Zymogen activation in a reconstituted pancreatic acinar cell system

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Pancreatic zymogens are activated by extracellular stimuli, including proteolytic enzymes, autocrine peptides, and cholecystokinin. This activation occurs in zymogen-granule and microsomal cellular compartments, but the mechanistic aspects of these processes are poorly understood. To examine these mechanisms, we developed a reconstituted pancreatic acinar cell system, which exhibits ATP-dependent and ATP-independent mechanisms of zymogen activation. We showed that activation of digestive zymogens within the pancreatic acinar cell initiates acute pancreatitis. Cytosolic events regulate this activation within intracellular compartments of unclear identity. In an in vivo model of acute pancreatitis, zymogen activation was observed in both zymogen granule-enriched and microsomal cellular fractions. The percentage of the pool of trypsinogen and chymotrypsinogen activated was about twofold and sixfold greater, respectively, in the microsomal than in the zymogen granule-enriched fraction. Activation of zymogen granule-enriched but not trypsinogen was significantly enhanced by ATP (5 mM) but not by the inactive ATP analog AMP-PNP. The processing of procarboxypeptidase B to its mature forms also demonstrated a requirement for ATP and cytosol. E64d, an inhibitor of cathepsin B, a thiol protease that can activate trypsin, did not affect chymotrypsin activation. These studies demonstrate that ATP-dependent and independent mechanisms of zymogen activation can take place in both zymogen granule and microsomal cellular fractions. Two studies have suggested that initial zymogen activation might occur in zymogen granules (13, 20). The latter studies used antibodies that selectively label the trypsinogen activation peptide, a surrogate for trypsinogen activation, in confocal and immunoelectron microscopy to detect the site of trypsinogen activation in the caerulein hyperstimulation model of acute pancreatitis. Trypsinogen activation peptide was initially localized to small vesicles in a supranuclear region of the acinar cell that contained markers of lysosomes and recycling endosomes. Whether the dissimilar findings relating to the site of zymogen activation are due to differences in detection methods or experimental systems remains unclear.

Materials and Methods

In Vivo Studies

All experiments and procedures using animals were approved by the Veterans Affairs International Animal Care and Use Committee (West Haven, CT; approved protocol FG0006). Experimental pancreatitis was initiated in 150- to 200-g Sprague-Dawley rats (anesthetized intraperitoneally with ketamine-xylazine) via a tail vein injection of 40 µg/kg caerulein (hyperstimulatory dose), a cholecystokinin analog used to cause pancreatitis, and pathologic pancreatic zymogen activ-
Pancreatic Fractionation: Isolation of Zymogen Granule-Enriched, Microsomal and Cytosolic Fractions

Pancreatic fractionation followed the protocol of Tartakoff and Jamieson (28) with several modifications. The pancreas was divided in two; one-half was homogenized on ice in 10 vol (≈5 ml) of 0.3 M sucrose and 1 mM DTT in the absence of protease inhibitors and used for isolation of the zymogen granule-enriched and microsomes (preparation A). The other one-half was homogenized in 10 vol (≈5 ml) of 0.3 M sucrose, 1 mM DTT, 25 mM Tris (pH 7.2), 5 mM aprotinin, soybean trypsin inhibitor (0.1 mg/ml), 5 mM benzamidine, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and complete EDTA-free protease inhibitor cocktail (20 μl/ml of 25% stock solution). All reagents were purchased from Sigma, except for the Roche protease inhibitor cocktail, used for isolation of cytosol (cytosolic buffer; denoted preparation B). The high-molecular-weight protease inhibitors were added to inhibit enzyme activity in the cytosol and to ensure that enzyme activity observed in the assay was within the organelles. Homogenates were centrifuged at 500 g for 5 min. All preparations and storage were at 4°C unless otherwise stated.

