Mechanisms of plasma membrane protein degradation: Recycling proteins are degraded more rapidly than those confined to the cell surface

(biotinylation/membrane compartmentation/membrane turnover)

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ABSTRACT Plasma membrane proteins of intact mouse 3T3 fibroblasts and H4-II-E-C3 hepatoma cells were separated into two groups based on their compartmentation between the cell surface and an intracellular compartment accessible at 20°C but not at 0°C. One group was derivatized at 0°C with sulfo-NHS-biotin or 2-(biotinamido)ethyl-1,3-dithiopropionate but not at 20°C. The second group was derivatized at 20°C as well as at 0°C. Derivatized proteins were isolated from 35S-labeled cells on streptavidin/agarose and resolved by twodimensional PAGE. With few exceptions, pulse-chase experiments revealed that those proteins confined exclusively to the cell surface turned over slowly (t1/2, >75 h), while those bimodally compartmentalized between the cell surface and the 20°C accessible compartment were degraded more rapidly (t1/2, <31 h). These observations suggest a mechanism to explain the varied metabolic stability of plasma membrane proteins in which the half-life of each protein is determined by the proportion of time spent in the endocytic compartment.

Proteins expressed at the surface of cells are degraded at different rates (1, 2). Changes in the stability of plasma membrane proteins may form the underlying basis for certain forms of cancer, diabetes, and other diseases. In most cases, cell-surface-expressed proteins must internalize into the cell for degradation to occur, since endosomes and lysosomes have been implicated in their proteolysis (reviewed in ref. 2). Possible explanations for differential stability of plasma membrane proteins include the following: (i) Each possesses an inherent susceptibility for selective removal from the membrane. This susceptibility is based on a structural feature of the protein that controls its protease sensitivity, rate of diffusion, or some other physical characteristic. (ii) Rate of turnover is simply a consequence of individualized recycling rates: rapidly degraded proteins recycle more frequently or spend a greater portion of their life-span inside the cell, thus exhibiting a greater probability for interaction with lysosomes, intracellular proteolytic enzymes, or membrane fission/fusion events. Support for the latter notion stems from the relatively rapid degradation rate for receptor proteins (t1/2, 2-20 h), known to undergo frequent recycling between the cell surface and endosomes (3), compared to other membrane glycoproteins readily visualized by surface-labeling methodologies (t1/2, >50 h) (4, 5).

To differentiate between different mechanisms of membrane turnove, we have adapted the recently described biotinylation/recovery assay (6–8) to analyze the degradation and recycling rates for a large number of plasma membrane proteins. In this method, metabolically labeled cultured cells are derivatized at either 0°C or 18°C–20°C with a membrane impermeant, biotinylating reagent. Only cell-surface proteins are derivatized at 0°C, while at 20°C plasma membrane proteins in an intracellular compartment, accessible to the medium only at the higher temperature, become derivatized (7, 8). Cells derivatized at 20°C are then exposed to glutathione or avidin at 0°C. Proteins resistant to stripping or blocking surface biotinyl groups from cells derivatized at 20°C are those plasma membrane proteins that are in an intracellular compartment not accessible to the cell surface. Proteins are recovered for analysis by affinity isolation on streptavidin/agarose and are released from the biotinyl group on the solid support by disulfide bond reduction (Fig. 1). In this case, we have analyzed the degradation rate and extent of intracellular compartmentation for a large number of surface-exposed plasma membrane proteins on fibroblast and epithelial cell lines. We find that, with perhaps only four exceptions, 35–40 proteins restricted to the cell surface are slowly degraded. On the other hand, plasma membrane proteins bimodally distributed between cell surface and endosomal pools are degraded with half-lives of 31 h or less. These results strongly support a mechanism of plasma membrane protein turnover in which the frequency of protein recycling between the cell surface and intracellular pools determines the stability of the protein.

MATERIALS AND METHODS

Cells and Cell Labeling. Mouse 3T3 fibroblasts were grown to confluence on 75-cm² culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. H4-II-E-C3 hepatoma cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and 4 mM glutamine. Cells were labeled in 3 ml of methionine-free minimal essential medium supplemented with 10% dialyzed fetal bovine serum and 400 μCi of [35S]methionine (1000 Ci/mmol; 1 Ci = 37 GBq) for 24 h in the case of 3T3 cells or for 12 h in the case of hepatoma cells. DMEM (0.01 vol) was added to the labeling medium so that the final specific activity of [35S]methionine was 100 Ci/mmole. Chases as specified in the text were carried out in the appropriate medium supplemented with 10% fetal bovine serum, 2 mM methionine, and 2 mM cysteine.

