RESEARCH COMMUNICATION

Formation of secretory vesicles in permeabilized cells: a salt extract from yeast membranes promotes budding of nascent secretory vesicles from the *trans*-Golgi network of endocrine cells

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The mechanism of secretory-vesicle formation from the *trans*-Golgi network (TGN) of endocrine cells is poorly understood. To identify cytosolic activities that facilitate the formation and fission of nascent secretory vesicles, we treated permeabilized pituitary GH₃ cells with high salt to remove endogenous budding factors. Using this cell preparation, secretory-vesicle budding from the TGN required addition of exogenous cytosol and energy. Mammalian cytosols (GH₃ cells and bovine brain)

promoted post-TGN vesicle formation. Most significantly, a salt extract of membranes from the yeast *Saccharomyces cerevisiae*, a cell lacking a regulated secretory pathway, stimulated secretory vesicle budding in the absence of mammalian cytosolic factors. These results demonstrate that the factors which promote secretory-vesicle release from the TGN are conserved between yeast and mammalian cells.

INTRODUCTION

The transport of macromolecules in eukaryotic cells follows a path in which cargo molecules are transported in membrane-bound vesicles from one compartment and fuse with an acceptor compartment in a highly ordered fashion. In the past 2 years it has become apparent that many of the components in the secretory pathway that mediate recognition of intracellular transport vesicles and their fusion with target membranes are functionally conserved between mammalian and yeast cells [1]. However, the mechanism whereby endocrine cells discriminate between proteins destined for the regulated or constitutive secretory pathways is still not well understood, although selective protein aggregation, initiated in the *trans*-Golgi network (TGN) has been implicated in this process [2,3]. Similarly, the molecular components that effect formation and release of different classes of secretory vesicle from the TGN have not been characterized. Recently, a phosphatidylinositol-transfer protein (PtdIns-TP), previously found to be involved in fusion of mature secretory granules with the plasma membrane of endocrine cells [4], was implicated in formation of nascent secretory vesicles from the TGN [5].

To identify cytosolic factors that mediate formation of nascent secretory vesicles in endocrine cells and hence selective protein compartmentalization in the late secretory pathway, we established a permeabilized cell system derived from rat pituitary GH₃ cells transfected with cDNA encoding preprosomatostatin (preproSRIF) [6]. We have now modified the permeabilized-cell system using a procedure which renders it completely dependent on exogenously added cytosol. As expected, cytosolic extracts derived from mammalian cells (GH₃ and bovine brain) stimulated secretory-vesicle formation. Here, we demonstrate that a high-salt extract from yeast (*Saccharomyces cerevisiae*) cell membranes can replace mammalian cytosol in promoting formation of post-TGN secretory vesicles from endocrine cells.

EXPERIMENTAL

Materials

[³⁵S]Methionine was obtained from Amersham International. Rat GH₃ cells expressing human preproSRIF, GH₃.Hu.S, were grown as described previously [7]. Rabbit anti-(growth hormone) (anti-GH) [6] and rabbit antisera to the free propeptide region of human proSRIF have been described [7].

Preparation of GH₃ cytosolic extract

GH₃.HuS cells were incubated in swelling buffer [8], followed by cell scraping, homogenizing and trituration in 'breaking buffer' (90 mM potassium acetate/2.5 mM magnesium acetate/20 mM Hepes, pH 7.2) once or twice through a 20-gauge needle and once through a 25-gauge needle. The homogenate was centrifuged at 800 *g* for 5 min at 4 °C to remove nuclei and unbroken cells, followed by centrifugation of the supernatant at 100 000 *g* for 60 min. The high-speed supernatant was dialysed overnight against breaking buffer and concentrated using a Centriprep-10 concentrator (Amicon). Aliquots were frozen in liquid N₂ and stored at −80 °C.

Preparation of yeast H1 membrane extract

A salt extract was prepared from yeast membranes based on a cytosol preparation method described by Vida et al. [9] using the yeast strain RS334 (*Matα*, *leu2-3, 112*, *gal1*, *ura3-52*, *reg1-501*, *pep4-3*, *prb1*) with the following modifications. The spheroplast suspension was vortex-mixed with glass beads twice for 1 min, each interspersed with a 1 min incubation on ice, followed by centrifugation at 3000 *g* for 15 min at 4 °C. The pellet containing crude membranes was resuspended in 250 mM sorbitol/500 mM potassium acetate/5 mM magnesium acetate/1 mM

dithiothreitol/20 mM Hepes, pH 6.8, and centrifuged at 3000 *g* for 20 min, followed by centrifugation of the supernatant at 40000 *g* for 15 min. The supernatant from this step was centrifuged further at 100000 *g* for 60 min at 4 °C. The 100000 *g* supernatant, designated as 'H1 extract', was dialysed overnight against breaking buffer, and the dialysed material was frozen in aliquots and stored at -80 °C.

