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Many cell surface proteins are internalized via dileucine- or tyrosine-based motifs within their cytoplasmic domains by the heterotetrameric adaptor protein complex, AP-2. In this study we have examined how AP-2 mediates internalization of large cell surface receptors, such as the eight-chain TCR:CD3 complex. Although most receptors have a single signal that drives internalization, the TCR complex has two (D/E)xxxL(L/I) motifs and 20 YxxØ motifs. Using 293T cells, we show that AP-2 is completely dependent on both signals to mediate TCR internalization, because deletion of either completely blocks this process. Significant plasticity and redundancy were observed in the use of the YxxØ motifs, with a clear hierarchy in their use (CD3γ > CD3ζ ∼ CD3ε > CD3ζ > CD3ε). Remarkably, a single, membrane-distal YxxØ motif in CD3δ could mediate ∼75% of receptor internalization, whereas its removal only reduced internalization by ∼20%. In contrast, significant rigidity was observed in use of the (D/E)xxxL(L/I) motif in CD3γ. This was due to an absolute requirement for the position of this signal in the context of the TCR complex and for a highly conserved lysine residue, K128, which is not present in CD3ζ. These contrasting requirements suggest a general principle by which AP-2 may mediate the internalization of large, multichain complexes. The Journal of Immunology, 2005, 174: 4153–4160.

Materials and Methods

Plasmids

Mutant and wild-type TCR:CD3 chains were generated by recombinant PCR and cloned in the murine stem cell virus-based vector, MSCV.IRES.GFP, as
Yeast two-hybrid

CD3:AP-2µ interaction was assessed using a galactosidase-4 (GAL4)\(^{4}\)-based yeast two-hybrid system (provided by S. Elledge, Baylor College of Medicine, Houston, TX). The entire cytoplasmic domains of wild-type or mutant CD3 chains were generated by PCR and cloned into pAS2, which confers leucine auxotrophy, AP-2µ in pACT, which confers tryptophan auxotrophy, was provided by J. Bonifacino (National Institutes of Health, Bethesda, MD). The day before transformation, 400\(\mu\)l of AP-2µ-transfectants received 2\(\mu\)g peptide-linked 3A9.TCR transfection reagent (Roche) for 5 min at room temperature. Each group of pACT and pAS2 vectors with 100 \(\mu\)g denatured salmon sperm DNA (Sigma-Aldrich), 30% polyethylene glycol 4000, and 100 \(\mu\)m lithium acetate/TE. Yeast were shaken for 30 min at 30\(^\circ\)C and heat-shocked for 15 min at 42\(^\circ\)C. Yeast were spun, resuspended in TE, and plated out on plain solid dropout plates, SD-TPR-Leu and SD-TPR-Leu-His + 3AT (Bio 101). All yeast grew on the former. Interaction was determined by growth on the latter (histidine auxotrophy) and β-GAL activity, as determined by a lacZ assay. Liquid cultures were set up by inoculation of early SD-TPR-Leu broth and were grown at 30\(^\circ\)C overnight to 1\(\times\)10\(^7\) cells/ml in SD-TPR-Leu broth. Four hundred microliters of culture was placed onto nitrocellulose filters using dot-blot apparatus (Bio-Rad), snap-frozen in liquid \(N_2\), placed on 3M filter paper saturated in Z buffer (60 \(\mu\)M Na\(_2\)HPO\(_4\), 40 \(\mu\)M NaH\(_2\)PO\(_4\), 10 \(\mu\)M KCl, 1 \(\mu\)M MgSO\(_4\), 7\(\alpha\)H\(_2\), and 50 \(\mu\)M 2-ME, pH 7.0) containing 1 mg/ml X-GAL (Sigma-Aldrich), and incubated overnight at 30\(^\circ\)C. 

