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A Role for Acidic Residues in Di-leucine Motif-based Targeting to the Endocytic Pathway*

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Recent reports have suggested that major histocompatibility complex class II molecules load peptide through a specialized compartment of the endocytic pathway and are targeted to this pathway by association with invariant chain (Iip31). Therefore we used a site-directed mutagenesis approach to determine whether Iip31 possesses novel protein targeting signals. Our results indicate that two di-leucine-like pairs mediate Iip31 targeting and that an acidic amino acid residue four or five residues N-terminal to each Iip31 di-leucine-like pair is required for endocytic targeting. Results from additional testing with hybrid Iip31 molecules indicate that the acidic residues N-terminal to di-leucine pairs are critical for accumulation of these molecules in large endocytic vesicles and in some cases provide a structure favorable for internalization. The acidic residues N-terminal to di-leucine pairs are important in some sequence contexts in providing a structure favorable for internalization, whereas in other contexts an acidic residue is critical for targeting to, and formation of, large endocytic vesicles. Although our results do not support the idea that Iip31 possesses unique protein targeting motifs, they do suggest that di-leucine motifs may be recognized as part of a larger secondary structure. In addition, our data imply that the targeting motif requirements for internalization may differ from the requirements for further transport in the endocytic pathway.

The major histocompatibility complex (MHC)[1] class II molecules are expressed on antigen-presenting cells such as B cells, macrophages, and dendritic cells and present primarily antigenic peptides derived from exogenous proteins to helper T cells (for review see Neefjes and Ploegh, 1992; Germain and Margulies, 1993; Germain, 1994; and Cresswell, 1994). The Iip31 molecule is a type II transmembrane protein that associates with MHC class II molecules in the endoplasmic reticulum and limits binding of these molecules to antigenic peptides (Roche and Cresswell, 1990; Teyton *et al.*, 1990) while escorting them (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990) to a specialized endosomal compartment (Peters *et al.*, 1991; Amigorena *et al.*, 1994; Tulp *et al.*, 1994; West *et al.*, 1994; Qui *et al.*, 1994). In this compartment Ii is released, and class II molecules bind peptides before appearing at the cell surface. We wished to determine the precise targeting information in the Iip31 cytoplasmic tail and in particular whether Iip31 contains a novel targeting motif that may explain the transport of MHC class II molecules to a specialized endocytic compartment dedicated to peptide loading.

Previous studies have demonstrated that information contained in the Iip31 cytoplasmic tail is both necessary and sufficient for Iip31 targeting to the endosomal/lysosomal compartment. Deletion analyses showed that the first 16 amino acid residues were required for Iip31 endosomal/lysosomal targeting (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990). Site-directed mutagenesis localized two signals in the Iip31 cytoplasmic tail which could independently target invariant chain to the endosomal system; one signal contains leucine and isoleucine at positions 7 and 8 of the tail (Pieters *et al.*, 1993). The second signal contains methionine and leucine at positions 16 and 17 (Odorizzi *et al.*, 1994; Bremnes *et al.*, 1994).

These Ii targeting motifs are part of a larger family of di-leucine-related targeting motifs that mediate lysosomal targeting and internalization from the cell surface (for review see Sandoval and Bakke, 1994). Di-leucine-related signals have been described for T lymphocyte antigen receptor CD3 γ and δ chains (Letourneur and Klausner, 1992), cation-dependent and -independent mannose 6-phosphate receptors (CD-M6PR and CI-M6PR, respectively; Johnson and Kornfeld, 1992a, 1992b; Chen *et al.*, 1993), interferon-γ receptor (Farrar and Schreiber, 1993), lysosomal integral membrane protein (LIMP) II (Ogata and Fukuda, 1994; Sandoval *et al.*, 1994), glucose transporter 4 (Verhey and Birnbaum, 1994), T lymphocyte cell surface CD4 molecule (Aiken *et al.*, 1994), and Iip31 (Odorizzi *et al.*, 1994; Bremnes *et al.*, 1994). Di-leucine-related variants that permit different degrees of LIMP II lysosomal targeting include Leu-Ile, Leu-Val, and Ile-Ile, whereas Val-Ile does not target LIMP II to lysosomes (Ogata and Fukuda, 1994; Sandoval *et al.*, 1994). Substitution of alanine for one or both hydrophobic residues abrogates lysosomal localization and internalization.

In this report we use alanine scan mutagenesis to ascertain the sequence requirements for Iip31 targeting of class II molecules to a specialized endosomal compartment. Our analysis

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[1] The abbreviations used are: MHC, major histocompatibility complex; CD-M6PR and CI-M6PR, cation-dependent and -independent mannose 6-phosphate receptor, respectively; LIMP, lysosomal integral membrane protein; PBS, phosphate-buffered saline; TGN, trans-Golgi network.

confirmed earlier studies demonstrating the importance of two di-leucine-like motifs for internalization of Ii from the cell surface and intracellular targeting to large vesicular endosomal structures. In addition, we uncovered a second critical component: an acidic amino acid residue four or five residues N-terminal to each of these di-leucine-like signals which is required for efficient targeting. By constructing hybrid molecules of Iip31 containing heterologous di-leucine-based signals and using alanine substitution analyses we have shown that acidic residues are required for the formation of the large endocytic vesicles induced by Iip31 expression. Thus, the di-leucine-based pair is part of a larger internalization and endocytic targeting motif that includes an N-terminal acidic residue. In accordance with the tyrosine-based internalization motifs, we suggest that the secondary structure of the di-leucine-containing cytoplasmic tails is an important part of the recognition motif. Targeting of Ii to the compartment for peptide loading is discussed in light of these findings.

EXPERIMENTAL PROCEDURES

Recombinant cDNA Constructs—The cDNAs encoding full-length Iip31 and the Δ2–16Ii N-terminal deletion mutant in the pCMU IV expression vector are as described (Lotteau *et al.*, 1990). All additional cDNAs encoding human Iip31 with mutant cytoplasmic tails were constructed by oligonucleotide cassette cloning using the *Sal*I site introduced at codon 19 of the wild-type Iip31 cDNA (Lotteau *et al.*, 1990). The first 57 nucleotides of this cDNA were removed by cleaving with *Xba*I and *Sal*I, and the resulting 4.7-kilobase fragment was electrophoresed and purified using the GeneClean II kit (Bio 101, Vista, CA) according to the manufacturer's instructions. Oligonucleotides encoding the mutant sequences were then ligated with this fragment. Oligonucleotides were synthesized using a Gene Assembler (Pharmacia Biotech Inc.) and purified by fast protein liquid chromatography using a Pre RPC/HR5/5 column (Pharmacia) according to the manufacturer's instructions. Alternatively, oligonucleotides in 1 ml of ammonium hydroxide were applied to an NAP-10 column (Pharmacia) and eluted with 1.4 ml of H_2O. All cDNA constructs in pCMU IV were sequenced by fluorescent dye terminator sequencing.

HeLa Cell Transfection—HeLa cells were grown in cDMEM (Dulbecco's modified Eagle's medium (Biowhittaker, Walkersville, MD) supplemented with fetal bovine serum (8%; Biowhittaker), glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml)). Transfections were done using the calcium phosphate method essentially as described (Schutze *et al.*, 1994).

