The Leucine-based Sorting Motifs in the Cytoplasmic Domain of the Invariant Chain Are Recognized by the Clathrin Adaptors AP1 and AP2 and their Medium Chains

(Received for publication, June 11, 1999, and in revised form, August 19, 1999)

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Recognition of sorting signals within the cytoplasmic tail of membrane proteins by adaptor protein complexes is a crucial step in membrane protein sorting. The three known adaptor complexes, AP1, AP2, and AP3, have all been shown to recognize tyrosine- and leucine-based sorting signals, which are the most common sorting signals within membrane protein cytoplasmic tails. Although tyrosine-based signals are recognized by the μ-chains of adaptor complexes, the subunit recognizing leucine-based sorting signals is less clear.

In this report we show by surface plasmon resonance that the two leucine-based sorting signals within the cytoplasmic tail of the invariant chain bind independently from each other to AP1 and AP2 but not to AP3. We also show that both motifs can be recognized by the μ-chains of AP1 and AP2. Moreover, by using monomeric as well as trimeric invariant chain constructs, we show that adaptor binding does not require trimerization of the invariant chain.

The major histocompatibility complex (MHC) class II molecules are expressed on antigen-presenting cells and present primarily antigenic peptides from exogenous proteins to T helper cells. The invariant chain (Ii), a type II transmembrane protein, associates with MHC class II molecules in the endoplasmic reticulum and prevents binding of immunogenic peptides to these molecules while escorting them to endosomal compartment where antigen-loading may take place (for review, see Refs. 1–3).

 Newly synthesized Ii is transport-competent as a trimeric complex (4), which is formed by its luminal domains (5, 6). Deletion analysis has shown that information contained in the cytoplasmic domain of Ii is necessary and sufficient for targeting of the protein to endosomal/lysosomal compartments (7, 8).

Site-directed mutagenesis localized two signals in the cytoplasmic tail that independently are able to sort Ii to endocytic compartments; one signal contains a leucine and isoleucine (LI) residues at positions 7 and 8 (9), and the second contains a methionine and leucine (ML) residues at positions 16 and 17 of the tail (10, 11). Additionally, the signals are part of an α-helix/turn, and acidic residues N-terminal of both sorting motifs are required for efficient sorting (12, 13).

Whether Ii reaches the endocytic pathway directly from the trans-Golgi network or indirectly via the plasma membrane (PM) by fast internalization is still a matter of debate. A dynamin mutant led to accumulation of MHCII/Ii at the plasma membrane (14), and each of the sorting motifs allowed a fast internalization from the PM (10, 15), suggesting an indirect transport route. On the other hand a direct pathway is supported by the finding that only 20% of a fusion protein with the Ii tail were ever exposed on the cell surface (11) and by the observation that the transport of Ii-MHC-II complexes to late endosomes is not inhibited by concanamycin B, which blocks trafficking between early and late endosomes (16). In conclusion the present data support a dual route to endosomes involving both direct transport and routing via the plasma membrane.

Both leucine- and tyrosine-based sorting motifs mediate internalization of membrane proteins from the PM and may sort membrane proteins to endosomes/lysosomes and to the compartment for peptide loading (for review see Refs. 3, 17, and 18). Different experimental approaches have shown that the medium chains of the three known adaptor complexes, AP1, AP2, and AP3, recognize tyrosine-based sorting motifs (19, 20). Recognition of leucine-based sorting signals by adaptors is less well characterized. It was recently proposed that these signals bind to the β-subunits of AP1 and AP2 (21). When peptides containing leucine-based signals and a photoactivatable cross-linker were incubated with adaptors, they were found to cross-link to the β-subunits of AP1 and AP2. In addition, also the μ-chains of AP1 and AP2 have been reported to bind to leucine-based signals. This was found using a random phage display library and by incubating recombinant adaptor μ-chains with immobilized peptides that were derived from the Ii cytoplasmic tail (22, 23). Apart from AP1 and AP2, leucine-based sorting motifs also bind to AP3, e.g. the leucine-based sorting motifs of the lysosomal membrane protein LIMP-II and of the melanosomal membrane protein tyrosinase bind to AP3 but not or only poorly to AP1 and AP2 (24). However, it is not known which of the AP3 subunits mediates binding.

