A Di-leucine Signal in the Ubiquitin Moiety

POSSIBLE INVOLVEMENT IN UBQUITINATION-MEDIATED ENDOCYTOSIS*

Received for publication, September 23, 1999, and in revised form, May 12, 2000
Published, JBC Papers in Press, May 18, 2000, DOI 10.1074/jbc.M907720199

Fubito Nakatsu, Machie Sakuma, Yo Matsuo‡, Hisashi Arase, Sho Yamasaki, Nobuhiro Nakamura§, Takashi Saito, and Hiroshi Ohno¶

From the Department of Molecular Genetics, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan, the 3Bioinformatics Group, Genomic Sciences Center, RIKEN, The Institute of Physical and Chemical Research, Wako 351-0198, Japan, and the 4Division of Molecular Membrane Biology, Cancer Research Institute, Kanazawa University, Kanazawa 920-8643, Japan

Some plasma membrane receptors in yeast are known to be internalized and degraded in lysosomes upon ligand-dependent ubiquitination. However, the role of ubiquitination in endocytosis and lysosomal degradation in higher eukaryotes has been controversial. In order to directly assess this question, we investigated the fate of chimeric molecules in which ubiquitin moiety was fused in-frame to the cytoplasmic region of membrane proteins. The chimeric proteins with the wild-type ubiquitin were endocytosed and delivered to lysosomes efficiently. Mutant ubiquitin with lysine-to-arginine substitution could still mediate endocytosis, suggesting that polyubiquitination is not required for the endocytosis. We next searched for the existence of an endocytosis signal(s) in the ubiquitin moiety, and identified a di-leucine signal, Leu^43-Ile^44. The Leu^43-Ile^44 sequence mediated endocytosis and lysosomal sorting in a Leu^43-dependent manner. These results suggest that the di-leucine signal in ubiquitin can be involved in ubiquitination-mediated endocytosis and lysosomal targeting of membrane proteins.

Selective endocytosis of plasma membrane proteins usually requires endocytosis signals within the cytoplasmic tail of the internalized proteins (1–7). Tyrosine-based (Y signal)1 and di-leucine-based (LL signal)2 signals are two of the most extensively investigated among them. Both signals are recognized by the AP-2 adaptor protein complex, a component of clathrin-coated pits, at the plasma membrane, and upon recognition, proteins having these endocytosis signals within their cytoplasmic tails are recruited to the clathrin-coated pits, which is then followed by endocytosis.

Ubiquitin is a 76-amino acid polypeptide expressed in all eukaryotic cells and is highly conserved from yeast to man (8–10). Ubiquitination, a covalent modification of proteins, has been long been known to mediate degradation of misfolded and/or proteins by proteasomes. The process usually requires polyubiquitination of degraded proteins. The expression of proteins such as cell cycle regulators and transcription factors, which has to be strictly controlled by rapid kinetics, is also regulated by polyubiquitination followed by proteasomal degradation.

Recently, other functions of ubiquitination have been unraveled; one is the degradation of proteins retained in the endoplasmic reticulum (11–14). This process has long been known as “endoplasmic reticulum degradation,” which serves as a quality control mechanism in the endoplasmic reticulum. Mutant and/or misfolded proteins in the endoplasmic reticulum, for both luminal and transmembrane proteins, are retrotranslocated into the cytosol, where they are degraded by proteasomes following polyubiquitination in most cases.

Ubiquitination has also been implicated in the internalization and degradation of plasma membrane proteins (11–13). In yeast, it has been revealed that ubiquitination serves to trigger the internalization of plasma membrane proteins into the endocytic pathway and subsequent degradation in the vacuole (12, 13). In animal cells, by contrast, the situation is not as clear. A number of plasma membrane proteins are known to be ubiquitinated. Many of them are receptor-tyrosine kinases or receptors associated with tyrosine kinases, and tyrosine phosphorylation upon ligand binding is required for their ubiquitination and subsequent internalization (11–14). In many cases, however, the ubiquitinated membrane proteins appear to be degraded through both proteasomes and lysosomes, and the role of ubiquitination in these processes has remained unclear.