Internalization of ^{125}I-Labeled BU45—Monoclonal antibody BU45 (Research Diagnostics, Flanders, NJ), recognizing an epitope in the human Ii lumenal domain, was labeled with ^{125}I using the chloramine-T method as follows. The antibody was transferred to PBS by adding 200 μl (40 μg) of BU45 to 2.3 ml of PBS and applying the antibody to a Centricon 30 filtration device (Amicon, Beverly, MA) to recover 50 μl of BU45. For the iodination reaction, 50 μl of BU45 was incubated with 1 mCi of $Na^{125}I$ (10 μl, Amersham Corp.) and 10 μl of chloramine T (4.2 mg/ml in 250 mM sodium phosphate, pH 7.0) for 1 min, then with 5 μl of sodium bisulfide (4.3 mg/ml in 250 mM sodium phosphate, pH 7.0) for 2 min. Five hundred μl of KI (100 mg/ml in PBS, pH 7.4) was added, and the mixture was applied to a PD-10 column (Pharmacia) equilibrated with PBS containing 1% bovine serum albumin (Sigma). Fractions (300 μl each) were collected, and fractions 10–12, containing the ^{125}I-labeled BU45, were pooled. Typically, the reaction yielded 50–70% ^{125}I incorporation, with a specific activity of about 2×10^7 cpm/μg of BU45.

Two days after transfection, HeLa cells in 100-mm dishes were treated with 1 ml of trypsin-EDTA (Life Technologies, Inc.) and resuspended with 9 ml of cDMEM. One-ml aliquots were distributed into six-well plates (Corning, Corning, NY), and cells were cultured for 12–18 h. Cells were washed on ice three times with 3 ml of ice-cold PBS containing Ca^{2+} and Mg^{2+} (Irvine Scientific, Santa Ana, CA), then incubated with 5×10^6 cpm of ^{125}I-labeled BU45 in cold PBS with 1% bovine serum albumin, 1 ml/well, for 1 h. Cells were washed four times with 3 ml of cold PBS, then incubated with Dulbecco's modified Eagle's medium containing 1% bovine serum albumin, 2 mM glutamine, 10 mM HEPES at 37 °C, 5% CO_2, or on ice. After incubation at 37 °C, plates were placed on ice, and the cells were washed once with cold PBS. Incubation with cold acid (0.5 M acetic acid in 0.15 M NaCl, pH 2.5; 0.5 ml/well) for 7 min removed the remaining cell surface-bound ^{125}I-labeled BU45. Addition of 2 ml of PBS neutralized the acid. The acid wash was removed, and cells were washed with 1 ml PBS, which was added to the original acid wash. Cells were lysed with 1 N NaOH. For each transfectant at each time point, the percentage of internalized ^{125}I-BU45 was calculated as $((A - B)/(A - B + C)) \times 100$, where A = cpm in the cell lysate of acid-washed cells incubated at 37 °C, B = cpm in the cell lysate of the corresponding acid-washed cells incubated at 4 °C, and C = cpm in the acid wash of cells incubated at 37 °C. In all cases, the acid wash removed >95% of the initial cell surface-bound ^{125}I-BU45. Background binding of ^{125}I-labeled BU45 to nontransfected HeLa cells in 35-mm dishes at 4 °C was always less than 1,000 cpm.

Indirect Immunofluorescence Microscopy—HeLa cells were transiently transfected as described above. To detect Ii, indirect immunofluorescence microscopy was done essentially as described (Jackson *et al.*, 1990), except that antibodies were diluted in PBS with 0.2% bovine skin gelatin (Sigma) and 0.2% saponin (Sigma), and coverslips were washed three times between staining steps with PBS/gelatin/saponin solution. Cells were stained with rabbit antiserum C351 (generously provided by Dr. Vito Quaranta, the Scripps Research Institute) to the Ii lumenal domain sequence KESLELEDPSSGLGVTKDL, followed by fluorescein-conjugated, affinity-purified goat antibodies to rabbit IgG (Cappell Laboratories, Malvern, PA).

RESULTS

The Iip31 cytoplasmic tail contains two independent di-leucine-based pairs, Leu-Ile and Met-Leu, which direct internalization and endocytic targeting of Iip31 or heterologous extracytoplasmic domains[2] (Odorizzi *et al.*, 1994; Bremnes *et al.*, 1994). To define further the sequence requirements for di-leucine-based internalization and endocytic targeting, we selectively altered the Iip31 cytoplasmic tail sequence and used two assays to measure the targeting of Iip31 cytoplasmic tail mutants to the endocytic compartment. First, we measured Iip31 internalization from the cell surface of transiently transfected HeLa cells by quantitating the uptake of cell surface-bound ^{125}I-labeled BU45 antibody to Ii as described under "Experimental Procedures." We used intact BU45 antibody, as it has been shown that internalization of ^{125}I-labeled BU45 and of ^{125}I-labeled BU45 Fab fragments is equivalent (Roche *et al.*, 1993). Second, we analyzed the subcellular localization of wild-type and mutant Iip31 molecules by indirect immunofluorescence microscopy. The precise location in the endosomal/lysosomal pathway in which Iip31 accumulates at steady state remains controversial. The observation that Iip31 colocalizes with endocytic markers (Romagnoli *et al.*, 1993) and with lysosomal-associated membrane protein (Pieters *et al.*, 1993)[3] suggests that it is located in late endosomes or lysosomes.

Amino Acid Residues in Addition to the Leu-Ile or Met-Leu Pair Are Required for Iip31 Internalization and Endosomal Targeting

We tested deletion mutants for internalization and vesicular targeting to determine if the di-leucine-like pair forms a complete targeting motif or if additional residues are required. The Δ2–7Ii deletion mutant (Table I), which retains the Met-Leu pair and contains the di-leucine-like pair Met-Ile at its N terminus, was internalized at a rate similar to wild-type Iip31 (Fig. 1). The Δ2–11Ii deletion mutant (Table I) retains the Met-Leu pair, and its internalization rate was also similar to wild-type Iip31 (Fig. 1). In contrast, the Δ2–13Ii, Δ2–16Ii, and Δ2–26Ii deletion mutants were not internalized (Fig. 1). As expected, the Δ2–26Ii mutant lacking both di-leucine-based pairs was not internalized. In the Δ2–16Ii mutant, placement of the Met-Leu pair at the cytoplasmic tail N terminus might compromise the internalization signal, as was the case with the Leu-Ile pair at the C terminus of the LIMP II cytoplasmic tail (Ogata and Fukuda, 1994). The Δ2–13Ii mutant, however, contained the Met-Leu pair four residues from the N terminus,

[2] L. Pond, unpublished results.

[3] L. Pond, unpublished observation.

TABLE I

Summary of internalization and large vesicle formation by Ii cytoplasmic tail deletion and alanine scan mutants

Data from Figs. 1, 2, 3, and 4 are summarized. Dashes denote identity with the wild-type Iip31 amino acid sequence. +, indicates that internalization and large vesicle formation are similar to wild-type Iip31. −, indicates no internalization or large vesicle formation.

