Degradation of transplanted rat liver mitochondrial-outer-membrane proteins in hepatoma cells

Susan M. RUSSELL and R. John MAYER*

Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K.

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Reductively [^3H]methylated ^3H mitochondrial-outer-membrane vesicles from rat liver and vesicles where monoamine oxidase has been derivatized irreversibly by [^3H]pargyline have been deliberately miscompartmentalized by heterologous transplantation into hepatoma (HTC) cells by poly(ethylene glycol)-mediated vesicle–cell fusion. Fluorescein-conjugated mitochondrial-outer-membrane vesicles have also been used to show that transplanted material is patched, capped and internalized. Reductively methylated outer-membrane proteins and monoamine oxidase are destroyed at the same rate ($t_{\frac{1}{2}}$ 24 h). Mitochondrial-outer-membrane proteins are not degraded at the same rate as HTC plasma-membrane proteins, endogenous cell protein, or endocytosed protein. Transplanted radiolabelled mitochondrial-outer-membrane proteins accumulate intracellularly in structures that are distinct from plasma membrane and lysosomes. However, when mitochondrial-outer-membrane vesicles derivatized with [^{14}C]sucrose are transplanted, the acid-soluble degradation products accumulate in the lysosomal fraction. [^{14}C]Sucrose-conjugated HTC cell plasma membrane accumulates in intracellular structures that are again distinct from plasma membrane and lysosomes. In contrast with the above observations, homologously transplanted mitochondrial-outer-membrane proteins from rat liver are destroyed in hepatocytes at rates that are remarkably similar ($t_{\frac{1}{2}}$ 60–70 h) to the rates in rat liver *in vivo* [Evans & Mayer (1982) *Biochem. Biophys. Res. Commun.* **107**, 51–58].

Intracellular proteins are degraded at rates that are characteristic of cell type and that can be modulated nutritionally, hormonally and in pathological states.

We have recently proposed that the diversity of degradation rates within biosynthetically defined cytomorphological sites is more limited than generally supposed (Russell *et al.*, 1982). This view is supported by the unit-turnover hypothesis for the plasma membrane (Tweto & Doyle, 1977) and the similarity of degradation rates for proteins in the mitochondrial inner membrane (Druyan *et al.*, 1969;

Aschenbrenner *et al.*, 1970; Ip *et al.*, 1974). Limited populations (Russell *et al.*, 1982) of degradation rates within a single cytomorphological site may be due to the action of different degradation systems, i.e. selective and non-selective. A dual mechanism for protein degradation (lysosomal and non-lysosomal) has been proposed from studies with inhibitors and extracellular modulators of intracellular proteinolysis (Knowles & Ballard, 1976). Lysosomal autophagy may be (for reviews, see Dean & Barrett, 1976; Ballard, 1977) responsible for non-selective destruction of proteins in each biosynthetically defined subcellular site. However, little is known about the selective process(es). Features of protein structure have been proposed to determine degradation rate (Dehlinger *et al.*, 1971; Dice & Goldberg, 1975; Bohley *et al.*, 1977), but we could find no evidence relating either protein size or charge to degradation rate for proteins in defined cytomorphological sites in rat liver (Russell *et al.*, 1982).

Abbreviations used: MOM, mitochondrial outer membrane; HTC, hepatoma tissue culture; EMEM, Eagle's minimal essential medium; HBSS, Hanks balanced salt solution; PEG-1000, poly(ethylene glycol) 1000; EBSS, Earle's balanced salt solution; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

* To whom correspondence and requests for reprints should be addressed.

Therefore limited heterogeneity of degradation (populations of degradation rates) in subcellular sites may be determined by the biochemical characteristics of each cytomorphological site.

We decided to examine this possibility by the deliberate miscompartmentalization of prelabelled MOM (mitochondrial-outer-membrane) vesicle proteins into hepatoma plasma membrane by poly-(ethylene glycol)-mediated MOM-hepatoma-cell fusion. This heterologous transplantation of liver MOM into hepatoma cells allows elucidation of the information content of MOM and its proteins in determining intrinsic protein-destruction rates.

The results show that transplanted MOM is not retained in the hepatoma plasma membrane. The MOM is rapidly internalized by a process analogous to lymphocyte patching and capping. Subsequent degradation is more rapid than that observed for MOM proteins in rat liver *in vivo* (Russell *et al.*, 1980, 1982) or for MOM proteins homologously transplanted [Evans & Mayer, 1982; the preceding paper (Evans & Mayer, 1983)] into rat hepatocytes (which are degraded at rates comparable with those observed *in vivo*). However, heterologously transplanted MOM proteins are destroyed at significantly slower rates than proteins internalized by endocytosis.

Materials and methods

Materials

NaB^3H_4 (5–20 Ci / mmol), $[\text{U}-^{14}\text{C}]\text{sucrose}$ (>350 mCi / mmol), Na^{125}I and $[4,5-^3\text{H}]\text{leucine}$ (170 Ci / mmol) were purchased from Amersham International. $[\text{phenyl-3-benzyl-}^3\text{H}]\text{Pargyline}$ hydrochloride was obtained from New England Nuclear Corp., and unlabelled pargyline hydrochloride was obtained from Abbot Laboratories, Chicago, IL, U.S.A. Percoll was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were the highest grade available.

Cell culture

HTC cells (derived from Morris hepatoma 7288c) purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.) were cultured in EMEM supplemented with MEM non-essential amino acids (Gibco, Paisley, Renfrewshire, Scotland, U.K.). Growth medium contained 10% (v/v) heat-inactivated newborn-calf serum and was replaced every 48–72 h. Cells were maintained as monolayer cultures on 90 mm-diameter plastic culture dishes (Nunc, Roskilde, Denmark) at 37°C in an incubator under CO_2/air (1:19).

