Characterization of the Region Involved in CD3 Pairwise Interactions within the T Cell Receptor Complex

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Assembly of the six-chain T cell antigen receptor-CD3 complex takes place by pairwise interactions. Thus, CD3-ε interacts with either CD3-γ or CD3-δ, and these dimers then associate with the TCR heterodimer (αβ or γδ) and the CD3-ζ homodimer to constitute a full complex. We have now mapped the site in CD3-ε responsible for the interaction with CD3-γ and CD3-δ by analysis of a series of deletion mutants encompassing the most conserved regions. We found that the highly conserved juxtamembrane domain is mainly responsible for the interaction. Thus, deletion of this 16-amino acid extracellular sequence resulted in the inhibition of up to 95% of the CD3-ε/γ interaction. A highly conserved sequence is also present in both CD3-γ and CD3-δ, suggesting that the domain in these two chains may reciprocally be involved in the interaction with CD3-ε. Indeed, an immobilized synthetic peptide corresponding to the CD3-γ sequence specifically associated to a bacterially expressed CD3-ε protein, suggesting the 16-amino acid domain is sufficient to promote CD3-ε/CD3-γ assembly. The conservation of the motif in the CD3 chains suggests that, in addition to CD3-ε/CD3-γ and CD3-ε/CD3-δ interactions, it may also mediate homotypic interactions. Indeed, it is shown that it mediates the formation of disulfide-linked homodimers and that the formation of homo- and heterodimers are mutually excluded. Finally, this domain contains a Cys-X-X-Cys sequence that resembles that of p56lck, which is responsible for the interaction with the cytoplasmic tails of CD4 and CD8. Since the replacement of the two cysteines (Cys97 and Cys100) in CD3-ε by alanines strongly inhibited pairwise formation, the existence of a Cys-X-X-Cys motif involved in protein-protein interactions is suggested.

The T cell antigen receptor complex (TCR-CD3)1 is composed, in most mature T cells, of six subunits (TCR-α, TCR-β, CD3-γ, CD3-δ, CD3-ε, and CD3-ζ) that serve the dual function of peptide antigen/major histocompatibility complex recognition and signal transduction to the cytoplasm (for reviews, see Refs. 1–4). The TCR and CD3 components of the receptor have specialized roles in determining both functions; while antigen/major histocompatibility complex binding resides in the TCR-αβ heterodimer, the CD3 chains are able to interact with intracellular proteins involved in signal transduction. The expression of TCR-CD3 on the cell surface is regulated in mature T cells in such a way that when one subunit is absent the remaining complex is not expressed (for a review, see Ref. 4). However, the identity and the number of subunits that compose a minimal TCR-CD3 complex remains controversial. In addition to the existence of a TCR-γ-δ receptor that replaces the TCR-αβ, other alternative forms of the receptor have been found, first by identification of complexes containing an alternative splice form of CD3-ζ, CD3-η, and/or the γ-chain of the FceRI (5, 6). These two chains can form heterodimers with CD3-ζ and replace CD3-ζ partially or completely. Second, it has been suggested that the homologous chains CD3-γ and CD3-δ can also form alternative complexes and that, therefore, one can be expressed on the cell surface of T cells in the absence of the other (7).

A different TCR-CD3 complex has been revealed in immature thymocytes, where a surrogate TCR-α chain has been found associated to TCR-β and CD3 chains in thymocytes with prerearranged TCR-α (8). Complexes of CD3 subunits not associated to TCR chains have also been found in certain thymomas (9). Moreover, murine pre-T cells in RAG-1−/− mice also have been shown to contain TCR-independent CD3-γε dimers on the cell surface that can be stimulated by in vivo administration of anti-CD3 antibodies to differentiate into pre-T cells (10). In addition, γε dimers and, to a lesser extent, δε dimers have been found associated to calcineurin in SCID thymocytes and on early thymocytes from normal mice through the CD4−/CD8− stage (11, 12).

Assembly of the TCR-CD3 complex has been suggested to take place by pairwise interactions that allow the formation of CD3 γε, δε, and ζζ dimers as well as TCR-αβ (12). The association between TCR and CD3 chains seems to depend largely on the interaction among transmembrane domains, where the basic amino acid residues of the TCR chains and the acidic residues of the CD3 chains are involved, perhaps forming salt bridges (13, 14). The importance of the basic and acidic residues of the transmembrane domains is highlighted by the conservation of the lysine residue in TCR-β and TCR-γ and especially by the conservation of the arginine and lysine residues and their positions in TCR-α and its equivalents (TCR-δ and pTα) (15). The transmembrane domain has also been shown to be important in CD3-ζ dimerization (16). However, very little is known of the mechanisms that govern the assembly of the CD3-γ, -δ, and -ε chains. CD3-ε can interact with...

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‡‡ The abbreviations used are: TCR, T cell antigen receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; DTT, dithiothreitol; IP, immunoprecipitation.
either CD3-γ or CD3-δ, but CD3-γ and CD3-δ do not interact directly. Interestingly, the removal of the acidic amino acids in the transmembrane domain of CD3-γ or CD3-δ results in the formation of CD3-γ-δ heterodimers (17).