Transient transfection of 293T and TCR internalization assay

Transfections and internalization assays were performed as described with some modification (24). 293T cells (human embryonic kidney cells; provided by D. Baltimore, Caltech, and E. Vanin, St. Jude Children’s Research Hospital, Memphis, TN) were incubated in 10-cm plates at 2 \(\times\)10\(^6\)plate overnight at 37\(^\circ\)C. DMEM (450 \(\mu\)l) was mixed with 30 \(\mu\)l of FibriGene 6 transfection reagent (Roche) for 5 min at room temperature. Each group of transfected cells received 2 \(\mu\)g peptide-linked 3A9.TCR αβ-chains as well as the CD3 chains indicated (36). DNA (2 \(\mu\)g of each chain) was mixed with FuGene/DMEM and incubated for 15 min at room temperature. The mixture was incubated with the cells for 2 days. On day 3, cells were stained with anti-VP8.1/2-PE (F23.1) and anti-CD3-allophycocyanin (BD Pharmingen). GFP\(^{+}\)/TCR\(^{-}\)/CD3\(^{-}\) cells were sorted and cultured in 96-well, flat-bottom plates at 5 \(\times\)10\(^3\)/well overnight. Brefeldin A (BFA; Epicentre Technologies, Madison, WI) was added at 10 \(\mu\)g/ml for the times indicated. Cells were stained with anti-TCR-β-Chyome (H57-597) and analyzed for TCR modulation (MACSFLCalibur; BD Biosciences). The percent internalization was determined from the median fluorescence values using untreated controls as reference.

Results

AP-2µ specifically interacts with tyrosine residues in CD3\(\delta\) and to a lesser extent CD3\(\gamma\)

Yeast two-hybrid assays have been successfully used to determine the relative abilities of various YxxØ motifs to bind to the AP-2µ-chain (14). Although AP-2 has been shown to bind to the TCR:CD3 complex (8, 27), it is unclear which YxxØ motifs in CD3\(\gamma\) can be bound by AP-2µ (Fig. 1A). Initially, we used a GAL4-based, yeast two-hybrid assay to determine which CD3 cytoplasmic tails can bind directly to AP-2µ. The CTLA-4 cytoplasmic tail, which is known to interact with AP-2µ (4, 27), mediated significant growth on minimal medium lacking histidine and production of β-GAL in yeast transformed with the AP-2µ vector, but not an empty vector control (Fig. 1B). In contrast, no interaction was observed with the CD28 cytoplasmic tail, as expected.

Using this assay, AP-2µ was found to specifically interact with the cytoplasmic tail of CD3\(\delta\) and, to a lesser extent, with that of CD3\(\gamma\), but not of CD3\(\epsilon\) or CD3\(\zeta\). Importantly, mutation of the two tyrosine residues in CD3\(\gamma\) and CD3\(\delta\) to phenylalanine (Y-F) substantially reduced this interaction. A small amount of growth was observed with the CD3\(\delta\).Y1/2-F mutant. This growth does not appear to be due to any interaction with the dileucine motif in CD3\(\delta\), because mutation of these residues did not affect the growth or production of β-GAL in this assay (data not shown). Although this could be due to background growth or another as yet undefined motif, it is clear that the CD3\(\delta\).AP-2µ interaction is substantially reduced by these substitutions. Mutation of either γY1 or γY2 completely abrogated the interaction with AP-2µ, suggesting that both are required for the weak association seen with wild-type CD3\(\gamma\). In contrast, reduced, but significant, interaction with AP-2µ was observed with single tyrosine mutations in the CD3\(\delta\) cytoplasmic tail, suggesting that either CD3\(\delta\) YxxØ motif alone can mediate interaction with AP-2.

Internalization of TCR is mediated by tyrosine-based signals in CD3 chains

BFA blocks anterograde transport from the endoplasmic reticulum to the Golgi complex and causes tubulation and fusion of early

\(^{4}\) Abbreviations used in this paper: GAL, galactosidase; BFA, brefeldin A.
endosomes with the trans-Golgi network, essentially blocking recycling of receptors back to the cell surface (37, 38). We have previously exploited this property to study TCR internalization in resting and activated T cells (24). We also showed that TCR:CD3 complexes ectopically expressed on 293T cells were internalized with kinetics comparable to those of normal T cells. This is consistent with the idea that TCR internalization is mediated by a general biological process that uses basic cell machinery (e.g., AP-2, dynamin, clathrin, etc.) that is present in all cells.

