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Dissociation of Rab3A by Ca^{2+}/calmodulin from different kinds of synaptosomal membranes

Jae-Bong Park[1,2]

1 Department of Biochemistry, University of Washington Medical School, Seattle, Washington 98195-7370, USA
2 Present address: Department of Biochemistry, College of Medicine, Hallym University, Chunchon 200-702, Korea

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Abbreviations: CaM, calmodulin; LP2, second lysis pellet of synaptosomal membrane; GDI, GDP dissociation inhibitor; CPG, controlled-pore glass

Abstract

In the previous study, we have shown that Rab3A is dissociated from synaptosomal membranes by Ca^{2+}/calmodulin complex. In this study, we further fractionated the synaptosomal membranes by gradient centrifugation and determined the localization and translocation behavior of Rab3A by Ca^{2+}/calmodulin in the different membrane fractions. Two different membrane pools, light and heavy membrane fractions, were obtained from synaptosomal membranes by self-generating gradient of Iodixanol. Rab3A, Rab5, synaptophysin, and synaptobrevin were found to be distributed in the two membrane fractions. When the ratio of Rab3A/synaptophysin was compared in the two membrane fractions, it was found that the value of the ratio in the light fraction was higher than that in the heavy one. To examine whether the dissociation of Rab3A by Ca^{2+}/calmodulin depends on the different fractions of synaptosomal membranes, Ca^{2+}/camodulin was added to the membranes. When the extents of Rab3A dissociation by Ca^{2+}/calmodulin were examined among from unfractionated synaptosomal membrane, fractionated light and heavy membranes, Rab3A was dissociated from all the membrane. The extents of Rab3A dissociation from the two fractionated membrane pools by Ca^{2+}/calmodulin were similar, whereas Rab3A was more easily dissociated by Ca^{2+}/CaM from unfractionated synaptosomal membranes than from fractionated membranes. The results suggest that additional factor(s) is present on the synaptosomal membrane and involved in the dissociation of Rab3A induced by Ca^{2+}/CaM.

Keywords: calmodulin, Rab3A, synaptic vesicle, translocation

Introduction

The release of neurotransmitter via regulated exocytosis is the primary mode of communication in the nervous system. Neurotransmitters are stored in vesicular organelles, the synaptic veicles, and are released by Ca^{2+}-dependent exocytosis upon stimulation of the presynaptic cell. Docking and fusion of vesicles at the presynaptic membrane are proposed to occur through a series of protein-protein interactions (Bajjalieh and Scheller, 1995). Like all vesicles that mediate transport from one membrane compartment, synaptic vesicles must find the appropriate acceptor membrane. Targeting would be most easily accomplished if vesicles contains proteins that specifically interact with molecules localized to the acceptor membrane. Thus the hypothesis, recently named the SNARE hypothesis (Söllner *et al.*, 1993), states that each class of transport vesicle contains a specific targeting protein (v- or vesicle SNARE) that is capable of associating only with a receptor protein (t- or target SNARE) specific to the appropriate acceptor membrane. This membrane protein complex is composed of three synaptic proteins: the synaptic vesicle protein synaptobrevin (v-SNARE) and the presynaptic plasma membrane proteins syntaxin 1 and SNAP-25 (t-SNAREs). The majority of synaptobrevin is bound to the vesicle protein synaptophysin. Synaptophysin selectively interacts with synaptobrevin in a complex which excludes the t-SNAP receptors syntaxin I and SNAP-25, suggesting a role for synaptophysin in the control of exocytosis (Edelman *et al.*, 1995).

In brain a small GTP-binding protein, Rab3A, is exclusively associated with synaptic vesicles, the secretory organelles of nerve terminals. Rab3A and Rab3C dissociate quantitatively from the vesicle membrane after Ca^{2+}-dependent exocytosis (Fischer von Mollard *et al.*, 1991; Fischer von Mollard *et al.*, 1994). Rab3A can be dissociated from membranes *in vitro* by the GDP-dissociation inhibitor (GDI), an enzyme which is group-specific for Rab proteins containing bound GDP (Ullrich *et al.*, 1993). A potential role for small GTP-binding proteins is to prime or activate members of the targetting complex (Bajjalieh and Scheller, 1995). Among the known Rab GTPase, Rab5 is also of great interest because it appears to be rate-influencing for receptor-mediated and fluid-phase

endocytosis (Hoffenberg *et al.*, 1995). Lateral fusion between endocytic vesicles is stimulated by Rab5 both *in vitro* and *in vivo*, and antibodies against Rab5 inhibit fusion *in vitro* (Gorvel *et al.*, 1991; Li and Stahl, 1993).