In the present study, we used the trimeric invariant chain containing the two leucine-based sorting motifs to analyze...
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binding to adaptor complexes. We provide biochemical evidence that the two leucine-based signals can interact independently of each other with AP1 and AP2 but not with AP3. Furthermore, our experiments support the concept that the leucine-based signals of Ii are recognized by the adaptor \( \mu \)-chains.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—** Plasmid pET3a.IiTMHis (25) encodes a soluble C-terminal histidine-tagged Ii molecule lacking the transmembrane domain. A mutant IiTM molecule lacking the cytoplasmic do- main (IiTM\( \Delta CT \)) was generated by cleavage of pET3a.IiTMHis with NdeI and HindIII. A double-stranded oligonucleotide (5'-GATGGTTGCACTGACACTGACCTCCTTCAAGGACCACTGCA-3') was inserted to generate an ATG start codon in front of the complete Ii TM domain.

The two leucine-based motifs in the Ii cytoplasmic tail were changed by overlap extension using the polymerase chain reaction (26) on pET3a.IiTMHis. Leucine and isoleucine in positions 7 and 8 and methionine and leucine in positions 16 and 17 of IiTM were replaced by alanines generating LI \( \rightarrow \) AA and ML \( \rightarrow \) AC, respectively. A double mutant termed LI,ML \( \rightarrow \) AA was created. The resulting plasmids were used to produce soluble IiTM- molecules in Erichschia coli BL21(DE3) (Novagen, Madison, WI).

**Expression and Purification of the GST Fusion Proteins—** Ii plasmid pET3a.IiTMHis was transformed into E. coli BL21 cells. Expression was induced at 30 °C with 0.4 mM isopropyl-1-thio-\( \beta \)-D-galactopyranosidase for 2 h. Bacteria were harvested by centrifugation at 4,000 × g. IiHis and IiHis mutant (11) as a template. The primers in both cases were TC-CTGCTACCTGACCTGCGCTGCTTGGTGAATTC and TC-CTGCTACCTGACCTGCGCTGCTTGGTGAATTC. Amplified constructs were purified as recommended in the Amersham Pharmacia Biotech manual. Ii plasmids were transformed into E. coli BL21(DE3) (Novagen, Madison, WI). Express constructs containing a histidine tag at their N termini. The fusion proteins were released by a series of 15-s sonication bursts, purified on the GST-Sepharose (Amersham Pharmacia Biotech), and analyzed by SDS-polyacrylamide gel electrophoresis in the presence or absence of dithiothreitol. After Western blotting, the Ii polypeptides were visualized by using the QIAexpress detection system (Qiagen, Hilden, Germany).

**Preparation of Adaptors—** Clathrin-coated vesicles were prepared from bovine brain essentially as described by (27) except using Hepes instead of Mes buffer. The coat components were released from the membranes with 0.5 mM Tris/HCl, pH 7.2, 2 mM EDTA (28) and separated from the vesicles by centrifugation at 240,000 × g for 45 min at 4 °C. The extract was adjusted to 20 mM Hepes/KOH, pH 7.3, 100 mM potassium acetate, 2 mM MgCl\(_2\) and concentrated to 1–2 mg/mL protein using ultracentrifugation in the stirring cell with a 10-kDa cut-off filter (Amicon, Witten, Germany). This clathrin-coated vesicle extract was frozen in liquid nitrogen and stored at –80 °C.

For surface plasmon resonance studies, the clathrin adaptor complexes AP1 and AP2 were purified according to standard procedures from bovine or porcine brain (29). Briefly, clathrin-coated vesicles were purified from brain after homogenization and differential centrifugation. Adaptor complexes were released from clathrin-coated vesicles with 0.5M Tris, pH 7.8, plus 2 mM EDTA for 30 min at 4 °C. After concentration at 100,000 × g for 1 h, the membranes were fused in the presence of 0.5 mM dihydrofolate reductase was expressed from the control plasmid pQE16 supplied with the kit and purified according to manufacturer's recommendations. Protein concentration was determined by Coomassie-stained gels by comparison with protein standards.

