Myristoylation-facilitated Binding of the G Protein ARF1\(_{\mathrm{GDP}}\) to Membrane Phospholipid Is Required for Its Activation by a Soluble Nucleotide Exchange Factor* (Received for publication, October 10, 1995)

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We have investigated the role of N-myristoylation in the activation of bovine ADP-ribosylation factor 1 (ARF1). We previously showed that myristoylation allows some spontaneous GDP-to-GTP exchange to occur on ARF1 at physiological Mg\(^{2+}\) levels in the presence of phospholipid vesicles (Franco, M., Chardin, P., Chabre, M., and Paris, S. (1995) J. Biol. Chem. 270, 1337-1341). Here, we report that this basal nucleotide exchange can be accelerated (by up to 5-fold) by addition of a soluble fraction obtained from bovine retinas. This acceleration is totally abolished by brefeldin A (IC\(_{50} = 2\) \(\mu\)M) and by trypsin treatment of the retinal extract, as expected for an ARF-specific guanine nucleotide exchange factor. To accelerate GDP release from ARF1, this soluble exchange factor absolutely requires myristoylation of ARF1 and the presence of phospholipid vesicles. The retinal extract also stimulates guanosine 5'-3-O-(thio)triphosphate (GTP\(_7\)S) release from ARF1 in the presence of phospholipids, but in this case myristoylation of ARF is not required. These observations, together with our previous findings that both myristoylated and non-myristoylated forms of ARF\(_{\mathrm{GTP},\mathrm{S}}\) but only the myristoylated form of ARF\(_{\mathrm{GDP}}\) bind to membrane phospholipids, suggest that (i) the retinal exchange factor acts only on membrane-bound ARF, (ii) the myristate is not involved in the protein-protein interaction between ARF1 and the exchange factor, and (iii) N-myristoylation facilitates both spontaneous and catalyzed GDP-to-GTP exchange on ARF1 simply by facilitating the binding of ARF\(_{\mathrm{GDP}}\) to membrane phospholipids.

ADP-ribosylation factors (ARFs)\(^1\) are a family of small (\(\approx 20\) kDa) guanine nucleotide binding proteins, originally identified as cofactors of cholera toxin and more recently recognized as essential participants in intracellular vesicular transport (1-4). All ARFs contain the amino-terminal myristoylation consensus sequence and are believed to be myristoylated in vivo (5). The functions of ARFs in membrane traffic are linked to their guanine nucleotide-dependent interactions with membranes. Myristoylation is also crucial for these interactions, but the precise mechanism for the attachment of ARF to membranes is still unclear. According to the model initially proposed by Serafini et al. (6) and at present most commonly accepted, the GDP-bound form of ARF is cytosolic; upon interaction with a specific nucleotide exchange protein, described as membrane-bound by some authors (7-9) and soluble by others (10, 11), ARF\(_{\mathrm{GDP}}\) is converted to ARF\(_{\mathrm{GTP}}\). This conversion would promote a conformational change of the amino terminus, allowing exposure of the myristoyl group and its insertion into membranes. This model, referred to as "myristoyl-GTP switch" by analogy with the Ca\(^{2+}\)-myristoyl switch model proposed for recoverin (12), therefore implies that myristoylation of ARF is not necessary for its interaction with the nucleotide exchange enzyme (9, 13) but is required after the exchange reaction for the stable association of ARF\(_{\mathrm{GTP}}\) with membranes. Our recent observations do not support this model. First, we found that nonmyristoylated ARF\(_{\mathrm{GTP},\mathrm{S}}\) strongly binds to phospholipid vesicles (14), indicating that a protein-lipid interaction must also be involved in the association of ARF\(_{\mathrm{GTP}}\) with membranes rather than just a simple lipid-lipid interaction. Second, by comparing the properties of nonmyristoylated ARF1 (myr-ARF1) to those of myristoylated ARF1 (myr-rARF1) both produced in Escherichia coli, we found that myr-ARF\(_{\mathrm{GDP}}\) partially binds to phospholipid membranes while rARF\(_{\mathrm{GDP}}\) is totally water soluble (15). We therefore proposed that myr-ARF\(_{\mathrm{GDP}}\) is loosely attached to membranes by the myristate chain, whereas myr-ARF\(_{\mathrm{GTP}}\) is strongly bound to phospholipids via both the fatty acid and hydrophobic or electrostatic interactions between a protein domain and the membrane bilayer. In other words, we propose that nucleotide exchange increases the affinity of ARF for phospholipids through a "protein switch" rather than a "myristoyl switch."