Using this system, we first introduced a series of tyrosine to phenylalanine (Y-F) substitutions in the YxxØ motifs of all four CD3 chains to determine whether these tyrosine-based motifs mediated TCR complex internalization. Wild-type and mutant TCR:CD3 complexes were expressed on 293T cells by transient transfection with plasmids expressing all six TCR:CD3 chains, and the extent of TCR internalization was evaluated using BFA. All constructs generated gave TCR surface expression levels comparable to those of wild type, and any small differences were normalized in calculating the percentage of TCR internalization. As we have previously shown, the effect of BFA is not complete, and TCR levels reach a steady state equilibrium after ~20–30 min (24, 37, 38) (Fig. 1C). This appears to be remarkably consistent, as shown by the predominantly small error seen in all experiments in this study. As the data clearly show, mutation of all YxxØ tyrosine residues in the CD3 chains (CD3ζγδε, Y-F) completely abrogated TCR internalization, suggesting that one or more of these residues mediated this process (Fig. 1, C and D). These data confirmed previous suggestions that TCR internalization is mediated by motifs within the CD3 cytoplasmic domains (8, 24, 39). However, we were surprised that mutation of the tyrosine-based motifs was sufficient to completely block internalization, because previous studies with TAC:CD3 chimeras had suggested that the dileucine- and tyrosine-based motifs act independently to mediate endocytosis (11). Thus, even though these CD3ζγδε, Y-F mutant complexes have an intact CD3δ dileucine motif, they cannot be internalized, suggesting that experiments with chimeric molecules may not recapitulate the requirements for intact, multichain complexes.

**TCR internalization is mediated by a broad hierarchy of tyrosine-based motifs**

Additional analysis of the individual CD3 chains was performed in two ways. First, we expressed TCR complexes with Y-F mutations within the ITAMs of a single CD3 chain (e.g., CD3ε mutant, CD3ζγδε wild type; Fig. 2A, left). Second, we expressed TCR complexes in which ITAM tyrosine residues of a single CD3 chain remained intact, whereas all remaining YxxØ tyrosine residues in the CD3 complex were mutated (e.g., CD3ε wild type and CD3ζγδε mutant; Fig. 2A, right). The majority of the constructs mediated TCR surface expression levels comparable to those of wild type (Fig. 3).

For TCR containing single CD3 chain mutations, internalization appeared to be unaffected when tyrosine residues in CD3ε, CD3γ, or CD3ζ were mutated (Fig. 2B). However, an ~50% reduction in TCR internalization was seen after mutation of the CD3ζ YxxØ tyrosine residues. This suggests the CD3ζ YxxØ signals are strongly recognized by the AP-2 internalization machinery, consistent with data from the yeast two-hybrid experiments. However, TCR internalization was not completely abrogated as seen when all CD3 YxxØ tyrosine residues are mutated, indicating some cooperativity between CD3ζ and the other CD3 chains. To address this issue, TCR complexes were expressed that contained Y-F mutations of all YxxØ tyrosines except for one CD3 chain (Fig. 2A, right). Cells expressing TCR complexes in which only the CD3ζ tyrosine residues were intact showed internalization levels ~60% of wild-type transfectants (Fig. 2C). TCR complexes containing intact tyrosine residues only in CD3ζ or CD3ζ mediated 30–40% internalization compared with wild type. Because this represents two CD3ζ YxxØ signals vs 12 CD3ζ YxxØ signals, it is conceivable that CD3ζ contributes more to TCR internalization. In contrast, internalization was completely blocked in cells where only CD3ε remained the wild-type chain. These data demonstrate the importance of CD3ζ tyrosine residues in mediating internalization, a process that appears to involve cooperativity among CD3ε- and CD3ζ- and/or CD3ζ-chains, but not CD3ε.