		Internalization/large vesicles
Wild-type Iip31	MDDQRDLISNNEQLPMLGRRPGAPESKCSR	+
	1 10 20 30	
Ii mutants		
Δ2–7	M----------	+
Δ2–11	M----------	+
Δ2–13	M----------	−
Δ2–16	M----------	−
Δ2–26	M----	−
L7S,I8S	------SS----------	+
M16S,L17S	----------SS----------	+
L7S,I8S,M16S,L17S	------SS-------SS----------	−
D2A,M16S,L17S	-A----------SS----------	−
D3A,M16S,L17S	--A----------SS----------	−
Q4A,M16S,L17S	---A----------SS----------	+
R5A,M16S,L17S	----A----------SS----------	+
D6A,M16S,L17S	-----A----------SS----------	+
L7A,M16S,L17S	------A--------SS----------	−
I8A,M16S,L17S	-------A-------SS----------	−
S9A,M16S,L17S	--------A------SS----------	+
L7S,I8S,S9A	------SSA----------	+
L7S,I8S,N10A	------SS-A----------	+
L7S,I8S,N11A	------SS--A----------	+
L7S,I8S,E12A	------SS---A----------	−
L7S,I8S,Q13A	------SS----A----------	+
L7S,I8S,L14A	------SS-----A----------	+
L7S,I8S,P15A	------SS------A----------	+
D2N,M16S,L17S	-N----------SS----------	−
D3N,M16S,L17S	--N----------SS----------	−
D2E,M16S,L17S	-E----------SS----------	+
D3E,M16S,L17S	--E----------SS----------	+
L7S,I8S,E12Q	------SS---Q----------	−
L7S,I8S,E12D	------SS---D----------	+

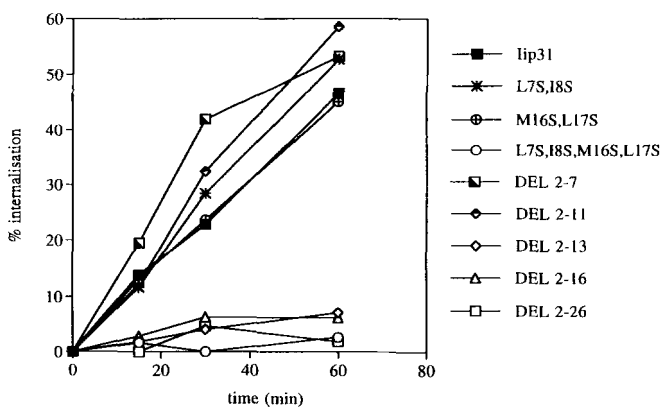

FIG. 1. **Internalization of Iip31 molecules containing cytoplasmic tail deletions or mutations of the di-leucine-based signals.** Internalization of ^{125}I-BU45 was measured as described under "Experimental Procedures." One representative experiment of three or more is shown for each construct. *Del*, delta.

and yet it was not internalized. These data suggest that amino acid residues N-terminal to the di-leucine-based pairs are necessary for Iip31 internalization.

Indirect immunofluorescence microscopy showed that the wild-type Iip31 molecules localized to large vesicles (Fig. 2*A*) and the cell surface (data not shown). The Δ2–7Ii molecules were present predominantly in small vesicles and the cell surface, although some cells contained Δ2–7Ii molecules in large vesicles (data not shown). The Δ2–11Ii molecules were present in small vesicles (data not shown) in accordance with previous reports for Δ2–11Ii molecules (Bakke and Dobberstein, 1990). The Δ2–13Ii, Δ2–16Ii, and Δ2–26Ii molecules were not detected in vesicles but were present at the cell surface (data not shown). The presence of Δ2–11Ii molecules in small but not large vesicles suggests that Iip31 cytoplasmic tail sequences, in addition to lumenal domain sequences[2] (Bremnes *et al.*, 1994), are necessary for large vesicle formation.

An Acidic Amino Acid Residue Four or Five Residues N-terminal to Each Di-leucine-based Pair Is Essential for Iip31 Internalization

To assess the role of Iip31 cytoplasmic tail amino acid residues in forming an internalization motif, alanine substitutions were made individually at positions 2–15 (Table I). Because the Iip31 tail contains two independent di-leucine-based pairs, we considered residues 1–8 and 9–17 separately. Therefore, to quantitate the effects of alanine substitutions at residues 1–8 with respect to the Leu-Ile pair, we mutated the Met-Leu pair to Ser-Ser. Similarly, to assess the effects of alanine substitutions at positions 9–17 with respect to the Met-Leu pair, we used as our parent molecule Iip31 with the Leu-Ile pair mutated to Ser-Ser. Mutation of either the Leu-Ile or Met-Leu pair to Ser-Ser had little or no effect on the Iip31 internalization rate (Fig. 1).

Substitution of alanine for Iip31 residues Asp^3, Leu^7, or Ile^8 abolished internalization, whereas mutation of Asp^2 to alanine dramatically decreased the internalization rate mediated by the Leu-Ile pair (Fig. 3*A*). In contrast, the internalization rates of Iip31 molecules containing an alanine substitution at residue Gln^4, Arg^5, Asp^6, or Ser^9 was comparable to the internal-

