A putative protein-sequestration site involving intermediate filaments for protein degradation by autophagy

Studies with transplanted Sendai-viral envelope proteins in HTC cells

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Reconstituted Sendai-viral envelopes (RSVE) were fused with hepatoma tissue-culture (HTC) cells, thereby introducing viral membrane glycoproteins into the plasma membrane [Earl, Billett, Hunneyball & Mayer (1987) *Biochem. J.* **241**, 801–807]. Fractionation of homogenized cells on Nycodenz gradients shows that much of the viral ^{125}I -labelled HN and F proteins were rapidly sequestered into a dense fraction distinct from fractions containing plasma membrane, lysosomes and mitochondria. Electron microscopy (results not shown) indicates that the dense fraction contains nuclear residues, multivesicular structures, dense bodies and fibrous structures. Both the dense fraction and a hexosaminidase-enriched fraction contain trichloroacetic acid-insoluble radioactivity, including intact ^{125}I -labelled viral proteins. The viral proteins are progressively transferred from the dense fraction to the hexosaminidase-enriched fraction; the transfer is retarded by 50 μg of leupeptin/ml. Trichloroacetic acid-soluble radiolabel is progressively released into the culture medium as the proteins are degraded. Within 5 h after transplantation of viral HN and F proteins into recipient cells, a proportion (approx. 45%) of the ^{125}I -labelled glycoproteins cannot be extracted by sequentially treating cells with digitonin (1 mg/ml), Triton X-100 (1%, w/v) and 0.3 M-KI. HN and F proteins in the non-extractable residue are tightly associated with nuclear-intermediate-filament (vimentin) material, as shown by Western blots and electron microscopy. The viral proteins are progressively transferred out of the nuclear-intermediate-filament residue; the transfer is slowed when cells are cultured with leupeptin. The data are consistent with the notion that transplanted viral HN and F proteins are sequestered to a perinuclear site in tight association with intermediate filaments before transfer into the autophagolysosomal system for degradation.

INTRODUCTION

The envelope of Sendai virus can be extracted by non-ionic detergents and reconstituted into membrane vesicles which contain both the haemagglutinin-neuraminidase (HN) and the fusion factor (F). We have used reconstituted Sendai-viral envelopes (RSVE), which are intrinsically able to attach to and fuse with tissue-culture cells, to implant HN and F proteins into the surface of hepatoma tissue-culture (HTC) cells to probe the mechanisms of plasma-membrane protein catabolism.

In the preceding paper (Earl *et al.*, 1987) we provide evidence that HN and F proteins are degraded like one group (Chu & Doyle, 1985) of endogenous plasma-membrane proteins in HTC cells. The viral proteins are degraded with long half-lives ($t_{1/2}$ 67 h) by a process which is strongly inhibited by 10 mM- NH_4Cl or 50 μg of leupeptin/ml, thus implying the involvement of lysosomes in their degradation. However, lysosomal degradation is but the final step in a sequence of events. We have studied some of the earlier events which ultimately direct viral membrane glycoproteins into the autophagolysosomal system.

MATERIALS AND METHODS

Materials

Na^{125}I and mouse anti-vimentin monoclonal antibody were purchased from Amersham International, Amer-

sham, Bucks., U.K. Nycodenz and Maxidenz were obtained from Nyegaard and Company, Oslo, Norway. Peroxidase-conjugated rabbit anti-mouse IgG antibody was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were the highest grade available.

Sendai virus and preparation of ^{125}I -RSVE

RSVE were prepared from Sendai-virus particles as described by Earl *et al.* (1987).

Cell culture and fusion with ^{125}I -RSVE

HTC cells (derived from the Morris hepatoma 7228c line) were cultured as described by Earl *et al.* (1987). The cells were fused with ^{125}I -RSVE in suspension.

