Activation of ZAP-70 through Specific Dephosphorylation at the Inhibitory Tyr-292 by the Low Molecular Weight Phosphotyrosine Phosphatase (LMPTP)*

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The ZAP-70 protein-tyrosine kinase plays a central role in signaling from the T cell antigen receptor. Recruitment and activation of ZAP-70 are transient and are terminated by phosphorylation of negative regulatory tyrosine residues and dephosphorylation of positively acting sites. We report that the low molecular weight protein-tyrosine phosphatase (LMPTP) specifically dephosphorylates the negative regulatory Tyr-292 of ZAP-70, thereby countering inactivation of ZAP-70. Expression of low levels of LMPTP resulted in increased ZAP-70 phosphorylation, presumably at the activating Tyr-493 and other sites, increased kinase activity, and augmented downstream signaling to the mitogen-activated protein kinase pathway. The ZAP-70 Y292F mutant was not affected by LMPTP. Our results indicate that LMPTP, like CD45, dephosphorylates a negative regulatory tyrosine site in a protein-tyrosine kinase and thereby strengthens T cell receptor signaling.

The molecular mechanisms of signal transduction and T cell activation have been intensely studied during the past few years. It has become evident that several protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPases) play crucial roles (1–6). Thus, one of the earliest biochemical events seen in T lymphocytes triggered through the TCR complex is the enhanced phosphorylation of a number of cellular proteins on tyrosine residues (7, 8). Inhibition of this phosphorylation by pharmacological agents prevents T cell activation as measured by both functional readouts and biochemical assays (9, 10).

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MATERIALS AND METHODS

Reagents and Plasmids—The 4G10 anti-phosphotyrosine mAb was from Upstate Biotechnology Inc. (Lake Placid, NY). The 12Ca5 mAb, which recognizes the hemagglutinin epitope, was from Roche Molecular Biochemicals, and the 9E10 mAb, which reacts with the Myc epitope, was from Zymed Laboratories Inc. (San Francisco, CA). Anti-Itk/Emt and the Itk/Emt expression plasmids were kind gifts from T. Kawakami. The cDNA for ZAP-70 (kindly provided by A. Rudd and A. Chan) and the ZAP-70-FF and ZAP-70-Y292F mutants (from A. Chan) were all cloned into the pEF/HA vector provided by C. Rudd and A. Chan) and the ZAP-70-FF and ZAP-70-Y292F mutants (from A. Chan) were all cloned into the pEF/HA vector

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Cells and Transfections—Jurkat T leukemia cells, the Lck-negative variant JCaM1, the ZAP-70-negative P116, and COS cells were kept at 24220 This paper is available on line at http://www.jbc.org

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logarithmic growth in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, l-glutamine, and antibiotics. Jurkat T cells were stimulated with 5 μg/ml of the anti-CD3 mAb, OKT3, in RPMI at 37 °C. COS-1 cells were transfected with 0.1–5 μg of plasmid by lipofection. Jurkat cells were transfected with a total of 5–10 μg of DNA by electroporation at 20 × 10^3 V/cm per sample at 960 microfarads and 260 V. Cells were used 48 h after transfection.

