RESEARCH COMMUNICATION

Stimulation of exocytotic membrane fusion by modified peptides of the rab3 effector domain: re-evaluation of the role of rab3 in regulated exocytosis

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We have shown previously that fusion between pancreatic zymogen granules and plasma membranes is stimulated by a peptide corresponding to the putative effector domain of rab3. Here we show that this stimulatory effect persists when the amino acid sequence of the peptide is substantially modified. We also show that an antibody raised against rab3a recognizes a protein of appropriate size on the zymogen-granule membrane, but has no effect on membrane fusion. We suggest that rab3 is not directly involved in the control of this membrane fusion event, and that the peptides are stimulating fusion by a mechanism unrelated to rab3.

INTRODUCTION

Small (20–30 kDa) monomeric GTP-binding proteins of the rab family have been implicated in the control of intracellular protein traffic (Gruenberg and Clague, 1992). Members of the rab family have been shown by immunocytochemistry to be present on the cytoplasmic surfaces of specific membrane compartments (Goud and McCaffrey, 1991), and there is considerable evidence that vesicular transport between compartments is controlled, at least in part, by these proteins. This evidence includes the inhibition of transport both by the non-hydrolysable GTP analogue, guanosine 5′-[γ-thio]triphosphate (GTP[S]) (Beckers and Balch, 1989; Melançon et al., 1990), and by synthetic peptides of the 'effector domains' of the rab proteins (Plutner et al., 1990). A model for the operation of the rab proteins that incorporates these experimental findings was proposed by Bourne (1988). According to this model, the GTP-binding proteins would act as molecular switches, with hydrolysis of GTP driving the vectorial transport of proteins from one compartment to another. GTP[S] would then block transport by preventing GTP hydrolysis; the synthetic effector domains would inhibit by binding to a GTPase-activating protein (GAP) (Calés et al., 1988; Vogel et al., 1988).

Secretory vesicles from several cell types have rab3 bound to their cytoplasmic surfaces. For example, in neurones, rab3a is found specifically on the membranes of synaptic vesicles (Fischer von Mollard et al., 1990) and, furthermore, dissociates from these vesicles during Ca^{2+}-dependent neurotransmitter release in synaptosomes (Fischer von Mollard et al., 1991). rab3-like proteins have also been shown to be present on the membrane of the pancreatic zymogen granule (Padfield and Jamieson, 1991; Schnefel et al., 1992). In the light of these findings, it has been proposed that rab3 is involved in the control of membrane fusion during regulated exocytosis (Fischer von Mollard et al., 1991). There are two significant differences, however, between this membrane fusion event and those involved in constitutive protein transport. First, GTP[S] almost always stimulates regulated exocytosis (Gomperts, 1990), and secondly, synthetic peptides of the effector domain of rab3 stimulate both secretion in permeabilized cells (Padfield et al., 1992; Senyshyn et al., 1992; Oberhauser et al., 1992) and exocytotic membrane fusion *in vitro* (Edwardson et al., 1993). These atypical features of this membrane fusion event have led to the proposal that rab3 functions in a different way from the other members of the family, such that the GTP-bound form is responsible for promoting fusion (Padfield et al., 1992). Further contortions are necessary to account for the stimulatory effect of the effector peptides. One possibility is that they keep rab3 in its GTP-bound state by binding to and inhibiting its GAP; another is that the peptides activate the normal (unidentified) downstream effector protein in place of rab3 (Padfield et al., 1992; Oberhauser et al., 1992).

We have recently provided evidence that the rab3 effector peptides stimulate fusion between pancreatic zymogen granules and plasma membranes through a mechanism distinct from that of GTP[S] (Edwardson et al., 1993). In the present study, we have examined the effects on this exocytotic membrane fusion event of modified rab3-effector domains, i.e. a rearranged form, a scrambled form and a truncated form. We show that the first two of these peptides are able to stimulate fusion, but that the third is not. We also show that a monoclonal antibody raised to rab3a recognizes a target of appropriate size on the membrane of the zymogen granule, but does not affect granule/plasma membrane fusion, either under basal conditions or in the presence of GTP[S]. We suggest that these results rule out the idea that the effector peptides act by mimicking the effects of an authentic form of rab3 on the zymogen-granule membrane, and in fact indicate that rab3 might not be involved at all in the control of exocytotic membrane fusion, at least in this cell type.

