Tyrosine-based sorting signals in the cytosolic tails of membrane proteins have been found to bind directly to the medium chain subunit (μ) of the adaptor complexes AP-1 and AP-2. For the leucine-based signals, an interaction with AP-1 and AP-2 has been reported, but no specific interacting subunit has been demonstrated. After searching for molecules interacting with the leucine-based sorting signals within the cytosolic tail of the major histocompatibility complex class II-associated invariant chain using a phage display approach, we identified phage clones with homology to a conserved region of the AP-1 and AP-2 μ chains. To investigate the relevance of these findings, we have expressed regions of mouse μ₁ and μ₂ chains on phage gene product III and investigated the binding to tail sequences from various transmembrane proteins with known endosomal targeting signals. Enzyme-linked immunosorbent binding assays showed that these phages specifically recognized peptides containing functional leucine- and tyrosine-based sorting signals, suggesting that these regions of the μ₁ and μ₂ chains interact with both types of sorting signals.

The cytosolic tails of membrane proteins contain the information for targeting to various intracellular destinations. Two main classes of endosomal sorting signals have been identified: one class characterized by an essential tyrosine and one by a double leucine (LL) motif or variants thereof (1, 2). There is increasing evidence that both classes of sorting signals interact directly with the adaptor complexes AP-1 and AP-2 for clathrin-dependent sorting from the trans-Golgi network (TGN) or the plasma membrane, respectively (3–11). Studies on adaptor complexes binding to tail columns indicate a multivalent attachment of aggregated complexes with a binding constant in the micromolar range (3, 4, 7). This is confirmed by recent data obtained by surface plasmon resonance (9), suggesting a model where binding to the signals is associated with clustering of the adaptors. In fact, adaptor complexes tend to aggregate and drive the formation of coats in vitro (12), and a requirement for AP clustering is in agreement with the current model where adaptors are first recruited to the membrane by a docking protein and then associate to form a coat to where the membrane proteins diffuse laterally.

A specific interacting subunit of the AP complexes was recently demonstrated for the tyrosine-based signals as they were shown to interact with both μ₁ and μ₂, the medium chain subunits of AP-1 and AP-2, respectively (10, 13). No such interaction was demonstrated for the leucine class signals in those assays, although there are data indicating that these also mediate binding to the AP complexes (9).

The latest information about how signals may be recognized by the sorting machinery was obtained using the yeast two-hybrid system (13), a library-based method for studying protein-protein interactions. We have used phage display technology as an alternative library-based approach to obtain more information about protein sequences recognizing leucine-based signals. Phage display of random peptides has in several cases been successfully applied to obtain detailed information about protein interactions (14). When using a 15-mer synthetic peptide encompassing the LI sorting motif of the major histocompatibility complex class II-associated invariant chain (II) (15–17) as a target for a random phage display library, we obtained phage clones with homology to a conserved region of the medium chain adaptor subunits (μ). In this report, we investigated these findings by expressing the corresponding μ domains at the phage surface and found that they are able to discriminate between wild-type and mutant leucine- and tyrosine-based signals.

**EXPERIMENTAL PROCEDURES**

**Phage Library and Bacteria**—The 10-mer fUSE5 library with a complexity of $2 \times 10^{12}$ primary phage clones has been described previously (18). The fUSE5 and fUSE2 phages and the Escherichia coli bacterial strains K91 and MC1061 were generous gifts from G. P. Smith (University of Missouri).

**Enrichment of fUSE5 Phage Binding to an Invariant Chain Tail Peptide**—The screening procedure was modified from that of Smith and Scott (19) and has been discussed in detail elsewhere (20). Briefly, the invariant chain tail peptide MDQVRDLISNNEQLK was biotinylated before binding to SoftLink avidin resin (Promega), a methacrylate polymeric matrix with covalently bound monomeric avidin. One round of panning was performed as follows. The SoftLink-bound peptide was blocked for 1 h in PBS, pH 7.4, containing 1% BSA before incubation with the fUSE5 phage for 1 h at room temperature. After extensive washing in PBS containing 0.1% BSA and 0.02% Tween (7–10 times), the bound phages were eluted with glycine HCl, pH 2.2. The eluate was amplified in R91K bacteria and polyethylene glycol-precipitated before a new round of panning was performed. Single clones were grown in LB medium containing 20 μg/ml tetracycline and examined for binding to the invariant chain peptide by ELISA (see below).