Preparation of MOM fraction from rat liver

MOM vesicles were prepared from 180–200 g Wistar rats exactly as described by Martinez &

McCauley (1977). The vesicles were washed after preparation in 1 M-NaCl by sonication for 20 s at 70 W (Soniprobe; Dawe, London W.3, U.K.), followed by centrifugation at 100 000 g_{av} for 30 min at 4°C. The membrane vesicles were labelled with $[\text{}^3\text{H}]\text{pargyline}$ (15.3 Ci / mmol) by incubating re-sonicated MOM (10 mg of protein) in 20 mM-potassium phosphate buffer, pH 7.4, with 30 μCi of $[\text{}^3\text{H}]\text{pargyline}$ for 60 min at 30°C. After the incubation, the MOM-vesicle suspension was exhaustively dialysed for 18 h at 4°C against HBSS without Ca^{2+} or Mg^{2+} , supplemented with 5 μM -pargyline. Reductively methylated MOM vesicles were prepared with NaB^3H_4 (5–20 Ci / mmol) by the method of Tack *et al.* (1980), modified by the use of 0.4 μmol of formaldehyde / mg of protein and 1 mCi of NaB^3H_4 / mg of protein. After reductive methylation the MOM vesicles were washed by centrifugation in 40 ml of phosphate-buffered saline (Dulbecco & Vogt, 1954). Pelleting of the MOM vesicles was prevented by a 1 ml cushion of 2.2 M-sucrose. The yellow layer of MOM vesicles was collected and exhaustively dialysed against HBSS without Ca^{2+} or Mg^{2+} for 18 h at 4°C. In experiments with MOM vesicles labelled with both $[\text{}^3\text{H}]\text{pargyline}$ and $[\text{}^{14}\text{C}]\text{sucrose}$, the vesicles were first labelled with $[\text{}^3\text{H}]\text{pargyline}$ then labelled with $[\text{}^{14}\text{C}]\text{sucrose}$ by the method of Pittman *et al.* (1979). The vesicles were dialysed against HBSS without Ca^{2+} or Mg^{2+} and containing 5 μM -pargyline. Fluorescein-labelled MOM vesicles were prepared by incubating vesicles (10 mg of protein) in 1 ml of 0.05 M- Na_2CO_3 buffer, pH 8.5, with 0.1 mg of fluorescein isothiocyanate isomer I (Sigma) for 30 min at 0°C. The membranes were washed in 40 ml of phosphate-buffered saline and collected by centrifugation on to a cushion (1 ml) of 2.2 M-sucrose. The fluorescein-labelled MOM was dialysed against HBSS without Ca^{2+} or Mg^{2+} at 4°C for 18 h.

Fusion

MOM vesicles were fused to HTC cells by a modification of the method used by Baumann *et al.* (1980). In a typical fusion procedure, monolayer cultures of hepatoma cells (yielding approx. 10^8 cells) were treated with trypsin/EDTA solution (Gibco; 1 mg of trypsin / ml; 0.4 mg of EDTA / ml) for 2 min at 37°C. The suspended cells were washed once with growth medium and three times with phosphate-buffered saline (10 ml). To the packed cell pellet was added 0.2 ml of freshly prepared and dialysed MOM suspension (1–3 mg of MOM protein / 10^8 cells per fusion). The components were mixed thoroughly by swirling and 0.6 ml of 50% (w/v) PEG-1000 (BDH Chemicals) in serum-free medium were added. The pH of the PEG-1000 solution was adjusted immediately before use to pH 7.2 with 0.1 M-NaOH. The cell-MOM fusion

mixture was incubated for 5 min at 37°C and then 15 ml of serum-free medium was added. After a further 2 min the cells were collected by centrifugation and washed twice with EMEM containing penicillin G (100 µg/ml) and streptomycin (60 µg/ml). The cells were then suspended in growth medium and plated out for monolayer culture. Viability of the cells after fusion (using the Trypan Blue exclusion test) was approx. 95%.

Measurement of hepatoma-cell protein degradation

Cellular protein degradation rate was measured by incubating 7×10^5 cells/plate with 0.05 µCi of [³H]leucine for 18 h, washing the cells (3 h) in growth medium supplemented with 2 mM-leucine and culturing the cells in the same medium. Plates of cells were taken at intervals for determination of trichloroacetic acid-soluble and -insoluble radioactivity. Growth medium was replaced every 48 h.

Measurement of hepatoma plasma-membrane protein degradation

Cells were iodinated by a modification of the method of Tweto *et al.* (1976). The initial density of the cells before iodination was 4×10^5 cells/plate. The cells were washed three times with EBSS (5 ml/plate) without Phenol Red, Ca²⁺ or Mg²⁺ (modified EBSS) and then labelled for 30 min at 37°C in modified EBSS (1 ml/plate) containing Tricine (0.1 M; pH 7.6), glucose (7 mM), 3 m-units of lactoperoxidase (Sigma), 1.75 m-units of glucose oxidase (Sigma, type II) and 0.05 mCi of Na¹²⁵I diluted to a specific radioactivity of 170 mCi/mmol with Na¹²⁷I. After labelling the cells were washed with modified EBSS (5 ml/plate) and incubated with growth medium (10 ml/plate). Plates were taken at intervals for the determination of trichloroacetic acid-soluble and -insoluble radioactivity.

Coupling of [¹⁴C]sucrose to plasma membrane

Cells (10^6 – 10^7) were harvested by treatment with trypsin, washed twice in HBSS without Phenol Red and then suspended in HBSS (0.2 ml) without Phenol Red. Cyanuric chloride-activated [¹⁴C]sucrose (100 µCi), prepared by the method of Pittman *et al.* (1979), was added and the suspension incubated for 30 min at 4°C. The cells were washed three times with HBSS (10 ml) without Phenol Red, resuspended in growth medium and cultured.