Isolation of Derivatized Proteins. After labeling and chasing, cell cultures were washed four times in phosphate-buffered saline (PBS; ref. 9) and derivatized for 2 h at 0°C or 20°C with gentle swirling in 500 μg of sulfo-NHS-biotin 2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-ss-biotin) per ml of PBS (3 ml per dish). Derivatized cultures were washed four times in PBS. Avidin-blocked cells were then incubated at 0°C for 1 h with avidin (1 mg/ml) (Sigma) in PBS. Glutathione stripping was carried out as described (10) at 0°C. Cells were scraped from the dishes in 0.25 M sucrose/0.01 M Abbreviations: IEF, isoelectric focusing; NHS-ss-biotin, sulfo-NHS-biotin 2-(biotinamido)ethyl-1,3-dithiopropionate.

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bound proteins were further washed by resuspension in solubilization buffer supplemented with 0.5 M NaCl and in solubilization buffer diluted with 10 vol of water. Washed beads were finally resuspended in 50 μl of two-dimensional gel solubilization buffer (11).

Electrophoresis. Solubilized streptavidin/agarose-bound proteins from unbiotinylated cells and from cells derivatized in the absence of chase were diluted so that the volume was proportional to the number of cpm present in solubilized membrane preparations for each sample prior to the streptavidin/agarose binding step. For samples chased for 1 or 25 h, samples were resuspended in 50 μl of sample buffer and the total cpm in each sample was estimated by counting a small aliquot prior to electrophoresis. Thirty micro liters of each sample was separated in two dimensions by isoelectric focusing (IEF) (11) and SDS/PAGE in 10% acrylamide/0.27% bisacrylamide (12). The gels were fixed, treated with Fluoromation (Research Products International) according to the manufacturers’ instructions, and exposed at -80°C to Kodak XR-5 film. Identified spots on the developed x-ray film were used to locate the appropriate spot on the dried gel and the excised gel pieces were incubated individually in glass scintillation vials with 0.3 ml of 30% hydrogen peroxide and 0.1 ml of 60% perchloric acid overnight at 50°C. Solubilized gel was diluted with 10 ml of scintillation fluid and assayed on a liquid scintillation counter.

Calculation of Membrane Protein Half-Lives. Six dishes of 35S-labeled cells were chased for 0–48 h and membrane preparations were solubilized and precipitated with 10% trichloroacetic acid; precipitated protein was counted by scintillation spectrometry. A first-order decay curve from the data showed a t1/2 of 59 h (Kd, 0.0117) for 3T3 cells and t1/2 of 136 h (Kd, 0.0051) for hepatoma cells.

In the chase experiments of Fig. 3 and Tables 1 and 2, cells were chased for 1 or 25 h. For 3T3 cells, the cpm recovered in the solubilized membrane fraction was 81.45 × 106 (1 h) and 71.09 × 106 (25 h). Based on the calculated decay constant for this preparation, the cpm recovered in the solubilized membranes from the 25-h chase was calculated to be 61.65 × 106 if both dishes incorporated the same amount of label and if the recovery of membrane was identical. The cpm recovered in each protein spot from cells chased for 25 h was then multiplied by 0.87 [(61.5 × 106)/(71.09 × 106 = 0.87)] to generate an adjusted value based on the measured decay. On the assumption that the degradation of each spot was first order, Kd and t1/2 were calculated for each from the equation

\[ C = U - K_d t \]

where t is 24 h, C is adjusted cpm recovered from proteins in the gel of 25-h chased cells, and U is the cpm recovered from proteins in the gel of 1-h chased cells.

In hepatoma cells, the cpm recovered in the solubilized membrane fraction were 35.5 × 106 (1 h) and 42.7 × 106 (25 h). The cpm recovered from cells chased for 25 h was calculated to be 31.6 × 106 based on the experimentally determined decay curve. The cpm recovered for each protein from 25-h chased cells was then multiplied by 0.74 [(31.6 × 106)/(42.7 × 106)] to generate the adjusted value. Kd and t1/2 values were determined for each protein as for 3T3 cells.