Moreover, we have reported that interaction of myr-ARF\(_{\mathrm{GDP}}\) with phospholipids allows a significant spontaneous nucleotide exchange at physiological (mM) levels of Mg\(^{2+}\) conditions under which GDP release from rARF\(_{\mathrm{GDP}}\) is undetectable (15). Here, we report that a soluble ARF-specific guanine nucleotide exchange activity can be obtained from bovine retinas and that stimulation of GDP release from ARF1 by this activity also requires myristoylation of ARF1 and the presence of phospholipids. In addition, we present evidence that the myristate is not necessary for the protein-protein interaction between ARF and the exchange factor but simply facilitates the binding of ARF\(_{\mathrm{GDP}}\) to the phospholipids, where it can interact with the exchange factor.

**EXPERIMENTAL PROCEDURES**

Materials—Nucleotides were purchased from Boehringer Mannheim. Azolecin, brefeldin A, trypsin (type XIII), and lima bean trypsin inhibitor (type II-1) were obtained from Sigma. \(^{35}\)S]GTP\(_7\)S was from...
Expression and Purification of Nonmyristoylated and Myristoylated ARF1—Bovine ARF1 was expressed in E. coli and purified by a single QAE-Sepharose chromatography as described previously (14). myr-ARF1 was prepared from bacteria coexpressing yeast N-myristoyltransferase (16) by a different procedure including a precipitation at 35% saturation of ammonium sulfate and sequential chromatography on DEAE-Sepharose and MonoS columns (15). As judged by Coomassie staining after SDS-PAGE, both ARF preparations were >95% pure, and the contamination of myr-ARF1 by the nonmyristoylated species was <20%. Both rARF1 and myr-rARF1 were obtained in a GDP-bound form, as demonstrated by nucleotide analysis.

Binding of ARF Proteins to Retinoid Outer Segment (ROS) Membranes—Bovine retinal ROS were prepared under dim red light as described by Kühn (17) and stored as pellets at −80°C. After thawing, a pellet of ROS was resuspended under dim red light in isotonic buffer (20 mM Tris/HCl, pH 7.5, 120 mM NaCl, 1 mM MgCl₂) at a concentration of 40 μM rhodopsin and homogenized in a Teflon glass tissue grinder, yielding an homogeneous suspension of permeabilized ROS fragments. Half of this suspension was used as “total ROS.” The other half was diluted to an homogeneous suspension of permeabilized ROS fragments. Half of the same buffer without sucrose, and sedimented at 6,000 g for 20 min. The floating ROS were harvested, diluted 2.5-fold in the same buffer and centrifuged at 20,000 g for 60 min. The resulting supernatant was further clarified by centrifugation at 80,000 × g for 90 min. The resulting supernatant was saved, diluted 3-fold in an isotonic buffer (20 mM Tris/HCl pH 7.5, 120 mM NaCl, 1 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol) supplemented with 45% (w/v) sucrose and centrifuged at 200,000 × g as described for the preparation of bovine ROS (18). The floating ROS were harvested, diluted 2.5-fold in the same buffer without sucrose, and sedimented at 6,000 × g for 20 min. The resulting supernatant was saved, diluted 3-fold in an isotonic buffer (20 mM Tris/HCl pH 7.5, 120 mM NaCl, 1 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol) to further dilute the residual sucrose, and centrifuged at 14,000 × g for 60 min. The pellet was discarded, and the supernatant was precipitated at 40% saturation of ammonium sulfate and centrifuged again at 14,000 × g for 60 min. The pellet was resuspended in the same isotonic buffer, dialyzed against this buffer, and clarified by centrifugation at 150,000 × g for 30 min. The final supernatant (2–3 mg of protein/ml) was divided into small aliquots and stored at −70°C.

