Communication

Secretory Vesicle Budding from the Trans-Golgi Network Is Mediated by Phosphatidic Acid Levels*

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Phospholipid metabolism plays a central role in regulating vesicular traffic in the secretory pathway. In mammalian cells, activation of a Golgi-associated phospholipase D activity by ADP-ribosylation factor results in hydrolysis of phosphatidycholine to phosphatidic acid (PA). This reaction has been postulated to stimulate nascent secretory vesicle budding from the trans-Golgi network. It is unclear whether PA itself or diacylglycerol (DAG), a metabolite implied in yeast secretory vesicle formation, regulates budding. To distinguish between these possibilities we have used a permeabilized cell system supplemented with phospholipid-modifying enzymes that generate either DAG or PA. The data demonstrate that in mammalian cells accumulation of PA rather than DAG is a key step in regulating budding of secretory vesicles from the trans-Golgi network.

Recent observations from several laboratories have shown that phospholipid metabolism regulates intracellular vesicle transport, particularly in distal steps of the secretory pathway (1). Studies from Emr and colleagues (2) demonstrated that in yeast Saccharomyces cerevisiae the VPS34 gene encodes a PI³ 3-kinase, which is required for protein transport from the late Golgi apparatus to the vacuole. Recently, a novel PI³ 3-kinase, thought to be complexed with the trans-Golgi network marker TGN38, was shown to stimulate release of poly(IgA)-receptor-containing constitutive secretory vesicles from liver Golgi membranes (3). Two enzymes, phosphatidylinositol-4-phosphate 5-kinase and phosphatidylinositol transfer protein (PI-TP), respectively (4), required for the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP₂), also regulate exocytosis of synaptic vesicles (5). Although it is unclear how PI metabolism modulates vesicle traffic, several proteins involved in synaptic vesicle endocytosis interact with PIP₂, e.g. dynamin, a GTPase involved in fission of endocytic vesicles from the plasma membrane (6).

Members of the phospholipase D (PLD) family of phospholipid-modifying enzymes have also been implicated in vesicle trafficking (7). PLD, which hydrolyzes phosphatidylcholine to phosphatidic acid (PA), is a downstream effector of the small GTP-binding protein ADP-riboylation factor (ARF-1) (8, 9) and is present on Golgi membranes (10). Interestingly, mammalian PLDs also require PIP₂ as an essential cofactor for enzymatic activity (11). Activation of PLD by ARF-1 potentiates coatomer (COP-I) binding to Golgi membranes in vitro (12). Studies from our laboratory have demonstrated that ARF promotes release of nascent secretory vesicles from the TGN and that this occurs via enhancement of PLD activity in the Golgi apparatus (13, 14). Because PA promotes a variety of physiological responses (7), it might enhance vesicle budding either by activating a signal transduction pathway or by altering the local charge distribution on the membrane.

Another class of enzymes that regulate phosphoinositide synthesis, PI-TPs, also function in the formation and release of secretory vesicles (1). Elegant studies from Bankaitis et al. (15) showed that the yeast SEC14 gene, which is essential for viability, encodes a PI-TP that modulates phosphatidylinositol levels in the Golgi apparatus and is required for secretory vesicle formation (16). Significantly, a mammalian homologue of Sec14p, as well as the yeast enzyme, promotes the budding of nascent secretory vesicles from the TGN in vitro (17). Recent evidence has shown that defects in sec14 can be bypassed by mutations in the SAC1 gene (18). Sac1-22 mutants appear to rescue sec14p defects by increasing the levels of DAG in the Golgi apparatus (18). To determine whether the production of PA or DAG in the TGN is required for budding of nascent secretory vesicles, we added phospholipid-modifying enzymes (PI-specific phospholipase C (PI-PLC) and diacylglycerol kinase (DAG K)) to permeabilized rat anterior pituitary GH3 cells. Here we show that in endocrine cells the production of PA rather than DAG enhances vesicle release from the TGN.

