Molecular Cloning and Functional Characterization of Brefeldin A-ADP-ribosylated Substrate

A NOVEL PROTEIN INVOLVED IN THE MAINTENANCE OF THE GOLGI STRUCTURE*

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Brefeldin A (BFA) is a fungal metabolite that disassembles the Golgi apparatus into tubular networks and causes the dissociation of coatomer proteins from Golgi membranes. We have previously shown that an additional effect of BFA is to stimulate the ADP-ribosylation of two cytosolic proteins of 38 and 50 kDa (brefeldin A-ADP-ribosylated substrate (BARS)) and that this effect greatly facilitates the Golgi-disassembling activity of the toxin. In this study, BARS has been purified from rat brain cytosol and microsequenced, and the BARS cDNA has been cloned. BARS shares homology with two known proteins, C-terminal-binding protein 1 (CtBP1) and CtBP2. It is therefore a third member of the CtBP family. The role of BARS in Golgi disassembly by BFA was verified in permeabilized cells. In the presence of dialyzed cytosol that had been previously depleted of BARS or treated with an anti-BARS antibody, BFA potently disassembled the Golgi. However, in cytosol supplemented with purified BARS, or even in control cytosols containing physiological levels of BARS, the action of BFA on Golgi disassembly was strongly inhibited. These results suggest that BARS exerts a negative control on Golgi tubulation, with important consequences for the structure and function of the Golgi complex.

The Golgi complex, which plays a key role in intracellular trafficking and sorting, is composed of a constellation of stacks of flat cisternae bound together through tubular-reticular connecting zones into an overall ribbon-like shape. There has always been great interest among cell biologists in understanding the molecular mechanisms responsible for Golgi architecture and dynamics. Unfortunately, although significant progress has recently been made by studying the process of disassembly and reassembly of the Golgi complex that occurs during treatments with toxins such as ilimaquinone (1) and brefeldin A (BFA) (2) or during mitosis (3), the present knowledge of these processes is still fragmentary.

The focus of this study is on the molecular factors involved in the Golgi disassembly induced by BFA, a fungal toxin that causes the massive transformation of Golgi stacks into a tubular-reticular network. The effects of BFA have been attributed to at least two mechanisms. One is the release of coat proteins, including the coatomer (a major protein complex involved in coat protein I (COPI)-coated vesicle formation) and the small GTP-binding protein ARF (ADP-ribosylation factor) (4, 5) from Golgi membranes. The second mechanism is the activation of the endogenous ADP-ribosylation of two cytosolic proteins of 38 kDa (glyceraldehyde-3-phosphate dehydrogenase, a multifunctional protein involved in several cellular processes), and 50 kDa (BARS, a protein of unknown function) (6–8). The role of coatomer in preserving the Golgi structure has been attributed to its function as a major membrane scaffold protein (2), but the significance of the ADP-ribosylation of BARS and glyceraldehyde-3-phosphate dehydrogenase is less well understood. In a previous report, we provided evidence that the cytosol contains factors that prevent Golgi disassembly by BFA. Because the activity of such factors appeared to be abolished by ADP-ribosylation, we suggested that the inhibitory components of the cytosol might be identical with the ADP-ribosylation substrates, glyceraldehyde-3-phosphate dehydrogenase and BARS (9).

In this study, we have undertaken the purification and molecular cloning of BARS. We report the primary sequence of BARS and the characterization of the function of this protein in the Golgi disassembly induced by BFA. BARS appears to be involved in controlling the equilibrium between tubular and stacked structures in the Golgi complex.

EXPERIMENTAL PROCEDURES

ADP-ribosylation Assay—Cytosol and membranes were prepared from fresh rat brains (300–400-g male Sprague-Dawley rats) according to Malhotra et al. (10), except that the 60% ammonium sulfate precipitation was omitted. Aliquots were frozen in liquid nitrogen and stored at –80 °C. The ADP-ribosylation assay included 5 mg/ml cytosol (substrate source), 1.2 mg/ml membranes (enzyme source), and 60 μg/ml BFA incubated for 2 h with 250 μM NAD* ([NAD]^*), as described previously (6).

