Volume 320, number 1, 52–56 FEBS 12265 March 1993

Synthetic peptides of the rab3 effector domain stimulate a membrane fusion event involved in regulated exocytosis

J. Michael Edwardson[a], Carol M. MacLean[a] and Greg J. Law[b]

[a]*Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK* and [b]*AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, UK*

Received 27 January 1993; revised version received 12 February 1993

We have developed a system in which the fusion of pancreatic zymogen granules with plasma membranes can be studied in vitro. Here we show that this membrane fusion event is stimulated specifically by peptides of the effector domain of rab3, a small, monomeric GTP-binding protein. In addition, we demonstrate that the stimulatory effect of the peptides involves their binding to a target on the plasma membrane, and is both qualitatively and quantitatively different from the effect of GTPγS, which also enhances membrane fusion. We suggest that regulated exocytosis in the pancreatic acinar cell may be under the control of more than one type of GTP-binding protein.

rab3; GTP-binding protein; Membrane fusion; Exocytosis

1. INTRODUCTION

Members of the super-family of small (20–30 kDa), monomeric GTP-binding proteins are known to be involved in the control of intracellular membrane traffic [1,2]. These proteins include the products of the yeast Sec4 [3] and YPT1 [4] and the various rab proteins, each of which is localized to a specific membrane compartment [5]. The control of membrane fusion during regulated exocytosis appears to involve rab3. This protein is found, for example, on the membranes of synaptic vesicles [6], and has been shown to dissociate from these vesicles during exocytosis [7]. In addition, several monomeric GTP-binding proteins have been found on the membrane of the pancreatic zymogen granule [8], one of which appears to be rab3-like [9,10]. Finally, it has recently been reported that peptides of the rab3 effector domain stimulate exocytosis in pancreatic acini [9], in chromaffin cells [11] and in mast cells [12]. These findings have been taken to indicate that G_E, the elusive GTP-binding protein believed to be involved in the control of exocytosis [13], might be a rab-like protein. We have developed a system in which the fusion of zymogen granules with plasma membranes can be studied in vitro [14,15]. Here we show that this membrane fusion event is stimulated specifically by peptides of the rab3 effector domain, but that their effects are both qualitatively and quantitatively different from that of GTPγS.

Correspondence address: J.M. Edwardson, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK. Fax: (44) (223) 334040.

2. MATERIALS AND METHODS

Peptide synthesis was performed on an SMPS Multiple Peptide Synthesis machine (Zinsser Analytic, Maidenhead, UK) using Fmoc chemistry. Purity was demonstrated by analytical HPLC (Waters, Milford, MA, USA). Sequence identity was verified by Edman degradation on an automated gas-phase protein sequencer (Applied Biosystems, Warrington, UK).

Zymogen granules, plasma membranes and zymogen granule membranes were prepared from rat pancreas as described previously [14]. Granules prepared from a single rat (300 μl suspension in 280 mM sucrose, 5 mM MES buffer, pH 6.0; total protein concentration approx 5 mg/ml) 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 and granule membranes were stored in aliquots at −20°C and thawed immediately before use. De-quenching assays were carried out using a Perkin-Elmer (Beaconsfield, UK) 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 sucrose/MES buffer, pH 6.5, at 37°C and a steady baseline was obtained. Peptides were then added, followed approximately 30 s later by a sample of target membranes. The fluorescence signal was usually followed for 5 min. De-quenching was expressed as a percentage of that achieved after solubilization of membranes by addition of 0.2% Triton X-100. All errors given are standard errors of the mean. Initial quenching was typically 95%. As explained previously [15], de-quenching values underestimate the extent of membrane fusion, and can be regarded essentially as arbitrary units.

3. RESULTS

In this study, membrane fusion was measured directly using a fluorescence dequenching technique [16]. The membranes of isolated pancreatic zymogen granules were loaded with the lipid-soluble fluorescent probe octadecylrhodamine B-chloride, at a concentration that resulted in the self-quenching of its fluorescence. The

Published by Elsevier Science Publishers B.V.