Park *et al.* (1996) previously observed that Rab3A dissociates from crude synaptic vesicles by Ca^{2+}/calmodulin (CaM). Ca^{2+}/CaM forms 1:1 complex with Rab3A. It has not been investigated whether other factor(s) is involved in the dissociation of Rab3A caused by Ca^{2+}/CaM. In this study we examined if the dissociation of Rab3A by Ca^{2+}/CaM from membrane fractions of crude synaptic vesicles separated by the self-forming gradient of Iodixanol is different.

Materials and Methods

Materials

CaM was obtained from Calbiochem and freshly dissolved in 50 mM Hepes, pH 7.4, for each experiment. $CaCl_2$, suprapur grade, was from EM Science. Anti-Rab3A, Rab5A antibody was from Santa Cruz. Anti-synaptophysin antibody was from Boeringer Manheim. Anti-synaptobrevin antibody was from Chemicon. Iodixanol (Opti-Prep) was from Nycomed. Micro BCA solutions were from Pierce. All other chemicals were reagent grade from Sigma.

Preparation of synaptosomes

Synaptosomes were prepared from cerebral cortex of nonhuman primates (*Macaca nemestrina*) obtained from the tissue distribution program of the Regional Primate Research Center at the University of Washington. The methods were a modification (Park *et al.*, 1996) of those of Fisher von Mollard *et al.* (1991) and Barrie *et al.* (1991). Each animal was anesthetized with Halothane and its brain case was opened. Then its spinal cord was severed and its brain removed within 90 s of death. About 50 g of cerebral cortex was quickly sliced in ice-cold buffer A (320 mM sucrose; 4 mM Hepes, pH 7.4; 2 mM EGTA; 0.1 mM phenylmethyl-sulfonyl fluoride; 1 μg/ml each of leupeptin, aprotinin, and pepstatin A; and 2 μg/ml *p*-aminobenzamidine). A gray matter-enriched portion was diluted with 10 vol. buffer A and blended three times for 5 s in an Oster blender. The suspension was homogenized with a Potter-Elvehjem homogenizer and centrifuged for 10 min at 800 *g*. The supernatant was recentrifuged for 10 min at 9,200 *g*, the resulting pellet was resuspended in 60 ml of buffer A, and aliquots (10 ml) were loaded onto discontinuous step gradients containing Ficoll 400 in 38 ml tubes. The gradients consisted of 9 ml of 13% Ficoll, 9 ml of 8.5% Ficoll, and 10 ml of 5% Ficoll (w/v). After centrifugation for 45 min at 58,000 *g* in a Beckman SW27 rotor, the synaptosome-enriched carpet above the 13% Ficoll cushion was collected, diluted with 4 vol. buffer A, and centrifuged for 10 min at 24,000 *g*. The pellets were resuspended in 120 ml of buffer A and recentrifuged for 10 min at 7,800 *g*.

Preparation of synaptosomal lysates and synaptosomal membranes

The washed synaptosomes were resuspended in buffer A, lysed with 9 vol. of ice-cold water (Milli-Q, Millipore), and homogenized with a Potter-Elvehjem homogenizer. EGTA and Hepes, pH 7.4, were added to final concentrations of 2 mM and 7.5 mM, respectively, and lysate was incubated for 30 min on ice. A low-speed membrane fraction (LP1, first lysis pellet) was removed by centrifugation for 20 min at 25,000 *g*, and a fraction containing Rab3A-enriched membranes (REM or LP2, second lysis pellet of synaptosomal membrane) was isolated by centrifugation of the supernatant for 90-120 min at 176,000 *g* in a Beckman 60Ti rotor. The LP2 were resuspended in 2-3 ml buffer containing 300 mM glycine, 0.1 mM EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 5 mM Hepes, pH 7.4. The 176,000 *g* supernatant was concentrated 10-fold with a PM-10 membrane (Amicon).

