Internalization of Serratia Protease into Cells as an Enzyme-Inhibitor Complex with $\alpha_2$-Macroglobulin and Regeneration of Protease Activity and Cytotoxicity*

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Extracellular serratial protease (56,000 Da) is known to be cytotoxic. Fluorescein isothiocyanate-labeled protease was found to form a complex with human $\alpha_2$-macroglobulin ($\alpha_2$M), and this enzyme-inhibitor complex was purified. The protease was found to be internalized by fibroblasts in culture as a complex with $\alpha_2$M, which resulted in cell destruction. Regeneration of enzyme activity was confirmed in cells after 2–3 h of incubation. Chicken egg-white ovomacroglobulin, a homolog of human $\alpha_2$M, formed a complex with this enzyme similarly and more tightly but failed to exhibit protease activity, cytotoxicity, and internalization into cells.

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The abbreviations used are: $\alpha_2$M, $\alpha_2$ macroglobulin from human serum; ovoM, ovomacroglobulin; FITC, fluorescein isothiocyanate; PBS, 0.01 M phosphate-buffered 0.15 M saline; pH 7.0; Krebs-Ringer solution, Krebs-Ringer 15.6 mM phosphate-buffered solution, pH 7.4; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

The inhibitor (15–17). The receptor for $\alpha_2$M is known to be located on membranes of fibroblasts and macrophages (6, 16–18).

For the present experiments we examined the kinetics of protease binding to cells and the internalization as related to the enzyme-inhibitor complex. We then determined that the internalization process and the regeneration of protease activity are closely associated with cytotoxicity to fibroblasts. Furthermore, the usefulness of ovoM for such studies is shown.

MATERIALS AND METHODS

Protease and Proteins—The serratial protease used was described in our previous publication and is referred to as 56,000 Da protease (1, 2). It was purified as described (1); purity was more than 98% based on gel electrophoresis. $\alpha_2$M and ovoM were gifts from Dr. M. Okada (Chemotherapeutics Institute, Kumamoto, Japan) and Dr. T. Mizuta (Japan Immunoresearch Laboratories, Takasaki, Japan); the purity of $\alpha_2$M and ovoM was more than 92 and 98%, respectively. The former was purified by the method of Barrett (19).

Fluorescein Isothiocyanate (FITC) Labeling of Protease—FITC labeling was described previously (5, 20). Briefly, 10 mol of excess FITC over the protease was reacted for 2–3 h at pH 8.0, 20 °C, and 0.96 mol of FITC was coupled per mol of protease.

Purification of Enzyme-Inhibitor Complex—The protease-$\alpha_2$M and protease-ovoM complexes were prepared as described previously (5). Briefly, 0.14 mg of FITC-labeled protease was incubated with about 2.0 mg of $\alpha_2$M or ovoM in 0.5 ml of 50 mM Tris-HCl buffer containing 0.15 M NaCl, pH 7.6, as 20 °C for 4 h or 5 min, respectively; the mixture was then applied to a column of Ultroge A 22 (LKB) (see Fig. 1, legend).

Cell Binding and Internalization—Human embryonic lung fibroblasts were cultured in RPMI-1640 medium with 10% fetal calf serum and plated at a density of $1 \times 10^5$ cells/well (16 mm in diameter) (Falcon 24-well, 3047), with Dulbecco's modified Eagle's minimal essential medium with 10% fetal calf serum, and cells were allowed to grow until saturation density. Then the cells were washed three times with 0.01 M phosphate-buffered 0.15 M saline, and the medium was replaced with Krebs-Ringer solution, pH 7.4. This medium was routinely utilized in all binding assays. A final volume of 1.0 ml containing a constant amount of FITC-labeled protease or its complex with macroglobulins was used for binding studies at 4 or 27 °C, for different incubation times and at different concentrations. At the end of the incubation period, supernatant was discarded and unbound material was rapidly removed by washing the plates three times with phosphated-buffered saline. Cells were then lysed in 1 ml of 29 mM Tris-HCl buffer, pH 8.5, containing 0.2% sodium dodecyl sulfate. The fluorescence intensity of the solubilized cell solution was measured on a fluorescence spectrophotometer (Hitachi model 650-40) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Protein content was quantified by the method of Lowry et al. (21), with bovine serum albumin as the standard. The amount of cell-bound FITC-labeled protease or its enzyme-inhibitor complex was determined from the relationship between the concentration and the fluorescence intensity of the standard FITC-labeled protease.

