Sendai-viral HN and F glycoproteins as probes of plasma-membrane protein catabolism in HTC cells

Studies with fusogenic reconstituted Sendai-viral envelopes

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Reconstituted Sendai-viral envelopes (RSVE) were produced by the method of Vainstein, Herschkovitz, Israel & Loyer [(1984) *Biochim. Biophys. Acta* **773**, 181–188]. RSVE are fusogenic unilamellar vesicles containing two transmembrane glycoproteins: the HN (haemagglutinin–neuraminidase) protein and the F (fusion) factor. The fate of the viral proteins after fusion-mediated transplantation of RSVE into hepatoma (HTC) cell plasma membranes was studied to probe plasma-membrane protein degradation. Both protein species are degraded at similar, relatively slow, rates ($t_{1/2} = 67$ h) in HTC cells fused with RSVE in suspension. Even slower degradation rates for HN and F proteins ($t_{1/2} = 93$ h) were measured when RSVE were fused with HTC cells in monolayer. Lysosomal degradation of the transplanted viral proteins is strongly implicated by the finding that degradation of HN and F proteins is sensitive to inhibition by 10 mM-NH₄Cl (81%) and by 50 µg of leupeptin/ml (70%).

INTRODUCTION

Sendai virus is an enveloped RNA virus. The envelope consists of lipids obtained from host cells, and two viral glycoproteins which mediate virus–cell interactions. The HN protein is involved in adsorption and desorption of the virus to and from cell-surface receptors, and the F protein induces fusion of the viral envelope with the plasma membrane at neutral pH (Scheid & Choppin, 1974).

Reconstituted envelopes (RSVE), in which the biological activities of HN and F proteins are preserved, can be produced by selectively extracting the envelopes from virus particles with Triton X-100 and then removing the detergent by agitating the extract in the presence of Biobeads SM-2 (Vainstein *et al.*, 1984). RSVE have been used to introduce macromolecules into animal cells in culture; in so doing, the RSVE vector fuses with the plasma membrane of target cells (Henis *et al.*, 1985). We have studied the fate of the viral glycoproteins implanted into the plasma membrane of hepatoma (HTC) cells as a consequence of fusion with RSVE. In hepatoma cells, native plasma membrane proteins are apparently degraded in a bimodal manner (Chu & Doyle, 1985). We were therefore interested to study whether implanted viral proteins would be treated like either group of endogenous proteins, and thus act as probes of the mechanism of degradation of such proteins in HTC cells.

MATERIALS AND METHODS

Materials

NaB³H₄ (5–10 Ci/mmol), Na¹²⁵I and deoxycytidine 5'-[α-³²P]triphosphate (3.0 × 10⁸ Ci/mmol) were purchased from Amersham International, Amersham, Bucks., U.K. Biobeads SM-2 were obtained from

Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were the highest grade available. Pathogen-free fertile chicken eggs were purchased from S.P.F. Farms, Wickham Laboratories, Hants., U.K.

Cell culture

HTC cells (derived from the Morris hepatoma 7288c) purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.) were cultured in Dulbecco's minimal essential medium (Gibco, Paisley, Renfrewshire, Scotland, U.K.) containing 10% (v/v) newborn-calf serum, penicillin G (100 units/ml) and streptomycin (100 µg/ml). Cells were maintained as monolayer cultures on 90 mm-diameter plastic culture dishes (Nunc, Roskilde, Denmark) at 37 °C in an incubator under CO₂/air (1:9).

Sendai virus

Sendai virus was propagated *in ovo* by injection of virus [0.2 haemagglutinating unit (HAU)/egg] into the allantoic space of 10-day-old embryonated chicken eggs. The eggs were incubated for a further 54–72 h before the allantoic fluid containing free virus particles was aspirated. Debris was removed by centrifuging the allantoic fluid at 3000 *g* for 15 min, and the virus was collected by re-centrifuging the supernatant at 20000 *g* for 30 min. The viral pellet was resuspended in solution I (160 mM-NaCl/20 mM-Tricine/NaOH, pH 7.4), and its haemagglutinating activity was determined by the ability of 25 µl of serially diluted virus preparation (or RSVE) to agglutinate sheep erythrocytes (0.5 ml of packed cells in 99.5 ml of PBS). A typical end-point in the assay of 10⁴ HAU/mg virus was usually obtained. The haemolytic potential of the virus preparation (or RSVE) was determined by the method of Toister & Loyer (1973).