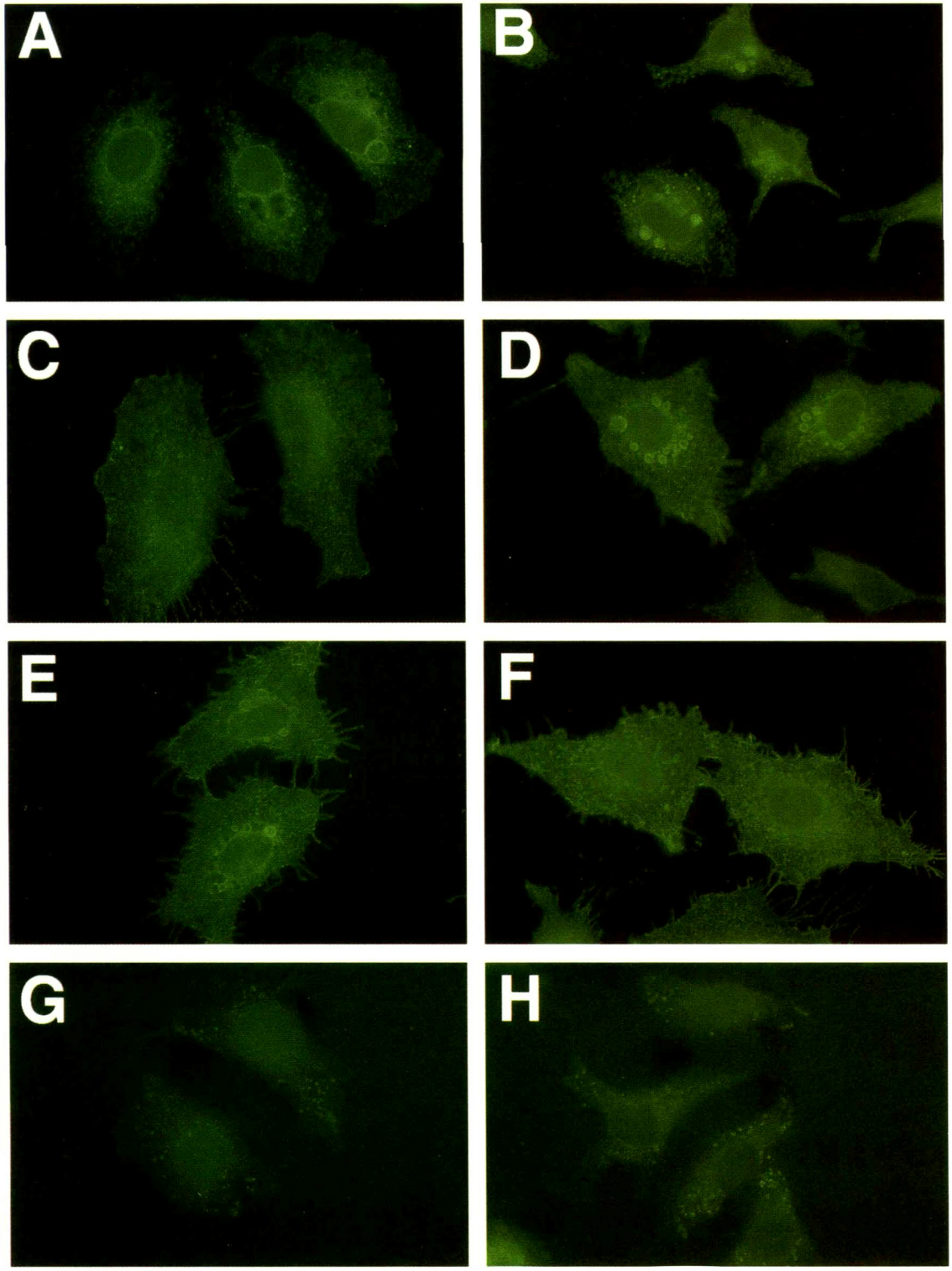

FIG. 2. **Indirect immunofluorescence microscopy of HeLa cells expressing Iip31 molecules containing wild-type (*panel A*) or mutant cytoplasmic tails (*panels B–H*).** Cells were stained with C351 rabbit antibodies to the Ii lumenal domain followed by fluorescein-conjugated antibodies to rabbit IgG. *Panel B*, hybrid LIMP II-Ii molecules. The first 17 residues of the cytoplasmic tail are MDERAPLISNNEQLPSS. *Panel C*, Ii molecules with the first 17 cytoplasmic tail residues mutated to MAARAPLISNNEQLPSS. *Panel D*, hybrid CI-M6PR-Iip31 molecules. The first 17 cytoplasmic tail residues are MDDRVGLVSNNEQLPSS. *Panel E*, hybrid CD3 γ-Ii molecules. The first 17 cytoplasmic tail residues are MSDQKTLLSNNEQLPSS. *Panel F*, hybrid CD3 γ-Ii molecules with Asp^3 mutated to alanine. The first 17 cytoplasmic tail residues are MSAQKTLLSNNEQLPSS. *Panel G*, hybrid CD4-Ii molecules. The first 17 cytoplasmic tail residues are MSQIKRLLSNNEQLPSS. *Panel H*, hybrid CD4-Ii molecules with Ser^2 and Gln^3 mutated to aspartic acid residues.

ization rate of the parent M16S,L17S Iip31 molecule (Fig. 3*A*). Similarly, for the Met-Leu pair, replacement of residue Glu^{12} with alanine eliminated internalization, but replacement of residue Ser^9, Asn^{10}, Gln^{13}, or Leu^{14} with alanine resulted in mutant Iip31 molecules that were internalized (Fig. 3*B*). Replacing residue Asn^{11} or Pro15 with alanine decreased the L7S,I8S Ii internalization rate by 40–50% relative to wild-type Iip31 (Fig. 3*B*). Indirect immunofluorescence showed that all

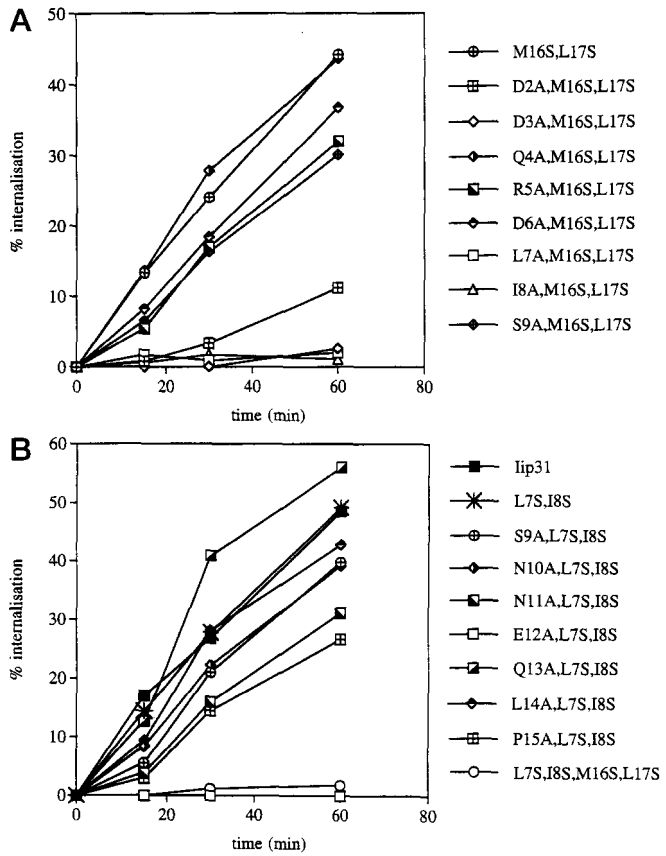

FIG. 3. **Internalization of Iip31 molecules containing alanine substitutions at residues 2–9 with the Met-Leu pair mutated to Ser-Ser (*panel A*) or at residues 9–15 with the Leu-Ile pair mutated to Ser-Ser (*panel B*).** Internalization of ^{125}I-BU45 was measured as described under "Experimental Procedures." One representative experiment of two or more is shown for each construct.

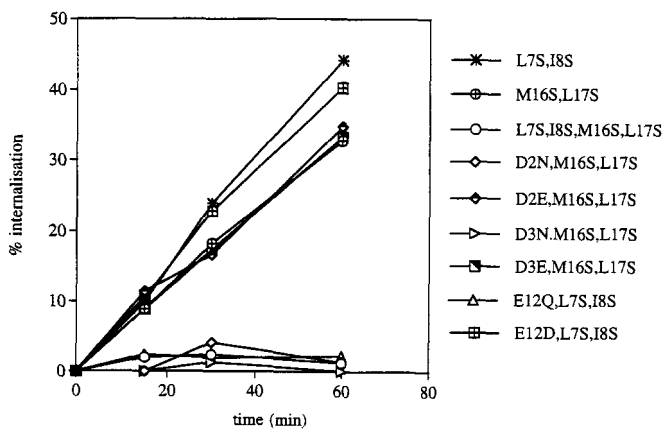

FIG. 4. **Internalization of Iip31 molecules containing substitutions of the acidic residues at position 2 or 3 with mutation of the Met-Leu pair to Ser-Ser, and at position 12 with mutation of the Leu-Ile pair to Ser-Ser.** Internalization of ^{125}I-BU45 was measured as described under "Experimental Procedures." One representative experiment of two or more is shown for each construct.

internalized mutant Iip31 molecules in the alanine scan series were present in large vesicles (data not shown), whereas non-internalized mutant Iip31 molecules concentrated at the cell surface and were not detected in vesicles (data not shown). Thus, to function as internalization and endosomal targeting signals for Iip31, residues Asp^2 and Asp^3 are essential for the Leu-Ile pair, and residue Glu^{12} is essential for the Met-Leu pair.

TABLE II

DI-leucine-based motifs and N-terminal acidic amino acid residues

Cytoplasmic tail di-leucine-based motifs known to mediate internalization and/or lysosomal targeting are shown. The di-leucine-like pairs and acidic amino acid residues four or five residues N-terminal to di-leucine-based motifs are underlined. References for the cytoplasmic tail sequences are as follows: Iip31, Claesson *et al.* (1983); LIMP II, Ogata and Fukuda (1994), Sandoval *et al.* (1994); CD3 γ and δ, Letourneur and Klausner (1992); CD-M6PR, Johnson and Kornfeld (1992a); CI-M6PR, Johnson and Kornfeld (1992b), Ogata and Fukuda (1994); CD4, Aiken *et al.* (1994); IFN-γR, Farrar and Schreiber (1993); GLUT4, Verhey and Birnbaum (1994). GLUT4, glucose transporter 4; IFN-γ R, interferon-γ receptor.

