Molecular Characterization of the Di-leucine-based Internalization Motif of the T Cell Receptor*

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Several cell surface receptors including the T cell receptor (TCR) are phosphorylated and down-regulated following activation of protein kinases. We have recently shown that both phosphorylation of Ser-126 and the presence of the di-leucine sequence Leu-131 and Leu-132 in CD3γ and for TCR down-regulation, alanine scanning of CD3γ was done. Mutations of Arg-124, Ser-126, Lys-128, and Gln-129 inhibited both phosphorylation and TCR down-regulation, whereas mutation of Asp-127 only inhibited down-regulation. Further analyses demonstrated a discrepancy between the ability to be phosphorylated on CD3γ and to down-regulate the TCR in several transfectants. Phosphorylation was not as strictly dependent on the nature and position of the phosphoacceptor group and basic residues as were the subsequent steps involved in TCR down-regulation. Our results suggest that PKC-mediated TCR down-regulation may be regarded as a two-step process. 1) Recognition and phosphorylation of CD3γ by PKC. In this process Arg-124, Ser-126, Lys-128, and Gln-129 are important. 2) Recognition of phosphorylated CD3γ by molecules involved in receptor internalization. In this process Ser(P)-126, Asp-127, Leu-131, and Leu-132 are important.

The T cell receptor (TCR)1 is a mitogenic receptor coupled to protein tyrosine kinases (1, 2). Following stimulation of the TCR, signals that lead to T cell activation and proliferation are transmitted across the T cell membrane (2, 3). Both TCR down-regulation and antigen unresponsiveness can be induced following stimulation with specific anti-TCR antibodies (4–6), with supraoptimal doses of antigen (7, 8), or with phorbol esters (9, 10). Physiological stimulation of the TCR leads to serine phosphorylation of CD3γ (11), and recently, it was found that T cell activation correlates with the degree of TCR down-regulation (12). Thus, down-regulation of the TCR appears to play a physiologically important role in the regulation of T cell function.

The TCR is a multimeric receptor composed of four dimers: the clonotypic TI heterodimer, generally Tiαβ, the CD3γδ and CD3ε, and the ζ homodimer (13–17). The disulfide-linked Tiαβ heterodimer is responsible for ligand recognition (18, 19), and the activation signals are delivered through the associated CD3 and ζ chains (reviewed in Ref. 20). After TCR stimulation, a number of cellular proteins including phospholipase Cγ1 become phosphorylated on tyrosine residues (21–23). Tyrosine phosphorylation of phospholipase Cγ1 amplifies its enzymatic activity, resulting in the production of inositol 1,4,5-trisphosphate and diacylglycerol (24). These second messengers are responsible for an increase in intracellular Ca\(^{2+}\) concentration and an activation of protein kinase C (PKC) (25, 26).

Among other substrates, the activated PKC phosphorylates the CD3γ chain (27, 28). Furthermore, activation of PKC leads to internalization and down-regulation of the TCR at the cell surface (9–11, 27, 29, 30), and we have recently shown that in addition to the di-leucine sequence Leu-131 and Leu-132 (31), phosphorylation of Ser-126 in the cytoplasmic tail of CD3γ is required for PKC-mediated down-regulation of the TCR (32, 33) (amino acid numbering of CD3γ according to Ref. 34). The role of the amino acids that surround Ser-126 as substrate specificity determinants for PKC and their role in the molecular mechanisms involved in TCR down-regulation following Ser-126 phosphorylation are unknown.

Based on the amino acid sequences that render synthetic peptides optimal substrates for protein kinases and on the sequence features that surround known phosphorylation sites on protein substrates, consensus sequences have been proposed as substrate specificity determinants for protein kinases (35–38). PKC requires basic amino acids near the phosphoacceptor group of the substrate, and phosphoacceptor groups surrounded by basic amino acids at both the N- and C-terminal seem to be better substrates for PKCa, -β, and -γ than phosphoacceptor groups surrounded by basic amino acids at only the N- or C-terminal side (37). Most studies describing substrate specificity determinants for PKC are based on in vitro analyses of synthetic peptides (39–44). Such peptides are powerful investigative tools, but their small size and random conformation may limit their ability to mimic the intracellular proteins they are intended to model. Furthermore, in vivo experiments are required to analyze the consequences of substrate phosphorylation.

