Characteristics of a cell-free assay for the delivery of proteins to the plasma membrane

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We have previously described the reconstitution, in a cell-free system, of the constitutive delivery of a newly synthesized protein, influenza neuraminidase, to the plasma membrane in BHK cells. Here we report some of the characteristics of this in vitro membrane fusion event. We show that fusion requires ATP hydrolysis, and exploit this requirement to distinguish the time-course of fusion from that of neuraminidase action. In addition, we present evidence for the occurrence of multiple fusions between hybrid membrane vesicles.

Membrane fusion; Plasma membrane; Influenza neuraminidase

1. INTRODUCTION

It has been pointed out many times that the molecular basis of intracellular protein transport will become accessible to biochemical analysis only when the various transport steps have been reconstituted in cell-free systems [1]. To this end, cell-free assays have been developed for many of the major transport steps, including endoplasmic reticulum-to-*cis* Golgi [2] and intra-Golgi [3,4] transport, constitutive [5] and regulated [6–8] delivery to the plasma membrane, and membrane fusion events on the endocytic pathway [9–13]. These assays are now being used to elucidate the molecular details of intracellular protein transport [1].

We have previously reported the reconstitution, in a cell-free system, of the insertion of a newly synthesized protein into the plasma membrane of BHK cells [5]. In this system, membrane vesicles containing influenza neuraminidase, initially present in a 'donor' post-nuclear supernatant, fuse with vesiculated plasma membranes from an 'acceptor' post-nuclear supernatant. Fusion is monitored through the ability of neuraminidase to cleave ^{3}H-labelled sialic acid residues from the envelope proteins of Semliki Forest virus (SFV) particles, that are bound to the outside surface of the plasma membrane before vesiculation. A membrane fusion event occurs that is dependent on the presence of ATP, that is specific (in that fusion activity is maximal when neuraminidase is concentrated at a late stage in the exocytic transport pathway) and that is abolished by proteolytic treatment of the cell extracts. This paper describes a further characterization of this in vitro membrane fusion event.

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2. MATERIALS AND METHODS

The sources of all cells, viruses and chemicals, the preparation of donor and acceptor post-nuclear supernatants and the procedure for the cell-free assay were as described previously [5], with the following two modifications: the buffer solution used in the preparation of the post-nuclear supernatants was 10 mM Tris-HCl, pH 7.5, 1 mM magnesium acetate (i.e. no sucrose was added) and a cocktail of protease inhibitors (benzamidine 17 μg/ml, pepstatin, 1 μg/ml, phenylmethylsulphonylfluoride 1 mM, antipain 1 μg/ml, soybean trypsin inhibitor 10 μg/ml and bacitracin 50 μg/ml) was added to all incubations. ATP-generating cocktails contained, as before (final concentrations) ATP 1 mM, creatine phosphate 8 mM, creatine phosphokinase 7 IU/ml; ATP-depleting cocktails contained (final concentrations) hexokinase 25 IU/ml, glucose 5 mM. Typically, about 50 μg of both donor and acceptor membrane protein and 50 μg cytosolic protein was added to an incubation volume of 250 μl. Radioactivity in acceptor preparations was about 5000 dpm sample.

3. RESULTS AND DISCUSSION

Membrane fusion in the cell-free system has been shown to depend on the presence of ATP [5]. In was not clear, however, whether ATP hydrolysis was required. To answer this question, the ability of a non-hydrolysable analogue of ATP, 5′-adenylylimidodiphosphate (AMP-PNP), to support fusion was tested. Normal incubations, with ATP-generating and ATP-depleting cocktails, were carried out, together with a third incubation in which AMP-PNP was added to a sample that had been depleted of ATP. Table I shows

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Table 1

Test of the ability of AMP-PNP to support membrane fusion

Experimental condition	Release of [^{3}H]sialic acid (%)
blank donor - ATP + AMP-PNP	0.0 ± 0.4
blank donor + ATP	0.0 ± 0.4
donor - ATP + AMP-PNP	0.0 ± 0.4
donor + ATP	1.5 ± 0.6

Donor (or blank donor) and acceptor post-nuclear supernatants were incubated together 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% trichloroacetic acid followed by centrifugation, and the [^{3}H]sialic acid present in the supernatant determined. AMP-PNP (1 mM) was added to some ATP-depleted samples. Release of [^{3}H]sialic acid, expressed as a percentage of the total present in the sample, is corrected for the background release obtained in the absence of ATP.