Density-gradient fractionation of HTC cells after fusion with ^{125}I -RSVE

At intervals after fusion with RSVE, cells on eight 9 cm-diam. dishes were washed once with phosphate-buffered saline (pH 7.4; Doherty & Mayer, 1985) and once with homogenization medium (0.2 M-sucrose/3 mM-KCl/0.3 mM-EDTA/5 mM-Tris/HCl, pH 7.4), before being scraped into 4 ml of homogenization medium. The cells were homogenized at 4 °C with ten strokes of a Dounce glass homogenizer (16–25 μm clearance), then centrifuged (1000 g for 10 min at 4 °C) to yield the supernatant (S_1) and pellet (P_1). The pellet was

Abbreviations used: HTC, hepatoma tissue culture; RSVE, reconstituted Sendai-viral envelopes; HN, haemagglutinin-neuraminidase protein; F, fusion protein.

suspended in 1 ml of homogenization medium, homogenized again and re-centrifuged, yielding supernatant (S_2) and pellet (P_2). Pellet P_2 contained considerable radioactivity, but was too gelatinous to apply to gradients, and was therefore re-homogenized for a third time, yielding a supernatant (S_3) and pellet (P_3). A 4 ml sample of combined supernatants ($S_1 + S_2 + S_3$) was applied to each self-generated Nycodenz (10–50%, w/v) gradient, constructed as in Doherty *et al.* (1987). After centrifugation at 84000 g for 30 min in an MSE 6 $\times$ 16.5 ml swing-out rotor, gradients were displaced with Maxidenz and fractions (0.5 ml) were collected. Radioactivity was determined in each fraction by using a Mini-Assay type 620 gamma counter, and each fraction was assayed for subcellular markers.

Detergent and salt extraction of RSVE-fused cells

This procedure was carried out exactly as described in Doherty *et al.* (1987).

Polyacrylamide-gel electrophoresis in the presence of SDS and autoradiography

SDS/polyacrylamide-gel electrophoresis was carried out as described by Laemmli (1970), by using 5–15% (v/v) polyacrylamide gradient gels. Protein bands were detected by silver staining (Wray *et al.*, 1981). Radioactivity was detected by autoradiography (Bonner & Laskey, 1974).

Western blotting

Western blotting was carried out essentially as described by Towbin *et al.* (1979). Nitrocellulose was pre-blocked by the method of Johnson *et al.* (1984), then incubated with antibody (mouse anti-vimentin monoclonal) as described by De Blas & Cherwinski (1983). The blots were washed five times over a 30 min period, and then incubated with second antibody, rabbit anti-mouse IgG conjugated to horseradish peroxidase, washed and stained as described by Adams (1981).

Biochemical assays

Leucine aminopeptidase was determined by the method of Peters *et al.* (1972), lactate dehydrogenase (EC 1.1.1.27) by the method of Johnson (1960), succinate dehydrogenase (EC 1.3.99.1) by the method of Pennington (1961), acid phosphatase (EC 3.1.3.2) by the method of de Duve *et al.* (1955), and *N*-acetylhexosaminidase (EC 3.2.1.52) by the procedure of Löffler *et al.* (1984). DNA was determined spectrofluorimetrically (Labarca & Paigen, 1980).

RESULTS

Nycodenz-density-gradient fractionation of HTC cell homogenates after fusion with ^{125}I -RSVE

When cell-free homogenates were prepared by repeated homogenization of RSVE-fused cells (see the Materials and methods section), approx. 50% of ^{125}I radioactivity was released into the supernatant fractions, but the remaining radioactivity could not be released from the gelatinous (P_3) pellet. Electrophoretic analysis of the pellet demonstrated the presence of nuclear histones and vimentin in this material (results not shown). Fractionation of the pooled supernatants on Nycodenz (10–50%, w/v) density gradients (three experiments) separated ^{125}I radioactivity into three discrete peaks, corresponding to

Fig. 1. Distribution of ^{125}I radioactivity in fractions collected from Nycodenz density gradients loaded with cell-free homogenates prepared from HTC cells 18 h (Δ), 40 h ($\blacksquare$) and 66 h ($\blacktriangle$) after fusion with ^{125}I -RSVE

Details of fractionation are described in the Materials and methods section.

fractions 8–9, 18–19 and 23–24 respectively (Fig. 1); the least ^{125}I was found in fractions 8–9 at each time point. Radioactivity in cells harvested 18 h after fusion was found predominantly in the peak of greatest density, but subsequently the proportion in this peak decreased, concomitant with an increase in the proportion of radioactivity in the intermediate-density peak. Total cell-associated radioactivity decreased with time as viral proteins were degraded (Earl *et al.*, 1987).

