**ADP-ribosylation Factor-1 Stimulates Formation of Nascent Secretory Vesicles from the trans-Golgi Network of Endocrine Cells**

(Received for publication, December 29, 1995)

Ye-Guang Chen§ and Dennis Shields†¶
From the Departments of Developmental and Molecular Biology and Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461

ADP-ribosylation factor (ARF) is a small GTP-binding protein that has been implicated in intracellular vesicular transport. ARF regulates the budding of vesicles that mediate endoplasmic reticulum to Golgi and intra-Golgi transport. It also plays an important role in maintaining the function and morphology of the Golgi apparatus. Using a permeabilized cell system derived from GH3 cells, we provide evidence that ARF-1 regulates the formation of nascent secretory vesicles from the trans-Golgi network. Both myristoylated and non-myristoylated forms of recombinant human ARF-1 enhanced vesicle budding from the trans-Golgi network. ARF-1 also stimulated vesicle budding from the trans-Golgi network, in marked contrast to its inhibitory effect on vesicular transport from the endoplasmic reticulum to Golgi. These data demonstrate that in endocrine cells, ARF-1 and in particular its N terminus play an essential role in the formation of secretory vesicles.

ADP-ribosylation factor (ARF) is a Mr 20,000 polypeptide that is a member of the Ras superfamily of small GTP-binding proteins. It was originally discovered as a cofactor in cholera toxin-mediated ADP-ribosylation of the Gsα subunit of heterotrimeric G-proteins (1). In the past several years, genetic and biochemical studies have shown that ARF also plays an essential role in intracellular vesicular transport (reviewed in Refs. 2–4). Deletion of one of two ARF genes (ARF1) from yeast Saccharomyces cerevisiae led to impaired processing and secretion of invertase, and deletion of both genes was lethal (5). Subsequent studies using in vitro systems have demonstrated that ARF is a necessary component for vesicular transport between the ER and Golgi apparatus and in the endocytic pathway (6–8). ARF is also required for the assembly of the coatamer complex (COP I-coated vesicles) and may therefore function in facilitating intra-Golgi and retrograde transport (9). Other in vitro studies have shown that ARF-1 is required for the binding of γ-adaptin to isolated Golgi membranes (10, 11), suggesting that it functions in the assembly of clathrin coats at the TGN. Experiments in which native and mutant ARF polypeptides were overexpressed in Chinese hamster ovary and normal rat kidney cells have also established a key role for ARF in ER to Golgi, intra-Golgi transport, and maintenance of Golgi morphology in vivo (12, 13). More recently, it was demonstrated that ARF activates phospholipase D activity (14, 15), and this enzyme has now been shown to be present in Golgi membranes (16). Furthermore, the ARF-stimulated phospholipase D activity was shown to affect β-COP binding to isolated Golgi membranes, raising the exciting possibility that changes in the membrane lipid composition (via phospholipase D activity) may influence coatamer recruitment (17).

Recently, we established a permeabilized cell system that supports prohormone processing and secretory vesicle formation from rat anterior pituitary growth hormone (GH) and prolactin (PRL) secretory vesicles (18). To determine if nascent secretory vesicle budding from the TGN might also be ARF-1-regulated, we used recombinant human ARF-1 in our permeabilized cell system. Here we provide evidence that ARF-1-stimulated vesicle formation from the TGN and that the N terminus of the polypeptide was essential for this function.

**EXPERIMENTAL PROCEDURES**

ARF-1 Peptide—A 16-amino acid peptide (GNIFANFLKGLFGKKE) corresponding to residues 2–17 of the ARF-1 N terminus and a random peptide of the same composition (FLKANGIGLNEKKGFF) were synthesized in the Laboratory for Macromolecular Analysis, Albert Einstein College of Medicine. The peptides were purified by high pressure liquid chromatography and their integrity confirmed by mass spectrometry.

Cell Culture—Rat anterior pituitary GH3 cells were grown as described previously (18). Cytosol Preparation—GH3 cells were swollen in 15 mM NaCl and 10 mM Heps pH 7.2 for 5 min and broken by scraping with a rubber policeman in breaking buffer (100 mM KCl, 25 mM Heps pH 7.2, and 100 units/ml Trasylol). Broken and unbroken cells were removed by centrifugation at 800 × g for 5 min, and the supernatant was centrifuged at 100,000 × g for 30 min, concentrated using a Centricron 10 concentrator (Amicon), and stored at −70 °C.

