Structure-Activity Relationship of the Leucine-based Sorting Motifs in the Cytosolic Tail of the Major Histocompatibility Complex-associated Invariant Chain*

(Received for publication, June 12, 1995, and in revised form, August 2, 1995)

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The cytosolic tail of the major histocompatibility complex-associated invariant chain protein contains two Leu-based motifs that both mediate efficient sorting to the endocytic pathway. Nuclear magnetic resonance data on a peptide of 27 residues corresponding to the cytosolic tail of human invariant chain indicate that in water at pH 7.4 the membrane distal motif Leu<sup>14</sup>-Ile<sup>15</sup> lies within a nascent helix, while the membrane proximal motif Met<sup>16</sup>Leu<sup>17</sup> is part of a turn. The presence of a small amount of methanol stabilizes an α-helix from Gin<sup>4</sup> to Leu<sup>27</sup> with a kink on Pro<sup>15</sup>. Point mutations of the cytosolic tail of the protein suggest that amino-terminal residues located in spatial proximity to the Leu motifs contribute to efficient internalization and targeting to endosomes in transfected COS cells. Residues on the spatially opposite side of the Leu motifs were, on the other hand, mutated with no measurable effect on targeting. Structural and biological data thus suggest that the signals are not continuous but consist of "signal patches" formed by the three-dimensional structure of the cytosolic tail of invariant chain.

In transmembrane proteins, specific signals for endosomal or lysosomal sorting have been identified within the cytosolic tails. In a number of proteins sorted to the endosomal pathway, a Tyr-containing motif seems to mediate internalization from the plasma membrane (for review, see Refs. 1 and 2) and form a tight β turn functionally comparable to the internalization signal found in cell surface receptors (3-6). However, recent NMR analysis of a synthetic peptide corresponding to the extreme 21 carboxyl-terminal amino acid residues of the cytosolic domain of trans-Golgi network (TGN) protein TGN38/41 (which is routed from the plasma membrane and back to TGN) shows that this Tyr-containing internalization signal lies within a nascent helix (7).

Tyrosine signals are not universal for sorting of membrane proteins to the endosomal/lysosomal pathway. In fact, the cytosolic tail of the lysosomal membrane protein LIMP II contains no Tyr but a Leu-Ile signal, located two residues from the carboxylic end, which mediates efficient sorting (8, 9). Letourneau and Klausner (10) reported that a Leu-Leu motif could mediate lysosomal targeting of the CD3-γ and -δ chains of the T-cell receptor complex. This signal was functional, even if located at the carboxy-terminal end, and together with a Tyr-based motif they were individually sufficient to induce endocytosis and delivery to lysosomes. Two similar signals were also reported in the cytosolic tail of the mannose 6-phosphate-receptor by Johnson and Kornfeld (11). The above studies may suggest that the Tyr signal is primarily mediating sorting to the endocytic pathway via internalization from the plasma membrane, whereas the Leu signal alone, or in combination with the Tyr signal, mediates direct sorting from the TGN to the endocytic pathway. However, both signals internalize efficiently plasma membrane proteins, and further information is needed to clarify the requirements for selecting the pathway from the TGN to endosomes (for further discussion see 2).

The invariant chain (Ii), which efficiently targets the associated major histocompatibility complex class II molecules to the endosomal/lysosomal pathway, is another example of a protein with cytosolic Leu-sorting motifs (12-14). Invariant chains from different species contain in their cytosolic tails pairs of Leu-Ile and Met-Leu (Ile-Leu), and mutational analysis of the cytosolic ii tail fused to reporter molecules have shown that these signals can independently mediate endosomal targeting (15, 16). Distribution and internalization studies also show that a significant fraction of the II molecules, alone or in complex with major histocompatibility complex class II, were transported to endosomes via the plasma membrane (17). Furthermore, the Leu-Ile and Met-Leu motifs individually mediate rapid internalization of a chimeric protein (INA) obtained by fusing neuraminidase (NA) to the cytosolic tail of II (15). In another study the ii tail was fused to the transferrin receptor, but the measured sorting via the plasma membrane was not sufficient to account for the amount of protein synthesized, indicating sorting also directly from the TGN (16). The accumulated data thus suggest that Leu-based motifs are actively engaged in the sorting of membrane proteins to the endosomal/lysosomal pathway.
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The NMR Analysis—In (1–27) peptide was studied at pH 7.4 in phosphate buffer at 283 K and pH 7.4. Assignment of proton spin systems was obtained with the sequential methodology outlined by Wüthrich (29). From the amide protons, TOCSY experiments allowed identification of the α and the β protons of almost all of amino acids. Residues with long side chains were identified by a combination of TOCSY and NOE experiments. Individual spin systems were placed in the primary structure by identification of characteristic short and medium range NOE connectivities.

