Volume 298, number 1, 61–64 FEBS 10694 February 1992

Identification of some of the brain G_n27 as the *ral* gene product

Comparison between the brain and platelet G_n-proteins

Rajinder P. Bhullar

Departments of Oral Biology, and Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, Canada

Received 21 November 1991

Two major G_n-proteins, G_n27 and G_n26, were detected in the 100,000 × g_{av} particulate fraction of rabbit and bovine brain. The G_n26 protein was also present in significant amounts (~50% of total) in the brain supernatant fraction. An antiserum raised against recombinant simian *ral*A recognized a 27-kDa brain protein with the same apparent molecular mass as the G_n27 protein. In further analysis by two-dimensional polyacrylamide gel electrophoresis, the brain particulate G_n-proteins were resolved into 6 major forms, four of 27 kDa (G_n27a–d) and two of 26 kDa (G_n26a and G_n26b). Minor GTP-binding components were also observed at 25 kDa and 24 kDa. The *ral*A antibody reacted strongly with the brain G_n27b form and weakly with the G_n27a and G_n27c but not with G_n27d or any of the other G_n-proteins. In addition, comparison of human platelet and bovine brain particulate G_n-proteins by two-dimensional polyacrylamide gel electrophoresis demonstrated a tissue/cell-type specific expression of the various forms of G_n-proteins.

G_n-protein; GTP-binding protein; G_n27; Brain; Platelet; *ral*

1. INTRODUCTION

In the past five years more than thirty *ras* p21 related GTP-binding proteins with molecular mass between 17–27 kDa have been identified in mammalian cells [2]. Included in this group is a unique family of 23–27 kDa GTP-binding proteins termed, the G_n-proteins [1]. These proteins are specifically labelled by [α-^{32}P]GTP upon incubation of nitrocellulose blots containing cellular proteins separated by SDS-polyacrylamide gel electrophoresis [1]. The G_n-proteins are most abundant in the platelet and brain with platelet containing a major 27 kDa (G_n27) membrane-bound form and the brain containing two membrane-bound (G_n27 and G_n26) and a soluble (G_n26) form [1]. Now, G_n-like proteins have been detected in a wide variety of cells [3–5] and have been shown to be present on the secretory granules of adrenal chromaffin cells [6,7], rapid transport vesicles of neurons [8], on rough endoplasmic reticulum in pancreas [9] and more recently, a 24 kDa GTP-binding protein (G_n24) has been shown to be preferentially associated with the platelet α-granule membrane [10].

Previously, it has been demonstrated that the platelet G_n27 protein can be resolved by two-dimensional polyacrylamide gel electrophoresis into seven distinct forms (G_n27a–g) and that the five most basic G_n27 species (a–e) are coded for by a *ral*-related gene [11]. However, the degree of heterogeneity and the identity of the gene coding for brain G_n27 has not been established. The present study was undertaken to answer these questions. The results presented in this paper demonstrate that the brain particulate G_n27 was resolved by two-dimensional polyacrylamide gel electrophoresis into four distinct forms (G_n27a–d) and that the three most basic species (a–c) were recognized by an antibody prepared against the recombinant *ral*A protein.

Abbreviations: G_n-proteins, 23–27 kDa proteins detected by binding of [α-^{32}P]GTP to nitrocellulose blots [1].

Correspondence address: R.P. Bhullar, Department of Oral Biology, University of Manitoba, 780 Bannatyne Avenue, Winnipeg, Manitoba, Canada, R3E OW3. Fax: (1) (204) 888 4113.

2. EXPERIMENTAL

2.1. *Materials*

[α-^{32}P]GTP (~3000 Ci/mmol) was from ICN Radiochemicals (Irvine, CA, USA). ^{125}I-labelled protein A (2–10 μCi/μg) was obtained from New England Nuclear (Lachine, Quebec, Canada). Phenylmethylsulfonyl fluoride (PMSF) and bovine haemoglobin was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nitrocellulose membrane (0.20 μm pore), ampholytes for isoelectric focusing (Bio-Lytes 3/10) and prestained protein markers for use on SDS-polyacrylamide gels were obtained from Bio-Rad Laboratories (Canada) (Mississauga, Ontario, Canada).

