Degradation of proteins in rat liver mitochondrial outer membrane transplanted into different cell types

Evidence for alternative processing

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The degradation of proteins in reductively [^3H]methylated mitochondrial outer membrane (MOM) transplanted into cells by a poly(ethylene glycol)-mediated process has been studied. The average rate of degradation (t_i 24–28 h) of MOM proteins transplanted into HTC cells was not the same as for endogenous MOM proteins (t_i 56 h), mitoplast proteins (t_i 120 h), plasma membrane proteins (t_i approx. 90 h) or cytosol proteins (t_i 75 h). The degradation of transplanted MOM proteins was inhibited to the same extent (30–45%) as that of endogenous mitochondrial and plasma membrane proteins by leupeptin and NH_4Cl . No inhibition of HTC cell cytosol protein degradation by NH_4Cl was observed. NH_4Cl differentially inhibited the degradation of endogenous MOM and mitoplast protein subunits as shown after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Proteins in MOM transplanted into tissue culture cells were degraded either with t_i 24–28 h (MRC-5, B82 and A549 cells) or with t_i 55–70 h (CHO-K1 and 3T3-L1 cells) similar to that of proteins in MOM transplanted into rat hepatocytes [Evans & Mayer (1983) *Biochem. J.* **216**, 151–161]. The data suggest that membrane protein destruction is but the end part of a fundamental intracellular membrane recognition process.

Microinjection of prelabelled soluble proteins into cells in culture is an ideal method for the study of soluble protein degradation without the complicating factors of general protein labelling methods (Loyter *et al.*, 1975; Zavortnik *et al.*, 1979; Neff *et al.*, 1981; Dice, 1982). Transplantation of membranes is used to introduce membranes isolated from one group of cells to other cells so that the degradation of membrane proteins can be studied (Baumann *et al.*, 1983; Evans & Mayer, 1983; Russell & Mayer, 1983). Previous work has shown the degradation rates of rat liver MOM proteins, including monoamine oxidase, to be similar to rates *in vivo* (t_i 55–70 h) when transplanted into hepatocytes (Evans & Mayer, 1983, 1984) and to be different to the rate *in vivo* (t_i 24–28 h) when transplanted into Morris hepatoma (HTC) cells (Russell & Mayer, 1983). The present study was undertaken in an effort to elucidate the factor(s) controlling the

degradation rate of rat liver MOM proteins when transplanted into cells. We have further characterized the degradation of transplanted rat liver MOM proteins in HTC cells by comparison with the degradation of endogenous mitochondrial and plasma membrane proteins and characterized the degradation of rat liver MOM transplanted into several cultured cell lines. We have also studied the mechanism of uptake of transplanted membrane into the different cell types by use of fluorescein-conjugated rat liver MOM.

Materials and methods

Cell lines and culture

All cell lines were maintained in Eagle's minimum essential medium supplemented with minimum essential medium non-essential amino acids, penicillin G (100 $\mu\text{g}/\text{ml}$), streptomycin (50 $\mu\text{g}/\text{ml}$) and fungizone (3 ng/ml; Gibco, Paisley, Renfrewshire, Scotland, U.K.). HTC (rat hepatoma) and 3T3-L1 (Swiss mouse fibroblast) cell growth

Abbreviations used: MOM, mitochondrial outer membrane; SDS, sodium dodecyl sulphate.

medium contained 10% (v/v) heat-inactivated newborn calf serum, MRC-5 (human lung fibroblast) and CHO-K1 (hamster ovary) cell growth medium contained 10% (v/v) heat inactivated foetal-calf serum and growth medium for RAG (mouse adenocarcinoma), A549 (human lung fibroblast) and B82 (mouse L cell subclone) cells contained 5% (v/v) foetal-calf serum. Cells were maintained as monolayer cultures on 90mm-diameter plastic culture dishes (Nunc, Roskilde, Denmark) at 37°C in an incubator under air/CO₂ (19:1). Cultures were fed every 24–48 h.

Preparation of MOM fraction from rat liver

MOM fraction was prepared as described previously (Russell & Mayer, 1983). Reductively methylated MOM vesicles were prepared by using NaB³H₄ (2–20 Ci/mmol; Amersham International) and fluorescein-labelled MOM vesicles were prepared with fluorescein isothiocyanate isomer I (Russell & Mayer, 1983).