Subfractionation of HTC cells after fusion

Subfractionation of the cells after fusion was achieved by (i) Percoll-gradient centrifugation of sonicated cells and (ii) by differential centrifugation of homogenized cells.

(i) *Percoll gradient centrifugation of sonicated cells.* Cells were washed twice with serum-free medium and harvested by scraping into 0.25 M-

sucrose (0.5–1 ml). In preliminary experiments, several sonication conditions ranging from no sonication to 80 W for 30 s (Dawe Soniprobe) were tested to establish conditions giving optimum cell breakage with minimal damage to subcellular structures. Sonicated cells (70 W for 15 s) were layered on to 10 ml of Percoll in 0.25 M-sucrose (mean density 1.075 g/ml) and centrifuged for $1.3 \times 10^6 g_{av}$ -min in rotor no. 43114-121 on the MSE 75 centrifuge (MSE Ltd., Crawley, Sussex, U.K.). The gradient was collected in 19–20 fractions and densities determined by fractionating an equivalent gradient containing density marker beads (Pharmacia, Uppsala, Sweden).

(ii) *Differential centrifugation of homogenized cells.* Cells were harvested as described above and washed by centrifugation in 0.25 M-sucrose/3 mM-imidazole buffer, pH 7.4. The cell pellet was resuspended in the same solution to a density of 2×10^7 cells/ml. Experiments were performed with 10^7 – 10^9 cells. Cells were homogenized by 30–40 strokes of a 3 ml ground-glass homogenizer (Jencons, Hemel Hempstead, Herts., U.K.). Fractionation by differential centrifugation was performed as described by Lopez-Saura *et al.* (1978).

Biochemical assays

Monoamine oxidase (EC 1.4.3.4) activity was assayed in duplicate by adding 50 µl samples to 100 µl samples of 20 mM-potassium phosphate buffer, pH 7.4, containing 0.5 mM-[¹⁴C]tyramine (1 mCi/mmol) and incubating at 30°C for 30 min. The reaction was carried out in plastic scintillation-vial inserts (Hughes and Hughes, Romford, Essex, U.K.) and terminated by the addition of 100 µl of 2 M-HCl. Blank vials were prepared by adding the acid before incubation. A 3 ml portion of 1% (w/v) diphenyloxazole in toluene/ethyl acetate (1:1, v/v) was added to each vial insert and the radioactive product was extracted into the upper non-aqueous phase by vortex-mixing for 1 min. Extraction efficiency of the products into the upper organic phase was >98% and was not corrected for. Assays were linear with time and protein concentration.

The following subcellular markers were also assayed: alkaline phosphodiesterase I (EC 3.1.4.1) (Beaufray *et al.*, 1974), acid phosphatase (EC 3.1.3.2) (De Duve *et al.*, 1955), succinate dehydrogenase (EC 1.3.99.1) (Pennington, 1961) and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) (Sellinger *et al.*, 1960). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Gel electrophoresis

Analysis of the labelled MOM proteins was exactly as described by Laemmli (1970) and the radioactivity was detected by fluorography (Bonner & Laskey, 1974).

Fluorescence microscopy

Hepatoma plasma-membrane proteins were labelled with fluorescein by incubating cells for 1 min at 4°C with 1 ml of 0.05 M- Na_2CO_3 buffer, pH 8.5, containing 0.1 mg of fluorescein isothiocyanate isomer I (Sigma). The cells were then washed by centrifugation four times in HBSS (10 ml) before plating out. Cells for fluorescence microscopy were fixed by immersing them in glutaraldehyde (2%, v/v) for 5 min and then in glycerol (50%, v/v). Fluorescence microscopy was carried out with a Leitz SM-Lux microscope.

Electron microscopy

Cells in suspension were collected by centrifugation and resuspended in 20 mM-potassium phosphate buffer, pH 7.4, containing 0.15 M-NaCl and glutaraldehyde (2%, v/v). Cells in monolayer culture were fixed on the culture plate in the same buffer. After at least 2 h fixation the cells were washed thoroughly in phosphate-buffered saline and postfixed with OsO_4 (1%) in 0.05 M-Veronal acetate/HCl buffer, pH 7.4. This was followed by dehydration in alcohol and embedding in Araldite. Sections were cut with an LKB Ultratome III equipped with a diamond knife and double stained with lead acetate and uranyl acetate. Electron microscopy was carried out with a Philips EM300 microscope.

Radioactivity measurements

Degradation measurements were made on samples (1 ml) of aspirated media made 10% (w/v) in trichloroacetic acid by adding 50% (w/v) trichloroacetic acid or on cells harvested in 10% (w/v) trichloroacetic acid (3 ml/plate) at 0°C. Acid-soluble radioactivity was measured in samples (200 μl) of the supernatant obtained by centrifuging medium or cell suspensions treated with trichloroacetic acid (10%, w/v). Acid-insoluble pellets were washed twice with 5 ml of 10% (w/v) trichloroacetic acid, solubilized in 50% (v/v) formic acid (500 μl) and 200 μl samples taken for radioactivity measurements. Radioactivity present as ^{14}C or ^3H was measured after adding 3 ml of Fiso fluor in a Packard 460 CD liquid-scintillation counter, and ^{125}I radioactivity was measured in a Mini-Assay type 6.20 gamma counter.

Trichloroacetic acid used in the experiments with pargyline contained 5 μM -pargyline hydrochloride, in the experiments with leucine, 2 mM-leucine, in the experiments involving reductive methylation, 10 mM-lysine and in the experiments with ^{125}I , 25 mM-sodium metabisulphite and 5 mM-NaI.

Results

Radiolabelling of the MOM proteins

Reductive [^3H]methylation and [^{14}C]sucrose coupling labelled many MOM proteins (Fig. 1).