**Cloning of μ Inserts into fUSE5**—The μ, phage was made by making the oligonucleotide 5'-TAGGCGCGCGGAGGCAAAGTATACGAGGCGCGCG-3' double-stranded by polymerase chain reaction, cutting with SfiI, and cloning into a SfiI site in the phage gpIII gene (underlined regions are flanking sequences, and coding region are shown as triplets). The μ₁ and μ₂ 12N phages and the μ₁ alanine mutant phage were defrayed in part by the payment of page charges. This article must be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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were made the same way with the sequences 5′-aaaa aat att ttt ct\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{a} t\textsuperscript{t} at\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{t} g\textsuperscript{t} a\textsuperscript{t} t\textsuperscript{g} at\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{t} g\textsuperscript{t} g\textsuperscript{g} 3′, 5′-aaa aat att ttt ct\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{a} t\textsuperscript{t} at\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{t} g\textsuperscript{t} a\textsuperscript{t} t\textsuperscript{g} at\textsuperscript{t} t\textsuperscript{t} t\textsuperscript{t} g\textsuperscript{t} g\textsuperscript{g} 3′, respectively. Flanked by the same sequences as underlined above. All oligonucleotides were synthesized at the Biotechnology Center (Ole, Norway).

**Sequencing of Phage Clones**—Polymerase chain reaction was carried out on 5′-ult mutants from single colonies of phage-infected K91K using Vent polymerase (New England Biolabs Inc.). The primers for polymerase chain reaction were biotin-5′-tcgaacagctgtgaacag-3′ and 5′-tcgaacagctgtgaacag-3′. Single-stranded templates were isolated on magnetic streptavidin beads (M280, Dynal, Inc.), and single-stranded sequencing was done using the Sequenase 2.0 kit (Amersham Pharmacia Biotech) and the sequencing primer 5′-ctctgatagtcagtgaag-3′ (19).

**fUSE5-Peptide Binding Assay**—MaxiSorp wells (Nunc) were coated with peptide in PBS, pH 7.4, overnight at 4 °C or at room temperature at concentrations of 500 μg/ml for free peptides and 250 μg/ml for BSA-coupled peptides before blocking in PBS containing 1% BSA for 1 h at room temperature. 20% methanol was added to free peptides during coating as this seemed to improve both binding and specificity in the assays for some of the peptides. Phage was added in PBS and 1% BSA for 2 h at room temperature at different concentrations. 10\textsuperscript{15} phages, calculated as transducing units of E. coli bacteria, correspond to 1 phage concentration (in transducing units of 133 pm). Phage concentrations above 300 pm were not used in these assays as they only resulted in a marked increase in background problems. The plates were washed extensively in PBS containing 0.05% Tween 20 before horseradish peroxidase-conjugated anti-M13 IgG (Amersham Pharmacia Biotech) was added for 1 h at room temperature (1:5000 in PBS and 1% BSA). Another extensive wash was performed in PBS/Tween 20 before the addition of 100 μl of substrate (1 mg/ml ABTS (Boehringer Mannhein) in citrate buffer, pH 4.0), and the plates were read at 405 nm in a TiterTec Multiskan plate reader.

**Peptides**—Table I gives an overview of all peptides used and their abbreviations and sequences. The invariant chain peptides Ii Li wt (where \(\text{wt}\) is wild type) and Ii DQ-AAA/AA were synthesized at the Biotechnology Center. The BSA-coupled peptides used for the tail of the transferrin receptor (TR) were (TR wt and TR Y-A). TGN38 (TGN38 wt and TGN38 Y-A), and the invariant chain (II ML wt and II ML-AA) as well as the BSA-conjugated C5 control sequence and un conjugated C1 (μ10) and C2 (μ15) were kindly provided by Dr. G. Banting (Bristol, United Kingdom).

The Lamp-1 (lysosomal associated membrane protein 1) peptides Lamp-1 wt and Lamp-1 Y-A are gifts from Dr. G. Banting (Lausanne, Switzerland), and the CD3\textsuperscript{y} peptides CD3\textsuperscript{y} wt and CD3\textsuperscript{y} Y-A were gifts from Dr. C. Geisler (Copenhagen, Denmark). The control peptides C3 and C4 were synthesized by Genosys Biotechnologies Inc. (Cambridge, MA). Amino acid analysis of the BSA conjugates was performed by Dr. K. Sletten (Biotechnology Center), showing that similar amounts of peptide were coupled per BSA for the wild type and its corresponding mutant. This was done to ensure that lack of reduced binding in the assays not was due to different amounts of peptides. To ensure that free peptides equally coated the MaxiSorp wells and gave similar results as BSA-coupled peptides, we performed a binding assay with free II ML wt and II ML-AA peptides. These were biotinylated and HPLC-purified (Dr. G. Banting), and the coating efficiency for the wild type compared with the mutant was measured using horseradish peroxidase-streptavidin. These experiments showed that similar binding curves were obtained with free and BSA-coupled peptides and that the two peptides equally coated the MaxiSorp wells. This was as expected since large differences in coating efficiency due to small differences in sequence are usually a problem only with very short peptides (5–6 residues).