2.2. *Preparation of brain particulate and supernatant fractions*

Fresh rabbit and bovine brain were used in these studies. After removal of white matter, the cerebral tissue obtained was homogenized using a Polytron homogenizer with 4 volumes of a buffer containing 10 mM Tris-HCl, pH 7.5, 0.2 mM PMSF and 10% sucrose [12]. The homogenate was filtered through four layers of cheesecloth and the particulate and supernatant fraction prepared by centrifugation at 100,000×g_{av} for 60 min at 4°C. The particulate fraction was resuspended in buffer containing 100 mM Tris-HCl, pH 7.5, 5 mM EDTA and 0.2 mM PMSF and stored along with the supernatant fraction at −50°C until used.

2.3. *Preparation of platelet particulate fraction*

Human platelets were washed three times in a medium containing 13 mM trisodium citrate, 5 mM dextrose and 135 mM NaCl (pH 6.5) [1] and were finally resuspended at 3×10^9/ml in buffer at 0°C containing 100 mM Tris-HCl, pH 7.5, 5 mM EDTA and 0.2 mM PMSF. These suspensions were frozen and thawed twice (with solid CO_2/methanol). The platelet cytosolic and particulate fractions were then prepared by centrifugation at 100,000×g_{av} for 60 min at 4°C. Membranes were resuspended in the above buffer and frozen at −50°C until used.

2.4. *Polyacrylamide gel electrophoresis*

Analysis of proteins in various fractions by one-dimensional SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere [13]. For analysis of proteins by two-dimensional polyacrylamide gel electrophoresis, the method of O'Farrell [14] was used with modifications described in [11]. After isoelectric focusing was complete, gels were extruded from the tubes and subjected to SDS-polyacrylamide gel electrophoresis in the second dimension.

2.5. *Nitrocellulose blotting and detection of proteins binding [α-^{32}P]GTP or ral*A *antibody*

For transfer of proteins on to nitrocellulose after SDS-polyacrylamide gel electrophoresis, gels were incubated for 30 min in a transfer buffer containing 0.05% (w/v) SDS before electroblotting [1,15]. The detection of G_n-proteins on nitrocellulose blots using [α-^{32}P]GTP has been described in detail elsewhere [1]. Bound ^{32}P was quantitated by counting Cerenkov radiation. To probe blots with the *ral*A antibody, the nitrocellulose sheets were shaken for 1 h at room temperature in a solution containing 10 mM Tris-HCl, pH 7.5, and 0.154 mM NaCl (buffer A) to which 1% (w/v) bovine haemoglobin had been added. The nitrocellulose blots were then incubated for 3 h in buffer A containing 1% bovine haemoglobin and *ral*A antibody (at a 1:250 dilution). After washing three times (10 min each) with buffer A, the blots were incubated for a further 1 h in buffer A containing 1% bovine haemoglobin and ^{125}I-labelled protein A (0.2 μCi/ml). After further washing of the blots in buffer A (five times for 5 min each), bound ^{125}I-labelled protein A was detected by autoradiography for 12–18 h at −50°C, using a Cronex intensifying screen (Picker International, Brampton, Ontario, Canada).

2.6. *Protein assay*

Samples containing protein were precipitated with 10% (w/v) trichloroacetic acid and the protein determined by the method of Lowry et al. [16], using a solution that contained 5% (w/v) human albumin and 3% (w/v) human globulin.

3. RESULTS

The presence of G_n-proteins in the platelet particulate, and the rabbit and bovine brain homogenate and the 100,000×g_{av} supernatant and particulate fractions was assessed by incubation with [α-^{32}P]GTP of nitrocellulose blots containing polypeptides separated by SDS-polyacrylamide gel electrophoresis. The platelet particulate fraction contained a major 27 kDa G_n-protein and minor amounts of 23, 24 and 25 kDa species (Fig. 1, lane a). In the brain, two major GTP-binding proteins of molecular mass 27 kDa (G_n27) and 26 kDa (G_n26) were detected in the homogenate (Fig. 1, lanes b and e) and particulate (Fig. 1, lanes d and g) fractions. However, the G_n26 protein detected in the particulate fraction was also present in significant amounts (~50% of total) in the brain supernatant fraction (Fig. 1, lanes c and f).

