A synthetic peptide of the rab3a effector domain stimulates amylase release from permeabilized pancreatic acini

(rab protein/GTP-binding protein/exocytosis/regulated secretion)

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ABSTRACT In this study we have employed a synthetic peptide of the rab3a effector domain, rab3AL, to examine whether a rab-like low molecular weight GTP-binding protein is involved in protein release from the rat pancreatic acinar cell. The peptide was found to be a potent stimulator of amylase release from streptolysin-O-permeabilized pancreatic acini, with an EC_{50} of ≈ 60 μM. Stimulation of amylase discharge by rab3AL did not occur using either intact acini or permeabilized acini depleted of ATP. In contrast, a different effector domain peptide of the rab2 protein, rab2AL, a peptide with distinct sequence homology to rab3AL, was unable to stimulate amylase release, suggesting the specificity of the rab3AL response to rab3-like proteins. rab3AL stimulated release at $[Ca^{2+}]$ that were nonstimulatory in the absence of the peptide (10 nM). rab3AL potentiated the effect of guanosine 5'-[γ-thio]triphosphate on amylase secretion and decreased the amount of guanosine 5'-[γ-thio]triphosphate required for maximal secretion, suggesting that these two agents interact to modulate a distal step(s) of secretion. The above results provide functional evidence for the role of a rab-like low molecular weight GTP-binding protein and its effector protein(s) in the control of protein release from pancreatic acini. Because the discharge response to rab3AL is near the maximal obtainable from permeabilized acini, our results would suggest that rab3-like proteins control an important step in regulated secretion of amylase.

rab proteins are members of the ras superfamily of low molecular weight GTP-binding proteins that are important elements in the control of vesicular transport from the endoplasmic reticulum (ER) to the Golgi and between the Golgi cisternae (1–8). Studies examining the *SEC* mutations of yeast have identified a low molecular weight GTP-binding protein, Sec4p, which when absent leads to the accumulation of secretory vesicles in the cells' cytoplasm (9). Although the exact function of Sec4p is unknown, it is thought to regulate the targeting and/or fusion of the secretory vesicles with the yeast plasma membrane (10). As a low molecular weight GTP-binding protein is required for constitutive exocytosis in yeast, it is plausible that a similar protein may control regulated exocytosis in mammalian cells.

Studies employing nonhydrolyzable analogues of GTP to examine the mechanism of regulated secretion in neutrophils were the first to indicate that GTP-binding proteins influence exocytosis at two distinct biochemical sites (11). The first is at the level of the receptor and is involved in the activation of membrane phospholipases. The second is believed to directly activate the exocytotic machinery. Subsequent studies in mast cells using neomycin to inhibit phospholipase C confirmed the fact that a GTP-binding protein distinct from the receptor-linked classical guanine nucleotide binding reg-

ulatory proteins was involved in the regulation of exocytosis (12). This GTP-binding protein activity has been termed G_E. Little is known about how G_E influences exocytosis. However, data obtained from permeabilized cells has suggested that G_E stimulates exocytosis through interaction with a calcium-binding protein, possibly by activation of a phosphatase (13). The activity of G_E may itself be controlled by phosphorylation, possibly by protein kinase C.

The identity of G_E is unknown. This protein could be homologous to the classical heterotrimeric guanine nucleotide-binding regulatory proteins that transduce receptor-mediated signals across the plasma membrane. Alternatively, G_E could be a ras-like low molecular weight GTP-binding protein. Low molecular weight GTP-binding proteins have been found associated with secretory vesicles from a number of tissues (14, 15), including pancreatic acinar cells (16). Most of these proteins are as yet unidentified although a member of the rab family of low molecular weight GTP-binding proteins, rab3a, has been found principally located with synaptic vesicles (17, 18). These observations plus the findings that rab3a dissociates from synaptic vesicles during exocytosis (19) has led to the suggestion that this protein may be involved in regulated exocytosis.

rab proteins contain a conserved region consisting of amino acid residues 33–48 (Ha-ras numbering), which is referred to as the effector domain. Although the function of this domain is unknown, it is thought to work like its counterpart in ras (20–22). The ras effector domain is required for the regulation of GTP hydrolysis and is essential for the interaction of ras with an effector protein, the GTPase activating protein (GAP), which stimulates GTP hydrolysis (23–28). Structural analysis has shown that the ras effector domain undergoes conformational changes on GTP hydrolysis (29). Previous work on both ras and rab proteins have demonstrated that synthetic peptides to the effector domain are likely to block interaction with upstream or downstream effector proteins. These effector domain peptides were found to be potent inhibitors of transport from the ER to the cis Golgi and between the Golgi stacks. Inhibition of transport by the effector domain peptides was rapid and irreversible, and kinetic analysis indicated that the peptides blocked a late step in transport, coincident with a Ca^{2+}-dependent prefusion step (1).

