Evidence that a kinase distinct from protein kinase C and phosphatidylinositol 3-kinase mediates ligation-dependent serine/threonine phosphorylation of the T-lymphocyte co-stimulatory molecule CD28

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The CD28 cytoplasmic tail contains several potential phosphorylation sites for the serine/threonine kinase protein kinase C (PKC) and/or proline-directed serine/threonine kinases, such as extracellular signal-regulated kinases. We demonstrate that ligation of CD28 by B7.1 results in strong serine/threonine phosphorylation of CD28. It is unlikely that ligation-stimulated phosphorylation of CD28 is mediated via activation of PKC, since it was not prevented by pre-treatment of Jurkat cells with inhibitors of PKC, and it was not mimicked by treatment with PKC activators such as PMA. Nevertheless, despite the lack of detectable effects of PMA treatment on CD28 phosphorylation, PMA did partially inhibit the association of CD28 with the putative signalling molecule phosphatidylinositol 3-kinase (PI 3-kinase) and the subsequent accumulation of PtdIns(3,4,5)P₃. PI

3-kinase exhibits dual specificity as both a lipid kinase and a protein serine kinase, and site-specific mutagenesis of the Tyr¹⁷³ residue in the CD28 cytoplasmic tail, which abolishes CD28 coupling to PI 3-kinase [Pages, Ragueneau, Rottapel, Truneh, Nunes, Imbert and Olive (1994) *Nature* (London) **369**, 327–329], also prevents ligation-stimulated phosphorylation of CD28. However, the two PI 3-kinase inhibitors wortmannin and LY294002 had no effect on phosphorylation of CD28 after ligation by B7.1. This study therefore demonstrates that (1) a CD28-activated serine/threonine kinase distinct from both PKC and PI 3-kinase mediates ligation-stimulated CD28 phosphorylation, and (2) the PMA-stimulated down-regulation of the coupling of CD28 to PI 3-kinase is not due to PMA-stimulated phosphorylation of CD28.

INTRODUCTION

Considerable attention has focused on characterizing the biochemical events that comprise the ‘second signal’ provided by the CD28 molecule after ligation by anti-CD28 monoclonal antibodies (mAbs) or the natural ligands B7.1 (also termed CD80) or B7.2 (CD86). This can lead to cyclosporin-A-insensitive interleukin-2 (IL-2) production and cellular proliferation in the presence of additional signals provided by the CD3–T-cell antigen receptor complex or the phorbol ester PMA [1,2]. Co-stimulation via CD28 also mediates strong up-regulation of IL-4, IL-5, IL-13, interferon- γ , tumour necrosis factor- α and granulocyte-macrophage colony-stimulating factor [3,4], up-regulation of the IL-2 receptor [5], CD40 ligand expression [6] and up-regulation of the chemokines IL-8 [7] and regulated on activation, normal T-cell expressed (RANTES) [8].

CD28 can couple to a number of signalling molecules including phospholipase C, p21^{ras}, sphingomyelinase and phosphatidylinositol 3-kinase (PI 3-kinase) (reviewed in [9] and [10]). Activation of protein tyrosine kinases such as Lck [11,12], Fyn [11,13] and Itk [14] have all been implicated in early CD28-dependent signalling events, and ligation of CD28 induces tyrosine phosphorylation of a number of substrates [15,16]. It is likely that these protein tyrosine kinases facilitate the coupling of CD28 to downstream signalling cascades. Indeed, CD28 is itself tyrosine phosphorylated and this appears to be necessary to allow binding of CD28 to PI 3-kinase [17]. The association between CD28 and PI 3-kinase occurs via a binding motif [(p)Tyr¹⁷³-Met-Asn-Met] within the CD28 cytoplasmic tail that binds to the *src* homology 2 (SH2) domains of the p85 kDa regulatory subunit of PI 3-kinase [17,18]. This subsequently leads

to concomitant increases in PI 3-kinase activity and accumulation of PtdIns(3,4,5)P₃, which is believed to be an important intracellular signal involved in the regulation of diverse physiological events (reviewed in [9] and [19]). In particular, the putative signalling pathway mediated by PI 3-kinase has been suggested to play a pivotal role in CD28-mediated events such as IL-2 production from normal T-cells, although this remains a contentious issue in leukaemic T-cell lines (reviewed in [9]). PI 3-kinase has also been reported to be necessary for CD28-mediated up-regulation of β 1-integrin adhesiveness [20] and CD28-dependent killing by human cytotoxic T-cells [21].

The 110 kDa catalytic subunit of PI 3-kinase also exhibits protein serine kinase activity [22], although the significance of this activity with respect to CD28 signal transduction is unknown. However, recent evidence has demonstrated that other protein serine/threonine kinases such as protein kinase C (PKC) may play an important function in CD28 signal transduction, since phorbol ester treatment of T-cells, which results in PKC activation, also stimulates threonine phosphorylation of CD28 under *in vitro* conditions [23]. Interestingly, the CD28 cytoplasmic tail contains several potential phosphorylation sites around Ser¹⁶³, Ser¹⁶⁶, Thr¹⁷⁷ and Thr¹⁸⁴ for PKC [24]. In addition, Thr¹⁷⁷ is adjacent to a proline residue and it may therefore also be a target for phosphorylation by proline-directed serine/threonine kinases, such as extracellular signal-regulated kinases (ERKs), which are members of the mitogen-activated protein (MAP) kinase family [25]. The importance of these phosphorylation sites in CD28 signalling has not been determined, although PMA treatment of T-cells inhibits ligation-stimulated tyrosine phosphorylation of CD28 [23], as well as the association with, and activation of, PI 3-kinase [26]. Since PMA can supply the

Abbreviations used: CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; IL-2, interleukin-2; mAb, monoclonal antibody; MAP, mitogen-activated protein; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C.

