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LEUCINE-SPECIFIC BINDING OF PHOTOREACTIVE LEU$_7$-MAP TO A HIGH MOLECULAR WEIGHT PROTEIN ON THE PLASMA MEMBRANE OF THE ISOLATED RAT HEPATOCYTE

Glenn E. Mortimore*, John J. Wert, Jr., Giovanni Miotto§, Rina Venerando§, and Motoni Kadowaki‡

Department of Cellular and Molecular Physiology, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 17033

§Dipartimento di Chimica Biologica, Università Degli Studi di Padova, 35121 Padova, Italy

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Leu$_8$-MAP (Multiple Antigen Peptide) is an effective inhibitor of macroautophagy and proteolysis in the isolated rat hepatocyte, having an apparent K_m (0.1 mM) equaling leucine. Since it is not transported into the cytosolic compartment, it very likely mediates its effect through a plasma membrane site. In an attempt to identify the site we photoreacted intact cells with a biologically active, iodinatable azide derivative of Leu$_7$-MAP. A ≈340,000 M_r protein whose labeling was protected 83% with 20 mM Leu was found in plasma membrane fractions when electrophoresed in 7.5-20% gradient gels under nonreducing conditions; addition of 20 mM dithiothreitol generated smaller m.w. products, possibly subunits, of consistent size. No specific labeling was observed with photoreactive derivatives of Ile$_7$-MAP or Val$_7$-MAP.

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The breakdown of cellular proteins by macroautophagy in eukaryotic cells is the principal endogenous source of free amino acids for energy and protein renewal early in starvation (reviewed in 1,2). In the hepatic parenchymal cell, vacuole formation is primarily controlled by the concerted action of eight regulatory amino acids of which leucine plays the dominant role (2-6). Although actively sought, a central mechanism of amino acid inhibition has

* To whom correspondence should be addressed.

‡ Present address: Department of Applied Biochemistry, Faculty of Agriculture, Niigata University, Niigata, Japan.

Abbreviations are: MAP, multiple antigen peptide; Fmoc, 9-fluorenylmethoxycarbonyl; Dde, N_ε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl derivative; HOBT, N-hydroxybenzotriazole; BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate; TFA, trifluoroacetic acid; OPfp, pentafluorophenyl ester; DMF, N,N-dimethylformamide; ASA, 4-azidosalicylic acid; NHS-ASA, N-hydroxysuccinimidyl-4-azidosalicylic acid; SDS, Na dodecylsulfate; DTT, dithiothreitol; PAGE, polyacrylimide gel electrophoresis.

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not been established (2). However, recent studies with the leucine analogue isovaleryl-L-carnitine (7) and with phenylalanine and its keto acid (8), have clearly pointed to the possibility that regulatory amino and keto acids are recognized by specific proteins at the plasma membrane. Conceivably, the recognition process generates information of some undetermined type that directly inhibits nascent vacuole formation.

Current studies with Leu_8-MAP[1], an octaleucine derivative of MAP (9), and other leucine analogues (10) strongly support this notion and, in addition, provide a feasible approach to identifying the leucine recognition protein(s). The findings show that Leu_8-MAP, which is nontransportable and remains outside the cytosolic compartment[1], inhibits macroautophagy through a single low-concentration site with an apparent K_m (0.10 mM) equal to that of leucine (7,11). Because the number of leucine residues on the MAP peptide is greater than that required for maximal inhibition[1], a photolabile group such as the heterobifunctional azido adduct reported by Ji and Ji (12) could be substituted for one of them to provide a potentially useful photoprobe for determining the presence of the putative leucine binding protein or proteins.

The results of such photoaffinity labeling experiments in the isolated rat hepatocyte are described in the following communication.