EXPERIMENTAL PROCEDURES

PI-PLC from Bacillus cereus (~118 units/ml), Escherichia coli recombinant sn-1,2-diaclylglycerol kinase (~61 units/ml), and the class II diacylglycerol kinase inhibitor (R 59949) were purchased from Calbiochem. Octyl ß-glucoside was purchased from Pierce. [γ-32P]ATP (specific activity: 3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Rabbit anti-growth hormone and anti-prolactin sera have been described previously (19, 20).

Cell Culture—Rat anterior pituitary GH3 cells were grown as described previously (19).

In Vitro Vesicle Budding—Cells were pulse-labeled with Tran³5S-label for 10 min at 37 °C, chased for 2 h at 20 °C, and permeabilized as described (14). Control incubations contained ~5 x 10⁶ permeabilized cells, 20 mM Hepes, pH 7.3, 125 mM KCl, 2.5 mM MgCl₂, 1 mM ATP, 200 μM GTP, 10 mM creatinine phosphate, 160 μg/ml creatinine phosphate kinase, and 5 μg/ml Trasylol. Incubation for 2 h at 37 °C under these conditions reconstitutes both prohormone processing and nascent secretory vesicle release from the TGN (19). To determine the effects of phospholipid modification on vesicle budding, permeabilized cells were pretreated for 10 min at 4 °C with or without the indicated concentration of PI-PLC and/or DAG kinase and then incubated under vesicle budding conditions. It should be noted that the in vitro reaction conditions for vesicle budding are suboptimal for PI-PLC and DAG kinase.

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activities. The assay for nascent secretory vesicle budding was based on quantifying the release of radiolabeled growth hormone (GH) and prolactin (PRL) into a 15,000 × g supernatant following an in vitro incubation (19, 20). Following the incubation, permeabilized cells were pelleted and lysed, and the lysates and supernatants containing nascent secretory vesicles were treated sequentially with anti-PRL and anti-GH (20). Immunoreactive material was resolved by SDS-PAGE and detected by fluorography. Band intensities were quantitated using a computing densitometer (Molecular Dynamics, Model 300A) and Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

Phosphatidic Acid Determination—Non-radioactive permeabilized GH3 cells were incubated under vesicle budding conditions with 30 μCi/ml [γ-32P]ATP in the presence or absence of PI-PLC, DAG kinase, and the DAG kinase Inhibitor II as indicated. Following incubation, samples were placed on ice, and the lipids were extracted with methanol:chloroform:water (1:1:0.8). After vortex mixing, the organic phase containing phospholipids was separated by centrifugation and dried under vacuum. The dried samples were resuspended in chloroform: methanol (19:1), and the phospholipids were resolved on oxalic acid:impregnated Whatman LK5DF TLC plates (Whatman, Clifton, NJ) by developing with an organic phase consisting of chloroform:methanol:acetic acid:water (55:45:3:4) (21). Radiolabeled phosphatidic acid was detected by autoradiography, and its identity was confirmed by comparison of its Rf value with a phosphatidic acid standard chromatographed on the same plate. Band intensities were quantitated by a computing densitometer, as above.

Subcellular Localization of Radiolabeled Phosphatidic Acid—Permeabilized GH3 cells were incubated in the presence of [γ-32P]ATP as described above. Following incubation, samples were centrifuged in a Microfuge for 20 s. The pellets were homogenized using a ball bearing homogenizer, and the entire homogenate was loaded on an equilibrium sucrose density gradient designed to separate Golgi membranes from microsomes (19, 20). Gradients were centrifuged in a SW41.2 Ti rotor (Beckman) for 4 h at 39,000 rpm, after which 1-ml fractions were collected. The lipids were extracted from each gradient fraction and analyzed by TLC as described above. In parallel, an aliquot of each gradient fraction was analyzed by SDS-PAGE and immunoblotted with anti-TGN38 antibody to identify Golgi membrane fractions enriched in the trans-Golgi network (20).