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additional signal(s) necessary for CD28-dependent IL-2 production, it may also modulate the enzymic activity or physical association of signalling proteins coupled to the CD28 cytoplasmic tail.

In this study, we demonstrate that ligation of CD28 by B7.1 results in strong serine/threonine phosphorylation of CD28. Although PMA treatment inhibited the B7.1-stimulated association of CD28 with PI 3-kinase, PMA did not stimulate detectable phosphorylation of CD28. Moreover, ligation-stimulated CD28 phosphorylation was not prevented by pre-treatment of Jurkat cells with serine/threonine kinase inhibitors known to have varying selectivity towards PKC. Furthermore, even though disruption of the PI 3-kinase binding motif around Tyr¹⁷³ abolished ligation-stimulated CD28 phosphorylation, inhibitors of PI 3-kinase had no effect on this CD28 phosphorylation. Our study therefore demonstrates that a protein serine/threonine kinase distinct from both PKC and PI 3-kinase mediates ligation-stimulated CD28 phosphorylation and that the PMA-stimulated down-regulation of CD28 coupling to PI 3-kinase is not due to direct phosphorylation of CD28.

MATERIALS AND METHODS

Reagents and antibodies

CD28 mAb 9.3 was kindly provided by Carl June (Naval Medical Research Institute, Bethesda, MD, U.S.A.); the p85 α mAb was from Doreen Cantrell (ICRF, London, U.K.); ionomycin, 8-bromo-cAMP, 8-bromo-cGMP, staurosporine, H9, calphostin C and Ro-31/8220 were purchased from Calbiochem-Novachem (Nottingham, U.K.). LY294002 was purchased from Affiniti Biomol (Exeter, Devon, U.K.). Wortmannin, PMA, 4 α -phorbol and PtdIns lipids were purchased from Sigma (Poole, Dorset, U.K.). SB203580 and PD098059 were generously given by Peter Young (SmithKline Beecham) and Dr. Alan Saltiel (Parke Davis, Ann Arbor, MI, U.S.A.) respectively.

Cell culture

The leukaemic T-cell line, Jurkat, was grown in RPMI-1640 supplemented with 10% (v/v) foetal-calf serum and the antibiotics streptomycin (50 μ g/ml) and penicillin (50 units/ml) at 37 °C [27]. Parental Chinese hamster ovary (CHO) cells and CHO cells transfected with B7.1 cDNA (CHO-B7.1⁺), were established and maintained as previously described [28]. The murine T-cell hybridoma DC27.1 was grown in Dulbecco's modified Eagle's medium containing 10% foetal-calf serum, supplemented with sodium pyruvate (1 mM), β -mercaptoethanol (50 μ M) and antibiotics [29].

Immunoprecipitation, immunoblotting and *in vitro* lipid kinase assays

Jurkat cells (2×10^7 except where stated) were gently co-sedimented with 10^7 CHO-B7.1⁺ cells in a volume of 1 ml by centrifugation at 200 *g* for 10 s, incubated at 37 °C in RPMI-1640 for the times indicated in the Figure legends and the pellets lysed in 1 ml of lysis buffer [1% (v/v) Nonidet P40/100 mM NaCl/20 mM Tris (pH 7.4)/10 mM iodoacetamide/10 mM NaF/1 mM PMSF/leupeptin (1 μ g/ml)/antipain (1 μ g/ml)/chymostatin (1 μ g/ml)/pepstatin A (1 μ g/ml)/1 mM sodium orthovanadate]. Lysates were pre-cleared and immunoprecipitates performed for 2 h at 4 °C as described [27] using CD28 mAb 9.3 (1 μ g/ml lysate) coupled to Protein A-Sepharose beads (Pharmacia). Immunoprecipitated samples for immunoblotting were electrophoresed through acrylamide gels [7–17% (v/v)

gradient] by SDS/PAGE and transferred by electroblotting on to PVDF membranes (Immobilon P, Millipore, Bedford, MA, U.S.A.) using 10 mM (cyclohexylamino)propanesulphonic acid buffer, pH 11, for 20–24 h at 0.3 mA [27]. Blots were probed with p85 α mAb (0.5 μ g/ml) and proteins visualized by an enhanced chemiluminescence detection system (Amersham), with 0.1 μ g/ml sheep anti-mouse Ig conjugated with horseradish peroxidase as a secondary antibody (Amersham). Alternatively, anti-CD28 immunoprecipitates were washed and subjected to *in vitro* lipid kinase assays as described [27] using a lipid mixture of 100 μ l of 0.1 mg/ml PtdIns and 0.1 mg/ml phosphatidylserine dispersed by sonication in 20 mM Hepes (pH 7.0)/1 mM EDTA. The reaction was initiated by adding 20 μ Ci of [γ -³²P]ATP (3000 Ci/mmol, DuPont-NEN, Stevenage, Herts., U.K.) and 100 μ M ATP to the immunoprecipitates suspended in 80 μ l of kinase buffer. The reaction was terminated after 15 min and phospholipids were then separated by TLC [27].