The aim of this study was to identify amino acids in the cytoplasmic tail of CD3γ required in vivo for PKC-mediated phosphorylation of CD3γ and for down-regulation of the TCR.

**EXPERIMENTAL PROCEDURES**

CdIs, Antibodies, and Chemicals—J GN cells, a TCR cell surface negative variant of the human T cell line Jurkat that synthesizes no

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1 The abbreviations used are: TCR, T cell receptor; PKC, protein kinase C; FCS, fetal calf serum; PDB, phorbol 12,13-dibutyryl; MFI, mean fluorescence intensity; WT, wild type; FACs, fluorescence-activated cell sorter.
CD3γ (45) and the original Jurkat cell line J76, were cultured in RPMI 1640 medium (Life Technologies, Inc., Paisley, UK) supplemented with penicillin, 0.5 IU/liter (Leo, Ballerup, Denmark), streptomycin, 500 mg/liter (Novo, Bagsværd, Denmark), and 10% (v/v) FCS (Sera-Lab Ltd., Sussex, UK) at 37 °C in 5% CO2. UCHT1 mouse monoclonal antibody directed against human CD3ε was obtained purified and phycoerythrin-conjugated from Dakopatts A/S (Glostrup, Denmark). F101.01 mouse monoclonal antibody against a conformational epitope on the TCR was produced in our own laboratory (46). The phorbol ester phorbol 12,13-dibutyrate (PDB) was from Sigma.

Constructions, Transfection, and TCR Down-regulation—All mutations were constructed as described previously (32, 47, 48) by polymerase chain reaction using Vent DNA polymerase containing 3′→5′ proofreading exonuclease activity (New England Biolabs) and the plasmid pG3T3−2 (34) as template. Mutations were confirmed by complete DNA sequencing. Transfections were performed using the Bio-Rad Gene Pulser at a setting of 270 V and 960 microfarads with 40 μg of plasmid per 2 × 107 cells. After 3–4 weeks of selection, G418-resistant clones were expanded and maintained in medium without G418. For TCR down-regulation, cells were adjusted to 1 × 107 cells per ml of medium (RPMI 1640 and 10% FCS) and incubated at 37 °C with various concentrations of phorbol esters. At the indicated time cells were transferred to ice-cold phosphate-buffered saline containing 2% FCS and 0.1% NaN3 and washed twice. The cells were stained directly with phycoerythrin-conjugated UCHT1 and analyzed in a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA). Mean fluorescence intensity (MFI) was recorded and used in the calculation of percent anti-CD3 binding: (MFI of phorbol ester treated cells) divided by (MFI of untreated cells) × 100%. For each construct at least three different clones were analyzed.

Phosphorylation and TCR Internalization—Phosphorylation assays were performed as described previously (32). The phosphorylated CD3γ chain with a molecular mass of 26–30 kDa was coprecipitated with CD3ε (20 kDa) using the anti-CD3ε antibody UCHT1. For each construct at least two different clones were analyzed. To determine TCR internalization, cells were incubated in RPMI 1640/10% fetal calf serum and immediately treated with 300 μM NaCl, 0.5 M acetic acid, pH 2.2. After centrifugation at 20,000 g for 20 s, the percentage of internalized Fab to total Fab was subsequently measured in a Cobra Autocount gamma counter. The percentage of internalized Fab to total Fab was subsequently calculated.