**Expression and Purification of the GST Fusion Proteins—** Cytoplasmic tails of the wild type Ii (M) and its L7A.L7A mutant were fused in-frame to the C terminus of the GST protein. The cytoplasmic tails of the Ii and the L7A.L7A mutant were amplified by the polymerase chain reaction using the full-length Ii and the double alanine mutant (11) as a template. The primers in both cases were TC-CCGGGATCCATGATGACCGAAGCGCGC (primer 1) and ATGATGACCGAAGCGCGC (primer EcoRI site). The amplified constructs were cloned into pGEX-2t vector (Amer- sham Pharmacia Biotech) using the BamHI-EcoRI sites, and the frame was verified by sequencing. Fusion proteins were expressed and purified as recommended in the Amersham Pharmacia Biotech manual. Briefly, BL21 cells carrying the constructs of interest were induced with 0.25 mM isopropyl-1-thio-\( \beta \)-D-galactopyranosidase for 3 h and collected by centrifugation. The fusion proteins were released by a series of 15-s sonication bursts, purified on the GST-Sepharose (Amersham Pharmacia Biotech), and analyzed by SDS-polyacrylamide gel electrophoresis in the presence or absence of dithiothreitol. After Western blotting, the Ii polypeptides were visualized by using the QIAexpress detection system (Qiagen, Hilden, Germany).

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100 nM in running buffer) were then immobilized at a flow rate of 5 µl/min until the base-line shift was around 2000 response units, corresponding to 2 ng/mm².

GST fusion proteins of the li tail were immobilized to a CM5 sensor surface that was first coated with an anti-GST antibody (BIAcore AB) to a density of 5000 response units. GST-li fusion proteins were immobilized at a density of 1000 response units.

All interaction experiments were performed with buffer A (see above) at a flow rate of 20 µl/min. When using isolated adaptor µ-chains, buffer A was adjusted to 0.5% Triton X-100. Association for 2 min was followed by dissociation for 2 min, during which buffer A was perfused. A short pulse injection (15 s) of 20 mM NaOH, 0.5% SDS was used to regenerate the sensor chip surface after each experimental cycle. The peptide-derivatized sensor chips remained stable and retained their specific binding capacity for more than 100 experimental cycles of association/dissociation and regeneration. AP1 and AP2 were used at 200 nM unless otherwise stated.

**Determination of Kinetic Constants**—The rate constants (ka for association and kd for dissociation) of the interaction between li construct and purified AP1 or AP2 were calculated by using the evaluation software of the BIAcore 2000. Association was determined 15–20 s after switching to buffer flow. The dissociation rate constants were determined 5–10 s after switching to buffer flow. After a first dissociation phase for around 30 s further dissociation of adaptors was very low. The association constant ka, the dissociation constant kd, and the calculation of the equilibrium constant Kd = ka/kd were determined by using the BIAevaluation software version 1.2, assuming a first order kinetic A + B = AB. The model calculates the association rate constant ka and the steady state response level Req by fitting data to the equation,

\[ R = Req(1 - \exp(-kaCt/kd)) \]  

(Eq. 1)

where t is the time in s, Req is the steady state response level, and C is the molar concentration of adaptors in the injection solution. The steric interference factor N that describes the valency of the interaction between the adaptors and the li constructs was set to 1. The dissociation rate constant kd was determined by fitting data to the equation,

\[ R = Req \exp(-kdCt) \]  

(Eq. 2)

where Req is the response level at the beginning time t0 of the dissociation phase. This model, which has recently been applied to describe adaptor tail interaction (32), is described in more detail elsewhere (33, 34). It should be noted that the above-described models allow the determination of rate constants without reaching equilibrium during the experimental cycle.