We have screened a collection of human CD3-e deletional mutants for their ability to interact with CD3-γ and CD3-δ. We found that a region in the extracellular domain of CD3-e, highly conserved in CD3-γ and CD3-δ, is responsible for CD3-γ-e and CD3-δ-e dimer formation as well as the formation of homodimers.

**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies**—The COS-7 African green monkey cell line was grown in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum (Life Technologies, Inc.). Mouse monoclonal antibodies APA1/1 and APA1/2, specific for human CD3-ε and CD3-δ, respectively, were obtained from purified CD3 proteins isolated from human thymus (7). The anti-CD3 antibody UCHT1 was generously donated by Dr. P. Beverley (Imperial Cancer Research Fund, London). HMT3.2 is a hamster monoclonal antibody reactive toward human CD3-γ and murine CD3-γ and CD3-δ (7) that was generously given by Dr. R. Kubo (National Jewish Center, Denver, CO).

**Plasmids**—The pSR-γ, pSRα, and pSR-ε constructs were made as described (7). Deletions 1–13 in CD3-ε were generated by loop-out mutagenesis as described previously (18). The CD3-ε mutant 14 was constructed after two PCR amplifications. The first amplification utilized primer 8 (Xhol restriction site) as the 5′-primer (CCTGTCCAAGATGCTCTCTGATT). Primer 61 corresponds to the mixture (Amersham Pharmacia Biotech) were added for 4 h. Afterward, the transmembrane domain of CD3-γ was amplified using a 5′-primer (CCCCCTCGAGGACATGGAACAGGGGAAG) as the 5′-primer and oligonucleotide GGGGGATCCTATCAC-CAAGAACTGACTTGTG that incorporate the mutant alanines followed by amplification of the complete CD3-γ with oligonucleotide 73 (CATCTCATGGCGTTCTCAGCCACTCTTCG) containing alanine substitutions as the 3′-primer. The second part of the molecule was amplified using a 5′-primer complementary to 126 (GCTCGGGCGCCTGGGATGGAACAGGGAAG) together with oligonucleotide 61 (containing a BamHI restriction site) as the 3′-primer (GGG-GATCTCAATGATGCTTCTGATT). Primer 61 corresponds to the most carboxyl-terminal region. After hybridization of the previously amplified fragments, a second round of PCR was performed with the external primers 8 and 61. This fragment was cloned into the Xhol and BamHI sites of the expression vector pSRα. The double cysteine substitution in CD3-ε was made as described in the oligonucleotide 121 (CATCCTCAGGGCATGACTGACCATGGC) and its complementary form as internal primers. CD3-ε-deletion A was generated with the internal primers 125 (CATCAGGCTGACTGACCATGACTGGATGC) containing alanine substitutions as the 3′-primer. The second part of the molecule was amplified using a 5′-primer complementary to 126 (GCTCGGGCGCCTGGGATGGAACAGGGAAG) that incorporate the mutant alanines followed by amplification of the complete CD3-γ with oligonucleotide 73 (CCTGTCCAAGATGCTCTCTGATTGC) and its complementary form as internal primers. CD3-ε-deletion A was generated with the internal primers 125 (CATCAGGCTGACTGACCATGACTGGATGC) containing alanine substitutions as the 3′-primer. Because CD3-γ DNA has an internal Xhol site, it was cloned into the pSRα vector by partial digestion. All constructs described were sequenced on an automated DNA Sequencer (Perkin-Elmer, Norwalk, CT, USA). The plasmid mixture was added as well as 20 μl of PUC-19 plasmid DNA (Promega) as a carrier. Cells were electroporated at 200 μF for 1 h and incubated with the desired antibody, diluted in washing solution (PBS plus 0.1% Tween 20), for 1 h. The membranes were washed five times with PBS-Tween and incubated for another hour with a peroxidase-labeled species-specific anti-lg antibody (Amersham Pharmacia Biotech) diluted in PBS-Tween. Last, the membrane was washed five times with PBS-Tween, and protein bands were visualized with the enhanced chemiluminescence method (ECL, Amersham Pharmacia Biotech). Densitometry analysis was performed in a Computing Densitometer model 300A.

**Immobilized Ligand Binding Assay**—A total of 1.4 mg of a 19-mer synthetic peptide (LQVYVRMCNQCIELNGL) that corresponds to amino acids 79–93 (LQVYVRMCNQCIELN) of the human mature CD3-γ protein were covalently coupled to a 1-ml HiTrap NHS-activated agarose column (Amersham Pharmacia Biotech) through a 4-aminoo acid spacer (GSKK). A control column was prepared by coupling a 19-mer nonpeptide (ERRRGGHDGYQGLSTATKDYTD) that corresponds to an intracellular sequence of CD3-γ. The efficiency of coupling was estimated to be 80% in both cases by optical absorbance.

