SH3 Binding Sites of ZG29p Mediate an Interaction with Amylase and Are Involved in Condensation−Sorting in the Exocrine Rat Pancreas†

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ABSTRACT: ZG29p, a novel pancreas-specific zymogen granule protein, has been proposed to act as a 'helper protein' in granule formation. To address its function in more detail, we searched for putative binding partners of ZG29p. In zymogen complexes isolated by nondenaturing isoelectric focusing, ZG29p was associated with a protein complex consisting of amylase and cationic trysinogen. Amylase also coeluted with ZG29p after immunoaffinity chromatography using an antibody to recombinant ZG29p. Cross-linking experiments with granule content proteins revealed a direct interaction between recombinant ZG29p and amylase. An interaction was also observed when purified amylase was used, whereas no interaction with recombinant or purified cationic trypsinogen was seen. ZG29p could also be cross-linked to three membrane proteins with molecular masses of 40, 18, and 16 kDa. The binding of ZG29p to amylase and to the membrane proteins was inhibited in the presence of synthetic peptides matching the consensus sequence of proline-rich SH3 binding sites present in ZG29p. The synthetic peptides could be cross-linked to amylase and to three yet unidentified acidic content proteins with molecular masses of about 30 kDa. The peptides also interacted with purified or recombinant amylase, but not with recombinant or purified cationic trypsinogen. In a condensation−sorting assay, the binding (sorting) of zymogen complexes to the granule membrane was reduced in the presence of the peptides. Our results indicate that the interaction of ZG29p with amylase is mediated by SH3 binding domains and that these domains are involved in the sorting of amylase to the granule membrane.

In endocrine and exocrine cells, secretory proteins are stored at high concentrations in secretory granules and are released in response to external stimuli. During the course of granule formation, proteins destined for storage and regulated secretion are segregated from those constitutively secreted (for review, see refs *1*−*3*). The specific milieu of the TGN,[1] e.g., a slightly acidic pH and a high Ca^{2+} concentration, triggers the initial step in granule formation, the selective aggregation of regulated secretory proteins into dense cores excluding constitutively secreted proteins. The condensing aggregates interact with components of the TGN membrane representing a second sorting event (*4*).

Granins are a family of proteins expressed in most endocrine and neuroendocrine cells that aggregate in a pH- and calcium-dependent manner (for reviews, see refs *5*, *6*). Granins have been proposed to act as 'helper proteins' which support aggregate formation and assist sorting of prohormones (*7*). Since granins and other secretory proteins exist as membrane-bound isoforms, it has been suggested that these isoforms interact in a homo- or heterophilic manner with soluble secretory proteins and thus function as 'nucleation receptors' during the process of aggregation. For granins and some other secretory proteins of endocrine cells, a disulfide-bonded loop at the N-terminus was identified as a sorting signal (*7*). However, in exocrine cells neither a common sorting signal nor a sorting receptor have been found so far.

In pancreatic acinar cells, approximately 20 different digestive enzymes are synthesized (*8*). Some of these enzymes form protein complexes within the lumen of the ER (*9*, *10*). These complexes have distinct protein compositions consisting mainly of certain zymogens in association with yet unidentified proteins (*9*). The complexes progressively aggregate in a pH-dependent manner and form dense core aggregates which are able to bind to the TGN membrane (*11*). It is therefore very likely that some of the unidentified proteins function as 'helper proteins' in complex formation, aggregation, and/or sorting to the membrane.

Recently, we reported on the cloning and expression of ZG29p, a novel pancreas-specific zymogen granule protein. ZG29p is associated with an amylase-containing protein complex (*12*), suggesting that ZG29p may act as a 'helper protein' in amylase complex formation and/or sorting. In pancreatic acinar cells, amylase is expressed in a tissue-specific manner and accounts for 30−35% of the total rate of enzyme synthesis in the rat pancreas (*13*). Mammalian α-amylases, which catalyze the hydrolysis of α-(1,4)-

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[1] Abbreviations: PPII, polyproline type II helix; RER, rough endoplasmic reticulum; SH3, src homology 3 domain binding motif; TGN, trans-Golgi network; ZG, zymogen granule(s); ZGC, zymogen granule content protein(s); ZGM, zymogen granule membrane.

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glucosidic linkages of starch components, glycogen, and various oligosaccharides (for review, see ref *14*), are present in pancreatic and salivary secretions and show a high degree of homology. Rat pancreatic amylase (55 kDa) shows a homolgy of 85% to porcine pancreatic amylase of which the tertiary structure has been solved (*15*−*17*). The enzyme consists of a large N-terminal domain divided into domains A and B, and of a small C-terminal region forming domain C (*15*, *16*). Domains A and B are in close contact, and the active site cleft is located between these two domains. Domain C is connected to domain A by a single polypeptide chain and forms a distinct globular unit which does not participate in substrate binding or hydrolysis (15).

ZG29p possesses a proline-rich amino acid stretch at positions 232−241 (LPLRPPPPAP) which exactly matches the consensus sequences of SH3 domain binding sites, XPXXPPPΦXP (*18*) or XpΦPpXP (*19*). A second amino acid stretch at positions 80−88 (HPRPPKPDP) shows a more limited overlap. SH3 domains are found in many intracellular signaling proteins, in cytoskeletal components, in enzymes, and in adaptor proteins (for review, see refs *20*−*22*). Although the sequences of SH3 domains are in general widely divergent, their three-dimensional structures are similar and are proposed to be protein modules mediating protein−protein interactions. SH3 binding sites are proline-rich motifs of approximately 10 amino acids which adopt a left-handed polyproline type II helix (PPII) with 3 residues per turn and bind to a hydrophobic pocket formed by conserved aromatic residues of the SH3 domain (*18*, *19*, *23*).

