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CD3γ contains a phosphoserine-dependent di-leucine motif involved in down-regulation of the T cell receptor

Jes Dietrich, Xiaohong Hou, Anne-Marie K.Wegener and Carsten Geisler[1]

Institute of Medical Microbiology and Immunology, University of Copenhagen, The Panum Institute, Building 18.3, Blegdamsvej 3C, DK-2200 Copenhagen, Denmark
[1]Corresponding author

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Several cell surface receptors including the T cell receptor (TCR) are phosphorylated and down-regulated following activation of protein kinase C (PKC). Among other substrates the activated PKC in T cells phosphorylates the CD3γ subunit of the TCR. To investigate the role of CD3γ phosphorylation in PKC-mediated TCR down-regulation, point mutated CD3γ cDNA was transfected into the CD3γ-negative T cell line JGN and CD3γ transfectants were analysed. Phosphorylation at S126 but not S123 in the cytoplasmic tail of CD3γ was required for PKC-mediated down-regulation of the TCR. Furthermore, analysis of a series of CD3γ truncation mutants indicated that in addition to S126 phosphorylation a motif C-terminal of S126 was required for TCR down-regulation. Point mutation analyses confirmed this observation and demonstrated that a membrane-proximal di-leucine motif (L131 and L132) in the cytoplasmic tail of CD3γ was required for PKC-mediated TCR down-regulation in addition to phosphorylation at S126. Incubation of T cells in hypertonic medium known to disrupt normal clathrin lattices severely inhibited PKC-mediated TCR down-regulation in non-mutated T cells, indicating that the TCR was down-regulated by endocytosis via clathrin coated pits. Based on the present results and previously published observations on intracellular receptor sorting, a general model for intracellular sorting of receptors containing di-leucine- or tyrosine-based motifs is proposed.

Key words: CD3γ/down-regulation/LL-motif/phosphorylation/TCR

Introduction

The efficient internalization of several transmembrane receptor proteins requires a signal sequence in the cytoplasmic tail of the protein (Vaux, 1992). One group of receptors internalize bound ligand upon interaction thereby delivering nutrients to cells. This group includes the low density lipoprotein receptor (LDLR) and the transferrin receptor (TfR). Studies of LDLR cytoplasmic tail mutants, and of sequence conservation among LDLRs from different species, have led to the proposal that a tyrosine-containing motif NPXY (where X is any amino acid) constitutes a signal for internalization (Davis *et al.*, 1987; Chen *et al.*, 1990). Similarly detailed studies of the TfR have demonstrated that the TfR internalization signal also consists of a critical four amino acid motif, namely YXRF (Collawn *et al.*, 1990). The internalization sequences of both LDLR and TfR most probably adopt a tight turn conformation (Collawn *et al.*, 1990; Ktistakis *et al.*, 1990; Bansal and Gierasch, 1991). Similar tyrosine-based internalization motifs have been described in the mannose 6-phosphate/insulin-like growth factor-II receptor (Man-6-P/IGF-IIR) and in lysosomal acid phosphatase (LAP) (Peters *et al.*, 1990; Carnfield *et al.*, 1991; Eberle *et al.*, 1991).

A second group of receptors transmit activation signals across the membrane upon ligand binding. This group includes receptors for growth factors, hormones and neurotransmitters. Several of these receptors [e.g. the epidermal growth factor receptor (EGFR), the interleukin 2 receptor (IL-2R) and the insulin receptor (IR)] also undergo internalization upon ligand binding (Beguinot *et al.*, 1985; Robb and Greene, 1987; Smith and Jarett, 1988; Hatakeyama *et al.*, 1989; Smith *et al.*, 1991; Duprez *et al.*, 1992). Both the EGFR and IR contain a tyrosine-based internalization motif which is involved in ligand-induced internalization of the receptors (Rajagopalan *et al.*, 1991; Carpentier *et al.*, 1993; C.Chang *et al.*, 1993). The IR motif probably also adopts a tight turn conformation (Chen *et al.*, 1990). Whether internalization plays a role in signal transduction for any of these receptors is unknown. However, internalization may alter the function of a particular receptor system by removing the receptor from the cell surface in a negative feedback fashion and/or by removing the growth factor or hormone from circulation.

The T cell antigen receptor (TCR) belongs to the group of signal transducing receptors. After TCR stimulation, activation signals are transmitted across the membrane (Abraham *et al.*, 1992) and both TCR internalization and antigen unresponsiveness can be induced by specific anti-TCR antibodies (Reinherz *et al.*, 1982; Telerman *et al.*, 1987; Boyer *et al.*, 1991), by supra-optimal doses of antigen (Lamb *et al.*, 1983; Zanders *et al.*, 1983) or by phorbol esters (Ando *et al.*, 1985; Cantrell *et al.*, 1985). This raises the interesting possibility that physiologically important regulation of T cell function may be mediated through control of the level of TCR expression at the cell surface. The TCR expressed on the surface of T cells is a multimeric protein complex composed of at least eight chains assembled from six different subunits. Current data suggest that this complex is composed of four dimers: the clonotypic Ti heterodimer, generally αβ; two CD3 dimers, γε and δε, and the ζ homodimer (Koning *et al.*, 1990; Manolios *et al.*, 1991; Kuhlmann and Geisler, 1993). The disulfide-linked αβ heterodimer is responsible for antigen recognition (Dembic *et al.*, 1986; Saito *et al.*, 1987b) and the activation signals are delivered through the associated CD3 chains (Malissen and Schmitt-Verhulst, 1993). Recent studies have provided definitive evidence for a signal transduction function of the

CD3ε and ζ chains (Irving and Weiss, 1991; Romeo and Seed, 1991; Letourneur and Klausner, 1992a; Wegener *et al.*, 1992; Hermans and Malissen, 1993); however, the specific role of the CD3γ and δ chains in TCR function is still unknown. After TCR stimulation, a number of cellular proteins become phosphorylated on tyrosine residues, among them being ζ (Baniyash *et al.*, 1988), ZAP-70 (Chan *et al.*, 1991), the proto-oncogene *vav* (Margolis *et al.*, 1992) and phospholipase Cγ1 (PLCγ1) (Park *et al.*, 1991; Secrist *et al.*, 1991; Weiss *et al.*, 1991). Tyrosine phosphorylation of PLCγ1 has been shown to augment its enzymatic activity, resulting in the production of inositol 1,4,5-trisphosphate and diacylglycerol (Nishibe *et al.*, 1990). These two second messengers are responsible for an increase in intracellular Ca^{2+} concentration and an activation of protein kinase C (PKC), respectively (Berridge and Irvine, 1984; Imboden and Stobo, 1985). Among other substrates the activated PKC phosphorylates the CD3γ chain at serine 126 (S126) and maybe at S123 (Davies *et al.*, 1987; Alexander *et al.*, 1992). These serines are located in a basic sequence 10 and seven residues from the cytoplasmic face of the plasma membrane, respectively [numbering the CD3γ amino acids according to Krissansen *et al.* (1986)].

