rab15, a Novel Low Molecular Weight GTP-binding Protein Specifically Expressed in Rat Brain*

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rab3A is a low molecular weight (LMW) GTP-binding protein specifically expressed in brain and localized to synaptic vesicles. rab3A has been proposed to play a role in neurotransmitter release by regulating membrane flow in the nerve terminal. In an attempt to define other LMW GTP-binding proteins that may regulate neurotransmitter release, seven cDNA clones encoding new members of the rab family of LMW GTP-binding proteins were isolated from a rat brain cDNA library. The rab proteins contain the four conserved structural domains essential for GTP binding in addition to domains required for membrane localization and effector protein interactions. One protein, rab16, is closely related to members of the rab3 subfamily, whereas two others are assigned as the rat homologs of canine rab8 and rab10. Four additional clones, rab12, rab13, rab14, and rab15, revealed unique sequences and are new members of the rab family of LMW GTP-binding proteins. The patterns of expression of rab15 and rab3A closely overlap but differ from that observed for all other known LMW GTP-binding proteins. This data suggests that rab15 may act in concert with rab3A in regulating aspects of synaptic vesicle membrane flow within the nerve terminal.

Neurons communicate with their target cells through the regulated release of neurotransmitters. Neurotransmitters such as acetylcholine are packaged and stored within the nerve terminal in small clear synaptic vesicles. Synaptic vesicle proteins are synthesized in the cell body and are rapidly transported to the nerve terminal by microtubule-based fast axonal transport. In response to the appropriate signal, the synaptic vesicles fuse with a specialized region of the presynaptic membrane and release their contents. Following exocytosis, the synaptic vesicle membrane and its associated proteins are actively retrieved from the presynaptic membrane in a highly coordinated process (1–3). GTP hydrolysis has been implicated as a regulatory switch controlling the targeting of transport vesicles (4). Biochemical and genetic studies have supported the involvement of GTP hydrolysis in mediating a variety of defined steps in intracellular membrane trafficking and secretion (5–9). Using cell-free systems it has been demonstrated that AlF₃ and the nonhydrolyzable GTP analog, GTPγS, inhibit vesicle-mediated transport between the ER and Golgi compartments. Similarly, the addition of GTPγS to in vitro transport assays disrupts the transport of mannose 6-phosphate receptors from endosomes to the trans-Golgi network (10). The ability of GTP analogs to stimulate Ca²⁺-independent exocytosis when introduced into mast cells (11) and the dependence of secretory vesicle budding on GTP hydrolysis in vitro (12) further implicates a role for GTP in multiple steps of secretion.

The rab family of LMW GTP-binding proteins has been proposed to be involved in many aspects of vesicular trafficking and secretion. They are postulated to mediate the fidelity of these processes through the binding and hydrolysis of GTP (4, 13). In keeping with the hypothesis that specific GTP-binding proteins regulate defined steps in membrane trafficking, several groups have recently reported the presence of rab proteins associated with the membranes of intracellular compartments. rab1 and rab6 are localized to the Golgi (8, 14, 15), rab2 is associated with an intermediate compartment between the ER and Golgi (16), rab3A is present on synaptic vesicles (17), rab5 is associated with early endosomes, and rab7 is associated with late endosomes (16). Using a cell-free system, Gorvel et al. (18) have shown that rab5 is essential for the fusion of early endosomes. Endosome fusion was inhibited by a rab5 mutant which is unable to bind and hydrolyze GTP and by antibodies against rab5, but not with antibodies against rab2 or rab7. The inhibition mediated by the rab5 mutant and rab5 antibodies was rescued by excess amounts of wild-type rab5 (18).

Evidence for a more direct role for members of the rab family of LMW GTP-binding proteins in secretion has come from genetic studies performed in yeast. Temperature-sensitive mutations in the ypt1 and sec4 loci disrupt yeast secretion at restrictive temperatures of growth (7, 19). Both loci encode LMW GTP-binding proteins which share the highest degree of sequence identity with the mammalian rab proteins. Whereas mutations in ypt1 interfere with vesicle trafficking between the ER and Golgi, the sec4 mutants are characterized by an accumulation of secretory vesicles which fail to fuse with the plasma membrane. Mutational analysis of sec4 suggests that in its GTP bound state, sec4 is activated to bind to the membrane of the secretory vesicle (20). Once bound, sec4 directs the vesicle to a putative docking site at the plasma membrane where the vesicle fuses with the plasma membrane to release its contents. It has been proposed that the hydrolysis of GTP to GDP releases sec4 from the vesicle membrane, making it available for another round of vesicle trafficking.