RESULTS

Compartmentation of Hepatoma Plasma Membrane Proteins. As shown in previous studies with other cell lines (6–8, 13), proteins from hepatoma cells derivatized with NHS-ss-biotin can be isolated on streptavidin/agarose beads (Fig. 2). Proteins that are nondifferentially adsorbed onto streptavidin/agarose from underderivatized cells (Fig. 2C) were excluded.
from consideration of compartmentation/turnover. It is unlikely that these latter proteins all contain endogenous biotin since only four biotinylated proteins are present in eukaryotic cells (14).

To identify proteins that are confined to the cell surface, dishes of cells were derivatized at 0°C or 20°C. Those proteins that were absent from IEF–SDS/PAGE resolved proteins after glutathione stripping at 0°C (Fig. 2B) or avidin treatment at 0°C (results not shown) are restricted to the cell surface. These are indicated in Fig. 2A (arrowheads). Five of the most abundant of these cell-surface-restricted proteins were removed from polyacrylamide gels of proteins from both 0°C derivatized and 20°C derivatized/glutathione-stripped cells. Scintillation counting showed that 89% or more of the cpm in this group of proteins was removed by glutathione treatment. A second group of proteins appear in significant concentrations on two-dimensional gels of membranes from glutathione-treated cells and are shown in Fig. 2B (large arrowheads). The cpm remaining after glutathione stripping ranged from 26% to 56% of total cpm recovered from 0°C biotinylated plus that recovered from stripped cells. In contrast to the first group of cell-surface-exposed proteins, this group exhibited a large intracellular pool as well as a cell-surface pool and were thus bimodally compartmentalized in the cell.

To determine whether the intracellular pools of bimodally distributed proteins were localized to the plasma membrane, cells were derivatized at 20°C, treated with glutathione, and fractionated on a Percoll gradient; 35S-labeled proteins were separated on IEF–SDS/PAGE (results not shown). All seven bimodally localized proteins were identified in the plasma membrane-rich fraction of the gradient. Thus, in these cells it is unlikely that proteins specifically isolated from cells biotinylated at 0°C or 20°C were derived from other cellular membrane compartments (e.g., lysosomes, rough endoplasmic reticulum) since these migrate to different locations on Percoll gradients (15) and are not accessible in intact cells to exogenous agents at <20°C.

**Fig. 2.** IEF–SDS/PAGE separation of hepatoma cell membrane proteins biotinylated under different conditions. Hepatoma cells were labeled with [35S]methionine for 16 h and either derivatized at 20°C (A) or at 0°C and then stripped with glutathione at 0°C (B), or they were left underivatized (C). Membrane proteins were isolated on streptavidin/agarose from bulk membrane preparations and separated in two dimensions. Proteins unique to 0°C derivatized cells are marked with small arrowheads, while those isolated from glutathione-treated as well as 0°C derivatized cells but absent or greatly reduced in concentration in preparations from underivatized cells are marked with large arrowheads. Spots enclosed by brackets were those of identical size but that exhibited graduated differences in charge. The latter were considered to be polypeptides of identical sequence that differ in charge due to posttranslational modifications. The measured pH gradient and size markers are shown, respectively, on the horizontal and vertical edges for A only.

Turnover of Hepatoma Cell Membrane Proteins in Different Cellular Compartments. Proteins isolated from cells that have been chased for 1 and 25 h prior to derivatizing at 20°C are shown in Fig. 3. Sixteen of the cell-surface-restricted proteins and all seven of those that were found to be bimodally compartmentalized were identified and excised from gel separations of proteins obtained from cells labeled and chased in the absence of label for 1 or 25 h before derivatizing at 20°C. The half-lives for these proteins were calculated (Table 1). Six of seven bimodally compartmentalized proteins exhibited half-lives of 22 h or less. Of these, the one protein that was not rapidly degraded exhibited many more cpm in the 25-h chased sample than in the 1-h chased sample. Although this protein is not present to a significant extent in the streptavidin/agarose-bound proteins from unbiotinylated cells, it may be a protein that nonspecifically binds to streptavidin/agarose in variable and unpredictable amounts. All but two of those proteins confined mostly to the cell surface showed half-lives of >79 h. Of those two that appear to turn over more rapidly, one (no. 9) appears to be degraded rapidly on the autoradiogram. We conclude from these data that there is a clear difference in the degradation rate between those hepatoma membrane proteins that are bimodally compartmentalized and those that are confined to the cell surface.