In the present study, we have investigated a possible role of ubiquitination of membrane proteins by using chimeric proteins in which ubiquitin or its mutants fused with the cytoplasmic region of the invariant chain (Ii) or the interleukin-2 receptor α chain (Tac). Our results show that a single ubiquitin moiety can mediate endocytosis of these chimeric proteins, and that Leu^43-Ile^44 in the ubiquitin moiety may serve as the LL signal involved in the ubiquitin-mediated endocytosis.

EXPERIMENTAL PROCEDURES

Antibodies—An anti-lamp2 (igg110) antibody was kindly provided by Dr. Eiki Kominami (Juntendo University). 7G7.B6, a monoclonal antibody recognizing Tac, was from the American Type Culture Collection (Rockville, MD). Normal mouse IgG was purified in this laboratory. The following were purchased from commercial sources: an unlabeled and a fluorescein isothiocyanate (FITC)-conjugated anti-Ii monoclonal antibody (LN2 and M-B741, respectively) and a phycoerythrin (PE)-conjugated anti-Tac monoclonal antibody (M-A251) were from Pharmingen (San Diego, CA); a PE-conjugated anti-mouse IgG antibody was from Southern Biotechnology Associates Inc. (Birmingham AL); an FITC-conjugated anti-Tac monoclonal antibody (33B3.1) and an FITC-conjugated anti-transferrin receptor (TfR) monoclonal antibody (YD31.2.2) were from Immunotech S. A. (Westbrook, ME); Cy3-conjugated anti-

*This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to H. O. and T. S.) and also, in part, by the Hokkoku Cancer Research Promotion Foundation (to H. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed. Tel.: 81-76-265-2721; Fax: 81-76-234-4519; E-mail: hohn@kenrokou.kanazawa-u.ac.jp.

1 The abbreviations used are: Y signal, tyrosine-based signal; LL signal, di-leucine-based signal; Ii, the invariant chain; FITC, fluorescein isothiocyanate; PE, phycoerythrin; TfR, transferrin receptor; ENAc, epithelial Na+ channel; FACs, fluorescence-activated cell sorter.
mouse IgG, Cy3-conjugated anti-rabbit IgG and FITC-conjugated anti-rabbit IgG antibodies were from Jackson ImmunoResearch (West Grove, PA).

**Plasmid Construction**—pcDNA3-Ii was a kind gift from Dr. Paul A. Roche (National Institutes of Health). The expressible constructs for ΔIi and Ub-ΔIi chimeric proteins were prepared by polymerase chain reaction-oriented site-directed mutagenesis as described elsewhere (15), based on pcDNA3-Ii as the template. Ubiquitin cDNA was first obtained by reverse transcriptase-polymerase chain reaction. Tac chimeras were made by ligating the synthetic oligonucleotides as described previously (16). Sequences of polymerase chain reaction-oriented constructs were confirmed by dideoxynucleotide sequencing.

**Cell Culture and Transfection**—HeLa cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma) (regular medium). HeLa cells stably expressing Ii or Tac-CD35vLL were grown in regular medium supplemented with 0.5 mg/ml genetin (Life Technologies, Inc.). Transfections were performed using calcium phosphate precipitation as described previously (17).

**Immunofluorescence Staining**—For antibody uptake, 36–40 h after transfection, HeLa cells grown on coverslips were cultured with appropriate antibodies in regular medium for indicated periods, washed with phosphate-buffered saline and fixed with 2% formaldehyde at room temperature for 20 min. Cells were then stained with the Cy3-anti-mouse IgG antibody as described (18). For studying co-localization in human cells, Ii or Tac-CD35vLL were grown in regular medium supplemented with 0.5 mg/ml genetin (Life Technologies, Inc.). Transfections were performed using calcium phosphate precipitation as described previously (17).

