Metabolic Behavior of Cell Surface Biotinylated Proteins†

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ABSTRACT: The turnover of proteins on the surface of cultured mammalian cells was measured by a new approach. Reactive free amino or sulfhydryl groups on surface-accessible proteins were derivatized with biotinyl reagents and the proteins solubilized from culture dishes with detergent. Solubilized, biotinylated proteins were then adsorbed onto streptavidin–agarose, released with sodium dodecyl sulfate and mercaptoethanol, and separated on polyacrylamide gels. Biotin–ε-aminocaproic acid *N*-hydroxysuccinimide ester (BNHS) or *N*-biotinoyl-*N'*-(maleimidohexanoyl)hydrazine (BM) were the derivatizing agents. Only 10–12 bands were adsorbed onto streptavidin–agarose from underivatized cells or from derivatized cells treated with free avidin at 4 °C. Two-dimensional isoelectric focusing–sodium dodecyl sulfate gel electrophoresis resolved >100 BNHS-derivatized proteins and >40 BM-derivatized proteins. There appeared to be little overlap between the two groups of derivatized proteins. Short-term pulse–chase studies showed an accumulation of label into both groups of biotinylated proteins up until 1–2 h of chase and a rapid decrease over the next 1–5 h. Delayed appearance of labeled protein at the cell surface was attributed to transit time from site of synthesis. The unexpected and unexplained rapid disappearance of pulse-labeled proteins from the cell surface was invariant for all two-dimensionally resolved proteins and was sensitive to temperature reduction to 18 °C. Long-term pulse–chase experiments beginning 4–8 h after the initiation of chase showed the disappearance of derivatized proteins to be a simple first-order process having a half-life of 115 h in the case of BNHS-derivatized proteins and 30 h in the case of BM-derivatized proteins. Pulse–chase analysis of biotinylated proteins by double labeling with [^{3}H]methionine and [^{35}S]methionine and their resolution on two-dimensional polyacrylamide gels allowed the rate of disappearance for a number of plasma membrane proteins to be examined. Four BNHS-reactive proteins examined by this approach between 8 and 48 h of chase demonstrated similar slow turnover, mirroring the long half-life determined for bulk BNHS-derivatized proteins. The turnover rates for eight BM-derivatized proteins were more heterogeneous than that for BNHS-derivatized proteins. Four of the latter exhibited $T_{1/2} \leq 22$ h.

A variety of approaches are available to study the degradation of plasma membrane proteins, but each has inherent shortcomings. Cells may be labeled with ^{125}I by lactoperoxidase with H_2O_2 generation systems (Morrison, 1975) or with other radiolabeled compounds that only react with surface-accessible proteins (Baumann & Doyle, 1978; Markwell & Fox, 1978). The subsequent turnover of these modified proteins can be followed by gel electrophoresis and autoradiography. The extent to which labeled proteins behave differently after their chemical modification with these reagents is not known. Cells labeled by these approaches exhibit a first-order but biphasic degradation of labeled proteins, the first phase being (in our hands) variable but always exhibiting a half-life of <24 h. This rapid phase of degradation may occur as a result of membrane or protein damage incurred upon oxidation. Another approach to study membrane turnover is to metabolically label cells and then isolate plasma membrane or immunoprecipitate a known plasma membrane protein from cell lysates. Studies in which the plasma membrane has been isolated (Horst & Roberts, 1979; Kaplan & Moskowitz, 1975) suffer from the uncertainty of not knowing whether the proteins isolated and visualized by gel electrophoresis and autoradiography are plasma membrane specific, peripheral, or integral, or are exposed to the endoplasmic or ectoplasmic surface. Antibody isolation of pulsed and chased proteins allows the metabolic behavior of only one or a few proteins to be assessed in a single study. An alternative approach to study membrane turnover was described by Kaplan et al (1979). These investigators metabolically labeled macrophages with [^{35}S]methionine, reacted exposed lysines on the labeled cells with membrane-impermeant trinitrobenzenesulfonate, and then precipitated derivatized proteins from lysates with anti-dinitrophenol antibodies. The success of this approach, however, depends on antibody specificity and access to derivatized proteins and could not be adapted (in our hands) to epithelial cells or fibroblasts.

In the approach described below, we reacted surface-accessible proteins from pulse-labeled cells with biotin derivatives and then precipitated reactive proteins from lysates with streptavidin–agarose. The advantages are several. (a) Cell membrane proteins are metabolically labeled, and thus labeling should not affect protein half-life. (b) Labeled cells can be chased for any length of time prior to derivatization. (c) A variety of biotinylated reactive compounds are available to derivatize glycoproteins (biotin hydrazide reaction after periodate oxidation, binding to biotinylated concanavalin A), proteins with free sulfhydryl groups (BM),[1] or proteins with reactive amino groups (BNHS). In this paper we show that BM and BNHS can be used as derivatizing reagents to study the degradation of hepatoma membrane proteins and describe

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[1] Abbreviations: BM, *N*-biotinoyl-*N'*-(maleimidohexanoyl)hydrazine; BNHS, biotin–ε-aminocaproic acid *N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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the heretofore unreported behavior of a large cohort of proteins at the cell surface.

Materials and Methods

Materials. BM and BNHS were purchased from Sigma Chemical Co. [^{35}S]Methionine (701 Ci/mmol) and [^{3}H]-methionine (80 Ci/mmol) were obtained from New England Nuclear.

