Codistribution of TAP and the granule membrane protein GRAMP-92 in rat caerulein-induced pancreatitis

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Codistribution of TAP and the granule membrane protein GRAMP-92 in rat caerulein-induced pancreatitis. Am. J. Physiol. 38: G999–G1009, 1998.—The pathological activation of zymogens within the pancreatic acinar cell plays a role in acute pancreatitis. To identify the processing site where activation occurs, antibodies to the trypsinogen activation peptide (TAP) were used in immunofluorescence studies using frozen sections from rat pancreas. Saline controls or animals receiving caerulein in amounts producing physiological levels of pancreatic stimulation demonstrated little or no TAP immunoreactivity. However, after caerulein hyperstimulation (5 µg·kg⁻¹·h⁻¹) for 30 min and the induction of pancreatitis, TAP immunoreactivity appeared in a vesicular, supranuclear compartment that demonstrated no overlap with zymogen granules. The number of vesicles and their size increased with time. After 60 min of hyperstimulation with caerulein, most of the TAP reactivity was localized within vacuoles ≥1 µm that demonstrated immunoreactivity for the granule membrane protein GRAMP-92, a marker for lysosomes and recycling endosomes. Pretreatment with the protease inhibitor FUT-175 blocked the appearance of TAP after hyperstimulation. These studies provide evidence that caerulein hyperstimulation stimulates trypsinogen processing to trypsin in distinct acinar cell compartments in a time-dependent manner.

pancreas; trypsin; protease inhibitor; trypsinogen activation peptide; trypsin

The pathological proteolytic activation of trypsinogen and its activation of other digestive zymogens within the pancreas are hypothesized to be critical steps in the initiation of acute pancreatitis. Active enzymes have been found in the pancreatic parenchyma (15, 30), and a marker for trypsinogen activation has been detected in the plasma of patients with acute pancreatitis (23). Aberrant zymogen processing may play a role in hereditary pancreatitis; in this disease a mutation in cationic trypsinogen eliminates a potential proteolytic degradation site in the molecule (27). The mutation might block the degradation of active trypsin within the pancreatic acinar cell and make it easier for active enzymes to overwhelm protective mechanisms and initiate pancreatitis. Although it is possible that some zymogen activation may take place within the pancreatic duct or in the interstitium, recent studies suggest that the earliest activation occurs within the pancreatic acinar cell. For example, exposure of isolated pancreatic acini to hyperstimulation by CCK, a condition that causes pancreatitis in vivo, results in the rapid (≤ 15 min) conversion of procarboxypeptidase A₁ to carboxypeptidase A₁ (14) and to enhanced trypsin activity (15, 24). Although zymogens can be processed to active forms within the acinar cell, the precise intracellular site of zymogen activation and mechanisms of activation remain unknown.

A probe has been developed that can provide direct evidence of trypsinogen processing to trypsin. The NH₂-terminal amino acid sequence of trypsinogen contains a short peptide sequence (7–10 amino acids depending on the species and isoform) that is responsible for maintaining trypsinogen in its inactive state and is known as the trypsinogen activation peptide (TAP). The proteolytic cleavage of TAP from trypsinogen results in the generation of trypsin and the release of TAP. The five amino acids (Asp-Asp-Asp-Asp-Lys) at the COOH terminal of TAP are conserved in all mammalian trypsinogen. Antibodies generated to the Asp-Asp-Asp-Lys peptide demonstrate at least a 10⁶-fold selectivity for TAP over trypsinogen by ELISA and radioimmunoassay (10). A recent study has used antibodies to TAP to detect its generation within the pancreatic acinar cell in the early phases of experimental pancreatitis (16).

