitory effect (data not shown). The only treatments that resulted in significant inhibition of AMC release were chymostatin and $iPr_2P$-F addition. Incubation of the membranes with 1 mM $iPr_2P$-F for 90 min inhibits the rate of hydrolysis to 47% of the control rate. Incubation of the membranes for 60 min with 2 mM $iPr_2P$-F inhibits the activity 75%. Thus, the membrane endopeptidase is inhibited by $iPr_2P$-F but is not highly sensitive to this specific inhibitor of serine proteases, since, under these conditions, chymotrypsin is inhibited 90–100%.

**Membrane Endopeptidase Distinct from Other Phenylalanine-Hydrolyzing Proteins.** Chymotrypsin and the cellular proteases cathepsin G and chymase are also capable of cleaving the COOH-terminal side of phenylalanine in synthetic substrates and several groups have reported the effects of synthetic and naturally occurring inhibitors on the hydrolytic capacity of these enzymes (17–25). To determine whether the RSVCEF membrane endopeptidase was an enzymatic activity similar or different from cathepsin G, chymase, and chymotrypsin, the effects of several highly specific inhibitors on the membrane-catalyzed hydrolysis of Suc-Ala-Ala-Phe-AMC were determined. As shown in Table 3, the RSVCEF endopeptidase has an inhibitor pattern distinct from that of the other three enzymes. The membrane endopeptidase is most strongly inhibited by chymostatin but considerable inhibition of the activity also is obtained with the tripeptide chloromethylketone, Cbz-Gly-Leu-Phe-$CH_2Cl$. Bz-Phe-al, Tos-Phe$CH_2Cl$, and α-1-antichymotrypsin have little or no effect on the membrane endopeptidase. In contrast, chymotrypsin is markedly inhibited by all five compounds; chymase is sensitive to α-1-antichy-