Fig. 1. Autoradiography of sodium dodecyl sulphate/polyacrylamide gels of MOM proteins

Proteins were labelled by (a) reductive [^3H]methylation, (b) [^3H]pargyline and (c) [^{14}C]sucrose conjugation. M_r standards are indicated on the left and right. [^{14}C]Sucrose-conjugated MOM proteins (c) were run on a different gel. The two forms of monoamine oxidase are indicated by A and B.

Pargyline labelled only the two forms (A and B) of monoamine oxidase (Fig. 1). Specific radioactivities of $(1-2) \times 10^8$ d.p.m./mg of protein were achieved by reductive [^3H]methylation, $(2-3) \times 10^5$ d.p.m./mg of protein by [^3H]pargyline labelling and $(0.5-1.0) \times 10^6$ d.p.m./mg of protein by [^{14}C]sucrose coupling.

Reductive methylation modified (to give mono- and di-methyl-lysine) 19.3% of the MOM-vesicle lysine groups (amino acid analyses; results not shown) and resulted in a $5.3 \pm 3.3\%$ ($n = 6$) inhibition of monoamine oxidase activity. Fractionation of the labelled membranes into a protein fraction, a glycolipid fraction and a phospholipid fraction (Bligh & Dyer, 1959) resulted in 86.5% of the radioactivity remaining in the protein fraction (results not shown). [^3H]Pargyline is a suicide inhibitor of monoamine oxidase and becomes covalently bound to the flavin moiety of the enzyme, which is also bound covalently to one of the enzyme polypeptide chains (Swett *et al.*, 1963; Cawthon *et al.*, 1980).

Fusion of MOM vesicles to HTC cells

The conditions employed for the fusion of MOM vesicles to HTC cells are similar to those used by others to incorporate biologically active plasma-

Table 1. *Effect of PEG, temperature and metabolic inhibitors on the extent of MOM-vesicle-cell fusion*

Fusion of reductively [^3H]methylated MOM vesicles to hepatoma cells was analysed by measuring the radioactivity insoluble in trichloroacetic acid (10%, v/v) recovered with the cells 4 h after mixing 0.1 mg of MOM vesicles with 10^7 cells. Each set of fusion conditions was tested in duplicate. Values given are means. Normal fusion conditions are described in the Materials and methods section. Conditions were varied by omitting PEG from the fusion mixture (no PEG), by an additional three washes in serum-free medium after PEG treatment and before addition of the [^3H]methylated MOM (PEG/wash/[^3H]MOM), by fusing at 0°C and by pretreating the cells for 30 min at 37°C with 5 mM- NaN_3 and 50 mM-deoxyglucose in serum-free medium. After fusion the cells were plated out in growth medium at a density of $10^6/90\text{ mm-diameter plate}$ and adherent cells were harvested 4 h later.

Fusion conditions	MOM-vesicle protein incorporated/ 10^8 cells (ng)	Fusion efficiency (%)
Normal fusion conditions	197.0	100
No PEG	0.2	0.1
PEG/wash/[^3H]MOM	43.3	22
0°C	193.1	98
Cells pretreated with NaN_3 + deoxyglucose	354.6	180

membrane proteins from rat liver into the plasma membrane of mouse fibroblasts (Doyle *et al.*, 1979; Baumann *et al.*, 1980). In a typical fusion, 1–3 mg of MOM protein was presented to 10^8 cells. The protein content of the cells was 800 pg/cell, which is higher than for cells growing in suspension (Leroy-Houyet *et al.*, 1979; McDonald & Gelehrter, 1981). After fusions the cells were washed and plated out. Approx. 90% of the cells remained adherent to the culture plates at 4 h after fusion.

The trichloroacetic acid-insoluble radioactivity associated with these cells was approx. 20% of that in labelled MOM presented to the target cells in the fusion. Transplanted MOM protein constituted approx. 6% of the protein content of the hepatoma cell plasma membrane (Leroy-Houyet *et al.*, 1979). The fusion was PEG-dependent and was not inhibited at 0°C (Table 1). Pretreatment of the cells with PEG and subsequent removal before adding labelled MOM gave a much lower fusion efficiency. Depletion of intracellular ATP by incubating the cells for 30 min in the presence of NaN_3 and deoxyglucose, which effectively inhibits endocytosis and micro-pinocytosis (Karnovsky, 1962; Ryser, 1968; Poste & Papahadjopoulos, 1976) apparently increased fusion efficiency (Table 1). However, the ATP-depleted cells were extremely difficult to

Table 2. *Distribution of radioactivity in cells and medium after fusion of methylated [^3H]MOM to HTC cells*

[^3H]Methylated MOM (1.36 mg, $2.44 \times 10^8\text{ d.p.m.}$) was fused to 0.78×10^8 cells as described in the Materials and methods section. The cells were diluted in growth medium (after washing) to a density of 4×10^6 cells/ml. Four samples (1 ml) of cell suspension were taken to determine the trichloroacetic acid (10%, w/v)-soluble and -insoluble radioactivity (see the Materials and methods section) immediately after fusion. The remaining cell suspension was split into plates containing 9 ml of prewarmed (37°C) growth medium (1 ml/90 mm-diameter plate) and cultured at 37°C in an incubator under CO_2/air (1:19). At 4 h after fusion the medium was collected and centrifuged at 200 g·min. Pelleted material and media were assayed for trichloroacetic acid (10%, w/v)-soluble and -insoluble radioactivity. Recovery of radioactivity at 4 h was 92.3%. All measurements were carried out in quadruplicate and the values given are means $\pm$ S.D.