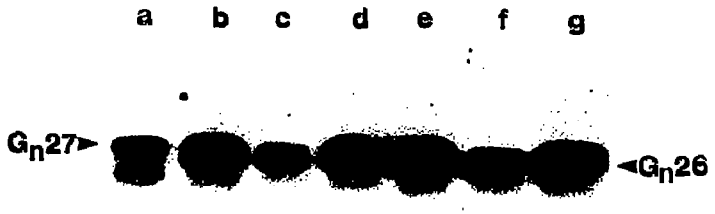

Fig. 1. Detection of G_n-proteins in platelet and brain. Samples of protein (100 μg) from platelet particulate (lane *a*), rabbit (lane *b*) and bovine (lane *e*) brain homogenate, rabbit (lane *c*) and bovine (lane *f*) brain 100,000 × g_{av} supernatant fraction and rabbit (lane *d*) and bovine (lane *g*) brain 100,000 × g_{av} particulate fraction were electrophoresed and blotted on to nitrocellulose. To detect G_n-proteins the blot was incubated with [α-^{32}]GTP and the above autoradiograph developed.

Previously, it has been demonstrated that the platelet G_n27 can be resolved into seven distinct forms (G_n27*a–g*) by two-dimensional polyacrylamide gel electrophoresis and that only the five most basic G_n27 species (*a–e*) are coded for by a *ral*-related gene [11]. As demonstrated in Fig. 1, the brian also contains a major 27 kDa (G_n27) GTP-binding protein. To determine if the brain G_n27 is coded for by a *ral*-related gene, two identical blots containing platelet and bovine brain particulate proteins separated using SDS-polyacrylamide gel electrophoresis were prepared. One of the blots was treated with [α-^{32}P]GTP and the second identical blot was probed with the *ral*A antibody. Results demonstrate that the 27 kDa GTP-binding protein (G_n27) present in the platelet and brain particulate fraction (Fig. 2A, lanes a and b) was also recognized by the *ral*A antibody (Fig. 2B, lanes a and b). Recombinant *ral*A protein also

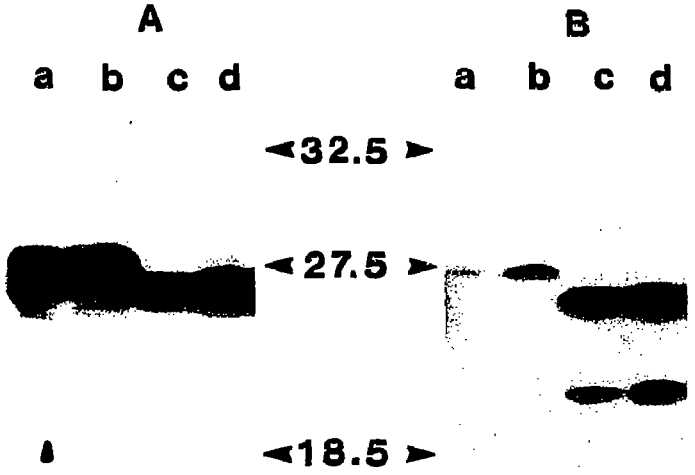

Fig. 2. Analysis of G_n-proteins and recombinant *ral*A by one-dimensional SDS-polyacrylamide gel electrophoresis. Proteins (100 μg) from human platelet (lane a) and bovine brain (lane b) particulate fraction and varying amounts of purified recombinant *ral*A protein (lane c, 0.1 μg; lane d, 0.2 μg) were electrophoresed and blotted on to nitrocellulose. The above autoradiograph shows: A, [α-^{32}P]GTP-binding proteins and B, proteins that bound *ral*A antibody, as detected by ^{125}I-labelled protein A. The position of prestained molecular mass marker proteins used to align the autoradiographs are shown in the centre of the figure.

