Medium Chains of Adaptor Complexes AP-1 and AP-2 Recognize Leucine-based Sorting Signals from the Invariant Chain*

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Interactions between tyrosine- and leucine-based sorting signals in the cytoplasmic tails of transmembrane proteins and adaptor complexes AP-1 and AP-2 are believed to be the first step in the formation of clathrin-coated vesicles that deliver these proteins to their destination. Medium chains of AP-1 and AP-2 have been reported to interact with tyrosine-based sorting signals in a number of in vitro assays. In the present study we found that recombinant medium chains could interact with leucine-based sorting signals from the cytoplasmic tail of the invariant chain. Medium chains may therefore be responsible for the proper recognition of both tyrosine and leucine sorting signals by AP-1 and AP-2 complexes.

Endosomal sorting signals are currently classified as tyrosine- and leucine-based signals (for a review, see Ref. 1). Transmembrane proteins containing such signals in their cytoplasmic tails are transported to their destination in clathrin-coated vesicles (CCVs) (2). Distinct adaptor protein (AP) complexes are important components of CCVs: they are thought to mediate CCV assembly by binding to the cytoplasmic tails of proteins containing endosomal sorting signals and subsequently recruiting clathrin (2, 3). AP-1 positive CCVs are associated with the trans-Golgi network, whereas AP-2 positive CCVs are mostly found on the plasma membrane although other intracellular locations have been reported (4, 5). AP complexes consist of two heavy, one light, and one medium (μ) chain each. Medium chains are able to bind tyrosine sorting signals in vitro (reviewed in Ref. 6), and this is believed to be the basis for the interactions between AP complexes and proteins containing such signals. However, no interaction between individual components of AP-1 or AP-2 and any of the leucine signals has been reported so far.

The invariant chain (Ii) contains two independent leucine signals in its cytoplasmic tail that are responsible for directing the major histocompatibility class II-Ii complexes to the endocytic compartments (7–9). The cytoplasmic tail of Ii has been shown to be important for AP-1 recruitment to the major histocompatibility class II-Ii complexes at the trans-Golgi network (10). Furthermore, a recent phase display-based study from our laboratory‡ has identified a short conserved sequence in the medium chains of the adaptor complexes that recognized a variety of tyrosine and leucine sorting signals, including both li signals. We therefore decided to test the ability of recombinant μ1 and μ2 to interact with the endosomal sorting signals from Ii.

MATERIALS AND METHODS

Reagents—The QIAexpress Type IV kit, Qiaex II DNA purification kit, and monoclonal anti-His antibody were from Qiagen. Materials for polyclonal rabbit IgA, goat anti-mouse IgG, and horseradish peroxidase-conjugated goat anti-mouse IgG were from Bio-Rad. The PDVF membrane and ECL reagents were from Amersham Pharmacia Biotech. Oligonucleotides were synthesized by Medprobe (Norway). Dynabeads coated with streptavidin were from Dynal. Other reagents were from Sigma.

Peptides—Peptides containing the first leucine-based (L1) signal from Ii and its alanine mutant were synthesized at the Biotechnology Center of Oslo (Norway). Their sequences were M1-DDQDLIRRR20 and M1-DDQDLIARRR20, respectively. Aliquots of these peptides were biotinylated (1:1 molar ratio of peptide and biotin) for use in binding assays. Biotinylated peptides containing the second (L2) signal and its mutant were kindly provided by Dr. G. Banting (University of Bristol, United Kingdom). Their sequences were biotin-N10NEQLPMLGRR20 and biotin-N10NEQLPAAGRR20, respectively (numbers are given according to the position of the residue in the p33 form of Ii).

Expression and Purification of Histidine-tagged Proteins—DNA coding for mouse μ1 and rat μ2 was kindly provided by Dr. T. Kirchhausen (Harvard Medical School). The full-length medium chains were cloned in frame into the Type IV pQE30 vector (Qiagen) to express constructs containing a histidine (His) tag at their N termini. The oligonucleotides used for PCR amplification were 5'-TCCGGGGATCCCATGTCGCCCA-GCCCGGCTCAG-3' and 5'-CTAGGCGGTACCATGATCGGAGGCTT-GAGCTCGGTACCATGATCGGAGGCTT-3', respectively. Amplified constructs were purified with Qiagex II kit (Qiagen) and cloned into BamHI and HindIII (for μ1) or KpnI and HindIII (for μ2) sites of pQE30. Proteins were expressed in the bacterial strain M15[pREP4] (Qiagen) according to the manufacturer’s protocol. Both proteins formed inclusion bodies, which were solubilized in 6 M guanidinium hydrochloride containing 10 mM β-mercaptoethanol. Proteins were purified in one step under denaturing conditions on nickel-nitrilotriacetic acid resin (Qiagen) according to specifications of the manufacturer. Purified proteins were dialyzed to a concentration of 10–20 μg/ml and refolded in the Binding buffer (0.1 M Tris, 5 mM EDTA, 0.1% Triton X-100, pH 7.5). Prior to the binding assay, proteins were centrifuged for 1 h at 100,000 × g (Airfuge) to remove the insoluble matter. His-tagged dihydrofolate reductase was expressed from the control plasmid pQE16 supplied with the kit and purified according to manufacturer’s recommendations. Protein concentration was determined from Coomassie-stained gels by comparison with protein standards.

