T Cell Receptor ζ Allows Stable Expression of Receptors Containing the CD3γ Leucine-based Receptor-sorting Motif*

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Jes Dietrich‡ and Carsten Geisler§

From the Institute of Medical Microbiology and Immunology, University of Copenhagen, The Panum Institute, DK-2200 Copenhagen, Denmark

The leucine-based motif in the T cell receptor (TCR) subunit CD3γ constitutes a strong internalization signal. In fully assembled TCR this motif is inactive unless phosphorylated. In contrast, the motif is constitutively active in CD4/CD3γ and Tac/CD3γ chimeras independently of phosphorylation and leads to rapid internalization and sorting of these chimeras to lysosomal degradation. Because the TCRζ chain rescues incomplete TCR complexes from lysosomal degradation and allows stable surface expression of fully assembled TCR, we addressed the question whether TCRζ has the potential to mask the CD3γ leucine-based motif. By studying CD4/CD3γ and CD16/CD3γ chimeras, we found that CD16/CD3γ chimeras associated with TCRζ. The CD16/CD3γ–TCRζ complexes were stably expressed at the cell surface and had a low spontaneous internalization rate, indicating that the leucine-based motif in these complexes was inactive. In contrast, the CD4/CD3γ chimeras did not associate with TCRζ and the leucine-based motif in these chimeras was constitutively active resulting in a high spontaneous internalization rate and low expression of the chimeras at the cell surface. Thus, our data demonstrate that TCRζ allows stable cell surface expression of receptors containing CD3γ leucine-based motifs by its potential to mask such motifs.

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§ Member of The Biotechnology Center for Cellular Communication.

The T cell receptor (TCR) is a multimeric receptor composed of the ligand binding Tiaβ dimer and the signal transducing subunits CD3γ and CD3δε and the TCRζζ dimer (1, 2). Only completely assembled octameric TiaβCD3γδεζζ complexes are efficiently expressed at the cell surface of mature T cells to ensure proper TCR functions. Thus, very selective mechanisms that only allow expression of completely assembled and functional TCR must exist in T cells. Several studies have indicated that the TCRζ chain plays important roles in such mechanisms. Thus, the TCR is not or is only very weakly expressed at the cell surface of T cells from TCRζ knockout mice and in the TCRζ-deficient T cell variant MA 5.8 (3–6). In the MA 5.8 variant, hexameric TiaβCD3γδεζζ complexes are assembled in the endoplasmic reticulum in the absence of TCRζ and subsequently transported via the Golgi apparatus to the lysosomes for degradation (6). Furthermore, association of TCRζ to the rest of the TCR seems to be critically dependent on the assembly of hexameric TiaβCD3γδεζζ complexes, and TCRζ does not associate with partial TCR complexes in T cell variants lacking Tia, β, or CD3γ (7–10). Thus, the selective expression of only completely assembled TCR at the T cell surface seems to be ensured by the TCRζ chain (11, 12). However, it still remains to be explained how the TCRζ chain redirects the sorting of incomplete TCR from a degradative pathway to the cell surface.

The observation that incompletely assembled TCR complexes are sorted to a degradative compartment and not expressed at the cell surface suggests that receptor-sorting motifs with the capacity to sort receptors to the lysosomes must be active in incomplete TCR (6). We and others have recently described a leucine-based (L-based) receptor-sorting motif (SΔ29DKQTLLΔ132) in the cytoplasmic tail of CD3γ (13–15). When active, the L-based motif is recognized and bound by clathrin-coated vesicle adaptor proteins either at the trans-Golgi network or at the plasma membrane (15–17). This leads to sorting of receptors to the lysosomes and to rapid receptor internalization, respectively. In completely assembled TCR, the CD3γ L-based motif is inactive and not accessible for adaptor proteins unless phosphorylated (14). In contrast, in chimeric Tac/CD3γ and CD4/CD3γ molecules, the motif is constitutively active independently of phosphorylation, and like hexameric TiaβCD3γδεζζ complexes, these chimeras are rapidly transported to the lysosomes for degradation (6, 13, 15). From these observations it may be suggested that the CD3γ L-based motif is active in incompletely assembled TCR and that TCRζ allows cell surface expression of completely assembled TiaβCD3γδεζζ complexes by masking this motif. In this study, we addressed the question whether TCRζ has the potential to mask the CD3γ leucine-based motif.