**Conjugation of Peptides to Alkaline Phosphatase and Binding Assay of Tail Peptides**—Conjugation of peptides to alkaline phosphatase (ALP) was essentially done as described previously (18). Briefly, the μ peptides μ10 and μ15 and the control peptide C3 were dissolved to 140 μg/ml in PBS, pH 7.4, before ALP (25 mg/ml) was added to a molar ratio of 1:20 (ALP/peptide) for 1 h at room temperature. 50 μl of glacial acetic acid was added, and the tubes were further incubated for 2 h at room temperature before adding glycine (1 M). The conjugates were purified by filtration with Centricon 100 spin columns (Amicon, Inc., Beverly, MA) and concentrated to a final concentration of 0.25 mg/ml. Amino acid analysis of the conjugates showed that, on average, seven peptides were coupled per ALP molecule. The binding assay was performed as follows. MaxiSorp plates were coated and blocked as described for phage binding assays. ALP-peptide conjugate was added in PBS and 1% BSA to a final concentration of 10 μg/ml. Free ALP and ALP-C3 were added at the same concentration to control wells.

**Competition Assay with Biotinylated Phages**—Phages were biotinylated with a large molar excess of biotin for 3–4 h at room temperature and then dialyzed overnight in PBS, pH 7.4, to remove free biotin. Biotin-labeled phages at a fixed concentration were allowed to interact with MaxiSorp plates coated with tail sequences in the presence or absence of an excess of unlabeled phage (up to 300 pm; see above). Binding of biotin-labeled phage was detected using horseradish peroxidase-streptavidin and horseradish peroxidase substrate.

**RESULTS**

**Identification of a fUSE5 Clone Binding to an Invariant Chain Tail Peptide**—After screening a random fUSE5 display library against a peptide derived from the 15 N-terminal amino acids of Ii including an Ii sorting signal, we identified phage-displayed peptides with homology to a region of the medium chain adaptor subunit (μ) (20). One phage clone expressing the residues GFPQ in its gpIII insert (Fig. 1, Phage clone 1) was found to interact strongly with the Ii peptide used in the screening (II LI wt), suggesting that this clone could recognize the Ii-type endosomal sorting signal of Ii. Homology searches in the SwissProt data base with sequences derived from the interacting phage led us to a conserved region of the medium chain adaptor subunit (μ) (Fig. 1). Other isolated phage clones from the screening also showed similarity to the same part of the Ii chain region (Fig. 1, Phage clones 2–4).

**fUSE5 Clones Expressing a Chain Regions of 24 Amino Acids as gpIII Fusion Proteins**—We wanted to investigate the relevance of the homologies between the phage clones and the μ chains by expressing a region from mouse μ\textsubscript{1} in fUSE5 gpIII and testing its ability to recognize the invariant chain LI signal. A secondary structure prediction on mouse μ\textsubscript{1} using the Chou and Fasman method (Ref. 22; see Ref. 21) and the SPRED program\textsuperscript{2} indicated an amphipathic helix preceding a turn containing the GYPQ motif. By running the ANTIGENIC algorithm (23) on the protein, the region was predicted to be antigenic, suggesting that it is surface-exposed. Based on the sequence comparisons and these structural predictions, region 104–127 from mouse μ\textsubscript{1} could form a functional domain at the phage surface. The phage clone was made by inserting an