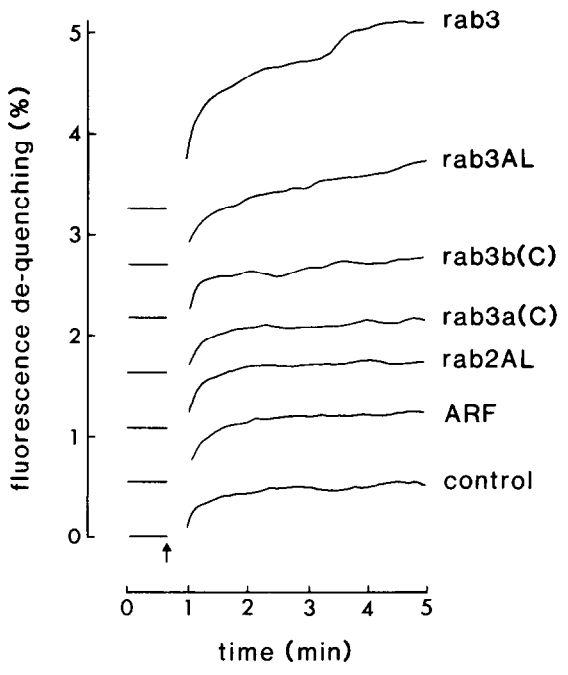

Fig. 1. Effects of peptides derived from small GTP-binding proteins on fusion between pancreatic zymogen granules and plasma membranes. Membrane fusion was measured through the de-quenching of the lipid-soluble probe octadecylrhodamine B-chloride, loaded into the membranes of the zymogen granules. Peptides are: ARF, effector domain of ADP-ribosylation factor (IPTIGFNVETVQYKNI, single letter amino acid code); rab2AL, effector domain of rab2 with AL substituted for TI at positions 3 and 4 (DLALGVEFGARMITID); rab3a(C), C-terminal domain of rab3a (TDQQAPPHGDCAC); rab3b(C), C-terminal domain of rab3b (SDTPPLLQQNCSC); rab3AL, effector domain of rab3 with AL substituted for TV at positions 3 and 4 (VSALGIDFKVKTIYRN); rab3, authentic effector domain of rab3 (VSTVGIDFKVKTIYRN). All peptide concentrations were 100 μM. Pancreatic plasma membranes (10 μg/ml protein) were added at the time indicated by the arrow. The basal fluorescence at the time of addition of membranes is indicated for each trace by the bar.

granules were then incubated at 37°C with unlabelled target membranes and fusion was measured through the dilution-dependent de-quenching of the fluorescence of the probe. We have shown previously [15] that granules fuse with pancreatic plasma membranes, but not with plasma membranes from liver or chromaffin cells. Granules will, however, also fuse with unlabelled granule membranes. Membrane fusion is unaffected by variations in Ca^{2+} concentration, but is stimulated by GTPγS over the same concentration range as that which stimulates exocytosis from permeabilized acini [17].

Fig. 1 illustrates the effects of several peptides, all at a concentration of 100 μM, on the fluorescence de-quenching signal given by plasma membranes. In this typical experiment, four of the six peptides tested – the effector domain of the small GTP-binding protein ADP-ribosylation factor (ARF), rab2AL (a modified effector domain of the protein rab2) and the C-terminal domains of rab3a and rab3b – had no detectable effect on the de-quenching caused by the membranes. In contrast, the effector domain of rab3 and the closely related peptide rab3AL both enhanced dequenching, with rab3 giving the bigger enhancement. Mean values for the effects of the peptides on de-quenching, as a percentage of the total fluorescence, are: rab3, 1.1 ± 0.3% (n = 4); rab3AL, 0.5 ± 0.1% (n = 4); ARF 0.1 ± 0.1% (n = 4); rab2AL, 0.1 ± 0.1% (n = 4); rab3a (C), 0.0% (n = 2) and rab3b(C) 0.2% (n = 2). None of the peptides had any significant effect on the basal fluorescence given by labelled granules alone.

The dependence of the stimulation of de-quenching by rab3 and rab3AL on peptide concentration, at a single plasma membrane concentration, is shown in Fig. 2. The effects of both peptides were maximal at about 50 μM, with rab3 causing a larger maximal stimulation. Peptide concentrations giving half-maximal stimulation (EC_{50}s) were 20 μM for rab3 and 15 μM for rab3AL. Fig. 3 shows the effects of the two peptides, at 50 μM, on the relationship between de-quenching and plasma membrane concentration. Both peptides increased the maximal de-quenching caused by the membranes, without significantly affecting the EC_{50} for membranes (4 μg/ml, as found previously [15]); once again, rab3 caused a larger stimulation than rab3AL. This effect of the peptides on the concentration–response relationship for membranes contrasts with that of GTPγS, which does not affect the maximal signal, but reduces the EC_{50} [15].