By analyzing receptor expression and sorting of mutated TCR and chimeric CD4/CD3γ and CD16/CD3γ molecules, we found that similar to the TCR, the CD16/CD3γ chimera was stably expressed at the cell surface in association with TCRζ. Furthermore, both the TCR and the CD16/CD3γ–TCRζ complexes had low spontaneous internalization rates indicating that the CD3γ L-based motif in these multimeric complexes was inactive. As is true for the TCR, the CD3γ L-based motif in the CD16/CD3γ–TCRζ complexes was activated following protein kinase C (PKC) activation, which resulted in a rapid internalization of the complexes from the cell surface. In contrast, the CD4/CD3γ chimera did not associate with the TCRζ chain and the CD3γ L-based motif in these chimeras was constitutively active resulting in a high spontaneous internalization rate and low cell surface expression of the chimera. Thus, our data demonstrate that the TCRζ chain allows stable...
cell surface expression of receptors containing a CD3\gamma L-based motif most probably by masking this motif.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**JGN, a TCR cell surface negative variant of the human T cell line Jurkat that synthesizes no CD3\gamma, was produced in our own laboratory (10). Cells were cultured in RPMI 1640 medium supplemented with penicillin 2 \times 10^5 units/liter (Leo Pharmaceutical Products, Ballerup, Denmark), streptomycin 50 mg/liter (Merck, Darmstadt, Germany), and 10% (v/v) FCS (Life Technologies, Paisley, UK) at 37 °C in 5% CO\(_2\), Phycocerythrin (PE)-conjugated and purified mouse anti-human CD3e (UCHT1), rat anti-mouse CD4 (L3T4), and mouse anti-human CD16 (3G8) monoclonal antibodies (mAb) were from Pharmingen (San Diego, CA). Mouse anti-human TCR\gamma mAb (6B10.2) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-rat immunoglobulin and peroxidase-conjugated rabbit anti-mouse immunoglobulin were from Dakopatts A/S (Glostrup, Denmark). The phorbol-ester phorbol 12,13-dibutyrate (PDB) was from Sigma.

**Constructions, Transfection, and Western Blotting—**The truncated CD3\gamma and the chimeric CD4/CD3\gamma and CD16/CD3\gamma molecules were constructed as described previously (14, 15, 18) by polymerase chain reaction using the plasmids pJ6T3\gamma-2 (19), pCD-L3T4.25 (20), or FcRIII-2 (21) as templates. Mutations were confirmed by DNA sequencing. Transfections were performed using the Bio-Rad Gene Pulser at a setting of 270 V and 960 microfarad with 40 μg of plasmid \(2 \times 10^7\) cells. After 3–4 weeks of selection, G418-resistant clones were expanded and maintained in medium without G418. Western blotting was performed as described previously (22).

