Intracellular Autoactivation of Human Cationic Trypsinogen Mutants Causes Reduced Trypsinogen Secretion and Acinar Cell Death⁎

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Mutations in the activation peptide of human cationic trypsinogen have been found in patients with chronic pancreatitis. Previous biochemical studies demonstrated that mutations p.D19A, p.D22G, and p.K23R strongly stimulate trypsinogen autoactivation. In the present study, we characterized the cell biological effects of these mutants using human embryonic kidney 293T and AR42J rat acinar cells. We found that relative to wild-type trypsinogen, secretion of the mutants from transfected cells was markedly decreased. This apparent secretion defect was completely rescued by inhibition of autoactivation via (1) inclusion of the small molecule trypsin inhibitor benzamidine in the growth medium; or (2) cotransfection with the physiological trypsin inhibitor SPINK1; or (3) by mutation of the catalytic Ser200 residue in trypsinogen. In contrast, extracellularly added SPINK1 or other nonpermeable proteinaceous trypsin inhibitors did not restore normal secretion of the mutants, indicating that intracellular autoactivation is responsible for the observed secretion loss. Acinar cells expressing the p.D22G mutant detached from the culture plate over time, became terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive, and exhibited elevated levels of the proapoptotic transcription factor CHOP. The observations indicate that activation peptide mutants of human cationic trypsinogen undergo autoactivation intracellularly, which leads to decreased trypsinogen secretion and eventual acinar cell death. The results thus define a novel pathological pathway for parenchymal injury in hereditary chronic pancreatitis.

Hereditary chronic pancreatitis is an autosomal dominant disorder associated with homozygous mutations in the serine protease 1 (PRSS1) gene, which codes for human cationic trypsinogen (1, 2). Approximately 70% of the PRSS1 mutation-positive cases are due to mutation p.R122H, ~25% are caused by p.N29I and about 4% are linked with p.A16V. Other rare and private mutations are found in the remaining cases (2 and references therein). An interesting subset of rare mutations affects the trypsinogen activation peptide; an eight-amino acid long sequence extending from Ala16 to Lys23 at the N terminus of trypsinogen (see Fig. 1) (3–5). Mammalian trypsinogen activation peptides contain a characteristic tetra-Asp motif before the Lys23-Ile24 peptide bond, which is cleaved during activation (5). This acidic stretch enhances proteolytic activation by the serine protease enteropeptidase (enterokinase), the physiological activator of trypsins in the duodenum (6). We found, however, that in the case of human cationic trypsinogen, the tetra-Asp sequence plays only a limited role in enteropeptidase recognition, but it is essential for suppression of trypsin-mediated trypsinogen activation, commonly referred to as autoactivation (7).

Compared with trypsinogens from other species, human cationic trypsinogen exhibits an unusually high propensity for autoactivation, which may result in an increased risk for pancreatitis in humans (8, 9). This notion is supported by a number of biochemical studies demonstrating that the majority of hereditary pancreatitis-associated mutations increase autoactivation of cationic trypsinogen (10–12, 5). The stimulatory effect is particularly strong in the case of the activation peptide mutations p.D19A, p.D22G, and p.K23R (Fig. 1) (5). The p.D19A and p.D22G mutations eliminate Asp residues from the tetra-Asp motif and thereby mitigate its inhibitory effect. The p.K23R mutation changes the P1 Lys residue to Arg, which is preferred by trypsin owing to a favorable electrostatic interaction in the specificity pocket of the protease. The plausible mechanistic basis for the strongly autoactivating phenotype of the activation peptide mutants provided compelling evidence that increased autoactivation of cationic trypsinogen is one of the fundamental pathological pathways in hereditary pancreatitis (8). Indeed, mutations p.N29I, p.N29T, p.R122C, and p.R122H were also found to stimulate autoactivation albeit to a lesser degree than the activation peptide mutations (10–12). Mutation p.A16V has no direct effect on autoactivation (13); however, it increases N-terminal processing of the activation peptide by chymotrypsin C, which, in turn, leads to increased autoactivation of cationic trypsinogen (14).

Despite our understanding of the mechanism of pancreatitis-associated trypsinogen mutations at the biochemical level, the potential cellular effects of increased trypsinogen autoactivation have remained unappreciated. In the present study, we investigated whether premature autoactivation can occur in the secretory pathway intracellularly in transiently transfected cells expressing the activation peptide mutants of cationic trypsinogen.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1–S3.

