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Recycling of 5′-nucleotidase in a rat hepatoma cell line

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Intracellular movement of cell surface 5′-nucleotidase was studied in H_4S cells, a rat hepatoma cell line. Surface labelled cells were incubated for various periods at 37°C and treated with neuraminidase at 0°C. Removal of sialic acid residues from glycoproteins results in a change of their isoelectric points. Analysis with isoelectric focusing was then used to distinguish between cell surface and intracellular 5′-nucleotidase. Incubation of ^{125}I-surface-labelled cells at 37°C resulted in a gradual decrease of labelled 5′-nucleotidase at the plasma membrane until, at 60 to 90 min, a steady state was reached with 52% of the label on the cell surface and 48% intracellular. Pretreatment of the cells with the weak base primaquine had no influence on this distribution while at the same time uptake of iron via the transferrin receptor was inhibited. Using immunoelectron microscopy 5′-nucleotidase was found on the cell surface, in multivesicular endosomes and the Golgi complex. Preincubation of the cells in the presence of cycloheximide caused a reduction of labelling in the Golgi complex, whereas the label in the other compartments was retained. These results lead to the conclusion that 5′-nucleotidase does not recycle through the Golgi complex and that in contrast to the transferrin receptor the recycling of 5′-nucleotidase is not inhibited by primaquine.

Key words: membrane transport/5′-nucleotidase/primaquine/transferrin receptor

Introduction

5′-Nucleotidase (5′N) is a plasma membrane glycoprotein of many cell types (for review, see Luzio *et al.*, 1986). A portion of rat liver 5′N could be released by treatment with phosphatidylinositol-specific phospholipase C, indicative for the presence of a phosphatidylinositol anchor to the plasma membrane (Shukla *et al.*, 1980; Low *et al.*, 1986). The enzyme has its active site at the external face of the plasma membrane (Newby *et al.*, 1975). In the hepatocyte, 5′N is predominantly located at the bile canalicular front. Intracellularly, 5′N is present in endosomes, lysosomes and the Golgi complex as measured by its enzymatic activity after subcellular fractionation; in addition, it was shown by histochemistry and immunocytochemistry (for review, see Luzio *et al.*, 1986). Exchange of 5′N between the cell surface and intracellular compartments has been established (Widnell *et al.*, 1982; Stanley *et al.*, 1980). However, pathways taken by the enzyme are largely unknown. In order to get more insight in the itinerary of 5′N through the cell we have studied the behaviour of the enzyme molecules present at the cell surface. Previously we used the rat hepatoma cell line H_4S and we established that 5′N, synthesized in these cells, contains both complex and hybrid N-linked oligosaccharide chains (Van den Bosch *et al.*, 1986) and thus may contain one or more sialic acid residues. Removal of sialic acids with neuraminidase reduces the negative charge of 5′N resulting in an increase of its isoelectric point. This provides us with a useful tool to discriminate between cell-surface and intracellular 5′N. In the present study we demonstrate that 5′N rapidly recycles between the cell surface and an intracellular compartment.

Intracellular transport of one class of membrane proteins in particular can easily be studied, i.e. cell surface receptors. Therefore, we have compared the recycling of the transferrin receptor with that of 5′N. Cell-surface receptors like the transferrin receptor recycle between the cell surface and the endosomal compartment with a recycle time in the range of 16 min in human hepatoma cells (Ciechanover *et al.*, 1983). Studies have demonstrated acidification of the endosomal compartment (Tycko and Maxfield, 1982), coated vesicles (Stone *et al.*, 1983; Forgac *et al.*, 1983) and (*trans*) Golgi compartments (Schwartz *et al.*, 1985; Orci *et al.*, 1987) as well as lysosomes (Ohkuma and Poole, 1978). Weak bases neutralize the pH of these compartments and interfere with receptor recycling (Basu *et al.*, 1981; Kaplan and Keogh, 1981). We have previously shown that both receptor recycling and protein secretion is inhibited to the same extent by the weak base primaquine, an 8-amino-quinolone similar to chloroquine, in human hepatoma cells (Schwartz *et al.*, 1984; Strous *et al.*, 1985). The effect of weak bases on two such different processes suggests that vesicle acidification is a basic feature of membrane protein sorting in the secretory pathway and in receptor recycling. However, a satisfactory explanation for all of the effects of weak bases is not yet available (Krogstad and Schlessinger, 1987). We have defined the effect of primaquine on the recycling of 5′N, and we have compared this with the effect on the recycling of the transferrin receptor.