RESULTS AND DISCUSSION

Data from several laboratories have demonstrated that ARF-1 stimulates endogenous phospholipase D-1 activity in Golgi membranes (8, 10, 13, 14). Most recently, we have shown that addition of exogenous human PLD-1a to a permeabilized cell system derived from growth hormone and prolactin-secreting rat anterior pituitary GH3 cells stimulated release of nascent secretory vesicles from the TGN (14). We postulated that either accumulation of PA in the TGN or metabolites implicated in signal transduction pathways, e.g. DAG, would enhance vesicle release. To determine whether PA or DAG might promote vesicle release, we added phospholipid-hydrolyzing enzymes to permeabilized GH3 cells directly to generate either DAG or PA independently of PLD activity and then analyzed their effects on the budding of nascent secretory vesicles (Fig. 1). As previously demonstrated (14), in the presence of an energy-generating system, vesicle release from the TGN was 25–30% efficient (lanes 3 and 4). Addition of PI-PLC, an enzyme which hydrolyzes PI to DAG, enhanced release of GH- and PRL-containing vesicles from 28% to 40% (compare lanes 3 and 4 with 7 and 8) suggesting that accumulation of DAG modulates vesicle budding. However, addition of bacterial DAG kinase, which phosphorylates DAG to generate PA, stimulated vesicle budding to approximately 45% (lanes 11 and 12).
significantly, addition of PI-PLC together with DAG kinase, an enzyme combination expected to generate significant levels of PA (Fig. 2), stimulated release of nascent secretory vesicles by 100% over the control (Fig. 1B; compare lanes 5 and 6 with 13 and 14). This suggested that production of PA rather than DAG was the significant determinant for vesicle budding. Consistent with a requirement for PA accumulation, addition of a DAG kinase inhibitor reduced this stimulated vesicle budding (Fig. 1B). Vesicle budding in the presence of PI-PLC and DAG kinase was itself energy-dependent since the absence of ATP and GTP budding was minimal (compare lanes 1 and 2 with 15 and 16). We interpret these results to suggest that the generation of PA in the TGN results in stimulation of nascent secretory vesicle release.

To test that the combined activities of PI-PLC and DAG kinase increased PA levels, permeabilized cells were incubated with \(\gamma\text{-}^{32}\text{P}}\text{ATP}\) and PI-PLC in the absence or presence of DAG kinase to measure formation of radiolabeled PA (Fig. 2). Addition of either PI-PLC or DAG kinase alone increased the level of PA but only slightly above that of control permeabilized cells (Fig. 2B). Most significantly, when both enzymes were present at concentrations that resulted in maximal vesicle budding (Fig. 1), PA levels were approximately 4-fold above controls (Fig. 2, lanes 1 and 5). It is noteworthy that the level of PA accumulation that correlated with enhanced vesicle budding was similar to that observed for ARF stimulation of Golgi PLD activity (approximately 3-fold (14)) which also promoted maximal vesicle budding.

It was possible that PA accumulated in all membranes possessing PI and would not be enriched in the TGN, the site of vesicle budding. To test this possibility, permeabilized cells were incubated under budding conditions with both enzymes and \(\gamma\text{-}^{32}\text{P}}\text{ATP}\.\) Following incubation, the Golgi membranes were separated from total microsomes by flotation on a sucrose density gradient (19), total phospholipids were extracted from each fraction, and the level of radiolabeled PA was determined (Fig. 3). As expected, PA was present in the gradient load zone and in the rough microsomes (Fractions 7–10 (Ref. 20)); however significant levels were evident at the top of the gradient where Golgi membranes are enriched (Fractions 2 and 3; Fig. 3, A and C). In the absence of PI-PLC and DAG kinase, there was minimal PA accumulation (Fig. 3B). These data suggest that the accumulation of PA in Golgi membranes results in enhanced vesicle release from the TGN.