To study the inhibition of binding, a different concentration of unlabeled free $\alpha_2$M or free ovoM was added to the FITC-labeled complexes; the total volume was kept at 1.0 ml. After the indicated
period of incubation, the amount of cell-bound FITC-labeled protein was determined by measuring the fluorescence intensity. Nonspecific binding was determined by subtraction of the binding data for FITC-labeled protease or its enzyme-inhibitor complex in the presence of 50-fold molar excess of unlabeled free inhibitor. However, incubation with an excess of unlabeled protease or enzyme-inhibitor complex was not possible because total destruction of cells resulted. In all cases, nonspecific binding was less than 15% of the total binding.

**Measurement of Protease Activity**—Activity was measured by an MAC-II polarization spectrophotometer (Japan Immunoresearch Laboratories, Takasaki, Japan) under strict temperature control as described (20). For measuring fluorescence polarization FITC-labeled gelatin was used as the substrate (20); as little as 0.05 μg/ml of protease can thus be detected. A typical assay medium contained 4 μg/ml FITC-labeled gelatin in 50 mM Tris-HCl buffer containing 1 mM ZnCl₂, pH 7.5, at 30 °C.

Regenerated protease activity in the cells was measured in homogenates of monolayered fibroblasts after incubating with unlabeled protease or its α₉M complex at 4 or 37 °C for different times (see legend of Fig. 6 for details). The treated cells, in a tissue culture tube (1.6 x 12.5 cm) (Falcon 3033), were rinsed with Krebs-Ringer solution three times at 4 °C; cells were then repeatedly frozen and thawed at −70 and 37 °C, respectively, after which they were sonicated (Tomy Seiki sonicator, 150W, Tokyo) under ice-cold conditions. The homogenates (four or five tubes) were pooled for assay of protease activity by the above method. Using FITC-labeled protease, cell homogenates were also applied to a column of Ultrogel AcA-22 to reveal the presence of FITC-labeled protease, or its complex with α₉M, in which enzyme-inhibitor complex was eluted as the first peak and was well separated from free FITC-labeled protease in the subsequent peak.

**RESULTS AND DISCUSSION**

**Purification of Enzyme-Inhibitor Complex and Its Cellular Uptake**—The enzyme-inhibitor complexes of the serratial protease with α₉M or ovoM were purified as shown in Fig. 1, A and B. Complex formation was completed within 4.0 h or 5 min, respectively, as revealed by the fluorescence polarization method (5). Therefore, each mixture of inhibitor and protease was applied after the appropriate incubation time to a column of Ultrogel AcA-22. When the FITC-labeled protease-α₉M complex was incubated for a longer period (6 h) at 20 °C without exposure to cells and was applied to the column, the elution pattern was different from that after a short incubation time. This indicates that α₉M was degraded into several fragments by the protease (Fig. 1C); ovoM showed only one peak even after 24 h of incubation (not shown). The results from gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate confirmed this fragmentation, as published previously (5).

The uptake of each complex into cells was measured by following the time course and dose response (Fig. 2, A–D) at 4 and 37 °C using the fibroblasts. The complex with α₉M was taken up to the greatest extent at 37 °C, followed by that at 4 °C (Fig. 2A). A plateau was approached for cell binding in about 1 h (Fig. 2B). When apparent binding was measured for different dose levels only the α₉M complex showed a prominent increase, which reached a plateau at about 15–25 μg/ml (Fig. 2A). The net intracellular uptake was estimated by subtracting the value at 4 °C from that at 37 °C (Fig. 2, C and D).
observed during 1 h of incubation, which proves endocytotic enzyme-inhibitor complexes at different incubation times. OvoM or protease alone showed a slight or no change of pH. Furthermore, this time course is fairly close to that seen in the complex into the lysosomal acidic milieu is apparent.

