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J Immunol 1999; 163:25-31; ;
http://www.jimmunol.org/content/163/1/25

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The CD3ε Subunit of the TCR Contains Endocytosis Signals

Aldo Borroto,* Juan Lama,*† Florence Niedergang, † Alice Dautry-Varsat, † Balbino Alarcón,* and Andrés Alcover‡†

Ligand binding to TCR induces its internalization and cell surface down-modulation. These phenomena contribute to the extinction of activation signals. Due to the multicomponent nature of the TCR-CD3 complex, its internalization may be mediated by one or several of its subunits. Although it has been reported that CD3γ and CD3δ contain endocytosis motifs involved in the internalization of the TCR-CD3 complex, other subunits could also be involved in this process. For instance, CD3ε and CDζ display amino acid sequences reminiscent of internalization motifs. To investigate whether CD3ε bears endocytosis signals, we have analyzed the internalization capacity of a panel of deletion and point mutants of CD3ε that were expressed on the cell surface independently of other TCR-CD3 subunits. Here we report that CD3ε displays endocytosis determinants. These data indicate that CD3ε could contribute to the internalization and cell surface down-regulation of TCR-CD3 complexes. Moreover, the existence of endocytosis signals in this polypeptide could serve to retrieve unassembled CD3ε subunits or partial CD3 complexes from the plasma membrane, thus restricting the expression on the cell surface to fully functional TCR-CD3 complexes.

have escaped retention in the endoplasmic reticulum (ER)\(^4\) and reached the cell surface independently of a fully formed TCR-CD3 complex.

**Materials and Methods**

**Reagents**

Chemicals were from Sigma Chemical (St. Louis, MO). \(^{125}\)I was from Amersham-Pharmacia Biotech (Piscataway, NJ). Human transferrin (Sigma Chemical) was loaded with iron and coupled to lissamine rhodamine (Eastman Kodak Co., Rochester, NY) or to fluorescein isothiocyanate (Molecular Probes, Eugene, OR) as previously described (17, 18). The mouse anti-CD3e mAb (SP34, IgG3) has been previously described (19). F(ab\(^9\))\(_2\) fluorescein-labeled rabbit anti-mouse Ig was from Dako (Glostrup, Denmark), and F(ab\(^9\))\(_2\) PE-labeled goat anti-mouse Ig Fc was from Immunotech (Marseille, France). The antisera to rat growth hormone (GH) for immunocytochemistry (lot AFP4115) was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health, Bethesda, MD). The mouse mAb against human CD8a (OKT8, IgG2a) was from Ortho Diagnostic Systems (Raritan, NJ).

**DNA constructs**

All chimeras and mutations were constructed by PCR using Taq DNA polymerase (New England Biolabs, Beverly, MA) essentially as previously described (20, 21). Human CD8 and CD3e cDNAs were used as templates, with appropriate oligonucleotides designed to introduce the amino acid changes. All of the PCR products were digested with XhoI and BamHI and cloned in the pSR expression vector (22). The constructs were all checked by complete DNA sequencing.

**Cell lines and transfections**

COS cells were grown in DMEM containing 4 g/L glucose and 10% FCS. COS cells were transfected by electroporation. Routinely, one 100-mm plate of cells grown to subconfluence was used for each transfection. Cells were resuspended in 200 \(\mu L\) of DMEM supplemented with 10% FCS and 10 nM HEPES buffer (pH 7.2) and gently mixed with the DNA transfection mixture (6 \(\mu g\) of pSR-CD3e vector, 17 \(\mu g\) of pSK plasmid used as carrier DNA, and 5 \(\mu L\) of 1.5 M NaCl) and then electroporated in 0.4-cm cuvettes at 200 V, 900 \(\mu F\), using an EasycjetT apparatus (Eurogentec, Seraing, Belgium). The cells were carefully resuspended by pipetting and plated in a 100-mm petri dish. After 24 h of culture, the cells were transferred to 24-well plates. Rat basophilic leukemia (RBL) cells were grown in RPMI 1640 supplemented with 10% FCS and 10 nM HEPES buffer (pH 7.2). RBL cells were transfected with 20 \(\mu g\) of pSR-CD3e (R183S) by electroporation at 260 V, 900 \(\mu F\). Stably transfected cells were selected in 1 mg/ml G418 (Life Technologies, Paisley, U.K.). Positive clones were selected by immunofluorescence and flow cytometry using the anti-CD3 mAb (SP34, IgG3) has been previously described (19). F(ab\(^9\))\(_2\) fluorescein-labeled rabbit anti-mouse Ig was from Dako (Glostrup, Denmark), and F(ab\(^9\))\(_2\) PE-labeled goat anti-mouse Ig Fc was from Immunotech (Marseille, France). The antisera to rat growth hormone (GH) for immunocytochemistry (lot AFP4115) was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health, Bethesda, MD). The mouse mAb against human CD8a (OKT8, IgG2a) was from Ortho Diagnostic Systems (Raritan, NJ).