To prepare the 35S-labeled extracellular domain of CD3-ε, a sequence encoding for amino acids 1–105 of the mature protein was obtained by PCR using oligonucleotide GCCCTCGAGCATATGGATGGTAATGAG and GCCCGGCCCGGCGCAGGGGAAG as the 5′-primer and oligonucleotide 112 (GGGGATCTTATGATGCTTCTGATTCT) as the 3′-primer. Because CD3-γ DNA has a nested Xhol site, it was cloned into the pSRα vector by partial digestion. All constructs described were sequenced on an automated DNA Sequencer (Perkin-Elmer, Norwalk, CT, USA). The plasmid mixture was added as well as 20 μl of ECL, Amersham Pharmacia Biotech). Densitometry analysis was performed in a Computing Densitometer model 300A.

**Immunoprecipitation and Deglycosylation Procedure**—In immunoprecipitation studies, 1% Nonidet P-40 or radioligand precipitation buffer (20 mM Tris, pH 8.0, 0.15 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml concentration of leupeptin, aprotinin, and chemostatin) lysates were precleared three times by incubation for 1 h with nonimmune mouse serum coupled to protein A- and protein G-Sepharose beads (Sigma) followed by centrifugation at 12,000 g in an Eppendorf centrifuge. The precleared supernatants were subsequently incubated for 4 h at 4 °C with 2–4 μg of the specific antibodies coupled to protein A- or protein G-Sepharose beads. Then the beads were washed five times with 1 ml of lysis buffer and resuspended in Laemmli sample buffer. SDS-polyacrylamide gel electrophoresis was performed on 13% polyacrylamide gels. Two-dimensional electrophoresis under nonreducing/reducing conditions was performed as described (7).

In deglycosylation studies, the immunoprecipitates were resuspended after the last wash in 60 μl of a 0.15 mM sodium citrate buffer, pH 5.5, containing 1 mM phenylmethylsulfonyl fluoride and 0.25% SDS. The samples were boiled for 2 min, and 1 milliunit of endo-β-asparaginyl-aminopeptidase from hog pancreas (Boehringer Mannheim) was added to half of each sample. The samples were incubated overnight at 37 °C and electrophoresed.

**Western Blot Analysis**—Immunoprecipitated samples were run on 13% acrylamide gels and were transferred onto nitrocellulose membrane (Bio-Rad). The membrane was blocked in 10% nonfat milk in PBS for 1 h and incubated with the desired antibody, diluted in washing solution (PBS plus 0.1% Tween 20), for 1 h. Afterward, the membrane was washed five times with PBS-Tween, and protein bands were visualized with the enhanced chemiluminescence method (ECL, Amersham Pharmacia Biotech). Densitometry analysis was performed in a Computing Densitometer model 300A.

**Characterization of the CD3 Association Domain**

The cells were washed with PBS and lysed in 1% Nonidet P-40-containing lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.8, 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and a 1 μg/ml concentration each of leupeptin and aprotinin).
Characterization of the CD3 Association Domain