PKC-mediated phosphorylation has been implicated in the internalization of numerous receptors including the EGFR, CD4, TfR, and the asialoglycoprotein receptor (ASGPR) (Klausner *et al.*, 1984; McCaffrey *et al.*, 1984; Beguinot *et al.*, 1985; Fallon and Schwartz, 1988; Shin *et al.*, 1991). It has been demonstrated that PKC phosphorylates the EGFR at T654 (Hunter *et al.*, 1984). This threonine is in a basic sequence nine residues from the cytoplasmic face of the plasma membrane very similar to S123 and S126 in the CD3γ tail. Studies in which substitution of A654 for T654 was performed demonstrated that phosphorylation of T654 was required for PKC-mediated down-regulation of the EGFR (Lin *et al.*, 1986). Likewise, phosphorylation of the membrane-proximal S408 in the cytoplasmic tail of CD4 is required for PKC-mediated internalization of CD4 (Shin *et al.*, 1991). Several studies have demonstrated that activation of PKC leads to internalization and down-regulation of the TCR (Ando *et al.*, 1985; Cantrell *et al.*, 1985, 1987, 1989; Davies *et al.*, 1987; Minami *et al.*, 1987), and it has been suggested that this is mediated through CD3γ phosphorylation (Cantrell *et al.*, 1985, 1989; Krangel, 1987; Minami *et al.*, 1987; Boyer *et al.*, 1991). However, the question of whether it is the phosphorylation of the CD3γ chain or another undefined cellular substrate for PKC that leads to PKC-mediated internalization of the TCR has not yet been determined. In this study, we demonstrate that phosphorylation of S126 in the cytoplasmic tail of CD3γ is required for PKC-mediated TCR down-regulation. Furthermore, we define a di-leucine motif in the cytoplasmic tail of CD3γ, which, in addition to S126 phosphorylation, is required for PKC-mediated down-regulation of the TCR.

Results

PKC-mediated TCR down-regulation is intact in JGN-WT cells

The production of the CD3γ-negative T cell variant JGN (Geisler, 1992) allowed us to study the consequences of various mutated CD3γ chains in TCR function. JGN cells do not express the TCR at the cell surface but upon transfection of wild-type CD3γ cDNA into JGN cells they become TCR cell surface positive (Figure 1F). As the JGN T cell variant was produced by γ-irradiation and cloning (Geisler, 1992), we were concerned whether the internalization machinery in JGN was intact. Wild-type CD3γ cDNA was transfected into JGN cells and TCR positive clones (JGN-WT) were selected. PKC-mediated down-regulation of the TCR by these clones was compared with down-regulation of the TCR by the unmutated Jurkat cell line J76. TCR down-regulation was measured by binding of anti-CD3 or anti-Vβ8 monoclonal antibodies as previous studies have demonstrated that PKC-mediated internalization of the TCR correlates with the reduction in TCR cell surface expression (Minami *et al.*, 1987; Boyer *et al.*, 1991). Incubation of JGN-WT and J76 cells for 1 h with different concentrations of the phorbol esters phorbol 12,13-dibutyrate (PDB) or phorbol 12-myristate 13-acetate (PMA) demonstrated that JGN-WT and J76 cells were equally sensitive to these PKC activators with respect to TCR down-regulation (Figure 1A and B). That the reduction of anti-CD3 binding was not caused by an altered affinity between the monoclonal antibody and its epitope following phorbol ester treatment was demonstrated by analysing anti-Vβ8 binding in parallel with binding of two different anti-CD3 monoclonal antibodies (Figure 1A and C). TCR down-regulation could be recorded already after 2 min of PDB treatment and reached a maximum after ~60 min (Figure 1D). TCR down-regulation was partially inhibited at 18°C and completely inhibited at 4°C (Figure 1E). From these data it was concluded that the mechanisms behind down-regulation of the TCR were not affected by the procedures performed in order to generate JGN cells.

PKC induced TCR down-regulation is probably mediated by endocytosis via clathrin coated pits

Previous studies have demonstrated that TCR internalization induced by monoclonal anti-TCR antibodies is mediated via clathrin coated pits (Telerman *et al.*, 1987; Boyer *et al.*, 1991). However, the pathway of TCR entry following PKC-mediated internalization has not been described. Incubation of cells in hypertonic medium disrupts normal clathrin lattices (Heuser and Anderson, 1989; Hansen *et al.*, 1993) and thereby inhibits endocytosis via clathrin coated pits whereas clathrin independent endocytosis is largely left unaffected (Oka *et al.*, 1989; Hansen *et al.*, 1993). Incubation of JGN-WT cells in hypertonic medium containing 0.45 M sucrose inhibited PKC-mediated TCR down-regulation (Figure 2). Replacing the cells in isotonic medium partially reversed the inhibition of TCR down-regulation (Figure 2) in agreement with the observation of recreation of normal clathrin lattices following replacement of cells from hypertonic to isotonic media (Heuser and Anderson, 1989). Likewise, acidification of the cytosol, also known to inhibit endocytosis via clathrin coated pits (Heuser, 1989; Hansen *et al.*, 1993), inhibited TCR down-regulation but to a lesser extent than incubation of cells in hypertonic medium (data not shown). Similar results were obtained with J76 cells. These results indicated that following PKC activation the TCR is endocytosed via clathrin coated pits.