One early feature of acute pancreatitis is the appearance of cytoplasmic vacuoles within pancreatic acinar cells (26). These vacuoles have been classified by their morphological appearance, content, and pH. In the caerulein (a CCK analog) hyperstimulation model of acute pancreatitis, many of the vacuoles appear to be autophagic in origin and contain membrane and content from several organelles. Immunocytochemical studies have indicated that at least some of these vacuoles contain both secretory zymogens and lysosomal markers (28). These vacuoles appear within 30 min after induction of pancreatitis with intravenous caerulein (6). In other studies, caerulein hyperstimulation has been associated with the appearance of acidic cytoplasmic vacuoles (20). These acidic compartments were present after 3 h of caerulein hyperstimulation; earlier times were not examined. Although the formation of acinar cell vacuoles is characteristic of caerulein-induced acute pancreatitis, their origins, mechanisms of formation, and contribution to the pathogenesis of acute pancreatitis remain unclear.

In the present study we use newly generated affinity-purified TAP antibodies to examine the initial phases of acute pancreatitis during in vivo caerulein hyperstimulation.
iation. The antibodies initially detect TAP immunoreactivity in a small (<1 µm) vesicular compartment, the membranes of which often demonstrated immunoreactivity for granule membrane protein (GRAMP)-92, a marker of lysosomes and recycling endosomes. After 1 h of hyperstimulation, most TAP immunoreactivity was present in larger vacuoles (≥1 µm). The appearance of TAP is blocked by pretreatment with a protease inhibitor. These studies confirm that trypsinogen can be proteolytically processed within the pancreatic acinar cell. Trypsinogen processing appears to take place in a compartment that overlaps with a marker of lysosomes and endosomes and not in zymogen granules.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats (150–300 g) were obtained from the Charles River Breeding Laboratories, Wilmington, MA. Caerulein was obtained from Pharmacia, Erlangen, Germany; all other chemicals and supplies were from Sigma Chemical, St. Louis, MO, unless otherwise noted.

The synthetic protease inhibitor 6-amidino-2-naphthyl-4-guanidinobenzoate dimethanesulfonate (nafamostat mesylate, FUT-175, Futhan) has a molecular mass of 540 Da. It inhibits a variety of serine proteases, including trypsin and chymotrypsin (5). It is likely that FUT-175 enters pancreatic acinar cells because a structurally related synthetic protease inhibitor has been shown to be cell permeable (21). FUT-175 was a kind gift from Dr. Masateru Kurumi, Research Laboratories of Torii Pharmaceutical, Chiba, Japan.

Animals and tissue preparation. The experiments were carried out on rats fasting overnight with free access to water. Animals were anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine (Ketaset; Aveco, Fort Dodge, IA) and 10 mg/kg xylazine (Rompun; Miles, Elkhart, IN). Caerulein was injected into the inferior vena cava as a bolus at a dose of 0.01 µg/kg for physiological stimulation or 5 µg·kg⁻¹·h⁻¹ for hyperstimulation. In control experiments, the corresponding volume of 0.9% sodium chloride was injected.

After 30, 45, and 60 min of stimulation, rats were subjected to in vivo perfusion fixation with a fixation buffer of 100 mM NaCl, 50 mM HEPES, 1 mM EGTA, 5 mM benzamidine, and 10 µM α-phenylmethylsulfonyl fluoride (PMSF), pH 7.2, that contained 0.05% glutaraldehyde-2% paraformaldehyde for 5 min. Then the pancreas was removed, cut into 10-µm sections, and stored in the buffer with 1% paraformaldehyde at room temperature. Primary antibodies were detected with rabbit anti-TAP IgG (1:400) and mouse anti-GRAMP-92 (13), were kind gifts from Dr. J. David Castle, University of Virginia, Charlottesville, VA. FITC-conjugated goat anti-rabbit F(ab')2 and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse F(ab')2 were used as secondary antibodies (Biosource, Burlingame, CA).

