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Regulation of Tyrosine Phosphorylation in Isolated T Cell Membrane by Inhibition of Protein Tyrosine Phosphatases

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Jurkat T cells activated by the phosphotyrosine phosphatase inhibitors H2O2 or vanadate were found to have a similar pattern of tyrosine phosphorylation when compared with T cells stimulated by anti-CD3 Ab cross-linking, suggesting that protein tyrosine phosphatase (PTP) inhibitors affect the early steps of TCR signaling. To study the role of PTPs in the most proximal membrane events of tyrosine phosphorylation, subcellular fractions of T cells were treated with the PTP inhibitors in the presence of ATP. In the membrane fraction, tyrosine phosphorylation of Lck, Fyn, and CD3ζ can be induced by PTP inhibitors, but not by anti-CD3. Detailed characterization of this cell-free system showed that the pattern and the order of induced tyrosine phosphorylation is similar to that induced in intact cells. Upon removal of the PTP inhibitor, the tyrosine-phosphorylated proteins, including Lck, Fyn, Syk, Zap70, and CD3ζ, are rapidly dephosphorylated. Preliminary characteristics indicate that a PTP distinct from CD45, SHP1, and SHP2 is present in T cell membranes and the inhibition of this yet unidentified PTP is most likely responsible for the Lck-dependent tyrosine phosphorylation triggered by PTP inhibitors. The Journal of Immunology, 1998, 161: 1743–1750.

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Abbreviations used in this paper: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; TBS, Tris-buffered saline; TBST, TBS 0.01% Tween-20; PNS, postnuclear supernatant; NF-kB, nuclear factor κB; anti-phosphotyrosine Ab; NP-40, Nonidet P-40.
target proteins in the T cell membrane fraction became tyrosine phosphorylated, whereas proteins in a cytosolic fraction did not. The inhibition of the membrane-associated PTPs triggered a cascade of tyrosine phosphorylation, which resembled that induced by TCR/CD3 stimulation in intact T cells. The tyrosine phosphorylation of the proteins in the T cell membrane fraction, including CD3-associated PTKs and CD3ζ, was rapidly reversed upon removal of H₂O₂. Lck is required for the H₂O₂ induction of tyrosine phosphorylation. Preliminary characterization indicates that a PTP distinct from CD45, SHP1, and SHP2 is present in T cell membranes, and may play a critical role in the negative regulation of TCR signaling.

Material and Methods

Cells and antibodies

Jurkat T cell line J77, a variant of clone E6-1 (American Type Culture Collection (ATCC), Rockville, MD), was cultured in RPMI 1640 medium supplemented with 10% FCS at 37°C in a 5% CO₂ humidified atmosphere. JCAM (J.CaM1.6) and JCD45 (J45.01) Jurkat T cells were obtained from the ATCC. Total thymocytes were obtained from 12- to 14-day-old normal and me/me mice (C3HeB/FeJ-lea-ala-me strain; the Jackson Laboratory, Bar Harbor, ME). Anti-phosphotyrosine (anti-ptyr) (RC20), anti-SHP1, anti-SHP2, and anti-Zap70 mAbs were purchased from Transduction Laboratories (Lexington, KY). Anti-CD3ζ monoclonal, anti-Fyn polyclonal, anti-Syk polyclonal, and anti-Zap70 polyclonal Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Lck polyclonal Ab was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-CD3 Ab (OKT3) was prepared from a hybridoma obtained from the ATCC.

Immunoprecipitation and Western blot analysis

Jurkat T cells (2 x 10⁶) were washed and resuspended in 1 ml of PBS. For CD3 stimulation, cells were incubated with OKT3 (2 μg/ml) for 5 min on ice, cross-linked by rabbit anti-mouse IgG (5 μg/ml) on ice for a further 5 min, then incubated at 37°C for 3 min. For H₂O₂ stimulation, cells were incubated with 5 mM H₂O₂ at 37°C for 3 min. After washing with PBS, cells were lysed in 1 ml of Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 0.5 mM sodium deoxycholate, 50 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin, and 2 μg/ml antipain) at 4°C for 30 min. The NP-40 lysate was centrifuged at 12,000 × g for 15 min at 4°C. Immunoprecipitation was carried out at 4°C overnight or at room temperature for 4 h with protein A-Sepharose beads. The beads were washed twice with 0.1% Triton X-100/TBS and once with TBS. The protein was eluted from the beads by boiling for 5 min in 50 μl of Laemmli reducing SDS sample buffer. Proteins from about 10⁷ cells were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 3% BSA/TBST and incubated with Abs in TBST for 2 h at room temperature. Following four 15-min washes with TBST, the membranes were incubated with second Ab for 30 min, washed three times for 5 min with TBST, and developed by enhanced luminal reagent (Amersham, Arlington Heights, IL).

