Signal-binding Specificity of the μ4 Subunit of the Adaptor Protein Complex AP-4*

Received for publication, November 22, 2000
Published, JBC Papers in Press, January 3, 2001, DOI 10.1074/jbc.M010591200

Ruben C. Aguilar‡§, Markus Boehm‡§§, Inna Gorskhova¶¶, Robert J. Crouch,‡
Kazuhiro Tomita‡‡, Takashi Saito‡‡, Hiroshi Ohno§§, and Juan S. Bonifacino†††

From the ‡Cell Biology and Metabolism Branch and the §Laboratory of Molecular Genetics, NICHD, National Institutes of
Health, Bethesda, Maryland 20892, the ¶¶Department of Molecular Genetics, Chiba University Graduate School of
Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan, and the §§Division of Molecular Membrane Biology, Cancer
Research Institute, Kanazawa University, 13-1 Takaramachi, Kanazawa 920-0934, Japan

The medium (μ) chains of the adaptor protein (AP) complexes AP-1, AP-2, and AP-3 recognize distinct subsets of tyrosine-based (YXXØ) sorting signals found within the cytoplasmic domains of integral membrane proteins. Here, we describe the signal-binding specificity and affinity of the medium subunit μ4 of the recently described adaptor protein complex AP-4. To elucidate the determinants of specificity, we screened a two-hybrid combinatorial peptide library using μ4 as a selector protein. Statistical analyses of the results revealed that μ4 prefers aspartic acid at position Y+1, proline or arginine at Y+2, and phenylalanine at Y−1 and Y+3 (Ø). In addition, we examined the interaction of μ4 with naturally occurring YXXØ signals by both two-hybrid and in vitro binding analyses. These experiments showed that μ4 recognized the tyrosine signal from the human lysosomal protein LAMP-2, HTGYEQF. Using surface plasmon resonance measurements, we determined the apparent dissociation constant for the μ4-XXYØ interaction to be in the micromolar range. To gain insight into a possible role of AP-4 in intracellular trafficking, we constructed a Tac chimera bearing a μ4-specific YXXØ signal. This chimera was targeted to the endosomal-lysosomal system without being internalized from the plasma membrane.

The heterotetrameric adaptor protein (AP) complexes AP-1, AP-2, AP-3, and AP-4 are components of protein coats that associate with the cytosolic face of organelles of the secretory and endocytic pathways (reviewed in Refs. 1–4). AP-2 is associated with the plasma membrane and mediates rapid interconversion of endocytic receptors, whereas AP-1, AP-3, and AP-4 are associated with the trans-Golgi network and/or endosomes and mediate intracellular sorting events. AP complexes are thought to participate in protein sorting by inducing the formation of coated vesicles as well as concentration of cargo molecules within the vesicles. Concentration of integral membrane proteins is mediated by direct interaction of the AP complexes with sorting signals present within the cytosolic tails of the proteins. Several types of cytosolic sorting signals have been described, the most common of which are referred to as “tyrosine-based” or “dileucine-based” depending on which residues are critical for activity (5, 6).

The four AP complexes have a similar structure and are composed of two large chains (α/γδε and β1–4, 90–130 kDa), a medium chain (μ1–4, ~50 kDa), and a small chain (σ1–4, ~20 kDa), each of which suberves a different function. Extensive analyses of the α chain of AP-2 have shown that it interacts, either directly or indirectly, with many regulators of coat assembly and/or vesicle formation (7). By analogy, the γδε chains are presumed to interact with other proteins that play similar regulatory roles. β1, β2, and β3 interact with the scaffolding protein, clathrin (8–10). In addition, β1 and β2 have been found to bind a subset of dileucine-based sorting signals (11). The μ chains, on the other hand, function as recognition molecules for signals conforming to the YXXØ consensus motif (Y is tyrosine, X is any amino acid, and Ø is leucine, isoleucine, phenylalanine, methionine, or valine) (12–20). The exact role of the σ chains is unknown, although σ1 and σ3 are required for the functional integrity of the AP-1 and AP-3 complexes, respectively (21, 22).