	$10^{-3} \times$ Radioactivity (d.p.m.)	
	Acid-insoluble	Acid-soluble
Fused cells	24.72 ± 0.93	4.01 ± 0.16
Adherent cells 4 h after fusion	14.33 ± 0.36	2.45 ± 0.20
Sedimentable (200 g·min) radioactivity in culture medium	2.60 ± 0.28	1.97 ± 0.03
Medium supernatant	0.90 ± 0.11	4.28 ± 0.16

resuspend between washings and the apparent increased fusion efficiency was due in part to incomplete washing of the more aggregated cells.

Preliminary experiments showed that the radioactivity associated with the cells immediately after fusion was due to several components (Table 2). At 4 h after fusion, approx. 18% of the radioactivity associated with the cells immediately after fusion could be recovered by centrifuging the medium at 200 g·min. Phase-contrast microscopy of the pelleted material showed the presence of cells, a large proportion (>75%) of which were not viable by the Trypan Blue-exclusion test. Acid-insoluble radioactivity was also present in the medium supernatant, which may be due to unfused MOM vesicles released from the cell surface as the cells adhere to the culture dish (Table 2). Medium used to culture the cells for 24 h (after the initial 4 h period) contained little radioactivity in either a 200 g·min pellet (<1% of the initial cell-associated radioactivity) or in unpelleted trichloroacetic acid-insoluble radioactivity (<0.1% of the initial cell-associated radioactivity). Total acid-insoluble radioactivity converted into acid-soluble radioactivity over 4 h was 19%. However, it was not possible to determine what proportion of this was attributable to non-viable cells; therefore in all subsequent experi-

Fig. 2. *Degradation of transplanted MOM proteins*
Loss of trichloroacetic acid (10%, w/v)-insoluble radioactivity (—) and appearance of trichloroacetic acid (10% w/v)-soluble radioactivity (----) after transplanting (a) reductively [³H]methylated MOM or (b) [³H]pargyline-labelled MOM (—), [³H]pargyline/fluorescein-labelled MOM (----) into HTC cells or (c) loss of trichloroacetic acid (10% w/v)-insoluble ³H radioactivity after transplanting MOM labelled with both [³H]pargyline (0.23×10^6 d.p.m./mg of protein) and [¹⁴C]sucrose (6×10^3 d.p.m./mg of protein, 5.55 mCi/mmol of sucrose) into HTC cells. Degradation measurements were begun 4 h after fusion of 0.2 mg of MOM to 2×10^7 cells. Cells were placed out in monolayer culture at a density of 10^5 cells/90 mm-diameter plate for the HTC cells fused with reductively [³H]-methylated MOM, and 4×10^5 cells/90 mm plate for HTC cells fused with [³H]pargyline-labelled MOM. Four plates were taken at each time point for determination of radioactivity (values are means $\pm$ S.D.).

ments the 4 h-after-fusion medium was discarded and degradation measurements were started from 4 h.

Degradation of MOM proteins after fusion to HTC cells

Degradation of reductively [³H]methylated MOM fused with HTC cells to acid-soluble radioactivity in the medium approximates a single first-order decay curve with a half-life ($t_{1/2}$) of 26 h (Fig. 2a). The mean half-life obtained for ten separate fusion experiments using [³H]methylated MOM was 24.9 ± 3.3 h (mean $\pm$ S.D.). [³H]Pargyline-derivatized monoamine oxidase in MOM vesicles fused to the cells showed a similar rate of degradation, with a half-life of 24 h (Fig. 2b). The mean half-life for six separate fusion experiments using [³H]pargyline-labelled MOM was 25.1 ± 5.2 h (mean $\pm$ S.D.). Release of [³H]pargyline-containing acid-soluble degradation products into the medium lagged behind the loss of trichloroacetic acid-insoluble radioactivity within the cell; correspondingly, [³H]pargyline-containing acid-soluble radioactivity accumulated within the cell (results not shown).

Degradation of endogenous HTC cell proteins

Endogenous cell protein is degraded with a half-life of 91 h when measured after labelling cell proteins for 18 h in the presence of [³H]leucine (Fig. 3a).

Degradation of HTC-cell plasma-membrane proteins

After [¹²⁵I]iodination of the plasma-membrane proteins (Baumann *et al.*, 1980), acid-insoluble ¹²⁵I is lost from the membrane with a half-life of 95 h (Fig. 3b). This is in excellent agreement with the half-life of 100 h obtained by Baumann & Doyle (1978) for the plasma-membrane proteins of HTC cells in suspension culture.

Fluorescent localization of transplanted MOM

Fluorescein-labelled MOM vesicles were used to assess the distribution and subcellular fate of transplanted MOM material. Fluorescence microscopy of cells 15 min after fusion with fluorescein-labelled MOM is shown in Fig. 4(a). The patches of fluorescein-labelled MOM material observed in cells after transplantation (Fig. 4a) are probably due to aggregation of the MOM material during, or immediately after, fusion. Similar patches of fluorescent transplanted liver glycoproteins are observed on the surface of L929 fibroblasts (McDonald & Gelehrter, 1981). In contrast, fluorescence microscopy of hepatoma cells labelled briefly (1 min) with fluorescein isothiocyanate showed a diffuse labelling of the entire cell surface (results not shown). No background fluorescence was observed in untreated cells or cells treated with PEG alone (results not

Fig. 3. Degradation of total endogenous and plasma-membrane proteins in HTC cells

(a) Loss of trichloroacetic acid (10%, w/v)-insoluble radioactivity from HTC cells (7×10^5 cells/plate) prelabeled for 18 h with 0.05 mCi of [4,5- ^3H]-leucine. After labelling, the cells were washed and cultured with growth medium supplemented with 2 mM-leucine. Four plates of cells were taken at each time point for determination of radioactivity. Growth medium was replaced every 48 h. (b) Loss of trichloroacetic acid (10%, w/v)-insoluble radioactivity from HTC cells (4×10^5 HTC cells/plate) after radioiodinating plasma-membrane proteins with 0.05 mCi of ^{125}I by a modification of the method of Tweto *et al.* (1976) (see the Materials and methods section). Four plates of cells were taken at each time point for determination of radioactivity (results are means $\pm$ s.d.). (c) Loss of trichloroacetic acid (5%, w/v)-insoluble [^{14}C]sucrose radioactivity after labelling HTC cells (10^7) for 30 min at 4°C with cyanuric chloride-activated [^{14}C]sucrose (100 μCi).

