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RESEARCH COMMUNICATION

Reconstitution *in vitro* of a membrane-fusion event involved in constitutive exocytosis

A role for cytosolic proteins and a GTP-binding protein, but not for Ca^{2+}

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The fusion of post-Golgi transport vesicles with the plasma membrane is perhaps the least well understood step in the network of intracellular membrane traffic. We have used an '*in vitro*' system to study this membrane-fusion event. We show here that fusion requires the presence of cytosolic proteins, but not Ca^{2+}, and is inhibited by the non-hydrolysable GTP analogue guanosine 5'-[γ-thio]triphosphate, which indicates the involvement of a GTP-binding protein.

INTRODUCTION

Transport of membranes and proteins in eukaryotic cells is accomplished by the specific targeting and fusion of vesicles. Over the last few years it has become apparent that the mechanisms involved in various intracellular membrane-fusion events are basically similar (Rothman & Orci, 1992). Perhaps the least well understood step in the intracellular transport network is the membrane-fusion event responsible for the delivery of proteins to the plasma membrane. This step has been studied in both intact (Turner *et al.*, 1992) and permeabilized (de Curtis & Simons, 1988; Gravotta *et al.*, 1990; Miller & Moore, 1991) cells. However, it has proved difficult, using this approach, to distinguish requirements for membrane fusion from earlier events, such as vesicle budding and transport.

We have previously reported the reconstitution *in vitro* of the delivery of a newly synthesized protein to the plasma membrane of baby-hamster-kidney (BHK) cells (Woodman & Edwardson, 1986). In this system, membrane vesicles containing influenza neuraminidase fuse with vesiculated plasma membranes. Fusion is assayed through the ability of neuraminidase to cleave [^{3}H]sialic acid residues from the envelope proteins of Semliki-Forest virions (SFV) bound to the external surface of the plasma membrane before vesiculation. A membrane-fusion event can be identified that requires ATP hydrolysis (Edwardson & Daniels-Holgate, 1990), that is specific and that is abolished by proteolytic treatment of the cell extracts (Woodman & Edwardson, 1986). In the present paper we show that fusion requires cytosolic proteins, but not Ca^{2+}, and is inhibited by the non-hydrolysable GTP analogue guanosine 5'-[γ-thio]triphosphate (GTP[S]), which indicates the involvement of a GTP-binding protein.

METHODS

The sources of cells, viruses and chemicals were as described previously (Woodman & Edwardson, 1986). Preparation of neuraminidase-containing 'donor' post-nuclear supernatants (from influenza-virus-infected cells), of corresponding blank donors (from non-infected cells) and of 'acceptors' containing [^{3}H]SFV, as well as the procedure for the '*in vitro*' membrane-fusion assay were also as described (Woodman & Edwardson, 1986), with the modifications detailed by Edwardson & Daniels-Holgate (1990). Membranes were obtained from post-nuclear supernatants by centrifugation at 8000 *g* for 30 min. Membrane pellets were washed twice and then resuspended in assay buffer to a protein concentration of about 1 mg/ml. Cytosol was prepared by centrifugation of a post-nuclear supernatant from untreated cells at 100000 *g* for 1 h. A typical protein concentration of a cytosol sample was 5 mg/ml. The final concentrations (in the incubation mixtures) of donor and acceptor membrane protein, and of cytosolic protein, were all approx. 200 μg/ml. Radioactivity in acceptor preparations was typically 5000 d.p.m./sample. All incubations were at 37 °C for 2 h. Triplicate determinations were made for each condition in al experiments.

RESULTS AND DISCUSSION

Extent of fusion

As explained previously (Woodman & Edwardson, 1986; Edwardson & Daniels-Holgate, 1990), membrane fusion is measured through the transfer of influenza-virus neuraminidase, initially present in vesicles in the donor post-nuclear supernatant, to vesicles in the acceptor post-nuclear supernatant, which contain SFV with [^{3}H]sialic acid-labelled envelope proteins. Transfer is routinely expressed as the percentage of the total [^{3}H]sialic acid in the acceptor that is cleaved from the labelled proteins in an ATP- and neuraminidase-dependent manner. All incubation mixtures contain the sialic acid analogue 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, which cannot cross membranes and inhibits any neuraminidase that is not protected within vesicles. Consequently, we can be confident that the signal detected in the cell-free system is indeed measuring membrane fusion. It has been shown (Woodman & Edwardson, 1986) that the [^{3}H]SFV present in sealed 'inside-out' plasma-membrane vesicles represents 16% of the total, and that the maximum possible cleavage of [^{3}H]sialic acid from free [^{3}H]SFV by neuraminidase is 60%. Hence, if all of the [^{3}H]SFV present within

Abbreviations used: GTP[S], guanosine 5'-[γ-thio]triphosphate; BHK, baby-hamster kidney; [^{3}H]SFV, ^{3}H-labelled Semliki-Forest virus (virions); MDCK, Madin–Darby canine kidney; CHO, Chinese-hamster ovary.