**Immunofluorescence labeling, flow cytometry, and confocal microscopy**

Experiments were conducted essentially as previously described (8). Fluorochrome-labeled transferrin was used at 150 nM.

**Internalization of radiolabeled anti-CD3e mAb**

Experiments were conducted 48 h after transfection on COS cells growing in 24-well plates. Cells were cooled on ice for 3 min and washed once at 4°C with DMEM supplemented with 10% FCS and 10 mM HEPES buffer (pH 7.2). The medium was thoroughly removed, and 75 \(\mu L\) of the same cold medium containing \(^{125}\)I-labeled anti-CD3e mAb (SP34) at a final concentration of 40 nM was added per well. Cells were incubated for 30 min at 4°C to allow binding of the Ab and then at 37°C for the appropriate times to allow internalization. At the end of each time point, cells were washed three times at 4°C with DMEM. The remaining surface-bound Ab was then removed by two successive acid washes (8 min with 800 \(\mu L\) of DMEM containing 25 mM sodium acetate, pH 1.8). At the end, the cells were lysed in 400 \(\mu L\) of 0.1 M NaOH solution and the wells washed with 800 \(\mu L\) of distilled water. Acid treatment removed the surface-bound Ab with an efficiency of 80–95%. Nonspecific internalization was determined using cells incubated in the presence of a 10-fold excess of unlabeled Ab or using the same amount of mock-transfected COS cells. Both techniques gave similar results. Data were corrected taking into account nonspecific internalization values as well as the efficiency of the acid wash, and the percentage of internalized receptors was calculated as previously described (24).

**Down-regulation experiments**

Cell surface expression of CD3e was measured by immunofluorescence and flow cytometry at various times after binding of soluble or plastic-bound Abs as previously described (25).

**Results**

**CD3e is internalized in the absence of the other TCR-CD3 subunits**

To analyze the capacity of CD3e to be internalized, this chain was expressed independently of other TCR-CD3 subunits. Isolated CD3e cannot reach the cell surface due to the presence of an ER retention signal in its cytosolic region (20, 26). We therefore expressed a CD3e form containing a mutation in its ER retention signal (R183S). As shown in Fig. 1A, this mutant was stably expressed on the cell surface of RBL cells. Immunoprecipitation experiments showed that CD3e is mainly expressed in these cells as disulfide-linked dimers, with monomeric and trimeric forms also present (data not shown). Disulfide-linked CD3e homodimers have been reported to exist in T lymphocytes, where they coexist with CD3e-\(\gamma\) and CD3e-\(\delta\) heterodimers (27). Likewise, homodimeric and trimeric forms of CD3e have been observed in COS cells coexpressing several CD3 subunits (28), as well as in vitro translated and assembled TCR-CD3 complexes (29). This suggests that the folding and self-assembly of CD3e chains in the presence or absence of other TCR-CD3 subunits are similar. Therefore, these polypeptides are suitable for investigating the presence of internalization determinants in CD3e.