Cells and Cell Labeling. H4-11-E-C3 hepatoma cells were grown on 10-cm dishes in modified Eagle's medium with Earle's salts supplemented with 10% fetal bovine serum (referred to as complete medium) at 37 °C under a 5% CO_2 atmosphere. Cells were labeled with 0.05–0.5 mCi of [^{35}S]-methionine (>700 Ci/mmol, New England Nuclear)/dish in 2 mL of modified Eagle's medium without methionine, supplemented with 10% dialyzed fetal bovine serum for 0.5–2.0 h, and then chased in complete media supplemented with 2 mM methionine.

Isolation of Biotinylated Protein. Labeled and chased cells were washed three times with PBS and reacted with 50 μg of BM (Sigma) or 100 μg of BNHS (Sigma)/mL of PBS for 30 min at 4 °C with gentle, circular agitation. The BNHS was first dissolved in dimethylformamide at 10 mg/mL. Derivatized cells were washed three times with PBS and lysed in 1% Triton X-100, 40 μg/mL phenylmethanesulfonyl fluoride, and 0.1 μg/mL each of the following protease inhibitors: leupeptin, antipain, and *p*-tosyl-L-lysine chloromethyl ketone. In some experiments (see Results) lysates were then made 0.1% in SDS. Lysates were centrifuged at 27000*g* for 30 min, and to the supernatant was added 50 μL of lysis buffer washed streptavidin–agarose (Bethesda Research Laboratories). Lysates were rotated end over end at 4 °C overnight, and the streptavidin–agarose centrifuged down on a microfuge and washed three times with lysis buffer, once with lysis buffer made 1 M in NaCl, once with 10% lysis buffer in distilled water, and once with lysis buffer made 0.1% in SDS. Adsorbed proteins were solubilized in 0.1 mL of SDS sample buffer (Maizel, 1969) by heating at 95 °C for 3 min.

Gel Electrophoresis. Proteins in SDS sample buffer were run on one-dimensional 8 or 10% acrylamide and 0.21 or 0.27% bis(acrylamide) (Maizel, 1969) or two-dimensional isoelectric focusing–SDS electrophoresis gels (O'Farrel, 1975; Baumann & Doyle, 1979), fixed, treated with EnHance (New England Nuclear), dried, and exposed for usually 1 week at −80 °C to Kodak XR-5 film.

Estimation of Protein Half-Lives. Two 10-cm dishes were pulse-labeled for 2 h with 1.0 mCi of [^{3}H]methionine (80 Ci/mmol) in 2.0 mL of methionine-free media containing 5% dialyzed fetal bovine serum. Two other 10-cm dishes were pulse-labeled for 2 h with 0.5 mCi of [^{35}H]methionine (701 Ci/mmol) in 2.0 mL of methionine-free media containing 5% dialyzed fetal bovine serum. For BNHS-derivatized cells, the following sequence was followed. Both [^{3}H]methionine-labeled dishes were chased for 8 h in complete media supplemented with 2 mM cold methionine while one [^{35}S]methionine-labeled dish was chased for 8 h and the other for 48 h under identical conditions. After the chase all four dishes were treated with BNHS for 30 min and extracted as described above. Lysates were kept frozen until the end of the experiment. At that time all four lysates were thawed and the two ^{3}H lysates (38×10^6 cpm each) were mixed with the two ^{35}S lysates (42.4×10^6 cpm for the 8-h chase; 21.7×10^6 cpm for the 48-h chase). The two mixed lysates (8 h, 48 h) were centrifuged at 27000*g* for 30 min, treated with streptavidin–agarose as above, centrifuged, and washed as described above, and adsorbed protein was run on two-dimensional isoelectric focusing–SDS electrophoresis polyacrylamide gels. Spots as identified in Figure 4 were removed from the dried gel with a paper punch and solubilized in 0.75 mL of 30% H_2O_2 for 3 h at 70 °C, mixed with scintillation fluid, and counted for the double label (^{35}S channel efficiency was 40% with 0.01% overlap from the ^{3}H channel; ^{3}H channel efficiency was 35% with 7.0% overlap from the ^{35}S channel). The ^{35}S/^{3}H for each spot was determined. The ^{35}S/^{3}H for total 48 h chased and precipitated proteins was experimentally determined to be 1.07. The ^{35}S/^{3}H for 8 h chased proteins (U) was calculated to be 1.34 from the equation:

$$\ln C = \ln U - K_d T \quad (1)$$

where C is the determined ^{35}S/^{3}H for 48 h chased, streptavidin–agarose-precipitated proteins, K_d is the graphically determined decay constant over the 8–48-h chase period [$T_{1/2} = (\ln 2)/K_d$], and T is the time of the chase (40 h). U should be nearly identical for all polypeptides. To determine the $T_{1/2}$ for individual protein spots, U and C were determined by counting ^{35}S and ^{3}H cpm for excised spots from 8 and 48 h chased gels, respectively, and the K_d was calculated from (1). C for all spots that showed ^{3}H cpm >100 were analyzed. To assess the turnover of BM-derivatized proteins, the same protocol was used except the two chase periods were reduced to 4 and 34 h. In this case, the ^{35}S/^{3}H for streptavidin-precipitated protein at 4 h was 0.70 and at 34 h was 0.38.

