Regulation of T Cell Receptor- and CD28-induced Tyrosine Phosphorylation of the Focal Adhesion Tyrosine Kinases Pyk2 and Fak by Protein Kinase C

A ROLE FOR PROTEIN TYROSINE PHOSPHATASES*

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The T cell receptor (TCR)-CD3 complex and the costimulatory molecule CD28 are critical for T cell function. Both receptors utilize protein tyrosine kinases (PTKs) for the phosphorylation of various signaling molecules, a process that is critical for the function of both receptors. The PTKs of the focal adhesion family, Pyk2 and Fak, have been implicated in the signaling of TCR and CD28. We show here evidence for the regulation of TCR- and CD28-induced tyrosine phosphorylation of the focal adhesion PTKs by protein kinase C (PKC). Thus, treating Jurkat T cells with the PKC activator phorbol 12-myristate 13-acetate (PMA) rapidly and strongly reversed receptor-induced tyrosine phosphorylation of the focal adhesion PTKs. In contrast, PMA did not affect TCR-induced tyrosine phosphorylation of CD3ζ or the PTKs Fyn and Zap-70. However, PMA induced a strong and rapid dephosphorylation of the linker molecule for activation of T cells, PMA failed to induce the dephosphorylation of proteins in PKC-depleted cells or in cells pretreated with the PKC inhibitor Ro-31-8220, confirming the role of PKC in mediating the PMA effect on receptor-induced protein tyrosine phosphorylation. The involvement of protein tyrosine phosphatases (PTPases) in mediating the dephosphorylation of the focal adhesion PTKs was confirmed by the failure of PMA to dephosphorylate Pyk2 in cells pretreated with the PTPase inhibitor orthovanadate. These results implicate PKC in the regulation of receptor-induced tyrosine phosphorylation of the focal adhesion PTKs in T cells. The data also suggest a role for PTPases in the PKC action.

Optimal T cell activation requires signals initiated by the TCR-CD3 complex and the T cell costimulatory molecule CD28 (1, 2). Thus, the concurrent ligation of these receptors triggers signals that synergistically regulate the function of T cells. Signals initiated following TCR and CD28 ligation include the stimulation of protein tyrosine kinases (PTKs), the generation of inositol trisphosphate and diacylglycerol, the activation of protein kinase C (PKC), the increase in intracellular Ca2+, the activation of the Ras and Rho family of GTPases, and the activation of the mitogen-activated protein kinase (MAPK) cascades (1–3). Stimulated MAPKs translocate from the cytoplasm to the nucleus where they phosphorylate and in turn activate transcription factors such as c-Fos and c-Jun. The increase in intracellular Ca2+ stimulates the serine/threonine phosphatase calcineurin, leading to the dephosphorylation of the transcription factor c-NAF and in turn to its activation and translocation to the nucleus. Activated transcription factors bind to promoters of genes, leading to the initiation of gene transcription.

In contrast to the extensive data available concerning the signals involved in promoting receptor function, much less is known about the mechanisms that down-regulate receptor signaling in T cells. There is evidence, however, implicating the serine/threonine kinase PKC in regulating receptor-initiated signaling in T cells. For example, PKC activation by PMA has been shown to modulate profoundly TCR- and CD28-mediated interleukin-2 production and T cell proliferation (4–8). Interestingly, the effect of PKC stimulation by PMA in different types of cells, including T cells, is bidirectional; thus, the stimulation of PKC delivers a synergistic auxiliary signal for cell activation and a negative signal for down-regulating receptor function (4, 5, 7, 9–11). The nature and the sequence of the signals downstream of PKC that modulate receptor function are poorly understood. PKC activation by PMA has been shown to induce the serine/threonine phosphorylation of several proteins, including TCR and CD28, in T cells (7, 9, 12–15). Such phosphorylation is thought to play a role in modulating CD28 and TCR functions by blocking the activity of signaling molecules and by modulating protein-protein interaction (9, 10, 16, 17). Recently, PMA-activated PKC has also been shown to serine phosphorylate PTPases in vivo and in vitro, leading to the modulation of their enzymatic activity (13, 18–22). However, the role of PKC-activated PTPases in receptor signaling in T cells has not been reported.

The phosphorylation of proteins on tyrosine residues by PTKs is a mechanism of signaling for various receptors, including receptors on T cells, and is critical for many cellular processes, including cell differentiation, proliferation, adhesion, and migration (1, 3). Recently, a new family of PTKs has been identified as the focal adhesion PTK family. This family consists of the non-receptor, proline-rich PTKs Fak (Focal adhesion kinase) and Pyk2 (Proline-rich tyrosine kinase 2, also designated CAKβ, RAFTK, FAK2, or CADTK) (23–30). These kinases have a molecular mass of 110–125 kDa and are closely related in their overall structures. Fak is expressed in most tissues, whereas Pyk2 is expressed mainly in the central nerv-
ous system and in cells and tissues derived from hematopoietic lineages. Fak and Pyk2 become tyrosine-phosphorylated and activated after the stimulation of various receptors including TCR (23–25, 28, 30–44), and both kinases have been linked to the signaling pathways that regulate MAPKs (31, 33, 35, 45).

