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Inhibition of Macroautophagy and Proteolysis in the Isolated Rat Hepatocyte by a Nontransportable Derivative of the Multiple Antigen Peptide Leu_8-Lys_4-Lys_2-Lys-βAla*

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The multiple antigen peptide derivative, Leu_8-Lys_4-Lys_2-Lys-βAla (Leu_8-MAP), was synthesized by attaching the carboxyl of leucine to the NH_2 termini of a branched lysine core, termed MAP, creating a molecule of about 1900 Da with 8 leucine residues. On a molar basis (independent of the number of leucine substitutions), Leu_8-MAP was as effective as leucine in suppressing macroautophagy and proteolysis; moreover, it exhibited the same apparent K_m (about 0.1 mM). The effect was specific for leucine since Ile_8-MAP was inactive. It is of interest, though, that Leu_8-MAP did not elicit the multiphasic response typical of leucine but instead evoked the single site inhibition normally seen with leucine plus the coregulator alanine. Some free leucine was produced from Leu_8-MAP during hepatocyte incubations, but the amounts were insufficient to account for the inhibition. Although this degradation created species of Leu-MAP that had lost 1–3 residues of leucine, their inhibitory effectiveness was not diminished. Because the extracellular/intracellular distribution ratio of [^{3}H]-Leu_8-MAP was 100:1 or greater, the direct transport of Leu_8-MAP across the plasma membrane into the cytosolic compartment can be excluded. Hence, cytosolic concentrations of Leu_8-MAP will be at least 100-fold smaller than those of leucine under conditions of comparable proteolytic inhibition. For these and related reasons, effects attributable to the recognition of Leu_8-MAP cannot be explained by signals generated within the cytosol. They could, however, be mediated from site(s) on the plasma membrane or within associated vesicles.

In liver, macroautophagy is the principal mechanism for degrading intracellular protein and is a major source of endogenous amino acids for gluconeogenesis and other critical pathways early in starvation (reviewed in Refs. 1, 2). The fact that autophagy is an ongoing process and subject to immediate feedback control by specific regulatory amino acids (3–5) attests to its importance in cellular homeostasis. Although the effects of amino acids are strongly modulated by insulin and glucagon in the perfused liver (6), amino acids as well as their keto derivatives are believed to represent prime regulators since the full range of deprivation-induced autophagy can be elicited by these agents in the absence of hormones (3, 5, 6). Of the 20 amino acids that turn over in protein, only 8 as a group (Leu, Tyr, Phe, Gln, Pro, Met, His, and Trp) possess direct inhibitory activity at physiological concentrations while the rest are without effect, even at 10 times normal plasma levels (5, 7). Leucine is the dominant regulator since it is the most potent and is required in all mixtures for maximal effectiveness (4, 5).

How amino and keto acids inhibit autophagy and long-lived proteolysis in animal tissues is not clear. Past studies in muscle with leucine and its α-keto acid favored the possibility that the inhibition is mediated via products of oxidation (8, 9). This view, however, has been disputed on the basis of evidence from rat heart that appears to dissociate the oxidation of the keto acid from its inhibitory activity (10). In liver, where leucine transamination activity is very low (5), leucine and α-ketoisocaproate are believed to exert their respective proteolytic effects through separate, nonmetabolic pathways. This contention is strongly supported by findings that submaximal responses to the amino and keto acids at upper physiologic concentrations are equal but nonadditive (5).

As an alternative possibility, it is reasonable to propose that amino and keto acids mediate their effects from specific recognition sites at the plasma membrane. Results of a recent investigation with the leucine analogue isovaleryl-L-carnitine, for example, have shown that it mimics leucine's complex multiphasic response in the perfused rat liver at equal external concentrations but at widely differing intracellular levels (11), evidence that strongly suggests a plasma membrane site. In addition, findings from a parallel study have revealed that the inhibitory response to phenylpyruvate, which is identical to that of phenylalanine, can be obtained when the keto acid is added externally but not when it is generated within the cell (12). These results clearly exclude the possibility that the keto acid had initiated its autophagic inhibition from an intracellular locus.