Preparation of sections and immunofluorescence. After fragments were rinsed with the perfusion buffer without fixatives, pancreatic fragments were equilibrated in a 15% sucrose-phosphate buffer solution at 4°C for 2 h. The tissue was embedded in OCT compound (Miles, Elkhart, IN), then sectioned in liquid nitrogen and isopentane. Approximately 5-µm frozen sections were obtained at −40°C and placed on alum-gelatin-coated slides. After sections were washed, they were rinsed with a buffer of 200 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.05% saponin, 1 mM benzamidine, 0.1 mM PMSF, pH 7.4, and protease inhibitors as described in Ref. 17. All subsequent incubations were performed in this buffer. Nonspecific labeling was reduced by quenching the sections with 3.0% goat serum.

For single labeling, tissues were incubated with the rabbit anti-TAP IgG (1:400) for 2 h at room temperature. Primary antibodies were detected with FITC-labeled goat anti-rabbit fragments (1:500) for 30 min.

For double labeling, tissues were incubated simultaneously with the rabbit anti-TAP IgG (1:400) and mouse anti-GRAMP-92 tissue culture supernatant (1:20) for 2 h at room temperature. Primary antibodies were detected with a mixture of FITC-labeled goat anti-rabbit F(ab')2 fragments (1:500) and TRITC-labeled goat anti-mouse F(ab')2 fragments (1:500) for 30 min.

After sections were washed, they were mounted in 15 µl of ProLong Antifade Kit (Molecular Probes, Eugene, OR) and photographed with a Zeiss Axiophot 2 microscope equipped with epifluorescence illumination and barrier filters to prevent overlap of the TRITC and FITC signals (Thornwood, NY). Photomicrographs were prepared using Kodak Tmax100 or Ektachrome (ASA 100) films at a magnification of ×630. Fluorescence micrographs were exposed for 20 s, developed using constant conditions, and digitized using a Nikon Coolscan slide scanner. Images were processed identically in Adobe Photoshop. Quantitative analysis was done by projecting photomicrographs of randomly photographed fields, and analysis was performed blinded to the treatment condition.

Electron microscopic immunolocalization of TAP and GRAMP-92. After fragments were rinsed with the perfusion buffer without fixatives, pancreatic fragments were dehydrated and infiltrated with Lowicryl K4M at −60°C (1). The polymerized block was cut to ultrathin sections (~0.07 µm) and mounted on 200 mesh nickel grids having a carbon-parlodion film. Thin grids were incubated with anti-GRAMP-92 IgG (1:5) for 30 min and treated with 5 nm protein A-gold (PAG) complex coupled to secondary antibody. Then, they were incubated with anti-TAP (1:40) for 30 min.
followed by 10 nm PAG coupled to a secondary antibody. They were finally stained with 0.05% uranyl acetate and embedded in 2% methylcellulose before viewing through a Phillips 300 electron microscope at 60 kV.

Electron microscopy for ultrastructure. The pancreatic fragments were fixed with 0.1 M sodium cacodylate containing 3% glutaraldehyde, dehydrated, and embedded in Epon. The ultrathin sections were examined through a Phillips 300 electron microscope at 60 kV.

RESULTS

Caerulein hyperstimulation results in the appearance of TAP in the acinar cell. The distribution of TAP in the pancreas was examined after in vivo caerulein treatment and perfusion fixation. There was little to no TAP immunoreactivity observed in unstimulated controls or after 60 min of physiological caerulein stimulation (0.01 µg·kg⁻¹·h⁻¹) in vivo (Fig. 1, A and B). However, intense TAP immunoreactivity was detected within acinar cells 60 min after caerulein hyperstimulation (5 µg·kg⁻¹·h⁻¹) in vivo (Fig. 1C). TAP was localized to structures that were restricted to acinar cells and was not found in endocrine cells, duct cells, or in the pancreatic duct or interstitial space. The TAP immunoreactivity appeared to be concentrated in a supranuclear region of the acinar cell that demonstrated little or no overlap with zymogen granules (Fig. 1, C and F, and Fig. 2, E and F). The specificity of the TAP labeling was demonstrated by the absence of labeling with either the secondary antibody alone or antibody that had been preabsorbed with the TAP peptide (Fig. 1, G–I). Detection of the TAP immunoreactivity in the hyperstimulated condition required both glutaraldehyde and paraformaldehyde in the fixative; after hyperstimulation, perfusion fixation in the absence of glutaraldehyde resulted in a loss of immunoreactivity (not shown). The loss of TAP immunoreactivity in the