ZAP-70 Kinase Assays, Autophosphorylation, and Dephosphorylation—Immunoprecipitations were performed as described previously (11–13, 37, 38). Briefly, cells were lysed in 1 ml of TNE buffer (1% Nonidet P-40, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 μg/ml aprotinin and leupeptin, and 100 μg/ml soybean trypsin inhibitor). ZAP-70 was immunoprecipitated from clarified lysates with 2 μl of specific antiserum or mAb, followed by agarose-conjugated goat anti-rabbit, anti-mouse IgG, or protein G-Sepharose. Immune complexes were washed three times in TNE, once in TNE with 0.5 M NaCl, and twice in TNE. The ZAP-70 kinase assay was carried out in 20 μl of 50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MnCl2, and 100 μM Na3VO4, containing 2 μg of GST-Vav fusion protein and 1 μM [γ-32P]ATP (10 μCi/sample), for 10 min at 30 °C. ZAP-70 autophosphorylation reactions consisted of incubating 0.3 μg of recombinant ZAP-70 (Upstate Biotechnology Inc.) or immunoprecipitated ZAP-70 incubated for 15 min at 30 °C in 25 μl of 10 mM HEPES, pH 7.5, 5 mM MnCl2, 25 mM MgCl2, 0.1% Nonidet P-40, 1 mM diethiothreitol, 1 μM [γ-32P]ATP (10 μCi/sample). Dephosphorylation by LMPTP was done by terminating the autophosphorylation reaction with 10 μl of 100 mM EDTA and adding the indicated concentration of recombinant LMPTP. After incubation for 15 min at 30 °C, the reaction was terminated by addition of SDS sample buffer and boiling. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose filters and exposed to film. Erk2 kinase assays were performed as described previously (12, 13, 37, 38). Immunoblots were developed by the enhanced chemiluminescence technique (ECL, Amersham Biosciences).

Tryptic Peptide Mapping—These experiments were performed as described previously (12, 39). Briefly, a piece of nitrocellulose filter containing the radiolabeled protein was blocked with polyvinyl pyrrolidone-360 and digested with three additions of 10 μg of Tosylphenylalanyl chloromethyl ketone-treated trypsin. The released phosphopeptides were separated by electrophoresis on cellulose thin layer plates at 1,000 V in pH 1.9 buffer for 27 min followed by ascending chromatography in n-butyl alcohol/pyridine/acetic acid/water (75:50:15:70) and exposed to film.

RESULTS

LMPTP Causes Hyperphosphorylation of ZAP-70—We recently reported that expression of LMPTP caused an augmentation of TCR-induced NFAT/AP-1 reporter gene activation (34). This effect of LMPTP was unique among PTPases in that it was expressed in Jurkat cells without affecting the NFAT/AP-1 activity. The effect of LMPTP was seen at physiological levels of LMPTP (0.1–0.3 μg of plasmid DNA); at higher concentrations LMPTP became less stimulatory and then inhibitory, presumably due to nonspecific effects.

LMPTP Activates ZAP-70—To test whether the LMPTP-induced hyperphosphorylation of ZAP-70 depends on the activation loop tyrosine 493, we expressed the Y492F/Y493F mutant of ZAP-70 (here termed ZAP-70-FF) in COS cells alone or together with LMPTP, or LMPTP-C12S as a control. Anti-phosphotyrosine immunoblots of the immunoprecipitated ZAP-70 molecules demonstrate that this mutant of ZAP-70 was not inducibly phosphorylated on tyrosine by a co-expressed LMPTP, while the wild-type ZAP-70 was (Fig. 2a, upper panel). Anti-HA blots of the same immunoprecipitates showed a comparable level of ZAP-70 in each sample (lower panel). Thus, the small PTPase, VHR, potently dephosphorylated ZAP-70 (lane 3). Finally, LMPTP did not cause any augmentation of the Lck-dependent phosphorylation of Itk/Emt (42) (Fig. 1c, upper panel). Thus, the positive effect of LMPTP on ZAP-70 seems to be relatively specific to this PTPase-PTK pair. Importantly, the effect was seen at physiological levels of LMPTP (0.1–0.3 μg of plasmid DNA); at higher concentrations LMPTP became less stimulatory and then inhibitory, presumably due to nonspecific effects.
LMPTP-induced hyperphosphorylation of ZAP-70 depends on the presence of Tyr-492 and/or Tyr-493.

Since phosphorylation of ZAP-70 at Tyr-493 has been shown to cause its enzymatic activation, we measured the ability of LMPTP to enhance the kinase activity of ZAP-70 against a Vav-derived peptide containing Tyr-174. Indeed, ZAP-70 immunoprecipitated from COS cells expressing wild-type ZAP-70 or ZAP-70-FF alone or together with LMPTP or LMPTP-C12S, as indicated. The lower panel is an anti-HA tag blot of the same samples. b, autoradiogram of in vitro kinase assays of ZAP-70 immunoprecipitates from COS cells expressing wild-type ZAP-70 or ZAP-70-FF alone or together with LMPTP or LMPTP-C12S, as indicated. In lanes 8–11, Lck was co-expressed. The substrate is a GST fusion protein with a Vav-derived peptide (amino acids 161–191) containing Tyr-174.