The transfected cells were then stained with UCHT1, an anti-CD3-ε antibody, corresponding to amino acids 89–105 of the human mature CD3-ε body in cells cotransfected with these mutants (Fig. 3, panels A and B). These experiments suggested that deletions 1, 5, 6, and 14 prevented the association of CD3-ε with CD3-γ and CD3-δ or, alternatively, that they altered the binding site for antibodies UCHT1, Leu 4, etc. In order to distinguish between both possibilities, an immunoprecipitation experiment with subunit-specific antibodies was performed. COS cells were transfected with different CD3-ε constructs, together with CD3-γ, and were metabolically labeled with a [35S]methionine and [35S]cysteine mixture. For these experiments, a double deletional mutant of regions 5 and 6 was produced that, as expected, was not stained by conformation-dependent antibodies when cotransfected with either CD3-γ or CD3-δ (data not shown). Radioimmunoprecipitation buffer lysates from doubly transfected cells were immunoprecipitated with antibodies HMT3.2 and APA1/1 that recognize isolated CD3-γ and CD3-ε chains, respectively. Half of each immunoprecipitate was treated with endo-β-acetylgalactosaminidase H to better distinguish between glycosylated CD3-γ and the nonglycosylated CD3-ε chain. As shown in Fig. 3A, the immunoprecipitation with APA1/1 from cells cotransfected with CD3-δ and wild type CD3-ε resulted in the coprecipitation of CD3-γ, which characteristically runs as 25 kDa in untreated samples and as a 17-kDa protein when it is deglycosylated (dγ). Upon deglycosylation, the 23-kDa CD3-ε chain remained unmodified. Of note, the mobilities of CD3-ε mutants were frequently different from that of the wild type form, and their position is indicated with arrowheads in Fig. 3. Only two deletions, deletion 5 + 6 and deletion 9, seemed to affect binding to CD3-δ, since immunoprecipitation with APA1/1 did not coprecipitate any detectable CD3-γ; however, the reverse immunoprecipitation with anti-CD3-γ antibody HMT3.2 showed similar levels of deletion 9 and eυ, suggesting that, indeed, deletion 9 did not affect the association with CD3-γ (data not shown). Furthermore, the immunoprecipitation with APA1/1 had not shown any detectable CD3-ε, strongly suggesting that the sequence eliminated in deletion 9 constitutes the recognition site for APA1/1. Interestingly, deletion 5 + 6 was the only deletion that considerably affected the eγ interaction, as shown by immunoprecipitation with anti-ε and anti-γ antibodies (Fig. 3, A and B). Deletion mutants 1 and 14 were clearly coprecipitated by the anti-γ antibodies (Fig. 3B), and conversely, CD3-γ was clearly coprecipitated by the anti-ε antibody in cells cotransfected with these mutants (Fig. 3A). These results suggest that the only deletion that had a major effect on the interaction with CD3-δ corresponded to regions 5 + 6. Nevertheless, because region 6 contains two of a total of four methionines and two of a total of five cysteines, it is difficult to quantitate the amount of protein relative to the wild type control in the experiments shown in Fig. 3. In order to generate quantifiable results, the immunoprecipitation with subunit-specific anti-ε and anti-γ antibodies was followed by immunoblotting with the opposite antibody. As shown in Fig. 4, immu-
noprecipitation with anti-γ antibody (HMT3.2) resulted in less deletion 5 to 6 coprecipitated as compared with that of the wild type. To control the efficiency of transfection, the membrane was stripped and reprobed with the anti-γ antibody. Densitometric scans of the bands corresponding to CD3-ε and CD3-γ showed that the level of the coprecipitated deletion 5 to 6 mutant was one-third of that of the wild type, whereas deletions 1 and 14 had only a marginal effect. The immunoprecipitation with the anti-ε antibody followed by immunoblotting with the anti-γ antibody showed comparatively similar results, although the effects were more pronounced. CD3-γ was barely coprecipitated in cells transfected with deletion 5 to 6, although the levels of CD3-ε detected after reprobing the membrane with APA1/1 showed in all cases similar levels of transfected CD3-ε. A densitometric scan of the CD3-ε and CD3-γ bands revealed that deletions 1 and 14 inhibited the association to CD3-γ by

**Fig. 2.** Immunofluorescence analysis of the association of CD3-ε with CD3-γ and CD3-δ. COS cells were transfected with expression vectors encoding for wild type or the indicated mutants of CD3-ε and either CD3-γ (A) or CD3-δ (B). Indirect immunofluorescent staining was performed with antibodies APA1/1 (anti-CD3-ε), HMT3.2 (anti-CD3-γ), APA1/2 (anti-CD3-δ), and UCHT1 (specific for CD3-εγ and CD3-εδ dimers). Photographs were taken at a ×400 magnification.
2–3-fold, whereas deletion 5 + 6 inhibited it by 20-fold. The fact that deletion 5 + 6 causes a 3-fold reduction in formation of CD3-εCD3-γ heterodimers when immunoprecipitation was carried out with anti-γ antibody and a 20-fold reduction when immunoprecipitation was performed with anti-ε could be due to an excess of expression of one transfected product over the other. As a control for the specificity of the immunoprecipitations, COS cells were transfected with ε wt and a CD4 chimera that is endoplasmic reticulum-retained (18). The immunoprecipitation with either anti-CD4 or anti-CD3-ε antibodies did not show any evidence of association (data not shown).

Dietrich et al. (27) have described that two sites in human CD3-γ mediated binding to CD3-ε. One, that they named site 17, corresponds to part of regions 1 and 2 of CD3-ε and involved five amino acids (Fig. 5A). The other site, named 56, of CD3-ε homologous to region 3 of CD3-ε involved four amino acids (Fig. 5A). The sites were involved in CD3-ε binding. To confirm this hypothesis, a synthetic peptide corresponding to the CD3-ε sequence was covalently coupled to an agarose column, and a [35S]methionine-labeled bacterial extract expressing the full extracellular domain of the mature CD3-ε protein (amino acids 1–105) was passed through the column. As a control of binding, a column made with an irrelevant peptide was incubated, rinsed, and eluted in the same conditions. As shown in Figs. 6B, CD3-ε bound efficiently and specifically to the CD3-ε sequence. To determine whether the immobilized CD3-ε peptide actually

FIG. 3. Deletion of regions 5 and 6 inhibit the interaction with CD3-γ. COS cells were transfected with expression vectors encoding for wild type CD3-ε (ε wt) or the indicated mutants of CD3-ε plus wild type CD3-γ. Cells were metabolically labeled with a [35S]methionine-cysteine mixture, and proteins were immunoprecipitated with antibodies against CD3-ε (APA1/1; panel A) or anti-CD3-γ (HMT3.2; panel B). Half of each sample was incubated with endo-β-acetylgalactosaminidase H (+) or left untreated (−). The positions of the CD3-ε mutants are indicated by arrowheads. dγ, deglycosylated CD3-γ; nms, nonimmune serum.
In Fig. 7, the double cysteine mutant associated much less CD3-$\gamma$. These results further support the idea that regions 5 and 6 of the inhibition obtained with the deletional mutant 5 was in the region of the inhibition obtained with the double cysteine mutation was in the CD3-$\gamma$, CD3-$\delta$, and immunoglobulin light chain (L) are indicated.