MATERIALS AND METHODS

Octadecylrhodamine B chloride was obtained from Molecular Probes (Eugene, OR, U.S.A.). M_r markers and horseradish peroxidase (HRP)-conjugated goat anti-(mouse IgG) were from Bio-Rad (Hemel Hempstead, Bucks., U.K.). GTP[S] was from Boehringer–Mannheim, Germany. All other reagents were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Abbreviations used: GTP[S], guanosine 5′-[γ-thio]triphosphate; HRP, horseradish peroxidase; GAP, GTPase-activating protein.

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Peptide synthesis was carried out on an SMPS multiple peptide synthesis machine (Camden Biologicals, Cambridge, U.K.) using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry. Purity was demonstrated by analytical h.p.l.c. (Waters, Milford, MA, U.S.A.). Sequence identity was verified by Edman degradation on an automated gas-phase protein sequencer (Applied Biosystems, Warrington, Cheshire, U.K.).

Zymogen granules, zymogen-granule membranes and plasma membranes were prepared from rat pancreas as described previously (Nadin et al., 1989). Cytosol was prepared by high-speed centrifugation (100000 *g* for 30 min) of pancreatic homogenate.

For fluorescence de-quenching assays, granules from a single rat [300 μl of suspension in 280 mM sucrose/5 mM Mes buffer, pH 6.0; protein concentration approx. 5 mg/ml (Bradford, 1976)] were loaded with octadecylrhodamine B chloride (100 μM) by incubation at 37 °C for 5 min. Labelled granules were recovered by centrifugation at 900 *g* for 10 min and resuspended in the original volume of buffer. Plasma membranes were stored in aliquots at −20 °C and thawed immediately before use. De-quenching assays were carried out using a Perkin–Elmer (Beaconsfield, Bucks., U.K.) LS-3 luminescence spectrometer connected to a pen recorder. Wavelengths used were 560 nm (excitation) and 590 nm (emission). Samples of labelled granules (10 μl) were added to 700 μl of sucrose/Mes buffer, pH 6.5, at 37 °C, and a steady baseline was obtained. Peptides or GTP[S] were then added, followed 30 s later by plasma membranes (either 15 or 30 μg/ml protein). The fluorescence signal was followed for 6 min. De-quenching was expressed as a percentage of that achieved after solubilization of membranes by addition of 0.2% (v/v) Triton X-100. All errors are S.E.M.s. Initial quenching was typically 95%. As explained previously (MacLean and Edwardson, 1992), the relationship between de-quenching and the percentage of granules undergoing fusion is complex, so that de-quenching values can be regarded essentially as arbitrary units.

For immunoblotting, proteins from fractions of the exocrine pancreas (50 μg/lane) were separated on an SDS/polyacrylamide gel (12.5%, w/v), transferred to nitrocellulose and probed with a mouse monoclonal antibody raised against rab3a (1:500 dilution of raw ascites; overnight incubation at 4 °C), followed by HRP-conjugated goat anti-(mouse IgG) (1:1000; 2 h incubation at room temperature). The antibody raised against rab3 was kindly supplied by Dr. R. Jahn of the Howard Hughes Medical Institute, Yale University, U.S.A.

RESULTS

Rab3-derived peptides

The sequences of the peptides used in this study are shown in Figure 1. They are based on the sequence of rab3AL, the putative effector domain of rab3 (16-mer), with Ala-Leu substituted for Thr-Val at positions 3 and 4. This peptide has been shown to stimulate regulated exocytosis in pancreatic acini (Padfield et al., 1992), chromaffin cells (Senyshyn et al., 1992) and mast cells (Oberhauser et al., 1992), and also to stimulate fusion between pancreatic zymogen granules and plasma membranes *in vitro* (Edwardson et al., 1993). The modified peptides used were: 'rearranged rab3AL', in which the 16-mer was notionally divided into three regions which were then re-ordered, 'scrambled rab3AL', in which the amino acids of rab3AL were placed in a random order, and 'rab3AL 5-mer', which is composed of the first five amino acids of the effector domain, and which has been shown to trigger exocytosis in mast cells (Oberhauser et al., 1992).