**RESULTS**

**Isolation of Soluble Trimeric li**—To study the interaction between the cytoplasmic tail of the li and adaptor complexes, we generated soluble li molecules devoid of their membrane-spanning region (liTM). Furthermore we generated soluble li molecules devoid of their membrane-spanning region (liTM). We also generated soluble li molecules devoid of their membrane-spanning region (liTM). We also generated soluble li molecules devoid of their membrane-spanning region (liTM). We also generated soluble li molecules devoid of their membrane-spanning region (liTM). We also generated soluble li molecules devoid of their membrane-spanning region (liTM).

**liTM forms under nonreducing conditions mainly dimers, even in the absence of the cross-linker. In contrast, liTMCT forms monomers. After cross-linking with 0.1 mM DTSSP, both liTM and liTMCT migrated as dimers and trimers.**

**AP1 and AP2 Binding to the Cytoplasmic Tail of Immobilized li Polypeptides**—To analyze the interaction between the li cytoplasmic tail and AP1 or AP2, we used a biosensor system monitoring surface plasmon resonance. This method has been used successfully in other studies on the interaction between adaptors and cytoplasmic tails of the epidermal growth factor receptor, hemagglutinin, lysosome-associated membrane protein-1, and mannose 6-phosphate receptor (24, 29, 32, 36, 37). The different li forms were immobilized via their hexahistidine tag to the surface of a NTA sensor. When binding of purified AP1 and AP2 to liTM and liTMCT were tested, very low binding was found for the tailless mutant, whereas both adaptors bound with high affinity to liTM. An equilibrium constant of 50 nM was determined for AP1 and 200 nM for AP2. The association constant (ka, s⁻¹) was 4.4 × 10⁴ for AP1 and 3.1 × 10⁴ for AP2; the dissociation constant (kd, s⁻¹) was 2.2 × 10⁻⁴ for AP1 and 6.2 × 10⁻⁴ for AP2 (Fig. 3).

The liTM leucine mutants showed reduced binding in comparison with liTM (see Table IA). When both leucine-based motifs were mutated, binding to AP1 and AP2 was too low to allow calculation of the rate constants. The on-rates for AP1 and AP2 binding to the ML → AA or LI → AA mutants were 1.3 to 4 times slower as compared with those determined for liTM. The off-rates for these mutants were 1.5 times faster for AP2 and 0.8 times slower for AP1 as compared with liTM. These experiments demonstrate that either of the leucine-based motifs can mediate high affinity binding of AP1 and AP2 and that their substitution by alanine residues results in complete loss of AP1 and AP2 binding.

**Binding of GST-li to AP1 and AP2**—The experiments described above for adaptor binding to li were performed with soluble li trimers immobilized to the sensor surface. We also analyzed binding of adaptors with monomeric GST-li tail fusion proteins immobilized to the sensor surface. AP-1 bound to a wild-type GST-li tail fusion protein with a KD of 114 nM and to AP-2 with a KD of 250 nM. Two controls, a GST-li tail fusion protein in which both leucine-based motifs were replaced by...
alanines and GST alone, did not bind AP-1 nor AP-2 (see Fig. 4 and Table IB). In conclusion, oligomerization of Ii into trimers is not necessary for the interaction with AP1 or AP2; also Ii monomers can bind to AP1 and AP2.

Binding of Ii to the \( \mu \)-Chains of AP1 and AP2—Although AP-1 and AP-2 have been shown to bind to the leucine-based sorting motifs of several membrane proteins such as cation-independent mannose 6-phosphate receptor, CD3-\( \gamma \), CD4, and glucose transporter 4 (21, 37, 38), it is still a matter of debate which of the adaptor subunits mediates this interaction. We have tested the binding of \( \mu \)-chains by passing recombinant \( \mu_1 \) and \( \mu_2 \) over biosensor surfaces derivatized with Ii. Both \( \mu_1 \) and \( \mu_2 \) bound to the wild-type Ii tail with kinetics very similar to those obtained for the fully assembled AP-1 and AP-2 complexes (Table I, compare C with A). Binding to the Ii tail mutant L1,ML \( \rightarrow \) AA was not detectable, pointing to the specificity of \( \mu_1 \) and \( \mu_2 \) binding. Thus, the leucine-based motifs of Ii are recognized by the adaptor \( \mu_1 \)- and \( \mu_2 \)-chains.

