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Evidence that the 50-kDa substrate of brefeldin A-dependent ADP-ribosylation binds GTP and is modulated by the G-protein βγ subunit complex

MARIA DI GIROLAMO*, MARIA GIUSEPPINA SILLETTA*, MARIA ANTONIETTA DE MATTEIS†, ANGELA BRACA*, ANTONINO COLANZI†‡, DARIUSZ PAWLAK*§, MARK M. RASENICK¶, ALBERTO LUINI‡, AND DANIELA CORDA*

*Laboratory of Cellular and Molecular Endocrinology, †Unit of Physiopathology of Secretion and ‡Laboratory of Molecular Neurobiology, Istituto di Ricerche Farmacologiche Mario Negri, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Chieti), Italy; and ¶Department of Physiology and Biophysics, University of Illinois, College of Medicine, Chicago, IL 60612-7342

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ABSTRACT **Brefeldin A, a fungal metabolite that inhibits membrane transport, induces the mono(ADP-ribosyl)ation of two cytosolic proteins of 38 and 50 kDa as judged by SDS/PAGE. The 38-kDa substrate has been previously identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We report that the 50-kDa BFA-induced ADP-ribosylated substrate (BARS-50) has native forms of 170 and 130 kDa, as determined by gel filtration of rat brain cytosol, indicating that BARS-50 might exist as a multimeric complex. BARS-50 can bind GTP, as indicated by blot-overlay studies with [α-^{32}P]GTP and by photoaffinity labeling with guanosine 5′-[γ-^{32}P][β,γ-(4-azidoanilido)]triphosphate. Moreover, ADP-ribosylation of BARS-50 was completely inhibited by the βγ subunit complex of G proteins, while the ADP-ribosylation of GAPDH was unmodified, indicating that this effect was due to an interaction of the βγ complex with BARS-50, rather than with the ADP-ribosylating enzyme. Two-dimensional gel electrophoresis and immunoblot analysis shows that BARS-50 is a group of closely related proteins that appear to be different from all the known GTP-binding proteins.**

The fungal metabolite brefeldin A (BFA) inhibits protein secretion and has dramatic effects on the organization of the Golgi complex (for review, see ref. 1). An early event in the action of BFA is the dissociation of a small GTP-binding protein, known as ADP-ribosylation factor (ARF), and coatomer from the Golgi membrane. This effect is at least in part mediated by the BFA-dependent inhibition of an ARF-specific, membrane-bound GTP/GDP exchange factor (2). The effect of BFA on the dissociation of coatomer from the Golgi membrane can be prevented by pretreatment with the nonhydrolyzable GTP analogue guanosine 5′-[γ-thio]triphosphate (GTP[γS]) or AlF_4^- (3, 4). This and other lines of evidence (5–9) indicate that not only small GTP-binding proteins, such as ARF, but also heterotrimeric GTP-binding proteins (G proteins) are involved in coat dynamics and vesicular transport (3–9).

Recently, we have shown (10, 11) that BFA is also able to activate an endogenous mono(ADP-ribosyl)transferase that specifically modifies the 38- and 50-kDa substrates on an amino acid residue different from those (arginine, cysteine, asparagine, and diphthamide) used by all the known ADP-ribosyltransferases (12). BFA activates this enzyme by binding to a site(s) with a ligand selectivity identical to that shown for the BFA-dependent inhibition of ARF binding (10, 11). From this and other lines of evidence (13) the ADP-ribosylation reaction appears to play a role in the *in vivo* action of BFA and in membrane traffic.

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The 38-kDa substrate is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (10, 11). Only 1–3% of GAPDH is modified by the BFA-dependent ADP-ribosyltransferase, suggesting either that GAPDH enzyme is not a functionally relevant substrate or that the ADP-ribosylated protein acquires new properties. Interestingly, in CHO cells a mutation in GAPDH causes a decrease in endocytic recycling associated with unusual tubular extensions emanating from late endocytic compartments (A. R. Robbins, personal communication). The 50-kDa substrate, BARS-50, instead is extensively ADP-ribosylated by the BFA-dependent ADP-ribosyltransferase (see below).

Here we show that BARS-50 is a group of closely related proteins that exist in mostly cytosolic complexes of 170 and 130 kDa, bind GTP, and interact with the G-protein βγ subunit complex (Gβγ) but are different from all other known G-protein α subunits. It is suggested that BARS-50 is one of the GTP-binding proteins involved in membrane transport.

MATERIALS AND METHODS

BFA, phenylmethylsulfonyl fluoride, leupeptin, GTP, and ATP were from Sigma. Tissue culture materials were from GIBCO. [^{32}P]NAD was from DuPont/NEN. Guanosine 5′-[γ-^{32}P][β,γ-(4-azidoanilido)]triphosphate ([^{32}P]AAGTP) was synthesized (14). BFA analogues B36 and B5, bovine brain Gβγ, and pertussis toxin were kindly provided by J. Donaldson (National Institutes of Health, Bethesda, MD), P. Gierschik (University of Ulm, Germany), and R. Rappuoli (Istituto Ricerche Immunobiologiche, Siena, Italy), respectively.