Fusion

Fusion of rat liver MOM vesicles to HTC cells has been described previously (Russell & Mayer, 1983). Essentially the same method was used to fuse either reductively methylated or fluorescein-labelled MOM vesicles to all cell types. In a typical fusion, monolayer cultures of cells were harvested by trypsin/EDTA solution (Gibco; 1 mg of trypsin/ml, 0.4 mg of EDTA/ml) for 2–5 min at 37°C. The suspended cells were washed once with growth medium and three times with phosphate-buffered saline (Dulbecco & Vogt, 1954). To the packed cell pellet was added 0.2 ml of freshly prepared and dialysed MOM suspension (0.5–2 mg of MOM protein/10⁸ cells per fusion). The components were mixed thoroughly by swirling and 0.6 ml of 50% (w/v) poly(ethylene glycol)-1000 (BDH) in serum-free medium was added. The cell/MOM mixture was incubated for 5 min at 37°C and then washed three times with growth medium. The cells were then plated out for culture. Three plates of cells were taken at each time point for the determination of acid-soluble and acid-insoluble radioactivity in the degradation experiments. Inhibitor studies used NH₄Cl and leupeptin at final concentrations of 10 mM and 50 µg/ml respectively. For fluorescent experiments the cells were viewed after fixation in 3.7% formaldehyde in phosphate-buffered saline and mounted under a circular 25 mm coverslip in buffered glycerol. Fluorescence was observed and photographed with a Leitz SM-Lux microscope.

Measurement of degradation of proteins synthesized in the presence of cycloheximide

HTC cells were harvested by trypsinization, plated out at a density of 0.5 × 10⁶ cells/plate in

growth medium and allowed to adhere to the culture plates and recover overnight. The medium was replaced with cycloheximide (1 µg/ml)-supplemented growth medium and the cells cultured for 30 min. [4,5-³H]Leucine (170 Ci/mmol; Amersham International) was added to the dishes at 2 µCi/ml. Control dishes of cells without cycloheximide also received [³H]leucine. After a labelling period of 3 h, cells were washed by incubating three times for 10 min in growth medium supplemented with L-leucine (2 mM). Cycloheximide inhibited the incorporation of [³H]leucine into trichloroacetic acid (10%, w/v)-insoluble material by 94.9%. Preliminary studies showed that on differential centrifugation of the cell homogenates the radioactivity incorporated in the presence of cycloheximide had a similar distribution to the mitochondrial enzyme, cytochrome oxidase. Four plates of cells were taken at each time point for the determination of cell acid-insoluble radioactivity.

Measurement of the degradation of endogenous HTC proteins in the absence and presence of NH₄Cl (10 mM)

Endogenous protein degradation was measured by a double-isotope procedure (Russell *et al.*, 1982) with [³⁵S]- and [³H]-methionine. One 90 mm plate of HTC cells (approx. 90% confluent) was washed twice in growth medium, without L-methionine, and incubated in the same medium containing 50 µCi of [³⁵S]methionine/ml (Amersham International) for 3 h. The labelled cells (test cells) were then washed by incubation three times for 30 min in growth medium, harvested by trypsinization, the cell number was determined and the cells were cultured after plating out equally onto eight culture plates. Another 90 mm plate of HTC cells was treated as the test cells, except that no addition of [³⁵S]methionine was made, and plated out at exactly the same cell density. These cells were termed the control cells. Both sets of cells were cultured for 3 days. At 3 days two plates of test and two plates of control cells were labelled for 3 h with [³H]methionine (40 µCi/ml; Amersham International) in growth medium without L-methionine. Two plates of control cells were also labelled for 3 h with 50 µCi of [³⁵S]methionine/ml. After labelling, both test and control plates were washed by incubating three times for 30 min in growth medium. Test and control cells were harvested by trypsinization and the cell number was determined. The number of control cells labelled after 3 days in culture by incubation with [³⁵S]methionine was made equal to the number of test cells labelled with [³⁵S]methionine before the 3 days in culture by discarding excess cells. All test cells were pooled and all remaining control cells were pooled. Cells were washed twice in 10 ml of 0.25 M-sucrose/3 mM

