The Effect of Site-Directed Mutagenesis of the Ambient Amino Acids of Leucine-Based Sorting Motifs on the Localization of Chicken Invariant Chain

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ABSTRACT Two Leu residues and their ambient amino acid residues are known to exist in the cytosolic tail of chicken invariant chain (Ii), and these play an important role as motifs in mediating the sorting endocytic pathway. We performed 20 mutations via site-directed mutagenesis by the PCR megaprimer method to study the effect of some ambient amino residues of both Leu on the localization of chicken Ii. These mutated fragments were ligated to the vector pEGFP-C1. The recombinant plasmids were transiently transfected into COS-7 cells with Lipofectamine 2000. Furthermore, the fluorescence of located fusion proteins (green fluorescent protein-Ii) was observed with a fluorescence microscope. Our results indicated that 2 Leu-based motifs are required for chicken Ii intracellular localization, and both motifs independently mediate this function of the Ii. The other amino acid residues surrounding both Leu also influence Ii-induced endosomal vacuolation. In addition, we found that Pro^{19}, which is near the Val^{17}-Leu^{18} motif, was a key residue for chicken Ii intracellular localization. Not only is it critical for endocytic targeting to each Leu, but its unique mutation can also result in altering the function of chicken Ii.

Key words: chicken invariant chain, site-directed mutagenesis, neighboring amino acid residue, localization

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INTRODUCTION

The major histocompatibility complex (**MHC**) class II molecules (also referred to as human leukocyte antigen molecules in humans) are expressed on antigen-presenting cells such as B cells, macrophages, and dendritic cells. They associate with the chaperones and present primarily antigenic peptides derived from exogenous proteins to $CD4^+$ T cells (Neefjes and Ploegh, 1992; Germain and Margulies, 1993; Cresswell, 1994; Germain, 1994). The MHC class II-associated invariant chain (**Ii**), a type II transmembrane glycoprotein that has 30 amino N-terminal residues exposed at the cytoplasmic side of the membrane, spans the membrane between residues 30 and 60 and has a large luminal carboxy (**C**)-terminal domain (Claesson and Peterson, 1983; Strubin et al., 1984). It is well known to act as a chaperone for MHC class II protein expression and facilitate antigen presentation (Bertolino and Rabourdin-Combe, 1996). Previous studies have demonstrated that the information contained in the Ii cytoplasmic tail is both necessary and sufficient for Ii targeting to the endosomal compartment and that a significant fraction of the Ii molecules, alone or in the complex MHC class II, are transported to endosomes via the plasma membrane (Bakke and Dobberstein, 1990; Freisewinkel et al., 1993; Rudensky et al., 1994; Vogt et al., 1995). Deletion analysis also showed that the first 16 amino acid residues were required for Ii endosomal targeting (Lotteau et al., 1990). Site-directed mutagenesis localized 2 signals in the Ii cytoplasmic tail that could independently target Ii to the endosomal system. One signal was the Leu-Ile motif, containing Leu and Ile at positions 7 and 8 of the tail. The other signal was the Met-Leu motif, containing Met and Leu at positions 16 and 17 (Bremnes et al., 1994).

Invariant chain from different species containing pairs of Leu-Ile and Met-Leu or Val-Leu in their cytosolic tails is conserved (Pieters et al., 1993; Bremnes et al., 1994; Sandoval et al., 1994). Alanine-scanned mutagenesis demonstrated differential requirements for clathrin-associated adaptor protein (**AP**) complexes AP1 and AP2 binding to Ii at the level of residues around the critical Leu residues (Thomas et al., 2002). In addition, an acidic amino acid residue containing 4 or 5 residues N-terminal to each of these di-Leu-like signals is required for efficient targeting (Pond et al., 1995).