shown). At 1 h after fusion (cells maintained at 37°C) the patches appeared to have formed into larger caps (Fig. 4b). Subsequently, the caps disappeared (1–8 h) and no strong punctate fluorescence

Fig. 4. Fate of transplanted fluorescein-conjugated MOM. HTC cells were fused with fluorescein-conjugated MOM (a) 15 min after fusion, (b) 1 h after fusion and (c) 18 h after labelling with cyanuric chloride-activated [^{14}C]sucrose (Pittman *et al.*, 1979) and fluorescein isothiocyanate. The bar represents 15 μm .

could be observed within the cells. Fluorescein-labelled MOM could be treated differently from radiolabelled MOM; however, no significant difference in the degradation rate of single-([^3H]pargyline) labelled MOM (Fig. 2) or MOM labelled with both [^3H]pargyline and fluorescein (Fig. 2b) was measured after transplantation into HTC cells.

Electron microscopy

Electron microscopy demonstrates close association of MOM vesicles of similar diameter (0.1 μm) with transversely sectioned filopodia (Fig. 5) immediately after vesicle–cell fusion. No evidence for

Fig. 5. *Electron micrograph of MOM-HTC-cell fusion*
Washed HTC cells 5 min after fusion of MOM vesicles (0.1 mg of protein) to 10^7 cells. Note close association of light vesicles (arrows) with the HTC cell surface. The bar represents 0.1 μ m.

cell-cell fusion was observed by phase-contrast microscopy or electron microscopy during the course of the experiments.

Subcellular distribution of the transplanted MOM material

(i) *Percoll-gradient centrifugation.* After fusion with reductively [3 H]methylated MOM or [3 H]-pargyline-labelled MOM, cells were sonicated and fractionated on Percoll gradients. From 24–28 h after fusion (Figs. 6b–6e) with reductively [3 H]-methylated MOM, two peaks of radioactivity corresponding to structures having densities of approx. 1.04 g/cm³ and 1.09 g/cm³ were obtained. The proportion of radioactivity at the two densities varied with time. In general, the proportion of radioactivity at low density decreased and the proportion of radioactivity at high density increased with time. In a similar way, [3 H]pargyline-derivatized monoamine oxidase in MOM vesicles also show a bimodal distribution when sonicated cells (10–48 h after fusion) are fractionated on a Percoll gradient (Figs. 6g–6j) with peaks at densities of 1.04 g/cm³ and 1.09 g/cm³. Cells show a broad distribution on Percoll-gradient centrifugation between densities 1.04 g/cm³ and 1.1 g/cm³. MOM vesicles show a single peak of density 1.04 g/cm³.

(ii) *Fractionation by differential centrifugation.* Cells (10^8) were fractionated immediately after fusion and at 48 h after fusion (Figs. 7a–7d). The latter time was chosen from the results of the Percoll fractionation, which showed that after this time a large shift in density of the radioactive material occurs and there is still sufficient radioactivity remaining to make accurate measurements possible. Homogenization was achieved with repeated strokes of a ground-glass homogenizer until no intact cells are visible by phase-contrast microscopy. Immediately after fusion, reductively [3 H]methylated or [3 H]pargyline labelled MOM radioactivity (Figs. 7a

and 7b) was distributed in a similar pattern to the plasma-membrane marker enzyme alkaline phosphodiesterase (Fig. 7f). The highest specific radioactivity of material 48 h after fusion of either [3 H]pargyline or reductively [3 H]methylated MOM was present in the 'M' fraction (Figs. 7c and 7d). Although mitochondrial (monoamine oxidase) and lysosomal (acid phosphatase and *N*-acetyl- β -glucosaminidase) marker enzymes are found in the 'M' fraction, the relative-specific-activity profiles for these marker enzymes are not the same as the radioactivity distributions.

Fate of [14 C]sucrose coupled to MOM vesicles and hepatoma plasma membrane

The distribution of acid-insoluble radioactivity in subcellular fractions 10 min after fusion with [14 C]-sucrose-labelled MOM vesicles or in hepatoma cells labelled with [14 C]sucrose (Tack *et al.*, 1980) is similar (Figs. 8a and 8b) to the distribution of the plasma-membrane marker (Fig. 7f). The distribution of acid-insoluble radioactivity in cells fused with [14 C]sucrose-labelled MOM 48 h (Fig. 8d) after fusion is not the same as acid-insoluble radioactivity derived from reductively [3 H]methylated or [3 H]pargyline-labelled MOM (Figs. 7c and 7d), but resembles the distribution of lysosomal marker enzymes (Figs. 7g and 7h). This may be explained by the acid precipitation of some degradation products of [14 C]sucrose-labelled MOM proteins (Pittman *et al.*, 1979).

The distribution of acid-insoluble radioactivity in subcellular fractions 48 h after labelling the hepatoma-cell surface with [14 C]sucrose (Fig. 8c) is not the same as acid-insoluble radioactivity derived from [14 C]sucrose-labelled MOM (Fig. 8d), with more than 50% of the acid-insoluble radioactivity in the 'N' fraction. The acid-insoluble 14 C radioactivity is clearly not associated with the cell plasma membrane (Fig. 7f), but is rapidly internalized within the cell (Fig. 4c). The acid-soluble [14 C]-sucrose-containing degradation products of [14 C]-sucrose-conjugated proteins accumulate lysosomally (Pittman *et al.*, 1979). Therefore lysosomal involvement in the degradation of proteins can be inferred from the distribution of acid-soluble [14 C]-sucrose label. Both [14 C]sucrose-conjugated MOM and plasma-membrane proteins are degraded to acid-soluble products (Figs. 8e and 8f), which distribute on cell homogenization like acid phosphatase (Fig. 7g). Acid-soluble products account for 50% of the total cellular radioactivity 48 h after fusion of [14 C]sucrose-conjugated MOM and 30% of the total cellular radioactivity 48 h after labelling plasma-membrane proteins.