imidazole, pH 7.4, and set in 0.25 ml of 2% (w/v) agar in the same solution at 30°C, mixed and cooled to 0°C. The cells were homogenized in a ground-glass homogenizer and subfractionated by differential centrifugation as described by Lopez-Saura *et al.* (1978). The N and L fractions were discarded. The S fractions were retained for further analysis. The M fractions, containing the mitochondria, were taken up in 3 ml of 10 mM-Tris/phosphate buffer, pH 7.5, and kept at 0°C for 10 min; then 1 ml of 1.8 M-sucrose/4 mM-ATP/4 mM-MgSO₄ was added. The suspension was mixed well and allowed to remain for 10 min on ice. The suspension was sonicated for 15 s at an amplitude setting of 8 μ m peak-to-peak (MSE sonicator) and layered over 5 ml of 1.2 M-sucrose. The discontinuous sucrose gradient was centrifuged for 20 min at 25000 g_{av} in rotor no. 43114-1250 on an MSE 75 centrifuge (MSE Ltd., Crawley, Sussex, U.K.). The interface layer containing the MOM was collected and the pellet (termed the mitoplast fraction) was resuspended in 0.2 ml of 0.25 M-sucrose/3 mM-imidazole buffer, pH 7.4.

Preliminary experiments established that the MOM fraction was purified 12-fold over the cell homogenate as measured by monoamine oxidase activity. The MOM fraction contained only 0.46% of the total acid phosphatase activity present in the homogenate. The mitoplast fraction was purified 11.5-fold over the homogenate with regard to the cytochrome oxidase activity. Mitoplast and MOM fractions were further analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

The effects of NH₄Cl (10 mM) on the degradation of endogenous HTC membrane proteins was again studied by the double-isotope method (Russell *et al.*, 1982). Two 90 mm plates of HTC cells (90% confluent) were incubated in L-methionine-free medium; to one plate (test cells) was added 50 μ Ci of L-[³⁵S]methionine/ml and both plates were incubated for 3 h. The two plates of cells were washed three times by incubation for 30 min in growth medium, harvested and cell numbers were determined. Test and control cells were then both plated out equally onto eight culture plates in growth medium containing 10 mM-NH₄Cl and cultured for 3 days. Little or no cell detachment, but some slowing of growth, occurred in these conditions. At 3 days, three 90 mm plates of untreated HTC cells (90% confluent) were incubated in L-methionine-free growth medium. To one plate was added 50 μ Ci of L-[³⁵S]methionine/ml (control cells) and to the remaining two plates was added 40 μ Ci of L-[³H]methionine/ml (test and control cells). The cells were incubated for 3 h, washed three times for 30 min in growth medium, harvested and cell

numbers were determined. The number of L-[³⁵S]-methionine-labelled control cells was made equal to the number of test cells labelled with L-[³⁵S]-methionine before the 3 days in culture. Test and control cells grown for 3 days in NH₄Cl-supplemented medium were also harvested. Test cells, i.e. [³⁵S]methionine-labelled cells (3 h labelled and cultured in NH₄Cl for 3 days) and [³H]methionine-labelled cells (3 h labelled and cultured for 90 min), were pooled. Control cells, i.e. unlabelled cells grown in the presence of NH₄Cl for 3 days, cells labelled with [³⁵S]methionine (3 h labelled and cultured for 90 min) and cells labelled with [³H]-methionine (3 h labelled and cultured for 90 min) were mixed. Test and control cells were subfractionated and analysed as described above.

Effect of leupeptin and NH₄Cl on the degradation of hepatoma plasma membrane protein

Cells (approx. 5×10^7) were harvested by scraping with a sterilized plastic spatula, washed twice in 2 ml of modified Earle's salt solution without Phenol Red, Ca²⁺ or Mg²⁺, supplemented with Tricine {N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine} (0.1 M, pH 7.6) and glucose (7 mM) and suspended for labelling in 2 ml of modified Earle's salt solution. Iodination was performed as described in Hubbard & Cohn (1972) with 1 mCi of Na¹²⁵I (Amersham International). The reaction was allowed to continue for 30 min at 25°C before terminating by washing the cells twice in Earle's salt solution, suspending the cells in 8 ml of growth medium and plating out at a density of approx. 2×10^6 cells/plate. After overnight incubation the medium was removed. Cells were further incubated with either growth medium, leupeptin-supplemented (50 μ g/ml) growth medium or with NH₄Cl (10 mM)-supplemented growth medium.