In this study we have used a synthetic peptide of the rab3 effector domain, rab3AL (1), to obtain evidence that a rab-like protein is required for amylase release from permeabilized pancreatic acini. Incubation of the permeabilized cells with the peptide was observed to dramatically stimulate amylase discharge. Controls showed the response to be specific, requiring energy in the form of ATP, and not due to the peptide causing general lysis of amylase-containing or-

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Cell Biology: Padfield *et al.*

Proc. Natl. Acad. Sci. USA 89 (1992) 1657

ganelles. The strength of the rab3AL response indicates that the peptide is influencing an important regulatory mechanism in the control of amylase release. The findings of this study provide evidence that a rab-like protein and its potential upstream or downstream effector protein may function in regulated protein secretion from a mammalian cell.

MATERIALS AND METHODS

Materials. Streptolysin O was obtained from Wellcome Diagnostics. Collagenase (CLSPA) was purchased from Worthington. ATP and EGTA were obtained from Boehringer Mannheim. All other chemicals were from Sigma.

Isolation of Pancreatic Acini. One male Sprague–Dawley rat (80–100 g, starved overnight) was killed by cervical dislocation. The pancreas was removed, cleaned free of fat and connective tissue, and finely diced using a razor blade. Acini were prepared by a modification of the method of Bruzzone *et al.* (30). The pancreatic slices were then placed in a 25-ml siliconized conical flask with 5 ml of oxygenated medium containing 98 mM NaCl, 4.8 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.1% bovine serum albumin, 0.01% soybean trypsin inhibitor, and 25 mM Hepes (pH 7.4; dissociation buffer) containing 500 units of collagenase. The flask was capped and placed in a 37°C shaking water bath. After 5 min the flask was removed and the contents was poured into a 15-ml siliconized Corex tube; the tube was then capped and vigorously shaken by hand for 10 min to complete the dissociation of the pancreatic tissue. The acinar suspension was then filtered twice: (*i*) through surgical gauze to remove undissociated tissue and (*ii*) through a 200-μm nylon mesh to remove the larger clumps of acini. The filtered acinar suspension was then poured into a clean 15-ml siliconized Corex tube and diluted with the incubation buffer [139 mM potassium glutamate, 0.01% soybean trypsin inhibitor, and 20 mM Pipes (pH 6.6)]. The acini were left to settle out and after 3 min the buffer was removed and the acinar pellet was resuspended in 14 ml of incubation buffer. This process of sedimentation and resuspension was repeated three times to remove all traces of collagenase. The final acinar pellet was resuspended in 3–4 ml of permeabilization buffer.

Acinar Permeabilization and Stimulation. For a secretion experiment, 50 μl of the acinar suspension was added to 50 μl of incubation buffer containing 0.4 mM EGTA, 4 mM MgATP, streptolysin O (1.0 international unit/ml), and the desired concentration of the peptide. The buffer also contained enough $MgCl_2$ and $CaCl_2$ to give 2 mM free Mg^{2+} and 10 nM free Ca^{2+}. The acini were then incubated, with gentle agitation, at 37°C. After 7.5 min, amylase release was stimulated by the addition of 100 μl of the incubation buffer containing 2 mM MgATP, 3.8 mM EGTA, and enough $MgCl_2$ and $CaCl_2$ to give 2 mM free Mg^{2+} and the indicated free Ca^{2+} concentrations. After incubation at 37°C for 25 min, the acini were pelleted by centrifugation at 2000 × *g* for 2 min in an Eppendorf centrifuge. The supernatant (100 μl) was carefully removed so that the acinar pellet was not disturbed and assayed for amylase. The acini were resuspended in 1.5 ml of a buffer containing 120 mM NaCl, 0.5 mM $CaCl_2$, 20 mM Mops (pH 6.8), and 0.02% Nonidet P-40, then lysed by sonication (50 W at 5 sec on ice) prior to assay for amylase. For experiments where the cells were metabolically inhibited, the original acinar suspension was preincubated for 30 min with 5 μM antimycin A and 6 mM 2-deoxyglucose (glucose is absent from the medium) prior to dividing the suspension into 50-μl samples. This procedure was found to deplete the intracellular ATP by >99% and abolish the Ca^{2+}-dependent amylase release response (P.J.P., unpublished observations).