**Fig. 2.** Apparent binding of FITC-labeled serratial protease or its complex to human fibroblasts. A, dose response. O—O, enzyme-inhibitor complex of α2M incubated at 37°C; O—-O, complex with α2M at 4°C; □—□ complex with ovoM at 37°C; □—-□, complex with α2M at 4°C; Δ—Δ, FITC-protease at 37°C; Δ—-Δ, FITC-protease alone at 4°C. B, time course of binding. C, net uptake into cells at various doses. Values at 4°C were subtracted from those at 37°C. D, net uptake into cells at different times. Values at 4°C were subtracted from those at 37°C. All symbols are the same as in A. See text for details.

**Fig. 3.** Measurement in fibroblasts of microenvironmental pH of the fluorescent probe with FITC-labeled protease or its enzyme-inhibitor complexes at different incubation times. O, FITC-labeled protease-α2M complex at 37°C; □, ovoM complex at 37°C; Δ, α2M complex at 4°C; ∆, FITC-labeled protease alone at 37°C.

To identify the endocytotic process, we then examined the microenvironmental pH of the fluorescent probe (FITC-labeled protease) by measuring the ratio of fluorescence intensity with excitation at 490 and 450 nm. The results, shown in Fig. 3, clearly indicate that the protease-α2M complex exhibited the greatest change in apparent pH. The lowest pH observed was 5.8 at 40 and 50 min after incubation with cells at 37°C (Fig. 3). Although this was based on a continuous rather than a pulse exposure of the complex, entrapment of the complex into the lysosomal acidic milieu is apparent. Furthermore, this time course is fairly close to that seen in Fig. 2, B and D. In contrast, a pH change at 4°C was not observed during 1 h of incubation, which proves endocytotic internalization at 37°C. The enzyme-inhibitor complex with ovoM or protease alone showed a slight or no change of pH at about 7.0 or 7.4, respectively, even at the maximum at 4 and 37°C.

**Binding and Internalization through the α2M Receptor: Competition by Macroglobulins**—When cell binding of the protease or the enzyme-inhibitor complexes was examined in the presence of unlabeled α2M, the binding of both complexes was inhibited in a dose-dependent manner (Fig. 4A). These results indicate that both complexes share the same α2M-binding site (receptor), but only the α2M complex is internalized through a specific receptor (Fig. 3). When both enzyme-inhibitor complexes were examined for binding and competed with the unlabeled ovoM, only the binding of ovoM complex was inhibited by ovoM in a dose-dependent manner; that with α2M exhibited little inhibition (Fig. 4B). The presence of an ovoM receptor on the cell surface was thus suggested, but the binding of fluorescent α2M complex was not effectively blocked by the unlabeled ovoM (Fig. 4B), indicating different receptors for these two macroglobulins. These two results in Fig. 4, A and B are not consistent, however: the ovoM complex was not internalized effectively (Fig. 3) for reasons that are unclear. OvoM appears to have no thioester or sulfhydryl groups (23), but it forms a tighter complex with serratial protease than does α2M, and it does so more rapidly (5). Both macroglobulins undergo a similar conformational change and yield complexes in which ovoM had a larger radius than that of α2M (5, 9).

**Regeneration of Protease Activity in Cells and Cytotoxicity**—We have previously reported that serratial protease is potently cytotoxic to fibroblasts, but ovoM inhibits both protease activity and cytotoxicity (4). Furthermore, there is no potential inhibitor of this protease in serum; α1 protease inhibitor was rapidly degraded, and α2M in the complex was also degraded but much more slowly. Cultured cells were killed in the presence of serum but were killed to a much lesser degree.
Uptake of Serratia Protease-α2M Complex and Cytotoxicity

A, binding competition with free α2M. O, complex with α2M; •, complex with ovoM. B, binding competition with free ovoM. O, complex with α2M; •, complex with ovoM. Complex concentration was 5 nM for all cases. See text for details.

FIG. 4. Competition of binding of enzyme-inhibitor complexes with macroglobulins. A, binding competition with free α2M. O, complex with α2M; •, complex with ovoM. B, binding competition with free ovoM. O, complex with α2M; •, complex with ovoM. Complex concentration was 5 nM for all cases. See text for details.