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Enteropetidase cathepsin B trypsin
N-Ala\textsuperscript{16}-Pro-Phe-Asp\textsuperscript{16}-Asp-Asp-Asp\textsuperscript{22}-Lys\textsuperscript{23}-Ile\textsuperscript{24}.

*p*A16V  p*D19A  p*D22G  p*K23R

**FIGURE 1.** Primary structure of the activation peptide in human cationic trypsinogen. Note that the N-terminal amino acid of trypsinogen is Ala\textsuperscript{16} because the first 15 amino acids of pretrypsinogen form the secretory signal peptide, which is removed in the endoplasmic reticulum. All known activators of trypsinogen (enteropetidase, cathepsin B, and trypsin) cleave the Lys\textsuperscript{23}-Ile\textsuperscript{24} peptide bond. The tetra-Asp motif is underlined, and the positions of the pancreatitis-associated mutations are indicated.

**EXPERIMENTAL PROCEDURES**

**Materials**—The AR42J cell line was purchased from ATCC (CRL-1492). Cell culture media and Lipofectamine 2000 transfection reagent were obtained from Invitrogen. Human SPINK1 was expressed in HEK 293T cells and purified from the conditioned medium using a bovine trypsin affinity column. Ecotin and human α1-antitrypsin were expressed in *Escherichia coli* and purified as described previously (15–17). Soybean trypsin inhibitor was from Fluka and was further purified on a bovine trypsin affinity column. Benzamidine and N-Cbz-Gly-Pro-Arg-p-nitroanilide\textsuperscript{2} were purchased from Sigma-Aldrich. The source of antibodies used for Western blotting is listed in supplemental Table S1.

**Plasmid Construction and Mutagenesis**—Eukaryotic expression plasmids pcDNA3.1(−) PRSS1; pcDNA3.1(−) PRSS1 with a Glu-Glu affinity tag at the C terminus; pcDNA3.1(−) PRSS1 p*A16V; pcDNA3.1(−) PRSS1 p*S200A and pcDNA3.1(−) SPINK1 were constructed previously (14, 18, 19). Construction of the pcDNA3.1(−) CPA1 plasmid will be reported elsewhere. Missense mutations p*D19A, p*D22G, and p*K23R were generated by overlap extension PCR mutagenesis and cloned in the pcDNA3.1(−) PRSS1 plasmid. Double mutants p*A16V-p*S200A, p*D19A-p*S200A, p*D22G-p*S200A, p*K23R-p*S200A, and the p*D22G mutant in the Glu-Glu tagged PRSS1 background were constructed by cut-and-paste using the Xhol-PfMI or the PfMI-BamHI restriction sites and the appropriate parent plasmids. Prokaryotic expression plasmids pTrapT7 PRSS1 carrying wild-type cationic trypsinogen or mutants p*D19A, p*D22G, or p*K23R were reported previously (5).

**Adenovirus Construction**—Recombinant adenovirus carrying wild-type human cationic trypsinogen or the p*D22G mutant was generated using the Adeno-X Expression Systems 2 (Clontech). First, the Glu-Glu-tagged versions of wild-type and p*D22G human cationic trypsinogen (PRSS1) were subcloned from the appropriate pcDNA3.1(−) plasmids into the pDNR-CMV donor vector using the XhoI and BamHI restriction sites. Donor vectors carrying trypsinogen inserts were then added to the Adeno-X LP Reaction Mix containing Cre recombinase and pLP-Adeno-X viral DNA. The mixture was incubated at room temperature for 15 min followed by heat inactivation at 70 °C for 5 min. Supercharge EZ10 ElectroCompetent *E. coli* cells (Clontech) were transformed with two μl of the reaction mix. Bacterial colonies harboring recombinant clones were identified by PCR using the Adeno-X LP primer mix and colony-screening protocol (Clontech). Positive colonies were used to inoculate 5 ml LB medium with ampicillin/chloramphenicol, which was incubated for 6–8 h at 37 °C and transferred to 100 ml LB with ampicillin/chloramphenicol, and bacteria were grown for 12 h at 37 °C. Plasmids carrying the wild-type and p*D22G cationic trypsinogen inserts were purified and plasmid integrity was verified by the characteristic banding pattern obtained after XhoI digestion. Infectious adenovirus particles were produced by transfecting HEK 293 cells in a 25-cm\textsuperscript{2} flask containing 5 ml of growth medium with 5 μg Pacl-digested, linear adenovirus DNA using the Lipofectamine method. To prevent the potentially harmful effects of trypsinogen activation, cells were cotransfected with 5 μg SPINK1 expression plasmid. Cells were harvested when ~50% of the cells became detached, typically after 3–4 days. Cells were collected by centrifugation, washed with phosphate-buffered saline (PBS) and resuspended in 500 μl PBS. Adenovirus particles were released by lysing the cells with three consecutive freeze-thaw cycles. Half of this primary lysate (250 μl) was then used to re-infect HEK 293 cells (pretransfected with 5 μg SPINK1 plasmid) in a 75-cm\textsuperscript{2} flask containing 15 ml of growth medium to obtain a higher titer secondary lysate. The virus was further amplified by repeating the re-infection procedure using the secondary lysate but without SPINK1 pretransfection. Finally, recombinant adenoviruses were purified with the Adeno-X virus mini purification kit (Clontech) and stored at −80 °C in aliquots. The infectious titer, expressed as plaque-forming units (pfu) per ml, was determined using the Adeno-X rapid titer kit (Clontech). Typical yields were 0.4 ml of purified adenovirus at 5 × 10\textsuperscript{8} pfu/ml concentration.