Postnuclear Supernatant (PNS) Preparation. Confluent Fischer rat thyroid (FRTL5) cells (15) were detached in Hanks' balanced salts solution (without Ca^{2+} and Mg^{2+}) plus 5 mM EGTA, lysed (10^8 cells per ml) by sonication in 5 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/1 mM EGTA/1 mM phenylmethylsulfonyl fluoride/0.5 mM *o*-phenanthroline/1 μM pepstatin with leupeptin at 10 μg/ml, and centrifuged at 600 × *g* for 15 min at 4°C. The PNS was rapidly frozen in aliquots. Cytosol and membranes were separated from PNS at 100,000 × *g* for 90 min at 4°C (10, 11). Rat brain cytosol and membranes were prepared as described (11). Protein concentration was determined by a dye-based assay (Bio-Rad).

ADP-ribosylation Assay. This assay was performed as described (10, 11). Samples of 50 μg of PNS or 50 μg of cytosolic proteins and 10 μg of membrane proteins were analyzed by

Abbreviations: AAGTP, guanosine 5′-[β,γ-(4-azidoanilido)]triphosphate; ARF, ADP-ribosylation factor; BFA, brefeldin A; Gβγ, G-protein βγ subunit complex; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDP[βS], guanosine 5′-[β-thio]diphosphate; GTP[γS], guanosine 5′-[γ-thio]triphosphate; IEF, isoelectric focusing; PNS, postnuclear supernatant; 2D, two-dimensional.

§Present address: Department of Pharmacodynamics, Medical Academy 2C Mickiewicza Strasse 15-230, Bialystok, Poland.

SDS/PAGE (8% acrylamide and 4 M urea, unless otherwise specified) and autoradiography. Two-dimensional (2D) isoelectric focusing (IEF)–SDS/PAGE followed Bravo's procedure (16). Pertussis and cholera toxin-catalyzed ADP-ribosylations were carried out as described (17). ADP-ribosylation was quantified by Instantimager (Packard).

GTP Overlay. Modifications of a described procedure (18) were a 2-hr incubation with [α-^{32}P]GTP and up to six 10-min washes of the nitrocellulose filters. This resulted in a pronounced labeling of small GTP-binding proteins in all cases and in a weak but detectable labeling of G proteins in 50% of total experiments. The weakness and variability of this labeling might be due to the inability of these proteins to partially refold on nitrocellulose (18).

Other Methods. Snake venom phosphodiesterase digestion (19), immunoblot analysis (17), and photoaffinity labeling (14) were performed as reported.

RESULTS

BFA-Dependent Mono(ADP-ribosyl)ation of a 50-kDa protein(s) in FRTL5 Cell Extracts. BFA, in the presence of [^{32}P]NAD, stimulated a dose- and time-dependent ADP-ribosylation of two cytosolic proteins of 38 kDa and 46–50 kDa (Fig. 1; refs. 10 and 11). The apparent size of the latter (BARS-50) in SDS/polyacrylamide gels was 46 or 50 kDa in the absence or presence of urea in the gel, respectively. The labeled protein (occasionally visible as a tight doublet after SDS/PAGE in the presence of urea; Fig. 1*A*) became detectable by 15 min, with maximal labeling by 2 hr (Fig. 2). The lowest effective concentration of BFA was 2 μg/ml and the BFA EC_{50} was 15 μg/ml, as previously reported for GAPDH (10, 11). In most experiments BFA stimulated the incorporation of radioactivity from [^{32}P]NAD into two other proteins of 45 and 41 kDa (as evaluated in urea-containing gels, p45 and p41; see Fig. 1*B*). The 45- and 41-kDa bands were more evident when the cell extract was prepared in the absence of protease inhibitors (Fig. 1*B*) or when the ADP-ribosylated proteins underwent long purification procedures (or freezing and thawing; Fig. 1*C*). The appearance of p45 was concomitant with, and roughly proportional to, a decrease in intensity of the labeled BARS-50 band (Fig. 1*C*). These observations, together with the fact (see below) that BARS-50 and p45 exhibited marked and very similar migration shifts in SDS/PAGE (depending on the presence of urea) and had identical pI values, suggest that p45 and p41 are degradation products of BARS-50.

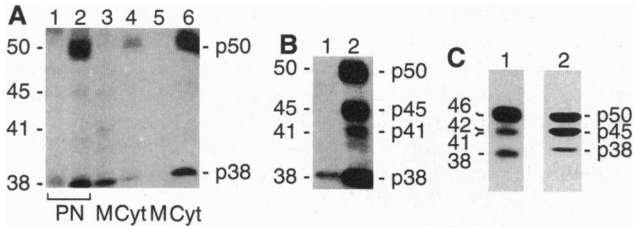

FIG. 1. BFA-induced ADP-ribosylation of GAPDH and BARS-50 in FRTL5 cells. (*A*) PNS (lanes 1 and 2), membranes (lane 3), and cytosol (lane 4) were ADP-ribosylated with [^{32}P]NAD in the absence (lane 1) or presence (lanes 2–4) of BFA. Membranes (lane 5) and cytosol (lane 6) obtained by centrifugation of PNS after the ADP-ribosylation reaction are also shown. (*B*) PNS prepared without protease inhibitors were ADP-ribosylated in the absence (lane 1) or presence (lane 2) of BFA. (*C*) Cytosol obtained by centrifugation of ADP-ribosylated PNS was precipitated with ammonium sulfate at 40% saturation. The pellet containing labeled BARS-50 was resuspended in 25 mM Tris (pH 7.5) and analyzed by SDS/PAGE and autoradiography, either immediately (lane 1) or after one cycle of freezing and thawing (lane 2). Similar results were obtained in five (*A* and *B*) and three (*C*) experiments performed in duplicate. The molecular masses (kDa) obtained by comparison with standards (Pharmacia) are indicated at left.