In the present study, we have investigated the interaction of ZG29p with amylase in more detail, focusing in particular on the role of proline-rich domains resembling SH3 domain binding sites. Using several different approaches, we show that ZG29p is part of a complex consisting mainly of amylase and cationic trypsinogen. Cross-linking experiments indicate that ZG29p directly interacts with amylase via its SH3 domain binding sites. We propose that these domains are involved in the sorting of zymogens to the granule membrane.

MATERIALS AND METHODS

Isolation of Zymogen Granules. Zymogen granules were isolated as described (*11*). Briefly, male Wistar rats (Charles River, Sulzfeld, Germany) weighing 200−230 g were fasted overnight and killed by exsanguination. The pancreas was removed, homogenized in 9 volumes of ice-cold homogenization buffer [0.25 M sucrose, 5 mM 2-*N*-morpholinoethanesulfonic acid, pH 6.25, 0.1 mM $MgSO_4$, 1 mM dithiothreitol, 10 μM Foy-305 (Sanol Schwarz, Monheim, Germany), 2.5 mM Trasylol (Bayer, Leverkusen, Germany), 0.1 mM phenylmethylsulfonyl fluoride] using a brendle type homogenizer (Thomas, Philadelphia, PA), and homogenates were centrifuged at 500*g* for 10 min. The resulting postnuclear supernatant (PNS) was further centrifuged at 2000*g* for 10 min. The brownish layer of mitochondria on top of the white pellet composed of zymogen granules (ZG) was removed. ZG were resuspended in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 8.0, and lysed by three cycles of freezing and thawing. The zymogen granule membrane (ZGM) was separated from the soluble zymogen granule content proteins (ZGC) by ultracentrifugation (100000*g* for 30 min), resuspended in 50 mM HEPES, pH 8.0, and stored at −20 °C.

Glycogen Precipitation of Amylase. Amylase was specifically precipitated and separated from other ZGC proteins as an insoluble glycogen−enzyme complex in 40% ethanol according to Loyter and Schramm (*24*).

Immunoblotting. Protein samples were separated by SDS−PAGE according to Laemmli (*25*), transferred to nitrocellulose using a semi-dry apparatus, and analyzed by immunoblotting (*26*). Immunoblots were processed using either enhanced chemiluminescence reagents (Amersham Corp., Arlington Heights, IL) or diaminobenzidine (DAB) reagent (0.05% DAB, 0.04% $CoCl_3$, 0.05% H_2O_2 in 10 mM Tris-HCl, pH 7.5).

Antibodies. Antibodies to purified pig amylase were raised in rabbits; pc Ab ZG29 to recombinant rat ZG29p is described in (*12*). Species-specific anti-IgG antibodies conjugated to HRP were obtained from BioRad (Richmond, CA).

Affinity Chromatography. For immunoaffinity chromatography, an IgG fraction was prepared from 3 mL of dialyzed antiserum directed against recombinant ZG29p (*12*) using a DEAE-Affi-Gel Blue gel (BioRad). The antibody was covalently bound to a solid agarose matrix (Affi-Gel Hz column) via the carbohydrate moieties at the F_c part of the antibody according to the manufacturer's instructions (BioRad). Two milligrams of ZGC was applied to the affinity column which was then washed 2 times with 50 mM HEPES, pH 8.0, and 2 times with 0.15 M NaCl in HEPES buffer; 0.2 M glycine, pH 2.5, was used for elution.

Isoelectric Focusing of Native Zymogen Complexes and 2D Gel Electrophoresis. For isolelectric focusing of native protein complexes, 0.1 g of IsoGel agarose (FMC BioProducts, Maine) and 1 g of D-sorbitol were completely dispersed in 8 mL of distilled water and heated in a microwave oven. The gel solution was cooled to 60 °C, ampholytes (pH 3.5−11.5) were added to a final concentration of 3%, and the gels were casted on GelBond (FMC BioProducts). Gels were allowed to cool and then chilled at 4 °C for at least 1 h. Protein samples (100−200 μg) were directly applied to the gels; 0.5 M acetic acid and 1 M NaOH were used as anolyte and catholyte, and focusing was done for 2 h at 10 W and 500 V. Gels were stained with Coomassie Brillant Blue solution (40% methanol, 10% acetic acid, 0.25% Coomassie Brillant Blue R-250) and destained in 20% methanol/10% acetic acid. Separation of proteins by 2D gel electrophoresis was according to Scheele (*27*) under denaturing conditions using 8 M urea for isoelectric focusing.