Gradient ultracentrifugation with Iodixanol

Generation of concentration gradient was performed by the manufacturer's instruction. 60% Iodixanol solution was diluted with solution II (8% sucrose, 6 mM EDTA, 120 mM Hepes, pH 7.4, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5 μg/ml pepstatin A) to be 50% of working solution. Working solution was diluted again with solution I (8% sucrose, 1 mM EDTA, 20 mM Hepes, pH 7.4, 5 μg/ml leupeptin, 5 μg/ml aprotinin, pepstatin A 0.5 μg/ml) to be 18% Iodixanol. LP2 membranes were resuspended in 18% Iodixanol solution and centrifuged at 350,000 *g* with VTi 65 rotor (Beckman) for 2 h at 4°C. The sample was fractionated to 18 tubes of 0.3 ml. Small parts of fractions were used for determination of protein concentration and Western blot. For dissociation of Rab3A, the membrane fractions were washed and dissolved with buffer B.

Purification of RabGDI

RabGDI was purified from bovine brain as descrived (Sasaki *et al*, 1990), except that all buffers used after the ammonium sulfate precipitation step contained 10% glycerol, 0.25 mM PMSF, 2.5 μg/ml each of aprotinin and leupeptin, and 1 μg/ml pepstatin A.

Dissociation of Rab3A from membranes

LP2 membranes were incubated for 30 min at 30°C with Ca^{2+} and CaM in 50 μl of buffer B (50 mM Hepes, pH 7.4, 0.5 μM $MgCl_2$, 1 mM dithiothreitol, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). The reaction mixtures were centrifuged for 30 min at 100,000 *g* in a Beckman TLA 45 rotor. The supernatants were saved and the

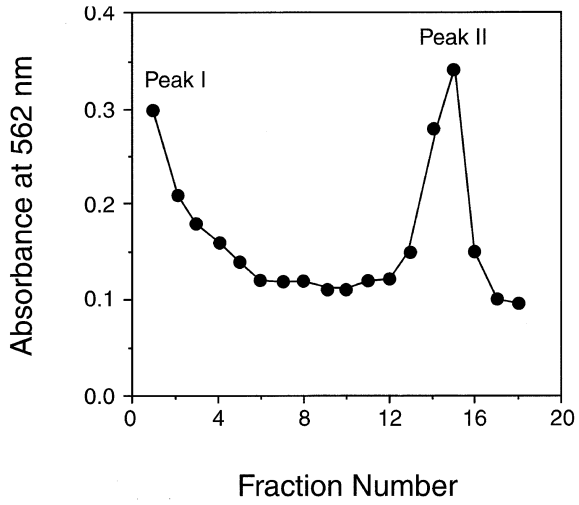

Figure 1. Fractionation of crude synaptic vesicles on ultracentrifugation of self-generated gradient of Iodixanol. Fractions were collected by using peristaltic pump from the bottom of tubes. Number 1 is lowest fraction. Protein was determined from 10 µl aliquot in the presence of 0.1% SDS by micro BCA method. Peak I and II were heavy and light membrane fractions, respectively.

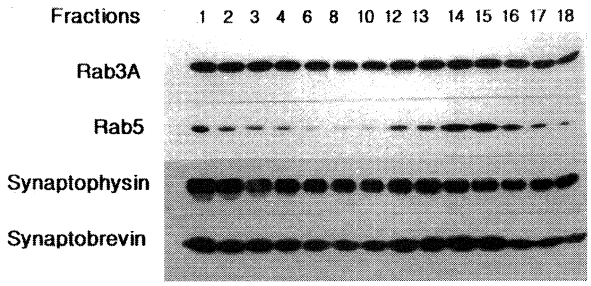

Figure 2. Western blot of each fraction. 30 µl of aliquots of each fraction were used for SDS-PAGE. After transfer of proteins to PVDF membranes, the membranes were blotted with anti-Rab3A, -Rab5, -synaptophysin, or -synaptobrevin antibodies.

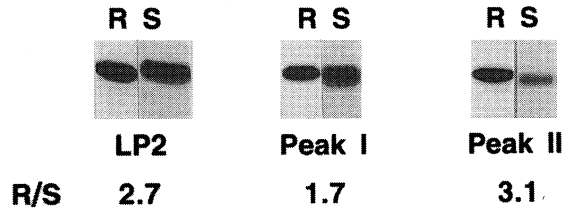

Figure 3. Comparison of synaptophysin of LP2 membrane, Peak I, and Peak II membrane fractions. The same PVDF membrane was blotted with anti-synaptophysin, and -Rab3A antibodies. The ratio of Rab3A (R)/synaptophysin (S) were calculated from (absorbance of Rab3A)/ (absorbance of synaptophysin) on X-ray film measured by laser densitometer.

pellets were resuspended in 50 µl of buffer B with brief sonication. Aliquots of each pellet, and the supernatant fraction were examined by SDS-PAGE and Western blot analysis using anti-Rab3A antibodies. After scanning the Western blots with a imaging densitometer (Bio-Rad model GS-670), absorbance values for the Rab3A protein band were obtained for each treatment sample. A standard curve was generated from the untreated membrane serial dilution samples. The amount of Rab3A dissociated into the supernatant fraction was expressed as dissociation values (Rab3A in supernatant/Rab3A in supernatant and pellet).

