CD3 and CD2 antigen-mediated CD3 γ -chain phosphorylation in permeabilized human T cells

Regulation by cytosolic phosphatases

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The role of cytosolic and membrane-associated phosphatases in regulating dephosphorylation of the CD3 antigen γ -chain has been investigated using streptolysin-O-permeabilized T lymphoblasts and Jurkat T leukaemia cells. Permeabilization of T cells caused a rapid extrusion of cytosolic type 2A phosphatases, but a membrane-associated phosphorylase phosphatase activity remained inside the cells. This activity had the properties characteristic of type 2A phosphatases, being resistant to inhibition by type 1 phosphatase inhibitors, though it was inhibited in a time-dependent manner by ATP or by non-hydrolysable ATP analogues, but not by GTP, CTP, ITP or PP_i. The membrane-associated type 2A phosphatase in permeabilized cells did not dephosphorylate the CD3 antigen γ -chain, suggesting that cytosolic phosphatases dephosphorylate the γ -chain *in situ*. Cross-linking the CD2 and CD3 antigens with a bivalent monoclonal antibody in the absence of cytosolic phosphatases induced marked phosphorylation of the CD3 γ -chain, immunoprecipitated using a novel γ -chain peptide analogue directed antiserum (TG1). Phosphorylation was inhibited by a protein kinase C (PKC) pseudosubstrate inhibitor, indicating that CD2/CD3-induced γ -chain phosphorylation is a PKC-mediated event. Activation of T cells either with phorbol 12,13-dibutyrate or by CD2–CD3 cross-linking caused [³²P]P_i incorporation into the same γ -chain Ser residues. The site-mapping data suggested that PKC *in situ* may incorporate phosphate at the CD3 γ -chain Ser-123 and Ser-126 residues, but that phosphate is rapidly lost from Ser-123 by cytosolic phosphatase action. Our findings underline the importance of the dual actions of kinases and phosphatases as potential regulators of T cell antigen–receptor complex function.

INTRODUCTION

Responses of T lymphocytes to antigen are mediated by the T cell antigen–receptor complex (TCR), which consists of a clonotypic α – β heterodimer, the γ – ϵ and δ – ϵ heterodimers that together comprise the CD3 antigen, and either a ζ – ζ homodimer or a ζ – η heterodimer [1–4]. Coupling of the TCR to signalling pathways is thought to require interactions of intracellular proteins with specific cytoplasmic domains of the invariant ζ , η and CD3 $\gamma\delta\epsilon$ polypeptides, and to involve the regulation of one or more tyrosine kinases [5–7]. The TCR ϵ and ζ chains appear to couple the TCR to specific pathways of protein tyrosine phosphorylation [8–11]. The CD2 antigen mediates similar activation signals to those induced via the TCR, possibly by utilizing a common tyrosine kinase [11,12], and CD2–CD3 cross-linking amplifies CD3-induced intracellular signals [13,14].

Another consequence of the binding of mitogenic ligands to the TCR is the activation of protein kinase C (PKC) [15], an event implicated in the regulation of the interleukin-2 and interleukin-2 receptor genes [16–19]. PKC activation in response to the binding of antigen [20], or as a result of addition of phytohaemagglutinin (PHA) or phorbol ester to T cells [21], results in phosphorylation of the CD3 antigen γ -chain [22]. Site-mapping studies have revealed detectable phosphate incorporation only at the γ -chain Ser-126 residue following activation of T cells with phorbol 12,13-dibutyrate (PdBu) or PHA *in vivo*

[23], although phosphate incorporation at both Ser-123 and Ser-126 was observed following phosphorylation of the γ -chain with purified PKC *in vitro* [24]. The possible physiological relevance of CD3 γ -chain phosphorylation is not yet known, though it is likely to play a key role in regulating the cell surface expression of the TCR [25,26] or in modulating the coupling of the TCR to mitogenic signalling pathways. We have previously shown that activation of T cells with PdBu, followed by its removal, leads to a rapid reversal of γ -chain phosphorylation, pointing to the action of phosphatases [24]. Dephosphorylation of the γ -chain also occurred in T cell microsomes [24]. We have further reported that purified oligomeric isoforms of type 2A phosphatases show selectivity towards the phosphorylated γ -chain as substrate in comparison with other purified phosphatases [24], and that type 2A phosphatases are abundant both in the T cell cytosol and in purified T cell plasma membrane fractions [27]. It was therefore of interest to know whether γ -chain dephosphorylation is regulated by cytosolic or membrane-associated phosphatases *in situ*, and also whether TCR-mediated signals regulate the activity of such phosphatases.

In the present work we show that cytosolic type 2A phosphatases rapidly leak out of T cells permeabilized with streptolysin-O, but that a pool of type 2A phosphatase activity remains associated with permeabilized cell membranes. However, membrane-associated phosphatases in permeabilized cells do not dephosphorylate the CD3 γ -chain, suggesting that the γ -chain *in*

situ is dephosphorylated by cytosolic type 2A phosphatases. We have also shown, for the first time, that CD2-CD3 cross-linking induces γ -chain phosphorylation, by using a novel anti-peptide antiserum for γ -chain immunoprecipitation and carrying out the phosphorylation step in permeabilized T cells in the absence of cytosolic phosphatases. Inhibition with a pseudosubstrate PKC inhibitor showed that CD2/CD3-induced γ -chain phosphorylation is a PKC-mediated event, and this was confirmed by demonstrating that the γ -chain Ser residue(s) phosphorylated in response either to CD2/CD3 or to PdBu activation are identical. Site mapping of the CD3 γ -chain, phosphorylated under conditions where no dephosphorylation is occurring, suggests that phosphate incorporation may occur at both Ser-123 and Ser-126.