Results

Characterization of cell surface 5′N

5′N is a glycoprotein with at least three N-linked oligosaccharide chains of the complex type (Van den Bosch *et al.*, 1986). These chains contain sialic acids, which can be removed by the enzyme neuraminidase. Removal of the negatively charged sialic acids changes the charge of the glycoprotein which can be measured on isoelectric focusing gels. Neuraminidase treatment at 0°C provides us with a biochemical method to measure internalization and recycling of membrane components containing cleavable sialic acid residues. Internalized 5′N is no longer accessible

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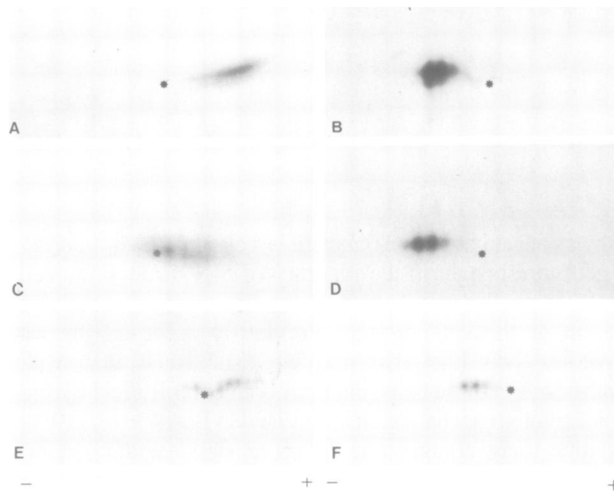

Fig. 1. Two-dimensional gel electrophoresis of immunoprecipitated 5′N. **Panel A** and **B**: cells were labelled with [^{35}S]methionine for 2 h, and lysed in 1% Triton X-100; the lysate was treated with (B) or without (A) neuraminidase. **Panel C** and **D**: cells were surface-labelled with ^{125}I and then treated (D) with or (C) without neuraminidase at 0°C. The cells were lysed, 5′N was quantitatively immunoprecipitated and analysed. **Panel E** and **F**: ^{125}I-labelled cells were lysed and mixed with a lysate of unlabelled, neuraminidase-treated cells. (E) Immunoprecipitated 5′N was analysed. (F) ^{125}I-labelled control cells after treatment with neuraminidase. In the isoelectric focusing dimension the basic side is to the left. The gel was stained with Coomassie Brilliant Blue to identify the position of BSA (*) (pI = 4.7).

to enzymatic release of sialic acid and can therefore be distinguished qualitatively from 5′N remaining on the cell surface. First it was important to determine whether neuraminidase treatment caused sufficient charge difference for a clear separation on isoelectric focusing gels. To test this, cells were labeled with [^{35}S]methionine and lysed in detergent. The lysate was split into two portions. One half was incubated on ice without and the other half with 80 mU/ml neuraminidase for 24 h. After two-dimensional gel electrophoresis of the immunoprecipitates, 5′N is present as multiple radioactive spots (Figure 1A). Treatment with neuraminidase resulted in a shift to the cathodic side of the gel with a simultaneous reduction of the number of spots compared to the control (Figure 1B). Albumin was added as a standard (pI = 4.7). To ascertain that neuraminidase treatment is also useful for cells growing in monolayer, H_4S cells were surface-iodinated on ice using lactoperoxidase and then treated with 80 mU/ml neuraminidase at 0°C for 1 h. Again 5′N was analysed by two-dimensional gel electrophoresis. This resulted in a similar electrophoretic pattern compared to neuraminidase digestion of the [^{35}S]methionine-labelled lysate, indicating the same susceptibility for the enzyme (compare Figures 1B and 1D). On one-dimensional SDS−PAGE only a slight difference in mol. wt of ±2000 is present between control and treated 5′N (not shown).

