Low Molecular Weight GTP-binding Proteins Are Associated with Neuronal Organelles Involved in Rapid Axonal Transport and Exocytosis*

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Recent evidence suggests that low molecular weight GTP-binding proteins may play important roles in a variety of membrane transport processes. In order to address the question of whether these proteins are involved in transport processes in the nerve axon, we have assessed their presence in rapid transport membrane vesicles. We report the characterization of a group of low molecular weight GTP-binding proteins which are constituents of rapid transport vesicles. Although these proteins are components of rapid transport vesicles, they are apparently not major rapidly transported species. They are found in cytosolic as well as in membrane fractions of axons, and the membrane-associated form behaves as an integral membrane protein(s). These proteins are also found in association with a variety of vesicular and organelar components of neurons including coated vesicles, synaptic vesicles, synaptic plasma membranes, and mitochondria. We discuss the possible roles of these proteins in rapid axonal transport and exocytosis.

Recently the growing family of GTP-binding proteins has expanded to include a set of low molecular weight proteins, some of which seem to be involved in directing membrane traffic within the cell. For example, recent evidence suggests that a 23-kDa GTP-binding protein isolated from yeast, Ypt1, plays a role in the transport of proteins through the Golgi (Segel et al., 1988). Sec 4, another yeast GTP-binding protein with a molecular mass of 23.5 kDa, has been implicated as a regulatory element in the secretory pathway between the Golgi and plasma membrane (Goud et al., 1988). The mechanism of action of such proteins is not known, but one hypothesis states that secretory GTP-binding proteins may mediate vectorial transport of individual vesicles, i.e. that these proteins could specify the direction traveled by each secretory vesicle between cellular compartments (Bourne, 1988).

We are investigating the role of GTP-binding proteins in a specialized mammalian transport system, the rapid axonal transport system of the optic nerve. Rapid transport vesicles represents one component of the axonal transport system which is utilized by retinal ganglion cells to transport essentially every constituent of the axon from the cell body. All together, five separate components of axonal transport have been identified (for review, see Grafstein and Forman, 1980). The slower components, of which there are two, are composed of structural cytoskeletal and contractile proteins as well as various enzymes of intermediary metabolism; these components move down the axon at a rate of 1 mm/day. Two intermediate components have been observed, but the data on these are conflicting. Karlsson and Sjostrand (1971) report a mitochondrial component moving at a velocity of 6-12 mm/day, while others (Willard et al., 1974; Lorenz and Willard, 1978) report an intermediate component of contractile proteins moving at a rate of 4-8 mm/day, as well as a mitochondrial component moving at 34-68 mm/day. The most recent data concerning mitochondrial movement suggests that these organelles are transported intermittently, but at a rate indistinguishable from that of other, smaller membranous particles (Forman, 1987).

Rapid transport velocity is typically measured at between 100 and 500 mm/day. The protein composition of the rapid component is very complex. Two-dimensional gel electrophoresis indicates that hundreds of proteins are rapidly transported (Stone et al., 1983). These rapidly transported proteins seem to be segregated into at least three distinct vesicle populations, one of which represents a class of synaptic vesicle precursor bound for the nerve terminal (Morin, 1989). Rapid transport vesicles are thought to be translocated toward the nerve terminal along microtubules via an ATP-dependent motor, kinesin, a recently discovered ATPase (Vale et al., 1985).

In this paper we report the characterization of a group of low molecular weight GTP-binding proteins (LMW-GBPs)1 which are constituents of isolated rapid transport vesicles. Our results show, that although these proteins are components of highly purified rapid transport vesicles, they are apparently not a major rapidly transported species. The LMW-GBPs are localized in cytosolic as well as in membrane fractions of axons, behave as integral membrane proteins when localized in membranes, and are found in association with a variety of vesicular and organelar components of neurons including coated vesicles, synaptic vesicles, synaptic plasma membranes, and mitochondria.

1 The abbreviations used are: LMW-GBP, low molecular weight GTP-binding protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)] tetracetic acid; GTPyS, guanosine 5′-O-thiotriphosphate.
EXPERIMENTAL PROCEDURES

Isolation of Axonal Vesicles—Subcellular fractionation was done using a modification of the method of Lorenz and Willard (1976). Within 10 min of killing by lethal injection, optic nerves and tracts were rapidly dissected and placed in 7 ml of ice-cold homogenization buffer (1 mM triethanolamine, 320 mM sucrose, pH 7.4, containing 30 μg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin) and immediately loaded on top of three successive 150,000 g at 4°C. The LMW-GBPs are components of purified rapid transport vesicles. Previously, we isolated rapid transport vesicles from rabbit optic nerve (Morin, 1989). When the optic nerve was prelabeled with [3H]methionine/cysteine and the rapidly transported proteins were separated by SDSPAGE, a number of labeled proteins were visualized, including one abundant species with an apparent molecular mass of 24 kDa. Since this rapidly transported 24-kDa membrane protein was in the molecular mass range of known secretory GTP-binding proteins, we decided to investigate whether this protein was in fact a GTP-binding protein.