Our laboratory has been particularly interested in the role of the μ chains in signal recognition. We have previously demonstrated that μ1 and μ2 display a bipartite structure, with the amino-terminal one-third being involved in interactions with the corresponding β chains and the C-terminal two-thirds being involved in recognition of YXXØ-type signals (23). X-ray crystallography revealed that the YXXØ-binding domain of μ2 consists of a banana-shaped all-β structure to which the signals bind in an extended conformation (19). The Tyr and Ø residues fit into hydrophobic pockets on this domain. Both crystallographic (19) and binding (13–18) studies have suggested that the identities of the Ø residue and the residues surrounding the critical Tyr residue are important determinants of the specificity of interaction. Although the subsets of YXXØ signals recognized by μ1, μ2, and μ3A overlap to a significant extent, each chain nonetheless exhibits certain preferences for residues neighboring the critical Tyr residue (14). For example, μ1, μ2, and μ3A prefer Leu, Val, and Ile residues at the Ø positions and neutral, basic, and acidic residues at the X positions, respectively. We have argued that these preferences alone are unlikely to account for the functional specificity of each AP complex.

This paper is available on line at http://www.jbc.org

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* This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (to H. O. and T. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.

§ Supported by a grant from the German Academic Exchange Service (DAAD).

¶¶ Supported by a National Research Council LMG-NICHD senior research associateship.

††† To whom correspondence should be addressed: Cell Biology and Metabolism Branch, NICHD, Bldg. 18T, Rm. 101, NIH, Bethesda, MD 20892. Tel.: 301-496-6368; Fax: 301-402-0078; E-mail: juan@helix.nih.gov.

1 The abbreviations used are: AP, adaptor protein; Gal4AD, Gal4 transcription activation domain; Gal4BD, Gal4 DNA-binding domain.
complex (14). However, they probably contribute to the selectivity and efficiency of specific signal recognition events.

Although much has been done to characterize the signal-binding specificity of μ1, μ2, and μ3A, little is known about sequence preferences for the more recently described μ4 (also known as μ-ARP2) (24). Previous studies have shown that μ4 interacts weakly with YXX0 signals from the lysosomal membrane proteins LAMP-1 (AGYQTL) (18) and CD63 (SGYEVM) (25) and the trans-Golgi network protein TGN38 (SDYQRL) (18). To determine whether μ4 might be able to recognize with higher affinity a defined subset of YXX0 signals, we have undertaken a yeast two-hybrid screening of a combinatorial YXX0 library. The results show that μ4 prefers signals with Phe at position Y-1, Asp at Y+1, Pro or Arg at Y+2, and Phe at Y+3 (Ø). A signal that fits this latter preference is found in the lysosomal membrane protein LAMP-2, and indeed, we found that the LAMP-2 signal binds to the lysosomal membrane protein bearing a μ4-specific YXX0 signal. We have undertaken a yeast two-hybrid screening of a combinatorial YXX0 library. The results show that μ4 prefers signals with Phe at position Y-1, Asp at Y+1, Pro or Arg at Y+2, and Phe at Y+3 (Ø). A signal that fits this latter preference is found in the lysosomal membrane protein LAMP-2, and indeed, we found that the LAMP-2 signal binds to the lysosomal membrane protein bearing a μ4-specific YXX0 signal. We have undertaken a yeast two-hybrid screening of a combinatorial YXX0 library. The results show that μ4 prefers signals with Phe at position Y-1, Asp at Y+1, Pro or Arg at Y+2, and Phe at Y+3 (Ø). A signal that fits this latter preference is found in the lysosomal membrane protein LAMP-2, and indeed, we found that the LAMP-2 signal binds to the lysosomal membrane protein bearing a μ4-specific YXX0 signal. 

