Differential Phosphorylation of the T Lymphocyte Costimulatory Receptor CD28

ACTIVATION-DEPENDENT CHANGES AND REGULATION BY PROTEIN KINASE C*

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Treatment of T lymphocytes with phorbol ester and anti-CD28 monoclonal antibody (mAb) can induce proliferation and interleukin 2 production by triggering still undefined intracellular signaling pathways. We have developed a deglycosylation procedure that allows the precise identification of a distinct CD28 protein band, facilitating the analysis of activation-dependent changes in the phosphorylation state of CD28. Phorbol 12-myristate 13-acetate (PMA) treatment induced the in vitro phosphorylation of CD28 on threonine as detected in immune complex kinase assays. This effect of PMA was (i) rapid, preceding a PMA-induced increase in CD28 surface expression; (ii) occurred using kinase buffer containing either manganese or magnesium; and (iii) was found in human peripheral T cells, Jurkat T cells, and in a Jurkat subclone, J.Cam1, that is deficient in Lck tyrosine kinase activity. In contrast, anti-CD28 monoclonal antibody stimulation led to a decrease in PI3 kinase activity or its association with CD28 when manganese-dependent increase in CD28 on threonine that was manganese-dependent and required Lck tyrosine kinase activity, as it was undetectable in J.Cam1 cells. Importantly, CD28 was phosphorylated on tyrosine in vivo as detected with anti-phosphotyrosine antibodies after stimulation with anti-CD28 monoclonal antibody. The in vivo tyrosine phosphorylation of CD28 was inhibited by PMA treatment and was absent in J.Cam1 cells. Thus, the CD28 coreceptor can trigger different intracellular signaling pathways, depending upon the nature of the initial costimulatory signal.

While the signaling pathway triggered by ligation of CD28 is poorly defined, it appears to be resistant to inhibition by the immunosuppressive agents cyclosporin A and FK506 that inhibit the serine/threonine phosphatase, calcineurin (reviewed in Ref. 4). Herbimycin A, a selective inhibitor of tyrosine kinases, can block the enhancement of IL-2 production and cell proliferation following stimulation with anti-CD28 mAb and PMA (5, 6), demonstrating a requirement for protein-tyrosine kinase activity for these events. The cytoplasmic protein-tyrosine kinases Lck, Fyn, and Itk/Emt/Tsk have been implicated in early CD28-dependent signaling events (7–9). Stimulation of cells with anti-CD28 mAb alone can trigger the phosphorylation of CD28 on tyrosine residues (7) with the concomitant recruitment and activation of the phosphatidylinositol 3-kinase (PI3 kinase) (10–15). Surprisingly, however, there was no increase in P13 kinase activity or its association with CD28 when Jurkat cells were treated with PMA and anti-CD28 mAb, conditions able to stimulate IL-2 production (15, 16).

We have identified early changes in protein phosphorylation that can occur following ligation of CD28. We have previously shown (7) that stimulation of human peripheral T lymphocytes or Jurkat T cells with anti-CD28 mAb led to a rapid increase in the tyrosine phosphorylation of CD28 as detected in immune complex kinase assays and in vivo [32P]orthophosphate labeling studies. Notably, this induction of tyrosine kinase activity was readily apparent in the absence of phorbol ester treatment. Incubation of the cells with PMA did, however, have dramatic effects on the in vivo phosphorylation profile of CD28 immune complexes. In particular, there was a pronounced increase in the basal levels of CD28 phosphorylation in the absence of CD28 ligation. Thus, triggering of an initial signaling cascade by PMA visibly altered the CD28 signaling complex.

To characterize further these PMA-induced and activation-dependent changes, we have developed a procedure for the enzymatic deglycosylation of CD28 that permits identification of the receptor following separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Identification of the deglycosylated CD28 protein was confirmed by cell-surface biotinylation, [35S]methionine/cysteine metabolic labeling and silver staining studies. This technique has allowed us to assess stimulation-dependent changes in the phosphorylation state of CD28 in human peripheral T lymphocytes, Jurkat T cells, and the Jurkat subclone, J.Cam1, that is deficient in Lck tyrosine kinase activity. We found that PMA treatment caused a rapid and Lck-independent increase in the in vitro phosphorylation of CD28 on threonine residues. A CD28 mAb stimulation of the CD28 receptor resulted in manganese-and Lck-dependent phosphorylation of CD28 on tyrosine residues. No evidence was found for serine or threonine phosphorylation of CD28 by its associated signaling protein, PI3 kinase. Stimulation of Jurkat cells with anti-CD28 mAb induced the in vivo phosphorylation