We reasoned that the PI concentration in the TGN was likely to be relatively low since PI constitutes only a small fraction of total cellular phospholipids. Consequently, it should be possible to demonstrate a dose-response relationship between PI-PLC and DAG kinase and the extent of vesicle budding (Fig. 4). At low concentrations of both enzymes, there was a linear increase in the level of vesicle budding (Fig. 4A, compare lanes 1 and 2 with 9-12) that reached a plateau at approximately 2-fold enhancement of vesicle release (Fig. 4B). Consistent with the idea that PI levels might be limiting, addition of increasing enzyme concentrations had no significant effect on vesicle budding from the TGN. In agreement with the data of Fig. 1, addition of maximal concentrations of either PI-PLC or DAG kinase alone stimulated vesicle release but only approximately 30% (Fig. 4B). Together with the data of Fig. 1, these results suggested that the phosphorylation of DAG by exogenously added DAG kinase promoted vesicle release, presumably as a result of elevated PA levels in the TGN.

Previous work from our laboratory showed that PLD-mediated PC hydrolysis increased PA levels and stimulated vesicle release from the TGN (14). However, the observation that \(S.\) \textit{cerevisiae sac1-22} mutants, which bypass sec14 PI-TP, were found to accumulate DAG in the Golgi apparatus (18) prompted us to test whether PA or DAG levels regulate vesicle budding from the TGN. The data presented here suggest that in mammalian endocrine cells accumulation of PA rather than DAG is required for secretory vesicle release from the TGN. A possible explanation for the difference between our results and those of Kearns et al. (18) might be related to the amount of DAG in sec1-22 mutant yeast, which is 3- to 6-fold above wild type levels. A small fraction of this pool could be converted to PA via endogenous DAG kinase, thereby restoring vesicle formation.

The observation that overexpression of bacterial DAG kinase in the \textit{sec1-22} background exacerbated the sec14 phenotype (18) could result from highly elevated PA levels in all organelles, presumably this would be toxic to the cell. It is also possible that yeast and mammalian cells may utilize different phospholipids to generate secretory vesicles.

Several hypotheses might explain how PA accumulation enhances vesicle budding. As suggested originally (22) to explain...
the requirement for VPS34p activity in vesicle transport from the late Golgi apparatus to the yeast vacuole, the accumulation of transient, local, high concentrations of negatively charged lipids in the outer lipid bilayer could either promote specific coat protein recruitment or itself induce changes in the curvature of the membrane leading to vesicle formation. Similarly the accumulation of negatively charged PA in the outer leaflet of the membrane bilayer could cause vesicle budding by a similar mechanism. In support of the former idea, Kistakis et al. (12) have demonstrated that the β-COP subunit of coatomer binds to PA-containing liposomes about 5- to 7-fold more efficiently than to those lacking this phospholipid; interestingly, when PIP2 was also present, coat binding was even more efficient.

An alternate possibility is that, since PA has been shown to stimulate the activity of phosphatidylinositol-4-phosphate 5-kinase, a soluble enzyme (23) in the final step of the PIP2 biosynthetic pathway, PIP2 production could promote coat binding to the membrane. Consistent with this possibility, recent data from Tüscher et al. (24) showed that PI-TP and bacterial PLD synergize in promoting vesicle budding; however, addition of geneticin, an aminoglycoside that binds PIP2, inhibited the stimulation of vesicle budding. Because the bacterial PLD is resistant to geneticin and unlike mammalian enzymes does not require PIP2 for activity, this suggests that PIP2 might be essential for vesicle budding. Several enzymes, some of which possess pleckstrin homology domains, bind PIP2 and are involved in vesicle trafficking. For example, PIP2 is an essential cofactor for PLD activity (11), it enhances the GDP-GTP exchange activity of the ARF guanine exchange factor (ARNO) (25), plays a key step in the fusion of synaptic vesicles with the plasma membrane (5), and regulates dynamin recruitment to membranes and dynamin GTPase activity via interaction with its pleckstrin homology domain (6). The recent demonstration that dynamin-II is Golgi-associated and is required for constitutive secretory vesicle budding (26) is consistent with this idea and may indicate that PIP2 is a key regulator of the exocytic vesicle budding machinery. We speculate that both PA and PIP2 synthesis are part of a feedback mechanism to regulate the recruitment of proteins involved in Golgi vesicle budding.