Fluorescence Microscopy. Cells were grown on plastic cover slips and biotinylated as described above. After three washes with ice-cold PBS, cells were treated with 20 μg of fluorescein-derivatized avidin (Vector Laboratories) in 0.15 M NaCl and 0.01 M Hepes (pH 8.2) for 30 min at 4 °C. Cells were then washed in PBS, fixed in 4% formaldehyde in PBS for 20 min, mounted, and photographed with a Leitz Diavert microscope equipped with epifluorescence optics.

Results

We used the derivatization reagents BM, which reacts with free sulfhydryl groups, and BNHS, which reacts with free amino groups. These were chosen because they both have spacer between biotin and reactive moieties and in preliminary screenings showed good reactivity with hepatoma cells. In order to maximize the number and specific activity of radiolabeled proteins precipitated from biotinylated cells while minimizing that from unreacted cells, the following parameters were tested: cell derivatization temperature (4 and 25 °C), biotinyl reagent concentration (10–200 μg/mL), lysis buffer composition (0.5–2.0% Triton X-100, 1.0% Triton plus 0.25% deoxycholate, 2.0% Triton X-100 plus 0.1–0.5% SDS), streptavidin–agarose wash protocol prior to elution of adsorbed proteins, and incubation time of lysate with streptavidin–agarose (2–16 h). The optimum conditions are described under Materials and Methods. In some later experiments 0.1% SDS was added to the lysate as it was found to reduce the levels but not the number of proteins adsorbed onto streptavidin from underivatized cells without affecting proteins adsorbed from derivatized cells.

On one-dimensional SDS/8% polyacylamide gels, >20 bands of M_r >25 000 were detected from cells pulsed for 1 h with [^{35}S]methionine, chased for 2 h, derivatized with BNHS, lysed, and adsorbed onto streptavidin–agarose (Figure 1). In underivatized control cells 9–10 of the above proteins are also precipitated although with three exceptions these proteins are found in much lower concentrations than in derivatized cells. Adsorption of underivatized cell lysate proteins to streptavidin–agarose was not decreased by agarose or bovine serum

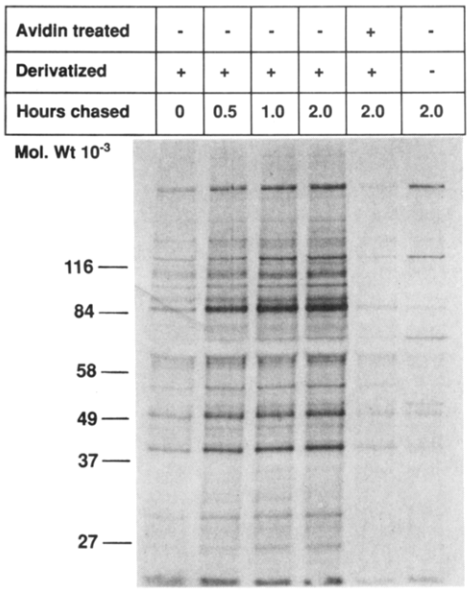

FIGURE 1: SDS/polyacrylamide gel electrophoresis of [^{35}S]-methionine-labeled cell lysates from cells derivatized with BHNS and precipitated with streptavidin–agarose. Cells were pulse-labeled for 30 min with 100 μCi of [^{35}S]methionine/dish and chased in complete media supplemented with 2 mM cold methionine for 0, 0.50, 1.0, or 2.0 h. Cells were reacted with PBS alone or 100 μg/mL BNHS in PBS and, in the case of one dish, reacted with 1 mg of avidin/mL of PBS before washing (3×). All dishes were lysed, treated with streptavidin–agarose as described under Materials and Methods, and electrophoresed on SDS/10% polyacrylamide gels.

albumin–agarose pretreatment. Two possible reasons for increased levels of nonspecifically adsorbed proteins in biotinylated cells are the following. (1) Proteins adsorbed from underivatized cell lysates comigrate with derivatized proteins. (2) Adsorbed proteins from underivatized cells are also surface-exposed, plasma membrane proteins and are more completely precipitated after derivatization. Since further resolution of streptavidin-adsorbed proteins seen on two-dimensional polyacrylamide gels (see below) increased the total number of proteins from biotinylated cells but not that from underivatized cells, the former explanation appears to be the more likely. Incubation of BNHS-reacted cells with 1 mg/mL avidin for 30 min followed by washing reduces precipitation of labeled protein by streptavidin–agarose close to that seen with underivatized cells. A 30-min pulse followed by short (0–2-h) chases results in increasing levels of precipitated protein which we attribute to transit time for newly translated proteins to reach the cell surface. Peak levels of specifically precipitated radiolabeled proteins appear 2 h after the 30-min pulse. Immediately thereafter the levels of all derivatized proteins examined decrease uniformly over the next several hours (see below). Similar results were seen upon reaction of cells pulsed for 30 min with [^{35}H]methionine and reacted with BM, a reagent that reacts with free sulfhydryl groups (results not shown).