The average rates of degradation of both transplanted MOM proteins and endogenous plasma-

Fig. 6. Percoll-gradient fractionation of homogenates of HTC cells

Enzyme and radioactivity distributions of sonicated HTC cells fused with [^3H]methylated or [^3H]pargyline-labelled MOM after fractionation on Percoll gradients (mean starting density 1.075 g/cm^3). Cytochrome oxidase (—) and acetyl- β -D-glucosaminidase (----) (a) and monoamine oxidase (—) and alkaline phosphodiesterase (----) distributions (f) are given as percentages of the total activity recovered from the gradient. Recoveries ranged from 88 to 95%. HTC cells were fractionated (b) 10 min, (c) 10 h, (d) 24 h and (e) 48 h after fusion with [^3H]methylated MOM and (g) 10 min, (h) 10 h, (i) 24 h and (j) 48 h after fusion of [^3H]pargyline-labelled MOM. Values for the radioactivity of the first two fractions from HTC cells fractionated after fusion of [^3H]pargyline-labelled MOM are not included.

membrane proteins were not altered by [^{14}C]-sucrose conjugation (cf. Figs. 2c and 3c with Figs. 2b and 3b). Some leakage of acid-soluble [^{14}C]-sucrose label into the culture medium occurs in a manner similar to that shown by acid-soluble [^3H]pargyline. At 48 h after labelling, 25–40% of the

initial MOM radioactive [^{14}C]sucrose and 8–15% of the plasma membrane label is found in the medium.

Degradation of endocytosed protein

HTC cells (8×10^7) were cultured in the presence of 1 mg of reductively [^3H]methylated bovine serum

Fig. 7. Differential centrifugation of homogenates of ^3H -labelled HTC cells

Distribution of trichloroacetic acid (10%, w/v)-insoluble radioactivity and enzyme markers for homogenized HTC cells after fusion with $[^3\text{H}]$ methylated MOM or $[^3\text{H}]$ pargyline MOM and fractionation by differential centrifugation. Homogenates were divided into five successive fractions: N, M, L, P, S. Results are presented exactly as described by Lopez-Saura *et al.* (1978). Cells were fractionated (a) 10 min and (c) 48 h after fusion of methylated $[^3\text{H}]$ MOM and (b) 10 min and (d) 48 h after fusion of $[^3\text{H}]$ pargyline MOM. n = Number of experiments. Results represent means $\pm$ standard deviation.

albumin for 18 h. After washing, the rate of destruction of endocytosed bovine serum albumin (0.21% of added bovine serum albumin) was measured (Fig. 9). Approx. 50% of the endocytosed radiolabelled material was degraded to acid-soluble products in 6 h.

Discussion

The transplantation of MOM-vesicle proteins in the hepatoma-cell plasma membrane was entirely

dependent on the presence of PEG (Table 1). The procedures used in this deliberate miscompartmentalization are similar in principle to those employed by Doyle *et al.* (1979) and Baumann & Doyle (1980) to transfer rat liver plasma-membrane proteins into the surfaces of hepatoma cells and mouse fibroblasts.

The purpose of transplantation [Evans & Mayer, 1982; the preceding paper (Evans & Mayer, 1983)] or microinjection of proteins (Loyter *et al.*, 1975;

Fig. 8. Differential centrifugation of homogenates of ^{14}C -labelled HTC cells

Distribution of radioactivity after fractionation of (i) homogenized HTC cells labelled with activated ^{14}C sucrose or (ii) HTC cells fused with ^{14}C sucrose-conjugated MOM (5.8×10^5 d.p.m./mg of protein) is shown. Homogenates are divided into five successive fractions, N, M, L, P and S, as described in Fig. 8. Patterns (a–d) show the distribution of trichloroacetic acid (10%, w/v)-insoluble radioactivity and (e, f) show the distribution of trichloroacetic acid (10%, w/v)-soluble radioactivity. ^{14}C sucrose introduced into the cells from either ^{14}C sucrose-labelled-MOM-vesicle-cell fusion (b, d, f) or direct labelling of the HTC cell plasma membrane (a, c, e) amounted to $1.65\text{--}2.75$ nmol/ 10^6 cells.

Schlegel & Rechsteiner, 1975) is to introduce labelled proteins into unlabelled cells so that their subsequent degradative fate can be studied unhindered by the complexities of radiolabelling all cell proteins by use of radiolabelled amino acids. Great care must be taken to minimally alter the transplanted or microinjected proteins. In the present study [and in the preceding paper (Evans & Mayer, 1983)] we have initially used two methods of covalent protein derivatization: reductive ^3H -methylation of MOM proteins and specific derivatization of monoamine oxidase in MOM vesicles by the suicide inhibitor ^3H pargyline. The advantage of reductive ^3H methylation is that it modifies predominantly ϵ -amino groups of lysine in MOM proteins (Evans & Mayer, 1982). Such a modification is infrequently critical for activity (for

MOM, reductive methylation only inhibits monoamine oxidase by 5%) or protein conformation; indeed, the pK of lysine is only altered slightly (0.4–0.6 pH unit) by the formation of dimethyl-lysine residues (Means, 1977). Reductive ^3H -methylation of pigeon erythrocyte proteins in conditions similar (Jones & Vidaver, 1981) to those described in the present study did not perturb ATP-dependent $^{45}\text{Ca}^{2+}$ uptake, Na^+ -dependent ^{14}C glycine uptake, membrane resealing or iso-electric-focusing patterns of erythrocyte membrane proteins. ^3H Pargyline is a potent irreversible inhibitor of monoamine oxidase which only binds to the FAD prosthetic group of the enzyme (Swett *et al.*, 1963; Cawthon *et al.*, 1980), thus minimizing enzyme modification.