Radioactivity measurements

Degradation measurements were made on samples (1 ml) of aspirated media or on cells harvested and sonicated (70 W for 5 s; Dawes Soniprobe) in phosphate-buffered saline (2 ml/plate) by adding 50% (w/v) trichloroacetic acid (final concentration 10%, w/v). Acid-soluble radioactivity was measured in samples (200 μ l) of the supernatant obtained by centrifuging medium or cell suspensions treated with trichloroacetic acid (final concentration 10%, w/v). Acid-insoluble pellets were washed once with 2 ml of 10% (w/v) trichloroacetic acid and solubilized for counting in 30% (v/v) formic acid (300 μ l). Radioactivity present as ³⁵S or ³H was measured after adding 3 ml of Fisofluor in a Packard 460 CD liquid-scintillation counter and ¹²⁵I radioactivity was measured in a Mini-Assay type 620 gamma counter. Radioactivity in

polyacrylamide-gel slices was measured as described by Russell *et al.* (1980).

Results

The rate of destruction of proteins in reductively [^3H]methylated mitochondria and MOM transplanted into hepatocytes is approximately equal to the rate of destruction of endogenous rat liver mitochondrial proteins (Russell *et al.*, 1980; Evans & Mayer, 1983). The relatively rapid rate of degradation (t_1 24–28 h) of reductively [^3H]methylated MOM proteins transplanted into hepatoma cells (Russell & Mayer, 1983) could reflect the relatively rapid rate of destruction of endogenous hepatoma mitochondrial proteins (t_1 36 h) in non-proliferating cells (Hare & Hodges, 1982*a,b*).

Fig. 1 shows the degradation of proteins synthesized in the presence of cycloheximide (mitoribosomally synthesized proteins; Knecht *et al.*, 1980). After an initial rapid disappearance of acid-insoluble radioactivity (0–2 h) the half-life of labelled proteins is 90 h, similar to the degradation of rat liver mitochondrial proteins *in vivo* (Russell *et al.*, 1980). The rapid degradation phase (0–2 h, Fig. 1) may be the destruction of mitoribosomally synthesized proteins without cytoplasmically synthesized partners, part of the proteolytic control of mitochondrial assembly (Galkin *et al.*, 1980). The normal growth of the cells after labelling confirmed the complete removal of cycloheximide (results not shown).

Half-lives measured (by the double-isotope method) for endogenous MOM proteins, the mitoplast fraction proteins and the S fraction proteins from homogenized HTC cells in the presence and absence of NH_4Cl (10 mM) are given in Table 1. No inhibition was observed for the degradation of the S-fraction proteins. MOM and mitoplast fraction proteins were also separated by SDS/polyacrylamide-gel electrophoresis and the calculated half-lives for polypeptides in successive gel slices are given in Table 2. The average half-life for proteins

in the MOM fraction and mitoplast fraction (t_1 56 h and 120 h respectively) are similar to values for these proteins in rat liver *in vivo* (Russell *et al.*, 1980). The average inhibition of protein degradation by NH_4Cl (10 mM) is similar for both MOM and mitoplast fraction proteins (28–37%, Tables 1 and 2). However, there is great variation in the inhibition by NH_4Cl of the degradation of the separated protein subunits (Table 2).

Release of acid-soluble radioactivity from iodinated HTC plasma membrane proteins over a 22 h period is inhibited 28% by leupeptin (50 $\mu\text{g}/\text{ml}$) and 32% NH_4Cl (10 mM, Table 3). The degradation of proteins in reductively [^3H]methylated MOM transplanted into HTC cells was inhibited by 35%

Fig. 1. Loss of acid-insoluble radioactivity from HTC cells labelled for 3 h in the presence of cycloheximide (1 $\mu\text{g}/\text{ml}$) with [^3H]leucine (2 $\mu\text{Ci}/\text{ml}$, 170 Ci/mmol).

Before labelling cells were at a density of approx. 10^6 cells/plate. After labelling cells were washed three times with growth medium. At each time point, quadruplicate plates of cells were taken for the determination of acid-insoluble radioactivity in the cells. Results represent means $\pm$ S.D.