\textsuperscript{2}Available on the World Wide Web at \url{http://www.embl-heidelberg.de}.
sequences). Oligonucleotide encoding the region 104–127 of mouse μ1 into phage gene III as described under “Experimental Procedures” (referred to as the μ1 clone; see Table II). As shown in Fig. 2A, the μ1 clone recognized the wild-type LI-containing peptide in a saturation-dependent fashion, whereas binding to the alanine mutant was not significantly higher than to BSA alone. Interestingly, the μ1 clone discriminated between the two peptides better than the GFPQ clone isolated after screening (data not shown), indicating that the μ1 clone is more adapted to the leucine signal than the clone from a limited random library. To test if the μ1 clone could bind to a broader range of leucine-based sorting signals, we also used peptides based on the second leucine signal in the Ii cytosolic tail (Ii ML wt) (15, 17) and the LL signal in the CD3γ tail (24). As shown in Fig. 2 (B and C), the μ1 sequence could also discriminate these wild-type tail sequences from their corresponding alanine mutants. The μ1 clone showed a higher interaction above background levels only when methanol was present during coating. The signal corresponding region in the μ2 homologue, region 102–125, with the differences being mostly conservative changes (Fig. 1). To test the mouse μ2 homologue, region 102–125 was cloned into phage gpIII (μ2 clone) (Table II). As shown in Fig. 3, the characteristics of the binding between the μ2 clone and the tail sequences are almost identical to what we obtained for the μ1 clone. This suggests that the region we have identified is involved in signal recognition by both AP-1 μ1 and AP-2 μ2.

Neither a fUSE2 control phage nor irrelevant fUSE5 clones gave signals in ELISA, even at high titers, confirming that other domains on the phage surface do not bind tail sequences. Microtiter wells coated with irrelevant control peptides gave signals below the BSA background level, even though some contained double leucines, tyrosines, or other residues usually present in signals (Table I). This shows that the context of the residues is important for binding and that the binding is not due to nonspecific interactions with bulky hydrophobic residues.

The Tyr-based sorting motifs are the most studied, and it is well established that proteins with these motifs bind adaptors. We therefore decided to include some Tyr-based signals in our assay, and we found that the μ1 phage clone could discriminate wild-type Lamp-1 (25), TGN38 (26), and TfR (27) from their corresponding alanine mutants (Fig. 2, D–F). The binding curves showed saturation characteristics at the higher concentrations of phages.

The μ1 region (amino acids 104–127) is very similar to the corresponding region in μ2 (amino acids 102–125), with the differences being mostly conservative changes (Fig. 1). To test the mouse μ2 homologue, region 102–125 was cloned into phage gpIII (μ2 clone) (Table II). As shown in Fig. 3, the characteristics of the binding between the μ2 clone and the tail sequences are almost identical to what we obtained for the μ1 clone. This suggests that the region we have identified is involved in signal recognition by both AP-1 μ1 and AP-2 μ2.

NMR data on the Ii tail has earlier demonstrated that a nascent α-helix in the N-terminal part of the Ii tail is stabilized in 20% methanol (17). Other peptides have also been shown to stabilize their folding in the presence of organic cosolvents (28, 29). Interestingly, we found that methanol added to 20% during the coating of wells with unconjugated peptides increased the specificity of binding between free tail peptides and the μ clones (data not shown). This was observed in particular for the CD3γ peptides, for which we observed clear differences in binding only when methanol was present during coating. The signal obtained against I wt seemed to increase with methanol added during the coating, whereas the lack of binding to the mutant Ii peptide was very clear both with and without methanol. Based on these observations, we suggest that methanol could
Sequences Interacting with Endosomal Sorting Signals in Vitro

Sequences are marked with corresponding residue numbers in the native proteins. Changed residues are underlined in the mutants.

| Abbreviation | Sequence | Protein derivation | Binds \( \mu \) clones |
|--------------|----------|--------------------|------------------------|
| Leucine-based signals | Li LI wt | 1MDQRLISNNQEQL14K | Invariant chain | +++ |
| | DQ-AA/LI-AA | 1MDAARDASNNQEQL14K | – | – |
| | ML wt | BSA-C111NQEQLMGLGR26 | Invariant chain | +++ |
| | ML-AA | BSA-C111NQEQLFAAGRR | – | – |
| | CD3\( \gamma \)et | BIO-C111DGVQRQRSAKDQTL131 | CD3\( \gamma \) chain | ++ |
| | CD3\( \varepsilon \)L- AA | BIO-C111DGVQRQRSAKDQTA5131 | – | – |
| Tyrosine-based signals | Lamp-1 wt | 357RKSHAGYQTT407 | Lysosomal Lamp-1 | +++ |
| | Lamp-1 Y-A | 357RKSHAGYQTT | Transferrin receptor | ++ |
| | TIR Y-A | BSA-C131EPLYATFRSLAR27 | – | – |
| | TGN38 wt | BSA-C336RASK6YQRNLKL357 | Golgi TGN38 | +++ |
| | TGN38 Y-A | BSA-C336RASK6YQRNLKL | +/− | – |
| Control peptides | C1 (\( \mu \)10) | 1MDFGYPQTDT127 | Mouse \( \mu \)_1 chain | – |
| | C2 (\( \mu \)15) | 11LLDEMDFGYPQTDT127 | Mouse \( \mu \)_1 chain | – |
| | C3 | SAWRHPSGG | None | – |
| | C4 | PWNLGISAT | None | – |
| | C5 | BSA-CRKLVLPAELLPNQEK | None | – |