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Furthermore, like the mammalian orthologs, the cytosolic tail of chicken Ii was found to contain 2 endosomal sorting signals (Leu^{8}-Ile^{9} and Val^{17}-Leu^{18}; Bremnes et al., 2000), but the functional properties of the amino acids around the 2 motifs are unclear in chicken Ii at present. Thus, after validating the feasibility of the experimental system to investigate the roles of amino acids surrounding 2 Leu-based motifs within the cytoplasmic tail of chicken Ii in Ii-induced endosomal vacuolation or Ii intracellular localization, we applied the green fluorescent protein (**GFP**)-fused Ii to definitively detect 2 Leu-based motifs mediating Ii endosomal targeting by intuitionistic fluorescence microscope. We demonstrated in this study that Ii-induced intracellular localization required specific neighboring amino acid residues of di-Leu motifs.

MATERIALS AND METHODS

Expression Vector

The expression vector pEGFP-C1 encodes a red-shifted variant of wild-type GFP (Prasher et al., 1992; Chalfie et al., 1994; Inouye and Tsuji, 1994) that has been optimized for brighter fluorescence and greater expression in mammalian cells. The enhanced green fluorescent protein (**EGFP**) fusion protein expressed from pLP-EGFP-C1 can be used to monitor gene expression and protein localization for the gene of interest. Fusions to the C-terminus of EGFP retain the fluorescence properties of the native protein, allowing the fusion protein to be localized in vivo. The recombinant EGFP vector can be transfected into mammalian cells by using any standard transfection method.

Recombinant cDNA Constructions

The cDNA fragment encoding the recombinant plasmid of chicken Ii has been described previously (Zhong et al., 2004). An overview of the mutants used in this study is presented in Table 1. Alanine-scanned mutagenesis was performed, and point mutations in the cytoplasmic tail of Ii were introduced by PCR-based megaprimer mutagenesis using the Ii expression vector pEGFP-Cl^{-}Ii as template. The forward primers containing the point mutations are listed in Table 2. A 100-μL volume was amplified with the mutated primers and the primer of *Bgl*II-F at a low annealing temperature. Polymerase chain reaction-based megaprimer mutagenesis was performed with an FTC-312 thermocycler (Barloworld Scientific, Stone, Staffordshire, UK), and the temperature profile was set as follows: 1 cycle of 94°C for 4 min, 42 to 46°C for 1 min, 72°C for 1 min; 24 cycles of 94°C for 40 s, 42 to 46°C for 1 min, 72°C for 1 min; and 1 cycle of 94°C for 40 s, 42 to 46°C for 1 min, 72°C for 5 min. After the forward round of PCR was ended, the high-anneal primer of C1-R (Table 2) was added and the temperature profile was set as follows: 25 cycles of 94°C for 40 s, 72°C for 90 s, 1 cycle of 72°C for 5 min. The PCR reactions were performed by using Pyrobest DNA polymerase (Takara, Dalian, China) for primer extension. The routine PCR amplification for the truncated form of wild-type Ii gene was performed with the primers Ii-83-F and Ii-83-R. The final PCR products were cloned into pEGFP-Cl. All mutants were verified by DNA sequencing.

Cells and Cell Culture

The COS-7 cells, derived from CV1 cells, were transformed with an origin-defective mutant of SV40 coding for the wild-type T antigen (Gluzman, 1981). The cell lines were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Sigma, Steinheim, Germany) at 37°C in 5% CO_2.

Transient Transfection of COS-7 Cells

The transfection procedure has been described previously by Huylebroeck et al. (1988). Briefly, 70% confluent cells were split 1:5 into 24 wells (the coverslips were previously placed in the wells) 1 d before transfection. The cells were seeded in 24-well plates with 3×10^4 cells/well in Dulbecco's modified Eagle's medium (Gibco) without serum. Transfection was performed by using Lipofectamine 2000 (2.0 μL/well, Invitrogen, Carlsbad, CA) for transient transfections, according to the manufacturer's instructions.

Fluorescence Microscopy

Expression of GFP was used as a marker of positively transfected cells. After transfection for 24 to 48 h, viable cells grown on coverslips were fixed with 4% paraformaldehyde. The cells were visualized with an Olympus fluorescence photomicroscope (Olympus, Tokyo, Japan).