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Table 1. Requirement for cytosolic proteins

Donor and acceptor membranes were used at a concentration of approx. 50 μg/ml). Cytosol concentration was also 50 μg/sample (200 μg/ml). Trypsin treatment of cytosol was at 100 μg/ml for 1 h on ice; soybean trypsin inhibitor (100 μg/ml) was then added, before the cytosol was used in incubations. Samples were incubated for 2 h at 37 °C in the presence or absence of ATP. At the end of the incubation, protein was precipitated by addition of 10% (w/v) trichloroacetic acid, followed by centrifugation, and the [^{3}H]sialic acid present in the supernatant was determined. Values are means (±S.E.M.) from several experiments of the ATP-dependent release of [^{3}H]sialic acid, corrected for any release given by blank donor membranes; the number of experiments is given in parentheses.

Experimental condition	Release of [^{3}H]sialic acid (%)
Membranes only	0.6±0.2 (8)
Membranes+cytosol	2.0±0.5 (8)
Membranes+trypsin-treated cytosol	0.3±0.3 (3)

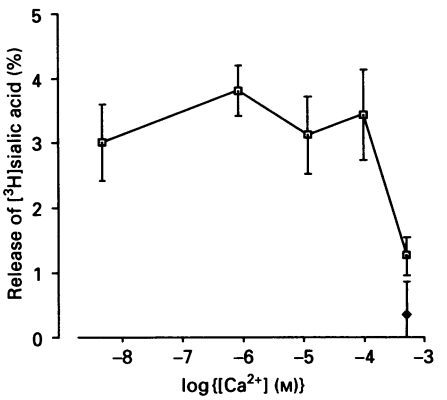

Fig. 1. Concentration–effect relationship for Ca^{2+}

Ca^{2+} was buffered with EGTA (1 mM). Free Ca^{2+} concentrations were calculated by using a program devised by Fabiato & Fabiato (1979). Donors (⊡), or blank donors (◆), and acceptors were incubated together in the presence or absence of ATP. Values are the means (±S.E.M.) for the ATP-dependent release of [^{3}H]sialic acid obtained in a single experiment. The small release given by blank donor was unaffected by Ca^{2+} concentration.

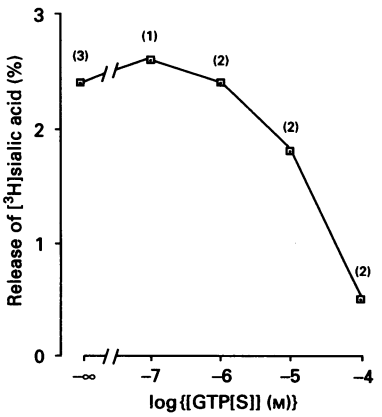

Fig. 2. Effect of GTP[S]

Donor and acceptor post-nuclear supernatants were incubated together in the presence of various concentrations of GTP[S]. Values are for ATP-dependent release of [^{3}H]sialic acid, corrected for any release given by blank donor. Results are means for up to three experiments (values in parentheses above each point).

sealed 'inside-out' vesicles becomes exposed to neuraminidase, approx. 10% of the total [^{3}H]sialic acid will be released. The percentage of the fusion-competent acceptor vesicles that receive donor vesicles, therefore, will be approx. 10 times the percentage of the [^{3}H]sialic acid released in the assay.

Requirement for cytosolic proteins

To assess the dependence of exocytotic membrane fusion on cytosolic proteins, membranes were centrifuged out of donor and acceptor post-nuclear supernatants, and incubations carried out with membranes alone and with membranes supplemented with cytosol (200 μg/ml). The results obtained are shown in Table 1. It was found that membranes incubated alone had 30% of the activity of membranes supplemented with cytosol. The most reasonable interpretation of this result is that factors in the cytosol are required for fusion (so that cytosol is required for full activity), but that these factors are able to bind to some extent to the donor/acceptor membranes (so that membranes centrifuged out of post-nuclear supernatants retain some activity). When membranes were supplemented with cytosol that had been treated with trypsin (100 μg/ml for 1 h on ice), activity was not significantly different from that obtained with membranes alone. Hence, at least some of the necessary components in the cytosol are likely to be proteins.