EXPERIMENTAL PROCEDURES

Isolated Hepatocytes--Male rats of the Lewis strain (Harlan Sprague Dawley, Indianapolis, IN), weighing 200-300 g at the time of experiment, were used as hepatocyte donors. They were housed and fed a 35% casein diet in a synchronous feeding regimen as detailed earlier (13). Cells were isolated 18 h after the start of the last feeding by the collagenase method of Seglen (14) as modified slightly by Venerando et al. (11).

Hepatocyte Proteolysis--Rates of long-lived protein degradation in isolated heptocytes were measured as detailed by Venerando et al. (11). In brief, cells (1.0-1.2 x 10^{-6}/ml) were incubated 30 min under experimental conditions described in the text. Cycloheximide (10 μM) was then added and samples of the cell suspension taken at 33 and 41 min for the chromatographic analysis of released valine determined after dansylation (15).

Synthesis of (^{125}I-ASA)Leu_7-MAP--(Fmoc-Leu)$_7$-MAP was synthesized by standard solid phase techniques (16) on β-Ala substituted resin (Bachem, Philadelphia) by the following procedure: (a) A 4-8x excess of Fmoc-Lys(Dde), N-methylmorpholine, HOBT, and BOP in molar ratios of 1:2:1:1 was reacted with the resin for 2 h after which the Fmoc groups were selectively removed by 20% piperidine in DMF; (b) A 4-8x excess of Fmoc-Lys(Fmoc)-OPfp (Novobiochem) was coupled to the free amine with one equivalent of HOBT in DMF for 2 h. Dde was then selectively removed with 2% hydrazine in pyridine. Steps (a) and (b) were repeated 2x to give (Fmoc-Leu)$_7$/NH_2-Lys_4-Lys_2-Lys-βAla (termed Leu-MAP peptide). The synthesizer solvents were extensively flushed

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[1] Miotto, G., Venerando, R., Marin, O., Siliprandi, N., and Mortimore, G.E. (1994) J. Biol. Chem., in press.

between steps to assure purity of reagents during the couplings. The peptide was removed from the resin by a mixture of 95% TFA, 3% 1,2-ethanedithiol, 1% thioanisole, and 1% anisole; it was then precipitated and washed with ether. Synthesis of the corresponding Ile- and Val-MAP peptides was achieved in the same way except for substituting the appropriate amino acids.

The photoreactive group ASA was coupled to the free NH_2 of the foregoing Leu-MAP peptide by reacting it with NHS-ASA (Pierce) in 0.1 M KH_2PO_4, pH 6.8, at room temperature overnight at a molar excess ratio of 20:1. The pH of the solution was raised to 9-10 with NaOH to remove the Fmoc groups; after 3-4 min the pH was lowered to 2-3 with HCl. The product was then purified by NH_4OH from a Macro-Prep 50 S column (BioRad) and dried under N_2. The attached ASA on 4.09 nmoles of Leu_7-MAP was then iodinated with 10 mCi of $Na^{125}I$ (17 mCi/μg, DuPont NEN). The reaction was carried out with Iodogen (Pierce) in 0.5 M KH_2PO_4, pH 7.0, for 60 min. After oxidation with H_2O_2, the unreacted ^{125}I was extracted with $CHCl_3$ and the aqueous layer dried under N_2.

Photolabeling of Hepatocytes and Cellular Fractionation--Isolated cells at a density of 10^7/ml were incubated with gentle shaking (orbital, 100 rpm) at 37°C under 95% O_2-5% CO_2 in Krebs-Ringer bicarbonate buffer containing 0.5% bovine albumin and 0.02 mM poly-L-lysine as a wetting agent. The photoligand (117 pmol/ml) was added, ± protective agents, and the cells incubated 2 min at 37°C. The medium and cells were then transferred to 60 mm plastic Petri dishes on ice and the contents irradiated with UV (330-380 nm) for 3 min at 0.6 mW/cm^2; the cells were washed twice with buffer and the above cycle was repeated. No specific labeling was found in the absence of UV irradiation or when the photoligand was preincubated with the cells at 0°C. The pelleted cells then were homogenized and the mitochondrial/lysosomal fraction prepared as described by Kadowaki et al. (17). A portion of the fraction was dispersed in colloidal silica-povidine gradient material and centrifuged as described (17). A whitish, viscous, 5'-nucleotidase-enriched band (density, ≈1.075 g/ml) was removed by pipet, dispersed in 5 ml of 0.25 M sucrose-3 mM EDTA, and spun at 100,000 g for 15 min. Typically, 300 μg of the pellet protein was dissolved in SDS for PAGE; conditions for individual experiments are given in the figures and text.