The concentration range over which the rab3 effector peptides stimulate membrane fusion (10–100 μM) is identical to the range that has been reported to stimulate amylase secretion in permeabilized acini [9]. This result indicates that the same process is being studied in the two systems, and that the effect of the peptides on secretion is through a direct effect on granule–plasma membrane fusion. In contrast to the results obtained with the permeabilized cell system, however, the authentic effector domain, and not the peptide with the AL modification, consistently produces the larger effect on membrane fusion. In order to obtain further information about how both the rab3 peptide and GTPγS act on the membranes, their effects on granule–plasma membrane fusion were compared with their effects on granule–granule membrane fusion. Optimal concentrations of both agents were used. In the experiment shown in Fig. 4a, GTPγS (100 μM) caused a small stimulation of the de-quenching produced by plasma membranes, whereas rab3 (50 μM) caused a larger stimulation. Mean effects on de-quenching, as a percentage of total fluorescence, were 0.2 ± 0.0% (n = 6) for GTPγS and 1.0 ± 0.2% (n = 6) for rab3. The stimulation produced by GTPγS is in good agreement with that reported previously for the concentration of plasma membranes used [15], and the enhancement of de-quenching by rab3 is similar to that reported above (see Fig. 1). A different

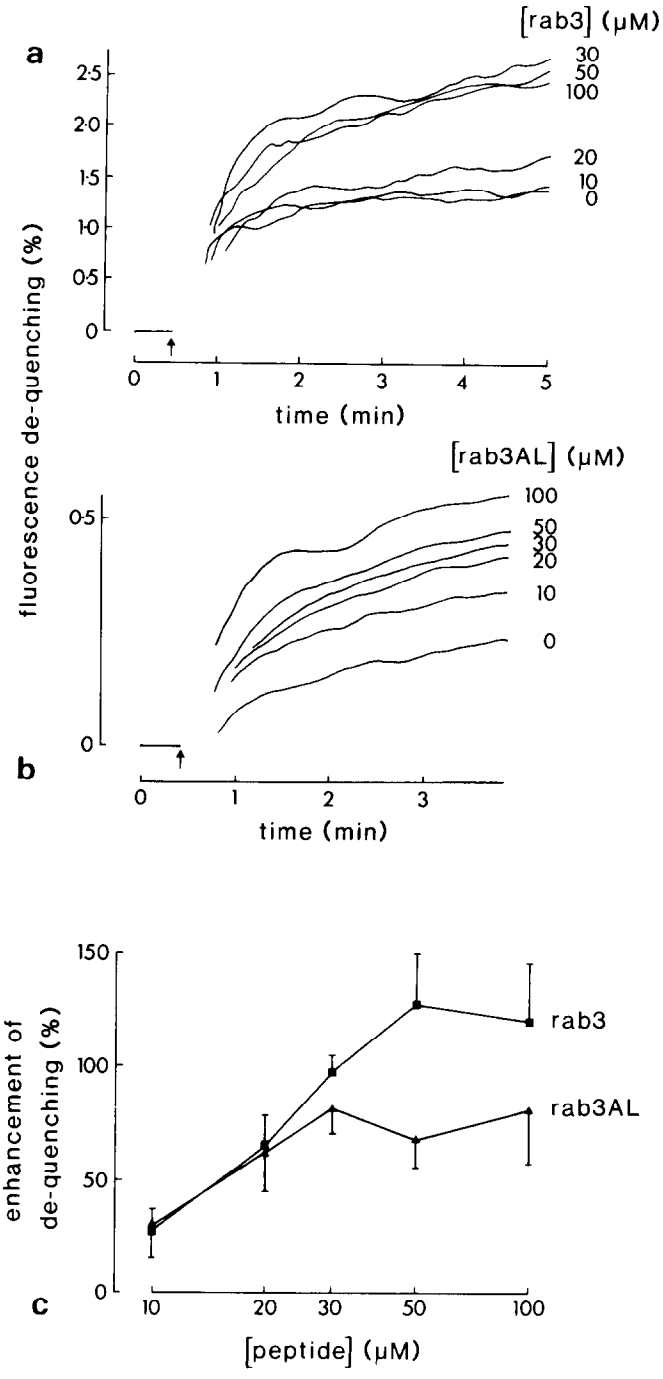

Fig. 2. Concentration–effect relationships for rab3 and rab3AL effector peptides. (a). Typical traces for rab3. (b) Typical traces for rab3AL. (c) Combined data for rab3 (■, $n = 5$) and rab3AL (▲, $n = 4$). Plasma membrane protein concentration was constant at 5 μg/ml.

