

A Cell-free Assay for the Insertion of a Viral Glycoprotein into the Plasma Membrane

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Abstract. A cell-free assay has been developed for the delivery of influenza virus neuraminidase to the plasma membrane. Two types of postnuclear supernatant, which acted as donor and acceptor of the enzyme, were prepared from baby hamster kidney cells. Donor preparations were obtained from cells infected with influenza virus and containing neuraminidase en route to the plasma membrane. Acceptor preparations were obtained from cells containing, bound to their plasma membranes, Semliki Forest virus with envelope glycoproteins bearing [³H]N-acetylneuraminic acid. Fusion between vesicles from these two preparations permits access of the enzyme to its substrate, which results in the release of free [³H]N-acetylneur-

aminic acid. This release was detected through the transfer of radioactivity from a trichloroacetic acid-insoluble to a trichloroacetic acid-soluble fraction. An ATP-dependent component of release was found, which appears to be a consequence of vesicle fusion. This component was enhanced when the donor was prepared from cells in which the enzyme had been concentrated in a compartment between the Golgi complex and the plasma membrane, which indicates that a specific exocytic fusion event has been reconstituted. The extent of fusion is greatly reduced by pre-treatment of donor and acceptor preparations with trypsin, which points to the involvement of proteins in the fusion reaction.

IN eukaryotic cells transport of proteins between organelles is believed to be mediated by vesicles that bud off one compartment and fuse with another (Palade, 1975). To avoid randomization of membrane components as a consequence of this extensive vesicular traffic, vesicles must be able to select and package proteins that are to be transported out of an organelle, leaving behind resident proteins, and then diffuse towards and fuse specifically with the target organelle. The elucidation of the molecular details of this protein transport system will require the reconstitution of its various components in cell-free preparations. This approach has already yielded important information about the transport of membrane proteins through the Golgi complex (Balch et al., 1984a, b; Braell et al., 1984). In this paper we describe a cell-free assay for the final step in the intracellular transport pathway of a newly synthesized viral glycoprotein, that is, its delivery into the plasma membrane.

In cells infected with influenza virus, the viral envelope glycoproteins, haemagglutinin and neuraminidase, are synthesized in large amounts (Rodriguez-Boulan and Pendergast, 1980; Fuller et al., 1985) and are transported from their site of synthesis in the endoplasmic reticulum to the plasma membrane via the Golgi complex (Rodriguez-Boulan et al., 1984; Rindler et al., 1984; Edwardson, 1984). They have, therefore, been used extensively as model plasma membrane proteins. We decided to exploit the enzyme activity of neuraminidase in designing a cell-free assay for fusion of post-Golgi vesicles with the plasma membrane.

Materials and Methods

Cells and Virus

Baby hamster kidney (BHK-21) cells were grown as monolayers in Glasgow's minimum essential medium (GMEM)¹ supplemented with 10% newborn calf serum, 10% tryptose phosphate broth, and 160 µg/ml gentamicin. All cells, medium, and supplements were supplied by Flow Laboratories, Rickmansworth, Herts.

The WSN (H1N1) strain of influenza virus, initially obtained from Dr. S. C. Inglis (Department of Pathology, University of Cambridge, UK), was grown in fertile hens' eggs, purified by centrifugation on a continuous sucrose density gradient (15–60% wt/vol in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl) for 2 h at 100,000 g and assayed by plaquing on Madin-Darby canine kidney (MDCK) cell monolayers. Stocks of virus were stored in liquid nitrogen.

Semliki Forest virus (SFV), initially obtained from Dr. T. R. Hesketh (Department of Biochemistry, University of Cambridge, UK), was grown on BHK-21 cells. Cells (10⁸ at 50% confluence on 5 × 14-cm diam tissue culture dishes) were washed twice with Eagle's minimum essential medium containing 0.2% (wt/vol) bovine serum albumin (BSA; Sigma, Poole, Dorset) and 20 mM Hepes, pH 6.8, and infected with 0.01 plaque-forming units (pfu) of SFV/cell in the same medium for 1 h at 37°C. The medium was then replaced with 50 ml complete GMEM. After 15 h at 37°C the medium was harvested and centrifuged at 5,000 g for 30 min to sediment cell debris. SFV was sedimented by centrifugation at 60,000 g for 2.5 h and purified by centrifugation for 2 h at 100,000 g on a gradient consisting of 10–20% (wt/vol) sucrose in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl above 25–50%

1. *Abbreviations used in this paper:* DD-NANA, 2,3-dehydro-2-desoxy-N-acetylneuraminic acid; GMEM, Glasgow's modified Eagle's medium; NANA, N-acetylneuraminic acid; pfu, plaque-forming unit; SFV, Semliki Forest virus; STM, sucrose, Tris, magnesium acetate.