Ii Does Not Bind to AP3 in Vitro—In addition to binding of
leucine-based sorting signals to AP1 and AP2, the leucine-based motifs in the cytoplasmic tails of the lysosomal membrane protein LIMP-II and the melanosomal enzyme tyrosinase are known to bind AP3 but not AP1 or AP2 (24). We tested the possibility of AP-3 binding to Ii by incubating the immobilized Ii tail constructs with cytosolic fractions enriched in either AP-1/AP-2 or AP-3. When IiTMT was incubated with a cytosolic fraction enriched in AP-1 and AP-2, we observed strong binding. In contrast, no binding was observed when IiTMT was incubated with an AP-3-enriched fraction (Fig. 5). As a control for the binding activity of the AP-3-enriched fraction, we show that tyrosinase strongly binds AP-3. We therefore conclude that Ii, although it contains two leucine-based sorting motifs, does not bind AP-3.

**DISCUSSION**

In this study we show that the Ii is able to bind the cytosolic adaptors AP1 and AP2 with high affinity. The binding of AP1 is about 4-fold stronger as compared with AP2, mainly due to a lower dissociation rate. The rate constants were determined using a Biosensor surface to which soluble forms of the Ii lacking the transmembrane domain were immobilized. Deletion of the cytoplasmic tail abrogated the binding of adaptors, clearly showing that the cytoplasmic tail of Ii mediated binding.

Ii is transported as a homotrimer and, together with MHC class II α- and β-chains, as a heterononameric complex to endosomal compartments (4). The soluble forms of the Ii used in this study behave in solution also as trimers. However, trimerization of Ii is not required for adaptor binding. Monomeric fusion proteins of the cytoplasmic tail of Ii with GST bound AP1 and AP2 almost as strong as the trimeric forms. Thus, in vivo more than one adaptor molecule may bind to the oligomeric Ii complexes. The binding curves obtained by surface plasmon resonance fitted best when assuming binding of a single adenovirus complex per Ii trimer, suggesting a 1:1 stoichiometry for adaptor- Ii complexes.

The membrane distal LI (position 7 and 8) and membrane proximal ML (position 16 and 17) signal have been shown in in vivo studies to be critical for the sorting of the Ii to the endosomal pathway (9–11). The two signals were therefore likely candidates for mediating AP1 and AP2 binding. Indeed, substitution of the LI and ML signals by alanine residues abolished binding of adaptors to the soluble trimeric form of the Ii as well as to the monomeric GST-Ii tail fusion protein. When either one of the two signals was substituted, the affinity to AP1 and AP2 decreased only moderately. This is in line with the observation that both signals function independently. In vivo studies had further indicated that the LI signal is more efficient than the ML signal in mediating rapid internalization from the plasma membrane (12). This is reflected in the rate constants for binding of AP2 (and AP1) to trimeric Ii, such that substitution of the LI signal had a greater effect than mutation of the ML signal.

Leucine-based signals in the lysosomal membrane protein LIMP-II and the melanosomal enzyme tyrosinase have recently been found to interact with AP3 (24), an adaptor complex found in association with trans-Golgi network membranes as well as with more peripheral membranes, including part of the endosomal system (39, 40). Neither of the two leucine-based signals in the Ii bound AP3. This is in agreement with in vivo data showing that down-regulation of AP3 by microinjection of antisense DNA did not alter the localization of Ii and MHC class II. The structural features that specify the selective affinity of leucine-based signals for AP3 (as in LIMP-II and tyrosinase (24)) or for AP1 and/or AP2 (as in Ii, CD4, CD3-γ, glucose transporter 4 or the mannose-6-phosphate receptors (21, 37, 41)) remain to be defined.