Table 1. Mean half-lives derived from isotope ratios for subcellular fractions from HTC cells grown in the presence or absence of NH_4Cl

HTC cells (approx. 5×10^6 cells) were labelled for 3 h with [^{35}S]methionine (50 $\mu\text{Ci}/\text{ml}$), washed three times with growth medium and grown in the presence or absence of 10 mM- NH_4Cl for 3 days. Parallel cultures of HTC cells were labelled for 3 h with [^3H]methionine (40 $\mu\text{Ci}/\text{ml}$) and appropriate cells were pooled to give test and control cell mixtures which were homogenized and fractionated (see the Materials and methods section for details). Analysis of data is as described in Russell *et al.* (1980).

Cell fraction	Mean half-life (h)		
	Untreated cells	NH_4Cl -treated cells	Inhibition (%)
MOM fraction	59.0	84.6	30.3
Mitoplast fraction	117.7	181.4	35.1
S fraction	76.8	71.5	-7.0

Table 2. *Effect of NH₄Cl on the half-lives of HTC cell proteins in MOM and mitoplast fractions separated by SDS/polyacrylamide-gel electrophoresis*

MOM fraction and mitoplast fraction samples from Table 1 were separated by 10% (w/v) SDS/polyacrylamide-gel electrophoresis and sequential 0.5 cm slices of gel were solubilized and counted for radioactivity and half-lives were determined from the isotope ratios (Russell *et al.*, 1980). Radioactive M_r markers were co-electrophoresed on the same gel as the samples.

Subunit M_r	Half-life (h)			
	MOM		Mitoplast	
	From untreated cells	From NH ₄ Cl-treated cells	From control cells	From NH ₄ Cl-treated cells
200	49.6	63.1	65.2	102.4
92.5	47.3	26.7	66.5	80.9
	52.9	209.5	85.7	94.7
	53.4	96.4	74.3	171.7
68	48.6	71.8	58.8	298.7
	51.8	300.0	118.8	211.9
	59.8	183.0	148.5	360.0
43	58.0	125.8	107.3	277.2
	62.8	78.9	126.0	174.0
	61.8	136.3	169.7	303.5
	58.6	214.4	136.3	181.6
	58.8	78.3	141.0	302.5
25	59.0	79.4	158.4	235.9
	54.7	110.1	121.4	116.0
	53.0	59.4	241.1	226.0
	42.3	66.5	107.3	110.8
	40.7	61.6	115.5	94.9
18	66.8	137.6	117.1	360.0
12	60.5	79.7	143.4	61.0
	36.2	127.1	—	360.0
Mean $\pm$ S.D.	54.1 $\pm$ 7.6	119.8 $\pm$ 27.0	121.2 $\pm$ 43.4	200.4 $\pm$ 94.0

Table 3. *Effect of NH₄Cl and leupeptin on the degradation of HTC plasma membrane proteins*

HTC cells (approx. 5×10^7 cells) were harvested and iodinated by using 1 mCi of Na¹²⁵I. The labelled cells were plated out onto 12 plates in growth medium overnight. At 24 h after labelling cells were washed in growth medium, three plates were taken for radioactivity measurements and the remaining plates were incubated in growth medium, growth medium supplemented with 10 mM-NH₄Cl or growth medium supplemented with leupeptin (50 μ g/ml). After 22 h triplicate plates were harvested for radioactivity determination on medium and cells. Results are expressed as means $\pm$ S.E.M.

Inhibitor	Degradation (%·h ⁻¹)	Inhibition (%)
Untreated	1.13 $\pm$ 0.04	—
NH ₄ Cl	0.77 $\pm$ 0.05	32
Leupeptin	0.85 $\pm$ 0.15	28

by both leupeptin and NH₄Cl (Fig. 2). Slightly greater inhibition of protein degradation (30–45%) was estimated with leupeptin and NH₄Cl if the calculations are performed by using the release of acid-soluble radioactivity into the medium (results not shown).

The average rate of degradation of proteins in reductively [³H]methylated MOM transplanted by poly(ethylene glycol)-mediated transfer into several cell types is summarized in Table 4. Degradation rates of proteins in [³H]methylated MOM transplanted into CHO-K1 (Chinese hamster ovary cell line) and 3T3-L1 (murine fibroblasts) were first-order and resembled the average degradation rates of proteins in [³H]methylated MOM transplanted into hepatocytes (Evans & Mayer, 1983). The average rates of degradation of proteins in [³H]methylated MOM transplanted into MRC-5 (human fibroblast), B82 (mouse L-cell subclone) and A549 (human lung carcinoma) were also first-order and resembled the average degradation rate of proteins in [³H]methylated MOM transplanted into HTC cells (Fig. 2 and Dice, 1982). RAG cells (mouse renal adenocarcinoma) degraded proteins in transplanted [³H]methylated MOM with complex kinetics (Fig. 3). Approx. 10% of the RAG cells after fusion were observed as multinucleated cells, not observed in any other cell types. Association of [³H]methylated MOM in mock fusions without poly(ethylene glycol)-1000 was 3–20% of the radioactivity associated in normal fusions (cf. Evans & Mayer, 1983; Russell & Mayer, 1983).