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The abbreviations used are: mAb, monoclonal antibody; PI3 kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; PNGase F, peptide N-glycosidase F; PVDF, polyvinylidene difluoride; PKC, protein kinase C; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; RIPT, radiolabeled precipitation buffer.
of CD28 on tyrosine residues; this in vivo tyrosine phosphorylation of CD28 was inhibited by PMA treatment and was completely absent in J.Cam1 cells. Thus, treatment of T lymphocytes with PMA and anti-CD28 mAb, conditions sufficient to stimulate IL-2 production, triggers two distinct phosphorylation pathways, one involving PKC-dependent stimulation of threonine kinase activity, the other involving tyrosine kinase activity. We propose that both of these signaling pathways are important for CD28-dependent lymphokine production.

MATERIALS AND METHODS

Cells—Human peripheral blood T lymphocytes were isolated by centrifugation through Ficoll (Organon Teknika, Durham, NC), plastic adherence, and nylon wool filtration. Contaminating red blood cells were lysed with Tris-buffered ammonium chloride. These T cells were generally >92% CD3+, 88% CD28+, 96% CD2−, and 100% major histocompatibility complex class I+, as determined by flow cytometry (data not shown). The T cell leukemia cell line U266 was kindly provided by Kendall Smith (Cornell University, New York) and the Lck−/− J.Cam1 cell line was from the American Type Culture Collection (ATCC, Rockville, MD). Cells were routinely cultured at 37°C with 5% CO₂ in 10% RPMI (RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal calf serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mg/ml fungizone (Life Technologies, Inc.), 100 μg/ml sodium orthophosphate (Life Technologies, Inc.), 10 μg/ml heparin, 7.2 (M. A. Bioproducts, Bethesda, MD), 2 mM glutamine (Life Technologies, Inc.), and 50 μM 2-mercaptoethanol (Sigma)).

Antibodies—The anti-CD28 mAb 9.3 (Bristol Myers Squibb, Seattle, WA) was used in most experiments. The anti-CD28 mAb 2E12 (the kind gift of R. Mittler, Bristol Myers Squibb), the anti-CD5 mAb Leu 1 (Becton Dickinson, Mountain View, CA), the polyclonal antiserum raised against an N-terminal peptide of human p56lck (Upstate Biotechnology, Inc., Lake Placid, NY) and the anti-CD3 mAb OKT3 (ATCC) were used where indicated. The anti-phosphotyrosine mAb 4G10 was a gift from T. Roberts (Dana-Farber Cancer Institute, Boston, MA).

Kinase Assays—Cells were washed twice with ice-cold PBS and resuspended in labeling media (1×10⁷ cells/ml in a freshly prepared 1 mg/ml solution of sulfosuccinimidyl-6-(biotinamido)hexanoate-biotin (Pierce) in phosphate-buffered saline, pH 7.2, and 2 mM glutamine). The cells were then incubated for 2 h at 37°C in labeling medium containing 1 μCi/ml [35S]methionine (Amersham Corp.), and detected by autoradiography. The immunoprecipitates were collected and deglycosylated as described below. The radiolabeled proteins were separated by SDS-PAGE. The gels were then incubated for 30 min in protein fixation buffer (25% methanol and 10% acetic acid), washed three times with water, and incubated with Autofluor (National Diagnostics, Atlanta, GA) for 1 h. The [35S]-labeled proteins were detected by autoradiography of dried gels.

Deglycosylation—The immunoprecipitates were washed twice with RIPA (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-Cl, pH 8, 1 mM sodium orthovanadate, 100 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), twice with RIPA containing 1 mM NaCl, twice more with RIPA, and once with Buffer D (0.1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 1 mM sodium orthovanadate, 100 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The immunoprecipitated proteins were denatured by boiling for 10 min in a solution of 0.5% SDS and 1% β-mercaptoethanol, then adjusted to 50 mM sodium phosphate, pH 7.5, and 1% Nonidet P-40. The proteins were incubated with 1,000 units of PNase F (New England Biolabs, Inc., Beverly, MA) for 1 h at 37°C. PNase F hydrolyzes N-glycan chains from proteins. The deglycosylated proteins were separated on 10.5% SDS-PAGE gels and visualized as indicated.

