Unconventional Secretion of AcbA in Dictyostelium discoideum through a Vesicular Intermediate

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The acyl coenzyme A (CoA) binding protein AcbA is secreted unconventionally and processed into spore differentiation factor 2 (SDF-2), a peptide that coordinates sporulation in Dictyostelium discoideum. We report that AcbA is localized in vesicles that accumulate in the cortex of prespore cells just prior to sporulation. These vesicles are not observed after cells are stimulated to release AcbA but remain visible after stimulation in cells lacking the Golgi reassembly stacking protein (GRASP). Acyl-CoA binding is required for the inclusion of AcbA in these vesicles, and the secretion of AcbA requires N-ethylmaleimide-sensitive factor (NSF). About 1% of the total cellular AcbA can be purified within membrane-bound vesicles. The yield of vesicles decreases dramatically when purified from wild-type cells that were stimulated to release AcbA, whereas the yield from GRASP mutant cells was only modestly altered by stimulation. We suggest that these AcbA-containing vesicles are secretion intermediates and that GRASP functions at a late step leading to the docking/fusion of these vesicles at the cell surface.

An understanding of the regulation of SDF-2 signaling is now emerging. During culmination, prespore cells respond to a steroid signal by rapidly releasing GABA, which binds to the GABA\textsubscript{A}\textsubscript{γ} receptor GrIE and stimulates a signal transduction pathway leading to the release of AcbA by prespore cells (3, 5). AcbA is processed into SDF-2 by TagC protease, which is displayed on the surface of prestalk cells in response to GABA. The 34-amino-acid peptide SDF-2 binds to the receptor histidine kinase DhkA, leading to elevated levels of intracellular cyclic AMP (cAMP), which induces spore encapsulation (2, 47). Low levels of SDF-2 also trigger the release of additional AcbA proteins, forming a positive-feedback loop (2, 6). Although only 1 to 3% of the total AcbA is secreted, the levels of SDF-2 in the spores are far above that required to rapidly induce sporulation (5, 19).

The release of AcbA is a critical step in this cascade, but the mechanism of its secretion is largely unknown. The Golgi-associated protein GRASP (Golgi reassembly stacking protein) appears to play an essential role in the process since grpA-null mutants lacking GRASP fail to produce SDF-2 (19). To further explore the role of GRASP and understand the regulation of AcbA secretion, we have determined the subcellular localization of AcbA before and after stimulating its release. Secreted AcbA appears to be localized within membrane-bound vesicles, which accumulate in the cortex of prespore cells during culmination. When AcbA secretion is stimulated by GABA or SDF-2, the cortical vesicles containing AcbA are lost from wild-type cells but remain in cells lacking GRASP. It appears that GRASP is not involved in the production or positioning of AcbA within the cortical vesicles, but it is essential for events leading to their regulated release.

MATERIALS AND METHODS

Strain construction, cell growth, and development. All strains were grown in HL-5 liquid medium supplemented with streptomycin (50 μg/ml) and penicillin (50 U/ml) (20, 45). Those carrying the cotB/lacZ construct were grown in me-
dium supplemented with 20 μg/ml G418 (Geneticin; Gibco). Cells were grown in HL-5 medium without drugs for 36 h prior to the assay. Cells were plated for synchronous development on nitrocellulose filters as described previously (45). Laboratory strain AX4, the Δatg mutants, the gpaA− strain, the atga− strain, and the KP strain were previously described (1, 2, 19, 20, 30, 31).

A point mutant of AcbA was generated by standard techniques to generate a tyrosine-to-alanine substitution at position 72 of the coding sequence. The modified coding sequence was cloned into Esherichia coli and Dictyostelium expression vectors as described previously for the wild-type gene (2). Briefly, the mutant and wild-type proteins were expressed in E. coli strain BL21(DE3) from the pET22a (Novagen) vector, affinity purified on nickel-chelating beads (Talon), and cleaved from the protein tag with enterokinase. The mutant protein was also expressed in Dictyostelium under the control of the actin 15 promoter in the pDNeo2 vector.

The transformation of Dictyostelium cells was performed according to a method described previously by Manstein and Hunt by using a BTX 600 electrotration device (26). Plasmid Cs4 5.03 containing the NSFls2 gene was kindly provided by Mark Bretscher (MRC, Cambridge, United Kingdom). KP cells were transformed with 10 μg of plasmid DNA that was linearized with ScaI and selected with 10 μg/ml blastidicin S for 2 weeks. Individual clones were grown and tested for the replacement of V-ethylmaleimide-sensitive factor (NSF) by the ts2 vector on Southern blots as previously described (46). The resulting KPNsf1a− strain remained sporogenous at 22°C but failed to form spores when incubated for 18 h at 30°C. The act15[Y72A] strain was made by the transformation of plasmid pDNeO2-act15Y72A into an act15 mutant strain described previously (2).