RESULTS

Previous studies have shown that 2 Leu-based motifs within the cytoplasmic tail of human Ii are necessary and sufficient for its endosomal targeting. To validate the effect of both Leu-based motifs and other amino acids close to them in the chicken Ii cytoplasmic tail, as described in Table 1, we constructed 20 mutants containing a fusion gene of mutagenesis (M1 to M20) and GFP and transfected them into the COS-7 cell strain. The mutations M3, M8 to M14, and M17 to M20 resulted in plasma membrane staining, whereas the wild type, M1, M2, M4 to M7, and M15 were all associated with vesicle staining.

Mutated Ii Gene

Twenty Ala-scanned mutants were obtained by performing 2 sets of PCR-based point mutations using

Table 1. Cellular distribution and internalization of the recombinants[1]

No.	Mutation at position	Ii cytoplasmic tail amino acid sequences	Localization
WT	Wild type	MAEEQRDLISSDGSSGVLP—	V
M1	L8A	MAEEQRD**A**ISSDGSSGVLP—	V
M2	L18A	MAEEQRDLISSDGSSGV**A**P—	V
M3	L8A, L18A	MAEEQRD**A**ISSDGSSGV**A**P—	PM
M4	L8A, S11A	MAEEQRD**A**IS**A**DGSSGVLP—	V
M5	L8A, D12A	MAEEQRD**A**ISS**A**GSSGVLP—	V
M6	L8A, G13A	MAEEQRD**A**ISSD**A**SSGVLP—	V
M7	L8A, S14A	MAEEQRD**A**ISSDG**A**SGVLP—	V
M8	L8A, S15A	MAEEQRD**A**ISSDGS**A**GVLP—	PM
M9	L8A, G16A	MAEEQRD**A**ISSDGSS**A**VLP—	PM
M10	L8A, V17A	MAEEQRD**A**ISSDGSSGV**A**P—	PM
M11	L8A, P19A	MAEEQRDAISSDGSSGVL**A**—	PM
M12	L18A, E3A	MA**A**EQRDLISSDGSSGV**A**P—	PM
M13	L18A, E4A	MAE**A**QRDLISSDGSSGV**A**P—	PM
M14	L18A, Q5A	MAEE**A**RDLISSDGSSGV**A**P—	PM
M15	L18A, R6A	MAEEQ**A**DLISSDGSSGV**A**P—	V
M16	L18A, D7A	MAEEQR**A**LISSDGSSGV**A**P—	V
M17	L18A, I9A	MAEEQRDL**A**SSDGSSGV**A**P—	PM
M18	L18A, S10A	MAEEQRDLI**A**SDGSSGV**A**P—	PM
M19	L18A, P19A	MAEEQRDLISSDGSSGV**AA**—	PM
M20	P19A	MAEEQRDLISSDGSSGVL**A**—	PM

[1]Mutations of amino acids to Ala are indicated by a boldface **A**. Localization of vesicle or plasma membrane staining is indicated by V and PM, respectively.

megaprimer. A segment of 120 nucleotides was obtained by the first round of PCR with mutant primers and *Bgl*II-F as primer, then by the second round of PCR, and the final 250 nucleotides were amplified with the former segment and C1-R as primer (Figure 1). The final PCR products were inserted into pEGFP-Cl. The recombinants were identified by *Sal*I-*Bgl*II digestion and sequencing confirmation. The resultant plasmids were named M1 to M20. The segment of the truncated form of the wild-type Ii gene that was amplified with the primers Ii-83-F and Ii-83-R (with 250 nucleotides of approximately 83 amino acids) was inserted into pEGFP-C1 and named WT.

Two Leu-Based Motifs Are Required for Chicken Ii Intracellular Localization by Fluorescence Microscopy

To validate that 2 motifs in the chicken are required for Ii-induced endosomal vacuolation, we first detected the intracellular localization of GFP expression to es-