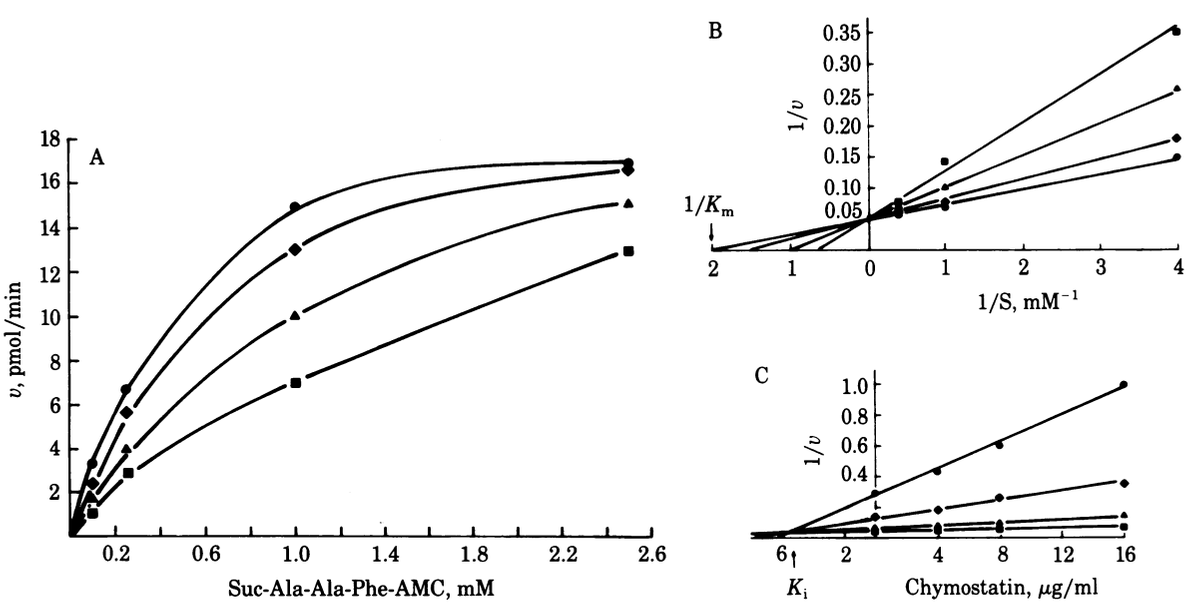


FIG. 2. Endopeptidase activity at different substrate concentrations in the absence and presence of chymostatin. (*A*) Chymostatin at 0 (●), 4 (◆), 8 (▲), and 16 (■) μg/ml was incubated with various concentrations of Suc-Ala-Ala-Phe-AMC (S) in 0.5 ml of a mixture containing 5% $Me_2SO$/0.05 M Hepes, pH 7.4. Twenty-five micrograms of membrane protein was added, the release of AMC was monitored for 5 min, and the rate of hydrolysis ($v$) was calculated as pmol of AMC released per min. (*B*) A double reciprocal plot of the data yielding a $K_m$ value of 0.5 mM. (*C*) Dixon (16) plot of the data (Suc-Ala-Ala-Phe-AMC: ●, 0.10; ◆, 0.25; ▲, 1.0; ■, 2.5 mM); the intercept of the lines yields a $K_i$ value for chymostatin of 5.4 μg/ml.

Table 2. Effects of various protease inhibitors on the endopeptidase-catalyzed hydrolysis of Suc-Ala-Ala-Phe-AMC

| Addition | Concentration | % control |
|---|---|---|
| None | | 100 |
| Dithiothreitol | 5 mM | 108 |
| Iodoacetamide | 10 mM | 98 |
| EDTA | 5 mM | 113 |
| EGTA | 5 mM | 109 |
| Benzamidine | 5 mM | 95 |
| ε-Aminocaproic acid | 5 mM | 114 |
| Nitrophenylguanidino benzoate | 40 μM | 100 |
| Leupeptin | 200 μg/ml | 94 |
| Elastatinal | 200 μg/ml | 108 |
| Bestatin | 200 μg/ml | 94 |
| Chymostatin | 50 μg/ml | 14 |
| | 200 μg/ml | 3 |
| $iPr_2P$-F | | |
| 30 min | 1 mM | 69 |
| 90 min | | 47 |
| 60 min | 2 mM | 25 |

Solutions of inhibitors were prepared such that addition of equal volumes of inhibitor solutions to the assay mixture established the indicated concentration. Inhibitors were added directly to the substrate (0.4 mM) along with 50–200 μg of membrane protein and the rate of AMC release was monitored. To measure the effect of $iPr_2P$-F, 150 μg of membrane protein was first incubated at room temperature with 1 or 2 mM $iPr_2P$-F. At the indicated times, an aliquot (50 μg of protein) was added to the substrate and the rate of AMC release was monitored.

motrypsin and Tos-Phe$CH_2$Cl; and cathepsin G, although inhibited by chymostatin and Cbz-Gly-Leu-Phe-$CH_2$Cl, is also sensitive to Tos-Phe$CH_2$Cl and α-1-antichymotrypsin.

**Endopeptidase Activity in Normal Chicken Embryo Fibroblasts and RSVCEF.** The endopeptidase activities in cell lysates prepared from parallel cultures of normal chicken embryo fibroblasts and RSVCEF are shown in Table 4. The level of enzyme in normal chicken embryo fibroblasts is increased approximately 3-fold following transformation by RSV. The activity in both cultures is inhibited 90% by chymostatin at 50 μg/ml.

## DISCUSSION

The inhibition by chymostatin of PA release from cultures of RSVCEF (13) has led to the identification and partial characterization of a chymostatin-sensitive enzyme activity in RSVCEF. This activity appears to be due to a membrane endopeptidase that cleaves on the COOH-terminal side of phenylalanine in synthetic peptide substrates (Table 1). The inhibitor profile indicates that the endopeptidase is not a thiol or metallo enzyme but rather a serine peptidase inhibitable by $iPr_2P$-F (Table 2). The enzyme activity, when compared with those of purified serine proteases, does not exhibit a high sensitivity to $iPr_2P$-F. However, the heterogeneous nature of the membrane fraction that contains the endopeptidase may influence its reactivity with $iPr_2P$-F. The membrane-associated form of PA isolated from these cells likewise requires relatively high levels of $iPr_2P$-F for inhibition compared with the levels required for the purified soluble form of PA (26).