Metabolic radiolabelling of Jurkat cells and phosphoamino acid analysis

Jurkat cells (4×10^8) were labelled with 1 mCi of [³²P]P_i (8500–9120 Ci/mmol, Life Sciences Products) as described [30]. Jurkat cells (2×10^7 /0.5 ml) were then co-sedimented with 10^7 CHO-B7⁺ cells or parental CHO-B7[−] cells in a volume of 1 ml by centrifugation at 200 *g* for 10 s, and incubated at 37 °C for appropriate times as indicated in the Figure legends. Cell lysates were immunoprecipitated with anti-CD28 mAb and immunoprecipitates were washed 7–10 times in ice-cold lysis buffer. Proteins were then electrophoresed through acrylamide gels [7–17% (v/v) gradient] by SDS/PAGE under reducing conditions. Proteins were visualized by autoradiography at −70 °C. For phosphoamino acid analysis, ³²P-labelled protein was electroblotted on to a PVDF filter after SDS/PAGE, which was then autoradiographed to determine the position of the labelled bands. Portions of the filter that contained ³²P-labelled protein were excised, soaked in methanol, and then washed in distilled water. Bound protein was hydrolysed in 6 M HCl (150–250 μ l) for 1 h at 110 °C. The hydrolysate was dried *in vacuo* and redissolved in 5 μ l of 0.1 M acetic acid containing 3 mg each of unlabelled phosphothreonine, phosphoserine and phosphotyrosine as standards. The phosphoamino acids were separated at 20 mA for 80 min on cellulose TLC (Polygram Cell 300, Macherey-Nagel, Duren, Germany) in acetic acid/pyridine/water (10:1:189, by vol.). The labelled phosphoamino acids were visualized by autoradiography at −70 °C.

PtdIns lipid labelling, extraction and HPLC separation

Phospholipids were extracted from 2×10^7 [³²P]P_i-labelled Jurkat cells with 750 μ l of chloroform/methanol/water (32.6:65.3:2.1, by vol.) as described [30,31]. The samples were deacylated and the ³²P-labelled glycerophosphoryl derivatives of PtdIns lipids were analysed by anion-exchange HPLC analysis using a Partisphere SAX column (Whatman) [28,30,31].

RESULTS

B7.1 ligation of CD28 stimulates serine/threonine phosphorylation of CD28

To investigate whether CD28 is itself a target for serine/threonine kinases, we utilized a [³²P]P_i metabolic labelling approach, whereby CD28 was immunoprecipitated from [³²P]P_i-labelled Jurkat cells. Accordingly, ligation of CD28 by B7.1 stimulated rapid phosphorylation of CD28 and these levels of increased phosphorylation exhibited sustained kinetics between 1–10 min,

Figure 1 Time course of ligation-stimulated CD28 phosphorylation in Jurkat cells

(A) [32 P] P_i -labelled Jurkat cells (2×10^7) were co-sedimented with 10^7 CHO cells (lane 1), or with 10^7 CHO-B7.1 $^{+}$ cells (lanes 2–4) for the times indicated (1' = 1 min, etc.). After incubation at 37 °C, cells were lysed and all lysates were subjected to immunoprecipitation with CD28 mAb 9.3 as described in the Materials and methods section. After washing the immunoprecipitates, proteins were resolved by SDS/PAGE and visualized by autoradiography at -70 °C. Molecular mass markers are shown on the left. (B) After autoradiography, the p44 phosphorylated band in (A) (lane 2) was excised, and bound protein was hydrolysed in HCl. Phosphoamino acids (P-Ser, P-Thr and P-Tyr) were resolved by TLC as described in the Materials and methods section and are indicated on the left.

followed by a small decrease in CD28 phosphorylation at 15 min (Figure 1A). Phosphoamino acid analysis revealed that phosphorylation of CD28 after ligation by B7.1 occurred predominantly on serine/threonine residues (Figure 1B). This phosphorylation occurred preferentially on serine and was approx. 2-fold greater than the observed phosphorylation on threonine residues (Figure 1B). In contrast, approx. less than 1% of the phosphorylation of CD28 was detected to occur on tyrosine residues (Figure 1B).

PKC activators and inhibitors do not alter basal or B7.1-stimulated phosphorylation of CD28

Given the presence of potential PKC phosphorylation sites within the CD28 cytoplasmic tail, we investigated whether or not PMA treatment of Jurkat cells was able to mimic ligation-stimulated CD28 phosphorylation and/or potentiate this outcome of B7.1 engagement of CD28. However, PMA treatment of Jurkat cells did not generally result in detectable phosphorylation of resting or B7.1-stimulated CD28 (Figure 2A), although the data presented do indicate a slight potentiation of ligation-stimulated CD28 phosphorylation by a single concentration (100 ng/ml) of PMA (Figure 2A). However, at least four other experiments revealed that this observation was not reproducible, and the observed potentiation in response to 100 ng/ml PMA may reflect discrepancies between individual samples in the amount of immunoprecipitated protein loaded on to the gel. In addition, pre-treatment with Ro-31/8220, a staurosporine-related PKC inhibitor [32], generally had no effect on the ligation-stimulated phosphorylation of CD28 (Figure 2B), although certain experiments revealed a very slight potentiation of CD28 phosphorylation, depending on the concentration used. For

Figure 2 Effect of PMA and Ro-31/8220 on ligation-stimulated CD28 phosphorylation

(A) [32 P] P_i -labelled Jurkat cells (2×10^7) were incubated at 37 °C with either vehicle (lane 1), PMA at the concentrations indicated (lanes 6 and 7), or, after co-sedimentation with 10^7 CHO-B7.1 $^{+}$, in the absence (lane 2) or presence of PMA at the concentrations indicated (lanes 3–5). Where appropriate, PMA was added 5 min before the addition of CHO-B7.1 $^{+}$. (B) [32 P] P_i -labelled Jurkat cells (2×10^7) were incubated at 37 °C either alone (lane 7) or after co-sedimentation with 10^7 CHO-B7.1 $^{+}$ in the absence (lane 1) or presence of Ro-31/8220 at the concentrations indicated (lanes 2–6). Ro-31/8220 was added 5 min before the addition of CHO-B7.1 $^{+}$. After appropriate incubation at 37 °C, cells [(A) and (B)] were lysed and all lysates were subjected to immunoprecipitation with CD28 mAb 9.3, as described in the Materials and methods section. After washing the immunoprecipitates, proteins were resolved by SDS/PAGE and visualized by autoradiography at -70 °C. Molecular mass markers are shown on the left.

instance, Figure 2(B) demonstrates a slight potentiation of the ligation-stimulated CD28 phosphorylation in the presence of 0.5–5 μ M Ro-31/8220, although these potentiating effects of Ro-31/8220 were not observed at higher concentrations (> 50 μ M) and were not consistently observed in at least four other experiments. Hence, the apparent enhancement of ligation-stimulated CD28 phosphorylation by certain concentrations of Ro-31/8220 observed in Figure 2(B), may also reflect minor discrepancies in the amount of immunoprecipitated protein loaded onto the gel.

