Stimulation of inositol 1,4,5-trisphosphate production by peptides corresponding to the effector domain of different Rab3 isoforms and cross-linking of an effector domain peptide target

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Rab3 proteins are localized on secretory vesicles and appear to be involved in regulated exocytosis. We have previously shown that a modified peptide corresponding to the effector domain of the small molecular mass GTP-binding protein Rab3A, Rab3A^{AL}, stimulates inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] production and amylase release in digitonin-permeabilized pancreatic acini. Experiments using monoclonal antibodies reveal that the Rab3-like protein present in pancreatic acini is not the Rab3A isoform. However, since the putative effector domains of the four as yet known Rab3 proteins (A, B, C and D) differ only in the C-terminal four amino acid residues, Rab3A effector domain peptide could mimic the action of the pancreas-specific Rab3 isoform. In the present study we report that peptides corresponding to the different Rab3 isoforms stimulate both Ins(1,4,5)P_3 production and amylase secretion with an order of potency Rab3B/D > Rab3A^{AL} > Rab3A = Rab3C. For Rab3A, B/D and C effector domain peptides the concentrations causing half-maximal response (EC_{50}) were 3, 0.2 and 3 nM for Ins(1,4,5)P_3 accumulation and 0.3, 0.02 and 0.3 nM for amylase release, respectively. A Rab1A effector domain peptide, Rab1A^{AL}, and a scrambled peptide of Rab3A^{AL} were less potent by several orders of magnitude in eliciting these responses compared with native Rab3 effector domain peptides. None of the peptides influenced Ins(1,4,5)P_3 production and amylase release in intact acini. Cross-linking of ^{125}I-Rab3B/D peptide to pancreatic acinar membranes showed a band at 70 to 75 kDa with maximum intensity at 75 kDa. Radiolabelling of the substrates could be displaced by unlabelled Rab3B/D peptide, and to a lesser extend by Rab3A peptide, whereas the scrambled peptide of Rab3A^{AL} had no effect. These data suggest that phospholipase C and exocytosis might be regulated by Rab3B- or Rab3D-like proteins in pancreatic acinar cells. A 75 kDa protein that preferentially cross-linked to ^{125}I-Rab3B/D effector domain peptide is a potential candidate as an effector protein of Rab3 effector domain peptides.

INTRODUCTION

Rab proteins belong to the family of Ras-related small-molecular-mass GTP-binding proteins (smg-proteins) and are important elements of vesicular transport within the cell [1–4]. It appears that each step in exocytosis and endocytosis is regulated by an individual member of the Rab family. Rab3 proteins are implicated in the regulation of exocytosis [5]. Four different Rab3 isoforms (A, B, C and D) have been described. The expression of these Rab3 proteins is tissue-specific, suggesting that different Rab3 isoforms control exocytosis in different cell types. Rab3A has been found only in neurons and neuroendocrine cells. In neurons Rab3A is localized to synaptic vesicles [6–8], and undergoes intracellular redistribution during exocytosis [9]. However, the physiological role of Rab3A is still unclear. Recent evidence suggests that Rab3A inhibits exocytosis [10,11]. Rab3B has been found in divergent cells such as mast cells, platelets, endothelial cells and the anterior pituitary [12–14]. In anterior pituitary cells, expression of Rab3B, but not of Rab3A, is required for calcium-induced exocytosis [13]. Rab3C has been shown to be expressed in the brain [15], where it is co-localized with Rab3A on synaptic vesicles [16]. Rab3D is enriched in adipose tissue and may regulate exocytosis of vesicles containing a glucose transporter in these cells [17].

Similar to Ras proteins Rab proteins contain a region of conserved sequence among all Rab proteins, termed the effector domain (ED; residues 33–48, according to H-Ras numbering). Rab proteins are likely to interact with effector proteins through the putative ED [18]. Synthetic peptides corresponding to the proposed ED of Rab3A proteins [19] stimulate exocytosis in different cell types [20–24], including pancreatic acini [25–27]. In mast cells the Rab3A^{AL} ED-peptide stimulates exocytosis in a similar fashion to polybasic peptides such as mastoparan [22], which directly activates G-proteins [28]. Moreover, these ED-peptides stimulate *in vitro* the fusion between zymogen granules and plasma membranes isolated from rat pancreas [29]. This effect, however, appears to be due to the physicochemical character of the peptides, since a specific sequence of amino acid residues of the peptides was not required [30].

We have shown recently that a modified Rab3A ED-peptide (Rab3A^{AL}) elicits accumulation of Ins(1,4,5)P_3 through hydrolysis of phosphatidylinositol 4,5-bisphosphate in permeabilized rat pancreatic acini [26,27], indicating that this peptide regulates exocytosis via activation of phosphoinositide-specific phospholipase C. However, the Rab3 protein isoform present in the exocrine pancreas is not yet known. Since the four subtypes of Rab3 proteins (A, B, C and D) share in part an identical sequence in the ED (residues 33–44, according to H-Ras numbering) [15,17,31], Rab3A ED-peptides could mimic the ED of all Rab3 isoforms. Experiments performed exclusively with Rab3A ED-peptides do not provide information concerning involvement of a specific isoform of Rab3 protein.

In the present study we investigated the effect of different isoforms of Rab3 ED-peptides on Ins(1,4,5)P_3 production and

Abbreviations used: CCK8, cholecystokinin octapeptide; EC_{50}, concentration giving half-maximal response; ED, effector domain; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; smg-protein, small molecular mass GTP-binding protein.

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amylase release in order to obtain information concerning Rab3 isoform preference for the target of the Rab3 ED-peptides in pancreatic acini. The results show that the Rab3B/D ED-peptide was the most potent at inducing Ins(1,4,5)P_3 production and amylase release. Furthermore, cross-linking of radiolabelled Rab3B/D ED-peptide to pancreatic membranes revealed specific labelling of a substrate banding at 75 kDa.