The abbreviations used are: GTPγS, guanosine 5'-3-O-(thio)triphosphate; kb, kilobase(s); LMW, low molecular weight; ER, endoplasmic reticulum; SDS, sodium dodecyl sulfate.
Since secretory vesicles are correctly targeted in sec4 mutants, sec4 must function at a latter stage of secretion. Sec4 may mediate the docking of the vesicle to putative receptor proteins on the plasma membrane or the fusion event itself (20). In addition to constitutive pathways, some mammalian cells also contain specialized forms of secretion such as regulated secretion at the presynaptic terminals of neurons (2) and polarized protein trafficking to distant plasma membrane domains in neurons and epithelial cells (21, 22). Although there is indirect evidence that rab proteins are involved in such specialized processes, the members of this family and their precise functions remain largely uncharacterized. In this paper, we report the isolation and characterization of rat brain cDNA clones encoding several rab proteins, which are highly related to yeast sec4. Two of these proteins are the rat homologs of previously identified rab proteins whereas five are unique. One of these proteins, rab15, is expressed within the rat central nervous system in a pattern that closely overlaps that observed for rab3A, suggesting that these rab proteins may act in, and differentially affect aspects of the vesicle trafficking flow. These rab proteins are part of a large family of rat proteins, which reflects the diversity of events required to mediate intracellular trafficking and secretion.

EXPERIMENTAL PROCEDURES

Materials—A whole rat brain cDNA library prepared in Lambda Zap II was purchased from Stratagene. The filters used for cDNA screening and Northern hybridization analysis were BA85 nitrocellulose and Nytran (Schleicher and Schuell), respectively. 5'-32P-dCTP and alkaline phosphatase were purchased from Du Pont-New England Nuclear. All chemicals and RNA molecular size markers were from Sigma or Bethesda Research Laboratories. Male Sprague-Dawley rats (100-150 g) were purchased from Simonsen Laboratories (CA).

Isolation of cDNA Clones—cDNA fragments containing the entire coding regions of the Discopyge ommata ora-1 (GenBank accession number M39830; nucleotides 1-883) and ora-2 (GenBank accession number M39831; nucleotides 1-669) LMW GTP-binding proteins were labeled by hexamer priming (24) and used to screen 3 x 10^6 recombinant clones in a rat brain Lambda Zap II cDNA library. Duplicate filters were hybridized under low stringency conditions in 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate), 5 x Denhardt's, 0.1% SDS, and 0.1 mg/ml E. coli tRNA at 50 °C and washed at 50 °C in 2 x SSC, 0.1% SDS. 47 positive clones were identified and the Lambda Zap II plasmid containing the cDNA liberated at 42 °C essentially as described by the manufacturer. The positive clones were further grouped under conditions of high stringency (6 x SSC, 5 x Denhardt's, 0.1% SDS, and 0.1 mg/ml E. coli tRNA at 65 °C) according to their patterns of cross-hybridization and hybridization to oligonucleotide probes specific for the following LMW GTP-binding proteins: rab1B, 5'-GGTCCGGAGACACGCTGGAGTGAAAGCTC3'; rab2, 5'-CAAGAGGGG-GTCTTGGACATTAATAATGAGGCAAAC3'; rab4, 5'-TCAG-GTGAAGCTGACCCGAGGATGGCTTGCTG T2'; and human rab5, 5'-AATCCAGGACAGAATTTCTGCGAGGAGAGGAG- GTA 3'. Overlapping deletions of the clones were generated in both directions and sequenced using Sequenase according to the manufacturer's specifications (U. S. Biochemical Corp.). The nomenclature used in sequencing of the cDNA clones is as follows: rab8 (clone LR8B); rab9 (clone LR9B); rab12 (clone LR12B); rab13 (clone LR13B); rab14 (clone LR14B); and rab16 (clone LR16B). The rab3A cDNA clone used in subsequent Northern analysis was identified from the above screen by sequence analysis.

Sequencing Analysis—Analysis of amino acid sequence homologies was performed using the FASTA and BESTFIT programs (Genetics Computing Co., Madison, WI) and the GenBank data base.