Adenovirus carrying the cDNA for the enhanced green fluorescent protein (GFP) was purchased from Virquest, Inc. (North Liberty, Iowa). Recombinant adenovirus with the human SPINK1 cDNA was a generous gift from Arul M. Chinnaiyan (University of Michigan, Ann Arbor, MI). For consistency, the GFP-adenovirus and SPINK1-adenovirus constructs were also re-amplified, purified, and titrated in our laboratory, as described above.

**Cell Culture and Transfection**—HEK 293T cells were cultured in six-well tissue culture plates (10\textsuperscript{5} cells per well) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin at 37 °C in 5% CO\textsubscript{2}. Transfections were performed using 4 μg expression plasmid and 10 μl Lipofectamine 2000 in 2 ml Dulbecco’s modified Eagle’s medium. After overnight incubation at 37 °C, cells were washed, and the transfection medium was replaced with 2 ml Opti-MEM I reduced serum medium. Where indicated, trypsin inhibitors were added to the Opti-MEM I medium. Time courses of expression were measured starting from this medium change and were followed for 24 h. AR42J rat pancreatic acinar cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal...
Intracellular Trypsinogen Autoactivation

bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin at 37 °C in 5% CO₂. Prior to transfection, cells were seeded in 24-well plates (2.5 × 10⁶ cells per well) and grown in the presence of 100 nM dexamethasone (final concentration) for 48 h to induce differentiation. Viral infections were performed using 10⁶, 3 × 10⁶, or 10⁷ pfu/ml final adenovirus concentrations in a total volume of 300 μl Opti-MEM I medium supplemented with 100 nM dexamethasone (final concentration). Time of trypsinogen expression was measured from the addition of adenovirus and conditioned medium and/or cells were harvested at 24 h after infection, unless indicated otherwise.

Preparation of Cell Lysates—Cells were washed twice with PBS. An aliquot (200 μl) of reporter lysis buffer (Promega, Madison, WI) and 4 μl protease inhibitor mixture (Sigma-Aldrich, P8340) were added to each well; the cells were scraped and briefly vortexed. For the analysis of eIF2α phosphorylation, 2 μl Halt phosphatase inhibitor mixture (Thermo Scientific, Waltham, MA; 78420) was also included. After a 15-min incubation at room temperature, the lysates were cleared by centrifugation. The protein concentration of the supernatant was measured with the MicroBCA protein assay kit (Thermo Scientific, Waltham, MA).

Western Blot Analysis—Aliquots of conditioned media (5 μl or 15 μl per lane, as indicated) or cell lysates (10 μg protein per lane) were electrophoresed on Tris-glycine mini-gels and transferred onto Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked with 5% milk powder solution at 4 °C overnight; and incubations with primary and secondary antibodies were performed at room temperature for 1 h. Horseradish peroxidase was detected using the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Primary and secondary antibodies and the working dilutions used are listed in supplemental Table S2.