EXPERIMENTAL

Materials

Collagenase type III (*Clostridium histolyticum*), soybean trypsin inhibitor, digitonin, reagents for amylase assay, alkaline phosphatase-conjugated goat anti-(mouse IgG) antibody, Protein A–Sepharose purification kit, and reagents for alkaline phosphatase stain (Sigma *FAST*-tablets containing Nitro-blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate) were from Sigma Chemical (Deisenhofen, Germany). The Ins(1,4,5)P_3 radioreceptor assay kit was purchased from DuPont–New England Nuclear (Dreieich, Germany). The Rab3(33–48)- and Rab1(33–48)-derived ED-peptides were synthesized utilizing fluoren-α-ylmethoxycarbonyl (Fmoc) chemistry and were purified by C_{18} reversed-phased HPLC. Purity exceeded 95% in each case (Neosystem, Strasbourg, France and Biometra, Göttingen, Germany). Monoclonal antibodies (clones 42.1 and 42.2) were generated against recombinant Rab3A [32]. Clone 42.2 was demonstrated to be specific for Rab3A, while clone 42.1 probably recognizes all Rab3 isoforms. The chemical cross-linker, disuccinimidyl suberate, was from Pierce Chemical Co. (Rockford, IL, U.S.A.). Reagents for electrophoresis were from Biometra (Göttingen, Germany).

Preparation of Rab3B/D ED-peptide-specific antiserum

Rab3B/D ED-peptide was used to generate polyclonal antibodies in rabbits. Rab3B/D ED-peptide was coupled to keyhole-limpet haemocyanin with glutaraldehyde [33]. The keyhole-limpet haemocyanin–peptide conjugate was used in complete Freud's adjuvant to immunize rabbits by intradermal injection. Rabbits were bled 2 weeks after the booster. Crude antisera were purified by Protein A–Sepharose affinity chromatography [33].

Preparation of pancreatic acini

Pancreatic acini were isolated from rat pancreas by collagenase digestion as described [34,35]. According to the Trypan Blue dye exclusion method, viability of the cells exceeded 95%. Then, the acini were suspended in K^+-Krebs Ringer Hepes-buffer containing 145 mM KCl, 2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 0.01 mM $CaCl_2$, 10 mM glucose, 0.2% (w/v) BSA, 0.01% (w/v) soybean trypsin inhibitor, 10 mM Hepes/NaOH, pH 7.4, and 10 μg/ml (w/v) digitonin. This procedure led to permeabilization of $>95\%$ of the acini as estimated by the Trypan Blue dye exclusion method and does not damage the secretory apparatus [34].

Subcellular fractionation

Isolated pancreatic acini were homogenized in 6 vol. of ice-cold homogenization buffer containing 25 mM Hepes, pH 7.0, 0.3 M sucrose, 0.5 mM $MgCl_2$, 1 mM benzamidine, 0.5 mM PMSF, 0.01% (w/v) aprotinin, 0.01% (w/v) leupeptin, and 0.01% (w/v) trypsin inhibitor by 20 strokes in a glass/Teflon Potter–Elvehjem homogenizer. The homogenate was centrifuged at 8000 *g* for 15 min, and the supernatants were pelleted by centrifugation at 50000 *g* for 25 min. This cellular subfraction contains membranes derived from the plasma membrane and the endoplasmic reticulum. Membranes from pancreatic acinar zymogen granules were prepared as described previously [36]. Protein was determined according to Bradford [37] using BSA as standard.

Immunoblot analysis

Samples (50 μg of protein) were boiled in Laemmli sample buffer [38], and clarified by centrifugation at 10000 *g* for 5 min. Proteins were resolved on SDS/12.5%-PAGE and electrotransferred to nitrocellulose sheets (0.2 μm). The blots were blocked for 30 min at room temperature in blocking buffer [20 mM Tris/HCl, pH 7.4, 140 mM NaCl, 2% (w/v) BSA, 0.1% (w/v) Tween 20] and were incubated with the Rab3-specific antibodies (1:1000 dilution in blocking buffer) for 2 h at room temperature. Antigen–antibody complexes were visualized using an alkaline phosphatase-conjugated goat anti-(mouse IgG) antibody and 5-bromo-4-chloro-3-indolyl phosphate and Nitro-blue tetrazolium.

Measurement of amylase release

The detection of amylase released into the medium from digitonin (10 μg/ml)-permeabilized rat pancreatic acini during 30 min incubation at 37 °C under a continuous supply of 100% O_2 was performed as described recently [39].

Ins(1,4,5)P_3 production

Ins(1,4,5)P_3 production in pancreatic acini was measured by displacement of bound [^{3}H]Ins(1,4,5)P_3 using an Ins(1,4,5)P_3-specific radioreceptor assay as described previously [39]. Briefly, acini from two rats were suspended in 30 ml of K^+-based Hepes-buffered Ringer solution. Aliquots (2 ml) were incubated in a continuously stirred cuvette at 37 °C. Subsequently, digitonin (10 μg/ml) and Rab3 ED-peptide or vehicle (water) were added. At specified times (0 s, 5 s, 15 s, 30 s, 1 min, 5 min), 200 μl aliquots were removed and mixed with an equal volume of ice-cold trichloroacetic acid (20%, w/v) and stored at 4 °C for 1 h. After centrifugation for 15 min at 5000 *g* (4 °C), 300 μl of the supernatants were extracted with 800 μl of 1,1,2-trichloro-1,2,2-trifluoroethane/trioctylamine (3:1, v/v). The Ins(1,4,5)P_3 content of the upper aqueous phase was determined in duplicate using the radioreceptor assay for Ins(1,4,5)P_3 and was performed according to the assay protocol.

Iodination of Rab3B/D ED-peptide

Rab3B/D ED-peptide (0.4 μg) was radioiodinated using the chloramine T method [33]. Radioiodinated Rab3B/D ED-peptide was separated from free reagent by reversed-phased HPLC. Specific radioactivity of ^{125}I-Rab3B/D ED-peptide was 74 TBq/mmol (2000 Ci/mmol).