Abbreviations used: RSVE, reconstituted Sendai-viral envelopes; HTC, hepatoma tissue culture; HN, haemagglutinin–neuraminidase protein; F, fusion protein; PBS, phosphate-buffered saline (Doherty & Mayer, 1985), pH 7.4; HAU, haemagglutinating unit, DNAase, deoxyribonuclease; FITC, fluorescein isothiocyanate.

Preparation of RSVE

Reconstituted envelopes (RSVE) were prepared from Sendai-virus particles by the method of Vainstein *et al.* (1984). The RSVE vesicles were isolated from non-reconstituted elements by centrifugation at 16000 *g* for 30 min at 4 °C so as to avoid damaging the vesicle integrity (Bartzatt & Volsky, 1984). The RSVE pellet was resuspended in solution I (1 mg/ml) for immediate use.

Protein radiolabelling procedures

Sendai-virus particles (1 mg) were labelled by reductive methylation with NaB³H₄ (5–10 Ci/mmol) by the method of Tack *et al.* (1980), modified by the use of 6 µmol of formaldehyde/mg of protein and 5 mCi of NaB³H₄/mg of protein. Radioiodination of Sendai virus used Na¹²⁵I and the chloramine- τ procedure of Brown *et al.* (1979). Quantities of 1 mg of radiolabelled virus were mixed with 5 mg of unlabelled virus prior to detergent solubilization for fusion studies.

FITC-labelled RSVE

FITC-labelled Sendai-virus particles were prepared as described by Chejanovsky *et al.* (1984). Fluoresceinated virus was mixed with unlabelled virus (1:1, w/w) before envelope solubilization and preparation of fluorescein-labelled RSVE (FITC-RSVE).

Cells treated with FITC-RSVE were grown on glass coverslips. At each time point, three coverslips were washed and the cells fixed by treatment with methanol/acetic acid (3:1, v/v). Fixed cells were dehydrated with an ethanol series and mounted in glycerol/PBS (9:1, v/v), pH 8.0, containing 1 mg of *p*-phenylenediamine/ml.

Trapping of [³²P]DNA by RSVE

pBR322 DNA was isolated from cultures of *Escherichia coli* H101 by alkaline lysis (Birnboim, 1983). The plasmid was purified and confirmed as pBR322 DNA by restriction analysis as described by Maniatis *et al.* (1982). The DNA was quantified by the method of Labarca & Paigen (1980), and was nick-translated by the method of Weinstock *et al.* (1979) using [α -³²P]dCTP (3.0 × 10³ Ci/mmol) at 15 °C for 2 h. ³²P-labelled pBR322 was loaded in RSVE by mixing 25–40 µg of DNA/mg of Triton X-100-solubilized envelope protein before reconstitution. Both RSVE reconstituted in the presence of [³²P]DNA and preformed RSVE mixed with [³²P]DNA were treated with bovine pancreatic DNAase I (type 4; Sigma) at 1 mg of enzyme/mg of RSVE protein for 45 min at 25 °C to remove nuclease-accessible DNA.

Fusions

RSVE vesicles were fused either with cells in suspension or in sub-confluent cell monolayers. Suspension fusions were conducted by treating monolayer cultures of cells with trypsin/EDTA solution (Gibco; 0.5 mg of trypsin/ml and 0.2 mg of EDTA/ml in Ca²⁺- and Mg²⁺-free PBS) for 2–4 min at 25 °C to yield approx. 5 × 10⁷ cells per fusion. The cells were washed once with growth medium and once with solution I (20 ml), then resuspended in 1 ml of ice-cold fusion buffer (solution I containing 0.15 mM-LaCl₃), to which was added 0.25 ml of fusion buffer containing 50–100 µg of vesicle protein. The fusion mixture was mixed, then incubated at 4 °C for 15 min to allow RSVE to bind to cell receptors, then vortex-mixed for 10 s to disassociate vesicle-cell aggre-

gates, and warmed at 37 °C for 30 min with mixing at intervals to allow fusion to occur. The cells were collected by centrifugation (800 *g* for 10 min), washed once with 1 ml of solution I, twice with 1 ml of growth medium and finally resuspended in 18 ml of growth medium before plating out for monolayer culture (1 ml of cell suspension/90 mm dish). A further 4 ml of medium was added per dish and the cells were cultured at 37 °C for 96 h, with growth medium being replaced every 24 h. Monolayer fusions were conducted in 16 mm-diameter 24-well plates (Costar, Cambridge, MA, U.S.A.). Each well (containing 6 × 10⁴ cells) was washed once with 1 ml of solution I before receiving 5–7.5 µg of vesicle protein in 0.2 ml of ice-cold fusion buffer. The cells were incubated at 4 °C for 15 min and were then warmed at 37 °C for 30 min. Vesicles which had not fused were removed by washing the cells in each well once with 1 ml of solution I and then twice with 1 ml of growth medium. The cells were then cultured in growth medium at 37 °C.

