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EXAMINATION OF INTRAMOLECULAR HETEROGENEITY OF PLASMA MEMBRANE PROTEIN DEGRADATION IN CANINE RENAL TUBULAR EPITHELIAL CELLS AND IN RAT LIVER

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Summary

We have examined the hypothesis that hydrophilic portions of membrane-bound proteins which lie on either side of the phospholipid bilayer may be degraded at a different rate than are the hydrophobic portions of membrane proteins which are within the bilayer. Plasma membrane fractions from cells of the Maden-Darby canine kidney cell line and rat liver were digested with papain and pronase to cleave a mixture of peptides which is enriched in hydrophilic amino acids. It is proposed that these peptides are derived from regions of membrane-bound proteins which lie outside the bilayer. The residual particulate protein is enriched in hydrophobic amino acids and presumably contains the portion of membrane-bound proteins which are in direct contact with the bilayer. A double-isotope method was used to assess the relative degradation rates of these two protein fractions. There was no measurable difference in protein degradation rates between the two fractions and the initial plasma membranes. These results suggest that the intramolecular heterogeneity which results from insertion of membrane-bound proteins into a bilayer is not a factor in their degradation.

Introduction

The concentration of any protein in a mammalian cell represents a balance between its rate of synthesis and degradation [1,2]. Soluble and endoplasmic reticulum proteins have heterogenous rates of degradation [3,4]. Whether

Abbreviation: MDCK, Maden-Darby canine kidney.

plasma membrane proteins are degraded individually or internalized by endocytosis has not been established. Evidence consistent with both possibilities has been presented. Several experiments suggest that the first-order rates of degradation of plasma membrane proteins differ by factors of 2—4 [5—7]. These results resemble those obtained with soluble proteins; however, Tweto and Doyle [2] did not observe heterogenous rates of degradation of plasma membrane proteins of HTC cells and concluded that these proteins are removed from the membrane in large structural units, presumably endocytotic vesicles.

Plasma membranes consist of a 40 Å wide phospholipid bilayer into which membrane-bound proteins are inserted [8]. Certain membrane proteins, such as the erythrocyte anion carrier [9], $(Na^+ + K^+)$-ATPase [10] and leucine aminopeptidase [11] span the membrane. It has been proposed that the surfaces of membrane-bound proteins which lie external to the two planes which define the bilayer are enriched in hydrophilic amino acids, and that surfaces within the bilayer are enriched in hydrophobic amino acids [12]. The possibility exists that cytoplasmically oriented hydrophilic portions of membrane proteins are degraded at a different rate than are the hydrophobic fragments within the membrane bilayer.

It is not presently possible to purify the hydrophobic and hydrophilic portions of specific membrane-bound proteins to study their separate rates of degradation. All membrane proteins may not be degraded by an identical mechanism and results obtained from study of a single membrane protein may neither detect nor measure the scope of intramolecular heterogenous degradation. For these reasons, we used a double-isotope amino acid method to pulse-label many, if not all, plasma membrane proteins in MDCK cells and in rat liver [4]. Leucine isotope ratios were determined in plasma membrane protein preparations before and after partial proteolytic digestion which permitted separation of membrane-bound protein into a particulate fraction containing peptides enriched in hydrophobic amino acids, and a soluble fraction containing peptides enriched in hydrophilic amino acids. If there were intramolecular heterogeneity in membrane protein degradation of the type postulated, different isotope ratios should have been observed in these membrane subfractions. No difference in isotope ratios was detected between these two fractions.

Materials and Methods

Chemicals and enzymes. Pepsin, trypsin, chymotrypsin and pronase were obtained from Sigma. Papain was a gift from Dr. Daniel Louvard, Department of Biology, UCSD.