A requirement for cytosolic proteins has been found for several other steps on the intracellular membrane-transport pathway, and some of these proteins have now been identified (Rothman & Orci, 1992). Furthermore, the involvement of cytosolic components specifically in the transport of proteins from the Golgi complex to the plasma membrane has recently been demonstrated in permeabilized Madin–Darby canine kidney (MDCK) cells (Gravotta *et al.*, 1990) and in permeabilized Chinese-hamster ovary (CHO) cells (Miller & Moore, 1991).

Requirement for Ca^{2+}

The effect of varying the concentration of free Ca^{2+} on exocytotic membrane fusion is shown in Fig. 1. It can be seen that fusion is unaffected by changes in Ca^{2+} concentration over the range 5 nM to 100 μM, but is inhibited at 500 μM. This result indicates that Ca^{2+} is not required for the membrane-fusion event involved in the constitutive delivery of proteins to the plasma membrane, at least in this cell type.

Although the role of Ca^{2+} in regulated exocytosis is well-established (Knight *et al.*, 1990), the Ca^{2+}-sensitive step in this process has not yet been identified. Work from this laboratory has in fact shown that, although regulated exocytosis in (for example) permeabilized pancreatic acinar cells requires Ca^{2+} (Edwardson *et al.*, 1990), the fusion of zymogen granules with plasma membranes *in vitro* does not (Nadin *et al.*, 1989; MacLean & Edwardson, 1992), which suggests that the Ca^{2+}-dependent step is upstream of the final membrane-fusion event. The involvement of Ca^{2+} in the constitutive delivery of proteins to the plasma membrane is also somewhat controversial. It has been reported, for example, that Ca^{2+}, at a physiological concentration of about 100 nM, is necessary for the delivery of the SFV envelope protein p62 to the plasma membrane in permeabilized BHK cells (de Curtis & Simons, 1988), although it is not clear whether Ca^{2+} is required for membrane fusion itself or simply to prevent post-Golgi vesicles from escaping from the cells. In contrast with this result, neither the transport of proteins from the Golgi complex to the plasma membrane in permeabilized CHO cells (Miller & Moore, 1991) nor the constitutive secretion of proteins from intact mammary epithelial cells (Turner *et al.*, 1992) showed any dependence on Ca^{2+}. A requirement for Ca^{2+} (100 nM) has been reported for endoplasmic-reticulum–Golgi transport in permeabilized CHO cells (Beckers & Balch, 1989),

although it has been suggested that Ca^{2+} may be required for correct protein folding rather than for transport itself (Helms *et al.*, 1990). Our result provides direct evidence that membrane fusion in constitutive exocytosis occurs independently of Ca^{2+}.

Involvement of a GTP-binding protein

The effect of the non-hydrolysable GTP analogue GTP[S] on exocytotic membrane fusion is shown in Fig. 2. GTP[S] inhibited fusion, with 50% inhibition occurring at a concentration of about 10 μM.

It is now generally accepted that vesicular transport between organelles is under the control of a family of GTP-binding proteins, and that GTP hydrolysis is required for membrane-fusion events on the intracellular transport pathway (Bourne, 1988; Goud & McCaffrey, 1991). In mammalian cells, GTP[S], at concentrations in the micromolar range, blocks several steps on the intracellular transport pathway, for example, endoplasmic-reticulum–Golgi transport (Beckers & Balch, 1989), intra-Golgi transport (Melançon *et al.*, 1987), fusion of endocytic vesicles (Mayorga *et al.*, 1989) and recycling of the mannose 6-phosphate receptor (Goda & Pfeffer, 1988). A similar situation exists in yeast cells, where GTP-binding proteins, such as Sec4p and Ypt1p, have been shown to be involved in membrane transport at different stages of the exocytotic pathway (Salminen & Novick, 1987; Segev *et al.*, 1988).

Transport of protein from the Golgi complex to the plasma membrane has been shown to be inhibited by GTP[S] in permeabilized MDCK (Gravotta *et al.*, 1990) and CHO (Miller & Moore, 1991) cells. Since it is also known that budding of vesicles from the *trans*-Golgi network is inhibited by GTP[S], however (Tooze *et al.*, 1990), it is not clear from this work on permeabilized cells whether exocytotic membrane fusion itself involves a GTP-binding protein. Our result demonstrates that this is indeed the case.

The '*in vitro*' assay for constitutive exocytosis described here has enabled us to add this membrane-fusion event to the growing list of steps on the intracellular transport pathway that require cytosolic proteins and involve a GTP-binding protein. In addition, we have provided further evidence that this step occurs independently of Ca^{2+}.

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