In Vitro Vesicle Budding—Permeabilized cells were prepared as described previously and used directly without prior high salt treatment (18). Briefly, ~1 × 10⁶ cells were pulse-labeled with Tran³²P-S-labeled for 12 min and chased at 19 °C for 2 h to accumulate GH and PRL in the TGN after which permeabilized cells were prepared. The permeabilized cells were incubated at 37 °C for 2 h in a 100-μl reaction containing either no additions, native ARF, mutant ARF, or the ARF peptide as indicated and 10 mM Heps pH 7.2, 2.5 mM MgCl₂, 0.5 mM CaCl₂, 35 mM KOAc, 110 mM KCl, and the energy-regenerating system (1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, and 80 μg/ml creatine phosphokinase). Following incubation, the samples were centrifuged at 13,000 × g for 15 s. The supernatant containing nascent secretory vesicles and cell lysates were immunoprecipitated with anti-prolactin.
and anti-growth hormone antisera (18). The immunoprecipitates were analyzed by SDS-PAGE followed by fluorography and quantitated using a Molecular Dynamics densitometer.

Mutagenesis and Expression of Recombinant ARF Proteins—Human ARF-1 cDNA subcloned into the pET11d expression vector and yeast N-myristoyl transferase (pBB131) (19) were kindly provided by G. Tanigawa and J. E. Rothman and J. Gordon, respectively. Mutagenesis was conducted using polymerase chain reaction (PCR). The ΔNT ARF-1 mutant in which residues 1–17 were deleted and Arg9 changed to Gly was generated using primers 5′-GAT-CAA-GCT-ATC-GTC-ATC-CTT-AG-GG-3′ and 5′-GCT-AGT-TAT-TCG-TCA-GCC-GC-3′ (T7 terminus primer). The T31N and Q71L mutants were introduced using two-step PCR. The first PCR reaction was completed with primers 5′-GAT-CGT-CTT-CCT-CCC-TGC-AGC-ATC-3′ (for T31N) or 5′-CTT-GTC-CAG-GCC-ACC-CAC-GTC-CCA-3′ (for Q71L) and 5′-TAA-TAC-GAC-TCA-CTA-TAG-GG-3′ (T7 promoter primer). The second PCR reaction was performed with the first PCR products and the T7 terminus primer. All mutations were confirmed by DNA sequencing. Human ARF-1 and the mutant proteins were expressed in and purified from Escherichia coli strain BL21 (DE3) (Novagen) according to Tanigawa et al. (20). Myristoylation for native and the T31N and Q71L mutants was accomplished by co-expressing N-myristoyl transferase with the ARF constructs.

RESULTS AND DISCUSSION

Human ARF-1 was expressed in and the protein purified from E. coli transformed with appropriate expression vectors encoding either ARF-1 or ARF-1 and yeast N-myristoyl transferase. In addition three ARF-1 mutants were also constructed, expressed, and purified; these corresponded to ARF-1 lacking the first 17 N-terminal amino acids (ΔNT), which could not serve as a myristic acid acceptor; T31N defective in GTP binding; and Q71L defective in GTP hydrolysis and corresponding to an activated ARF-1. Permeabilized GH3 cells were incubated in the absence and presence of the ARF-1 polypeptides, and nascent secretory vesicles were separated by brief centrifugation and analyzed for immunoreceptible GH (Fig. 1). Surprisingly, both the myristoylated and non-myristoylated forms of native ARF-1 enhanced vesicle budding approximately 2-fold (Fig. 1, A, lanes 5–8, and B). Interestingly, the two mutants, ΔNT and T31N, did not stimulate vesicle budding above background levels (lanes 9–12). In contrast, the Q71L mutant corresponding to GTP-activated ARF-1 enhanced vesicle formation to the same extent as the native polypeptide (lanes 13 and 14). The concentration needed for 50% stimulation was about 1 μM (data not shown), which is close to that required for phospholipase D stimulation (14) and promotion of coatamer binding to Golgi membranes (21).

Earlier work has shown that the N terminus of ARF-1 is required for its activity in vesicular transport and that a peptide corresponding to residues 2–17 prevented ER to Golgi transport in vitro (7). The ΔNT mutant also failed to activate cholera toxin-catalyzed ADP-ribosylation of Gαs or to rescue the yeast arfa1− arf2− lethal mutant (7). To determine if secretory vesicle budding from the TGN might be similarly inhibited, we used the same peptide in our permeabilized cells. In marked contrast to its inhibitory effects in other vesicular transport systems (6–8), the ARF-1 peptide stimulated formation of GH-containing vesicles from the TGN about 2-fold (Fig. 2A, compare lanes 3 and 4 with 7 and 8); identical results were obtained for prolactin-containing vesicles (Fig. 2B). Since our permeabilized cell system is not cytosol-dependent unless pretreated with high ionic strength buffers (18), ARF-1 stimulation did not require addition of cytosol (lanes 7–10). The specificity of the ARF-1 effect was demonstrated by incubating permeabilized cells with a peptide having the identity composition as the ARF-1 peptide but possessing a random sequence. Even at a concentration of 200 μM (4–8-fold higher than that used for the native peptide) there was no stimulation of GH or prolactin vesicle budding (Fig. 2, C and D). ATP and GTP were required for vesicle formation (lanes 1 and 2), and in their absence the ARF-1 peptide did not promote vesicle budding from the TGN (lanes 11 and 12).