Inhibitor studies

Studies on the role of lysosomes in RSVE-protein degradation used either 10 mM-NH₄Cl or 50 µg of leupeptin/ml in growth medium throughout the period of culture. To investigate whether phagocytosis was important for uptake of RSVE, cells were incubated in serum-free growth medium containing 5 mM-NaN₃ and 50 mM-2-deoxyglucose for 30 min before fusion, and throughout the fusion procedure.

Measurement of degradation of transplanted proteins in HTC cells

At each time point, three dishes of cells cultured from the suspension fusion or three wells of monolayer-fused cells were taken for determination of cell-associated radioactivity. Growth medium was collected and the cells were washed once with 1 ml of solution I before being harvested by hydrolysis *in situ* with Pronase, a mixture of proteinases isolated from *Streptomyces griseus* (1 mg/ml) in 0.25 M-potassium phosphate buffer, pH 7.4, for 1 h at 37 °C to maximize recovery of cell radioactivity (Silverman & Amenta, 1984). The cells were collected by scraping and each well or dish was washed with 0.5 ml of Pronase solution. The washings and scraped cells were pooled (5 ml/90 mm dish or 2 ml/well).

Radioactivity measurements were made on samples of aspirated media, or on Pronase-harvested cells which had been sonicated (70 W for 5 s; Dawe Soniprobe). Media samples were made 10% (w/v) in trichloroacetic acid and incubated for 30 min at 4 °C, then centrifuged at 1200 *g* for 15 min. Acid-soluble radioactivity was measured in 0.3 ml samples of the supernatant, and acid-insoluble pellets were each washed with 1 ml of 10% (w/v) trichloroacetic acid before being solubilized for radioactivity counting in 30% (v/v) formic acid (0.3 ml). Total cell-associated radioactivity was measured in sonicated cell samples. ³H radioactivity was measured in a Packard 460 CD liquid-scintillation counter with FisoFluor as scintillant, and ¹²⁵I radioactivity was measured with a Mini-Assay type 620 gamma counter.

Estimation of t_{1/2} values for protein degradation

The degradation rates of radiolabelled HN and F proteins transplanted into HTC cells were calculated from the rate of loss of cell-associated radioactivity

(CAR), where the initial value (CAR_0) was obtained from the expression:

$$CAR_0 = CAR_{final} + MAS_{total} \quad (1)$$

i.e. the radiolabelled protein available for degradation at the start of the experiment (CAR_0) is equal to the residual CAR at the final time point (96 h) plus the cumulative total trichloroacetic acid-soluble radioactivity released into the medium by 96 h (MAS_{total}). Thereafter, values of CAR at time t were obtained from:

$$CAR_t = CAR_0 - MAS_t \quad (2)$$

Gel electrophoresis, autoradiography, fluorography

Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (1970). Radioactive polypeptides were detected by autoradiography with calcium tungstate intensifying screens for ^{125}I (Bonner & Laskey, 1974), and by fluorography using sodium salicylate as the fluor for 3H (Chamberlain, 1979).

Antisera

Anti-Sendai-virus antibodies were raised by immunizing rabbits with u.v.-inactivated Sendai-virus particles by a schedule described by Mayer & Walker (1980).

Protein assay

Protein was determined by the method of Lowry *et al.* (1951) in the presence of 0.1% (w/v) SDS, with bovine serum albumin as a standard.

RESULTS

Analysis of RSVE by SDS/polyacrylamide-gel electrophoresis demonstrated that RSVE vesicles contain predominantly two protein species (Fig. 1), namely the haemagglutinin-neuraminidase (HN) and fusion factor (F). Electron-microscopic examination revealed that RSVE are unilamellar vesicles with diameters of 200–300 nm (results not shown). The biological activities of both HN and F proteins were largely conserved. RSVE agglutinated washed sheep erythrocytes at approx. 60% of the activity of the virus particles from which they were derived, and induced haemolysis of aged human erythrocytes almost as efficiently (> 80%) as intact virus when compared on a per-HAU basis.