Preparation of Membrane Fractions

The pellet from the 500-g centrifugation was discarded, and the resulting postnuclear supernatant was centrifuged at 3,000 g for 10 min over a 2 M sucrose cushion. The sucrose cushion was necessary to prevent organelle rupture during centrifugation. After the 3,000-g centrifugation, the layer at the interface of preparation A was retained, diluted with 2 vol of 0.3 M sucrose, and washed at 3,000 g over a 2 M sucrose cushion (further described below in KCl and Sucrose Wash). The layer at the interface was retained; this was the zymogen granule-enriched fraction, which was subsequently diluted with distilled water to give a concentration of 0.3 M sucrose (using a refractometer; Leica, Buffalo, NY). The supernatant from the initial 3,000-g centrifugation (preparation A) was then centrifuged at 15,000 g for 15 min over a 2 M sucrose cushion. The layer at the interface (mitochondrial fraction) was retained. The remaining supernatant was centrifuged at 180,000 g for 60 min over a 2 M sucrose cushion. The layer at the interface was removed, diluted with 2 vol of 0.3 M sucrose, and washed at 288,000 g over a 2 M sucrose cushion. The layer at the interface was retained; this was the microsomal fraction, which was subsequently diluted with water to give a concentration of 0.3 M sucrose.

KCl and Sucrose Wash

To eliminate associated proteins from the organelle surface, zymogen granule-enriched and microsomal fractions were diluted fivefold in 0.3 M sucrose (control) or 0.5 M KCl (21). They were centrifuged at 3,000 g and 180,000 g, respectively, over a 2 M sucrose cushion. The layer at each interface was collected and diluted with water to 0.3 M sucrose. These were denoted as “washed” fractions.

Preparation of the Cytosolic Fraction

After the 500-g centrifugation, the supernatant (preparation B) was retained and centrifuged at 180,000 g for 60 min on a 2 M sucrose cushion. The supernatant was removed, and the layer at the interface was discarded. Endogenous ATP and low-molecular-weight protease inhibitors were removed from the cytosol by dialysis (molecular weight cut-off 3,500) in 0.3 M sucrose, 25 mM Tris (pH 7.2), and 100 mM KCl for 60 min with a dialysis cassette (Slide-A-Lyzer dialysis cassette, 3,500 molecular weight cut off, 0.5- to 3-ml capacity; Pierce Biotech). After dialysis, the fraction was centrifuged at 288,000 g for 15 min in tubes precoated with cytosolic buffer to remove small vesicles.

The zymogen granule-enriched fraction and the microsomal fraction from preparation A (lacking protease inhibitors) and the cytosolic fraction (containing high-molecular-weight protease inhibitors) from preparation B were used in activation assays described elsewhere.

Zymogen Activation Assays

In this study, the conversion of zymogens to mature enzymes is taken as either an increase in enzyme activity, as determined with fluorogenic assays (16, 17), or the appearance of mature enzyme forms, as determined with immunoblot analysis (15).

Enzymatic Assays

Assay buffer (350 μl) [50 mM Tris (pH 7.6) and 150 mM KCl] was added to each well of a 24-well plate (Greiner Bio-one Cellstar TC-plate; Greiner Bio-One, Monroe, NC) at room temperature (25°C). Zymogen granule-enriched or microsomal fractions (50 μl), previously stored on ice, were then added to the assay buffer in each well at room temperature. Next, 50 μl of 400 μM enzyme substrate (trypsin or chymotrypsin substrate; trypsin was MAAl315-v and chymotrypsin was MAAl311-v, from Peptides International, Louisville, KY) diluted in assay buffer at room temperature (40 μM final) were added to each well. The plate was lightly agitated from side to side to mix the contents of each well and then left at room temperature under normal lighting conditions. In preliminary studies, incubation times of 0–60 min were used to allow uptake of fluorogenic substrate by the organelles; 15 min was found to be the optimal value. For in vivo experiments, stimulated zymogen granule-enriched and microsomal fractions were assayed alone, in the absence of any cytosol or ATP, after immediate addition of substrate.