Preparation of bovine brain cytosolic extract

Fresh bovine brain was homogenized in 250 mM sucrose/50 mM Tris/HCl (pH 7.2)/2 mM magnesium acetate/100 mM potassium acetate/1 mM PMSF using a Waring blender for 3 min at 4 °C. The homogenate was centrifuged at 1000 *g* for 10 min to remove nuclei and unbroken cells; the post-nuclear supernatant was centrifuged at 100000 *g* for 60 min and dialysed against breaking buffer before being frozen in aliquots in liquid N₂. The protein concentration in the each extract was determined using the Bio-Rad assay.

Preparation of permeabilized GH₃ cells

Cytosol-dependent permeabilized cells were prepared after a 10 min pulse-labelling with [³⁵S]methionine at 37 °C and a 2 h chase at 19 °C as described previously [6]. The permeabilized cells were washed once with breaking buffer and twice with a modified high-salt 'wash buffer' (250 mM potassium acetate/2.5 mM magnesium acetate/250 mM sucrose/20 mM Hepes, pH 6.8), followed by resuspension in assay buffer (90 mM potassium acetate/2.5 mM magnesium acetate/250 mM sucrose/20 mM Hepes, pH 6.8).

Vesicle budding from the TGN and immunoprecipitation

Aliquots of control and salt-washed permeabilized cells ($\approx 1.5 \times 10^5$ cells/sample) resuspended in assay buffer were incubated in a final volume of 100 μ l containing the indicated amount of extract and an energy-regenerating system [6]. To monitor secretory-vesicle formation, aliquots of the reaction mix were incubated on ice for 30 min with 25 mg/ml proteinase K and 4 mM tetracaine in the presence or absence of 0.5% Triton X-100. Following incubation, samples were centrifuged in a microfuge to separate nascent secretory vesicles (S) from the cell pellet (P). Samples were immunoprecipitated with antisera to GH or proSRIF as described previously [6,7]. Immunoreactive material was analysed by SDS/PAGE and fluorography. Fluorographs were quantified using a Molecular Dynamics computing densitometer [6]. All experiments were performed at least three times.

RESULTS

Preparation of cytosol-dependent permeabilized GH₃ cells

We have described previously a permeabilized-cell system where prohormone processing could be uncoupled from the formation of secretory vesicles from the TGN [6]. The permeabilized cells treated with 400 mM potassium acetate were unable to release nascent secretory vesicles from the TGN unless supplemented with cytosol [6]. However, treatment with such high-ionic-strength buffers frequently lead to lysis of cell nuclei, making it difficult to measure vesicle formation or prohormone processing. To circumvent this, we have stabilized the permeabilized cells by addition of isotonic concentrations of sucrose in conjunction with moderate salt (250 mM) treatment. This procedure rendered secretory-vesicle budding from the TGN cytosol-dependent with-

Figure 1 Immature secretory-vesicle formation requires cytosol

[³⁵S]Methionine-labelled permeabilized GH₃ cells [6] pre-treated with 250 mM potassium acetate were incubated with the indicated concentrations of extracts isolated from GH₃ cells (A), bovine brain (B) or *S. cerevisiae* (C) for 90 min at 30 °C in the presence of an energy-regenerating system. At the end of the reaction, the supernatant (S), corresponding to nascent secretory vesicles, and pellet (P) fractions were separated by brief centrifugation. Each fraction was incubated with anti-GH antibodies [6], followed by resolution of the immunoprecipitates by SDS/PAGE, using a 15% gel. (D) Quantification of vesicle-budding efficiency. Fluorographs from incubations containing an energy-regenerating system and extracts from GH₃ cells (●), bovine brain (○), yeast H1 extract (■), heated GH₃ cytosol (□), proteinase-treated GH₃ cytosol (▲) and post-ribosomal supernatant from yeast (△) were analysed using a Molecular Dynamics densitometer. Percentage GH budding = [intensity of GH in the supernatant (S)/total GH material in supernatant (S) + pellet (P)] × 100. Data are the averages from three separate experiments.

out causing cell lysis. In the absence of added cytosol, the salt-treated permeabilized GH₃ cells were unable to form nascent secretory vesicles (Figure 1, lanes 1 and 2). Addition of cytosol from GH₃ cells restored budding efficiency to a maximum of ≈ 35 –40% (Figure 1A, lanes 7–10), a level similar to that obtained in control permeabilized cells [6]. Addition of heat-treated or proteinase-digested GH₃ cell cytosol, by contrast, did not support vesicle formation above background levels (Figure 1D), indicating that the budding factor(s) are most likely proteins. Studies from other laboratories have demonstrated that bovine brain is a rich source of factors required for vesicle budding and fusion in the secretory pathway [10]. We therefore compared the ability of a brain cytosolic extract with that of GH₃ cytosol for its ability to stimulate formation of post-TGN secretory vesicles. Similar to GH₃ cytosol, the bovine brain extract also promoted vesicle budding in a concentration-dependent manner (Figure 1B).