When interaction of FITC-RSVE with HTC cells was observed by fluorescence microscopy immediately after fusion, discontinuous punctate fluorescence was seen at the periphery of cell processes and over the surface of the cells. Within the first 24 h of culture, the fluorescence appeared to cluster into 'patches', which were localized intracellularly either side of the cell nucleus, and fluorescence was rarely seen on cell processes (results not shown).

RSVE as vehicles for the introduction of [^{32}P]DNA into hepatoma cells

Evidence for the RSVE-cell fusion was provided by their ability to act as vehicles for the delivery of plasmid DNA into hepatoma cells. After reconstitution of RSVE in the presence of ^{32}P -labelled pBR322 DNA, approx. 50% of the [^{32}P]DNA was immunoprecipitable with anti-Sendai-virus antiserum after DNAase I treatment of the RSVE (Table 1). The experiment was designed to

Fig. 1. Fluorograms showing polypeptide composition of radio-labelled Sendai virus and RSVE analysed by SDS/polyacrylamide (15%) gel electrophoresis

(a) Track 1: polypeptide composition of intact Sendai virus reductively methylated with NaB^3H_4 showing five major bands, namely P (polymerase), HN (haemagglutinin-neuraminidase), NP (nucleoprotein), F (fusion factor) and M (matrix protein). Track 2: composition of RSVE derived from 3H -labelled Sendai-virus particles. (b) Track 1: polypeptide composition of intact Sendai virus radioiodinated with $Na^{125}I$. Track 2: polypeptide composition of RSVE derived from ^{125}I -labelled Sendai virus. M_r values ($\times 10^{-3}$) of marker proteins are shown.

show that DNA was inside an immunoprecipitable RSVE: we did not attempt to immunoprecipitate fully all the RSVE-entrapped DNA. By contrast, in the same experiments < 12% of the [^{32}P]DNA added externally to RSVE, and negligible amounts of free [^{32}P]DNA, could be precipitated by the antiserum. These observations imply that the DNA present during the reconstitution process is entrapped within RSVE. When [^{32}P]DNA-loaded RSVE, which had been treated with pancreatic DNAse I to remove nuclease-accessible DNA, were fused with HTC cells in suspension, > 20% of added radioactivity became cell-associated in a manner which was resistant to washing of the cells or treatment of targeted cells with DNAase I (Table 1). By contrast, when pre-formed RSVE mixed with [^{32}P]DNA externally and treated with DNAse I were targeted at HTC cells, 7.2% of the external (but nuclease-resistant) DNA was delivered to the cells. When [^{32}P]DNA was mixed with HTC cells in suspension in the absence of RSVE, however, only 1.7% of added radioactivity became associated with the cells in a manner resistant to washing procedures and DNAase I treatment.

Effect of metabolic inhibitors on the extent of RSVE-cell fusion

The possibility that cells may internalize RSVE by endocytosis or phagocytosis was investigated. Cells treated with 5 mM- NaN_3 and 50 mM-2-deoxyglucose in Dulbecco's medium for 30 min before fusion and

Table 1. RSVE delivery of ^{32}P -labelled pBR322 DNA to hepatoma cells in suspension

Association of [^{32}P]DNA with HTC cells was done as follows: 100 μg of RSVE protein containing 0.15 μg of [^{32}P]DNA either entrapped (DNA-loaded RSVE) or externally added (DNA mixed with unloaded RSVE), or 0.15 μg of free [^{32}P]DNA, was added to 4×10^7 HTC cells as described in the Materials and methods section. After 30 min incubation at 37 °C the cells were washed, then treated with bovine pancreatic DNAase I (type III) (2 mg/ml) in solution I containing 5 mM-MgCl₂ for 20 min at 25 °C, washed twice in solution I and sonicated for 30 s, after which samples were taken for radioactivity counting by dissolution in 33% (v/v) formic acid and addition of Fisofluor scintillant. Incubation with anti-SV (Sendai virus) antiserum was as follows: 60 μl of antiserum was incubated with DNA-loaded RSVE or DNA mixed with RSVE (30 μg of viral protein), or with free [^{32}P]DNA (50 ng), for 1 h at 20 °C, then for 3 h at 4 °C. The precipitate obtained was pelleted by centrifugation, washed with 1 ml of solution I and solubilized in 33% formic acid before liquid-scintillation counting of radioactivity.