RESULTS

Alanine Scanning Revealed Five Residues That Influenced PKC-mediated TCR Down-regulation—The CD3γ-negative T cell variant JGN (45) allowed us to study the potentials of various mutated CD3γ chains to be substrates for PKC and to analyze their ability to function in PKC-mediated TCR down-regulation at the T cell surface. JGN cells do not express the TCR at the cell surface, but, upon transfection of wild-type (WT) CD3γ+ cDNA into JGN cells, they become TCR cell surface-positive (45). We have recently shown that phosphorylation of CD3γ at Ser-126 is required for PKC-mediated down-regulation of the TCR (32). In addition to phosphorylation of Ser-126, two leucines, Leu-131 and Leu-132, are required for TCR down-regulation following PKC activation (32). These leucines are not CD3γ determinants for PKC as C-terminal truncations of the CD3γ cytoplasmic tail up to Thr-130 do not affect PKC-mediated phosphorylation of CD3γ (32). To examine the role of individual CD3γ membrane-proximal amino acids in PKC-mediated phosphorylation of CD3γ and TCR down-regulation, an alanine scanning was performed from Arg-121 to Thr-130. The different constructs (Fig. 1A) were separately transfected into JGN cells, and TCR cell surface-positive clones were isolated. The ability of the CD3γ-transfected cells to down-regulate the TCR following PDB-mediated PKC activation was analyzed.

Substitution of alanine for Arg-124, Ser-126, Asp-127, Lys-128, and Gln-129 inhibited PKC-mediated TCR down-regulation as shown with an asterisk. JGN-A125A is identical to JGN-WT.

TCR down-regulation (Fig. 1B). Ser-126, Asp-127, and Lys-128 seemed to be absolutely required for PKC-mediated TCR down-regulation as down-regulation of the TCR in JGN-S126A, JGN-D127A, and JGN-K128A cells was abolished even at the highest concentrations of PDB tested. In contrast, the inhibitory effect of substitution of alanine for Arg-124 and Gln-129 could be compensated for by increasing the concentration of PDB tested (Fig. 1B). The Arg-124, Ser-126, Asp-127, Lys-128, and Gln-129 residues were subsequently studied in greater detail.

A Basic Residue at Position 124 Renders CD3γ an Optimal Substrate for PKC, whereas an Acidic Residue at this Position Acts as a Negative Substrate Determinant—The role played by the amino acid at position 124 in the cytoplasmic tail of CD3γ as a substrate determinant for PKC was further studied by transfecting the R124E and R124K constructs (Fig. 2A) into JGN cells. JGN-R124K cells down-regulated the TCR as efficiently as JGN-WT cells, whereas higher concentrations, approximately 3–5-fold, of PDB were required to induce TCR down-regulation in JGN-R124A cells (Fig. 2, B and D). With increasing concentrations of PDB, JGN-R124A cells could down-regulate the TCR almost as efficiently as JGN-WT cells. In contrast, substitution of the acidic glutamic acid for the basic arginine severely affected TCR down-regulation even at the highest concentrations of PDB tested (Fig. 2, B and D).

To directly examine the role of the different mutations in PKC-mediated CD3γ phosphorylation, cells were loaded with 32P and incubated with PDB for 10 min. Following cell lysis and immunoprecipitation, the samples were run on SDS-PAGE and analyzed by autoradiography. JGN-WT and JGN-R124K CD3γ became phosphorylated following incubation with 22.5 nM PDB, whereas JGN-R124A CD3γ became weakly phosphorylated and JGN-R124E CD3γ did not become phosphorylated at this PDB concentration (Fig. 2C, and data not shown). Increasing the concentration of PDB to 225 nM resulted in an...
increased phosphorylation of both the J GN-WT, J GN-R124K, and J GN-R124A CD3γ chains, and a weak phosphorylation of the J GN-R124E CD3γ became apparent (Fig. 2C, and data not shown).

PKC Is Less Dependent on the Nature and Position of the Phosphoacceptor Group Than the Subsequent Mechanism Involved in TCR Down-regulation—To examine whether a threonine could act as a phosphoacceptor in the 126-position of the CD3γ tail and whether it could function in PKC-mediated down-regulation of the TCR, the construct S126T (Fig. 3A) was transfected into J GN cells. J GN-S126T cells did not down-regulate the TCR as efficiently as J GN-WT cells even at the highest concentration of PDB tested (Fig. 3, B and D). This indicated that Thr-126 was not as good a phosphoacceptor as Ser-126 or that phosphorylated Thr-126 did not function as efficiently as phosphorylated Ser-126 in the subsequent mechanisms involved in TCR down-regulation. To examine whether Thr-126 became phosphorylated following PKC activation, cells were loaded with 32P and incubated with PDB for 10 min. Following PKC activation, J GN-S126T CD3γ was phosphorylated as readily as J GN-WT CD3γ (Fig. 3C), indicating that S126T-CD3γ was as good a substrate for PKC as WT-CD3γ.