Fig. 1. Trypsinogen activation peptide (TAP) immunoreactivity is specifically detected in pancreatic acinar cells after in vivo caerulein hyperstimulation. Fluorescence micrographs of TAP immunoreactivity from in vivo treatments: unstimulated control (A), 60 min after physiological stimulation with caerulein (0.01 µg/kg iv; B), and 60 min after hyperstimulation with caerulein (5 µg·kg⁻¹·h⁻¹ iv; C) and corresponding phase images (D, E, and F) are shown. There was virtually no TAP immunoreactivity in unstimulated control or after 60 min of physiological stimulation (A and B). Note intense TAP immunoreactivity in small vesicles (arrowheads) and large vacuoles (arrows) within acinar cells 60 min after hyperstimulation (C). Most TAP immunoreactivity filled vacuoles, but some were localized at margins (C and F). There was no immunoreactivity detected in absence of primary antibody in unstimulated tissue (G) or after 60 min of hyperstimulation (H). Preabsorption of TAP antibody with the peptide Asp-Asp-Asp-Asp-Lys eliminated labeling of hyperstimulated (60 min) pancreas (I). Original magnification, ×630.
that excluded zymogen granules. Original magnification,

structures in acinar cells

Table 1. Quantitation of TAP-immunoreactive structures in acinar cells

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<th>%Acinar cells with TAP</th>
<th>Physiological Stimulation (60 min)</th>
<th>Hyperstimulation</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>TAP (+) structures/100</td>
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<td>cells</td>
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Values were determined by counting number of trypsinogen activation peptide (TAP)-positive (+) structures in 100 acinar cells from 2 or 3 rats for each condition.

Although TAP immunoreactivity was in small vesicles (<1 µm) after 30 min of hyperstimulation, most labeling was in vacuoles (≥1 µm) after 60 min (Fig. 2, A, C, and E). Compared with phase images, TAP labeling was concentrated in a supranuclear region that demonstrated little or no overlap with the apical zymogen granule compartment (Fig. 2, E and F). To identify the compartments in which TAP is localized, additional labeling studies were performed.

Caerulein hyperstimulation results in the redistribution of GRAMP-92. The distribution of the membrane protein GRAMP-92 was examined after in vivo treatments. In unstimulated controls, GRAMP-92 immunoreactivity was associated with small, faintly labeled vesicles throughout the acinar cell as described elsewhere (13). Intense GRAMP-92 immunoreactivity was occasionally found in irregular vesicles <1 µm that were distributed in a supranuclear region (Fig. 3, A–C). During 60 min of hyperstimulation, GRAMP-92 became enriched in vacuoles with a diameter of ≥1 µm (Fig. 3, D–L). The number of these vacuoles (≥1 µm) increased with the duration of hyperstimulation (Table 2: 44, 88, and 136 vacuoles per 100 acinar cells at 30, 45, and 60 min, respectively). GRAMP-92 immunoreactivity was concentrated at the margin of these vacuoles (Fig. 3, F, I, and L). Although GRAMP-92 uniformly localized to the margins of small vesicles, the antigen appeared to be irregularly distributed on most large vacuoles. The findings suggest that during hyperstimulation there is redistribution of GRAMP-92 and fusion among compartments enriched in this protein. Notably, the size, morphology, and distribution of some GRAMP-92-associated structures were similar to those observed for TAP.