State of [^{32}P]DNA	Radioactivity precipitated by anti-SV antiserum (% of total)	[^{32}P]DNA associated with HTC cells (5 of total radioactivity)
[^{32}P]DNA-loaded RSVE	50.30	20.3
[^{32}P]DNA mixed with unloaded RSVE	11.40	7.2
Free [^{32}P]DNA	0.03	1.7

Table 2. Fusion of ^{125}I -RSVE vesicles with hepatoma cells treated with metabolic inhibitors in suspension

HTC cells (1.5×10^7) were harvested from monolayer cultures by trypsin treatment and were incubated in suspension in serum-free growth medium containing 5 mM- NaN_3 and 50 mM-2-deoxyglucose for 30 min at 37 °C. The cells received 20 μg of vesicle protein during the fusion process, which was conducted in the presence of the inhibitors but otherwise as described for a normal fusion. Cells which were not treated with metabolic inhibitors also received 20 μg of RSVE in control fusions. The ^{125}I -RSVE had a specific radioactivity of 1.56×10^8 c.p.m./ μg of protein.

Inhibitors	^{125}I associated with cells (c.p.m.)	Fusion (%)
—	8131	26
+	13152	42

throughout the fusion procedure to deplete intracellular ATP were fused with ^{125}I -RSVE, as were untreated control cells. Depletion of cellular ATP in this manner, which effectively inhibits endocytosis and micropinocytosis (Karnovsky, 1962; Ryser, 1968; Poste & Papahadjopoulos, 1976) did not decrease radioactivity associated with ATP-depleted cells, and apparently increased fusion efficiency with respect to untreated cells (Table 2), as previously shown in mitochondrial membrane-HTC-cell fusion (Russwell & Mayer, 1983).

Degradation of RSVE glycoproteins in hepatoma cells

When ^3H - or ^{125}I -RSVE were targeted at HTC cells, between 25 and 40% of the radiolabelled vesicles remained associated with the cells immediately after fusion. After 5 h incubation in growth medium, 65–85% of RSVE-fused cells were attached to the substratum, and non-adherent cells, debris from lysed cells and unfused RSVE were removed by replacing the medium. Adherent cells had attained a sub-confluent density (approx. 1.25×10^5 cells/cm²) by the end of each experiment. Throughout each experiment there was a progressive loss of radioactivity from RSVE-fused cells, which was matched by a concomitant increase in radioactivity in the growth medium. The loss of cell-associated radioactivity with time yielded a biphasic semi-logarithmic plot (Fig. 3). The initial rapid loss of cell-associated radioactivity (0–5 h) corresponds mainly to losses of detached and lysed cells, which are removed from the culture plates by aspiration 5 h after fusion; for this reason the 5 h time point was defined as t_0 (h) for protein-degradation measurements. However, rapid degradation of some RSVE after internalization by phagocytosis cannot be completely excluded. The initial loss of radiolabel owing to removal of detached cells was followed by a slow degradative phase corresponding to the degradation of RSVE proteins implanted into the hepatoma-cell plasma membranes. Radiolabel released into the medium had both trichloroacetic acid-soluble and -insoluble components. The cumulative acid-soluble radioactivity and acid-insoluble radioactivity released into the medium over the course of an experiment accounted for 65–70% and 30%, respectively, of the loss of radioactivity from cells over that period (results not shown). Since adherent cells were harvested with Pronase, the measured cell-associated radioactivity comprised both trichloroacetic acid-soluble and -insoluble components. However, at any time point cells harvested by brief treatment with trypsin/EDTA solution, sonicated and made 10% (w/v) in trichloroacetic acid as described by Russell & Mayer (1983), contained < 5% of radiolabel in a trichloroacetic acid-soluble form (results not shown).

Treatment of cell monolayers with trypsin/EDTA solution to obtain a population of single cells in suspension changes cell morphology and may also affect the surface properties of the cells in ways which cause alterations in the rates of degradation of (plasma-membrane) proteins. For this reason cells maintained in monolayer culture were also fused with RSVE vesicles. Interestingly, a difference was obtained in the rate of degradation of radiolabelled HN and F proteins fused in suspension, compared with cells that had been fused with RSVE in monolayer. In suspension-fused cells, ^{125}I -labelled HN and F proteins were degraded with an average $t_{1/2}$ of 67.0 h, whereas in cells fused in monolayer the $t_{1/2}$ was longer (average 93.0 h). When the experiment was repeated with ^3H -labelled RSVE, HN and F proteins were degraded at similar rates in suspension-fused cells ($t_{1/2} = 64.7$ h) and in monolayer-fused cells, ($t_{1/2} = 91.8$ h). Thus the $t_{1/2}$ value was independent of the method by which the proteins were radiolabelled.