SDF-1, SDF-2, and AcbA assays. The bioassays for detecting SDF-1 and SDF-2 were carried out by using KP cells as previously described (1). For the SDF-2 assay, 1 ml of exponentially growing KP cells was harvested by centrifugation at 2,000 × g, resuspended in 1 ml cAMP buffer (20 mM MES [morpholinopropanesulfonic acid] [pH 6.2], 20 mM NaCl, 20 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 5 mM cAMP), and counted. A total of 4.5 × 106 cells were diluted into 12.5 ml cAMP buffer. Aliquots of 500 μl of the cell suspension were incubated for 1 h at 23°C in the wells of a 24-well dish (1 × 105 cells/well). Serial dilutions of supernatants were added to the wells and uninduced cells were counted 1 h later. A sample was considered positive if it caused the conversion of 40 to 50% of the cells into spores over the background level of sporulation. Samples were quantified by serial dilution, with 1 unit defined as the lowest dilution giving a full induction of spore formation, and units were normalized to 105 producing cells whenever applicable.

To test for SDF-2 release from the KPNsf1a− strain, cells were plated and incubated for 18 h in the same way as described above for KP cells in the SDF-2 assay. To test for the release of SDF-2 from the KPNsf1a− cells at the nonpermissive temperature, cells were incubated at either 22°C or 30°C for 30 min and then treated for 5 min with 0.1 pM SDF-2 or 1 nM GABA. Culture supernatants were harvested, and the levels of SDF-2 were then determined with fresh KP cells in a standard SDF-2 assay (1).

The Δatg mutants have various developmental defects and do not develop synchronously on filters, so they were handled differently in order to assess SDF-1 and SDF-2 production and release. Mutant cultures were harvested from HL-5 medium and developed by plating 107 cells onto nonnutrient agar plates (2% Difco agar in PDF buffer) and incubating them at 22°C for at least 20 h. To account for asynchronous development, aggregates, and slugs were removed from the agar plates with a needle by using a dissecting microscope, leaving mainly early culminants on the plate. The cells were monitored regularly and collected at the early culminant stage, when stalks became apparent under the nascent sori. The cells were resuspended in 1 ml cAMP buffer, centrifuged at 4,000 rpm for 1 min in a microcentrifuge, and resuspended in fresh cAMP buffer. After another round of washing, the cells were counted and plated at a density of 106 cells/ml in 24-well plates. The cells were then induced for 5 min with either 1 pM SDF-2 peptide or 10 nM GABA, supernatants (100 μl) were recovered, and the levels of SDF-2 were quantified. Purified recombinant AcbA proteins were assayed for [3H]palmitoyl-CoA binding as described previously (2).

Immunostaining and fluorescence microscopy. Immunostaining was carried out on cells fixed by using a two-step method described previously by Fukuji et al. (18). Cells were fixed with 2% formalin in 15 mM NaCl phosphate buffer (pH 6.5) at room temperature for 5 min, followed by a 5-min incubation at −10°C in 1% formaldehyde in methanol. Cells were washed in phosphate-buffered saline (PBS) and then sedimented onto slides by using a cytospin centrifuge (Cytospot 7620; Wescor) for 5 min at 2,000 rpm. The cells were then incubated in 100 μl of PBS containing 10 μg/ml bovine serum albumin (BSA) (PBS-BSA) with 1 μl of affinity-purified anti-AcbA rabbit polyclonal antibodies and/or various mouse monoclonal antibodies (2). After overnight incubation at 4°C, the cells were washed three times in 100 μl PBS at 37°C (5 min each) and then incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and rhodamine red-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories Inc.) at 1:2,000 dilution in PBS-BSA. Prespore vesicles (PSVs) were stained with mouse monoclonal MUD102 primary antibody (1:500) (48), followed by Alexa Fluor 594-conjugated goat anti-mouse IgM (Molecular Probes) as the secondary antibody. Stained cells were washed four times in 100 μl PBS at 37°C, with DAPI (4′,6-diamidino-2-phenylindole) included in the third wash.