Calcium Buffers. The composition of the calcium buffers was formulated using a computer program kindly supplied by

W-P Instruments (New Haven, CT). This program uses algorithms for calculating free $[Ca^{2+}]$ and $[Mg^{2+}]$ according to ref. 31.

Amylase Assay. Amylase was measured using the assay developed by Bernfeld (32) except that the assay buffer was modified to contain 120 mM NaCl, 0.5 mM $CaCl_2$, and 20 mM Mops (pH 6.8). Amylase discharge is expressed as percent of total cellular amylase released in 25 min.

Peptide Synthesis. Peptides used in this study were synthesized by solid-phase synthesis on an Applied Biosystems automated peptide synthesizer (model 430). Deprotection and removal of the peptide from the resin support was effected by treatment with HF. All peptides (C-terminal amide derivatives) were purified using preparative HPLC on a reverse-phase C_{18} silica column (Vydac, 10 mm × 250 mm, 218TP) using a 0–65% (vol/vol) gradient of acetonitrile containing 0.05% trifluoroacetic acid. Homogeneity was demonstrated by analytical HPLC, and identity was confirmed by amino acid and sequence analysis.

Statistical Analysis. Statistical significance was determined using a two-tailed unpaired Student's *t* test. All statistical analysis was done using a computer program supplied by GraphPad (San Diego).

RESULTS

Influence of rab3AL on Ca^{2+}-Dependent Amylase Release. To gain access to the secretory machinery for biochemical analysis, acini were permeabilized using streptolysin O as described in our previous studies (33). To permit the peptides to equilibrate into the permeabilized cells prior to stimulation of amylase release, the acini were preincubated with the permeabilization agent and the peptide for 7.5 min before addition of the high-Ca^{2+} (stimulation) buffers. These conditions yielded an optimal peptide response without compromising the responsiveness to Ca^{2+}.

Incubation of the permeabilized acini with rab3AL, a peptide of the rab3a effector domain, was found to strongly stimulate Ca^{2+}-dependent amylase release. The concentration dependence of the rab3AL effect is illustrated in Fig. 1. The peptide had a maximal effective concentration of 100 μM with a EC_{50} of 60 μM. Maximally, rab3AL increased amylase discharge 150–230% over control values. The size of this response would indicate that the peptide is influencing an important regulatory step in amylase release. As the peptide produced such a pronounced effect on amylase release, and
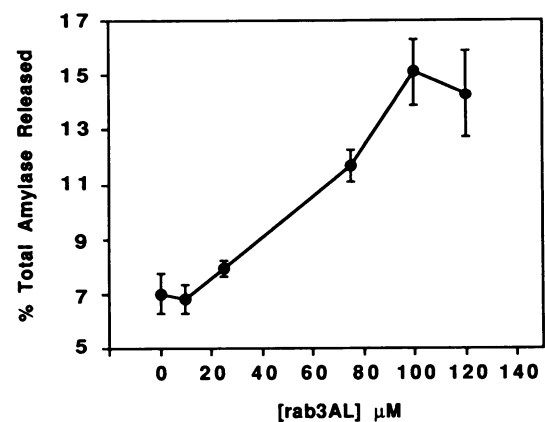

FIG. 1. Effect of rab3AL on amylase release elicited by 3 μM free Ca^{2+}. The peptide was preincubated with permeabilized pancreatic acini for 7.5 min prior to stimulation of amylase release by addition of Ca^{2+} stimulation buffer. The experiment shown is representative of four experiments conducted on four preparations of pancreatic acini. Data represent the mean ± SEM of five replicates.

to conserve peptide, all subsequent experiments were conducted using 60 μM (half maximal) peptide.