To exclude the possibility that in spite of several washings some remaining neuraminidase activity was present in the cell lysate we mixed the lysate of iodinated cells with that of neuraminidase-treated unlabelled cells. As seen in Figure 1E no shift can be noticed indicating that, under the experimental conditions used, neuraminidase is no longer active after cell lysis and that only sialic acid residues from

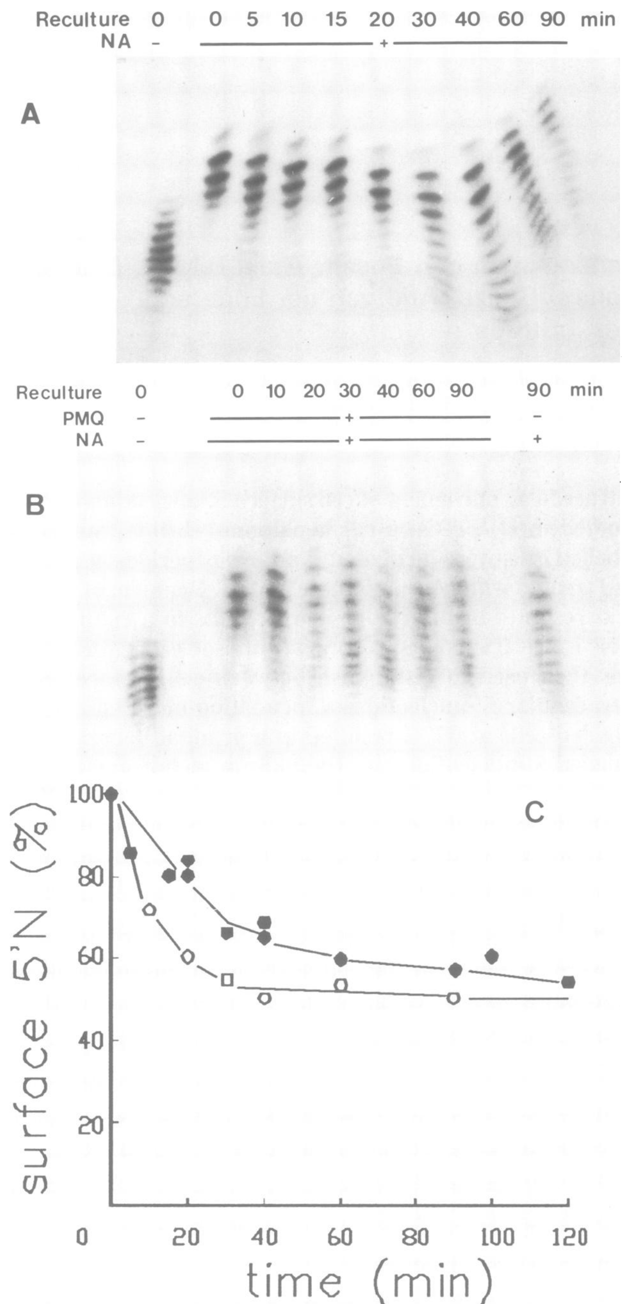

Fig. 2. Time course of internalization and recycling of 5′N. Cells were iodinated at 0°C, cultured for the indicated times at 37°C to allow internalization of 5′N, chilled on ice and treated with neuraminidase (NA). At t = 0 one control sample was not treated with neuraminidase. Cells were lysed, 5′N was immunoprecipitated and analysed by isoelectric focusing. (**A**), control cells, (**B**), effect of primaquine on internalization. After labelling with ^{125}I on ice cells were recultured in the presence of 0.3 mM primaquine (PMQ). One sample was incubated for 90 min without primaquine to compare directly with the primaquine incubated sample. The basic side of the gel is at the top. (**C**) Lanes of the gels in (A and B) were scanned and the ratios of neuraminidase-sensitive (i.e. surface 5′N) to total 5′N were calculated as percentage of total 5′N and plotted. ●—●, control cells; ○—○, cells treated with primaquine.

cell-surface 5′N are removed. As immunoprecipitation of 5′N is quantitative and provides us with a completely pure glycoprotein (Figure 1), one-dimensional isoelectric focusing analysis was used in further experiments.

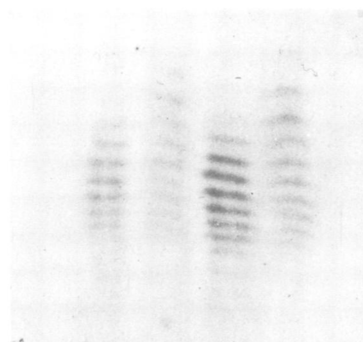

Fig. 3. Steady state distribution of metabolically labelled 5′N. Cells were labelled with [^{35}S]methionine for 2 h and chased for either 4 or 20 h. Thereafter cells were treated with neuraminidase at 0°C (NA). Control cells were not treated with neuraminidase (NA). Cells were lysed, 5′N was immunoprecipitated and analysed by isoelectric focusing. The basic side of the gel is at the top.