Images were obtained with a Deltavision deconvolution system by using a Nikon Eclipse TE 200 inverted microscope with a 100× oil immersion objective. AcbA proteins were visualized by using an FITC-coupled antibody with the green filter set. Other proteins were visualized by using a rhodamine-coupled antibody with the red filter set. Images were deconvolved by using Softworks software, and images of single 1-μm optical sections were used for the figures.

To ensure that the immunofluorescent signals reported on the correct antigen, negative controls were carried out with all antibodies. In all cases, antibodies were tested under conditions that should result in an absence of a visible antigen signal, such as in the absence of primary antibody (all), neutralization of reactivity by incubation with the relevant peptide antigen (AcbA), or staining of a mutant strain for the protein (AcbA). The residual staining observed for the AcbA mutant is likely due to cross-reactivity to the AcbB protein, the only other protein in Dictyostelium known to contain an acyl-CoA binding motif. All figures were derived from representative cells taken from multiple experiments with at least two biological samples that were always performed side-by-side with the requisite negative controls.

AcbA localization assay. Cells were allowed to develop on filters until early culmination, typically between 20 and 22 h, and developing structures were then harvested and dissociated to single cells by passage through an 18-gauge needle. To test for changes in AcbA localization after the induction of SDF-2 production, cell suspensions were treated with 0.1 pM SDF-2 peptide or with 1 nM GABA for 5 min at room temperature before fixation and antibody staining. To quantify the localization of AcbA antigen to cortical puncta, at least 200 cells were counted for cells that contained a “ring” of AcbA staining (AcbA). The residual staining observed for the AcbA mutant is likely due to cross-reactivity to the AcbB protein, the only other protein in Dictyostelium known to contain an acyl-CoA binding motif. All figures were derived from representative cells taken from multiple experiments with at least two biological samples that were always performed side-by-side with the requisite negative controls.

Purification of AcbA-containing vesicles. Cells developing on standard nitrocellulose filters (45) were harvested just as fruiting bodies were beginning to undergo terminal morphogenesis, usually after 22 h of starvation. All subsequent steps were carried out either on ice, in a cold room, or in a refrigerated centrifuge at 4°C. A total of 2 × 1010 cells were scraped from 40 filters into 2 ml of breaking buffer (250 mM sucrose, 10 mM HEPES-KOH, 1 mM diithiothreitol [DTT] [pH 7.4], and an EDTA-free Complete protease inhibitor cocktail table [Roche]). Multicellular structures were disrupted into cell clumps and single cells by two passages through a 1.5-inch-long 18-gauge needle using a 5-ml syringe. The cells were brought to 10 ml with breaking buffer and disrupted by five rounds of Dounce homogenization, followed by passage through a 5-μm-porosity Nuclepore filter (Corning Nuclepore, Track-Etch membrane) by using a 5-ml syringe. The crude extract was centrifuged at 2,500 × g for 10 min to remove whole cells, large fragments of broken cells, and nuclei. The clarified cell extract was centrifuged for 1 h at 100,000 × g in a tabletop ultracentrifuge (Beckman TL100.4 rotor at 40,000 rpm) to pellet vesicles (P100

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The pellet was resuspended in 0.5 ml of breaking buffer and brought up to 20% with Optiprep density gradient medium (Sigma) in the bottom of a 13-ml ultracentrifuge tube. An Optiprep step gradient of 20 to 0% was overlaid (2% increments of 1 ml each) on top of the sample, and the gradient was centrifuged in a Beckman SW40 rotor at 27,000 rpm for 12 h. Fractions (0.5 ml) were collected from the bottom of the gradient. To monitor the purification of the AcbA-containing vesicles, the amount of total protein in each fraction was determined, and the amount of the AcbA protein was estimated by semiquantitative Western blotting using 2-fold dilutions to compare samples. Fractionations were also carried out with Ficoll gradients, and similar results were obtained.

RESULTS

Localization of AcbA to cortical puncta at the time of SDF-2 signaling. SDF-2 signaling occurs at the midpoint of culmination of fruiting body development at the time that the ameboid prespore cells begin to encapsulate into spores in a wave of differentiation from the top to the bottom of the nascent sorus (38). Extracellular AcbA is processed by the TagC protease to produce the SDF-2 peptide, which causes rapid encapsulation (2). For this signaling to coordinate terminal cell differentiation with morphogenesis, it is expected that the AcbA protein is released over a short time interval, possibly within minutes of an initial triggering event. We examined the subcellular localization of AcbA late in development by indirect immunofluorescence with affinity-purified polyclonal antibodies (2). Using a procedure that enhances the detection of cytoskeletal structures (18), we found punctate staining of AcbA that was concentrated at the periphery of the cells (Fig. 1A). The specificity of the anti-AcbA antibodies was shown by the ability of chemically synthesized SDF-2 to block all staining and by the absence of staining in \( \text{acbA}^{-/H11002} \) cells (Fig. 1B).