Fig. 1 reports the summary of NOE information for In (1–27) in water at 283 K and pH 7.4. Together with the strong αCH$_i$-NH$_{i+1}$ connectivities, a number of NOEs were observed between the NH resonances of sequential amino acids in the regions before and after Pro$_{15}$, the strongest being observed in the Asp$_6$–Asn$_{11}$ region and between Met$_{16}$ and Leu$_{17}$. Proximity of NHs of adjacent residues requires a kink in the backbone, a conformation associated with a β turn or with an α helix (42). Accordingly, the NOEs between pairs of NHs in the region Arg$_8$–Glu$_{12}$ suggest that consecutive turns are present in this part of the peptide and that Met$_{16}$–Leu$_{17}$ is part of a turn. An ensemble of consecutive turns resembles a helix-like conformation, since distance constraints in tight turns are similar to those in helical segments (29). This is confirmed by αCH$_i$-NH$_{i+2}$ connectivities (Arg$_2$–Leu$_3$, Asp$_5$–Ile$_6$, Leu$_2$–Ser$_3$, Ile$_6$–Asn$_{10}$, and Ser$_9$–Asn$_{11}$), and by βCH$_i$-NH$_{i+3}$ cross-peaks (Gln$_6$–Arg$_9$, Leu$_1$–Ile$_6$, Ser$_9$–Asn$_{10}$, and Glu$_{12}$–Gln$_{13}$). However, the contemporary presence of αCH$_i$-NH$_{i+1}$ and NH$_{i+1}$-NH$_{i+2}$ NOEs (Fig. 1) indicates that the local helix-like structure in the region at the amino-terminal side of Pro$_{15}$ dynamically transforms into extended conformations.

The absence of a stable helical structure is confirmed by the lack of characteristic NOEs and by the $3^J_{HN}$α coupling constant values. We did not observe loop to loop interproton NOEs (e.g. α of residue i to the NHs of residues i + 3 and i + 4), although several residues show the NH to NH interaction. On the other hand, only the amides of residues Leu$_7$ and Ser$_9$ have coupling constants (5.9 Hz) similar to that expected for a stable helical structure (43), while the $3^J_{HN}$α coupling constants for the remaining residues in that region all are > 7 Hz. These results (stretch of sequential and short range NOEs, the absence of helix-defining NOEs within a conformation ensemble giving rise to largely averaged coupling constants) argue for the presence of “nascent helix” structure (44), including the Leu$_2$-Ile$_6$ sorting signal of invariant chain. The term nascent helix refers to an ensemble of interconverting extended chain and turn-like structures existing over a peptide sequence at the earliest stages of helix initiation.

From Pro$_{25}$ onward, the peptide also assumes turn-like conformations up to Ala$_{25}$, as suggested by NH$_1$-NH$_{1+1}$, βCH$_i$-NH$_{i+2}$, and αCH$_i$-NH$_{i+2}$ NOE connectivities (Fig. 1). In particular, the presence of strong NOEs between the amide protons of Met$_{16}$ and Leu$_{17}$ and between αCH$_i$ and NH$_{i+2}$ NOEs (Fig. 1) indicate that the second signal Met$_{16}$–Leu$_{17}$ is part of a turn. A nascent helix can be stabilized by small amounts of organic co-solvent (44). Upon addition of methanol at a concentration of 20% (v/v) to the In (1–27) aqueous solution, all resonances needed to be reassigned because one-to-one comparison between the two solvents was not possible. Complete sequential assignment was made as described above. Stabilization of the helical structure was confirmed by the sequential αCH$_i$-NH$_{i+1}$ and NH$_{i+1}$-NH$_{i+2}$ and medium-range (αCH$_i$-NH$_{i+
u}$, ν > 2, and αCH$_i$-βCH$_{i+
u}$) NOEs (45), slowly exchanging amide protons (29), and $3^J_{HN}$α coupling constants (43). Fig. 2 summarizes the observed NOEs, the relative exchange rates of amide protons and the apparent $3^J_{HN}$α coupling constants for In (1–27) at 283 K and pH 7.4 in the presence of 20% methanol. The fact that in the region Gln$_{6}$–Leu$_{14}$ the NH$_{i}$-NH$_{i+1}$ NOEs are intense, while the αCH$_i$-NH$_{i+1}$ NOEs are much weaker, implies a generally helical structure (42). The observation of several unambiguous αCH$_i$-NH$_{i+2}$, αCH$_i$-βCH$_{i+3}$, and a single αCH$_i$-NH$_{i+4}$ cross-peaks (Fig. 2) supports the presence of a helix.