Other methods

SDS-PAGE was performed according to Laemmli (1970). Proteins were transferred from SDS-PAGE gels to PVDF membrane at 80 V for 30 min at 4°C for Western analysis of Rab3A. Immunoblots were performed as described (Aepfelbacher *et al.*, 1994). Protein concentration was determined using the micro BCA method following manufacturer's instruction for membrane samples containing 0.1% SDS. All procedures were performed at 4°C unless otherwise indicated.

Results

Fractionation of synaptosomal membranes

When LP2 membranes were fractionated by self-generating gradient of Iodixanol and the protein concentration of each fraction was determined, two main peaks of high protein concentration were identified (Figure 1). The distribution of Rab3A, Rab5, synaptophysin, and synaptobrevin in each frction were analyzed by Western blot with antibodies as probes. Among the proteins, Rab5 and synaptobrevin were mainly localized in two fractions whereas the Rab3A and synaptophysin were distributed in all fractions (Figure 2). The amounts of Rab3A and synaptophysin in light (Peak II) and heavy fraction (Peak I) were compared by Western blot (Figure 3). Synaptophysin is an integral membrane protein, which has 4 transmembrane domains (Leube *et al.*, 1985) in synaptic vesicles. It is usually used for marker protein of synaptic vesicles. It was found that heavy membrane fraction had much more synaptophysin than light one whereas contents of Rab3A of both membrane fractions were similar. It means that more rab3A was localized in light membrane fraction than heavy one. Arbitrary ratio of Rab3A/synaptophysin, which was absorbance ratio determined from X-ray film by laser densitometer, of LP2 membranes, heavy and light membrane fractions were 2.7, 1.7, and 3.1, respectively.