**TABLE II**

Phage clones and ALP conjugates

Flanking regions of USES gpIII are underlined. See Table I for abbreviations used for peptides coupled to ALP.

| Clone/conjugate | Sequence | Binding |
|-----------------|----------|---------|
| Phage clones | \( \mu \)_1 clone | NH2-ADGARDNFMVIYELLDELMDFGYQTTDGAAGE | +++ |
| | \( \mu \)_1 AAAA | NH2-ADGARDNFMVIYELLDELMDFAAAAADTDGAAGE | ++ |
| | \( \mu \)_2 clone | NH2-ADGAKNFVLIYELLDELMDFGYQNSEGAAGE | +++ |
| | \( \mu \)_2 12N | NH2-ADGAKNFVLIYELLDELMDFGYQNSEGAAGE | – |
| ALP conjugates | ALP-\( \mu \)_15 | ALP-(LLDEMDFGYPQTDT)_4 | +++ |
| | ALP-\( \mu \)_10 | ALP-(MDFGYPQTDT)_2 | ++ |
| | ALP-\( \mu \)_C3 | ALP-(SAWRHPQSGG)_2 | – |
| | ALP | ALP-(no peptide) | – |

\( ^n \) denotes the average number of peptides coupled per ALP molecule and was estimated by amino acid analysis to be in the range 5–9 for all conjugates.

give the peptides a more native conformation when locked on the surface of the wells. Methanol added to the BSA conjugates seemed to enhance nonspecific interactions, possibly due to misfolding of these proteins.

BSA coupling is an approach used to ensure equal binding when coating a solid phase with small proteins or peptides (30). Amino acid analysis of our BSA conjugates showed that similar amounts of peptides were coupled for a wild type and its corresponding mutant. We can therefore rule out that the lack of reduced binding to mutant peptides was due to the lack of primary attachment of these to the MaxiSorp wells or lack of peptide on the conjugates. Furthermore, the free ALP wt and Li ML wt and Li ML-AA peptides not coupled to BSA gave binding curves similar to those shown for Li ML peptides in Fig. 2B. The free Li ML peptides (which were biotinylated and HPLC-purified) were found to coat the MaxiSorp wells at equal efficiencies (see “Experimental Procedures”), showing that changing the ML residues to alanine did not affect the ability to attach to MaxiSorp wells.

**Binding of Alkaline Phosphatase Conjugates of \( \mu \) Chain-derived Peptides to Tail Sequences**—To investigate whether binding could also be obtained with a shorter sequence containing the GYPQ motif and when not expressed as a phage fusion product, we coupled the \( \mu \) peptides \( \mu \)_10 and \( \mu \)_15 (Table I) to alkaline phosphatase as described. Amino acid analysis of the ALP-\( \mu \) conjugate showed that, on average, seven peptides were coupled to each ALP molecule, giving a multivalency corresponding to that in the fUSE5 phage (four copies of gpIII). Fig. 4 (A and B) shows ALP-\( \mu \)_10 binding to the tail sequences Li ML wt and TGN38 wt, to their corresponding mutants, and to BSA. Similar results were obtained for other tail sequences tested, both for ALP-\( \mu \)_10 and ALP-\( \mu \)_15 (data not shown). As shown, neither unconjugated ALP nor ALP coupled to an irrelevant control peptide (C3 in Table II) interacted with the tail peptides. In conclusion, the ALP conjugates bound to both tyrosine- and leucine-based signals, confirming that shorter sequences than the 24-residue region in our \( \mu \) phage clones containing the GFPQ sequence could recognize the signals and that the peptide sequences do not necessarily have to be expressed on the phage.