Electrophoresis--PAGE was performed with 3 mm gels: 12% slab or 7.5-20% gradient gels (4% stacking) using the Laemmli buffer system (18); minor modifications in the protocol are described under the individual experiments. Following the runs, the gels were fixed, stained with Coomassie Blue, sliced in 1 mm sections, and the slices counted in a Beckman Gamma 5500B system.

RESULTS AND DISCUSSION

The coupling of NHS-ASA to a free ε-amino group of the Leu-MAP peptide (see "Experimental Procedures") yielded the photolabile derivative depicted schematically in Fig. 1. As shown in the chromatogram of Fig. 2 the final product was apparently homogeneous. The result indicates that very few amino acid deletions had occurred in the peptide synthesis and all or nearly all the molecules in the preparation contained only a single photoreactive group (^{125}I-ASA). Although the exact position of the adduct among the 4 terminal lysine residues of the MAP molecule could not be predetermined, the fact that only one substitution was possible proved advantageous in that it virtually eliminated the potential for photocrosslinking between proteins.

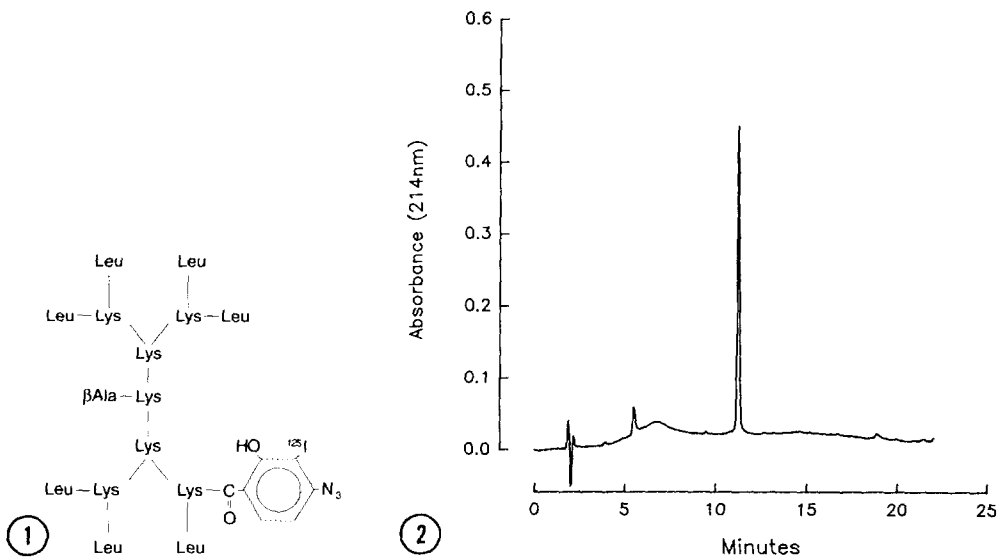

Fig. 1. Schematic structure of the photoreactive MAP derivative (^{125}I-ASA)Leu$_7$-MAP, formed by coupling NHS-ASA to the free NH_2 of the Leu-MAP peptide followed by removal of the Fmoc protective groups and iodination of ASA (see "Experimental Procedures"). The branched lysine structure terminating in β-alanine represents the MAP core.