Next we assessed the relative contributions of the individual YxxØ signals in the CD3ζ, CD3γ, and CD3ζ cytoplasmic domains. Although mutation of the CD3ζ ITAM tyrosine residues in the presence of an otherwise intact TCR:CD3 complex had little

![FIGURE 2. Multiple CD3 YxxØ signals mediate TCR internalization. 293T cells were transfected and sorted as described in Fig. 1. Cells were treated with BFA for 30 min, and TCR internalization was determined. A. Schematic of the two types of transfectants generated where one of the CD3 chains is mutated (left) or one of the CD3 chains is wild type (right). B–F, The mean ± SE from three to 15 experiments are shown as a percentage of the CD3 wild-type (WT) transfectants. Y-F, YxxØ Tyr residues mutated to Phe. TCR expression levels relative to wild type are depicted in Fig. 3.](http://www.jimmunol.org/DownloadedFrom/4155A/4155A.jpg)
The dileucine-based signal in CD3γ, but not CD3δ, mediates TCR internalization

It is well established that the dileucine-based motif in CD3γ can mediate TCR internalization. The majority of studies have focused on protein kinase C-mediated internalization of the TCR in which the aspartic acid (D127), the two leucines (L131/132), and phospho-

ylation of a serine residue proximal to the motif (S126) were found to be essential (29, 39). However, less is known about the requirements for constitutive internalization of the TCR in resting cells. Recently, it was found that mutation of the human CD3γ dileucine residues completely abolished TCR internalization in an S126 phosphorylation-independent manner (28). It is not known whether the CD3δ dileucine-based signal participates in constitutive TCR internalization, although there are some data from experiments with Tac:CD3 chimeras that it is less effective at mediating lysosomal targeting than its CD3γ counterpart (11). In these studies we wanted to assess the roles of the CD3γ and CD3δ dileucine-based signals, in the context of the whole TCR:CD3 complex, in mediating TCR internalization.

Cells were transfected with wild-type or dileucine mutant (LL-AA) CD3γ- and CD3δ-chains in the presence of the remaining wild-type TCR:CD3 chains (Fig. 4A). Mutation of the CD3γ dileucine residues significantly reduced TCR internalization (Fig. 4, B and C). In contrast, TCR internalization was not affected by mutation of the CD3δ dileucine residues. Interestingly, coexpression of the CD3γ:LL-AA and CD3δ:Y1/2-F mutants did not further reduce TCR internalization, suggesting that the defects incurred by these mutations were not additive, but cooperative (Fig. 4B). Collectively, these data raised two interesting issues. First, as mutation of the CD3γ dileucine residues did not abrogate internalization, were there other proximal residues that contributed to its function? Second, why is the CD3δ (D/E)xxL/L/I motif unable to mediate TCR internalization?

**FIGURE 4.** The dileucine residues in CD3γ, but not CD3δ, are required for optimal TCR internalization. A, Cytoplasmic domain amino acid sequence of the CD3γ and CD3δ constructs analyzed. TM, transmembrane domain; CY, cytoplasmic domain. ITAM is boxed. B, and C, 293T cells were transfected and sorted as described in Fig. 1. Cells were treated with BFA for 30 min (B) or for the times indicated (C), and TCR internalization was determined. B, The mean ± SE from seven experiments are shown as a percentage of the CD3 WT transfectants. C, A representative experiment is shown. LL-AA, Leu residues (L131/L32 in CD3γ and L142/143 in CD3δ) mutated to Ala. TCR expression levels relative to wild type (WT) are depicted in Fig. 6. D, Sequence alignment of the dileucine region of CD3γ and CD3δ. TM, transmembrane domain; CY, cytoplasmic domain; h, hydrophobic residues. Residues that differ between CD3γ and CD3δ are in bold; the dileucine-based signal used by CD3γ is boxed; nonconserved residues are indicated by a dot; acidic residues are indicated by a dash.
Key parameters for TCR internalization by the CD3γ dileucine-based signal: position and K128

It was particularly surprising that the CD3δ dileucine-based motif did not appear to mediate TCR internalization even though the dileucine and negatively charged residues in the (D/E)xxL(L/I) motif were present (CD3γ, DKQTLL; CD3δ, EVQALL). This inferred that there were other parameters that determined the ability of the CD3γ dileucine-based motif to mediate TCR internalization.