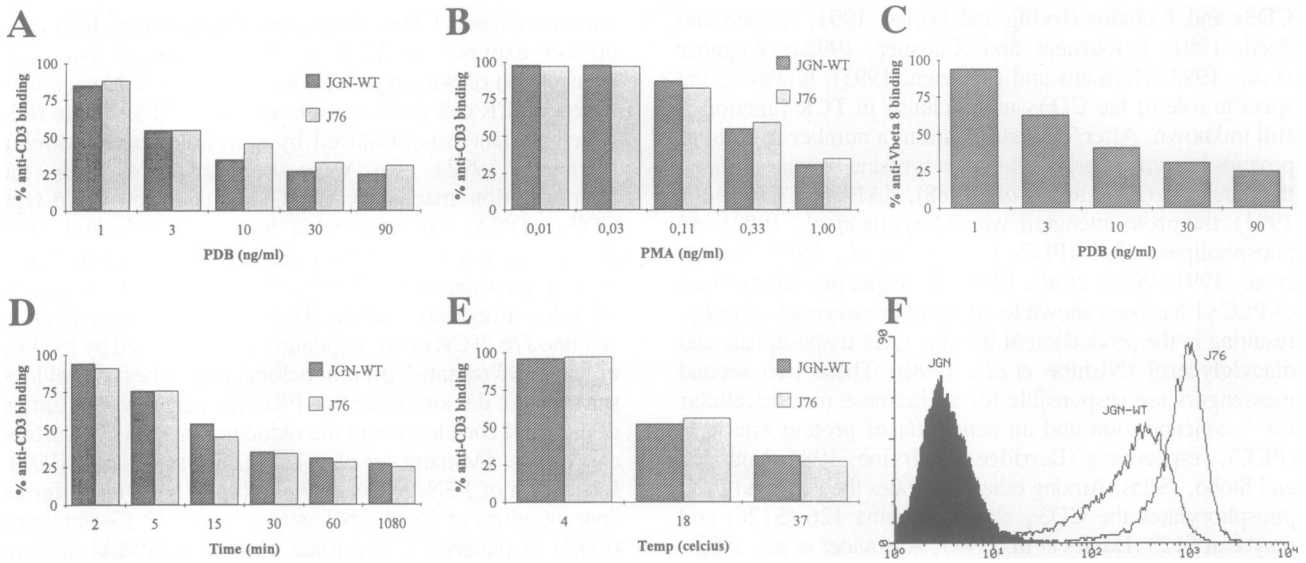


Fig. 1. PKC-mediated down-regulation of the TCR is intact in JGN-WT cells. (**A**) JGN-WT and J76 cells were incubated with different concentration of PDB for 1 h and TCR down-regulation was determined by staining with anti-CD3 monoclonal antibodies and flow cytometry comparing the mean fluorescence intensity (MFI) of PDB-treated cells with MFI of untreated cells. (**B**) JGN-WT and J76 cells were incubated with different concentrations of PMA for 1 h and TCR down-regulation was determined by staining with anti-CD3 monoclonal antibodies and flow cytometry comparing MFI of PMA-treated cells with MFI of untreated cells. (**C**) JGN-WT cells were incubated with different concentration of PDB for 1 h and TCR down-regulation was determined by staining with anti-Vβ8 monoclonal antibodies and flow cytometry comparing MFI of PDB-treated cells with MFI of untreated cells. (**D**) JGN-WT and J76 cells were incubated with PDB (20 ng/ml) for different time intervals 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. (**E**) JGN-WT and J76 cells were incubated with PDB (20 ng/ml) for 1 h at either 4, 18 or 37°C 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. (**F**) FACS histogram of the CD3γ-negative cell line JGN, JGN transfected with wild-type CD3γ cDNA (JGN-WT) and the original Jurkat cell line J76. The cells were stained with phycoerythrin conjugated anti-CD3 monoclonal antibodies. The abscissa gives the fluorescence intensity in a logarithmic scale. The ordinate gives the relative cell number.

Phosphorylation of CD3γ S126 is required for PKC-mediated TCR down-regulation

Early reports demonstrated that PKC activators caused a down-regulation of the TCR (Ando *et al.*, 1985) and a concomitant phosphorylation of CD3γ (Cantrell *et al.*, 1985). Later it was demonstrated that CD3γ was also phosphorylated after physiological stimulation of T cells with specific antigen–MHC complexes, with mitogens, or with anti-CD3 monoclonal antibodies (Cantrell *et al.*, 1987; Alexander *et al.*, 1992). To examine the role of CD3γ serine phosphorylation in PKC-mediated down-regulation of the TCR, the serines in the cytoplasmic tail of CD3γ at positions 123 and 126 were mutated to valines resulting in three different constructs, SV123, SV126 and SV123/126 (Figure 3A). These constructs were transfected into JGN cells and TCR-positive clones were isolated. After loading cells with ^{32}P the ability of the CD3γ chain to become phosphorylated was measured. As demonstrated in Figure 3B the CD3γ chains from JGN-WT and JGN-SV123 were phosphorylated following PKC activation, whereas the CD3γ chains from JGN-SV126 and JGN-SV123/126 cells were not. These results indicated that the predominant substrate for PKC in the CD3γ tail is S126.

The ability of the CD3γ transfected cells to down-regulate the TCR following activation of PKC was analysed. As shown in Figure 3C and D, JGN-SV123 cells down-regulated the TCR as efficiently as JGN-WT cells, whereas TCR down-regulation in JGN-SV126 and JGN-SV123/126 cells was strongly inhibited but not totally absent. At high PDB concentrations (90 ng/ml) a 10–15% reduction in TCR

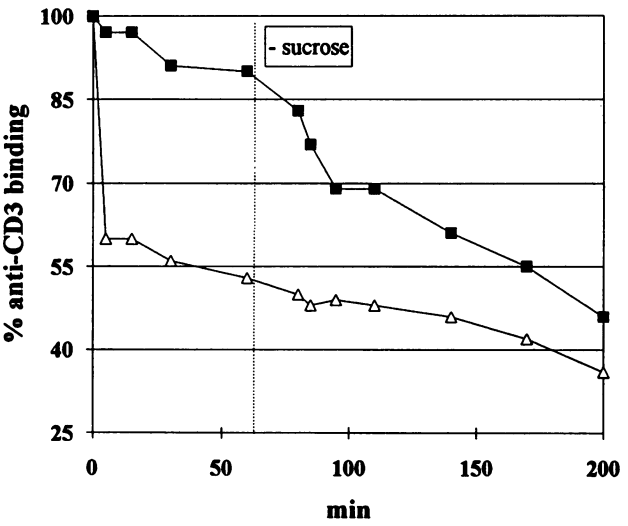


Fig. 2. PKC-mediated TCR down-regulation is inhibited by incubation in hypertonic medium. JGN-WT cells were incubated with PMA (20 ng/ml) in DMEM with (■) or without (△) 0.45 M sucrose. Following 60 min of incubation the cells were washed twice in DMEM and resuspended in DMEM containing PMA (20 ng/ml) without sucrose. At indicated times cell aliquots were transferred to ice-cold PBS, 2% FCS, 0.1% NaN_3 and TCR down-regulation was subsequently determined by staining with anti-CD3 monoclonal antibodies and flow cytometry.

expression in both JGN-SV126 and JGN-SV123/126 cells was seen in several experiments. These results suggested that phosphorylation of CD3γ at S126 but not at S123 is required