Values are means ± SE from two separate experiments ($n = 6$).

the values for release of [^{3}H]sialic acid given by both a blank donor (prepared from uninfected cells) and a neuraminidase-containing donor. It is apparent that while ATP supported a neuraminidase-dependent release of [^{3}H]sialic acid above background, AMP-PNP did not. This result indicates that hydrolysis of ATP is required to drive membrane fusion in this cell-free system. It has been shown [5] that latent [^{3}H]SFV (i.e. that present in sealed 'inside-out' plasma membrane vesicles) typically 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 sealed 'inside-out' vesicles becomes exposed to neuraminidase, approximately 10% of the total [^{3}H]sialic acid will be released. The 1.5% release of [^{3}H]sialic acid shown in Table I, therefore, indicates that 15% of the acceptor vesicles have fused with donor vesicles.

The time-course of ATP-dependent release of [^{3}H]sialic acid is sigmoid, with a maximum rate of release at 30 min and saturation at about 60 min [5]. The most resonable interpretation of this result is that delivery of neuraminidase to its substrate is complete after 30 min (the point at which the rate of product release peaks) and that the enzyme-substrate interaction then continues to completion over the next 30 min. In order to test this idea, it was necessary to devise a method for measuring membrane fusion itself. The method chosen exploits the fact that fusion requires ATP whereas neuraminidase activity does not. Samples were incubated initially in the presence of an ATP-generating cocktail. At various times, an ATP-depleting cocktail was added and the incubation continued for a further 60 min to allow enzyme-substrate interaction to proceed to completion. The time-course of the

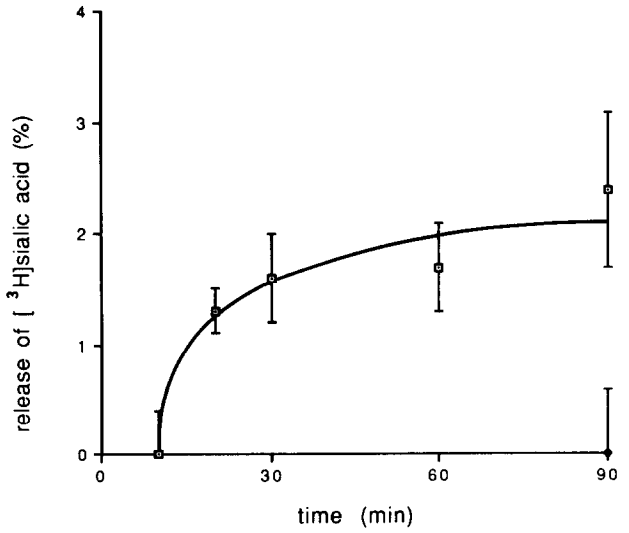

Fig. 1. Time-course of fusion. Samples of acceptor were incubated with either donor (⊡) or blank donor (♦) for various times in the presence of ATP. An ATP-depleting cocktail was then added and incubation continued for a further 60 min. Values are means ± SE of triplicate determinations and are corrected for [^{3}H]sialic acid release occurring in the absence of ATP.

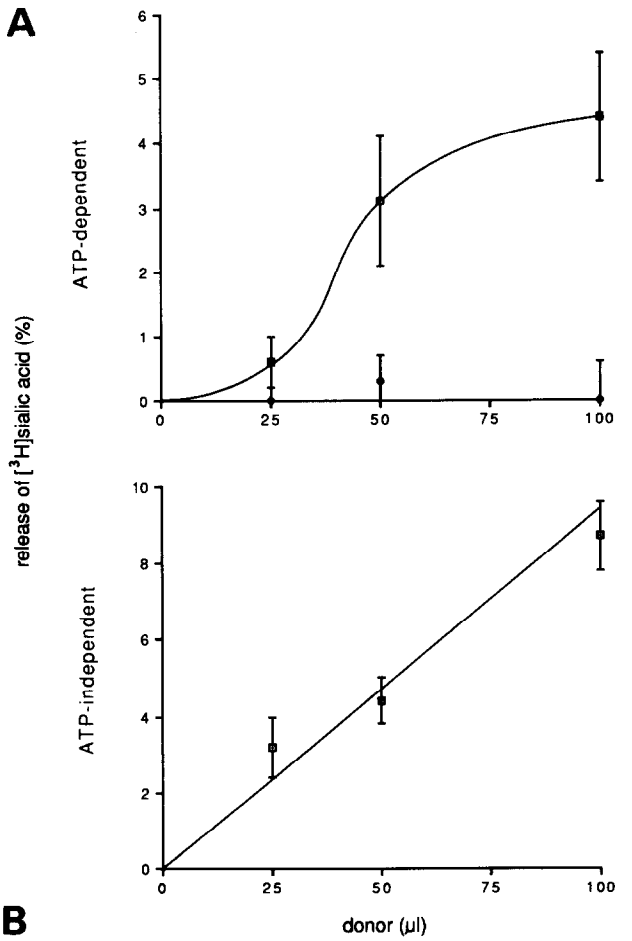

Fig. 2. Dependence of [^{3}H]sialic acid release on donor concentration. Samples of acceptor (25 µl) were incubated for 2 h at 37°C with various volumes of donor (⊡) or blank donor (♦). (A) ATP-dependent release of [^{3}H]sialic acid. (B) ATP-independent release given by donor, corrected for release occurring with blank donor. Values are means ± SE of triplicate determinations.