Figure 2 Kinetics of vesicle formation from the TGN

Permeabilized cells pretreated with 250 mM potassium acetate were incubated with 6 mg/ml of either yeast H1 extract (A) or GH₃ cytosol (B) in the presence of an energy-regenerating system for the indicated times. At each time point, GH present in the supernatant (S) and pellet (P) fractions was determined by immunoprecipitation using anti-GH antibodies, followed by resolution of the immunoprecipitates by SDS/PAGE. (C) Quantification of vesicle budding efficiency. Fluorographs from incubations in which salt-washed permeabilized cells were incubated with 6 mg/ml of GH₃ cytosol (●), yeast H1 extract (○) or no cytosol (■), all in the presence of an energy-regenerating system, or 6 mg/ml of H1 extract in the absence of an energy-regenerating system (□).

Extract from yeast cells promotes vesicle budding from mammalian TGN

Many of the proteins that mediate recognition, budding and fusion of intracellular transport vesicles are conserved between mammalian and yeast cells [1,11]. We used a membrane extract (H1) from *S. cerevisiae* (see the Experimental section) to determine if yeast components could support vesicle budding from the TGN of mammalian cells (Figure 1C). The yeast H1 extract promoted secretory vesicle budding ≈ 5 -fold above background levels in a reaction that was concentration-dependent (Figure 1D). In contrast, a post-ribosomal supernatant from yeast gave only moderate stimulation (Figure 1D), suggesting that active yeast budding factors associate with the membranes and can be removed by salt extraction. Similar to the GH₃ cell cytosol, boiled or proteinase-treated H1 extract had no effect on vesicle formation (results not shown). In addition, a post-ribosomal supernatant from *Escherichia coli* or increasing concentrations of BSA did not stimulate vesicle budding (results not shown). Similar to the two mammalian cytosolic extracts, vesicle formation generated by H1 extract was also energy- and time-dependent (Figure 2). In the absence of ATP and GTP there was minimal secretory-vesicle budding from the TGN. The kinetics of vesicle formation in the presence of GH₃ cytosol (Figures 2B and 2C) were similar to that of control permeabilized GH₃ cells [6], with maximum budding at ≈ 45 –60 min of incubation. However,

Figure 3 GH is compartmentalized into nascent secretory vesicles

Permeabilized GH₃ cells, pre-treated with high salt, were incubated with 4.5 mg/ml yeast H1 extract for 90 min at 30 °C in the presence of an energy-regenerating system. Following the incubation, samples were treated with (lanes 3, 4, 7 and 8) or without (lanes 1, 2, 5 and 6) 25 mg/ml proteinase K in the presence (lanes 5–8) or absence (lanes 1–4) of 0.5% Triton X-100 for 30 min at 4 °C. Proteinase digestion was terminated by addition of 0.1 mM PMSF, and the pellet and supernatant fractions were separated by brief centrifugation (see the Experimental section). Each fraction was treated with anti-GH serum and the immunoprecipitates resolved by SDS/PAGE.

vesicle budding in the presence of H1 extract reached a plateau after ≈ 90 min and was only $\approx 50\%$ of the level obtained with the GH₃ cell extract (Figures 2A and 2C). These data suggest that H1 may lack specific budding components that are present in GH₃ cell cytosol.

Cargo proteins in nascent vesicles are resistant to proteinase treatment

To exclude the possibility that GH was released as a result of membrane lysis following high-salt treatment or the presence of lipases in H1 extract, aliquots of the incubation were digested with and without proteinase K in the presence and absence of Triton X-100 (Figure 3). In the presence of H1 extract where the budding efficiency was approx. 25% efficient (lanes 1 and 2), a major portion of the GH-immunoreactive material in the supernatant fraction was resistant to proteinase K treatment (lanes 3 and 4). When the membranes were solubilized with Triton X-100, the GH-immunoreactive material in the supernatant was now sensitive to proteinase K digestion (lanes 7 and 8). Together these results indicate that H1 yeast extract did not damage the integrity or function of the mammalian TGN and that GH and proSRIF (results not shown) were present in membrane-bound vesicles.