GenBank Accession Numbers—The nucleotide sequences of rab8, rab12, rab10, rab13, rab15, rab14, and rab16 have been submitted to the GenBank data base as accession numbers M58675-M58681, respectively.

Northern Analysis—Total RNA was extracted from rat tissues using CsCl/guanidinium HCl as described by Chirgwin et al. (23) and the poly(A) fraction purified by passage on poly(A) Quick push columns (Stratagene, La Jolla, CA) according to the manufacturer's specifications. The RNAs were fractionated on 1% agarose gels containing 2.2 M formaldehyde, transferred to Nytran (Schleicher and Schuell), and immobilized by baking at 80 °C for 1 h. Hybridizations were performed under conditions of high stringency (55 °C in 50% formamide, 5 x Denhardt's, 0.1% SDS, 5 x SSC, and 0.1 mg/ml E. coli RNA) using 1 x 10^6 cpm of each cDNA fragment labeled by hexamer priming (24). Final washings were performed at 70 °C in 0.1 x SSC, 0.1% SDS. RNA quantity and integrity were verified by reversibly staining the filters with methylene blue prior to hybridization (25).

RESULTS

Isolation of rab cDNA Clones—In a previous paper (26), we reported the isolation of two cDNA clones (ora-1 and ora-2), which encode LMW GTP-binding proteins with significant homology to the yeast sec4 protein. In a search for LMW GTP-binding proteins involved in regulating neurotransmitter release, the coding regions of the sec4-like ora-1 and ora-2 cDNA clones were used to screen a rat brain cDNA library under conditions of low stringency (50 °C). 47 positive cDNA clones were isolated and grouped according to their pattern of hybridization to oligonucleotide probes specific for previously identified rab proteins (see "Experimental Procedures") under conditions of high stringency (65 °C). Of the 47 clones, 8 non-hybridizing cDNA clones were further grouped into seven classes based on their pattern of cross-reactivity to each other, and the cDNA inserts of these clones were completely sequenced. The nucleotide and deduced amino acid sequences are presented in Fig. 1. Assignment of the initiator AUG codons was based on computer-assisted DNA analysis and by comparison of the amino acid sequences deduced from the largest open reading frames with known mammalian rab and yeast sec4 protein sequences. The encoded proteins range in length from 134 to 215 amino acids. The extreme NH2 termini of the cDNA clones encoding rabs8, rab12, and rab13 are not present, and subsequent attempts to isolate full-length cDNA clones proved unsuccessful. The rab protein sequences presented in Fig. 1 share 40-90% amino acid identity with ora-1, ora-2, and sec4, and between 33-99% amino acid identity with rabs proteins identified from marine ray, rat, human, and bovine brain, and canine Madin-Darby canine kidney cells (Table I). The rab protein sequences predicted from two of the clones demonstrate 89 and 99% amino acid identity with canine rab8 and rab10, respectively (27) and are considered the rat homologs of these proteins. One of these protein sequences determined in this study, rab16, shares the highest level of sequence identity and similarity with members of the rab3 subfamily (Table I). Three highly related forms of rab3 denoted A, B, and C have been previously identified from rat, human, and bovine brain which share 76-82% amino acid identity with each other (Table I) and demonstrate 95-97% amino acid identity between the mammalian homologs (28, 29). In addition, the mammalian rab3 proteins share 77% amino acid identity and 85-88% amino acid similarity with the orb3A homolog identified in marine rays. Since rab16 shares 73% amino acid identity with rab3A, rab3C and demonstrates 76% amino acid identity with orb3B, it is considered a closely related rab protein rather than an additional member of this subfamily. The protein sequences encoded by the four remaining cDNAs demonstrate 34-60% amino acid identity with existing rab protein sequences (Table I) and so are considered novel members of this family of LMW GTP-binding proteins. In keeping with current nomenclature, the proteins encoded by these cDNA clones were named rab12, rab13, rab14, and rab15 (refer to "Experimental Procedures"). Whereas rab15 and rab13 share the highest level of sequence identity with canine rab10 (51 and 61%,
Fig. 1. Nucleotide and deduced amino acid sequences of rab proteins. The nucleotide and predicted amino acid sequences of the cDNA clones encoding the proteins rab8, rab10, rab12, rab13, rab14, rab15, and rab16 are shown.