Effect of other serine/threonine kinase inhibitors and activators on ligation-induced CD28 phosphorylation

In an attempt to further elucidate the identity of the protein serine/threonine kinase that phosphorylates CD28 after stimulation by B7.1, we have used 0.001–1 μ M staurosporine [32,33], 0.1–100 μ M H9 [34] or 0.5–50 μ M calphostin C [35], which are known to inhibit several kinases including PKC, cAMP-dependent protein kinase and/or cGMP-dependent protein kinase. To date, however, we have been unable to modify ligation-stimulated CD28 phosphorylation with these inhibitors (results not shown). Moreover, treatment of Jurkat cells with a variety of agents known to activate cAMP-dependent protein kinase (e.g. 5 μ M forskolin in combination with 500 μ M isobutylmethylxanthine for 1 h; or 0.1–100 μ M 8-bromo-cAMP for 5–15 min) and cGMP-dependent protein kinase (e.g. 100 μ M sodium nitroprusside or 0.1–100 μ M 8-bromo-cGMP for 5–15 min), had no effect on either basal or ligation-stimulated CD28 phosphorylation (results not shown). Since at least one serine/threonine residue within the CD28 cytoplasmic domain (e.g. Thr¹⁷⁷) lies within a potential site for phosphorylation by the proline-directed ERK serine/threonine kinases, we also examined the effect of PD098059, which inhibits MAP kinase kinase and thus ERK activation [36]. However, 1 h pre-treatment with PD098059 (0.1–50 μ M) had no effect on the phosphorylation of CD28 after ligation by B7.1. Similarly, SB203580 (30 μ M), which inhibits the related, but distinct p38 MAP kinase [37], also had no effect on ligation-stimulated CD28 phosphorylation (results not shown).

PMA pre-treatment inhibits ligation-induced CD28 association with, and activation of, PI 3-kinase

Hutchcroft et al. have previously correlated the inhibition of CD28 coupling to PI 3-kinase after PMA treatment of T-cells [26] with PMA-stimulated threonine phosphorylation of CD28 under *in vitro* conditions [23]. Our observation that PMA treatment of Jurkat cells had no detectable effect on CD28 phosphorylation under *in vivo* conditions was therefore in marked contrast with the findings of Hutchcroft et al. Hence, we investigated whether or not PMA was able to disrupt CD28 coupling to PI 3-kinase under conditions where no detectable effects on CD28 phosphorylation were observed. Accordingly, we performed immunoblotting experiments on CD28 immunoprecipitates using an antibody to the p85 subunit of PI 3-kinase. These experiments revealed that the association of the p85 subunit with CD28 after stimulation by B7.1, was partially inhibited by treatment with PMA at concentrations (e.g. 100 ng/ml) that did not stimulate CD28 phosphorylation (Figure 3A). The decreased association of p85 with CD28 in the presence of PMA, correlated with a decrease in the amount of PI 3-kinase activity present in CD28 immunoprecipitates derived from Jurkat cells stimulated by B7.1 (Figure 3B). The inhibition by PMA of both the ligation-stimulated CD28 association with PI 3-kinase (Figure 3C) and the lipid kinase activity associated with CD28 immunoprecipitates (Figure 3D) was prevented by the pre-treatment of Jurkat cells with the PKC inhibitor Ro-31/8220. It should be noted, however, that Ro-31/8220 exerted a greater degree of protection against PMA-stimulated down-regulation of p85 association with CD28 (Figure 3C) than it did for PMA-stimulated down-regulation of *in vitro* lipid kinase activity associated with CD28 immunoprecipitates (Figure 3D).

In addition, we also determined the effect of PMA treatment on *in vivo* PI 3-kinase activity by measuring the accumulation of PtdIns(3,4,5) P_3 after CD28 stimulation by B7.1. Thus [³²P]P_i-labelled Jurkat cells were co-sedimented with CHO-B7.1⁺ (Figure 4A) in the presence of various concentrations of PMA. Ligation

Figure 3 Effect of PMA on recruitment of PI 3-kinase by CD28

(A) and (B) Jurkat cells (2×10^7) were incubated at 37 °C with vehicle or PMA (100 ng/ml) for the times indicated before sedimentation with CHO-B7.1⁺ cells (10^7). (C) and (D) Jurkat cells were incubated with Ro-31/8220 (0.5–50 μ M) for 5 min before the addition of 100 ng/ml PMA. These cells were then incubated for a further 5 min in the presence of both Ro-31/8220 and PMA, after which the Jurkat cells were co-sedimented with CHO-B7.1⁺. After co-sedimentation, cells were incubated again at 37 °C for 5 min, after which they were lysed (A), (B), (C) and (D). All lysates were subjected to immunoprecipitation with CD28 mAb 9.3. After washing the immunoprecipitates, proteins were resolved by SDS/PAGE, transferred to PVDF membranes and immunoblotted with p85 α mAb (indicated by the arrow) (A) and (C), or assayed for associated *in vitro* lipid kinase activity using PtdIns as a substrate (B) and (D), as described in the Materials and methods section. The resulting PtdIns(3,4,5) P_3 lipid product is indicated by the arrow.