MATERIALS AND METHODS

Materials

PdBu and other biochemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Purified PHA and streptolysin-O were from Burroughs-Wellcome. Protein A-Sepharose was from Pharmacia, Uppsala, Sweden, and endo- β -N-acetylglucosaminidase F (Endo-F) was from New England Nuclear. [γ - 32 P]ATP and 125 I were obtained from Amersham International. Phosphoinhibitor-1 and inhibitor-2 were generously provided by Professor Philip Cohen, University of Dundee, Scotland, U.K., and dephosphorylation was from Dr. Jozef Goris, University of Leuven, Belgium. The UCHT1 (IgG1) mouse monoclonal antibody (mAb) against the CD3 antigen was kindly given by Professor Peter Beverley, University College and Middlesex School of Medicine, London, U.K., and the anti-CD2 mAb MAR206 was provided by Dr. A. Moretta, Ludwig Institute, Lausanne, Switzerland. The OKT3-OKT3-OKT11 bivalent heteroconjugate mAb was made from Fab' fragments and purified as described [28]. Cell culture materials were from Gibco, Paisley, Scotland, U.K.

Cell culture

Human T lymphoblasts were prepared by stimulating peripheral blood mononuclear cells [10^6 cells/ml of RPMI 1640/10% (v/v) foetal calf serum] with 5 μ g of PHA/ml for 72 h. After washing of cells, growth was maintained for up to 14 days by supplementing the medium every 2 days with 0.1 nm-interleukin-2, as previously described [21]. Cells were washed free of interleukin-2 for 2-4 days before experiments, in order to induce quiescence. The Jurkat 6 human T leukaemia cell line was cultured in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (50 μ g/ml) and 2% foetal calf serum.

Peptides

Peptides were synthesized as previously described [23]. Peptide T4, an analogue of inhibitor-1, has the sequence Arg-Arg-Ala-Thr-Val-Ala, and was phosphorylated as described [29]. Peptide GS, an analogue of glycogen synthase, has the sequence Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, and peptide PS, an analogue of the PKC pseudosubstrate inhibitor [30], has the sequence Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val.

Buffers

The intracellular buffer used during cell permeabilization has been described previously [22]. Concentrations of free Ca^{2+} and

Mg^{2+} were calculated by the Chelate computer program, by using appropriate dissociation constants for Ca^{2+} , Mg^{2+} and H^+ [31]. Free Ca^{2+} was at 150 nM for the buffer used in Jurkat cells and at 400 nM for the buffer used in T lymphoblasts during phosphorylation assays. Phosphatase assays were carried out in intracellular buffer containing a resting free Ca^{2+} concentration of 100 nM.

Enzyme assays

Phosphopeptide T4 phosphatase activity was assayed by incubating 1 μ M-phosphopeptide in 20 mM-Tris/HCl, pH 7.0, for 2 min at 30 °C prior to estimation of released [32 P] P_i as previously described [27]. 32 P-labelled phosphorylase *a* was prepared as described [32] and phosphorylase phosphatase activity in streptolysin-O-permeabilized cells was measured using 1 μ M-phosphorylase *a* in the presence of intracellular buffer at 37 °C. Reactions were stopped with trichloroacetic acid after 2 min for Jurkat cells or after 5 min for T lymphoblasts, the cells were pelleted and released [32 P] P_i in the supernatant was measured as described [33]. PKC assays in streptolysin-O-permeabilized cells were carried out using peptide GS as substrate, as previously described [34].

Characterization of CD3 γ -chain TG1 antiserum

Rabbit antiserum (TG1) was raised against a thyroglobulin-conjugated C-terminal peptide analogue of the CD3 γ -chain with the sequence Cys-Leu-Gln-Gly-Asn-Gln-Leu-Arg-Arg-Asn. Microsomes were prepared from T lymphoblasts sheared with a Stansted cell-breaking pump and microsomes or intact cells were then labelled with 1 mCi of 125 I/ 5×10^7 cell equivalents or cells respectively by lactoperoxidase-catalysed iodination [35]. Cells were lysed at 10^7 /ml for 30 min at 0 °C in 1% digitonin, 10 mM-Tris/HCl, pH 7.4, 0.15 M-NaCl and proteinase inhibitors. Immunoprecipitates were prepared from 10^7 cell equivalents with 20 μ l of TG1 antiserum and 5 μ l of Protein A-Sepharose, as described [35], and washed three times with lysis buffer. Where applicable, immunoprecipitates were digested with Endo-F [35] prior to analysis on an SDS/17%-polyacrylamide gel under reducing conditions. For immunoblotting, unlabelled immunoprecipitates were prepared as above and separated on an SDS/17% polyacrylamide gel under non-reducing conditions, and immunoblotting was carried out with TGI antiserum, diluted 1:100, as described [36].

Phosphorylation of the CD3 γ -chain

Phosphorylation of the CD3 γ -chain with [32 P]ATP in streptolysin-O-permeabilized T lymphoblasts or Jurkat cells and subsequent immunoprecipitation with the anti-CD3 mAb UCHT1 was performed as described previously [22]. For CD3 γ -chain immunoprecipitation with TG1, Nonidet P40 detergent cell lysates were adjusted to a final concentration of 0.2% SDS, heated for 5 min at 105 °C, precleared for 15 min using *Staphylococcus aureus* Protein A, incubated with 20 μ l of TG1 antiserum and 30 μ l of Protein A-Sepharose at 4 °C for 1 h, and the immunoprecipitates were then washed three times with lysis buffer containing 0.1% SDS. Separation of the phosphorylated CD3 γ -chain was carried out on 12.5% polyacrylamide gels under non-reducing conditions.