**EXPERIMENTAL PROCEDURES**

Recombinant DNA Constructs—The constructs Gal4AD-μ1, Gal4AD-μ2, and Gal4AD-μ3A in the pACTII(LEU2) plasmid (CLONTECH, Palo Alto, CA) have been described previously (12, 13). The Gal4AD-μ4 construct was prepared by ligating a BamHI-StuI polymerase chain reaction fragment corresponding to the 5′-part of μ4 and a SacI-PstI cDNA fragment corresponding to the 3′-part of μ4 into the BamHI-Xhol site of the pACTII(LEU2) vector using a PstI-Xhol adaptor. As previously described (14), a DNA fragment encoding the 33-amino acid cytoplasmic tail of TGN38 engineered to contain an Esg1 site (by introduction of silent mutations in place of the codons for Arg41 and Pro52 from the TGN38 cytoplasmic tail) was used to prepare the pGBT9-TGNΔ-Esg1 construct by ligation into the EcoRI and Xhol sites of the pGBT9(ΔTRP1) vector (CLONTECH). Oligonucleotides encoding either a combinatorial XXXXXXX peptide library (14) or different YXX0-type signals were digested with Esg1 and PstI and then ligated into pGBT9-TGNΔ-Esg1 cut with Esg1 and PstI. The amino acid sequence encoded by the resulting constructs was Gal4BD-HNKRIIAFALEGKRKSVTRPPKXXX0. The construct Gal4BD-B2 was kindly provided by Dr. M. S. Robinson (University of Cambridge, Cambridge, United Kingdom). All of the other two-hybrid constructs were made by ligation of polymerase chain reaction products into the pGBT9 or pACTII vector. The construct pET28a-μ4(156–453) was obtained by cloning nucleo-}

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auxotrophy and β-galactosidase activity) to test the binding specificity of library clones.

Cell Culture and Transfection—HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Biofluids, Inc., Rockville, MD) (regular medium). Primary cultures of skin fibroblasts from AP-3-deficient (regular medium) mice (Jackson Laboratory, Bar Harbor, ME) were obtained ing to the manufacturers' instructions. In brief, 500 ng of the pET28a-

Statistical Analyses—The experimental (observed) frequency for each residue at each position of the XXX0 signal was calculated using the sequences selected by the μ4 subunit from the combinatorial library. Preferences were evaluated by calculating the difference between the observed and expected frequencies (ΔF) in standard error units (14). Any ΔF value above 1 (i.e., favored) or below −1 (i.e., disfavored) was considered to be significantly different from 0 (random).

Site-directed Mutagenesis—Single amino acid substitutions were made using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Briefly, 50 ng of plasmid carrying the target CDNA was incubated with two complementary primers (2 μM each) containing the desired mutation in the presence of 2 μM dNTP mixture and 2.5 units of Pfu DNA polymerase for 16 cycles according to the following temperature profile: 0.5 min at 95 °C, 1 min at 55 °C, and 8 or 16 min at 68 °C. After replication of both vector strands, the methylated parental DNA was digested for 1 h at 37 °C with 10 units of DpnI endonuclease, and the nicked vector with the desired mutation was transformed into Escherichia coli.

Expression and Purification of μ4 (156–453)‐containing elution fractions were pooled; dialyzed 4-(156–453)-containing elution fractions were pooled; dialyzed 4-(156–453). The binding of μ4 (156–453) to the ETLYRRF peptide.

In Vitro Binding Assays—[35S]labeled μ4 (156–453) protein was obtained by in vitro transcription/translation using the TNT T7 Quick coupled transcription/translation system (Promega) and EasyTaq™ expression protein labeling mixture (PerkinElmer Life Sciences) according to the manufacturers’ instructions. In brief, 500 ng of the pET28a-[35S]labeled μ4 (156–453) construct was incubated with 20 μl of TNT Quick Master Mix and 11 μCi of [35S]methionine in a total volume of 25 μl at 30 °C for 90 min. The transcription/translation reaction mixture (containing [35S]-labeled μ4 (156–453)) was diluted 1:100 in binding buffer and centrifuged (180,000 × g, 15 min, 4 °C). 500 μl of supernatant was applied to peptide-coupled beads and incubated for 12 h at 4 °C. The beads were washed three times at 4 °C with binding buffer without bovine serum albumin, boiled in Laemmli sample buffer, and separated by SDS-polyacrylamide gel electrophoresis. The SDS gel was soaked in sodium salicylate and subjected to autoradiography.

Dilution of μ4 (156–453) was made using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). Briefly, 50 ng of plasmid carrying the target CDNA was incubated with two complementary primers (2 μM each) containing the desired mutation in the presence of 2 μM dNTP mixture and 2.5 units of Pfu DNA polymerase for 16 cycles according to the following temperature profile: 0.5 min at 95 °C, 1 min at 55 °C, and 8 or 16 min at 68 °C. After replication of both vector strands, the methylated parental DNA was digested for 1 h at 37 °C with 10 units of DpnI endonuclease, and the nicked vector with the desired mutation was transformed into Escherichia coli.