**Role of the GYPQ Sequence in the Interaction between the \( \mu \) Domain and Sorting Signals**—To investigate if the GYPQ residues are involved in binding to the tail sequences, we constructed a fUSE5 \( \mu \)_1 clone with these 4 residues changed to alanines (\( \mu \)_AAA) (Table II). The ELISA binding curves for the wild-type \( \mu \)_1 clone versus the alanine mutant showed a reduced binding for the alanine mutant compared with the wild type (Fig. 5A). To quantitate the difference in binding between the wild-type \( \mu \)_1 clone and \( \mu \)_AAA, we performed a titration at a point on the binding curve at the first point of saturation.
FIG. 3. ELISA binding curves for the μ2 clone and various tail sequences. MaxiSorp wells coated with various tail sequences were blocked and incubated with increasing concentrations (in transducing units) of FUSE5 phage expressing residues 104–127 from mouse μ2 (μ2 clone). Binding of phage was detected by adding horseradish peroxidase-conjugated anti-M13 antibody and horseradish peroxidase substrate as described under “Experimental Procedures.” A, binding to Li LI wt (●), Li DQ-AA/LI-AA (○), and BSA (×). B, binding to Li ML wt (●), Li ML-AA (○), and BSA (×). C, binding to Lamp-1 wt (●), Lamp-1 Y-A (○), and BSA (×). D, binding to TGN38 wt (●), TGN38 Y-A (○), and BSA (×) (see Table I for abbreviations and sequences). Abs, absorbance.

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Figures and tables have been replaced with text-based descriptions. The text contains a detailed analysis of binding curves and competition assays, along with discussion on the significance of these findings.
brane proteins have been shown to be active in type II membrane proteins and vice versa (27, 38, 39). Consistent with this, the phage clones in our study were shown to recognize sorting signals from both type I and II proteins.

The hypothesis that adaptor preclustering is required for binding tail sequences (12) is in agreement with results from in vitro studies of adaptor complexes binding to tail columns (3, 4, 7, 9). These data indicate a multivalent attachment of aggregated complexes with a binding constant in the micromolar range and the requirement of a large (1000-fold or more) excess of competing peptide. In our system, it is possible that the multivalence of the expressed domains on the phage surface with four to five copies of the gpIII fusions per phage contributes to an enhanced binding to the signals.

The number of binding sites for AP complexes on a chip used for surface plasmon resonance was estimated in one study to be 500-fold lower than the number of peptides coupled, indicating that these sites are made up of more than one peptide simultaneously interacting with the AP complexes (9). This observation is in agreement with the requirements for relatively high concentrations of peptides in our phage-peptide assays. Peptides in the required arrangement may be rare on the surface of our microtiter wells, as is also supposed to be the case on the chips used for surface plasmon resonance.

Not much is known about the structure of the \( \mu_1 \) chains, but comparison of \( \mu_2 \) chains from different species suggests that the protein is divided into two regions of similar length, but with different characteristics (40). Region I (230 amino acids) is more conserved and is separated from region II (190 amino acids, containing several sequences specific to \( \mu_2 \) interspersed with other strongly conserved sections) by a linker region of variable length (10–42 amino acids, linking the two functional domains together). Limited tryptic cleavage of AP-2 complexes bound to clathrin coats results in the cleavage and degradation of the \( \mu_2 \) C terminus (41). Since the N terminus of \( \mu_2 \) remains intact, it has been suggested that the interactions determining the association with the other subunits may be located in this part (region I). Our results suggest that there is also a recognition site for sorting signals in region I, which is the more conserved. This does not rule out the possibility that other regions of the \( \mu_2 \) chains also interact with the same sorting signals. The specificity in binding of full-length \( \mu_2 \) chains to the various signals may be determined by more than one region, and our results suggest that one binding motif is common or general. Other binding sites may reside in the less conserved C-terminal part of the \( \mu_2 \) chains, which also could give different recognition characteristics for \( \mu_1 \) and \( \mu_2 \). A role for a more C-terminal interaction site is supported by the fact that not only the full-length mouse \( \mu_2 \) clone, but also a truncated mouse \( \mu_2 \) clone (\( \Delta \mu_2 \))
expressing residues 121–435 has been shown to interact with tyrosine signals (10, 13). The sequence we have studied spans residues 102–125 of the NP2 chain and recognizes a tail sequence. The sequence we have studied spans residues 121–435 has been shown to interact with the plasma membrane and most likely also at other intracellular localization sites (1, 43, 44). To account for this complexity and to assure specific sorting of the various molecules, an endosomal sorting signal might have more than one site of interaction with the cellular sorting machinery. As the site we have identified is conserved between μ chains and recognizes a relative broad spectrum of tyrosine- and leucine-based signals, it is tempting to speculate that this interaction takes place at one of the earlier stages in the sorting process. However, only further elucidation of this intricate process, which must be solved both in time and space, will allow us to make any firm conclusions.

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