Figs. 2(b)–2(e) show the distribution of subcellular markers on the Nycodenz gradients. The activity of leucine aminopeptidase (plasma membrane) was restricted to two low-density peaks (fractions 2 and 6 respectively), distinct from the least dense peak of radioactivity (centred on fraction 9; Fig. 2a). The activities of the lysosomal markers *N*-acetylhexosaminidase (Fig. 2b) and acid phosphatase (Fig. 2d) were found in two peaks centred on fractions 9 and 18–19 respectively, which were coincident with the least-dense and intermediate-density peaks of viral ^{125}I radioactivity. Succinate dehydrogenase (mitochondria) was also coincident with the intermediate-density peak of radioactivity (Fig. 2e), indicating a mixture of organelles at this density. Some DNA was found associated with the highest-density peak of radioactivity, but most DNA was found in the gelatinous pellet obtained after cell homogenization (results not shown). ^{125}I -RSVE added to pre-homogenized cells, or added to cells at 4 °C and then homogenized, or RSVE alone had the distribution shown in Fig. 2(f).

Fig. 2. Distribution of radioactivity and marker enzymes in fractions collected from Nycodenz density gradients loaded with cell-free homogenates prepared from HTC cells 40 h after fusion with ^{125}I -RSVE

Control cells (■); leupeptin-treated cells (▲). (a) ^{125}I radioactivity; (b) *N*-acetylhexosaminidase activity; (c) leucine aminopeptidase; (d) acid phosphatase; (e) succinate dehydrogenase; (f) ^{125}I -RSVE alone (□); ^{125}I -RSVE mixed with cell-free homogenates at 4°C (○); ^{125}I -RSVE mixed with cells at 4°C, then homogenized (△).

Fig. 3. Densitometry of autoradiograph obtained after SDS/polyacrylamide-gel electrophoresis of material from the highest-density peak of radioactivity obtained when HTC cells were fractionated 24 and 48 h after fusion with ^{125}I -RSVE

(a) ^{125}I -RSVE; (b) control cells at 24 h; (c) control cells at 48 h; (d) leupeptin-treated cells at 48 h. Positions of F and HN proteins are indicated. Autoradiograms were scanned with white light with a Helena Quick Scan R+D Scanner (Helena Laboratories, Beaumont, TX, U.S.A.).

Leupeptin treatment caused changes in the distribution both of ^{125}I radioactivity and of some marker-enzyme activities (Fig. 2). In particular, there was an increase in the radioactivity in the intermediate-density peak, which was also shifted to greater density (Fig. 2a). Coincident with this change was a shift to greater density of the lysosomal-marker dense peak (Figs. 2b and 2d).

Radioactivity in the peaks of intermediate and high density (Fig. 2a) was > 90% precipitable in trichloroacetic acid (final concn. 10%, v/v). Electrophoretic analysis of material from the high-density peak revealed the presence of intact ^{125}I -labelled HN and F proteins (Fig. 3), which were also detectable in the intermediate-density peak (results not shown). Prolonged exposure of cells to leupeptin delayed the transfer of HN and F glycoproteins from the high-density peak (Figs. 3c and 3d).

Electron-microscopic investigation of material from the high-density peak of radioactivity in Nycodenz

gradients revealed the presence of nuclear residues, nuclear envelope vesicles, occasional disrupted mitochondria, multivesicular bodies and electron-dense fibrous material (results not shown).

Approx. 50–55% of radioactivity in cell-free homogenates (the combined supernatants; see the Materials and methods section) applied to gradients was recovered in fractions displaced by Maxident. Up to 35% of loaded radiolabel was evenly adherent to the walls of the plastic centrifuge tubes.

Detergent/salt extraction of cultured cells after fusion with ^{125}I -RSVE

The possible association of transplanted viral proteins with cytoskeletal elements was investigated by sequentially extracting cells with digitonin, Triton X-100 and KI solutions. These procedures sequentially release cytosolic

Fig. 4. Histograms showing the extractability of (a) marker enzymes and DNA from HTC cells by treatment sequentially with digitonin (D), Triton X-100 (TX) and KI, (b) trichloroacetic acid-insoluble radioactivity from cells at intervals after fusion with ^{125}I -RSVE, and (c) of the radioactivity from leupeptin-treated cells

Abbreviations: LNA, leucine aminopeptidase; LDH, lactate dehydrogenase; AP, acid phosphatase.