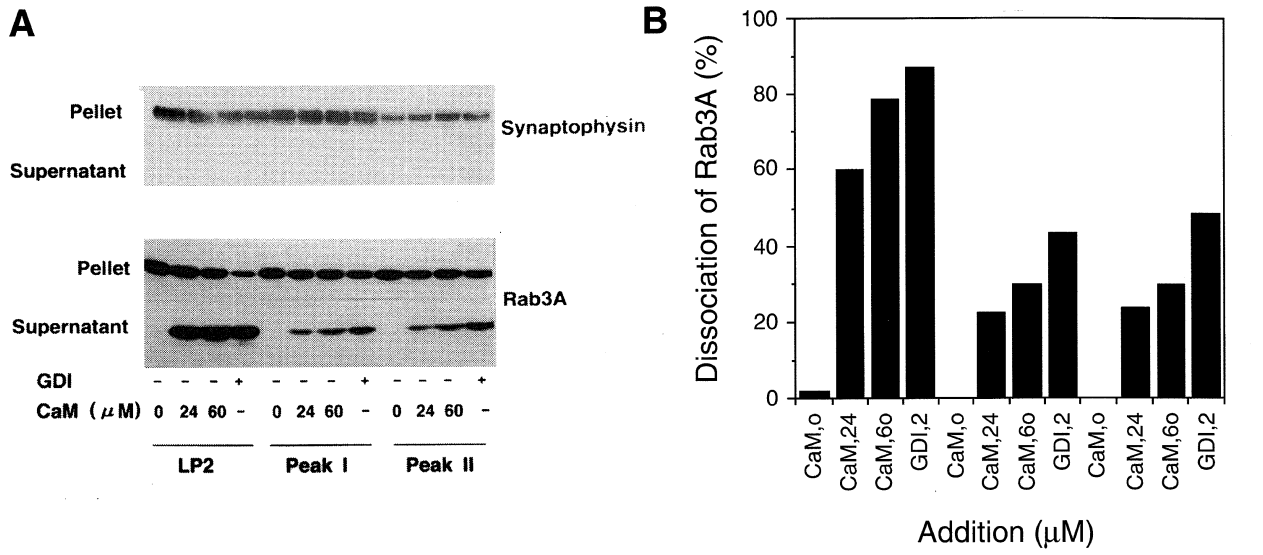

Figure 4. Dissociation of Rab3A from LP2 membrane, peak I and peak II membrane fractions. Peak I and Peak II membranes (Figure 1) were washed and resuspended in buffer B. Unfractionated LP2 membranes, fractionated peak I and peak II membrane fractions (15 μg proteins) were incubated with 0.1 mM Ca^{2+} and CaM for 30 min at 30oC in 50 ul of buffer B. Membranes were separated from supernatant media by table-top ultracentrifugation for 30 min at 100,000 *g*, 4°C. Pellet membranes were resuspended in 50 μl buffer B. Aliquots of pellet and supernatant solutions were analyzed on SDS-PAGE and by Western blot with anti-Rab3A and -synaptophysin antibodies. No synaptophysins were detected in supernatant, suggesting that membrane pellets and supernatants were separated completely (A). Rab3A bands were quantitated by laser densitometer and dissociation values were expressed as the ratio: Rab3A of supernatant/Rab3A of supernatant and pellet (B).

Dissociation Rab3A from LP2 membranes, light and heavy fraction of LP2 membranes

It was preveously observed that Ca^{2+}/CaM induces Rab3A to dissociate from LP2 membranes (Park *et al.*, 1996). To compare the extents of dissociation of Rab3A by Ca^{2+}/CaM or RabGDI from LP2 membranes and fractionated membranes, each type of membrane was incubated with Ca^{2+}/CaM or RabGDI and membranes and supernatant media were separated by ultracentrifugation. Then Rab3A of each pellet and supernatant was analyzed by Western blot. Rab3A was pretty easily dissociated by Ca^{2+}/CaM or RabGDI from LP2 membranes whereas was not from both fractionated membranes (Figure 4). It was found that the extents of dissociation of Rab3A by Ca^{2+}/CaM from light and heavy membrane fractions were similar. It could be considered that feasibility of dissociation of Rab3A by Ca^{2+}/CaM was decreased for unkown reason(s) during fractionation of membranes on ultracentrifugation. When LP2 membranes was preincubated for 1 h at 37°C, Ca^{2+}/CaM could not dissociate Rab3A from LP2 membranes (Park *et al.*, 1996).

Discussion

Rab3A seems to be localized on several types of organellar membranes in neurons. Synaptic vesicles were purified by controlled-pore glass (CPG) chromatography (Huttner *et al.*, 1983). They showed three membrane peaks on CPG chromatogram and the second peak membranes have a high content of synapsin I which is a typical protein of synaptic vesicle. We observed that three Rab3A peaks from each membrane fractions were obtained on CPG chromatography, thus Rab3A was localized on those three microsome membrane fractions (data not shown). Nerve terminals typically contain two types of regulated secretory vesicles: small synaptic vesicles and large dense-core vesicles. Synaptic vesicles contain low molecular weight neurotransmitters. Upon exocytosis, the membranes recycle and the contents of new vesicles are filled locally from the cytoplasm. Large dense-core vesicles contain neuropeptides that are synthesized at the endoplasmic reticulum and transported through the Golgi. Assembly of these vesicles involves the biosynthetic secretory apparatus. Thus, regulated secretory vesicles can be classified into two types according to their mode of assembly: recycling type and the biosynthetic type (Miller and

Moore, 1990). Little is known about the structures of neuropeptide-containing vesicles (Südhof and Jahn, 1991). Synaptic vesicles are synthesized at a rapid rate in nerve terminals to compensate for their rapid loss during neurotransmitter release. Their biogenensis involves endocytosis of synaptic vesicle membrane proteins from the plasma membrane. Vesicle formation is time- and temperature-dependent, requires ATP, is calcium-independent, and is inhibitied by GTPγS (Desnos *et al.*, 1995). Synaptic vesicles probably recycle by going through an endosomal intermediate. The existence of an endosomal intermediate is a conjecture based on the appearence of internal spaces labeled with endocytotic markers after synaptic vesicle endocytosis (Heuser and Reese, 1973). It is not obvious whether the two membrane fractions (Peak I and II) are small synaptic vesicles, large dense-core vesicles, or large endosomal intermediate membranes. More analysis such as electron microscopy should be required to get detailed informations of two membrane fractions.