TAP immunoreactivity is localized to GRAMP-92-positive structures. To determine whether TAP immunoreactivity was distributed in GRAMP-92-immunoreactive structures, pancreatic sections were double labeled. After 30 min of hyperstimulation, there were few TAP-positive structures, but more than one-half of these also demonstrated GRAMP-92 immunoreactivity (Fig. 4, A and B, and Fig. 5). As shown (Fig. 4, A, C, D, F, G, I), TAP is localized to the juxtanuclear region of the acinar cell. In the same cells, GRAMP-92 immunoreactivity is localized to the inner margin of small vesicles and vacuoles distributed throughout the acinar cell but concentrated in a supranuclear region (Fig. 4, B, C, E, F, H, I). Although most of the structures that demonstrated TAP immunoreactivity were also positive for GRAMP-92, the majority of GRAMP-92-positive structures did not contain TAP (Fig. 4, G and H). The distribution of TAP in GRAMP-92-positive structures in shown to better advantage in Fig. 5. Although TAP usually filled the content of GRAMP-92-positive structures (Fig. 5, A and B, C and D), TAP was sometimes observed in only a part of the structure (Fig. 5, E and F). As previously noted, GRAMP-92 uniformly labeled the margin of small vesicles (Fig. 5D) but irregularly labeled the margins of larger structures (Fig. 5, B and F). The association of TAP with GRAMP-92 was quantitated using projections of fluorescence micrographs.
After 30 min of hyperstimulation, 75% of TAP-positive structures demonstrated GRAMP-92 immunoreactivity, but after 45 and 60 min of hyperstimulation the number increased to 86 and 99%, respectively (Table 2). The size of structures demonstrating both TAP and GRAMP-92 immunoreactivity also changed with time (Fig. 6). Although TAP immunoreactivity predominantly localized in vesicles of <1 µm after 30 and 45 min of hyperstimulation, its distribution shifted to vacuoles >1 µm after 60 min of treatment. Notably, TAP was rarely found in vesicles of <1 µm after 60 min of hyperstimulation (Fig. 6). Although GRAMP-92-positive vacuoles of >1 µm were present after 30 min of hyperstimulation (and not found in the unstimulated or the physiologically stimulated conditions), only 3% contained TAP immunoreactivity; the value increased to 27 and 58% for 45 and 60 min, respectively. These findings demonstrate that the association of TAP with GRAMP-92 increases in a time-dependent manner. This association increases with the duration of hyperstimulation and is more likely to be observed when GRAMP-92 is associated with vacuoles >1 µm.

The distribution of TAP in GRAMP-92-positive structures was examined by immunoelectron microscopy.

Table 2. Quantitation of TAP- and GRAMP-92-immunoreactive structures in acinar cells

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<th>Physiological Stimulation (60 min)</th>
<th>Hyperstimulation</th>
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<tr>
<td></td>
<td>Control 30 min 45 min 60 min</td>
<td></td>
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<tr>
<td>GRAMP-92 vacuoles (≥1 µm)</td>
<td>0 44 88 136</td>
<td></td>
</tr>
<tr>
<td>TAP- and GRAMP-92-positive structures ≥1 µm</td>
<td>0 1 12 78</td>
<td></td>
</tr>
<tr>
<td>TAP- and GRAMP-92-positive structures &lt;1 µm</td>
<td>0 0 0.5 12 1</td>
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Values were determined by counting number of TAP- and granule membrane protein (GRAMP)-92-positive structures per 100 acinar cells from 2 or 3 rats for each condition.
using tissues that were embedded in Lowicryl and labeled with primary antibodies followed by colloidal gold coupled to protein A and secondary antibodies. This hydrophobic embedding medium provided much lower background labeling than labeling performed on frozen sections (not shown), but membranes are difficult to visualize. Similar to the immunofluorescence studies, there was virtually no labeling over zymogen granules (Fig. 7A), but labeling for both TAP and GRAMP-92 was detected over small irregular vesicles (Fig. 7, A and B) and at the margins of large vacuoles (Fig. 7C). These observations confirm those made using immunofluorescence.