bound the connecting peptide region of CD3-$\epsilon$, a similar experiment to the one shown in Fig. 6B was carried out. In this case, a 125I-labeled peptide corresponding to the connecting peptide region of CD3-$\epsilon$ was passed through the CD3-$\gamma$ column. As control for specificity, the same peptide was passed through the irrelevant peptide (\(z\)) column. As shown in Fig. 6C, the CD3-$\epsilon$-peptide bound to the CD3-$\gamma$ and not to the irrelevant column. An additional control was established by passing a 125I-labeled preparation of the irrelevant peptide through both columns. The CD3-$\gamma$ column bound the CD3-$\epsilon$-peptide but not the irrelevant one (Fig. 6C). In summary, the data shown in Fig. 6, B and C, demonstrate that the region in CD3-$\gamma$ equivalent to 5 + 6 in CD3-$\epsilon$ is also involved in CD3-$\epsilon$ binding and that the sequence is by itself sufficient to promote binding. Therefore, the region 5 + 6 delineates a conserved sequence in the CD3-$\epsilon$-$\delta$ interaction.

Two Cysteines in Region 6 of CD3-$\epsilon$ Are Involved in the Association with CD3-$\gamma$ and CD3-$\delta$ and May Form an Intrachain Disulfide Loop—The two cysteines of the CD3 association region are conserved not only in all described CD3-$\epsilon$-$\delta$ chains (Fig. 1) but also in all CD3-$\gamma$ and CD3-$\delta$ chains (not shown). To prove the possible involvement of the two cysteines in CD3-$\epsilon$ pair formation, we made a double mutation of the two cysteines (positions 97 and 100 of the human mature protein) to alanines (see Figs. 4 and 7). This is consistent with the idea that DTT affected the interaction via the involvement of Cys$^{97}$ and Cys$^{100}$. Although the DTT/iodoacetamide treatment

![Fig. 4. Quantitation of $\epsilon/\gamma$ interactions by Western blot analysis. Deletions 1, 5, 6, and 14 were cotransfected with CD3-$\gamma$ in COS cells. The samples were divided, and immunoprecipitation was performed with anti CD3-$\gamma$, HMT3.2 (left) or anti CD3-$\epsilon$, APA1/1 (right), followed by immunoblotting with the indicated antibody. The quantity of CD3-$\gamma$ and CD3-$\epsilon$ specifically immunoprecipitated in each case was estimated by reprobing the membranes with the antibodies used for immunoprecipitation. The ratios of coprecipitated proteins were analyzed by densitometry and are shown at the bottom as percentages of the coprecipitating chains obtained with wild type CD3-$\epsilon$. The positions of CD3-$\gamma$, CD3-$\epsilon$, and immunoglobulin light chain (L) are indicated.](image)

![Fig. 5. Effect of mutation of site 17 in CD3-$\gamma$ on the association with CD3-$\epsilon$. A. Amino acid sequence of human CD3-$\gamma$, indicating sites 17 and 56 (boldface type) according to Dietrich et al. (27) and their corresponding CD3-$\epsilon$ deletions. The five amino acids of site 17 were replaced by alanines (CD3-$\gamma$A). B. COS cells were transfected with the expression vector encoding for wild type CD3-$\epsilon$ plus CD3-$\gamma$A and metabolically labeled with $^{35}$S-methionine/cysteine mixture. Antibodies APA1/1 (anti-CD3-$\epsilon$) and HMT3.2 (anti CD3-$\gamma$) were used for immunoprecipitation. Half of the samples were untreated (+) or treated (-) with endo-$\beta$-N-acetylglucosaminidase II (endoH) and resolved on 13% acrylamide gels. Positions of CD3-$\epsilon$, CD3-$\gamma$, and deglycosylated CD3-$\delta$ (\(d\)) are indicated.](image)
Cys100 to alanine (mut). Samples were immunoprecipitated with irrelevant peptide (z-cetamide treatment on other regions of CD3-γ) and CD3-δ. Residues that are conserved in at least two subunits are shown in boldface type. B, the CD3-γ connecting peptide is sufficient to bind the extracellular portion of CD3-ε. A, 35S-labeled bacterial cell lysate expressing the extracellular domain of CD3-ε was passed through a column containing a covalently bound peptide corresponding to the connecting peptide of CD3-γ (lanes 2 and 4) or containing an irrelevant control peptide (lanes 1 and 3). The columns were rinsed, and equal volumes of the unbound material were loaded in lanes 1 and 2, whereas the bound material was eluted from both columns and equal volumes were loaded in lanes 3 and 4. The column with immobilized CD3-γ peptide bound 16% of the loaded radioactivity, whereas the control column bound only a 3%. C, the CD3-γ connecting peptide binds the equivalent region in CD3-ε. 125I-Labeled peptides corresponding to amino acids 89–105 of CD3-ε and an irrelevant sequence (ζ) were passed through columns containing immobilized the CD3-ε peptide (γ) or the irrelevant peptide (ζ). The columns were rinsed, and the bound material was eluted and counted. A total of 1 × 10^6 cpm of labeled peptides were loaded per column.