RESULTS

Phosphatases in permeabilized T cells

It has previously been shown that T cells contain both cytosolic and membrane-associated type 2A phosphatases [27] and that the purified type 2A PCS_H oligomer is more potent than the

Fig. 1. Release of cytosolic phosphatases from permeabilized Jurkat cells

Washed aliquots of 10^7 Jurkat cells were permeabilized with streptolysin-O with or without 100 ng of PdBu/ml. At the times shown, 10^6 cells were removed, pelleted for 10 s in a Microfuge, and the supernatants placed on ice. The pellets were suspended in 40 μ l of 20 mM-Tris/HCl, pH 7.0, and the cells fragmented by 20 passages through the needle of a Hamilton syringe. Phosphopeptide T4 phosphatase activity was then assayed in samples of the supernatants (Δ , $\square$) and fragments ($\square$, $\blacksquare$) from cells permeabilized in the absence (Δ , $\square$) or presence ($\blacktriangle$, $\blacksquare$) of 100 ng of PdBu/ml. Further details are as in the Materials and methods section. Comparable results were obtained in two separate experiments.

other type 2A oligomers in dephosphorylating the CD3 antigen γ -chain [24], as well as an artificial phosphopeptide substrate called T4 [27] *in vitro*. Fig. 1 shows that T4 phosphopeptide phosphatase activity was rapidly released from Jurkat T cells following permeabilization with streptolysin-O, phosphatase extrusion being complete within 10 min. This is consistent with our previous finding that cytosolic marker enzymes such as lactate dehydrogenase are released from T cells following incubation with streptolysin-O [22]. However, in contrast to the complete release of lactate dehydrogenase from permeabilized T cells [22], much of the total T4 phosphatase activity measured remained cell-associated (Fig. 1). The total T4 phosphatase activity recovered (cell-associated + extruded) increased from 60 to 100 pmol/min per 10^6 cells during the time course of the experiment, presumably due to increased activity of the extruded phosphatase upon dilution in the permeabilization buffer. Based on the cell-associated activity data alone, about 50% of the T4 phosphatase remained inside the cells after 10 min of permeabilization. Activation of PKC by adding PdBu to cells during permeabilization did not affect the levels of T4 phosphatase activity that remained cell-associated or was released from the cells. These results show that one or more phosphoserine phosphatases remained inside T cells following permeabilization, presumably in a membrane-associated form.

We next characterized this membrane-associated phosphatase activity using [32 P]phosphorylase *a*, previously shown to be a substrate only for the type 1 and type 2A phosphatases [37]. To demonstrate that the [32 P] P_i measured was due to the actions of a phosphatase rather than a proteinase, assay mixtures were extracted with isobutanol/toluene (1:1, v/v) in the presence of 5% ammonium molybdate. Radioactivity was found in the organic phase (results not shown), consistent with the release of [32 P] P_i by the actions of a phosphatase, since the products of a proteinase would either be removed by trichloroacetic acid

Table 1. Phosphorylase phosphatase activities of permeabilized Jurkat cells

Jurkat cells were permeabilized with streptolysin-O in intracellular buffer containing 100 nM free Ca^{2+} for 10 min as described in the Materials and methods section. The cells were then centrifuged at 1200 *g* for 5 min, the supernatant removed, and the permeabilized cells gently resuspended in the same buffer at 4×10^7 cells/ml. Assays were carried out on aliquots of 3×10^6 cells with the additions as shown. The results shown were obtained from four separate experiments in which the control phosphorylase phosphatase values were in the range 12.3–14.3 pmol/min per 3×10^6 cells.

Additions	Phosphorylase phosphatase activity (% of control)
Mg $^{2+}$ (3–12 mM)	50–70
Ca $^{2+}$ (100–475 nM)	89–115
Phosphoinhibitor-1 (30 nM)	119
Inhibitor-2 (100 nM)	130
De-inhibitor (3.3 units/ml)	113

precipitation or, if released as small peptides, be recovered in the aqueous phase. The properties of phosphorylase phosphatase activity detected in permeabilized Jurkat cells were found to be characteristic of type 2A but not of type 1 phosphatases (Table 1). Similar results (not shown) were obtained with T lymphoblasts. No inhibition of activity was observed with the specific inhibitors of type 1 phosphatases, inhibitor-1 and inhibitor-2 [37], even when these inhibitors were preincubated with permeabilized cells for 10 min prior to the phosphorylase phosphatase assay (results not shown). The deinhibitor polypeptide, previously shown to be effective in activating a latent membrane-associated form of the type 1 phosphatase [38], did not change the activity in these assays. However, Mg $^{2+}$ inhibited the activity by up to 50%, similar to the reported inhibitory effects of Mg $^{2+}$ on purified type 2A phosphatases [39]. Furthermore, Fig. 2 shows that ATP or adenosine 5'-[γ -thio]triphosphate (ATP[S]), but not GTP, inhibited phosphorylase phosphatase activity in a time-dependent manner. Inhibition was only apparent after 3–5 min of incubation with the adenosine nucleotides. In five separate experiments the maximum inhibition observed with 100 μ M-ATP was $41 \pm 7\%$ (range 36–53%). A similar time-dependent inhibitory effect was found with the non-hydrolysable ATP analogue adenosine 5'-[β -imidazo]triphosphate, but no inhibition was observed when 100 μ M-CTP, -ITP, -GTP[S] or -PP $_i$ was added to assays performed as in Fig. 2 (results not shown), suggesting that the effect was specific for ATP and its non-hydrolysable analogues. Similar results have previously been described for purified type 2A phosphatases using phosphorylase *a* as substrate [40,41]. It was shown that ATP induces a time-dependent inhibition of the purified PCS $_L$ type 2A phosphatase [40], as well as of other type 2A oligomers, but not of the type 1 phosphatase [41]. The results shown in Fig. 2 therefore support the conclusion that the membrane-associated phosphorylase phosphatase activity remaining in permeabilized T cells represents one or more oligomeric type 2A phosphatases.