Isotopes. L-[^{14}C]Leucine, uniformly labeled, specific activity 312 mCi/nmol and L-4,5-[^{3}H]leucine, specific activity 650 mCi/mol were obtained from Schwarz-Mann. Isotope solutions were neutralized and added to cell cultures or injected intraperitoneally into rats in a total volume of 1 ml of 0.9% NaCl. Radioisotope counting was by liquid scintillation spectrometry. Protein samples were precipitated with 10% trichloroacetic acid, collected on glass filter membranes, and washed three times with 5 ml of 5% trichloroacetic acid. They were solubilized in 5 ml of Aquasol-2 (New England Nuclear). Channels were used in which the ^{3}H window contained 20% of ^{14}C counts and the ^{14}C window contained essentially no ^{3}H counts. Counting was performed to 0.2%

accuracy. Efficiencies were about 25% for ^{3}H and 50% for ^{14}C. Quenching was monitored by a channels-ratio technique and the addition of an internal standard. No differential quenching among samples was encountered.

Cell culture. Maden-Darby canine kidney (MDCK) cells were provided by Dr. Milton Saier, Jr., Department of Biology, UCSD. When grown in culture, these cells have morphological and functional properties of distal tubular epithelial cells and exhibited polarity, microvilli, and tight junctions [13]. Cells ($4 \cdot 10^5$) were plated on glass culture dishes (150 nm diameter) in 20 ml of Dulbecco Modified Eagle's Medium with 6.25% horse serum, 1.25% fetal calf serum, streptomycin and penicillin, and were maintained at 37°C in an atmosphere of 5% carbon dioxide. Unless otherwise stated, the culture medium was changed on the fourth day and cells were harvested just prior to confluency, usually on the sixth or seventh day. Each dish was washed three times with phosphate-buffered saline and scraped with a rubber policeman.

The preparation of plasma membranes from MDCK cells was adapted from that of Jorgensen et al. [14]. 20—30 dishes, yielding $3 \cdot 10^7$ cells/dish, were scraped into 12 ml of 0.25 M sucrose, 1.0 mM EDTA, 30 mM histidine, pH 7.1, (histidine/sucrose buffer). Cells were homogenized with five strokes of a motor-driven, Teflon pestle homogenizer, decanted into tubes, and centrifuged at 7000 rev./min for 15 min in a Sorval SS-34 rotor. The resulting supernatant was centrifuged at 20 000 rev./min for 30 min. The supernate from this latter centrifugation was added to an equal volume of 0.5 M sucrose, pH 7.4, and centrifuged at 4°C at 100 000 × *g* for 60 min. This pellet was collected and resuspended in 1 ml of 0.25 M sucrose as the microsomal fraction. The supernate was the supernatant fraction. The 20 000 rev./min pellets were resuspended in 5 ml of histidine/sucrose buffer using a glass homogenizer and kept on ice. An aliquot of this suspension containing 28 mg protein was mixed with a solution prepared such that the final concentrations was25 mM imidazolium chloride, pH 7.0, 2 mM EDTA, 0.6 mg/ml deoxycholate, and 3% sucrose in 40 ml (Sharkey, R., personal communication). The membrane fraction was added last and the mixture was rapidly stirred and permitted to stand for 60 min at room temperature. This mixture layered over an exponential gradient (10—35% sucrose) in Ti-14 zonal rotor (Beckman Instruments) at 3000 rev./min. The gradient was constructed so that the sucrose concentration was a linear function of rotor radius. Centrifugation was for 90 min at 4°C and 40 000 rev./min. Fractions containing $(Na^+ + K^+)$-ATPase activity were combined, dialyzed at 4°C against 0.01 M sodium phosphate, pH 7.2, and concentrated by centrifugation at 100 000 × *g* for 90 min. The pellet was suspended in a total volume of 2.0 ml phosphate buffer for subsequent incubation with proteolytic enzymes and amino acid analysis. The yield of plasma membrane protein obtained by these procedures ranged between 450 and 800 μg from 20 to 30 dishes of confluent cells.