Subcellular fractionation of Jurkat T cells

After washing in PBS, T cells (2 x 10⁶) were incubated in 2 ml of hypotonic buffer (42 mM KCl, 10 mM HEPES (pH 7.4) and 5 mM MgCl₂) for 15 min at 4°C. The cells were passed through a 30-gauge needle 10 times. The extract was centrifuged at 250 x g for 10 min to remove the nuclei and intact cells. The postnuclear supernatant (PNS) was centrifuged at 150,000 x g for 30 min at 4°C to separate the cytoplasm from the membrane fraction.

Induction of T cell membrane by PTP inhibitors and dephosphorylation assay

Membranes from 10⁷ T cells were resuspended in 0.5 ml hypotonic buffer containing 1 mM ATP, then stimulated with 10 mM H₂O₂ or 1 mM vanadate at 37°C for 3 min. The reaction mixtures were centrifuged at 12,000 x g for 5 min at 4°C. The membrane pellets were washed once with hypotonic buffer at 4°C. For the dephosphorylation assay, pelleted membranes were resuspended in 0.5 ml hypotonic buffer, incubated at 37°C for varying times. After centrifugation, membranes were solubilized in NP-40 lysis buffer.

In vitro kinase assay

Immunoprecipitates were incubated together with 0.1 μg of enolase and 1 μCi of [γ-³²P]ATP in 50 μl of kinase reaction buffer (20 mM HEPES, pH 7.4, 10 mM MnCl₂) at room temperature for 30 min. The reaction was stopped by adding 25 μl of 2 x SDS sample buffer and boiling for 5 min. Proteins were then resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and autoradiographed.

Results

Tyrosine phosphorylation induced by H₂O₂, a PTP inhibitor, is similar to that induced by TCR stimulation

To determine the dose dependence of H₂O₂-induced tyrosine phosphorylation, Jurkat T cells were incubated with H₂O₂ at concentrations ranging from 0.1 mM to 10 mM for 2 min or for 5 min. Tyrosine-phosphorylated proteins were immunoprecipitated by an anti-ptyr (4G10). After SDS-PAGE and transfer, proteins were immunoblotted with another anti-ptyr (RC20). H₂O₂ at 0.2 mM induced detectable tyrosine phosphorylation while 5 to 10 mM induced maximal tyrosine phosphorylation. The time of incubation had little effect on tyrosine phosphorylation (Fig. 1A). H₂O₂-induced tyrosine phosphorylation is a rapid event that can be detected within 1 min (data not shown). The major tyrosine-phosphorylated proteins observed have the apparent molecular weights of 18 kDa, 38 kDa, 56 kDa, 70 kDa, and 100 kDa. To compare the patterns of tyrosine phosphorylation induced by H₂O₂ to that induced by CD3 stimulation, Jurkat T cells were stimulated either by 10 mM H₂O₂ or by anti-CD3 cross-linking followed by immunoprecipitation with anti-ptyr or Abs specific for Lck, Fyn, Syk, Zap70, and CD3ζ. The tyrosine phosphorylation induced by H₂O₂ and by anti-CD3 appeared quite similar (Fig. 1B). Both modes of stimulation increased the tyrosine phosphorylation of Lck, Fyn, Syk, Zap70, and CD3ζ. Immunoprecipitation of Zap70 PTK with CD3ζ, as well as the coinmunoprecipitation of Zap70 with Lck, was also observed, indicating that both types of stimulation resulted in the association of Zap70 with CD3ζ and the association of Zap70 with Lck.