Rabbits were given an intravitreal injection of 0.5 mCi of [3H]methionine/cysteine in each eye. After 2.5 h, the optic nerves and tracts were dissected and the membranes were prepared and run on an SDS gel. Membranes were prepared in the same way from unlabeled optic nerve. Both membranes were run on the same SDS gel, transferred onto nitrocellulose, and the unlabeled membranes subjected to [32P]GTP binding. As shown in Fig. 1a, a major band of 24 kDa is seen in a [32P]labeled blot of axonal membranes, as well as in metabolically labeled axonal membranes. Two other minor bands with slightly smaller molecular weights are also visualized by [32P]GTP binding. To show that the nucleotide-binding capacity of these proteins was specific for GTP, [32P]GTP blots were performed in the presence of unlabeled GTP or ATP. A 1000-fold excess of unlabeled GTP totally abolished the signal, whereas a 1000-fold excess of ATP had no effect (Fig. 1b).

To determine whether GTP-binding proteins were identical to the major rapidly transported 24-kDa species, two-dimenional gel electrophoresis was performed. The GTP-binding proteins formed a streak at the origin of the first dimension while the 24-kDa labeled species focused as a series of discrete spots in the pH 4–5 region. (data not shown). Therefore, although the 24-kDa GTP-binding protein may be a constituent of rapid transport vesicles, it is not a major labeled, rapidly transported protein species.

The LMW-GBPs are components of purified rapid transport vesicles. It was essential to obtain a more highly purified preparation of rapid transport vesicles to ascertain if the GTP-binding proteins are truly components of rapid transport vesicles. One means of purifying small (800 Å) rapid transport vesicles is to run labeled vesicles through an agarose gel according to the method of Rubenstein et al. (1981); the purified vesicles localize in a tight band of radioactivity (Morin, 1989). Another means of purifying these vesicles is to immunoadsorb vesicles with a monoclonal antibody to synaptophysin, a protein which has already been demonstrated to be a component of rapid transport vesicles (Morin, 1989).

Labeled axonal vesicles were run on two lanes of an agarose gel. One lane was dried and autoradiographed (Fig. 2a). The
24-kDa GTP-binding Protein on Rapidly Transported Membranes

**FIG. 1.** SDS-polyacrylamide gel electrophoresis and \[^{32}P\]GTP binding of axonal membrane proteins. Rabbits were given an intravitreal injection of 0.5 mCi of \[^{35}S\]methionine/cysteine in each eye. After 2.5 h, the optic nerves and tracts were dissected, membranes were prepared and analyzed by electrophoresis on 12% SDS-polyacrylamide gels. Membranes from unlabeled optic nerve membranes were prepared and analyzed by electrophoresis on 12% SDS-PAGE, transfer to nitrocellulose, and subjected to \[^{32}P\]GTP binding. a, comparison of \[^{32}P\]GTP binding with SDS-PAGE profile of labeled axonal membrane proteins. Lane 1, autoradiograph of 120,000 cpm of labeled axonal membrane; lane 2, \[^{32}P\]GTP blot of 36 \(\mu\)g of unlabeled axonal membrane. b, competition of \[^{32}P\]GTP binding by GTP and ATP. Unlabeled axonal membranes (24 \(\mu\)g/lane) were subjected to SDS-PAGE, transfer to nitrocellulose, and \[^{32}P\]GTP binding in the presence of no competing nucleotide (lane 1), a 1000-fold excess of unlabeled GTP (lane 2), and a 1000-fold excess of unlabeled ATP (lane 3). The numbers on the left or right of this and subsequent figures refer to the molecular mass in kilodaltons of the marker proteins.

other lane was cut up into 0.5-cm slices; the slices were subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with \[^{32}P\]GTP (Fig. 2b). The \[^{32}P\]GTP blot in Fig. 2b shows that most of the GTP-binding protein migrated to a wide band centering around four to five half-centimeters from the origin. This corresponds exactly to the location of the rapid transport vesicles seen in Fig. 2a.