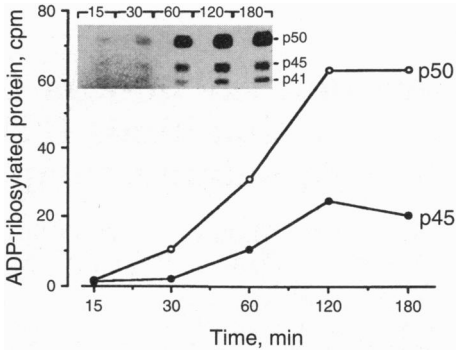

FIG. 2. Time course of BFA-induced ADP-ribosylation of BARS-50 and p45. Membranes (10 μg per sample) and cytosol (50 μg) were incubated with [^{32}P]NAD in the presence of BFA (30 μg/ml) for the indicated times at 37°C. Samples were analyzed by SDS/PAGE and the bands corresponding to p45 and BARS-50 (p50) proteins were quantified by an InstantImager (Packard); autoradiograph (*Inset*) is also shown. Similar results were obtained in five experiments.

Protein labeling in the presence of [^{32}P]NAD may be due to several reactions, including poly(ADP-ribosyl)ation and non-enzymatic ADP-ribosylation (20). Like GAPDH (10), BARS-50 appears to be mono(ADP-ribosyl)ated in the presence of BFA, since the ^{32}P-labeled protein, when separated by SDS/PAGE, transferred to nitrocellulose, and digested with snake venom phosphodiesterase, produced [^{32}P]5′-AMP (identified by TLC and HPLC; data not shown), a result considered diagnostic of mono(ADP-ribosyl)ation (19). Non-enzymatic mono(ADP-ribosyl)ation by ADP-ribose (21, 22) could be excluded because BARS-50 was not ADP-ribosylated when the BFA-dependent reaction was carried out in the presence of [^{32}P]ADP-ribose instead of [^{32}P]NAD (data not shown; ref. 10).

Several lines of evidence indicate that the ADP-ribosylation of BARS-50 is induced by the same ADP-ribosylating enzyme that is active on GAPDH. Both proteins were ADP-ribosylated in the presence of BFA with a similar time course and dose response (Fig. 2; refs. 10 and 11). The linkage of the [^{32}P]ADP-ribose to BARS-50 was stable to NH_2OH and $HgCl_2$ treatment, as already shown for GAPDH (10, 11). The inactive BFA analogues B36, B5, B2, and B17 were unable to induce ADP-ribosylation of GAPDH and BARS-50, whereas B30 was active on both proteins with the same low potency (data not shown and ref. 11). Finally, the BFA-dependent ADP-ribosyltransferase, solubilized and purified severalfold from rat brain, induced the ADP-ribosylation of both GAPDH and BARS-50 (unpublished observation).

Biochemical Characteristics of BARS-50. BARS-50 (or, more precisely, the form of BARS-50 that can be ADP-ribosylated) is largely cytosolic. A minor fraction of the protein (<5%) is found in the membrane pellet washed with low-salt (20 mM) buffers, indicating that BARS-50 may associate reversibly with membranes. Since the BFA-dependent ADP-ribosyltransferase is instead membrane-associated (10, 11), the ADP-ribosylation of BARS-50 requires the presence of both membrane and cytosol (Fig. 1*A*).

BARS-50 is not abundant. A 200-fold enrichment was necessary to detect a well-resolved band on silver-stained gels, identifiable as ADP-ribosylated BARS-50 (Fig. 3*C*). The same sample was then analyzed on a 2D gel and the ADP-ribosylated spots (visualized by autoradiography; see below) coincided with well-resolved silver-stained spots (data not shown). BARS-50 represents 0.005% of the cytosolic proteins as evaluated by the incorporated [^{32}P]ADP-ribose under saturating conditions (and assuming a 1:1 reaction). Roughly the same concentration was obtained by evaluating the amount of BARS-50 from the silver-stained proteins in one-dimensional

SDS/polyacrylamide gels. This is an indication that a large fraction of BARS-50 is ADP-ribosylated in the presence of BFA. In addition, in 2D gels the ADP-ribosylated and silver-stained spots precisely coincided, suggesting again extensive ADP-ribosylation, since ADP-ribosylated proteins would have noticeably different migration properties.

