Structural Determinants of Interaction of Tyrosine-based Sorting Signals with the Adaptor Medium Chains

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Many integral membrane proteins contain tyrosine-based signals within their cytoplasmic domains that mediate internalization from the cell surface and targeting to lysosomal compartments. Internalization depends on an interaction of the tyrosine-based signals with the clathrin-associated adaptor complex AP-2 at the plasma membrane, whereas lysosomal targeting involves interaction of the signals with an analogous complex, AP-1, at the trans-Golgi network. Recent studies have identified the medium chains μ2 of AP-2 and μ1 of AP-1 as the recognition molecules for tyrosine-based signals. We have now investigated the structural determinants for interaction of the signals with μ2 and μ1. The position of the signals was found to be an important determinant of interactions with μ2 and μ1; signals were most effective when present at the carboxyl terminus of a polypeptide sequence. Another important determinant of interaction was the identity of residues surrounding the critical tyrosine residue. Mutation of some residues affected interactions with μ2 and μ1 similarly, whereas other mutations had differential effects. These observations suggest that both the position and the exact sequence of tyrosine-based sorting signals are major determinants of selectivity in their interaction with clathrin-associated adaptor complexes.

The cytoplasmic domains of some integral membrane proteins contain tyrosine-based sorting signals that mediate internalization from the cell surface and targeting to intracellular compartments such as endosomes and lysosomes (reviewed in Refs. 1–3). Many tyrosine-based signals conform to the motif YXXØ, where X is any amino acid and Ø is an amino acid with a bulky hydrophobic side chain (Leu, Ile, Phe, Val, and Met). The Tyr and Ø residues are known to be critical for function, whereas the importance of the X residues is less well defined.

Early morphological studies demonstrated that internalization from the cell surface and targeting to lysosomes occur through clathrin-coated areas of the plasma membrane and the trans-Golgi network (TGN), respectively (reviewed in Ref. 4). These observations led to the hypothesis that tyrosine-based signals are recognized by components of clathrin coats. Several studies provided experimental evidence for such recognition, as the cytoplasmic domains of proteins that have tyrosine-based signals were shown to bind to either both of the clathrin-associated adaptor complexes AP-2 and AP-1 (5–14). The AP-2 complex is a component of plasma membrane clathrin coats and consists of two large chains (α-adaptin and either β2- or β-adaptin), one medium chain (μ2, also known as AP50), and one small chain (σ2, also known as AP17) (reviewed in Refs. 15–17). The AP-1 complex localizes to TGN clathrin coats and is also composed of two large chains (γ-adaptin and either β1- or β-adaptin), one medium chain (μ1, also known as AP47), and one small chain (σ1, also known as AP19) (reviewed in Refs. 15–17). The analogous subunits of AP-2 and AP-1 are structurally related to each other and are thought to subserve similar functions at the plasma membrane and the TGN, respectively. Recent studies have demonstrated that the medium chains, μ2 and μ1, of the AP-2 and AP-1 complexes, respectively, are capable of interacting with tyrosine-based sorting signals, suggesting that they may be the signal recognition components of the clathrin coats (13).

The findings described above imply that tyrosine-based signals can be recognized in at least two distinct sites within the cells (the plasma membrane and the TGN), and this recognition results in transport to different end compartments (endosomes and lysosomes, respectively). This raises the question of how certain tyrosine-based sorting signals can mediate only internalization while others are additionally capable of mediating lysosomal targeting. A likely explanation is that the position of the signal, the nature of the amino acids that surround the critical tyrosine, or a combination of both factors determines different affinities and fine specificities of interactions with μ2 and μ1. The use of a quantitative two-hybrid assay and an in vitro competition assay, developed in the course of our previous work (13), has now allowed us to assess the importance of these factors. The results of the experiments presented here demonstrate that both the position of the signal and its exact amino acid composition are indeed major determinants of interaction with the medium chains of adaptor coat proteins.