Reverse Transcription-PCR Analysis—RNA was isolated from transfected cells using the RNeasy kit (Ambion, Austin, TX); 1 μg RNA was reverse-transcribed with M-MLV reverse transcriptase (Ambion) in 20 μl reaction volume, and 1 μl cDNA was used as template in PCR reactions (50 μl final volume). PCR amplicons were run on 2% agarose gels, and bands were visualized by ethidium bromide staining. Primer sequences are given in supplemental Table S2.

TUNEL Staining—TUNEL detection of DNA fragmentation was carried out using the In Situ Cell Death Detection kit (Roche Applied Science) according to the manufacturer’s instructions. Briefly, AR42J cells transfected with given adenovirus constructs were cultured in 24-well plates. At 24 h, after infection cells were harvested and fixed in suspension with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature, followed by a 2-min incubation with 0.1% Triton X-100 in 0.1% sodium citrate on ice to permeabilize the cells. TUNEL reagent (100 μl) was added to air dried cells and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. To visualize all nuclei, cells were then incubated with 4’,6-diamidino-2-phenylindole (2 μg/ml final concentration in PBS) for 5 min and washed twice with PBS. Cells were examined under a Nikon Eclipse TE 300 inverted microscope with a TE-FM epifluorescence attachment.

Trypsin Activity Measurements—Conditioned medium from HEK 293T cells (20 μl) or AR42J cells (10 μl) was supplemented with 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂ to 50 μl volume, and trypsinogen was activated with 1 μl 1.4 μg/ml human enteropeptidase (R&D Systems, Minneapolis, MN) for 1 h at 37 °C. Trypsin activity was then measured by adding 150 μl of the chromogenic substrate, N-Cbz-Gly-Pro-Arg-p-nitroanilide to 0.14 mM final concentration. One-min time courses of p-nitroaniline release were followed at 405 nm in 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂ at room temperature using a Spectramax Plus 384 microplate reader (Molecular Devices).

Expression and Purification of Human Cationic Trypsinogen—Human cationic trypsinogen and mutants p.D19A, p.D22G, and p.K23R were expressed in E. coli Rosetta (DE3) as cytoplasmic inclusion bodies. Refolding and purification of trypsinogen on immobilized ectoen was carried out as reported previously (5, 10, 11, 20).

RESULTS

Decreased Secretion of Activation Peptide Mutants from Transfected HEK 293T Cells—Initially, we performed experiments using the HEK 293T cell line, which can be efficiently transfected with expression plasmids and thus allows for rapid testing of several mutants. For this study, we selected the strongly autoactivating mutants p.D19A, p.D22G, and p.K23R and used p.A16V as a control mutant, which does not alter autoactivation (5, 13). Secretion of cationic trypsinogen in the growth medium was measured by activity assays, SDS-PAGE with Coomassie Blue staining, and Western blotting. Fig. 2, A and B, show that secretion of mutants p.D19A, p.D22G, and p.K23R was reduced to ~30% of the wild type, whereas mutant p.A16V was normally secreted. Decreased trypsinogen secretion was not the result of intracellular accumulation, as cell lysates also contained lower amounts of mutants p.D19A, p.D22G, and p.K23R (Fig. 2C).

Suppression of Trypsinogen Secretion Is Caused by Intracellular Autoactivation of the Activation Peptide Mutants—To determine whether the reduced secretion of the mutants was related to their autoactivation, we tested the effect of trypsin inhibition using three different approaches. First, we supplemented the growth medium with 1 mM benzamidine, a small, relatively cell-permeable molecule, which competitively inhibits trypsin (Kᵢ ~ 25 μM). Under these conditions, secretion of wild-type and mutant trypsinogens was comparable (Fig. 2D). Second, we cotransfected the cells expressing wild-type or mutant trypsinogens with SPINK1, also known as pancreatic secretory trypsin inhibitor, the physiological inhibitor of trypsin in the pancreas. Similarly to benzamidine, SPINK1 completely rescued the secretion loss of mutant trypsinogens (Fig. 2D). Finally, we made catalytically inactive versions of wild-type and mutant trypsinogens by mutating Ser²⁰⁰ (which corresponds to Ser¹⁹⁵ in classic crystallographic numbering). As shown by Fig. 2D, all p.S200A mutants were secreted to comparable levels, and mutations p.D19A, p.D22G, and p.K23R had no detrimental effect in this background. Importantly, this experiment also ruled out the possibility that the secretion loss might be related to
Intracellular Trypsinogen Autoactivation