Enzyme assays. $(Na^+ + K^+)$-ATPase assays [15] were performed on zonal gradient fraction following preincubation in 0.4 M KCl, 0.3 mg/ml deoxycholate, 5 mM Tris/sulfate, and 5 mM $MgSO_4$, pH 7.6, for 10 min on ice. Activity was determined by difference, using 10^{-5} M strophanthidin as an inhibitor. Acid phosphatase activity was determined by the method of Gianetto and de Duve [16]. Glucose-6-phosphatase activities were determined according to

Swanson [17]. NADH-cytochrome *c* reductase (NADH : (cytochrome *c*) oxidoreductase, EC 1.6.99.3) was assayed by the method of Dallner [18] and cytochrome oxidase (ferrocytochrome *c* : oxygen oxidoreductase, EC 1.9.3.1) according to the method of Wharton and Tzagoloff [19]. RNA was determined by the orcinol method [20]. The method of Lowry et al. [21] was used for determination of protein.

Proteolytic digestion membranes. Plasma membrane aliquots containing 50 μg of protein in a final volume of 1.0 ml were incubated alone and in various combinations with trypsin, chymotrypsin, papain or pronase at room temperature. The procedure used for most of the experiments described was 30 min incubation with 2 μg/ml papain followed by 30 min incubation with 3 μg/ml pronase (Guidotti, G., personal communication). The samples were then centrifuged at 100 000 × *g* to obtain pellet (residual) and supernatant (cleaved) fractions.

Amino acid analysis. Each sample was brought to 300 μl by addition of buffer and 100 μl of norleucine as an internal standard; was added with 0.5 ml of 6 N HCl. The samples were hydrolyzed at 110°C for 24 h. Amino acid analysis was performed using a Beckman Model 118C analyzer. The results were corrected for loss of norleucine standard. The proteolytic enzymes were also subjected to amino acid analysis, and their compositions were subtracted from the analysis of the supernatant fraction. This correction always amounted to less than 7% of the total amino acids. Tyrosine, alanine, valine, methionine, leucine, isoleucine and phenylalanine were considered hydrophobic in calculating the hydrophobic percentages. Tryptophane was not measured.

^{14}C and ^{3}H were counted in the plasma membrane fraction, pellet and supernatant subfractions, and in a 10 μl aliquot of cell homogenate. A turnover index was calculated by dividing the isotope ratio in a specific fraction by the isotope ratio in the total homogenate protein. This was done to control partially for changes in cell growth and isotope pools [22].

Studies with rat liver fractions. Rats were decapitated and livers were perfused in situ with chilled 0.9% NaCl to remove blood and plasma proteins, and were divided in half by weight. A plasma membrane-rich fraction was obtained from half of the liver using the method of Neville [23] as modified by Emmelot et al. [24]. The remaining half of the liver was homogenized in 0.25 M sucrose, pH 7.4, using a motor-driven, Teflon pestle and glass homogenizer, and microsomal and supernatant fractions were prepared according to the method of Novikoff and Hues [25].

Results

Plasma membrane fractions from MDCK cells showed 3—6-fold enrichment in $(Na^+ + K^+)$-ATPase activity when compared with homogenates. Glucose-6-phosphatase, acid phosphatase, NADH-cytochrome *c* reductase and cytochrome oxidase activities and RNA were not detected in plasma membrane fractions from MDCK cells or from rat liver. Transmission electron microscopic studies of the rat liver plasma membranes revealed sheets of membrane, bile canalicular structures and vesicles; contamination by other definable organelles was infrequent. Plasma membranes fractions were incubated with proteolytic

enzymes to release exposed portions of membrane-bound proteins. Preliminary studies indicated that the greatest amount of protein was cleaved following sequential incubation with papain and pronase which also resulted in greatest enrichment in hydrophobic amino acids in the pellet. The results with plasma membranes from MDCK cells and rat liver are shown in Table I. Proteolytic treatment resulted in removal of approximately 35% of membrane-bound protein. The material released consisted of peptides significantly enriched in hydrophilic amino acids with respect to the intact plasma membrane fraction. The residual particulate material was correspondingly enriched in hydrophobic amino acids.