Production of Recombinant Proteins. Recombinant ZG29p was produced as previously described (*12*). The following primer sequences were used to amplify either the complete coding sequence of rat amylase (1−496) or a truncated amylase (1−421) by PCR: 5′ACAGGATCCTATGACCCACACACTGCGGATG3′ (forward primer ramyl.up); as reverse primer either 5′ACAAAGCTTACAACTTTGAGTCGGCATGGATTGC3′ (full length) or 5′ACAAAGCTTCTGCTAAAAGCCACTTGGTTGC3′ (partial) were used (the underlined sequences show those sequences introducing restriction sites into the PCR products). To amplify rat cationic trypsinogen (residues 23−246) 5′TTGCATGCGACAAGATTGTTGGAGGCTACACC3′ was used as forward primer while 5′CAAAGCTTGGCAGCGACGGTCTGCT-

GAATCC3′ was used as reverse primer. Using the restriction sites at the ends of the PCR products, the cDNAs were cloned in-frame with the 6xHIS-tag into the pQE vector (Quiagen, Hilden, Germany). Induction of expression and purification of recombinant proteins under denaturing conditions were according to the manufacturer's instructions.

Cross-Linking. Recombinant proteins were desalted before use. Purified pig amylase and bovine trypsinogen were purchased from Sigma. The proline-rich peptides RQSQAL-PLRPPPPAPVNDEPC (peptide I, residues 227−246 of ZG29p plus an additional cysteine) and ETHPRPPKPD-PVKSSSSC (peptide II, residues 78−94 of ZG29p plus an additional cysteine) were chemically synthesized (Institute for Molecular Biology and Tumor Research, Marburg, Germany). Similar results were obtained when cross-linking experiments were performed with peptide I, peptide II, or a mixture of both. One milligram of the recombinant proteins was incubated with 0.6 mg of the trifunctional cross-linker sulfo-SBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)hexanoamido]ethyl-1,3′-dithiopropionate; Pierce, Rockford, IL) at room temperature for 30 min in the dark in order to couple the cross-linker to the protein via the *N*-hydroxysuccinimide ester group. One milligram of the peptides was incubated with 0.5 mM of the cross-linker SASD [sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate; Pierce] which was radioiodinated using IODO-GEN (Pierce) and $Na^{125}I$ according to the manufacturer's instructions. To remove unbound cross-linker, the reaction mixture was applied to a desalting column (PD-10; Amersham Pharmacia Biotech, Freiburg, Germany). Then 100 μg of protein carrying the cross-linker was added either to 2 mg of ZGC or to ZGM (corresponding to 100 μg of protein) in a volume of 400 μL of 50 mM HEPES, pH 8.0. After incubation for 30 min in the dark, the samples were split, and the pH was shifted to either 7.5 or 5.9 by addition of an equal volume of 0.1 M MES, pH 6.75 or 4.7, respectively. After incubation at room temperature for 2 h, the samples were exposed to UV light (265 nm) for 15 min to cross-link interacting proteins to the photoactivated aryl acid group of the cross-linkers. Samples were subjected to SDS−PAGE under reducing conditions which led to cleavage of the disulfide bridge within the cross-linkers leaving the biotin moiety of the sulfo-SBED or the radiolabel of SASD at the binding partner. For detection of biotin, the proteins were blotted to nitrocellulose membranes, incubated with NeutrAvidin (Pierce) according to the manufacturer's instructions, and processed using SuperSignal Ultra (Pierce, Rockford, IL). For detection of the radiolabel, Coomassie-stained gels or protein blots were subjected to autoradiography.

In Vitro Condensation−Sorting Assay. ZGC was adjusted to a protein concentration of 5 μg/μL by dilution with 50 mM HEPES, pH 8.0, and then prespun at 13000*g* at room temperature for 10 min as described recently (*11*). To 200 μL of this sample were added 1 million counts of [^{35}S]-methionine/cysteine-labeled ZGC as a tracer, and the pH was adjusted to 5.9 by adding the same volume of 100 mM MES, pH 4.7. To study sorting, 100 μg of ZGM was applied. The final protein concentration in the assay was 2.5 μg/μL; 100−200 μg of synthetic peptides (see *Cross-Linking*) was added to study their effect on condensation and sorting of zymogens. After incubation under rotation for 2 h at room temperature, the mixture was centrifuged at 13000*g* for 30 min. The resulting supernatants were further centrifuged at 400000*g*. All pellets were resuspended in HEPES buffer, and condensation−sorting was quantitated by liquid scintillation counting (Raytest, Straubenhardt, Germany).

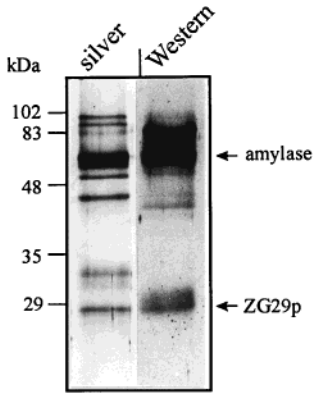

FIGURE 1: Immunoaffinity chromatography of ZGC proteins using an antibody to ZG29p. A polyclonal antibody to recombinant ZG29p was coupled to the resin of an affinity column, and ZGC proteins were applied. Bound proteins were eluted with 0.2 M glycine, pH 2.5. The eluate was analyzed by silver staining (lane 1) and immunoblotting (lane 2) using a mixture of antibodies directed against purified amylase and recombinant ZG29p. The arrows indicate the positions of amylase and ZG29p.