MATERIALS AND METHODS

Recombinant DNA Constructs—The structures of some of the basic constructs used in this study are shown in Fig. 1. The constructs GAL4bd-TGN38 Tail (Fig. 1), GAL4bd-TGN38 Tail (Tyr → Ala), GAL4bd-(SYQRL)₃, GAL4bd-(STYQRL)₃, GAL4bd-(AGYQTD)₃, GAL4bd-(ISYKGL)₃, GAL4bd-(ISYKGL)₃, GAL4bd-μ1, GAL4bd-μ2, GAL4ad-μ1, GAL4ad-μ2, pGEX(SDYQL)₃, and pcDNA3-μ2 have been described previously (13). GAL4bd-TGN38 Tail Δ1 and mutants of this construct, GAL4bd-TGN38 Tail Δ2 and GAL4bd-TGN38 Tail Δ3 (Fig. 1), were made by ligation of polymerase chain reaction fragments into the EcoRI and SalI sites of the vector pGBT9 (CLONTECH, Palo Alto, CA). GAL4bd-(ISYKGL)₃ was made by ligating synthetic oligonucleotides into the EcoRI and SalI sites of pGBT9. Constructs having the GAL4ad fused to a-adaptin (18), γ-adaptin (19), β2-adaptin (20), σ2 (21), σ1 (21), or p47A (22) were made by ligation of polymerase chain reaction-amplified cDNAs into the pACT2 vector. The Tac (a chain of the inter-
Externalization Assays—Internalization of 125I-labeled anti-human Tac monoclonal antibody (clone 7G7.B6; Upstate Biotechnology, Inc., Lake Placid, NY) by transiently transfected HeLa cells was analyzed as described previously (24). Internalization results are expressed as the means ± S.D. of triplicates. All experiments were repeated at least twice.

RESULTS

Reporter Assays Used to Study Signal-Adapter Interactions—Interactions of signals with adaptor subunits were analyzed using the yeast two-hybrid system (25) as described previously (13). Sequences containing sorting signals were fused to the GAL4bd (the "bait"), and the adaptor subunits were appended to the GAL4ad (the "prey"). The occurrence of interactions was evidenced by the ability of yeast cells cotransformed with both GAL4 constructs to grow on histidine-deficient plates and to express β-galactosidase activity. The growth assays were only qualitative, but more sensitive, allowing detection of interactions in some cases in which the β-galactosidase assays gave marginal or no activity. On the other hand, the β-galactosidase assays were less sensitive, but allowed quantitative comparisons among different signals. Therefore, both assays were used in order to detect a wider range of interactions.

Exposure of a Tyrosine-based Sorting Signal at the Carboxyl Terminus of a Bait Construct Enhances Its Interaction with Both µ2 and µ1—Our first experiments were aimed at determining the importance of the position of a signal for interaction with adaptor subunits in the yeast two-hybrid system. We used the cytoplasmic tail of TGN38, which has the tyrosine-based signal SDYQRL, as the basis for the initial constructs. The SDYQRL signal mediates internalization of TGN38 from the cell surface and its retrieval to the TGN (23, 26). We had previously demonstrated that the full-length cytoplasmic tail of TGN38 interacts with both the µ2 and µ1 adaptor medium chains (13). Analysis of β-galactosidase activity showed that the interaction of the full-length tail with the medium chains was relatively weak (Fig. 2). A µ2 construct lacking the first 120 amino acids from the amino terminus (3M9) displayed better reactivity with the TGN38 tail than full-length µ2 (3M2) (Fig. 2, TGN38 Tail); the same was true for all of the other bait constructs tested (see, for example, Figs. 5 and 6). In all cases, mutation of the critical Tyr residue decreased the β-galactosidase activity to background levels (Fig. 2, TGN38 Tail ∆Y). The SDYQRL sequence is located four amino acids away from the carboxyl terminus of the 34-amino acid cytoplasmic tail of TGN38 (Fig. 1). To assess whether the placement of the signal influences the strength of the interactions, we tested the effect of deleting the last four residues of the TGN38 tail (NLKL). This deletion increased the interaction with µ2 by 2–3 orders of magnitude and the interaction with µ1 by ~5-fold (Fig. 2, TGN38 Tail ∆1). These experiments thus demonstrate that exposure of the SDYQRL signal at the carboxyl terminus of the bait construct enhances its interaction with the medium chains.

Dependence on the Spacing between the Signal and the GAL4bd—We next examined whether the spacing of the SDYQRL signal from the GAL4bd influenced its recognition by µ2 and µ1. This was accomplished by removing varying portions of the TGN38 tail sequence between the GAL4bd and the SDYQRL signal. Shortening the spacer sequence from 27 (TGN38 Tail ∆27) to 18 (TGN38 Tail ∆28) residues had little effect on the interactions, but shortening to eight residues (TGN38 Tail ∆32) caused a 10-fold or greater decrease in interactions with both µ2 and µ1 (Fig. 3). This observation suggests that the