Preparation of Retinal Isosmotic Extract (RIE)—In method I, thawed pellets of ROS were resuspended at 25 μM rhodopsin in an isotonic buffer supplemented with protease and phosphatase inhibitors (20 mM Tris/HCl, pH 7.5, 120 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM iodoacetamide, 2 μM α-protinin, 1 μM pepstatin, 1 μM leupeptin, and 3 mM β-glycerophosphate). The suspension was centrifuged at 8,000 × g for 90 min. The resulting supernatant was further clarified by centrifugation at 400,000 × g for 20 min and concentrated 25–30-fold on a Centricon-10 (Amicon), to a final protein concentration of ~3 mg/ml. This preparation was kept at 4°C. In method II, bovine retinas were shaken in a buffer (200 mM Hepes, pH 7.4, 120 mM KC1, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol) supplemented with 45% (w/v) sucrose and centrifuged at 20,000 × g as described for the preparation of bovine ROS (18). The floating ROS were harvested, diluted 2.5-fold in the same buffer without sucrose, and sedimented at 6,000 × g for 20 min. The resulting supernatant was saved, diluted 3-fold in an isotonic buffer (20 mM Tris/HCl pH 7.5, 120 mM NaCl, 1 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol) to further dilute the residual sucrose, and centrifuged at 14,000 × g for 90 min. The pellet was discarded, and the supernatant was precipitated at 40% saturation of ammonium sulfate and centrifuged again at 14,000 × g for 60 min. The pellet was resuspended in the same isotonic buffer, dialedyzed against this buffer, and clarified by centrifugation at 150,000 × g for 30 min. The final supernatant (2–3 mg of protein/ml) was divided into small aliquots and stored at −70°C.

Trypsin Treatment of RIE—RIE, prepared by method I above, was incubated with 100 μg/ml trypsin for 15 min at 37°C, followed by 1 mg/ml lima bean trypsin inhibitor for 10 min at 37°C. Mock treatment was performed by adding trypsin and trypsin inhibitor to the same concentrations from a mixture of the two that had been incubated for 10 min at 37°C and then incubating the RIE sample for 25 min at 37°C.

Preparation of Phospholipid Vesicles—Large unilamellar vesicles of azoectin (soybean lipids) were prepared as described (14).

GTPγS Binding Assay—rARF1 GDP or myr-rARF1 GDP (1–2 μM) was incubated at 37°C in 50 mM Hepes-NaOH, pH 7.5, 1 mM dithiothreitol, 10 μM [35S]GTPγS (1000 cpm/pmol), 1 μM ATP (to prevent the degradation of GTPγS by RIE), and 1.5 mg/ml phospholipid 3:S with RIE at a final concentration of 0.6 mg of protein/ml or an equal volume of the corresponding isotonic buffer, and MgCl₂ to a final concentration of 1 mM free Mg²⁺. Samples of 50 μl were diluted into 2 ml of ice-cold 200 mM Hepes, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, and filtered on 25-nm BA 85 nitrocellulose filters (Schleicher & Schuell). Filters were washed twice with 2 ml of the same buffer, dried, and counted.

Results

GTPγS-dependent Binding of myr-rARF1 to ROS Membranes Is Enhanced by a Retinal Soluble Factor—Our recent studies of the interaction between ARF1 and transducin subunits on retinal ROS membranes (19) led us to examine the binding of myr-rARF1 to ROS membranes in the presence of an excess of GDP or GTPγS. Fig. 1A shows that the binding of myr-rARF1 to ROS membranes was enhanced in the presence of GTPγS, as expected if some myr-rARF1GDP was formed during the incubation and if the GTPγS-bound form of myr-rARF1 interacted more strongly with ROS membranes than the GDP-bound form, as was observed with artificial phospholipid vesicles (15). Surprisingly, however, the GTPγS-dependent binding of myr-rARF1 to ROS membranes was found to be greater when a suspension of freeze-thawed ROS fragments...
Myristoylation Is Required for Activation of ARF1