**Sorting Signal Competition Assay**—The assay was basically performed as described previously (17). Briefly, chimeric proteins were overexpressed in HeLa cells by transient transfection using 5 μg of plasmid DNA in a single well of 6-well plates. Ub-ΔIi constructs were transfected into HeLa cells stably expressing Tac-CD35vLL, while Tac constructs were transfected into those stably expressing Ii. Thirty-six to forty h after transfection, cells were harvested in phosphate-buffered saline containing 20 mM EDTA. When Ub-ΔIi chimeras were used as competitors, cells were incubated with anti-Ii antibody followed by PE (polyclonal rabbit IgG antibody) for 45 min on ice, washed, and analyzed by the FACS-Calibur. It is established that the overexpression of a given protein with endocytosis signals causes underestimation of the internalization rate of the protein because of the saturation of a component(s) for internalization (17). To avoid this possibility, a population of cells expressing the chimeric proteins at high levels, as judged by a high level expression of the GFP, was excluded from the analysis, and only the data for cells expressing GFP at intermediate levels were collected. Amounts of each chimeric protein remaining on the cell surface were defined as the specific fluorescence value, which was obtained by subtracting the mean PE fluorescence value of the GFP negative (i.e. untransfected population) from that of the GFP intermediate (i.e. the population with a moderate expression of chimeric proteins). The same

**RESULTS**

**Ubiquitin Mediates Endocytosis of a Chimeric Protein in HeLa Cells**—In order to examine the direct role of ubiquitin in endocytosis, we made chimeric molecules of ubiquitin with a membrane protein, Ii. Ii is a type II transmembrane protein that contains two LL signals in its cytoplasmic tail (19) (Fig. 1). We first made a deletion mutant of Ii, which lacked most of the cytoplasmic tail of Ii including the two LL signals (Fig. 1, ΔIi). We then fused the wild-type ubiquitin sequence to the N terminus of ΔIi to construct Ub-ΔIi (Fig. 1). Ii, ΔIi, or Ub-ΔIi was transiently expressed in HeLa cells, and their internalization was examined by the anti-Ii antibody uptake for 4 h followed by indirect immunofluorescence staining (see “Experimental Procedures” for details). Ii was internalized from the cell surface as reported previously (19) (Fig. 2B). When most of the cytoplasmic tail of Ii was deleted, the internalization was abolished (Fig. 2A, ΔIi), consistent with the requirement of LL signals for the internalization (19). In contrast, Ub-ΔIi was internalized and gave a punctate staining like Ii (Fig. 2C). It is known that Lys65 of ubiquitin is a major polyubiquitination site for proteasomal recognition (9, 10). In order to rule out the involvement of proteasomes in the internalization, Lys65 in the Ub-ΔIi chimeric molecule was changed to Arg (Fig. 1, Ub(K48R)-ΔIi). As shown in Fig. 2D, this chimera was also internalized. Furthermore, a chimeric molecule in which all of the 7 Lys residues in ubiquitin were substituted with Arg still underwent endocytosis (data not shown). Since the transfection efficiency was consistently low when this molecule was used, we decided to use Ub(K48R)-ΔIi for the rest of this study. Ubiquitin also mediated the endocytosis of another chimeric protein, in which ubiquitin was appended at the C terminus of Tac, a type I integral membrane protein (data not shown).

We next tested whether ubiquitin-mediated endocytosis leads the ubiquitinated proteins to endosomal/lysosomal pathways. As shown in Fig. 2, the internalized Ub(K48R)-ΔIi partially colocalized both with TIR (Fig. 2, E-G) and lgp110 (lamp2) (Fig. 2, H-J), suggesting that ubiquitin delivers the protein to the endosomal/lysosomal compartment. Taken together, the above results suggest that monoubiquitination could serve as an endocytosis as well as a lysosomal targeting signal for the ubiquitinated plasma membrane proteins in mammalian cells, as was the case in yeast.