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*Notice that the β-galactosidase activity values in Figs. 2–6 are represented on a logarithmic scale.*
bait sequence has to be placed at a certain minimum distance from the GAL4bd for it to be able to interact optimally with the SDYQRL signal. In these analyses, the signals were presented either at the end of a TGN38 tail-derived segment or as a triple repeat; SDYQRL was found to be about an order of magnitude higher than interactions with either $\mu_2$ or p47A (Figs. 5 and 6). An alanine scan mutagenesis of the SDYQRL sequence revealed that both the Tyr and Leu residues are essential for interaction with all three chains (Fig. 5). Mutation of the Ser residue, on the other hand, had little or no effect on interactions with the three medium chains. Other mutations had differential effects on interactions with each of the three chains. For instance, mutation of the Asp or Glu residue to Ala did not affect interaction with $\mu_2$, but significantly decreased interaction with both $\mu_1$ and p47A. In contrast, the Arg residue was required for optimal interaction with $\mu_2$, but not for interactions with $\mu_1$ and p47A. Mutation of Tyr to Phe, Arg to Asp, or Leu to Val decreased interactions with all three chains.

Thus, while some residues are critical for interaction with all three medium chains, others are required only for interaction with some chains, but not with others. These observations suggest that each medium chain possesses a characteristic pattern of recognition for residues surrounding the critical tyrosine.

**Analysis of the Interaction with Other Tyrosine-based Signals**—The experiments described above were done using the SDYQRL signal from TGN38 as the basis for the bait constructs. To extend these observations to other tyrosine-based signals, we analyzed the interaction of signals derived from various integral membrane proteins with the adaptor medium chains. In these analyses, the signals were presented either at the end of a TGN38 tail-derived segment or as a triple repeat; the SDYQRL signal displayed strong, tyrosine-dependent interactions in both contexts (Fig. 6). The STYQPL signal from
the mouse lysosomal membrane protein CD68 (27) also interacted well with all three medium chains in both bait contexts (Fig. 6). In contrast, the AGYQTI signal from the lysosomal membrane protein Lamp-1 (28, 29) interacted poorly with the threemedium chains when placed at the end of the TGN38 tail sequence, but more strongly when presented as a triple repeat. The LSYTRF signal from the transferrin receptor (30, 31) exhibited a different pattern of interactions: it interacted with m2 when positioned at the end of the TGN38 tail sequence, but not at all in the context of a triple repeat. In addition, the LSYTRF sequence did not bind to μ1 or p47A under any of the conditions tested (Fig. 6). Finally, the ISYKGL sequence, which is one of two elements involved in the localization of furin to the TGN (32, 33), only interacted weakly with the 3M9 form of μ2 when expressed as a triple repeat (Fig. 6).

From these experiments, it is apparent that (a) there are great disparities in the strength of interaction of different tyrosine-based signals with the medium chains; (b) different signals display a substantial degree of selectivity toward particular medium chains; and (c) the interactions are highly dependent on the context in which the tyrosine-based signals are inserted. Regarding this last point, it is important to bear in mind that these experiments entailed placement of the signals in a context that is different from their native context. Even with this caveat, though, the experiments demonstrate clearly the principle that the composition of the signal is an important determinant of interactions.

### Phosphorylation of the Critical Tyrosine Residue Inhibits Interaction with m2

All the experiments described above indicate that tyrosine is the only residue that is absolutely required for strong interactions with the medium chains. Because tyrosine residues can be post-translationally modified by addition of phosphate groups, we were interested in determining whether phosphorylation affected binding to the medium chains. This question is particularly relevant because tyrosine-based sorting signals bear a striking resemblance to phosphotyrosine motifs that bind SH2 domains of signal transduction molecules (reviewed in Ref. 34). To address this issue, we resorted to an in vitro binding assay in which peptides were used to compete the binding of in vitro translated, labeled m2 (3M9) to a GST-(SDYQRL)3 fusion protein (13). The competition curves shown in Fig. 7 demonstrate that whereas an SDYQRL peptide inhibited the binding in a dose-dependent fashion, no competition was observed with a peptide in which the Tyr residue was replaced by a nonhydrolyzable phosphotyrosine analog (SDpYQRL) at concentrations of up to 667 μM.

The results of this experiment suggest that phosphorylation of the tyrosine residue is not only unnecessary for interactions, but actually inhibits recognition of the tyrosine-based signal by m2.