Phosphoamino Acid Analysis—Anti-CD28 mAb immunoprecipitates were phosphorylated in vitro and deglycosylated as described above. Following separation on 10.5% SDS-PAGE gels, the phosphoproteins were transferred to PVDF membranes and detected by autoradiography. The radiolabeled CD28 phosphoprotein bands were excised from the membrane and incubated in 5.7 nHCl for 90 min at 110°C. The resulting phosphoamino acids were separated by one-dimensional thin-layer electrophoresis (17) and visualized by autoradiography. The migration positions of phosphotyrosine, phosphothreonine, and phosphoserine standards (Sigma) were determined with ninhydrin (Sigma).

Anti-phosphotyrosine Western Blotting—Anti-CD28 mAb immunoprecipitates were deglycosylated as described above, separated on 10.5% SDS-PAGE gels, and then transferred to PVDF membranes. The membranes were incubated sequentially with the anti-phosphotyrosine mAb 4G10, then HRP-coupled sheep anti-mouse secondary antibody (Amersham Corp.). The phosphotyrosine-containing protein bands were detected by autoradiography after enhanced chemiluminescence (Amersham Corp.) development.

RESULTS

 Pretreatment of Cells with PMA Induces a Dramatic Alteration in the Phosphorylation Profile of CD28 and Associated Proteins—Protein phosphorylation plays a key role in the regulation of both enzymatic activity and protein-protein interactions. Since resting human peripheral T lymphocytes are not amenable to in vivo orthophosphate labeling studies, we have used in vitro kinase assays to develop procedures that can be used to examine the early biochemical interactions between CD28 and its associated complex of signaling proteins. CD28 immune complexes were prepared from Brij 96 detergent lysates of J urkat T cells and then incubated in vitro with [γ-32P]ATP. The resulting phosphoproteins were separated by SDS-PAGE, transferred to PVDF membranes, alkali-treated to enrich for tyrosine phosphorylation, and detected by autor-
conditions as detected following and 50 kDa following separation by SDS-PAGE under reducing conditions. The broad, poorly defined protein band between 40 and 50 kDa, not limited to PKC, caused visible changes in the CD28 signal.

Thus, triggering of a signaling cascade by PMA, involving but not limited to cell lysates, led to increased phosphorylation of several CD28-associated proteins (Fig. 1, lanes 1 and 2) including the cytoplasmic protein tyrosine kinases Lck and Fyn (7, 8). Also, there was a notable increase in the phosphorylation of a broadly diffuse protein band migrating between 40 and 50 kDa. The identification of this phosphoprotein as CD28 was confirmed by two-dimensional nonreducing-reducing gel electrophoresis studies (7). Pretreatment of the cells with 5 ng/ml PMA for 18 h had a pronounced effect on the phosphorylation profiles displayed in CD28 immune complexes (Fig. 1, lanes 3 and 4). There was a dramatic increase in overall levels of protein phosphorylation, particularly of CD28 itself, even in the absence of anti-CD28 mAb stimulation (Fig. 1, lanes 1 and 3). However, a small but detectable activation-dependent increase in phosphorylation was still seen in immune complexes prepared using the anti-CD3 mAb OKT3 (data not shown). Identical results were obtained when 1 mM iodoacetamide was included in the lysis, wash, and SDS-samples prepared from detergent lysates of Jurkat T cells that had been metabolically labeled with a mixture of [35S]methionine and [35S]cysteine (Fig. 2B). Once again, PMA treatment of CD28 immunoprecipitates converted the ill-defined 22-kDa protein into a sharply focused band migrating with an apparent molecular mass of 22 kDa following deglycosylation (Fig. 2A, lane 6) corresponding to CD5. Coomassie blue staining of the proteins bound to the PVDF membranes revealed that the PNGase F treatment shifted completely the migration patterns of the immunoglobulin heavy chains without altering that of the light chains (data not shown). This result confirmed the efficiency of the deglycosylation protocol as well as the absence of detectable proteolysis. Similar results were obtained using CD28 immunoprecipitates prepared from Jurkat T cells and with different CD28 mAbs (data not shown).

The identification of the 22-kDa protein as a deglycosylated form of CD28 was confirmed in studies using immunoprecipitates prepared from detergent lysates of Jurkat T cells that had been metabolically labeled with a mixture of [35S]methionine and [35S]cysteine (Fig. 2B). Once again, PNGase F treatment of CD28 immunoprecipitates converted the ill-defined 40-50 kDa CD28 protein into a sharply focused band migrating with an apparent molecular mass of 22 kDa (Fig. 2B, lane 1 and 2). This band was not present in the absence of CD28 mAb nor in immunoprecipitates prepared using the anti-CD3 mAb OKT3 (data not shown). Identical results were obtained when 1 mM iodoacetamide was included in the lysis, wash, and SDS-sample buffers during the preparation of the CD28 immunoprecipitates to prevent the formation of spurious disulfide-dependent protein interactions (Fig. 2B, lanes 3 and 4).