picture emerged when granule membranes were used as the unlabelled target (Fig. 4b). Now GTPγS caused a clear stimulation, whereas rab3 did not (in fact, in the experiment shown, de-quenching was reduced). Mean effects on de-quenching were 0.3 ± 0.2% ($n = 5$) for GTPγS and 0.0 ± 0.2% ($n = 5$) for rab3.

Evidence has been presented previously [14,15] that GTPγS has its effect through GTP-binding proteins located both on the granule membrane and the plasma membrane, and the data shown here are consistent with this idea. The fact that the rab3 peptide stimulates only fusion between granules and plasma membranes suggests that its target is found only on the plasma membranes. A clear prediction of this hypothesis is that pre-incubation of plasma membranes with the peptide should enhance the de-quenching signal produced, but that pre-incubation of granule membranes with the peptide should be ineffective. This prediction was tested in the experiment shown in Fig. 4c and d and found to be upheld. Mean values for the effects of pre-incubation of the membranes with rab3 on the size of the de-quenching signal were a 0.3 ± 0.1% ($n = 5$) increase for plasma membranes and a 0.1 ± 0.1% ($n = 5$) decrease for granule membranes. The fact that pre-incubation of the plasma membranes with the rab3 peptide was less effective than adding the peptide to the fusion assay mix (compare Fig. 4c with Fig. 1) can be accounted for either by a rapid dissociation of the peptide from its binding site following the dilution of the membranes or by a deterioration in the state of the membrane during the pre-incubation period. Since pre-incubation caused no significant reduction in the size of the de-quenching signal given by membranes alone, however, the former explanation seems more likely.

4. DISCUSSION

According to the model for vesicular transport originally proposed by Bourne [1], vesicles bearing a monomeric GTP-binding protein in its GTP-bound form dock with the target membrane; GTP hydrolysis then occurs, allowing membrane fusion; finally, the GDP-bound form of the protein dissociates from the membrane and recycles to initiate another round of transport. The GTPase activity of the GTP-binding protein is believed to be stimulated by a protein present on the target membrane, known as a GTPase activating protein, or GAP [18,19]. In support of this model, it has been shown, for example, that both ER-Golgi [20] and intra-Golgi [21] membrane transport steps on the constitutive pathway are blocked by GTPγS, presumably because the bound GTPγS cannot be hydrolysed. The rab3AL effector peptide also blocks ER-Golgi transport [22], perhaps by binding to a GAP and inhibiting its action on the GTP-bound rab protein. In order to extend this model to the membrane fusion event involved in regulated exocytosis, which in the exocrine pancreas is stimulated by both GTPγS and rab effector peptides, one would have to propose that the GTP-bound form of rab promotes fusion and that GTP hydrolysis terminates its action. Such a proposal has already been outlined in an attempt to explain the effects of these peptides on exocytosis [9,11,12].

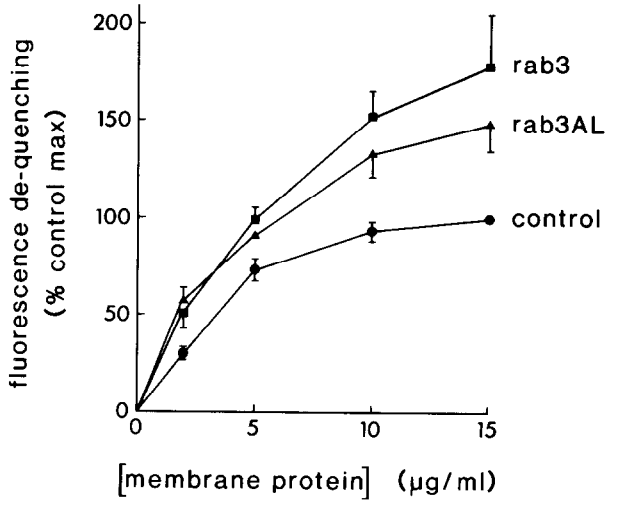

Fig. 3. Concentration–effect relationships for plasma membranes in the absence and presence of the rab3 and rab3AL effector peptides. (●) control ($n = 6$), (■) rab3 ($n = 3$) and (▲) rab3AL ($n = 4$). Peptide concentrations were constant at 50 μM.