### Effect of Substitutions in the SDYQRL Signal on Endocytosis

The ability to measure the interactions of variants of the SDYQRL signal with μ2 using the yeast two-hybrid system provided us with an opportunity to compare these results with...
the function of the signal variants in vivo. This was done by examining the effect of various mutations on the ability of the SDYQRL signal to mediate internalization from the plasma membrane, a process that is amenable to quantitative analysis. Antibody internalization assays were performed on HeLa cells transfected with Tac constructs having different cytoplasmic tails as described previously (24). In addition to normal Tac, which has no internalization signals, the two basic constructs used in these studies were ID5 and TGG1 (23), having the SDYQRL signal after 8 and 22 residues of the TGN38 tail, respectively (Fig. 1). We observed that whereas normal Tac was incapable of mediating antibody internalization, both the ID5 and TGG1 chimeras were efficiently internalized relative to Tac (Fig. 8, A–C). Mutation of either the Tyr or Leu residue in the SDYQRL signal to Ala completely abrogated the internalization activity of the signal (Fig. 8A). In contrast, mutation of either the Arg or Asp residue to Ala had no effect on internalization (Fig. 8, B and C). Substitution of the Arg residue with Asp or of the Tyr residue with Phe decreased the rate of endocytosis by ∼50–60% (Fig. 8, B and C). Overall, these results are consistent with the observations made using the two-hybrid system (Fig. 5). However, some of the mutations (i.e. Arg to Ala, Arg to Asp, and Tyr to Phe) caused more substantial decreases in the two-hybrid analyses than in the internalization assays. This latter observation suggests that the internalization machinery is capable of utilizing signals that have a relatively low affinity for the recognition molecules.

DISCUSSION

The Problem of Fine Specificity in the Function of Tyrosine-based Signals—The concept that tyrosine-based signals are involved in various sorting processes has received overwhelming experimental support over the past decade. It is now well established that processes such as internalization and lysosomal targeting (reviewed in Refs. 1–3), localization to specialized endosomal/lysosomal organelles such as antigen-processing compartments (24, 35), transport to the basolateral surface of polarized epithelial cells (reviewed in Ref. 36), and localization to the TGN (23, 26) all depend to a large extent on the function of tyrosine-based signals. The number and diversity of the cellular compartments that rely on tyrosine-based signals for sorting pose a formidable problem of fine specificity, an issue of which we currently have a very limited understanding.

Some of the sources of specificity have been gleaned from studies of protein trafficking in intact cells. The evidence gathered to date suggests that the exact position of the signal within the cytoplasmic domain (30, 37) and the nature of residues neighboring the critical tyrosine (23, 29, 38, 39) are major determinants of both activity and specificity. Other factors, such as the oligomeric state of the signal-bearing proteins, post-translational modifications at or near the signals, and the presence of additional signals such as dileucine motifs (40, 41) or acidic clusters (32, 33), are also likely to influence the role of particular tyrosine-based signals. The recent finding that tyrosine-based signals interact with the medium chains of the clathrin-associated adaptor complexes AP-2 and AP-1 (13) has opened new avenues of experimentation into the problem of specificity, enabling a dissection of the determinants of specificity at a molecular level.

Differences in the Apparent AviAvidity of the Three Medium Chains for Tyrosine-based Signals—The use of quantitative β-galactosidase assays in this study allowed us to confirm that tyrosine-based signals interact with the μ2 and μ1 chains in a tyrosine-dependent manner. In contrast to μ2 and μ1, the other chains of the AP-2 and AP-1 complexes did not show detectable interaction with tyrosine-based signals in the two-hybrid system.

The μ2 chain displayed higher levels of interaction with most tyrosine-based signals relative to μ1 and p47A. This could be a peculiarity of the two-hybrid system; however, it is tempting to speculate that these differences might have physiological significance. The localization of μ2 to the TGN, for instance, could ensure that only those proteins that have the strongest signals are recognized intracellularly for transport to lysosomal compartments. Proteins having weaker signals, on the other hand, would proceed to the plasma membrane, where they could be recognized by μ2. The different affinities of interaction with μ2 and μ1 could thus be one of the factors that determine the role of signals in internalization versus lysosomal targeting.

The Importance of the Position of the Signal—Two positional parameters have been revealed by our studies as being important for determining the strength of interactions. One is the placement of the signal with respect to the carboxyl terminus of the bait sequence; the other is the distance from the signal to the GAL4bd. Placement of the signal at the carboxyl terminus rather than internally within a polypeptide sequence was found to augment markedly the interactions with the medium chains. This effect may be due to the exposure of the terminal carboxyl group of the bulky hydrophobic residue, which could provide an additional attachment point to the medium chains. Alternatively, the increase could be due to an enhanced conformational flexibility that would permit adoption of a favorable configuration for binding to the medium chains. This finding is probably relevant to the fact that most, although not all, lysosomal membrane proteins, such as CD68 (27), Lamp-1 (42, 43), Lamp-2 (43), and CD63 (44), have their tyrosine-based signals at the carboxyl terminus. In line with this idea postulated above, we think that an increased affinity caused by the presence of the signal at the carboxyl terminus of these proteins might allow recognition by μ2 at the TGN and, consequently, promote transport to lysosomes.