BARS Purification—All purification steps (see Table I) were performed at 4 °C by fast protein liquid chromatography (Amersham Phar-
macia Biotech) starting from ADP-ribosylated cytosol (240 mg) precipitated by 35% ammonium sulfate. The amount of BARS present at each step was evaluated by 10% SDS-PAGE followed by autoradiography (Instant Imager, Packard). The precipitate was applied to a hydrophobic column (Phenyl-Sepharose H.P., Amersham Pharmacia Biotech) followed by a hydroxyapatite column (Bio-Gel HTP, Bio-Rad). The recovered fractions were concentrated (Centriplus Concentrators 10, Amicon), and loaded onto a gel filtration column (Superose 12, Amersham Pharmacia Biotech). The BARS-containing fractions (eluted at an apparent molecular mass of 170 kDa) were concentrated and subjected to 2D isoelectric focusing-SDS-PAGE (11). The radiolabeled spots stained with Coomassie Blue were subjected to in situ trypsin digestion. Peptides were separated by reverse-phase high pressure liquid chromatography and sequenced by the Protein Structure Laboratory, University of California, Davis. The obtained peptide sequences were compared with sequence data banks using the BLAST similarity search algorithm at the National Center for Biotechnology Information site.

Polyclonal Antibodies—Peptides were synthesized as described previously (12). After the collection of preimmune sera, male HVC/CR rabbits were immunized with the BARS synthetic peptide conjugated through the MAP system (Novabiochem) via subcutaneous injections, following a previously described procedure (13). IgGs were purified by affinity chromatography on protein A-Sepharose (Amersham Pharmacia Biotech). Anti-peptide-specific antibodies were purified by affinity chromatography on peptide-coupled NHS-activated columns (HiTrap, Amersham Pharmacia Biotech).

Construction of BARS Probes and Screening of a Rat Brain cDNA Library—mRNA was obtained from male rat brain using a Quickprep mRNA purification kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Poly(A) mRNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using dT_{18} or random hexameric primers (First Strand cDNA synthesis kit, Amersham Pharmacia Biotech). The resulting cDNA first strand was subjected to polymerase chain reaction. Degenerate primers were designed on the basis of the sequences of peptide 36-2 (probe 1 sense primer, CCYTCCACGAGCDGCDGDGTAC) and peptide 55-1 (probe 2 antisense primer, CCTAGAAVAGCACGTTRAABCC), peptide 55-2 (probe 2 antisense primer, CCYTCACGAGCDGCDGDGTAC), and peptide 61 (probe 1 antisense primer, CCYTCCACGAGCDGCDGDGTAC). A ZAP II rat brain cDNA library (Stratagene) was screened essentially as indicated by the manufacturer. The polymerase chain reaction products and cDNA inserts were subjected to automated nucleotide sequencing (Nucleic Acid Facility, Istituto Dermatomatologico Dell’Immacolata, Rome).

Transient Transfection of BARS (CtBP3/BARS, see below) in COS7 Cells—The 1290-base pair coding sequence of BARS cDNA was amplified by reverse-phase high pressure liquid chromatography and sequenced through polymerase chain reaction using specific primers and passed through a series of chromatographic columns. After each step, the radiolabeled DNA was quantified in each chromatographic fraction by 32P-labeled protein was quantified in each chromatographic fraction by SDS-PAGE followed by electronic autoradiography (Packard Instant Imager). ADP-ribosylated cytosol was subjected to precipitation with 35% ammonium sulfate, then dissolved in Buffer A (25 mM Hepes, pH 8, 5% glycerol, 0.5 mM ammonium sulfate, 1 mM dithiothreitol), applied to a Phenyl-Sepharose H.P. column, and eluted with a decreasing linear gradient of ammonium sulfate in Buffer B (25 mM Hepes, pH 8, 5% glycerol, 0.2 mM sodium phosphate, 1 mM dithiothreitol). Fractions containing BARS were pooled, concentrated, and applied to a gel filtration column (Superose 12 H.R.) pre-equilibrated with Buffer B (25 mM Hepes, pH 8, 5% glycerol, 150 mM NaCl). Superose 12 H.R. was calibrated with the following molecular mass standard proteins (Bio-Rad): γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.350 kDa). The void volume of the column was determined with blue dextran.