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against 20 mM Tris-HCl (pH 7.0), 250 mM NaCl, 5 mM EDTA, and 0.5 mM dithiothreitol; and concentrated by centrifugation in a Centriprep-3 device (Amicon, Inc., Beverly, MA).

Preparation of Peptide-coupled Beads and Surface Plasmon Resonance Sensor Chips—The following peptides were obtained from Zymed Laboratories Inc. (South San Francisco, CA): CWKRHHTGYEQF, CWKRHHTGAEQF, CWKRHHTGYEQA, CWRPKETLRRF, and CWRPKETLYRA. Peptide-coupled beads for in vitro binding assays were prepared by coupling the Cys residue of the peptides to EZ-Link™ PEO-maleimide-activated biotin (Pierce) in phosphate-buffered saline (pH 6.9) at peptide and biotin concentrations of 1 mM and 1.67 mM, respectively. The reaction was quenched by the addition of phosphate-buffered saline (pH 6.9), incubated overnight with 300 µl of immunopure immobilized streptavidin beads (Pierce) was washed twice with phosphate-buffered saline (pH 6.9), incubated overnight with 300 µl of biotinylation reaction, and washed three times with binding buffer (0.05% (w/v) Triton X-100, 50 mM HEPES (pH 7.3), 10% (v/v) glycerol, 100 mM KC1, 2 mM MgCl2, 0.1 mM CaCl2, 50 µM dithiothreitol, and 0.1% bovine serum albumin). Surface plasmon resonance experiments were carried out on a Biacore 1000 instrument (Biacore AB, Uppsala) at 25 °C using SA sensor chips with streptavidin covalently immobilized on a carboxymethylated dextran matrix. The chips were conditioned by bovine serum albumin. Surface plasmon resonance experiments were repeated twice on two different chips. Data transformation and overlay sensorgrams were prepared using Biacal Evaluation Version 3.9 software (Biacore AB). The response from the reference surface was subtracted from the other three flow cells to correct for refractive index changes, matrix effects, nonspecific binding, injection noise, and baseline drift. Using nonlinear least-squares fitting, the equilibrium dissociation constant (Kd) was evaluated by fitting data to a single site interaction model (Equation 1),

\[ [RU]_n = RU_{max} + (Kd/C) \]  

where [RU]n is the steady-state response level, RUmax is the maximal capacity of the surface (which was floated during the fitting procedure), and C is the concentration of µ4 in micromolar.

Antibodies and Immunofluorescence Microscopy—Immunofluorescence microscopy of fixed permeabilized cells and antibody internalization microscopy experiments were done as previously described (27, 29). Among the residues at the Ø position, the only preference was for Tyr, whereas Val was strongly disfavored (Fig. 1B). Among the residues at the Ø position, the only preference was for Tyr, whereas Val was strongly disfavored (Fig. 1B). Among the residues at the Ø position, the only preference was for Tyr, whereas Val was strongly disfavored (Fig. 1B). Among the residues at the Ø position, the only preference was for Tyr, whereas Val was strongly disfavored (Fig. 1B).

Fig. 4. Estimation of the affinity of the ETLYRRF sequence for µ4-(156–453) by surface plasmon resonance spectroscopy. A, various concentrations of µ4-(156–453) as indicated were injected onto streptavidin-coated flow cells previously loaded with the biotinylated CWRPKETLYRRF peptide. B, nonlinear least-squares fitting of the data shown in A yielded an equilibrium dissociation constant (Kd) of 7.0 ± 2.5 µM for the binding of µ4-(156–453) to the CWRPKETLYRRF peptide. RU, response units.
growth on histidine-deficient plates revealed that μ4 interacted only with the YXXØ signal from human LAMP-2 (HTGYEQF) (Fig. 2A). The LAMP-2 signal was not recognized only by μ4 though, as it bound even more strongly to μ2 and μ3A (Fig. 2, A and B). A salient feature of this signal is the presence of Phe at the Ø position, which fits the μ4 preferences deduced from