In isolated T cell membranes, protein tyrosine phosphorylation is also induced by PTP inhibitors

The above results demonstrated that incubation with H₂O₂ induces tyrosine phosphorylation of PTKs and TCR subunits involved in the early steps of TCR signaling, which suggested that H₂O₂ may be targeted at molecules on T cell membranes. To assess this possibility, Jurkat T cells were homogenized in a hypotonic buffer, then fractionated by differential centrifugation. The PNS, a fraction obtained by 250 × g centrifugation to remove the nuclei from homogenates, was further separated into crude membrane and cytosolic fractions by 150,000 × g centrifugation. After H₂O₂ stimulation, these fractions were subsequently solubilized by NP-40 lysis buffer and immunoprecipitated with an anti-ptyr Ab. The results show that H₂O₂ induces strong protein tyrosine phosphorylation in the PNS, very weak tyrosine phosphorylation in the cytosolic fraction (Fig. 2A). When the cytosolic fraction was mixed with the membrane fraction, H₂O₂-induced tyrosine phosphorylation was reconstituted. Considering that some essential elements for tyrosine phosphorylation such as ATP and divalent ions, could be missing in the membrane fraction, it was supplemented with 1 mM ATP and 5 mM Mg²⁺. In the presence of 1 mM ATP, H₂O₂ induced strong tyrosine phosphorylation in the crude membrane fraction (Fig. 2B). In the PNS and cytosolic fractions, H₂O₂-induced tyrosine phosphorylation was not affected by adding exogenous ATP. In contrast, CD3 stimulation does not induce tyrosine phosphorylation.
phosphorylation in analogous experiments using subcellular fractions.

Two other PTP inhibitors, vanadate and pervanadate, were also used to treat T cell membrane fractions in the presence of ATP (Fig. 3). Vanadate (0.1 mM) was as potent as 2 mM H$_2$O$_2$ in the induction of tyrosine phosphorylation. Pervanadate was extremely potent, with 0.1 mM inducing maximal tyrosine phosphorylation. The pattern of tyrosine phosphorylation induced by H$_2$O$_2$, vanadate, and pervanadate appears quite similar.

To determine the proteins phosphorylated in fractionated T cell membranes by inhibition of PTPs, we specifically examined the phosphorylation of CD3-associated PTKs and the CD3$\zeta$ subunit (Fig. 4A). In the membrane fraction, tyrosine phosphorylation of Lck, Fyn, and CD3$\zeta$ was induced by H$_2$O$_2$. In the presence of cytosolic components, as shown in the PNS fraction, the tyrosine phosphorylation of Zap70 and the coimmunoprecipitation of Zap70 with CD3$\zeta$ and Lck were observed. This indicated that Zap70 can be recruited into the TCR/CD3 complex in cell-free conditions in response to H$_2$O$_2$ stimulation. When the cytoplasmic fraction was stimulated by H$_2$O$_2$, tyrosine phosphorylation of Zap70 was not observed (Fig. 4B). These data indicate that H$_2$O$_2$ does not induce Zap70 tyrosine phosphorylation directly but, instead, through the activation of PTKs in the T cell membrane which, in turn, phosphorylate Zap70. Thus, the tyrosine phosphorylation induced by PTP inhibition is a membrane-related event.

PTP inhibitor-induced tyrosine phosphorylation in isolated T cell membrane is rapidly reversed upon removal of inhibitors

To study the inhibition of PTPs in the T cell membrane, we designed a dephosphorylation assay by using the fractionated T cell membranes. T cell membranes were stimulated by H$_2$O$_2$/ATP for 3 min, then H$_2$O$_2$ was removed from the reaction mixture by adding catalase and washing. Following additional incubation, the
The presence of $H_2O_2$ triggers the tyrosine phosphorylation in T cells. As in Jurkat T cells, $H_2O_2$ and vanadate-induced tyrosine phosphorylation was observed in CD45$^-$ T cells and their membrane preparation, indicating that CD45 is not involved in $H_2O_2$-induced tyrosine phosphorylation (Fig. 7, A and B).

When a similar experiment was performed by using JCAM, a Lck$^-$ variant of the Jurkat T cell line, it was very interesting that neither $H_2O_2$ nor vanadate induced strong tyrosine phosphorylation (Fig. 7, A and B). This indicates that, in the absence of Lck, inhibition of PTPs is not sufficient to trigger tyrosine phosphorylation. This was confirmed by showing that tyrosine phosphorylation in a JCAM cell line transfected with Lck was $H_2O_2$ inducible (data not shown). These data suggested that the PTPs inhibited in T cell membranes could be involved in the negative regulation of Lck signaling.