Further support for our identification of the 22-kDa deglycosylated form of CD28 came from studies in which the proteins present in CD28 immunoprecipitates were visualized directly by silver staining (Fig. 2C) (19). A 22-kDa silver-stained protein was present in CD28 immunoprecipitates prepared from Jurkat T cells using either of two different anti-CD28 mAb, 9.3 (Fig. 2C, lanes 3 and 4) and 2E12 (lane 5). The 22-kDa protein was not present upon incubation of cell lysates with protein A-Sepharose in the absence of anti-CD28 mAb (Fig. 2C, lane 1), incubation of anti-CD28 mAb with protein A-Sepharose without cell lysate (Fig. 2C, lane 2) or in anti-CD5 immunoprecipitates (Fig. 2C, lane 6). The disperse, glycosylated form of CD28 was not detectable by silver stain (data not shown). The silver-stained protein corresponding to CD5 was identified by its phosphorylation as detected by autoradiography (data not shown).

Having established the effectiveness of the deglycosylation procedure, we next examined the effect of treating in vitro phosphorylated CD28 immunoprecipitates with PNGase F (Fig. 2D). Human peripheral T cells were incubated for 2 days...
with 5 ng/ml PMA, then harvested and stimulated with anti-CD28 mAb. The CD28 immune complexes (corresponding to 2 × 10^6 cells each) were phosphorylated in vitro, washed stringently, and then treated without (Fig. 2D, lane 1) or with (Fig. 2D, lane 2) PNGase F. The immunoprecipitates were separated on a 10.5% SDS-PAGE gel, transferred to PVDF membranes, and alkali-treated, and the phosphoproteins were detected by autoradiography. The diffusely migrating CD28 phosphoprotein (Fig. 2D, lane 1, indicated by the bracket) was indeed converted by PNGase F treatment into a well defined, faster migrating form (Fig. 2D, lane 2).

These studies demonstrate that CD28 migrates as a sharply focused 22-kDa protein band after in vitro deglycosylation, as confirmed using cell surface biotinylation, [35S]methionine/cysteine metabolic labeling, and silver staining techniques. The development of this procedure has permitted us to continue the characterization of activation-dependent biochemical modifications of CD28. Importantly, there are specific protein bands in the 40–50 kDa region of CD28 immunoprecipitates whose migration patterns are not altered following the deglycosylation
CD28 Phosphorylation

Fig. 3. Effect of PMA on levels of CD28 expression and phosphorylation. A, silver stain. Jurkat T cells were harvested following treatment with 5 ng/ml PMA at 37 °C for 2, 24, or 48 h. Cells were stimulated with anti-CD28 mAb and lysed in Brij 96 buffer, and immunoprecipitates (corresponding to 3 × 10^6 cells each) were incubated with [γ-^32P]ATP. The immunoprecipitates were washed stringently and then treated with PNGase F as described under "Materials and Methods." The proteins were separated by SDS-PAGE and silver-stained (19). B, in vitro phosphorylation. The phosphoproteins from the gel in A were detected by autoradiography. C, in vitro phosphorylation. Jurkat T cells were harvested following treatment with 5 ng/ml PMA at 37 °C for 15, 30, or 60 min and prepared as described in A. The phosphoproteins were detected by autoradiography. The migration positions of CD28 and molecular mass markers (kDa) are indicated.

The increased phosphorylation of CD28 following PMA stimulation is not due to changes in the levels of CD28 surface expression—Preincubation of cells with PMA differentially affects the expression levels of several receptors found on T lymphocytes; PMA has been shown to increase CD28 surface expression while decreasing CD3 expression (2) (data not shown). A PMA-induced increase in CD28 expression could have accounted for the PMA-dependent stimulation of CD28 phosphorylation detected in our in vitro kinase assays (Fig. 1). To examine this possibility, we compared the kinetics of these two PMA-dependent effects. CD28 immunoprecipitates were prepared from detergent lysates of Jurkat T cells that had been treated for varying lengths of time with 5 ng/ml PMA and then stimulated with anti-CD28 mAb. As shown in Fig. 3A, incubation of the cells for 2 h with PMA led to a barely detectable increase in immunoprecipitated CD28 protein; longer incubation times resulted in 2-fold (24 h) and 3-fold (48 h) increases in CD28 expression levels as determined by densitometry. Autoradiography of the same gel (Fig. 3B) revealed a strikingly different time course for the effect of PMA on the in vitro phosphorylation of CD28. Although the increase in CD28 protein expression occurred gradually over a 2-day period, PMA treatment caused an abrupt and sustained increase in CD28 phosphorylation, evident within 2 h and continuing for 2 days (Fig. 3B). The kinetics of phosphorylation was rapid: pretreatment of Jurkat T cells with 5 ng/ml PMA for 15 min was sufficient for maximal stimulation of CD28 phosphorylation (Fig. 3C) without discernable changes in the expression levels of CD28 protein (data not shown). In separate studies we have shown that Jurkat T cells had to be pretreated with 5 ng/ml PMA for at least 12 h before an increase in CD28 surface expression was detectable by cell surface immunofluorescence staining (data not shown). Thus, the PMA-dependent increase in CD28 phosphorylation is a rapid event, occurring within minutes of drug treatment and was not the result of increased expression levels of CD28 protein.