Cross-linking assay

The cross-linking assay was performed by a similar procedure as described for the cross-linking of Rab3A protein to bovine brain membranes [40]. Radioiodinated Rab3B/D ED-peptide (approximately 10^6 c.p.m.) was incubated with 200 μg of membrane protein in a buffer containing 20 mM Hepes/NaOH, pH 7.2, 5 mM $MgCl_2$, 200 mM NaCl, 0.05% (w/v) sodium cholate for 20 min at 30 °C in a final volume of 45 μl. After the incubation, disuccinimidyl suberate (dissolved in dimethyl sulphoxide) was added to a final concentration of 1.5 mM and incubation was continued for a further 30 min at 30 °C. The reaction was stopped by addition of 30 μl of 100 mM Tris/NaOH, pH 7.2.

The samples were subjected to SDS/PAGE (7.5% polyacrylamide gel) according to Laemmli [38], gels were subsequently dried and autoradiographed.

Statistical analysis

The data shown are means ± S.E.M. of at least three independent experiments. The statistical significance was calculated using Student's *t*-test for unpaired values.

RESULTS

Immunoblot analysis with a monoclonal antibody that recognizes probably all Rab3 protein isoforms detected a protein banding at 27 kDa in membranes from rat pancreatic acinar zymogen granules (Figure 1). In the homogenate only tiny amounts of this protein were detectable. An antibody specific for Rab3A failed to recognize a substrate in pancreatic acinar homogenate or zymogen granule membranes, but labelled a 26 kDa protein in a rat brain homogenate. These results confirm that Rab3-like proteins, but not the Rab3A isoform, are present in pancreatic acini and are mainly localized to the zymogen granular membrane [36,41].

The synthetic Rab3 ED-derived peptides used in the present study are shown in Table 1. The amino acid sequences of the ED of Rab3B and Rab3D are identical. The Rab3A, Rab3B/D, and Rab3C ED-peptides differ only in the four C-terminal amino acid residues. In Rab3A^{AL} ED-peptide residues 35 and 36 of native Rab3A were changed from threonine and valine to alanine and isoleucine, respectively. This modification has been reported to increase the potency of Rab3A ED-peptide to inhibit vesicular transport and to stimulate exocytosis [19,25]. As controls, a peptide corresponding to the effector domain of Rab1A^{AL} and a scrambled ED-peptide of Rab3A^{AL} were used.

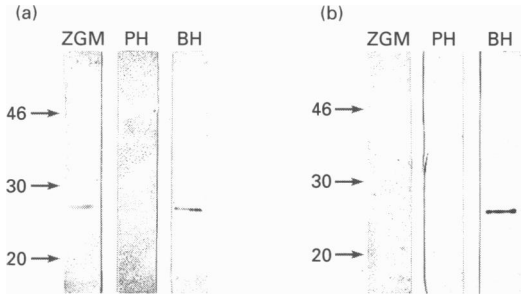

Figure 1 Identification of a Rab3-immunoreactive protein in subcellular fractions of rat pancreatic acinar cells

Western blotting was performed with homogenates of rat pancreatic acini (PH), rat brain (BH) and zymogen granule membranes (ZGM). The blots were probed with an antibody that recognizes all Rab3 isoforms (**a**) or with a Rab3A-isoform-specific antibody (**b**). Antibody–antigen complexes were visualized using an alkaline phosphatase-conjugated antibody.

Table 1 Amino acid sequences of the H-Ras, Rab1A and Rab3 effector domain-derived peptides (residues 33–48 according to H-Ras numbering)

Position (according to H-Ras numbering) ...	33 48
H-Ras	D-P-T-I-E-D-S-Y – R-K-Q-V-V-I-D
Rab3A	V-S-T-V-G-I-D-F-K-V-K-T-I-Y-R-N
Rab3B/D	V-S-T-V-G-I-D-F-K-V-K-T-V-Y-R-H
Rab3C	V-S-T-V-G-I-D-F-K-V-K-T-V-F-K-N
Rab3A^{AL}	V-S-A-L-G-I-D-F-K-V-K-T-I-Y-R-N
Scrambled Rab3A^{AL}	Y-N-R-V-L-S-A-D-F-K-V-G-K-I-I-T
Rab1A^{AL}	I-S-A-L-G-V-D-F-K-I-R-T-I-E-L-D

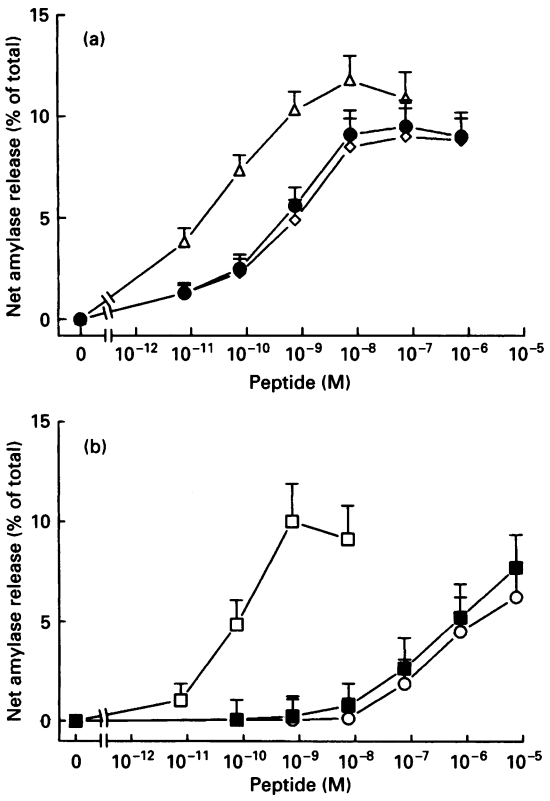

Figure 2 Dose–response relationships for the stimulation of amylase secretion in response to Rab ED-derived peptides from digitonin-permeabilized rat pancreatic acini

Acini were suspended in 3 ml of K^+-Krebs Ringer Hepes-buffer, permeabilized with digitonin (10 μg/ml) and incubated with the indicated concentration of a Rab-derived ED-peptide for 30 min at 37 °C. Amylase release into the medium was determined as described in the Experimental section. Data are means ± S.E.M. of five independent experiments. (Δ) Rab3B/D, (●) Rab3A, (◇) Rab3C, (□) Rab3A^{AL}, (○) Rab1A^{AL}, (■) scrambled Rab3A^{AL} ED-peptide.