**Ubiquitin Possesses LL Signal Activity**—The ability of ubiquitin to trigger endocytosis of the chimeric proteins raised the possibility that ubiquitin itself has an active endocytosis sig
signal (RASDKQTLL) from CD3 which the cytoplasmic tail of Tac was replaced with the LL expression of Tac-CD3 was analyzed. FACS analysis showed that the level of surface overexpression of Ii but not D (Fig. 3, B-D) did not affect the expression level of TfR on the cell surface. In competition assay, we first needed to establish a HeLa cell marker protein with an LL signal suitable for a sorting signal of the same type to the cell surface (17). In another words, for example, the competition occurs among Y (or LL) signals but not between Y and LL signals. We took advantage of this system to test whether signals possess a Y signal in the cytoplasmic tail, was used as an endogenous marker protein. Since there is no endogenous marker protein with a Y signal, TfR, ubiquitin possesses Y or LL signals. For the Y signal, TfR, Ii also enhanced the cell surface expression of Tac-CD3γLL without affecting the expression of TfR (Fig. 3, C, D, G, and H). These results indicate that ubiquitin has an LL signal(s) which competes with the LL signal of Tac-CD3γLL.

**Leu^42-Ile^44** but Not Leu^69-Val-Leu^71 Acts as an Active Di-leucine Signal**—The above results prompted us to search for LL signals in the amino acid sequence of ubiquitin, and two candidate sequences for LL signals, Leu^42-Ile^44 and Leu^69-Val-Leu^71, were found (Fig. 4A). To examine whether these putative LL signals actually possess endocytosis ability, we constructed a Tac chimera in which the cytoplasmic tail of Tac was replaced with either Ub(K48R)-Ii (Tac-LI) or with Ub(K48R)-Ii (Tac-LVL) (Fig. 4B). These chimeric proteins were transiently expressed in HeLa cells, and their subcellular localization was determined by the anti-Tac antibody uptake (Fig. 5). Confocal microscopic analysis revealed that Tac-LI (Fig. 5B), but not Tac-LVL (Fig. 5C), internalized the anti-Tac antibody into the cell. When Leu of Tac-LI was substituted with Ala (Tac-AL in Fig. 4B) to destroy the LL signal, the cells did not internalize the antibody anymore (Fig. 5D), further confirming that Leu^42-Ile^44 has the LL signal activity. The internalized Tac-LI colocalized both with lgp110 (lamp2) (Fig. 5, E-G) and with TfR (Fig. 2, H-J), suggesting that the LI sequence delivers the proteins to the endosomal/lysosomal compartment.

Next, we performed a sorting signal competition assay using these chimeric proteins. HeLa cells stably expressing Ii were transfected with these constructs and the surface expression of Ii (for the LL signal) or TfR (for the Y signal) was examined (Fig. 6). Overexpression of Tac-LI but neither Tac-LVL nor Tac-AL increased the surface expression of Ii. Since the level of

---

**Fig. 2. A ubiquitin moiety directs the endocytosis and endosomal/lysosomal localization of Ub-ΔIi chimeric proteins.** HeLa cells transfected with pcDNA3-ΔIi (A), Ii (B), Ub-ΔIi (C), or Ub (K48R)-ΔIi (D-J) were cultured in the presence of anti-Ii antibody for 4 h. After fixation, the cells were stained with Cy3-anti-mouse IgG antibody. In E-J, the cells were double-stained as described under “Experimental Procedures.” E and H show the localization of Ub(K48R)-ΔIi. F depicts the localization of lgp110 in the same cell as shown in E, whereas G depicts the merged image of E and F. Similarly, I depicts the localization of TfR in the same cell as shown in H, whereas J depicts the merged image of H and I. All samples were analyzed by confocal microscopy. Scale bar, 10 μm.
the surface expression of TfR was not affected upon the over-expression of any of these chimeric proteins, this effect was specific for the LL signal (Fig. 6).