Fig. 4. Characterization of the effects of PMA on CD28 phosphorylation. A, anti-CD28 immune complexes were prepared from untreated (lanes 1 and 2) or PMA-treated (5 ng/ml for 5 min on ice) (lanes 3 and 4) Jurkat T cells. Anti-CD28 mAb was incubated with intact cells (lanes 2 and 4) or added to cell lysates (lanes 1 and 3). Cells were lysed in Brij 96 buffer, the immunocomplexes collected and incubated with [γ-^32P]ATP. The immunoprecipitates were stringently washed and then treated with PNGase F. The phosphoproteins were separated by SDS-PAGE, transferred to PVDF, and detected by autoradiography. Migration positions of CD28 and molecular mass markers (kDa) are indicated. B, the radiolabeled CD28 phosphoprotein bands from A were subjected to one-dimensional phosphoamino acid analysis and visualized by autoradiography as described under "Materials and Methods." The migration positions of phosphotyrosine, phosphothreonine, and phosphoserine standards are indicated.

PMA induces the in vitro phosphorylation of CD28 on threonine residues—We performed one-dimensional phosphoamino acid analyses to characterize the effect of different stimulation conditions on the in vitro phosphorylation state of CD28. Anti-CD28 immune complexes (corresponding to 5 × 10^6 cells) were prepared from Brij 96 lysates of Jurkat T cells that were either untreated (Fig. 4A, lanes 1 and 2) or were pretreated with 5 ng/ml PMA for 5 min on ice (Fig. 4A, lanes 3 and 4). Anti-CD28 mAb was used to activate intact cells (Fig. 4A, lanes 2 and 4) or added to cell lysates (Fig. 4A, lanes 1 and 3). There is little in vitro phosphorylation of CD28 immunoprecipitated from unstimulated cells (Fig. 4A, lane 1). Pretreatment with PMA led to dramatically increased levels of CD28 phosphorylation (Fig. 4A, lane 3). Activation of intact cells with anti-CD28 mAb increased the level of CD28 phosphorylation of both untreated and PMA-treated cells (Fig. 4A, lanes 2 and 4). Comparable results were obtained in the absence of PNGase F treatment (Fig. 1)

The radiolabeled 22-kDa CD28 phosphoprotein bands were then excised from the PVDF membrane, subjected to acid hydrolysis, separated by thin-layer electrophoresis, and visualized by autoradiography. We found that activation of Jurkat T cells with anti-CD28 mAb stimulated the in vitro phosphorylation of CD28 on tyrosine residues; minimal phosphorylation
on threonine residues was observed (Fig. 4B, lane 2). Treatment of the cells with 5 ng/ml PMA for 5 min on ice caused a striking phosphorylation of CD28 on threonine residues (Fig. 4B, lane 3). The effects of PMA and anti-CD28 mAb treatments were not synergistic, resulting in increased phosphorylation of CD28 on both tyrosine and threonine (and serine) residues (Fig. 4B, lane 4). Identical phosphorylation profiles were obtained from the CD28 phosphoproteins in Fig. 3B, where the PMA pretreatment ranged from 2 to 48 h (data not shown).

Phosphorylation of CD28 on Threonine Residues Is Independent of P13 Kinase Activity—These changes in the in vitro phosphorylation profile of CD28 following treatment of T cells with PMA and anti-CD28 mAb could reflect the differential regulation or recruitment of protein kinases and/or phosphatases to the CD28 signaling complex. P13 kinase, which has serine/threonine kinase activity in addition to lipid kinase activity, is a well-characterized enzyme responsible for the phosphorylation of CD28 on threonine residues. We found that pretreatment of J urkat T cells with 100 nm Wortmannin for 30 min at 37 °C prior to harvest had no effect on the phosphorylation of CD28 in in vitro kinase assays, either alone (Fig. 5A, lanes 1 and 3) or when used in combination with PMA (Fig. 5A, lanes 2 and 4).