Upon gel filtration (Superose 12 HR 10/30 size-exclusion column, Pharmacia) the cytosolic ADP-ribosylated BARS-50 protein was reproducibly eluted in two main peaks at ≈170 kDa and ≈130 kDa, with a minor peak at ≈100 kDa (data not shown), suggesting that native BARS-50 exists as a homooligomer or as part of a heterocomplex. A remarkable characteristic of BARS-50 is that its mobility during SDS/PAGE is markedly affected by the presence of urea. The apparent size of the protein was ≈46 kDa in 8% polyacrylamide without urea and 50 kDa with 4 M urea (Fig. 3 *A* and *B*). The mobility of other proteins (see G proteins and GAPDH as an example; Fig. 3) is not, or very slightly, affected by urea. The degradation product p45 showed a urea-dependent shift very similar to that of BARS-50 (42 kDa in gels without urea; see Figs. 1 and 3).

To determine the pI of BARS-50, the ADP-ribosylated protein was subjected to 2D IEF–SDS/PAGE. Autoradiography revealed a complex pattern. The tight doublet at 50 kDa was resolved into a cluster of at least 11 distinguishable spots with pI values from 6.55 to 6.1 (identified by progressive numbers, the most abundant being 3, 4, 8, 9, and 10; Fig. 4*A*). p45 generated a similar cluster (13, 14, and 16 being the most abundant spots) containing at least 7 spots with pI from 6.65 to 6.2 (Fig. 4*A*). p41 was resolved into 2 spots with pI values of 6.5 and 6.35 (Fig. 4*A*). The spatial patterns of BARS-50 and p45 clusters were very similar, further indicating that p45 derives from BARS-50 by loss of a peptide of ≈5 kDa.

BARS-50 Binds GTP and Is Modulated by Gβγ. The apparent molecular mass of BARS-50 (similar to that of the α subunits of G proteins), its ability to be ADP-ribosylated, and the involvement of G proteins in BFA-sensitive steps of vesicular traffic suggested that BARS-50 might possess characteristics of a GTP-binding protein.

Both GTP[γS] and guanosine 5′-[β-thio]diphosphate (GDPβS) at 0.1–2 mM were able to inhibit, although to different extents (20% and 50% inhibition, respectively), the BFA-induced ADP-ribosylation of BARS-50 (Fig. 5*A*), whereas ATP was ineffective (data not shown). These nucleotide effects were similar to those shown for cholera toxin-induced ADP-ribosylation of G_s, which was also decreased to a similar extent by GDP[βS] or GTP[γS] at the above concentrations (data not shown and ref. 23). The effect of guanine nucleotides on ADP-ribosylation appears to be specific for

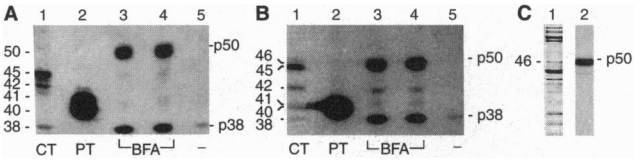

FIG. 3. (*A* and *B*) Urea-dependent shift of BARS-50 and p45. ADP-ribosylation of membrane proteins from FRTL5 cells catalyzed by cholera toxin (CT) (lane 1) and pertussis toxin (PT) (lane 2) and of FRTL5 PNS in the absence (lane 5) or presence (lanes 3 and 4) of BFA was analyzed by SDS/8% PAGE without (*A*) or with (*B*) 4 M urea. Similar results were obtained in 20 experiments. (*C*) Identification of silver-stained, partially purified BARS-50. ADP-ribosylated rat brain cytosol proteins were precipitated with ammonium sulfate at 35% saturation, chromatographed sequentially on hydrophobic (phenyl-Sepharose CL-4B; Pharmacia) and hydroxylapatite (Bio-Gel HT; Bio-Rad) columns, and separated by SDS/PAGE. The ADP-ribosylated BARS-50 detected by autoradiography (lane 2) corresponds to the silver-stained band (lane 1) of the same sample. Similar results were obtained in three experiments.

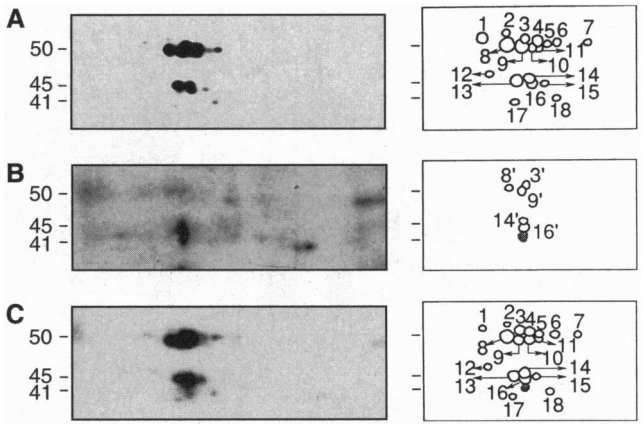

FIG. 4. Analysis of ADP-ribosylated (*A*) and AAGTP-labeled (*B*) BARS-50 by 2D IEF–SDS/PAGE. Membranes (10 μg) and cytosol (50 μg) from FRTL5 cells were incubated with [^{32}P]NAD plus BFA (*A*) or with [^{32}P]AAGTP (*B*), or samples separately labeled as in *A* and *B* were combined and analyzed (*C*). Proteins were analyzed by IEF–SDS/PAGE and autoradiography. Panels on the right are schematic representations of labeled spots, which were resolved by autoradiography at different exposure times to obtain the best resolution of spots labeled with different efficiencies. Namely, spots with heavier labeling were resolved after a short exposure time, and the autoradiography was then repeated for a longer time to resolve weaker labeling (e.g., spots 1 and 12). Each experiment was repeated four times, with similar results.