Fig. 5. SDS/polyacrylamide-gel electrophoresis of detergent- and salt-insoluble residues of HTC cells after fusion with ^{125}I -RSVE

Gels were stained with silver, showing: track 1, protein M_r ($\times 10^{-3}$) standards; tracks 2(a) and 2(b), cell residues; track 3, nuclear protein standards (histones) obtained from sonicated chicken erythrocyte nuclei. Note that histone H5 is avian-specific.

proteins, membrane proteins and vesicle contents, disrupt microtubules and remove actin filaments, leaving a residue enriched in intermediate filaments (Mackall *et al.*, 1979; Heuser & Kirschner, 1980; Friedman *et al.*, 1984; Doherty *et al.*, 1987).

Treatment of cells with digitonin (1 mg/ml) for 2 min at 4°C preferentially released lactate dehydrogenase (55%), a cytosolic enzyme (Fig. 4a), but also extracted approx. 25% of the plasma-membrane marker (leucine aminopeptidase) and approx. 30% of lysosomal acid phosphatase activity. Despite solubilization of some membrane components, only a small proportion ($< 10\%$) of trichloroacetic acid-insoluble ^{125}I -labelled viral proteins was released even at 5 h after fusion (Fig. 4b). When cells were then extracted with Triton X-100 (1%, w/v) for 1 h at 4°C , substantial solubilization of membranes was effected, as indicated by the release of 66% of leucine aminopeptidase activity, $> 40\%$ of lactate dehydrogenase activity, approx. 60% of acid phosphatase activity and approx. 55% of DNA. Treatment with Triton X-100 at 4°C (which also disrupts microtubules)

extracted 45–50% of trichloroacetic acid-insoluble radioactivity from the cells, but, importantly, neither detergent extraction nor exposure to 0.3 M-KI (which causes disruption of microfilaments) released a substantial proportion of the radiolabelled proteins from cells (Fig. 4b). The radiolabel contained in the cell residue accounted for approx. 45% of radiolabelled protein recovered from cells 5 h after fusion with RSVE and for approx. 35% of radioactive protein recovered 68 h after fusion. Leupeptin treatment tended to increase the proportion of radioactivity recovered in the residue (Fig. 4c).

Electrophoretic analysis of residue samples in the presence of SDS and 2-mercaptoethanol (the sample buffer extracts $> 80\%$ of endogenous residue proteins) showed multiple polypeptides (Fig. 5) including histones, and a protein band with an apparent M_r of approx. 58000, which was shown to be vimentin by Western blotting (results not shown). Autoradiography of the electrophoresed residue revealed that both HN and F

Fig. 6. Autoradiograph obtained of the detergent- and salt-insoluble residue from HTC cells (fused with ^{125}I -RSVE) after SDS/polyacrylamide-gel electrophoresis

Track 1, ^{14}C -labelled protein M_r ($\times 10^{-3}$) standards; track 2, residue obtained from leupeptin-treated cells in the presence of mercaptoethanol; track 3, residue obtained from leupeptin-treated cells in the absence of mercaptoethanol; track 4, residue from control cells electrophoresed in the presence of mercaptoethanol; track 5, residue from control cells electrophoresed in the absence of mercaptoethanol. Positions of HN and F proteins are indicated.

proteins were present (Fig. 6). If 2-mercaptoethanol was omitted from the Laemmli sample buffer, the two proteins migrated with apparent M_r values intermediate between those of the fully denatured polypeptides. In the presence or absence of 2-mercaptoethanol there were aggregated or conjugated viral proteins which did not enter the resolving gel, which indicates that disulphide cross-linking is probably not the cause of the aggregation (Fig. 6; cf. Doherty *et al.*, 1987).

Electron microscopy revealed the presence of 9–12 nm-wide truncated filaments associated with electron-dense bodies in the residue material (results not shown).

Taken together, the data suggest that, after fusion of HTC cells with RSVE, the viral proteins become in some way tightly associated with an intermediate-filament-enriched (Friedman *et al.*, 1984) cell residue.