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REFERENCES

1. De Camilli, P., Emer, S. D., McPherson, P. S. & Novick, P. (1996) Science 271, 1533–1539
2. Stack, J. H., Herman, P. K., Schu, P. V. & Emer, S. D. (1993) EMBO J. 12, 2185–2204
3. Jones, S. M. & Howell, K. E. (1997) J. Cell Biol. 139, 339–349
4. Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukuaki, K., Takenawa, T., Anderson, R. A. & Martin, T. F. J. (1995) Nature 374, 173–177
5. Martin, T. F. J. (1997) Trends Cell Biol. 7, 271–276
6. Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gorg, R., Smith, C. I. E., Driscoll, P. C., Waterfield, M. D. & Panayotou, G. (1996) EMBO J. 15, 6241–6250
7. Exton, J. H. (1997) J. Biol. Chem. 272, 15579–15582
8. Brown, H. A., Gutowski, S., Moonaw, C. R., Slaughter, C. & Sternweis, P. C. (1993) Cell 75, 1137–1144
9. Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, R. G., Gout, I., Hiles, J., Totty, N. F., Truong, O. & Hauan, J. J. (1994) Science 263, 523–526
10. Kistakis, N. T., Brown, H. A., Sternweis, P. C. & Roth, M. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4952–4956
11. Pertile, P., Liscovitch, M., Chait, V. & Cantley, L. C. (1995) J. Biol. Chem. 270, 5130–5135
12. Kistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C. & Roth, M. (1996) J. Cell Biol. 134, 295–306
13. Chen, Y.-G. & Shields, D. (1996) J. Biol. Chem. 271, 5287–5300
14. Y.-G. Chen, S. Ishihata, A., Austin, C. D., Hammond, S. M., Sung, T. C., Frohman, M. A., Morris, A. J. & Shields, D. (1997) J. Cell Biol. 138, 495–504
15. Bankaitis, V. A., Malehorn, D. E., Emer, S. D. & Greene, R. (1989) J. Cell Biol. 108, 1271–1281
16. McGee, T., Skinner, H. B., Whitters, K. A., Henry, S. A. & Bankaitis, V. A. (1994) J. Cell Biol. 124, 273–287
17. Ohashi, M., De Vries, K. J., Frank, R., Snoek, G., Bankaitis, V., Wirtz, K. & Huttner, W. B. (1995) Nature 377, 544–547
18. Kearns, B. G., McGee, T. P., Mayinger, P., Gedvilaite, A., Phillips, S. E., Kagwada, S. & Bankaitis, V. A. (1997) Nature 387, 101–105
19. Xu, H. & Shields, D. (1995) J. Cell Biol. 122, 1169–1184
20. Austin, C. D. & Shields, D. (1996) J. Cell Biol. 135, 1471–1483
21. Henderson, R. J. & Tocher, D. R. (1992) in Lipid Analysis, A Practical Approach (Hamilton, R. J., and Hamilton, S., eds) pp. 65–111, IRL Press, New York
22. Herman, P. K., Stack, J. H. & Emer, S. D. (1992) Trends Cell Biol. 2, 363–368
23. Jenkins, G. H., Fisette, P. L. & Anderson, R. A. (1994) J. Biol. Chem. 269, 11547–11554
24. Tüscher, O., Lorra, C., Bouma, B., Wirtz, K. W. A. & Huttner, W. B. (1997) FEBS Lett. 419, 271–275
25. Cardin, P., Paris, S., Antony, B., Robineau, S., Beraud-Dufour, S., Jackson, C. L. & Chabre, M. (1996) Nature 384, 481–484
26. Jones, S. M., Howell, K. E., Henley, J. R., Cao, H. & McNiven, M. A. (1998) Science 279, 573–577