Figure 2 shows the effect of Rab3 ED-peptides on amylase release from digitonin-permeabilized pancreatic acini. Basal amylase release was 6.7 ± 1.6% (n = 7) of total amylase present in the acini at the beginning of the experiment. All Rab3 ED-peptides increased amylase output from the acini. The EC_{50} values of ED-peptide-induced amylase release were 0.3 nM, 0.08 nM, 0.02 nM and 0.3 nM for Rab3A, Rab3A^{AL}, Rab3B/D and Rab3C respectively, indicating that the Rab3B/D ED-peptide was most potent ($P < 0.01$ Rab3B/D versus Rab3A and C). Maximum effective concentrations of each ED-peptide increased amylase output by 10–12% of total cellular amylase content (n = 5). Thus, Rab3 ED-peptides caused amylase release almost as effective as maximally effective concentrations of cholecystokinin octapeptide (CCK8) (0.1 nM), which increased amylase release by 11.8 ± 1.6% of total amylase content (Table 2).

Compared with the Rab3 ED-peptides, a scrambled Rab3A^{AL} ED-peptide and the Rab1A^{AL} ED-peptide were several orders of magnitude less potent in stimulating amylase release (Figure 2b). The scrambled Rab3A^{AL} ED-peptide and Rab1A^{AL} ED-peptide

Table 2 Effect of Rab3B/D-peptide antiserum on amylase release in response to CCK8

Acini were suspended in 2 ml of K^+-Krebs Ringer Hepes buffer, permeabilized with digitonin (10 μg/ml), and incubated with 0.1 nM CCK8 or vehicle (water) and the indicated concentration of Rab3B/D-peptide antiserum or pre-immune serum. Other experimental conditions were identical to those described in Figure 2. The basal amylase secretion (7.2±1.2% of total) in the presence of vehicle was subtracted from each value. Data are means±S.E.M. of three independent experiments.

	Pre-immune serum (μg/ml)			Immune serum (μg/ml)		
	0	1.5	15	0	1.5	15
Net amylase release (% of total)	11.8±1.6	11.2±1.5	13.8±1.9	11.8±1.6	11.6±1.8	14.4±1.9

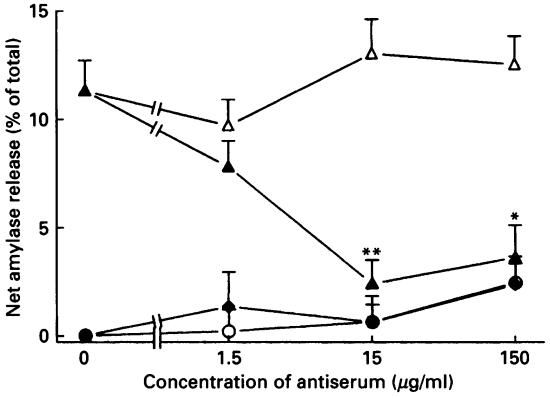

Figure 3 Effect of Rab3B/D ED-peptide antiserum on amylase release in response to Rab3B/D ED-peptide

Acini were suspended in 2 ml of K^+-Krebs Ringer Hepes-buffer, permeabilized with digitonin (10 μg/ml), incubated with Rab3B/D ED-peptide (5 nM) or vehicle (water) and the indicated concentration of Rab3B/D ED-peptide antiserum or pre-immune serum. Other experimental conditions were identical as described in Figure 2. Data are means±S.E.M. of three independent experiments. Asterisks indicate significant differences between amylase release in the presence of pre-immune serum and immune serum, respectively. $^*P < 0.05$, $^{**}P < 0.01$. (△), Rab3B/D + pre-immune serum, (▲), Rab3B/D + immune serum, (◆), pre-immune serum, (○), immune serum.

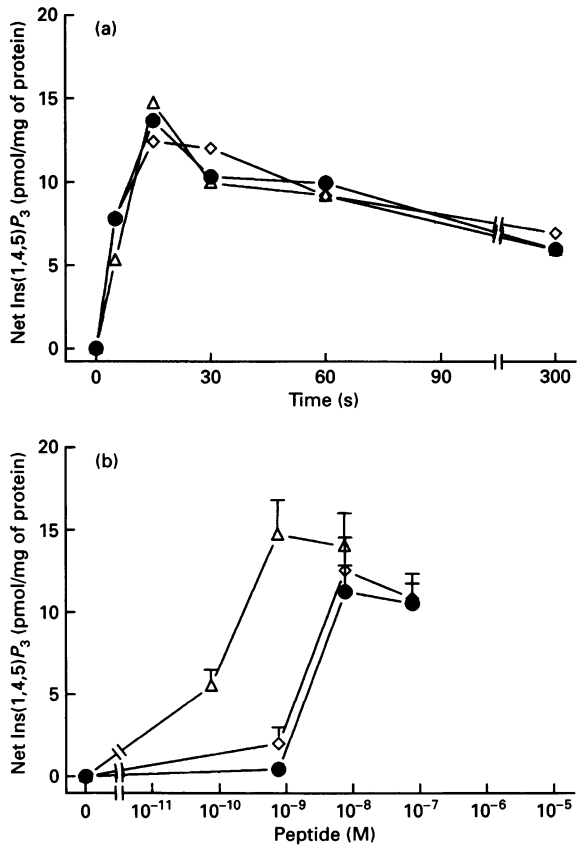

Figure 4 Rab3 ED-peptide-stimulated Ins(1,4,5)P_3 accumulation in digitonin-permeabilized rat pancreatic acini

Rat pancreatic acini were suspended in 2 ml of K^+-Krebs Ringer Hepes buffer, and permeabilized with digitonin (10 μg/ml). (**a**) The acini were incubated with maximally effective concentrations of Rab3 ED-peptides (7.5 nM) for the indicated time at 37 °C. (**b**) Ins(1,4,5)P_3 production of the acini was measured after a 15 s incubation with the Rab3 ED-peptide. The Ins(1,4,5)P_3 content of the samples was determined in 200 μl aliquots as described in the Experimental section. The experiments shown are representative for three different experiments (**a**) or are depicted as mean±S.E.M. of four independent experiments (**b**). (△) Rab3B/D, (●) Rab3A and (◇) Rab3C ED-peptide.

increased amylase release only at high concentrations (> 50 nM). In intact acini none of the Rab3 ED-peptides had any effect on amylase release (data not shown).