To further analyze the role of the Leu$^{43}$-Ile$^{44}$ signal in ubiquitin quantitatively, we measured the internalization rate of the surface-attached anti-Tac antibody. Fig. 7 showed that Tac-LI was rapidly internalized, whereas the internalization rate of Tac-AI was reduced to the background level of Tac, indicating that the internalization was totally dependent on the Leu$^{43}$-Ile$^{44}$ signal. Taken together, it became clear that Leu$^{43}$-Ile$^{44}$ in ubiquitin serves as an active LL signal in cells.

Leu$^{43}$-Ile$^{44}$ Mediates Internalization in the Context of Ubiquitin Moiety—Since Leu$^{43}$-Ile$^{44}$ has the LL signal activity, we wanted to determine whether the Leu$^{43}$-Ile$^{44}$ signal is active in the context of the ubiquitin sequence. To this end, we made Ub(K48R/L43A)-ΔII and Ub(K48R/I44A)-ΔII, in which either Leu$^{43}$ or Ile$^{44}$ was substituted with alanine in addition to K48R substitution (Fig. 8A). The constructs were transiently expressed in HeLa cells stably expressing Tac-CD3γLL, and the

---

**FIG. 3.** Ub-ΔII chimeric proteins affect the surface expression of membrane proteins with the LL signal but not with the Y signal. HeLa cells transiently transfected with ΔII (A and E), II (B and F), Ub-ΔII (C and G), or Ub(K48R)-ΔII (D and H) were subjected to sorting signal competition assay as described under “Experimental Procedures.” The intensity of FITC for the transfected population (unfilled curve) was compared with that of the untransfected population (filled curve) in the same samples.

**FIG. 4.** Amino acid sequence of ubiquitin (A) and a schematic representation of Tac chimeras containing putative LL signals (B). A, amino acid sequence of ubiquitin deduced from GenBank accession number M26800. Two putative LL signals are underlined. B, schematic representation of Tac chimeras. C-terminal 4 amino acids (out of the 10-amino acid-long putative cytoplasmic tail) of Tac were replaced with the amino acid sequences containing either of the putative LL signals, IPPDQQRLI or ESTLHLVL, as depicted in A. TM, transmembrane domain.

---

**FIG. 5.** Leu$^{43}$-Ile$^{44}$ but not Leu$^{43}$-Val-Leu$^{44}$ has endocytosis activity. HeLa cells transiently expressing Tac (A), Tac-LI (B, E–J), Tac-LVL (C), or Tac-AI (D) were cultured in the presence of anti-Tac antibody for 4 h, fixed, and incubated with Cy3 anti-mouse IgG antibody. In E–J, the cells were double-stained as described under “Experimental Procedures.” E and H show the localization of Tac-LI. F depicts the localization of lgp110 in the same cell as shown in E, whereas G depicts the merged image of E and F. Similarly, I depicts the localization of TfR in the same cell as shown in H, whereas J depicts the merged image of H and I. All samples were analyzed by confocal microscopy. Scale bar = 10 μm.
sorting signal competition assay was employed. As shown in Fig. 8B, the overexpression of either Ub(K48R/L43A)-ΔII or Ub(K48R/I44A)-ΔII failed to increase the surface expression of Tac-CD3gLL (Fig. 8B, d and e), suggesting that Leu43-Ile44 indeed acts as an LL signal in the context of the ubiquitin sequence.

We next measured the internalization rates of Ub(K48R/L43A)-ΔII, Ub(K48R/I44A)-ΔII, and Ub(K48R)-ΔII in HeLa cells to examine the effect of the L43A and I44A mutations on the endocytosis mediated by ubiquitin moiety (Fig. 8C). Although a substantial internalization was observed for both Ub(K48R/L43A)-ΔII and Ub(K48R/I44A)-ΔII, their internalization rates were significantly lower than that of Ub(K48R)-ΔII at all time points measured.