We also examined the divalent cation requirements for the in vitro phosphorylation of CD28. The PMA-dependent phosphorylation of CD28 on threonine occurred with kinase buffer containing either MnCl2 or MgCl2 (Fig. 5B). Since the serine/threonine kinase activity associated with P13 kinase is manganese-dependent, this finding, in combination with our Wortmannin data, indicates that P13 kinase is not responsible for the threonine phosphorylation of CD28. Interestingly, the anti-CD28 mAb-dependent phosphorylation of CD28 on tyrosine residues was detected only when the kinase reactions were performed in the presence of MnCl2 (Fig. 5B, lane 1). This tyrosine phosphorylation occurred readily in kinase buffer containing a combination of manganese and magnesium, so the presence of magnesium ions was not inhibitory (data not shown).

Differential CD28 in Vitro Phosphorylation Profiles in J urkat and J Cam1 Cell Lines—To further characterize the early phosphorylation events associated with CD28-dependent signaling, we compared the in vitro phosphorylation profiles obtained using CD28 immune complexes prepared from J urkat T cells with those prepared from J Cam1 cells. The J Cam1 cell line was derived following chemical mutagenesis of Jurkat T cells and does not express an enzymatically active form of the intracellular protein tyrosine kinase Lck.

Fig. 5. Effect of Wortmannin and divalent cations on CD28 phosphorylation. A, J urkat T cells were treated with either dimethyl sulfoxide (lanes 1 and 2), 100 nm Wortmannin (lanes 3 and 4) or 5 ng/ml PMA (lanes 2 and 4) for 20 min at 37 °C. Following stimulation with anti-CD28 mAb, the cells were lysed with Brij 96 buffer, and the CD28 immune complexes (corresponding to 4 × 106 cells) were incubated with [γ-32P]ATP. After stringent washing, the immunoprecipitates were treated with PNGase F, and the phosphorylated proteins were separated by SDS-PAGE and transferred to PVDF. The radiolabeled CD28 phosphoprotein bands were subjected to one-dimensional phosphoamino acid analysis and visualized by autoradiography as described under "Materials and Methods." B, CD28 immune complexes (corresponding to 3 × 106 cells) were prepared from J urkat T cells that had been treated for 1 h with 5 ng/ml PMA prior to stimulation with anti-CD28 mAb. The cells were lysed with Brij 96 buffer, and the CD28 immune complexes were incubated in kinase buffer containing [γ-32P]ATP and either 10 mM MnCl2 (lane 1) or 10 mM MgCl2 (lane 2). The phosphorylated proteins were then treated as described in A and subjected to phosphoamino acid analysis. The migration positions of phosphoserine, phosphothreonine, and phosphoserine standards are indicated.

pronounced increases in protein phosphorylation (Fig. 6A, lanes 3 and 4). In contrast, stimulation of J Cam1 cells with anti-CD28 mAb yielded little enhancement of protein phosphorylation, particularly of CD28 (Fig. 6A, lanes 5 and 6). PMA treatment, however, was still able to stimulate the phosphorylation of CD28 in the J Cam1 T cells (Fig. 6A, lanes 7 and 8).

Phosphoamino acid analyses were then performed on the 22-kDa deglycosylated form of CD28 immunoprecipitated from the J Cam1 cells. Stimulation of these cells with anti-CD28 mAb did not induce appreciable levels of tyrosine phosphorylation on CD28, in the presence or absence of PMA (Fig. 6B). However, treatment of the J Cam1 cells with PMA was still able to stimulate the phosphorylation of CD28 on threonine and serine residues. Thus, the PMA-dependent changes in CD28 phosphorylation in vitro can occur in the absence of an enzymatically active form of the intracellular protein tyrosine kinase Lck.