For in vitro experiments, after the 15-min incubation of substrates with organelles, 50 μl of buffer (control) or cytosol (50 μl) were added to each well and incubated for a further 15 min. In preliminary studies, these conditions resulted in a linear increase in fluorometric emission. Preliminary studies also demonstrated that the cytosol-dependent generation of protease activity was linear when 50 μl was added to the assay; this amount was used unless otherwise stated. After a total incubation of 30 min, ATP (5 mM) or AMP-PNP (5 mM) was added, and fluorescence emissions were recorded on an excitation wavelength of 380 nm and an emission of 440 nm for 11 min (20 readings) with an HTS 7000 fluorimeter (Perkin-Elmer Analytical Instruments, Shelton, CT). The fluorometric emission slope (relative fluorescence units/s) represented enzymatic activity. Organelles were lysed with Triton X-100, and amylase content was determined (Pheroabas kit; Pharmacia, Rochester, NY). The enzyme activity was normalized to amylase content (expressed as fold activation vs. the zymogen granule/microsomal fraction plus cytosol plus ATP condition). Because preliminary studies demonstrated that normalization to amylase or protein content were equivalent, data are normalized to amylase content.

Pancreatic Zymogens

For determination of total pancreatic trypsinogen and chymotrypsinogen in the zymogen granule-enriched and microsomal fractions, samples were incubated with excess enterokinase (5 μg/100 μl) in the presence of 10 mM CaCl2 at 37°C for 60 min, allowing for complete activation of trypsinogen and chymotrypsinogen in the sample (18). The samples were assayed with fluorogenic substrate for either trypsin or chymotrypsin (values for total activity determined as relative fluorescence units per second as a function of total protein content, with protein determined by the Bradford assay). To determine the percentage activation induced by cytosol and ATP in the zymogen
granule-enriched and microsomal fractions, the rfu/sec values obtained in these samples were expressed as a function of total protein content and the values divided by the total activity in that fraction.

**Organelle Lysis**

To determine whether active enzymes from lysed organelles contributed to measured enzyme activities, the cytosolic preparation was modified. High-molecular-weight protease inhibitors (0.1 mg/ml soybean trypsin inhibitor, 5 mM aprotinin) were replaced with lower-molecular-weight inhibitors (5 mM benzamidine, 5 mM leupeptin). Before dialysis, the soybean trypsin inhibitor (0.1 mg/ml) and 5 mM aprotinin were added to one-half of the preparation. Both cytosolic preparations were dialyzed to remove low-molecular-weight inhibitors, centrifuged at 288,000 g, and used in trypsin and chymotrypsin activity assays. Hence, the cytosolic preparation with soybean trypsin inhibitor has the capacity to inhibit free protease and the other does not. Furthermore, cytosol treated with membrane-permeable complete EDTA-free protease inhibitor cocktail (20 μl/ml of 25X stock solution; Roche) was used in additional assays to demonstrate that zymogen activation was occurring within membrane-bound compartments.

**Proteasomal Inhibitors**

Proteasomal inhibitors lactacystin (20 μM) and MG-132 (0.75 μM) (Calbiochem) were added at the same time as substrate in enzyme assays to examine the possible involvement of the proteasome in chymotrypsin activation.

**Immunoblot Assays for Zymogen Activation**

Zymogen granule-enriched fraction or microsomal fraction was incubated alone, in the presence of cytosol, or in the presence of cytosol and ATP. After 1 min, 10 μl of complete EDTA-free protease inhibitor cocktail and 100 μl of 6X Laemmli sample buffer were added to each well. Because carboxypeptidase degraded rapidly in this system, procarboxypeptidase processing was assayed after 1-min incubations. Samples were heated to 95°C for 5 min. Equal protein was loaded as determined by the Bradford assay, and proteins were separated by SDS-PAGE (12.5% polyacrylamide) and processed for immunoblot analysis on polyvinylidene difluoride membranes as described (15). To detect proteolytic zymogen activation, membranes were probed with an antibody that detected the zymogen and active forms of procarboxypeptidase B (gift from G. Scheele).