Further corroborative data come from slowly exchanging amides; except for Gln$_{13}$, all the amide protons in the Gln$_6$–Leu$_{14}$ region are in slow exchange. The slow exchange most likely indicate hydrogen bonding, since it is unlikely that a slowly exchanging proton is buried in the interior of a biomolecule as small as the cytosolic tail of In. $3^J_{HN}$α < 6 Hz in the Gln$_6$ to Leu$_{14}$ region also supports the presence of a helix (43). Furthermore, αCH$_i$-NH$_{i+2}$ cross-peaks, suggestive of a 3$_{10}$ helix (45), were not detected in the middle region of the peptide...
and we conclude that the Gln4-Leu14 region of I-1(1-27) forms an α helix.

From Pro15 onward, we observed strong αCH-NH NOE connectivities, meaning that the conformation is essentially extended. However, the presence of NH1-NH2, βCH-NH1,2, two αCH-NH2,3 (Pro15-Leu17 and Met16-Gly21) and a single small αCH-NH1,2 (Leu14-Leu17) NOE connectivities (Fig. 2) is indicative of local structures and short range order. βCH-NH2,3 connectivities are commonly observed in type I or type III turns (42), but while type III turns are very similar to type I, the presence of a Pro in the trans isomer is compatible with both type I and type II classes of turns (46). In principle, they can be distinguished on the basis of

\[ \text{HN} \] and \[ \text{CH} \] connectivities, meaning that the conformation is essentially extended. The average root mean square deviation for backbone atoms of the polypeptide and the variability in its relative position with respect to the helix. The average root mean square deviation for backbone atoms of residues 16–25 is 0.293 nm. No unique conformation could be determined for the Met1-Asp2-Asp3 and the Ser26-Lys27 segments.

The presence of an αCH-NH NOE connectivity between Pro15 and Met16 suggests that the region Leu14 to Leu17 forms a type I β turn, since the αCH-NH distance is between 0.19 and 0.35 nm (47). The presence of an αCH-NH NOE between Leu14 and Leu17 suggests that the type I turn can actually prolong the helix up to Leu17. For Arg20 to Ala21 and Ala23 to Ser26, the observation of αCH-αCH NOE connectivities between Arg20 and Pro21, and Ala23 and Pro24 for the trans isomer of both regions, suggests the presence of two type II β turns.

Methanol is known to induce structure in peptides (see, for example, Ref. 48). However, at the used ratio of 80% water/20% methanol (v/v), the mixture has a helix-promoting ability slightly higher than that of pure water (48), indicating that the structure is only stabilized by the presence of methanol. The possibility that the secondary structure arises through aggregation was ruled out by investigating a 10-fold diluted sample of I-(1–27). No differences in chemical shift and line width of the NH resonances were observed in one-dimensional spectra, and NOESY experiments confirmed all the connectivities, and thus the structure, described above.

From NMR data in water/methanol, 100 randomly selected starting conformations were generated by means of distance-geometry calculations. The best nine structures, in terms of smallest target function values, were subjected to restrained energy minimization. Before minimization, they fulfilled quite well the whole set of NMR restraints with no violations of the upper bounds of the distance restraints greater than 0.05 nm, and of dihedral angle restraints greater than 5°. The energy of the refined structures were all in the narrow range from −1877 to −2080 kJ mol⁻¹; the maximal distance constraint violation was 0.050 ± 0.001 nm, and the average sum of distance constraint violations was 0.293 ± 0.001 nm. None of the selected structures presented additional short interproton distances not experimentally observed. Fig. 3A shows a superposition of all nine structures for the region covering residues Met1-Pro15, all compatible with NOE data, since calculations of structures from NOES data is a means to assess possible and favored conformational states and not single structures. The convergence achieved over the well defined α helix from Gln4-Leu14 was good, with a root mean square deviation for its backbone atoms of 0.090 nm. The region Pro15-Glu25 (Fig. 3B) did not converge to a consensus structure, reflecting the absence of structurally significant NOEs for this region of the polypeptide and the variability in its relative position with respect to the helix. The average root mean square deviation for backbone atoms of residues 16–25 is 0.293 nm. No unique conformation could be determined for the Met1-Asp2-Asp3 and the Ser26-Lys27 segments.