Effects of peptides on fusion between pancreatic zymogen granules and plasma membranes

The lipid-soluble fluorescent probe octadecylrhodamine B chloride was loaded into the membranes of the zymogen granules at a concentration that resulted in the self-quenching of its fluorescence. Fusion between the labelled granules and unlabelled plasma membranes was then measured through the consequent dilution-dependent de-quenching (Hoekstra et al., 1984; MacLean and Edwardson, 1992; Edwardson et al., 1993). Typical de-quenching traces obtained in the presence of the various peptides are shown in Figure 2. Plasma membranes alone (30 μg/ml protein) gave a de-quenching signal of 1.2%. In the presence of rab3AL (50 μM) the same concentration of membranes gave a signal of 2.7%. Both rearranged rab3AL and scrambled rab3AL

VSTVGIDFKVKTIYRN	rab3
VSALGIDFKVKTIYRN	rab3AL
DFKVKVSALGITIYRN	Rearranged rab3AL
YNVLSADFKVGKIRIT	Scrambled rab3AL
VSALG	rab3AL 5-mer

Figure 1 Rab3-derived peptides

Amino acid sequences (single-letter code) of the four peptides used in this study, compared with the sequence of the authentic rab3-effector domain. Amino acids that are different in rab3 and rab3AL are shown in bold type. Residues that are likely to be electrically charged at pH 6.5 are indicated.

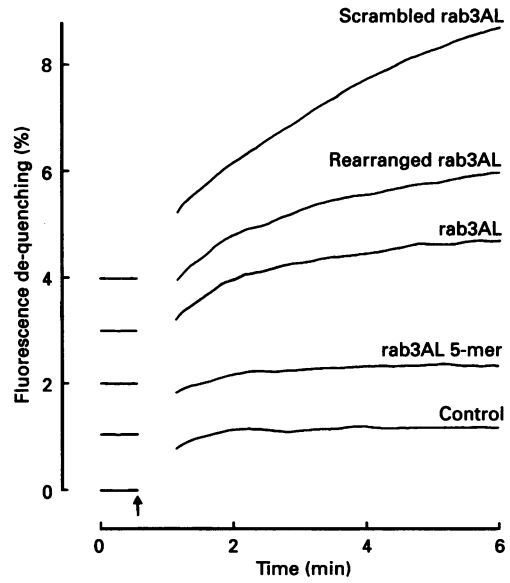

Figure 2 Effects of peptides on fusion between pancreatic zymogen granules and plasma membranes

Membrane fusion was measured through the de-quenching of the lipid-soluble probe octadecylrhodamine B chloride, loaded into the membranes of the zymogen granules. All peptide concentrations were 50 μM. Peptides were added to the suspension of labelled granules, and pancreatic plasma membranes (30 μg/ml protein) were added 30 s later, at the point indicated by the arrow. The basal fluorescence at the time of addition of membranes is indicated for each trace by the bar. Traces are separated by 0.5% de-quenching units for clarity.

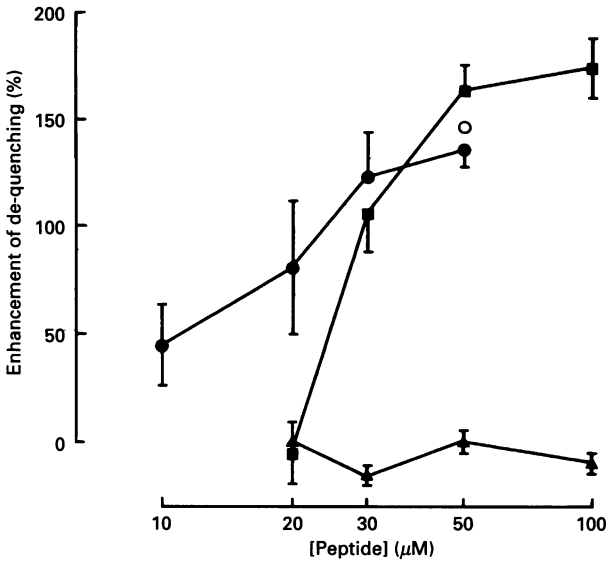

Figure 3 Concentration-dependence of effects of peptides on membrane fusion

Combined data from three separate experiments at a constant plasma membrane protein concentrations (30 μg/ml). Symbols: ●, rearranged rab3AL; ■, scrambled rab3AL; ▲, rab3AL 5-mer. The point shown for rab3AL (○) represents the maximal effect of the peptide (data taken from Edwardson et al., 1993).