Activation-dependent, in Vivo Phosphorylation of CD28 on Tyrosine in J urkat but Not J Cam1 Cells—We next examined the effect of anti-CD28 and PMA stimulation on the in vivo phosphorylation state of CD28, having established that the deglycosylation protocol allowed us to monitor specific changes in the phosphorylation (Figs. 2D and 3–6) of a precisely identified CD28 protein band (Fig. 2, A–C). Stimulation of J urkat cells with anti-CD28 mAb led to a dramatic increase in the tyrosine phosphorylation of CD28 (Fig. 7A, lane 2) as detected with the anti-phosphotyrosine mAb 4G10. The phosphorylation
of CD28 on tyrosine was almost completely inhibited by pre-
treatment of the Jurkat cells with 5 ng/ml PMA for 5 min on ice
(Fig. 7A, lane 3). We also assessed whether activation-depend-
ent phosphorylation of CD28 could be detected in vivo in the
absence of the Src-family tyrosine kinase Lck. There was no
detectable phosphorylation on tyrosine of CD28 immunopre-
cipitated from J.Cam1 cells, regardless of the stimulation con-
ditions (Fig. 7A, lanes 4–8). In vitro kinase assays confirmed
the absence of Lck activity in J.Cam1 cells. As expected, enzy-
matically active Lck, capable of auto-phosphorylation, could be
immunoprecipitated from Jurkat cells (Fig. 7B, lane 1) but not
from J.Cam1 cells (Fig. 7B, lane 2). The phosphorylation of
CD28 on tyrosine appears to require active Lck tyrosine kinase
activity. Demonstrable tyrosine phosphorylation of CD28, how-
ever, may not be required for IL-2 production of Jurkat cells
following stimulation with anti-CD28 mAb and PMA.

**DISCUSSION**

Activation of T lymphocytes requires the triggering of two
disparate signal transduction cascades. Engagement of the
CD28 costimulatory receptor by anti-CD28 mAb can lead to
IL-2 production and cellular proliferation in the presence of an
additional signal; this second signal can be provided by the
phorbol ester, PMA (22). Our studies demonstrate that engage-
ment of the CD28 receptor can initiate distinct phosphorylation
pathways. One pathway involved the PKC-dependent stimula-
tion of threonine kinase activity; the other required protein-
tyrosine kinase activity. PMA treatment rapidly induced the in
vitro phosphorylation of CD28 (Figs. 1, 3, B and C, 4A, and 6A)
on threonine and serine residues (Figs. 4B and 5A), in the
presence of either manganese or magnesium ions (Fig. 5B) and
was detected in human peripheral T cells, Jurkat T cells, and in
the Jurkat subclone, J.Cam1, that is deficient in Lck tyrosine
kinase activity (Figs. 1, 2D, 4B, and 6, A and B). In contrast,
anti-CD28 mAb stimulation of the in vitro phosphorylation of
CD28 on tyrosine residues was manganese-dependent (Fig. 5B)
and was not detectable in J.Cam1 cells (Figs. 6, A and B). Stimulation of Jurkat cells with anti-CD28 mAbs led to the in
vivo phosphorylation of CD28 on tyrosine (Fig. 7A, lanes 1 and
2); this activation-dependent, tyrosine phosphorylation of
CD28 in vivo was abrogated by a short pretreatment of the
Jurkat cells with 5 ng/ml PMA (Fig. 7A, lanes 3 and 4) and was
dependent on the Src family tyrosine kinase Lck (Fig. 7B, lanes
5–8).

Protein phosphorylation is involved in the regulation of enzy-
matic activity and protein-protein interactions. An accurate
assessment of the phosphorylation state of CD28 required the
development of an effective in vitro deglycosylation procedure
that yielded a distinct, well focused 22-kDa form of CD28. This
is in stark contrast to the diffuse migration pattern of the
heavily glycosylated CD28 protein following one-dimensional
reducing SDS-PAGE (Figs. 1, 2, A and B, and 6A), or two-
dimensional nonreducing/reducing SDS-PAGE analysis (7).

We used several different protein labeling techniques to con-
firm our identification of the 22-kDa deglycosylated form of
CD28. The cell surface biotinylation method (Fig. 2A) allowed
detection of small amounts of compartmentalized (cell surface)
CD28 protein without requiring radioactive labeling. Interest-
ingly, the deglycosylation of CD28 consistently enhanced its
detection. This may be due to a variety of causes, including the
sharper focusing of the protein band, more efficient transfer of
the protein out of the SDS-PAGE gel and onto the PVDF
membrane, and greater accessibility of the streptavidin to the
bation of purified human T lymphocytes or Jurkat T cells with anti-CD28 mAb led to the increased phosphorylation of CD28 on tyrosine residues, as detected in vitro kinase assays. There was a definite divalent cation preference; many cytoplasmic protein-tyrosine kinases prefer manganese over magnesium, as was the case in our CD28 in vitro kinase assays (Fig. 5B). Importantly, we also demonstrated unequivocal activation-dependent in vivo tyrosine phosphorylation of CD28 as detected with anti-phosphotyrosine antibodies (Fig. 7A).