Alignment of the CD3γ and CD3δ sequences revealed 11 amino acid residues between the transmembrane domain and the ITAM motif that differ between the two chains (Fig. 4D). Initially, we generated mutants in which only these nonconserved residues were swapped between the two proteins (Fig. 5A, CD3γ-δL11-γ and CD3δ-γL11-δ). This was preferred over exchanging the entire cytoplasmic tails to avoid altering the contribution of the tyrosine-based signals. TCR containing CD3γ-δL11-γ instead of CD3γ-WT, which essentially contained two CD3δ dileucine-based motifs (one in the wild-type CD3δ-chain and one placed in the context of CD3γ), were completely refractory to internalization (Fig. 5B). This was surprising, because mutation of the dileucine residues had only reduced TCR internalization by ~60% (Fig. 4, B and C).

Do TCR containing two CD3γ dileucine-based motifs, where CD3δ-WT has been replaced with CD3δ-γL11-δ, exhibit enhanced internalization? Interestingly, the data infer the opposite, with a ~30% reduction in TCR internalization observed in the presence of CD3δ-γL11-δ (Fig. 5B, third bar). TCR internalization was prevented in this complex when the wild-type CD3γ was replaced with the CD3γ LL-AA mutant, suggesting that the internalization seen with CD3γ-WT/CD3δ-γL11-δ was mediated by the dileucine signal in CD3γ, rather than the CD3γ dileucine motif inserted into CD3δ (data not shown). Likewise, when both regional mutants were expressed instead of the wild-type CD3γ-γ and CD3δ-chains (Fig. 5B, fourth bar), essentially transposing the CD3γ dileucine-based signal from CD3γ to CD3δ, minimal TCR internalization was observed. Collectively, these data suggest the following. First, additional residues surrounding the two leucines in CD3γ, which are not present in CD3δ, are necessary for TCR internalization. Second, the position of the active dileucine-based signal relative to the rest of the TCR:CD3 complex is important. Third, placement of this signal in the wrong location appears to reduce internalization, suggesting that it may act as a dominant negative by encouraging AP-2 to engage the TCR in a less productive orientation.

Additional mutants were generated to determine which CD3γ-specific residue(s) was responsible for the efficacy of the CD3γ dileucine-based signal (Fig. 5A). Substitution of CD3γ residues S126/K128/T130 with the equivalent residues in CD3δ (A137/ V139/A141) gave essentially the same results as those observed above (Fig. 5C). Single-point mutations of these CD3γ residues suggested that T130 was not required (Fig. 5D). Furthermore, mutation of the S126 residue, which is phosphorylated after phorbol ester treatment, has little effect on TCR internalization, consistent with recent findings using CD3γ-deficient Jurkat T cells (28). Remarkably, mutation of a single lysine residue (K128) completely prevented TCR internalization (Fig. 5D). Conservative substitution...
of the lysine with arginine reduced internalization by ~30%, inferring preference for lysine and an absolute requirement for a positively charged amino acid. This was a surprising finding, because this residue had not previously been shown to be required for AP-2 binding and TCR internalization. Previous studies had shown a strong requirement for a negatively charged residue (D or E) four amino acids N-terminal to the dileucine residues (8). This was confirmed by the complete abrogation of TCR internalization by mutation of D127 (Fig. 5D). It is unclear whether D127 and/or K128 are directly or indirectly involved in AP-2 binding. However, it is clear that the lysine residue is an essential part of the CD3γ dileucine-based internalization signal. Furthermore, it is clearly essential, because the K128 residue is not present in CD3δ, whereas the negatively charged residue (D/E) at 127 is present in both CD3γ and CD3δ. Interestingly, all but one of the constructs (CD3γ,T130A) described in this study and above gave TCR surface expression levels comparable to or above wild-type TCR levels and were inversely proportional to their effects on internalization (i.e., if TCR internalization was reduced, expression was increased; Fig. 6). For reasons that are not clear, this correlation was only observed with dileucine-based signal mutants.

Lastly, we questioned whether this lysine residue was responsible for the inability of CD3γ, D11,L,γ to replace CD3γ,WT in mediating TCR internalization. Although mutation of K128 in CD3γ completely prevented TCR internalization, the lack of this lysine residue in CD3δ is not sufficient, as demonstrated with TCR expressing CD3γ, D127,V128K,γ (Fig. 5E). This suggests that additional residues and/or structural constraints are required. Taken together, these data suggest that there is significant rigidity in the requirements for AP-2-mediated internalization of the TCR via the dileucine-based motif.