Addition of the fungal metabolite brefeldin A (BFA) did not affect the spontaneous phospholipid-dependent GTP-γ-S binding to myr-ARF1 but totally abolished the RIE-catalyzed exchange, with half-maximal inhibition at 2 μM BFA (Fig. 3A). BFA is known to inhibit a wide variety of membrane traffic pathways (20) and has been reported to inhibit an ARF-specific guanine nucleotide exchange activity present in Golgi membranes (7–9) or in brain cytosol (10). The exact target of BFA is not yet known. It was proposed to be not the exchange factor itself but rather an associated protein because the sensitivity to BFA of the soluble exchange activity from bovine brain was lost after partial purification (11). The exact mechanism notwithstanding, the complete inhibition by BFA strongly suggests that the retinal extract contains an ARF-specific guanine nucleotide exchange factor. Moreover, this factor is protease sensitive, since trypsin treatment of RIE totally abolished its stimulatory effect on the activation of myr-ARF1 (Fig. 3B). Trypsin sensitivity was also demonstrated for the Golgi membrane-bound exchange activity (7–9). This trypsin control is important in light of the recent report (21) that acid phospholipids such as phosphatidylinositol 4,5-bisphosphate can greatly increase the rate of GDP dissociation from ARF1. In fact, two additional observations further argue against a possible role for acid phospholipids in our RIE effects: (i) the exchange activity remained soluble after a centrifugation at 400,000 × g, which excludes the presence in RIE of membrane vesicles, and (ii) inclusion of 20% (w/w) phosphatidylinositol 4,5-bisphosphate in azolectin vesicles had only a marginal (2-fold) stimulatory effect on the GDP dissociation rate from myr-ARF1 at 0.5 μM levels of Mg(2+) and no effect at all at mM levels of Mg(2+) (data not shown), consistent with the prediction that under the latter conditions most of the negative charges of phosphatidylinositol 4,5-bisphosphate should be masked (22). Thus, the most straightforward interpretation for our data is that a soluble protein is responsible for the RIE-catalyzed guanine nucleotide exchange on myr-ARF1.

("total ROS") was used rather than thoroughly washed ROS membranes (Fig. 1A, lanes 3 and 4). In contrast, the binding of myr-ARF1 to ROS membrane did not decrease upon washing of the membranes (Fig. 1A, lanes 1 and 2). Under the same conditions (1 mM Mg(2+)), nonmyristoylated ARF1 did not bind significantly to ROS membranes in the presence of either GDP or GTP-γ-S, whether the membranes were washed or not (Fig. 1A, lanes 5–8), suggesting that no nucleotide exchange occurred on ARF1 in either condition.

It was possible to restore a strong binding of myr-ARF1 to washed ROS membranes in the presence of GTP-γ-S by addition of the soluble fraction resulting from the first isotonic wash (Fig. 1B, lanes 1–3), while reconstitution with the subsequent hypotonic washes did not enhance myr-ARF1 binding (not shown). The simplest interpretation of these data is that washing of ROS membranes eliminates a soluble factor required for optimal GTP-γ-S-dependent binding of myr-ARF1 to ROS membranes. This factor is mostly recovered in the first isotonic wash, referred to as RIE.

Retinal Isotonic Extract Contains a Guanine Nucleotide Exchange Protein for myr-ARF1—Since an increased GTP-γ-S-dependent binding of myr-ARF1 to ROS membranes is probably caused by an accelerated GDP-to-GTP-γ-S exchange, we directly investigated the effect of RIE on the time course of [35S]GTP-γ-S binding to ARF1 and myr-ARF1 in the presence of phospholipid vesicles and at 1 mM Mg(2+) (Fig. 2). As previously reported (15), myr-ARF1 slowly took up GTP-γ-S under these conditions, with a t1/2 ~ 30 min, but the exchange was markedly accelerated (~5-fold) in the presence of RIE, which by itself bound only a small amount of nucleotide (Fig. 2A). In contrast, the retinal extract had no stimulatory effect on the binding of GTP-γ-S to nonmyristoylated ARF1 (Fig. 2B). In fact, the binding of GTP-γ-S to ARF1 was very low and was even not additive with the binding to RIE when the two components were incubated together, which indicates that no significant nucleotide exchange occurred on ARF1, whether RIE was present or not. Thus, the retinal extract appears to accelerate the nucleotide exchange selectively on the myristoylated protein.
Phospholipids and Myristoylation of ARF Are Both Required for the Interaction between ARF<sub>GDP</sub> and the Retinal Nucleotide Exchange Factor—The finding that the retinal nucleotide exchange factor is soluble while myr-arf<sub>1GDP</sub> is loosely attached to membranes (15) raises the question as to whether the interaction between the two proteins occurs in solution or on the membrane. This question cannot be answered by measuring GDP-to-[<sup>35</sup>S]GTP<sub>S</sub> exchange because phospholipids are absolutely necessary to stabilize the GTP<sub>S</sub>-bound form at mM Mg<sup>2+</sup> levels (15). However, it is possible to measure GDP dissociation via the [<sup>3</sup>H]GDP-to-GTP exchange, a reaction that can be monitored with or without phospholipids. We therefore investigated [<sup>3</sup>H]GDP dissociation from rARF1 and myr-rARF<sub>1</sub> in a medium containing a large excess of unlabeled GDP in the presence or absence of retinal extract (Fig. 4).