Table 2. Primers for amplification of the mutation and wild-type Ii gene

Primer	Sequence[1] (5′→3′)
E3A-R	GATGAGGTCCCGCTGCTCGGCAGCCAT
E4A-R	GATGAGGTCCCGCTGGGCCTCAGCCAT
Q5A-R	GAGATGAGGTCCCGAGCCTCCTCAGCC
R6A-R	GGAGGATGAGGTCGCCCTGCTCCTCAGC
D7A-R	GGAGGAGATGAGAGCCCGCTGCTCCTC
L8A-R	CGGAGGAGATGGCGTCCCGCTGCTCCT
I9A-R	GCTGCCATCGGAGGAAGCGAGGTCCCG
S10A-R	CTGCTGCCATCGGAGGCGATGAGGTC
S11A-R	CCACTGCTGCCATCGGCGGAGATGAG
D12A-R	CCCACTGCTGCCAGCGGAGGAGATGAG
G13A-R	CACCCCACTGCTAGCATCGGAGGAGAG
S14A-R	GAGCACCCCACTAGCGCCATCGGAGGAG
S15A-R	GGAGCACCCCAGCGCTGCCATCGGAG
G16A-R	CCAATGGGGAGCACAGCACTGCTGCC
V17A-R	CCCAATGGGGAGAGCCCCACTGCTGCC
L18A-R	TCCCAATGGGGGCCACCCCACTGCTG
P19A-R	CTGTTCCCAATAGCGAGCACCCCACTG
*Bgl*II-F	GAAGATCTCGAGCTCAA (low-anneal *Bgl*II)
C1-R	GCGTCGACAGGCATCTTCCTCTGCAGCGACTCC (high-anneal *Sal*I)
Ii-83-F[2]	GAAGATCTCGAGCTCAAGCTTCGAAT (*Bgl*II)
Ii-83-R[3]	GCGTCGACAGGCATCTTCCTCTGCAGCGACTCC (*Sal*I)

[1]F = forward; R = reverse.

[2]Ii-83-F = forward primer for amplification of the truncated form of the wild-type Ii gene.

[3]Ii-83-R = reverse primer for amplification of the truncated form of the wild-type Ii gene.

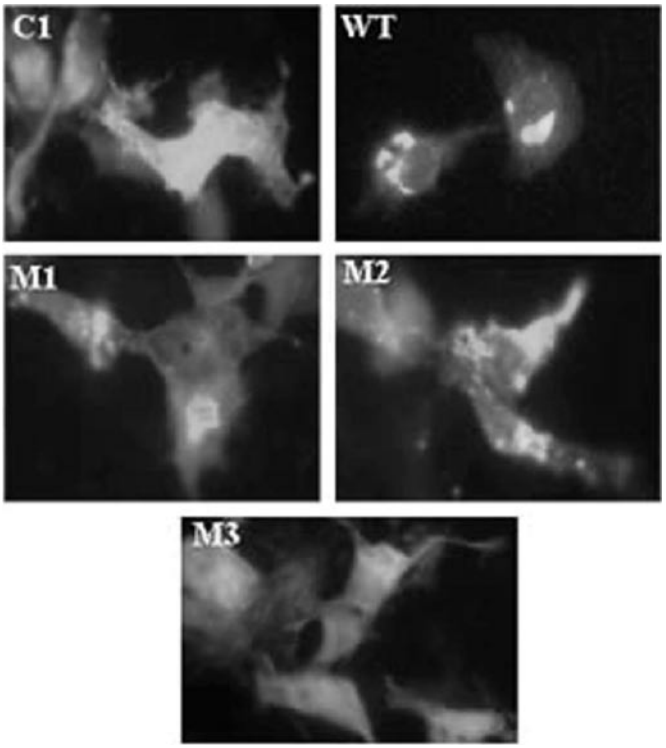

Figure 2. Localization of invariant chain (Ii) wild types and mutants in COS-7 cells transiently transfected with pEGFP-C1 (C1), wild-type Ii (WT), L8A (M1), L18A (M2), and L8A, L18A (M3) were visualized by fluorescence microscopy.

timate the effect of M1 to M3 constructs (Table 1). The results (Figure 2) indicated that Leu is a key element of the targeting motif. In the control cells transfected with pEGFP-Cl alone, there was a strong plasma membrane fluorescence staining in COS-7 (C1 in Figure 2). When other constructs were transfected into COS-7 cells, different results were observed by fluorescence microscopy. Fluorescence staining was found in the intracellular vesicles of the cells transfected by wild-type constructs (WT in Figure 2). A site mutation of Leu^{8} (M1) or Leu^{18} (M2) to Ala did not abolish this endosomal localization (M1 to M2 in Figure 2). However, in the mutation of both Leu^{8} and Leu^{18}, fluorescence detection revealed strong plasma membrane staining (M3 in Figure 2). Therefore, mutated Ii molecule lost the ability to internalize to endosome transport. The above-mentioned events indicated that the chicken Ii cytoplasmic tail might contain 2 independent Leu-based sorting signals.