(wt/vol) sucrose in the same buffer. Stocks of virus were stored in liquid nitrogen.

Preparation of [³H]SFV

Preparation of [³H]SFV was as described above for SFV, except that the post-infection maintenance medium contained 5 mCi [³H]N-acetyl-D-mannosamine (30 Ci/mmol; New England Nuclear, Stevenage, Herts). The envelope glycoproteins of shed virions consequently contained [³H]N-acetylneuraminic acid ([³H]NANA) (Monaco and Robbins, 1973).

Preparation of Donor Postnuclear Supernatant

BHK cells (10^8 at near-confluence on 3×14 -cm diam tissue culture dishes) were washed twice with 30 ml GMEM containing 0.2% (wt/vol) BSA and infected with 10 pfu influenza virus/cell in the same medium for 1 h at 37°C. The medium was replaced with GMEM containing 2% newborn calf serum. After a further 4 h at 37°C, the cells were either harvested at once or incubated for a further 1 h at 20°C. The medium was discarded and the cells were washed twice with 30 ml infection medium without virus and twice with 30 ml STM buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM magnesium acetate). The dishes were drained and the cells were scraped off using a rubber scraper. The cell suspension (volume ~2 ml) was homogenized in a Dounce homogenizer, and a postnuclear supernatant was prepared by centrifugation at 500 g for 5 min. Donor preparations were stored in 250 μ l samples in liquid nitrogen.

Preparation of Acceptor Postnuclear Supernatant

BHK cells (10^8 at near-confluence on 3×14 -cm diam tissue culture dishes) were cooled to 4°C and washed twice at this temperature with 30 ml GMEM, pH 6.8, containing 0.2% (wt/vol) BSA. [³H]SFV ($\sim 2 \times 10^6$ disintegrations per minute [dpm]) was then added in 15 ml of the above medium, and the cells were rocked gently for 2 h at 4°C. The binding medium was removed and the cells were washed twice with 30 ml of the above medium and twice with 30 ml of STM buffer, each time at 4°C. The dishes were then drained and the cells were scraped off, homogenized, and centrifuged as described above. A typical preparation contained 5×10^5 dpm. Acceptor preparations were stored in 250- μ l samples in liquid nitrogen.

Determination of Acceptor Latency

Samples of [³H]SFV (4,000–8,000 dpm) or acceptor preparation (50 μ l) were incubated at 37°C for 2 h with either neuraminidase from *Clostridium perfringens* (Sigma Type V; 400 mU/ml), or influenza virus (4×10^7 pfu/ml) in 250 μ l of STM buffer containing 25 mM potassium chloride, 25 mM Hepes-KOH, pH 7.0, 1.5 mM magnesium acetate, and 1 mM dithiothreitol (Sigma). In some cases incubation mixes contained 0.1% (wt/vol) Triton X-100. At the end of the incubation the tubes were cooled on ice and 5 μ l 10% (wt/vol) Triton X-100 in 250 mM K₂EDTA, pH 6.9 was added to solubilize the membranes. Protein was precipitated by the addition of 250 μ l 20% (wt/vol) TCA. After 1 h on ice the tubes were centrifuged for 1 min in an Eppendorf microfuge and the supernatant fluids were removed. Radioactivity was counted by liquid scintillation spectrometry using 0.6% (wt/vol) butyl PBD/70% (vol/vol) toluene/30% (vol/vol) Triton X-100 as scintillation fluid. All determinations were made in either duplicate or triplicate.

Cell-free Assay

For standard assays 50 μ l of donor preparation was mixed with 50 μ l of acceptor preparation ($>10,000$ dpm), 100 μ l of STM buffer, 25 μ l of a solution containing 250 mM potassium chloride, 250 mM Hepes-KOH, pH 7.0, 15 mM magnesium acetate and 10 mM dithiothreitol (Sigma), 10 μ l of 25 mM 2,3-dehydro-2-desoxy-NANA (Boehringer Mannheim GmbH, Mannheim, FRG), an inhibitor of neuraminidase (Meindl et al., 1974), and either 5 μ l of an ATP-depleting cocktail (1,250 IU/ml hexokinase [Sigma Type C-300, from baker's yeast] in 250 mM glucose) or 7.5 μ l of an ATP-generating cocktail (2.5 μ l 100 mM ATP [Sigma, from equine muscle, disodium salt], 2.5 μ l 800 mM creatine phosphate (Boehringer Mannheim GmbH), and 2.5 μ l 700 IU/ml creatine phosphokinase (Sigma Type I, from rabbit muscle)). The total volume of the mixture was made up to 250 μ l with water. The mixture was incubated at 37°C, usually for 1 h. At the end of the incubation the tubes were cooled on ice and processed as above. All determinations were made in either duplicate or triplicate.