Fig. 5. Intracellular distribution of a Tac chimera bearing a μ4-specific signal. A–I, HeLa cells stably transfected with a Tac-DLYYDPM chimeric construct were treated with (B–I) or without (A) leupeptin (Leup; 1 mg/ml) for 4 h. J–L, primary cultures of fibroblasts from AP-3-deficient mocha mice were transiently transfected with Tac-DLYYDPM. Fixed permeabilized cells were incubated with rabbit antiserum to the luminal domain of Tac and monoclonal antibodies to the lysosomal membrane proteins CD63 (D–F) and LAMP-2 (G–L), followed by incubation with Alexa 488-conjugated anti-rabbit and Cy3-conjugated anti-mouse IgG antibodies.
the combinatorial analyses. The Tyr-to-Ala and Phe-to-Ala variants of the LAMP-2 signal (HTGAEQF and HTGYEQA, respectively) were unable to interact with μ4 or with any other μ chain (Fig. 2B).

To verify the yeast two-hybrid results, we performed a binding assay using in vitro transcribed/translated μ4-(156–453) and chemically synthesized and biotinylated LAMP-2 peptides. The peptides were bound to streptavidin beads and incubated with radioactively labeled μ4-(156–453). Bound μ4 was revealed by SDS-polyacrylamide gel electrophoresis and fluorography. As shown in Fig. 2C, μ4-(156–453) bound well to the wild-type LAMP-2 sequence (HTGYEQF), but only barely to the Tyr-to-Ala (HTGAEQF) and Phe-to-Ala (HTGYEQA) variants of the sequence.

The resolution of the crystal structure of the μ2 signal-binding domain allowed identification of residues that are directly involved in interactions with the critical tyrosine residue of the signals (19). Several of those residues are conserved in the other μ chains, including μ4 (3). To determine whether interactions of μ4 with YXXØ signals involved conserved residues in the tyrosine-binding pocket, we mutated the conserved Asp190 or Lys138 residue of μ4 to Ala. Two-hybrid assays revealed that these mutations abrogated interactions of μ4 with the tyrosine-based signal from LAMP-2 (Fig. 2D). Thus, the structural bases for the recognition of YXXØ signals by μ4 appear to be similar to those of μ2.

Characterization of μ4-YXXØ Interactions by Surface Plasmon Resonance Spectroscopy—μ4-YXXØ interactions were further characterized by surface plasmon resonance spectroscopy. In these studies, we used three biotinylated peptides: CWRPKETLYRRF, corresponding to one of the sequences selected from the combinatorial library (Fig. 1B), and its Tyr-to-Ala (CWRPKETLARRF) and Phe-to-Ala (CWRPKETYRRA) variants. Preliminary in vitro binding experiments showed that the CWRPKETLYRRF peptide bound radiolabeled μ4-(156–453) (Fig. 3A) in a concentration-dependent manner (Fig. 3B), whereas CWRPKETLARRF and CWRPKETYRRA did not (Fig. 3, A and B). The three biotinylated peptides were loaded onto separate flow cells of a streptavidin-coated chip. Recombinant μ4-(156–453) was then applied, and binding of the protein was measured by an increase in response units. The signal for the CWRPKETLYRRF peptide at a concentration of 13.4 μM reached a plateau at ~1000 response units, whereas that of the two variant peptides only reached 100–150 response units. This was in the range of the nonspecific binding of μ4-(156–453) to the biotinylated streptavidin surface without any peptide bound, as shown by the blank curve. After ending the injection of protein solution at 5 min, the value for the signal dropped sharply for all samples, indicating that the binding process was mostly reversible. However, ~20% of the binding could not be reversed even after washing for 10 min (data not shown). We performed an analysis of the interaction of different concentrations of μ4-(156–453) with the CWRPKETLYRRF peptide (Fig. 4A). As expected, the signal amplitude was dependent on the amount of μ4-(156–453) applied. The response approached a plateau value (a steady-state level, RU∞ (Equation 1)) after ~4.5 min. A plot of RU∞ against the concentration of μ4 is presented in Fig. 4B. Nonlinear regression analysis of these data yielded an apparent equilibrium dissociation constant of 7.0 ± 2.5 μM and a maximum binding capacity (RUmax) of the surface of 1550 ± 165 response units. Although these values should be considered only estimates, it is nonetheless clear that the interactions are of low affinity.