RESULTS

ZG29p Is Associated with an Amylase-Containing Protein Complex. To identify binding partners of ZG29p among the soluble proteins of ZGC, immunoaffinity chromatography was performed using a polyclonal antibody to recombinant ZG29p. Soluble ZGC proteins were applied to the affinity column, and bound proteins were eluted at pH 2.5 and separated by SDS−PAGE. Silver staining of the eluate revealed a prominent protein band of about 55 kDa coeluting with ZG29p (Figure 1, lane 1) which was recognized by a monospecific antibody to pig amylase after immunoblotting (Figure 1, lane 2). In addition to amylase, other minor proteins were eluted (Figure 1, lane 1), including ZG29p which was identified by immunoblotting (Figure 1, lane 2). Coelution of amylase and ZG29p was also obtained when recombinant ZG29p carrying a HIS-tag was incubated with ZGC proteins and purified by Ni−NTA (data not shown).

Recently, we reported on the isolation of five distinct zymogen complexes from pancreatic acinar cells using nondenaturing agarose gel electrophoresis (*9*). Amylase was found in only one of these complexes, in conjunction with the cationic form of trypsinogen and other minor cationic proteins. When the native complex was isolated and analyzed by immunoblotting following electrophoresis under denaturing conditions, ZG29p was also found in this complex (*12*). The buffer conditions in the nondenaturing agarose gel electrophoresis system could have potentially disrupted interactions between ZG29p and other proteins. To exclude this possibility, we performed native isoelectric focusing. This technique allowed the separation of protein complexes due to their net charge in the absence of a running buffer and revealed a more refined separation of ZGC in a variety of different protein complexes (Figure 2a). The amylase-containing complex (Figure 2a, arrow) was isolated and further analyzed under denaturing conditions by 1D and 2D

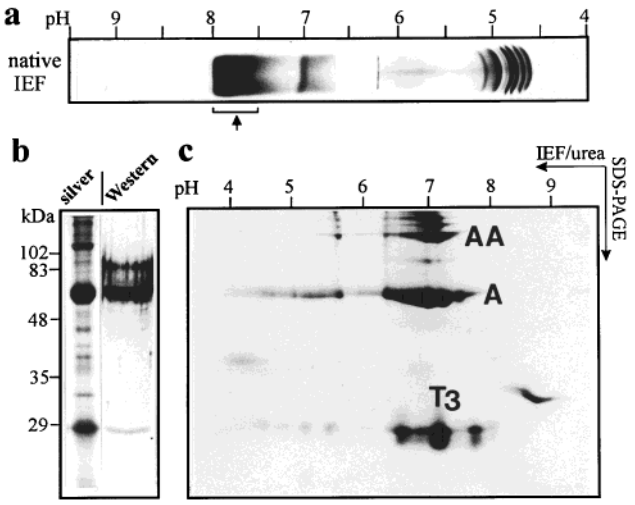

FIGURE 2: Isolation and separation of a zymogen complex containing amylase and ZG29p. (a) Content proteins were subjected to isoelectric focusing in IsoGel agarose under nondenaturing conditions, and protein complexes were stained by Coomassie brilliant blue. (b) The complex with a p*I* of 7.5−8.0 (see arrow in 2a) was cut out and separated by SDS−PAGE on a 12.5% acrylamide gel. Proteins were silver stained (lane 1) or analyzed by immunoblotting using a mixture of antibodies directed against purified amylase and recombinant ZG29p (lane 2). (c) The isolated complex was separated by 2D gel electrophoresis under denaturing conditions and silver stained (A = amylase; AA = amylase aggregates; T3 = cationic form of trypsinogen).

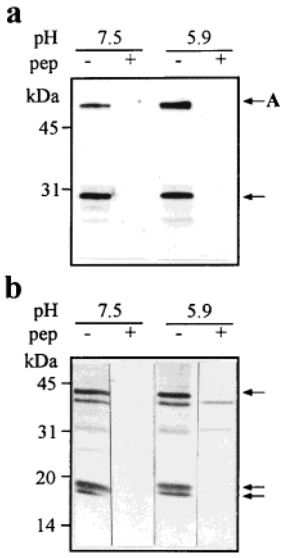

FIGURE 3: Cross-linking of ZG29p to ZGC and ZGM proteins. The trifunctional cross-linker sulfo-SBED was coupled to recombinant ZG29p and incubated either with ZGC proteins (a) or with isolated ZGM (b) in the absence (−) or presence (+) of synthetic peptides matching the consensus sequence of SH3 binding sites (pep). The pH was adjusted to 7.5 or 5.9. Cross-linked samples were separated on 12.5% (a) or 15% (b) acrylamide gels and blotted to nitrocellulose. Biotinylated proteins were detected by ECL using streptavidin−peroxidase. The major protein bands are labeled by arrows. A = amylase.

gel electrophoresis (Figure 2b,c). Silver staining revealed that the isolated complex consisted of different proteins (Figure 2b, lane 1) with two prominent protein bands at 55 and 30 kDa. Immunoblotting showed that ZG29p and amylase were present in the complex (Figure 2b, lane 2). 2D gel electrophoresis followed by silver staining indicated that according to the molecular weights and the isoelectric points of the protein spots, amylase as well as the cationic form of trypsinogen were the major constituents of the complex (Figure 2c, see also *9*). In addition, some yet unidentified protein spots with an alkaline p*I* were detected to be part of the complex.