Fig. 2. Effect of NH_4Cl (10mM) and leupeptin (50 $\mu\text{g/ml}$) on the degradation of proteins in reductively [^3H]methylated MOM transplanted into HTC cells as described in the Materials and methods section

At each time point triplicate plates were taken for the determination of acid-insoluble radioactivity. Cells were maintained in growth medium (○), growth medium plus NH_4Cl (●) or growth medium plus leupeptin (▲). Results represent means $\pm$ S.D.

Table 4. Degradation of reductively [^3H]methylated rat liver MOM transplanted into several cell types

Reductively [^3H]methylated MOM was transplanted into six cell lines by using the method described in the Materials and methods section. Half-lives were calculated from triplicate determinations of acid-insoluble and -soluble radioactivity in cells and medium for five time points at 24h intervals. Degradation rates were calculated from the cell acid-insoluble radioactivity from first-order rate plots for one or two experiments and are given where appropriate as mean $\pm$ S.E.M. Degradation rates were also calculated from the release of acid-soluble radioactivity into the medium over each 24h interval and are given as means $\pm$ S.D. for measurements over the four intervals in each experiment.

Cell type	Half-life for transplanted membrane (h)	
	Computed from acid-insoluble radioactivity	Computed from acid-soluble radioactivity
CHO-K1	63 $\pm$ 7	57 $\pm$ 2
3T3-L1	63 $\pm$ 7	59 $\pm$ 2
MRC-5	29	30 $\pm$ 3
B82	28	27 $\pm$ 12
A549	29	27 $\pm$ 3
RAG	80% degraded in 24h	80% degraded in 24h

Fluorescent micrographs of 3T3-L1, MRC-5 (Fig. 4), A549 and RAG (Fig. 5) cells were taken immediately after transplantation (results not shown), 6h after transplantation and 24h after

Fig. 3. Loss of cell acid-insoluble radioactivity from RAG cells after transplantation of reductively [^3H]methylated MOM as described in the Materials and methods section

At each time point triplicate plates were taken for the determination of acid-insoluble radioactivity. Results represent means $\pm$ S.D.

transplantation of fluorescein-conjugated rat liver MOM. Patching and capping of fluorescent material was not observed in any cell type at any time after fusion (cf. HTC cells; Russell & Mayer, 1983). No punctate perinuclear fluorescence was observed, in contrast to that observed 2h after fusion of fluorescein-conjugated MOM to hepatocytes (Evans & Mayer, 1983). Comparison of phase-contrast (results not shown) and fluorescent fields revealed that the majority of phase-lucent vacuoles within 3T3-L1, MRC-5 and A549 cells at 24h after fusion excluded fluorescent material.

Discussion

The studies described in this paper constitute an attempt to understand why proteins in rat liver MOM are degraded at *in vivo* rates (Russell *et al.*, 1980) when transplanted into hepatocytes (Evans

Fig. 4. Fluorescent micrographs of MRC-5 and 3T3-L1 cells after transplantation of fluorescein-conjugated MOM MRC-5 cells at (a) 6 h and (b) 24 h, and (c) control cells showing endogenous fluorescence. 3T3-L1 cells at (d) 6 h and (e) 24 h, and (f) control cells showing endogenous fluorescence.

Fig. 5. *Fluorescent micrographs of RAG and A549 cells after transplantation of fluorescein-conjugated MOM* RAG cells at (a) 6h and (b) 24h, and (c) control cells showing endogenous fluorescence. A549 cells at (d) 6h and (e) 24h, and (f) control cells showing endogenous fluorescence.