Relative rates of degradation of HN and F proteins in hepatoma cells

Cells fused with ^{125}I -RSVE in suspension were harvested and processed for SDS/polyacrylamide-gel

Fig. 2. Densitometric scan of an autoradiogram obtained after SDS/polyacrylamide-gel-electrophoretic analysis of HTC cells fused in suspension with ^{125}I -RSVE showing the relative intensity of bands corresponding to viral HN (haemagglutinin-neuraminidase) and F (fusion) glycoproteins

Fluorograms were scanned with white light with a Helena Quick Scan R + D Scanner (Helena Laboratories, Beaumont, TX, U.S.A.)

electrophoresis. Densitometric analysis of the relative intensities of the autoradiographic bands corresponding to ^{125}I -labelled HN and F proteins from cells grown in the presence or absence of leupeptin demonstrated that the relative proportions of the two proteins remained approximately constant with time (Fig. 2). This indicates that HN and F proteins are degraded at similar rates and that leupeptin does not preferentially inhibit the degradation of either protein, although leupeptin clearly decreased their rate of degradation.

Effects of leupeptin and NH_4Cl on degradation of implanted RSVE glycoproteins in hepatoma cells

The presence of leupeptin in cell culture medium significantly decreased the rate at which ^{125}I -labelled HN

and F proteins were degraded (70% inhibition; Fig. 3), but NH_4Cl treatment resulted in an even more marked inhibition (81%) of the average rate of HN- and F-protein degradation in these cells (Fig. 3).

DISCUSSION

RSVE contain the two proteins, HN and F, which are needed to attach to and fuse with target cells (Fig. 1). The interaction of fluorescein-conjugated RSVE with HTC cells can be observed by fluorescence microscopy, but unequivocal evidence that fusion of RSVE with cells had occurred (i.e. the introduction of envelope components into the target cell plasma membranes) is difficult to provide. Chejanovsky & Loyter (1985) have demonstrated

Fig. 3. Graphs showing the loss of cell-associated radioactivity from cells with time after fusion of HTC cells in suspension with ^{125}I -RSVE

After fusion of $100\text{ }\mu\text{g}$ of ^{125}I -RSVE with 5×10^7 cells, the cells were grown in growth medium (●) or in growth medium containing either leupeptin (▲; $50\text{ }\mu\text{g/ml}$) or $10\text{ mM-NH}_4\text{Cl}$ (■). At each time point cell and medium samples were taken from three 90 mm-diam. dishes and were analysed in triplicate to obtain mean values. Errors were less than 10%.

that fluorescent lipid analogues inserted into RSVE undergo fluorescence dequenching and a decrease in the efficiency of fluorescence energy transfer between fluorophore pairs when RSVE are fused with target cells, but otherwise RSVE-cell fusion has to be inferred from indirect evidence. For instance, RSVE vesicles may be used as vehicles to deliver macromolecules into the cytosol of target cells. When RSVE loaded with radiolabelled pBR322 DNA are targeted at HTC cells in suspension, the entrapped DNA is delivered into the cells almost 12 times more efficiently than transfer of free DNA (Table 1), and is presumably the consequence of fusion-mediated microinjection.

The possibility that hepatoma cells internalize substantial amounts of RSVE by phagocytosis or endocytosis of bound non-fused vesicles during the $37\text{ }^\circ\text{C}$ incubation period of the fusion procedure was investigated. Cells in which intracellular ATP had been depleted by pre-treatment with 2-deoxyglucose and NaN_3 to inhibit endocytosis (Karnovsky, 1962; Ryser, 1968; Poste & Paphadjopoulos, 1976) were fused with ^{125}I -RSVE. No impairment of ^{125}I -RSVE-cell association was observed under these conditions (Table 2). This suggests that endocytosis of bound non-fused RSVE is not a major

mechanism by which RSVE become associated with HTC cells. The reasons why cells treated with metabolic inhibitors actually received more ^{125}I -RSVE than did control cells are not immediately clear, but may reflect the inability of ATP-depleted cells to internalize surface receptors for RSVE as efficiently as control cells after treatment with trypsin/EDTA solution before the fusion.