To test whether CD3e bears internalization signals, cells were incubated in the presence of anti-CD3e mAb and rhodamine-labeled transferrin and then fixed, permeabilized, and stained with fluorescein-labeled secondary Abs. As shown in Fig. 1B, cells incubated at 37°C, but not those incubated at 4°C, internalized the anti-CD3e mAb, which colocalized with transferrin (Fig. 1B). Transferrin accompanies its receptor through the endocytic and recycling pathway and is therefore widely used as a marker for early endocytic organelles (30, 31). Therefore, our data indicate that CD3e is internalized and reaches endocytic intracellular compartments. Furthermore, cross-linking with a surface-bound mAb induced the down-regulation of CD3e from the cell surface, as assessed by flow cytometry (Fig. 1C). Altogether, these data show that CD3e can be internalized in the absence of other components of the TCR-CD3 complex, thus indicating that this subunit displays internalization sequences.

**CD3e contains endocytosis sequences in its cytosolic region**

To localize putative endocytosis sequences in the cytosolic tail of CD3e, a panel of mutants encompassing the whole cytosolic region was generated (Fig. 2A). All of these mutants were expressed on the cell surface of transfected COS cells at comparable levels (Fig. 2B). The capacity of these mutants to be internalized was then measured using a \(^{125}\)I-labeled anti-CD3e mAb. Consistent with the results shown in Fig. 1, three CD3e mutants containing single-residue changes in the ER retention sequence (L180S, R183K, and R183S) were internalized equally well (Fig. 3A). A panel of truncated CD3e molecules was then tested. As shown in Fig. 3B, the

\(^4\) Abbreviations used in this paper: ER, endoplasmic reticulum; ITAM, immunoreceptor tyrosine-based activation motif; GH, growth hormone; RBL, rat basophilic leukemia.
deletion of residues 181–185 (Δ1) did not inhibit CD3ε internalization, as compared with single-residue mutants described in Fig. 3A. However, a larger deletion involving residues 166–185 (Δ2) significantly reduced the capacity of CD3ε to be internalized. This suggests that some sequences contained in the 166–180 region could be involved in CD3ε internalization. Interestingly, this region contains two tyrosine-based motifs (Y166EPI and Y177SGL), which could support CD3ε internalization (12). These motifs are part of the ITAM of this subunit that is involved in signal transduction.

Although the deletion of residues 166–185 (Δ2) inhibited CD3ε internalization, the inhibition was only partial (Fig. 3B), indicating that CD3ε bears additional internalization sequences. To determine whether the cytosolic tail of CD3ε contains additional internalization sequences, the effect of additional truncations in CD3ε internalization was analyzed. Thus, CD3ε molecules lacking residues 166–185 and other residues of the cytosolic region were tested (see Fig. 2B, Δ3–Δ6). As shown in Fig. 3C, none of the additional deletions of the CD3ε cytosolic tail had a further inhibitory effect on the internalization capacity of the Δ2 mutant.

Altogether, these data indicate that no other endocytosis sequences were present in the cytosolic tail of CD3ε, besides the endocytosis signals present between amino acids 166 and 180. These results suggest that CD3ε could also contain endocytic signals outside the cytosolic region.

Mutants of CD3ε lacking tyrosine residues of the cytosolic region are internalized

The region between residues 166 and 180 contains two consensus tyrosine-based motifs (Y166EPI and Y177SGL) that could support CD3ε internalization (12). To investigate whether any of these motifs is actually a tyrosine-based internalization signal for CD3ε, we replaced both tyrosine residues by serine. These amino acid substitutions were shown to abolish internalization of other receptors containing tyrosine-based endocytic signals (11). As shown in Fig. 3D, the substitution of both tyrosine residues by serine did not inhibit CD3ε internalization. These results indicate that the two tyrosine-based sequences of CD3ε do not behave as bona fide tyrosine-based internalization signals for this molecule. Nevertheless, the deletion analysis described above strongly suggests that this region of the molecule is involved in its endocytosis.