**Receptor Internalization and Recycling—**To determine the spontaneous internalization rates, cells were incubated in RPMI 1640 + 10% FCS at a cell density of \(2 \times 10^5\) cells/ml at 37 °C or 4 °C with PE-conjugated anti-CD3e, anti-CD16, or anti-CD4 mAb. At the time indicated, aliquots of cell suspension were washed in ice-cold RPMI 1640 + 10% FCS, divided in two equal parts, and subsequently treated with 300 μg/ml NaCl, 0.5 M acetic acid, pH 2.2, for 10 s or left untreated. The fluorescence of the cells was measured by flow cytometry in a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The percentage of internalized mAb to cell surface bound mAb was subsequently calculated using the equation: (MFI of phorbol ester treated cells) divided by (MFI of untreated cells). The MFI was recorded and used in the calculation of percent mAb internalization. For PKC-induced receptor internalization, cells were adjusted to \(2 \times 10^5\) cells/ml medium (RPMI 1640 + 10% FCS) and incubated at 37 °C with various concentrations of the phorbol ester PDB. At the time indicated, cells were transferred to ice-cold PBS containing 2% FCS and 0.1% NaN\(_3\), and washed twice. The cells were stained directly with PE-conjugated anti-CD3e or anti-CD16 mAb and analyzed by flow cytometry. MFI was recorded and used in the calculation of percent mAb binding. For PKC-induced internalization, the MFI of untreated cells was divided by (MFI of untreated cells) \(\times 100\%\).

**RESULTS AND DISCUSSION**

The CD16/CD3\gamma Chimera Is Stably Expressed at the Cell Surface in Association with TCR\gamma—We and others have previously demonstrated that the CD3\gamma L-based motif is constitutively active in chimeric CD4/CD3\gamma and Tac/CD3\gamma molecules independently of phosphorylation (13, 15). The active L-based motif results in very low expression of these chimeras at the cell surface. To determine whether the TCR\gamma chain had the potential to mask the CD3\gamma L-based motif and thereby allow stable receptor cell surface expression, we took advantage of the observation that the Fc receptor FcRIIIA-α chain (CD16) is only expressed on the cell surface in association with TCR\gamma (23). In contrast to CD16, CD4 is expressed as a monomer at the cell surface (24). By comparing the chimeric CD4/CD3\gamma and CD16/CD3\gamma molecules either with or without an intact CD3\gamma L-based motif, this enabled us specifically to examine if and how the TCR\gamma chain influenced the activity of the CD3\gamma L-based motif. Six different constructs were made. CD3\gamma-tS126 and CD3\gamma-tP133 coded for the CD3\gamma chain with a truncated cytoplasmic tail immediately before and after the L-based motif, respectively. CD4/CD3\gamma-tS126 and CD4/CD3\gamma-tP133 coded for chimeric molecules composed of the extracellular and transmembrane domains of CD4 and the cytoplasmic tail of CD3\gamma truncated immediately before and after the L-based motif, respectively. CD16/CD3\gamma-tS126 and CD16/CD3\gamma-tP133 coded for chimeric molecules composed of the extracellular and transmembrane domains of CD16 and the cytoplasmic tail of CD3\gamma truncated immediately before and after the L-based motif, respectively (Fig. 1A). These constructs were separately transfected into the CD3\gamma negative Jurkat variant JGN (10) and G418-resistant transfected clones were analyzed for cell surface expression of the transfected molecules by FACS analysis. As shown in Fig. 1B, the TCR and the CD16/CD3\gamma chimera were all highly expressed at the cell surface independent of the presence or absence of the CD3\gamma L-based motif.
motif. In agreement with previous studies, the CD4/CD3γ-tS126 chimera was highly expressed, whereas the CD4/CD3γ-tP133 chimera with an active CD3γ L-based motif was only weakly expressed at the cell surface although highly expressed intracellularly (Fig. 1B and data not shown) (15). To analyze whether the chimeras actually associated with TCRζ, cells were lysed in digitonin lysis buffer and immunoprecipitated with either anti-CD4 or anti-CD16 mAb. The precipitates were resolved by SDS-polyacrylamide gel electrophoresis, and Western blot analysis was performed using the anti-TCR mAb. As shown in Fig. 1C, TCRζ clearly co-precipitated with the CD16/CD3γ chimeras but did not co-precipitate with the CD4/CD3γ chimeras.