It would be possible to test the foregoing hypothesis by determining the inhibitory effectiveness of a nontransportable leucine derivative on macroautophagy. Since there is no positional requirement of the carboxyl group of leucine for biological activity (13), the amino acid could be attached from this point to a number of possible molecular structures that might impede or block leucine's transport (11, 13). One such possibility is MAP,[1] a dense, branched lysine core to which peptides have been coupled to enhance antibody production (14). In this

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[1] The abbreviations used are: MAP, multiple antigen peptide; Leu_8-MAP, Leu_8-Lys_4-Lys_2-Lys-βAla; Ile_8-MAP, Ile_8-Lys_4-Lys_2-Lys-βAla; HPLC, high performance liquid chromatography; AVi, autophagosome; AVd, autolysosome (the number followed by x indicates multiples/fractions of the amino acid concentrations in the standard normal plasma mixture (23)); Fmoc, *N*-(9-fluorenyl)methoxycarbonyl.

TABLE I

Purification and characterization of Leu-MAP subfractions

The initial product of Leu-MAP solid-phase synthesis was fractionated into three separate peaks by C18 reverse-phase HPLC as described under "Experimental Procedures." The subfractions, identified by their retention times, and their major characteristics are listed below; the 19.40-min peak (bottom) is Leu_8-MAP. The inhibitory effectiveness of the three fractions on deprivation-induced proteolysis in the isolated rat hepatocyte was determined at a dose of 100 μM as described by Venerando *et al.* (16); the values are means ± S.E. of three to five experiments.

Retention time	Proportion of total Leu-MAP	Amino acid composition			Inhibition of induced proteolysis
		β-Ala	Lys	Leu	
min	%		*Ratios*		%
18.34	10.2	1	5.10	5.48	31.8 ± 6.9
19.12	26.5	1	5.53	6.20	32.6 ± 5.3
19.40	63.4	1	6.80	7.70	30.7 ± 4.6

article we describe experiments in which leucine was attached to MAP in this way. The results show that the resultant Leu_8-MAP is nontransportable and biologically active and, hence, could serve as an effective probe for testing the hypothesis.

EXPERIMENTAL PROCEDURES

Animals—Male rats of the Wistar strain, weighing 140–160 g at the time of experiment, were maintained on standard laboratory chow and water *ad libitum*. All animals were nonstarved, and the experiments were started at 1100 h. The rats were heparinized (500 units/100 g body weight) and anesthetized by the intraperitoneal injection of ketamine (16 mg/100 g body weight).

Isolation and Incubation of Hepatocytes—Liver parenchymal cells were isolated by the collagenase perfusion method of Seglen (15), modified slightly by Venerando *et al.* (16). Cell viability was tested both by lactate dehydrogenase release and trypan blue exclusion and was usually 90% or better. The yield of hepatocytes after purification was about 1.5–2.0 × 10^8 cells/liver. Incubations were performed in 10-ml conical flasks containing 3 ml of cell suspension (1.0–1.2 × 10^6 cells/ml) in Krebs-Ringer bicarbonate buffer with 0.5% bovine serum albumin. The incubation system was devised to maintain a constant, humidified atmosphere of 5% CO_2, 95% O_2. For efficient gas exchange, the level of cell suspension in the incubation flasks was kept below 10 mm. Temperature was maintained at 37 °C in a recirculating water bath.

Intracellular Protein Degradation—Rates of long-lived proteolysis were determined as described by Venerando *et al.* (16). After first incubating the cells for 30 min with the additions described in the individual experiments, 10 μM cycloheximide was then added and samples of the hepatocyte suspension collected at 33 and 41 min for the chromatographic determination of dansylated valine as detailed earlier (16). Rates were calculated as percentages of the maximal deprivation response based on the difference between valine release with and without 10 times normal plasma amino acids (16); valine was omitted from the amino acid mixture, and glutamine was reduced to less than 3 mM to prevent the rise in intralysosomal pH that occurs with high levels of glutamine (7). Overall, the rates averaged 0.40 and 0.980 nmol min^{-1}/10^6 cells with and without the amino acid addition, respectively.