There is evidence implicating the focal adhesion PTKs Fak and Pyk2 in receptor-initiated activation of T cells. Thus, Pyk2 became tyrosine-phosphorylated after CD28 and after TCR ligation, whereas Fak was phosphorylated after TCR but apparently not after CD28 ligation (30, 32, 43, 46, 47). Importantly, co-ligating CD28 and TCR increased the tyrosine phosphorylation of Pyk2, suggesting that this kinase may have a role in the costimulatory process in T cells (46, 47). We show in the present article that PKC activation by PMA reverses receptor-induced tyrosine phosphorylation of the focal adhesion PTKs. PMA added to cells activated for 5 min by TCR or CD28 ligation led to the dephosphorylation of Pyk2 and Fak. In contrast, PMA did not affect TCR-induced tyrosine phosphorylation of CD3ζ and of the PTKs Fyn and Zap-70. However, PMA induced strong and rapid dephosphorylation of the linker molecule LAT. The dephosphorylation of the focal adhesion PTKs by PMA required PKC, as PMA failed to dephosphorylate proteins in PKC-depleted cells or in cells pretreated with the PKC inhibitor Ro-31-8220. The PT-Pase inhibitor orthovanadate completely abolished PMA-induced dephosphorylation of the focal adhesion PTKs, thereby suggesting a role for PT-Pases in the PMA effect. Together, the data implicate PKC in the regulation of receptor-induced tyrosine phosphorylation of the focal adhesion PTKs in T cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sodium orthovanadate, PMA, and 4α-phorbol were from Sigma. LumiGLO chemiluminescent substrate kit was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Ro-31-8220 was obtained from Calbiochem. Anti-Zap-70 mAb, anti-PTP1C mAb, and anti-CD3 mAb as above. PKC depletion was as described previously (47).

**Results**

**Regulation of TCR-induced Tyrosine Phosphorylation of the Focal Adhesion PTKs by PMA**—To investigate whether PKC regulates TCR-induced tyrosine phosphorylation of the focal adhesion PTKs in T cells, we examined the effect of the PKC activator PMA on receptor-induced tyrosine phosphorylation of these PTKs. Thus, we stimulated purified normal resting human T cells and Jurkat T cells with anti-CD3 mAb for 5 min to allow proteins to become tyrosine-phosphorylated and then added PMA. As previously reported, stimulating normal resting human T cells (Fig. 1A) or Jurkat T cells (Fig. 1B) with anti-CD3 mAb for 5 min induced protein tyrosine phosphorylation, determined by blotting WCL with anti-phosphotyrosine mAb. Interestingly, the addition of PMA to the cells in the continuous presence of the mAb for an additional 5 min (Fig. 1, A and B, compare lanes 3 and 4) or 10 min (Fig. 1, A and B, compare lanes 5 and 6) markedly decreased the phosphorylation of certain proteins. In normal human T cells and in Jurkat T cells tyrosine phosphorylation of a 110-, a 70-, and a 40-kDa protein was reproducibly and most prominently affected by PMA. The dephosphorylation of the proteins was rapid and strong, as it was apparent within 5 min of the addition of PMA and was marked at 10 min. PMA effect was dose-dependent and was not mimicked by the biologically inactive 4α-phorbol (Fig. 1C, compare lanes 7 and 8).

To examine the effect of PKC activation on receptor-induced tyrosine phosphorylation of Pyk2 and Fak, Jurkat T cells were stimulated with anti-CD3 mAb for 5 min and then treated with PMA as described above. Pyk2 and Fak were immunoprecipitated and transferred to membranes, and the membranes were then immunoblotted with anti-phosphotyrosine mAb or with the specific antibodies. As reported previously, ligating CD3 with a mAb increased tyrosine phosphorylation of Pyk2 and Fak (Fig. 2, A and B, *upper panel*). Such phosphorylation declined with time after receptor ligation, reaching background values within 30–60 min (not shown). Strikingly, the addition of PMA to cells already stimulated for 5 min with anti-CD3 mAb almost completely reversed Pyk2 and Fak tyrosine phosphorylation (Fig. 2, A and B, compare lanes 3 and 4). The dephosphorylation of Pyk2 and Fak by PMA was rapid and strong, as the phosphorylation of the PTKs was markedly reduced within 5 min of the addition of PMA. Reprobing the membranes with anti-Pyk2 mAb (Fig. 2A, *lower panel*) or anti-Fak mAb (Fig. 2B, *lower panel*) showed equal amounts of proteins in all lanes, ruling out the possibility that incubating the cells with PMA induced a selective degradation of the PTKs.