An important advantage of the in vitro fusion assay used here is that it permits a study of the effects of these agents on each of the two interacting membranes. From this study it is apparent that GTPγS and the rab effector peptides do not have the same effect, which indicates that membrane fusion may be under the control of more than one GTP-binding protein. We have reported previously [14] that the effect of GTPγS on zymogen granule–plasma membrane fusion is mimicked by the $[AlF_4]^-$ ion. This is now known to be a characteristic of the involvement of heterotrimeric GTP-binding proteins, that are active in the GTP-bound state [23]. We propose, therefore, that at least a major part of the effect of GTPγS on membrane fusion is mediated through an effect on a heterotrimeric GTP-binding protein. The evidence available further indicates that the effect of GTPγS involves its binding to both the granule membrane and the plasma membrane. The effect of the rab peptides, on the other hand, is apparently restricted to the plasma membrane. This would of course be expected if its target were a GAP-like protein, although this has not yet been demonstrated. Further work will be necessary both to identify this target and to understand fully how these peptides are producing their effects.

Acknowledgements: This work was supported by project grants from the Wellcome Trust and the Medical Research Council to JME and by an AFRC grant to GJL. We are grateful to Mr. A.J. Northrop of AFRC IAPGR, Babraham, UK for expert technical assistance and to Mr. C.J. Littlewood of Zinsser Analytic, Maidenhead, UK for synthesis of the peptides.