**Compartmentation and Turnover of Mouse 3T3 Cell Plasma Membrane Proteins.** Membranes from 3T3 cells were prepared from sonically disrupted cells by differential centrifugation. Eighteen proteins were identified as having a pool resistant to avidin treatment at 0°C after being derivatized at 20°C. Twenty-two additional proteins were derivatized at 0°C but were absent from avidin-blocked or glutathione-stripped cells. Nineteen cell-surface localized proteins and 13 proteins distributed between the cell surface and the 20°C accessible compartment were excised from gels of cells chased for 1 and 25 h and the half-lives were calculated (Table 2). Other proteins not listed in Table 2 did not incorporate sufficient radioactivity for quantitation (<50 cpm above background in 1-h chased samples). The half-lives of proteins confined to the cell surface ranged from 36 to >100 h with the exception of one protein (h11, 21 h). This particular spot, upon visual inspection, however, showed no significant decrease in spot intensity between 1 and 25 h of chase, suggesting that there was some overlap in the gel between a rapidly degraded
protein and the protein of interest or that recovery of radiolabel in the spot from 25-h chased cells was incomplete. Of the 13 proteins from 20°C derivatized, avidin-blocked cell-membrane proteins that contained enough radiolabel to calculate a half-life, 11 exhibited a half-life of ≤31 h. One slowly degraded protein (8A in Table 2), however, showed a significant decrease in radiolabel in the autoradiogram from 25-h chased proteins compared with 1-h chased proteins. There thus appears to be minimal, if any, overlap in the half-lives of those proteins confined to the surface of 3T3 cells and those found in the 20°C accessible compartment.

**DISCUSSION**

The results demonstrate a relationship between the compartmentation and stability of plasma membrane proteins. In both fibroblast and epithelial cells, proteins restricted to the cell surface are slowly degraded while proteins localized at steady state to a compartment resistant to avidin/glutathione treatment.

Table 2. Half-lives of 3T3 cell plasma membrane proteins confined only to the cell surface or bimodally distributed between the cell surface and endosomes (derivatized at 20°C prior to stripping away biotinyl groups with glutathione at 0°C)

<table>
<thead>
<tr>
<th>Cell-surface-restricted proteins</th>
<th>Bimodally distributed proteins</th>
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<tr>
<td>1-h chase, cpm</td>
<td>25-h chase, cpm</td>
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<tr>
<td>No.</td>
<td>cpm</td>
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<td>1</td>
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<td>273</td>
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The cpm for chased proteins was multiplied by 0.76 to adjust the recovered cpm to compensate for total cpm recovered in 25-h chase membrane lysate.

**Fig. 3.** IEF-SDS/PAGE separation of [35S]methionine-labeled, biotinylated hepatoma cell membrane proteins isolated from avidin-blocked cells chased for different intervals. Cells were labeled with [35S]methionine overnight and then chased for 1 or 25 h before derivatizing intact cells at 20°C. Large arrowheads point to those proteins identified in Fig. 2 as having large intracellular pools, while small arrowheads point to those confined largely to the cell surface.

ments at 0°C are degraded significantly more rapidly. Those biotinylated proteins isolated from avidin-blocked or glutathione-stripped cells likely reside in recycling endosomes. This conclusion is supported by the following data. Glutathione-resistant proteins from hepatoma cells cofractionate in Percoll gradients with plasma membrane markers. Glutathione has been demonstrated to strip the biotinyl group from all cell-surface-accessible proteins, even those that may be inaccessible to macromolecular probes (16). Thus, proteins that are resistant to glutathione stripping are in an intracellular compartment of the same density as plasma membrane and are accessible to biotinylation reagent at 20°C. The 20°C block prevents access to late compartments on the endocytic pathway but allows access to recycling endosomes (17–21). Lastly, the low density lipoprotein receptor displays an intracellular pool accessible to NHS-s-s-biotin at 20°C that is the same size as its recycling pool (8). The notion that...
proteins localized to endosomes at steady state are in the act of recycling is based on measurement of the distribution and internalization rates of receptors for extracellular ligands (e.g., transferrin, low density lipoprotein, asialoglycoprotein, mannose-6-phosphate) that undergo extensive recycling (22-25). By a number of criteria, a large pool for each of these model recycling proteins is localized to endosomal membranes in addition to the cell-surface pool. Moreover, there is a direct correlation between receptor distribution and rates of internalization for the mannose-6-phosphate receptor and its site-directed mutants that are less efficiently internalized than the normal receptor protein (25). Therefore, plasma membrane proteins exhibiting large endosomal pools would be expected to recycle frequently. Those proteins restricted to the cell surface, on the other hand, would be expected to recycle far less frequently than those having significant intracellular pools.