Vesicle formation but not prohormone processing is cytosol-dependent

Proteolytic processing of prohormones requires the activity of endocrine-cell-specific proteinases, designated prohormone convertases (PCs), [12]. Data from other laboratories has shown that GH₃ cells possess PC2, an enzyme whose substrates include proSRIF [13]. We have demonstrated that proSRIF cleavage can be achieved by incubating permeabilized cells at a pH of ≈ 6.1 with micromolar levels of free calcium [6,14,15]. Since yeast H1 extract promotes vesicle budding (Figures 1 and 2), we examined whether prohormone processing occurred independently of these budding components. Permeabilized GH₃/Hu.S cells expressing human proSRIF [7] were salt-washed and incubated in the presence and absence of H1 extract for indicated times (Figure 4). The efficiency of prohormone processing and vesicle budding were determined simultaneously by treating the vesicle fraction and residual permeabilized cells with antiserum against the SRIF propeptide. In agreement with the data of Figure 1, vesicle formation required yeast H1 extract (lanes 1–16), and no budding

Figure 4 Vesicle budding, but not prohormone processing, is cytosol-dependent

(A) Permeabilized cells radiolabelled with [35 S]methionine were incubated in the presence of an energy-regenerating system at 30 °C with 6 mg/ml yeast H1 extract for the indicated times (lanes 1–16) or without added extract for 120 min (lanes 17 and 18). At each time point, aliquots of the incubation mixture were separated into supernatant and pellet fractions, which were treated with an antibody directed against the proregion of SRIF [7]. The immunoprecipitable material was resolved by SDS/PAGE using a 20% gel, followed by fluorography. The arrowhead depicts the 11 kDa propeptide. (B) Densitometric analysis of proSRIF processing and vesicle budding efficiency. The gel in (A) was analysed by densitometry and the efficiency of vesicle formation and proSRIF processing determined. Percentage budding = [intensity of SRIF in (S)/total SRIF material in (S) + (P)] $\times$ 100; percentage processing = [intensity of free propeptide in (S + P)/total SRIF-immunoreactive material in S + P] $\times$ 100. ●, ○, Vesicle budding in the presence (●) or absence (○) of H1 extract. ■, □, ProSRIF processing in the presence (■) or absence (□) of H1 extract.

was observed even after 2 h incubation in the absence of H1 (lanes 17 and 18). Consistent with our previous observations [6], proSRIF processing occurred independently of added yeast extract (compare lanes 16 and 18) and therefore could be initiated in the TGN in the absence of secretory-vesicle formation.

DISCUSSION

Prohormone processing appears to be tightly coupled to the formation of nascent immature secretory granules, although these two processes can be uncoupled *in vitro* [6,15]. The biochemical components that mediate nascent secretory-vesicle formation from the TGN of endocrine cells are not well characterized, although clathrin-coated nascent secretory vesicles have been identified morphologically [16]. In an attempt to identify cytosolic activities involved in this process, we depleted permeabilized cells of endogenous cytosol by treatment with high-ionic-strength buffers (250 mM potassium acetate) and restored vesicle budding activity by addition of heterologous extracts. A cytosolic extract [6; Figures 1 and 2) as well as a high-salt extract [6] from GH₃ cells supported vesicle formation

in a concentration-, energy- and time-dependent manner. Indeed, the vesicle budding efficiency ($\approx 40\%$) was indistinguishable from that of control non-salt-treated permeabilized cells [6,15]. In addition, a cytosolic extract from bovine brain also stimulated vesicle budding approx. fivefold above background (Figure 1).

Many of the components that mediate endoplasmic-reticulum-to-Golgi transport and vesicle fusion with the plasma membrane are remarkably conserved in function between yeast cells and mammalian neurons [1,11]. Recently, Ohashi et al. [5] demonstrated that, in the presence of limiting concentrations of mammalian cytosol, purified mammalian PtdIns-TP can be replaced by yeast SEC14p in an *in vitro* vesicle budding assay which promotes both regulated and constitutive vesicle formation [5]. By contrast, in our system, factors released from *S. cerevisiae* membranes alone could support secretory-vesicle budding from the TGN in the absence of mammalian cytosol. However, the yeast extract had only $\approx 50\%$ of the GH₃ cytosol activity (Figures 1C and 2), suggesting that SEC14p, which is a cytosolic protein [17], alone is not sufficient for the formation of both regulated and constitutive vesicles, but rather additional factors, absent in yeast, are also required. In this context it is noteworthy that the budding efficiency of vesicles containing SRIF, a hormone that is efficiently sorted to the regulated pathway in GH₃ cells [18], was relatively low in the presence of yeast membrane salt extract (Figure 4). In contrast, the budding of nascent vesicles containing GH, which is poorly stored in these cells [18], was quite efficient. A possible explanation for these observations is that the yeast extract preferentially promoted constitutive vesicle formation, whereas GH₃ cytosol stimulated release of both regulated and constitutive secretory vesicles. To test this hypothesis, we are currently: (1) purifying fractions from yeast extracts which stimulate constitutive vesicle budding; and (2) supplementing these partially purified activities with cytosolic fractions from endocrine cells to identify factors that are specific for the formation of regulated vesicles.

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