Our data are consistent with either a direct or indirect role for Src family cytoplasmic protein-tyrosine kinases in the tyrosine phosphorylation of CD28 in response to anti-CD28 mAb stimulation. There was no detectable in vivo or in vitro phosphorylation of CD28 on tyrosine residues in J.Cam1 cells that lack enzymatically active Lck (Figs. 6 and 7). We have previously shown that Lck and Fyn can associate with CD28 (7), and activation of Lck after CD28 ligation has been reported (8). Furthermore, recent coexpression studies demonstrated that Lck or Fyn (but not Zap-70 or Itk) could provide the necessary signal for the association of p85-PI3 kinase with CD28 (23, 24). J.Cam1 cells produce only minimal (10-fold less than wild-type J urkat cells) or no (cell line-dependent variation) IL-2 in response to anti-CD28 mAb and PMA.2 However, it has been reported that J.Cam1 cells can make IL-2 in response to combined treatment with anti-CD28 mAb, PMA, and ionomycin (12). It remains to be shown whether CD28 is an in vivo substrate of Lck.

PMA treatment has multiple effects on the CD28 costimulatory receptor, including up-regulation of CD28 surface expression (Fig. 3A) and the stimulation of a CD28-associated threonine kinase activity (Figs. 4B and 5A). Although these are kinetically separable events (Figs. 3, A–C), it is possible that the increased surface expression of CD28 protein in response to PMA treatment allows differential association with downstream effector proteins. This idea is supported by the finding that the PMA-dependent induction of CD28-associated threonine kinase activity continued for at least 2 days after a single treatment with phorbol ester (Fig. 3B and data not shown). Further studies are under way to determine whether new protein synthesis is required for this effect.

PMA is a direct activator of several protein kinase C isoforms (24); however, as our in vitro kinase conditions were not permissive of PKC activity, it is likely that the PMA-induced phosphorylation of CD28 on threonine residues in vitro was an indirect result of PKC activation. We propose that PMA treatment of T lymphocytes induces the in vivo phosphorylation of CD28 by one or more PKC isoforms; this in turn allows the recruitment or activation of a kinase responsible for the in vitro phosphorylation of CD28 on threonine residues. PKA kinase, which has serine/threonine kinase activity in addition to lipid kinase activity, is a candidate for the kinase responsible for the in vitro phosphorylation of CD28 on threonine residues. However, the in vitro phosphorylation of CD28 on threonine was unaffected by the PKA inhibitor Wortmannin and, unlike the serine/threonine PK13 kinase activity, was not manganese-dependent.

The cytoplasmic domain of CD28 does contain a consensus site for PKC phosphorylation, although direct phosphorylation of this site by PKC remains to be demonstrated. Interestingly, the presumptive site of PKC phosphorylation is close to the pY-M-N-M site of p85-PI3 kinase association (10, 11, 14). Phosphorylation of CD28 by PKC could alter the ability of p85-P13 kinase or other signaling proteins to associate with CD28.

Importantly, we and others have recently shown that PMA treatment inhibits the association of PKA kinase with CD28 and that Wortmannin does not inhibit IL-2 production following stimulation of J urkat cells with anti-CD28 antibody and PMA, 

15, 16, 25). PKA kinase has been shown only to bind to tyrosine-phosphorylated CD28 (11, 13, 14). In our current studies, we observed that PMA treatment consistently decreased the in vitro phosphorylation of CD28 on tyrosine residues after stimulation with anti-CD28 mAb (Figs. 4B and 5A). Furthermore, we found that PMA treatment also inhibits the in vivo phosphorylation of CD28 on tyrosine (Fig. 7), suggesting that PMA treatment results in an actual decrease in the phosphorylation of CD28 on tyrosine, and that it is not a nonspecific effect caused by limiting amounts of divalent cations or ATP in the kinase reaction mix.

Thus, in the presence of PMA, anti-CD28 mAb stimulation of J urkat T cells does not lead to appreciable phosphorylation of CD28 on tyrosine (Fig. 7A) and there is little (or no) recruitment or activation of PKA kinase. Nevertheless, an intracellular signaling cascade has been triggered that ultimately results in IL-2 production. Biochemical and mutational analyses are currently underway to identify the kinase responsible for the phosphorylation of CD28 on threonine residues and to determine the potential role of this kinase activity in CD28- and PMA-dependent IL-2 production. While phosphorylation of CD28 on tyrosine may not be required for lymphokine production, it may play a key role in other CD28 effector functions, such as prevention of antigen unresponsiveness following engagement of the T cell antigen receptor.

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