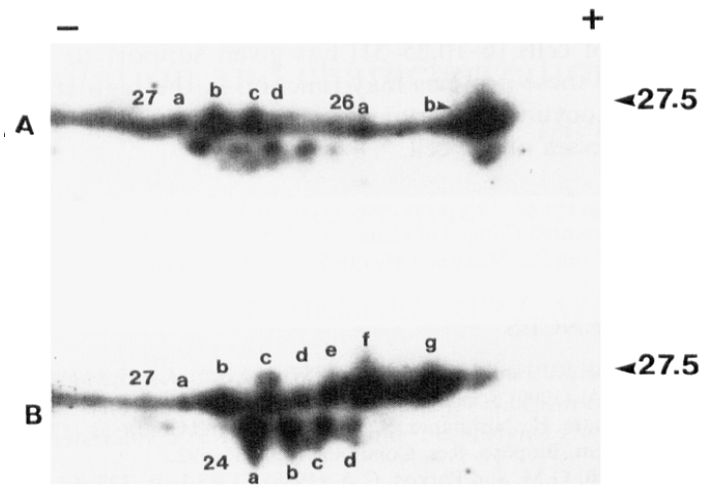

Fig. 3. Comparison of brain and human platelet G_n-proteins by two-dimensional polyacrylamide gel electrophoresis. Samples of proteins (200 μg) from bovine brain (A) and human platelet (B) particulate fractions were subjected to isoelectric focusing with protein applied to the high pH (cathode) end of the gel. SDS-polyacrylamide gel electrophoresis in the second dimension was carried out using 13% (w/v) acrylamide and the gels were blotted on to nitrocellulose. To detect G_n-proteins, the blots were treated with [α-^{32}P]GTP and an autoradiograph developed as shown above. The major G_n-proteins detected are labelled. The orientation of the cathode (−) and anode (+) during isoelectric focusing is indicated, along with the mobility of a prestained 27.5 kDa protein during electrophoresis in the second dimension.

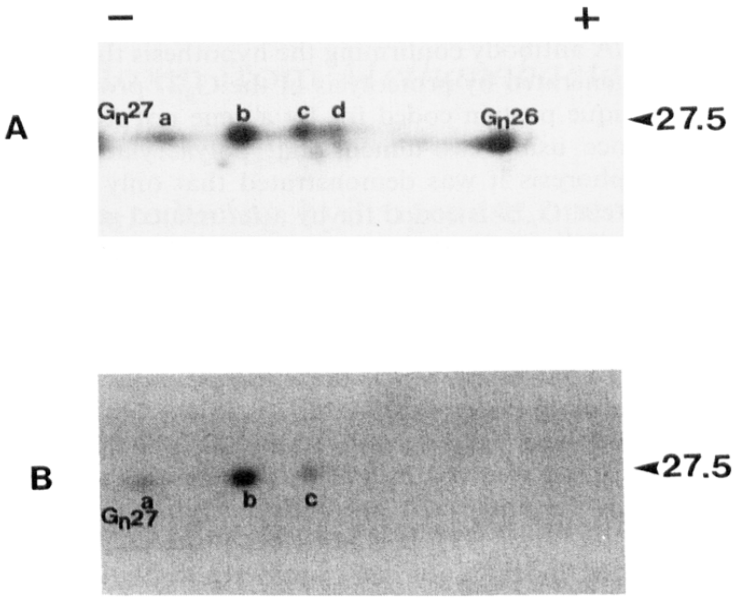

Fig. 4. Identification using two-dimensional polyacrylamide gel electrophoresis of brain G_n-proteins recognized by anti-*ral*A antibody. Bovine brain particulate proteins (100 μg) were subjected to isoelectric focusing with the protein applied to the high pH (cathode) end of the gel. SDS-polyacrylamide gel electrophoresis in the second dimension was carried out using 13% (w/v) acrylamide. Two identical gels were then blotted on to nitrocellulose. To detect G_n-proteins and proteins recognized by the *ral*A antibody, one blot (A) was treated with [α-^{32}P]GTP and another (B) with *ral*A antibody followed by ^{125}I-labelled protein A. Autoradiographs are shown. The major proteins detected with [α-^{32}P]GTP are labelled G_n27*a–d* and G_n26. The orientation of the cathode (−) and anode (+) during isoelectric focusing is indicated, along with the mobility of a prestained 27.5 kDa protein during electrophoresis in the second dimension.

bound [α-^{32}P]GTP (Fig. 2A, lanes c and d) and was recognized by the *ral*A antibody (Fig. 2B, lanes c and d).

The brain and platelet particulate G_n-proteins were compared by two-dimensional polyacrylamide gel electrophoresis. Results demonstrate that the G_n-proteins detected in the brain particulate fraction were resolved into six major species, four of 27 kDa (G_n27*a–d*) and two of 26 kDa (G_n26*a* and G_n26*b*) (Fig. 3A). Minor GTP-binding components were also observed at 25 and 24 kDa (Fig. 3A). In contrast, the platelet particulate G_n-proteins were separated into 11 major forms, seven of 27 kDa (G_n27*a–g*) and four of 24 kDa (G_n24*a–d*) (Fig. 3B). To determine if all species of brain G_n27 are coded for by a *ral*-related gene, two identical blots containing brain particulate proteins separated by two-dimensional polyacrylamide gel electrophoresis were prepared. One of the blot was treated with [α-^{32}P]GTP (Fig. 4A) and the second identical blot was treated with the *ral*A antibody (Fig. 4B). The results demonstrate that of the four G_n27 *a–d* forms detected using [α-^{32}P]GTP binding (Fig. 4A), the *ral*A antibody reacted strongly with G_n27*b*, weakly with G_n27*a* and G_n27*c* (Fig. 4B). None of the other G_n-proteins were recognized by the *ral*A antibody.