To quantitate the turnover kinetics of bulk BM- and BNHS-reactive proteins for subsequent analysis of individual proteins by double labeling and two-dimensional polyacrylamide gel resolution, we pulse-labeled cells for 2 h with [^{35}S]methionine and chased cultures for 3–70 h. Immediately thereafter, cultures were biotinylated and lysed, and then biotinylated proteins were precipitated with streptavidin–agarose. At each time point, control cultures (unreacted with biotinyl reagents) were also precipitated. Adsorbed proteins were removed from the agarose beads in SDS sample buffer. Radiolabel in each lysate and precipitate were counted and

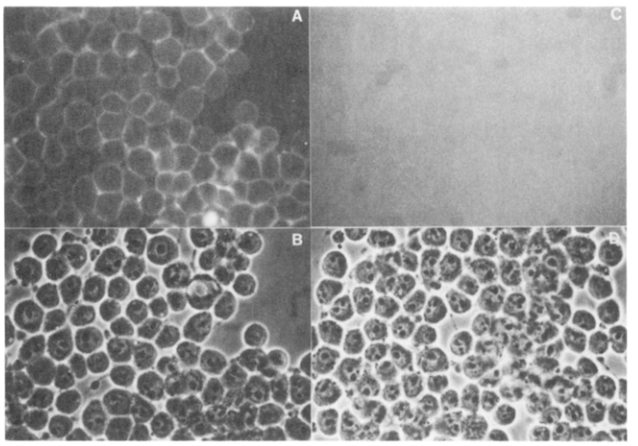

FIGURE 2: Fluorescence microscopy of BNHS-derivatized cells reacted with fluorescein–avidin. Cells were derivatized (A and B) or left underivatized (C and D) and reacted with fluorescein-labeled avidin as described under Materials and Methods. (B) and (D) are phase micrographs; (A) and (C) are fluorescence micrographs.

the cpm specifically bound to streptavidin–agarose determined from the equation:

$$[(\text{cpm in control lysate})/(\text{cpm in biotinylated lysate})] \times (\text{cpm precipitated from biotinylated lysate}) - (\text{cpm precipitated from control lysate}) = \text{cpm specifically bound}$$

This is to normalize for small differences in the total cpm between lysates from control and biotinylated cells at each time point and to subtract out cpm from proteins that are precipitated from control cells and are therefore not necessarily present at the cell surface. BM-reacted proteins showed a monophasic first-order decay curve ($T_{1/2}$ = 30 h) between 8 and 65 h of chase. BNHS-reactive proteins decayed more slowly ($T_{1/2}$ = 115 h) from 8 to 65 h of chase. In both cases specifically precipitated radiolabeled protein peaked after 2 h of chase and then rapidly decreased ($T_{1/2}$ = 2–3 h) from 2 to 8 h. Over this same time period from 0 to 65 h loss of total labeled protein in cell lysates exhibited a monophasic first-order decay ($T_{1/2}$ = 42 h).

Binding of fluorescein–avidin to BNHS and underivatized cells is shown in Figure 2. Fluorescence microscopy of derivatized cells showed evenly distributed fluorescence on the surface of the cells with no internal reaction. Underivatized cells were unreactive. Derivatized cells that were returned to media at 37 °C for 30 min and then reacted with fluorescein–avidin also showed no reaction (results not shown). The latter results suggest that biotinylation causes a rapid internalization of derivatized protein.

The poor resolution of proteins specifically adsorbed from biotinylated cells on one-dimensional gels and the presence of bands found in underivatized cells which appear to intensify in biotinylated cells suggested that one-dimensional resolution was not sufficient to identify individual proteins. Two-dimensional isoelectric focusing–SDS/polyacrylamide gels resolved adsorbed proteins from underivatized cells into 8–10 spots (Figure 3A). A total of >100 spots were detected from cells similarly pulsed with [^{35}S]methionine for 2 h and chased for 2 h but derivatized with BNHS before adsorption onto streptavidin–agarose (Figure 3B). These results demonstrate that there are a large number of cell surface proteins that expose derivatizable amino groups while the number of nonspecifically adsorbed proteins is limited to about the same number seen on one-dimensional gels. BNHS-derivatized proteins appeared heterogeneous with respect to both size and isoelectric point. Imposition of an 8-h chase between the 2-h

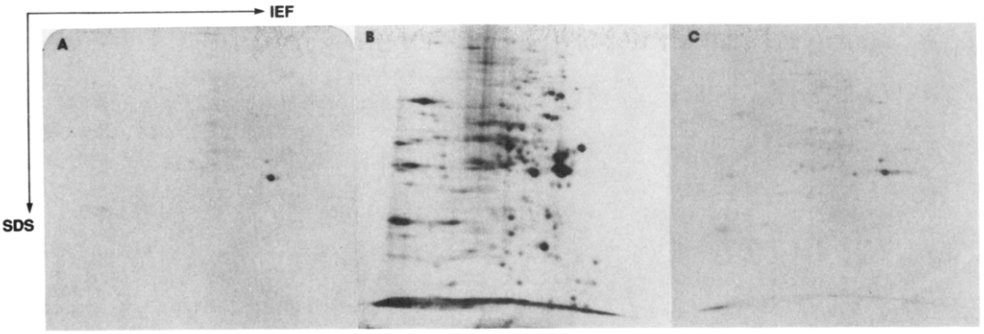

FIGURE 3: Two-dimensional isoelectric focusing–SDS/polyacrylamide gel electrophoresis of BNHS-reactive proteins chased for 2 or 8 h. [^{35}S]Methionine-pulsed cells from underivatized (A) or BNHS-derivatized cultures (B and C) were chased for 2 h (A and B) or 8 h (C) and lysed; streptavidin–agarose-adsorbed proteins were run on two-dimensional polyacrylamide gels as described under Materials and Methods. Gels were processed for fluorography and exposed for 15 days.