Cross-Linking Reveals an Interaction of ZG29p with Amylase Which Is Mediated by SH3 Binding Sites. (A) Cross-Linking Studies with Recombinant ZG29p. To identify the direct binding partner(s) of ZG29p, cross-linking experiments with recombinant ZG29p carrying the trifunctional cross-linker sulfo-SBED were performed. Sulfo-SBED-labeled ZG29p was incubated with ZGC proteins (Figure 3a) or with ZGM (Figure 3b) at pH values of 5.9 or 7.5 to mimic the slightly acidic milieu of the TGN or the conditions found in the pre-Golgi compartments. The slightly acidic pH of 5.9 triggers the selective aggregation of zymogens in vitro, leading to the formation of dense cores (*11*). Samples were analyzed by SDS−PAGE and immunoblotting (Figure 3a,b). Under reducing conditions, the disulfide bridge of the cross-linker was cleaved, and the biotin moiety remained attached to interacting proteins which were detected by ECL using streptavidin-conjugated peroxidase. Cross-linking in the presence of ZGC proteins revealed predominantly two biotinylated protein bands with molecular masses of 55 and 30 kDa (Figure 3a, lanes 1 and 3) which were characteristic of amylase and trypsinogen.

When the cross-linking was carried out in the presence of isolated ZGM (Figure 3b), three putative binding partners with molecular masses of 16−19 kDa and 40 kDa were detected. In all cases, the cross-linking reaction appeared to be pH-independent and could be inhibited completely by the addition of ZG29p lacking the cross-linker (not shown), supporting the specificity of the interactions.

To test whether the putative SH3 binding sites of ZG29p were involved in the interactions with its binding partners, cross-linking experiments with ZGC and ZGM were performed in the presence of synthetic peptides which exactly matched the SH3 binding sites of ZG29p. Addition of the peptides inhibited the cross-linking of ZG29p to the ZGC proteins (Figure 3a, lanes 2 and 4) and to the proteins at the ZGM (Figure 3b, lanes 2 and 4). In the presence of unrelated control peptides, cross-linking was not influenced (not shown). These data indicate that proline-rich peptides compete with ZG29p for its binding partners, and that ZG29p apparently interacts with its ligands via SH3 domain binding sites.

The results obtained by cross-linking using a ZGC fraction suggested that according to their molecular mass amylase as well as cationic trypsinogen were the binding partners of ZG29. To examine whether both proteins interacted directly with ZG29p, cross-linking experiments with sulfo-SEBD-labeled ZG29p and commercially available, purified amylase or cationic trypsinogen from porcine and bovine pancreas were performed. Interestingly, purified trypsinogen was not cross-linked to ZG29p (Figure 4, lanes 1 and 3), whereas cross-linking with purified amylase revealed strong labeling with a slight increase in intensity at pH 5.9 (Figure 4, lanes 2 and 4). When recombinant cationic trypsinogen from rat was used instead of bovine trypsinogen, similar results were obtained (not shown).

(B) Cross-Linking Studies with a Synthetic Peptide Matching the Consensus Sequence of SH3 Binding Sites. Our data

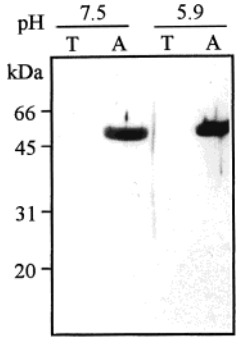

FIGURE 4: Cross-linking of ZG29p to purified amylase. The cross-linker sulfo-SBED was coupled to recombinant ZG29p and incubated either with purified pig amylase (A) or with bovine trypsinogen (T) at a pH of 7.5 or 5.9. Cross-linked samples were separated on 12.5% acrylamide gels. Biotinylated proteins were blotted and detected as in Figure 3.

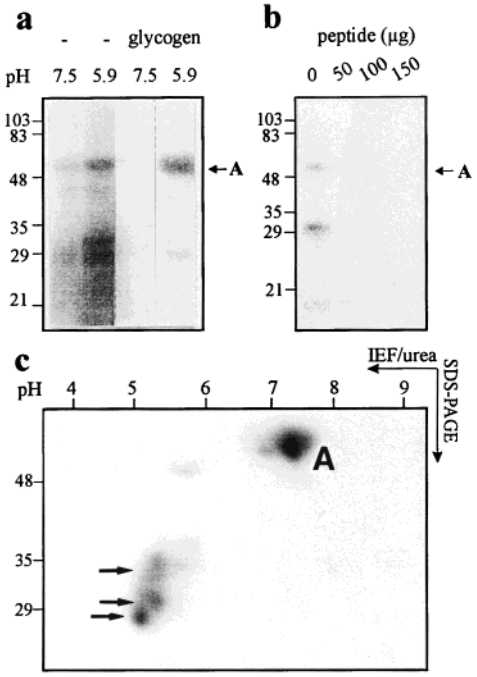

FIGURE 5: Cross-linking of synthetic peptides matching the SH3 binding sites of ZG29p to ZGC proteins. The iodinated cross-linker SADS was coupled to synthetic peptides overlapping with the SH3 binding sites of ZG29p. (a) SADS-labeled peptides were incubated with ZGC proteins at a pH of 7.5 or 5.9. After cross-linking, amylase was specifically precipitated in half of the samples by the addition of glycogen. Samples were separated on 12.5% acrylamide gels, blotted to nitrocellulose, and analyzed by autoradiograpy. (b) SASD-labeled peptides were incubated with ZGC proteins at pH 5.9 in the presence of increasing amounts of unlabeled peptides to compete for binding. Samples were analyzed as above. (c) Cross-linked ZGC proteins corresponding to Figure 5, lane 2 were separated by 2D gel electrophoresis under denaturing conditions and analyzed as above. Autoradiographs of the blots are shown. Arrows in (c) indicate the postions of unidentified acidic content proteins. A = amylase.