Fig. 2c shows a \[^{32}P\]GTP blot of vesicles which had been immunoadsorbed with anti-synaptophysin (lane 1) and control antibody (lane 2) attached via an anti-mouse IgG to Sepharose beads. As seen in this blot, anti-synaptophysin specifically adsorbs vesicles which contain the GTP-binding protein. From this result and the one preceding, it is clear that highly purified rapid transport vesicles contain LMW-GBPs.

Distribution of the GTP-binding Protein in Cytosolic and Membrane Fractions of Axons—We were interested in determining whether the GTP-binding proteins were present in axonal cytosol as well as in membranes and whether the membrane-bound form was peripherally or integrally associated with the membrane. Homogenates of axons were either untreated or treated with various agents, then separated into cytosolic and membrane fractions by sedimentation at 100,000 x g. The fractions were then run on a SDS gel, transferred to nitrocellulose, and subjected to \[^{32}P\]GTP binding.

Fig. 3 shows the results of such an experiment. As shown in the untreated fractions (section 1), the GTP-binding protein is present in both cytosol and membrane. Treatment with NaCl (section 2), \(\mathrm{Na}_{2} \mathrm{CO}_{3}\) (section 3), urea (section 5), or Triton X-114 (section 6), agents which should cause preferentially associated membrane proteins to become soluble, does not shift the distribution of the GTP-binding protein, although Triton X-100 treatment (section 4) does shift the distribution slightly from membrane to cytosol. Thus, there are GTP-binding proteins associated with both cytosol and membraneous organelles; the membrane-bound protein(s) form seems to behave like an integral membrane protein.

LMW-GBPs Are Components of Synaptic Vesicles, Synaptic Plasma Membrane, Coated Vesicles, and Mitochondria but Not of Lysosomes—To further explore the cellular distribution of the GTP-binding proteins, synaptic vesicles, synaptic plasma membranes, clathrin-coated vesicles, mitochondria, and lysosomes were isolated from rabbit brain, by methods described.
under "Experimental Procedures." Synaptic vesicles and synaptic plasma membranes were chosen because they are likely targets for delivery of rapidly transported material. LMW-GBPs may also play a functional role in synaptic vesicle mediated neurotransmitter release (see Bourne, 1988). Clathrin-coated vesicles were examined since they have been implicated in the exocytotic transport of rapidly transported proteins (Stone et al., 1984). Mitochondria and lysosomes were analyzed since mitochondria are transported through axons to the nerve ending (Forman, 1987), while lysosomes are very rarely found in either axons or nerve endings (Grafstein and Forman, 1980).

Synaptic vesicles were isolated by the procedure of Huttner et al. (1983), substituting agarose gel electrophoresis for controlled pore glass bead chromatography as the final step. As shown in Fig. 4a the vesicles migrate as a single band having a considerably slower mobility than either clathrin-coated vesicles or rapid transport vesicles. Fig. 4b demonstrates that this band is highly enriched in synaptophysin, a marker protein for synaptic vesicles (Wiedenmann and Franke, 1985). Also this band contains the vast majority of the GTP-binding activity (Fig. 4c). One discrepancy was noted. Fraction 2 contained a high amount of GTP binding, while containing much less protein than fractions 3 and 4. Also synaptophysin was not detected in fraction 2.

We subjected aliquots of each subcellular fraction to SDS-PAGE followed by [32P]GTP binding. As shown in Fig. 5, a major 24-kDa GTP-binding protein together with the two minor components are present in synaptic vesicles (lane 2), synaptic plasma membrane (lane 3), coated vesicles (lane 4), and mitochondria (lane 5), but not in lysosomes (lane 6). Thus, the 24-kDa GTP-binding protein is found not only in rapid transport vesicles, but in vesicular compartments which are expected to contain precursors (coated vesicles) or targets (synaptic vesicles, synaptic plasma membranes) for rapid transport vesicles.

**DISCUSSION**

We have examined the protein composition of rapid transport vesicles purified from rabbit optic nerve. The technique of [32P]GTP blotting revealed a major GTP-binding protein of 24-kDa molecular mass, together with two minor components. Although this GTP-binding protein corresponded in mobility to a metabolically labeled, rapidly transported protein, two-dimensional electrophoresis proved that the two species were not identical. The GTP-binding protein(s) is also located on synaptic vesicles, synaptic plasma membrane, coated vesicles, and mitochondria but not on lysosomes. LMW-GBPs have been implicated as regulatory elements in the secretory pathway of the yeast Saccharomyces cerevisiae (Segev et al., 1988; Schmitt et al., 1988; Goud et al., 1988). Studies conducted in a mammalian system also implicate GTP-binding proteins as having a functional role in the transport of vesicles within the Golgi apparatus (Melancon et al., 1987). Our finding that GTP-binding proteins are components of rapid transport vesicles suggests that they may play functional roles in the process of rapid axonal transport. Also, by analogy with the role of the GTP-binding protein, elongation factor Tu, in the protein synthetic machinery, the GTPase activity of the LMW-GBP may serve as an internal kinetic constant ensuring targeting accuracy (Thompson, 1988). For example, the GDP-bound form of the protein might recognize a protein component of the donor membrane. The substitution of GTP for GDP may lead to vesicle budding and promote its specific attachment to a docking protein on the appropriate acceptor membrane. Following GTP hydrolysis, vesicle fusion may take place, while the GTP-binding protein might return through the cytoplasm to target another vesicle for transport as proposed by Bourne (1988).