The transfer of reconstituted viral membrane to cells in a fusion event may be envisaged to result in implantation of an exogenous membrane 'patch' (in which HN and F glycoproteins are integral membrane proteins), into the cell surface. Chejanovsky & Loyer (1985) and Henis *et al.* (1985) have provided evidence that both phospholipid and protein components of RSVE fused with target cell plasma membranes are mobile and diffuse laterally in the plane of the membrane. It may be argued, therefore, that transplanted viral membrane components can be considered as part of the recipient membrane, and that the HN and F proteins may be treated in a similar way to endogenous plasma-membrane proteins. (Indeed, in Sendai-virus-infected cells both proteins are inserted into the plasma membrane after synthesis.) Thus the implantation of two well-defined (sequenced), but exogenous, membrane glycoproteins into the plasma membrane of HTC cells provided model proteins by which the mechanism(s) of plasma-membrane protein degradation may be investigated. To this end we studied the fate of HN and F proteins and their rate of degradation in hepatoma cells.

Transplanted HN and F proteins were degraded with long half-lives in HTC cells ($t_{1/2} = 67\text{--}93\text{ h}$, depending on whether cells were fused in suspension or in monolayer). Our method for calculation of protein $t_{1/2}$ values assumes that trichloroacetic acid-insoluble radioactivity released into the medium over the course of the experiment was not available for degradation (McElligott & Dice, 1983). This assumption is reasonable, since the precise origin of this material is unclear, e.g. debris from occasional lysed cells or shedding of HN and F from the surface of growing cells may both contribute. If degradation rates for HN and F are calculated solely from the loss of cellular radioactivity, a shorter $t_{1/2}$ value is obtained (49.5 h for suspension-fused cells). This value is greater than that obtained by Hare & Huston (1985a,b), who measured the loss of trichloroacetic acid-insoluble radiolabel from hepatoma cells fused in suspension with RSVE by using poly(ethylene glycol) as a fusogen. The value obtained by Hare & Huston (1985b) for degradation of HN and F proteins ($t_{1/2} = 46\text{ h}$) after fusion of cells with hybrid RSVE-band-3 virosomes is more similar to the values reported here.

The measured half-life of HN and F proteins reported in the present paper is clearly the average of the rates of degradation of the two proteins. However, electrophoretic analysis indicates that both proteins are degraded at very similar rates (Fig. 2). This contrasts with the findings of Hare & Huston (1985b), who report that HN ($t_{1/2} = 37\text{--}40\text{ h}$) is degraded more slowly than F ($t_{1/2} = 21\text{--}23\text{ h}$) after poly(ethylene glycol)-mediated fusion of virosomes with hepatoma cells. We suggest that this discrepancy may arise from the fact that, whereas in virosomes the F protein is non-fusogenic (Hare & Huston, 1985a), in our hands both HN and F proteins are biologically active and the RSVE are intrinsically fusogenic.

When hepatoma-cell plasma membranes are labelled externally with ^{125}I by lactoperoxidase/glucose oxidase-catalysed iodination, degradation of the labelled proteins occurs in a bimodal manner (Hare & Huston, 1984; Chu & Doyle, 1985). This is also true for plasma-membrane proteins in other cell lines (Baumann & Doyle, 1978; Kaplan *et al.*, 1979). Hare & Huston (1984) report that surface-iodinated plasma-membrane proteins fall into two categories: those degraded with $t_{1/2} = 16$ h and those degraded more slowly ($t_{1/2} > 40$ h). The long half-lives of implanted Sendai-viral envelope glycoproteins in HTC cells suggest that HN and F proteins are treated like the slowly degraded group of proteins described by Hare & Huston (1984). These categories are more closely defined by Chu & Doyle (1985), who found in hepatoma cells that, whereas total externally oriented plasma-membrane proteins were degraded with an average $t_{1/2}$ of 87 h, concanavalin A-bound glycoproteins were degraded more rapidly (average $t_{1/2} = 42$ h), leading the authors to the view that 'the bulk of glycoproteins are turned over faster than the minimally glycosylated proteins'. Transplanted Sendai-viral glycoproteins are degraded at rates similar to the average half-life of concanavalin A-binding glycoproteins of the hepatoma cell surface, when calculated solely from the loss of cell-associated radioactivity, i.e. as in Chu & Doyle (1985). The division of the degradation rates of proteins in a defined cytomorphological site, e.g. plasma membrane, into two groups such as these is predicted by the hypothesis of limited groups (populations) of degradation rates in each cytomorphological site, which we have previously proposed (Russell *et al.*, 1982).