A serine protease inhibitor blocks caerulein-induced generation of TAP and vacuole formation. To provide evidence that the generation of TAP is associated with protease activation, rats received the synthetic protease inhibitor FUT-175 (nafamostat mesylate) intravenously during the period of caerulein hyperstimulation (19). FUT-175 blocked the appearance of TAP induced by caerulein hyperstimulation and appeared to reduce the number of vacuoles (Fig. 8). To examine the effects of the protease inhibitor on vacuole formation in greater detail, tissues were examined by transmission electron microscopy. Acinar cells from the unstimulated control and after physiological stimulation with caerulein contained few cytoplasmic vacuoles (Fig. 8, A and B). In contrast, acinar cells from hyperstimulated pancreas demonstrated a time-dependent increase in the size and number of vacuoles (Fig. 9, C–E). However, FUT-175 pretreatment dramatically reduced the number and size of vacuoles generated by caerulein hyperstimulation (Fig. 9F and Table 3). Thus FUT-175 appears to block both TAP generation and vacuole formation induced by hyperstimulation.

DISCUSSION

There is compelling evidence that active enzymes are generated within the pancreas early in the course of
acute pancreatitis (15, 18, 30). However, the cellular site of this activation remains unclear. The present study uses antibodies that selectively detect the cleaved activation peptide from the NH₂ terminus of trypsinogen as a marker for the proteolytic processing of trypsinogen to trypsin. TAP immunoreactivity appears in acinar cell vesicles within 30 min of exogenous caerulein hyperstimulation. The number of vesicles, the size of the vesicles, and the number of cells that demonstrate TAP immunoreactivity increased with time. No TAP immunoreactivity was observed within the pancreatic duct during the treatment period. These observations suggest that trypsinogen is processed to trypsin within the acinar cell and may be retained in the acinar cell.

The major goal of this study was establishing that trypsinogen processing occurs in the pancreatic acinar cell by examining the distribution of TAP immunoreactivity using immunofluorescence. TAP immunoreactivity was concentrated in vesicles that were distributed in supranuclear regions of acinar cells that did not overlap with the zymogen granules. The appearance of TAP immunoreactivity within acinar cells after caerulein hyperstimulation has been reported (16). However, that study did not find that the TAP was localized to distinct cellular structures but found TAP to be diffusely distributed in the apical region of the acinar cell. The differences in TAP localization between that study and the present report are likely to be due to the method used to detect the antigen (a secondary antibody coupled to horseradish peroxidase vs. immunofluorescence), the duration of hyperstimulation (1–4 h vs. 30–60 min), and the method of fixation. The reaction products generated by the horseradish peroxidase-labeled probe can easily diffuse and result in a lower resolution than indirect immunofluorescence. Furthermore, we observed that glutaraldehyde must be included in the fixative to detect TAP in membrane-bound compartments. Presumably, glutaraldehyde fixation is required to limit the diffusion of the TAP. We also found that after 2 h of hyperstimulation TAP immunoreactivity increased diffusely in the acinar cell (not shown) and had a similar appearance to that reported for the other study (16). Although our study confirms that trypsinogen can be processed to trypsin in the pancreatic acinar cell, it suggests that this processing is initiated in distinct juxtanuclear structures and not at the apical pole of the acinar cell as reported (16). Our conclusion that TAP is generated in a distinct subcellular compartment is supported by a report that used immunoelectron microscopy to demonstrate that TAP generation in the caerulein hyperstimulation model is localized to cytoplasmic vesicles and not zymogen granules (9).

Although there were few TAP-positive structures after 30 min of hyperstimulation, the number increased steadily up to 60 min. These findings suggest that there

![Fig. 5. High-magnification micrograph demonstrating codistribution of TAP and GRAMP-92. Pancreatic sections from 45 or 60 min of caerulein hyperstimulation were double labeled for TAP (A, C, and E) and GRAMP-92 (B, D, and F). As shown in paired micrographs, TAP immunoreactivity was found within GRAMP-92-enriched structures with either irregularly labeled margins (B, F) or with a regular margin (D). Arrows indicate corresponding regions in paired micrographs. Original magnification, ×1,000.](http://ajpgi.physiology.org/ Downloaded from 10.220.33.5 on July 7, 2017)