The endocytosis signals of CD3ε can be transferred to another protein

A further proof for the presence of endocytic signals in a receptor can be obtained by analyzing whether the graft of the region containing the putative endocytic sequence confers to another protein the capacity to be endocytosed. Most of the previously described endocytosis motifs have been localized in the cytosolic region of receptors (12), although several reports have shown that transmembrane regions could also support receptor internalization (32–37). Therefore, we generated two chimeric molecules in which the extracellular region of CD3ε was replaced by the rat GH. These chimeras contained either the transmembrane region alone, or both the transmembrane and the cytosolic region of CD3ε (Fig. 4A). The capacity of these chimeras to be internalized was then analyzed in transiently transfected COS cells using anti-GH Abs. As shown in Fig. 4B, both chimeras were internalized, as revealed by down-regulation experiments, although the one lacking the cytosolic region of CD3ε was less efficiently down-regulated. Moreover, both chimeras were found in intracellular vesicular compartments, as assessed by immunofluorescence and confocal microscopy (Fig. 5). Double labeling microscopy using rhodamine-coupled secondary Abs to detect the GH chimera and fluorescein-coupled transferrin showed colocalization between GH/CD3ε chimera and transferrin labeling (Fig. 5), indicating that the chimeras reached the endocytic organelles. This vesicular staining was not observed in cells incubated with irrelevant Abs, implying that the entry of the anti-GH Ab was mediated by the GH/CD3ε chimeras and not by fluid-phase endocytosis (data not shown). These data provide additional proof for the presence of endocytic signals in CD3ε and indicate that the transmembrane region of CD3ε could also contribute to its internalization.

The internalization signals of CD3ε are functional in T lymphocytes

Because all of the data described above were obtained in non-T cells, it was necessary to verify whether the internalization signals...
borne by isolated CD3ε molecules are also functional in T lymphocytes. To this end, the same cDNA constructs mutated in the ER retention signal described above were transfected in Jurkat T cells lacking TCR-CD3 cell surface expression. However, CD3ε constructs carrying the extracellular and transmembrane regions did not reach the cell surface. This is likely due to its association with other CD3 subunits, which are expressed intracellularly in these cells. This clone lacks TCRβ expression but expresses intracellularly all of the other subunits of the complex (23). Although the CD3ε constructs transfected lack the ER retention signal, association of these mutants with other CD3 subunits in the ER likely leads to the retention of the complexes via the ER retention signals of other subunits (4). Because the assembly into complexes of TCR-CD3 subunits involves mainly the extracellular and transmembrane regions (4, 28), we made a construct in which the extracellular and transmembrane regions of CD3ε were substituted by those of CD8α. This chimera was readily expressed in TCR-CD3-negative cells. Moreover, it was internalized as efficiently as CD3ε molecules expressed in non-T cells, as assessed by down-modulation experiments (Fig. 6), thus indicating that CD3ε carries internalization determinants that are functional in T lymphocytes.

Discussion

In this report we show that CD3ε bears endocytosis signals that could contribute to the internalization and cell surface down-regulation of TCR-CD3 complexes.

Previously reported data indicated that internalization of TCR-CD3 complexes may involve determinants from one or several of its subunits, depending on the kind of stimulus inducing internalization. For instance, phorbol ester-induced TCR-CD3 internalization depends on the presence of a di-leucine-based motif located in the cytosolic region of CD3γ (13, 14). In contrast, TCR-CD3 internalization induced by superantigens or mAbs occurs even in the absence of the entire cytosolic region of CD3γ (9, 15, 16). Moreover, TCR-CD3 internalization induced by an anti-CD3 mAb can take place in the absence of the cytosolic region of either CD3γ or CD3δ, but it is blocked when both of these cytosolic regions are

**FIGURE 2.** Cell surface expression of a panel of CD3ε mutants transiently expressed in transfected COS cells. A, Schematic representation of mutations and deletions performed in the cytosolic region of CD3ε. The amino acid sequence of the entire cytosolic region of CD3ε is shown in single-letter code. Point mutations are underlined by stars. Deletions are shown by linesinterrupting the sequence. B, Expression of CD3ε on the cell surface was measured 48 h after transfection by immunofluorescence and flow cytometry. Staining was performed with anti-CD3ε mAb, SP34, and fluorescein-coupled secondary Abs. The figure shows a representative experiment of three independent experiments conducted.
deleted (15). However, the absence of the cytosolic regions of CD3δ and CD3γ still allows TCR-CD3 internalization induced by peptide Ag. Therefore, under physiologic conditions, TCR-CD3 internalization likely involves determinants present in TCR-CD3 subunits other than CD3γ and CD3δ. The data we present here demonstrate that CD3ε itself bears endocytosis determinants and could therefore support the internalization of TCR-CD3 complexes.