of CD28 by B7.1 resulted in the accumulation of PtdIns(3,4,5) P_3 (Figure 4A) as previously described [28,38]. However, pre-treatment with PMA (5–500 ng/ml) inhibited the CD28-stimulated accumulation of PtdIns(3,4,5) P_3 (Figure 4A). The highest concentration of PMA examined was 500 ng/ml, which induced $68(\pm 5.5)\%$ inhibition ($n = 5$) of CD28-stimulated PtdIns(3,4,5) P_3 accumulation after ligation by B7.1. The non-PKC-activating phorbol ester 4 α -phorbol was without effect on CD28-stimulated PtdIns(3,4,5) P_3 accumulation (Figure 4A). Optimal inhibition by PMA of the CD28-stimulated PtdIns(3,4,5) P_3 accumulation was induced after 5–10 min of pre-treatment and was sustained for at least 30 min (Figure 4B). Pre-treatment of Jurkat T-cells with the PKC inhibitor Ro-31/8220 (5 μ M) before the addition of PMA, considerably reduced the inhibitory effects of PMA on CD28-stimulated PtdIns(3,4,5) P_3 accumulation (Figure 4C), although some inhibition was still detectable. Neither PMA nor Ro-31/8220 had any detectable effect on basal levels of PtdIns(3,4,5) P_3 (Figure 4C).

Site mutagenesis of Tyr¹⁷³ prevents ligation-stimulated CD28 phosphorylation

PI 3-kinase is a unique kinase in that it exhibits dual specificity as both a lipid kinase and a protein serine/threonine kinase [22], and its coupling to CD28 after CD28 ligation is now well established [9]. We reasoned therefore that PI 3-kinase bound to the (p)Tyr¹⁷³-Met-Asn-Met motif may mediate ligation-stimulated phosphorylation of the CD28 cytoplasmic tail. To further characterize ligation-stimulated phosphorylation of CD28, we

Figure 4 Effect of PMA on the CD28-stimulated accumulation of PtdIns(3,4,5)P₃

utilized the DC27.1 murine T-cell hybridoma, which had been transfected with either wild-type human CD28 or a site-specific CD28 mutant in which Tyr¹⁷³ was mutated to Phe (Tyr¹⁷³ → Phe), since previous studies have reported that mutation of Tyr¹⁷³ is sufficient to completely abolish binding of PI 3-kinase to CD28 [17]. In murine T-cell hybridomas transfected with human wild-type CD28, stimulation of CD28 by B7.1 resulted in the phosphorylation of CD28 (Figure 5A). As demonstrated in Jurkat cells, phosphorylation occurred predominantly on serine/threonine residues with equal stoichiometry (results not shown). In contrast with the serine/threonine phosphorylation observed in Jurkat, ligation-stimulated CD28 phosphorylation was of much shorter duration, such that phosphorylation was at a maximum at 1 min post-stimulation and had declined to basal levels at 10 min post-stimulation. Accordingly, subsequent experiments were performed using the optimum time of 1 min stimulation of CD28 by B7.1. The kinetics of ligation-stimulated CD28 serine/threonine phosphorylation, correlate with the previously described kinetics of CD28-stimulated accumulation of PtdIns(3,4,5)P₃ in both Jurkat cells and murine T-cell hybridomas [29,38], suggesting that these two events may indeed be related. Interestingly, we were unable to detect ligation-stimulated phosphorylation of CD28 in DC27.1 cells expressing Tyr¹⁷³ → Phe mutants (Figure 5B). Immunoblotting experiments confirmed previous observations [17] that whereas PI 3-kinase associated with CD28 following ligation by B7.1 in cells expressing wild-type CD28, there was no detectable association of PI 3-kinase with CD28 in the Tyr¹⁷³ → Phe mutants (results not shown).

Evidence that phosphorylation of CD28 on serine/threonine residues is independent of PI 3-kinase activity

Since mutation of Tyr¹⁷³ has previously been shown to abolish CD28 coupling to PI 3-kinase [17,29], our demonstration that engagement of B7.1 did not stimulate CD28 phosphorylation in the Tyr¹⁷³ → Phe mutant initially suggested the possible involvement of PI 3-kinase in modulating CD28 serine/threonine phosphorylation. To further investigate this possibility, we utilized another site-specific CD28 mutant in which Tyr²⁰⁰ was mutated to Phe (Tyr²⁰⁰ → Phe) and expressed in the murine T-cell hybridoma. Although Tyr²⁰⁰ does not lie within any recognized consensus binding motifs for the SH2 domains of the p85 subunit of PI 3-kinase, mutation of this residue has previously been reported to inhibit PI 3-kinase association with CD28 by approx. 90 % [29]. However, the precise role of Tyr²⁰⁰ in the recruitment of PI 3-kinase to CD28 remains unclear. Given our observation that mutation of Tyr¹⁷³ disrupted both PI 3-kinase coupling to CD28 and ligation-stimulated CD28 phosphorylation, it was surprising to note that mutation of Tyr²⁰⁰ to Phe (Tyr²⁰⁰ → Phe) had no effect on the phosphorylation of CD28 after ligation by B7.1 (Figure 6A). Immunoblotting experiments confirmed previous observations [29] that the ligation-stimulated association of PI 3-kinase with CD28 in the Tyr²⁰⁰ → Phe mutants was

[³²P]P_i-labelled Jurkat cells (2×10^7) were incubated at 37 °C (A) with PMA (solid bars) or 4α-phorbol (hatched bars) for 20 min; (B) with PMA (100 ng/ml) for the times indicated; (C) with Ro-31/8220 (5 μM) as indicated below the histogram for 5 min followed by vehicle/PMA for 20 min. Cells (10^7) were co-sedimented with CHO-B7.1⁺ and were incubated again at 37 °C for 5 min. Vehicle controls were added to the other treatments where appropriate. (A), (B) and (C) After incubation with CHO-B7.1⁺, PtdIns(3,4,5)P₃ was extracted, deacylated and the glycerophosphorylinositol derivative of PtdIns(3,4,5)P₃ [GroPIns(3,4,5)P₃] analysed by HPLC separation, as described in the Materials and methods section. Data are: (A) and (C) from a single experiment representative of at least four others; (B) means ± standard errors from at least three experiments.