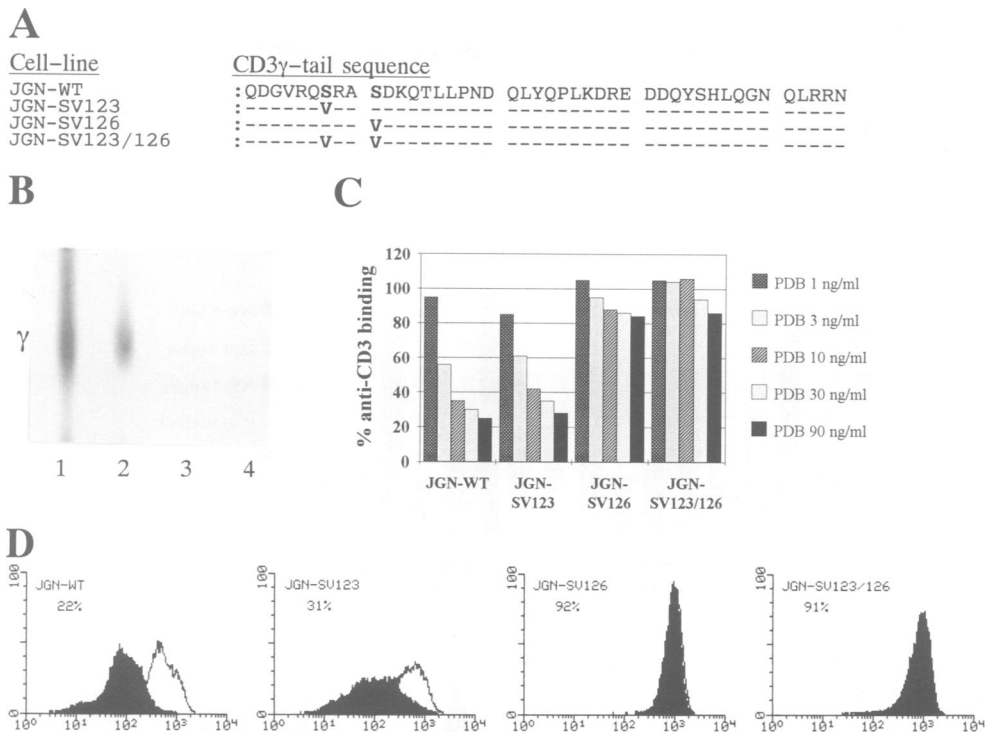


Fig. 3. S126 is required for PKC-mediated down-regulation of the TCR. (**A**) Schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the JGN-WT, JGN-SV123, JGN-SV126 and JGN-SV123/126 cell lines. (**B**) Phosphorylation analysis of CD3γ from JGN-WT (lane 1), JGN-SV123 (lane 2), JGN-SV126 (lane 3) and JGN-SV123/126 (lane 4) cells. (**C**) JGN-WT, JGN-SV123, JGN-SV126 and JGN-SV123/126 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. (**D**) FACS histograms of untreated cells (white) and cells treated with PDB (30 ng/ml) (black) for 1 h. The cell line and the percent anti-CD3 binding following PDB treatment are given in the upper left corner of each histogram. The ordinate gives the relative cell number. The abscissa gives the fluorescence intensity in a logarithmic scale in arbitrary units. Mean fluorescence intensity of the cell lines stained with irrelevant monoclonal antibodies varied between two and five arbitrary units (data not shown).

for normal down-regulation of the TCR following activation of PKC.

The tyrosine-based motif including Y138 in the CD3γ tail is not required for TCR down-regulation

In several receptors, a tyrosine-based motif required for receptor internalization has been described (Davis *et al.*, 1987; Chen *et al.*, 1990; Collawn *et al.*, 1990; Carnfield *et al.*, 1991; Peters *et al.*, 1990; Eberle *et al.*, 1991). Furthermore, studies of chimeric proteins consisting of the external and transmembrane parts of the human Tac antigen (IL-2R α chain) fused to fragments of mouse CD3γ or δ cytoplasmic tails have demonstrated a tyrosine-based motif (YQPLK) including mouse CD3γ Y137, corresponding to human CD3γ Y138, which is sufficient to induce both internalization and delivery to lysosomes of Tac (Letourneur and Klausner, 1992b). To determine whether the tyrosine-based motif including Y138 in the CD3γ tail was required for PKC-mediated down-regulation of the TCR, the following constructs containing the external, transmembrane and cytoplasmic parts of CD3γ with truncation at P140 [tP140, corresponding to truncation of the CD3γ tail encoded by exon six (Saito *et al.*, 1987a; Tunnacliffe *et al.*, 1987)], truncation at Q139 and mutation of Y138 to F (tYF138) or Y138 to A (tYA138), and truncation at Y138 (tY138) (Figure 4A) were transfected into JGN cells. TCR cell surface positive clones were isolated and their ability to down-regulate the TCR following activation of PKC was determined. As shown in Figure 4B and C all of the cells down-regulated the TCR as efficiently as JGN-WT cells. Furthermore, the truncations and mutations did not influence the ability of the CD3γ chain to become phosphorylated following PKC activation (data not shown).

Evidence for an internalization motif including L132

Since the tyrosine motif was not required for PKC-mediated TCR down-regulation we suspected that a motif further N-terminal in the CD3γ cytoplasmic tail was involved in the association between the TCR and the internalization machinery. Therefore, we next attempted to localize internalization sequence(s) further membrane-proximal in the cytoplasmic tail of CD3γ by successive truncations from Y138 (Figure 5A). Truncations up to L132 did not affect the ability of the transfectants to down-regulate the TCR after PKC activation (Figure 5B and C). However, truncations from L132 and on strongly inhibited TCR down-regulation. Phosphorylation analysis demonstrated that the ability to phosphorylate the CD3γ chain following PKC activation was preserved in JGN-tL132 and JGN-tL131 cells, whereas truncation at D127 and A125 abolished CD3γ phosphorylation (data not shown). These results indicated that in addition to phosphorylation at S126 an internalization motif including L132 was required for PKC-mediated down-regulation of the TCR.

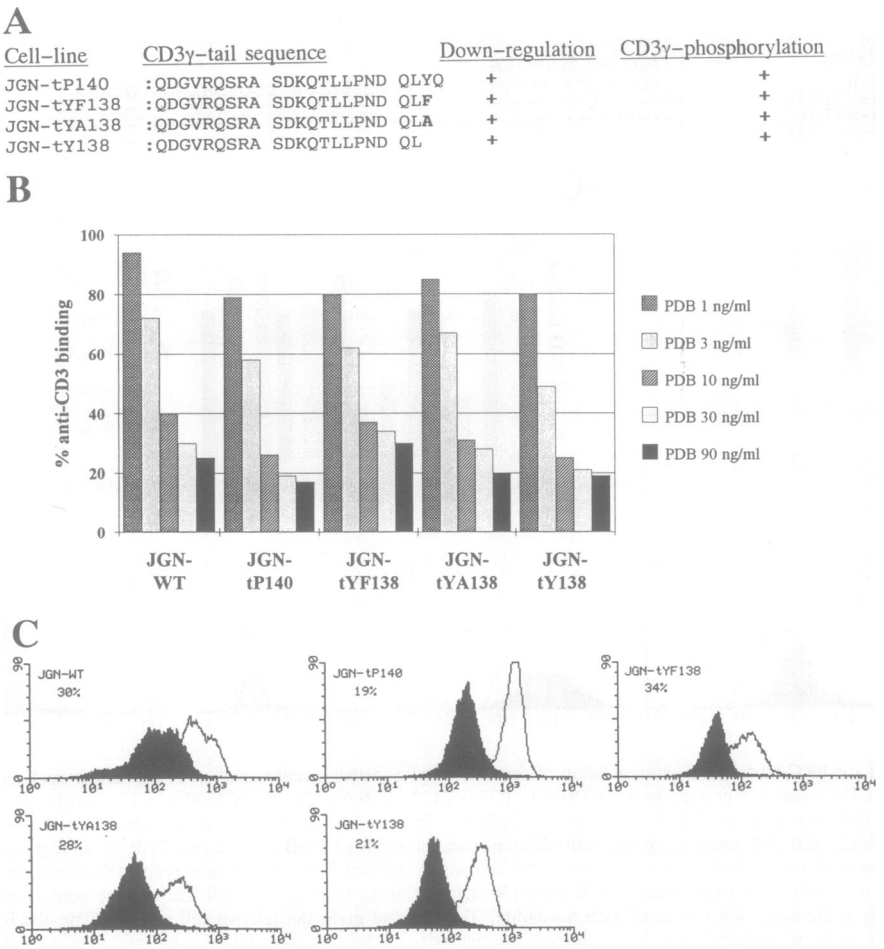


Fig. 4. The Y138-based motif is not required for PKC-mediated TCR down-regulation. (**A**) Schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the JGN-tP140, JGN-tYF138, JGN-tYA138 and JGN-tY138 cell lines and the results from down-regulation and CD3γ phosphorylation analysis. (**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**) FACS histograms of untreated cells (white) and cells treated with PDB (30 ng/ml) (black) for 1 h. The cell line and the percent anti-CD3 binding following PDB treatment are given in the upper left corner of each histogram. The ordinate gives the relative cell number. The abscissa gives the fluorescence intensity in a logarithmic scale in arbitrary units. Mean fluorescence intensity of the cell lines stained with irrelevant monoclonal antibodies varied between two and five arbitrary units (data not shown).