Our experiments also demonstrate that the spacing of the signal from the GAL4bd is critical for interaction. Although the spacing requirements for interactions in the two-hybrid system are likely to be different from those in the physiological environment of the signals, it is apparent that the close proximity of the signal to another domain (i.e. the GAL4bd or the transmembrane domain) hinders recognition by the adaptors. The elegant studies of Rohrer et al. (37) have emphasized the im-
portance of the spacing of the signal from the membrane by demonstrating that moving the tyrosine-based signal of Lamp-1 by just one residue closer to or farther away from the transmembrane domain decreases lysosomal targeting without affecting internalization. Once again, this suggests that placing the tyrosine-based signal at the appropriate distance from the membrane makes for a strong signal capable of interacting with \( \mu_2 \), and of mediating lysosomal targeting. Our data suggest that \( \mu_2 \) may recognize weaker signals because of its higher intrinsic avidity; this probably explains why the effects of signal positioning on internalization are less noticeable.

Sequence Requirements—Some amino acid substitutions in the SDYQRL signal affected interactions with all of the medium chains equally, whereas other substitutions had differential effects (Fig. 5). In addition, different signals displayed characteristic preferences for interaction with each medium chain (Fig. 6). These observations confirm the notion that the identity of residues surrounding the critical tyrosine is a major determinant of interactions. Indeed, it is the nature of these residues that determines (a) the vastly different avidities with which signals are recognized and (b) the preference of each medium chain for some signals over others. How these preferences relate to the function of different signals in physiological settings remains to be established for specific sorting events in vivo.

Correlation with Endocytosis—As a first step toward examining the correlation between signal-adaptor interactions and protein sorting in intact cells, we compared the internalization rates in transfected HeLa cells of constructs carrying mutations in the SDYQRL signal. Most of the results obtained in the internalization assays were concordant with the interactions with \( \mu_2 \) detected using the two-hybrid system. For example, mutation of Tyr to Ala and of Leu to Ala completely blocked internalization, and mutation of Asp to Ala had no effect. Other mutations, such as Arg to Ala, Arg to Asp, and Tyr to Phe, caused more substantial decreases in interaction with \( \mu_2 \). These observations are consistent with the idea that mutations that are sufficient to ablate lysosomal targeting from the TGN (a process that may be mediated by the low-affinity \( \mu_1 \) chain) have little effect on internalization (a process mediated by the high-affinity \( \mu_2 \) chain) (29, 37).

The apparently lower sensitivity of the two-hybrid assays relative to the internalization assays may be due to the distinct geometry of the interacting species in each system. The interactions are truly bimolecular in the two-hybrid system, whereas they probably involve multivalent attachment in the cells. This is due to the fact that the integral membrane proteins often exist as oligomers (45) and that the adaptor complexes become incorporated into a two-dimensional lattice-like structure (15–17).

Concluding Remarks—In summary, we have demonstrated that both the position of the tyrosine-based sorting signals and the identity of residues surrounding the critical tyrosine are important determinants of interaction with the adaptor medium chains. The avidity with which a particular signal interacts with each adaptor must thus be the result of a combination of these factors. We have also presented evidence suggesting that the avidity of \( \mu_1 \) for tyrosine-based signals is generally lower than that of \( \mu_2 \). These factors are likely to be the major determinants of function of tyrosine-based signals in internalization and lysosomal targeting.

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FIG. 8. Effect of mutations in the SDYQRL signal on internalization of \(^{125}\text{I}\)-labeled anti-Tac antibody from the cell surface. HeLa cells were transiently transfected with Tac, ID5, ID5 (Tyr\(^{333}\)→Ala), or ID5 (Leu\(^{336}\)→Ala) (A); Tac, ID5, ID5 (Arg\(^{335}\)→Ala), or ID5 (Arg\(^{335}\)→Asp) (B); and Tac, TTG31, TTG31 (Asp\(^{332}\)→Ala), or TTG31 (Tyr\(^{333}\)→Phe) (C).
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