FIG. 6. The equivalent in CD3-γ to region 5 + 6 of CD3-ε is sufficient to promote the association of CD3-ε. A, sequence comparison of region 5 + 6 of CD3-ε with its counterpart in CD3-γ and CD3-δ. Residues that are conserved in at least two subunits are shown in boldface type. B, the CD3-γ connecting peptide is sufficient to bind the extracellular portion of CD3-ε. A, 35S-labeled bacterial cell lysate expressing the extracellular domain of CD3-ε was passed through a column containing a covalently bound peptide corresponding to the connecting peptide of CD3-γ (lanes 2 and 4) or containing an irrelevant control peptide (lanes 1 and 3). The columns were rinsed, and equal volumes of the unbound material were loaded in lanes 1 and 2, whereas the bound material was eluted from both columns and equal volumes were loaded in lanes 3 and 4. The column with immobilized CD3-γ peptide bound 16% of the loaded radioactivity, whereas the control column bound only a 3%. C, the CD3-γ connecting peptide binds the equivalent region in CD3-ε. 125I-Labeled peptides corresponding to amino acids 89–105 of CD3-ε and an irrelevant sequence (ζ) were passed through columns containing immobilized the CD3-ε peptide (γ) or the irrelevant peptide (ζ). The columns were rinsed, and the bound material was eluted and counted. A total of 1 × 10^6 cpm of labeled peptides were loaded per column.

FIG. 7. Role of Cys97 and Cys100 of CD3-ε in the association with CD3-γ and CD3-δ. COS cells were transfected with CD3-γ or CD3-δ and either wild type CD3-ε or CD3-ε doubly mutated in Cys97 and Cys100 to alanine (mut). Samples were immunoprecipitated with APA1/1 (anti-ε), and blotting was performed with HMT3.2 (anti-γ) or APA1/2 (anti-δ). The two panels at the bottom show the result of stripping and reprobing the same membrane with APA1/1 as a control for the efficiency of transfection. The percentage of coprecipitated CD3-γ and CD3-δ chains is calculated in reference to the amount of transfected CD3-ε and considering the level obtained with wild type CD3-ε as 100%.

could affect cysteines other than those of region 6, we have shown that deletions involving the other cysteines (deletions 2, 4, and 13) did not have an effect on the εγ and εδ interactions (see Figs. 2 and 3). Nevertheless, an effect of the DTT/z-dodecylsulfate treatment on other regions of CD3-γ could not be dismissed.

CD3-ε Homodimerization Depends on the Same Region In-volved in the Formation of εγ and εδ Dimers—Given the homology in regions 5 and 6 between CD3-ε and CD3-γ and -δ, it would not be surprising if the region involved in the association of CD3-ε with CD3-γ and CD3-δ also promotes the formation of homodimers. CD3-ε contains five cysteines that could participate in the formation of disulfide-linked homodimers. Two, found in regions 2 and 4, are supposedly implicated in intrachain loop formation, another one is in the transmembrane domain, and the remaining two cysteines are in region 6. To identify the cysteine residues that participate in homodimer formation, detergent lysates were obtained from COS cells transfected with wild type CD3-ε or with deletion mutants 2, 4, 6, and 13 that had been metabolically labeled with [35S]methionine and [35S]cysteine. CD3-ε proteins were immunoprecipitated from the lysates with antibody APA1/1 and run in two-dimensional diagonal gels under nonreducing/reducing conditions. In the wild type sample, as well as in deletions 2, 4, and 13, dimers and trimers of CD3-ε were identified as protein spots that ran below the diagonal (Fig. 9). In the sample corresponding to deletion 6, no oligomers of CD3-ε were detected, although the monomer was readily detected on the diagonal of the gel, suggesting that the cysteines of region 6 participate in theimerization of CD3-ε.

The above data suggest that the same region mediates the association of CD3-ε with CD3-γ and CD3-δ and the formation of homodimers. This result would imply that the formation of CD3-ε homodimers is an alternative to the formation of εγ and εδ heterodimers and that CD3-ε could either associate with itself or with CD3-γ or CD3-δ. As shown in Fig. 10, in cells cotransfected with wild type CD3-ε and CD3-γ, and immunoprecipitated with CD3-ε- and CD3-γ-specific antibodies, dimers, trimers, and other oligomers of CD3-ε and CD3-γ were...
observed. However, only monomers of CD3-γ were coprecipitated with the anti-e antibody, and, conversely, only monomers of CD3-e were coprecipitated with the anti-γ antibody. These results suggest that CD3 homodimers do not associate with the other CD3 chains and reinforces the idea of the existence of a competition between homodimer and heterodimer formation of the CD3-γ, -δ, and -e chains, since regions 5 and 6 are involved in both processes.