The specificity of Rab3B/D ED-peptide-induced amylase release was confirmed by a polyclonal antibody raised against the Rab3B/D ED-peptide. This antibody abolished Rab3B/D ED-peptide (7.5 nM)-induced amylase release in a concentration-dependent fashion, whereas it had no effect on basal or CCK8 (0.1 nM)-stimulated amylase secretion (Figure 3). These results confirm that the Rab3 ED-peptide response is specific. However, this antibody raised against the synthetic peptide did not recognize a substrate in Western blot analysis of either pancreatic homogenate or zymogen granule membranes (data not shown).

Since Rab3A^{AL} ED-peptide stimulates Ins(1,4,5)P_3 production in rat pancreatic acini [26,27], we investigated the effect of ED-peptides corresponding to the different isoforms of Rab3 on Ins(1,4,5)P_3 accumulation in digitonin (10 μg/ml)-permeabilized rat pancreatic acini. Figure 4(a) shows the time course for the effect of native Rab3 ED-peptides (Rab3A, B/D, C) on Ins(1,4,5)P_3 production. Each ED-peptide (7.5 nM) caused a rapid increase in the Ins(1,4,5)P_3 content of the acini. Ins(1,4,5)P_3 levels were maximal 15 s after beginning of the incubation with the peptide and then declined. In the absence of secretagogue the Ins(1,4,5)P_3 content of the acini did not change significantly during a 5 min incubation. None of the peptides influenced Ins(1,4,5)P_3 production in intact acini (data not shown).

To investigate the potency of the Rab3-isoform-derived ED-peptides to increase Ins(1,4,5)P_3 production, dose–response

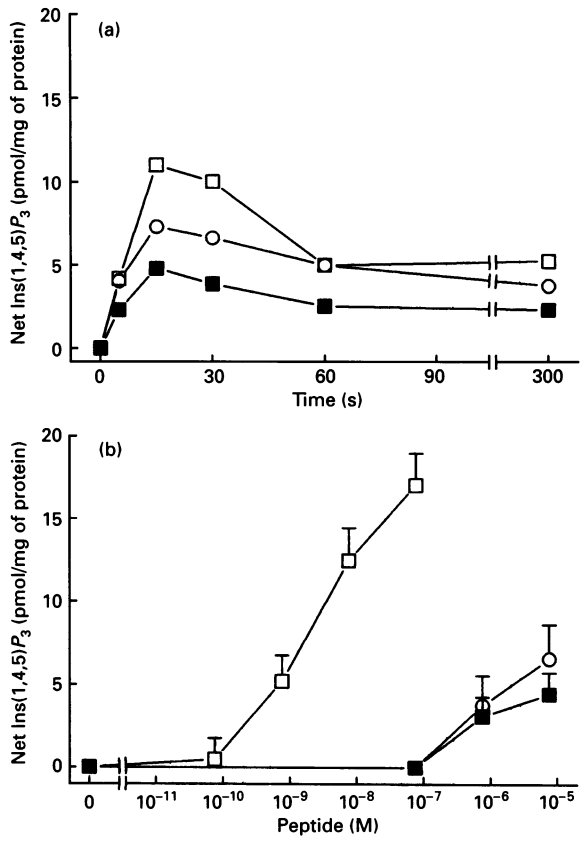

Figure 5 Effect of Rab1- and Rab3 ED-related peptides on Ins(1,4,5)P_3 production

Isolated rat pancreatic acini were permeabilized with digitonin and were incubated with the indicated Rab1- or Rab3-related ED-peptide. (**a**) Time course of Rab1A^{AL} (75 μM)-, Rab3A^{AL} (7.5 nM)-, and of the scrambled ED-peptide of Rab3A^{AL} (75 μM)-induced Ins(1,4,5)P_3 accumulation. (**b**) Dose–response curves of Ins(1,4,5)P_3 production in response to Rab1-/Rab3-related ED-peptides. The experiments shown are representative for three different experiments (**a**) or are depicted as mean ± S.E.M. of four independent experiments (**b**). (□) Rab3A^{AL}, (○) Rab1A^{AL} ED-peptide and (■) scrambled ED-peptide of Rab3A^{AL}.

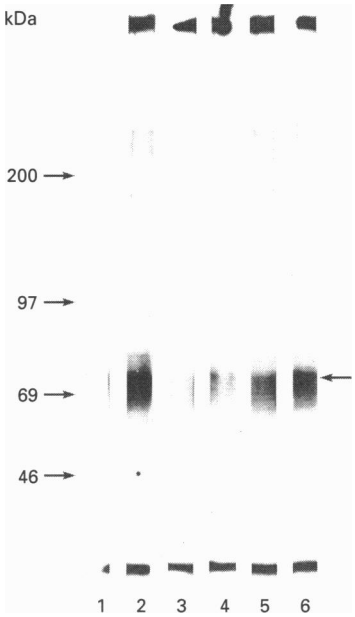

Figure 6 Cross-linking of the ^{125}I-Rab3B/D effector domain to rat pancreatic acinar membranes

Isolated rat pancreatic membranes (200 μg of protein) were incubated with ^{125}I-Rab3B/D ED-peptide in the absence (lanes 1 and 2) and presence of 0.75 μM of unlabelled Rab3B/D ED-peptide (lane 3), 0.75 μM Rab3A ED-peptide (lane 4), or 0.75 μM (lane 5) or 7.5 μM (lane 6) of scrambled Rab3A^{AL} ED-peptide. After 20 min, disuccinimidyl suberate was added except for the sample run in lane 1 as described in the Experimental section. The experiment shown is representative of five independent experiments.

curves for the effect of Rab3-derived ED-peptides on Ins(1,4,5)P_3 accumulation were performed (Figure 4b). The EC_{50} values for Rab3B/D, A and C ED-peptides to increase the Ins(1,4,5)P_3 content of the acini were 0.2 nM, 3 nM and 3 nM respectively. Thus, the Rab3A and C ED-peptides were at least ten times less potent at increasing Ins(1,4,5)P_3 production than Rab3B/D ED-peptide ($P < 0.05$ for Rab3B/D versus Rab3A and C, respectively). These data demonstrate that among the Rab3 ED-peptides, Rab3B/D ED-peptide is the most potent activator of phosphoinositide-specific phospholipase C.