Figure 5 Ligation-stimulated phosphorylation of wild-type and mutant human CD28 in murine T-cell hybridomas

(A) [32 P] P_i -labelled murine T-cell hybridomas (2×10^7) expressing wild-type human CD28 were sedimented alone (lanes 1 and 6), or co-sedimented with 10^7 CHO-B7.1 $^{+}$ cells (lanes 2–5) or with 10^7 parental CHO cells (lanes 7–9) for the times indicated (1' = 1 min, etc.). (B) [32 P] P_i -labelled murine T-cell hybridomas (2×10^7) expressing wild-type human CD28 or Tyr 173 $\rightarrow$ Phe site-specific CD28 mutants were incubated at 37 °C either alone (lanes 1 and 5), with 100 ng/ml PMA (lanes 3 and 7), or after co-sedimentation with CHO-B7.1 $^{+}$ in the absence (lanes 2 and 6) or presence of 100 ng/ml PMA (lanes 4 and 8). Where appropriate, PMA was preincubated with the cells 10 min before co-sedimentation with CHO-B7.1 $^{+}$ cells. After incubation for 1 min (1') at 37 °C with either CHO-B7.1 $^{+}$ (A) and (B) or parental CHO (A), cells were lysed and subjected to immunoprecipitation with CD28 mAb 9.3, as described in the Materials and methods section. After washing the immunoprecipitates, proteins were resolved by SDS/PAGE and visualized by autoradiography at -70 °C. Molecular mass markers are shown on the left.

reduced by approx. 90% compared with that observed in cells expressing wild-type CD28 (results not shown).

The effects of wortmannin and the structurally unrelated LY294002 on ligation-stimulated CD28 phosphorylation were also examined, since they act as inhibitors of both the protein kinase and the lipid kinase activities of PI 3-kinase [39,40]. However, concentrations of wortmannin and LY294002, which are known to inhibit CD28-stimulated PtdIns(3,4,5) P_3 accumulation [30,38], had no effect on phosphorylation of CD28 after

Figure 6 Effect of site-specific mutagenesis of Tyr 200 and PI 3-kinase inhibitors on CD28 phosphorylation

(A) [32 P] P_i -labelled murine T-cell hybridomas (2×10^7) expressing wild-type human CD28 or Tyr 200 $\rightarrow$ Phe site-specific CD28 mutants were incubated at 37 °C after sedimentation alone (lanes 1 and 3) or after co-sedimentation with CHO-B7.1 $^{+}$ (lanes 2 and 4). (B) [32 P] P_i -labelled Jurkat cells (2×10^7) were incubated at 37 °C either alone (lane 1), with wortmannin (lane 6), LY294002 (lane 10), or after co-sedimentation with CHO-B7.1 $^{+}$ in the absence (lane 2) or presence of either wortmannin (lanes 3–5) or LY294002 (lanes 7–9). Where appropriate, cells were preincubated at 37 °C with wortmannin or LY294002 at the concentrations indicated for 5 min (5') before co-sedimentation and incubation with CHO-B7.1 $^{+}$. After incubation with CHO-B7.1 $^{+}$ cells for 1 min (1') (A) or 5 min (B) at 37 °C, cells were lysed and subjected to immunoprecipitation with CD28 mAb 9.3, as described in the Materials and methods section. After washing the immunoprecipitates, proteins were resolved by SDS/PAGE and visualized by autoradiography at -70 °C. Molecular mass markers are shown on the left.

ligation by B7.1 in either Jurkat cells (Figure 6B) or murine T-cell hybridomas expressing wild-type CD28 (results not shown). Appropriate control experiments were routinely performed to verify that working batches of these inhibitors prevented CD28-stimulated accumulation of PtdIns(3,4,5) P_3 or *in vitro* lipid kinase activity present in either CD28 or p85 immunoprecipitates, as previously described [30].

DISCUSSION

The data presented here show that stimulation of CD28 by its natural ligand B7.1 results in heavy serine/threonine phos-

phorylation of CD28. The presence of several potential PKC phosphorylation sites suggested that the observed serine/threonine phosphorylation of CD28 in response to B7.1 ligation might be mediated by activation of PKC. However, phosphorylation of CD28 after ligation by B7.1 in Jurkat cells was not mimicked by treating cells with PMA, a phorbol ester that activates both conventional (e.g. α , β and γ) and novel (e.g. δ , ϵ and η) isoenzymes of PKC. One possibility is that atypical PKC (e.g. ζ) isoenzymes, which are insensitive to PMA, may mediate ligation-stimulated serine/threonine phosphorylation of CD28. However, the PKC inhibitor Ro-31/8220, which can exhibit a limited degree of selectivity towards PKC isoenzymes [33], was used at concentrations sufficient to inhibit both PMA-activated and PMA-resistant PKC isoforms [33,41,42] and it had no reproducible effect on CD28 phosphorylation in response to B7.1 ligation. Taken together, these results indicate that PKC is not involved in mediating ligation-stimulated serine/threonine phosphorylation of CD28. Nevertheless, PMA treatment of Jurkat cells did prevent the association of PI 3-kinase with the cytoplasmic tail of CD28 and the *in vivo* accumulation of PtdIns(3,4,5) P_3 after stimulation of CD28 by B7.1. Since these effects of PMA were not mimicked by the non-PKC-activating phorbol ester 4 α -phorbol and were prevented by Ro-31/8220, the inhibitory effects of PMA on CD28 coupling to PI 3-kinase are likely to be mediated by activation of PKC.