FIGURE 2. Secretion of activation peptide mutants from transiently transfected HEK 293T cells and the effect of trypsin inhibitors. See under “Experimental Procedures” for details. Representative gels and blots of three independent experiments are shown. The arrow indicates the trypsinogen band. A, time course of trypsinogen secretion as measured by trypsin activity in the conditioned media after enteropeptidase activation. Activity was expressed as percentage of the 24 h wild-type trypsin activity, which corresponded to an ∼100 mOD/min reading under the assay conditions described under the “Experimental Procedures.” The average of three independent experiments is shown. For clarity, the error bars have been omitted; the S.E. was always within 15%. Note that the data points for the p.D22G and p.K23R mutants overlap. B, trypsinogen levels in the growth medium at 24 h after transfection. Condicioned medium (400 μl) was precipitated with trichloroacetic acid (10% final concentration) and analyzed by SDS-PAGE and Cooomassie Blue staining (upper panel). The identity of trypsinogen was confirmed by Western blotting (5 μl medium per lane) using an antibody against human cationic trypsinogen (R&D Systems; lower panel). C, trypsinogen levels in cell lysates at 24 h after transfection. Western blotting was performed on cell lysates (10 μg total protein per lane) using an antibody against human cationic trypsinogen (R&D Systems). Actin was measured as loading control. D, effect of benzamidine, cotransfected SPINK1, and mutation of the catalytic Ser200 on trypsinogen secretion, as judged by SDS-PAGE and Cooomassie Blue staining. E, effect of extracellularly added SPINK1 (0.2 μM final concentration) on trypsinogen secretion, assessed by SDS-PAGE and Cooomassie Blue staining (upper panel) and Western blotting (lower panel).

To confirm that trypsinogen autoactivation responsible for suppression of trypsinogen secretion occurred intracellularly and not extracellularly, we added SPINK1 trypsin inhibitor to the growth medium of HEK 293T cells expressing wild-type or mutant trypsinogens. We used 0.2 μM final inhibitor concentration, because we estimated the concentration of secreted wild-type cationic trypsinogen at or below 0.1 μM. As shown in Fig. 2E, SPINK1, a non-permeable, proteinaceous trypsin inhibitor, was unable to correct the decreased trypsinogen secretion. Using the p.D22G mutant as a test case, we confirmed this finding with two other proteinaceous trypsin inhibitors, ecotin and α1-antitrypsin (supplemental Fig. S1), and we concluded that extracellularly added nonpermeable trypsin inhibitors cannot rescue the suppressed secretion of trypsinogen mutants. Taken together, these results clearly establish that intracellular autoactivation of the mutants is responsible for the phenomenon of reduced trypsinogen secretion.

Intracellular Trypsin Activity in HEK 293T Cells Expressing the p.D22G Mutant—Autoactivation of trypsinogen should result in measurable trypsin activity. Surprisingly, however, we could not detect trypsin activity in the conditioned medium or lysates of cells expressing trypsinogen mutants using the high turnover substrate N-Cbz-Gly-Pro-Arg-p-nitroanilide at 140 μM final concentration (Km ~ 15 μM). A trypsin band was not visible on immunoblots either (Fig. 2, B and C). Using intact cells, we also tested the cell-permeable fluorescent trypsin substrate bis-(Cbz-Ile-Pro-Arg)-rhodamine 110 (BZiPar, Invitrogen, R6505), with similarly negative results. These observations suggest that trypsin generated through autoactivation of the mutants becomes rapidly degraded inside the cell.

To demonstrate that trypsin activity is present in the secretory pathway, even if transiently or at very low levels, we cotransfected wild-type cationic trypsinogen and the p.D22G mutant with human procarboxypeptidase A1 (pro-CPA1). Trypsin is the physiological activator of pro-CPA1, and it cleaves the Arg110-Ala111 peptide bond releasing the 94-amino acid long CPA1 activation peptide. As shown in Fig. 3, activated CPA1 was detected by Western blot in lysates of cells expressing the p.D22G mutant but not in lysates of cells transfected with wild-type trypsinogen. Cotransfection with SPINK1 abolished CPA1 activation, confirming the role of trypsin in the activation reaction.

misfolding and consequent degradation of the activation peptide mutants. This was of concern, because we recently identified two cationic trypsinogen mutants (p.R116C and p.C139S), which exhibited reduced secretion from HEK 293T cells due to misfolding and intracellular degradation (18).