Since vesicle budding in our system was ATP- and GTP-dependent (Figs. 1 and 2) (18), it is unlikely that the ARF-1 peptide had detergent-like properties that caused membrane lysis resulting in leakage of GH and prolactin into the supernatant (22). However, to exclude this possibility, protease protection studies were performed (Fig. 3A). Proteinase K digestion of the released vesicle fraction (supernatant) and residual permeabilized cells (pellet) showed that in control and ARF-1-treated permeabilized cells, PRL was largely protease-resistant (lanes 1–4); in the presence of Triton X-100, it was degraded quantitatively (lanes 5 and 6); identical results were obtained for GH-containing vesicles. The ARF-1 peptide stimulation of vesicle formation was further confirmed by high speed centrifugation (Fig. 3B). Significantly more PRL was recovered in the high speed pellet (nascent secretory vesicles) isolated from ARF-1 peptide-treated permeabilized cells than from control incubations (lanes 2 and 5). The residual immunoreactive PRL present in the 150,000 × g supernatant corresponded to the small fraction of hormone that had leaked from disrupted Golgi membranes and was not therefore sedimentable (lanes 3 and 6). Together these results indicate that the released hormones were present in membrane-bound vesicles.

Kinetic analysis (Fig. 4, inset) showed that the ARF-1 peptide enhanced both the rate and level of vesicle budding. We therefore determined at which stage in the budding reaction
the ARF-1 peptide might act (Fig. 4). Permeabilized cells were incubated in the absence and presence of cytosol, and the ARF-1 peptide was added at various times after initiating the reaction. In the absence of cytosol, the ARF-1 peptide stimulated vesicle budding only early during incubation (within the first 30 min); thereafter its addition had little effect. Conversely, in the presence of cytosol, the peptide continued to stimulate vesicle budding up to about 50 min. We suggest that ARF-1 acts early in the budding reaction and may interact with a factor(s) that in the absence of cytosol is rapidly consumed and is therefore no longer available for vesicle formation. In addition, the time dependence of the ARF-1 reaction and our

**Fig. 2.** The ARF-1 N-terminal peptide stimulates budding of nascent secretory vesicles from the TGN. A, the release of nascent secretory vesicles (S) containing GH was used to measure formation of immature secretory granules from permeabilized cells (P). Similar results were obtained for prolactin not shown. B, quantitation of growth hormone and prolactin vesicle budding from the TGN by densitometry. C, formation of growth hormone-containing vesicles in the presence of 50 or 200 μM random ARF-1 peptide (R, M, lanes 5–8) or 50 μM native peptide (lanes 9 and 10). D, densitometric analysis of vesicle formation shown in C. ARFp, human ARF-1 peptide; ERS, energy-regenerating system. Budding efficiency was calculated as in Fig. 1.

**Fig. 3.** The ARF-1 peptide stimulates budding of intact secretory vesicles. A, resistance of PRL-containing vesicles to proteolysis. Following the budding assay performed without or with the ARF-1 peptide (ARFp), samples were incubated with 25 μg of proteinase K/ml at 4 °C for 30 min in the absence (lanes 1–4) or presence of 1% Triton X-100 (lanes 5 and 6). The pellet (P) and supernatant (S) fractions were separated by brief centrifugation and analyzed by SDS-PAGE following immunoprecipitation with anti-PRL or anti-GH (not shown) antibodies. B, sedimentation of nascent secretory vesicles containing PRL. Following incubation at 37 °C, permeabilized cells (lanes 1 and 4, CP) were separated by centrifugation (21,000 × g for 20 s) from the vesicle-containing supernatant. This was further centrifuged in a Beckman Airfuge (150,000 × g, 10 min) to separate nascent vesicles (lanes 2 and 5, SP) from supernatant material (lanes 3 and 6, SS). All the samples were treated with anti-PRL antibodies and analyzed by SDS-PAGE.
observation (not shown) that prohormone cleavage occurred in the presence of the ARF peptide, a reaction that requires generation of an acidic pH via a vacuolar H^+ pump in the TGN and nascent vesicles (23), argue against membrane leakiness having induced hormone release. Instead, the data of Figs. 2, 3, and 4 strongly suggest that ARF-1 stimulated nascent secretory vesicle release from the TGN.