Synthesis and Purification of Leu_8-MAP—The synthesis of Leu_8-MAP was accomplished by coupling *N*-(9-fluorenyl)methoxycarbonylFmoc-protected leucine to $Fmoc_8$-Lys_4-Lys_2-Lys-βAla NovaSyn KA 100 Resin (Novabiochem) with the use of standard solid-phase techniques and the continuous flow variant of "Fmoc-polyamide" chemistry (17). The Fmoc protecting group on the branched Lys core was removed with a 20% solution of piperidine in *N*,*N*-dimethylformamide. A 5-fold excess of Fmoc-leucine then was introduced and activated *in situ* by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in the presence of *N*-methylmorpholine. Maximal acylation, checked on the resin sample by a qualitative ninhydrin reaction (18), was achieved by double coupling. Finally, the branched peptide was removed from the polyamide resin support with aqueous 95% trifluoroacetic acid. For some experiments radiolabeled Leu_8-MAP (specific radioactivity, 8.4 mCi/mmol) was synthesized from Fmoc-[^{3}H]Leu prepared from [^{3}H]Leu (specific radioactivity, 70 Ci/mmol and 9-fluorenylmethoxycarbonyl chloride (19)).

The crude peptide was desalted on a Sephadex G-10 column equilibrated with 10 mM HCl and purified by semipreparative HPLC on a C18 reverse-phase column (Beckman Ultraspere ODS, 10 × 250 mm, 5-μm particle size) with a linear gradient of acetonitrile ranging from 0 to 30% (25 min at 3 ml min^{-1}) in the presence of 0.1% aqueous trifluoroacetic acid. The principal peaks were collected and lyophilized twice from 10 mM HCl to obtain the chloride derivatives. The peak corresponding to Leu_8-MAP (Table I and Fig. 1) had a purity of 98% by HPLC, and the ratios of Leu and Lys to β-Ala, analyzed by HPLC as the dansylated derivatives (20) after acid hydrolysis, were within 3–4% of the theoretical values. The yield of Leu_8-MAP was approximately 60–65% of the crude peptide as some lysine deletions commonly occur in the synthesis of the MAP core. These secondary species of Leu-MAP comprise the remaining fractions in Table I.

Materials—Dansyl chloride was obtained from Pierce; bovine serum albumin (fraction V, protease free), amino acids, and collagenase (type IV) were from Sigma; radiochemicals were purchased from Amersham Corp.; all other reagents were of the highest grade commercially available.

RESULTS

Inhibition of Macroautophagy and Proteolysis by Leu_8-MAP—Fig. 1 depicts the structure of Leu_8-MAP, a peptide consisting of the branched lysine core MAP (14) to which 8 leucines were coupled through their carboxyl groups to the α- and ε-amino termini. The peptide has a net positive charge since it has 8 amino groups (leucine) and only a single carboxyl (β-alanine). The spatial configuration of the MAP peptide is not known, but owing to the repelling effect of similar charges, it could be one in which the leucine residues radiate from a central core, positioned as well-separated units at the periphery of the molecule.

As shown in Fig. 2*A*, Leu_8-MAP strongly inhibited deprivation-induced proteolysis. In comparison with previous effects of leucine in perfused livers of fed rats, which ranged from 60 to 89% of the total suppressive effect of 4 × plasma amino acid mixtures (5, 6, 11), the value observed at 0.8 mM (4 × normal plasma leucine) was remarkably similar. One curious difference in the response to Leu_8-MAP, though, was noted: no zonal loss of inhibition was observed. The regulatory amino acids leucine, tyrosine, and glutamine all exhibit characteristic multiphasic response curves in which inhibition is lost within a narrow zone centered around 1 × plasma concentrations. Because the zonal effect is abolished by 0.5 mM alanine (5, 6, 21, 22), alanine is believed to serve as a specific co-regulator for the expression of inhibition by the regulatory amino acids at *normal* levels (21, 22). In the case of Leu_8-MAP, the dose response appeared as though alanine were present. The reason for this is not known but the phenomenon will be dealt with later under "Discussion."

Since leucine expresses its inhibition through a single-site mechanism when co-regulation by alanine is manifest, it is of interest that V/S *versus* V plots of dose responses for leucine + alanine yield linear slopes with an apparent K_m of about 0.1 mM, half of leucine's normal plasma concentration (11, 16). In keeping with these findings the regression of a similar plot for Leu_8-MAP (Fig. 2*B* was also linear ($r = 0.99$), and the apparent K_m was the same (0.10 mM).