At \( \sim 22 \) h of development, about half of the cells displayed punctate cortical AcbA localization (Table 1). To test whether the AcbA in the cortical puncta is released to generate SDF-2, we took advantage of the fact that both GABA and low levels of SDF-2 trigger the rapid release of AcbA (2, 3). Cells were dissociated from culminants after 22 h of development and stimulated with either 1 nM GABA or 0.1 pM SDF-2 for 5 min before fixing and staining with anti-AcbA antibodies. Both of anti-AcbA antibodies (green), anti-protein disulfide isomerase (PDI) antibodies (red), and DAPI (blue). (C) \( \text{grpA}^{-}/H11002 \) cells that lack GRASP were harvested after 22 h of development on filters and immunostained with affinity-purified polyclonal antibodies for AcbA (2). Using a procedure that enhances the detection of cytoskeletal structures (18), we found punctate staining of AcbA that was concentrated at the periphery of the cells (Fig. 1A). The specificity of the anti-AcbA antibodies was shown by the ability of chemically synthesized SDF-2 to block all staining and by the absence of staining in \( \text{acbA}^{-} \) cells (Fig. 1B).

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these treatments resulted in the disappearance of the cortical rings of stained material, suggesting that the peripheral AcbA had been released (Fig. 1A and Table 1).

We have recently demonstrated a requirement for the GRASP protein in the unconventional secretion of AcbA (19). The cells from culminating structures of developing grpA− cells were stimulated with SDF-2 or GABA and stained with anti-AcbA antibodies. In the unstimulated grpA− cells, AcbA was found in cortical puncta as in the wild-type cells (Fig. 1C and Table 1). However, the AcbA puncta in the grpA− cells were unaltered by either treatment and were indistinguishable from the untreated sample in appearance and frequency (Fig. 1C and Table 1). The defect in the apparent release of the cortical puncta from the grpA− cells might be an indirect result of GRASP’s involvement in some earlier extracellular signaling event required for SDF-2 biogenesis. To explore this possibility, we also developed grpA− cells admixed with an equal number of wild-type cells to test if the behavior of the AcbA puncta could be restored in the mutant cells. When the cells from these chimeras were treated with GABA to stimulate SDF-2 production, all of the GRASP mutant cells retained the AcbA staining pattern of untreated cells (Table 2). Thus, the requirement for GRASP in reducing the number of AcbA puncta is cell autonomous. The observations that SDF-2 and GABA both cause a loss of AcbA-containing puncta in wild-type cells, but not in grpA− cells, correlate with these cells’ SDF-2 release properties and further implicate the cortical AcbA-containing puncta in SDF-2 biogenesis.

To explore the possible identity of the AcbA puncta, we used a variety of antibodies directed against proteins that mark specific subcellular compartments that might contain AcbA. Since AcbA is prespore specific late in development, we examined whether AcbA was associated with prespore-specific vesicles (PSVs). PSVs contain spore coat proteins and fuse with the plasma membrane during sporulation (13, 43). We immunostained 18-h-developing cells with the PSV-specific antibody MUD102 and found that most cells were stained in the characteristic pattern expected for a PSV marker (Fig. 2). The AcbA antibodies produced a labeling pattern distinct from that of MUD102, suggesting that AcbA is not a component of PSVs. Contractile vacuoles (CVs) are osmoregulatory organelles that collect water from the cytoplasm and expel it outside the cell and so could provide a possible route for AcbA secretion (9). The vacuolar H+−ATPase (V-ATPase) is present on CVs at 10-fold-higher levels than on endolysosomal membranes and is a good CV marker (10). We stained cells with antibodies directed against the 100-kDa membrane subunit of the V-ATPase, VatM, and observed no significant overlap with the AcbA puncta (Fig. 2). This finding suggests that CVs do not contain AcbA and therefore are unlikely to be a route for the release of AcbA. The p80 protein is found in endocytic compartments and is most prevalent in late phagosomes, while p25 is found at the plasma membrane and is specific to recycling endosomes (7, 37). No significant overlap was observed between AcbA and either p25 or p80 at any time during development (Fig. 2 and data not shown). Similarly, the immunostaining pattern of the endoplasmic reticulum, obtained with antibodies directed against protein disulfide isomerase (PDI), showed no significant overlap with the AcbA puncta (Fig. 1). These results suggest that AcbA may be sequestered in a novel secretory compartment, since AcbA puncta do not colocalize with PSVs, CVs, the endoplasmic reticulum, or p25- or p80-associated endosomes.