BARS-50, since ADP-ribosylation of GAPDH was not affected by guanine nucleotides under these conditions (Fig. 5*A*).

Two approaches were then taken to assess directly the binding of guanine nucleotides to BARS-50. First, proteins separated by SDS/PAGE and blotted onto nitrocellulose were overlayed with [α-^{32}P]GTP. A protein precisely comigrating with ADP-ribosylated BARS-50, both in the presence and in the absence of urea, was able to bind GTP (Fig. 5 *B* and *C*). Second, to examine the ability of native BARS-50 to bind GTP, experiments involving photoaffinity labeling with AAGTP were carried out. By one-dimensional SDS/PAGE, an AAGTP-photolabeled protein which comigrated with BARS-50 was resolved (Fig. 5 *D* and *E*). The labeling of this protein was inhibited by GTP[γS] and guanosine 5′-[β,γ-imido]triphosphate, but not by ATP (Fig. 5*E*), suggesting a specific interaction with guanine nucleotides. In 2D gels, the major AAGTP-labeled spots had precisely the same pI and apparent size as some of the major ADP-ribosylated proteins at 50 and 45 kDa. AAGTP labeling (Fig. 4*B*, spots 3′, 8′, 9′, 14′, and 16′) precisely corresponded to the ADP-ribosylated spots (nos. 3, 8, 9, 14, and 16). To confirm the identity of the ADP-ribosylated and AAGTP-labeled proteins, they were analyzed on the same 2D gel (Fig. 4*C*); indeed, the above proteins precisely overlapped. Thus, the AAGTP-labeled proteins are very likely to correspond to some isoforms of BARS-50, p45, and p41 (Fig. 4). The efficiency of AAGTP binding appears to be higher for the degradation products p45 and p41 than for BARS-50; perhaps the proteolytic cut facilitates GTP binding. In some cases, the two 45-kDa AAGTP-labeled proteins appeared to migrate slightly faster (in the second dimension) than the BARS-50 proteolytic fragments. However, the spatial disposition and relative intensity of the GTP labeled spots strongly suggest that they are in fact the BARS-50 degradation products. This discrepancy might be explained by an effect of the presence of bound AAGTP on the process of proteolytic degradation of BARS-50, which would sometimes result in slightly different migration rates of the AAGTP-labeled and ADP-ribosylated fragments. Alternatively, GTP-bound BARS-50 fragment might preserve a degree of folding during the electrophoretic separation and thus

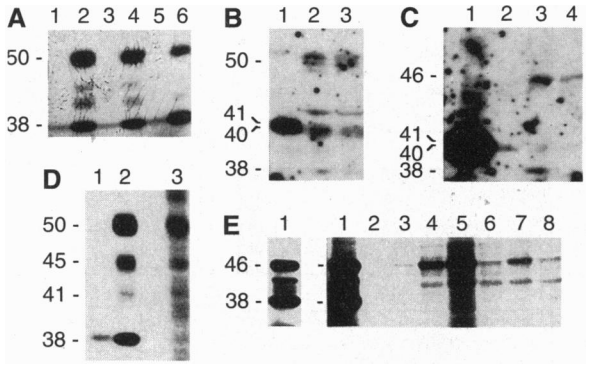

FIG. 5. Binding and effect of guanine nucleotides on BARS-50. (*A*) Inhibitory effect of 500 μM GDP[βS] (lanes 5 and 6) and of 100 μM GTP[γS] (lanes 3 and 4) on BFA-induced ADP-ribosylation of BARS-50. The assay was carried out with FRTL5 PNS in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of BFA. By densitometric analysis (LKB Ultroscan-XL), the ADP-ribosylation of BARS-50 was 4.02 in control, 3.22 in GTP[γS]-treated, and 2.12 in GDP[βS]-treated samples (arbitrary units). The experiment shown is representative of four. (*B* and *C*) Binding of [α-^{32}P]GTP to FRTL5 cytosolic proteins separated by SDS/PAGE in the presence (*B*) or absence (*C*) of urea. [α-^{32}P]GTP-labeled proteins (*B* lane 3 and *C* lane 4), ADP-ribosylated BARS-50 (*B* lane 2 and *C* lane 3), membrane proteins from FRTL5 cells ADP-ribosylated by pertussis toxin (lanes 1), and [α-^{32}P]GTP-labeled FRTL5 membrane proteins (*C* lane 2) were analyzed. The experiment shown is representative of four. (*D*) Photoaffinity labeling of FRTL5 cell cytosol (200 μg of protein per lane) with 100 nM AAGTP (lane 3) and ADP-ribosylation of PNS from FRTL5 cells in the absence (lane 1) or presence (lane 2) of BFA, analyzed in the same gel containing urea. (*E*) Photoaffinity labeling of FRTL5 cytosol (200 μg of protein per lane) with AAGTP at 10 nM (lane 2), 50 nM (lane 3), 100 nM (lanes 4 and 6–8), and 500 nM (lane 5). Samples also included 100 μM GTP[γS] (lane 6), 1 mM ATP (lane 7), and 100 μM guanosine 5′-[β,γ-imido]triphosphate (lane 8). FRTL5 PNS, ADP-ribosylated by BFA is in lanes 1 (same sample at two exposure times). Proteins were then analyzed by SDS/PAGE (without urea) and autoradiography. The experiment shown is representative of four.