To determine whether the peptide stimulated a physiologically relevant pathway, rab3AL was incubated with either intact acini or permeabilized acini rendered secretory incompetent by depletion of ATP. In three experiments, rab3AL (60 μM) was found not to significantly increase amylase discharge from acini whose ATP levels were depleted by >99% (data not shown). Similarly, rab3AL (60 μM) was also found to have no significant influence on amylase discharge from nonpermeabilized acini (observations from four experiments; data not shown). This demonstrates that the site of action of the rab3AL is intracellular and that the peptide affects an ATP-dependent process, consistent with the requirement for ATP in secretagogue-stimulated secretion from intact pancreatic acinar cells (34). The findings of the ATP-depletion experiments also indicate that rab3AL is not increasing amylase discharge by initiating lysis of amylase-containing compartments (i.e., zymogen granules) or by causing spontaneous nonspecific fusion of amylase-containing organelles with the acinar cell plasma membrane. Furthermore, the peptide has no amphipathic structure that would confer detergent-like properties (W.E.B., unpublished data).

Specificity of rab3AL Response. To investigate the specificity of the rab3AL response, we examined the influence of various rab-effector-domain-related peptides on amylase release from permeabilized acini. The peptides tested are shown in Fig. 2. The rab3 peptide has the native sequence of the rab3a effector domain (residues 33–48, Ha-ras numbering) of the intact protein. rab3AL is identical to the effector domain except that alanine and leucine are substituted, respectively, for the native threonine and valine. In ER-to-Golgi transport, these substitutions were found to markedly enhance the inhibitory properties of the peptide (1). rab1AL and rab2AL are peptides corresponding to the effector domains of rab1 and rab2, respectively, but like rab3AL both peptides have alanine and leucine substituted, respectively, for the native threonine and isoleucine.

The influence of the various peptides on amylase release is shown in Fig. 3. At 50 μM only rab3AL produced a significant increase in amylase discharge. rab1AL, a peptide with a homologous sequence to rab3AL, and the rab3 peptide were also observed to slightly elevate amylase discharge. rab2AL, a peptide of the rab2 protein with low homology to rab3AL, produced no detectable change in release. When the peptide concentration was increased to 100 μM (Fig. 3B), rab2AL again was found to have no detectable influence on secretion. However, at this higher concentration, the rab3 peptide did produce a significant increase in amylase release, this increase being ≈50% of that obtained with rab3AL. Due to the relative insolubility of the rab1AL peptide in the permeabilization buffer it was not possible to test the effect of this peptide at the higher concentration. The finding that rab2AL (100 μM) and rab1AL (50 μM) could not influence amylase release suggests that the response to rab3AL is specific.

Ca^{2+} Dependence of the rab3AL Response. Examination of the Ca^{2+} dependence of the rab3AL effect (Fig. 4) showed that the peptide elicited amylase release at nonstimulatory concentrations of free Ca^{2+} (10 nM). Since the increases in

FIG. 3. Influence of various rab-effector-domain-related peptides on amylase release stimulated by 1 μM free Ca^{2+}. (*A*) Effect of 50 μM rab1AL, 50 μM rab2AL, 50 μM rab3AL, and 50 μM rab3 on amylase release. (*B*) Effect of 100 μM rab2AL, 100 μM rab3AL, and 100 μM rab3 on amylase release. In both sets of experiments, the peptide was preincubated with the permeabilized pancreatic acini for 7.5 min prior to stimulation of amylase release by addition of the Ca^{2+} stimulation buffer. The experiment shown is representative of three experiments conducted on three preparations of pancreatic acini. Data represent the mean ± SEM of five replicates. Results of two-tailed Student's t test comparing response to specific peptide vs. the control are shown. $*$, $P < 0.05$; $**$, $P < 0.01$.

amylase release produced by elevating the [Ca^{2+}] from 10 nM to 5 μM or 10 μM in the presence of 60 μM peptide are not statistically significantly different from each other, we are unable to determine whether or not rab3AL alters the Ca^{2+} sensitivity of the secretory response. At higher concentrations of free Ca^{2+} (10 μM and above), the peptide did not have any significant effect on the Ca^{2+}-dependent response.