**Requirement of vesicular trafficking for SDF-2 release.** To test whether the release of AcbA from the cell involves membrane trafficking, we determined whether secretion is dependent on the general membrane trafficking protein N-
ethylmaleimide-sensitive factor (NSF) (24, 50). A temperature-sensitive variant of NSF that allowed us to examine the requirement for NSF in SDF-2 production was characterized previously (46). To carry out this test, we used KP cells, wild-type cells engineered to overexpress the catalytic subunit of cAMP-dependent protein kinase that were used in an SDF-2 bioassay (1). We replaced the wild-type copy of the NSF gene with the same results.

| Cell line + condition | SDF-2 production at 22°C | SDF-2 production at 30°C |
|-----------------------|-------------------------|-------------------------|
| KP + 0.1 pM SDF-2     | +                       | +                       |
| KP + 1 nM GABA        | +                       | +                       |
| KP NSF60 + 0.1 pM SDF-2| +                       | +                       |
| KP NSF60 + 1 nM GABA  | +                       | −                       |

* Developed KP cells and KP NSF60 cells were treated as indicated prior to determinations of SDF-2 activity. “+” indicates >5,000 U SDF-2/10^3 cells; “−” indicates <10 U of SDF-2/10^3 cells. Each experiment was repeated three times with the same results.

Suggested that the mutants were defective in SDF-2 production, so we tested this with two of the mutants directly by stimulating atg7 and atg8 mutant cells with SDF-2, or GABA, and measured the amount of SDF-2 produced. No detectable SDF-2 production was observed in these experiments, suggesting that components of the autophagic pathway are indeed required for SDF-2 production (Table 4).

The autophagy mutants’ SDF-2 production deficit provided another means to test the potential functional significance of the AcbA-containing cortical puncta in SDF-2 biogenesis by examining whether the mutants produce cortical AcbA. Since the atg mutants display developmental defects ranging from near-complete arrest during aggregation (atg1) to poor spore encapsulation (atg8) that precluded us from examining them as pure populations, we codelivered each of the mutants with wild-type cells marked with β-galactosidase and β-galactosidase-positive cells were scored for the presence of cortical AcbA-containing puncta. Control experiments demonstrated that 1 to 2% of the Acs [cob/LacZ] prespore cells fail to stain with anti-β-galactosidase antibodies (data not shown). ND, not done.

**TABLE 4. Genetic requirements for SDF-2 production**

| Strain | Signal production in fruiting bodies (%)a | Induced SDF-2 productionb | % of cells with cortical AcbA puncta*c |
|--------|------------------------------------------|---------------------------|-------------------------------------|
| Ax4 (wild type) | 100 | 100 | + 48 |
| atg1 | 100 | <0.01 | ND | ND |
| atg3 | ND | ND | 3.0 |
| atg6 | 100 | <0.01 | ND | 5.0 |
| atg7 | 100 | <0.01 | − | 2.0 |
| atg8 | 100 | <0.01 | − | 10 |
| acbA [act15-achA] | 100 | 10 | + | 42 |
| acbA [act15-achA Δ72A] | 100 | <0.01 | − | <0.4 |

* SDF-1 and SDF-2 were purified from fruiting bodies using cation and anion exchange, respectively, and then quantified (see Materials and Methods). Determination of SDF-2 production was performed by using cells disaggregated from mid-culminants (~22 h) and stimulated with either 1 pM SDF-2 peptide or 10 nM GABA. Both inducers gave the same results with these strains. „−“ indicates that no SDF-2 was detected (<0.2 units per 10^3 cells), while a “+” indicates that >5,000 units per 10^3 cells were produced.