Table I: Cell Surface Turnover of BNHS-Derivatized, Streptavidin-Adsorbed Proteins

protein from 2D gel	adsorbed from nonbiotinylated cells	$^{35}S/^{3}H$ from 8-h chase	$^{35}S/^{3}H$ from 48-h chase	$^{35}S/^{3}H$ from 48-h chase	$T_{1/2}$ (h)
1	+	1.44		1.24	>125
2	+	1.33		0.78	48
3	+	1.23		0.36	20
4	−	1.56	1.14		>125
5	−	1.44	1.13		>125
6	−	1.30	1.00		>125
7	−	1.14	1.11		>125
$\chi \pm$ SD		1.35 ± 0.14	1.12 ± 0.02		

pulse and derivatization resulted in greatly decreased intensity for all spots (Figure 3C). This decrease was blocked by temperature reduction to 18 °C but not by lysosomotropic amines such as 20 mM methylamine (results not shown). Both of these conditions inhibit the lysosomal degradation of membrane proteins (Hare & Huston, 1986; Hare, 1988). The total radiolabel in 2- and 8-h chase lysates from derivatized cells and that in the 2-h chase lysate from underivatized cells were nearly identical ($\sim 100 \times 10^6$ cpm). Thus, derivatized proteins uniformly disappear or become unreactive after a relatively short chase.

The double-labeling protocol described under Materials and Methods was carried out on BNHS- and BM-derivatized proteins to see if half-life values could be assigned to individual protein spots resolved on two-dimensional polyacrylamide gels. Because superficial inspection of two-dimensionally resolved BNHS-derivatized proteins at 2 and 8 h of chase showed the loss of label to be relatively uniform, we decided to examine the degradation of BNHS-derivatized proteins from 8 to 48 h of chase. Loss of ^{35}S label from cell lysates between 8 and 48 h was consistent with the turnover of bulk cell lysate proteins showing a $T_{1/2} = 42$ h. Total ^{35}S cpm in the lysates decayed from 42 384 at 8 h to 21 665 at 48 h of chase. The $^{35}S/^{3}H$ for bulk streptavidin–agarose-eluted proteins from the 48-h chase was 1.07. Starting $^{35}S/^{3}H$ was then calculated, as described under Materials and Methods, to be 1.34. As the ^{35}S label was >25 times more efficient at blackening the film, ^{3}H label was invisible. Rapidly degraded proteins should thus be absent or greatly reduced in the 48-h chase gel relative to the 8-h chase gel. No protein exhibiting rapid degradation could be identified since all spots seen in the 8-h chase sample were also seen in the 48-h chase sample (Figure 4). Twenty-five spots from the 48 h chased gel and the identical spots from the 8 h chased gel were removed, and the $^{35}S/^{3}H$ ratio for each was determined. Only seven proteins show ^{3}H cpm >100 above background, and these are listed in Table I along with their $^{35}S/^{3}H$ ratios and $T_{1/2}$ calculated as described under Materials and Methods. The $^{35}S/^{3}H$ for isolated proteins from

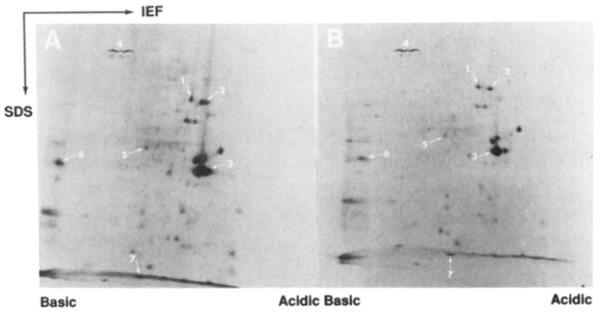

FIGURE 4: Two-dimensional isoelectric focusing–SDS/polyacrylamide gel electrophoresis of BNHS-reactive proteins chased for 8 or 48 h. Cells were labeled with [^{3}H]methionine or [^{35}S]methionine as described under Materials and Methods for estimation of protein half-lives. Mixed [^{3}H]methionine- and [^{35}S]methionine-pulsed cells chased for 8 h (A) or 48 h (B) were run on two-dimensional gels as described under Materials and Methods. Numbers designate those proteins whose half-lives were determined as described under Materials and Methods and are shown in Table I.

8 h chased cells should be nearly identical: the mean was calculated to be 1.35 ± 0.14, in close agreement with 1.34 calculated above. Three of the seven proteins were those also recovered from underivatized cells and these exhibited strikingly different $^{35}S/^{3}H$ ratios after 48 h of chase (Table I). Protein 3 was turned over particularly rapidly ($T_{1/2} = 20$ h). The four proteins examined which were found only in biotinylated cells exhibited nearly identical $^{35}S/^{3}H$ ratios (mean = 1.12 ± 0.18) corresponding to a $T_{1/2} > 100$ h, consistent with that seen for bulk BNHS proteins (115 h). Thus these results show that four BNHS-derivatized proteins exhibit rather slow rates of removal from the cell surface between 8 and 48 h.