It is still a matter of debate by which subunit(s) of the adaptor complexes the leucine-based signals are recognized. The leucine-based signals of CD3γ was shown to interact with the β2-chain of AP2 using a photocross-linking approach (21), whereas the reports on the binding to the μ1- and μ2-chains of AP1 and AP2 are conflicting. Ohno et al. (42) failed to see an interaction between μ1 and μ2 and a leucine-based signal in the yeast two-hybrid system, whereas Rodionov and Bakke (23) demonstrated binding of μ1 and μ2 to immobilized peptides containing the leucine-based signals of Ii. Here we show that isolated μ1- and μ2-chains bind to soluble trimeric forms of the

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**TABLE I**

**Kinetic rate constants for the interaction of Ii with adaptor complexes and isolated adaptor μ-chains**

The immobilized Ii forms (soluble trimeric complexes in A and C; GST fusion proteins in B) were immobilized to the sensor surface at equivalent densities (variation less than 10%). The wild type sequence of the Ii cytoplasmic tail is designated wt, LI → AA, ML → AA, LI,ML → AA; L7A,L17A is the trimerization of Ii is not required for adaptor binding. Mono-meric fusion proteins of the cytoplasmic tail of Ii with GST were used at concentrations ranging from 20 to 250 nM. The rate constants ($k_a$, $k_d$, and the equilibrium rate constant $K_D$) were determined as described under “Experimental Procedures.”

|                | $k_a$  | $k_d$  | $K_D$ |
|----------------|--------|--------|-------|
| **AP1**        |        |        |       |
| A Trimeric Ii  | $4.4 \times 10^4$ | $2.2 \times 10^{-3}$ | 50    |
| Li → AA       | $1.0 \times 10^4$ | $1.8 \times 10^{-3}$ | 180   |
| ML → AA       | $2.1 \times 10^4$ | $1.8 \times 10^{-3}$ | 86    |
| LI,ML → AA    | Below detectability | Below detectability |       |
| IiTMT(CT)     | Below detectability | Below detectability |       |
| B Monomeric GST-Ii fusion protein |        |        |       |
| wt            | $3.7 \times 10^4$ | $4.2 \times 10^{-3}$ | 114   |
| L7A,L17A      | Below detectability | Below detectability |       |
| **AP2**        |        |        |       |
| A Trimeric Ii  | $4.4 \times 10^4$ | $2.2 \times 10^{-3}$ | 50    |
| Li → AA       | $1.0 \times 10^4$ | $1.8 \times 10^{-3}$ | 180   |
| ML → AA       | $2.1 \times 10^4$ | $1.8 \times 10^{-3}$ | 86    |
| LI,ML → AA    | Below detectability | Below detectability |       |
| IiTMT(CT)     | Below detectability | Below detectability |       |
| B Monomeric GST-Ii fusion protein |        |        |       |
| wt            | $3.7 \times 10^4$ | $4.2 \times 10^{-3}$ | 114   |
| L7A,L17A      | Below detectability | Below detectability |       |

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2. B. Hoflack, personal communication.
Ii with affinities strikingly similar to those found for the fully assembled heterotetrameric AP1 and AP2. The binding is strictly dependent on the two leucine-based signals. Loss of a single signal has differential effects on $\mu_1$ and $\mu_2$ binding, which were also seen for the binding of AP1 and AP2 and for Ii sorting in vivo. Thus, binding of leucine-based signals to medium chains can fully account for their binding to AP1 and AP2.

Our data show that binding of Ii to AP1 and AP2 was dependent on the two leucine-based signals that are also the major determinants for sorting of Ii in vivo. In this context it has been noted that in vivo studies on the role of AP1 for sorting of Ii have provided conflicting data. Although overexpression of dominant negative forms of the clathrin heavy chain failed to affect Ii localization (43), overexpression of Ii has to be noted that this is due to differential binding or local factors at the different sorting stations where these adaptors are present.

**Acknowledgments**—We are grateful to Brigitte Pesold and Gaby Sonnenmoser for excellent technical assistance.

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