PEPTIDE	SEQUENCE
rab1AL	I S **A L** G V D F K I R T I E L D
rab2AL	D L **A L** G V E F G A R M I T I D
rab3AL	V S **A L** G I D F K V K T I Y R N
rab3	V S T V G I D F K V K T I Y R N

FIG. 2. Sequences of rab peptides used in permeabilized pancreatic acinar cells. The substituted amino acids are denoted by the boldface type.
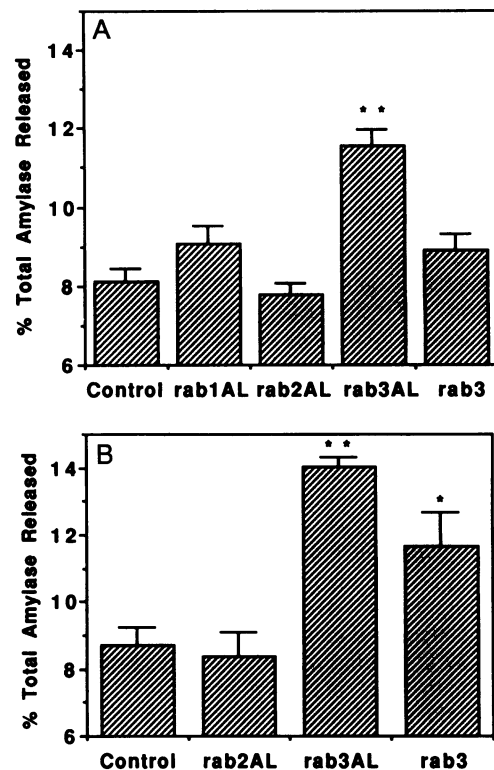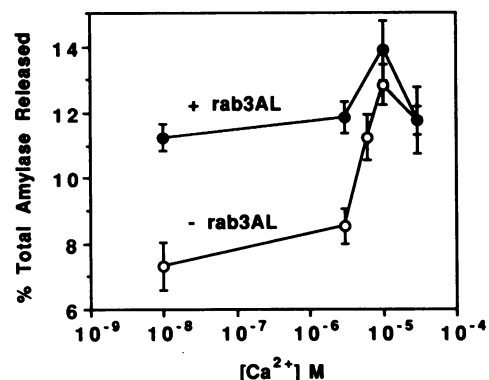

FIG. 4. Influence of 60 μM rab3AL on Ca^{2+}-dependent amylase release. Permeabilized pancreatic acini were incubated with or without the peptide for 7.5 min prior to stimulation of amylase release by addition of the Ca^{2+} stimulation buffer. The experiment shown is representative of four experiments conducted on four preparations of pancreatic acini. Data represent the mean ± SEM of five replicates.

Influence of rab3AL on Guanosine 5'-[γ-thio]Triphosphate (GTP[γS])-Stimulated Amylase Release. Recent work from our laboratory has demonstrated that GTP[γS], the nonhydrolyzable analogue of GTP, can also stimulate amylase release from acini at nonstimulatory concentrations of free Ca^{2+}. These results suggest that both rab3AL and the GTP[γS] may act through a common GTP-binding protein to increase amylase discharge. To test this hypothesis, we examined the influence of 60 μM rab3AL (about half maximal) on GTP[γS]-dependent amylase release. The results of these studies are shown in Fig. 5A. They show that rab3AL decreased the maximal concentration of GTP[γS] required for release, shifting the EC_{50} for the nucleotide from 30 μM to <10 μM GTP[γS]. At 10 μM GTP[γS], the effect of the peptide was not purely additive but significantly potentiated the GTP[γS] response. In contrast, at supramaximal GTP[γS] concentrations, the increase in amylase release produced by addition of the peptide was significantly diminished and not different from that obtained with GTP[γS] alone. The maximal responses obtained with GTP[γS] and the peptide shown in Fig. 5 do not significantly exceed the maximal responses to the peptide in the absence of GTP[γS] shown in other experiments; variability of responses of different prepara-
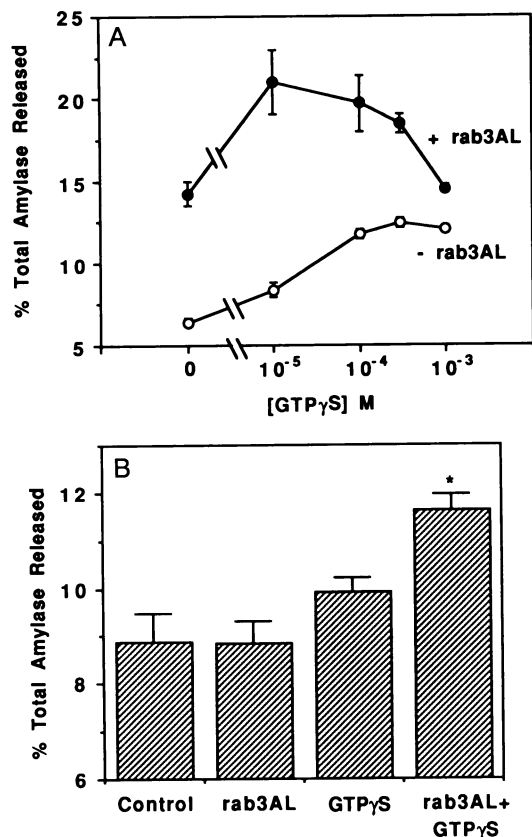
tions of acini account for the apparent increase in extent of release seen here.