Because macroautophagy is the intracellular process believed to be responsible for deprivation-induced protein degradation in liver (1, 2), it is reasonable to expect that the proteolytic inhibition resulting from the addition of Leu_8-MAP would be associated with a corresponding suppression of macro-

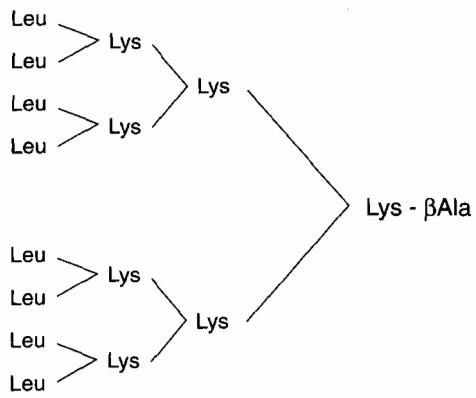


FIG. 1. **Schematic structure of Leu_8-MAP.** Eight leucine residues are attached by their carboxyl groups to the α- and ϵ-amino termini of a compact, branched lysine core (MAP), giving a dendritic peptide with a molecular weight of about 1900. The peptide is then cleaved from the resin, leaving β-alanine as the terminal carboxyl residue.

autophagy. In Table II, the percentage inhibition by Leu_8-MAP of the volume density of macroautophagic vacuoles and the rate of deprivation-induced protein degradation were nearly the same. Although the effects of leucine were somewhat smaller than those of the MAP peptide, similar agreement between the two parameters was also obtained. The stereologic data also identify the affected step in the autophagic pathway. Inasmuch as the aggregate volume of AVi was decreased to the same extent as that of AVd, one may conclude that the rate of sequestration (AVi formation) was inhibited. This is the only explanation that would account for both the morphologic effects and the proteolytic inhibition. Such a site of action has been previously described for leucine (1, 2).

Inhibitory Effectiveness of Other MAP Derivatives—Replacing the leucine residues of Leu_8-MAP with isoleucine, an amino acid devoid of regulatory activity in rat liver (4, 5), yielded a peptide that was totally ineffective as an inhibitor (Fig. 2*A*). This evidence suggests that the branched lysine core of MAP is not directly involved in the observed inhibition and that the specificity of the peptide resides in the nature of the attached amino acid. A second aspect of the inhibitory effectiveness of Leu_8-MAP concerns the relationship between the number of leucine residues and biological activity. As shown in Table I no difference in inhibitory potency was observed when the two Leu-MAP subfractions in the crude MAP peptide were directly compared with Leu_8-MAP at a concentration of 0.1 mM. As these species contained roughly 5 and 6 leucine residues/molecule (Table I), it seems clear that optimal activity of the Leu-MAP derivatives can be attained with at least 5 leucine residues and possibly less. Moreover, since lysine deletions were present, activity must be independent of specific structural features of the branched core.

Stability of Leu_8-MAP—The metabolic stability of Leu_8-MAP during incubation with fresh hepatocytes was ascertained from an HPLC analysis of MAP peptide fractions before and after 60 min of incubation (Fig. 3) and from the release of free [^{3}H]leucine generated from labeled Leu_8-MAP (Table III). It is apparent from Fig. 3 that Leu_8-MAP undergoes limited hydrolysis during hepatocyte incubation with the loss of up to 3 residues of leucine by 60 min. However, the lysine core and its attached β-alanine remained intact as judged from amino acid analyses of the HPLC peaks (Fig. 3). Owing to the small quantities produced, the Leu_n-MAP species produced could not be assayed for biological activity. Nevertheless, it is reasonable to conclude from the results in Table I that the loss of up to 3 leucine residues would not diminish