The turnover of protein in these two fractions was examined by a double-isotope technique. The assumptions involved in the use of this method to measure protein turnover were previously discussed [4] and require experimental verification for each series of experiments.

(i) At the time cells were harvested or, for in vivo experiments, when rats were killed, the specific activity of labeled proteins must be decreasing with time. This is a particular problem with proteins which are synthesized elsewhere in the cell and incorporated into the plasma membrane before decreased protein specific activity can begin. In the case of MDCK cells, measurements were made on plasma membrane fractions at intervals of 1, 2, and 4 days after addition of the second isotope. Between the first and second day, there was a loss of 35% of radioactivity and, between the second and fourth day, only 14%. We assume that, by the second day, the remaining labeled membrane proteins re in a state of decreasing specific activity. Therefore, a two day time interval between the addition of [^{3}H]leucine to cultures and harvesting of the cells was chosen. For studies involving administration of labeled leucine to rats, previously published data provide evidence that labeled plasma membrane proteins reveal decreased specific activity after a 6 h interval [4].

(ii) The isotope must not be converted into other amino acids, lipids, or carbohydrates. A double-labeled MDCK plasma membrane fraction was

TABLE I

DISTRIBUTION OF HYDROPHOBIC AMINO ACID RESIDUES IN PLASMA MEMBRANE FRACTIONS FROM MDCK CELLS AND RAT LIVER BEFORE AND AFTER SEQUENTIAL INCUBATION WITH PAPAIN AND PRONASE

Results with MDCK cells are expressed as means of four experiments ± S.E. and have been corrected for amino acid contents of the proteolytic enzymes used. Norleucine was added prior to amino acid analysis as an internal standard and 82—96% was recovered in all experiments.

	Fraction of total protein	Mole% hydrophobic amino acid residues	Fraction of total hydrophobic amino acid residues
MDCK cells plasma membrane	1.00	37.4 ± 6.5	1.00
Residual	0.63 ± 0.18	40.3 ± 5.9	0.84 ± 0.09
Cleaved	0.34 ± 0.12	16.9 ± 4.3	0.16 ± 0.09
Rat liver plasma membrane	1.00	33, 40	1.00
Residual	0.61, 0.67	42, 49	0.82, 0.86
Cleaved	0.93, 0.33	16, 18	0.20, 0.17

extracted by the procedure of Siekevitz [26] to remove lipids, nuclei acids and other contaminants. Neither the cpm of ^{3}H nor ^{14}C were changed by these procedures indicating that label was not present in lipids, nucleic acids of carbohydrates. Double-labeled MDCK plasma membranes were hydrolyzed for

TABLE II

RELATIVE TURNOVER OF PROTEIN PRESENT IN FRACTIONS PREPARED FROM MDCK CELLS AND RAT LIVER

Expt. 1. 25 μCi of uniformly labeled L-[^{14}C]leucine and 50 μCi of L-4,5-[^{3}H]leucine were added simultaneously to each of 25 dishes of MDCK cells 2 days prior to harvesting the cells at confluence. There was no subsequent change of medium. Details regarding processing of cells, preparation of cell fractions, proteolytic digestion of plasma membranes and radioisotope technology are presented under Materials and Methods and apply to Expts. 1—4. Expt. 1. 25 μCu of L-[^{14}C]leucine was added to each of 25 dishes of MDCK cells 5 days prior to harvesting, medium was changed 3 days prior to harvesting, and 50 μCi of L-[^{3}H]leucine was added to each dish 2 days prior to harvesting. Expt. 3. 25 μCi of L-[^{14}C]leucine was added to each of 25 dishes of MDCK cells 5 days prior to harvesting the medium was changed 18 h later and 50 μCi of L-[^{3}H]leucine was added to each dish 2 days prior to harvesting. Expt. 4. 25 μCi of L-[^{14}C]leucine was added to each of 25 dishes of MDCK cells 5 days prior to harvesting medium was changed 24 h later and 50 μCi of L-[^{3}H]leucine was added to each dish 2 days before harvesting and the medium was changed 24 h later. Expt. 5. Two male rats weighing 150—170 g each were given 50 μCi of L-[^{14}C]leucine intraperitoneally. Two days later, each rat received an intraperitoneal injection of 100 μCi of L-[^{3}H]leucine. The rats were killed 4 h later. The livers were combined, homogenized and microsomal, supernatant and plasma membrane fractions were prepared as described in Materials and Methods. Plasma membranes were treated with papain and pronase as described in Materials and Methods.