Fig. 2. HPLC analysis of (ASA)Leu$_7$-MAP. The derivative tested was the same as that shown in Fig. 1 except that it was not iodinated and the azide group was photoreacted with methyl amine. The analysis was carried out with a C18 Vydac column (4 x 150 mm, 10 μm particle size). Eluant A was composed of H_2O + 0.05% TFA and B, acetonitrile + 0.05% TFA. The flow was 1 ml min^{-1} from 5% to 80% of B in 16 min. The peptide appeared at 12 min; the small peak at 5.5 min was an artifact.

The photoreactive product, tested before iodination, was biologically active, inhibiting proteolysis to the same extent as leucine. The effect, determined in the dark at a concentration of 0.4 mM (equivalent to 2x plasma leucine), was 94.8 ± 13.4% (mean ± S.E., n = 3) of the maximal suppression by leucine at 1 mM (176 nmol Val min^{-1}/10^6 cells). A similar effect was reported earlier for Leu$_7$-MAP[1].

Our initial photoaffinity experiments were designed to determine whether any membrane protein(s) could be specifically labeled with (^{125}I-ASA)Leu$_7$-MAP. In parallel runs hepatocytes were photolyzed with the ligand ± 20 mM unlabeled leucine; crude membrane fractions were then isolated, solubilized in Laemmli sample buffer (1% SDS) under mild reducing conditions (2.5% β-mercaptoethanol), and subjected to 12% PAGE. We observed labeled bands in the first 3 gel slices (1 mm) that were consistently protected by 20 mM leucine. Although the exact slice position (≈250,000 M_r) and magnitude of protection varied in different runs, statistical significance was readily established by pairing: each effect individually had a p value of ≤0.05 and the overall protection in 7 such experiments was 44.8 ± 8.8% (mean ± S.E.;

$p<0.005$); variance in the separate experiments was evaluated by recycling the slices through the counter 10 times. Protein labeling at the top of the 4% stacking gel was also suppressed to an equal extent, indicating the presence of relevant protein(s) that did not enter the gel. The degree of protection was similar to that reported for glucose against ^{125}I-ASA photolabeling of the glucose transporter in human erythrocyte ghosts (19).

Because the Laemmli sample buffer (18) contained β-mercaptoethanol, it seemed likely that the ≈250,000 M_r protein band in the 12% resolving gels was derived by partial disulfide bond reduction of a larger protein complex regularly observed at the top of the 4% stacking gels (see above). In an attempt to the extract the unreduced complex we omitted the mercaptoethanol, increased the SDS from 1% to 2%, and electrophoresed the sample in a 7.5-20% gel gradient. A single, prominent ≈340,000 M_r band entered the first fraction of the gradient (Fig. 3), leaving a clear zone between the band and a sharp demarcation (change in refraction) between the stacking and gradient gels. Labeling of the band was decreased 83% with 20 mM leucine, yielding a signal-to-noise ratio of nearly 6:1. The enhanced signal can be explained by a selective increase in protein extraction combined with a decrease in nonspecific labeling following omission of the reductants. Some leucine-protected photolabel remained at the top of the stacking gel, although 68% of the total effect appeared in the gradient gel. No labeling of the ≈250 k protein(s) or other leucine-specific fractions was found.

The effect of more extensive disulfide reduction with 20 mM DTT on the distribution of leucine-specific labeling among gel fractions is shown in Fig. 4. Owing to variable increases in nonspecific labeling that appeared after reduction, we chose to illustrate the response to DTT by plotting the protective effects of 20 mM leucine as mean pair differences between parallel slices; positive values denote protection. In addition to the ≈250,000 M_r band that was observed in our initial experiments (see above), we found two prominent components at M_r 73-65k and 49-46k. Similar results were obtained in three comparable experiments. No leucine-specific photolabeling was noted at the top of the stacking gels in these experiments indicating that the ≈340,000 M_r protein complex in Fig. 3 was effectively broken into smaller units. As to the nature of the M_r 73-65k and 49-46k fractions, it is reasonable to assume that they represent subunits of the higher molecular weight proteins; however, other explanations cannot be ruled out. It would be possible to obtain more definitive information by characterizing the fragments that appear after reduction of the isolated M_r ≈340k complex.