**Discussion**

The TCR:CD3 complex has 20 YxxØ motifs and two (D/E)xllx(L/I) motifs. In this study we have defined which motifs constitute signals that are used by AP-2 to mediate TCR internalization. In so doing, we have also examined the general principle that AP-2 may use to mediate the internalization of large, multichain transmembrane complexes. Contrary to previous expectations (11), our studies have clearly shown that AP-2 requires both tyrosine- and dileucine-based signals to mediate TCR internalization and revealed both plasticity and rigidity in the engagement of these signals. It should be noted that these experiments were performed in 293T cells, and it remains to be determined whether the same requirements for TCR internalization will apply to lymphoid cells. However, we have previously shown that the TCR is internalized and recycled in a manner comparable to that in normal T cells (24), and others have shown that TCR internalization is mediated by a general biological process that uses basic cell machinery (i.e., AP-2, clathrin, etc.) that is present in all cells (14).

There appears to be significant plasticity in the use of YxxØ motifs by AP-2, which is consistent with the broad specificity of AP-2µ (3). The YxxØ sequences within CD3γ, CD3δ, and, in particular, CD3δ, appear to mediate TCR internalization. Generally, there was a good correlation between data obtained from the yeast two-hybrid AP-2µ binding assay and the 293T TCR internalization assay. In both instances, the CD3δ YxxØ signals were clearly the most efficient. The only difference was our observation that CD3γ tyrosines alone could mediate some TCR internalization in 293T cells, but we could not detect any binding to AP-2µ. This could be due to an affinity that is below the threshold of this assay. Our use of the entire CD3 cytoplasmic domains, rather than tandemized repeats of the YxxØ motifs, may also yield different results (14). Alternatively, this difference could simply be due to the duplication of CD3γ in the TCR complex.

Our studies suggest that there is a definable hierarchy in the ability of different YxxØ signals to mediate TCR internalization: δY2 >> δY1 ≈ γY1 ≈ γY1−6 ≈ γY2 = εY1/2. The proposed binding motif for AP-2µ suggests a strong preference for arginine (R) or proline (P) at Y + 2 and a preference for leucine (L) rather than isoleucine (I) at Y + 3 (Fig. 7) (40). This is consistent with δY2, δY1, and γY1 being particularly favored and may explain why εY1 is not used.

Inherent in this plasticity was marked redundancy in the use of different YxxØ signals. Remarkably, ~75% of wild-type TCR internalization was mediated by a single tyrosine residue in CD3δ of the 20 in the TCR:CD3 complex. However, mutation of this residue only slightly reduces TCR internalization. This infers that AP-2 only requires a single, optimal YxxØ signal to efficiently mediate TCR internalization, but can use several YxxØ signals in the TCR:CD3 complex.

In stark contrast, use of the dileucine-based motif exhibited remarkable rigidity. Even though both CD3γ and CD3δ have (D/E)xllx(L/I) motifs, TCR internalization is mediated by the
former, but not the latter. AP-2 has been shown to bind to a variety of sequences, including some that do not entirely conform to this motif (3). Thus, it is particularly surprising that for TCR internalization via this motif, AP-2 should exhibit such rigidity. The majority of data concerning this motif has come from its requirement for protein kinase C-mediated TCR internalization (8, 29, 39, 41, 42). In this instance, phosphorylation of the unique CD3γ S126 defines this specificity. However, for constitutive TCR internalization, we and others have clearly shown that this residue is not required (28). Our study clearly showed that the aspartic acid (D127) and the two leucine residues (L131/L132) in CD3γ were required for TCR internalization, consistent with previous studies (8, 43).

So what is the basis for use of the (D/E)xxL(L/I) motif in CD3γ, but not in CD3δ? Our studies have shown that this specificity is manifest at two levels. First, the position of this motif within the complex appears to be critical, because the transposition of the CD3γ (D/E)xxL(L/I) motif into CD3δ, in the presence of an inactive CD3γ mutant, abrogated TCR internalization. Second, there is an absolute dependency on the highly conserved, CD3γ-specific lysine at LL-3 (K128). We have shown that a single mutation of either K128 or D127 completely abrogates TCR internalization. However, the latter can be efficiently substituted for by glutamic acid, which is the residue in the analogous position in CD3δ. We have shown that a single mutation of either K128 or D127 completely abrogates TCR internalization. In this instance, phosphorylation of the unique CD3γ S126 defines this specificity. However, for constitutive TCR internalization, we and others have clearly shown that this residue is not required (28). Our study clearly showed that the aspartic acid (D127) and the two leucine residues (L131/L132) in CD3γ were required for TCR internalization, consistent with previous studies (8, 43).