Mutant cells were mixed 1:1 with Ax4 [cob/LacZ], disaggregated from mid-culminants, and immunostained for AcbA and β-galactosidase, and β-galactosidase-negative cells were scored for the presence of cortical AcbA-containing puncta. Control experiments demonstrated that 1 to 2% of the Ax4 [cob/LacZ] prespore cells fail to stain with anti-β-galactosidase antibodies (data not shown). ND, not done.
Requirement of AcbA binding to acyl-CoA for SDF-2 release. Acyl-CoA binding proteins such as AcbA bind acyl-CoA with high affinity to act as lipid shuttles within cells. *Dictyostelium* AcbA binds palmitoyl-CoA with an apparent $K_d$ (dissociation constant) of 0.35 $\mu$M, which is similar to data for the fungal and animal proteins (2). Therefore, we tested whether acyl-CoA binding by AcbA is required for SDF-2 production. Alanine substitutions of the conserved tyrosine 74 of bovine ACBP result in a severe reduction in acyl-CoA binding without affecting the stability of the protein (21, 22). Based on these findings, we generated a point mutation at the analogous position of the AcbA coding sequence (AcbAY72A) and expressed the modified protein in bacteria and *Dictyostelium* to test its biochemical and physiological properties. We purified the AcbAY72A protein and the wild-type AcbA protein from *E. coli* and tested their ability to bind palmitoyl-CoA, as previously described (2). The lipid binding of the mutant protein was too low to measure. At 3 $\mu$M $[^{14}C]$palmitoyl-CoA, the maximum concentration that could be used in the assay, only 10% of the AcbAY72A protein bound the lipid, while 100% binding of wild-type AcbA was obtained at this concentration. The recombinant proteins were also tested for their abilities to generate the SDF-2 peptide. Starved KP cells were activated with 10 nM GABA for 5 min, washed, and incubated for 30 min with 10 pmol the wild-type or AcbAY72A protein, and SDF-2 activity was quantified by a bioassay. The same amount of SDF-2 activity ($\sim 10^4$ units/10^5 cells) was produced by the AcbA and AcbAY72A proteins in this assay.

Next, we tested the SDF-2 signaling function of the AcbAY72A protein in *Dictyostelium* cells. The expression of the wild-type protein in acbA mutant mutants under the control of an actin promoter (acbA [act15/acf4]) rescued the developmental defects of the acbA mutant, restored normal spore viability (data not shown), and restored SDF-2 production both in fruiting bodies and after stimulation in *vitro* (Table 4). In contrast, the expression of the AcbAY72A protein in acbA mutant mutants (acbA [act15/acf4]) did not rescue spore viability or SDF-2 production (Table 4). Still, these cells did produce a stable AcbAY72A protein throughout development, and we could produce substantial quantities of SDF-2 signal when we disrupted the cells and treated the resulting lysate with trypsin (data not shown). The observations that AcbAY72A produced in *E. coli* or *Dictyostelium* can be proteolytically processed into SDF-2 while AcbAY72A-expressing cells cannot produce SDF-2 suggest that acyl-CoA binding is required for the normal biogenesis of SDF-2 during development. If the AcbA-containing cortical puncta are in fact specialized vesicles involved in SDF-2 production, then lipid binding might be required for AcbA’s incorporation into the puncta. Consistent with this idea, developing AcbAY72A-expressing mutant cells did display scattered AcbA-positive puncta, but we did not detect any cells with rings of cortical puncta, whereas about half of the cells expressing wild-type AcbA did display cortical puncta (Table 4).

Purification of AcbA-containing vesicles. If the AcbA-containing puncta are secretory vesicles that undergo regulated fusion with the plasma membrane, or exocytosis, we should be able to fractionate and characterize them as AcbA-containing vesicles from cells. We attempted this using a standard vesicle purification scheme consisting of cell breakage under isosmotic conditions and low-speed sedimentation to remove unbroken cells, nuclei, and mitochondria, followed by high-speed sedimentation of smaller particles and vesicles into a pellet (P100) fraction that was then subjected to equilibrium density gradient centrifugation in Optiprep or Ficoll medium (see Materials and Methods). The AcbA protein in the various fractions was monitored by Western blotting, and yields were estimated by 2-fold dilution series of key fractions.

As expected for a soluble protein, most of the AcbA in cells was recovered in the supernatant, and about 1% of the total cellular AcbA was recovered in the P100 fraction. After the P100 fraction was resuspended, AcbA could be quantitatively repelleted into a P100 fraction (data not shown). This is consistent with a small amount of AcbA being associated with cellular components larger than ribosomes but smaller than organelles such as mitochondria and nuclei. After isopycnic separation on an Optiprep gradient, all of the AcbA in the P100 fraction was recovered in a single peak on the gradient with a relative low density (Fig. 3A). By staining the Western blots with PDI and MUD102 antibodies, we could determine that the endoplasmic reticulum and prespore vesicles were well resolved from the fractions containing AcbA (data not shown). In addition, using quantitative PCR (qPCR) as a means of identifying mitochondrial and nuclear DNA, we could not detect any nuclei on the gradient, suggesting that they were removed by the low-speed sedimentation step, while the mitochondrial DNA resolved into a peak of high density, near the pellet (data not shown).