The foregoing photobinding appears to be specific for leucine since no protection was seen in any gel slice when (^{125}I-ASA)Ile_7-MAP and (^{125}I-ASA)-Val_7-MAP (4 trials each) were tested against their respective 20 mM unlabeled

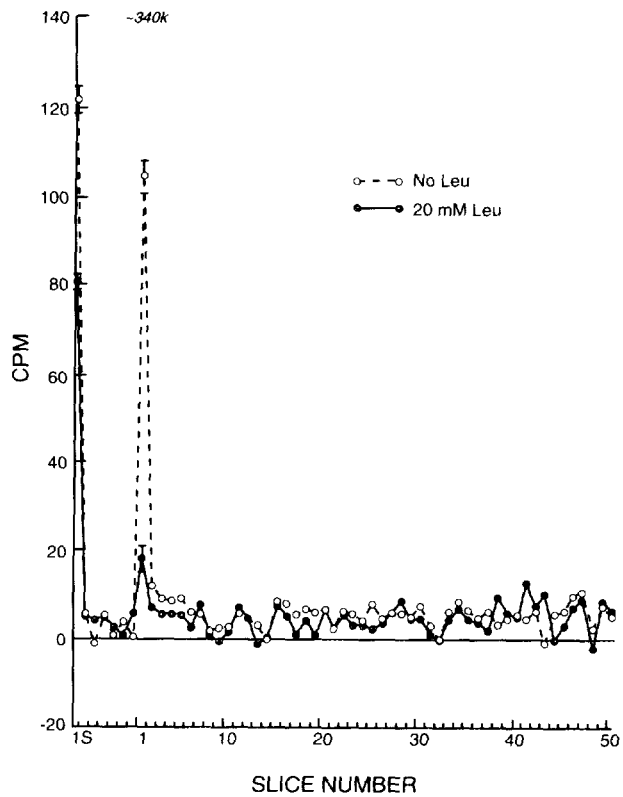

Fig. 3. Nonreducing PAGE showing a high molecular weight protein of M_r ≈340,000 that had entered a 7.5-20% gradient gel and was protected 83% with 20 mM leucine; in addition, some partially protected protein was present above the 4% stacking gel (above 1S). Fresh hepatocytes were photolyzed with (^{125}I-ASA)Leu$_7$-MAP ± 20 mM leucine as detailed under "Experimental Procedures". A plasma membrane enriched fraction was solubilized in sample buffer for 90 min at 25°C with 2% SDS but without β-mercaptoethanol or DTT. Radioactivity in the slices of each experiment was normalized to a total cpm value (sum of 50 slices) that represented an average of the 3 runs; all slices were counted 10 times in rotation. Values shown are means of 3 separate experiments; bars in slice 1 and above 1S depict ± 1 S.E. The molecular weight markers were myosin, β-galactosidase, and phosphorylase B.

amino acids. In addition, 20 mM valine provided no protection against leucine photolabeling. Results with 20 mM isoleucine, though, were less certain since an effect was obtained on one but not two other occasions. Thus while it is conceivable that very high levels of isoleucine can compete with leucine binding, no specific binding of the isoleucine or valine photoligands was evident. In these experiments and the ones below, the samples were electrophoresed as described for the initial photoaffinity experiments.

In the case of leucine photolabeling, it is important to note that the binding was saturable and protection of the ≈250k M_r labeled band was within the physiologic range of leucine: labeling was decreased 40 ± 19% with 1 mM leucine and 50 ± 18% with 1 mM Leu$_8$-MAP (pooled effect: 45 ± 11%, $p<0.05$, n = 4). With regard to affinity, one can only roughly estimate the K_d.