Results

Principle of the Assay

Fig. 1 illustrates the principle of the cell-free assay. Two types of postnuclear supernatant, which acted as donor and acceptor of neuraminidase, were prepared from BHK-21 cells. Donor preparations were obtained from cells infected with influenza virus, and containing neuraminidase en route to the plasma membrane. Acceptor preparations were obtained from cells containing, bound to their plasma membranes, SFV with envelope glycoproteins bearing [³H]NANA. Fusion between vesicles from these two preparations permits access of the enzyme to its substrate, which results in release of free [³H]NANA. This release was detected through transfer of radioactivity from a TCA-insoluble to a TCA-soluble fraction. The NANA analogue 2,3-dehydro-2-desoxy-

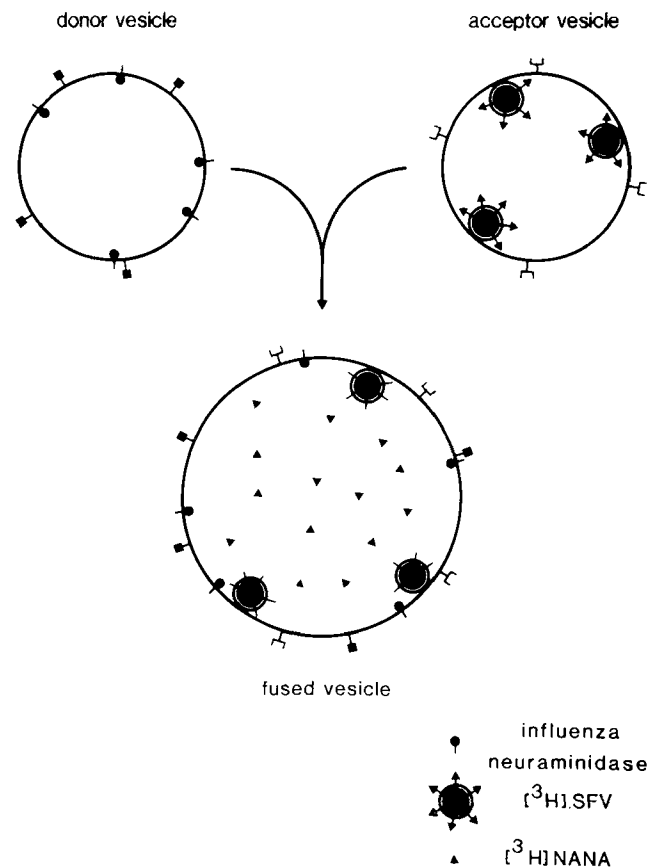


Figure 1. Principle of the cell-free assay. Two types of postnuclear supernatant, which act as donor and acceptor of neuraminidase, are prepared from BHK-21 cells. Donor preparations are obtained from cells infected with influenza virus and containing neuraminidase en route to the plasma membrane. Acceptor preparations are obtained from cells containing, bound to their plasma membranes, SFV with envelope glycoproteins bearing [³H]NANA. Fusion between vesicles from these two preparations results in access of the enzyme to its substrate and release of free [³H]NANA. This release is detected through transfer of radioactivity from a TCA-insoluble to a TCA-soluble fraction. Trivial release of [³H]NANA as a consequence of contact between vesicles bearing externally exposed enzyme and substrate is minimized by inclusion in the incubation mix of DD-NANA, an inhibitor of neuraminidase. Vesicles are shown bearing outward-facing "recognition markers" that are presumed to mediate the specific fusion reaction.

NANA (DD-NANA), an inhibitor of neuraminidase, was included routinely in the assays, to prevent trivial release of [³H]NANA consequent on contact between vesicles bearing externally exposed enzyme or substrate.

A similar approach has been used by Davey et al. (1985) in the cell-free reconstitution of an endocytic fusion event.

Characterization of the Acceptor Preparation

To permit an estimation of the efficiency of any vesicle fusion reaction, it was necessary to determine the percentage of the [³H]SFV in an acceptor preparation that was located within sealed vesicles, a figure that we term acceptor latency. Latency was determined by comparing the abilities of neuraminidase to cleave [³H]NANA from [³H]SFV and from samples of acceptor preparation. Soluble neuraminidase from *Clostridium perfringens* was used to remove the possibility that unsealed vesicles, that were nevertheless able to exclude influenza virions, would give an artificially high value for acceptor latency. In a typical experiment neuraminidase cleaved maximally 79% of the total radioactivity from [³H]SFV and 68% from a sample of acceptor preparation. It was confirmed by chromatography on Bio-Gel P2 (Bio-Rad, Watford, Herts; data not shown) that the radioactivity released was associated with NANA. The percentage of the total [³H]SFV in the acceptor preparation that is exposed to enzyme is, then, $100 \times \% \text{ cleavage from acceptor} / \% \text{ cleavage from } [^3\text{H}]\text{SFV}$, which equals $100 \times 68/79$, or 86%. Hence, 14% of the total [³H]SFV in this acceptor preparation is protected inside sealed vesicles. The mean latency value (\pm SEM) obtained was $16 \pm 4\%$ ($n = 12$).