Structural Features of the rab Proteins — The amino acid sequences of rab8, rab10, rab12, rab13, rab14, rab15, and rab16 were aligned with protein sequences for previously identified rab proteins, including yeast sec4, ora-1, and ora-2. As shown in Fig. 2, the proteins all display the structural features characteristic of LMW GTP-binding proteins (30, 31, 40). Four domains originally identified in the LMW GTP-binding protein p21 ras that are involved in the binding of GTP/GDP (region I-IV) are highly conserved in the rab proteins. These include the highly conserved DTAGQE motif (region I) which functions to stabilize binding to the γ-phosphate. This domain is preceded by a conserved tryptophan residue in all members of the rab family (Fig. 2) but not in p21 ras (30).

The guanine specificity region characterized by the motif NKXD (region II) is also highly conserved and has been shown by mutational analysis of p21 ras, sec4, and ypt1 to regulate the binding of GTP. The fourth GTP-binding domain which contributes to interactions with the guanine base is highly conserved in all the rab proteins and is characterized by the motif EXSAK/L (region IV). Although the phosphate binding loop characterized by the motif GXXXGK (region I) is the most variable of the four domains required for GTP binding, the lysine residue which mediates the interaction with the β- and γ-phosphates of GTP, is highly conserved in all rab proteins. Comparison of the rab protein sequences revealed two additional regions of high homology: (i) a region following the second GTP-binding domain which contains a conserved arginine residue surrounded by flanking sequences also displaying a high level of conservation; and (ii) a conserved phenylalanine residue, adjacent to the fourth GTP-binding domain (Fig. 2). Interestingly, the protein sequences surrounding the phenylalanine residue display limited homology, highlighting the persistence of this residue at this position.

A less conserved region is the putative effector domain (Fig. 2). The corresponding domain in p21 ras (amino acids 32-40) was characterized as the site of interaction for the protein, GAP (31, 32). GAP is a regulatory protein which functions to stimulate the intrinsic GTPase activity of LMW GTP-binding proteins. Whereas the corresponding domains of the rab proteins contain an invariant threonine residue as the first amino acid of this region, the rest of this region is clearly more divergent. The putative effector sequences of rab8, rab10, and rab12 are identical to the corresponding region in yeast sec4, ora-1, and ora-2. In addition, rab16, rab12, and rab15 contain conservative amino acid changes at positions 2 and 4 of the motif. In contrast, the putative effector domain of rab14 is identical to the corresponding regions in rab4 and canine rab9 and rab11. The high level of sequence conservation observed between the effector domains of these rab proteins suggests that various members of the rab family may exhibit overlapping specificity for GAP proteins.

Although the rab proteins diverge significantly in their respective carboxyl-terminal domains, all of these proteins have 1 or 2 cysteine residues at their COOH termini. Muta
tional analysis of p21-ras, sec4, and rab3A has revealed that...
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RAB14

Classification of Rab proteins by amino acid sequence comparison

Rab protein sequences were compared with yeast sec4, and rab protein sequences from marine ray (M. n.), rat (R.), bovine (B.), canine (C.), and human (H.). The values above the diagonal refer to percentage of amino acid identity shared between any two sequences, whereas the numbers below the diagonal describe percentage of amino acid similarity, taking into account conservative amino acid substitutions.

Table I

Classification of rab proteins by amino acid sequence comparison

The COOH-terminal cysteine residues are essential for proper membrane localization and normal function of these proteins (20, 33, 34). Whereas rab8 and rab13 terminate in the -CAAX motif characteristic of ras-like GTP-binding proteins, rab14, rab15, and rab16 terminate in a -CXC motif, which in rab3A is polyprenylated to expedite attachment to synaptic vesicles. The -CC motif, which is essential for the proper localization and normal functioning of yeast sec4, is present at the COOH termini of rab10 and rab12.