Intracellular Localization of a Chimeric Protein Bearing a μ4-specific Signal—To gain insights into the possible function of AP-4, we took advantage of the identification of a YXXØ signal (DLYYDPM) that was apparently specific for μ4 (Fig. 1B). This signal, as well as its corresponding Tyr-to-Ala mutant (DLYADPM), was appended to the cytosolic tail of the transmembrane protein Tac (29). The constructs were stably expressed in HeLa cells, and their intracellular distribution at steady state was examined by immunofluorescence microscopy using antibodies to the Tac luminal domain. We observed that the Tac-DLYYDPM chimera was present in the Golgi complex and plasma membrane (Fig. 5A). Treatment with the lysosomal

![Fig. 6. Analysis of the internalization of Tac and Tac signal chimeras.](http://www.jbc.org/) Live HeLa cells stably expressing Tac (no signal) or Tac signal chimeras were incubated with anti-Tac antiserum for 1 h at 4 °C. After washing off the unbound antibody with phosphate-buffered saline, half of the cells were incubated at 37 °C for 30 min (B, D, F, and H) to allow antibody internalization, and the rest were kept at 4 °C as controls (A, C, G, E, and G). All the cells were then fixed, and the internalized antibody was detected by incubation with Cy3-conjugated anti-mouse IgG. The presence of the proteins at the plasma membrane was evidenced by staining of the outline of the cells (A–E and G–H), whereas internalized proteins were detected as intracellular vesicles (F).
inhibitor leupeptin, however, resulted in accumulation of Tac-DLYYDPM in intracellular vesicles (Fig. 5B). Some of these vesicles colocalized with the lysosomal transmembrane proteins CD63 (Fig. 5, D–F) and LAMP-2 (Fig. 5, G–I), suggesting that a fraction of the Tac-DLYYDPM chimera was transported to late endosomes or lysosomes. The vesicular staining and colocalization of the chimera with LAMP-2 were not affected by the absence of the AP-3 complex in cells from the mocha mouse strain (Fig. 5, J–L) (30), consistent with the observation that the DLYYDPM signal does not interact with μ3A (Fig. 1C). The DLYYDPM signal did not mediate internalization of the chimera from the cell surface (Fig. 6, A and B), whereas a PL-SYTRF signal derived from the transferrin receptor did (Fig. 6, E and F). As expected, the Tyr-to-Ala mutant chimera (DLYADPM) and a Tac construct without any tyrosine-based sorting signal were not significantly internalized (Fig. 6, C and D, and J and K, respectively). These observations were in agreement with the inability of the DLYYDPM signal to interact with μ2 and suggested that the vesicular localization of the Tac-DLYYDPM chimera was not the result of internalization from the cell surface.

**DISCUSSION**

The results of the experiments reported here show that μ4 shares, with other members of the μ family of AP subunits, the ability to recognize a subset of YXXØ sorting signals. As is the case for other μ chains, interactions of μ4 with YXXØ signals require the Tyr and Ø residues (Figs. 2 and 3) and are saturable (Fig. 4). These properties emphasize the remarkable structural conservation of the μ chain family of proteins. Indeed, of 15 residues in μ2 known to be involved in interactions with Tyr and Ø residues (19), 14 are identical in μ4 (3), with the remaining one being a conservative Leu173 (μ2)-to-Val187 (μ4) substitution. Mutation of one of two of the identical amino acids, Asp190 or Lys398, to Ala abrogates interaction of μ4 with the signals (Fig. 2D), confirming that μ2 and μ4 recognize YXXØ signals in a similar fashion.

These structural similarities notwithstanding, the subset of YXXØ signals recognized by μ4 exhibits some characteristic features that distinguish it from that of other μ chains. The most salient feature of μ4 specificity is the preference for aromatic residues (Phe or Tyr) at various positions neighboring the critical Tyr residue. None of the other μ chains characterized to date exhibits this preference (14). The preference for Phe residues is particularly strong at the Y-1 and Y-3 (Ø) positions. In the case of the Ø position, this might be explained by the Leu173 (μ2)-to-Val187 (μ4) substitution. The smaller Val187 residue lining the hydrophobic pocket could allow accommodation of the large aromatic side chain of Phe while disfavoring binding of the smaller Val side chain. Another preference specific for μ4 is Asp at position Y+1, whereas other preferences are similar to those of other μ chains. For instance, the selectivity for Pro at Y+2 appears to be a general characteristic of all the μ chains. This suggests that a bend in the polypeptide chain imposed by Pro stabilizes the conformation of the signals for interaction with μ chains. μ4 also favors Arg at Y+2, a preference shared only with μ2 (14). In the case of μ2, this preference for Arg is due to the establishment of hydrophobic interactions of the Arg side chain with Trp421 and Ile419 of μ2 and a hydrogen bond between the guanidinium group of Arg and Lys420 of μ2 (19). Two of these residues in μ2 (Trp421 and Lys420) are conserved in μ4 (Trp429 and Lys440, respectively), but not in the other μ chains (3), which probably explains why only μ2 and μ4 favor Arg at Y+2.