In order to ascertain whether lysosomal degradation is an important mechanism for the breakdown of labelled HN and F proteins implanted into HTC-cell plasma membrane, the effects of the thiol proteinase inhibitor, leupeptin, and of a lysosomotropic agent, NH_4Cl , on the kinetics of viral protein degradation were investigated. Although it is recognized that neither agent specifically affects only lysosomal functions, it is clear that the rate of HN- and F-protein degradation is substantially inhibited by leupeptin and by NH_4Cl (Fig. 3). Inhibition was maintained throughout the experiments, strongly implicating lysosomes in the degradation of the viral proteins in HTC cells. These findings are in accord with the inhibitory effects of NH_4Cl and leupeptin reported by Huston & Hare (1984) on the degradation of surface-iodinated membrane proteins in dividing hepatoma cells. They found that in $\text{H}_4\text{-II-E}$ clone 3 hepatoma cells degradation of iodinated plasma-membrane protein was inhibited over the period 0–24 h by 21% by 50 μM -leupeptin and by 60% by 10 mM- NH_4Cl . More recently, they reported that degradation of HN and F proteins is > 90% sensitive to 20 mM- NH_4Cl after poly(ethylene glycol)-mediated virosome-hepatoma-cell fusion (Hare & Huston, 1985b).

In conclusion, therefore, Sendai-viral HN and F proteins implanted into the plasma membrane of HTC cells do appear to act as probes of the mechanism of

degradation of one population of HTC-cell plasma-membrane proteins, in terms of both their rates of catabolism and the lysosomal involvement in their degradation.

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REFERENCES

- Bartzatt, R. L. & Volsky, D. J. (1984) *Biosci. Rep.* **4**, 551–557
- Baumann, H. & Doyle, D. (1978) *J. Biol. Chem.* **253**, 4408–4418
- Birnboim, H. C. (1983) *Methods Enzymol.* **100**, 243–255
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Brown, J. P., Tamerius, J. D. & Hellstrom, I. (1979) *J. Immunol. Methods* **31**, 201–209
- Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132–135
- Chejanovsky, N. & Loyter, A. (1985) *J. Biol. Chem.* **260**, 7911–7918
- Chejanovsky, N., Beigel, M. & Loyter, A. (1984) *J. Virol.* **49**, 1009–1013
- Chu, F.-F. & Doyle, D. (1985) *J. Biol. Chem.* **260**, 3097–3107
- Doherty, F. J. & Mayer, R. J. (1985) *Biochem. J.* **226**, 685–695
- Hare, J. F. & Huston, M. (1984) *Arch. Biochem. Biophys.* **233**, 547–555
- Hare, J. F. & Huston, M. (1985a) *Exp. Cell Res.* **161**, 317–330
- Hare, J. F. & Huston, M. (1985b) *Exp. Cell Res.* **161**, 331–341
- Henis, Y. I., Gutman, O. & Loyter, A. (1985) *Exp. Cell Res.* **160**, 514–526
- Kaplan, J., Unkeless, J. C. & Cohn, Z. A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3824–3828
- Karnovsky, M. L. (1962) *Physiol. Rev.* **42**, 143–168
- Labarca, C. & Paigen, K. (1980) *Anal. Biochem.* **102**, 344–352
- Laemmli, K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp. 88–91, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Mayer, R. J. & Walker, J. H. (1980) *Immunological Methods in the Biological Sciences: Enzymes and Proteins*, Academic Press, London
- McElligott, M. A. & Dice, J. F. (1983) *Biochem. J.* **216**, 559–566
- Poste, G. & Papahadjopoulos, D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1603–1607
- Russell, S. M. & Mayer, R. J. (1983) *Biochem. J.* **216**, 163–175
- Russell, S. M., Burgess, R. J. & Mayer, R. J. (1982) *Biochim. Biophys. Acta* **714**, 34–45
- Ryser, H. J. P. (1968) *Science* **159**, 390–396
- Scheid, A. & Choppin, P. W. (1974) *Virology* **57**, 475–479
- Silverman, J. A. & Amenta, J. S. (1984) *Anal. Biochem.* **141**, 538–544
- Tack, B. F., Dean, J., Eilott, D., Lorenz, P. E. & Schechter, A. N. (1980) *J. Biol. Chem.* **255**, 8842–8847
- Toister, Z. & Loyter, A. (1973) *J. Biol. Chem.* **248**, 422–432
- Vainstein, A., Hershkovitz, M., Israel, S. & Loyter, A. (1984) *Biochim. Biophys. Acta* **773**, 181–188
- Weinstock, R., Sweet, R., Weiss, M., Cedar, H. & Axel, R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1299–1303