The experiments reported here characterize the

Fig. 9. Degradation of [^3H]bovine serum albumin in HTC cells

Loss of acid-insoluble radioactivity from endocytosed proteins. HTC cells (80×10^6) were cultured in the presence of 1 mg of reductively [^3H]methylated bovine serum albumin (180×10^6 d.p.m./mg of protein) for 18 h, washed and trichloroacetic acid (10%, w/v)-insoluble radioactivity subsequently determined.

fate of deliberately miscompartmentalized MOM proteins heterologously transplanted into hepatoma cells by PEG-mediated MOM-vesicle-cell fusion. The experiments were carried out in order to compare the intracellular fate and eventual destruction of this heterologously transplanted MOM material with MOM vesicles homologously transported by similar methods into rat hepatocytes (Evans & Mayer, 1982). In hepatocyte monolayers maintained under culture conditions that mimic rates of intracellular proteinolysis observed *in vivo* (Evans & Mayer, 1982) the average rate of destruction of reductively [^3H]methylated mitochondrial or MOM proteins and [^3H]pargyline-derivatized monoamine oxidase [the preceding paper (Evans & Mayer, 1983)] is remarkably similar (i.e. mitochondria, $t_{\frac{1}{2}}$ average, 72.5 h; MOM proteins, $t_{\frac{1}{2}}$ average, 60–70 h; monoamine oxidase, $t_{\frac{1}{2}}$ 55 h) to the measured rates in rat liver *in vivo* (Russell *et al.*, 1980; Russell *et al.*, 1982). In contrast, both heterologously transplanted reductively [^3H]methylated MOM proteins and [^3H]pargyline-derivatized monoamine oxidase are destroyed at a faster rate, with an approx. $t_{\frac{1}{2}}$ of 24 h.

The fate of the heterologously transplanted MOM was observed, after labelling MOM with fluorescein isothiocyanate, by fluorescence microscopy. The behaviour of the fluorescent material is analogous to the patching and capping of cross-linked membrane proteins observed in lymphocytes (Bourguignon & Singer, 1977), although in hepatoma cells no strong

fluorescence was observed in vacuoles after capping. This type of behaviour was not observed when fluorescein-conjugated rat liver MOM vesicles were homologously transplanted to rat hepatocytes (P. J. Evans & R. J. Mayer, unpublished work), where the fluorescent material accumulated in vesicles in a perinuclear position without initial surface capping. The different fate of fluorescent MOM in the hepatoma cells and the hepatocytes may reflect the heterologous and homologous transplantation to the target cells. The different response may be due to differences in the composition and surface micro-morphology of the plasma membrane of hepatoma cells and hepatocytes. The membrane of the hepatoma cells may have altered fluidity and relationship to the cytoskeleton. Several reports have described the altered lateral mobility of plasma-membrane molecules in transformed cells (Poste *et al.*, 1975; Ben-Bassat *et al.*, 1977; Eldridge *et al.*, 1980), including the capping of patches of aggregated membrane proteins (Zucker-Franklin *et al.*, 1979).

Accelerated degradation of fluorescein-conjugated proteins relative to their iodinated counterparts has been observed (Zavortnik *et al.*, 1979), but the rate of destruction of [^3H]pargyline-labelled monoamine oxidase (Fig. 2b) in transplanted doubly labelled (fluorescein and [^3H]pargyline) MOM was very similar to the rate of degradation of [^3H]pargyline-labelled enzyme (Fig. 2b) in singly derivatized MOM.

The data show that the average rate of degradation of transplanted reductively [^3H]methylated MOM proteins and the rate of degradation of [^3H]pargyline-labelled monoamine oxidase are very similar. The destruction rates ($t_{\frac{1}{2}}$ approx. 24 h) are not the same as: (a) in rat liver *in vivo* or when transplanted into hepatocytes ($t_{\frac{1}{2}}$ approx. 60–70 h); (b) endogenous average HTC protein degradation rate ($t_{\frac{1}{2}}$ approx. 90 h); (c) rate of degradation of HTC cell plasma membrane modified by [^{125}I]iodination or conjugation with [^{14}C]sucrose ($t_{\frac{1}{2}}$ approx. 95 h); (d) destruction of endocytosed bovine serum albumin (~50% destroyed in 6 h); (e) the average rate of destruction of hepatoma-cell mitochondrial proteins under serum-deprivation conditions ($t_{\frac{1}{2}}$ 36 h; Hare & Hodges, 1982). The trichloroacetic acid-soluble products of degradation of [^{14}C]sucrose-conjugated MOM products and HTC cell plasma-membrane proteins accumulate intracellularly in structures that fractionate on centrifugation like lysosomal acid phosphatase.

Different modifications of transplanted MOM proteins or endogenous HTC cell plasma-membrane proteins do not affect average protein degradation rates (Figs. 2, 3 and 5).

Reductively [^3H]methylated MOM proteins and [^3H]pargyline-labelled monoamine oxidase are

destroyed at rates comparable with those observed *in vivo* (Russell *et al.*, 1980, 1982) when transplanted into hepatocytes [Evans & Mayer, 1982; the preceding paper (Evans & Mayer, 1983)]. However, when transplanted into HTC cells the same preparations are destroyed approx. 2.5-fold faster. Therefore the information in the liver MOM that determines the rate of intrinsic protein destruction (Russell *et al.*, 1982) is interpreted differently.

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