In conclusion, we have shown that AP-2-mediated internalization of the TCR:CD3 complex requires both YxxØ and (D/E)xxL(L/I) signals. Indeed, previous studies with TAC/IL-2:CD3 chimeras had suggested that the dileucine- and tyrosine-based motifs act independently to mediate endocytosis (11). However, our data clearly show that both signals are required for AP-2-mediated internalization of the intact, cell surface TCR:CD3 complex. Why is this necessary, and how might the contrasting features of these signals, detailed above, assist in this process? One possibility is that the large size of the TCR complex necessitates the use of two distinct signals. Indeed, it has been shown that their binding sites do not overlap, inferring that AP-2 could bind both simultaneously (13). It seems unlikely that the requirement for both signals is due to their weak affinity per se, because comparable AP-2α interaction was observed with the cytoplasmic tails of CD3δ and CTLA-4, which is known to mediate rapid internalization. Furthermore, we have seen no additive effect of combining the CD3γ LL-3 and CD3δ Y-F mutants. It is also possible that with such a large complex, there is some steric hindrance in the ability of AP-2 to effectively associate with these signals. Thus, it is possible that binding to one signal induces a conformational change that facilitates interaction with the other.

If there is rigidity in AP-2 interaction with the CD3γ (D/E)xxL(L/I) signal, how might plasticity in interaction with YxxØ signals be accommodated? The length and flexibility of the large β subunit hinge coupled with conformational changes that occur after AP-2 phosphorylation imply that μ2 can orientate in any direction with respect to the rest of the AP-2 complex through its flexible linker, perhaps facilitating binding to different YxxØ signals (44–46). Our data suggested that the position of the CD3γ (D/E)xxL(L/I) signal in relation to the rest of the TCR:CD3 complex is critical. We were intrigued to find that transposition of the CD3γ (D/E)xxL(L/I) motif into CD3δ resulted in a 30% reduction in TCR internalization in the presence of wild-type CD3γ. These data infer that this mutant may act as a dominant negative, facilitating AP-2 association, but preventing TCR internalization. This suggests that the orientation of AP-2 docking with the TCR:CD3 complex may be important. Whether this relates to the position of preferred YxxØ signals (e.g., in CD3δ rather than CD3γ) or some other factor remains to be determined. We also cannot rule out the possibility that other, as yet undefined, motifs in the TCR:CD3 complex may be contributing to this process.

In conclusion, we have shown that AP-2-mediated internalization of the TCR:CD3 complex requires both YxxØ and (D/E)xxL(L/I) signals, a requirement not normally afforded to simple transmembrane receptors. Of particular note was our finding that a highly conserved lysine residue in CD3γ was essential for TCR internalization. Although this residue was crucial for TCR internalization, it was not found in all dileucine signals (~80% of dileucine signals had D/E at LL-4, whereas ~45% have K/R at LL-3) (3), suggesting that its use may be restricted to certain types of receptors. The identification of these signals will allow us to manipulate TCR internalization and determine the physiological importance of this process in T cell function, serial ligation, and TCR down-modulation (24, 26). Furthermore, there appears to be both plasticity and rigidity in the use of the YxxØ and (D/E)xxL(L/I) signals, respectively. This may highlight the difficulties associated with moving large multichain complexes in excess of 200,000 Da. In this instance, the AP-2 complex would need to effectively mediate internalization and thus would require multiple, but distinct, motifs while retaining the flexibility to internalize a variety of large cell surface complexes. It remains to be seen whether these observations form a general principle by which AP-2 and perhaps other adaptor proteins mediate the transport of large, multichain transmembrane complexes.

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Disclosures

The authors have no financial conflict of interest.

References