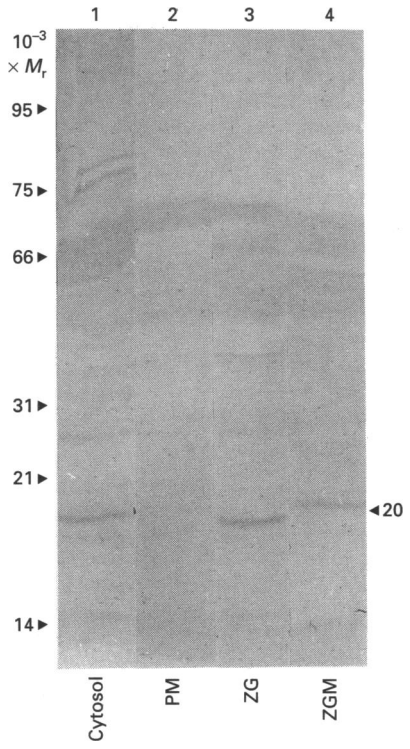

Figure 4 Detection of rab3 immunoreactivity in fractions from the exocrine pancreas

Proteins from fractions of the exocrine pancreas [cytosol, plasma membranes (PM), zymogen granules (ZG) and zymogen-granule membranes (ZGM)] were separated on an SDS/polyacrylamide gel (12.5%), transferred to nitrocellulose and probed with a mouse monoclonal antibody raised against rab3a (1:500 dilution of raw ascites), followed by HRP-conjugated goat anti-(mouse IgG) antibody (1:1000). M_r values of markers are shown on the left.

(50 μM) also enhanced the signal given by membranes (to 2.8% and 4.3% respectively). rab3AL 5-mer (50 μM), on the other hand, caused no significant enhancement of the signal given by membranes (1.3%).

The concentration-dependence of the effects of the modified peptides, at a single concentration of plasma membranes (30 μg/ml), is shown in Figure 3. We have shown previously that at high membrane concentrations, rab3AL produces a maximal enhancement of fusion of 150% at a concentration of 50 μM, with an EC_{50} of 15 μM (Edwardson et al., 1993). The stimulation produced by both rearranged rab3AL and scrambled rab3AL was also concentration-dependent, with maximal enhancements of 140% and 180% respectively, and EC_{50} values of approx. 20 μM and 30 μM. rab3AL 5-mer produced no enhancement at any of the concentrations tested. Figure 3 shows the results for concentrations of rab3AL 5-mer up to 100 μM; in a further experiment (results not shown) this peptide was found to be ineffective up to 400 μM.

Effect of anti-rab3a antibody on granule/plasma membrane fusion

A mouse monoclonal antibody raised against recombinant rab3a recognized a protein of M_r 20000 on immunoblots of pancreatic zymogen granules and granule membranes (Figure 4). Reactivity was also observed in cytosol but not in plasma membranes. Despite this ability to bind to a rab3-like protein on the zymogen-granule membrane, however, the antibody had no significant effect either on basal fusion between zymogen granules and plasma membranes or on the stimulation of fusion by GTP[S]. Values for de-quenching with plasma membranes (15 μg/ml) were $1.1 \pm 0.3\%$ ($n = 8$) in the control, $0.8 \pm 0.2\%$ ($n = 8$) in the presence of anti-rab3a ascites, at a 1:500 dilution, and $0.9 \pm 0.3\%$ ($n = 8$) in the presence of control ascites at the same concentration. Stimulation of de-quenching by GTP[S] (100 μM) was $0.2 \pm 0.0\%$ ($n = 3$) in the control, $0.3 \pm 0.1\%$ ($n = 3$) with anti-rab3 ascites and $0.1 \pm 0.0\%$ ($n = 3$) with control ascites.

DISCUSSION

We have shown previously (Edwardson et al., 1993) that peptides of the effector domain of rab3 stimulate fusion between pancreatic zymogen granules and plasma membranes *in vitro*. In the present study, we have demonstrated that rearranged and scrambled forms of the rab3AL peptide retain biological activity. This result indicates that activity does not require a particular sequence of amino acid residues, and casts considerable doubt on the idea that the peptides stimulate fusion as a result of specific binding to a protein that normally interacts with rab3. It is much more likely that the peptides share some physico-chemical feature that accounts for their activity. In fact, all of the active peptides have three positively charged amino acids and a net positive charge. In contrast, inactive peptides, such as the effector domains of ADP-ribosylation factor and rab2AL, the C-terminal domains of rab3a and rab3b (Edwardson et al., 1993), and also the rab3AL 5-mer (this study), have no, or at most one, positively charged amino acid and a net neutral or negative charge. This pattern persists when other peptides unrelated to rab3 are tested in the membrane-fusion assay. We have recently found, for example, that the peptides mastoparan and substance P, both of which have multiple positive charges, also enhance fusion between zymogen granules and pancreatic plasma membranes (C. M. MacLean, G. J. Law and J. M. Edwardson, unpublished work). Interestingly, it has been shown that not only mastoparan and substance P (Mousli et al., 1989), but also the rab3 peptides (Law et al., 1993), trigger mast-cell degranulation through a