In the presence of phospholipids, very similar results were obtained for [<sup>3</sup>H]GDP dissociation (Fig. 4A) and [<sup>35</sup>S]GTP<sub>S</sub> binding (Fig. 2). No dissociation of GDP could be detected from rARF1 with or without RIE, whereas the dissociation rate from myr-rARF<sub>1</sub> was increased 5-fold by the retinal exchange protein.

In the absence of phospholipids (Fig. 4B), there was still no measurable dissociation of [<sup>3</sup>H]GDP from unmyristoylated ARF<sub>1</sub> with or without RIE, but most importantly, there was also no acceleration of GDP release from myr-rARF<sub>1</sub> in the presence of RIE. In fact, the rate of [<sup>3</sup>H]GDP dissociation was even decreased (by 2-fold) by addition of the retinal extract, but the same effect was obtained with bovine serum albumin, which points to a nonspecific stabilizing effect of RIE proteins. Moreover, addition of BFA to the release medium did not affect the inhibitory effect of RIE in Fig. 4B, while it totally abolished its stimulatory effect in Fig. 4A (not shown). Altogether, these data indicate that the retinal exchange factor can recognize ARF<sub>GDP</sub> only if ARF is myristoylated and if phospholipid vesicles are present. In other words, the catalyzed exchange reaction occurs on the membrane and not in solution.

The Retinal Nucleotide Exchange Factor Promotes GTP<sub>S</sub> Release from Both Myristoylated and Nonmyristoylated Membrane-bound ARF<sub>GTP,S</sub>—Even though the physiological role of a guanine nucleotide exchange factor is to promote release of bound GDP and replacement with GTP, it has been reported for several Ras-specific exchange factors such as the yeast SCD25 (24) or CDC25 (25) gene products and the mammalian Sos1 gene product (26) that the release of GTP is also significantly stimulated by the exchange factor in vitro. This prompted us to examine the effect of RIE on [<sup>35</sup>S]GTP<sub>S</sub> release from both myr-rARF<sub>1</sub> and rARF<sub>1</sub> in an attempt to determine whether once membrane-bound (since both myristoylated and nonmyristoylated ARF<sub>GTP,S</sub> bind to phospholipids) the two proteins are still recognized differently by the exchange factor.

In the experiment described in Fig. 5, myr-rARF<sub>1</sub> and rARF<sub>1</sub> were preloaded with [<sup>35</sup>S]GTP<sub>S</sub> in the presence of phospholipids, and a large excess of unlabeled GTP<sub>S</sub> was added, at 1 mM Mg<sup>2+</sup>, to initiate the release assay. In the absence of RIE, spontaneous [<sup>35</sup>S]GTP<sub>S</sub> dissociation was very slow for myr-rARF<sub>1</sub> (Fig. 5A) but notably faster for rARF<sub>1</sub> (Fig. 5B) at the same concentration of phospholipid vesicles. This difference
reflects the different affinity of the two proteins for phospholipids. Indeed, increasing the concentration of phospholipids 3-fold reduced the GTP

$\gamma$S dissociation rate from rARF1 by 2-fold and also increased (up to 80% at equilibrium) the fraction of rARF1 bound to $^{35}$SGTP-$\gamma$S in the preloading step (data not shown). This is consistent with the view that phospholipids are absolutely required for stabilizing ARF$_{GTP,\gamma}$S, whether myristoylated or not, but with a different concentration dependence for the two proteins because the myristate increases the affinity of ARF$_{GTP,\gamma}$S for lipids (by at least 10-fold, as judged by sedimentation experiments, not shown).