SHP1 and SHP2, two SH2-containing PTPs, have been reported to be involved in the dephosphorylation of T cell membrane-associated proteins such as Lck, Zap70, and CD3z (23–27). As cytosolic PTPs, SHP1 and SHP2 may associate with phosphorylated membrane proteins, and therefore be present in T cell membrane. To study the role of SHP1 and SHP2 in the regulation of tyrosine phosphorylation, we examined the subcellular distribution of SHP1 and SHP2. The membrane and cytosolic fractions were prepared from T cells, as well as fibroblasts, as a control. Western blot analysis with anti-SHP1 and anti-SHP2 showed that both SHP1 and SHP2 were expressed in T cells and fibroblasts predominantly as cytosolic proteins with only a small amount of SHP1 detectable in the T cell membrane preparation (Fig. 8A). The presence of SHP2 in the T cell membrane fraction was not detected in our experiment. This suggested that SHP1 may be the membrane-associated PTP which, when inhibited, triggers tyrosine phosphorylation. Neither SHP1 nor SHP2 was detected in 3T3 cell membrane preparations. Therefore, the PTP activity observed in our membrane preparations is unlikely to be due to cytosolic contaminants.
To study whether the inhibition of T cell membrane-associated SHP1 is related to the initiation of tyrosine phosphorylation, T cell membranes were prepared from thymocytes of motheaten mice, a genetic model for SHP1 deficiency. *me/me* mice, which are homozygous at the motheaten locus do not express detectable SHP1 (24). The results showed that H2O2 and vanadate induced similar tyrosine phosphorylation in both *me/me* mice and normal littermate control mice (Fig. 8, B and C). Without stimulation, the basal tyrosine phosphorylation in *me/me* mice is at a low level, and is only slightly above that in normal littermate mice. The exception is an 18-kDa protein determined to be CD3ζ, which is constitutively tyrosine phosphorylated in *me/me* mice. In normal mice, tyrosine phosphorylation of CD3ζ was induced by H2O2 and vanadate stimulation. This indicates that SHP1 does play a role in the regulation of protein tyrosine phosphorylation of CD3ζ. However, it is clear that additional PTPs are present in *me/me* T cell membranes, whose activity affects the tyrosine phosphorylation observed in isolated T cell membranes.

**Discussion**

Although it is known that tyrosine phosphorylation can be induced by PTP inhibitors in T cells, the signals triggered by PTP inhibitors and the targets of PTP inhibitor have not been fully defined. We report here that the tyrosine phosphorylation of proteins induced by H2O2 or vanadate is very similar to that induced by TCR stimulation (Fig. 1). These results suggest that the PTP inhibitors H2O2 and vanadate specifically affect the early steps of TCR signaling. To further study this question, we established a cell-free system by subcellular fractionation of Jurkat T cells. In cell-free conditions, tyrosine phosphorylation of proteins in the T cell membrane fraction can be induced by PTP inhibitors, H2O2, or vanadate, but not by TCR/CD3 stimulation (Figs. 2 and 3). In cell-free conditions,
we found that membrane-associated Lck, Fyn, Zap70, and CD3ζ were phosphorylated by ATP/H2O2 (Fig. 4). Furthermore, the regulation of tyrosine phosphorylation in isolated membrane was studied by a dephosphorylation assay (Fig. 5). Lck and other TCR-associated PTKs and CD3ζ were rapidly dephosphorylated in T cell membranes upon removal of PTP inhibitors (Figs. 5 and 6). The dephosphorylation of Lck also resulted in the inactivation of Lck kinase activity (Fig. 6). Therefore, we established a cell-free system in which tyrosine phosphorylation may be regulated by the addition or removal of PTP inhibitors.

To our knowledge, this is the first report that regulation of tyrosine phosphorylation/dephosphorylation may be studied in cell-free conditions. Characterization of this new system indicates that isolated T cell membranes keep the dynamic balance between tyrosine phosphorylation and dephosphorylation and demonstrates an ordered pattern of tyrosine phosphorylation. We expect this system to prove useful to explore early signal transduction cascades. By reconstitution of membranes and cytosol from different cell types or different tissues, it may be possible to determine the specific cytosolic substrates of membrane-associated kinases and the interactions between cytosolic factors and membrane proteins. In cell-free conditions, these factors may be conveniently added into the assay mixture or specifically removed by Abs. Also, these cell-free systems may be useful to test drugs or reagents that are not permeable to the cell membranes.