ATP-dependence of release of [^{3}H]sialic acid generated in this way is shown in Fig. 1. This time-course, which represents the time-course of fusion, has a 10 min lag phase and then a rising phase of half-time approximately 10 min. Fusion is almost over after 30 min, when the rate of [^{3}H]sialic acid release in the earlier time-course [5] is maximal.

The relationship between donor vesicle concentration and [^{3}H]sialic acid release was investigated, in order to determine whether or not multiple fusion events between hybrid vesicles occur in the cell-free system. The fact that release of cleavable, latent [^{3}H]sialic acid is of the order of 10–20% at the normal donor:acceptor concentration ratio of 1:1 suggests that the number of fusion-competent donor vesicles in a typical incubation is limiting. If only single fusion events occur, then at a ratio of 1:1, half of the donor vesicles will fuse with labelled acceptor vesicles and half with 'blank' acceptor vesicles present in the donor post-nuclear supernatant. Now if the ratio is increased to 2:1, only one-third of the donor vesicles will fuse with labelled acceptor vesicles. However, since there are now twice as many donor vesicles the delivery of neuraminidase to latent [^{3}H]SFV will increase, maximally, by a factor of 1.33. For a concentration ratio of 4:1 this factor will be 1.6, and as the concentration ratio tends to infinity, it will tend to become 2-fold. Another effect of increasing the donor:acceptor ratio will be to increase the proportion of the labelled acceptor vesicles that fuse with active donor vesicles, rather than with 'blank' donor vesicles present in the acceptor post-nulear supernatant. Since only a small proportion (10–20%) of the acceptor vesicles fuse with either active or blank donor vesicles initially, however, the contribution of this effect to the predicted increase in the ATP-dependent release of [^{3}H]sialic acid will be small. If fusion between hybrid vesicles is permitted, the ATP-dependent release of [^{3}H]sialic acid should tend towards the maximum value of 10% as the donor:acceptor concentration ratio rises. In both cases the ATP-independent release, which is apparently a consequence of residual extravesicular neuraminidase activity and not related to fusion [5], should rise linearly with concentration ratio. In the experiment illustrated in Fig. 2, the donor:acceptor ratio was increased from 1:1 to 4:1 and ATP-dependent and independent release of [^{3}H]sialic acid was measured. It can be seen that the ATP-dependent release of [^{3}H]sialic acid increased from 0.6% at a ratio of 1:1 to 4.4% at a ratio of 4:1, a rise of more than 7-fold. This result indicates that multiple fusions occur, as has recently been reported for an in vitro fusion event occurring on the endocytic pathway [13], and that at high donor:acceptor ratios neuraminidase gains access to about half of the latent [^{3}H]SFV. ATP-independent release rises approximately linearly from 3.2% to 8.7%, as expected.

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REFERENCES

[1] Balch, W.E. (1989) J. Biol. Chem. 264, 16965–16968.
[2] Balch, W.E., Wagner, K.R. and Keller, D.S. (1987) J. Cell Biol. 104, 749–760.
[3] Balch, W.E., Dunphy, W.G., Braell, W.E. and Rothman, J.E. (1984) Cell 39, 405–416.
[4] Rothman, J.E. (1987) J. Biol. Chem. 262, 12502–12510.
[5] Woodman, P.G. and Edwardson, J.M. (1986) J. Cell Biol. 103, 1829–1835.
[6] Whitaker, M.J. and Baker, P.F. (1983) Proc. Roy. Soc. Lond. Ser. B 218, 397–413.
[7] Crabb, J.H. and Jackson, R.C. (1985) J. Cell Biol. 101, 2263–2273.
[8] Nadin, C.Y., Rogers, J., Tomlinson, S. and Edwardson, J.M. (1989) J. Cell Biol. 109, 2801–2808.
[9] Gruenberg, J.E. and Howell, K.E. (1986) EMBO J. 5, 3091–3101.
[10] Braell, W.A. (1987) Proc. Natl. Acad. Sci. USA 84, 1137–1141.
[11] Woodman, P.G. and Warren, G. (1988) Eur. J. Biochem. 173, 101–108.
[12] Diaz, R., Mayorga, L. and Stahl, P. (1988) J. Biol. Chem. 263, 6093–6100.
[13] Wessling-Resnick, M. and Braell, W.A. (1990) J. Biol. Chem. 265, 690–699.