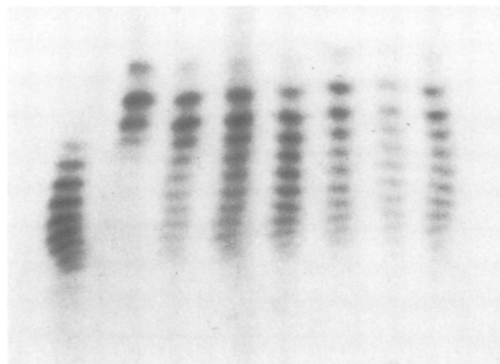

Fig. 4. Effect of primaquine on the steady-state distribution of 5′N. Cells were surface-labelled and recultured for 90′ to allow 5′N to reach a steady-state situation. Thereafter primaquine (PMQ) was added to the culture medium and cells were cultured for another 30 min. Cells were lysed, and treated with neuraminidase (NA); 5′N was immunoprecipitated and analysed by isoelectric focusing. The basic side of the gel is at the top.

Fate of cell surface 5′N

To study internalization of cell-surface 5′N, iodinated cells were cultured at 37°C to allow membrane transport. After various times, cells were chilled on ice, washed and treated with neuraminidase. As seen in Figure 2A, left lane, 5′N present at the cell surface consists of at least nine iso-proteins of which the upper two are present in low quantities. Upon treatment with neuraminidase the nine-band pattern is converted to a five-band pattern with some overlap. Upon incubation of the cells at 37°C the pattern gradually changes. After 60 min ~40% of 5′N was inaccessible to neuraminidase suggesting internalization. Scanning of the gel patterns revealed that labelled 5′N reached an equilibrium distribution with 48% intracellular and 52% (±3%, N = 9) at the cell surface (Figure 2C). Internalization occurs according to a (pseudo) first-order reaction (correlation coefficient, −0.968). The time needed to reach half-maximal effect is 21 min (Figure 2C). The presence of a steady state implies that the population of 5′N, initially present at the cell surface, recycles between intracellular compartments and the plasma membrane.

To examine if cell surface 5′N exchanges with other major 5′N-containing compartments, we compared the steady state of surface-labelled 5′N with that of metabolically labelled 5′N. Cells were labelled with [^{35}S]methionine for 2 h and then cultured for 4 or 20 h in medium without radioactivity. After 20 h it is conceivable that the distribution of radioactive 5′N is in equilibrium with that of unlabelled 5′N in all cellular membranes. The cells were treated with neuraminidase and 5′N was analysed by isoelectric focusing. Figure 3 shows that the amount of [^{35}S]methionine-labelled neuraminidase-sensitive 5′N is comparable to that of ^{125}I-labelled 5′N in the steady-state situation (Figure 2). These results indicate that both ^{125}I-cell surface labelled and [^{35}S]methionine metabolically labelled 5′N have the same distribution between the cell surface and intracellular membranes, which indicates that both classes of 5′N recycle through the same compartments.

An attempt was made to explore the possibility that the enzyme present at the plasma membrane can recycle between the cell surface and the Golgi complex. In that case neuraminidase-treated 5′N was re-examined for the presence of sialic acid residues. Cell-surface proteins were vectorially iodinated and treated with neuraminidase. The cells were then recultured for 20 h, and 5′N was isolated and analysed by isoelectroc focusing. Analysis by isoelectric focusing showed no change in IEF patterns between 5′N from cells before and after reculturing. Thus, cell-surface located 5′N does not recycle between the Golgi complex and the plasma membrane in significant amounts. As sialyltransferase is present in the Golgi complex this result also indicates that Golgi 5′N is indeed resident and probably does not recycle between this compartment and the plasma membrane.

Effect of primaquine treatment on plasma membrane 5′N and the transferrin receptor

Weak bases such as primaquine neutralize the pH of acidic intracellular compartments such as endosomes and lysosomes and interrupt recycling of receptors without affecting their internalization. This results in a consequent receptor accumulation in an intracellular compartment (Schwartz *et al.*, 1984). When labelled 5′N is allowed to enter the cells in the presence of primaquine, an equilibrium is reached at ~30 min (Figure 2B). Quantitation of the bands reveals a distribution of 5′N between intracellular and plasma membranes similar to the normal steady-state situation. Internalization in the presence of primaquine occurs also according to (pseudo) first-order kinetics (correlation coefficient, −0.998). The time required to get the half-maximal effect is 8.5 min, which is shorter than the situation in the absence of primaquine. This indicates that recycling of 5′N is not interrupted by primaquine but even accelerated.