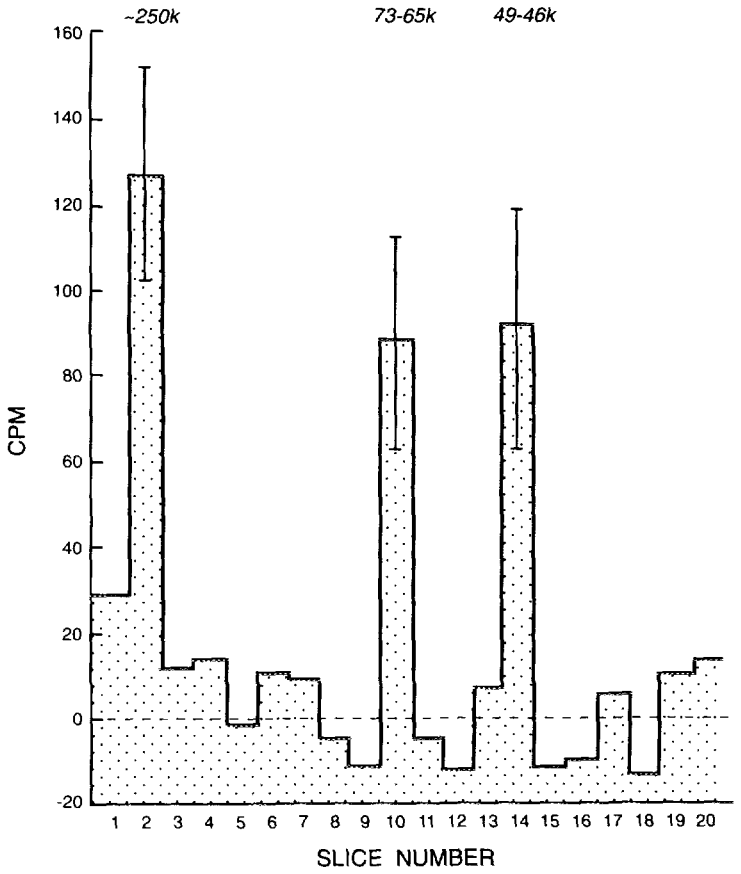

Fig. 4. The effect of disulfide bond reduction on the distribution of leucine-specific photolabeling in membrane fractions run on 12% resolving-4% stacking gels. Experimental conditions were similar to those in Fig. 3 except that the membrane samples from the cells were solubilized in standard Laemmli buffer (1% SDS) to which 20 mM DTT was added. For convenience of interpretation (see text) the graph displays the protective effects of 20 mM leucine (no Leu minus 20 mM Leu) as positive values. The values represent means ± S.E. of differences between the above parallel runs obtained from 10 counting cycles; results from 1 mm slices were pooled in groups of 3. Leucine protection in the 3 bands was significant to $p<0.01$.

Undoubtedly, it would exceed 10^{-5} M, the concentration of leucine generated proteolytically in one min by 10^{7} cells/ml (11); the upper limit, of course, would be less than 10^{-3} M (see above).

It should be mentioned that an attempt was made, without success, to photolabel the isolated plasma membrane prepared as described under "Experimental Procedures". It is possible that the leucine binding proteins are conformationally altered as a result of cell disruption.

In demonstrating specific, low affinity binding of leucine by the plasma membrane of the rat hepatocyte, this study provides a new approach for probing the amino acid regulation of macroautophagy. Although the relevance of the binding proteins to the process is not presently known, some answers

are now evident while others will be attainable in the future. For example, because 20 mM valine does not compete with the leucine photoderivative for photolabeling, it is reasonable to exclude the system L transporter as the site of binding. But one might also extend the recognition site hypothesis by determining the binding function of other regulatory amino acids coupled to photoreactive MAP. In a related study, we have found that products of leucine binding are internalized by the hepatocyte and appear in lysosomes within 5 min[2]. These results are strikingly reminiscent of the fate of [^{14}C]sucrose-insulin in the perfused rat liver (20) and raise the interesting possibility that the binding site is in fact a rapidly turning over receptor.

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