To confirm that trypsinogen autoactivation responsible for suppression of trypsinogen secretion occurred intracellularly and not extracellularly, we added SPINK1 trypsin inhibitor to the growth medium of HEK 293T cells expressing wild-type or mutant trypsinogens. We used 0.2 μM final
Intracellular Trypsinogen Autoactivation

**Figure 3. Intracellular activation of pro-CPA1 in cells expressing the p.D22G trypsinogen mutant.** HEK 293T cells were transfected with wild-type cationic trypsinogen or mutant p.D22G and cotransfected with pro-CPA1 and SPINK1 expression plasmids, as indicated. Cells were harvested at 24 h after transfection, and cell lysates (10 μg total protein per lane) were analyzed by Western blotting using an antibody against the Glu-Glu affinity tag engineered at the C terminus of CPA1. A representative immunoblot of two independent experiments is shown.

**Cathepsin B Plays No Role in the Intracellular Activation of the Activation Peptide Mutants**—The lysosomal cysteine protease cathepsin B can also activate trypsinogens by cleaving the same Lys23-Ile24 peptide bond as enteropeptidase and trypsin (Fig. 1) (21, 22). Cathepsin B-mediated activation of human cationic trypsinogen has a pH optimum of ~4.0 in the test tube (22), suggesting that this reaction is unlikely to be significant in the mildly acidic secretory pathway. Nonetheless, to rule out the remote possibility that cathepsin B, and not autoactivation, might be responsible for the observed intracellular activation of the activation peptide mutants, we expressed and purified the mutants from *E. coli* and characterized their activation by cathepsin B in vitro at pH 4.0. As shown in *supplemental Fig. S2*, none of the mutants exhibited increased activation by cathepsin B compared with wild-type cationic trypsinogen. Thus, mutant p.D19A was activated as well as wild-type cationic trypsinogen, mutant p.K23R was activated at a slower rate, and, surprisingly, mutant p.D22G was completely resistant to activation by cathepsin B. Similar findings were reported from a previous study using model peptides analogous to the trypsinogen activation peptide (23). The results indicate that enhanced intracellular activation of the activation peptide mutants cannot be catalyzed by cathepsin B.

**Adenovirus-mediated Expression of Wild-type and p.D22G Mutant Cationic Trypsinogens in AR42J Rat Acinar Cells**—The experiments with HEK 293T cells indicated that the activation peptide mutants of human cationic trypsinogen undergo intracellular autoactivation, which results in reduced trypsinogen secretion. To confirm and extend these observations to a more relevant cell type, we transfected dexamethasone-differentiated AR42J rat pancreatic acinar cells (24–28). These cells are poorly transfected by the commonly used plasmid-based methods; therefore, we generated recombinant adenovirus constructs harboring wild-type cationic trypsinogen and a representative activation peptide mutant, p.D22G. Transfection efficiencies of AR42J cells with recombinant adenovirus were consistently in the 90–100% range. Fig. 4A demonstrates that infection of acinar cells with increasing virus concentrations resulted in proportionally increased secretion of trypsinogen into the growth medium. Because AR42J cells express endogenous rat trypsinogens, we detected human cationic trypsinogen by Western blotting using a Glu-Glu epitope tag engineered at the C terminus. In agreement with our previous experiments on HEK 293T cells, secretion of the p.D22G mutant from AR42J cells was diminished relative to the wild type, as judged by immunoblots and by activity assays of the conditioned medium (Fig. 4B). In cell lysates, levels of the p.D22G mutant were also significantly lower than those of wild-type cationic trypsinogen (Fig. 4C). The secretion defect of the p.D22G mutant was completely rescued by benzamidine or cotransfection with SPINK1, whereas extracellularly added SPINK1, ecotin, soybean trypsin inhibitor, or α1-antitrypsin were ineffective (Fig. 4D). Importantly, endogenous rat chymotrypsinogens were secreted comparably by cells expressing wild-type or p.D22G mutant human trypsinogens, indicating that suppressed secretion is not a generalized phenomenon but specific for the p.D22G mutant trypsinogen. These results on AR42J acinar cells are in complete agreement with those obtained on HEK 293T cells and confirm that intracellular autoactivation of activation peptide mutants results in decreased trypsinogen secretion.