Addition of RIE caused a marked acceleration of $^{35}$SGTP-$\gamma$S release from both myr-rARF1 and rARF1 (Fig. 5, A and B). This indicates that (i) like Ras-specific exchange factors mentioned above, RIE can also recognize the GTP-bound form of its target protein and catalyze a GTP release, albeit to a much lesser extent than the GDP release (compare the dissociation rates of GDP and GTP-$\gamma$S from myr-rARF1 in the presence of RIE in Figs. 4A and 5A), and (ii) the retinal exchange factor can recognize nonmyristoylated about as well as myristoylated membrane-bound ARF$_{GTP,\gamma}$S. Therefore, this result strongly suggests that in RIE-catalyzed GDP release (Fig. 4), the myristate was required for binding ARF$_{GDP}$ to the phospholipids rather than directly involved in the interaction of ARF$_{GDP}$ with the exchange factor.

It is noteworthy that RIE-catalyzed GTP-$\gamma$S release from both ARFs was only partly inhibited by BFA, even at 300 $\mu$M (Fig. 5, A and B), whether BFA was preincubated with RIE or with ARF and phospholipids. The significance of this result is unclear since the exact mechanism of action of BFA is not known. The fact that the spontaneous release of GTP-$\gamma$S was unchanged in the presence of BFA confirms that BFA does not affect the interaction of ARF$_{GTP,\gamma}$S with phospholipids. It remains possible that this lipophilic drug somehow hinders the attachment of the exchange factor to the lipids, the inhibition being less pronounced when the target protein (ARF$_{GTP,\gamma}$S) is itself solidly membrane-bound.

Importantly, very similar results were obtained when GTP-$\gamma$S-to-$^{35}$SGTP-$\gamma$S exchange was measured (Fig. 6) instead of $^{35}$SGTP-$\gamma$S-to-GTP-$\gamma$S exchange (Fig. 5). Again, the spontaneous exchange was faster for nonmyristoylated ARF1 (Fig. 6B), RIE markedly accelerated the exchange on both ARFs, and BFA caused a partial inhibition (Fig. 6, A and B). Together, the results of Figs. 5 and 6 unambiguously demonstrate that RIE promotes GTP-$\gamma$S-GTP-$\gamma$S exchange on both ARFs, but it should be noted that measure of both GTP-$\gamma$S release (Fig. 5) and GTP-$\gamma$S loading (Fig. 6) was necessary to eliminate all possible artifacts. Indeed, as GTP-$\gamma$S release is tightly correlated with the dissociation of ARF$_{GTP,\gamma}$S from phospholipids, RIE-stimulated GTP-$\gamma$S release in Fig. 5 could have been due in part to a displacement of ARF$_{GTP,\gamma}$S from the lipids, possibly by proteins of RIE unrelated to the exchange activity. On the other hand, RIE-catalyzed GTP-$\gamma$S binding in Fig. 6 could have been due to some residual ARF$_{GDP}$, at least in the case of myr-rARF1. The symmetry of the data obtained by the two methods definitely rules out all of these possibilities.

**DISCUSSION**

In this study, we have analyzed the role of N-myristoylation of bovine ARF1 in its activation in vitro by a soluble, brefeldin A-sensitive guanine-nucleotide exchange activity obtained from bovine retinas. We demonstrate that stimulation by the retinal extract of GDP release from ARF1 strictly requires myristoylation of ARF1 and the presence of phospholipids. The retinal extract also stimulates to a lesser extent the release of GTP-$\gamma$S from ARF$_{GTP,\gamma}$S in the presence of phospholipids but, in that case, myristoylation of ARF is not required.