**Protein Kinase Inhibitors**

The general protein kinase inhibitors K252a (100 nM) and H-89 (100, 30, and 1 μM) (Calbiochem) and the phosphatidylinositol 3-kinase inhibitors wortmannin (100 nM) and LY-294002 (25 μM) (Sigma) were added at the same time as substrate in enzyme assays to determine the effects of protein kinases in trypsinogen and chymotrypsinogen activation. Controls were incubated with the same concentration of drug vehicle (DMSO).

**Role of Cathepsin B in Zymogen Activation**

To examine the effects of cathepsin B inhibition on zymogen activation, trypsin and chymotrypsin were assayed in the presence of the cathepsin B inhibitor E64d (Sigma). E64d (1 mM) was added at time 0 (24). Control experiments used an equal volume of the vehicle (1:1 methanol-water). Effects of E64d on 100 ng of pure trypsin and chymotrypsin (Sigma) were evaluated using the same assay technique as described above.

**Statistical Analysis**

Statistical significance was determined using the Wilcoxon rank sum test. Significance was assumed for P < 0.05. Deviation bars in all figures represent 25th and 75th quartiles.

**RESULTS**

Detection of Zymogen Activation in Both Zymogen Granule-Enriched and Microsomal Fractions In Vivo

Zymogen activation in particulate fractions. Zymogen granule-enriched and microsomal fractions were harvested from rat pancreas stimulated in vivo with supraphysiological concentrations of caerulein for 15 min. This treatment caused approximately threefold and fivefold increases in trypsin activity in both the zymogen granule-enriched and microsomal fractions, respectively, when compared with unstimulated pancreas. (Fig. 1A). Similarly, chymotrypsin activity was ~10- and 6-fold higher in the zymogen granule-enriched and microsomal fractions, respectively, when compared with controls from unstimulated pancreas (normalized to amylase content) (Fig. 1B). On the basis of evidence that zymogen activation might occur in distinct cellular organelles, we developed a cell-free assay system to determine whether activation could be reconstituted using isolated pancreatic fractions.

Reconstitution of Zymogen Activation in Particulate Fractions

Total zymogen and amylase content of particulate fractions. The relative trypsinogen, chymotrypsinogen, and amylase contents were similar between the zymogen granule-enriched and microsomal fractions (Fig. 2). Therefore, results were normalized to amylase content unless noted otherwise.

**Trypsinogen activation.** Zymogen granule-enriched, mitochondrial, and microsomal fractions were isolated from unstimulated pancreas and assayed for trypsin activity. The mi-

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**Fig. 1.** Zymogen activation is observed in both a zymogen granule-enriched (ZG) and microsomal (Mic) fraction following supraphysiological caerulein treatment in vivo. Rats were treated for 15 min with caerulein (40 μg/kg body wt) via tail vein injection. Enhanced trypsinogen (A) and chymotrypsinogen (B) activities are seen in both ZG and Mic fractions at 15 min. *P < 0.05 vs. ZG or Mic from unstimulated pancreas (control).
tochondrial fraction and cytosol showed negligible activities. A small amount of basal activity was observed in zymogen granule-enriched and microsomal fractions alone (Fig. 3). However, the activities in these fractions were significantly enhanced by the presence of cytosol (Fig. 3). Addition of 5 mM ATP did not affect trypsin activity in either the zymogen granule-enriched fraction or microsomes in the presence or absence of cytosol (Fig. 3), indicating that trypsinogen processing to trypsin is ATP independent.