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**LMPTP Dephosphorylates Tyr-292 of ZAP-70**—The simplest explanation for the effect of LMPTP on ZAP-70 would be the specific dephosphorylation of a negative regulatory site, analogous to the effect of CD45 on Lck. To test the notion that ZAP-70 might be a direct substrate for LMPTP, we expressed a substrate-trapping (44) LMPTP-D129A mutant in T cells and assessed the ability of this mutant to co-immunoprecipitate with ZAP-70. As shown in Fig. 3a, the substrate-trapping mutant LMPTP bound to ZAP-70 at both low and high salt conditions, while ZAP-70 was detectable in wild-type LMPTP immunoprecipitates performed only in very low salt buffer. Since high salt conditions prevent nonspecific or weak protein-protein interactions, these results suggest that LMPTP can bind ZAP-70 through its catalytic cleft.

To directly test whether LMPTP can interact with ZAP-70 and dephosphorylate a negative regulatory site, we analyzed the effects of LMPTP on ZAP-70 phosphorylation by tryptic peptide mapping. Recombinant ZAP-70 was incubated with [γ-32P]ATP to allow the kinase to autophosphorylate, including at the regulatory Tyr-292 residue (40, 45). Subsequently, phospho-ZAP-70 was incubated with different nanomolar concentrations of recombinant LMPTP. As seen in Fig. 3b, 20 nM (assuming 100% purity and activity) of LMPTP caused a selective decrease in a spot in comparison with other peptides on the maps. The spot contained 1,857 cpm in the control sample, 1,252 cpm in the presence of 20 nM LMPTP, and 633 cpm in the presence of 60 nM LMPTP. This spot was absent in maps of an immunoprecipitated Y292F mutant ZAP-70 allowed to autophosphorylate in the presence of [γ-32P]ATP, but was present in parallel maps of wild-type ZAP-70 (Fig. 3c). These experiments suggest that LMPTP preferentially dephosphorylates ZAP-70 at Tyr-292. In agreement with this notion, the substrate-trapping LMPTP-D129A mutant precipitated considerably more wild-type ZAP-70 than Y292F mutant from pervanadate-treated Jurkat cells and then allowed to autophosphorylate in vitro. Note that the spot corresponding to Tyr-292 is missing in the Y292F mutant.
noprecipitation and immunoblotting. As in previous experiments, LMPTP augmented the tyrosine phosphorylation of wild-type ZAP-70 (Fig. 4), although the expression levels were lower in these cells. In contrast, the Y292F mutant ZAP-70 was equally phosphorylated in the presence of LMPTP as in its absence. Quantitation of the bands indicated that Y292F mutant ZAP-70 contained 2.3 times as much phosphotyrosine as wild-type ZAP-70 in the absence of LMPTP. In the presence of LMPTP, the phosphotyrosine content of wild-type ZAP-70 was increased by 2-fold, while the Y292F mutant was unchanged. Thus, LMPTP can augment the tyrosine phosphorylation of ZAP-70 to a level that approaches that of Y292F mutated ZAP-70, suggesting that a substantial portion of phospho-Tyr-292 was dephosphorylated by LMPTP in the cells.

LMPTP Also Augments Signals Downstream of ZAP-70—
The positive effect of LMPTP on the phosphorylation and activity of ZAP-70 was also reflected in the downstream activation of the Erk2 mitogen-activated protein kinase (Fig. 5a). As specificity controls, we expressed two other PTPases normally found in T cells, HePTP and TCPTP. As expected (12, 37), the former inhibited Erk2 activation, while TCPTP had little effect. These results have been obtained in several independent experiments, and they fit well with our previous observation (34) that LMPTP augmented TCR-induced NFAT/AP-1 reporter gene induction.