Two-dimensional resolution of 2 h [^{35}S]methionine pulsed, BM-derivatized proteins after a 34-h chase is shown in Figure 5B. Greater than 40 spots were resolved after 4- (not shown) and 34-h chases. This group of biotinylated proteins comprises predominantly high molecular weight and basic proteins. Derivatized cells treated with avidin after a 4-h chase and prior

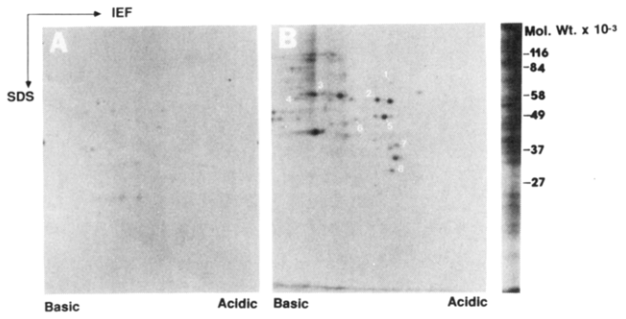

FIGURE 5: Two-dimensional isoelectric focusing–SDS/polyacrylamide gel electrophoresis of BM-reactive proteins chased for 34 h. Cells were labeled as described under Materials and Methods except that lysates were made 0.1% SDS before addition of streptavidin. (A) Four hour chase treated with avidin prior to lysis; (B) 34-h chase. To the right of (B) is a one-dimensional separation of 34 h chased proteins run in the same gel but only in the second dimension. Prestained molecular weight markers (Sigma) were also included in the second dimension. Numbers refer to excised proteins whose half-lives were determined and are shown in Table II.

Table II: Cell Surface Turnover of BM-Derivatized, Streptavidin-Adsorbed Proteins

protein from 2D gel	$^{35}S/^{3}H$ from 4-h chase	$^{35}S/^{3}H$ from 34-h chase	$T_{1/2}$ (h)
1	*a*	0.07	5
2	1.18	0.13	9
3	0.92	0.42	22
4	0.96	0.59	36
5	1.23	0.27	14
6	*a*	0.91	76
7	1.55	1.42	>100
8	1.38	1.55	>100
$\chi \pm$ SD	1.20 ● 0.24	0.67 ± 0.57	

[a] Too few CPM werre removed from these proteins in the 4-h chase gel. Therefore, the mean $^{35}S/^{3}H$, 1.20, was used to calculate $T_{1/2}$.

to cell lysis showed 7–8 spots (Figure 5A). We examined the turnover of eight spots from which >100 cpm of ^{3}H were recovered on both 4- and 34-h chase gels. The $^{35}S/^{3}H$ as well as the $T_{1/2}$ for each of these is shown in Table II. Half-lives range from 5 to >100 h with several proteins turning over rapidly and several turning over so slowly that no significant decay could be measured after 34 h. $^{35}S/^{3}H$ ratios for 4 h chased proteins were similar to one another (1.20 ± 0.24). If these eight proteins are representative of the remaining 32 proteins from which not enough cpm were recovered to analyze, it would appear that BM-derivatized proteins turn over more rapidly and heterogeneously at the cell surface than do BNHS-derivatized proteins.

Discussion

Adsorption of biotinylated proteins from the surface of derivatized cells onto streptavidin–agarose provides an opportunity to examine the metabolic behavior of several classes of reactive proteins with respect to their access to the cell surface. In this study we have examined two such classes: those which have reactive sulfhydryl and those with reactive amino groups. Each class comprises >40 different proteins. Two-dimensional protein maps of these two classes were clearly distinct from one another. Only quantitative and not qualitative differences in the proteins adsorbed by streptavidin–agarose were affected by reducing the concentration of derivatized agents. Thus proteins which react with BNHS or BM form two mostly nonoverlapping groups. We do not know if all proteins from biotinylated cells which adsorb onto streptavidin–agarose have convalently bound biotin. It seems likely that many of the absorbed proteins separated on two-dimensional polyacrylamide gels are noncovalently associated with biotinylated proteins in the presence of lysis buffer but do not themselves contain biotin. The limitations of the approach appear to be only two. (1) Loss of label from isolated proteins may be due to degradation or simply transfer of the proteins to an internal, surface-inaccessible compartment. We cannot distinguish between these two possible fates. Preparation of antibody to the adsorbed proteins may, however, provide an additional probe by which these possibilities could be differentiated. (2) Eight to ten proteins are precipitated from underivatized cells. These proteins must be excluded from turnover analysis of isolated proteins as they most probably reside in compartments other than the plasma membrane. Two of these appear to turn over more rapidly than BNHS-derivatized membrane proteins, suggesting they behave metabolically different than surface accessible proteins. A similar number of proteins react with horseradish peroxidase conjugated to avidin in cell lysates separated on one-dimensional polyacrylamide gels and blotted onto nitrocellulose. It seems likely that these are endogenous biotin-conjugated proteins. Despite these shortcomings, the biotinylation of pulse-labeled proteins appears to be a good alternative to direct substitution of plasma membrane proteins with radioisotopic compounds to detect their access to the cell surface. Since derivatization was done at 4 °C, it seems unlikely that the bulky biotinyl reagents would pass through the plasma membrane. An alternative entry to intracellular proteins could involve noncovalent binding of the reagents to the cell surface, perhaps through biotin binding proteins, and then covalent attachment to intracellular proteins after cell lysis. Both of these possibilities seem to be excluded by seeing greatly reduced adsorption of proteins onto streptavidin–agarose after treatment of intact, derivatized cells with free avidin and no intracellular reaction of either derivatized or underivatized cells by fluorescence microscopy of cells treated with fluorescein–avidin.