imply that amylase, but not cationic trypsinogen, is the direct binding partner of ZG29p. To verify this notion and to analyze the interaction of SH3 binding sites with ZGC proteins directly, synthetic peptides matching the SH3 binding sites of ZG29p were coupled to the radioiodinated cross-linker SASD and incubated with ZGC proteins at pH 5.9 or pH 7.5 (Figure 5a). After cross-linking, amylase was specifically precipitated in half of the samples by the addition

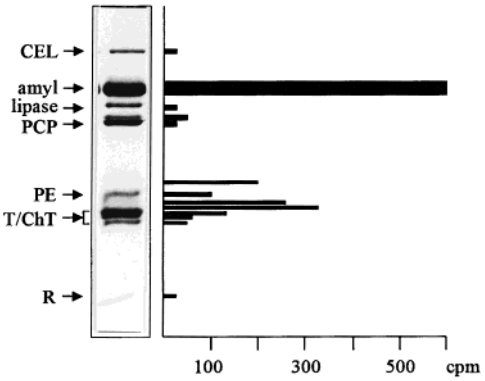

FIGURE 6: Determination of the protein-bound radioactivity of ZGC proteins after cross-linking to SASD-labeled synthetic peptides matching the SH3 binding sites of ZG29p. Cross-linking of ZGC proteins was performed at pH 5.9. Proteins were separated on 12.5% acrylamide gels and stained with Coomassie brilliant blue. Stained protein bands representing the major zymogens carboxy ester lipase (CEL), amylase (amyl), lipase, procarboxypeptidases (PCP), proelastase (PE), trypsinogens (T), chymotrypsinogens (ChT), and Rnase (R) were cut out and counted in a γ-counter.

of glycogen according to Loyter and Schramm (*24*), and samples were analyzed by SDS−PAGE and autoradiography. Two protein bands with molecular masses of 55 kDa and approximately 25−35 kDa were radiolabeled at both pH conditions, but signals obtained at pH 5.9 were more pronounced (Figure 5a, lanes 1 and 2). After glycogen precipitation of amylase, predominantly one radiolabeled protein of 55 kDa was detected, indicating that the synthetic peptides had bound directly to amylase (Figure 5a, lanes 3 and 4). Cross-linking was inhibited by the addition of increasing amounts of synthetic peptides lacking the cross-linker, indicating that the interactions were competable and specific (Figure 5b). When synthetic peptides were used which resembled but did not exactly match the corresponding SH3 binding sites, we obtained very similar results, whereas experiments with several unrelated peptides did not reveal any cross-linking (not shown).

To achieve a refined separation, ZGC proteins which were incubated with the SASD-labeled peptides at a pH of 5.9 and cross-linked afterward (see Figure 5a, lane 2) were separated by 2D gel electrophoresis (Figure 5c). Besides amylase (Figure 5c, A), several radiolabeled spots corresponding to yet unknown acidic proteins with molecular masses of 25−35 kDa were observed (Figure 5c, arrows). However, radiolabeled cationic trypsinogen could not be detected (compare to Figure 2c), suggesting that the synthetic peptides interacted mainly with amylase. Consistent with this, quantitation of the bound radioactivity of the major Coomassie-stained protein bands of the sample after SDS−PAGE showed that the highest amount of radioactivity was measured in the 55 kDa protein band corresponding to amylase, while the protein bands corresponding to other major ZGC proteins contained low amounts (Figure 6). We assume that the radiolabeled acidic proteins shown in Figure 5c do not correspond to one of the major ZGC proteins.

Cross-linking experiments confirmed that as for full-length ZG29p, SASD-labeled synthetic peptides were unable to directly bind to trypsinogen. When cross-linking with the SASD-labeled synthetic peptides was performed in the presence of purified bovine trypsinogen (Figure 7, lane 1) or recombinant cationic trypsinogen from rat (Figure 7, lane

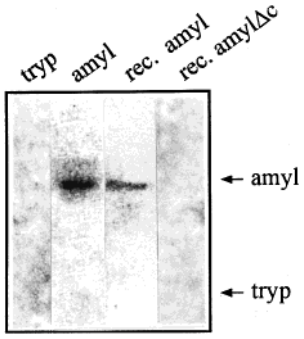

FIGURE 7: Cross-linking of synthetic peptides matching the SH3 binding sites of ZG29p to purified or recombinant amylase or trypsinogen. The iodinated cross-linker SADS was coupled to synthetic peptides overlapping with the SH3 binding sites of ZG29p. SADS-labeled peptides were incubated with recombinant cationic trypsinogen (tryp), purified pig amylase, recombinant full-length amylase, or a truncated form of amylase lacking domaine C (amylΔC) at a pH of 5.9. Cross-linked samples were analyzed as mentioned in Figure 5. An autoradiograph of the blot is shown.

2), no radiolabeling of trypsinogen was observed. However, purified amylase and recombinant full-length amylase from rat showed strong labeling (Figure 7, lanes 3 and 4). When a truncated form of amylase missing most of domain C was used, cross-linking was omitted (Figure 7, lane 5).