Membrane-associated phosphatases do not dephosphorylate the CD3 γ -chain.

We have previously shown that removal of PdBu from intact 32 P-labelled T cells causes a rapid reversal of PKC-induced phosphorylation of the CD3 γ -chain, pointing to the actions of phosphatases [24]. Furthermore, purified type 2A phosphatases *in vitro* show selectivity towards the phosphorylated γ -chain as substrate in comparison with other purified phosphatases [24]. It was therefore of interest to determine whether the CD3 γ -chain

Fig. 2. Phosphorylase phosphatase assays in pre-permeabilized Jurkat cells

Assays were performed as in Table 1 with addition of nucleoside phosphates at 100 μ M. Comparable results were obtained in two separate experiments. □, Control; ■, ATP; ●, ATP[S]; ▲, GTP.

Fig. 3. Phosphorylation of the CD3 γ -chain in streptolysin-O-permeabilized T lymphoblasts

Two portions (2×10^7 cells) of washed T lymphoblasts were permeabilized with streptolysin-O in the presence of [32 P]ATP as described in the Materials and methods section in the absence (track 1) or presence (track 2) of 20 ng of PdBu/ml. The cells were pelleted, solubilized and the CD3 antigen immunoprecipitated with the UCHT1 mAb. Two further portions of T lymphoblasts (2×10^7 cells) were permeabilized with streptolysin-O for 10 min in the same way, except that [32 P]ATP was excluded, in the absence (track 3) or presence (track 4) of 20 ng of PdBu/ml. The cells were pelleted, the supernatant removed, and the cells then incubated with [32 P]ATP as above, followed by immunoprecipitation with the UCHT1 mAb.

was dephosphorylated by the membrane-associated type 2A phosphatases detected in permeabilized T cells (Table 1), or whether the presence of cytosolic phosphatases was essential for γ -chain dephosphorylation to occur. Fig. 3 shows that the phosphorylation of the γ -chain immunoprecipitated with the anti-CD3 mAb UCHT1 from PdBu-treated T lymphoblasts was much greater (cf. track 4 and track 2) if the cells were pre-permeabilized with streptolysin-O to allow cytosolic proteins to leak from the cells prior to the phosphorylation step. Since

Fig. 4. Membrane phosphatases do not dephosphorylate the CD3 γ -chain in permeabilized Jurkat cells.

Jurkat cells (2×10^6) were incubated with intracellular buffer containing [32 P]ATP, streptolysin-O and 20 ng of PdBu/ml for 15 min to phosphorylate the CD3 γ -chain as described in the Materials and methods section. Cells were then pelleted, the supernatant removed, and the cells washed three times in 1 ml portions of intracellular buffer containing 1 mg of fatty-acid-free BSA/ml to remove PdBu. The washed cells were resuspended in 1.1 ml of intracellular buffer at 37 $^{\circ}$ C and 0.1 ml samples were removed at 0 min (track 1), 2 min (track 2), 4 min (track 3), 6 min (track 4), 8 min (track 5), 10 min (track 6), 15 min (track 7) and 20 min (track 8). The level of CD3 γ -chain phosphorylation was subsequently analysed by immunoprecipitating the CD3 antigen with the UCHT1 mAb from detergent lysates.

cytosolic phosphatases were no longer present in these pre-permeabilized cells (Fig. 1), it seemed likely that the increased γ -chain phosphorylation level observed was due to the removal of phosphatases that normally acted on the γ -chain *in situ*.

The experiment shown in Fig. 4 provides support for this interpretation. Jurkat T cells were treated with PdBu in the presence of streptolysin-O and [32 P]ATP to induce γ -chain phosphorylation, and the PdBu was then rapidly removed by washing the cells three times in fatty-acid-free BSA [24]. Control experiments (not shown) demonstrated that this procedure did not remove the membrane-associated phosphorylase phosphatase activity as characterized in Table 1. The cells were then re-incubated at 37 $^{\circ}$ C and the phosphorylation state of the γ -chain was estimated at times up to 20 min, a time at which the γ -chain had previously been found to be almost completely dephosphorylated *in vivo* under the same experimental conditions [24]. Fig. 4 shows that the membrane-associated type 2A phosphatases did not apparently have access to the phosphorylation sites on the γ -chain, since γ -chain phosphorylation did not detectably change (by densitometric scanning) during the 20 min incubation. However, we cannot formally exclude the possibility that the cell permeabilization procedure affected the efficacy of the γ -chain as a phosphatase substrate. Identical results (not shown) were found with T lymphoblasts. The implication of these findings is that the phosphorylated γ -chain is normally dephosphorylated by cytosolic phosphatases *in situ*, and that these phosphatases are removed during streptolysin-O permeabilization of the cells, as shown in Fig. 1.