The density gradient fractionation suggested that all of the pelletable AcbA in the cytoplasm of 22-h-developing cells is associated with particles that have a high lipid content. Therefore, we tested whether AcbA is sequestered within membrane vesicles by examining its protease sensitivity. The P100 fraction was subjected to digestion by proteinase K in the presence or absence of the nonionic detergent Triton X-100. The AcbA protein appeared to be completely resistant to proteolysis unless the fraction was treated with Triton X-100 (Fig. 4A). Identical results were obtained when this experiment was carried out with material from the peak fractions from the density gradient (Fig. 4B). Vesicles purified from the grpA mutant cells gave identical results in these experiments (data not shown). This suggests that the AcbA protein in 22-h-developing cells that is not freely soluble, or sequestered in large organelles, is present inside a relatively homogeneous population of lipid vesicles.

Of critical importance is whether the AcbA-containing vesicles that we can purify by cell fractionation are related to the cortical puncta observed by antibody staining of whole cells. We tested this relationship in two ways. First, we attempted to purify the AcbAY72A mutant protein from AcbA Y72A mutant cells. Since this mutant does not produce SDF-2, or AcbA-containing cortical puncta, we might expect to see the mutant protein’s purification properties altered if the AcbA-containing vesicles are related to SDF-2 production. In fact, the AcbAY72A protein was not recovered from the low-density fraction of the density gradient but was instead found in a higher-density fraction, well resolved from the wild-type AcbA fractions (Fig. 3B). Second, if the vesicles that we purified are the puncta that we observed by immunostaining, then we should find little or no AcbA in the gradient fractions after the
addition of GABA to wild-type cells, but there should be little reduction following the addition of GABA to cells lacking GRASP. To test this, we carried out a standard vesicle purification procedure separately with wild-type and _grpA_ cells that were treated with GABA just prior to cell disruption. The yield of AcbA-containing vesicles from wild-type cells was significantly reduced after treatment with GABA, but similar amounts were recovered from the GRASP mutant cells before and after treatment with GABA (Fig. 3C). These results suggest that the _grpA_ cells are blocked in the GABA-stimulated cellular release of these vesicles, consistent with their SDF-2 release phenotype. The distribution of the AcbA<sup>Y72A</sup> mutant protein on the density gradients as well as the disappearance of the AcbA protein after GABA treatment of wild-type cells prior to fractionation suggest that the cortical puncta identifiable by anti-AcbA antibody staining of fixed cells are the low-density AcbA-containing vesicles.

**DISCUSSION**

Acyl-CoA binding proteins (ACBPs) are small, highly conserved proteins that are found in all eukaryotes. Mammalian ACBP not only shuttles lipid intermediates around the cell but also serves as a precursor of DBI, a peptide that functions both in peripheral tissues and in the central nervous system (12, 36). In the brain, DBI binds to GABAA receptors and modulates their response to GABA (12). DBI can displace diazepam bound to the GABAA receptor and was named the diazepam binding inhibitor for this property (11, 12). In a wide variety of tissues, DBI binds to the peripheral receptor localized on mitochondria, where it modulates steroid synthesis, leading to the processing of cholesterol into pregnenolone, the precursor of all steroids (27, 33). Although DBI clearly acts as an intercellular signal, it is not clear how it or its precursor is released since neither one possesses a signal sequence that would direct it to the conventional endoplasmic reticulum-Golgi pathway.