migrate slightly differently. In summary, these results show that some isoforms of the native BARS-50 and of the degradation products can bind GTP. This conclusion is further supported by the observation that about 10% of native BARS-50 can be eluted from an agarose-GTP column (Sigma) by 1 mM GTP, but not by 1 mM ATP (data not shown).

BARS-50 did not comigrate with ADP-ribosylated α_s or α_i, and BARS-50 was not a substrate of pertussis or cholera toxin-induced ADP-ribosylation (Fig. 3 *A* and *B*). Specific α_s or α_i antibodies were unable to recognize BARS-50.

A well-known property of G protein α subunits is to bind G$\beta\gamma$. The BFA-induced ADP-ribosylation of BARS-50 was completely inhibited by brain G$\beta\gamma$ in a dose-dependent manner (Fig. 6). To address the question of whether G$\beta\gamma$ interacts with BARS-50, we evaluated the degree of inhibition of the ADP-ribosylation of BARS-50, by varying alternatively the amount of membranes (enzyme) or cytosol (BARS-50) in the assay (Table 1). Increasing the concentration of membranes in the ADP-ribosylation mixture did not affect the extent of inhibition, whereas concentrations of cytosol higher than 100 μg caused a loss of the G$\beta\gamma$-induced inhibition. This suggests that G$\beta\gamma$ interacts with a cytosolic rather than a membrane component and that >100 μg of cytosol is needed to abolish the G$\beta\gamma$-dependent inhibition. At lower cytosol concentrations G$\beta\gamma$ may be in excess compared with the cytosolic component inhibiting its effect. Moreover, G$\beta\gamma$ had no effect on the ADP-ribosylation of GAPDH (Fig. 6*B*), further indicating that G$\beta\gamma$ does not interact with the enzyme but that it binds (either directly or through some associated proteins) to BARS-50.

We could also exclude the possibility that G$\beta\gamma$ could have caused BARS-50 to translocate to the membrane, as reported for the cytosolic kinases (β-adrenergic receptor kinase; ref. 24) since, when BFA-induced ADP-ribosylation was analyzed in PNS in the presence of G$\beta\gamma$, no major change was found in the ratio between soluble and membrane-bound ADP-ribosylated BARS-50 (Fig. 6*B*).

DISCUSSION

In this paper we describe some biochemical features of a protein substrate of BFA-dependent ADP-ribosylation, BARS-50 (10, 11), that might be crucial to the understanding of the protein's cellular function(s).

BARS-50 represents 0.005% of the cytosolic proteins. The numerous clustered ADP-ribosylated spots that appear in 2D gels indicate that BARS-50 is a class of proteins comprising many isoforms or one protein undergoing different and multiple posttranslational modifications.

Both direct and indirect data (binding of GTP and effect of guanine nucleotides on ADP-ribosylation of BARS-50) indicate that BARS-50 is a GTP-binding protein. GTP binding was assessed by overlay with GTP and by photolabeling of the native protein with AAGTP (Fig. 5). The overlay technique has mainly been reported to detect small GTP-binding proteins, including those of the Ras, Rab, and ARF families. Presumably, the small GTP-binding proteins retain folding of the GTP binding site after SDS/PAGE and blotting. Indeed, the GTP labeling in the 20- to 25-kDa region (where small GTP-binding proteins migrate) was the most pronounced in the gel. However, the labeling of BARS-50, albeit lighter, was also detectable, indicating that a fraction of this protein may be able to refold after blotting. That the 50-kDa GTP-labeled protein is in fact BARS-50 is indicated by its electrophoretic properties. Not only did the GTP-labeled and ADP-ribosylated bands precisely comigrate in one-dimensional SDS/PAGE (in itself not a strong proof) but they also exhibited exactly the same 4-kDa shift in the presence of urea (Fig. 5 *B* and *C*). While a urea-induced change of migration properties in SDS/PAGE is a property of several proteins, a large (4-kDa) shift is so rare that the precise coincidence of the two 50-kDa electrophoretic bands strongly suggests that the bands represent the same protein.