Both BNHS- and BM-derivatized proteins reached the cell surface in 1–2 h after termination of the label. This is in reasonable agreement with the transit time for secretory or membrane proteins in several cell types (Fitting & Kabat, 1982; Strous & Lodish, 1980; Lodish et al., 1983). After reaching the cell surface, pulse-labeled proteins uniformly and quickly disappear over the next several hours. Removal of BNHS proteins from the cell surface between 2 and 8 h of chase was remarkably uniform as two-dimensional gels identified no proteins that were stably retained on the cell surface. Proteins present in the cell lysate showed little detectable decay over this same period of time as detected by total trichloroacetic acid precipitable radiolabel and by one-dimensional gel analysis of these proteins (results not shown). Three explanations for this rapid loss of reactivity appear most plausible. (1) Selected proteins may be rapidly degraded. Other studies (Baumann & Doyle, 1979; Kaplan et al., 1979; Hare & Huston, 1984) have documented a rapid phase of surface-labeled protein degradation of variable duration depending on the method of labeling, cell type, etc. Since the rapid loss of reactive proteins seen here was insensitive to lysosomotropic amines which prevent transfer of plasma membrane proteins to lysosomes for their subsequent degradation, this possibility seems unlikely. (2) A portion of externalized proteins may be internalized into the cell interior. A number of receptors have been shown to have large intracellular pools hidden from the cell surface (Lamb et al., 1983; Kaplan & Keough, 1983; Tietze et al., 1982; Weigel & Oka, 1984; Deutsch et al., 1982). Thus, pulse-labeled proteins may rapidly equilibrate between

internal and surface pools once they reach the cell surface. (3) Plasma membrane proteins may undergo a structural alteration once they reach the cell surface such that potentially reactive groups become refractory to biotinylation. To distinguish between these possibilities, it would be necessary to generate antibodies against derivatized proteins so that their intracellular metabolic fate could also be followed.

Our unproved assumption is that long-term removal of reactive proteins from the cell surface is due to their degradation. If so, degradation of bulk BNHS-derivatized proteins is much slower than that of BM-derivatized proteins. It is not clear why this is the case, but this may suggest that proteins which have exposed sulfhydryl groups are more unstable than those without. Perhaps these proteins are more readily cross-linked than average plasma membrane proteins. We were not able to detect any BNHS-reactive proteins that turned over rapidly between 8 and 48 h of chase either by removing the label from spots on two-dimensional gels or by close examination of the gel for ^{35}S-labeled spots that showed noticeably decreased radiolabel after 48 h of chase relative to 8 h of chase.

Previous studies have followed the degradation of plasma membrane proteins by direct isotopic labeling of surface-exposed proteins (Baumann & Doyle, 1978; Hare & Huston, 1984; Chu & Doyle, 1985; Hare, 1988) or by isolating pulse-labeled and chased membrane proteins by fractionation, derivatization (Horst & Roberts, 1979; Kaplan & Moskowitz, 1975; Kaplan et al., 1979), or immunoprecipitation (Mellman et al., 1983; Stoschek & Carpenter, 1984; Gardner & Fambrough, 1979; Sahagian & Neufeld, 1983; Krupp & Lane, 1982). In general, these studies agree that in secondary cultures many receptors and other membrane proteins exhibit half-lives of 25 h or less but the majority of plasma membrane proteins are degraded rather slowly ($T_{1/2} \geq 90$ h) and uniformly. Primary cultured hepatocytes seem to exhibit greater heterogeneity in the degradation of the exposed proteins (Chu & Doyle, 1985) perhaps because they are more differentiated and express greater numbers of receptors which are degraded more rapidly than nonreceptor proteins. Our studies of biotinylated protein turnover have generated results consistent with other studies. We find that, after the rapid loss of surface label over the first few hours of chase, BNHS-labeled proteins disappear slowly in a synchronous manner. BM-reactive proteins, however, disappear more rapidly. One explanation for the difference between these BM- and BNHS-derivatized proteins is that the latter group may include many receptor proteins as these are known to be rich in cysteine residues [e.g., insulin (Ullrich et al., 1985), low-density lipoprotein (Yamamoto, 1984), epidermal growth factor (Ullrich et al., 1984), and the insulin-like growth factor I (Ullrich et al., 1986) receptors] and exhibit half-lives of 25 h or less (Stoschek & Carpenter, 1984; Krupp & Lane, 1982).

In summary, we were able to demonstrate that derivatization with biotinyl reagents is an effective means of detecting cell surface access and isolation of a large number of plasma membrane specific proteins. The behavior of these proteins with respect to their presence at the cell surface was investigated. We find that both BNHS- and BM-reactive proteins reach the cell surface about 1–2 h after labeling and then uniformly disappear. We do not know if their disappearance results from internalization coupled to degradation or transfer to some internal pool. Those BNHS-derivatized proteins that remain on the cell surface then proceed to disappear more slowly than BM-reactive proteins. This slower disappearance is likely due to degradation as the half-life of bulk BNHS-reactive protein is similar to values reported for ^{125}I-labeled (Chu & Doyle, 1985) and metabolically labeled proteins (Kaplan et al., 1979). More importantly, biotinylation coupled with double labeling and two-dimensional resolution of derivatized proteins allows the metabolic behavior of a wide assortment of plasma membrane proteins to be examined.