Influenza virion-bound neuraminidase could cleave maximally only $61 \pm 2\%$ ($n = 4$) of [³H]NANA from [³H]SFV. Since acceptor latency is 16%, therefore, the maximum possible fusion-dependent release of [³H]NANA will be $\sim 10\%$ of the total.

The observation that soluble neuraminidase, from *C. perfringens*, is more effective than virion-bound enzyme suggests that steric hindrance of both enzyme and substrate is occurring. This idea is supported by the finding that both forms of enzyme cleaved off at least 90% of [³H]NANA in the presence of the detergent Triton X-100 (0.1% [wt/vol]). This effect of Triton X-100 renders it impossible to measure latency by comparing the cleavage produced by neuraminidase in the presence and absence of detergent. The near-complete cleavage produced by influenza neuraminidase in

the presence of Triton X-100 indicates, of course, that the specificity of the viral neuraminidase and the glycosylation of SFV in BHK-21 cells are complementary.

The Cell-free Assay

We expected that the fusion event in which we were interested would require energy. Consequently, donor and acceptor preparations were incubated together in the absence and presence of ATP. Table I shows the results obtained in a typical experiment. Donor prepared from influenza virus-infected cells gave both ATP-dependent and ATP-independent components of release of [³H]NANA. Blank donor, prepared from mock-infected cells, gave a small ATP-dependent component of release and no ATP-independent component. The ATP-dependent component of release given by the blank donor preparation was found to be time-dependent and sensitive to heat treatment of the extract (data not shown). At present the significance of this component is unclear.

DD-NANA, an inhibitor of neuraminidase, was included routinely in the assays. Any release occurring in the presence of DD-NANA should be a consequence of enzyme-substrate interaction within a compartment from which the inhibitor is excluded, provided that its concentration is sufficient to saturate the enzyme. In the absence of DD-NANA, the small ATP-dependent component of release of [³H]NANA was superimposed on a large background release of 30–60% of the total radioactivity, and so was difficult to measure reproducibly. The ATP-independent release of [³H]NANA given by donor preparations appears to be a result of incomplete inhibition of externally exposed enzyme by the DD-NANA. This component of release increases linearly over the 1-h incubation period, and its magnitude at the end of this period falls progressively as the concentration of DD-NANA is increased (data not shown). At a concentration of 5 mM DD-NANA, the highest that can reasonably be achieved, ATP-independent release falls to $\sim 8\%$.

Table II shows the pooled results from ten cell-free assays. As can be seen, the ATP-dependent component of release of [³H]NANA given by donor preparations is approximately fourfold greater than that given by blank donor preparations.

The ATP-dependent, Influenza Neuraminidase-dependent Component of Release of [³H]NANA Is a Consequence of Vesicle Fusion

The inclusion of the detergent Triton X-100 in the incubation mix, at a concentration (0.1% wt/vol) that should lyse vesicles, abolished the ATP-dependent, influenza neuraminidase-dependent component of release of [³H]NANA given by donor preparations but did not affect that given by blank donor preparations (Fig. 2). This result indicates that the ATP-dependent, influenza neuraminidase-dependent component of release of [³H]NANA is a consequence of vesicle

Table I. Results from a Typical Cell-free Assay

| Donor | ATP | Release of [³ H]NANA (%) | ATP-dependent release of [³ H]NANA (%) | ATP-dependent, influenza neuraminidase-dependent release of [³ H]NANA (%) |
|-------|-----|--------------------------------------|--|---|
| Blank | – | 0.0 ± 0.2 | – | – |
| | + | 0.7 ± 0.2 | 0.7 ± 0.2 | – |
| Donor | – | 10.8 ± 0.3 | – | – |
| | + | 15.3 ± 0.4 | 4.5 ± 0.4 | 3.8 ± 0.4 |

Samples of acceptor preparation were incubated at 37°C for 1 h with samples of either blank donor or donor preparations in the presence of either an ATP-depleting cocktail (–) or an ATP-generating cocktail (+). Protein was precipitated by the addition of TCA, and the percentage of the total [³H]NANA released into the supernatant fluid was determined. Values are means \pm SEM of triplicate determinations. All of the ATP-dependent components of release of [³H]NANA are statistically significant ($P < 0.01$).