Effect of Point Mutations on the 2 Internalization Signals

To elucidate the requirements for a structural context of the Leu^{8} and Leu^{18} and to observe the effect of residues in the region of both targeting motifs, we next performed a set of point mutations by changing several residues to Ala (see M4 to M19 in Table 1). The results indicated that these mutated residues had a different effect on Ii localization. Regarding the Leu^{8}-Ile^{9} signal,

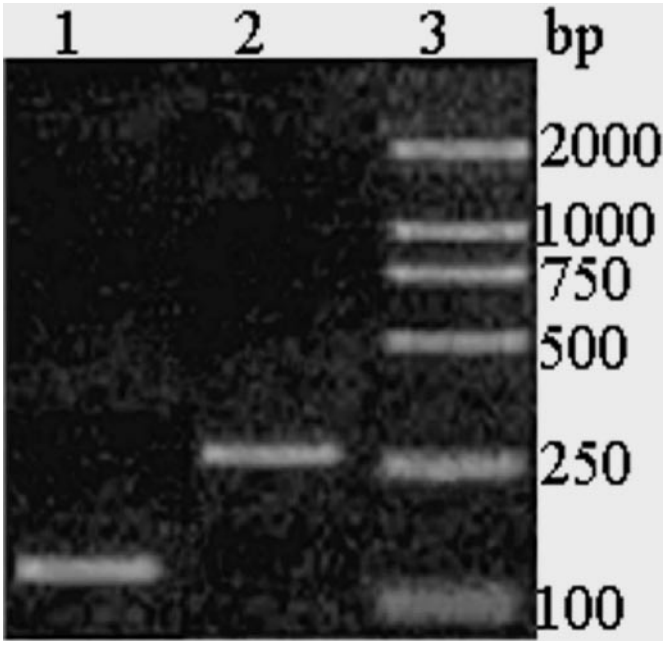

Figure 1. The amplified product of a mutated invariant chain (Ii) gene. Lane 1 = the amplified megaprimer by the mutated primer and the low-anneal *Bgl*II primer; lane 2 = the amplified PCR product using the megaprimer and the high-anneal C1-82-R as primer; lane 3 = DL2000 markers. bp = base pairs.

at a time-point mutation of Leu^{8} and Ser^{11} (M4), Asp^{12} (M5), Gly^{13} (M6), or Ser^{14} (M7) (see M4 to M7 in Table 1 and Figure 3), no change in internalization was detected, whereas Leu^{8} and Ser^{15} (M8), Gly^{16} (M9), Val^{17} (M10), or Pro^{19} (M11) (see M8 to M11 in Table 1 and Figure 3) prevented internalization. For the second signal, Val^{17}-Leu^{18}, mutations of Leu^{18} and Glu^{3} (M12), Glu^{4} (M13), Gln^{5} (M14), Ile^{9} (M17), Ser^{10} (M18), or Pro^{19} (M19) to Ala abolished internalization (see M12 to 14 and M17 to M19 in Table 1 and Figure 3), whereas the mutations of Leu^{18} and Arg^{6} (M15) or Asp^{7} (M16) to Ala had no effect on internalization (see M15 to M16 in Table 1 and Figure 3). These data suggest that 2 autonomous endosomal sorting signals function in internalization (Figure 2) in the cytosolic tail of chicken Ii. Moreover, the data also indicated that the functional Leu-based sorting signal required specific neighboring residues.