![Fig. 6. Number of GRAMP-92-enriched structures with TAP increases in a time-dependent manner. After in vivo caerulein hyperstimulation and processing as described in Fig. 4, GRAMP-92- and TAP-containing structures were classified as either those with a diameter of <1 µm (open bars) or those with a diameter of ≥1 µm (solid bars). Values shown per 100 acinar cells were obtained from 2 or 3 separate experiments.](http://ajpgi.physiology.org/ Downloaded from 10.220.33.5 on July 7, 2017)
was a delay between acinar cell stimulation and the initiation of trypsinogen processing. The morphology of the TAP-containing structures changed during hyperstimulation. Initially, TAP localized to small vesicles (~1 µm). However, after 60 min of hyperstimulation most TAP was localized to structures that were ≥1 µm.

The time-dependent generation of TAP in distinct subcellular compartments has been suggested by another study. With the use of the caerulein hyperstimulation model and an ELISA to detect TAP, the marker has been found to increase within the pancreas after 15 min of hyperstimulation (18). With the use of subcellular fractionation of the pancreas, the study also demonstrated that TAP initially appeared in a light fraction soon after hyperstimulation and later became associated with a heavier fraction. This observation is consistent with the present immunofluorescence study, which demonstrates a transition of TAP immunoreactivity from smaller to larger vesicles as a function of time.

To further define the compartment associated with the primary trypsinogen processing, we performed double labeling studies. The membrane glycoprotein protein GRAMP-92 has been observed to be enriched in lysosomes and recycling endosomes in several tissues (13). In the pancreatic acinar cell, GRAMP-92 is reported to be associated with membranes in the region of, but not overlapping with, the Golgi complex (13). GRAMP-92 immunoreactivity has been found on small irregular vesicles that were located in close proximity to zymogen granules but not on the zymogen granule membrane. In the present study, caerulein hyperstimulation was associated with a redistribution of GRAMP-92 immunoreactivity from diffusely distributed small vesicles to vacuoles that were concentrated in a juxtanuclear region. Much of the TAP was distributed in vesicles that were enriched with GRAMP-92; over one-half of the TAP-positive vesicles demonstrated GRAMP-92 immunoreactivity after 30 min of hyperstimulation, and virtually all of the TAP-positive structures were also positive for GRAMP-92 after 60 min of hyperstimulation (Table 2). Because it may be difficult to detect GRAMP-92 labeling on small vesicles, the number that exhibited immunoreactivity for both TAP and GRAMP-92 may be underestimated. However, at the earliest time we can visualize TAP generation (30 min), the majority of the structures are associated with GRAMP-92 immunoreactivity. Therefore, it is likely that much of the initial TAP generation takes place in a GRAMP-92-positive compartment.

There was a notable time-dependent increase in the size of the structures with both TAP and GRAMP-92. The present study does not resolve the relationship between the small and large vesicular compartments. It is possible that they are independent structures and that TAP is processed in at least two distinct compartments. Alternatively, the larger compartment may be formed by fusion of the smaller vesicles. Since we observed a redistribution of GRAMP-92 and a decrease in the number of small GRAMP-92 vesicles, we favor the later mechanism for the appearance of the large vacuoles. Although GRAMP-92 is found throughout the acinar cell, most GRAMP-92-positive structures demonstrating TAP immunoreactivity localized to the juxtanuclear region. This finding suggests trypsinogen processing might take place in a subset of GRAMP-92-positive structures that have the specific requirements necessary to support trypsinogen processing.
The identity of the compartment(s) involved in generating TAP remains unknown. In another paper (7), we report that procarboxypeptidase A₁ can be proteolytically processed to carboxypeptidase A₁ in a secretory compartment of the pancreatic acinar cell. Because this event is likely to require active trypsin, trypsinogen may also be processed in this secretory compartment.