Table II. Pooled Results from Ten Assays

| Donor | ATP-dependent release of [³ H]NANA (%) | ATP-dependent, influenza neuraminidase-dependent release of [³ H]NANA (%) |
|-------|--|---|
| Blank | 1.2 ± 0.2 | – |
| Donor | 5.1 ± 0.5 | 3.9 ± 0.5 |

See footnote to Table I.

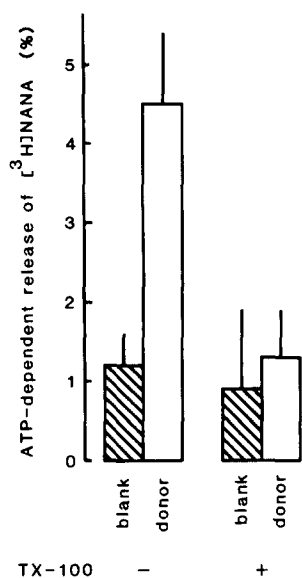


Figure 2. Effect on ATP-dependent release of free $^3\text{H}]\text{NANA}$ of the presence of Triton X-100 in the incubation mix. Incubations were carried out as usual either in the absence or the presence of Triton X-100 (0.1% wt/vol). Protein was precipitated by the addition of TCA, and the values of the ATP-dependent components of release of free $^3\text{H}]\text{NANA}$ were calculated. Values are means \pm SEM from five separate experiments.

fusion. Further evidence that this is indeed the case was obtained from an experiment in which the release of $^3\text{H}]\text{NANA}$ given by a standard preparation was compared with that given by blank preparations to which enzyme and substrate had been added at the start of the incubation (Table III). A statistically significant ATP-dependent, influenza neuraminidase-dependent component of release was given only by a combination of standard donor and acceptor preparations. Hence, enzyme and substrate must be present within sealed vesicles.

Time-course of the ATP-dependent, Influenza Neuraminidase-dependent Component of Release of $^3\text{H}]\text{NANA}$

The time-course of the ATP-dependent, influenza neuraminidase-dependent component of release of $^3\text{H}]\text{NANA}$ is shown in Fig. 3. The time-course has a distinct sigmoid shape. This may reflect the need for a finite time for the vesicle populations to fuse together and deliver enzyme to substrate. The relationship between the time-courses of release and of vesicle fusion is at present unclear, although the time-course of the action of enzyme on substrate clearly contributes significantly.

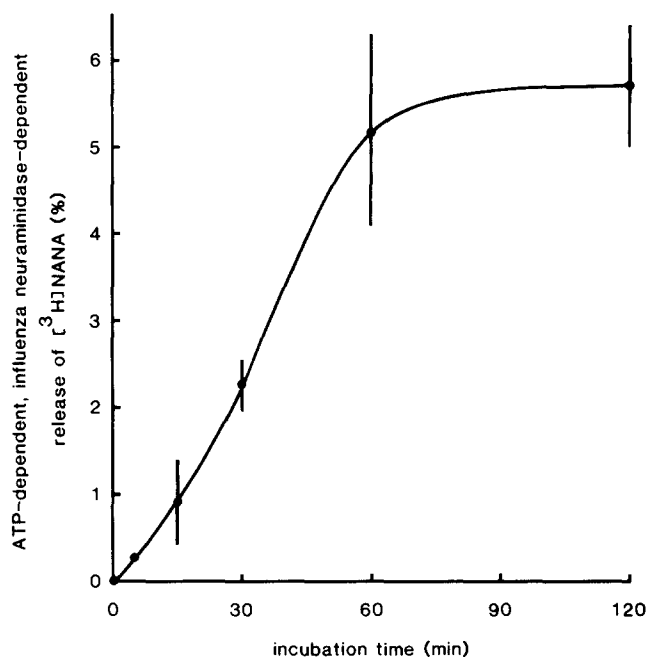


Figure 3. Time-course of the ATP-dependent, influenza neuraminidase-dependent component of release of $^3\text{H}]\text{NANA}$. Samples of acceptor preparation were incubated at 37°C for various times with samples of either blank donor or donor preparations in the presence of either an ATP-depleting cocktail or an ATP-generating cocktail. Protein was precipitated by the addition of TCA, and the values for the ATP-dependent, influenza neuraminidase-dependent component of release of free $^3\text{H}]\text{NANA}$ at the various times were calculated. Values are means \pm SEM from four separate experiments.