Pro^{19} Is Located C-terminal to the Val-Leu Motif, Possibly Maintaining the Overall Structure in Chicken Ii

In the next experiment, we investigated the role of the Pro residue at position 19 in chicken Ii C-terminal to the Met-Leu signal in endosomal sorting. During a substitution of Leu^{8} or Leu^{18} for Ala, a simultaneous mutation of Pro^{19} (M11 and M19) could cause Ii to abolish its internalization (M19 and M11 in Figure 3). Moreover, when neither mutated Leu^{8} nor Leu^{18} but only Pro^{19} (M20) was changed to Ala, a similar result was observed (M20 in Figure 4). Therefore, we pre-

sumed that the Pro^{19} might be located C-terminal to the Val-Leu motif, possibly acting as a key residue contributing to the required structure of the signal.

DISCUSSION

In this study, we carried out a series of experiments to investigate the effect of 2 Leu and the ambient amino residues on endosomal vacuolation in chicken Ii. Based on the results, there could be more potential targeting motifs in the chicken Ii molecule. In most conditions, a single mutation of an amino acid may not have an influence on endosomal localization of chicken Ii, whereas 2 residues together could easily change this targeting. However, as a key residue, the 19th Pro plays an important role in maintaining Ii structure, because a single substitution for it would cause Ii to lose its endocytic targeting.

The cytoplasmic tail of chicken Ii was found to contain 2 Leu as sorting signals, located at the 8th and 18th amino acid residues. Both caused efficient sorting to endosomes and internalization from the plasma membrane independently (Bremnes et al., 2000). Our study also showed that just one of them could maintain the basic intracellular localization of the chicken Ii molecule (M1 to M2 in Figure 2). These results are in agreement with previous observations about Leu-based targeting motifs in mammalian Ii (Bremnes et al., 1994). In accordance with an extrapolation of spatial structure (Figure 5) based on the amino acid sequence of chicken Ii, which was predicted by software I-Tasser (http://zhang.bioinformatics.ku.edu/I-TASSER/output/S9930), more helixes might exist within the chicken Ii molecule, and the first α-helix (residues 3 to 11) would contact the 2 other helixes (residues 31 to 43 and 46 to 56) with a hydrophobic structure. The hydrophobic residue Leu^8 is located right at the interface of the composed α-helix. There may also be a long loop (residues 12 to 30) at the cytoplasmic side of the membrane, with Leu^{18} situated within the segment of this loop. For all of them, a mutation of Leu^8 or Leu^{18} could change the first helix or the loop, but that is not enough to alter the entire spatial structure and thereby disrupt the localization of chicken Ii.

The function of a protein is known to be determined by its structure. Our results also indicate that in addition to Leu, which is a key residue for this targeting motif, some other amino acid residues are also necessary to retain the molecular spatial structure for endosomal localization of Ii. When we further investigated the influence of these residues on 2 Leu based on their targeting motifs, different effects were found. By comparing the results from chicken Ii with others from human Ii, we found that when the 15 amino acid residues of chicken Ii were mutated, 9 of them, namely, Glu^3, Glu^4, Gln^5, Ile^9, Ser^{10}, Ser^{15}, Gly^{16}, Val^{17}, and Pro^{19}, had an obvious cooperative effect with Leu^8 or Leu^{18} on localization, because the chicken Ii lost its endo-