FIG. 5. Regeneration of protease activity in cells treated with serratial protease-α2M complex. Protease activity was measured by fluorescence polarization (see text). Fibroblasts in test tubes were treated with protease-α2M complex at 10 nM (0.5 ml in volume) and incubated for 30 min. Cells were then washed three times to remove the free complex in Krebs-Ringer solution. Incubation then was continued in 10 mM Hepes buffer in 0.1 M NaCl containing 0.75% bovine serum albumin at pH 7.4 until the cell harvest for the enzyme assay. An average of four values was used for one point. See text for details.

FIG. 6. Elution pattern of FITC-labeled protease-α2M complex after treating fibroblasts. After treating the cells for a given time, cells were frozen and thawed three times, followed by sonication and centrifugation (at 10,000 × g for 2 min). Supernatants were applied to a column of Ultrogel AcA-22, and eluted similarly as described for Fig. 1. A, control, treated for 30 min at 4 °C. B, 30 min at 37 °C. C, 5 h at 37 °C. Fluorescence intensity of protease (○) and peak with enzyme activity (—) is shown. Black and white arrows show peaks of α2M and protease, respectively.

in the absence of serum. Consequently, the slow regeneration of protease activity from the α2M complex appeared to produce cell toxicity (4, 5). Thus, we concluded that protease activity is associated with cytotoxicity.

The present data have confirmed that serratial protease is effectively taken up into fibroblasts through α2M receptor-mediated endocytosis in 40–60 min (Figs. 2 and 3). Then the complex gradually degenerates to yield active enzyme in 1–5 h (Fig. 5). This time period agrees with that from in vitro data; more than 90% of the protease was shown to be regenerated in vitro (4). Thus, the cytotoxicity of the protease appears to depend greatly on the internalization of this semistable enzyme-inhibitor complex and subsequent regeneration of enzyme activity in cells. To confirm the degradation of the complex, we examined the complex by chromatography on an Ultrogel AcA-22 column after a long incubation period. The results in Fig. 6 indicate a considerable change in the chromatographic pattern in A and C in which prolonged incubation resulted in considerable degradation of enzyme-inhibitor complex.

Fluorescence Microscopy and Internalization of the Enzyme-Inhibitor Complex—After treatment with the complex or protease alone, fibroblasts were examined with a fluorescent microscope. As shown in Fig. 7, the protease complex with α2M could be visualized in the cytoplasm of treated cells; neither the serratial protease alone nor its complex with ovoM could be seen so prominently. These results agree with previous ones and with the above observations (Figs. 2, 3, and 5). Thus, regenerated protease activity in the cells appear to be associated with cytotoxicity.

Concluding Remarks—The present observations have demonstrated that there is a unique cytotoxic mechanism generated by serratial protease that is mediated by its semistable complex with α2M. The complex is internalized via α2M receptors on fibroblasts (Figs. 2–4, 6, and 7); after internalization, the protease activity is gradually regenerated (Fig. 5), and is associated with cytotoxicity in cells (Fig. 7). Protease alone and its complex with ovoM are not as toxic as the α2M complex because they are not efficiently internalized. This unique mechanisms of internalization of serratial protease
FIG. 7. Fluorescence and phase contrast microscopy for fibroblast cells treated with FITC-labeled protease or its enzyme-inhibitor complex. A–C, inverted fluorescence microscopy. D–F, inverted phase contrast microscopy. A, cells treated with 2.5 µg/ml of FITC-labeled protease-α2M complex. B, FITC-labeled protease alone (2.5 µg/ml). C, FITC-labeled protease-ovoM complex (2.5 µg/ml). D–F, same as A–C, only unlabeled complex was used. A–C, incubated at 37 °C for 2 h; D–F, incubated at 37 °C for 18 h. Concentrations of the protease and inhibitors in A–C and D–F were the same. Magnification × 600 (A–C); × 210 (D–F).

may also help explain the cytotoxicity of Pseudomonas protease. Work along these lines is in progress in our laboratory.

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REFERENCES