Specificity of the Fusion Reaction

If the component of release of free $^3\text{H}]\text{NANA}$ that is dependent on both influenza virus and ATP does indeed represent a membrane fusion event that is normally responsible for the delivery of influenza neuraminidase to the plasma membrane, then it should show specificity. In other words, only post-Golgi vesicles from the donor preparation should fuse with plasma membrane vesicles from the acceptor preparation. We set out to establish whether there was any evidence for this specificity.

Since the production of acceptor involves the binding of $^3\text{H}]\text{SFV}$ to BHK-21 cells at 4°C , a temperature at which no

Table III. Dependence of the Release of Free $^3\text{H}]\text{NANA}$ on the Position of Enzyme and Substrate Relative to Vesicle Membranes

| Donor | Acceptor | ATP-dependent release of $^3\text{H}]\text{NANA}$ (%) | ATP-dependent, influenza neuraminidase-dependent release of $^3\text{H}]\text{NANA}$ (%) |
|-------------------------|---------------------------------|---|--|
| Blank | Acceptor | 1.6 ± 0.7 | — |
| Blank + influenza virus | Acceptor | 2.4 ± 0.7 | 0.8 ± 1.0 |
| Donor | Acceptor | 5.9 ± 1.2 | 4.3 ± 1.4 |
| Blank | Blank + $^3\text{H}]\text{SFV}$ | 3.0 ± 0.9 | — |
| Blank + influenza virus | Blank + $^3\text{H}]\text{SFV}$ | 0.3 ± 2.6 | -2.7 ± 2.8 |
| Donor | Blank + $^3\text{H}]\text{SFV}$ | 0.8 ± 1.0 | -2.2 ± 1.3 |

Reactions were carried out using standard donor and acceptor preparations, and also blank donor preparations to which was added, at the start of the incubation, influenza virus (of neuraminidase activity equal to that of the standard donor), and blank acceptor preparations to which was added $^3\text{H}]\text{SFV}$ (containing an equal amount of radioactivity to that of the standard acceptor extract). Samples were incubated together in pairs, as shown, at 37°C for 1 h. Protein was precipitated by the addition of TCA, and the values for ATP-dependent release of free $^3\text{H}]\text{NANA}$ given by the various pairs were calculated. Values are means \pm SEM of triplicate determinations. Of the ATP-dependent, influenza neuraminidase-dependent components of release of $^3\text{H}]\text{NANA}$, only that given by the standard combination of donor and acceptor is statistically significant ($P < 0.01$).

endocytosis occurs (Fries and Helenius, 1979), radiolabeled virions in these preparations will be bound exclusively to the plasma membrane. However, there is no simple way of obtaining a donor preparation containing influenza neuraminidase exclusively in post-Golgi vesicles. Instead, an attempt was made to concentrate neuraminidase in a compartment late in the transport pathway. Such a preparation would be expected to give an enhanced ATP-dependent release of free [³H]NANA for a given amount of neuraminidase activity. The procedure used to obtain this preparation is based on the observation (Matlin and Simons, 1983; Rodriguez-Boulan et al., 1984; Fuller et al., 1985; Griffiths et al., 1985) that incubation of enveloped virus-infected cells at 20°C causes an arrest in the transport of the viral envelope glycoproteins in a reticular, membrane-limited compartment on the *trans* side of the Golgi complex. Specifically, cells were infected with influenza virus at 37°C for 5 h and then cooled to 20°C for 1 h before homogenization. In addition, a donor preparation was obtained from cells to which influenza virus had been bound for 2 h at 4°C. This preparation will contain influenza neuraminidase bound exclusively to the plasma membrane, and would be expected to give little or no ATP-dependent release of free [³H]NANA.

The neuraminidase activities of these two new donor preparations, and of a standard 37°C donor preparation, were determined by measuring the initial rates of release of [³H]NANA from [³H]SFV in the presence of 0.2% (wt/vol)

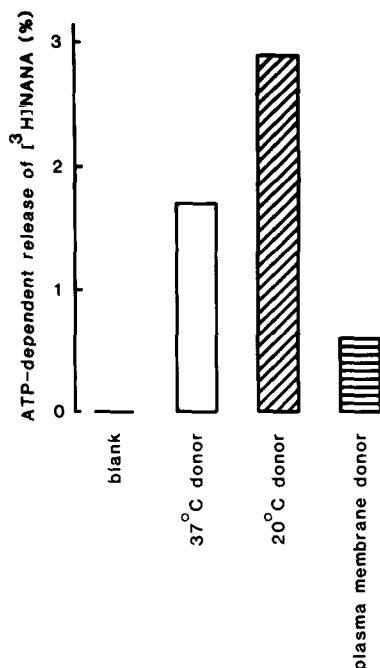


Figure 4. Specificity of the fusion reaction. In addition to the standard blank donor and donor preparations, postnuclear supernatants were prepared from infected cells that had been incubated at 37°C for 5 h and then at 20°C for 1 h, and from cells to which influenza virus had been bound for 2 h at 4°C. Samples of the donor preparations, containing equal amounts of enzyme activities and with their protein concentrations balanced by the addition of BSA, were incubated with samples of acceptor preparation at 37°C for 1 h. A sample of blank donor preparation was also included. Protein was precipitated by the addition of TCA, and the ATP-dependent release of [³H]NANA was determined. Values are means of duplicate determinations which differed by <10%.