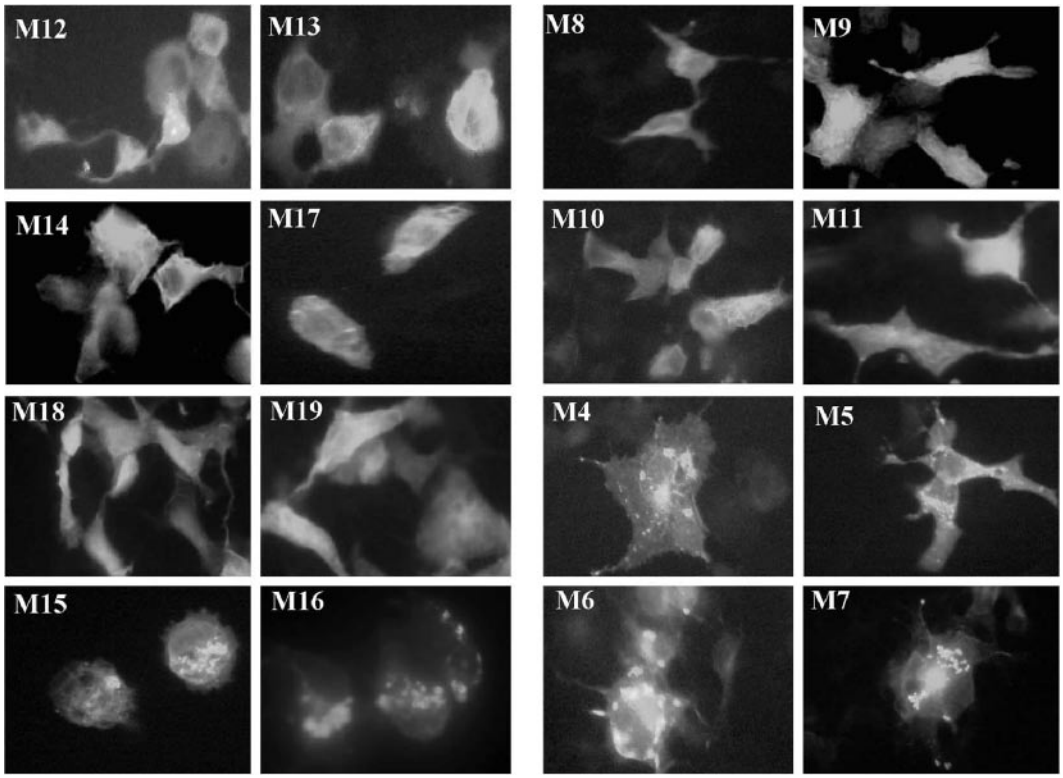

Figure 3. Localization of invariant chain (Ii) mutants in COS-7 cells transiently transfected with L18A, E3A (M12), L18A, E4A (M13), L18A, Q5A (M14), L18A, I9A (M17), L18A, S10A (M18), L18A, P19A (M19), L18A, R6A (M15), L18A, D7A (M16); and L8A, S15A (M8), L8A, G16A (M9), L8A, V17A (M10), L8A, P19A (M11), L8A, S11A (M4), L8A, D12A (M5), L8A, G13A (M6), L8A, S14A (M7) were visualized by fluorescence microscopy.

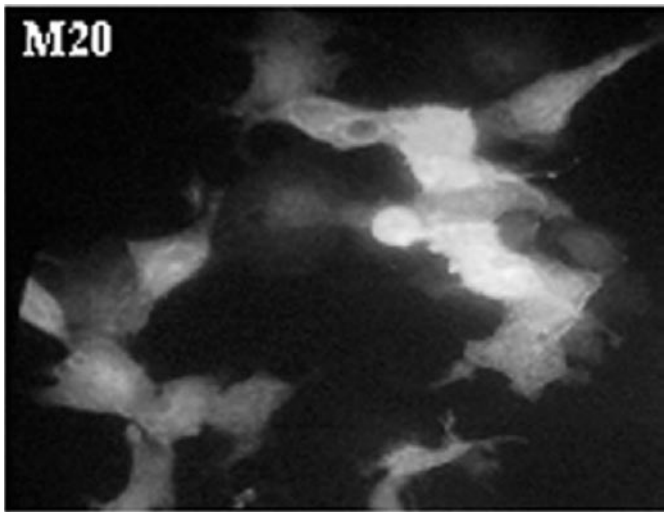

Figure 4. Localization of the invariant chain (Ii) mutant of P19A in COS-7 cells transiently transfected with P19A (M20) was visualized by fluorescence microscopy.

Figure 5. The structural forecast of chicken invariant chain (Ii).

somal localization, whereas the other 6 mutated residues, namely, Arg^{6}, Asp^{7}, Ser^{11}, Asp^{12}, Gly^{13} and Ser^{14}, showed no cooperative effect.