4. DISCUSSION

G_n-proteins represent a unique family of 23–27 kDa GTP-binding proteins that are highly abundant in the platelet and brain and are specifically labelled by [α-^{32}P]GTP on nitrocellulose blots of cellular proteins that have been separated by SDS-polyacrylamide gel electrophoresis [1]. The platelet membrane contains a major 27 kDa (G_n27) species and lesser amounts of 23, 24 and 25 kDa G_n-proteins [1]. The platelet soluble fraction contained much smaller amounts of G_n-proteins [1]. Using two-dimensional polyacrylamide gel electrophoresis, the platelet membrane G_n-proteins were demonstrated to be highly heterogenous, consisting of at least 12 major polypeptides, seven of 27 kDa (G_n27*a–g*), one of 26 kDa (G_n26) and four of 24 kDa (G_n24*a–d*) [11]. The five most basic forms of G_n27 (G_n27*a–e*) and G_n26 were shown to be coded for by genes closely related to *ral*A [11], a gene which was originally identified in a simian B-cell cDNA library [17] and more recently two related *ral* genes, *ral*A and *ral*B, have been cloned from a human pheochromocytoma library [18].

As demonstrated in the present study (Fig. 1) and reported previously [1], the brain contains two major membrane-bound G_n-proteins (G_n27 and G_n26) and a major soluble G_n-protein (G_n26). Using one-dimensional SDS-polyacrylamide gel electrophoresis, the brain G_n27 was shown to be coded for by a *ral*-related

gene (Fig. 2). The brain G_n26 was not recognized by the anti-*ral*A antibody confirming the hypothesis that G_n26 is not generated by proteolysis of the G_n27 protein but is a unique protein coded for by a gene not related to *ral*. Since using two-dimensional polyacrylamide gel electrophoresis it was demonstrated that only part of the platelet G_n27 is coded for by a *ral*-related gene [11], the possibility existed that not all of the brain G_n27 is coded for by a *ral*-related gene. The brain G_n27 was resolved by two-dimensional polyacrylamide gel electrophoresis into at least four polypeptides with widely different charges of which the anti-*ral*A antibody recognized only three (Fig. 4). However, the brain G_n27 was much less heterogenous than the platelet G_n27 which can be resolved into at least seven polypeptides of which the anti- *ral*A antibody recognized the five most basic forms [11]. It is not clear if the G_n27 forms recognized by the anti- *ral*A antibody in platelet and brain are identical or different. Clearly there are certain forms of the G_n27 (G_n27d and G_n27e) protein coded for by a *ral*-related gene that are present only in the platelet. G_n-proteins in the brain demonstrate a lower degree of hetrogeneity compared to those present in the platelet. The observed heterogeneity in the G_n27 and other G_n-proteins could be accounted for by post-translational modifications [19] and by differential splicing from the transcripts of a small number of distinct genes, including *ral* and other yet to be identified genes. In addition, results presented in this paper demonstrate that there is tissue specific expression of certain G_n-proteins. G_n26, a major brain G_n-protein is only detected in the brain and is absent from the platelet. The G_n26 protein detected in brain is likely to be coded for by a gene related to *rab*3, a gene which was initially cloned from a rat brain cDNA library [20]. This conclusion is supported by the following observations: (i) the *rab*3A protein is found in the highest concentration in neural tissue [21] as is G_n26; (ii) large amounts of the *rab*3A protein are present in the cytosol [22] as is the case for G_n26 (Fig. 1); (iii) *rab*A, also termed smg p25A, is able to bind GTP on nitrocellulose blots after SDS-polyacrylamide gel electrophoresis [23–25], a procedure used for the initial detection of brain G_n26 and other G_n-proteins [1]. Thus, taking into consideration these facts it is likely that the brain G_n26 is coded for by a gene related to *rab*3.