Triton X-100. Protein concentrations were determined by the method of Bradford (1976). Samples of these donor preparations, containing equal amounts of neuraminidase activity, and with protein concentrations balanced by addition of BSA, were then subjected to the usual fusion assay. The values obtained for ATP-dependent release of free [³H]NANA are shown in Fig. 4. The release produced by the 20°C donor was almost double that given by the 37°C donor and approximately fivefold greater than that given by the plasma membrane donor. In this particular experiment the blank donor gave no ATP-dependent release. Hence, the degree of release produced depends on the position of neuraminidase within the cell, as would be expected of a specific fusion event. There was no correlation between the amount of protein contained in the donor samples and the ATP-dependent release of [³H]NANA produced. For example, the 37°C donor sample contained 20% more protein than the 20°C donor sample but had less activity in the assay. Hence, the variation in donor activity observed is not simply a consequence of differences in the amounts of postnuclear supernatant used. In addition, there was no indication of a position-dependence of the ATP-independent component of release (data not shown), a result that would be expected of a trivial effect unrelated to fusion.

Efficiency of the Fusion Reaction

The ATP-dependent, influenza neuraminidase-dependent component of release of [³H]NANA produced after a 2-h incubation at 37°C amounts to 5.7% of total (Fig. 3). Given that the latent releasable [³H]NANA represents 10% of total, fusion is ~57% efficient at its end-point. Except where stated, the results presented in this paper were obtained using donor preparations obtained from cells in which intracellular transport of neuraminidase was blocked in the *trans* Golgi reticulum by incubation at 20°C for 1 h. As is shown in Fig. 4, fusion is less efficient if this procedure is not adopted.

Fusion Requires Proteins

It was expected that vesicle fusion would require the presence of proteins both on the cytoplasmic surface of the vesi-

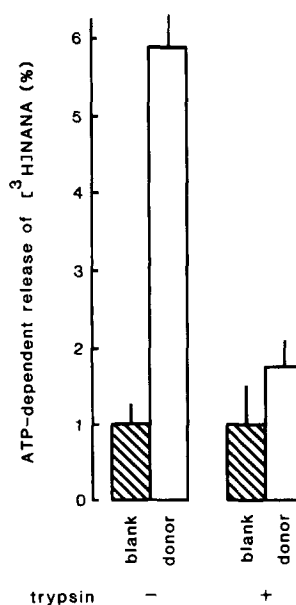


Figure 5. Trypsin sensitivity of the fusion reaction. Incubation mixes were treated with either trypsin-TPCK (Sigma Type XIII; 100 µg/ml) or trypsin-TPCK plus soybean trypsin inhibitor (Sigma; 100 µg/ml) for 1 h at 4°C. Soybean trypsin inhibitor was then added to trypsin-treated samples, and all samples were incubated at 37°C for 1 h as usual. Protein was then precipitated by addition of TCA, and the ATP-dependent release of [³H]NANA was determined. Values are means ± SEM of triplicate determinations.

cles themselves and in the cytosol. To test this prediction the effect of trypsin treatment of the preparations on the fusion reaction was investigated. Mixed donor and acceptor preparations were treated with trypsin-TPCK (100 µg/ml) at 4°C for 1 h. Soybean trypsin inhibitor (100 µg/ml) was then added and the preparations were incubated at 37°C for 1 h as usual. The results obtained are shown in Fig. 5. Trypsin treatment caused an 84% reduction in the size of the ATP-dependent, influenza neuraminidase-dependent component of release of [³H]NANA, which indicates that proteins are involved in the fusion reaction. Since crude postnuclear supernatants were used in this experiment, it is not possible at present to discriminate between proteins bound to the vesicle membranes and those free in the cytosol.

Discussion

In this paper we describe a cell-free assay for the insertion of influenza virus neuraminidase into the plasma membrane which exploits the enzyme activity of this glycoprotein. Enzyme and substrate (SFV with envelope glycoproteins containing [³H]NANA) have been placed in separate populations of vesicles and fusion between the two populations has been monitored by the appearance of the product, free [³H]NANA. The assay has revealed two influenza neuraminidase-dependent components of release of [³H]NANA, one ATP-independent and the other ATP-dependent. Only the latter component appears to be associated with vesicle fusion. Since vesicle-mediated intracellular transport of proteins is known to be a highly specific, energy-dependent process (Palade, 1975), we are encouraged by our identification of a fusion reaction that requires the presence of ATP in the incubation mix and that is sensitive to the position of neuraminidase in the cells from which the donor preparation is obtained.