Given the assumption that proteins derivatized at 20°C but resistant to avidin blocking or glutathione stripping at 0°C are recycling more frequently than those proteins derivatized at 0°C, there is a clear difference in the turnover rates for recycling proteins compared to cell-surface-restricted proteins that recycle infrequently. Eleven of 20 surface-restricted proteins in 3T3 cells and 14 of 16 of those in hepatoma cells exhibited no detectable loss of label in a 25-h chase (Table 1). With only one or two exceptions, the remaining cell-surface-restricted proteins in both cell types were also degraded slowly. Thus, the observed metabolic stability calculated for these proteins is strikingly different for those bimodally distributed between cell-surface and intracellular pools. The latter group of 7 proteins in hepatoma cells and 13 proteins in 3T3 cells all exhibited half-lives of 31 h or less, except for 3 that may have longer half-lives or, more likely, were incorrectly grouped with regard to their compartmentation properties. In both cell lines, some proteins were noted that did not show a clear-cut distribution between those confined solely to the cell surface and those found partly in the intracellular compartment. Proteins chosen for analysis, however, were all that showed a clear cell surface only or bimodal distribution between the cell surface and the intracellular compartment and that were absent or greatly reduced in concentration from among those proteins from underivatized cells that bound to streptavadin/agarose.

The conclusion we draw from the data presented is that the stability of plasma membrane proteins is determined by their frequency of recycling. Proteins that recycle frequently are degraded more rapidly. The clear implication from this conclusion is that plasma membrane protein turnover is purely stochastic: the greater time spent inside the cell increases the probability of encountering events that lead to the degradation pathway. These events could include proteolysis by endosome-specific proteases, membrane fission leading to vesicle maturation, or nonselective membrane transfer into separately generated late endosomes/lysosomes. It would follow that identical proteins may have strikingly different turnover rates in different cells. These rates would reflect differences in coated pit density, lysosome concentration, or other cell-specific properties. This, for example, is seen in the case of the low density lipoprotein receptor, which is degraded with a t1/2 of 15-20 h in fibroblasts (8, 24, 26, 27), but with a t1/2 of 2-3 h in macrophages (28) or a mannose-6-phosphate receptor, which is degraded with a t1/2 of 16 h in Chinese hamster ovary cells but with a t1/2 of 39 h in human leukemia cells (29). The sorting mechanism to separate slowly from rapidly degraded proteins within a given cell type would use signals to recycle frequently or infrequently. Recycling signals could include phosphorylation of critical residues or specific sorting sequences (30, 31).

The proposed mechanism to explain different rates of degradation for plasma membrane proteins is consistent with previous measurements of cell-surface protein turnover. Degradation rates for receptors undergoing frequent recycling such as those for low density lipoprotein, asialoglycoprotein, mannose-6-phosphate, tumor necrosis factor, epidermal growth factor, interferon, Fc, interleukin 2, and insulin are relatively rapid compared to bulk plasma membrane turnover (summarized in ref. 2). A receptor that is more slowly degraded is the transferrin receptor (t1/2, 60 h). As its degradation rate was measured in suspension cells, the unique properties of these cells may affect recycling rate and therefore, indirectly, degradation rate. The bimodal distribution of half-lives found previously for surface-localized proteins in hepatoma cells (5) may also be explained by the presence of nonoverlapping groups of rapidly and slowly recycling proteins. It is also interesting to note that bulk hepatoma membranes turn over more slowly (t1/2, 131 h) than do bulk 3T3 cell membranes (t1/2, 59 h) as the latter exhibit a greater number of bimodally compartmentalized proteins.

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