Binding Assay—Biotinylated peptides containing leucine signals containing are used for PCR amplification were 5'-TCCGGGGATCCCATGTCGCCCA-GCCCGGCTCAG-3' and 5'-CTAGGCGGTACCATGATCGGAGGCTT-GAGCTCGGTACCATGATCGGAGGCTT-3', respectively (numbers are given according to the position of the residue in the p33 form of Ii).

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RESULTS

We first examined the interactions between the two leucine signals from $\mathrm{Ii}$ and $\mu_1$. Equal amounts of peptides encoding the wild type and the mutant signal immobilized on Dynabeads were incubated with $\mu_1$. As shown in Fig. 1A, $\mu_1$ preferentially bound to the peptide containing the first signal (LI signal hereafter). Interactions between $\mu_1$ and the peptide-containing alanine mutant of LI signal were over 5-fold weaker as compared with the wild type peptide (Fig. 1B). Similarly, we found that $\mu_1$ interacted with the peptide containing the second leucine signal (ML signal hereafter) over 4-fold stronger than with the peptide containing mutated ML signal (Fig. 1, C and D). To rule out the possibility that the observed interactions were dependent on the short histidine tag and not on $\mu_1$ sequence itself, we investigated interactions between the peptides containing LI and ML signals and His-tagged dihydrofolate reductase. No binding to either LI or ML signal was observed (data not shown).

We then investigated the dependence of binding of $\mu_1$ to LI signal on the concentration of the medium chain. Equal amounts of the LI peptide immobilized on Dynabeads were incubated with the increasing amounts of $\mu_1$. As shown in Fig. 2, binding reached saturation at about 2 $\mu$g of $\mu_1$. To further demonstrate the specificity of interactions between $\mu_1$ and LI signal, we studied them in the presence of competing peptides. A fixed amount of $\mu_1$ (1 $\mu$g/assay) was incubated overnight with various concentrations of either wild type or mutant LI peptide (0–1,200 molar excess of a peptide over sequence itself). To $\mu_1$, we assayed. As shown in Fig. 3 (A and B), binding of $\mu_1$ to LI signal was strongly inhibited by the wild type LI signal at the background levels (data not shown).

We also studied the interactions between the leucine signals from $\mathrm{Ii}$ and $\mu_2$. As shown in Fig. 3 (A and B), binding of $\mu_2$ to the mutated LI signal was significantly less than to the wild type LI signal. However, no interactions between $\mu_2$ and ML signal could be detected in our system, as both the wild type ML signal and its alanine mutant bound $\mu_2$ at the background levels (data not shown).

DISCUSSION

In this study we demonstrated that the full-length medium chain of the AP-1 adaptor complex interacted with both leucine sorting signals from the invariant chain in an in vitro assay. We also demonstrated interactions between the LI signal from $\mathrm{Ii}$ and the full-length medium chain of the AP-2 adaptor complex. These interactions were specific as medium
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interactions between AP-1 and AP-2 complexes and both leucine and tyrosine sorting signals have been well documented (Ref. 2 and references therein and Refs. 13 and 14). Furthermore, it has been demonstrated that interactions between tyrosine signals and AP-2 could be improved by the addition of phosphoinositides that are phosphorylated at the D-3 position of the inositol ring or when AP-2 was in the clathrin coat (18). Medium chains of AP-1 and AP-2 were shown to recognize a variety of tyrosine-based sorting signals in yeast two-hybrid system and in a number of in vitro assays (19–23), and this recognition is believed to be the basis for interactions between the tyrosine signals and AP complexes although interactions between the β-chain of AP-2 and a signal from asialoglycoprotein receptor have also been reported (24). On the other hand, the leucine signal from CD3y failed to interact with the medium chains in the two-hybrid system (19), leading to an opinion that leucine signals might be recognized by a different AP subunit. However, a recent phage-display study2 has identified a sequence from the medium chains that was able to recognize a number of leucine-based signals. It is important to notice that this sequence was missing from some of constructs used in other studies (19, 20, 22, 23) that were still able to bind tyrosine signals. One could therefore hypothesize that the medium chains contain at least two sites that are able to interact with different endosomal sorting signals.

The results presented here are mainly in line with those of Bremnes et al.,2 but in contrast to the phage-display studies our approach did not demonstrate interactions between μ₂ and ML signal. At present we are not able to explain this difference, but in the cell both LI and ML signals are independently involved in the internalization from the plasma membrane (8), and this might indicate that the AP-2 complexes interact with both signals in vivo. Such interactions may require additional residues around ML signal that were not present on the peptide used in our experiments. We are also aware of possible pitfalls associated with extrapolation of in vitro results to an in vivo situation as other subunits of AP complexes may be involved in the recognition of endosomal sorting signals either directly or indirectly by influencing the conformation of medium chains in the complex or the degree of their exposure to the cytoplasm. Nevertheless, studies of potential interactions between individual chains of AP complexes and sorting signals is an essential first step in understanding the detailed mechanism of how sorting signals are recognized.

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