Next we wanted to study how critical the exact position of the phosphoacceptor group was for PKC-mediated phosphorylation and TCR down-regulation. Accordingly, J GN cells were transfected with constructs in which the serine was transposed to either one position N-terminal (Fig. 3A, construct 125SA) or one position C-terminal (Fig. 3A, construct 126DS). As in J GN-S126T cells, a discrepancy between the ability to function as a PKC substrate and the ability to function in TCR down-regulation was observed for both of the mutated CD3γ chains (Fig. 3). 125SA- and 126DS-CD3γ clearly became phosphorylated following PKC activation, although not as efficiently as WT-CD3γ. However, down-regulation of the TCR was completely abolished or severely inhibited in J GN-125SA and J GN-126DS cells, respectively.

Asp-127 Is dispensable for CD3γ Phosphorylation but Indispensable for TCR Down-regulation—Substitution of alanine for Asp-127 completely abolished PKC-mediated TCR down-regulation even at the highest concentrations of PDB tested (Fig. 4). To test whether this was caused by a lack of CD3γ phosphorylation, cells were loaded with 32P and incubated with PDB for 10 min. Following PKC activation, J GN-D127A CD3γ was phosphorylated as readily as J GN-WT CD3γ (Fig. 4C), indicating that D127A-CD3γ was as good a substrate for PKC as WT-CD3γ and that internalization mechanisms subsequent to CD3γ phosphorylation were dependent on Asp-127.

A Basic Residue C-terminal to Ser-126 Is a Prerequisite for PKC-mediated CD3γ Phosphorylation and TCR Down-regulation—Substitution of alanine for Lys-128 completely abolished PKC-mediated TCR down-regulation even at the highest concentrations of PDB tested (Fig. 4, B and D). To test whether
comparing MFI of PDB-treated cells with MFI of untreated cells. Concentrations of PDB for 1 h, and TCR down-regulation was determined by staining with anti-CD3 monoclonal antibodies and flow cytometry.

#### Fig. 3. Analyses of the phosphoacceptor group.

A, schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the indicated cell lines and a summation of the results from the CD3γ phosphorylation and TCR down-regulation analyses. CD3γ phosphorylation and TCR down-regulation were scored as described in the legend to Fig. 2. B, cells were incubated with different concentrations of PDB for 1 h, and TCR down-regulation was determined by staining with anti-CD3 monoclonal antibodies and flow cytometry comparing MFI of PDB-treated cells with MFI of untreated cells. C, phosphorylation analyses of CD3γ from JGN-WT (lanes 1 and 5), JGN-S126T (lanes 2 and 6), JGN-125SA (lanes 3 and 7), and JGN-126DS (lanes 4 and 8) cells. D, FACS histograms as described in the legend to Fig. 2.

This was caused by a lack of CD3γ phosphorylation, cells were loaded with 32P and incubated with PDB for 10 min. Following cell lysis and immunoprecipitation, the samples were run on SDS-PAGE and analyzed by autoradiography. JGN-K128A CD3γ did not become phosphorylated even after incubation with 225 nM PDB (Fig. 4C). The same results were obtained for JGN-K128E cells in which glutamic acid substituted for lysine at position 128 (data not shown). Substitution of arginine for glutamine showed that TCR down-regulation was completely abolished, indicating that the acidic aspartic acid.