A scrambled ED-peptide of Rab3A^{AL} has recently been shown to elicit fusion between isolated plasma membranes and zymogen granules *in vitro* [30]. In this study we investigated the effect of a scrambled Rab3A^{AL} ED-peptide on Ins(1,4,5)P_3 production in digitonin-permeabilized pancreatic acini. As shown in Figure 5, the dose–response curve of scrambled Rab3A^{AL} ED-peptide-induced Ins(1,4,5)P_3 production was by three orders of magnitude to the right compared with Ins(1,4,5)P_3 accumulation elicited by native Rab3A^{AL} ED-peptide. Similar results were obtained for a modified peptide corresponding to the effector domain of Rab1A (Rab1A^{AL}). These data show that native Rab3 ED-peptides stimulate phosphoinositide-specific phospholipase C by three orders of magnitude more than a scrambled ED-peptide of Rab3A^{AL} or Rab1A^{AL} ED-peptide.

In order to identify an effector for Rab3 ED-peptides, a radioiodinated Rab3B/D ED-peptide was cross-linked to pancreatic acinar membranes and a cytosolic fraction using a similar protocol as described for cross-linking of Rab3A protein to bovine brain membranes [40]. As shown in Figure 6, ^{125}I-Rab3B/D ED-peptide bound to a substrate in pancreatic acinar membranes (subfraction enriched in plasma membranes and endoplasmic reticulum) banding at 70 to 75 kDa on SDS/PAGE. The maximum intensity was always observed at the upper part of this band (75 kDa), indicating that only one target is specifically labelled by the radio-iodinated peptide. In the cytosolic fraction no specific labelling was detected. Labelling of the substrate was almost completely displaced by unlabelled Rab3B/D ED-peptide (0.75 μM), and less potently by unlabelled Rab3A ED-peptide (0.75 μM). The scrambled ED-peptide of Rab3A^{AL} caused no (0.75 μM) or only a minor (7.5 μM) displacement of the 70–75 kDa band, indicating that cross-linking of the ^{125}I-Rab3B/D ED-peptide to this substrate was specific. Labelling of all other proteins was unspecific.

DISCUSSION

Peptides corresponding to the putative effector domain of the smg-protein Rab3A have been used to investigate the function of Rab3A in the secretory pathway. A number of studies have shown that Rab3A ED-peptides (residues 33–48) stimulate exocytosis in different cells, including pancreatic acini [25–27]. However, the Rab3 protein expressed in the exocrine pancreas is not Rab3A isoform (Figure 1 and [41]).

In the present study, we report that peptides of the ED of the different Rab3 isoforms stimulate Ins(1,4,5)P_3 production and amylase release with an order of potency Rab3B/D > Rab3C = Rab3A > Rab3A^{AL} (scrambled). We showed recently that the Rab3A^{AL} ED-peptide increases Ins(1,4,5)P_3 production by phosphatidylinositol 4,5-bisphosphate hydrolysis and stimulates amylase secretion in digitonin-permeabilized rat pancreatic acini [26,27]. CCK8- as well as Rab3A^{AL} ED-peptide-stimulated amylase release were abolished by the Ins(1,4,5)P_3 receptor antagonist heparin [26], suggesting that Rab3 ED-peptides and calcium-mobilizing secretagogues share a common mechanism to stimulate amylase release from pancreatic acini.

In the present study, the dose–response curve for Rab3 ED-peptide-induced amylase release is shifted by several orders of magnitude to lower peptide concentrations compared with a recent study by Padfield et al. [25] (EC_{50} $\sim 60\ \mu M$ versus ~ 0.1 nM). The reason for this discrepant result might be due to different experimental conditions and/or a different mode of permeabilization (streptolysin O versus digitonin used in the present study).

The specificity of the effects of Rab3 ED-peptides has recently been discussed since in mast cells Rab3A^{AL} ED-peptide appears to stimulate exocytosis similar to the polybasic peptide mastoparan via pertussis toxin-sensitive G-proteins [22]. Moreover, Rab3A^{AL}, as well as a scrambled ED-peptide of Rab3A^{AL}, could induce fusion of pancreatic zymogen granules with plasma membranes *in vitro* [30]. It was concluded that the effect of Rab3A ED-peptides is due to their charge distribution rather than being related to the action of Rab3-like proteins. A different scrambled ED-peptide of Rab3A^{AL} was used in the present study and was less potent by several orders of magnitude in stimulating Ins(1,4,5)P_3 production and amylase secretion in digitonin-permeabilized pancreatic acini compared with the native Rab3 ED-peptides (see Figures 2 and 5). This shows that Rab3 ED-peptide-induced Ins(1,4,5)P_3 production and amylase release depends on a specific sequence of amino acid residues of the peptide. The specificity of the effects of Rab3 ED-peptides on amylase release and Ins(1,4,5)P_3 production is further confirmed by experiments using peptides from the different isoforms of Rab3 ED. Moreover, a polyclonal antibody raised against Rab3B/D ED-peptide specifically abolished Rab3B/D ED-peptide-induced amylase release, but had no effect on the basal or CCK8-induced amylase secretion (Figure 3).