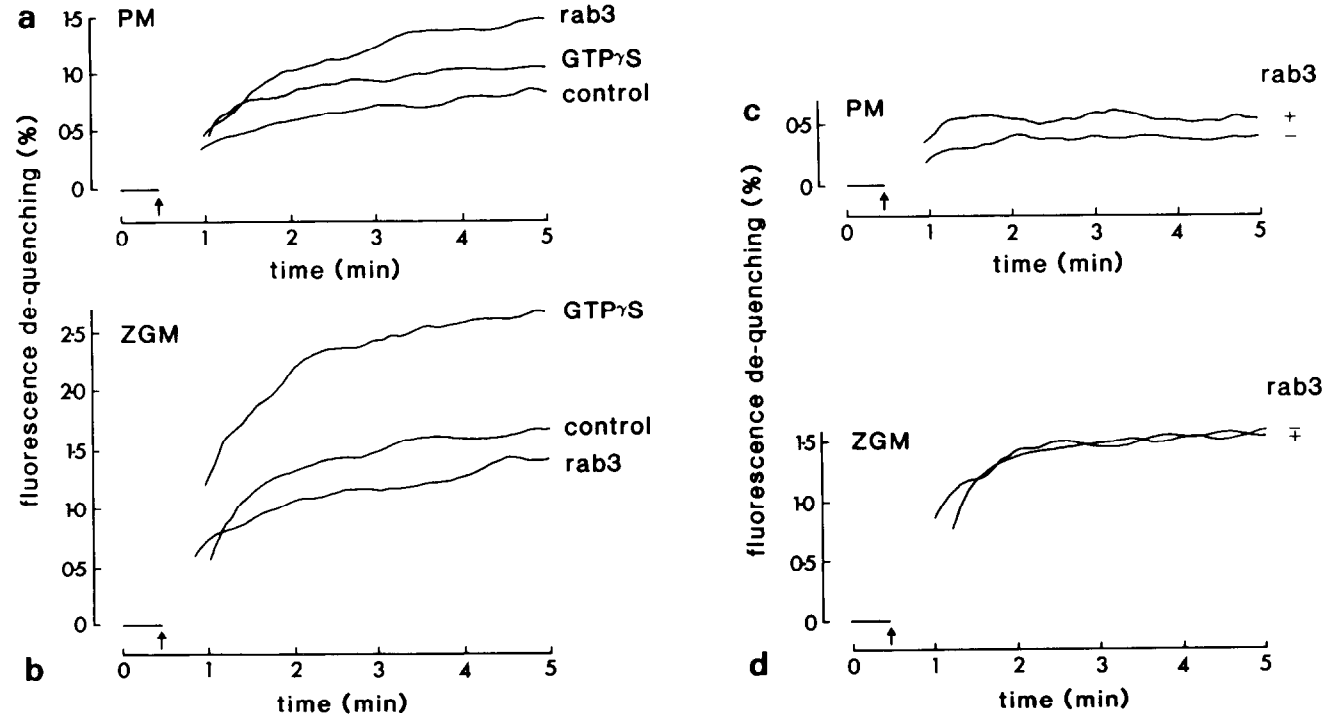

Fig. 4. Effects of rab3 effector peptide and GTPγS on membrane fusion. (a) Granule–plasma membrane fusion. Peptide (50 μM) and GTPγS (100 μM) were added 30 s before target membranes. (b) Granule–granule membrane fusion. (c) Fusion of granules with plasma membranes that had been pre-incubated with rab3 effector peptide (50 μM) for 15 min at 37°C. The final concentration of peptide in the incubation mix was 0.5 μM, which, without pre-incubation, will not enhance fusion (see Fig. 2). (d) Fusion of granules with granule membranes that had been pre-incubated with rab3 effector peptide as described above. Plasma membrane protein concentration was 10 μg/ml; granule membrane protein concentration was 5 μg/ml.

REFERENCES

[1] Bourne, H.R. (1988) Cell 53, 669–671.
[2] Gruenberg, J. and Clague, M.J. (1992) Curr. Opin. Cell Biol. 4, 593–599.
[3] Goud, B., Salminen, A., Walworth, N. and Novick, P. (1988) Cell 53, 753–768.
[4] Segev, N., Mulholland, J. and Botstein, D. (1988) Cell 52, 915–924.
[5] Goud, B. and McCaffrey, M. (1991) Curr. Opin. Cell Biol. 3, 626–633.
[6] 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. USA 87, 1988–1992.
[7] Fischer von Mollard, G., Südhof, T.C. and Jahn, R. (1991) Nature 349, 79–81.
[8] Padfield, P.J. and Jamieson, J.D. (1991) Biochem. Biophys. Res. Commun. 174, 600–605.
[9] Padfield, P.J., Balch, W.E. and Jamieson, J.D. (1992) Proc. Natl. Acad. Sci. USA 89, 1656–1660.
[10] Schnefel, S., Zimmerman, P., Pröfrock, A., Jahn, R., Aktories, K., Zeuzem, S., Haase, W. and Schulz, I. (1992) Cell Physiol. Biochem. 2, 77–89.
[11] Senyshyn, J., Balch, W.E. and Holz, R.W. (1992) FEBS Lett. 309, 41–46.
[12] Oberhauser, A.F., Monck, J.R., Balch, W.E. and Fernandez, J.M. (1992) Nature 360, 270–273.
[13] Gomperts, B.D. (1990) Annu. Rev. Physiol. 52, 591–605.
[14] Nadin, C.Y., Rogers, J., Tomlinson, S. and Edwardson, J.M. (1989) J. Cell Biol. 109, 2801–2808.
[15] MacLean, C.M. and Edwardson, J.M. (1992) Biochem. J. 286, 747–753.
[16] Hoekstra, D., de Boer, T., Klappe, K. and Wilschut, J. (1984) Biochemistry 23, 5675–5681.
[17] Edwardson, J.M., Vickery, C. and Christy, L.J. (1990) Biochim. Biophys. Acta 1053, 32–36.
[18] Beckers, C.J.M. and Balch, W.E. (1989) J. Cell Biol. 108, 1245–1256.
[19] Calés, C., Hancock, J.F., Marshall, M.S. and Hall, A. (1988) Nature 332, 548–551.
[20] Vogel, V.S., Dixon, R.A.F., Schaber, M.D., Diehl, R.E., Marshall, M.S., Scolnick, E.M., Sigal, L.S. and Gibbs, J.B. (1988) Nature 335, 90–93.
[21] Melancon, P., Glick, B.S., Malhotra, V., Weidman, P.J., Serafini, T., Gleason, M.L., Orci, L. and Rothman, J.E. (1990) Cell 51, 1053–1062.
[22] Plutner, H., Schwaninger, R., Pind, S. and Balch, W.E. (1990) EMBO J. 9, 2375–2383.
[23] Kahn, R.A. (1991) J. Biol. Chem. 266, 15595–15597.