Effect of Point Mutations on the Two Internalization Signals—To elucidate the requirements for a structural context of the Leu7-Ile6 and Met16-Leu17 signals, we performed a set of point mutations on the INA fusion protein changing several residues to alanine (Fig. 4). To monitor internalization from the plasma membrane, the transfected COS cells were incubated with 125I-labeled antibodies on ice and chased at 310 K. In order to study one signal at a time, the region of the other signal was either deleted or destroyed by mutating Leu7 or Leu17 to Ala and the time response for internalization of the bound antibody was measured as described earlier (15). The time to reach 25% internalization is noted in Fig. 4. Constructs were considered not actively internalized if they did not reach 15% internalization after 10 min, in analogy with INA constructs with both internalization signals deleted (15).

Regarding the Leu7-Ile6 signal, point mutation of Arg9 and Asp3 to Ala (construct 6, Fig. 4), no change of the internalization rate was detected, whereas both Gln9 (construct 5) and Asp9 (construct 4) prevented internalization. Alteration of amino acids at the carboxyl terminus of the signal can be changed to alanine without affecting the internalization signal; mutation of the potentially phosphorylatable Ser9 (construct 9), and Asn10 and Asn11 (construct 10), did not change the rate of internalization in line with earlier studies (15).

For the second signal, Met16-Leu17, mutation of Pro15 to Ala (construct 21) abolished internalization, whereas neither mutation of Glu13 nor Leu14 (constructs 19 and 20, respectively) altered the internalization efficiency. The negative residue Glu12 (construct 15), however, reduced internalization to background level. For all the INA mutations, immunofluorescence studies showed a strong plasma membrane staining for the constructs that were not internalized and vesicular staining (V, localization column in Fig. 4) corresponding to endosomes for the constructs that were actively internalized. When native li harboring the identical cytosolic tail mutations as INA was
expressed in COS cells, the corresponding Ii construct accumulated on the plasma membrane or in endosomes like the INA molecule (data not shown), verifying that the tetrameric NA is a reporter molecule that reflects the endosomal sorting properties of the trimeric native Ii molecule (15).

Deletion of the first 11 residues (construct 18) reached 25% internalization in 4.0 min, whereas elimination of the first signal by point mutations (construct 14) reached 25% internalization in 2.0 min. This may indicate that additional amino-terminal residues than those provided in construct 17 modulate the efficiency of the Met-Leu signal. Our biological data thus confirm that the cytosolic tail of Ii comprises two autonomous endosomal sorting signals that function in internalization (15, 16, 39) and in addition point out that a functional Leu-based sorting signal requires specific geometrically neighboring residues.

DISCUSSION

We have applied NMR spectroscopy to study the solution structure of a synthetic peptide corresponding to the cytosolic region of the protein Ii and containing the two internalization signals.

In aqueous solution at pH 7.4 we detected NOEs characteristic of a nascent helix involving the membrane distal motif Leu14-Ile16, while the membrane proximal motif Met35-Leu17 is part of a turn. In the presence of 20% methanol, the nascent helix was stabilized. We observed a regular α helix in the region Gin5-Leu14 and a segment of consecutive β turns between Leu14 and Ser26, namely a type I (Leu14-Leu17) and two type II (Arg20-Ala23 and Ala23-Ser26) β turns. The presence of a small NOE between the χCH of Leu14 and the NH of Leu17 suggests that the turn is actually part of the helix. Inspection of the calculated backbone φ and ψ dihedral angles of the segment Leu14-Pro15-Met16-Leu17 shows that except for minor variation in Leu14, all residues retain values appropriate for an α helix. The calculations thus indicate that the type I turn can be accommodated in the helix with minor conformational changes on Leu14 without the rest of the helical residues being perturbed. The resulting structure is an α helix from Gin4 to Leu17 with a bend at Pro15. Previous calculations on model helical polypeptides containing proline also confirm that it is sufficient to introduce a conformational change of only one residue in order to accommodate proline in a distorted helix (49, 50). Kinked proline α helices with minor conformational changes and minimal disruption of the helix hydrogen bonding have also been observed in crystal structures of proteins (51).

Comparison of the cytosolic tails of Ii from human, mouse, and rat shows that Pro15 is conserved (12), while in the chicken a Pro is present at site 17. This suggests that the proline conserved in that area might have a definite structural/functional role. It is noteworthy that mutation of Pro15 to Ala (construct 21, Fig. 4) abolishes internalization. Considering that Ala is the most helix favoring of the 20 commonly occurring amino acids (52, 53) such a substitution is expected to preserve the helix while avoiding the bend. In fact, energy minimization calculations (not shown) on Ala15 Ii-(1–27) peptide have found a regular helix from Gin4 to Leu17. Accordingly, it is tempting to speculate that a kinked helix is required for internalization of Ii.