Alternatively, this result can be explained as follows: in human hepatoma cells primaquine treatment causes a reduction of cell-surface and an increase of intracellular membranes (Zijderhand-Bleekemolen *et al.*, 1987). Under such conditions it is possible that endocytosis stops as there

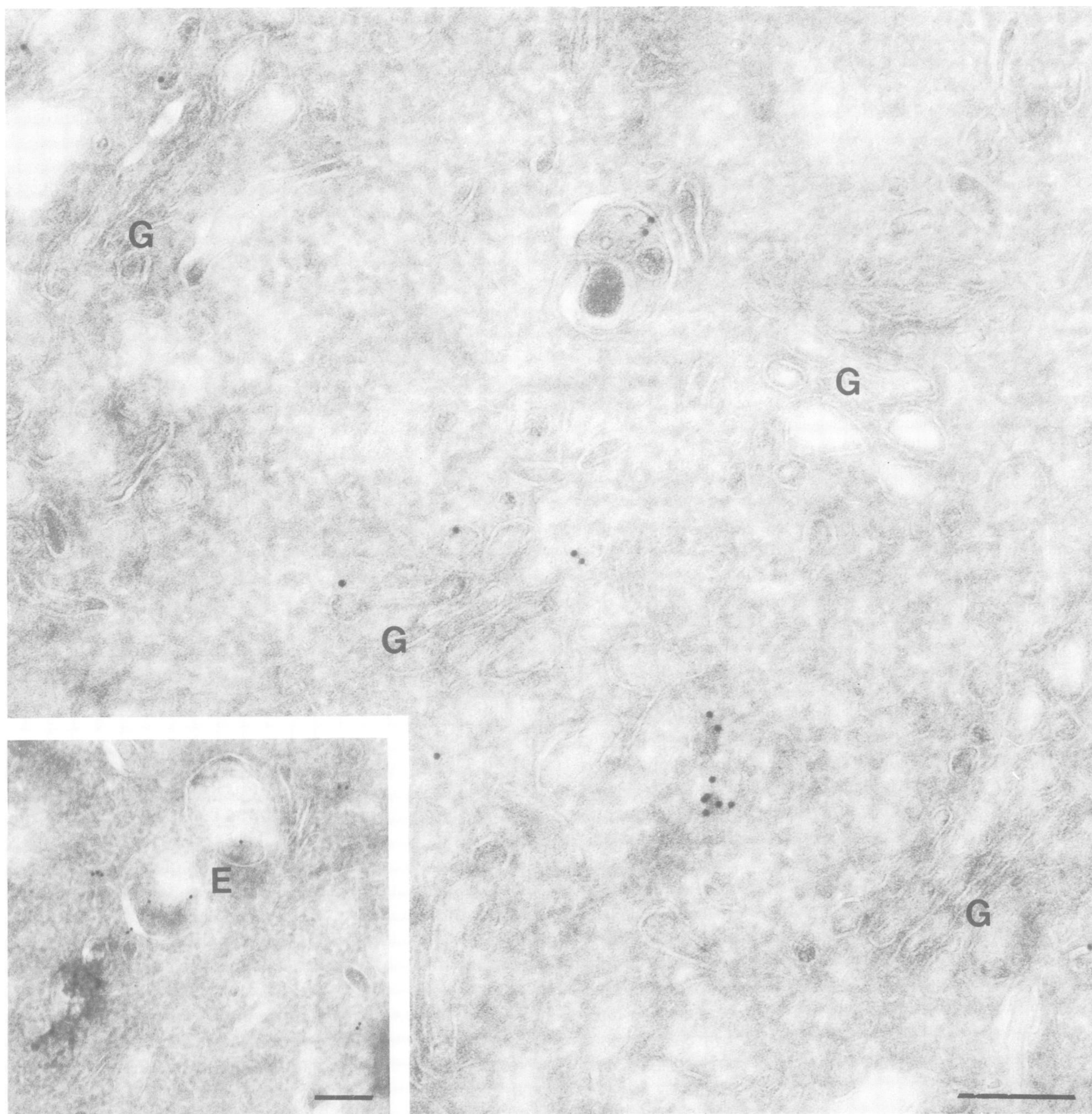

Fig. 5. Golgi complex (G) showing 5′N label in vacuoles and tubules near the Golgi stacks. Inset: multivesicular endosome. G, Golgi stack; E, endosome. Bars, 0.25 μm.