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The Acid-Triggered Entry Pathway of *Pseudomonas* Exotoxin A†

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ABSTRACT: In this study we examined the pH requirements and reversibility of early events in the *Pseudomonas* toxin entry pathway, namely, membrane binding, insertion, and translocation. At pH 7.4, toxin binding to vesicles and insertion into the bilayer are very inefficient. Decreasing the pH greatly increases the efficiencies of these processes. Acid-treated toxin exhibits pH 7.4 binding and insertion levels. This indicates that hydrophobic regions that become exposed upon toxin acidification become buried again when the pH is raised to 7.4. In contrast, the change in toxin conformation that occurs upon membrane binding is irreversible. Returning samples to pH 7.4, incubation with excess toxin, or dilution with buffer up to 1000-fold leads to very little loss of bound toxin. Bound toxin exhibits an extremely high susceptibility to trypsin compared to free toxin (at both pH 4 and pH 7.4). At pH 4, membrane-associated toxin slowly proceeds to a trypsin-protected state; neutralization halts this process. At low pH, toxin was found to bind and insert into DMPC vesicles very efficiently at temperatures both above and below 23 °C, the lipid melting point. With fluid targets, the proportion of bound toxin that was photolabeled from within the bilayer peaked rapidly and then decreased with time. With frozen targets, the efficiency of photolabeling peaked but then remained fairly constant. The results suggest that after insertion PTx can cross a fluid bilayer much more efficiently than it can a frozen one. We conclude that the reversible pH-triggered changes in toxin conformation [Farahbakhsh, Z. T., Baldwin, R. L., & Wisnieski, B. J. (1987) *J. Biol. Chem. 262*, 2256-2261] have a functional role in promoting membrane binding, insertion, and translocation. The kinetics of translocation is governed by the pH and the physical state of the target membrane.

PTx[1] is one of the extracellular products secreted by toxicogenic strains of *Pseudomonas aeruginosa* (Callahan, 1974; Liu, 1974). Its activity as an inhibitor of protein synthesis makes it a critical virulence factor during infection (Cryz, 1985). PTx is secreted as a single polypeptide chain with a molecular weight of 66 583 (Gray et al., 1984). Like diphtheria toxin (DTx), it catalyzes the ADP-ribosylation of elongation factor 2 (Iglewski & Kabat, 1975; Lory & Collier, 1980). Although the entry mechanisms of these two toxins exhibit some similarities, they do not appear to be identical (Gray et al., 1984; Middlebrook & Dorland, 1984; Zalman & Wisnieski, 1985; Sundan et al., 1984; Olsnes & Sandvig, 1988). The three-dimensional structure of PTx has recently been determined by X-ray crystallography (Allured et al., 1986) and shown to contain three structural domains. On the basis of this information, plasmids that code for specific portions of the toxin molecule have been used to generate peptides. Preliminary studies with these peptides indicate that the three domains of the toxin are required for cell recognition, translocation, and ADP-ribosylation, respectively (Hwang et al., 1987).

Despite the structural and functional characterization of the toxin, the mechanism by which the ADPr-transferase domain gains access to the target cell cytoplasm is still under intense investigation. The process seems to involve receptor-mediated endocytosis (Moehring & Moehring, 1983; Morris & Saelinger, 1986; Robbins et al., 1984). The importance of exposure to a low-pH environment derives from observations that compounds that increase the pH of normally acidic organelles protect the cells against PTx (FitzGerald et al., 1980; Sundan et al., 1984) and that this block can be overcome by lowering the external pH (Moehring & Moehring, 1983; Morris & Saelinger, 1986). We have noted that incubation of toxin below pH 6 leads to exposure of hydrophobic regions (Farahbakhsh et al., 1987). Most of the acid-induced conformational changes observed were reversible ($t_{1/2}$ <30 s), and they occurred within a narrow pH range (Farahbakhsh et al., 1987).

The goal of this investigation was to explore the functional role of such changes and any further changes that might occur upon membrane binding. Although we have shown that toxin inserts into the membrane bilayer upon acidification (Zalman & Wisnieski, 1985; Farahbakhsh et al., 1986), factors that govern its translocation have not yet been established. Photolabeling experiments were conducted to establish the effects

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[1] Abbreviations: PTx, *Pseudomonas* exotoxin A; DTx, diphtheria toxin; PC, egg phosphatidylcholine; CS, cholesterol; 12APS-GlcN, *N*-[12-(4-azido-2-nitrophenoxy)stearoyl][1-^{14}C]glucosamine; TSE, 20 mM Tris-HCl, 150 mM NaCl, and 1 mM ethylenediaminetetraacetic acid, pH 7.4; DMPC, dimyristoylphosphatidylcholine; ANS, 1-anilino-8-naphthalenesulfonic acid; T_m, membrane melting point; CHO, Chinese hamster ovary cells; SDS, sodium dodecyl sulfate.

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