Human Iip31				M	D	D	Q	R	D	L	I	S	
				N	N	E	Q	L	P	M	L	G	
Human LIMP II				A	D	E	R	A	P	L	I	R	T
Mouse CD3 γ				A	S	D	K	Q	T	L	L	Q	N
Mouse CD3 δ				A	A	E	V	Q	A	L	L	K	N
Human CD-M6PR	E	E	S	E	E	R	D	D	H	L	L	P	M
Bovine CI-M6PR				D	D	S	D	E	D	L	L	H	V
Bovine CI-M6PR				A	D	D	R	V	G	L	V	R	G
Human CD4				M	S	Q	I	K	R	L	L	S	E
Rat GLUT4				F	R	R	T	P	S	L	L	E	Q
Human IFN-γ R				S	I	I	L	P	K	L	I	S	V

To assess further the requirements for Iip31 internalization, we tested the effects of conservative substitutions, and substitution of polar but uncharged residues, for the acidic amino acid residues. The internalization rates of mutants D2E and D3E containing only the Leu-Ile pair, and of mutant E12D containing only the Met-Leu pair (Table I), did not differ from the parent M16S,L17S and L7S,I8S mutants, respectively (Fig. 4). Thus, aspartic acid and glutamic acid are functionally interchangeable at positions 2, 3, and 12 in the Iip31 cytoplasmic tail. The D2N,M16S,L17S and D3N,M16S,L17S mutants were not internalized (Fig. 4), indicating that an isoteric polar, uncharged residue is not permissible at position 2 or 3. Likewise, substitution of glutamine for Glu^{12} abolished internalization mediated by the Met-Leu pair (Fig. 4). Substitution of polar, uncharged residues for the acidic residues yielded molecules expressed predominantly at the cell surface with no detectable vesicular localization (data not shown). Thus, isoteric polar residues cannot substitute for the critical acidic residues necessary for recognition of the two Iip31 internalization and endosomal targeting signals.

Acidic Residues Are Important in Endosomal Targeting but Are Not Essential for Internalization

Seven of 10 di-leucine-based signals known to mediate internalization contain acidic residues N-terminal to the di-leucine pair (Table II). To determine whether acidic residues are required for internalization mediated by these di-leucine signals, we selectively replaced the five amino acid residues N-terminal to the Iip31 Leu-Ile pair. Because previous work has shown that the two Iip31 motifs can function when separated (Bakke *et al.*, 1993) and because there are only two amino acid residues C-terminal to the LIMP II Leu-Ile pair, we limited our analyses to N-terminal motif residues. In this way, we could compare different di-leucine-based signals in the context of one reporter molecule, Iip31. We chose sequences from three di-leucine-based signals which contain acidic residues N-terminal to the di-leucine pair: LIMP II, bovine CI-M6PR, and CD3 γ. We also included the CD4 signal, which has no acidic residue in the five residues N-terminal to the di-leucine pair. We made replacements without altering the Iip31 cytoplasmic tail length. In each hybrid the Met-Leu pair was mutated to Ser-Ser to eliminate any contribution of this pair to Iip31 sorting. We then assessed the effects of alanine substitutions within these se-

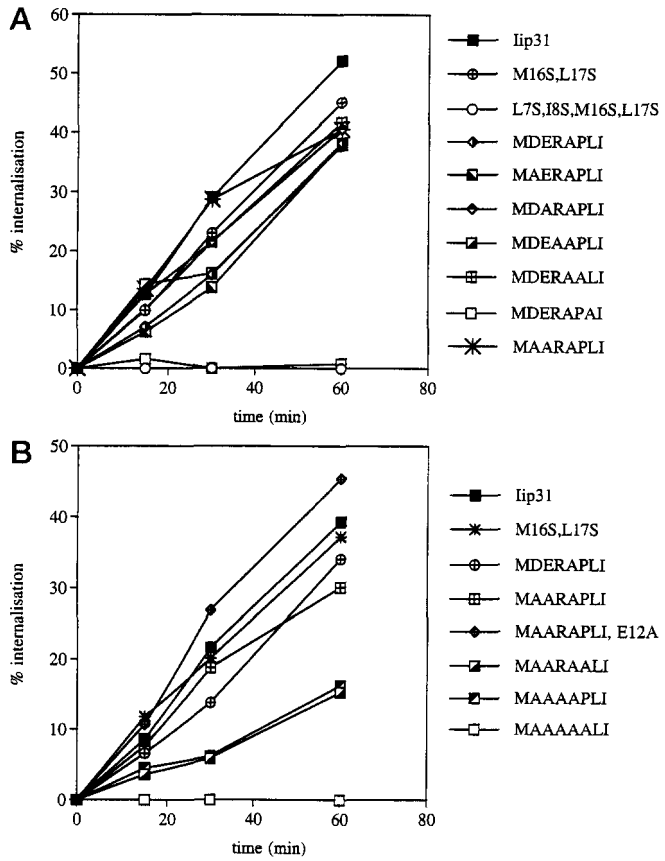

FIG. 5. *Panel A*, internalization of Iip31 molecules containing LIMP II-Ii hybrid cytoplasmic tails. *Panel B*, internalization of Iip31 molecules containing multiple alanine substitutions in the LIMP II-Ii hybrid cytoplasmic tail. In each case, amino acid residues 2–8 of the Iip31 sequence were replaced by the indicated sequence, and the Met-Leu pair was mutated to Ser-Ser. Internalization of ^{125}I-BU45 was measured as described under "Experimental Procedures." One representative experiment of two or more is shown for each construct.

quences on Ii internalization and endosomal targeting.

Targeting of LIMP II-Ii Hybrid Molecules—The cytoplasmic tail of the type I transmembrane protein LIMP II contains two acidic residues and a Leu-Ile pair in a sequence context similar to the Iip31 signals (Table II). To test whether acidic amino acid residues in the LIMP II cytoplasmic tail are required for di-leucine-based targeting, we made a LIMP II-Ii hybrid tail. To create the hybrid, we substituted part of the LIMP II cytoplasmic tail for residues 2–6 of the Iip31 cytoplasmic tail (Fig. 5*A* and Table III). We then assessed Ii internalization and endosomal targeting mediated by the transplanted wild-type LIMP II sequence and alanine scan mutants of this sequence. Substitution of the LIMP II cytoplasmic tail sequence ERAP for the Iip31 tail sequence DQRD, and mutation of Met-Leu to Ser-Ser, gave an internalization rate equal to that of the parent M16S,L17S mutant (Fig. 5*A*). Indirect immunofluorescence microscopy showed that this substitution mutant localized to large endosomal vesicles (Fig. 2*B*), similar to wild-type Iip31 (Fig. 2*A*). Furthermore, alanine substitution at LIMP II residue Asp^2, Glu^3, Arg^4, or Pro^6 had no effect on the Iip31 internalization rate (Fig. 5*A*) or localization to large endosomal vesicles (data not shown). Changing both N-terminal acidic amino acid residues to alanine in the MAARAPLISNNEQLPSS mutant also did not alter the internalization rate from that of the wild-type LIMP II sequence (Fig. 5, *A* and *B*). Interestingly, this mutant was localized to smaller vesicles present throughout the cytoplasm (Fig. 2*C*) but not to the large perinuclear vesicles seen with Iip31 (Fig. 2*A*). Our previous results showed

TABLE III
Di-Leucine-based motif substitution mutants

Substitutions were made at positions 2–8 of the Iip31 cytoplasmic tail, and in each case the Met-Leu signal was mutated to Ser-Ser. The presence of large or small vesicles containing the chimeric Ii molecules in transiently transfected HeLa cells was detected by indirect immunofluorescence with rabbit antiserum C351 raised against the Ii C terminus. The expression levels of the chimeric Ii molecules were the same or higher than the wild-type Iip31 molecules, as detected by ^{125}I-BU45 binding to transfected cells within each experiment (data not shown).