Previous studies have shown that ARF-1 peptides and full-length myristoylated ARF-1 potently inhibit transfer of vesicular stomatitis virus G protein from the ER to cis/distal Golgi and through the Golgi stacks and 4 strongly suggest that ARF-1 stimulated nascent vesicle budding from the TGN. In this model ARF-1 functions in concert with a negative regulator of its effector domain. A mutant lacking this domain, which normally exposes the ARF-1 N terminus affecting its membrane localization (27), consequently the N terminus of the T31N mutant would be inaccessible to and could not compete with those molecules regulating or binding to the ARF-1 effector domain. The identification of the putative ARF-1 interacting proteins will help distinguish between these models and enable us to dissect further the mechanism of secretory granule formation in endocrine cells; these studies are currently in progress. We speculate that these proteins in association with endogenous myristoylated ARF-1 enhance secretory vesicle budding via stimulation of phospholipase D activity (14, 15, 28) or recruitment of cavinin (10, 11) to the TGN or both.

Acknowledgments—We particularly thank Duncan Wilson, Cary Austin, and Paul Melancon for very helpful discussions; G. Tanigawa and J. E. Rothman for the ARF-1 plasmid; and J. I. Gordon for the N-myristoyl transferase plasmid.

REFERENCES
1. Kahn, R. A., and Gilman, A. G. (1984) J. Biol. Chem. 259, 6228–6234
2. Donaldson, J. G., and Klausner, R. D. (1994) Curr. Opin. Cell Biol. 6, 527–532
3. Boman, A. L., and Kahn, R. A. (1995) Trends Biochem. Sci. 20, 147–150
4. Moss, J., and Vaughan, M. (1995) J. Biol. Chem. 270, 12237–12233
5. Stearns, T., Kahn, R. A., Botstein, D., and Hoyt, M. A. (1990) Mol. Cell. Biol. 10, 6690–6699
6. Balch, W. E., Kahn, R. A., and Schwaninger, R. (1992) J. Biol. Chem. 267, 13053–13061
7. Kahn, R. A., Randazzo, P., Serafini, T., Weiss, O., Rulka, C., Clark, J., Amherdt, M., Roller, P., Org, L., and Rothman, J. E. (1992) J. Biol. Chem. 267, 13039–13046
8. Lenhard, J. M., Kahn, R. A., and Stahl, P. D. (1992) J. Biol. Chem. 267, 13047–13052
9. Rothman, J. E. (1994) Nature 372, 55–63
10. Starnes, M. A., and Rothman, J. E. (1993) Cell 73, 999–1005
11. Traub, L. M., Ostrom, J. A., and Kornfeld, S. (1993) J. Cell Biol. 123, 561–573
12. Dascher, C., and Balch, W. E. (1994) J. Biol. Chem. 269, 1437–1448
13. Zhang, C., Rosenwald, A. G., Willingham, M. C., Skuntz, S., Clark, J., and Kahn, R. A. (1994) J. Cell Biol. 124, 289–300
14. Brown, H. A., Gutowski, S., Moonaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 71, 1137–1144
15. Cockcroft, S., Thomas, G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) Science 263, 523–526
16. Ktistakis, N. T., Brown, H. A., Sternweis, P. C., and Roth, M. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4952–4956
17. Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1995) Mol. Biol. Cell 6, 119 (abstr.)
18. Xu, H., and Shields, D. (1993) J. Biol. Cell 122, 1169–1184
19. Duronio, R. J., Jackson-Machelski, E., Heuckeroth, R. O., Olins, P. O., Devine, C. S., Yonenoto, W., Silve, L. W., Taylor, S. S., and Gordon, J. I. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1506–1510
20. Tanigawa, G., Orci, L., Amherdt, M., Ravazzola, M., Heim, J. B., and Rothman, J. E. (1993) J. Cell Biol. 123, 1365–1371
21. Donaldson, J. G., Cassel, D., Kahn, R. A., and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6408–6412
22. Weidman, P. J., and Winter, W. M. (1994) J. Biol. Cell 127, 1815–1827
23. Xu, H., and Shields, D. (1994) J. Biol. Chem. 269, 22875–22881
24. Kahn, R. A., Clark, J., Rulka, C., Stearns, T., Zhang, C., Randazzo, P. A., Terui, T., and Cavenagh, M. (1995) J. Biol. Chem. 270, 143–150
25. Heins, J. B., Palmer, D. J., and Rothman, J. E. (1993) J. Cell Biol. 121, 751–760
26. Haun, R. S., Tsai, S.-C., Adamik, R., Moss, J., and Vaughan, M. (1993) J. Biol. Chem. 268, 7064–7068
27. Amor, J. C., Harrison, D. H., Kahn, R. A., and Ringe, D. (1994) Nature 372, 704–708
28. Liscovitch, M., Chalifa, V., Pertile, P., Chen, C. S., and Cantley, L. C. (1994) J. Biol. Chem. 269, 21403–21406