When the trypsin activity was normalized, the relative activity was about sixfold greater in microsomes than in the zymogen granule-enriched fraction, indicating more efficient processing in the microsomes. To validate this finding, trypsinogen content was estimated by its conversion to trypsin by enterokinase (Fig. 2). Values for the cytosol-dependent activation were compared with the total trypsinogen activation for each fraction. Cytosol stimulated activation of 2% and 5% of total trypsinogen in the zymogen granule-enriched fraction and microsomes, respectively, further suggesting that processing may be more efficient in the microsomes (Table 1). However, because the total trypsinogen content of the zymogen granule-enriched fraction was much greater, the 2% of the pool activated yielded higher overall activity than the 5% of the smaller microsomal trypsinogen pool. Thus, when trypsinogen activation was normalized to its distribution between the two compartments, the activities contributed by the zymogen-granule enriched fraction were about eightfold higher than those seen in microsomal compartments (not shown).

Chymotrypsinogen activation. Isolated zymogen granule-enriched, mitochondrial, and microsomal fractions from unstimulated pancreas were assayed for chymotrypsin activity. Similar to trypsin, no chymotrypsin activity was observed in the cytosolic fraction alone (Fig. 4). Furthermore, addition of ATP to each fraction did not affect enzyme activity. However, there was a prominent increase in chymotrypsin activity when cytosol was combined with either fraction (Fig. 4). Chymotrypsin activities increased further with the addition of ATP. Thus, when both ATP and cytosol were added, chymotrypsin activity increased 5-fold and 2.5-fold in zymogen granules and microsomes, respectively. In preliminary studies, the effects of 0.25, 0.5, 1, and 5 mM ATP were examined. Little enhancement of activation was seen until the concentration of 5 mM was reached; this value was used in all studies. Addition of the nonhydrolyzable ATP analog AMP-PNP did not enhance zymogen activation, confirming that ATP hydrolysis is required. Similar to trypsin, chymotrypsin activity (normalized to amylase content) was up to sixfold higher in the microsomal fraction than in the zymogen granule-enriched fraction (not shown). When normalized to the zymogen content, cytosol-dependent activation was 1 and 6% of total

<table>
<thead>
<tr>
<th>Zymogen Granule and amylase content (%)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
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<tr>
<td>Zymogen Granule enriched fraction</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>Microsomes</td>
<td>-</td>
<td>-</td>
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Fig. 2. Zymogen and amylase distribution in ZG and Mic pancreatic fractions. Trypsinogen, chymotrypsinogen, and amylase content were assayed as described in MATERIALS AND METHODS. Values are expressed as percentages of the total of these 2 fractions.

Table 1. Percent zymogen activation in reconstituted system

<table>
<thead>
<tr>
<th></th>
<th>ZG</th>
<th>ZG/Cyt</th>
<th>ZG/Cyt/ATP</th>
<th>Mic</th>
<th>Mic/Cyt</th>
<th>Mic/Cyt/ATP</th>
</tr>
</thead>
<tbody>
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<td>Trypsin activity</td>
<td></td>
<td>0</td>
<td>0.2±0.1</td>
<td>1.0±0.2</td>
<td>3.0±0.3</td>
<td>10.0±3.8</td>
</tr>
<tr>
<td>Chymotrypsin activity</td>
<td></td>
<td>0</td>
<td>0.6±0.2</td>
<td>7.0±1.1</td>
<td>6.0±3.1</td>
<td>10.0±3.8</td>
</tr>
</tbody>
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Values are means ± SE. Activities are calculated as rfu/s per total protein content from each assay condition and expressed as a percentage of the total activated zymogen as determined in Fig. 2. ZG, zymogen granule-enriched fraction; Mic, microsomal fraction; Cyt, cytosol fraction.
chymotrypsinogen content for zymogen granule-enriched and microsomal fractions, respectively. In the presence of cytosol and ATP, these values increased to 7 and 10%, respectively (Table 1). Similar to trypsinogen, more efficient processing of chymotrypsinogen was seen in microsomes than in the zymogen granule-enriched fraction. However, when chymotrypsinogen activation was normalized to the distribution of zymogens between the two fractions, the activities contributed by the zymogen granule-enriched fraction were about sixfold higher than those seen in microsomal compartments (not shown). In contrast to trypsinogen, these studies demonstrate that cytosol-dependent chymotrypsinogen processing in isolated organelles exhibits both ATP-independent and ATP-dependent components.