**Intracellular Autoactivation of the p.D22G Mutant Causes Apoptotic Death via CHOP Induction in AR42J Cells**—We observed that after 24–32 h, AR42J cells transfected with p.D22G adenovirus became detached from the tissue culture plates, whereas cells transfected with wild-type trypsinogen or a control adenovirus carrying GFP remained attached to the plastic support (Fig. 5A). Cells expressing the p.D22G mutant were positively stained with the TUNEL assay, an indicator of apoptotic cell death (Fig. 5B). Cotransfection with SPINK1 protected cells from apoptosis and detachment, whereas SPINK1 added to the growth medium was ineffective, indicating that cell death is caused by intracellular autoactivation of trypsinogens (Fig. 6A).

We hypothesized that trypsinogen autoactivation might cause endoplasmic reticulum (ER) stress, which in turn can result in apoptosis (29–31). However, we found no difference in XBP1 splicing, BiP levels, eIF2α phosphorylation, or IκBα levels in cells transfected with wild-type trypsinogen or the p.D22G mutant (supplemental Fig. S3). On the other hand, mRNA levels of the ER stress-inducible proapoptotic transcription factor CHOP were significantly higher in cells expressing the p.D22G mutant (supplemental Fig. S3). Measuring the time course of CHOP expression in AR42J cells transfected with the p.D22G mutant revealed that elevated CHOP mRNA could be detected as early as 6 h after transfection; levels increased further by 12 h and remained high up to 24 h. (Fig. 6B). The early induction of CHOP suggests that this is a cause, rather than a consequence, of acinar cell death. Taken together, the results indicate that intracellular autoactivation of the p.D22G mutant causes apoptosis through CHOP induction via a mechanism that is unrelated to ER stress.

**DISCUSSION**

In the present study, we demonstrated that activation peptide mutants of human cationic trypsinogen that exhibit robust autoactivation in the test tube can undergo autoactivation inside the cell as well. Somewhat unexpectedly, we found that the most readily apparent indication of intracellular autoacti-
Intracellular Trypsinogen Autoactivation

We found it remarkable that at relatively low expression levels of the p.D22G mutant and without measurable trypsin activity, cells would suffer apoptosis. This observation suggests that in the normal pancreas, where human cationic trypsinogen is the most abundant secretory protein, even limited autoactivation of trypsinogens might have significant pathological consequences. Our data are in agreement with those of a recent study, in which an artificial trypsinogen construct with a furin-sensitive activation peptide was shown to undergo activation in the secretory pathway and cause apoptotic death of pancreatic acinar cells (34). An earlier report also suggested that expression of pancreatitis-associated trypsinogen mutants may decrease viability of transfected acinar cells; however, the inadequate transfection method used renders interpretation of these data difficult (35).

FIGURE 4. Secretion of the p.D22G activation peptide mutant from AR42J rat acinar cells and the effect of trypsin inhibitors. Dexamethasone-differentiated AR42J cells were transfected with recombinant adenovirus carrying wild-type cationic trypsinogen or the p.D22G mutant. The arrow indicates the trypsinogen band. A, trypsinogen levels in the growth medium at 24 h after infection as a function of virus concentration added. Conditioned media (15 μl per lane) were analyzed by Western blotting using an antibody against the Glu-Glu tag at the C terminus of trypsinogens. B, trypsin activity of conditioned media (10 μl) after enteropeptidase activation. Activity was expressed as percentage of the highest activity measured, which corresponded to ~100 μMOD/min reading. The average of three independent transfection experiments is shown with S.E. indicated. Statistical analysis was performed with the Tukey-Kramer multiple comparisons test. *, p < 0.05 and ***, p < 0.001. Note that the high trypsin activity measured even in the absence of transfection is due to endogeneous rat trypsinogens secreted by the AR42J cells. C, trypsinogen levels in cell lysates at 24 h after infection, as a function of virus concentration added. Cell lysates (10 μg total protein per lane) were analyzed by Western blotting using an antibody against the Glu-Glu tag at the C terminus of trypsinogens. D, effect of trypsin inhibitors on the secretion of the p.D22G mutant. AR42J cells were transfected with a final virus concentration of 3 × 10⁶ pfu/ml. The inhibitors were included in the growth medium at the indicated final concentrations. Cotransfection of SPINK1 was achieved with an adenovirus construct used at 3 × 10⁶ pfu/ml final concentration. Conditioned media collected at 24 h after infection were analyzed by Western blotting. Representative immunoblots of two or three independent experiments are shown. As a control for the general secretory capacity of AR42J cells, the secretion of endogenous chymotrypsinogens was measured. Conditioned medium (20 μl) was supplemented with 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂ to 50 μl volume, and chymotrypsinogens were activated with 40 nM human cationic trypsin (final concentration) for 1 h at 37 °C. Chymotrypsin activity was then measured at room temperature by adding 150 μl substrate (0.2 mM N-Suc-Ala-Ala-Pro-Phe-p-nitroaniline dissolved in 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂) and following 1-min time courses of p-nitroaniline release at 405 nm in a microplate reader.