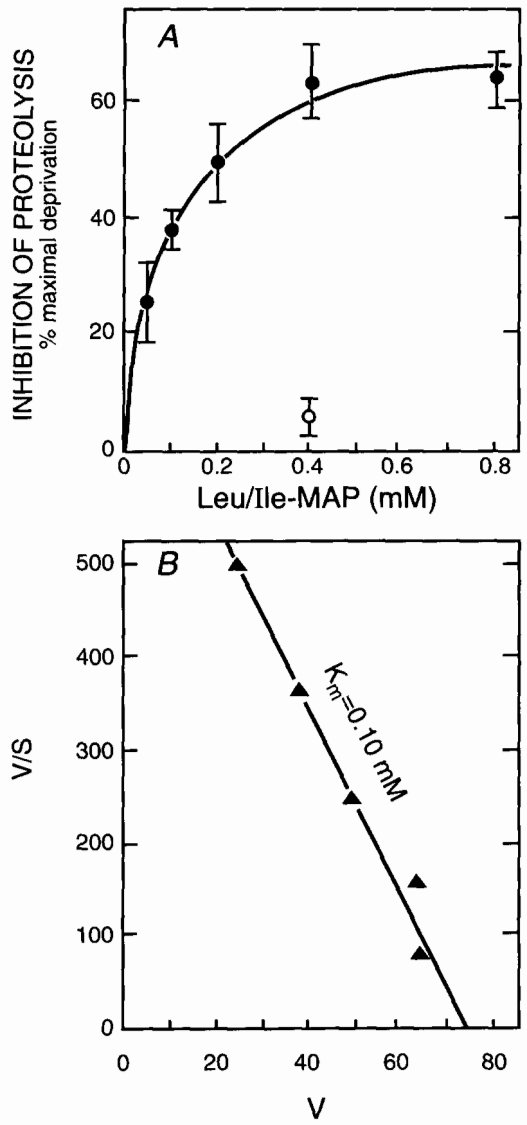


FIG. 2. **Inhibitory effectiveness of Leu_8-MAP and Ile_8-MAP on deprivation-induced proteolysis in the isolated rat hepatocyte.** Cells were incubated at a density of 1 × 10^6/ml as described under "Experimental Procedures." *A*, proteolytic inhibition with graded levels of Leu_8-MAP (●) and a single concentration (0.4 mM) of Ile_8-MAP (○); the values shown are means ± S.E. of three to six cell preparations analyzed in triplicate. *B*, *V*/*S versus V* plot of the Leu_8-MAP data from *panel A*, where *V* represents inhibition of proteolysis and *S*, the mM concentration of Leu_8-MAP.

inhibitory effectiveness.

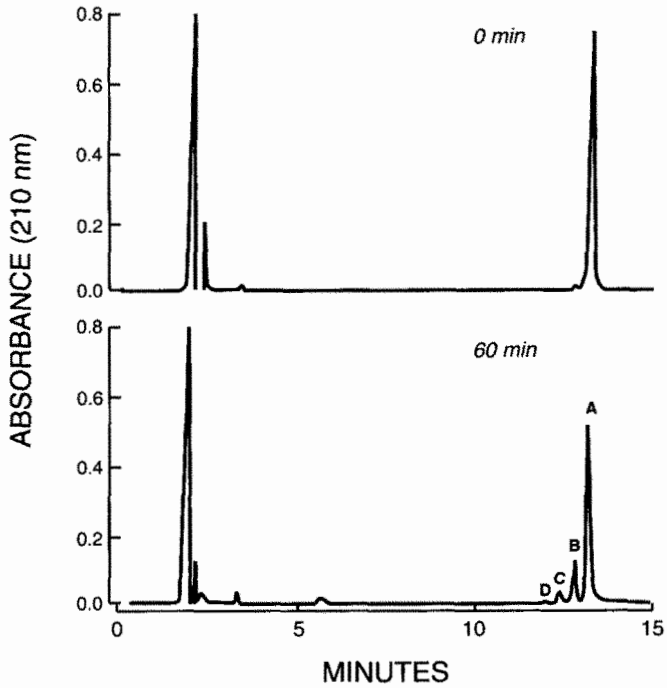
This conclusion, however, does not exclude the possibility that leucine released from the MAP peptide contributed to the proteolytic inhibition. The rate of leucine loss from Leu_8-MAP at different substrate concentrations is given in Table III. Release was concentration-dependent, and a plot of V/S *versus* V yielded a linear regression (r = 0.99) with an apparent K_m of 20 μM; when monitored at 10-min intervals, the rate of hydrolysis was constant with time (not shown). Thus, from data in Table III, the addition of 0.1 mM Leu_8-MAP to 10^6 cells/ml would elevate the concentration of leucine or leucine equivalents (Leu_8-MAP + free leucine) from 0.1 mM to about 0.13 mM over a 30-min period. If the cells had responded rapidly to this increase, proteolytic effects might have been overestimated. However, responses to changes in amino acids are known to lag by approximately 20 min (1, 3). Consequently, rates between 33 and 41 min will largely reflect amino acid levels present at the start of incubation. This was tested in two control experiments