These experiments demonstrated that in the absence of the CD3γ L-based motif both the TCR, the CD4/CD3γζ, and the CD16/CD3γ chimeras were highly expressed at the cell surface independent of their capacity to associate with TCRζ. In contrast, in the presence of an intact CD3γ L-based motif only molecules capable of forming association with TCRζ were expressed at the cell surface. Because the CD4/CD3γ-tP133 and CD16/CD3γ-tP133 chimeras had identical cytoplasmic tails with an intact CD3γ L-based motif and only differed intracellularly by their ability to associate with the TCRζ chain, these observations suggested that TCRζ masked and thereby inactivated the CD3γ L-based motif of the CD16/CD3γ-tP133 chimera. The CD3γ L-based Motif Is Inactive in CD16/CD3γ-TCRζ Complexes but Can Be Activated Following PKC Activation—We have previously demonstrated that CD4/CD3γ chimeras with an inactive CD3γ L-based motif have low spontaneous internalization rates and are highly expressed at the cell surface, whereas CD4/CD3γ chimeras with an active CD3γ L-based motif have high spontaneous internalization rates and are weakly expressed at the cell surface (15). To analyze whether the CD3γ L-based motif in the CD16/CD3γ-tP133-TCRζ complex was active at the cell surface, the spontaneous internalization rate was determined. As expected, the CD4/CD3γ-tP133 chimera had a high spontaneous internalization rate reflecting the active CD3γ L-based motif present in this chimera, and the TCR-tP133 had a low spontaneous internalization rate reflecting the inactive CD3γ L-based motif in the completely assembled TCR. Like the TCR-tP133, the CD16/CD3γ-tP133-TCRζ complex had a low spontaneous internalization rate, indicating that the CD3γ L-based motif in the CD16/CD3γ-tP133-TCRζ complex was inactive (Fig. 2A). Likewise, the CD4/CD3γ-tS126 that did not contain the CD3γ L-based motif had a low spontaneous internalization rate.

Following PKC-induced phosphorylation the CD3γ L-based motif in the context of the TCR is activated. This results in an increased internalization rate and a down-regulation of the TCR from the cell surface (14, 25). To analyze whether the CD3γ L-based motif in the CD16/CD3γ-tP133-TCRζ complex could be activated following PKC activation, cells were treated with the phorbol ester PDB and subsequently analyzed for receptor expression. Interestingly, similar to the TCR-tP133, the CD16/CD3γ-tP133-TCRζ complex was down-regulated from the cell surface following PKC activation (Fig. 2B). An approximately 5-fold higher PBD concentration was required to induce down-regulation of the CD16/CD3γ-tP133-TCRζ complex as compared with the TCR, which might indicate that the CD3γ L-based motif was not as accessible for PKC in the CD16/CD3γ-tP133-TCRζ complex as in the TCR-tP133.

Taken together, these experiments demonstrated that the CD3γ L-based motif is constitutively active in monomeric CD4/CD3γ-tP133 chimeras but inactive in CD16/CD3γ-tP133-TCRζ complexes. Furthermore, as seen for the CD3γ L-based motif in the TCR, the CD3γ L-based motif in the CD16/CD3γ-tP133-TCRζ complex was activated following phosphorylation. These observations strongly indicated that the TCRζ chain allows stable cell surface expression of receptors containing CD3γ L-based motifs by masking this motif and that phosphorylation directly influences the interaction between CD3γ and TCRζ in both TCR and CD16/CD3γ-TCRζ complexes. Previous studies have demonstrated that the cytoplasmic tails of ζ, η, CD3γ, CD3δ, and CD3ε are dispensable for TCR expression (18, 26–28). Thus, neither of these chains seems to be involved in masking any potential internalization/degradation motifs in the TCR. In contrast, to our knowledge the shortest TCRζ chain that allows TCR cell surface expression previously published contained a cytoplasmic tail of 26 amino acids (29, 30). These studies support our present results. Experiments with successive truncations of the TCRζ chain are presently being performed to determine the minimal length of the TCRζ cytoplasmic tail that allows TCR expression.

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