The simplest interpretation of these data is that the retinal exchange factor acts only on membrane-bound ARF. Its interaction with ARF$_{GDP}$ requires myristoylation of ARF because only myr-ARF$_{GDP}$ can significantly bind to phospholipids. In contrast, its interaction with ARF$_{GTP,\gamma}$S does not require myristoylation of ARF because both myristoylated and nonmyristoylated ARF$_{GTP,\gamma}$S strongly bind to phospholipids. In other words, the myristate is not directly involved in the interaction of ARF with the exchange factor; it is simply required to bring ARF$_{GDP}$ to the membrane where the catalyzed exchange reaction occurs. A similar role of the myristate in facilitating membrane binding has been ascribed to several other myristoylated proteins (27).

How could one explain that an apparently soluble exchange factor interacts only with membrane-bound ARF? At least two possibilities can be entertained. First, it can be questioned whether the exchange protein is totally cytosolic in the cell. Indeed, it is rather puzzling that in rat liver Golgi membranes the BFA-sensitive nucleotide exchange activity was described as tightly bound to membranes, being resistant to salt extraction but not to alkaline extraction (8). While it is of course possible that the Golgi-bound exchange activity is completely different from the soluble enzyme present in our retinal extract, it may well be also that the two enzymes are related peripheral proteins more or less tightly bound to membranes depending on the tissue. Thus, the retinal exchange factor might in fact loosely bind to membranes like myr-ARF$_{GDP}$, possibly also through a lipid modification or via a positively charged structure such as a pleckstrin homology domain frequently found in small G protein-specific guanine nucleotide exchange factors (28) and thought to be involved in interactions with membrane phospholipids (29). Accordingly, the probability of interaction between myr-rARF$_{GDP}$ and the exchange factor would be enhanced on the membrane surface as a result of an increased local concentration of the two proteins. A similar mechanism has been recently proposed to explain the lipid-dependent interactions of transducin $\alpha$ and $\beta$ subunits (30).

Alternatively, another possibility is that the insertion of the myristate chain into the lipid bilayer might induce a conformational change of the ARF protein, which would properly expose the interaction domain with the exchange factor. Indeed, sup-
port for a membrane-dependent conformational change of myr-
ARFGDP comes from our observation that the rate of GDP
release is increased by phospholipids (15). The amphiphatic
amino-terminal α-helix most likely participates in this confor-
mational change. This helix has been shown to be held by
hydrophobic forces in a cleft of the GDP-bound unmyristoylated
form of ARF1 (31), but its fate upon binding of the myristoyl
group to the membrane is not known. It seems reasonable to
predict that the helix could be displaced out of the cleft and
come in contact with the phospholipid bilayer. The interaction
with the exchange factor could somehow accentuate the confor-
mation and further open the nucleotide binding site. These two possible mechanisms are in fact not exclusive. Both
an increased local concentration and a correct orientation of
myr-ARFGDP and the exchange enzyme might well facilitate
their interaction.

Our finding that only myristoylated ARF can be activated (by
GDP-to-GTP exchange) by the retinal exchange factor contra-
dicts the conclusions of two previous studies on Golgi mem-
branes (9, 13) but provides a reasonable explanation for the
observation that myristoylation is required for many ARF
activities (32–34).

It should be stressed that only the GDP-to-GTP exchange
has a functional meaning. Our observation that in vitro release of
GTP−γS was stimulated by the retinal extract provides in-
sight into the role of myristoylation but is of questionable
physiological relevance. It is very likely that in vivo ARF GTP
will not remain bound to its exchange factor because of the
presence of effectors with higher affinity.

We have observed that breflidin A totally inhibits the retinal
extract-stimulated GDP release from myr-ARF with an IC50 of
2 μM, whereas it inhibits only partially the stimulated GTP−γS
release. The significance of this result is difficult to assess as
long as the molecular basis for breflidin inhibition is unknown.
The exact target of BFA is still not found, and we cannot
exclude that the nucleotide exchange activity of our retinal
extract involves in fact several proteins. Thus, in addition to
the exchange factor itself, an adaptor protein serving as a
membrane anchor might be required, which could be the recep-
tor for BFA. This would be consistent with the observation that
the sensitivity to BFA is lost during purification of the ex-
change protein (11). Obviously, the exact mechanism underly-
ing ARF activation is far from being understood, but if the
process proves to involve several components, it may help to
first describe the characteristics of a crude preparation before
trying to reconstitute the system with purified elements.

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