viation was reduced secretion of mutant trypsinogens. This secretion loss was dependent on intracellular autoactivation, as trypsin inhibitors delivered intracellularly rescued the defect, while trypsin inhibitors applied extracellularly were ineffective. Misfolding as a potential reason for the decreased secretion was ruled out by the normal secretion pattern of the catalytically inactive p.S200A forms of the mutants. Because secretion of other proteins was unchanged, and active trypsin was not secreted, the most straightforward explanation for the reduced trypsinogen secretion is that intracellular autoactivation of trypsinogen is followed by rapid degradation of active trypsin. Consistent with this interpretation, direct demonstration of trypsin activity in cell lysates was unsuccessful, indicating that steady state trypsin concentrations inside the cells are very low. On the other hand, an indirect method using cotransfection of the natural trypsin substrate pro-CPA1 demonstrated convincingly that autoactivation of the p.D22G mutant generated transient trypsin activity in the secretory pathway intracellularly.

The lysosomal protease cathepsin B has been shown to activate trypsinogen to trypsin (21, 22), and an early event in experimental pancreatitis is the increased colocalization of cathepsin B and trypsinogen in autophagic vacuoles, which results in intracellular trypsin activation and cell injury (32, 33). We ruled out the possibility that cathepsin B might play a role in the intracellular activation of the trypsinogen mutants tested here by demonstrating that p.D22G is resistant to cathepsin B-mediated activation, whereas mutant p.K23R exhibits decreased activation, and mutant p.D19A is activated normally. These results are consistent with our previous findings, that none of the hereditary pancreatitis-associated trypsinogen mutants exhibits increased sensitivity to cathepsin B (22).

With longer culture times (24–36 h), AR42J cells transfected with the p.D22G activation peptide mutant exhibited apoptotic cell death accompanied by prominent induction of the proapoptotic transcription factor CHOP. Because CHOP is typically induced under conditions of ER stress (29–31), we evaluated several ER stress markers (XBP1 splicing, BiP levels, eIF2α phosphorylation, and 1αβα levels) but observed no appreciable alterations.

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Taken together with recent studies, the present results indicate that human cationic trypsinogen (PRSS1) mutants fall into three distinct categories with respect to their cell-biological phenotype. (1) The activation peptide mutants p.D19A, p.D22G, and p.K23R exhibit strong autoactivation in the test tube (5) and also undergo intracellular autoactivation in HEK 293T cells and AR42J acinar cells, which results in reduced trypsinogen secretion and acinar cell death (this study). (2) Mutants p.A16V, p.N29I, p.N29T, p.E79K, p.R122C, and p.R122H are secreted normally from transfected HEK 293T cells (18), indicating that intracellular autoactivation does not occur, even though some of these mutants (p.N29I, p.N29T, p.R122C, and p.R122H) exhibit moderately increased autoactivation in the test tube (10–12). Therefore, these mutants seem to cause pancreatitis by a different...
mechanism than the activation peptide mutants; however, additional studies are needed in acinar cells, which may be more sensitive to intracellular autoactivation. (3) Finally, mutants p.R116C and p.C139S suffer misfolding, intracellular retention, and degradation, which elicit ER stress in HEK 293T cells (18). ER stress can result in CHOP induction and apoptotic cell death (29–31). Thus, even though the pathological pathways associated with given cationic trypsinogen mutations may differ at the molecular level; CHOP induction and acinar cell death may be a common end point of mutation-induced pancreatic injury. This notion is consistent with the clinical observation that disease characteristics of hereditary chronic pancreatitis caused by the various PRSS1 mutations are essentially indistinguishable (2).

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