Our findings that PMA disrupted PI 3-kinase coupling to CD28 after stimulation by the physiological ligand B7.1 are in agreement with earlier observations obtained using Jurkat cells stimulated with anti-CD28 mAb [26]. However, our findings that PMA does not stimulate CD28 phosphorylation are clearly in marked contrast with those of Hutchcroft et al., who have previously reported that PMA stimulated the phosphorylation of CD28 exclusively on threonine in Jurkat cells [23]. There are several reasons for the differences between our findings and those of Hutchcroft et al.: (i) the threonine kinase detected by Hutchcroft et al. co-associated with CD28 immunoprecipitates in *in vitro* immune-complex assays and may not therefore be functional under *in vivo* settings and thus may have no physiological role; (ii) *in vivo* PMA-stimulated phosphorylation occurs below detectable limits at very low stoichiometry; (iii) Hutchcroft et al. utilized the mild detergent Brij 96 in cell lysis buffers, which has previously been used to maintain low-affinity protein-protein associations [43] and which may have no physiological relevance; (iv) specific phosphatase(s) that may target phosphoserine/phosphothreonine residues probably operate under the *in vivo* conditions used in this study, but these may not be present in the *in vitro* immune-complex assay.

The observed inhibitory effects of PKC activation on CD28 coupling to PI 3-kinase may operate to limit CD28 signal transduction and CD28-dependent IL-2 production. Indeed, transient activation of PKC has previously been demonstrated to limit IL-2 production in response to mitogens in murine T-cells [44]. The physiological signal for PKC activation may be elicited by CD28, since levels of diacylglycerol are elevated following CD28 ligation [45] and this may be part of an auto-regulatory negative-feedback mechanism that limits CD28 activation of PI 3-kinase and/or other signalling cascades. Alternatively, the CD3-T-cell antigen receptor complex, which activates PKC [46], may mediate a possible 'cross-talk' mechanism that modulates CD28 signalling, although we have been unable to detect any effect of CD3 ligation on the interaction between CD28 and PI 3-kinase (S. Ward, unpublished work). Since we were unable to detect reproducible PMA-stimulated phosphorylation of CD28, our observations may be explained by effects of PMA-activated PKC on PI 3-kinase, rather than CD28. Certainly, the δ and ϵ

isoforms of PKC contain consensus sequence-recognition motifs for p85 SH2 domains and associate with PI 3-kinase [47]. Moreover, PMA treatment stimulates phosphorylation of the p110 catalytic and p85 regulatory subunits exclusively on serine and threonine residues respectively [48]. Since the p85 subunit may act primarily as a multi-functional adaptor molecule by virtue of its SH2, SH3 and/or proline-rich regions [9], PMA-stimulated phosphorylation of the p85 subunit may therefore disrupt protein-protein interactions involving the p85 subunit. Another possibility is that the reported PMA-stimulated phosphorylation of the p110 catalytic subunit may modulate PI 3-kinase activity. Certainly, PMA can induce modest accumulation of PtdIns(3,4,5) P_3 in fibroblasts [49] and platelets [50]. However, neither PMA nor PKC inhibitors have any significant effect on the basal levels of PtdIns(3,4,5) P_3 in Jurkat cells. This is an unexpected observation given that p21^{ras} is a potent downstream target of phorbol-ester-activated PKC in T-cells [46], and in other systems it can interact directly with the p110 catalytic subunit [51] and has been demonstrated to activate PI 3-kinase [52]. Thus T-cells may express distinct isoforms of PI 3-kinase whose kinase activity is independent of a regulatory input from p21^{ras}. Another unexpected observation was that although treatment with the PKC inhibitor Ro-31/8220 almost fully prevented PMA-stimulated down-regulation of p85 association with CD28, the levels of lipid kinase activity associated with CD28 immunoprecipitates were not similarly restored. The reasons for this discrepancy are not clear, but this observation may reflect the differential involvement of various PKC isoenzymes in the regulation of PI 3-kinase/CD28 protein interactions and/or PI 3-kinase activity. Although these isoenzymes may all be stimulated by PMA under the conditions employed in this study, they may exhibit different sensitivity to the PKC inhibitor Ro-31/8220 and this may be one possible explanation for these surprising observations. Alternatively, this discrepancy may be an *in vitro* artifact, since the PMA-stimulated down-regulation of *in vivo* CD28-stimulated PtdIns(3,4,5) P_3 accumulation that was observed in [³²P]P_i-labelled Jurkat cells was almost totally prevented by pre-treatment with Ro-31/8220.

The Thr¹⁷⁷ residue lies within a potential phosphorylation motif for proline-directed serine/threonine kinases such as ERK [25]. ERK has been shown to phosphorylate Sos and down-regulate the Ras activation pathway in a proposed negative-feedback mechanism [53]. One possibility therefore is that ERK mediates ligation-stimulated serine/threonine phosphorylation of CD28, which may regulate CD28 signalling. However, there are several reasons why it is unlikely that the known members of the MAP kinase family such as ERK or c-Jun N-terminal kinase are involved in mediating ligation-stimulated CD28 phosphorylation. Firstly, phosphorylation of CD28 after ligation by B7.1 is unaffected by pre-treatment with PD098059, an inhibitor of MAP kinase kinase that therefore inhibits activation of the ERK signalling cascade [36]. Secondly, although antibody stimulation of CD28 results in activation of the ERK signalling pathway, the natural ligand B7.1 does not [54]. Hence, the ERK signalling cascade would not be activated under the conditions used in this study. Similarly, CD28 ligation alone is not sufficient to activate the c-Jun N-terminal kinase subgroup of MAP kinases. Instead, CD28 activates c-Jun N-terminal kinase only in the presence of additional signals provided by either PMA/ionomycin or CD3 ligation [55,56].