The ability of LMPTP to augment TCR-induced activation of Erk2 was also observed in JCaM1 cells co-transfected with Y505F-mutated Lck, which cannot be activated by dephosphorylation. These experiments clearly demonstrated that the positive effect of LMPTP was independent of the phosphorylation of Lck at Tyr-505 (Fig. 5b). Thus, it is clear that LMPTP does not augment TCR signaling by activating Lck. Instead, the direct dephosphorylation of Tyr-292 of ZAP-70 is by itself sufficient to explain the effect of LMPTP.

DISCUSSION

We were initially puzzled by the finding that LMPTP, a phosphatase, caused increased phosphorylation of ZAP-70. However, the effect was observed repeatedly, and we decided to consider three different possibilities: (i) LMPTP may dephosphorylate and activate Lck to increase its phosphorylation of ZAP-70; (ii) another PTPase (e.g. SHP-1) is functionally inactivated by LMPTP-mediated dephosphorylation and subsequently reduces its dephosphorylation of ZAP-70; (iii) LMPTP may dephosphorylate a negative regulatory site on ZAP-70 leading to an allosteric activating effect or a reduced associa-
tion with a negative regulator, such as c-Bl (46), or another PTPase. Our data are consistent with the third model, although more complex or additional effects are difficult to exclude.

Our experimental results do not support the possibility that LMPTP augments ZAP-70 phosphorylation by activating Src family PTKs. First, we have been unable to detect any effects of LMPTP on Lck or Fyn. Second, LMPTP augmented the TCR-induced activation of the mitogen-activated protein kinase pathway in JCaM1 cells co-expressing the Y505F-mutated Lck, which cannot be activated through dephosphorylation of Tyr-505. Third, the tyrosine phosphorylation of TCR-ζ was unaltered in ZAP-70 immunoprecipitates. Fourth, Lck-mediated tyrosine phosphorylation of IκB/Emt in co-transfected COS cells was not augmented by LMPTP, but was counteracted by LMPTP. Fifth, although LMPTP was able to cause some dephosphorylation of Lck in vitro, the dephosphorylation was slow and showed no preference for Tyr-505 over Tyr-394 (not shown).

The possibility that LMPTP reduces the action of another PTPase has been suggested to dephosphorylate ZAP-70, namely SHP-1 (47, 48) that SHP-1 can indeed dephosphorylate ZAP-70, but that a cellular PTPase with this specificity must exist in resting T cells. In our hands, none of the nearly 20 different PTPases we have tested (11–15) could remove phosphate from Tyr-292 by LMPTP results in an extent that exceeds the loss of phosphate at Tyr-493) to an extent that exceeds the loss of phosphate at Tyr-292 by LMPTP. Thus, the potential dephosphorylation sites in SHP-1 had no effect. Thus, a potential dephosphorylation of SHP-1 by LMPTP would not suffice as a mechanism for the effect of LMPTP that we report.

The model we favor is that LMPTP directly dephosphorylates ZAP-70 at Tyr-292. As mutation of Tyr-292 causes a significant increase (45) in the total tyrosine phosphorylation of ZAP-70 (29), we propose that the removal of phosphate from Tyr-292 by LMPTP results in an increase of ZAP-70 phosphorylation at other sites (including Tyr-493) to an extent that exceeds the loss of phosphate at Tyr-292. This notion is supported by the lack of effects of LMPTP on Y292F-mutated ZAP-70 (Fig. 4).

The physiological relevance of LMPTP-mediated Tyr-292 dephosphorylation is difficult to address conclusively. The absence of phosphate at this site in resting T cells clearly indicates that a cellular PTPase with this specificity must exist in resting T cells. In our hands, none of the nearly 20 different PTPases we have tested (11–13, 34, 37, 38) has a positive effect on TCR signaling, except LMPTP and CD45. A physiological relevance is also suggested by the low level of LMPTP required for optimal stimulation of ZAP-70 and the membrane-proximal subcellular location of LMPTP. Finally, the opposite effect of the catalytically inactive LMPTP-C12S may be the result of a “dominant-negative” competition with endogenous LMPTP.

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