& Mayer, 1983) and much faster (approx. 2-fold) when transplanted into a related cell line, i.e. HTC cells (Russell & Mayer, 1983). The experiments were designed (a) to investigate whether rat liver MOM proteins transplanted into HTC cells were degraded at rates which were different from those measured for endogenous mitochondrial and plasma-membrane proteins, and (b) to investigate the degradative fate of rat liver MOM proteins transplanted into tissue-culture cell lines which were not derived from rat tissues. Two approaches have been employed to measure the degradation of mitochondrial protein in HTC cells undergoing cell proliferation in normal nutritional conditions. The first method used protein labelling in the presence of cycloheximide to restrict protein synthesis to essentially mitochondrially synthesized proteins (Knecht *et al.*, 1980) and followed the subsequent loss of labelled protein from the cells in the absence of cycloheximide. The results (Fig. 1) give a degradation rate for mitoribosomally synthesized proteins ($t_{1/2}$ 90 h) similar to the rate of degradation of mitochondrial proteins in rat liver *in vivo* (Russell *et al.*, 1980). The second method used a double-isotope labelling technique (Russell *et al.*, 1982) to measure the half-lives of endogenous HTC cell MOM ($t_{1/2}$ 56 h) and mitoplast proteins ($t_{1/2}$ 120 h, Tables 1 and 2). These results indicate that HTC cell mitochondrial proteins in proliferating cells grown in nutritionally complete medium are degraded at very similar rates to the mitochondrial proteins in rat liver *in vivo* (Russell *et al.*, 1980).

The rates of degradation of proteins in endogenous MOM and mitoplast of HTC cells are therefore much slower than reported for mitochondrial proteins ($t_{1/2}$ 36–39 h) in Reuber hepatoma cells (Hare & Hodges, 1982a,b). However, the experiments by Hare & Hodges (1982a,b) were performed on serum-deprived non-proliferating cells and it is widely known that serum deprivation causes accelerated degradation of cellular proteins (Amenta *et al.*, 1977, 1978). Proteins in transplanted MOM in HTC cells are degraded at a rate ($t_{1/2}$ 24–28 h) which is not the same as rates for proteins in endogenous HTC cell mitochondria (Tables 1 and 2).

Inhibition of the average rates of degradation (30–45%) of proteins in transplanted [3 H]-methylated MOM in HTC cells by the fungal proteinase inhibitor leupeptin and the lysosomotropic agent NH_4Cl (Fig. 2) was similar to the inhibition by these agents of the average rate of degradation of iodinated plasma membrane proteins (25–32%, Table 3) and the inhibition of degradation rate by NH_4Cl of endogenous mitochondrial membrane proteins (28–37%, Tables 1 and 2). This evidence, and our previous finding of [^{14}C]sucrose-

labelled peptides derived from either transplanted rat-liver [^{14}C]sucrose-conjugated MOM or [^{14}C]sucrose-conjugated plasma membrane in structures which were distributed on cell homogenization as acid phosphatase and hexosaminidase (Russell & Mayer, 1983), infers a lysosomal involvement in the degradation of both transplanted and endogenous membrane proteins in HTC cells. Lysosomal involvement in the degradation of mitochondrial inner membrane and matrix proteins is also suggested by the data in Tables 1 and 2 and the 60–70% inhibition by NH_4Cl of the degradation of [^3H]biotin-labelled pyruvate carboxylase, a matrix enzyme, in 3T3-L1 cells (Chandler & Ballard, 1983).

The results in Table 2, however, suggest that some caution is needed in the interpretive extrapolation of data on inhibition of average protein degradation rates in cells, since there is clear evidence for large differences in the inhibition of degradation of organelle protein subunits separated by SDS/polyacrylamide-gel electrophoresis.

It is noteworthy that NH_4Cl does not inhibit the degradation of HTC cytosol (S-fraction) proteins (Table 1). Soluble proteins microinjected into the cytosol of HeLa cells vary in the degree of inhibition of degradation caused by NH_4Cl , but the degradation of one microinjected protein, ^{125}I -labelled bovine serum albumin, is unaffected by NH_4Cl (Rote & Rechsteiner, 1983). Also, soluble proteins conjugated with [^{14}C]sucrose microinjected into L929 cells give rise to an accumulation of [^{14}C]sucrose-labelled peptides, mainly in the cytosol (Bigelow *et al.*, 1981). In contrast, degradation of [^{14}C]sucrose-labelled bovine serum albumin-poly(lysine) conjugate that enter HeLa cells by endocytosis is inhibited by 80% by NH_4Cl (Rote & Rechsteiner, 1983).