The ACBP homolog in _Dictyostelium_, AcbA, has been shown to bind palmitoyl-CoA with the same affinity as mammalian ACBP as well as to be proteolytically cleaved to generate a conserved intercellular signaling peptide (2). Most of the AcbA in developing _Dictyostelium_ cells is soluble, but about 15% could be pelleted with the total membrane fraction (19). While this is consistent with some of the AcbA being membrane bound, the fact that AcbA binds acyl-CoA in membranes could account for it being found in crude membrane fractions.
A small amount of intact, unprocessed AcbA can be found in the extracellular fluid throughout development, but SDF-2 appears only after 22 h of development as the cells are building a fruiting body (1; C. Anjard and W. F. Loomis, unpublished data). No SDF-2 can be recovered at any time in development from lysates of washed cells, suggesting that AcbA is not processed internally before secretion. During culmination, extracellular AcbA is rapidly cleaved by the TagC protease that is exposed on the surface of prestalk cells in response to GABA or priming with low levels of SDF-2 (2). The burst of SDF-2 starting 30 s following priming with low levels of SDF-2 indicates that some cellular AcbA is rapidly secreted and is immediately available for processing.

Over 20 proteins have been identified as being secreted by pathways other than the conventional endoplasmic reticulum-Golgi pathway (reviewed in reference 35). These proteins include the yeast mating factor, macrophage migration-inhibitory factor (MIF), the inflammatory cytokine interleukin-1β, and the fibroblast growth factors FGF1 and FGF2 (17, 28, 40, 41, 43, 44). The a-factor mating peptide of Saccharomyces cerevisiae as well as MIF in animals are secreted directly from the cytoplasm by specialized ABC transporters (17, 42). Since the inhibition of ABC transporters with vanadate, verapamil, or corticosterone did not block the secretion of AcbA, it is unlikely that AcbA release is mediated by one of the many ABC transporters in Dictyostelium (19).

Here we showed that AcbA release is dependent on the general membrane trafficking protein NSF, suggesting that membrane fusion is an integral step in this unconventional secretion pathway. Interestingly, NSF has been implicated in the regulation of exocytosis in a number of cell types (23, 49). The unconventional secreted protein Ku was also observed to accumulate in vesicles (34). We have now shown that a portion of AcbA is concentrated in membrane-bound vesicles that appear near the plasma membrane just prior to release. These vesicles are distinct from PVSs, CVs, the endoplasmic reticulum, or p25- or p80-associated endosomes. While we do not know the source of these vesicles, it is clear that lipid binding appears to be required for AcbA’s inclusion into these vesicles since the cells reconstituted with the AcbAY72A protein did not produce cortical puncta mid-culmination, and we could not recover low-density AcbA-containing vesicles. However, we cannot rule out the possibility that the AcbAY72A protein is incorporated into cortical vesicles but is rapidly degraded.

It is important that less than 5% of the cellular AcbA is secreted and processed into SDF-2 peptides (19). Our biochemical analyses have shown that >95% of the total cellular AcbA remains associated with 22-h-developed cells after they are stimulated to release AcbA (our unpublished observations), so this new secretion system operates on a small fraction of AcbA. Since AcbA-containing vesicles disappear after stimulation along with most other AcbA staining, it seems likely that most of the cellular AcbA is removed during our indirect immunofluorescent staining procedure, or else we would have observed AcbA staining throughout the cell. Thus, our ability to visualize this new secretion mechanism is probably due to our fortuitous selection of the formalin-methanol cell fixation protocol.

Several other general mechanisms of unconventional protein secretion that require membrane trafficking have been proposed, including the sequestration of proteins into vesicles through the autophagy pathway (29). Indeed, two recent reports demonstrated a requirement of autophagy proteins for the secretion of acyl-CoA binding proteins in Pichia pastoris and Saccharomyces cerevisiae, two highly divergent species of yeast (14, 25). We have demonstrated a similar requirement for the autophagosome machinery in the secretion of SDF-2 and the formation of AcbA-containing cortical vesicles. These results are consistent with the existence of a conserved unconventional secretion pathway for the acyl-CoA binding protein in eukaryotes.

The final step in the secretion pathway of AcbA appears to be the fusion of the cortical vesicles with the surface membrane, or exocytosis of the vesicles from multivesicular bodies (MVBs), in a reaction that requires the function of GRASP (14, 19). Furthermore, the secretion of AcbA in Saccharomyces cerevisiae requires the plasma membrane SNARE protein Sso1 (14). It was proposed previously that GRASP might tether vesicles of this unconventional secretion pathway to the plasma membrane (29). However, we observed the cortical localization of AcbA-containing vesicles in the absence of GRASP.

This observation suggests that GRASP either is directly involved in regulated vesicle (or MVB) fusion or is required for the correct localization of a fusion-regulating protein or protein complex to the vesicle (or MVB) or the plasma membrane. An understanding of the mechanism by which AcbA is captured into a vesicular element and then released in a signal-dependent and GRASP-dependent manner remains the obvious next challenge.

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