is no replenishment of plasma membrane. Our results would then reflect the rate of internalization of 5′N , as in this option there is no exocytosis of membrane. To discriminate between these two possibilities cells were iodinated on ice and then recultured for 90 min, i.e. the time at which a steady-state distribution of labelled 5′N had been established. The steady state implies that the internal and surface pools of labelled 5′N are in equilibrium. At this time-point primaquine was added at a concentration of 0.3 mM. If this drug had an inhibitory effect on the exocytosis of membrane, this treatment should cause a (further) decrease of the cell-surface label. No such effect was observed (Figure 4), and it can thus be concluded that recycling of 5′N is not inhibited by changing the pH of the endosomal compartment. In addition, it is apparent that membrane recycling continues in the presence of primaquine at higher recycling rates.

We have also determined the effect of primaquine on the recycling of the transferrin receptor. This receptor recycles continuously between the cell surface and intracellular organelles, both with and without ligand. To confirm the effect of primaquine on receptor recycling we have measured the uptake rate of [^{125}I]transferrin and [^{59}Fe]transferrin. Unfortunately the H_4S cells have only a few percent of their transferrin receptors exposed at the cell surface. This made a reliable measurement of the recycling kinetics impossible. However, transferrin receptor recycling results in iron accumulation in the cells. This uptake process, assayed using [^{59}Fe]transferrin, was almost completely (85%) impeded by

primaquine (not shown). By analogy with transferrin receptor recycling in other cell types (HepG2 cells, BeWo chorion carcinoma cells) it is likely that also in H_4S cells these receptors disappear from the cell surface in the presence of primaquine (Stoorvogel *et al.*, 1987; Van der Ende *et al.*, 1987; Ciechanover *et al.*, 1983; Schwartz *et al.*, 1984). Taken together, the results show that under conditions in which receptor recycling is inhibited the recycling of 5′N continues.

Immunocytochemistry

To get more insight in the role of intracellular compartments in the recycling process we used immunoelectron microscopy. At the cell surface 5′N label had a random distribution over the microvilli. Occasionally membrane specializations reminiscent of bile canaliculi were visible between the cells, containing a higher density of label. In the cell, label was clearly present in multivesicular endosomes (Figure 5, inset). Most of the Golgi labelling was found in vesicles close to the Golgi stack (Figure 5). Treatment of cells with cycloheximide for 2 h showed that the labelling of the Golgi stack was diminished. Some label was still present in the *trans*-most cisternae. Labelling of the plasma membrane and multivesicular endosomes was about the same as in control cells.

Discussion

Neuraminidase treatment of cells is a powerful tool to study the topology of membrane glycoproteins. In this way a distinction between glycoproteins present at the cell surface and in intracellular organelle membranes can be made without loss of signal. The reliability of the method is clearly demonstrated. To interpret our results we used the criterion that 5′N inaccessible for neuraminidase is intracellular. However, care must be taken in this interpretation. H_4S cells are derived from rat hepatocytes. This indicates that tight junctions and bile canicular domains can be formed. It is not known whether the canalicular domains and the lateral membranes are completely accessible for ^{125}I-labelling or neuraminidase treatment. Taking this into consideration, it is possible that part of 5′N protected against neuraminidase treatment is not present in the cell, but at the basal-lateral or bile canalicular membranes.

Kinetics of recycling

After labelling of the cell surface, subsequent membrane flow leads to a redistribution of label between the plasma membrane and intracellular membranes, until—in steady state—48% of the label is intracellular. This distribution is of the same magnitude as found in hepatocytes by Stanley *et al.* (1980). From our data we can conclude that the $t_{1/2}$ of the process to reach equilibrium is 21 min. In the presence of primaquine the $t_{1/2}$ was 8.5 min, while the equilibrium distribution was essentially the same (46% intracellular and 52% at the surface). This means that in the presence of the drug the rate of endocytosis as well as the internalization is increased. Once the steady-state distribution of labelled 5′N has been established, no further intracellular accumulation of label was observed, even after longer time periods, up to 24 h. We conclude, therefore, that both in the presence and absence of weak bases a bidirectional membrane flow exists between the plasma membrane and internal membranes. Wilcox *et al.* (1982) showed that monensin inhibits the endocytosis of 5′N in rat fibroblasts. This finding points to the Golgi complex as a major regulatory organelle in membrane recycling; inhibition by monensin of 5′N recycling is probably caused by its interference (as an ionophore) with this regulatory function. Primaquine primarily acts as a weak base on the endosomal compartment. As 5′N does not recycle via the Golgi complex it is expected that monensin and primaquine act differently on the intracellular transport of the enzyme.