Localization of a di-leucine-based internalization signal sequence

Studies of Tac-CD3γ and Tac-CD3δ chimeras have demonstrated that, in addition to the tyrosine-based motif, a di-leucine-based motif existed in fragments of mouse CD3γ and δ tails that, independently of the tyrosine-based motif, was sufficient to induce both endocytosis and delivery to lysosomes of Tac (Letourneur and Klausner, 1992b). The critical residues in the di-leucine-based motif included L130 and L131 in the mouse CD3γ tail, corresponding to L131 and L132 in the human CD3γ tail. Based on these observations and since the present truncation analysis indicated that L132 was required for down-regulation of intact TCR, we next wanted to examine whether L131 and L132 constituted parts of an internalization motif in full-length CD3γ. To analyse the role of L131 and L132 in PKC-mediated TCR down-regulation, the constructs LA131, LA132 and LA131/132 containing the entire CD3γ with substitution of alanine for leucine at positions 131, 132 and 131+132, respectively (Figure 6A), were transfected into JGN. TCR positive clones were isolated and the ability to down-regulate the TCR following PKC activation was analysed. As demonstrated in Figure 6B and C neither JGN-LA131, JGN-LA132 nor JGN-LA131/132 cells down-regulated the TCR after PKC activation indicating that both L131 and L132 were individually required for TCR down-regulation. Phosphorylation analysis demonstrated that in both JGN-LA131, JGN-LA132 and JGN-LA131/132 cells the CD3γ chain was phosphorylated following PKC activation although TCR down-regulation was abolished (Figure 6). These results indicated that L131 and L132 constitute parts of a phosphoserine-dependent internalization motif in the complete TCR.

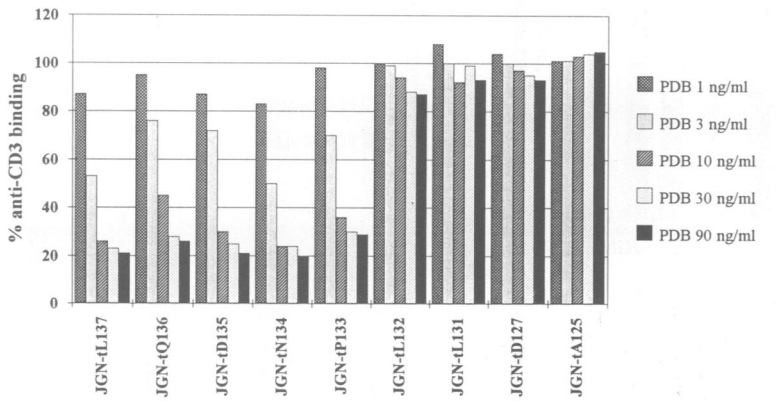
Discussion

Experiments have shown that phosphorylation of receptors can lead to down-regulation of receptor activity in at least two ways: by internalization and by desensitization (Hunter *et al.*, 1984; Hausdorff *et al.*, 1991; Shin *et al.*, 1991;

A

Cell-line	CD3γ-tail sequence	Down-regulation	CD3γ-phosphorylation
JGN-tL137	:QDGVRQSRA SDKQTLLPND Q	+	+
JGN-tQ136	:QDGVRQSRA SDKQTLLPND	+	+
JGN-tD135	:QDGVRQSRA SDKQTLLPN	+	+
JGN-tN134	:QDGVRQSRA SDKQTLLP	+	+
JGN-tP133	:QDGVRQSRA SDKQTLL	+	+
JGN-tL132	:QDGVRQSRA SDKQTL	−	+
JGN-tL131	:QDGVRQSRA SDKQT	−	+
JGN-tD127	:QDGVRQSRA S	−	−
JGN-tA125	:QDGVRQSR	−	−

B



C

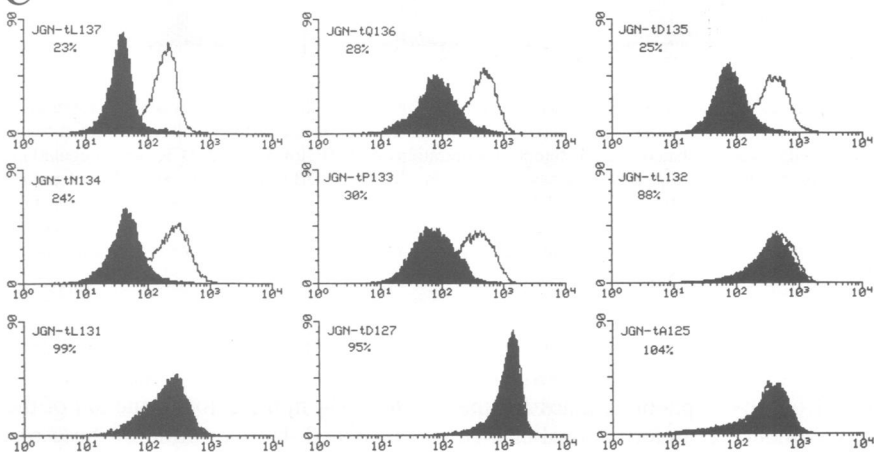


Fig. 5. Truncation analysis demonstrating the requirement of L132 for PKC-mediated TCR down-regulation. (**A**) Schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the indicated cell lines and the results from down-regulation and CD3γ phosphorylation analysis. (**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**) FACS histograms of untreated cells (white) and cells treated with PDB (30 ng/ml) (black) for 1 h. The cell line and the percent anti-CD3 binding following PDB treatment are given in the upper left corner of each histogram. The ordinate gives the relative cell number. The abscissa gives the fluorescence intensity in a logarithmic scale in arbitrary units. Mean fluorescence intensity of the cell lines stained with irrelevant monoclonal antibodies varied between two and five arbitrary units (data not shown).

Countaway *et al.*, 1992; Pelchen-Matthews *et al.*, 1993). Activation of T cells leads among other events to the phosphorylation of the CD3γ chain. The physiological relevance of CD3γ chain phosphorylation for TCR function is unknown. In the present study, by introducing point mutations at residues 123 and 126 in the CD3γ cytoplasmic tail, we demonstrated that phosphorylation of S126 is required for PKC-mediated TCR down-regulation, whereas phosphorylation of S123 is not required. These results are in agreement with results obtained by comparing tryptic peptide analyses of CD3γ chains phosphorylated *in vivo* with various synthetic peptides, corresponding to portions of the cytoplasmic tail of CD3γ that had been phosphorylated *in vitro* using purified bovine PKC (Davies *et al.*, 1987). However, from our results, we cannot exclude the possibility that S123 is phosphorylated and subsequently rapidly dephosphorylated following PKC activation as previously suggested (Alexander *et al.*, 1992). In fact such events could be responsible for the minimal TCR down-regulation observed in JGN-SV126 cells. A minimal TCR down-regulation was also observed in JGN-SV123/126 cells. This may be due to a rapid phosphorylation and dephosphorylation of the CD3δ chain which contains both the serine residue corresponding to CD3γ S123 and the di-leucine motif but