pertussis-toxin-sensitive mechanism. Furthermore, the rab3AL peptide has been found to stimulate inositol 1,4,5-trisphosphate production in pancreatic acinar cells at concentrations similar to those required to trigger amylase release (Piiper et al., 1993). One interpretation of these results is that the peptides are able to affect the behaviour of heterotrimeric GTP-binding proteins, such as G_i and G_q.

In addition to their effect on the extent of fusion between zymogen granules and plasma membranes, the rab3 peptides also had effects on the kinetics of fusion. For example, in the experiment illustrated in Figure 2, addition of the scrambled peptide increased the half-time of fusion from 30 s to almost 2 min. This effect on kinetics may indicate that the peptides are activating a time-dependent mechanism that eventually leads to membrane fusion. The peptides seem not to be able to cause membranes to fuse together randomly, since we have found that the rab3 effector peptide will not render a previously inactive membrane (liver plasma membranes) able to fuse with pancreatic zymogen granules *in vitro* (results not shown). It is possible that the two interacting membranes need to dock together before the peptides can produce their effect.

The lack of effect of the rab3AL 5-mer alluded to above contrasts with its positive effect on exocytosis in mast cells at similar concentrations (Oberhauser et al., 1992). This difference may be a consequence of the different techniques used to detect exocytotic membrane fusion. It is perhaps worth noting, however, that in the mast-cell system, degranulation is triggered by a surprising group of peptides. For example, even a 5-mer derived from the effector domain of rab1AL has activity when applied intracellularly under patch-clamp conditions.

Immunoblot analysis revealed the presence of a rab3-like protein on the membrane of the zymogen granule. This result is at variance with those of Schnefel et al. (1992), who identified a protein of M_r 27000 with an antibody that recognizes both rab3a and rab3b (clone 42.1), but found no reactivity with the antibody used here (clone 42.2), that recognizes only rab3a. We have no explanation for this discrepancy. We used the antibody at a higher concentration (1:500 dilution, compared with 1:1000), but we feel that it is unlikely that this twofold change could account for the qualitative difference in the results. Despite the presence of a rab3-like protein on the membrane of the zymogen granule, the anti-rab3a antibody had no effect in our functional assay. We cannot completely rule out the possibility that the antibody binds to the target without interfering with its function. There are, however, precedents for the inhibition of rab function by antibodies. For example, a monoclonal antibody to rab1b inhibits endoplasmic-reticulum-to-Golgi membrane traffic in permeabilized Chinese hamster ovary cells (Plutner et al., 1991) and an antiserum to rab5 inhibits early endosome fusion *in vitro* (Gorvel et al., 1991). Consequently, we would expect the anti-rab3a antibody also to block rab3 function. The fact that the antibody was ineffective strongly suggests that rab3 does not play any part in the control of membrane fusion, at least in the pancreatic acinar cell. It is, of course, still possible that rab3 is involved in the control of exocytosis at a point upstream of the final membrane fusion event.

It remains, then, to explain the mechanism of action of GTP[S] on exocytotic membrane fusion. We have shown previously (Edwardson et al., 1993) that the stimulatory effect of GTP[S] is qualitatively and quantitatively different from that of the rab effector peptides. If we rule out the possibility that rab3 is involved in the control of membrane fusion, we no longer need to modify the Bourne (1988) model for the operation of monomeric GTP-binding proteins in order to account for the stimulatory action of GTP[S] on regulated exocytosis. The effect of GTP[S] on this step is much more easily accommodated by a model involving heterotrimeric GTP-binding proteins, which are known to be active in the GTP-bound state. Evidence that heterotrimeric GTP-binding proteins are involved in intracellular membrane traffic is accumulating rapidly (see, for example, Stow et al., 1991; Pimplikar and Simons, 1993). The involvement of these proteins in exocytotic membrane fusion, however, remains to be directly demonstrated.

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