We also examined the effect of the mutation of Leu43-Ile44 on endocytosis morphologically. Ub(K48R/L43A)-ΔII was transiently transfected in HeLa cells, and its internalization measured by the anti-II antibody uptake was compared with that of Ub(K48R)-ΔII (Fig. 9). Ub(K48R/L43A)-ΔII gave little, if any, intracellular staining with 10-min uptake (Fig. 9b), whereas Ub(K48R)-ΔII was rapidly internalized to show a significant intracellular staining at the same uptake period (Fig. 9a). A longer uptake period, however, allowed Ub(K48R/L43A)-ΔII to internalize the antibody to give a substantial staining of the intracellular vesicular structures (data not shown). Taken together, our results suggest that Leu43-Ile44 acts as an internalization signal in the ubiquitin moiety.

**DISCUSSION**

In this study, we showed that a single ubiquitin moiety directs the endocytosis and endosomal/lysosomal localization of chimeric plasma membrane proteins fused in-frame with ubiquitin in their cytoplasmic region in mammalian cells. Several lines of evidence suggest that ubiquitination of the cytoplasmic tail of plasma membrane receptors is important for triggering their endocytosis in yeast (11–14). Especially, Terrel et al. (20) reported that a single ubiquitin moiety, rather than polyubiquitin chains, was sufficient to signal endocytosis.

In mammals, in contrast, the role of ubiquitination in endocytosis of many plasma membrane receptors has remained unclear. Colony stimulating factor-1 receptor is one of the few mammalian proteins whose ubiquitination is believed to be involved in their endocytosis (21). Recently, Lee et al. (21) reported that a single ubiquitin moiety, rather than polyubiquitin chains, was sufficient to signal endocytosis.

ENaC is another mammalian plasma membrane protein whose ubiquitination is thought to be associated with its internalization. ENaC is the epithelial Na⁺ channel whose defect causes Liddle's syndrome, an autosomal dominant form of hypertension (22, 23). It has been reported that the cytoplasmic tails of β and γ subunits of the channel contain a proline-rich PY motif that interacts with the WW domains in Nedd4, a widely expressed E3 ubiquitin-protein ligase (24). A recent study has shown that the binding of Nedd4 to ENaC alone is not sufficient, but Cys854 in the ubiquitin-ligase domain of Nedd4, which forms a thioester bond with ubiquitin, is required.
for the down-modulation of ENaC (25). In addition, Staub et al. (26) showed that mutation of Lys residues in the γ subunit leads to both a reduction of ubiquitination and an increased number of channels at the plasma membrane, which causes an elevated channel activity. These data suggest that the ubiquitination of ENaC is physiologically important for the internalization.

Ubiquitination of growth hormone receptor is also thought to be involved in its endocytosis (27, 28). In the case of growth hormone receptor, however, it has recently been suggested that its internalization requires the recruitment of the ubiquitin conjugation system to the cytoplasmic tail of the receptor rather than its ubiquitination itself (14, 29).

Taken together, it is likely that there are at least two mechanisms for ubiquitination-mediated endocytosis of plasma membrane proteins; one seems to involve the direct ubiquitination of the endocytosed proteins as suggested in the present study, whereas the other likely involves the ubiquitination of endocytic machinery rather than the endocytosed protein itself. Both pathways are probably required for a fully efficient, rapid endocytosis of the ubiquitinated proteins. It is also possible that cells evolve with more than one mechanism to ensure complete endocytosis followed by degradation (i.e. desensitization) of the proteins, as many of them are growth factor receptors, and failure in desensitization may lead to undesired proliferation and subsequent tumorigenesis.

We also showed that Leu43-Ile44 in the ubiquitin moiety possesses the LL signal ability through different approaches. This suggests that the LL signal may play a direct role in the ubiquitin-mediated endocytosis of plasma membrane proteins; one seems to involve the direct ubiquitination of the endocytosed proteins as suggested in the present study, whereas the other likely involves the ubiquitination of endocytic machinery rather than the endocytosed protein itself. Both pathways are probably required for a fully efficient, rapid endocytosis of the ubiquitinated proteins. It is also possible that cells evolve with more than one mechanism to ensure complete endocytosis followed by degradation (i.e. desensitization) of the proteins, as many of them are growth factor receptors, and failure in desensitization may lead to undesired proliferation and subsequent tumorigenesis.