Residues 1–8	Internalization	Large endocytic vesicles
LIMP II		
MDERAPLI	+	+
MAERAPLI	+	+
MDARAPLI	+	+
MDEAAPLI	+	+
MDERAALI	+	+
MDERAPAI	–	–
MAARAPLI	+	–
MAARAALI	+/–	–
MAAAAPLI	+/–	–
MAAAAALI	–	–
CI-M6PR		
MDDRVGLV	+	+
MADRVGLV	+	+
MDARVGLV	–	–
MDDAVGLV	+	+
MDDRAGLV	+	+
MDDRVALV	+	+
MDDRVGAV	–	–
MAARVGLV	–	–
CD3 γ		
MSDKQTLL	+	+
MADKQTLL	+	+
MSAKQTLL	+	–
MSDAQTLL	+	+
MSDKATLL	+	+
MSDKQALL	+	+
MSDKQTAL	–	–
CD4		
MSQIKRLL	+	–
MAQIKRLL	+	–
MSAIKRLL	+	–
MSQAKRLL	+	–
MSQIARLL	+	–
MSQIKALL	1/2+	–
MSQIKRAL	–	–
MDQIKRLL	+	–
MSDIKRLL	+	–
MDDIKRLL	+	–

that acidic residues C-terminal to di-leucine-like pairs appear not to be required for internalization (Fig. 3). Nevertheless, by mutating residue Glu^{12} of the Iip31 cytoplasmic tail to alanine, we determined the importance of its presence for internalization of the LIMP II-Ii hybrid molecule. The mutant Ii molecules containing the sequence MAARAPLISNNAQLPSS were internalized as efficiently as Ii molecules containing the wild-type LIMP II sequence (Fig. 5*B*) but were localized to smaller vesicles similar to those seen with the MAARAPLISNNEQLPSS mutant (data not shown). From these data, we conclude that the LIMP II sequence acts as an internalization signal in the context of the Iip31 cytoplasmic tail. Although the LIMP II N-terminal acidic amino acid residues are not required for internalization, they are important for formation of the large Ii-containing endosomal vesicles.

We made further mutations in the LIMP II signal to determine which features of this signal are critical for internalization. Having found that the sequence MAARAPLISNNEQLPSS gave the wild-type internalization rate, we made the mutants MAARAALISNNEQLPSS and MAAAAPLISNNE-

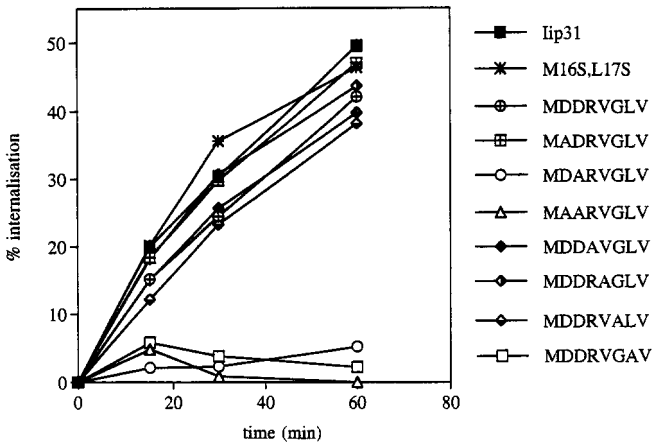

FIG. 6. **Internalization of Iip31 molecules containing the CI-M6PR-Ii hybrid cytoplasmic tail or alanine scan mutations.** See the Fig. 5 legend for details. One representative experiment of two or more is shown for each construct.

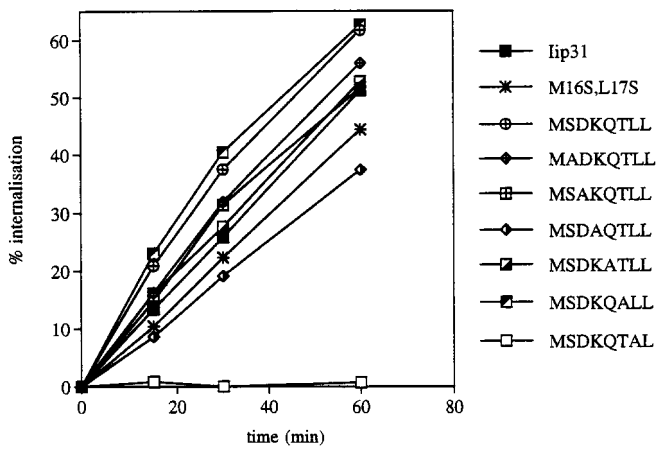

FIG. 7. **Internalization of Iip31 molecules containing CD3 γ chain-Ii hybrid cytoplasmic tail or alanine scan mutations.** See the Fig. 5 legend for details. One representative experiment of three or more is shown for each construct.

QLPSS and found that Ii hybrid molecules containing these mutant cytoplasmic tails were internalized at approximately half the rate of hybrid molecules containing the wild-type LIMP II signal (Fig. 5*B*). Mutation of residues 2–6 to alanines, resulting in the mutant MAAAAALISNNEQLPSS, abrogated internalization (Fig. 5*B*). Thus, MAARAP constitutes a permissive, minimal sequence N-terminal to the Leu-Ile pair required to achieve the wild-type Iip31 internalization rate.

Targeting of CI-M6PR-Ii Hybrid Molecules—We next replaced residues 2–8 of the Iip31 cytoplasmic tail with residues from one of the bovine CI-M6PR di-leucine-based signals, DDRVGLV (see Table II). Like the LIMP II di-leucine-based signal, this CI-M6PR signal contains acidic residues and a di-leucine signal in a sequence context similar to the Iip31 signals. The wild-type CI-M6PR signal confers Ii internalization (Fig. 6) and large vesicle formation (Fig. 2*D*). Alanine scan mutagenesis of this CI-M6PR signal showed that mutation of the Asp^3 or Leu^7 residues, but not the Asp^2, Arg^4, Val^5, or Gly^6 residues, abrogated internalization (Fig. 6) and large vesicle formation (Table III). Thus, the bovine CI-M6PR signal DDRVGLV is similar to the Iip31 di-leucine-based signals in that an acidic residue four residues N-terminal to the di-leucine pair is critical for its recognition, but different in that mutation of the Asp^2 residue to alanine had no effect on internalization or large vesicle formation.