| Step | Protein | BARS Purification Yield % |
|------|---------|---------------------------|
| Rat brain cytosol | 240 | 36 |
| Ammonium sulfate precipitate | 80 | 36 |
| Phenyl-Sepharose H.P. | 5.33 | 25 |
| Hydroxyapatite | 1.066 | 17.5 |
| Superose 12 H.R. | 0.1066 | 14 |

RESULTS

Purification, Microsequencing, and Molecular Cloning of BARS—The purification strategy of BARS is summarized in Table I. Cytosol was prepared from rat brain, where BARS is relatively abundant (Fig. 1A). Prior to purification, the cytosolic BARS was ADP-ribosylated with [32P]NAD in the presence of BFA. It was then precipitated with 35% ammonium sulfate and passed through a series of chromatographic columns. After each step, the [32P]ADP-ribosylated protein was identified by SDS-PAGE and autoradiography (see “Experimental Procedures”). This procedure achieved an overall 900-fold purification with a 40% yield. The last step was a gel filtration column, from which the protein eluted with an apparent molecular mass of about 170 kDa. BARS was then concentrated and subjected to 2D isoelectric focusing electrophoresis. Three well resolved spots (at a molecular mass of 46 kDa and isoelectric points of 6.05, 6.10, and 6.15; see Fig. 1, B and C (inset)) that were clearly [32P]ADP-ribosylated and Coomassie Blue-stained were isolated and subjected to microsequencing after in situ digestion (see “Experimental Procedures”). Nine non-overlapping peptides, corresponding to internal sequences, were obtained in three separate preparations (Fig. 2A). These sequences were compared with protein data bases. The comparison indicated strong homology with members of the CtBP family of proteins (14–18). The alignment of the BARS peptide with one such protein, CtBP1, was used to design two pairs of degenerate primers, which were then used to amplify a pool of rat brain mRNA by reverse transcriptase-polymerase chain reaction. The two stretches of DNA thus obtained were used as probes to screen a rat brain cDNA library. Five clones, strongly hybridizing with both probes, were isolated and sequenced. The longest of these cDNA clones was 2430 base pairs long. It contained a full-length ORF coding for a 430-amino acid
The GenBank/EBI accession numbers for the sequences reported are AF016507 (mouse CtBP1); AF059735 (mouse CtBP2); and AF016507 (human CtBP2).

To clarify this point. Provisionally, we will call the new protein (predicted mass 47 kDa). The deduced amino acid sequence (BARS; GenBank/EBI accession number AF067795) included the nine peptides (Fig. 2A) obtained from the microsequencing, with only one mismatch at residue 175 (Gly instead of Ser). The cloned protein was compared with known protein data bases. As expected, it was found to be highly similar to the two known mammalian members of the CtBP family (CtBP1 and CtBP2). Both of these proteins have been cloned in both human and mouse (14–16). The identity was 97% with human and mouse CtBP1 (GenBank/EBI accession numbers U37408 and AJ010483, respectively) and 79% with human and mouse CtBP2 (GenBank/EBI accession numbers AF016507 and AF059735, respectively). The only significant region of diversity between CtBP1 and BARS was the N-terminal stretch (Fig. 2B), where the two proteins differ markedly in sequence and length. At the nucleotide level, the BARS cDNA was 94% identical to mouse CtBP1 (86% to human CtBP1) and 72% identical to human and mouse CtBP2. Interestingly, a long sequence at the 5′ end of the BARS cDNA was absent in the CtBP1 and CtBP2 cDNAs. This sequence was present in two mouse sequences (GenBank/EBI accession numbers AA212717 and AI006262) in the EST data base. In fact, in the AA212717 sequence, a 449-base pair-long region (which included the 5′ untranslated region, the ATG start codon, and 273 base pairs of the BARS sequence) was 98% identical to the BARS cDNA. This strongly indicates that BARS is a third form of CtBP that exists both in rat and in mouse. BARS and CtBP1 may be encoded by an alternatively spliced gene or by two different genes. Studies are in progress to clarify this point. Provisionally, we will call the new protein CtBP3/BARS.