Table 4. Comparison of endopeptidase activities in normal and transformed chicken embryo fibroblasts

| | Endopeptidase-specific activity |
|---|---|
| Normal | 6.5 ± 2.6 |
| RSVCEF | 20.9 ± 4.1 |

Parallel cultures of normal and transformed fibroblasts were harvested during exponential growth. Cellular homogenates were prepared, and multiple aliquots of the homogenates were assayed for endopeptidase activity and protein content. Results are expressed as pmol of AMC released/μg of protein per hr and represent mean ± SD.

The RSVCEF endopeptidase appears to be distinct from chymotrypsin and two other phenylalanine-preferring chymotrypsin-like cellular enzymes, cathepsin G and chymase (Table 3). Although some similarities do exist between the inhibitor profile of the endopeptidase and the profile of these other proteolytic enzymes, distinct discrepancies are apparent. These data suggest that the RSVCEF endopeptidase represents an as yet undescribed enzyme activity; however, it is important to point out that a crude form of the endopeptidase was assayed in these studies and compared with highly purified forms of the other enzymes (Table 3). A truly valid comparison must await purification of the endopeptidase.

A chymostatin-sensitive membrane-associated endopeptidase also has been reported by Zimmerman and co-workers (27, 28). This activity was detected by deoxycholate-solubilize pancreatic microsomal membranes by using the same synthetic substrate, Suc-Ala-Ala-Phe-AMC. The microsomal endopeptidase, however, was reported to cleave the substrate between alanine and phenylalanine and to be inhibited by 1,10-phen-

Table 3. Effects of specific inhibitors on the RSVCEF membrane endopeptidase and other phenylalanine-preferring proteases

| Inhibitor | Conc. | % inhibition | | | |
|---|---|---|---|---|---|
| | | Endopeptidase | Chymotrypsin | Cathepsin G | Chymase |
| α-1-Antichymotrypsin | 7.6 μg/ml | 0 | 100 | Inhibition (17) | Inhibition (17) |
| Chymostatin | 0.1 mM | 94 | 100 | Inhibition (18) | — |
| ($R_1$-Gly-Leu-Phe-al) | 1.0 mM | 100 | 100 | | |
| Bz-Phe-al | 0.1 mM | 0 | 30 | — | — |
| | 1.0 mM | 6 | 93 | | |
| Cbz-Gly-Leu-Phe-$CH_2$Cl | 0.1 mM | 66 | 73 | Inhibition (19) | — |
| | 1.0 mM | 93 | 96 | | |
| Tos-Phe$CH_2$Cl | 0.1 mM | 0 | 33 | No inhibition (20) | Inhibition (21, 22) |
| | 1.0 mM | 3 | 94 | Inhibition (23, 24) | |

Inhibitors at the indicated concentrations (conc.) were incubated with membrane (10 μg) for 30 min at 22°C in 0.45 ml of 0.05 M Hepes/5% $Me_2SO$, pH 7.4. A control containing membranes but no inhibitor was incubated in parallel. Fifty microliters of 0.01 M Suc-Ala-Ala-Phe-AMC was added to initiate the reaction and the release of AMC was monitored. The effects of the inhibitors on chymotrypsin-mediated hydrolysis of Suc-Ala-Ala-Phe-AMC were measured in parallel experiments. Effect of the inhibitors on chymase and cathepsin G were taken from the indicated references. $R_1$, [(*S*)-(1-carboxy-2-phenylethyl)carbonyl]-α-[2-iminohexahydro-4(*S*)-pyrimidyl].

anthroline (28). In contrast, the RSVCEF endopeptidase cleaves on the COOH-terminal side of phenylalanine and is insensitive to 1,10-phenanthroline (Table 2) and thus is distinct from the microsomal endopeptidase.