CD2/CD3-mediated phosphorylation of the CD3 γ -chain

It was clearly of interest to investigate the regulation of TCR-induced CD3 γ -chain phosphorylation in the permeabilized T cell system, since receptor-mediated control of PKC activity is not necessarily mimicked accurately by direct activation of PKC with PdBu. In contrast to the experiments illustrated in Figs. 3

Fig. 5. Recognition of the CD3 γ -chain by TG1 antiserum

(a) Immunoblotting of immunoprecipitates from T lymphoblast lysates. Immunoprecipitates were prepared from cell lysates using the anti-CD2 mAb MAR206 (track 1), TG1 antiserum (track 2) or anti-(MHC class I) mAb W6/32 (track 3), followed by separation of precipitated proteins on an SDS/17%-PAGE gel and detection with the same antibodies for each track by immunoblotting as described in the Materials and methods section. (b) Endo-F treatment of CD3 immunoprecipitates. T lymphoblasts and T lymphoblast membranes were labelled with ^{125}I as described in the Materials and methods section and separated on an SDS/17%-PAGE gel under reducing conditions. Immunoprecipitates (tracks 1–3) were prepared from detergent lysates. A portion of each immunoprecipitate was digested with Endo-F as described in the Materials and methods section and the digested samples separated on the same gel (tracks 4–6). Track 1, OKT3 mAb immunoprecipitate from whole-cell lysate; track 2, TG1 immunoprecipitate from whole-cell lysate; track 3, TG1 immunoprecipitate from membranes; track 4, Endo-F-treated OKT3 immunoprecipitate; track 5, Endo-F-treated TG1 immunoprecipitate from whole-cell lysate; track 6, Endo-F-treated TG1 immunoprecipitate from membranes.

and 4, use of the UCHT1 or OKT3 anti-CD3 mAbs in such investigations to immunoprecipitate the CD3 γ -chain was problematic for two reasons. First, these mAbs bind to epitopes derived from CD3 $\gamma\epsilon$ and $\delta\epsilon$ chain heterodimers [42], so that the rigorous conditions required to destroy phosphatase activity in whole-cell lysates (e.g. heating at 105 °C) will disrupt the epitopes recognized by such mAbs. Second, use of the same anti-CD3 mAbs for both activating the cells and CD3 antigen immunoprecipitation can lead to technical problems during the immunoprecipitation procedure. A rabbit polyclonal antiserum (TG1) was therefore raised against a C-terminal peptide homologue of the γ -chain. Immunoblotting of mAb immunoprecipitates from T cell detergent lysates (Fig. 5a) revealed that TG1 specifically stained two proteins that were immunoprecipitated by an anti-CD3 mAb (track 2) but not by an anti-CD2 mAb (track 1) or a class I mAb (track 3). The lowest band shown in Fig. 5(a), track 1, was not stained with a specific CD3 δ -chain antiserum and is most likely an immature form of the γ -chain. Fig. 5(b)

shows that TG1 immunoprecipitated a protein from cell-surface- ^{125}I -labelled T cells (track 2) or, more clearly, from ^{125}I -labelled T cell membranes (track 3), that migrated on an SDS/PAGE gel at the same position as the polypeptides that comprise the CD3 complex immunoprecipitated with the OKT3 mAb (track 1). Treatment of the OKT3 immunoprecipitate with Endo-F, followed by SDS/PAGE analysis, revealed (Fig. 5b, track 4) the expected non-glycosylated 19 kDa ϵ -chain, as well as the deglycosylated 16 kDa γ -chain and 14 kDa δ -chain [43]. A faint band of 12 kDa was probably an intermediate form of the δ -chain digest. Endo-F digestion of the TG1 immunoprecipitates (tracks 5 and 6) generated the expected deglycosylated 16 kDa γ -chain product and the 19 kDa ϵ -chain. These findings show that the TG1 antiserum specifically recognized the CD3 γ -chain and appeared to immunoprecipitate a $\gamma\epsilon$ -chain heterodimer, in agreement with previous work demonstrating an interaction of these two polypeptides [42]. It is unlikely that TG1 recognized the ϵ -chain, since the γ -chain peptide sequence used for raising the TG1 antiserum is not found within the ϵ -chain cytoplasmic domain.

It is apparent from Fig. 5 (cf. tracks 1 and 2) that OKT3 mAb was more efficient than TG1 in immunoprecipitating CD3 antigen polypeptides. In initial experiments (results not shown) a very small increase in phosphorylation of the TG1-immunoprecipitated CD3 γ -chain was observed following activation of T lymphoblasts with the UCHT1 mAb, although phosphorylation of the γ -chain from PdBu-treated cells was readily detected (see Fig. 7, track 5). It therefore appeared likely that TG1 would be a useful reagent for detecting γ -chain phosphorylation only under conditions where marked PKC activation had occurred. Since cross-linking of the CD3 and CD2 antigens has been shown to amplify common signalling pathways [13,14], a bivalent heteroconjugate OKT3–OKT3–OKT11 mAb was used to cross-link the CD3 and CD2 antigens. Utilizing a sensitive assay that has previously been described for measuring PKC activation in permeabilized T cells [34], it was shown (Fig. 6a) that this reagent was highly effective in stimulating peptide GS phosphorylation in Jurkat cells, providing that the free Ca^{2+} concentration was maintained at 50 nM or above. Free Ca^{2+} alone at 400 nM or above caused substantial peptide GS phosphorylation in Jurkat cells (Fig. 6a), probably by directly mobilizing diacylglycerol via a Ca^{2+} -sensitive mechanism. The OKT3–OKT3–OKT11 mAb also markedly activated PKC in T lymphoblasts (results not shown), though in these cells the effects of free Ca^{2+} alone on PKC activation were much less marked, in agreement with previous findings [22]. Fig. 6(b) shows that incorporation of 10 μM pseudosubstrate peptide PKC inhibitor [22] into the permeabilized T cell assay was sufficient to completely block the CD3–CD2-induced increment of peptide GS phosphorylation. Furthermore, comparable concentrations of pseudosubstrate peptide inhibited to a similar extent both PdBu-induced and CD2–CD3-induced peptide GS phosphorylation (Fig. 6b). Taken together, the data shown in Fig. 6 strongly suggest that the OKT3–OKT3–OKT11 bivalent mAb was a highly effective reagent for activating PKC in T cells.