To verify that the cloned rat CtBP3/BARS is indeed BARS (operationally defined as the 50-kDa cytosolic substrate ADP-ribosylated by BFA), we used several experimental approaches. First, CtBP3/BARS was overexpressed in COS7 cells. The amount of BARS was then measured in the transfected population. If CtBP3/BARS is BARS, the ADP-ribosylatable 50-kDa protein should be increased after transfection. Fig. 3A shows that indeed the cytosol of CtBP3/BARS-transfected COS7 cells contains a much larger amount of the 50-kDa protein ADP-ribosylated by BFA than of mock-transfected controls. This finding indicates that CtBP3/BARS is BARS. To verify whether the other CtBPs might have BARS properties, human CtBP1 was also overexpressed in COS7 cells, and the cytosol from these cells was ADP-ribosylated. Again, high levels of the 50-kDa ADP-ribosylatable protein were found (data not shown). Similar data were obtained in cells overexpressing mCtBP2 (data not shown), which indicates that indeed the known CtBPs are BARS. Second, antibodies against CtBP proteins were obtained. Two were generated against peptides VSGQVALR (present also in CtBP1) and SVEQIREVASGAARIR (present also in CtBP1 and CtBP2), corresponding to regions 174–181 and 147–162 of CtBP3/BARS (denominated anti-BARS/9 and anti-BARS/14 antibodies, respectively). A third antibody, raised against the whole human CtBP1 protein (anti-CtBP/BARS) (14), was obtained as a gift from Dr. Chinnadurai (St. Louis University Medical Center, St. Louis, MO). All of these antibodies gave detectable signals in brain cytosol in immunoblotting experiments, and the antibody against the whole CtBP1 protein also efficiently immunoprecipitated all of the mammalian CtBPs (not shown). Brain cytosol was ADP-ribosylated and used to test whether the [32P]labeled cytosolic BARS was recognized by antibodies raised against the CtBP proteins. Fig. 3B, lane 4, shows that the anti-BARS/9 antibody recognized a 50-kDa band in Western blots. The band is more evident after enrichment of BARS by ammonium sulfate precipitation of the cytosol (Fig. 3B, lane 5). That this band corresponds to BARS is indicated by the fact that it precisely co-runs in SDS-PAGE with brain BARS shown in Fig. 2A, lane 4 in both the presence and absence of urea (we have previously reported that BARS exhibits the urea shift in SDS-PAGE; see Ref. 7). Similar results were obtained with the anti-BARS/9 antibody. Moreover, the anti-whole CtBP1 antibody quantitatively immunoprecipitated [32P]BARS from the [32P]ADP-ribosylated cytosol (Fig. 3C).

Finally, we used the known property of CtBP proteins of binding to the C terminus of the adenoviral protein E1A-243R (14, 19). A bacterially expressed GST-(C-ter)-E1A fusion pro-
tein (20) was linked to glutathione-agarose beads and used to selectively extract E1A C-terminal-binding proteins from brain cytosol ([32P]ADP-ribosylated in the presence of BFA. If the ADP-ribosylated BARS in this cytosol is a protein of the CtBP family, it should bind to E1A. Indeed, 70% of the [32P]ADP-ribosylated cytosolic BARS was found to specifically and tightly bind to the GST-(C-ter)-E1A (data not shown). Conversely, it was shown that cytosolic proteins that bind to the GST-(C-ter)-E1A are ADP-ribosylated in the presence of BFA (data not shown). Thus, the above collective evidence shows that the cloned rat CtBP3 is a BARS and suggests that all of the CtBPs have the features of BARS proteins. These proteins will thus henceforth be referred to as CtBP/BARS.