**PKA Fails to Induce the Dephosphorylation of CD3ζ or of the PTKs Fyn and Zap-70**—Following TCR ligation, Src PTKs (including Fyn) are rapidly activated, leading to the phosphorylation of the tyrosine residues in the CD3ζ subunit of TCR and to the subsequent activation of the PTK ZAP-70. To examine whether PKC activation under the same conditions that lead to the dephosphorylation of the focal adhesion PTKs also regulates tyrosine phosphorylation of molecules phosphorylated early in the TCR signaling cascade, Jurkat T cells were activated for 5 min with anti-CD3 mAb and then treated with

**Immunoprecipitation and Immunoblotting**—This was done as described previously with some modification (39, 47, 48). Brieﬂy, 10 µg of rabbit anti-mouse Ig were incubated for 2 h with 50 µl of protein A-agarose. After incubation, the beads were pelleted by centrifugation and washed with ice-cold solubilization buffer. The primary Ab was then added to the beads followed by the addition of cell lysates from 6 x 10⁶ cells. The mixture was gently rotated for 2 h at 4 °C. After incubation, the beads were pelleted by centrifugation and washed 5 x with ice-cold solubilization buffer. After the final centrifugation, the beads were resuspended in 2 x SDS-PAGE sample buffer and boiled for 5 min. Immunoblotting was as described previously (39, 47, 48).
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PMA. Proteins were then immunoprecipitated from cell lysates and examined for tyrosine phosphorylation by blotting with anti-phosphotyrosine mAb (α-PY). As shown in Fig. 3A, Fyn was strongly tyrosine-phosphorylated in unstimulated cells, and its phosphorylation increased only modestly upon CD3 aggregation. PMA treatment did not appear to affect the receptor-induced tyrosine phosphorylation of Fyn (Fig. 3A, compare lanes 3 and 4), although a slight decrease was seen in some experiments. Similarly, PMA did not affect tyrosine phosphorylation of CD3ζ, as at 10 min there was no difference in the tyrosine phosphorylation of CD3ζ in cells treated with or without PMA (Fig. 3B, compare lanes 3 and 4). In contrast to CD3ζ and Fyn, Zap-70 was not tyrosine-phosphorylated in unstimulated cells (Fig. 3C). Ligating CD3 markedly increased the tyrosine phosphorylation of the PTK. PMA addition to cells stimulated for 5 min with anti-CD3 mAb failed to dephosphorylate Zap-70, as the extent of Zap-70 tyrosine phosphorylation in untreated or in PMA-treated cells was similar (Fig. 3C, compare lanes 3 and 4). The failure of PMA to induce the dephosphorylation of CD3ζ, Fyn, and Zap-70 strongly suggests that PKC activation does not lead to general, nonspecific regulation of receptor signaling.

PMA Induces the Dephosphorylation of the Linker Molecule LAT—PMA strongly and reproducibly dephosphorylated ~40-kDa proteins (Fig. 1). Recently, a 36–40-kDa linker molecule has been identified as LAT (Linker for Activation of T cells), which becomes tyrosine-phosphorylated upon TCR and CD28 ligation (48, 50). Thus, we examined whether PMA down-regulates receptor-induced LAT phosphorylation. As previously reported, ligating CD3 with a mAb increased tyrosine phosphorylation of LAT (Fig. 3D, upper panel). Notably, the addition of

Fig. 1. PMA reverses TCR-induced protein tyrosine phosphorylation. 5 × 10⁶ purified normal human T cells (A) or Jurkat T cells (B) in RPMI containing 0.001% BSA were lysed immediately with boiling sample buffer (lanes 1) or were stimulated for 5 min at 37 °C with 1 μg/ml anti-CD3 mAb (lanes 2–6). After 5 min incubation, cells were either lysed immediately with boiling sample buffer (lane 2) or were treated with only media (lanes 3 and 5) or with media containing PMA (50 ng/ml; final concentration) (lanes 4 and 6), and the incubation was resumed for an additional 5 min (lanes 3 and 4) or 10 min (lanes 5 and 6). After boiling in sample buffer, proteins in WCL were separated by SDS-PAGE, transferred to membranes, and immunoblotted with anti-phosphotyrosine mAb (α-PY). Arrows indicate proteins that become dephosphorylated after PMA treatment. C, 5 × 10⁶ Jurkat T cells in RPMI containing 0.001% BSA were lysed immediately with boiling sample buffer (lane 1) or were stimulated for 5 min at 37 °C with 1 μg/ml anti-CD3 mAb (lanes 2–8). After 5 min incubation, cells were either lysed immediately with boiling sample buffer (lane 2) or were treated with only media (lane 3) or with media containing the indicated concentrations of PMA (lanes 4–7) or with 50 ng/ml of 4α-phorbol (lane 8), and the incubation was resumed for an additional 5 min. After boiling in sample buffer, proteins in WCL were separated by SDS-PAGE, transferred to membranes, and immunoblotted with anti-phosphotyrosine mAb (α-PY).