The 24-kDa GTP binding protein(s) that we describe is found in both cytosolic and membranous fractions of axons. Two explanations for these findings can be proposed. The first is that there are at least two proteins, one in the cytosol and one associated with membranes. The second is that there is a single 24-kDa GTP-binding species which is found in both a membrane-associated and cytosolic form. We favor the second explanation for several reasons. Takai and collaborators purified both a membrane and cytosolic 24-kDa GTP-binding protein from bovine brain and showed that they are identical (Yamamoto et al., 1988). Also Sec 4, a 24-kDa yeast GTP-binding protein essential for Golgi to plasma membrane vesicle transport, has been shown to be present in both membranes and cytosol (Goud et al., 1988). Very recently a
series of elegant experiments have demonstrated that recycling of Sec 4 between membrane and cytosol is necessary for secretion (Walworth et al., 1989). Finally our observation that the 24-kDa GBP is associated with rapid transport vesicles but apparently is not itself rapidly transported argues that this protein continually recycles between these vesicles and the cytosol. These observations are consistent with Bourne’s model in which the GTP-binding protein is continuously recycled from a cytosolic pool. Several LMW-GBPs have been shown to be myristylated or palmitylated (Chen et al., 1985; Buss and Setton, 1986), and this is thought to be their mode of attachment to membranes. Recently a group of 24-kDa GTP-binding proteins have been purified from bovine brain and their cDNAs cloned (Matsui et al., 1988). A carboxy-terminal consensus sequence for palmitylation has been found on all members of this family. Acylation and deacylation may therefore serve as the mechanism by which transport-regulating GTP-binding proteins are inserted, and removed from, vesicle membranes in transport.

Similar to the situation in yeast, where different GTP-binding proteins have been identified in different compartments of the secretory pathway, we observe several LMW-GBPs in several compartments of the rapid transport pathway, including coated vesicles, (possible precursors), synaptic vesicles, and synaptic plasma membrane (targets). One caveat should be noted with respect to the coated vesicle results. Since coated vesicles function in both exocytic and endocytic pathways, the possibility remains that the LMW-GBPs we see associated with coated vesicles result from the recycling of surface membrane.

Our finding that the 24-kDa GTP-binding protein is also associated with mitochondria may be explained by evidence that mitochondria are transported at an intermediate to rapid rate in axons along microtubules (Karlsson and Sjostrand, 1971; Willard et al., 1974; Lorenz and Willard, 1978). The 24-kDa GTP-binding protein(s) may thus be involved, not only with rapid transport of vesicles, but with axonal transport in general. Lysosomes, unlike mitochondria, appear to be excluded from axons (Grafstein and Forman, 1980); as expected, the LMW-GBPs were not found in association with lysosomes.

Another intriguing potential function for one or more of the LMW-GBPs is in presynaptic neurotransmitter release. Recently evidence for the direct role of a GTP-binding protein in Ca\(^{2+}\)-stimulated mast cell degranulation, an analogous process, has been reported (Howell et al., 1987). Also, two LMW-GBPs have been shown to be selectively ADP-ribosylated by botulinus neurotoxin D, a potent inhibitor of presynaptic neurotransmitter release (Matsuoka et al., 1989).

Our further experiments will decide if and how these LMW-GBPs are involved in the processes of rapid axonal transport and neurotransmitter release. An in vitro functional assay, which monitored fusion of vesicles or motility of vesicles along microtubules, would be useful in probing the GTP dependence of rapid transport; GTP-S (a nonhydrolyzable analog of GTP) or botulinus toxin (a potential specific probe for the GTP-binding protein) would be expected to block fusion and/or motility if it was GTP-dependent. Purification of the GTP-binding protein(s) in sufficient quantities to raise antibodies would allow an examination of its mode of synthesis and transport, and its definitive localization along the rapid transport-exocytic pathway.

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REFERENCES