The AAGTP photolabeling experiments support this conclusion and show that native BARS-50 can bind GTP, based on the identity of the electrophoretic properties in 2D gels of some GTP-labeled spots with those of some BARS-50 isoforms. A calculation of the number of AAGTP-labeled and ADP-ribosylated protein molecules in the same spot shows that ADP-ribosylation is at least 20-fold more efficient than

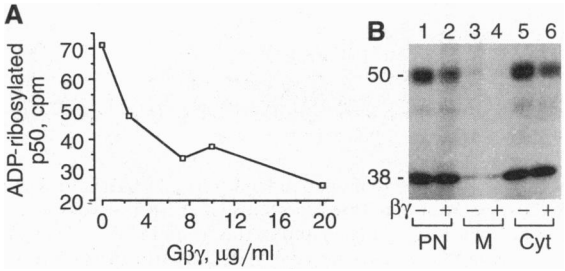

FIG. 6. Inhibitory effect of G$\beta\gamma$ on BFA-induced ADP-ribosylation of BARS-50. (*A*) Dose dependence of the brain G$\beta\gamma$-induced inhibition of BFA-dependent ADP-ribosylation. (*B*) ADP-ribosylated PNS (lanes 1 and 2) and membranes (lanes 3 and 4) and cytosol (lanes 5 and 6) obtained by centrifugation of ADP-ribosylated PNS. ADP-ribosylation was carried out in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of bovine brain G$\beta\gamma$ (10 μg/ml). Samples were analyzed by SDS/PAGE and autoradiography. Similar results were obtained in four (*A* and *B*) separate experiments.

Table 1. Effect of bovine brain Gβγ on BFA-induced ADP-ribosylation of BARS-50

Membranes		Cytosol	
Protein, μg	% inhibition	Protein, μg	% inhibition
5	40 ± 6	25	47 ± 15
8	52 ± 18	50	31 ± 8
25	60 ± 4	75	46 ± 18
50	45 ± 5	100	31 ± 11
100	48 ± 10	150	10 ± 10
200	52 ± 12	250	0

Samples prepared as described (*Materials and Methods*) in the presence of brain Gβγ (10 μg/ml) were analyzed by SDS/PAGE and autoradiography. The ADP-ribosylated lanes corresponding to BARS-50 were quantified by densitometric analysis (LKB Ultroscan-XL) and percent inhibition (mean ± SD) was calculated. Similar results were obtained in four experiments.

photolabeling for BARS-50. This is expected, since the efficiency of AAGTP photoincorporation is about 2.5% for tubulin (25) or Gα (26), and might in part explain why only some of the ADP-ribosylated spots appear to correspond to AAGTP-labeled proteins. Guanine nucleotides not only bind BARS-50 but also partially inhibit its BFA-dependent ADP-ribosylation. It cannot be completely excluded that GTP might act in part by inhibiting the ADP-ribosylating enzyme, but the observation that the labeling of GAPDH is unmodified by guanine nucleotides suggests that it is the binding of GTP to BARS-50 that is responsible for inhibition of BARS-50 ADP-ribosylation. This would be analogous to the well-described effects of guanine nucleotides on the ADP-ribosylation of G protein α subunits by toxins (27).

Gβγ also inhibits the BFA-dependent ADP-ribosylation of BARS-50, but not that of GAPDH. This result again suggests an effect on BARS-50 rather than on the ADP-ribosylating enzyme. A number of proteins have been shown to interact with Gβγ, including β-adrenergic receptor kinase, phospholipase Cβ, and adenylyl cyclase (28). Our observation is in line with the possibility of a degree of structural similarity between BARS-50 and classical α subunits, although BARS-50 was not recognized by Gα-specific antibodies (see *Results*). It is also possible that BARS-50 belongs to a class of proteins that contain the pleckstrin homology domain, which is involved in protein–protein interaction and is able, in particular, to interact with Gβγ (29, 30).

Other proteins with size similar to that of BARS-50 can bind GTP. Among these, elongation factor 1α can be excluded because it is markedly more basic than BARS-50 (31, 32). Tubulin can be excluded because the BFA-dependent ADP-ribosylation of BARS-50 was evident in cytosol deprived of α- and β-tubulin by pretreatment with paclitaxel (formerly taxol) (33). Antibodies raised against γ-tubulin, actin, and centractin did not recognize ADP-ribosylated BARS-50.

We have described a family of proteins, BARS-50, that can be efficiently ADP-ribosylated by an enzyme stimulated by BFA, through a site(s) endowed with the same ligand selectivity as that involved in the inhibition of membrane transport. BARS-50 proteins can bind GTP and, most likely, Gβγ; these events result in inhibition of BARS-50 ADP-ribosylation. In particular, the ability of BARS-50 to interact with Gβγ might be of functional significance in the context of membrane transport. G proteins have been implicated in the formation of coated buds and vesicles (3–9). More specifically, Gβγ can interfere with the activation and binding of ARF to Golgi membranes (4), an early event in vesicle formation. Moreover, ARF can interact directly with Gβγ (34). It can thus be imagined that BARS-50, by interacting with Gβγ, plays a regulatory role in ARF binding and that its state of ADP-ribosylation, which can be controlled by BFA, may alter its regulatory properties. Further, BARS-50 might be involved in regulating the protein matrix that confers on the Golgi apparatus its characteristic shape. The hypothesis of a role of BARS-50 in membrane transport is supported by the recent finding that inhibitors of *in vitro* BARS-50 ADP-ribosylation potently inhibit some of the cellular effects of BFA (13).