DISCUSSION

When RSVE fuse with target cells the two transmembranous viral glycoproteins, HN and F, are implanted into the plasma membrane (Henis *et al.*, 1985). We have shown in the preceding paper (Earl *et al.*, 1987) that the fluorescein-conjugated viral proteins are internalized, within 24 h of fusion, to a perinuclear location. Degradation of internalized viral proteins occurs only relatively slowly ($t_{1/2} = 67$ h) and is inhibited by both leupeptin and NH_4Cl (Earl *et al.*, 1987). These observations suggest that viral proteins which are implanted in the plasma membrane are internalized and maybe sequestered in a defined intermediate site before donation into the autophagolysosomal system. This notion was investigated by two methods of fractionation of cells at intervals after fusion with ^{125}I -RSVE.

Repeated homogenization of HTC cells leaves approx. 50% of ^{125}I -labelled viral proteins in a gelatinous pellet containing histones, DNA and some vimentin, indicating a strong association with the nuclear-cytoskeletal matrix. However, radioactivity in the cell-free homogenates fractionated on Nycodenz density gradients was resolved into three discrete fractions (Fig. 1). Although the radioactivity found in the least-dense and intermediate-density peaks could be ascribed to structures possessing lysosomal enzyme activities (Figs. 2a, 2b and 2d), coincidence of the peak of radioactivity of greatest density with an identifiable organelle cannot be shown (Fig. 2). Moreover, the intact viral proteins (Fig. 3) at this position on the gradient are at a higher density than is seen for intact RSVE or RSVE added to pre-homogenized cells (Fig. 2f) or for plasma-membrane marker (Fig. 2c). The viral components in the peak of greatest density are probably released from the tight perinuclear association (shown by the localization of internalized fluorescein-labelled HN and F proteins; see Earl *et al.*, 1987) by homogenization, leaving the gelatinous (nuclear) pellet, which is unamenable to fractionation. Precisely in what form the viral proteins might associate around cell nuclei is not easily defined (but see below).

The temporal redistribution of HN and F proteins (Fig. 3) from high- to intermediate-density peaks is consistent with a shift from one cytological site to a second site (whose density is coincident with that of secondary lysosomes). When cells are treated with

leupeptin, radioactivity accumulates in the intermediate-density (lysosomal) peak, which consequently shifts to slightly greater density (Kominami *et al.*, 1983; Fig. 2a), but no accumulation in the peak of greatest density is seen (Fig. 2a). It is therefore clear that viral proteins which at first were implanted into the plasma membrane and subsequently internalized to a perinuclear site (Earl *et al.*, 1987) are eventually degraded in lysosomes. If the capacity of lysosomes to degrade the viral proteins is rate-limiting, i.e. to give a $t_{1/2} = 67$ h (Earl *et al.*, 1987), it seems logical that proteins destined for degradation should be sequestered at an intermediate site where they will not impair normal cellular functioning.

The location of internalized viral membrane proteins was investigated further by treatment of cells sequentially with digitonin (Mackall *et al.*, 1979), Triton X-100 at 4 °C (Heuser & Kirschner, 1980) and finally with 0.3 M-KI (Friedman *et al.*, 1984). Cell markers, including DNA, were for the most part released by the detergent treatments (Fig. 4a), yet a substantial proportion of viral proteins was not released. Release of marker enzymes from membranes and membrane-bounded organelles argues against the continued existence of the viral proteins in membranes in the intractable residue. Instead it implies either aggregation (e.g. cross-linking), or a tight association with a nuclear-cytoskeletal (intermediate-filament-enriched) residue (Figs. 4–6), possibly through interaction with the cytoplasmic domains of the viral proteins (Hsu & Choppin, 1984; Blumberg *et al.*, 1985).

In 3T3-L1 cells, microinjected lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase similarly associate with a nuclear-cytoskeletal residue, as do endogenously radiolabelled proteins in these cells (Doherty *et al.*, 1987). Clearly, therefore, this is not a phenomenon restricted to transplanted viral membrane proteins in hepatoma cells. We believe that the data are consistent with the notion of a sequestration site for both transplanted and cellular proteins, involving intermediate filaments before autophagolysosomal protein degradation.

Finally, we should not lose sight of the fact that the observed intracellular processing of viral membrane glycoproteins may be a cellular response to stress, i.e. the implantation of viral proteins into the plasma membrane. Sequestration of viral proteins at an intracellular site may prevent the impairment of normal cellular functioning: the most obvious site would be a site concerned with the collection of proteins for autophagolysosomal degradation.

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