The Role of CtBP/BARS in the Golgi Disassembly Induced by BFA—We have previously shown that brain cytosol contains factors that prevent the Golgi disassembly induced by BFA. We have also proposed that BARS is one of these factors, based on the fact that the ADP-ribosylation of BARS by BFA correlates with the loss of inhibitory activity of the cytosol on Golgi disassembly (9). This hypothesis can now be tested directly by exploiting the molecular tools (anti-CtBP/BARS antibodies and purified CtBP/BARS) developed in the course of this study. To assay Golgi disassembly by BFA we used streptolysin O-permeabilized rat basophilic leukemia cells, which maintain a near normal Golgi morphology and respond to BFA under appropriate conditions in the presence of cytosol (9). The position and overall morphology of the Golgi was monitored by immunofluorescence using an antibody to mannosidase II, a well characterized Golgi resident protein. Cytosol was prepared from rat brain as described (9) and dialyzed extensively to remove NAD+. Because NAD+ is the ADP-ribose donor in all ADP-ribosylation reactions, its removal is absolutely necessary in these experiments to prevent the ADP-ribosylation of BARS and, therefore, the possible consequent inactivation of this protein (see above and Ref. 9). Fig. 4, B and C, illustrates the importance of removing NAD+ (see also Ref. 9). In dialyzed, NAD+-deprived cytosol, BFA lost its ability to disassemble the Golgi (compare panel A, showing a “normal” bright central Golgi spot, with panel F, showing the massive Golgi-disassembling action of the toxin in intact cells). When 400 μM NAD+ was re-added to this cytosol, BFA regained its activity. The same effect (regain of BFA activity) was observed when cytosol was pre-ADP-ribosylated (panels B and C, showing a completely diffuse Golgi fluorescence), confirming that NAD+ functions here as the ADP-ribose donor (see Ref. 9). These results are consistent with the idea that ADP-ribosylation inactivates the inhibitory effect of BARS on BFA (9). The role of CtBP/BARS in Golgi disassembly was then tested directly by quantitatively immunodepleting dialyzed cytosol of CtBP/BARS. The rationale of this experiment is that if CtBP/BARS is a cytosolic factor that prevents the tubular transformation of the Golgi by BFA, then depleting the cytosol of CtBP/BARS should greatly facilitate the Golgi disassembling action of the toxin. Fig. 4, D and E, shows that, indeed, although in control (mock-depleted) cytosol BFA was inactive, in CtBP/BARS-depleted cytosol it induced its full effect of Golgi disassembly. Thus, BARS is indeed an inhibitor of the tubular transformation of the Golgi induced by BFA. Notably, even though the extent of the disassembly by BFA under these conditions was comparable with that induced by the toxin in intact cells (shown in Fig. 4F for comparison), the toxin concentrations required for activity were somewhat higher than those effective in the intact system. Similar observations of loss of potency of BFA in permeabilized cells have been reported several times previously (see Ref. 9 and references therein). The origins of this effect are still unclear. Perhaps inhibitory factors other than BARS are present in the cytosol or permeabilization induces the partial loss of a component needed for the Golgi response to BFA. Next, CtBP/BARS-depleted cytosol was complemented with CtBP/BARS, using the chromatographically purified protein (a mixture of the cytosolic CtBPs/BARS) at a final concentration 5-fold higher than calculated to be present in control cytosol. The effect of BFA on the Golgi was nearly completely suppressed (Fig. 4G), again consistent with an inhibitory effect of CtBP/BARS on Golgi disassembly. Interestingly, in parallel experiments BARS did not inhibit another effect of BFA, namely, the dissociation of coatomer proteins from the Golgi complex (data not shown, see Ref. 9). This finding indicates that BARS does not generically block the action of BFA, but rather it exerts an opposing action on the Golgi tubular disassembly. Finally, mock-depleted, NAD+-depleted cytosol was treated with anti-BARS/9 antibody. If the antibody has neutralizing properties, it should inhibit CtBP/BARS and thereby abolish the ability of the cytosol to prevent the effects of BFA. Indeed, in cytosol treated with the anti-BARS/9 antibody (Fig. 4H), BFA potently disassembled the Golgi complex. Preimmune IgGs had no effect (data not shown). Similar experiments were carried out to check the ultrastructure of the Golgi com-
results indicate that ADP-ribosylation causes a conformational change in CtBP/BARS that results in homo- or hetero-oligomerization of the protein and in an altered exposition of hydrophobic surfaces. Such changes might explain the loss of activity of the protein in antagonizing the effect of BFA on Golgi disassembly.