The only information available so far about the donor compartment is that it appears to be situated late in the transport pathway of neuraminidase. Incubation of influenza virus-infected cells at 20°C could cause a concentration of neuraminidase in genuine vesicles that are competent to fuse with the plasma membrane only at 37°C. Alternatively, the homogenization procedure could artificially generate fusion-competent vesicles from the budding reticular network of membranes observed on the *trans* face of the Golgi complex in these cells (Griffiths et al., 1985). Answers to questions such as these must await the further characterization of the cell-free vesicle fusion reaction.

Under the conditions used at present, donor preparations cannot bring about the complete release of latent [³H]NANA. The most likely explanation of this incomplete release is that the amount of neuraminidase present in fusion-competent vesicles in a typical donor preparation is limiting. An increase in the amount of donor preparation used in the assay is unlikely to solve this problem because this will increase not only the number of fusion-competent donor vesicles, but also the number of competing unlabeled plasma membrane target vesicles. What is required is an increase in the percentage of the total neuraminidase that is contained in fusion-competent vesicles. Such an increase is apparently produced (Fig. 4) by blocking the intracellular transport of newly synthesized neuraminidase in the *trans* Golgi reticulum by incubation of cells at 20°C. It should be possible to produce a

further increase by exploiting the observation (Matlin and Simons, 1983; Rodriguez-Boulan et al., 1984; Fuller et al., 1985; Griffiths et al., 1985) that re-warming 20°C-blocked cells causes the rapid (5–30 min) appearance of the proteins at the cell surface. By choosing the correct time of incubation at 37°C a donor preparation further enriched in neuraminidase-containing, fusion-competent vesicles could in principle be obtained. At present, we are investigating the efficacy of this procedure.

A problem with the assay reported here is the production by blank donor preparations of a significant, and variable, ATP-dependent component of release of free [³H]NANA. A similar finding has been reported by Davey et al. (1985), who suggest that this component is caused by enzymes, other than neuraminidases, released during the homogenization. We are attempting to circumvent this problem by reconstituting the incubation mix from individual membrane preparations supplemented with cytosol preparations. Preliminary experiments indicate that conditions can be obtained in which all of the ATP-dependent component of release depends also on the presence of influenza neuraminidase. The use of membrane fractions supplemented with cytosol will eliminate some of the problems with experimental design that have been encountered using crude postnuclear supernatants. For example, in comparisons between the activities of different donor preparations, both membrane and cytosol protein concentrations of the preparations should ideally be equalized. However, this is impossible when crude postnuclear supernatants are used. In addition, the use of fractionated donor and acceptor preparations would make possible a more complete study of the requirements of the vesicle fusion reaction for cytosolic components and for proteins expressed on the cytoplasmic surfaces of the vesicles.

An advantage of the use of influenza neuraminidase as a model protein in these studies is the fact that this glycoprotein is known to be delivered asymmetrically to the plasma membrane of epithelial cells in culture. For example, in MDCK cell monolayers influenza envelope glycoproteins are delivered almost exclusively to the apical surface, whereas vesicular stomatitis virus G is delivered almost exclusively to the basolateral surface (Rodriguez-Boulan and Sabatini, 1978; Rodriguez-Boulan and Pendergast, 1980). It has been demonstrated by two different techniques (Rindler et al., 1984; Fuller et al., 1985) that these proteins reside in the same membrane domain until at least the *trans* cisternae of the Golgi complex. This raises the possibility that vesicles budding from the *trans* Golgi can package separately proteins destined for the two domains of the plasma membrane of the epithelial cell and then fuse specifically with the appropriate domain to deliver their cargo. Using the cell-free assay reported here it may be possible to determine whether post-Golgi vesicles containing influenza neuraminidase do indeed fuse specifically with the apical domain of the plasma membrane of MDCK cells, and if this is the case to elucidate the molecular basis of this specificity. However, it will be necessary first to devise methods for binding the target substrate, [³H]SFV, separately to both surfaces of MDCK cell monolayers.

We thank Professor G. Warren (Department of Biochemistry, University of Dundee, UK) for his advice during the development of this project.

This work was supported by the Wellcome Trust and the National Kidney

Research Fund. P. G. Woodman is a Science and Engineering Research Council scholar.

Received for publication 2 June 1986, and in revised form 28 July 1986.

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