Contribution of free enzyme from lysed organelles. To test the possibility that organelles were being lysed during the assay, an event that might lead to an underestimation of zymogen activation, additional studies were performed. With the use of two different cytosolic preparations, one with high-molecular-weight protease inhibitors and the other without, the contribution of enzyme from lysed organelles was determined. If significant lysis occurred, then there would be more activity in preparations lacking protease inhibitors in the cytosol than in those with the inhibitors. Because the effects of the two cytosol preparations were similar, it is unlikely that organelles are lysed during the assay (Table 2). As a control, membrane-permeable protease inhibitors did inhibit enzyme activity (Table 2).

**Effects of membrane-associated proteins and the proteasome.** The proteasome is an organelle with trypsin and chymotryptic activities that can be associated with membrane bound compartments. Although its major role is ATP-dependent protein degradation, it can sometimes catalyze limited proteolysis (1, 21). To exclude a role for the proteasome in zymogen activation or a direct contribution to our enzymatic assays, two studies were performed. First, to remove the proteasome and other peripherally associated proteins from membranes, the zymogen granule-enriched and microsomal fractions were washed in 0.5 M KCl or 0.3 M sucrose (control) (3). Neither wash condition affected enzyme activities (Table 3). Next, chymotrypsin activity was assayed in the presence or absence of the proteasomal inhibitors lactacystin (20 μM) and MG-132 (0.75 μM); neither reduced chymotrypsinogen activation (Table 4). Interestingly, lactacystin causes a slight but significant increase in activity in both zymogen granule-enriched and microsomal fractions when cytosol and ATP were present. This is not a direct effect on the enzyme because lactacystin had no effect on purified chymotrypsin (not shown). These studies demonstrate that membrane-associated proteins, including the proteasome, do not contribute to the ATP-dependent activation of chymotrypsinogen.

**Procarboxypeptidase B activation.** To confirm the results of assays that used fluorogenic protease substrates, the generation of carboxypeptidase B was assayed by immunoblot (Fig. 5). No carboxypeptidase B was seen in either the zymogen granule-enriched or microsomal fraction alone or in the presence of 5 mM ATP. Carboxypeptidase B was detected in cytosol alone; when we compared bands with densitometry, cytosol alone accounted for 6–9% of maximal activity (not shown). When cytosol was added to either particulate fraction, an immunoreactive band corresponding to carboxypeptidase B was observed. A more intense band was seen in the presence of cytosol and ATP (this condition denotes maximal activity; Table 2).
Effects of proteasome inhibitors on chymotrypsinogen activation. 100 nM, a concentration that inhibits many kinases, showed no inhibition of activity. However, the general protein kinase C inhibitor H-89 caused a concentration-dependent inhibition of cytosol-dependent or ATP-dependent chymotrypsin activity. These data indicate that ATP-dependent mechanisms in zymogen granule-enriched and microsomal fractions (Fig. 6). In control studies, E64d did not affect the activity of purified trypsin (not shown). These findings suggest that, similar to other models, cathepsin B converts procarboxypeptidase to its active form of procarboxypeptidase B (CP-B), the active form of procarboxypeptidase B, is generated in both ZG and Mic fractions with added Cyt and further enhanced by ATP. Results are representative of 3 separate experiments.