The mechanism of inhibition by chymostatin of the RSVCEF endopeptidase has been suggested by these studies. The data in Table 3 indicate that the effect of chymostatin on the enzyme is not just due to the terminal phenylalaninal residues since the simple phenylalaninal compound, Bz-Phe-al elicits no inhibitory effect. Similarly, the terminal phenylalanine chloromethyl ketone in Cbz-Gly-Leu-Phe-$CH_2Cl$ is not solely responsible for enzyme inhibition because the endopeptidase is insensitive to the simple phenylalanine chloromethyl ketone compound, Tos-Phe$CH_2Cl$. The inhibitory effects of both chymostatin and Cbz-Gly-Leu-Phe-$CH_2Cl$ therefore suggest that the tripeptide structure Gly-Leu-Phe is necessary to position the inhibitor at the active site of the enzyme. Once bound to the active site, either the aldehyde or chloromethyl ketone group can react to inactivate the enzyme. The kinetic data in Fig. 3 are consistent with a competition for active site binding between the Gly-Leu-Phe-al moiety of chymostatin and the Ala-Ala-Phe-AMC of the substrate. The time-dependent occurrence of irreversible inhibition is likewise consistent with aldehyde-mediated inhibition of the enzyme following chymostatin binding.

It is tempting to speculate that the RSVCEF endopeptidase activity described herein is involved in the mechanism of PA release. The release of PA from a firmly bound membrane state to a soluble extracellular form does not appear to proceed via the classical secretion pathway. The release of PA from these cells, however, is sensitive to chymostatin and the inhibitory effect of chymostatin on PA release appears to be due specifically to the antiproteolytic activity of chymostatin (13). The only detectable chymostatin-sensitive peptidase activity in RSVCEF cultures is the described endopeptidase (Table 1). The endopeptidase activity is enhanced after transformation of chicken embryo fibroblasts by RSV (Table 4), an event that also leads to increased production of PA (4). A number of other cell cultures have been examined including B16 melanoma and human fibrosarcoma HT-1080. Similar to the RSVCEF cultures, the release of PA from these mammalian cultures is inhibited by chymostatin and the cells also possess a chymostatin-sensitive endopeptidase activity (unpublished data). These observations provide further support for a role of the endopeptidase in PA release. However, there are some unresolved questions that prevent the establishment of a direct link between endopeptidase activity and PA release. First, the concentration of chymostatin that is required to completely inhibit the endopeptidase activity (50–100 $\mu$g/ml) is 15–30% of that needed in culture medium to inhibit PA release (300 $\mu$g/ml). This may be due to an inability to estimate the effective concentration of chymostatin at the cell surface because chymostatin is relatively insoluble in aqueous culture medium (13). Also, an inaccessibility of the cellular endopeptidase to exogenous chymostatin may require high concentrations of the compound in the extracellular medium. A second unresolved question is that the natural substrate for the endopeptidase has not been established. Although the cellular form of PA is firmly bound to RSVCEF membranes (5), the molecular mass of the released extracellular form of PA is identical (within 1,000 daltons) to that of the cell-associated form (10). Therefore, if the endopeptidase functions proteolytically to release PA from its membrane association, it would not appear to cleave a large membrane-bound segment of the PA molecule. Instead, the endopeptidase may cleave other membrane proteins that directly or indirectly interact with PA. The cleavage of these proteins could perturb the association of PA with the membrane, resulting in the release of soluble PA into the extracellular medium. However, until such a putative substrate is found, the link between endopeptidase activity and PA release remains circumstantial. Nevertheless, the circumstantial evidence that links the two enzymes—i.e., chymostatin sensitivity—has allowed for the detection of this apparently unique endopeptidase activity in transformed fibroblasts. Its purification, now feasible because of its specific inhibitor profile and reactivity toward defined synthetic substrates, should allow for both the identification of its natural substrate(s) and the elucidation of its role in the subtle, yet influential, protein perturbations that occur in normal and transformed cell membranes.

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