Rab3A-derived ED-peptides stimulate exocytosis in pancreatic acini despite the absence of the Rab3A isoform in these cells (Figure 1 and [36,41]). Similar results were obtained in mast cells [21,42]. However, the four subtypes (A, B, C and D) of Rab3 proteins share an identical sequence in the putative effector domain [15,17,31]. Thus, Rab3A ED-peptides could mimic function exerted by each of these proteins, and do not provide information concerning the involvement of a specific isoform of Rab3 protein. The data of the present study showing that in pancreatic acini the Rab3B/D ED-peptide is more potent in stimulating Ins(1,4,5)P_3 production and amylase release than Rab3A or Rab3C ED-peptide indicate that the putative Rab3 ED-peptide target preferentially binds the Rab3B/D ED-peptide and suggest that Rab3B- or D-like proteins might regulate exocytosis and phospholipase C in these cells. However, we cannot exclude the possibility that the Rab3 ED-peptide-induced activation of phospholipase C and amylase release is unrelated to Rab3 protein function.

The Rab3 protein isoform expressed in pancreatic acini is not known yet. In the present study, an antibody that probably recognizes all Rab3 isoforms bound to a 27 kDa protein that is mainly localized to the membrane of zymogen granules, whereas a Rab3A-specific antibody did not recognize a substrate. Similar data have been reported previously [36,41]. Thus a Rab3-like protein, but not Rab3A, is present in pancreatic acini. The polyclonal Rab3B/D ED-peptide-specific antibody did not recognize a substrate in pancreatic acini (data not shown).

Rab3A and Rab3B are closely related proteins, but appear to regulate exocytosis in a different manner. Loading anterior pituitary cells with antisense oligonucleotides directed against Rab3B, but not Rab3A, inhibited exocytosis [13]. By contrast, in adrenal chromaffin and PC12 cells Rab3A appeared to be a negative regulator of exocytosis [10,11]. The question whether a Rab3-like protein stimulates or inhibits exocytosis and phospholipase C in pancreatic acini cannot be answered from the data of the present study.

Concerning the mechanism of Rab3 ED-peptide stimulation of phospholipase C and amylase secretion, it is possible that the pancreas-specific Rab3 protein inhibits exocytosis and that the Rab3 ED-peptides act as an antagonist at a Rab3 binding site of a putative Rab3 protein effector, thereby preventing the effect of native Rab3 proteins [19]. Another possibility is that pancreatic Rab3 protein stimulates exocytosis and that Rab3 ED-peptides mimic the active GTP-bound form of the Rab3 protein. Alternatively, Rab3 ED-peptides might directly stimulate a target that is unrelated to Rab3 protein function.

Cross-linking of ^{125}I-Rab3B/D ED-peptide to pancreatic acinar membranes led to a specific labelling of a band ranging from 70 to 75 kDa with a maximum of intensity at 75 kDa (Figure 6). Rab3B/D and to a lesser extent Rab3A ED-peptide, but not a scrambled ED-peptide of Rab3A^{AL}, abolished binding of ^{125}I-Rab3B/D to 70–75 kDa substrate. Thus, the putative Rab3 target in pancreatic acini preferentially binds to the Rab3B/D effector domain. These data correlate with the potency of the Rab3-related ED-peptides to stimulate Ins(1,4,5)P_3 production and amylase secretion. We suggest that the 70–75 kDa target of Rab3 ED-peptides in pancreatic acini might be involved in Rab3 ED-peptide-induced stimulation of Ins(1,4,5)P_3 production and amylase secretion. However, we cannot decide whether the 70–75 kDa Rab3 ED-peptide binding protein is a target for pancreatic Rab3 proteins. Moreover, it is possible that other Rab3 ED-peptide targets bind to Rab3B/D ED-peptides which escaped the cross-linking assay.

Recently, Olszewski et al. [43] identified Rab3A ED-peptide binding proteins of 14 and 17 kDa using a cross-linking procedure in insulin-secreting HIT-T15 cells, whereas no Rab3A ED-peptide target of higher molecular mass was detected. In the present study, however, we could not detect a 14 and a 17 kDa Rab3 ED-peptide target in pancreatic acinar cell membranes. It appears possible that Rab3A and Rab3B/D ED-peptides recognize different targets in different cells. Since Rab3A and Rab3B/D ED-peptides show only minor differences in their amino acid sequence, it is unlikely that the 75 kDa Rab3B/D ED-peptide-preferring target is also present in HIT-T15 cells.

Shirataki et al. [40,44] identified a putative target for Rab3A, termed Rabphilin-3A, which preferentially interacts with the GTP-form of Rab3A in a crude membrane fraction of bovine and rat brain. Rabphilin-3A isolated from bovine brain is a protein with a molecular mass of 85 kDa on PAGE and has two repeated C_2 domains in the C-terminal region. Analogous C_2 domains are found in protein kinase C, synaptotagmin and phospholipase A_2 which bind calcium and phospholipid through this region. Cross-linking experiments and Northern blot analysis revealed that this protein is absent from rat exocrine pancreas [44]. Moreover, Rab3A^{AL} ED-peptide did not displace Rab3A protein in the cross-linking assay [18]. Thus, it is unlikely that Rabphilin-3A is the putative target for Rab3 ED-peptides in

pancreatic acini. As has been suggested for other smg-proteins (e.g. Ras) [45] multiple effector proteins may exist for Rab3.

In conclusion, the present study shows that the Rab3B/D isoform of the Rab3 ED-peptides stimulated Ins(1,4,5)P_3 accumulation and amylase release most potently, whereas a scrambled peptide of Rab3A effector domain and Rab1A^{AL} ED-peptide were much less potent in eliciting these responses. Moreover, cross-linking experiments revealed the presence of a Rab3B/D ED-peptide-preferring target in pancreatic membranes. These results suggest regulation of phospholipase C and exocytosis by Rab3B/D-like proteins and a Rab3B/D-preferring target in rat pancreatic acini.