TABLE II

Inhibition by Leu_8-MAP of deprivation-induced macroautophagic vacuole formation and proteolysis in the isolated rat hepatocyte

Isolated hepatic parenchymal cells were incubated for the determination of proteolysis in triplicate as described under "Experimental Procedures"; parallel incubations from the same cell batch were carried out for electron microscopy as described elsewhere (3, 24, 25). Thin sections were cut, stained (3), and examined with a Philips 300 or 400 electron microscope. The stereologic methods used were the same as those described earlier (3, 25) except for the method of sampling. To minimize sampling error, three areas were randomly selected from the blocks and, from each, 10–12 micrographs (×16,140) were systematically produced as in earlier studies (25). Fractional AVi (autophagosome) and AVd (autolysosome) volumes were then determined by the point method through a grid of intersecting lines (3, 25).

Additions	Macroautophagic volume densities				Induced proteolysis
	AVi	AVd	Total	Total	
	μl/ml of cytoplasm			*% inhibition*	
None	7.72	7.52	15.24	0	0
Leucine, 0.8 mM	3.63	3.91	7.54	50.5	50.3
Leu_8-MAP, 0.4 mM	2.98	2.35	5.33	65.0	63.6



Amino acids ratio of peaks

	A	B	C	D
β-Alanine	1	1	1	1
Lysine	6.80	6.73	6.78	7.03
Leucine	7.80	7.00	6.15	4.82

FIG. 3. **Degradation of Leu_8-MAP during its incubation with isolated rat hepatocytes.** Leu_8-MAP (0.2 mM) was incubated under the conditions described in Fig. 2 and samples taken at 0 and 60 min for HPLC analysis of the MAP substrate in 6% perchloric acid supernatants. The analysis was modified slightly from that detailed under "Experimental Procedures." A C18 Vydac column (4 × 150 mm, 10-μm particle size) was used, and the gradient conditions were changed as follows: eluant A was 0.05% aqueous trifluoroacetic acid and eluant B, acetonitrile with 0.05% trifluoroacetic acid; B was run at 10% for 5 min and then increased linearly over 10 min to 30% at a flow of 1 ml min^{-1}. The above chromatogram is a digitized laser scan of the original. Amino acid analyses of the separate peaks were run after acid hydrolysis as in Table I; ratios of the separate peaks are shown at the *bottom* of the graph.

by the addition of 0.02 mM leucine at 10, 20, and 30 min; proteolytic rates from 33 to 41 min were not diminished but remained the same (within 4%) as those with no additions. These findings thus rule out interference from the gradual release of leucine by endogenous proteolysis and explain why

TABLE III

Rate of leucine release from [^{3}H]Leu-MAP during incubation with isolated rat hepatocytes

Isolated hepatic parenchymal cells were incubated as described under "Experimental Procedures" for 30 min at the concentrations of [^{3}H]Leu_8-MAP listed below and a density of 1 × 10^6 cells/ml. Samples were deproteinized in ice-cold 6% perchloric acid and the [^{3}H]Leu separated from [^{3}H]Leu_8-MAP by reverese-phase HPLC as detailed under "Experimental Procedures." Radioactivity was determined in UltimaGold liquid scintillation cocktail (Packard) with the use of a Beckman LS 1800 liquid scintillation spectrometer; rates were computed from the counts divided by the specific radioactivity of [^{3}H]Leu_8-MAP. The results shown are means ± S.E. of three to four experiments.

Leu_8-MAP conc.	Rate of leucine release
μM	*nmol min^{-1}/10^6 cells*
10	0.358 ± 0.07
100	0.867 ± 0.02
400	1.074 ± 0.01
800	1.075 ± 0.04

consistent results were obtained in Fig. 2*B* with low initial concentrations of Leu_8-MAP.