The absence of TAP immunoreactivity on zymogen granules suggests that trypsinogen processing must proceed in another compartment. In this context, there is evidence for several pools of secretory proteins. Acinar cell secretory proteins can be divided into those that are newly synthesized and those that come from a storage pool (2). The pools both undergo regulated

Fig. 8. Pretreatment with protease inhibitor FUT-175 reduces generation of TAP and formation of vacuoles with GRAMP-92. Fluorescence light micrographs of double labeling of anti-TAP antibody (A) and anti-GRAMP-92 antibody (B) followed by FITC- and TRITC-conjugated secondary antibodies and phase-contrast micrograph (C) after 60 min of FUT-175 (50 µg·kg⁻¹·h⁻¹) pretreatment and 60 min of caerulein hyperstimulation (5 µg/kg iv). Serine protease inhibitor blocked appearance of TAP induced by hyperstimulation. Additionally, it reduced formation of vacuoles associated with GRAMP-92. Original magnification, ×630.

Fig. 9. Hyperstimulation is associated with a time-dependent increase in cytoplasmic vacuoles that is blocked by protease inhibitor FUT-175. Electron micrographs from in vivo treatments: unstimulated control (A); 60 min caerulein (0.01 µg/kg iv) physiological stimulation (B); 30 (C), 45 (D), and 60 min (E) caerulein (5 µg/kg iv) hyperstimulation; and 60 min of FUT-175 (50 µg·kg⁻¹·h⁻¹) pretreatment and 60 min of caerulein hyperstimulation (F). Cytoplasmic vacuoles were generated after caerulein stimulation, and size and number increased in a time-dependent manner (C, D, and E). FUT-175 pretreatment prevented vacuole formation (F). Bar = 1 µm.
secretion, but the nascent pool is smaller, has higher basal secretion, and has different relative amounts ofzymogens than the storage pool (25). Although secretion from the storage pool comes from zymogen granules, electron microscopic autoradiography studies suggest that secretion of nascent proteins may come from vesicles that bud from immature zymogen granules (25). Additional studies will be required to determine whether zymogen processing and the generation of TAP takes place in an early or late synthetic pool and whether it can be detected in a secretory compartment.

Recent studies that examine the trafficking of lysosomal proteins may also be relevant to the identity of the compartments involved in trypsinogen processing. Although most of the TAP appears to be generated in a GRAMP-92-enriched compartment, the distribution of the membrane protein in the physiological state and during hyperstimulation has not been fully defined. Immunocytochemical studies suggest that GRAMP-92 is associated with lysosomes, endosomes, and small vesicles that are found at the margins of zymogen granules (25). A recent study has demonstrated codistribution of TAP and the lysosomal enzyme cathepsin B in the caerulein model of pancreatitis (9). Some lysosomal enzymes have been localized to a small vesicular compartment associated with margins of zymogen granules (8) that have a similar appearance to that enriched in the caerulein model of pancreatitis (9). These divergent results indicate that additional studies will be required to determine the enzymatic mechanism of trypsinogen activation in the acinar cell.

Similarly to its effect on TAP generation, the protease inhibitor FUT-175 blocked the generation of cytoplasmic vacuoles generated by caerulein hyperstimulation in vivo. Although the formation of cytoplasmic vacuoles in the pancreatic acinar cell is a feature of acute pancreatitis, the importance of these structures in the pathogenesis of the disease remains unclear. Previous studies had demonstrated that a serine protease inhibitor that is structurally related to FUT-175 blocks the generation of cytoplasmic vacuoles generated in isolated pancreatic acini by caerulein hyperstimulation (21). The relationship between inhibiting the generation of TAP and blocking the formation of cytoplasmic vacuoles in acinar cells by protease inhibitors remains unclear.

In summary, this study provides evidence that hyperstimulation is associated with the generation of TAP within the pancreatic acinar cell. Because a protease inhibitor blocked the appearance of TAP, its generation is very likely due to the proteolytic processing of trypsinogen to trypsin. Much of the TAP is distributed in a vesicular compartment with a marker for lysosomes and recycling endosomes, GRAMP-92. This sug-
gests that an early step in trypsinogen processing may take place within lysosomes or endosomes.

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