**DISCUSSION**

In the present study, we have mapped the region in CD3-e that is responsible for binding to the CD3-γ and CD3-δ chains. Previous reports have shown that CD3-γ and CD3-δ compete for binding to CD3-e, suggesting that CD3-γ and CD3-δ are bound to the same site in CD3-e (7). This has been now confirmed using a set of deletional mutants of CD3-e. Four deletions resulted in inhibition of both CD3-γ and CD3-δ binding.

**FIG. 8.** Cysteines 97 and 100 in region 6 of CD3-e form an intrachain disulfide loop. COS cells were transfected with the expression vector encoding for wild type CD3-e and CD3-γ. Immunoprecipitations using anti-CD3-e (APA1/1) and anti CD3-γ (HMT3.2) antibodies were carried out followed by immunoblotting with APA1/1 antibody. A, cell lysates were incubated with 10 mM phenanthroline for 30 min before immunoprecipitation and compared with untreated samples. B, immunoprecipitations were performed from cell lysates that had been sequentially incubated for 10 min with 10 mM DTT and then with 25 mM iodoacetamide (IAA), incubated just with 25 mM iodoacetamide, or left untreated (NT). Afterward, immunoprecipitations were performed, the immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, and immunoblotting was performed with APA1/1 to detect CD3-e specifically precipitated with APA1/1 and coprecipitated with CD3-γ. The positions of CD3-e and immunoglobulin heavy (H) and light (L) chains are indicated.

**FIG. 9.** Formation of disulfide-linked homodimers by CD3-e mutants. COS cells were transfected with the indicated mutants of CD3-e, labeled with a [35S]methionine/cysteine mixture, and immunoprecipitated with APA1/1. The immunoprecipitates were resolved in diagonal gels under nonreducing (NR) and reducing (R) conditions. The monomeric, dimeric, and trimeric forms of CD3-e are indicated with arrows. The relative masses of the monomer, dimer, and trimer were calculated to be 23, 38, and 63 kDa, respectively.

**FIG. 10.** Association of CD3-γ with monomeric forms of CD3-e. COS cells were transfected with either CD3-e alone or CD3-e plus CD3-γ and were immunoprecipitated with antibodies APA1/1 (anti-e) or HMT3.2 (anti-γ). The immunoprecipitates were analyzed in diagonal gels under nonreducing/reducing conditions. The positions of monomeric and oligomeric forms of CD3-e and CD3-γ are indicated. The arrowheads show the positions of carbonic anhydrase (31 kDa) and trypsin inhibitor (21.5 kDa) used as molecular mass markers.

The four mutants corresponded to deletions in the extracellular domain of CD3-e, suggesting that this domain is responsible for the interaction with CD3-γ and CD3-δ. Indeed, deletions affecting the whole transmembrane domain or the cytoplasmic tail did not have an effect on CD3-e/γ and CD3-e/δ interactions. Of the four deletion mutants (deletions 1, 14, 5, and 6), two were contiguous and corresponded to what is known as the connecting peptide in other immunoglobulin superfamily members. This sequence is located between the putative β-barrel of CD3-e and the transmembrane domain. The combined deletion of
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regions 5 and 6 resulted in a major effect on the ε/γ interaction (64–95% inhibition), whereas the effect of the other two deletions (deletions 1 and 14) was minor (11–68% inhibition). These results point to the connecting peptide as the major region involved in ε/γ and ε/δ interactions, although other regions must also contribute, since the double deletion 5 + 6 did not result in a complete inhibition. Nevertheless, a peptide corresponding to the equivalent region in CD3-γ bound to CD3-ε, suggesting that this region is sufficient to promote CD3 interactions. Our results do not support those of Dietrich et al. (27). Using protein structure predictions, these authors made two mutants in segments located between the A and B β-sheets (site 17) and between the E and F sheets (site 56) of the putative immunoglobulin fold of CD3-γ. They found that these two mutants were unable to associate to the other CD3 subunits. Site 56 corresponds to our deletion 3 in CD3-ε, which did not have an effect on the interaction with CD3-γ, and site 17 corresponds to a sequence in CD3-ε found between our deletions 1 and 2. Deletion 2 did not affect the interaction with CD3-γ, whereas deletion 1 only had a minor effect. Indeed, in our system, a deletion of site 17 in CD3-γ did not alter the association with CD3-ε. The discrepancy between our results and those of Dietrich et al. could be explained by their utilization of UCHT1 as an anti-CD3-ε antibody when it has been previously established that it is a quaternary structure-dependent antibody that recognizes ε/γ and ε/δ dimers (26). Therefore, the possibility cannot be excluded that mutations in sites 17 and 56 alter the epitope recognized by the conformation-dependent antibodies. In fact, a sequence proximal to region 14 has been proposed in chicken CD3-ε as the site for antibody CT3 recognition (24).