To obtain more evidence that the peptide may potentiate the GTP[γS] response, we examined the effect of a lower concentration (20 μM) of rab3AL on amylase release elicited by 50 μM GTP[γS]. Under these conditions the peptide was found to potentiate the GTP[γS] response (Fig. 5B), although the degree of potentiation was variable among preparations (data not shown). These findings indicate that rab3AL and GTP[γS] interact positively to influence amylase release from permeabilized acinar cells.

DISCUSSION

The rab3AL peptide was found to be a potent stimulator of amylase release from streptolysin-O-permeabilized rat pancreatic acini over a 4-fold concentration range (25–100 μM), comparable to that found for inhibition of vesicular transport *in vitro* (1). This response does not appear to be due to the peptide either influencing amylase secretion from the small number of nonpermeabilized acinar cells present in the preparation or producing lysis of amylase-containing organelles. The rab3AL response is also not due to a nonspecific stimulation of release. This was clearly demonstrated by the inability of rab2AL to alter amylase release even at 100 μM and by the requirement of the response to rab3AL for ATP.

It is presently unclear why rab3AL is a more potent stimulator of amylase release than the rab3 peptide, which has the native sequence of the conserved region of the rab effector domain. In ER-to-Golgi transport, the alanine and leucine substitutions present in rab3AL were made because site-directed mutagenesis of ras suggested that these amino acid changes result in marked reduction of the ability of GAP to stimulate the inherent GTPase activity of ras. It was anticipated that these substitutions would produce a less-potent peptide (1). However, the converse has been found to be true, first for Golgi-related vesicular transport (1) and now for amylase secretion. There are several possible explanations for the potency of rab3AL. (*i*) The conformation of the substituted peptide may be more adaptable for binding the rab effector protein than the native effector domain sequence. (*ii*) There may be some subtle variations in the mechanism of the rab and ras effector domains function that are unknown at the present.

The specificity of the rab3AL response may reflect the possibility that rab3AL competitively inhibits the association of an endogenous rab3-like protein with a downstream effector protein that stimulates the intrinsic rate of GTP hydrolysis. Although the identity of this protein is unknown, using ras as a predictive model for rab function the effector protein may be a GAP-like protein. If so, the inhibition of rab–GAP interaction by the peptide would keep the rab protein in its GTP-bound conformation. However, according to the model of constitutive secretion proposed by Bourne (35), this should inhibit release. Because the rab3AL peptide and GTP[γS] both exert a positive regulatory effect on release, this model may not be valid for regulated secretion, which may require the rab-like protein to be in its GTP-bound active form to promote exocytosis. Therefore, it is possible that the postulated GTPase may normally exert an inhibitory effect on regulated secretion and the peptide may relieve this inhibition by blocking its action on the GTP-bound form of a rab3-like protein. The response to the peptide that we observe might then be due to rab3-like proteins that were activated after permeabilization and introduction of the peptide by binding cytosolic GTP or to an exchangeable pool of GTP-bound rab3-like proteins that were present in the cells prior to permeabilization. It is also possible that rab3AL directly stimulates a downstream effector that differs from a GAP-like protein. This possibility does not require that GTP