Experiment	Tissue fractions (cpm/10 ml aliquot ($\times 10^{-3}$))			Plasma membrane fractions (total cpm/fraction ($\times 10^{-3}$))		
	Homogenate	Microsome	Supernatant	Plasma membrane	Residual	Cleaved
1. Control: MDCK cells						
^{3}H	86.1	52.4	60.9	60.9	38.9	1.7
^{14}C	43.9	28.0	29.7	30.0	19.4	8.4
$^{3}H/^{14}C$	1.96	1.87	2.05	2.03	2.01	2.02
Turnover index	—	0.95	1.05	1.03	1.02	1.03
2. MDCK cells						
^{3}H	72.8	31.0	10.2	32.2	22.9	4.7
^{14}C	19.6	12.1	1.9	12.8	8.8	2.1
$^{3}H/^{14}C$	3.7	2.6	5.4	2.51	2.60	2.23
Turnover index	—	0.69	1.45	0.68	0.70	0.61
3. MDCK cells						
^{3}H	59.6	40.2	18.1	21.9	12.8	4.3
^{14}C	8.6	7.2	2.9	3.1	1.9	0.6
$^{3}H/^{14}C$	6.9	5.6	6.2	7.1	6.7	7.2
Turnover index	—	0.81	0.90	1.02	0.971	1.03
4. MDCK cells						
^{3}H	85.0	62.1	20.2	36.4	25.5	5.2
^{14}C	14.5	12.6	3.3	12.1	7.9	1.6
$^{3}H/^{14}C$	5.9	4.9	6.1	3.0	3.2	3.2
Turnover index	—	0.84	1.03	0.52	0.55	0.56
5. Rat liver						
^{3}H	92.6	71.2	19.6	62.2	3.0	2.0
^{14}C	19.7	12.1	3.3	21.4	1.1	0.7
$^{3}H/^{14}C$	4.7	5.9	5.9	2.9	2.7	2.9
Turnover index	—	1.25	1.26	0.62	0.58	0.61

24 h in 6 N HCl and the hydrolysate was added to an amino acid analyzer. The effluent from the colorimeter was collected in 5-ml aliquots. Radioactivity was counted and related to the elution position of each individual amino acid. Radioactivity for ^{3}H and ^{14}C was recovered only in the leucine fraction and 98% of total radioactivity placed on the analyzer was recovered. These results indicate that both ^{3}H and ^{14}C counts in these experiments represent only radioactive leucine.

(iii) The rates of synthesis of protein constituents of the plasma membrane must be the same at the time that each isotope is administered. In experiments using intact animals, control of diet, diurnal variation, environmental agents which induce protein synthesis, and other factors are critical. Because MDCK cells are in exponential growth following the administration of ^{14}C and approach confluence after administration of [^{3}H]leucine, the problem is more complex. In the present experiments, the double-isotope method was only used to compare isotope ratios in subfractions of the same plasma membranes. These experiments should provide valid comparisons even though the relative rates of synthesis of plasma membrane proteins may not be the same at the time each isotope is administered.