Although ligation-stimulated tyrosine phosphorylation of CD28 can be detected by immunoblotting with anti-phosphotyrosine mAb [17,23], phosphoamino acid analysis of immunoprecipitated ³²P-labelled CD28 after ligation by B7.1 revealed very small amounts of phosphotyrosine in CD28 immuno-

precipitates. Thus tyrosine phosphorylation of CD28 occurs at very low stoichiometry relative to serine/threonine phosphorylation. Interestingly, our study demonstrates that mutagenesis of the Tyr¹⁷³ residue, which lies within the PI 3-kinase binding motif [17], results in the complete loss of detectable serine/threonine phosphorylation of CD28. Mutation of Tyr¹⁷³ has previously been shown to result in an 80% reduction of the CD28 tyrosine phosphorylation that can be detected by immunoblotting [29], as well as the complete abolition of the binding of PI 3-kinase to CD28 [17]. Our data therefore strongly suggests that integrity of this PI 3-kinase binding motif is required for optimal CD28 phosphorylation. Since PI 3-kinase can act as both a lipid kinase and a protein serine/threonine kinase [22], we considered the possibility that PI 3-kinase might mediate serine/threonine phosphorylation of CD28. However, surprising and contradictory results were obtained from other experiments that were designed to further investigate this possibility. Firstly, previous studies have shown by immunoblotting that site-specific mutagenesis of Tyr²⁰⁰ is sufficient to reduce the association of PI 3-kinase with CD28 by approx. 90%, although this mutation has no detectable effect on ligation-stimulated tyrosine phosphorylation of CD28 [29]. The data presented in this study indicate that ligation-stimulated serine/threonine phosphorylation of CD28 is similarly unaffected by mutation of the Tyr²⁰⁰ residue, since overall phosphorylation of CD28 in the Tyr²⁰⁰ → Phe mutant is comparable with that observed in cells expressing wild-type CD28. Nevertheless, low-stoichiometry binding of PI 3-kinase to CD28 may occur in sufficient amounts in the Tyr²⁰⁰ → Phe mutant to mediate normal serine/threonine phosphorylation of CD28. Secondly, compelling evidence against PI 3-kinase directly phosphorylating CD28 is provided by the observation that the PI 3-kinase inhibitors wortmannin or LY294002 did not inhibit ligation-stimulated phosphorylation, and these findings correlate with those of other groups [23]. However, there are certain problems associated with the interpretation of the results obtained with the PI 3-kinase inhibitors. For instance, we have previously demonstrated that wortmannin does not prevent the ligation-stimulated recruitment of PI 3-kinase to CD28 [30]. Thus one possibility is that the inhibitors were not sufficiently effective to completely inhibit CD28-associated PI 3-kinase activity. This remaining PI 3-kinase activity may be below the sensitivity of the assays employed to detect lipid kinase activity, but it may be sufficient for maximal stimulation of CD28 phosphorylation. Furthermore, the proposed adaptor-molecule functions of the p85 subunit of PI 3-kinase [9] may mediate the recruitment of a serine/threonine kinase responsible for phosphorylating CD28, and this function of p85 would be insensitive to PI 3-kinase inhibitors, but would be disrupted by mutagenesis of Tyr¹⁷³. Alternatively, the adaptor molecule Grb-2 has also been reported to bind to CD28 via the motif around Tyr¹⁷³ [57], and may be involved in the recruitment of a serine/threonine kinase responsible for CD28 phosphorylation. In contrast with the effects on PI 3-kinase, it is possible that mutation of Tyr²⁰⁰ has no effect on Grb-2 association with CD28, which would explain the lack of effect of this mutation on CD28 phosphorylation. However, there is no detectable Grb-2 association with human CD28 transfected into murine hybridoma T-cells (D. Olive, unpublished work).

There are several outstanding questions regarding the observed ligation-stimulated phosphorylation of CD28. Firstly, the physiological role of ligation-stimulated serine/threonine phosphorylation of CD28 remains to be established, although the activation of serine/threonine kinase(s) by CD28 is very interesting since it may contribute to the mechanisms by which CD28 couples to and regulates downstream signalling cascades. Some of these

mechanisms, such as how CD28 couples to and activates acidic sphingomyelinase [10], are still far from fully understood. Secondly, the identity of the serine/threonine kinase responsible for ligation-dependent CD28 phosphorylation also remains unclear. Although CD28 has previously been reported to elevate intracellular levels of cGMP [58], suggesting that cGMP-dependent protein serine/threonine kinase may be activated by CD28, pharmacological agents known to inhibit or activate cGMP-dependent protein kinase or other known serine/threonine kinases have no detectable effect on the ligation-stimulated serine/threonine phosphorylation of CD28. One possibility is that the phorbol-ester-resistant, PKC-related serine/threonine kinase known as protein kinase N, which is activated by Rho [59,60], may be involved in the phosphorylation of CD28, since there is evidence that CD28 can couple to the Rho family of GTP-binding proteins [61]. It will therefore be interesting to examine the possible role of protein kinase N in ligation-dependent phosphorylation of CD28. One final possibility is that a so-far-unidentified novel serine/threonine kinase mediates CD28 phosphorylation. Given that manipulation of CD28 may be beneficial in several disease states such as protection against HIV infection [62] and multiple sclerosis [63], as well as acceptance of allografts [64], a CD28-activated serine/threonine kinase that phosphorylates CD28 may be a valuable future therapeutic target.

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