FIG. 5. (*A*) Influence of 60 μM rab3AL on GTP[γS]-dependent amylase release at 10 nM free Ca^{2+}. Permeabilized pancreatic acini were incubated with or without the peptide for 7.5 min prior to stimulation of amylase release by addition of the GTP[γS]-containing stimulation buffer. The experiment shown is representative of four experiments conducted on four preparations of pancreatic acini. Data represent the mean ± SEM of five replicates. (*B*) Influence of 20 μM rab3AL and/or 50 μM GTP[γS] on amylase release at 10 nM free Ca^{2+}. The experiment shown is representative of three experiments conducted on three preparations of pancreatic acini. Data represent the mean ± SEM of five replicates. Two-tailed Student's *t* test comparing the response to rab3AL (20 μM) plus GTP[γS] with that obtained for 50 μM GTP[γS] indicates that the difference is significant. *, $P < 0.05$.

be continuously present, which may be the case in permeabilized cells. If rab3AL mimics the active conformation of the native rab3-like effector region when GTP is bound, this may explain the apparently greater effectiveness of rab3AL compared to the rab3 peptide under limiting GTP levels.

Which of the rab proteins is rab3AL peptide influencing? From the specificity of rab3AL response one would assume that the affected protein would have an effector domain that has close sequence homology to that of rab3a. One possibility is rab3a itself. This protein has been localized to synaptic vesicles (17) and has been observed to translocate from the membrane to the cytosol during exocytosis (19). These findings have been interpreted as evidence for the involvement rab3a in regulated exocytosis. A rab3 protein has been detected in pancreatic acinar cells and appears to be principally associated with the membrane of zymogen (secretory) granules (B. P. Jena and J.D.J., unpublished observation). Another possible candidate is rab1, whose effector domain has >95% sequence homology and 75% identity to rab3AL. However, we feel that it is unlikely that rab1 is involved in amylase secretion. First, if rab1 does function in exocytosis, one would expect that the rab1AL peptide, whose amino acid sequence encompasses the effector domain of rab1, would be a more potent stimulator of amylase release. Second, the rab1 protein has been primarily localized to the ER and Golgi-related elements (1) and has not been detected on secretory vesicles. Another distinct possibility is that rab3AL is influencing an as yet uncharacterized member of the rab family of proteins. Studies from our laboratory have identified at least seven low molecular weight GTP-binding proteins associated with the plasma membrane and zymogen granule membranes from the rat exocrine pancreas (16). Since the majority of these proteins still remain unidentified, rab3AL could be influencing the activity of one of them.

The permeabilized cell assay for exocytosis allows one to examine the terminal step(s) in the secretory process (36). Although we are unable to define at precisely what stage in the amylase release process rab3AL influences discharge, the strength of the peptide response suggests that the target protein controls an important distal regulatory step. In fact the secretory responses to rab3AL are very similar to the levels of secretion caused by secretagogues such as caerulein in nonpermeabilized acini prepared by the same method (30). The fact that the effects of GTP[γS] and rab3AL on amylase release are synergistic suggests that both agents interact at an unknown but possibly common site to modulate secretion and accordingly that a cascade of GTP-binding proteins may interact to regulate the terminal stages of regulated exocytosis.

Is the GTP-binding protein that rab3AL influences G_E? Recent research from this (33) and other laboratories (37) has indicated that a G_E-like activity may be present in the exocrine pancreas. The findings of our study support the present model for G_E function (38) and, therefore, it is feasible that rab3AL is influencing G_E activity. This interpretation would infer that G_E is a rab-like low molecular weight GTP-binding protein. Further characterization of G_E and the rab3AL response in the pancreatic acinar cell is required to address this question.

In conclusion, the findings of this study provide functional evidence that a rab-like low molecular weight GTP-binding protein and a potential downstream effector protein may be involved in regulated exocytosis in mammalian cells. This protein appears to exert a positive regulatory influence over an important step in the process of amylase release.

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