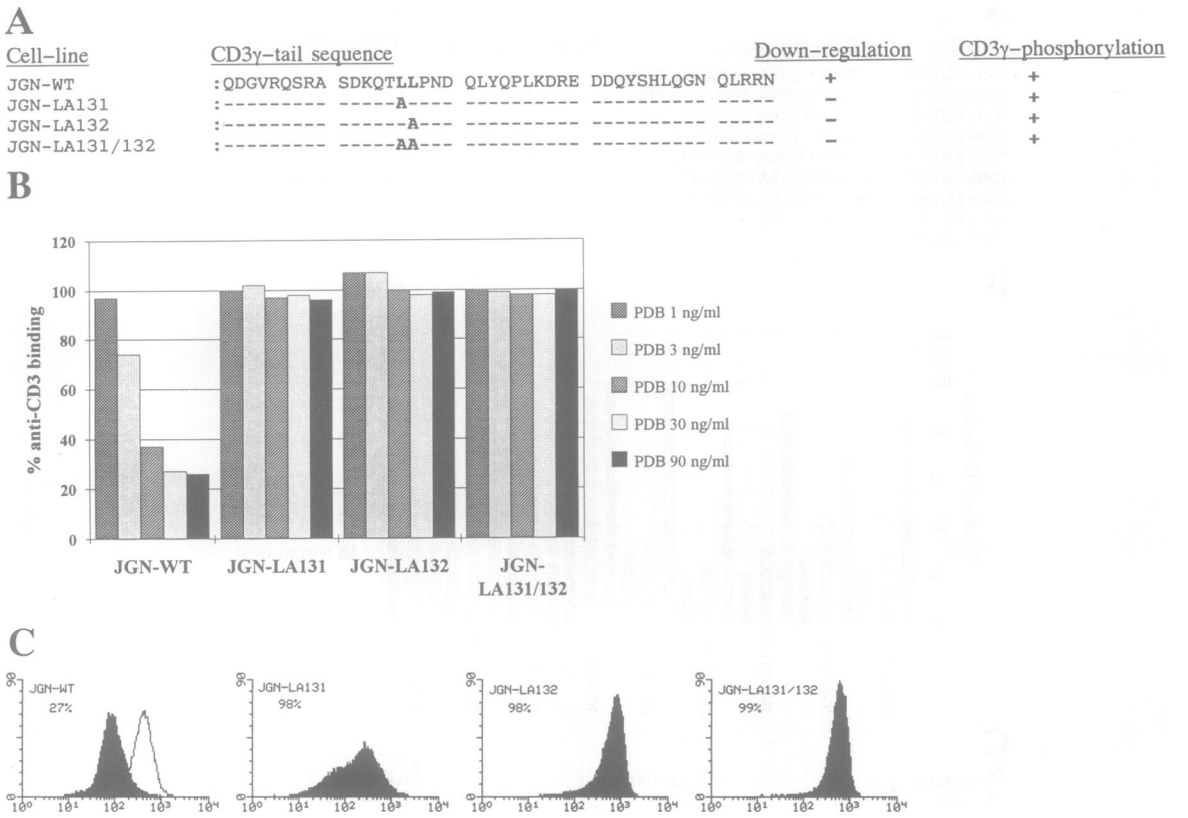


Fig. 6. L131 and L132 are individually required for PKC-mediated down-regulation of the TCR. (**A**) Schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the indicated cell lines and the results from down-regulation and CD3γ phosphorylation analysis. (**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**) FACS histograms of untreated cells (white) and cells treated with PDB (30 ng/ml) (black) for 1 h. The cell line and the percent anti-CD3 binding following PDB treatment are given in the upper left corner of each histogram. The ordinate gives the relative cell number. The abscissa gives the fluorescence intensity in a logarithmic scale in arbitrary units. Mean fluorescence intensity of the cell lines stained with irrelevant monoclonal antibodies varied between two and five arbitrary units (data not shown).

lacks the serine residue corresponding to CD3γ S126 (Figure 7A) (Krissansen *et al.*, 1986). Another possibility is that the substitution of two hydrophobic valines for the polar S123 and S126 may induce a conformational change of the CD3γ chain that slightly mimics the conformational change induced by S126 phosphorylation. PKC-mediated TCR down-regulation may be mediated by at least two mechanisms: by increasing the rate of TCR internalization as previously suggested (Minami *et al.*, 1987; Boyer *et al.*, 1991) and by decreasing the rate of TCR recycling to the plasma membrane. In addition to increasing the internalization rate, the phosphorylated CD3γ could be responsible for retaining the TCR inside the cell, a mechanism described for the phosphorylated ASPGR (Fallon and Schwartz, 1988). Although it seems unlikely, we cannot exclude the possibility that TCRs containing mutations of S126 and of the di-leucine motif in the CD3γ chain were in fact internalized and subsequently very rapidly recycled to the cell surface. However, we can conclude that both S126 and the di-leucine motif are required for PKC-mediated TCR down-regulation.

In parallel to the requirement of S126 phosphorylation of the CD3γ tail for PKC-mediated TCR down-regulation, at least two other receptors have been described which require phosphorylation of membrane-proximal serine or threonine residues for PKC-mediated internalization. Mutation of S408 to A408 in the cytoplasmic tail of CD4 and mutation of T654 to A654 in the cytoplasmic tail of the EGFR abolish PKC-mediated internalization of these receptors (Hunter *et al.*, 1984; Lin *et al.*, 1986; Shin *et al.*, 1991). Both of these receptors are endocytosed via clathrin coated pits (Beguinot *et al.*, 1985; Shin *et al.*, 1991; Pelchen-Matthews *et al.*, 1993). Likewise the TCR is endocytosed via clathrin coated pits following incubation with anti-TCR monoclonal antibodies (Telerman *et al.*, 1987; Boyer *et al.*, 1991) and this is probably also the pathway of TCR entry following PKC activation as indicated by the present experiments in which cells were incubated in hypertonic medium.

The cytoplasmic tails of several receptors have been described to contain motifs which determine the intracellular fate and endocytosis of the receptors. Most of these motifs contain a tyrosine and some of them are predicted to adopt a tight turn conformation (Davis *et al.*, 1987; Chen *et al.*, 1990; Collawn *et al.*, 1990; Ktistakis *et al.*, 1990; Peters *et al.*, 1990; Bansal and Gierasch, 1991; Carnfield *et al.*, 1991; Eberle *et al.*, 1991). Detailed analysis of fragments of the cytoplasmic tails of mouse CD3γ and δ fused to the external and transmembrane parts of human Tac have demonstrated two motifs which independently were sufficient to induce both internalization and delivery to lysosomes of

A

Membrane proximal/phospho-serine -threonine dependent di-leucine based internalization motifs:

```
CD3γ : Membrane|QDGVRQSRASDKQTLLPN--      → Functional domain (YX2LX7YX2L)
CD3δ : Membrane|HETGRLSGAADTQALLRN--      → Functional domain (YX2LX7YX2L)
CD4  : Membrane|CRHRRRQAERMSQIKRRLLSE--   → lck binding domain
IFNγR: Membrane|FYIKKINPLKEKSIILPKLISV--  → Functional domain (YDKPH)
EGFR : Membrane|RRRHIVRLRTLRRLLQE--       → PTK domain
```

Membrane distal/adaptor accessible di-leucine based sorting/internalization motifs:

```
Man-6-P/IGF-IIR : Membrane|----------------------------EDLLHV163STOP
CD-MPR          : Membrane|----------------------------DHLLPM67STOP
LIMP II         : Membrane|RGQGSTDEGTADERAPLIRT STOP
Invariant chain : Membrane|RSCKSEPAGPRRGLMPLQENNSILDRQDDM STOP
ASPGR (H1)      : Membrane|MTKEYQDLQHLDNEESDHHQLRKGPPPPQPLLQRLCSGPR STOP
```