The 16-amino acid region (connecting peptide) involved in deletion 5 + 6 is highly conserved among the CD3-γ, -δ, and -ε chains, both in terms of amino acid identity and of conservative replacements. This is particularly important, especially when considering that there is very little homology between the remaining extracellular domain of CD3-ε and the other CD3 chains. This region is not only involved in the formation of heterodimers but also, as a consequence of the sequence homology of CD3-γ and CD3-δ, in homodimerization. Indeed, as shown here, both processes occur in an alternate manner, because the dimers and oligomers of CD3-ε were not associated with CD3-γ.

According to their proximity in the genome (28, 29), the conservation of the exon/intron organization and sequence homology, it seems clear that CD3-δ, CD3-γ, and CD3-δ have originated from two duplication events, of which, the process that generated CD3-γ and CD3-δ is the most recent (30). Indeed, birds appear to have one chain that is homologous to both CD3-γ and CD3-δ (31). The utilization of the same region in CD3-ε for the formation of homodimers and heterodimers with CD3-γ and CD3-δ could be reminiscent of the evolutionary process, since, in a primitive ancestor when only one of the CD3 chains existed, the homodimers of this chain played the roles that the CD3-εCD3-γ and CD3-εCD3-δ dimers play in mammalian T cells.

If the formation of homodimers of CD3 chains is a consequence of the high degree of conservation existing in the region involved in CD3/CD3 heterologous interactions, then it is unclear why CD3-γ and CD3-δ do not interact directly. Although this interaction has not been detected in wild type proteins, it has been shown in CD3-γ and CD3-δ mutants lacking the glutamic and aspartic acid residues located in their transmembrane region (17). These results support the notion that CD3-γ and CD3-δ have the potential to associate but the interaction is impeded by the transmembrane domain acidic residues. It is more difficult to explain, however, the absence of such a negative effect in the CD3-ε/γ interaction, since CD3-ε also has an aspartic acid residue in the transmembrane domain. Furthermore, the aspartic acid in the transmembrane domain of CD3-γ has been shown to facilitate the formation of disulfide-linked homodimers (16). Therefore, it seems that the presence of acidic amino acid residues in the transmembrane domains of the CD3 chains has very different effects depending on the subunit involved, either enhancing or inhibiting CD3 interactions. This heterogeneity in the effects of the putatively charged residues could be due to differences in the involvement of the transmembrane domains in CD3 interactions.

The region responsible for homo- and heterodimerization in CD3-ε is equivalent to the connecting peptide region of TCR-α, -β, -γ, and -δ chains, which is involved in the formation of heterodimers. However, no obvious homology among the TCR chains and the CD3 association domain was found. Interestingly, the CD3-association sequence contains two cysteines separated by two amino acids, Cys-X-X-Cys, in an analogous fashion to the cysteines intervening in the interaction between p56lck and CD4 or CD8 (32, 33). Indeed, a five-amino acid sequence that includes the two cysteines, VCENC100, is found in human and pig CD3-ε and in human and murine p56lck. Although the CD3-ε chains from other species do not simultaneously have Glu and Asn in positions 98 and 99, they do have at least one of the two positions conserved (Fig. 1). Amino acids Val104, Cys97, and Cys100 are present in all CD3-ε chains. The sequence homology suggests that a similar stretch of amino acids could have been selected to maintain protein/protein interactions in two very different systems: in intracytoplasmic interactions between Lck and CD4 or CD8 and in extracellular interactions between CD3-δ and CD3-δ or CD3-γ. With regard to the implication of cysteine residues in the interaction between Lck and CD4 or CD8, it has been suggested that the four cysteine residues could be coordinated with a zinc ion in a zinc finger-like fashion (33). Although both cysteines in CD3-ε are important for the interaction with CD3-γ and CD3-δ, Zn2+ does not seem to be required, since sequestration of the ion with phenanthroline does not result in an impaired interaction. On the other hand, in light of the result of DTT treatment, it seems likely that Cys97 and Cys100 form an intrachain disulfide bridge. A Cys-X-X-Cys motif constitutes the active site of thioredoxin, a cytosolic reducing agent, and of protein-disulfide isomerase, an endoplasmic reticulum protein that catalyzes the formation of native disulfide bonds. Indeed, it is suggested that the Cys-X-X-Cys sequence forms an intramolecular disulfide bond that could help to prevent irreversible oxidation of the active sites of thioredoxin and protein-disulfide isomerase (34).

In summary, we show in this work that the connecting peptide region is the major contributor to the formation of both covalently linked homodimers and noncovalently bound heterodimers among the CD3-γ, CD3-δ, and CD3-ε chains of the TCR/CD3 complex. Two cysteines that conform to a Cys-X-X-Cys motif within the connecting peptide region are important, suggesting that these residues either directly participate in the interactions or are necessary to maintain an adequate conformation of the region, perhaps through the formation of an intrachain disulfide bridge. The determination of the tridimensional structure of the region will help to demonstrate which of the two possibilities is correct.

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