**DISCUSSION**

In this study, we have purified and cloned CtBP3/BARS, a protein substrate of BFA-dependent ADP-ribosylation. The primary sequence of CtBP3/BARS is highly similar to that of CtBP1, a protein originally identified based on its property to bind the C terminus of the transforming adenoviral protein E1A (14, 19). In fact, CtBP3/BARS and CtBP1 are nearly identical throughout most of their sequence, except that CtBP3/BARS lacks the 11 terminal amino acids of CtBP1. Whether this finding represents a functionally significant difference remains to be ascertained. While this work was in progress, another mammalian homologue of CtBP, CtBP2, was cloned in human and mouse (15, 16), which also differs from CtBP1 mostly at the N terminus. Moreover, at least two CtBP homologues have been reported in *Drosophila* (17, 18). Thus, the newly cloned protein can be considered as the third member of the mammalian CtBP family (see “Results”). To indicate this fact, we have called the protein CtBP3/BARS. Interestingly, CtBP1 and -2 can also be considered BARS because they are substrates of BFA-dependent ADP-ribosylation.

We have previously reported that BARS behaves as a cluster of proteins in 2D gels and that some of these proteins bind GTP (7). This is consistent with the presence of several BARS isoforms and/or post-translational modifications. CtBP3/BARS however is not a classical GTP-binding protein, because its sequence does not have homology to known G proteins, nor contains a canonical GTP-binding motif. This in itself is not surprising because proteins that bind GTP but do not possess this motif have already been reported (21). However, preliminary binding studies using the recombinant CtBP3/BARS have so far failed to reveal specific GTP binding (data not shown). The explanation for this failure could be that either not all of the BARS isoforms bind GTP, or an as yet undefined post-translational modification of BARS is required for GTP binding.

The function of the mammalian CtBP2 protein and the *Drosophila* CtBP have recently become partially understood. These proteins appear to function in the regulation of transcription as co-repressors interacting with a broad range of transcription factors. This finding of course raises the intriguing question as to whether the CtBP/BARS family might play two different roles, one in transcription and one in Golgi maintenance. In principle, it is possible that one of the CtBPs/BARS might have evolved a radically different function than that of other CtBPs/BARS and that such function might be limited to the Golgi. This hypothesis seems unlikely, however, in view of the high degree of homology among the CtBPs/BARS. The alternative is that the CtBPs/BARS might have a dual role, one in the nucleus and one in the cytoplasm. A multiple role for proteins (“moonlighting”, see Ref. 22) is not a new concept. Indeed, the examples are numerous, and it has even been proposed that “moonlighting” might be a common property of proteins (22). What might be the significance of a dual role in the case of CtBP/BARS? An attractive hypothesis is that this protein might provide a link, during mitosis, between transcriptional events and Golgi function. This conjecture would fit previous observations that the Golgi complex disassembles during mitosis (3) and that CtBPs/BARS are phosphorylated during the mitotic phase of the cycle (19). Further work is required to clarify this important point.
The central problem posed by the present findings in the context of the issue of Golgi dynamics and architecture is what might be the precise role of CtBP/BARS in Golgi maintenance. Our observation that this protein antagonizes the tubular-reticular disassembly of the Golgi complex by BFA suggests that the physiological role of CtBP/BARS is to stabilize this organelle. The inhibition of BFA is not the result of a trivial mechanism such as, for instance, direct binding and neutralization of the toxin. This possibility is ruled out by the facts that: (a) the concentrations of BFA used in our experiments largely exceed (at least by 150-fold) those of BARS; and (b) another effect of BFA, namely, the dissociation of coatomer proteins from the Golgi complex, is not antagonized by BARS (see also Ref. 9). Thus, BARS exerts a specific negative regulation on the tubular transformation of the Golgi complex. Our working hypothesis is that the physiological function of BARS is to exert a negative control on the formation of Golgi tubules. This premise could explain the ability of BARS to prevent the effect of BFA, and it might also have significant consequences in the physiology of the secretory pathway. Tubules are very abundant and dynamic structures, with a potentially important role in traffic; however, their precise function is still obscure. Elucidating the molecular mechanisms of action of CtBP/BARS might provide new insights into the mechanisms controlling the dynamics of Golgi tubules and the equilibrium between tubular and stacked structures in the Golgi complex.

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