Stanley *et al.* (1980) studied transport of 5′N in isolated rat hepatocytes. They used enzyme activity as a measure for the presence of the enzyme. 5′N at the cell surface was inactivated by binding Con A or antibodies at 0°C. One hour of reculturing increased cell surface activity as a result of redistribution of intracellular enzyme. From their data one can estimate that the ratio between intracellular and cell-surface 5′N equals that in cells not incubated with Con A after 2−3 h of reculturing. Using the same method Widnell *et al.* (1982) found that the equilibrium situation in fibroblasts was only established 15 h after quenching of cell-surface 5′N. However, in both cases a real steady-state distribution was not reached.

Pathway of recycling

In adult rat hepatocytes histochemical and biochemical evidence has been presented showing that 5′N is associated with Golgi vesicles, lysosomes and coated vesicles (Widnell and Little, 1977; Wanson *et al.*, 1980). In a rat hepatoma cell line immunofluorescence label had a punctate distribution with no indication of Golgi-associated anti-5′N label (Stanley *et al.*, 1980). Immunoelectron microscopy showed the presence of the enzyme in the Golgi complex of rat liver (reviewed in Luzio *et al.*, 1986). In accordance with these results we found 5′N labelling in multivesicular endosomes and a small amount in the Golgi complex. After 2 h incubation in cycloheximide 5′N was diminished but still detectable in the Golgi complex. This effect is different from results obtained in rat liver, where 5′N label completely disappeared from the Golgi complex after cycloheximide treatment (Geuze *et al.*, 1984). It indicates that in H_4S cells a portion of the 5′N is resident in the (*trans*) Golgi complex.

Due to the low amount of surface receptors it was not possible to quantitate the primaquine effect on the number of cell-surface transferrin receptor molecules present. However, a drastic redistribution of transferrin receptors is deduced from the observation that iron uptake is almost completely inhibited if primaquine is present. This is also found in HepG2 cells (Stoorvogel *et al.*, 1987; Ciechanover *et al.*, 1983). In these cells the amount of surface binding sites decreased to $\sim 20\%$ within minutes. In contrast to the effect on the transferrin receptor, primaquine does not prevent the recycling of surface 5′N. The recycle time is even shortened. A possible explanation for this result is that 5′N normally recycles through the cell via more than one route. In this option it is possible that primaquine forces 5′N to recycle exclusively via a faster—primaquine insensitive—pathway. As a result the mean circulation time is shortened and thus, the steady-state situation is reached in a shorter period of time. This path is probably identical with the so-called diacytotic route taken by receptor−ligand complexes. A similar finding is reported for the asialoglycoprotein receptor in hepatocytes: incubation in the presence of

chloroquine increased the portion of ligand–receptor complexes that entered the diacytosis route (Tietze *et al.*, 1982; Simmons and Schwartz, 1984; Chang and Kullberg, 1984; Stein and Sussman, 1986). Also the low-density-lipoprotein receptor returns a higher portion of its ligand to the cell surface in the presence of weak bases (Greenspan and St Clair, 1984). We suggest that recycling of membrane proteins (including receptor molecules) occurs via two different pathways, one of which is insensitive to weak bases.

Materials and methods

Cell culture

H_4S cells, originally obtained from Dr A.H.Tashjan (Harvard School of Public Health, Boston) (Strous and Lodish, 1980), were grown as monolayer in Eagle's minimal essential medium (MEM; Gibco Laboratories, Grand Island, NY) with 10% fetal calf serum (FCS; Boehringer, Mannheim Biochemicals, Indianapolis, IN). The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Labelling of cells

Lactoperoxidase iodination was done essentially as described by Hubbard and Cohn (1972). Confluent cultures (60 mm) were placed on ice and rinsed three times with cold phosphate-buffered saline (PBS). Then 2.5 ml of PBS containing 1 mg glucose (Merck, Darmstadt, FRG), 0.1 mg lactoperoxidase (Sigma Chemical Co, St Louis, MO), 50 μg glucose oxidase (Millipore B.V., Etten-Leur, The Netherlands) and 0.2 mCi ^{125}I (Amersham, UK) was added. Iodination was performed for 20 min on ice. The reaction was quenched by addition of 0.01 M tyrosine and sodium metabisulphite. Cells were then washed four times with cold PBS.