T lymphoblasts were therefore pre-permeabilized with streptolysin-O to remove cytosolic phosphatases, in the presence of OKT3–OKT3–OKT11 mAb or PdBu, so that no dephosphorylation of the CD3 γ -chain could occur following a subsequent phosphorylation step with [^{32}P]ATP. After removal of the nuclear pellet from detergent lysates of these cells, SDS was added to the lysates to a final concentration of 0.2% followed by heating at 105 °C for 5 min. Control experiments (results not shown) demonstrated that this procedure inactivated phosphatases released during cell lysis that otherwise caused partial dephosphorylation of the CD3 γ -chain during the immuno-

Fig. 6. PKC assays in permeabilized Jurkat cells

PKC was assayed by measuring peptide GS phosphorylation as described in the Materials and methods section. (a) Ca^{2+} -dependence of phosphorylation. Δ , No further additions; $\blacktriangle$, +5 μg of OKT3-OKT3-OKT11/ml. (b) Pseudosubstrate peptide inhibition of peptide GS phosphorylation. Δ , No addition; $\blacktriangle$, +5 μg of OKT3-OKT3-OKT11/ml; $\blacksquare$, +20 ng of PdBu/ml.

precipitation procedure, even in the presence of phosphatase inhibitors. The CD3 γ -chain was then immunoprecipitated from boiled lysates with TG1 antiserum. Fig. 7 shows that CD2-CD3 cross-linking induced phosphorylation of the TG1-immunoprecipitated γ -chain (track 1) that was completely inhibited by incorporation of the pseudosubstrate PKC inhibitor peptide in the assay (track 2). Direct activation of PKC with PdBu induced a higher level of γ -chain phosphorylation (track 5) that was also blocked with the pseudosubstrate (track 6), in agreement with our previous findings [22]. These results therefore demonstrate, for the first time, that a CD3/CD2-mediated PKC activation pathway results in the phosphorylation of the CD3 γ -chain.

Site mapping of the PdBu- and CD2/CD3-induced CD3 γ -chain phosphorylation sites

It has previously been shown that Ser-126 of the CD3 γ -chain was the only site phosphorylated *in vivo* when T lymphoblasts were activated with PdBu or PHA [23]. However, phosphorylation of the immunopurified CD3 complex *in vitro* with purified PKC resulted in phosphorylation of γ -chain Ser-123 and Ser-126 at a ratio of about 3:1 [24], and type 2A phosphatases also showed selectivity towards γ -chain peptide analogues phosphorylated at Ser-123 compared with analogues phosphorylated at Ser-126 [24]. We have therefore speculated previously that γ -chain Ser-123 might be phosphorylated *in vivo*, but that this phosphate could be removed from this site during immunoprecipitation due to the major release of active phosphatases that

Fig. 7. Phosphorylation of the CD3 γ -chain in pre-permeabilized T lymphoblasts with TG1 antiserum

Three portions of 4×10^7 T lymphoblasts were permeabilized for 5 min with streptolysin-O as described in the Materials and methods section with no further additions (tracks 3 and 4), 10 μg of OKT3-OKT3-OKT11 mAb/ml (tracks 1 and 2) or 50 ng of PdBu/ml (tracks 5 and 6). Each portion of cells was divided into two equal aliquots, pelleted and the supernatants removed. Phosphorylation reactions were then carried out as described in the Materials and methods section with (tracks 2, 4 and 6) or without (tracks 1, 3 and 5) the addition of 50 μM pseudosubstrate PKC peptide inhibitor. The CD3 γ -chain was immunoprecipitated with TG1 antiserum after boiling the detergent cell lysates for 5 min with 0.2% SDS.

occurs during cell lysis with Nonidet P-40 detergent [44]. Immunoprecipitation of the phosphorylated γ -chain with TG1 antiserum under conditions in which dephosphorylation is no longer occurring, as shown in the present work (Figs. 4 and 7), provides a way of testing this hypothesis. Fig. 8 combines the data from two separate site-mapping experiments in which T lymphoblasts were activated either with PdBu or with mAb OKT3-OKT3-OKT11. Phosphopeptides derived from trypsin digests of the TG1-immunoprecipitated CD3 γ -chain were separated on an f.p.l.c. Mono Q column. A single major peak of ^{32}P -labelled peptide eluted from the column at 13.0 ml, irrespective of whether the cells had been activated with PdBu or with OKT3-OKT3-OKT11, indicating that the same γ -chain Ser residue(s) had been phosphorylated in both cases (Fig. 8). These results therefore establish, for the first time, that CD3/CD2-induced CD3 γ -chain phosphorylation occurs at the same site(s) that are phosphorylated following direct activation of PKC by PdBu, consistent with previous findings suggesting that the γ -chain is a PKC substrate and that CD3-induced γ -chain phosphorylation is a PKC-mediated event [22,24].