The pathway of degradation of proteins of rat liver [^3H]methylated MOM transplanted into HTC cells and endogenous HTC cell membrane proteins is equally susceptible to leupeptin and NH_4Cl and the degradative mechanism appears to differ from the pathway of degradation of soluble cytosol-located proteins and endocytosed proteins.

Since membrane proteins, including monoamine oxidase, in rat liver MOM are degraded at such different rates when transplanted into rat hepatocytes and rat hepatomas (Evans & Mayer, 1983; Russell & Mayer, 1983) we decided to investigate the degradative fate of rat liver MOM proteins in several cell lines not of rat origin.

With the exception of RAG cells (Table 4), the rate of degradation of proteins in [^3H]methylated MOM transplanted into several cell types is first-order and corresponds to average half-lives of either 24–28 h or 56–66 h. The latter average half-life is similar to the average half-life *in vivo* of rat

liver MOM proteins (Russell *et al.*, 1980). Transplantations of [³H]methylated MOM into RAG cells results in a complex non-first-order degradation rate for the exogenous membrane, similar to the degradation of endocytosed proteins by HTC cells (Russell & Mayer, 1983). This may be related to the observation that RAG cells are easily fused by poly(ethylene glycol)-1000 treatment and are therefore very useful in the production of somatic cell hybrid cells (S. Brown, personal communication). The grouping of cells into those (MRC-5, B82, A549 and HTC; Evans & Mayer, 1983) which degrade rat liver MOM proteins relatively quickly ($t_{1/2}$ 34–28 h) and those (CHO-K1, 3T3-L1 and hepatocytes; Evans & Mayer, 1983) which degrade rat liver MOM proteins relatively slowly is not related to cell growth rate (results not shown). The grouping is not dependent on the method of MOM internalization in each cell type, since the previously observed patching and capping of fluorescein-conjugated MOM in HTC cells followed by the relatively fast rate of MOM protein degradation is not seen in MRC-5 and A549 cells (Figs. 4 and 5) which also degraded MOM proteins at the faster rate. Classification of cells into two protein degradation groups in response to challenge with a single type of protein-containing exogenous membrane implies strict alternative processing of proteins in transplanted membrane. A similar alternative destruction of a membrane protein (erythrocyte Band 3) has recently been shown after transplantation by reconstituted viral envelope (Beigel & Loyter, 1983) into target cells.

Retention of the same degradation rate for a soluble heterologous protein microinjected into several cell lines has been recorded. [¹²⁵I]iodinated bovine serum albumin microinjected into eight cell lines had similar degradation rates in all cells (Zavortnik *et al.*, 1979).

The data presented here are consistent with a model recently proposed which accounts for the biphasic decay curve of classically radiolabelled proteins in cells in culture (Mayer *et al.*, 1984). The model is basically a two-component system comprising a rapidly degrading pool of proteins (B) which is 25–30% of the total protein, and the organellar protein pool (A). Translocation of proteins from the A pool to the rapidly degrading B pool is required before degradation takes place. Translocation rates from A to B based on the fate of radiolabelled short- and long-lived proteins in cell cultures are approx. $1\% \cdot h^{-1}$ ($K_{A \rightarrow B} = 0.24 \text{ day}^{-1}$) and a degradative rate in B of $3\text{--}4\% \cdot h^{-1}$ ($K_{B \rightarrow \text{amino acids}} = 0.72\text{--}0.96 \text{ day}^{-1}$). Degradation rates of 0.24 day^{-1} for proteins in [³H]methylated MOM transplanted into hepatocytes, CHO-K1 and 3T3-L1 cells would result from loading the protein into the organellar pool (A) and degrada-

tion rates of 0.62 day^{-1} for HTC, MRC-5, A549 and B82 cells would result from loading the majority of membrane proteins into the rapidly degrading pool (B). A recognition process must be required to give rise to the two alternative treatments of proteins in the transplanted membrane in the different cell lines.

Our combined data from studies on hepatocytes, CHO-K1, 3T3-L1 cells and HTC, MRC-5, B82 and A549 cells suggest that the current lack of understanding of intracellular catabolic mechanisms for membrane proteins results from the fact that protein destruction is but the end part of a fundamental intracellular membrane-recognition process.

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