DISCUSSION

The pathological activation of digestivezymogens, particularly proteases, appears to have a central role in the initiation of acute pancreatitis. Our initial studies suggested that zymogen activation could occur in distinct cellular organelles. After the induction of caerulein pancreatitis, we observed enhanced protease activities in both zymogen granule-enriched and microsomal fractions (Fig. 7). These data indicate that ATP-dependent mechanisms in zymogen granule-enriched and microsomal fractions may be mediated by protein kinases.

Selective effects of E64d on zymogen activation. The thiol protease, cathepsin B, has been proposed as the mediator of pathologic trypsinogen activation, which initiates the proteolytic cascade activating otherzymogens in acute pancreatitis (5, 7, 23, 24). Fluorogenic protease activity assays were repeated in the presence of the cathepsin B inhibitor, E64d (9, 10, 12). The inhibitor blocked cytosol-dependent generation of trypsin activity in both the zymogen granule-enriched and microsomal fractions (Fig. 7). In control studies, E64d did not affect the activity of purified trypsin (not shown). These findings suggest that, similar to other models, cathepsin B converts procarboxypeptidase to trypsin in our reconstitution system. However, cytosol and ATP-dependent chymotrypsin activity and carboxypeptidase B generation in the particulate fractions were not inhibited by E64d (Figs. 8 and 9). The latter findings suggest that chymotrypsinogen and procarboxypeptidase activation might be independent from cathepsin B and trypsin.
cytosol is possibly due to contamination from light vesicles, as this activity was markedly diminished by very high-speed centrifugation. Furthermore, there is a lack of an effect on activity by removal of adherent proteins from membrane fractions with potassium chloride or treatment with proteasomal inhibitors. Because cytosol-dependent zymogen activation is observed with isolated organelles from unstimulated cells, it might represent basal cellular activity (as seen in whole cell studies; Refs. 16, 17), or it could result from activation of the machinery, such as protein kinases, required for zymogen activation during preparation of cytosol.

Second, cytosol-stimulated zymogen activation occurs in both zymogen granule-enriched and microsomal compartments, suggesting that more than one compartment might support zymogen activation. Although the levels of activation seen in these compartments represent a small percentage of the total zymogen content (Table 1), the values are consistent with those from studies that have used intact cells (9, 15, 24). Our data suggest a more efficient processing of zymogens in microsomes than with zymogen granule-enriched fraction and a more efficient processing of chymotrypsinogen than of trypsinogen (Table 1). The proportions of total zymogen in zymogen granule-enriched fraction are greater than those seen in the microsomal fraction (Fig. 2) and comparable to levels described in another study (18). Thus, when normalized to the distribution of active enzymes between the two compartments, the greater contribution to enzyme activity comes from the zymogen granule-enriched fraction. Whether a similar distribution is observed in intact cells remains unclear.

Studies from our laboratory and another group have both reported that initial zymogen processing is localized to a vesicular compartment containing markers of lysosomes and recycling endosomes (13, 20). In our present study, immunoblot analysis demonstrates that the lysosomal and endosomal marker GRAMP-92 is present predominantly in the zymogen granule-enriched fraction and to a lesser extent in the microsomal fraction (data not shown). Because these two have substantially different densities, it is unlikely that GRAMP-92 is associated with the same organelle in the zymogen granules and microsomes. Additional fractionation studies will be required to identify the compartments ultimately responsible for zymogen processing.

Third, on the basis of the effects of ATP and E64d in our system, the requirements for trypsinogen activation may be distinct from those necessary for activation of chymotrypsinogen and procarboxypeptidase. The ATP requirement for conversion of chymotrypsinogen and procarboxypeptidase and kinase inhibitor studies indicates a potential role for one or more protein kinases. Our data with protein kinase inhibitors indicates a lack of involvement for phosphatidylinositol 3-kinase in the cell-free system. The results with the inhibitor H-89 suggest that a protein kinase inhibited in the range from 30 to 100 μM may regulate processing. Protein kinase A and protein kinase G are inhibited at submicromolar concentrations of H-89 and are therefore unlikely candidates (11). However, other protein kinases, such as casein kinases I and II, calmodulin-dependent kinases, and protein kinase C, require inhibitory H-89 concentrations in the ranges observed in this study (11). Notably, preliminary studies suggest that H-89 also selectively inhibits chymotrypsin but not trypsin activity in acini simulated by supraphysiological concentrations of caerulein (unpublished data). Studies with selective protein kinase inhibitors and specific agonists may identify the protein kinases that mediate this activation.