The calibration of the f.p.l.c. Mono Q column using known peptide analogues of the CD3 γ -chain encompassing the putative Ser-123 and Ser-126 phosphorylation sites has been previously reported [23]. However, the elution position of the major phosphopeptide shown in Fig. 8 did not coincide with the elution position of any of the phosphorylated peptide analogues yet tested, which include γ -chain tryptic digest peptide analogues incorporating either SerP-123 or SerP-126 [23]. Our interpretation of these data is that the phosphopeptide that eluted at 13.0 ml shown in Fig. 8 is probably Gln-SerP-Arg-Ala-SerP-Asp-Lys, and that PKC therefore phosphorylates the CD3 γ -chain at both Ser-123 and Ser-126 *in situ*, phosphorylation at the Ser-123 site being detectable in the present work due to the inhibition of phosphatase activity during phosphorylation and subsequent isolation of the γ -chain. It has already been shown

Fig. 8. Anion-exchange f.p.l.c. analysis of tryptic peptides derived from the phosphorylated CD3 γ -chain immunoprecipitated with TG1 from pre-permeabilized T lymphoblasts

T lymphoblasts (2×10^8) were pre-permeabilized with streptolysin-O in the presence of 100 ng of PdBu/ml ($\square$) or 10 μ g of OKT3-OKT3-OKT11 mAb/ml ($\blacktriangle$), the cytosolic phosphatases removed, and the CD3 γ -chain was subsequently phosphorylated with 1.25–1.5 mCi of [32 P]ATP and then immunoprecipitated with TG1 antiserum, as in the legend to Fig. 6, followed by tryptic digestion and separation on a Mono Q column of 32 P-labelled peptides as described in the Materials and methods section. The peak of radioactivity that eluted at 7.5–9.0 ml in the tryptic digest derived from OKT3-OKT3-OKT11-treated cells has not been identified, but may contain an incompletely digested peptide. The arrows refer to the positions at which 32 P-labelled synthetic peptides elute from the column: a, Gln-SerP-Arg; b, Gln-SerP-Arg-Ala-Ser-Asp-Lys; c, Ala-SerP-Asp-Lys.

that PKC *in vitro* phosphorylates γ -chain peptide analogues at both Ser-123 and Ser-126, and that incorporation of phosphate into Ser-123 prevents digestion at the adjacent trypsin cleavage site [23], so under conditions in which both Ser-123 and Ser-126 are phosphorylated it is likely that this heptapeptide is highly resistant to digestion. However, it was not possible to calibrate the column with this peptide phosphorylated at both Ser residues, since the stoichiometry of phosphorylation of such peptides by PKC *in vitro* is low (about 0.2% [23]), and the chances of both Ser residues being phosphorylated in a single peptide molecule under such conditions are presumably even lower. Thus Edman degradation of the phosphopeptide shown in Fig. 8 with analysis of [32 P]P_i released at each cycle would be necessary to provide formal proof that both Ser-123 and Ser-126 are phosphorylated *in situ*. Insufficient radioactivity was recovered in the γ -chain tryptic digest phosphopeptides to investigate both Ser residues by this approach, even following phosphorylation with up to 1.5 mCi of [32 P]ATP (Fig. 8), so this approach was not pursued.

DISCUSSION

Four main conclusions may be drawn from these investigations. First, a membrane-associated phosphorylase phosphatase activity has been detected in permeabilized T cells that has the properties of type 2A phosphatases (Table 1, Fig. 2). Second, the CD3 γ -chain appears to be dephosphorylated by cytosolic phosphatases, since it is not dephosphorylated by these membrane-associated phosphatases (Figs. 1, 3 and 4). Third, cross-linking of the CD3 and CD2 antigens induces a marked level of PKC activation (Fig. 6) that causes phosphorylation of the CD3 γ -chain (Fig. 7) immunoprecipitated with a novel antiserum raised against a γ -chain peptide analogue (Fig. 5). Fourth,

activation of T cells by PdBu or by CD2–CD3 cross-linking in the absence of cytosolic phosphatases induces phosphorylation of the same γ -chain Ser residues, consistent with the proposed role for PKC in mediating CD3/CD2-induced γ -chain phosphorylation (Fig. 8).

The finding that membrane-associated phosphorylase phosphatase activity having the properties of type 2A phosphatases is present in permeabilized T cells (Figs. 1 and 2, Table 1) is consistent with our previous finding that the type 2A_c subunit common to all oligomeric forms of these enzymes [39] is present in T cell membrane fractions [27]. Furthermore, we have previously isolated three high-molecular-mass forms of type 2A phosphatases from Jurkat T cell microsomes [24]. It appears from the present work that these membrane-associated type 2A phosphatases do not have access to the CD3 γ -chain (Fig. 4), even though the putative Ser phosphorylation sites are close to the plasma membrane [2]. The dephosphorylation of the γ -chain in T cell microsomes that we have previously reported [24] was presumably caused by phosphatases that do not normally have access to the γ -chain *in situ*. In light of the selectivity of purified type 2A phosphatases in dephosphorylating the γ -chain compared with other phosphatases [24], it seems likely that dephosphorylation of the γ -chain *in vivo* is regulated by one or more cytosolic type 2A phosphatase oligomers.