Fig. 2. Down-regulation of TCR-induced tyrosine phosphorylation of the focal adhesion PTKs by PMA. A and B, 6 × 10⁶ Jurkat T cells in RPMI containing 0.001% BSA were lysed with ice-cold lysis buffer (lane 1) or were stimulated with 1 μg/ml anti-CD3 mAb for 5 min at 37 °C (lanes 2–4). At 5 min, cells were either lysed immediately with ice-cold lysis buffer (lane 2), were treated with only media (lane 3), or with media containing PMA (50 ng/ml; final concentration) (lane 4), and the incubation was resumed for an additional 5 min. After solubilizing with ice-cold lysis buffer, proteins in cell lysates were immunoprecipitated (IP) with the indicated mAb and were analyzed by immunoblotting with anti-phosphotyrosine mAb (α-PY, upper panel) or with the specific mAb (lower panel).

Fig. 3. PMA fails to induce the dephosphorylation of CD3ζ, Fyn, or Zap-70 but rapidly dephosphorylates LAT. A–D, 6 × 10⁶ Jurkat T cells in RPMI containing 0.001% BSA were lysed with ice-cold lysis buffer (lane 1) or were stimulated with 1 μg/ml anti-CD3 mAb for 5 min at 37 °C (lanes 2–4). At 5 min, cells were either lysed immediately with ice-cold lysis buffer (lane 2), were treated with only media (lane 3), or with media containing PMA (50 ng/ml; final concentration) (lane 4), and the incubation was resumed for an additional 5 min. Proteins in cell lysates were immunoprecipitated (IP) with the indicated mAb and were analyzed by immunoblotting with anti-phosphotyrosine mAb (α-PY, upper panel) or with the specific mAb (lower panel).
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Evidence for the Requirement of PKC for PMA Regulation of Receptor Signaling—PKC is the intracellular receptor for PMA and therefore is implicated in mediating PMA effects on cells. PKC is a diacylglycerol analogue that can permeate biological membranes and directly bind to a cysteine-rich region on PKC that is physiologically recognized by diacylglycerol, leading to the sustained activation of the kinase. To confirm further the role of PKC in mediating the down-regulation by PMA of receptor signaling, cells were depleted of PKC by incubating for 16 h with 400 nM PMA. Such treatment effectively reduces Ca2+-dependent and Ca2+-independent PKC (Fig. 4A). As shown in Fig. 4B, ligating CD3 in PKC-depleted cells induced an increase in protein tyrosine phosphorylation. Importantly, freshly added PMA to PKC-depleted cells failed to dephosphorylate these proteins (Fig. 4B, compare lanes 3 and 4, and 7 and 8). To examine the effect of PKC depletion on PMA regulation of Pyk2 tyrosine phosphorylation, lysates from untreated or from PKC-depleted cells were immunoprecipitated with anti-Pyk2 mAb and then immunoblotted with anti-phosphotyrosine mAb (Fig. 4C, upper panel) or with the specific mAb (lower panel). IP, immunoprecipitated.

PMA to cells already stimulated for 5 min with anti-CD3 mAb almost completely reversed LAT tyrosine phosphorylation (Fig. 4, lower panel). As shown in Fig. 4C, PMA failed to induce the dephosphorylation of Pyk2 in PKC-depleted cells (compare lanes 3 and 4, and 7 and 8). To confirm further the involvement of PKC in the PMA effect, Jurkat T cells were treated with the PKC inhibitor Ro-31-8220 and then treated with anti-CD3 mAb and PMA. As shown in Fig. 5 A and B, Ro-31-8220 blocked PMA-induced protein dephosphorylation, including Pyk2, in a dose-dependent manner (compare lanes 2 and 3, 5 and 6, and 8 and 9). These results strongly implicate PKC in mediating PMA effects on receptor-induced tyrosine phosphorylation of Pyk2.