The results of double-isotope studies using plasma membranes from MDCK cells and rat liver are shown in Table II. In experiments with MDCK cells, 21 ± 5% of radioactive leucine was found in the cleaved fractions in agreement with results in Table I. Expt. 1 is a control experiment in which both isotopes were simultaneously administered in a ratio of ^{3}H : ^{14}C of 2 : 1. The expected isotope ratio of 2 was measured in all fractions and a turnover index [22] of 1 was observed. This experiment established statistical reliability of the technique in subsequent experiments. In Expts. 2—4, [^{14}C]- and [^{3}H]leucine were administered for different time intervals or as shorter pulses. Regardless of experimental manipulations, no significant difference was observed in isotope ratios or turnover indices when the residual and cleaved plasma membrane fractions were compared. Expt. 5 is representative of two studies with rat liver plasma membranes after administration of the isotopes to rats in vivo. No significant differences in isotope ratio or turnover index were observed when residual and cleaved plasma membrane fractions are compared. Each of four experiments described could have detected a 25% difference in the mean rate of degradation between residual and cleaved fractions.

There was no significant difference in protein degradation rates between the residual and cleaved fractions. Since each membrane-bound polypeptide is synthesized intact, no difference in the synthesis rates of these two portions of each polypeptide is possible. It follows from these experiments that there is no difference in degradation rates between these two subfractions of plasma membrane proteins.

Discussion

The double-isotope method is well-suited for comparing the relative degradation rates of different proteins or groups of proteins in intracellular organelles [4]. The ease and reproducibility of the method have been demonstrated for many organs and for cells growing in culture. There are several assumptions

inherent in its use which have been discussed above. In addition, there are limitations in the interpretation of results which require comment.

(i) The ^{3}H : ^{14}C ratios observed largely reflect isotope incorporation into and degradation of membrane proteins with half-lives between 5 h and 10 days. In all probability, every plasma membrane protein is being examined, and the isotope ratios are largely determined by more abundant proteins having half-lives up to 10 days. Although the ratios of the hydrophilic and hydrophobic fractions were indistinguishable, some membrane proteins may exist in which intramolecular heterogeneity of degradation occurs but was not detected by this method. If less than 20% of the membrane-bound proteins are degraded by an intramolecularly heterogeneous process such that the degradation rate of hydrophilic portions is three times that of the hydrophobic portion, this process would have escaped detection.

(ii) It is assumed that all protein segments enriched in hydrophilic amino acid residues are detached from the membrane following partial proteolytic digestion. Some hydrophilic portions of membrane proteins may remain attached to the membrane. This would tend to decrease differences in the isotope ratio.

(iii) Although plasma membranes obtained from MDCK cells and rat liver have biochemical and morphological features of plasma membrane, there is never certainty regarding the homogeneity of such membrane preparations. Golgi, lysosomal, endoplasmic reticulum and soluble proteins may be present in all plasma membrane preparations and unduly influence the ratios.

(iv) Cleavage of plasma membrane proteins with extraneous proteolytic enzymes may not mimic the natural process by which these proteins undergo degradation in an intramolecularly heterogeneous fashion. Limited proteolysis of hydrophilic sequences on the cytoplasmic surface may mark a membrane protein for elimination even though none of the protein is cleaved from the membrane.

Some proteins are not inserted into the membrane bilayer but may be attached with variable affinity. These proteins probably do not contribute to the observed results because in single experiments with plasma membranes from MDCK cells and rat liver, repeated washing with phosphate-buffered saline and 0.1 M EDTA did not change the ^{3}H : ^{14}C ratio in the membrane fractions before or after proteolytic digestion.

The purity of plasma membrane fractions is important in interpretation of results of these studies. $(Na^{+} + K^{+})$-ATPase activity was enriched 2—6-fold, and enzyme activities related to lysosomes, mitochondria and endoplasmic reticulum were not detected. Although absolute purity of a plasma membrane fraction is impossible to establish, the enzyme and chemical studies indicate that contamination by other organelles is negligible. The plasma membrane fragments are vesicles [14] which are both right-side out and inside out. The permeability of different vesicles to proteolytic enzymes is unknown; however, we assume that the random distribution of plasma membrane vesicles is similar in each experiment and, therefore, comparison of isotope ratios is valid.

The results of the present study indicate that the turnover indices for pulse-labeled leucine in hydrophobic and hydrophilic portions of plasma membrane proteins are the same, and are consistent with a model of membrane protein

degradation involving interiorization and degradation of entire proteins if not structural membrane units.

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