Unlike many other receptors that display only one strong internalization signal in their cytosolic regions (11, 12), our data indicate that CD3ε internalization is determined by the additive effect of determinants located in the cytosolic and transmembrane regions of the molecule. One of these is located between amino acids 166 and 180 (Fig. 3). This determinant could be formed by the tyrosine-based motifs (YxxI/L) that constitute the ITAM of CD3ε. However, these sequences do not seem to behave as typical tyrosine-based motifs described for other receptors (11), because the mutations of both tyrosine residues to serine did not alter CD3ε internalization. Rather, this sequence seems to behave as a weak internalization signal, such as those found in the IL-2 receptor β and γ subunits (35, 37). The tyrosine-based motifs within the ITAM of other receptors have been shown to be involved in internalization. However, their particular features were different depending on the receptor and on the ligand inducing internalization. For instance, tyrosine-based motifs of the ITAMs of CD3γ and CD3δ, together with a di-leucine motif, mediate the internalization of chimeric molecules containing the cytosolic tail of these CD3 chains (38). Moreover, both tyrosine residues of the ITAM of FcγRIII were shown to be required for its internalization, because the mutation of each tyrosine residue to serine or valine abolished multimeric receptor internalization (39). In contrast, although one of the tyrosine-based motifs of the ITAM of Igα is required for B cell receptor constitutive internalization, this mutation does not impair the internalization induced by multivalent ligands (40).

Sequential deletions of the CD3ε cytosolic tail between the transmembrane region and aa 166 (Fig. 2A, Δ3–Δ6) did not inhibit the internalization capacity of the 166–185 (Δ2) deletion mutant (Fig. 3C). This suggests that, within the cytosolic tail of CD3ε, only the region between amino acids 166 and 180 contains endocytosis signals and that the other internalization determinants could be part of the transmembrane region. In support of this, a chimera containing only the transmembrane region of CD3ε fused to the rat GH was internalized, although less efficiently than a chimera displaying both the transmembrane and cytosolic regions.
of CD3ε (Figs. 4 and 5). The presence of signals for sorting in the endocytic pathway in both the cytosolic and transmembrane regions has been reported for other membrane proteins (32–37), although a transmembrane endocytic motif had not been described. Our data demonstrate that CD3ε has the capacity to be internalized, thus suggesting that this subunit could contribute to TCR-CD3 complex internalization. Moreover, considering, on the one hand, that CD3γ and CD3δ also carry internalization signals (14, 15, 38) and, on the other hand, that CD3γ and CD3δ are not the only subunits involved in TCR-CD3 internalization, it is likely that TCR-CD3 complex internalization utilizes endocytic signals from all CD3 subunits. This would not be surprising, because all CD3 subunits are involved in TCR signal transduction.

Nevertheless, from our analysis, we cannot determine whether all or only some of the CD3ε endocytic determinants shown here are involved in the internalization of the TCR-CD3 complex. In fact, some of these determinants may not be accessible to the endocytic machinery when this subunit is part of a complete TCR-CD3 complex. In this case, endocytosis signals exposed only in isolated CD3ε subunits could serve to retrieve CD3ε subunits or partial CD3 complexes that would have escaped the ER retention barrier and reached the plasma membrane independently of fully assembled TCR-CD3 complexes. In this regard, it is worth noting that only low amounts of CD3ε associated with CD3γ or CD3δ were found expressed on the surface of certain thymocytes as well as on immature CD4−CD8−thymocytes but not in mature T cells (41–43). The removal of isolated CD3ε molecules from the plasma membrane may represent an additional control mechanism to prevent the expression of unassembled CD3ε subunits on the cell surface.

Acknowledgments

We thank Dr. D. Ojcius for critical reading of the manuscript.

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