Evidence for the Involvement of PTPases in PKC-mediated Regulation of Receptor Signaling—The dephosphorylation of the focal adhesion PTKs by PMA implicates PTPases in the PMA effect. To study further the involvement of PTPases in the PMA effect, we pretreated Jurkat T cells for 30 min with the PTPase inhibitor sodium orthovanadate and then examined the effect of PMA on receptor-induced protein tyrosine phosphorylation. As shown in Fig. 6A, the inhibition of PTPases by orthovanadate led to a general increase in the basal level of protein tyrosine phosphorylation in unstimulated cells in a dose-dependent manner (compare lanes 1, 4, and 7). Ligating CD3 in orthovanadate-treated cells induced protein tyrosine phosphorylation. As shown in Fig. 6B, pretreatment with orthovanadate led to an increase in the basal level of protein tyrosine phosphorylation in unstimulated cells in a dose-dependent manner (compare lanes 1, 4, and 7). Ligating CD3 in orthovanadate-treated cells induced protein tyrosine phosphorylation. As shown in Fig. 6C, pretreatment with orthovanadate led to an increase in the basal level of protein tyrosine phosphorylation in unstimulated cells in a dose-dependent manner (compare lanes 1, 4, and 7). Ligating CD3 in orthovanadate-treated cells induced protein tyrosine phosphorylation.
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DISCUSSION

The recently identified focal adhesion PTKs have been implicated in the signal transduction pathways of TCR and CD28. In the present study we showed evidence for the down-regulation of receptor-induced tyrosine phosphorylation of the focal adhesion PTKs by activated PKC as follows: 1) PMA, a PKC activator, rapidly and strongly potentiated the dephosphorylation of the PTKs; 2) PMA failed to dephosphorylate the proteins in PKC-depleted cells or in cells treated with the PKC inhibitor Ro-31-8220.

The rapid and strong dephosphorylation of focal adhesion PTKs upon PKC activation strongly suggests that the tyrosine phosphorylation of these PTKs is tightly regulated by PKC. Recently, PMA-activated PKC has been shown to secrete phospholipase C in vivo and in vitro, leading in some cases to the modulation of their enzymatic activity (13, 18–22). Thus, it is possible that PKC activation is stimulating a PTPase(s) in Jurkat T cells that regulates receptor-initiated protein tyrosine phosphorylation. However, PMA treatment of Jurkat T cells did not lead to an increase in the PTPase activity that coprecipitates with Pyk2, as determined by subjecting Pyk2 immunoprecipitates to in vitro PTPase reactions using the Promega tyrosine phosphatase assay system (Madison, WI) (data not shown). Furthermore, we did not detect the PTPases PTP1C or PTP1D in Pyk2 immunoprecipitates, and Pyk2 was not detected in the immunoprecipitates of these PTPases (not shown).

Alternatively, PKC could be down-regulating tyrosine phospho-

FIG. 6. The PTPase inhibitor sodium orthovanadate (Na2VO3) blocks PKC-mediated regulation of receptor-induced protein tyrosine phosphorylation. A, 6 x 106 Jurkat T cells in RPMI containing 0.001% BSA were untreated (lanes 1–3), pretreated with 2 mM sodium orthovanadate (lanes 4–6), or with 4 mM sodium orthovanadate (lanes 7–9) for 30 min at 37°C. After incubation, the cells were lysed immediately (lanes 1, 4, and 7) or were stimulated with anti-CD3 mAb for 5 min at 37°C (lanes 2, 3, 5, 6, 8, and 9). At 5 min, the cells were treated with media only (lanes 2, 5, and 8) or with media containing PMA (50 ng/ml; final concentration) (lanes 3, 6, and 9), and the incubation was resumed for an additional 5 min. Phosphoproteins were detected with anti-phosphotyrosine mAb (α-PY) as described in Fig. 1. B, lysates from cells activated as described in A were precipitated with anti-Pyk2 mAb as described in the legend for Fig. 2. Pyk2 in the precipitates was analyzed by immunoblotting with anti-phosphotyrosine mAb (α-PY, upper panel) or with the specific mAb (lower panel). IP, immunoprecipitated.

PM-initiated signals. Stimulating Jurkat T cells with anti-CD28 mAb (Fig. 7A) for 5 min induced protein tyrosine phosphorylation, determined by blotting WCL with anti-phosphotyrosine mAb. Importantly, the addition of PMA to the cells in the continuous presence of the mAb for an additional 5 min (Fig. 7A, compare lanes 3 and 4) or 10 min (Fig. 7A, compare lanes 5 and 6) markedly potentiated the dephosphorylation of receptor-induced protein tyrosine phosphorylation. As shown in Fig. 7B, PMA rapidly reversed tyrosine phosphorylation of Pyk2 induced by CD28 ligation (compare lanes 3 and 4).