Our biological data show that leucine motifs are influenced by residues located at their amino-terminal side as Ser9, Asn10, and Asn11 can be changed to alanine without affecting the internalization signal. This is in line with other studies showing that the signals work independently from the carboxyl-terminal residues (8–10). In addition, amino acids spatially close to the signals are fundamental for their correct function and this can be rationalized by referring to the three-dimensional structure of Ii-(1–27). The presence of Ala instead of Gin4 hampers efficient internalization (Fig. 4). The helical wheel diagram (Fig. 5) indicates that Gin4 is positioned on the same side of the helix as Leu7 and Ile8, so that its mutation alters the surroundings of the signal. Accordingly, if residues pointing away from the signal are changed, the mutation is expected to be irrelevant. In fact, mutation of Arg6 and Asp6, which are found on the opposite side of the helix (Fig. 5), does not alter the sorting capabilities of the molecule. Mutation of the negatively charged Asp6 into Ala also abolishes internalization. This may be related either to the need of negatively charged residue to be located at the amino-terminal side of the signal (see below) and/or to the specific ability of Asp to stabilize α helical structures when flanking the amino terminus (54, 55). The side chain of Ala at site 3 cannot form a hydrogen bond with the free NH groups at the amino terminus of the helix as does Asp residue, resulting in a strong decrease of helicity, which may prevent internalization. An indirect confirmation of the relevance of the second hypothesis would be the finding that Asp4 becomes part of the helix when the concentration of Ii-(1–27) is increased.\(^2\) Altogether our results suggest that for a

\(^2\) S. Ness, personal communication.

\(^3\) A. Motta, B. Brennes, M. A. Castiglione Morell, R. W. Frank, G. Saviano, and O. Bakke, unpublished data.

### DISCUSSION

We have applied NMR spectroscopy to study the solution structure of a synthetic peptide corresponding to the cytosolic region of the protein Ii and containing the two internalization signals.

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correct functioning, the first signal requires the specific structural context generated by the helix and the amino-terminal residues with a specific spatial arrangement around Met$^7$-Leu$^8$. The segment Leu$^{14}$-Pro$^{15}$-Met$^{16}$-Leu$^{17}$ takes up a turn and forms the kinked part of the helix. Point mutations reveal that except for Leu$^{14}$ (construct 20 in Fig. 4), it is essential to have all the other residues unaltered for a functional second signal. By mutating Pro$^{15}$, Met$^{16}$, and Leu$^{17}$ (constructs 21, 22, and 23, respectively), no internalization was observed. It must be noted that substitution of residues within the helix with Ala does not modify the helix, rather it alters the chemical nature of the environment of the signal. In fact, only the substitution Ala for Pro has a structural explanation, since it destabilizes the turn (56) and/or removes the kink from the helix (see above), while both Ala and the native residues Met and Leu at sites 16 and 17 have low preferences for turns (56). As a partial confirmation to the relevance of the chemical properties of the side chains, we observed reduced internalization to background level through the second signal for the substitution Glu$^{22}$-Ala. This finding points out the necessity of a negatively charged residue on the amino-terminal side of the second signal, suggesting as a putative consensus signal for II two hydrophobic residues and the negatively charged amino-terminal residue: Asp$^2$(Glu$^{21}$)-Xaa-Xaa-Leu$^7$(Met$^{16}$)-Ile$^8$(Leu$^{17}$). The two signals might work differently at various stages of the intracellular pathway, requiring specific structural contributions. For Ii, and the fusion protein INA, it is not clear whether it is sorted to the endosomal pathway solely via the plasma membrane and/or directly to endosomes from TGN. The most likely interpretation of the accumulated data is that both pathways are functional (2).

Since endosomal localization is not detected when active internalization is destroyed, this would lead to the conclusion that both pathways are affected by the same point mutations. The internalization signal, indicate a nascent helix upstream of a low density lipoprotein receptor (4), containing a Tyr in the plasma membrane, whereas at least two intact tails in the structural elements.

The sorting machinery at TGN and plasma membrane might thus see elements of the same sorting signal or require common structural elements.

Acknowledgments—We are grateful to Prof. Bernhard Dobberstein (Zentrum für Molekularbiologie, Universität, Heidelberg) for helpful discussion and Antonio Maiello (Consiglio Nazionale delle Ricerche, Arco Felice) for technical assistance.

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Structure of Invariant Chain Sorting Signals

Fig. 5. Helical wheel representation of the Met$^1$-Leu$^{14}$ region of the Ii(1–27) peptide. Leu$^7$ and Ile$^8$ are boxed.
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