Lack of Intracellular Uptake by Leu_8-MAP—Its comparatively high molecular mass (~1900 Da) and unique branched structure should render Leu_8-MAP a poor candidate for transmembrane transport. As the primary aim of this study was to determine whether leucine or a leucine derivative could inhibit macroautophagy from a plasma membrane site, the question of Leu_8-MAP's ability to be transported across the plasma membrane into the cytosol became a principal point of consideration. The cellular distribution of [^{3}H]Leu_8-MAP is shown in Table IV. Freshly isolated rat hepatocytes were incubated for 30 min at 4 and 37 °C at the lowest concentration of peptide (0.4 mM) giving a maximal inhibitory response. In contrast to leucine, which equilibrates rapidly and establishes a 1:1 concentration ratio between extra- and intracellular water, the intracellular distribution of Leu_8-MAP was virtually nil under all conditions. Thus under conditions where proteolytic inhibition was the same, the apparent intracellular concentration of the MAP peptide would be at least 100 times smaller than that of leucine. Although some MAP peptide was undoubtedly internalized by fluid-phase endocytosis, the amount would have been too small to measure in these experiments.

Even if one takes the worst case as an hypothesis and assumes that 1% of the intracellular space had equilibrated with extracellular Leu_8-MAP, the resulting intracellular concentration of the MAP peptide at 30 min, where proteolysis is maximally inhibited, would be nearly 8-fold lower than that of free leucine generated by endogenous proteolysis, where proteolytic rates are maximally accelerated. Because Leu_8-MAP and leucine both inhibit autophagy equally, one must conclude that the site of recognition by Leu_8-MAP and very likely leucine as well is not within the cytosolic compartment but on the plasma membrane or an associated vacuole.

TABLE IV
Lack of intracellular uptake of Leu_8-MAP by the isolated rat hepatocyte

Hepatocytes (3×10^6/ml) were incubated in Krebs-Ringer bicarbonate buffer and in the presence of 400 μM [^{3}H]Leu_8-MAP under the conditions specified below. At the times indicated 1 ml of cell suspension was collected and the cells spun through silicone oil into a layer of 12% sucrose and 6% perchoric acid. Labeled Leu_8-MAP was separated from [^{3}H]Leu by the same HPLC procedure used for purifying Leu_8-MAP (see "Experimental Procedures") and the radioactivity in the sample measured as in Table III. Intracellular [^{3}H]Leu_8-MAP was determined by subtracting the amount of labeled peptide in extracellular water from the total Leu-MAP label in the pellet, the former computed from the distribution of [^{14}C]inulin in parallel incubations. The difference was then divided by the amount of intracellular water, taken to be 4.7 μl/10^6 cells (26), and corrected for the specific radioactivity of the added [^{3}H]Leu_8-MAP (8.2 μCi/μM).

Temperature	Leu_8-MAP concentration normalized to EC value of 400 μ		
	Extracellular	Intracellular	Intracellular/ extracellular
4°C			
0 min	100	0.073 ± 0.78	0.0007
10 min	100	0.205 ± 1.27	0.0021
30 min	100	0.063 ± 1.30	0.0006
37°C			
0 min	100	0.073 ± 0.78	0.0007
10 min	100	0.090 ± 1.26	0.0009
30 min	100	0.220 ± 1.70	0.0022

DISCUSSION

The present findings clearly demonstrate that Leu_8-MAP through its bound leucine residues is an effective inhibitor of macroautophagy and proteolysis in the hepatocyte in the absence of its plasma membrane transport. Hence, Leu_n-MAP and probably free leucine must be recognized at the plasma membrane or within an associated vacuole. In this regard, these findings represent a significant advance over results from an analogous study based on the cellular distribution of the leucine analogue isovaleryl-L-carnitine in the perfused rat liver (11). Although the intracellular/extracellular ratio of isovalerylcarnitine was low (about 1:10), it proved to be the consequence of the analogue's rapid breakdown in the cytosolic compartment following its inward transport from the medium (11). Thus, in contrast to the present study, a site of recognition within the cytosolic compartment could not be satisfactorily excluded.

The present findings with Leu_8-MAP are of additional interest because they can be utilized to evaluate the structural requirements for leucine recognition. It is clear, for example, that the carboxyl groups of the leucine residues are not necessary for macroautophagic inhibition as they are coupled to the α- and ε-amino termini of lysine. It should be pointed out that this aspect of leucine's recognition was predicted from the retention of biological activity in a leucine analogue in which the carboxyl group was moved two carbon atoms away from the amine (13). Second, the fact that biological activity is not retained when isoleucine is substituted for leucine is in keeping with an earlier conclusion (11) that the structure of the branched chain is an important feature in the recognition process.