It was observed that Ca^{2+}/CaM induced Rab3A to dissociate from membranes. It is considered that translocation of Rab3A by Ca^{2+}/CaM should be controlled by regulatory factor(s). First the dissociation of Rab3A by Ca^{2+}/CaM from different kinds of membranes of synaptosomes containing Rab3A were tested in this paper. Dissociation of Rab3A by Ca^{2+}/CaM from the two kinds of membrane fractions of LP2 membranes was similar although the concentrations of protein species (Rab3A and synaptophysin) of each membrane were differnt: the ratio of Rab3A/synaptophysin of peak I and peak II membranes was 1.7 and 3.1, respectively. It suggests that the dissociation of Rab3A from membrane by Ca^{2+}/CaM or by RabGDI, at least, is not affected by the presence of high concentration of synaptophysin in synaptic vesicles. Dissociation of Rab3A by Ca^{2+}/CaM from two fractionated membranes, however, was reduced compared to unfractionated LP2 membranes. First possibility of the decrease of dissociation of Rab3A is that guanine nucleotide may be released from Rab3A and second possibility is regulatory factor(s) for dissociation of Rab3A may be released from membranes during fractionation.

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References

Aepfelbacher, M., Vauti, F., Weber, P.C. and Glomset, J.A. (1994) Spreading of differentiating human monocytes is associated with a major increase in membrane-bound CDC42. *Proc. Natl. Acad. Sci. U.S.A.* 91: 4263-4267

Barrie, A. P., Nicholls, D. G., Sanchez-Prieto, J. and Sihra, T. S. (1991) An ion channellocus for protein kinase C potentiation of transmitter glutamate release from guinea pig cerebrocortical synaptosomes. *J. Neurochem.* 57: 1398-1404

Desnos, C., Clift-O'Grady, L. and Kelly, R. B. (1995) Biogenesis of synaptic vesicles *in vitro*. *J. Cell Biol.* 130: 1041-1049

Edelman, L., Hanson, P. I., Chapman, E. R. and Jahn, R. (1995) Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. *EMBO J.* 14: 224-231

Fischer von Mollard, G., Sudhof, T. C. and Jahn, R. (1991) A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. *Nature* 349: 79-81

Fischer von Mollard, G., Stahl, B., Khokhlatchev, A., Sudhof, T. C.and Jahn, R. (1994) Rab3C is a synaptic vesicle protein that dissociates from synaptic vesicles after stimulation of exocytosis. *J. Biol. Chem.* 269: 10971-10974

Gorvel, J. P., Chavrier, P., Zerial, M. and Gruenberg, J. (1991) Rab5 controls early endosome fusion *in vitro*. *Cell* 64: 915-925

Heuser, J. E. and Reese, T. S. (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57: 315-344

Hoffenberg, S., Sanford, J. C., Liu, S., Daniel, S. D., Tuvin, M., Knoll, B. J., Wessling-Resnick, M. and Dicky, B. F. (1995) Biochemical and functional characterization of a recombinant GTPase, Rab5, and two of its mutants. *J. Biol. Chem.* 270: 5048-5056

Huttner, W. B., Greengard, P. and De Camilli, P. (1983) Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicl preparation. *J. Cell Biol.* 96: 1374-1388

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriphage T4. *Nature* 227: 680-685

Li, G. and Stahl, P. D. (1993) Structure-function relatioship of the small GTPase rab5. *J. Biol. Chem.* 268: 24475-24480

Leube, R. E., Kaiser, P., Seiter, A., Zimbelmann, R., Franke, W. W., Rehm, H., Kn, P., Prior, P., Betz, H., Reinke, H., Beyreuther, K. and Wiedenmann, B. (1985) Synaptophysin: molecular organization and mRNA expression as determined from cloned cDNA. *EMBO J.* 6: 3261-3268

Miller, S. G. and Moore, H.-P. (1990) Regulated secretion. *Curr. Opin. Cell Biol.* 2: 642-647

Park, J. B., Farnsworth, C. C. and Glomset, J. A. (1996) Ca^{2+}/calmodulin causes Rab3A to dissociate from synaptosomal membranes. submitted for publication.

Sasaki, T., Kikuchi, A., Araki, S., Hata, Y., Isomura, M., Kuroda, S. and Takai, Y. (1990) Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to ras p21-like GTP-binding protein. *J. Biol.*

Chem. 265: 2333-2337

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Germanos, S., Tempst, P. and Rothman, J. E. (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362: 318-324

Südhof, T. C. and Jahn, R. (1991) Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron* 6: 665-677

Ullrich, O., Stenmark, H., Alexandrov, K., Huber, L. A., Kaibuchi, K., Sasaki, T., Takai, Y. and Zerial, M. (1993) Rab GDP dissociation inhibitor as a general regulator for the membrane association of Rab proteins. *J. Biol. Chem.* 268, 18143-18150