Tissue Distribution of the rab mRNAs—Expression of the
rab proteins was investigated by Northern analysis. To minimize the possibility of cross-hybridization between different rab mRNAs, hybridizations were performed under conditions of high stringency (see “Experimental Procedures”) using the probes. Variable levels of the rab transcripts were observed in total RNA prepared from whole brain, spinal cord, heart, kidney, and lung and to a lower level in muscle and liver. Highest levels of the minor transcript were observed in lung, kidney, whole brain, and spinal cord, as shown in Fig. 4, a 1.6 kb transcript for rab3A was expressed predominantly in total RNA prepared from whole brain as well as spinal cord. Northern analysis was performed on poly(A)+ RNA prepared from these samples. Northern analysis of poly(A)+ RNA detected a 1.4 kb rab13 transcript, whose expression closely paralleled that observed for rab8. Whereas high levels of rab13 expression were observed in lung, kidney, whole brain, and spinal cord, lower levels were detected in muscle, heart, and liver. The major 2.40 kb rab14 transcript was detected in high levels in whole brain, spinal cord, heart, kidney, and lung and to a lower level in muscle and liver. Highest levels of the minor 1.20 kb transcript were detected in heart and lung.

Previous studies have reported that the expression of rab3A is restricted to neural tissues (28, 29, 35, 36). The high level of sequence identity shared between rab3A and rab16 suggested that rab16 may also be expressed exclusively in neural tissues. To address this question, the pattern of rab3A and rab16 expression was investigated by Northern analysis. As shown in Fig. 4, a 1.6 kb transcript for rab3A was expressed predominantly in total RNA prepared from whole brain as well as spinal cord. Longer exposures of the filter revealed lower levels of rab3A mRNA in rat heart, muscle, and lung.
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FIG. 3. Tissue distribution of rab8, rab10, rab12, rab13, and rab14. Total RNA (15 μg) from rat spinal cord, whole brain, skeletal muscle, heart, kidney, liver, and lung was fractionated and probed with radiolabeled cDNA fragments for rab8, rab10, rab12, and rab14 as indicated. A radiolabeled cDNA fragment for rab13 was used to probe a filter containing 3 μg of poly(A+) RNA isolated from the corresponding tissues. The sizes of the transcripts are indicated on the left. In addition, the total RNA filters were reversibly stained with methylene blue to detect the presence of equal amounts of intact 28 and 18 S ribosomal RNAs, as indicated.

FIG. 4. Tissue-specific rab expression. Total RNA (15 μg) from spinal cord, whole brain, muscle, heart, kidney, liver, and lung was fractionated and probed with a radiolabeled cDNA fragment for rab5A. Radiolabeled cDNA fragments for rab15 and rab16 were used to probe a filter containing 3 μg of poly(A+) RNA from the corresponding tissues. The transcript sizes are shown on the left.

but not in liver or kidney (not shown). Conversely, two transcripts measuring 2.6 and 2.0 kb were detected for rab16 in poly(A+) RNA prepared from these tissues (Fig. 4). Whereas the 2.6 kb transcript was expressed predominantly in rat lung, lower levels of the transcript were detected in other tissues tested such as spinal cord and whole brain. Longer exposures revealed the presence of the minor 2.0 kb transcript in lung, spinal cord, and whole brain. The different patterns of tissue-specific expression observed for these closely related rab proteins further confirm that rab16 is not an additional member of the neural specific rab3 subfamily.

Interestingly, Northern analysis of rab15 revealed a pattern of expression which closely resembled that observed for rab3A. A single transcript measuring 3.6 kb was detected in poly(A+) RNA isolated from whole brain with lower levels in spinal cord (Fig. 4). Longer exposures of the filter revealed low levels of rab15 in rat heart, muscle, and lung but not in kidney or liver. These results demonstrate that rab15 is expressed predominantly in rat neural tissues in a pattern that closely overlaps that observed for rab3A. However, the inability to detect rab15 in total RNA suggests that rab15 is expressed at a lower level than rab3A in these tissues.

The enriched expression of rab3A and rab15 in rat neural tissues prompted us to further investigate the distribution of these rab species among the different regions of the rat central nervous system. Total and poly(A+) RNAs were prepared from dissected regions of rat brain and probed with radiolabeled cDNA fragments specific for rab3A and rab15, respectively. As shown in Fig. 5, rab3A mRNA is expressed throughout the brain and spinal cord at similar levels, with lower levels detectable in the pituitary gland. Although expressed throughout the brain, the levels of rab15 expression appear to be somewhat more variable in the different brain regions. The highest level of expression was detected in the cortex, hippocampus, and striatum, with lower levels present in the olfactory bulb, cerebellum, brainstem, and spinal cord. rab15 mRNA was barely detectable in the pituitary gland even following prolonged exposure of the filter. These experiments reveal that rab3A and rab15 are expressed in distinct but overlapping patterns in the rat brain.