The mechanisms whereby type 2A phosphatases may be regulated in T cells remain unknown. Signalling via the CD2 or CD3 antigens did not cause immediate (1–10 min) detectable changes in cytosolic type 2A phosphatase activity (D. R. Alexander, unpublished work). However, it is possible that discrete pools of type 2A phosphatases may be specifically regulated, resulting in changes in activity that are difficult to detect in whole cytosol. Furthermore, the CD2-mediated dephosphorylation of a 19 kDa protein has been reported in T cells which was blocked by okadaic acid, an inhibitor of type 1 and type 2A phosphatases [45]. This receptor-mediated phosphatase regulation event occurred 0.25–2 h after CD2 mAb binding, well beyond the time scale investigated in the present work. Another possible mode of regulation of the type 2A phosphatases is by targeting to selected sites of action, as suggested by the interactions of simian virus 40 and polyomavirus tumour antigens with specific type 2A phosphatase subunits [46,47]. Further work will be necessary to elucidate possible pathways of type 2A phosphatase regulation in T cells.

The striking increase in PKC activity upon CD2–CD3 cross-linking at intracellular Ca²⁺ concentrations of 50–150 nM (Fig. 6) is consistent with the up-regulation of CD3-induced signals by anti-CD2 mAbs previously reported [13,14]. This result underlines the potential importance of the CD2 antigen in amplifying TCR-mediated mitogenic signals, an event presumed to occur *in vivo* upon interaction of CD2 with LFA-3 [48]. There is considerable evidence that at least some of the coupling events that link CD2 to intracellular pathways are common to both CD2 and the TCR [12,49]. Our finding that CD2–CD3 cross-linking results in PKC-mediated phosphorylation of the CD3 γ -chain supports such a conclusion (Fig. 7). Since the physiological relevance of CD3 γ -chain phosphorylation for TCR function remains unknown, it is possible only to speculate on the role of PKC and cytosolic 2A phosphatases in regulating its phosphorylation state. Interestingly, it has been repeatedly noted that no CD3 γ -chain phosphorylation can be detected in resting 32 P-labelled intact T cells [20]; T cell activation is essential in order for detectable γ -chain phosphorylation to occur. However, it remains possible that the γ -chain is being continuously phosphorylated and dephosphorylated *in vivo* due to the potency of cytosolic type 2A phosphatases. It has been suggested that the γ -chain phosphorylation level regulates the distribution of the

TCR between the cell surface and intracellular pools [25,26]. This suggestion is supported by the finding that down-regulation of receptors from the cell surface correlates with increased γ -chain phosphorylation [21]. On the other hand, ionomycin was found to induce γ -chain phosphorylation without detectable changes in cell-surface expression of the CD3 antigen [50]. It is possible that phosphorylation of specific γ -chain Ser residues has specific effects. Since the CD3 ϵ -chain has been reported to couple to intracellular signals that are distinct from those mediated via the ζ -chain [10,11], and since $\gamma\epsilon$ heterodimers are apparently integral components of the TCR [51,52], it is likely that γ -chain phosphorylation plays an important role in coupling or uncoupling the receptor from intracellular signals. Interestingly, the putative Ser-123 and Ser-126 phosphorylation sites in the γ -chain are located immediately adjacent to a six-amino-acid motif which is also found in the cytoplasmic tails of other CD3 polypeptides, and which may therefore play a role in common intracellular coupling events [11]. From these data, it is clear that the opposing actions of PKC and type 2A phosphatases in regulating γ -chain phosphorylation may be crucial in determining the coupling of the TCR, and possibly CD2 as well, to intracellular signal pathways. For example, if PKC is partially active in primary quiescent T lymphocytes, as suggested for the CD45R0⁺ sub-set (A. Robinson & D. R. Alexander, unpublished work), the effects of PKC in mediating changes in TCR function may be kept below a threshold level by the actions of phosphatases. Inhibition of these phosphatases would be expected, for example, to amplify the actions of PKC in inducing expression of the interleukin-2 and interleukin-2 receptor genes.

For the first time, the site mapping of the Ser residues phosphorylated in the CD3 γ -chain has been carried out under conditions in which dephosphorylation was not simultaneously occurring (Figs. 4 and 8). Generation of the TG1 CD3 γ -chain antiserum (Fig. 5) was critical in obtaining this result, since it allowed immunoprecipitation of the γ -chain following phosphatase inhibition by the heating of detergent cell lysates (Fig. 7). The CD2/CD3- or PdBu-induced incorporation of [³²P]P_i under these 'zero dephosphorylation' conditions resulted in the generation of a novel phosphopeptide not previously observed in such studies (Fig. 8). These results confirm the finding of Fig. 7 that CD2/CD3-induced γ -chain phosphorylation is mediated by PKC. Should our tentative identification of the novel phosphopeptide as Gln-SerP-Arg-Ala-SerP-Asp-Lys be confirmed, this may resolve the apparent paradox that, whereas PKC *in vitro* phosphorylates Ser-123 and Ser-126 [24], PKC activation *in vivo* results in detectable [³²P]P_i incorporation only at Ser-126 [23]. Having previously shown that purified type 2A phosphatases dephosphorylate γ -chain phosphopeptide analogues at Ser-123 more efficiently than at Ser-126 [24], it is possible that [³²P]P_i incorporation at Ser-123 is not normally detected *in vivo* due to its rapid dephosphorylation. The reconstitution in intact cells of the TCR containing γ -chains mutated in the putative phosphorylation sites will be necessary to investigate these possibilities. In the light of the present work, the effects of mutating both Ser-123 and Ser-126 would be of interest.

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