B

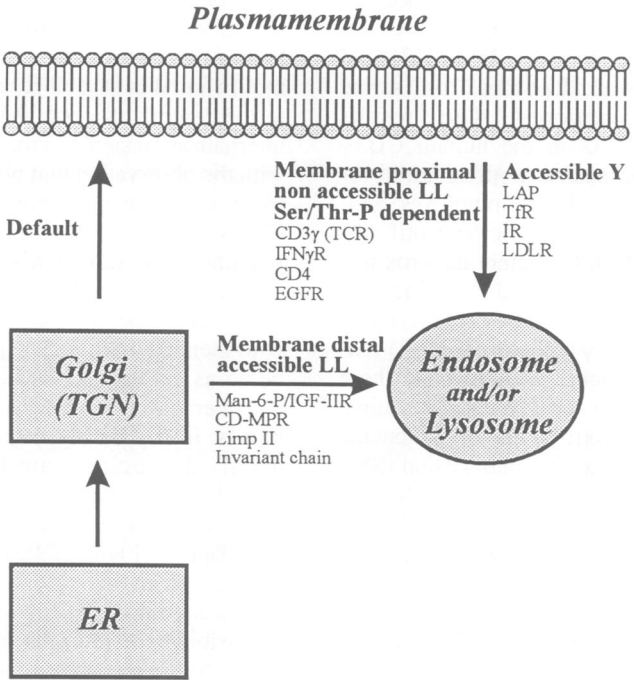


Fig. 7. (**A**) Amino acid sequences of known and possible di-leucine-based internalization/sorting motifs. Amino acid sequences of fragments or the entire cytoplasmic tail (LIMP II, invariant chain and ASPGR H1 subunit) of receptors containing known (CD3γ CD4, IFNγR, Man-6-P/IGF-IIR, CD-MPR and invariant chain) and possible (CD3δ, EGFR, LIMP II and the H1 subunit of ASPGR) di-leucine-based internalization/sorting motifs or variations (LI: IFNγR and LIMP II; IL: invariant chain) of this motif. The di-leucine-based motifs are underlined. The serine and threonine residues which become phosphorylated and are known to be required for PKC-mediated internalization are in bold and underlined. Serine residues which may be phosphorylated following PKC activation are underlined. The phosphoserine/threonine-dependent di-leucine motifs are all followed by known or putative functional domains, whereas the adaptor accessible di-leucine motifs all are located in the C-terminal end of the receptors as indicated. The amino acid number from the cytoplasmic face of the plasma membrane of the C-terminal amino acid in Man-6-P/IGF-IIR and CD-MPR is indicated. (**B**) Proposed general flow diagram for receptors containing different sorting/internalization motifs. Receptors containing membrane-distal adaptor accessible di-leucine motifs (Man-6-P/IGF-IIR, CD-MPR, Limp II and invariant chain) are transported directly from the trans-Golgi network (TGN) to lysosomes/endosomes, whereas receptors with membrane-proximal phosphoserine-threonine-dependent di-leucine motifs [CD3γ (TCR), CD4, IFNγR and EGFR] are transported via the default pathway to the plasma membrane. Following serine/threonine phosphorylation the phosphoserine/threonine-dependent di-leucine motifs become accessible for adaptors leading to internalization and transport of the receptors to lysosomes/endosomes. Receptors containing a tyrosine motif are not recognized in the TGN (Pearse, 1988; M.Chang *et al.*, 1993; Robinson, 1993) but are transported by the default pathway to the plasma membrane. In the plasma membrane the tyrosine motif is recognized (either spontaneously or following ligand binding and possible receptor dimerization) by adaptors leading to receptor internalization.

the chimeras (Letourneur and Klausner, 1992b). One of these motifs was tyrosine-based including Y137 corresponding to human CD3γ Y138. In contrast to these observations, our results demonstrated that the tyrosine-based motif including human CD3γ Y138 is neither required nor sufficient for PKC-mediated down-regulation of complete TCR complexes. These observations may seem conflicting; however, it is possible that an isolated fragment of the CD3γ tail adopts a different conformation than the full-length CD3γ tail in the complete TCR. The human CD3γ Y138 forms part of the $YX_2LX_7YX_2L$ motif found in several receptor associated subunits (Reth, 1989; Wegener *et al.*, 1992). This motif has been found to be critical for signal transduction by the CD3ε and ζ chains (Letourneur and Klausner, 1992a; Wegener *et al.*, 1992; A.-M.K.Wegener and B.Malissen, unpublished data), and probably plays a similar role in CD3γ. The di-leucine motif and the $YX_2LX_7YX_2L$ motif are separated by the structure-breaking [both α-helix and β-sheet (Chou and Fasman, 1974)] amino acids proline and asparagine, further supporting the idea that these two motifs exist in different secondary structures serving different functions.

The other internalization/sorting motif described by Letourneur and Klausner (1992b), included mouse CD3γ L130 and L131 corresponding to human CD3γ L131 and L132. Whether this motif was dependent on serine phosphorylation was not mentioned. Our results demonstrated that L131 and L132 in the human CD3γ cytoplasmic tail constituted a di-leucine motif required for the down-regulation of complete TCR complexes and, furthermore, it was shown that this di-leucine motif was dependent on phosphorylation of S126. Membrane-proximal internalization motifs based on di-leucine or isoleucine–leucine have been described in CD4 (LL) (Shin *et al.*, 1991) and in the interferon γ receptor (IFNγR) (LI) (Farrar and Schreiber, 1993) (Figure 7A). In CD4 these leucines are located at residues 413 and 414 just C-terminal of S408 and the function of this motif is, like the di-leucine motif in CD3γ, dependent on serine phosphorylation (Shin *et al.*, 1991). Whether the leucine–isoleucine internalization motif which closely follows a serine residue in the IFNγR is phosphoserine-dependent has not been described, but it is possible since the cytoplasmic tail of IFNγR is phosphorylated at serine residues following ligand binding and PKC activation (Hershey *et al.*, 1990; Mao *et al.*, 1990). Interestingly, the T654 in the EGFR required for PKC-mediated internalization of the EGFR is very closely followed by a di-leucine sequence (Figure 7A). To our knowledge, the role of these leucines in PKC-mediated EGFR internalization has not been described.