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1. Klausner, R. D., Donaldson, J. G. & Lippincott-Schwartz, J. (1992) *J. Cell Biol.* **116,** 1071–1080.
2. Donaldson, J. G., Cassel, D., Kahn, R. A. & Klausner, R. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6408–6412.
3. Donaldson, J. G., Lippincott-Schwartz, J. & Klausner, R. D. (1991) *J. Cell Biol.* **112,** 579–588.
4. Donaldson, J. G., Kahn, R. A., Lippincott-Schwartz, J. & Klausner, R. D. (1991) *Science* **254,** 1197–1199.
5. Pimplikar, S. W. & Simons, K. (1993) *Nature (London)* **362,** 456–458.
6. Ohashi, M. & Huttner, W. B. (1994) *J. Biol. Chem.* **269,** 24897–24905.
7. Leyte, A., Barr, F. A., Kehlenbach, R. H. & Huttner, W. B. (1992) *EMBO J.* **11,** 4795–4804.
8. Colombo, M. I., Mayorga, L. S., Casey, P. J. & Stahl, P. D. (1992) *Science* **255,** 1695–1697.
9. Hansen, S. H. & Casanova, J. E. (1994) *J. Cell Biol.* **126,** 677–687.
10. De Matteis, M. A., Di Girolamo, M., Colanzi, A., Pallas, M., Di Tullio, G., McDonald, L. J., Moss, J., Santini, G., Bannykh, S., Corda, D. & Luini, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1114–1118.
11. Colanzi, A., Di Girolamo, M., Santini, G., Sciulli, G., Santarone, S., Pallas, M., Di Tullio, G., Bannykh, S., Corda, D., De Matteis, M. A. & Luini, A. (1994) in *GTPase-Controlled Molecular Machines*, eds. Corda, D., Hamm, H. & Luini, A. (Ares-Serono, Rome), pp. 197–217.
12. Jacobson, M. K., Loflin, P. T., Nasreen, A.-E., Mingmuang, M., Moss, J. & Jobson, E. L. (1990) *J. Biol. Chem.* **265,** 10825–10828.
13. Santini, G., Sciulli, M. G., Colanzi, A., Mironov, A., Santarone, S., Innamorati, G., Fusella, A., Di Girolamo, M., Corda, D., De Matteis, A. & Luini, A. (1994) *Mol. Biol. Cell* **5,** 242 (abstr.).
14. Rasenick, M. M., Talluri, M. & Dunn, W. J., III (1994) *Methods Enzymol.* **237,** 100–110.
15. Ambesi-Impiombato, F. S., Parks, L. A. M. & Coon, H. G. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3455–3459.
16. Bravo, R. (1984) in *Two-Dimensional Gel Electrophoresis of Proteins*, eds. Celis, J. E. & Bravo, R. (Academic, Orlando, FL), pp. 3–36.
17. Di Girolamo, M., D'Arcangelo, D., Cacciamani, T., Gierschik, P. & Corda, D. (1992) *J. Biol. Chem.* **267,** 17397–17403.
18. Doucet, J.-P. & Tuana, B. S. (1991) *J. Biol. Chem.* **266,** 17613–17620.
19. Kots, A. Y., Skurat, A. V., Sergienko, E. A., Bulargina, T. V. & Severin, E. S. (1992) *FEBS Lett.* **300,** 9–12.
20. Ueda, K. & Hayaishi, O. (1985) *Annu. Rev. Biochem.* **54,** 73–100.
21. Hilz, H., Koch, R., Fanick, W., Klapproth, K. & Adamietz, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3929–3933.
22. Tanaka, Y., Yoshihara, K. & Kamiya, T. (1989) *Biochem. Biophys. Res. Commun.* **163,** 1063–1070.
23. Gill, D. M. & Woolkalis, M. J. (1991) *Methods Enzymol.* **195,** 267–280.
24. Lohse, M. J. (1993) *Biochim. Biophys. Acta* **1179,** 171–188.
25. Rasenick, M. M. & Wang, N. (1988) *J. Neurochem.* **51,** 300–311.
26. Gordon, J. H. & Rasenick, M. M. (1988) *FEBS Lett.* **235,** 201–206.
27. Moss, J. & Vaughan, M. (1988) *Adv. Enzymol.* **61,** 303–379.
28. Clapham, D. E. & Neer, E. J. (1993) *Nature (London)* **365,** 403–406.
29. Gibson, T. J., Hyvönen, M., Birney, E., Musacchio, A. & Saraste, M. (1994) *Trends Biochem. Sci.* **19,** 349–353.
30. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G. & Lefkowitz, R. J. (1994) *J. Biol. Chem.* **269,** 10217–10220.
31. Brands, J. H. G. M., Maassen, J. A., Van Hemert, F. J., Amons, R. & Möller, W. (1986) *Eur. J. Biochem.* **155,** 167–171.
32. Walsh, M. J. & Kuruc, N. (1992) *J. Neurochem.* **59,** 667–678.
33. Vallee, R. B. (1986) *Methods Enzymol.* **134,** 104–115.
34. Colombo, M. I., Inglese, J. C., D'Souza-Shorey, C., Beron, W., Lefkowitz, R. J. & Sthal, P. D. (1994) *Mol. Biol. Cell* **5,** 381 (abstr.).