A Polar Amino Acid Three Residues C-terminal to Ser-126 Optimizes CD3γ as a Substrate for PKC—As indicated by the alanine scanning, substitution of alanine for Gln-129 seemed to affect PKC-mediated TCR down-regulation. Incubation of cells with PDB demonstrated that approximately three times higher concentrations of PDB were required to induce down-regulation of the TCR in JGN-Q129A cells as compared with JGN-WT cells (Fig. 5, B and D). However, by increasing the amount of PDB, JGN-Q129A cells could down-regulate the TCR as efficiently as JGN-WT cells. To test whether this phenomenon was caused by a reduced CD3γ phosphorylation, JGN-Q129A cells were loaded with 32P and incubated with PDB for 10 min. JGN-Q129A CD3γ became phosphorylated but not to the same extent as JGN-WT CD3γ even at 225 nM of PDB, indicating that Gln-129 contributed as a substrate determinant for PKC (Fig. 5C). To test whether this was due to a unique characteristic of the glutamine side chain or if other amino acids that physically and chemically resemble glutamine could substitute for glutamine, the Q129E and Q129N constructs (Fig. 5A) were separately transfluoresc into JGN cells, and TCR cell surface-positive clones were isolated. JGN-Q129E and JGN-Q129N cells became CD3γ-phosphorylated and down-regulated the TCR as efficiently as JGN-WT cells following PKC activation (Fig. 5, B and D, and data not shown). This indicated that a polar amino acid, in contrast to an apolar amino acid, three residues C-terminal to the phosphoacceptor optimized the in vivo substrate recognition by PKC.

TCR Down-regulation Is Mediated by Increasing the Rate of TCR Internalization—Previous studies have indicated that the TCR is spontaneously internalized and recycled back to the cell surface and that the spontaneous TCR internalization rate is dependent upon a basal level of CD3γ phosphorylation (29, 49). This implies that the level of TCR expression, and thereby
probably T cell function, may be regulated by the activity of PKC and protein phosphatases. To directly study the role of CD3γ Ser-126 phosphorylation in spontaneous and PDB-induced TCR internalization, JGN-WT, JGN-S126A, and JGN-S126E cells were analyzed (Fig. 6). JGN-S126E cells were included to determine whether a negatively charged amino acid could mimic Ser-126 phosphorylation. Following activation of PKC with PDB neither JGN-S126A nor JGN-S126E cells down-regulated their TCR cell surface expression (Fig. 6, A-B) according to the observed lack of CD3γ phosphorylation in these transfectants. The spontaneous and PDB-induced TCR internalization was next analyzed by incubation of cells with 125I-labeled Fab fragments of the TCR monoclonal antibody F101.01(46) for various periods with subsequent measurement of the percentage of internalized acid-resistant Fab fragments. The spontaneous TCR internalization rate of JGN-S126A cells was reduced as compared with that of JGN-WT cells (Fig. 6 C) indicating that the spontaneous TCR internalization was dependent on Ser-126 phosphorylation. In contrast, the spontaneous TCR internalization rate of JGN-S126E cells was higher than that of JGN-WT cells, although not as high as the PDB-induced internalization rate of JGN-WT cells (Fig. 6 C). Incubation of JGN-S126A and JGN-S126E cells with PDB did not increase the TCR internalization rate above the spontaneous internalization rate (data not shown). These results strongly suggested that the TCR internalization rate is dependent on Ser-126 phosphorylation and that introduction of a negatively charged amino acid at the position of the phosphoacceptor group can partially mimic the effect of Ser-126 phosphorylation.

**DISCUSSION**

The present study demonstrated that, although not absolutely required, a basic amino acid (Arg-124) two residues N-terminal to the phosphoacceptor group (Ser-126) was optimal in rendering CD3γ a PKC substrate in vivo. Studies of synthetic peptides have suggested that in some instances PKC prefers arginine to lysine as substrate determinant (40). However, we could not detect any change in the level of CD3γ phosphorylation or TCR down-regulation by substitution of lysine for Arg-124, indicating that it is their common characteristic in form of a positive charge that makes arginine and lysine PKC substrate determinants. Interestingly, substitution of the acidic amino acid glutamic acid for Arg-124 had a stronger inhibitory effect on CD3γ phosphorylation and TCR down-regulation than substitution of an apolar amino acid for Arg-124. This showed that an acidic amino acid located two residues N-terminal to the phosphoacceptor group acted as a negative substrate determinant for PKC. Furthermore, it indicated that Arg-124 is involved in electrostatic contacts with acidic amino acids in the catalytic domain of PKC. This agrees with results obtained by model building of PKC in which Lys-23 (corresponding to CD3γ Arg-124) of the PKC pseudosubstrate makes electrostatic contacts with Asp-470 and Glu-533 in the catalytic domain of PKC (50). Thus, the consequence of the R124E mutation would be a local electrostatic repulsion in contrast to an electrostatic attraction. To our knowledge, this is the first description of the existence of a negative substrate determinant for PKC in vivo. That the inhibitory effect of R124A could be compensated for by increasing the concentration of PDB suggested that, although important for optimal
CD3γ phosphorylation, Arg-124 was not required for the subsequent steps in TCR internalization.