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REFERENCES

1 Goud, B., Salminen, A., Walworth, N. C. and Novick, P. (1988) Cell **53**, 753–768
2 Pfeffer, S. R. (1992) Trends Cell Biol. **2**, 41–46
3 Novick, P. and Brennwald, P. (1993) Cell **75**, 597–601
4 Wagner, A. C. C. and Williams, J. A. (1994) Am. J. Physiol. **266**, G1–G14
5 Fischer von Mollard, G., Stahl, B., Li, C., Südhof, T. C. and Jahn, R. (1994) Trends Biol. Sci. **19**, 164–168
6 Mizoguchi, A., Kim, S., Ueda, T. and Takai, Y. (1989) Biochem. Biophys. Res. Commun. **162**, 1439–1445
7 Darchen, F., Zahraoui, A., Hammel, F., Monteils, M. P., Tavitian, A. and Scherman, D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5692–5696
8 Fischer von Mollard, G., Mignery, G. A., Baumert, M., Perin, M. S., Hanson, T. J., Burger, P. M., Jahn, R. and Südhof, T. C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1988–1992
9 Fischer von Mollard, G., Südhof, T. C. and Jahn, R. (1991) Nature (London) **349**, 79–81
10 Holz, R. W., Brondyk, W., Senter, R. A., Kuizon, L. and Macara, I. G. (1994) J. Biol. Chem. **269**, 10229–10234
11 Johannes, L., Lledo, P. M., Roa, M., Vincent, J.-D., Henry, J. P. and Darchen, F. (1994) EMBO J. **13**, 2029–2037
12 Karniguian, S., Zahraoui, A. and Tavitian, A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7647–7651
13 Lledo, P.-M., Vernier, P., Vincent, J.-D., Mason, W. T. and Zorec, R. (1993) Nature (London) **364**, 540–543
14 Weber, E., Berta, G., Tousson, A., St John, P., Green, M. W., Gopalokrishna, T., Jilling, T., Sorgher, E., Elton, T. S., Abrahamson, D. R. and Kirk, K. L. (1994) J. Cell Biol. **125**, 583–594
15 Matsui, Y., Kikuchi, A., Kondo, J., Hishida, T., Teranishi, Y. and Takai, Y. (1988) J. Biol. Chem. **263**, 11071–11074
16 Fischer von Mollard, G., Stahl, B., Khokhlatchev, A., Südhof, T. C. and Jahn, R. (1994) J. Biol. Chem. **269**, 10971–10974
17 Baldini, G., Hohl, T., Lin, H. Y. and Lodish, H. F. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 5049–5052
18 McKiernan, C. J., Brondyk, W. H. and Macara, I. G. (1993) J. Biol. Chem. **268**, 24449–24452
19 Plutner, H., Schwaninger, R., Pind, S. and Balch, W. E. (1990) EMBO J. **9**, 2375–2383
20 Senyshyn, J., Balch, W. E. and Holz, R. W. (1992) FEBS Lett. **309**, 41–46
21 Oberhauser, A. F., Monck, J. R., Balch, W. E. and Fernandez, J. M. (1992) Nature (London) **360**, 270–273
22 Law, G. J., Northrop, A. J. and Mason, W. T. (1993) FEBS Lett. **333**, 56–60
23 Li, G., Regazzi, R., Balch, W. E. and Wollheim, C. B. (1993) FEBS Lett. **327**, 145–149
24 Richmond, J. and Haydon, P. G. (1993) FEBS Lett. **326**, 124–130
25 Padfield, P. J., Balch, W. E. and Jamieson, J. D. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 1656–1660
26 Piiper, A., Stryjek-Kaminska, D., Stein, J., Caspary, W. F. and Zeuzem, S. (1993) Biochem. Biophys. Res. Commun. **192**, 1030–1036
27 Zeuzem, S., Stryjek-Kaminska, D., Caspary, W. F., Stein, J. and Piiper, A. (1994) Am. J. Physiol. **267**, G350–G356
28 Higashijima, T. and Ross, E. M. (1991) J. Biol. Chem. **266**, 12655–12661
29 Edwardson, J. M., MacLean, C. M. and Law, G. J. (1993) FEBS Lett. **320**, 52–56
30 MacLean, C. M., Law, G. J. and Edwardson, J. M. (1993) Biochem. J. **294**, 325–328
31 Elferink, L. A., Anzai, K. and Scheller, R. H. (1992) J. Biol. Chem. **267**, 5768–5775
32 Matteoli, M., Takei, K., Cameron, R., Hurlbut, P., Johnston, P. A., Südhof, T. C., Jahn, R. and De Camilli, P. (1991) J. Cell Biol. **115**, 625–633
33 Harlow, E. and Lane, D. (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, New York
34 Piiper, A., Plusczyk, T., Eckhardt, L. and Schulz, I. (1991) Eur. J. Biochem. **197**, 391–397
35 Zeuzem, S., Feick, P., Zimmermann, P., Haase, W., Kahn, R. A. and Schulz, I. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6619–6623
36 Schnefel, S., Zimmermann, P., Pröfrock, A., Jahn, R., Aktories, K., Zeuzem, S., Haase, W. and Schulz, I. (1992) Cell. Physiol. Biochem. **2**, 77–89
37 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
38 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
39 Piiper, A., Stryjek-Kaminska, D., Stein, J., Caspary, W. F. and Zeuzem, S. (1994) Am. J. Physiol. **266**, G363–G371
40 Shirataki, H., Kaibuchi, K., Yamaguchi, T., Wada, K., Horiuchi, H. and Takai, Y. (1992) J. Biol. Chem. **267**, 10946–10949
41 Jena, B. P., Gumkowski, F. D., Konieczko, E. M., Fischer von Mollard, G., Jahn, R. and Jamieson, J. D. (1994) J. Cell Biol. **124**, 43–53
42 Oberhauser, A. F., Balan, V., Fernandez-Badilla, C. L. and Fernandez, J. M. (1994) FEBS Lett. **339**, 171–174
43 Olszewski, S., Deeney, J. T., Schuppin, G. T., Williams, K. P., Corkey, B. E. and Rhodes, C. J. (1994) J. Biol. Chem. **269**, 27987–27991
44 Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M. and Takai, Y. (1993) Mol. Cell. Biol. **13**, 2061–2068
45 Feig, L. A. and Schaffhausen, B. (1994) Nature (London) **370**, 5768–5775

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