The inhibitory effects of E64d on trypsinogen activation are consistent with the published effects of cathepsin B on trypsinogen activation, but its lack of effectiveness in inhibiting chymotrypsinogen or procarboxypeptidase B processing is
surprising. Together with the selective effects of ATP on chymotrypsinogen or procarboxypeptidase B activation, these findings strongly suggest that the activation of some zymogens is trypsin independent. Indeed, one study even suggests that trypsin has a more relevant role in autodegradation rather than in activation of itself and other zymogens (10). However, we cannot exclude the possibility that undetectable increases in trypsin activity might be responsible for the activation of the other zymogens. Most other studies with pancreatic cathepsin B and trypsinogen activation have not examined activation of other zymogens (10, 12, 24). In this context, in cathepsin B knockout mice, despite a significant reduction in trypsinogen activation, cell injury and other measures of pancreatitis are still prominent (9). Furthermore, in transgenic mice with increased levels of pancreatic secretory trypsin inhibitor 1, active trypsin is inhibited and the severity of caerulein-induced pancreatitis is reduced but not eliminated (19). One possible explanation for the persistent injury seen in both the cathepsin B knockout and the transgenic pancreatic secretory trypsin inhibitor 1 animals is that activation of proteases other than trypsinogen may not be reduced.

In summary, this report used an in vivo model of acute pancreatitis and a reconstituted system to examine the mechanisms of zymogen activation in the pancreatic acinar cell. From these studies, we conclude that pathological zymogen activation can occur in more than one cellular organelle and requires cytosolic factors. Furthermore, these studies demonstrate that zymogen activation is complex and that distinct pathways may lead to activation of specific subsets of proteases. In this context, activation within the acinar cell may not follow the pattern that has been established for intestinal activation, with trypsinogen activation leading to proteolytic activation of other zymogens. Elucidation of the cytosolic

![Fig. 7. The cathepsin B inhibitor, E64d, prevents Cyt-dependent trypsinogen activation in ZG (A) and Mic (B) fractions. Assays were performed as for Fig. 3, but some samples were preincubated with E64d (1 mM) for 30 min. Control experiments (i.e., in the absence of E64d) were preincubated with 1:1 methanol-water, the vehicle for E64d. Data are normalized to amylase content and expressed as fold vs. ZG/Mic + Cyt + ATP; n = 3. Activation in Cyt alone was negligible (data not shown). *P < 0.05 vs. Cyt or Cyt/ATP condition.

![Fig. 8. The cathepsin B inhibitor E64d does not prevent chymotrypsinogen activation in ZG (A) and Mic (B) fractions. Assays were performed as for Fig. 4, but some samples were preincubated with E64d (1 mM) for 30 min. Control experiments (i.e., in the absence of E64d) were preincubated with 1:1 methanol-water, the vehicle for E64d. Data are normalized to amylase content and expressed as fold vs. ZG/Mic + Cyt + ATP; n = 3. Activation in Cyt alone was negligible (data not shown).

![Fig. 9. CP-B generation in ZG and Mic fractions is not inhibited by preincubation with E64d. Immunoblot assay demonstrating that Cyt- and ATP-dependent generation of CP-B is not inhibited by preincubation with E64d (1 mM). Results are representative of 3 separate experiments.](http://ajpgi.physiology.org)]
factors and pathways that mediate zymogen activation may lead to the rational design of preventive therapies.

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GRANTS

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