FIG. 7. PMA-activated PKC regulates CD28-induced tyrosine phosphorylation of Pyk2. A, 5 x 106 Jurkat T cells in RPMI containing 0.001% BSA were lysed (lane 1) or were stimulated for 5 min at 37°C with 3 μg/ml anti-CD28 mAb (lanes 2–6). After 5 min incubation, cells were either lysed immediately with boiling sample buffer (lane 2) or were treated with only media (lanes 3 and 5) or with media containing PMA (50 ng/ml; final concentration) (lanes 4 and 6), and the incubation was resumed for an additional 5 min (lanes 3 and 4) or 10 min (lanes 5 and 6). After boiling in sample buffer, proteins in WCL were separated by SDS-PAGE, transferred to membranes, and immunoblotted with anti-phosphotyrosine mAb (α-PY). B, 6 x 106 Jurkat T cells in RPMI containing 0.001% BSA were lysed immediately with ice-cold lysis buffer (lane 1) or were stimulated with 3 μg/ml anti-CD28 mAb for 5 min at 37°C (lanes 2–4). At 5 min, cells were either lysed immediately with ice-cold lysis buffer (lane 2), were treated with only media (lane 3), or with media containing PMA (50 ng/ml; final concentration) (lane 4), and the incubation was resumed for an additional 5 min. After solubilizing with ice-cold lysis buffer, proteins in cell lysates were immunoprecipitated (IP) with anti-Pyk2 mAb and were analyzed by immunoblotting with anti-phosphotyrosine mAb (α-PY, upper panel) or with the specific mAb (lower panel).
rylation of the focal adhesion PTKs by inhibiting a signaling event upstream of the tyrosine phosphorylation of the focal adhesion kinase or by blocking the activity of PTKs responsible for the phosphorylation of the focal adhesion PTKs. This may prematurely terminate receptor signaling and, in turn, allow for the dephosphorylation of the focal adhesion PTKs by PT-Pases associated with these PTKs. Src kinases have been shown to associate with focal adhesion PTKs and therefore have been implicated in the tyrosine phosphorylation of the PTKs (30, 32, 33, 45, 51); however, serine/threonine phosphorylation of Src kinases by PMA-activated PKC has been studied extensively and has been shown not to affect significantly the kinase activity of the Src PTKs (52–55). Although in some of our studies, PMA induced a slight decrease in receptor-induced tyrosine phosphorylation of Fyn, the decrease did not correlate with the marked dephosphorylation of Pyk2 seen in all experiments. Our data showed that PMA induced the dephosphorylation of the linker molecule LAT. This molecule is thought to be important for linking molecules tyrosine phosphorylated early in the TCR signaling transduction pathways with downstream molecules (50). Thus, it is possible that the dephosphorylation of LAT is interrupting the flow of signals that lead to the tyrosine phosphorylation of the focal adhesion PTKs. However, at this time there is no evidence that LAT functions in the signaling pathways upstream of the focal adhesion PTKs. Thus, although our data strongly suggest that PT-Pases are involved in the PKC action, it is not clear at this time whether PKC activation is leading to the stimulation of PT-Pases or whether it is facilitating the function of basal level phosphatases by inhibiting a signaling event(s) upstream of the tyrosine phosphorylation of the focal adhesion PTKs.

TCR- and CD28-induced protein tyrosine phosphorylation declines with time after receptor ligation, indicating that a mechanism exists for down-regulating receptor signaling. Our data showed that PMA-induced activation of PKC potentiated the dephosphorylation of LAT and of the focal adhesion PTKs that became tyrosine-phosphorylated after TCR and CD28 ligation. TCR and CD28 ligation has also been shown to initiate signals that activate PKC. However, PMA is a global activator of PKC, which binds directly to PKC and leads to the persistent activation of the kinase. Thus, the potentiated dephosphorylation of LAT and of the focal adhesion PTKs following PMA treatment of CD3- and CD28-activated T cells could be due to the enhancement of PKC activation by PMA. This issue awaits further investigation.