The results presented in this paper provide the first evidence that brain contains protein products of a *ral*-related gene and that even though a large number of GTP-binding proteins are present in the cell, the method developed for the detection of G_n-proteins [1] selectively identifies only the protein products of genes closely related to *ral* and *rab*. It remains to be determined whether the remarkable ability of the G_n-proteins to bind [^{32}P]GTP on nitrocellulose blots implies similarities in the functions or regulation of these proteins. The function of G_n-proteins in the cell is not known, but the detection of G_n-proteins and the products of *rab* genes on the secretory granules and the Golgi of a wide variety of cells [6–10,25–31] has given support to the idea that these proteins may function in the regulation of the exocytic/secretory and other membrane trafficking processes of the cell.

Acknowledgements: This work was supported by a grant from the Medical Research Council of Canada (MA-10720) and a Scholarship to R.P.B. from the Manitoba Health Research Council.

REFERENCES

[1] Bhullar, R.P. and Haslam, R.J. (1987) Biochem. J. 245, 617–620.
[2] Hall, A. (1990) Science 249, 635–640.
[3] Nagahara, H., Nishimura, S., Sugimara, T. and Obata, H. (1987) Biochem. Biophys. Res. Commun. 149, 686–692.
[4] Bokoch, G.M. and Parkos, C.A. (1988) FEBS Lett. 227, 67–70.
[5] Wolfman, A., Moscucci, A. and Macara, I.G. (1989) J. Biol. Chem. 10820–10827.
[6] Burgoyne, D. and Morgan, A. (1989) FEBS Lett. 245, 122–126.
[7] Doucet, J-P., Fournier, S., Parluekar, M. and Trifaro, J-M. (1989) FEBS Lett. 247, 127–131.
[8] Bielenski, D.F., Morin, P.J., Dickey, B.F. and Fine, R.E. (1989) J. Biol. Chem. 264, 18363–18367.
[9] Nigam, S.K. (1990) Proc. Natl. Acad. Sci. USA 87, 1296–1299.
[10] Van der Meulen, J.A., Bhullar, R.P. and Chancellor-Maddison, K.A. (1991) FEBS Lett. 291, 122–126.
[11] Bhullar, R.P., Chardin, P. and Haslam, R.J. (1990) FEBS Lett. 260, 48–52.
[12] Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. 259, 13806–13813.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[15] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Chardin, P. and Tavitian, A. (1986) EMBO J. 5, 2203–2208.
[18] Chardin, P. and Tavitian, A. (1989) Nucleic Acids Res. 17, 4380.
[19] Grand, R.A. (1989) Biochem. J. 258, 625–638.
[20] Touchet, N., Chardin, P. and Tavitian, A. (1987) Proc. Natl. Acad. Sci. USA 84, 8210–8214.
[21] Zahraoui, A., Touchot, N., Chardin, P. and Tavitian, A. (1989) J. Biol. Chem. 264, 12394–12401.
[22] Burstein, E. and Macara, I.G. (1989) Mol. Cell. Biol. 9, 4807–4811.
[23] Kikuchi, A., Yamashita, T., Kawata, M., Yamamoto, K., Ikeda, K., Tanimoto, T. and Takai, Y. (1988) J. Biol. Chem. 263, 2897–2904.
[24] Matsui, Y., Kikuchi, A., Kondo, J., Hishada, T., Teranishi, Y. and Takai, Y. (1988) J. Biol. Chem. 263, 11071–11074.
[25] Fischer von Mollard, G., Mignery, G.A., Baumert, M., Perin, M.S., Hanson, T.J., Burger, P.M., Jahn, R. and Sudholf, T.C. (1990) Proc. Natl. Acad. Sci. USA 87, 1988–1992.
[26] Ngsee, J.K., Miller, K., Wendland, B. and Scheller, R.H. (1990) J. Neurosci. 10, 317–322.
[27] Matsuoka, I. and Dolly, J.O. (1990) Biochim. Biophys. Acta 1026, 99–104.
[28] Goud, B., Zahraoui, A., Tavitian, A. and Saraste, J. (1990) Nature 345, 553–556.
[29] Mizoguchi, A., Kim, S., Ueda, T., Kikuchi, A., Yorifuji, H., Hirokawa, N. and Takai, Y. (1990) J. Biol. Chem. 265, 11872–11879.
[30] Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K. and Zerial, M. (1990) Cell 62, 317–329.
[31] Darchen, F., Zahraoui, A., Hammel, F., Monteils, M-P., Tavitian, A. and Scherman, D. (1990) Proc. Natl. Acad. Sci. USA 87, 5692–5696.