ZG29p Is Involved in Condensation−Sorting of Zymogens. Our cross-linking experiments indicate that SH3 binding sites mediate the interaction of ZG29p with amylase and other unknown ZG proteins, suggesting that these binding sites might be involved in aggregation of zymogens into dense cores and/or in binding of dense cores to membrane components. Using a previously established assay (*11*), the influence of the synthetic peptides overlapping with SH3 binding sites on the condensation/aggregation of zymogens and on sorting of zymogen complexes to the membrane was analyzed. ZGC proteins were mixed with radiolabeled tracer proteins as well as ZGM and incubated in the absence or presence of the peptides at a pH of 5.9. After incubation, the samples were subjected to centrifugation at 13000*g* (low-speed pellet), and the resulting supernatants were centrifuged at 400000*g* (high-speed pellet). The radioactivity was determined in the low- and high-speed pellets. The sedimentable radioactivity in the low-speed pellets was indicative for the amount of aggregated zymogens bound to the granule membrane, while a loss of radioactivity measured in the high-speed pellets reflected an interference with the formation of dense core aggregates (see also *11*). The radioactivity determined in the low-speed pellets was reduced by 40% compared to control conditions (Table 1) in the presence of synthetic peptides. A reduction in the amount of sedimented ZGC proteins was further confirmed by SDS−PAGE and silver staining (not shown). However, a neglectable loss of radioactivity (<3%) was observed in the high-speed pellets (Table 1). These results indicate that SH3 binding sites mediate the association of aggregated zymogens with the ZG membrane, and are not primarily involved in the process of zymogen aggregation.

Table 1: Condensation−Sorting Assay[a]

	% of cpm in pellet fractions		
pellet fraction ZGC+ZGM, pH 5.9	control	+peptide (100 μg)	+peptide (200 μg)
13000*g*	100	60.3 ± 3.9	55 ± 5.5
400000*g*	100	97.5 ± 2.2	98 ± 3.1

[a]Condensation−sorting in the presence of synthetic peptides matching the SH3 binding sites of ZG29p. Radiolabeled ZGC proteins and ZGM were incubated at a pH of 5.9 in the absence (control) or presence of synthetic peptides (+peptide). After incubation, the samples were centrifuged at 13000*g* (low-speed pellet). The corresponding supernatants were further centrifuged at 400000*g* (high-speed pellet). Sedimentable radioactivity was determined in the pellet fractions and compared to controls. Controls performed without peptides or in the presence of unrelated peptides gave similar results. When unrelated instead of proline-rich peptides were used under the described experimental conditions, condensation-sorting was not affected. The data are from three independent experiments.

DISCUSSION

In this paper, we provided evidence that ZG29p, a putative 'helper protein' in granule formation, is associated with amylase in a protein complex. The interaction of the proteins is mediated by SH3 binding sites present in ZG29p. SH3 binding domains therefore may play a general role in the sorting and/or condensation of zymogens in the pancreas.

SH3 Domain-Mediated Binding of ZG29p to Amylase. In exocrine cells, such as acinar cells of the pancreas, the molecular mechanisms of aggregate formation of zymogens and the binding of the granule core to the membrane are poorly understood. We have continued the search for novel zymogen granule proteins which could be involved in both processes and have identified a pancreas-specific protein, ZG29p, which has a number of peculiarities. In a recent study, we showed that ZG29p is homologous to the C-terminal part of the nuclear protein mta1p. Its pancreas-specific expression is mediated by alternative transcription initation from an enhancer element present in the first intron of the mta1 gene (*12*). In contrast to the nuclear protein mta1p, ZG29p enters the secretory pathway and is present in the soluble ZGC fraction.

In the present study, we confirmed that ZG29p is associated with a protein complex consisting of amylase, cationic trypsinogen, and other minor components. Similar results were obtained either by an immunoaffinity approach using ZG29p antibodies (Figure 1) or via the isolation and separation of native zymogen complexes by agarose gel electrophoresis and native isoelectric focusing (Figure 2). Aggregates of amylase were frequently observed in 2D gels, indicating that proteins of the complex tend to form oligomeric structures even under denaturing conditions (Figure 2c) (*12*). This indicates that amylase, cationic trypsinogen, and ZG29p form a stable complex with tight interactions among the binding partners. In cross-linking experiments with recombinant ZG29p and soluble ZGC proteins, amylase as well as other content proteins with molecular masses of 25−30 kDa were cross-linked. We assume that the latter are mainly yet unidentified acidic content proteins (see Figure 5), and not cationic trypsinogen. In agreement with this assumption, recombinant ZG29p could not be cross-linked to purified or recombinant cationic trypsinogen, but could be cross-linked to purified or recombinant amylase, indicating that amylase is the direct binding partner of ZG29p. This is further supported by our cross-linking studies with synthetic peptides matching the SH3 binding sites of ZG29p. The peptides could be successfully cross-linked to amylase, but not to trypsinogen. Although cationic trypsinogen is a major

constituent of the complex, it does not appear to directly interact with the SH3 binding sites of ZG29p. Since trypsinogen is present in the same complex as ZG29p, minor amounts of trypsinogen could become cross-linked with ZG29p when a partially complexed and aggregated mixture of ZGC proteins is present. Cross-linking is not observed, however, when purified proteins are used, indicating that this interaction is indirect. In addition to amylase, ZG29p specifically interacted with other acidic ZGC proteins with molecular masses of about 30 kDa (Figure 2c). It is likely that these yet unidentified minor components participate in the formation of the amylase-containing protein complex. Alternatively, these proteins could mediate an indirect interaction of ZG29p and cationic trypsinogen.