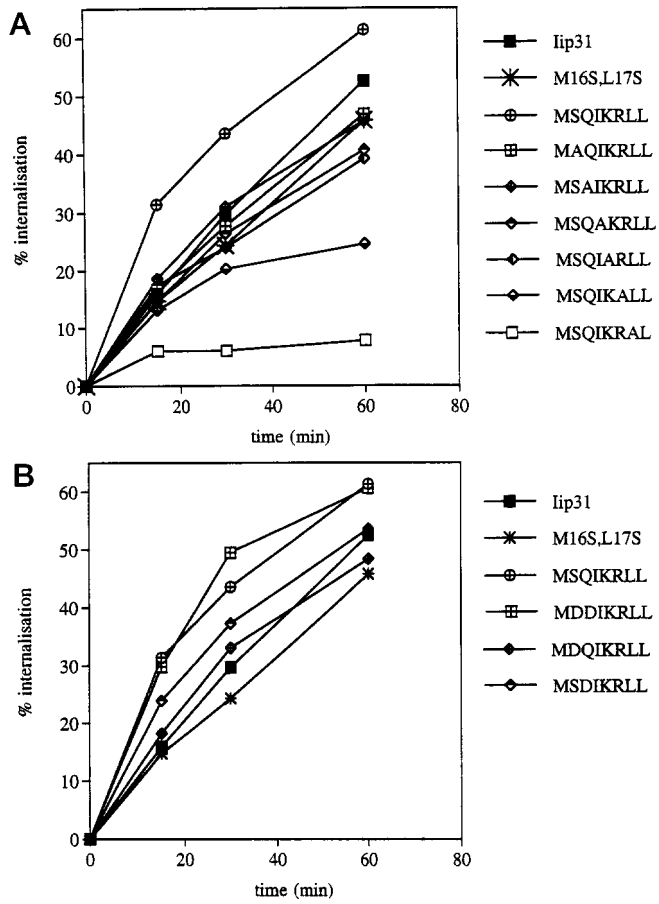

FIG. 8. *Panel A*, internalization of Iip31 molecules containing CD4-Ii hybrid cytoplasmic tail or alanine scan mutations. *Panel B*, internalization of CD4-Ii chimeric molecules containing aspartic acid substitutions in the CD4 di-leucine-based motif. See the Fig. 5 legend for details. One representative experiment of two or more is shown for each construct.

Targeting of CD3 γ-Ii Hybrid Molecules—The CD3 γ chain contains the di-leucine-based signal SDKQTLL, which mediates internalization and enhances lysosomal degradation of the Tac lumenal domain (Letourneur and Klausner, 1992). Replacement of the N-terminal Iip31 di-leucine-based signal with the wild-type CD3 γ signal showed that the CD3 γ signal mediates Ii internalization at approximately the wild-type Iip31 rate (Fig. 7) and allows for large vesicle formation (Fig. 2*E*). Alanine scan mutagenesis showed that individually mutating residues 2–6 had little or no effect on the internalization rate (Fig. 7). As expected, mutation of Leu^7 to alanine abolished internalization (Fig. 7). Mutation to alanine of residue Asp^3 or Leu^7, but not Ser^2, Lys^4, Gln^5, or Thr^6 also prevented large vesicle formation (Fig. 2*F* and Table III). These results show that single alanine substitutions N-terminal to the di-leucine pair in the CD3 γ signal do not affect the Ii internalization rate. Acidic residue Asp^3, however, is necessary for large vesicle formation but not for internalization mediated by the CD3 γ signal.

Targeting of CD4-Ii Hybrid Molecules—Lastly, we replaced the Iip31 N-terminal di-leucine-based signal with the CD4 sequence SQIKRLL. The resulting hybrid molecule was internalized slightly faster than the wild-type Iip31 molecule (Fig. 8*A*). Alanine scan mutagenesis showed that substitution of residues Ser^2, Gln^3, Ile^4, or Lys^5 with alanine had little effect on the internalization rate (Fig. 8*A*). Mutation of residue Arg^6 to alanine decreased the internalization rate by about half, compared with the wild-type CD4 sequence, and mutation of residue Lue^7 to alanine abrogated internalization (Fig. 8*A*).

Indirect immunofluorescence showed that hybrid Ii molecules containing the wild-type CD4 signal or its alanine substitution mutants were present on the cell surface and in small vesicles (Fig. 2*G* and Table III), but in no case were large vesicles detected (Table III). We therefore sought to reconstitute large vesicle formation by replacing one or both of the CD4 residues Ser^2 and Gln^3 with aspartic acid residues. Hybrid CD4-Ii molecules containing the modified CD4 signals were internalized at a rate similar to molecules containing the wild-type CD4 signal (Fig. 8*B*) but were localized to small vesicles and the cell surface, with no large vesicles detected (Fig. 2*H* and Table III). Thus, aspartic acid residues in this sequence context do not permit large vesicle formation.

DISCUSSION

In this report, we performed extensive alanine scan mutagenesis of sequences N-terminal to di-leucine-like pairs in the MHC class II-associated Iip31 molecule, and on four other cell surface receptors. Our results suggest that internalization and targeting within the endocytic pathway require different targeting motifs. Mutation to alanine of an acidic residue, but not the other residues immediately N-terminal to the di-leucine pair, abolished large endocytic vesicle formation and, in some cases, internalization. These results show that residues N-terminal to the di-leucine-based pairs are part of a larger motif recognized by the intracellular targeting machinery and that the sequences surrounding the di-leucine pair, in particular N-terminal acidic residues, are involved in targeting within the endocytic compartment.

Our data show that residues 2–8 of the Iip31 cytoplasmic tail constitute one internalization motif, and residues 12–17 constitute a second motif. Indeed, Iip31 cytoplasmic tail residues 1–11 and 12–30 can separately confer internalization to hybrid molecules with the neuraminidase transmembrane and lumenal domains (Bakke O. *et al.*, 1993). Furthermore, we note similarities in the two Iip31 signals (Table II). We have also shown that when the Met-Leu pair is mutated to Ser-Ser, mutation of amino acid residues Asp^2 or Asp^3 to alanine abrogates internalization with respect to the Leu-Ile pair. Although residue Glu^{12} is present, it apparently cannot substitute for the Asp^2 or Asp^3 residues. Similarly, when amino acid residue Glu^{12} is mutated to alanine and the Leu-Ile pair is mutated to Ser-Ser, residues Asp^2 and Asp^3 cannot provide the acidic residue needed for internalization mediated by the Met-Leu pair. Thus, the two Iip31 internalization motifs are functionally separate.

MHC class II and Iip31 molecules are targeted to a specialized endosomal compartment where class II molecules bind antigenic peptides (see the Introduction). We were interested in determining if the Iip31 cytoplasmic tail has a novel signal for targeting to this compartment. Our results show that the LIMP II and CI-M6PR di-leucine-based signals can mediate targeting to the same or similar vesicular compartments as wild-type Iip31. Therefore, we suggest that the Iip31 cytoplasmic tail does not contain novel sequence information for targeting to the compartment for peptide loading and therefore that other molecules with di-leucine-based signals have access to this compartment. Whether class II molecules influence targeting of class II molecule-Iip31 complexes to this compartment remains to be tested.