For metabolical labelling the cells were incubated at 37°C for 20 min in methionine-free MEM. Then medium was removed and methionine-free MEM containing 40 μCi/ml [^{35}S]methionine (~800–1000 μCi/mmol, Amersham Corp.) was added. Labelling was terminated by washing the cells with MEM. Thereafter, cells were chased in fresh medium containing 0.1 mM unlabelled methionine for the appropriate time periods. Cell lysis and immunoprecipitation of 5′N was done as described previously (Van den Bosch *et al.*, 1986).

Neuraminidase treatment of cells

Labelled cells were washed three times with ice-cold PBS and then 500 μl PBS containing 40 mU neuraminidase (*Vibrio cholerae*, Calbiochem-Behring Corp., La Jolla, CA) was added. After 60 min on a rocking platform at 0°C cells were washed five times with cold PBS without Ca^{2+} and Mg^{2+}, containing 1 mg/ml bovine serum albumin (BSA), 1 mg/ml glucose and 1 mM EDTA to inhibit further neuraminidase activity. Immunoprecipitates were analysed by isoelectric focusing.

Gel electrophoretic analysis

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (1975). In the first dimension proteins were separated by charge via isoelectric focusing. Ampholines pH 3.5 to 10 were employed. As a standard, BSA (pI 4.7) was added to the sample prior to isoelectric focusing. In the second dimension proteins were separated by SDS–PAGE (10% w/v) according to Laemmli (1970). For detection of albumin the gels were stained with Coomassie Brilliant Blue.

One-dimensional isoelectric focusing was performed using the following method: immune complexes were dissociated in 10 μl Laemmli sample buffer by heating the sample for 5 min at 90°C. Samples were cooled to room temperature, 50 μl urea sample buffer was added (57% urea, 2% Nonidet-P40, 2% ampholines (40%), 1.5% dithiothreitol) and several grains of extra urea. Total sample was applied to the gel and overlaid with a urea sample buffer diluted 1:1 with 2% Nonidet-P40.

Isoelectric focusing was done on a 4% polyacrylamide slab gel containing 55% urea, 2% Nonidet-P40, 6% of a mixture of 40% ampholines (pH 3.5 to 10), 0.02% ammonium persulphate, 0.1% phenylmethylsulphonyl floride (PMSF). The upper chamber was filled with 50 mM NaOH and the lower chamber with 20 mM H_3PO_4. The gel was run with unlimited amperage and a maximum of 1000 V during 18 h; the power was limited at 3 W.

Labelling of transferrin

Human transferrin (Sigma Chemical Co, St Louis, MO) was saturated with iron according to Klausner *et al.* (1983). Proteins were iodinated with ^{125}I (Amersham) using Iodo-beads (Pierce Chemical Company, Rockford, IL). Unbound ^{125}I was removed by gel filtration through a Sephadex G25 column (Pharmacia, Uppsala, Sweden). Loading transferrin with ^{59}Fe (Amersham) was performed according to the procedure of van der Heul *et al.* (1978) with specific radioactivity 5×10^6 d.p.m./mg.

Binding assays

Binding assays were performed with semiconfluent cultures. Cells were incubated with or without 0.3 mM primaquine. Cells were cooled to 0°C, incubated at pH 4.5 and pH 7.3 to remove all plasma membrane-bound transferrin. Subsequently, ice-cold binding medium (MEM containing 20 mM Hepes pH 7.3) containing 5 μg/ml [^{125}I]transferrin was added. Binding reactions were carried out on ice for 60 min. Nonspecific binding was determined in the presence of a 100-fold excess of unlabelled transferrin. After binding cells were washed four times, lysed in 1 M NaOH and counted in a gamma counter.

Uptake of transferrin and iron

Uptake of [^{59}Fe]ferrotransferrin was carried out generally in 30 mm dishes. Cells were incubated wth 20 μg/ml transferrin in 1 ml of binding medium at 37°C. After incubation the cells were washed with ice-cold PBS and dissolved in 1 M NaOH according to van der Ende *et al.* (1987). The one cycle experiments were carried out as described in detail previously (Ciechanover *et al.*, 1983; van der Ende *et al.*, 1987).

Electron microscopy

Cells were fixed in a mixture of 1% acrolein and 0.2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 and embedded in gelatin as described by Geuze and Slot (1980). Preparation of ultrathin cryosections, immunolabelling with 8 nm protein A–gold particles, staining and embedding of the sections was as described by Slot and Geuze (1983). To stop protein synthesis 0.1 mg/ml cycloheximide was added 2 h before fixation.

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