Membrane-distal di-leucine sorting signals have been identified in the mannose 6-phosphate/insulin-like growth factor-II receptor (Man-6-P/IGF-IIR) and the cation-dependent mannose-6-phosphate receptor (CD-MPR) (Johnson and Kornfeld, 1992a,b; Chen *et al.*, 1993). These receptors bind acid hydrolases in the Golgi apparatus. The ligand–receptor complexes are then concentrated in clathrin coated pits and bud off from the Golgi in coated vesicles that transport the complex to a prelysosomal compartment. The di-leucine motifs of the Man-6-P/IGF-IIR (EDLLHV) and the CD-MPR (DHLLPM) are both located at the very C-terminal end of the cytoplasmic tail (Figure 7A). Deletion of these motifs resulted in an impaired sorting function, indicating that the di-leucine-containing sequence represents a motif for mediating interaction with Golgi adaptor proteins (Johnson and Kornfeld, 1992a,b). In contrast to the membrane-proximal di-leucine motifs in CD3γ and CD4, the membrane-distal di-leucine motifs in Man-6-P/IGF-IIR and the CD-MPR are independent of serine and/or threonine phosphorylation (Johnson and Kornfeld, 1992a,b). Recent experiments have revealed an isoleucine–leucine motif in the invariant chain (Pieters *et al.*, 1993). As in the Man-6-P/IGF-IIR and CD-MPR receptors this motif is found in the membrane-distal part of the cytoplasmic tail of the invariant chain and functions as a Golgi sorting signal involved in the transport of the invariant chain from the Golgi to the endosomes (Pieters *et al.*, 1993). Possible adaptor accessible di-leucine-based sorting/internalization motifs or variants of this motif are also found in the cytoplasmic tail of the lysosomal integral membrane protein LIMP II (Vega *et al.*, 1991) and in the constitutively endocytosed H1 subunit of the ASPGR (Fuhrer *et al.*, 1991).

It is interesting that di-leucine-based motifs are found to function both as an internalization signal in the plasma membrane and as a sorting signal in the Golgi membrane. In contrast to this, the primary function of tyrosine-based motifs found in several receptors, but not in the CD3γ chain in the context of a complete TCR, seems only to be as an internalization signal in the plasma membrane, in agreement with the observation that plasma membrane adaptors but not Golgi membrane adaptors bind the tyrosine-based motifs (Pearse, 1988; M.Chang *et al.*, 1993; Robinson, 1993). It remains to be shown whether the di-leucine-based motifs interact directly with plasma and Golgi membrane adaptors and whether different types of adaptors are able to interact with identical motifs. The possibility exists that there could be an as yet undescribed component which is involved in the interaction between adaptors and the di-leucine-based motifs. It may be suggested that, in contrast to membrane-distal di-leucine motifs, the membrane-proximal phosphoserine-dependent di-leucine-based internalization motifs in resting cells with low PKC activity are inaccessible for adaptors (Figure 7B). Receptor-mediated cell activation leading to an increase in PKC activity and thereby to serine/threonine phosphorylation subsequently results in accessibility of phosphoserine-dependent di-leucine-based motifs for their adaptors and down-regulation of the receptor (Figure 7B). This mechanism may provide a means for negative feedback regulation of receptor function.

Materials and methods

Cells and medium

JGN cells, a TCR cell surface negative variant of the human T cell line Jurkat that synthesize no CD3γ (Geisler, 1992) and the original Jurkat cell line J76 were cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with penicillin, 0.5 IU/litre (Leo, Ballerup, Denmark), streptomycin, 500 mg/litre (Novo, Bagsværd, Denmark), and 10% (v/v) fetal calf serum (FCS; Sera-Lab Ltd, Sussex, UK) at 37°C in 5% CO_2.

Antibodies and chemicals

UCHT1 mouse monoclonal antibody directed against human CD3ε was obtained purified and phycoerythrin (PE) 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 (Geisler *et al.*, 1988). Fluorescein isothiocyanate (FITC) conjugated $F(ab)_2$ fragments of affinity purified goat anti-mouse Ig were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). FITC conjugated anti-Vβ8 was from T Cell Diagnostics, Inc. (Cambridge, MA, USA). The phorbol esters phorbol 12,13-dibutyrate (PDB) and phorbol 12-myristate 13-acetate (PMA) were from Sigma Chemical Co. (St Louis, MO, USA).

Constructs and transfection

All mutations and truncations were constructed as previously described (Geisler *et al.*, 1992; Wegener *et al.*, 1992) by the polymerase chain reaction (PCR) using Vent DNA polymerase containing 3′→5′ proofreading exonuclease activity (New England Biolabs, Inc., Beverly, MA, USA) and the wild-type human CD3γ cDNA containing plasmid pJ6T3γ-2 (Krissansen *et al.*, 1986) as template. PCR products were cut with *Xba*I and *Nco*I, cloned into the 4.1 kb *Xba*I−*Nco*I fragment of pBluescript-βWT (Geisler *et al.*, 1992) and confirmed by complete DNA sequencing. The 1.8 kb *Xba*I−*Bam*HI pBluescript fragment containing the PCR product was subsequently cloned into the 5.9 kb *Xba*I−*Bam*HI fragment of the expression vector pTβFneo (Ohashi *et al.*, 1985). Transfections were performed using the Bio-Rad Gene Pulser at a setting of 270 V and 960 μF with 40 μg of plasmid per 2×10^7 cells. Following electroporation, cells were maintained in RPMI medium for 24 h and plated at 1×10^4 and 5×10^4 cells/ml in 96-well tissue culture plates (Greiner GmbH, Frickenhausen, Germany) in medium containing 1 mg/ml G418 sulfate (Geneticin) (Gibco BRL). After 3−4 weeks of selection, G418-resistant clones were expanded and maintained in medium without G418.

TCR down-regulation

Cells were adjusted to 1×10^5 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 PBS containing 2% FCS and 0.1% NaN_3 and washed twice. The cells were stained directly with PE conjugated UCHT1, FITC conjugated Vβ8, or indirectly with F101.01 followed by FITC conjugated secondary antibody and analysed in a FACscan flow cytometer (Becton Dickinson, Mountain View, CA, USA). 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 analysis in hypertonic medium cells were adjusted to 1×10^5 cells/ml in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine and 20 mM HEPES without sodium bicarbonate (DMEM) with or without 0.45 M sucrose and incubated at 37°C with 20 ng/ml PMA. For acidification of the cytosol, 1 M acetic acid, pH 5.0, was added 1:100 to DMEM adjusted to pH 5.0, whereas control cells were incubated in DMEM adjusted to pH 5.0.

Phosphorylation, immunoprecipitation and electrophoresis

Cells (3×10^7) were washed twice in phosphate-free buffer (PFB) (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 20 mM Tris−HCl, pH 7.35) and incubated for 1 h at 37°C in 10 ml phosphate-free RPMI 1640 (Irvine Scientific, Santa Ana, CA, USA). The cells were washed once, resuspended in 10 ml phosphate-free RPMI 1640 containing 1 mCi [^{32}P]orthophosphate (Amersham Laboratories, Amersham, UK), and incubated at 37°C for 3.5 h. PBD (50 ng/ml final concentration) was added and the incubation continued for 10 min. Subsequently the cells were washed three times in ice-cold PFB containing 10 mM EDTA, 5 mM EGTA and 10 mM NaF and lysed in 1 ml lysis buffer (1% Triton X-100, 10 mM Tris−HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 5 mM EGTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The lysates were precleared three times with protein A−Sepharose (PA) beads, incubated with 2 μg UCHT1 for 1.5 h and subsequently incubated with PA for another 1.5 h. The PA beads were washed once in PFB with the addition of 0.5 M NaCl, once in PFB containing 0.1% SDS, and once in 0.1% NP-40, Tris−HCl, pH 7.4, before elution of the proteins from the beads by boiling for 5 min in sample buffer (50 mM Tris−HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol). Samples were electrophoresed in 10% polyacrylamide gels. Autoradiography of the dried gels was performed by using Hyperfilm-MP (Amersham Laboratories). ^{14}C-labelled proteins from Amersham were used as molecular weight markers.

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