The presence of Lck in T cell membranes is required for H2O2- and vanadate-induced tyrosine phosphorylation. Lck is activated when T cells are treated with H2O2 (Fig. 7). It has been hypothesized that H2O2 and other oxidants may activate Src family kinases by changing the intracellular redox state. Redox state is coupled to the oxidation of cysteine residues in proteins by a complex thiol/disulfide exchange mechanism that may result in the conformational change and activation of the Src family kinases (35–37). However, H2O2 and all other in vivo-effective oxidants and alkylating agents fail to activate Src family kinases in vitro (36, 37). Only HgCl2 can activate Src family kinases in vitro, presumably by bridging two adjacent sulfhydryl groups to form an R-S-Hg-S-R bond (36, 37). Currently, there is no evidence that such an intramolecular or intermolecular disulfide bridge could be formed through the H2O2 induction. Our results showed that tyrosine phosphorylation of Lck is induced by vanadate or H2O2 in a cell-free system, but is not induced when isolated Lck is treated with H2O2 or vanadate (data not shown). It supports the hypothesis that the activation of Src family kinases by H2O2 and vanadate is indirect, and a common mechanism, the inhibition of PTPs, is the most likely explanation for H2O2, and other oxidants such as phenylarsine oxide- and diamide-induced tyrosine phosphorylation in T cells (38).

The identity of the PTPs inhibited by H2O2 and vanadate that associate with T cell membranes and regulate Lck and other TCR-associated PTKs is currently under investigation. In JCAM T cells, which are deficient in Lck, tyrosine phosphorylation was not induced by H2O2 (Fig. 7) (8). Therefore, the membrane-associated
Western blotting with mAb to SHP1 or SHP2.

PTPs targeted by PTP inhibitors are most likely to be involved in negative regulation of Lck. This is consistent with previous reports showing that CD45-deficient T cells respond to H₂O₂ with a signal similar to J77 T cells (Fig. 7). A role for SHP1 and SHP2 in the negative regulation of T cell activation has been reported (23). T cells deficient in SHP1 are hypersensitive to TCR stimulation (26, 27). SHP1 can dephosphorylate and inactivate bacterially pro-

FIGURE 8. A. Distribution of SHP1 and SHP2 in the membrane and cytosolic fractions prepared from J77 T cells and 3T3 cells. The membrane or cytosolic fraction represents 5 × 10⁶ of cell was added by SDS gel sample buffer, boiled and electrophoresed in SDS-PAGE followed by Western blotting with mAb to SHP1 or SHP2. B and C, H₂O₂ (5 mM) or vanadate (100 μM) stimulation of membranes prepared from thymocytes of motheaten mice. The experiment was performed as described in Figures 3 and 7.

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motedLck (39). Lck, Zap70, and CD3ζ were dephosphorylated by SHP1 when cotransfected in a Spodoptera frugiperda cell line (40). SHP1, a primarily cytosolic PTP, could be present in T cell membranes via an interaction with CD3ζ and CD5 (25). SHP2 may also be present in the T cell membrane in association with tyrosine phosphorylated CTLA-4 (27). We have studied the subcellular distributions of SHP1 and SHP2 and found a small amount of SHP1, but not SHP2, in T cell membrane preparations. To further study the potential role of SHP1 in regulating tyrosine phosphorylation, the membranes from thymocytes of SHP1 deficient me/me mice were stimulated by H₂O₂ (Fig. 8). Strong tyrosine phosphorylation was induced by H₂O₂ in both me/me mice and normal littermate control mice, although CD3ζ was found to be constitutively tyrosine phosphorylated in me/me mice but not in normal control mice, consistent with previous reports (24, 25). These data indicate that, in addition to SHP1 and CD45, a novel PTP may be present in T cell membranes, the inhibition of which is more likely related to the overall tyrosine phosphorylation observed upon H₂O₂ stimulation. The further characterization and identification of this membrane-associated PTP is of great interest and will be facilitated by the ability to study the regulation of tyrosine phosphorylation in this cell-free system.

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