Another interesting phenomenon is that the same kinds of amino acid residues have different effects on the cooperative localization of Ii because of their different positions on the protein molecule. Pond et al. (1995) found that when an acidic amino acid residue 4 or 5 residues N-terminal on the cytoplasmic tail of human Ii was mutated, its intracellular localization, mediated by 2 Leu-based sorting signals, was abolished. This would be similar to the acidic residues in human Ii in that both mutated acidic amino acid residues (Glu^{3} and Glu^{4}) would play a cooperative and important role with mutated Leu^{18} in the internalization. However, another acidic residue, Gly^{13}, had no influence on intracellular localization of chicken Ii. An Ala in position 2 of chicken Ii is absent from human Ii, and 2 aspartic acidic residues in positions 2 and 3 in human Ii are exchanged with Glu in chicken Ii, which may result in a change of chicken Ii conformational structure and further affect its intracellular localization. Moreover, the effects of 4 Ser residues were similar to those of the acidic residues; the mutant Ser^{10} or Ser^{15}, but not Ser^{11} or Ser^{14}, would influence the function of Ii. The contrasting effect of Gly^{13} and Gly^{16} was similar to that of Ser. All these suggest that the function of a residue in the chicken Ii molecule might be dependent not only on its properties and position on the protein molecule, but also on its crucial position based on its changing the spatial structure.

Moreover, under a mutation of Leu^{18}, the substituted residue Glu^{3}, Glu^{4}, or Gln^{5}, which is located away from Leu^{8}, would change the internalization of chicken Ii. In contrast, under a mutation of Leu^{18}, the substituted residue, Arg^{6} or Asp^{7}, located in a neighboring position to Leu^{8}, showed no effect on endosomal internalization, whereas a mutated Ser^{11}, Asp^{12}, Gly^{13}, or Ser^{14} might interfere with the localization of Ii. According to Bakke and Dobberstein (1990) and Lotteau et al. (1990), point mutations of the amino acids around di-Leu-based motifs of human Ii revealed that amino-terminal residues located in spatial proximity to the Leu-based motifs contributed to efficient internalization and targeting of endosomes, whereas residues on the spatially opposite side of the motifs were mutated with no measurable effect on targeting. Our results indicated that the influence of other residues, whether they were close to Leu or not in the Ii amino acid sequence, might have various effects on localization. In addition to the effect of the spatial distance between residues of an amino acid and Leu, in the case of Ii localization mediated by 2 Leu as targeting motifs, some residues would act as an important signal together with Leu or as a necessary portion of the whole molecule structure to maintain its function, and some would simply be less important residues in the protein chain.

In addition, a Pro at the position of the 15th residue N-terminal in the human Ii molecule is believed to be important for the Met-Leu signal, possibly contributing to the required structure of the signal (Motta et al., 1995). This study revealed that after substitution of Ala for only Pro^{19}, chicken Ii abolished its internalization (Figure 4). In a comparison of the amino acid sequence of the cytosolic tail of humans with those of mice and rats, Pro^{15} was conserved (Bakke and Dobberstein, 1990). Based on the presumed molecular structure, Pro^{15} may be located at the bend of the α-helix formed by the residues from Gln^{4} to Leu^{17}. According to the presumed spatial structure of chicken Ii in Figure 5, Pro^{19} in chicken Ii might be located on a loop (residues 12 to 30) that was connected with the first helix (residues 3 to 11), which suggests Pro^{19} may be an important residue for structural stability. Previous studies on model helical polypeptides containing Pro also confirmed that it is sufficient to introduce a conformational change of only one residue to accommodate Pro in a distorted helix (Piela et al., 1987; Polinsky et al., 1992). Kinked Pro α-helices with minor conformational changes and minimal disruption of the helix hydrogen

bonding have also been observed in crystal structures of proteins (Barlow and Thornton, 1988). Thus, the substitution of Ala, a highly flexible residue, for Pro^{19}, a rigid residue, affected chicken Ii structural stability. This suggests that the Pro^{19} located C-terminal to the Val-Leu motif is a novel protein sorting signal, possibly maintaining the entire structure of the chicken Ii.

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