PKC activation by PMA appears to have different effects on the tyrosine phosphorylation of the focal adhesion PTKs in different types of cells (25, 34, 39, 41, 43, 47). PMA induced tyrosine phosphorylation of Pyk2 in rat pheochromocytoma (PC12) cells (25), in CMK megakaryotic cells (38), and in human embryonic kidney 293 cells (25). Similarly, PMA led to strong tyrosine phosphorylation of Fak in fibroblasts (41). In contrast, PMA failed to induce tyrosine phosphorylation of Pyk2 or Fak in nonadherent RBL-2H3 mast cells (34, 39) and in Jurkat T cells (43, 47). Interestingly, PMA increased the tyrosine phosphorylation of Pyk2 and Fak in RBL-2H3 cells adherent to fibronectin, suggesting that PMA enhances integrin-induced tyrosine phosphorylation of the PTKs (34, 39). Notably, although PKC depletion from PC12 cells or fibroblasts markedly decreased PMA-induced tyrosine phosphorylation of Pyk2, it did not affect receptor-induced tyrosine phosphorylation of the PTK in those cells (25, 41). In contrast, depletion or inhibition of PKC completely abolished SCF- and m1 muscarinic acetylcholine receptor-induced tyrosine phosphorylation of Pyk2 in CMK megakaryotic cells and in human embryonic kidney 293 cells, respectively (38, 44). These results strongly suggest the existence of PKC-dependent and PKC-independent pathways for the tyrosine phosphorylation of Pyk2. We have shown here that depletion of PMA-sensitive PKC did not block receptor-induced tyrosine phosphorylation of Pyk2 in Jurkat T cells and that PKC activation by PMA led to a marked reduction in TCR- and CD28-induced tyrosine phosphorylation of the kinase. Thus, it appears that in different types of cells, tyrosine phosphorylation of the focal adhesion PTKs is differentially regulated and the role of PKC in the tyrosine phosphorylation of focal adhesion PTKs is cell-specific.

Focal adhesion PTKs have been implicated in playing a role in linking receptor-initiated signaling pathways to the MAPK cascades (31, 33, 35, 45). The focal adhesion PTKs are also associated constitutively with cytoskeletal proteins such as paxillin and, therefore, could link receptor-initiated signaling pathways with the cytoskeleton (35, 38, 56, 57). Pyk2 and Fak do not contain SH2 and SH3 binding domains; therefore, the phosphorylation of these kinases on tyrosine is critical for their interaction with the SH2 domains of other signaling molecules. Accordingly, the tyrosine phosphorylation of Pyk2 and Fak has been reported to result in the association of the PTKs with several SH2-containing signaling molecules, including Src kinases and adaptor molecules such as Grb2 (29, 30, 32, 33, 44). Thus, the dephosphorylation of the focal adhesion PTKs upon PKC activation could provide a mechanism for controlling the interaction of these PTKs with other SH2-containing molecules and for regulating the function of the kinases in receptor-signaling in T cells.

In conclusion, our data identify a new mechanism for the regulation of TCR and CD28 signaling by PKC, namely the regulation of Pyk2 and Fak tyrosine phosphorylation. Our results also suggest that the regulation of CD28 and TCR signaling by PKC involves protein tyrosine phosphatases. These findings shed a light on our understanding of the molecular mechanisms that regulate receptor-initiated signaling in T cells.

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REFERENCES

1. Qian, D., and Weiss, A. (1997) *Curr. Opin. Cell Biol.* 9, 205–212
2. Chambers, C. A., and Allison, J. P. (1997) *Curr. Opin. Immunol.* 9, 396–404
3. Minami, Y., and Samelson, L. E. (1996) *Immunity* 5, 197–205
4. Werlen, G., Jacinto, E., Xia, Y., and Karin, M. (1998) *EMBO J.* 17, 3101–3111
5. Mills, G. B., May, C., Hill, M., Ebanks, R., Roifman, C., Mellors, A., and Gelfand, E. W. (1989) *J. Immunol.* 142, 1993–2003
6. Ledbetter, J. A., Imboden, J. B., Schleifer, G. L., Grosmaire, L. S., Rabinovitch, P. S., Lindsten, T., Thompson, C. B., and June, C. H. (1990) *Blood* 75, 1531–1539
7. Abraham, R. T., Ho, S. N., Barna, T. J., Rusovick, K. M., and McKean, D. J. (1988) *Mol. Cell. Biol.* 8, 5448–5458
8. Isakov, N., and Altman, A. (1987) *J. Immunol.* 138, 3100–3107
9. Park, D. J., Min, H. K., and Rhee, S. G. (1992) *J. Biol. Chem.* 267, 1496–1501
10. Ward, S. G., and Cantrell, D. A. (1990) *J. Immunol.* 144, 3523–3528
11. Cantrell, D. A., Lucas, S. C., Ward, S., Westwick, J., and Gullberg, M. (1989) *J. Immunol.* 143, 3653–3658
12. Hatahet, J. E., Tsai, B., and Bierer, B. E. (1996) *J. Biol. Chem.* 271, 13362–13370
13. Autero, M., and Gahmberg, C. G. (1987) *Eur. J. Immunol.* 17, 1503–1506
14. Minami, Y., Samelson, L. E., and Klausner, R. D. (1987) *J. Biol. Chem.* 262, 13342–13347
15. Cantrell, D. A., Davies, A. A., and Crumpton, M. J. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 8158–8162
16. Hatchett, J. E., Franklin, D. P., Tsai, B., Harrison-Findik, D., Varticovski, L., and Bierer, B. E. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 8808–8812
17. Parry, R. V., Boulougouris, G., Sansom, D. M., and Ward, S. G. (1997) *Biochem. Soc. Trans.* 25, 305
18. Zhao, Z., Shen, S. H., and Fischer, E. H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 5097–5101
19. den Hertog, J., Sap, J., Pals, C. E., Schlessinger, J., and Kruijver, W. (1995) *Cell Growth Diff.* 6, 303–307
20. Ellison, A., and Leder, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 12235–12239
21. Flint, A. J., Gebbink, M. F., Franzia, B. R. J., Hill, D. E., and Tonks, N. K. (1993) *EMBO J.* 12, 1837–1846
22. Yamada, A., Streuli, M., Saito, H., Rothstein, D. M., Schlossman, S. F., and Morimoto, C. (1990) *Eur. J. Immunol.* 20, 1655–1660
23. Hanks, S. K., Calab, M. B., Harper, M. C., and Patel, S. K. (1992) *Proc. Natl.
PKC Regulates Pyk2/Fak Tyrosine Phosphorylation in T Cells

24. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5192–5196

25. Lev, S., Moreno, H., Martinez, R., Tobioka, H., Kotani, K., and Sasaki, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5192–5196

26. Lev, S., Moreno, H., Martinez, R., Tobioka, H., Kotani, K., and Sasaki, T. (1995) J. Biol. Chem. 270, 5192–5196

27. Avraham, H., Hunter, D., Dawson, T. L., Wilm, M., Anderegg, R. J., Graves, L. M., and Earp, H. S. (1996) J. Biol. Chem. 271, 29993–29998

28. Yu, H., Li, X., Marchetto, G. S., Dy, R., Hunter, D., Calvo, B., Dawson, T. L., Wilm, M., Anderegg, R. J., Graves, L. M., and Earp, H. S. (1996) J. Biol. Chem. 271, 29993–29998

29. Li, J. Z., Avraham, H., Rogers, R. A., Raja, S., and Avraham, S. (1996) Blood 88, 417–428

30. Ganju, R. K., Hatch, W. C., Avraham, H., Ona, M. A., Druker, B., Avraham, S., and Groopman, J. E. (1997) J. Exp. Med. 185, 1055–1063

31. Tokiwa, G., Dikic, I., Lev, S., and Schlessinger, J. (1996) Science 273, 792–794

32. Qian, D. P., Lev, S., Vanoers, N. C., Dikic, I., Schlessinger, J., and Weiss, A. (1997) J. Exp. Med. 185, 1253–1259

33. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550

34. Okazaki, H., Zhang, J., Hamawy, M. M., and Siraganian, R. P. (1997) J. Biol. Chem. 272, 32443–32447

35. Ganju, R. K., Dutt, P., Wu, L. J., Hamawy, M. M., and Siraganian, R. P. (1997) J. Biol. Chem. 272, 30498–30503

36. Astier, A., Avraham, H., Manie, S. N., Groopman, J., Canty, T., Avraham, S., and Groopman, J. E. (1998) J. Biol. Chem. 273, 1055–1063

37. Hamawy, M. M., Swieter, M., Mergenhagen, S. E., and Siraganian, R. P. (1997) J. Biol. Chem. 272, 10804–10810

38. Fechner, J. H., Vargo, D. J., Graeb, C., Wang, J., Hanaway, M. J., Watkins, D. I., Fiekarzczuk, M., Neville, D. M. J., and Knecht, S. J. (1997) Transplantation 63, 1339–1345

39. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trihle, R. P., and Samelson, L. E. (1998) Cell 92, 83–92

40. Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., Shalloway, D., and Hunter, T. (1985) Cell 42, 849–857

41. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1995) Mol. Biol. Cell 6, 637–647

42. Astier, A., Avraham, H., Manie, S. N., Groopman, J., Canty, T., Avraham, S., and Groopman, J. E. (1998) J. Biol. Chem. 273, 10804–10810

43. Hamawy, M. M., Mergenhagen, S. E., and Siraganian, R. P. (1997) J. Biol. Chem. 272, 228–232

44. Hamawy, M. M., Swieter, M., Mergenhagen, S. E., and Siraganian, R. P. (1997) J. Biol. Chem. 272, 30498–30503

45. Felsch, J. S., Cachero, T. G., and Peralta, E. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5053–5056

46. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791

47. van Seventer, G. A., Mullen, M. M., and Vansevant, J. M. (1998) Eur. J. Immunol. 28, 3867–3877

48. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791

49. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) J. Cell Biol. 119, 905–912