Internalization Rates Mediated by One and Two Di-leucine-based Signals in the Iip31 Cytoplasmic Tail Are Equivalent—Using the Ii molecule in HeLa cells, we have shown that two di-leucine-based signals in the Iip31 cytoplasmic tail produced approximately the same internalization rates as single signals alone. Our results are generally in agreement with those of Bremnes *et al.* (1994), who found that mutation of the Leu-Ile pair to Ala-Ile decreased only slightly the internalization rate of Ii-neuraminidase hybrid molecules in the CV-1 simian fibroblast cell line. In contrast, two YTRF motifs at specific locations in the transferrin receptor cytoplasmic tail clearly confer twice the internalization rate of one motif (Collawn *et al.*, 1993). This result indicates that the sorting machinery can simultaneously recognize two aromatic residue-based motifs if there is a sufficient number of residues separating the motifs and if they are in structurally similar environments. Moreover, in chick embryo fibroblasts, mutation of one of the two Iip31 di-leucine-like pairs to Ala-Ala decreased by 50% the internalization rate of hybrid molecules containing the Iip31 cytoplasmic tail and transferrin receptor transmembrane and lumenal domains (Odorizzi *et al.*, 1994).

One hypothesis to explain these contrasting results is that different cell types have differing abilities to recognize two di-leucine motifs in one cytoplasmic tail. This theoretical difference regarding internalization motifs across cell types and species has been demonstrated experimentally. For example, the tyrosine motif analog NPVF mediates low density lipoprotein receptor internalization in Chinese hamster ovary cells (Davis *et al.*, 1987) but not in Madin-Darby canine kidney cells (Matter *et al.*, 1994). A second hypothesis is that the transferrin receptor and Ii lumenal or transmembrane domains themselves influence the internalization rate. Precedence for this hypothesis is suggested by data showing that the M6PR lumenal domain influences receptor recycling and residence time within endosomes (Dintzis *et al.*, 1994).

Acidic Residues N-terminal to Di-leucine-like Pairs Influence Targeting within the Endocytic Pathway—We examined internalization and large vesicle formation mediated by several di-leucine signals and their five adjacent N-terminal amino acid residues in the context of the Ii cytoplasmic tail. In the Iip31 and CI-M6PR signals, acidic residues four or five residues N-terminal to the di-leucine-like pairs were required for Ii internalization and large vesicle formation. In the LIMP II and CD3 γ chain signals, N-terminal acidic residues were required for large vesicle formation but not for internalization. Since substitution of acidic residues in the CD4 signal did not reconstitute large vesicle formation, we concluded that acidic residues alone are insufficient for formation of large endocytic vesicles. Together, these data show that internalization from the cell surface does not require acidic residues N-terminal to di-leucine-like pairs but that the acidic residues in certain sequence contexts influence sorting within the endocytic pathway. Thus, we hypothesize that acidic residues, in a specific structural context, combine with the di-leucine-like pair to signal vesicle targeting and that this same structure is appropriate for internalization. Our hypothesis suggests that part of one motif may be recognized for internalization and another part for vesicular targeting. This hypothesis is consistent with the possibility that recognition of di-leucine-based signals at the cell surface and within the endosomal pathway is mediated by different molecules. Targeting of internalized molecules to endocytic subcompartments may then depend on the amino acid sequences surrounding the di-leucine pair.

Cytoplasmic tail acidic residues in other sequence contexts may be involved in other intracellular targeting processes. For example, basolateral targeting, but not internalization, mediated by cytoplasmic tail sequences of the type I transmembrane protein low density lipoprotein receptor is influenced by acidic residues C-terminal to its tyrosine-based targeting motif (Matter *et al.*, 1994). This observation suggests that although basolateral targeting and internalization sequences may overlap, the motifs for the two processes are distinct. These results are analogous to our observations that N-terminal acidic residues are not always required for di-leucine-based internalization but

are required for large vesicle formation. Interestingly, basolateral targeting mediated by the Fc receptor II di-leucine-based signal does not appear to need acidic residues, as determined by analyses of truncation and alanine scan mutants (Hunziker and Fumey, 1994).

An alternative hypothesis is that the acidic residues are not required for Iip31 targeting to a specialized endocytic compartment *per se* but that they are required for large vesicle formation after Ii entry into an endocytic subcompartment. Immunofluorescence studies have shown previously that large vesicles staining for Iip31 are also enriched with late endosomal or lysosomal markers such as CI-M6PR or lysosomal-associated membrane protein molecules (Lotteau *et al.*, 1990; Romagnoli *et al.*, 1993; Pieters *et al.*, 1993). Large vesicle formation induced by Iip31 expression is time-dependent after Iip31 transfection (Pieters *et al.*, 1993), indicating that a critical level of Iip31 accumulation must be reached before large vesicles form. The mechanism by which these large endocytic vesicles form is not known. The Iip31 molecule may alter endosome/lysosome morphology, perhaps by multimerizing and interfering with protein trafficking as suggested previously (Romagnoli *et al.*, 1993). We note that overexpression of rab5 (Bucci *et al.*, 1992) or rab22 (Olkkonen *et al.*, 1993) proteins, which are localized to early and late endosomes and the plasma membrane, also causes large vesicle formation, perhaps by inducing endosomal clustering and fusion. Therefore, another possible mechanism for large vesicle formation in Iip31-expressing cells is that acidic residues in the Iip31 tail mimic a signal recognized for fusion of vesicles in the endosomal compartment. Knowledge of the mechanism by which these large vesicles form may provide insight into the regulation of protein trafficking within the endocytic compartment.

The Iip31 molecules might enter the endosomal/lysosomal compartment either directly from the TGN or by internalization from the cell surface. The relative contributions of these two routes for Iip31 is presently not known. Odorizzi *et al.* (1994) estimate that more than 80% of hybrid molecules containing the Iip31 cytoplasmic and transmembrane domains and the transferrin receptor lumenal domain are delivered intracellularly from the TGN to the endocytic pathway. For the CI-M6PR and CD-M6PR, which contain tyrosine-based and di-leucine-based targeting motifs, mutation or deletion of the di-leucine pair did not alter their internalization rates, but drastically reduced correct sorting of lysosomal enzymes (Lobel *et al.*, 1989; Johnson and Kornfeld, 1992a). These results suggest that di-leucine motifs mediate direct sorting of CD-M6PR from the TGN to late endosomes. In this report and others (Letourneur and Klausner, 1992; Ogata and Fukuda, 1994; Pieters *et al.*, 1993; Aiken *et al.*, 1994), di-leucine-based motifs were shown to mediate internalization from the cell surface. Moreover, internalization of the CD4 molecule and of the CD3 γ-Tac hybrid is likely to proceed via clathrin-coated pits (Pelchen-Matthews *et al.*, 1991; Letourneur and Klausner, 1992). Thus, it is likely that the di-leucine motif is recognized at the TGN and at the cell surface. This suggestion raises further questions. Are the proteins recognizing the same di-leucine motifs at the TGN as at the cell surface? Are these proteins the same as the adaptin complexes that recognize aromatic residue-based internalization motifs (Glickman *et al.*, 1989).

The results described here provide insight into the minimal requirements for Iip31 internalization and endocytic targeting mediated by its two di-leucine-based signals. Our results show that acidic residues are required for targeting of Ii within the endosomal compartment, that internalization is not determined solely by the di-leucine-like pairs, and that nearby residues also contribute to efficient internalization. If all di-leucine-based motifs are recognized by the same targeting molecules, it is probable that a common secondary structure exists. Future studies will determine the validity of this hypothesis. The sequence requirements for the di-leucine-based signals are strikingly different from the aromatic residue-based signals, although parallels may yet exist between the di-leucine-based motif structure and the aromatic residue-based motif β-turn or nascent α-helical structure. The discovery that di-leucine-based signals responsible for internalization and endosomal targeting involve a larger motif helps explain the specificity of the molecular recognition events required for protein sorting.

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