DISCUSSION

In this paper we report the isolation and characterization of cDNA clones encoding a complex family of rab proteins, some of which may function in regulating neurotransmitter release. The seven novel rab proteins identified in this study contain the four conserved domains essential for GTP binding in addition to domains required for membrane localization and effector interactions. Since it remains to be established that each of the rab proteins is regulated by its own GAP, it is noteworthy that several of the rabs share identical effector domain sequences. Given the high level of sequence conservation, it is tempting to speculate that some of the rab proteins may display overlapping specificity for GAPs, thereby requiring a smaller number of these proteins than previously suggested (37, 38). Although a GAP for rab3A has been identified from bovine brain (39), it is presently unclear whether this GAP will bind and activate the GTPase activities of other rab
proteins, which share identical GAP effector domain sequences. In addition to these domains, all of the rab proteins contain a conserved arginine and phenylalanine residue following the second and fourth GTP-binding domains, respectively. The sequence and three-dimensional structural analysis of p21 ras reveal that the arginine and phenylalanine residues are also conserved in p21 ras and reside within the second and fifth α-helical domains, respectively (40). Whereas these α-helical domains reside close to the surface of the GTP bound form of p21 ras, following GTP hydrolysis the second helical region undergoes considerable conformational change and is no longer detectable on the surface of the GDP bound form of p21 ras. Since the conservation of amino acid sequences is generally characteristic of an important functional property, these residues may be important in the conformational switch or interact with additional regulatory proteins. One candidate protein, GDI, has been shown to inhibit the dissociation of GDP from rab3A and yeast sec4, but not from the GDP bound form of p21 ras (41). Since GDI functions on both rab3A and yeast sec4, it may act as a general regulator of rab proteins and mediate its effects through either of these regions. However, direct proof will require functional studies.

The results of this study revealed no observable correlation between the level of amino acid sequence identity shared between the rab proteins and their patterns of expression. For example, rab3A and rab15 are distantly related and yet are expressed in similar patterns. Conversely, rab3A and rab16 are much more closely related and yet are expressed in very different patterns. Given the current state of knowledge, it is reasonable to assume that each of the rab proteins is localized to a different cellular compartment where they function to regulate membrane trafficking. Therefore, the complex patterns of expression evolved for many of the rab proteins will permit different cell types to express specialized combinations of these proteins. These unique combinations of rab proteins may fine tune the trafficking of membranes within the different tissues.

The specific expression of two different rab proteins within the brain suggests that these proteins regulate different steps in membrane flow within the nerve terminal. Many synaptic vesicle proteins such as VAMP, synapsin, and p65 (synaptotagmin) are members of small gene families which are differentially expressed in unique combinations throughout the brain (44–46). Although each neuron has been proposed to express at least one member of each gene family, it is presently unclear if different members of any one of these families are co-expressed within a single cell type. However, the overlapping patterns of rab3A and rab15 expression throughout the brain suggest that these rab proteins may be co-expressed within the same cell types. Subsequent experiments will be essential to determine the cellular localization of rab15 and to determine if it is co-localized with rab3A on synaptic vesicles.

Whereas a role for rab15 is presently unclear, rab3A has been proposed to play an important role in regulated secretion from neurons (1). Although a direct involvement of rab3A in regulating neurotransmitter release has yet to be demonstrated, the specific localization of rab3A to synaptic vesicles (17, 36, 42) and its ability to dissociate from these membranes during Ca**⁺**-induced exocytosis (43) further supports its involvement. Since the release of neurotransmitters from the nerve terminal is a highly coordinated, multistep process requiring the targeting of synaptic vesicles to the active zone of the presynaptic membrane, the fusion of these membrane compartments, and subsequent recycling of synaptic vesicles within the nerve terminal, additional LMW GTP-binding proteins may be required to operate on different stages of membrane flow within the nerve terminal. The enriched expression of rab15 throughout the brain in a pattern that closely overlaps that observed for rab3A makes rab15 a possible candidate for regulating these processes.

Acknowledgments—We thank Dr. Antonio Malgaroli for providing dissected rat brain tissues and Drs. Cornelis Elferink, Mark Bennett, and John Ngsee for critically reading this manuscript.

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