Despite the fact that μ4 prefers certain residues at positions neighboring the critical Tyr residue, the subset of YXXØ signals recognized by μ4 overlaps to a significant extent with those recognized by other μ chains (Fig. 1C). This further strengthens the previous conclusion that μ chains recognize distinct but overlapping sets of YXXØ signals (14). Therefore, the involvement of AP complexes in specific sorting events cannot depend solely on the specificity of signal recognition by their μ chains. Rather, the role of signal preferences is likely to “fine-tune” the efficiency of sorting.

A screening of several naturally occurring YXXØ signals revealed that the lysosomal targeting signal from LAMP-2 (HTGYEQF) (30) interacts with μ4 (Fig. 2). This signal has a Phe residue at the Ø position, which could explain why it binds to μ4 (Fig. 1B). Previous studies had demonstrated weak interactions of μ4 with two other lysosomal membrane proteins, LAMP-1 (18) and CD63 (25). Taken together, these observations suggest a possible role for the AP-4 complex in sorting to lysosomes. However, the signals from all of these lysosomal membrane proteins interact better with μ2 and μ3A than with μ4 (Fig. 2, A and B). To gain insight into the potential function of AP-4, we took advantage of the identification of a signal (DLYYDPM) that interacts exclusively with μ4 (Fig. 1C). This signal was placed at the cytosolic carboxyl terminus of a Tac chimeric construct devoid of other sorting signals (13). The resulting Tac-DLYYDPM chimera was expressed by stable transfection into HeLa cells, and its localization was determined by indirect immunofluorescence microscopy. In the absence of protease inhibitors, the protein exhibited a steady-state localization to the Golgi complex and plasma membrane. However, incubation with the lysosomal inhibitor leupeptin resulted in accumulation of the protein in lysosomes, as shown by colocalization with LAMP-2 (Fig. 5). This indicated that the Tac-DLYYDPM chimera was transported to and degraded in lysosomes. As expected, this accumulation was dependent on the critical Tyr residue of the signal. The Tac-DLYYDPM chimera was not efficiently internalized from the plasma membrane (Fig. 6), in accordance with its inability to interact with μ2 (Fig. 1C). In addition, the Tac-DLYYDPM chimera was still targeted to lysosomes in AP-3-deficient mocha cells, further demonstrating that AP-3 does not play a role in the recognition of the DLYYDPM signal. Even though our two-hybrid results indicated that there is no interaction between the DLYYDPM signal and μ1 (Fig. 1C), we cannot rule out the possibility that AP-1 could somehow be involved in sorting of the Tac-DLYYDPM chimera. However, Meyer et al. (31) have suggested that targeting of proteins to lysosomes is not affected in μ1-deficient cells. These observations are consistent with the possibility that μ4 and, by extension, the AP-4 complex are involved in targeting proteins from the trans-Golgi network to the endosomal-lysosomal system. This involvement could provide an alternative means of sorting proteins to lysosomes, the existence of which has been suggested by previous studies (31–34). The evidence for a role of AP-4 in targeting to the endosomal-lysosomal system presented here, however, is indirect and should be considered tentative until it becomes possible to study protein sorting in AP-4-deficient cells. Attempts to ablate expression of this complex in mice are underway.

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Signal-binding Specificity of the μ4 Subunit of the Adaptor Protein Complex AP-4
Ruben C. Aguilar, Markus Boehm, Inna Gorshkova, Robert J. Crouch, Kazuhiro Tomita, Takashi Saito, Hiroshi Ohno and Juan S. Bonifacino

J. Biol. Chem. 2001, 276:13145-13152. doi: 10.1074/jbc.M010591200 originally published online January 3, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010591200

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