PKC phosphorylates substrates at either serine or threonine. It is still not clear whether PKC prefers serine to threonine although two experiments have pointed in that direction (40, 51). It was therefore of interest to test the effect of the substitution of threonine for Ser-126. From the 32P analyses it was found that S126T-CD3γ was as good a PKC substrate as WT-CD3γ. However, TCR down-regulation was significantly inhibited in JGN-S126T cells. This indicated that threonine is as well accepted as a phosphoacceptor as serine by PKC but that the mechanisms involved in TCR down-regulation following CD3γ phosphorylation, Arg-124 was not required for the subsequent steps in TCR internalization.

**Fig. 5.** A polar amino acid three residues C-terminal to Ser-126 optimizes CD3γ as a substrate for PKC. A, schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the indicated cell lines and a summation of the results from the CD3γ phosphorylation and TCR down-regulation analyses. CD3γ phosphorylation and TCR down-regulation were scored as described in the legend to Fig. 2. B, cells were incubated with different concentrations of PDB for 1 h, and TCR down-regulation was determined by staining with anti-CD3 monoclonal antibodies and flow cytometry comparing MFI of PDB-treated cells with MFI of untreated cells. C, phosphorylation analyses of CD3γ from JGN-WT (lanes 1 and 3) and JGN-Q129A (lanes 2 and 4) cells. D, FACS histograms as described in the legend to Fig. 2.

**Fig. 6.** TCR internalization is dependent on Ser-126 phosphorylation that can be partially mimicked by introduction of a negatively charged amino acid. A, schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the indicated cell lines and a summation of the results from the CD3γ phosphorylation and TCR internalization analyses. CD3γ phosphorylation and PDB-induced TCR internalization were scored as described in the legend to Fig. 2. B, cells were incubated with different concentrations of PDB for 1 h, and TCR down-regulation was determined by staining with anti-CD3 monoclonal antibodies and flow cytometry comparing MFI of PDB-treated cells with MFI of untreated cells. C, cells were incubated with 125I-labeled Fab fragments of the monoclonal antibody F101.01 for the periods indicated. The acid-sensitive and the acid-resistant 125I-labeled Fab fragments were subsequently measured. The percentage of acid-resistant 125I to total 125I was calculated and taken as the percentage of TCR internalized.
CD3γ by molecules involved in receptor internalization. In this process Ser(P)-126, Asp-127, Leu-131, and Leu-132 seem to be important. Whether the pSD-LL motif is recognized as one large motif or phosphorylation of Ser-126 causes a conformational change that exposes the D-LL motif remains to be determined.

Di-leucine or leucine-isoleucine sorting signals have been identified in other molecules, e.g., the mannose 6-phosphate/insulin-like growth factor-II receptor (57, 58), the cation-dependent mannose-6-phosphate receptor (59), the lysosomal integral membrane protein II (60), CD4 (61, 62), and the invariant chain (63, 64). Interestingly, in these molecules an acidic amino acid or a serine is found four and five residues N-terminal to the di-leucine/leucine-isoleucine sequence, exactly corresponding to the position of Ser-126, Asp-127, and Leu-131/132 in CD3γ. This supports the hypothesis that molecules involved in sorting/internalization of receptors with di-leucine-based motifs recognize a motif composed of both acidic amino acids and the di-leucine/leucine-isoleucine sequence. That the spontaneous TCR internalization rate in JGN-S126E cells was higher than that of JGN-WT cells further indicated that the electrostatic effects of Ser-126 phosphorylation play an important role for recognition of the motif by molecules involved in receptor sorting/internalization. The existence of an internalization motif that becomes activated by protein kinase-mediated phosphorylation would afford cells a means to couple receptor-mediated activation with receptor desensitization. Alternatively, for T cells where ligands are scarce, down-regulation of activated receptors could allow additional receptors to be activated, thereby intensifying cell activation, as recently suggested (12).

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