An intriguing difference between dose responses to Leu_8-MAP and leucine is the absence of the sharp, zonal loss of proteolytic inhibition at 1× with Leu_8-MAP, a characteristic feature of regulatory amino acid responses (1, 5, 6, 11, 16, 22) in livers expressing the low concentration-sensitive mode of amino acid control (16, 22). This dose-response, termed multiphasic (6), is strongly modified by the co-regulator alanine and insulin (6, 16, 21, 22). With the addition of 0.5 mM alanine, for example, the zonal loss is abolished, and the response to leucine becomes a single-component inhibition that plots linearly with various Michaelis-Menten transforms (16, 22). Thus, the dose response to Leu_8-MAP (Fig. 2, *A* and *B*) is identical to the combined effect of leucine and alanine.

The multiphasic response cannot be explained without presupposing the existence of multiple sites of recognition with sharply differing affinities for leucine (6, 11). Two are particularly relevant to this discussion: (i) a low concentration site of inhibition and (ii) a higher concentration site (lower affinity) mediating a signal that nulls the inhibition at 1× plasma levels (6, 11). With leucine or a leucine analogue as the regulator, the presence of alanine would inactivate the second site, possibly through a secondary allosteric mechanism. Previous studies with leucine and its analogue isovaleryl-L-carnitine have demonstrated that each ligand reacts equally well at high and low sites, alone or in combination, and both interact with 1 × alanine (11). The possibility that the two sites are located at the cell membrane was suggested by a nearly 10-fold disparity that exists between isovaleryl-L-carnitine and leucine in their intracellular/extracellular concentrations (11). Whereas isovalerylcarnitine is rapidly metabolized within the cell and fails to attain internal concentrations much greater than 10% of its external values, the corresponding ratio for leucine is close to unity (11). Because both responses are concentration-dependent, the only cellular location that satisfies requirements for both ligands is one that is directly accessible to the exterior.

With regard to the above question of why Leu_8-MAP does not manifest a zonal loss of inhibition at 0.2 mM (1 ×), we have considered three possibilities. First, the second leucine site, which nulls proteolysis at 1 ×, might be inaccessible to a bulky molecule such as Leu_8-MAP. This could occur, for example, if the two sites lie close together within a restricted space that permits only a single molecule of Leu_8-MAP to enter. In this case the higher affinity inhibitory site would have a greater probability of being occupied at the effective concentrations, and the zonal loss of proteolytic inhibition (nulling effect) might not be expressed. Second, it is remotely possible that leucine is moved in some unknown way at its external concentration to a compartmentalized site where it is recognized. If so, the movement of Leu_8-MAP might also be impaired. This notion, however, suffers from overcomplexity. As a mechanism of regulation it would require a closely integrated network of signals between the sites simply to operate the multiphasic response normally.

Third, differences could exist between the sites in their ability to recognize leucine and leucine derivatives. If so, the site mediating the nulling (zonal) phenomenon might fail to recognize the MAP peptide at the appropriate concentration. It should be noted, however, that in evaluating dose responses to several different leucine analogues (11, 13), we have not yet encountered loss of the nulling effect where low concentration inhibition was evident. Moreover, the apparent lack of a carboxyl requirement applies to both sites (13). Of the three hypotheses, we believe the first, which invokes steric hindrance by Leu_8-MAP, is the most reasonable.

The findings of this study add considerably to earlier evidence that amino acid control of deprivation-induced autophagy in the rat hepatocyte is mediated through their recognition at the plasma membrane (11, 12). In the case of leucine, a logical next step will be to demonstrate specific leucine binding. Because recognition occurs at physiological concentrations, the binding affinity will be low and difficult to detect without the use of such adjuncts as photolabeling. The MAP peptide would be particularly useful in this respect for two reasons: (i) a photoreactive adduct can be substituted for a leucine residue without loss of biological activity and (ii) its binding will be restricted to the plasma membrane and the vacuolar system.

Indeed, photobinding of an iodinatable, azide derivative of Leu_7-MAP to a high molecular weight protein (M_r ~340,000) has been demonstrated in plasma membrane fractions of the isolated rat hepatocyte (27). Although the binding is low affinity in nature, it is specific for leucine. Its functional significance remains to be established.

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