Leu43 is a buried residue in the three-dimensional structure of ubiquitin whereas Ile44 is exposed (31), as Shih et al. (30) also mentioned. Therefore, it seems difficult for an interacting molecule to the LL signal, such as AP-2 (32, 33), to access Leu43-Ile44. The fold of ubiquitin is basically a single-layered five-stranded β-sheet, one side of which is covered by an α-helix and
a few loop structures. The other side of the sheet is totally exposed to solvents. Leu$^{43}$ is located on one of the five $\beta$-strands constituting the $\beta$-sheet, with its side chain buried in the hydrophobic core. The $\beta$-strand consists of six residues (Gln$^{40}$ to Phe$^{45}$), which is located at the edge of the $\beta$-sheet and exposed to solvents, is very short and does not seem to attach to the $\beta$-sheet firmly. In addition, ubiquitin is a relatively small protein. The short $\beta$-strand at the edge of the sheet might therefore be susceptible to conformational change induced by an interacting molecule, such as AP-2, resulting in Leu$^{43}$ to become relatively accessible to the interacting molecule. In accordance with this, an NMR structure of ubiquitin (PDB code 1D3Z) shows a slightly better exposure of Leu$^{43}$ than the crystal structure. Another possibility may be that Leu$^{43}$ does not participate in direct binding to AP-2 but rather stabilizes the structure required for AP-2 binding. Structural analysis of the interaction between the LL signal and AP-2 will answer this question.

The LL signal is one of the best characterized sorting signals found in the cytoplasmic tail of many plasma membrane proteins (1, 3). AP-2 directly binds the LL signal at the plasma membrane, and leads the proteins having the signal in their cytoplasmic tails into the endocytic pathway (32, 33). The human immunodeficiency virus type 1 accessory protein, Nef, also has an active LL signal and binds to AP-2 via its LL signal. It has been reported that Nef also binds to the cytoplasmic tail of CD4, a co-receptor on helper T cells, and formation of the ternary complex among CD4, Nef, and AP-2 is important for the down-modulation of CD4 (34–36). The interaction of Nef with CD4 (i.e., hydrogen bond) is different from the covalent modification of membrane proteins with ubiquitin. Nevertheless, in both cases, a cytosolic molecule containing an endocytosis signal connects to the plasma membrane receptor, which does not possess an endocytosis signal by itself, to endocytic machinery. This type of mechanism for endocytosis may play a role in a variety of aspects in regulated endocytosis.

In conclusion, we identified an active LL signal in ubiquitin, which may serve as an endocytosis signal for ubiquitinated plasma membrane receptors. Further experiments will be needed to attain a complete understanding of the mechanism of ubiquitination-mediated endocytosis and the subsequent degradation of plasma membrane proteins.

Acknowledgments—We thank Drs. E. Kominami (Juntendo University) and P. A. Roche (National Institutes of Health) for generously providing the reagents. We also thank Dr. J S. Bonifacino (National Institutes of Health) for reading the manuscript.
A Di-leucine Signal in the Ubiquitin Moiety: POSSIBLE INVOLVEMENT IN UBQUITINATION-MEDIATED ENDOCYTOSIS
Fubito Nakatsu, Machie Sakuma, Yo Matsuo, Hisashi Arase, Sho Yamasaki, Nobuhiro Nakamura, Takashi Saito and Hiroshi Ohno

J. Biol. Chem. 2000, 275:26213-26219.
doi: 10.1074/jbc.M907720199 originally published online May 18, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M907720199

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 13 of which can be accessed free at http://www.jbc.org/content/275/34/26213.full.html#ref-list-1