Cross-linking and competition experiments indicate that the interaction of ZG29p with amylase is mediated by at least one of the two proline-rich SH3 binding sites present in ZG29p. A rough estimation of the K_d values obtained from competition experiments with synthetic peptides (Figure 5) indicates that proline-rich peptides exactly matching the SH3 binding sites of ZG29p bind to amylase with an affinity of approximately 5−10 μM. Synthetic peptides just resembling the motif have a 10−20-fold lower affinity. Similar K_d values have been reported for other synthetic peptides interacting with SH3 domains (*19*, *28*, and references cited therein).

When a truncated form of amylase missing domain C was used in cross-linking experiments, an association of proline-rich peptides was omitted (Figure 7). On the basis of its molecular structure, domain C exhibits some features which make it a likely candidate for being a ligand binding site. Domain C forms a β-barrel comprised of two four-stranded β-sheets (*15, 16*). The cavity between the two sheets is filled with hydrophobic side chains including four conserved aromatic residues. In addition, the cleft formed by broad cohesive hydrophobic interfaces between domains A and C is filled with side chains of a large number of conserved aromatic residues (*17*). Proline-rich ligands bind to SH3 domains in the left-handed PPII helix conformation (*18, 19, 28*). This structural motif is common to many proteins (*29*). As PPII helices are not internally hydrogen bonded and depend on backbone solvation for stability, they are located on the surface of globular proteins presenting ideal mediators of protein−protein interactions. PPII helices are highly regular structures with three residues per turn and can be imagined as a trigonal prism which can be plugged into the surface of interacting proteins. Although SH3 domains are structurally diverse, the ligand binding sites are localized to conserved aromatic residues forming an exposed hydrophobic patch on the surface of the protein. The aromatic residues between domains A and C and/or within domain C might therefore be the ideal ligand binding sites mediating the interaction of ZG29p and amylase via the polyproline II helices of ZG29p. A refined structural analysis of the interacting proteins is required and will be part of future studies.

It is likely that domain C is also involved in homotypic aggregation of amylase. This domain shows a Greek key topology found in several lens proteins such as crystallin and enzyme crystallins (*30*−*38*), but also in nonlens proteins (*39*−*41*). The β- and γ-crystallins are supposed to play a role in organizing the highly ordered aggregation of lens proteins. The crystallins and other nonlens proteins possessing the Greek key stucture belong to a protein superfamily (*30, 34, 37*). It is assumed that this structure is inherited from an evolutionary ancestor as a protein module for protein−protein interactions to other proteins by domain swapping.

A Role for ZG29p in Condensation−Sorting. Our recent findings indicate that zymogen complexes are composed of a distinct set of known zymogens and several novel, yet unidentified proteins. These complexes initially formed in the RER (*9*) and completed aggregation into dense cores in the lumen of the TGN (*11*). As ZG29p is a constituent of an amylase-containing complex and directly interacts with amylase via its SH3 binding sites, it might fulfill important functions in complex formation, in the aggregation of amylase or the sorting of the complexes to the granule membrane. Furthermore, it is thought that proteins with SH3 domains or SH3 domain like structures organize the assembly of proteins or mediate their aggregation (*21*). Amylase, which was among the first enzymes to be crystallized (*42*), tends to form highly ordered structures by homotypic interaction (*17*). Under certain conditions, intracisternal crystals composed of a lattice of amylase can be formed in vivo within the lumen of the RER (*10, 43*). As amylase readily crystallizes by itself, it is unlikely that ZG29p plays an essential structural role in the formation of amylase-containing complexes or aggregates. In addition, a stoichiometric interaction of ZG29p and amylase as prerequisite for complex formation and aggregation seems unlikely due to the apparently low amount of ZG29p within the complex. This is supported by the finding that condensation of zymogens is not inhibited in the presence of synthetic peptides matching the SH3 binding sites of ZG29p, which are known to interact directly with amylase (Table 1).

However, we cannot exclude that binding of ZG29p (or corresponding peptides) to amylase triggers a structural alteration within the amylase molecule, which would then initiate homotypic interaction and consequently pH-dependent aggregation. In vitro experiments have shown that purified amylase must be in a complexed form (i.e., fixed to a substrate, slightly modified, or polymerized) to allow homotypic, pH-dependent aggregation (*44*). It was therefore postulated that a modification of amylase is required for homotypic interaction (*44*).

In cross-linking experiments with granule membranes, ZG29p interacted with three yet unidentified membrane proteins. This interaction is indicative for a role of ZG29p in sorting/binding of the amylase-containing complexes to the membrane. A role of ZG29p in sorting is supported by the fact that binding of amylase and other zymogens to the ZGM was reduced when the condensation−sorting assay was performed in the presence of the synthetic peptides mentioned above. With its two SH3 binding sites, ZG29p may act as a linker protein which would mediate the binding of amylase complexes to membrane proteins. When attached to the membrane, ZG29p-bound amylase could function as a nucleation site for further association and aggregation of amylase molecules.

Besides the interaction of ZG29p with amylase, SH3 binding sites may mediate other protein interactions among granule proteins. Interestingly, the sorting/binding of several zymogens to the granule membrane was inhibited in the presence of proline-rich peptides, and at least three unidentified acidic content proteins were cross-linked to them. We

propose that SH3 binding sites or polyproly helix II structures are involved in protein interactions of other granule proteins and might fulfill a general role in granule formation.

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