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Hematopoietic Cell Phosphatase (HCP) Regulates $p56^{LCK}$ Phosphorylation and ZAP-70 Binding to T Cell Receptor ζ Chain

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Ligation of the T cell receptor complex and CD4 leads to activation of the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ resulting in phosphorylation of TcRζ chain and the recruitment of ZAP-70. In this study, we have reconstituted $p56^{lck}$ phosphorylation of TcRζ and ZAP-70 recruitment in heterologous cells and examined the role of the tyrosine phosphatase HCP in regulating the process. Both $p56^{lck}$ and $p59^{fyn}$ induce significant phosphorylation of TcRζ. However, under conditions of comparable $p56^{lck}$ and $p59^{fyn}$ expression, $p56^{lck}$ was found to induce three to four fold greater *in vivo* phosphorylation of TcRζ. HCP dephosphorylated $p56^{lck}$, ZAP-70 and the TcRζ chain. Further, dephosphorylation of the different TcRζ isoforms results in disruption of the interaction between TcRζ and ZAP-70. These results indicate that HCP acts to negatively regulate signal transduction pathways in T cells.

In T cells, several PTKs, as well as PTPs, have been found to play key roles in early signaling events (1,2). Although termination of signals generated by PTKs most likely involve tyrosine dephosphorylation, little is known regarding the specific PTPs that regulate these pathways. One candidate is the phosphotyrosine phosphatase HCP (also named SHPTP1, PTP1C or SHP) which is expressed primarily in hematopoietic cells (3,4,5,6). Several lines of evidence suggest that HCP might be involved in early T cell signaling events. Mutations of HCP in the motheaten (null mutation) and viable motheaten mouse strains (deletion in the phosphatase domain) (7,8) show severe combined immunodeficiency and systemic autoimmunity as well as multiple other hematopoietic abnormalities (9). Studies using motheaten spleen cells have shown that the proliferative response of T and B cells to various mitogens (ConA, LPS, PHA) was reduced (Sidman et al., 1978; Davidson et al., 1978 (10,11). Further, HCP is tyrosine phosphorylated upon T cell receptor stimulation (12) and upon stimulation of macrophages with colony-stimulating factor 1 (13). However, the specific signal transduction pathways regulated by HCP are largely unknown.

Recent studies have suggested that HCP negatively regulates signaling through association with the IL-3 receptor β chain (14), with the FcγRIII complex (15,16) and with the EPO receptor (17).

Src-related protein-tyrosine kinases such as $p56^{lck}$ and $p59^{fyn}$ associate with the CD4/CD8 and TcRζ/CD3 complexes (18,19). Receptor ligation stimulates $p56^{lck}$ and $p59^{fyn}$(T) activity (20,21,22,23), resulting in the phosphorylation of TcRζ and the recruitment of ZAP-70 and Syk (24,25). Association of ZAP-70 with TCRζ is mediated by the two tandemly located SH2 motifs in ZAP-70 that interact with the two phosphotyrosine residues within a 17 amino acid T-cell activation motif (TAM) (25,26). Recently, interaction between src kinases and ZAP-70 has been described. $p56^{lck}$ and $p59^{fyn}$ have been reported to phosphorylate ZAP-70 (27) and bind via the SH2 domains to the ZAP-70 kinase (28). ZAP-70 itself can tyrosine phosphorylate on Tyr-292 (29). However, Tyr-492 and Tyr-493 are the principal sites of T cell antigen receptor-induced

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Abbreviations: HCP, hematopoetic cell phosphatase; TCR, T-cell antigen receptor; ZAP-70, 70-kDa ζ chain-associated protein; SH2 and SH3, Src-homology domains 2 and 3; GST, gluthatione-S-transferase; pTyr, phosphotyrosine; mAb, monoclonal antibody; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; ConA, concanavalin A; LPS, lipopolysaccharide; PHA, phytohemagglutinin.

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tyrosine phosphorylation and dependent on the presence of the src kinase $p56^{lck}$ (29,30,31). Phosphorylation of these sites results in increased catalytic activity of ZAP-70 (30,31).

In this paper, we have examined the role of HCP in regulating the early phosphorylation events of T cell activation. HCP was found to dephosphorylate the TcRζ chain as well as the $p56^{lck}$ and ZAP-70 kinases. HCP dephosphorylates the different TcRζ isoforms and in this process, disrupts the interaction between TcRζ and ZAP-70.

MATERIALS AND METHODS

Cells and antibodies. Spodoptera frugiperda (Sf) cell line IPLB-SF21 was obtained from Invitrogen and was propagated as a monolayer culture in Sf 900 insect medium (Gibco/BRL) supplemented with 10% Fetal Calf Serum and 50μg/ml gentamycin according to the procedure of Brown and Faulkner (1977)(32). Viral infections were performed at a multiplicity of infection of 5 for protein production or of 0.1 for virus production. Antibodies included anti-TCRζ (TIA-2, kindly provided by Dr. P. Anderson, DFCI, Boston, MA and N39, kindly provided by Dr. Cox Terhorst). Rabbit antisera against $p59^{fyn(T)}$ and $p56^{lck}$ were generated against synthetic peptides corresponding to residues 35–51 and 39–64, respectively. Antisera against ZAP-70 were kindly provided from Dr. T. Mustelin (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Anti-phosphotyrosine mAb, 4G10 was kindly provided by Dr. B. Druker (Dana-Farber Cancer Institute, Boston, MA). Rabbit anti-mouse immunoglobulin was obtained from Dako Corporation (Carinteria, Calif.).

Baculovirus expression system. cDNAs encoding full-length $p59^{fyn(T)}$, $p56^{lck}$, ZAP-70, TcRζ and HCP (kindly provided by Dr. R. Perlmutter, Seattle, WA, Dr. G. Raab, Boston, MA, Dr. B. Seed, Boston, MA, and Dr. James N. Ihle, Memphis, Ten, respectively) were either amplified by PCR with specific oligonucleotides that included restriction sites for subcloning into the transfer vector pVL1392 or pVL1393 or directely cloned. *Spodoptera frugiperda* (Sf21) cells were then transfected with a mixture of linear wild type baculoviral DNA (Invitrogen, San Diego, CA) and the pVL1392/pVL1393-DNA constructs and screened for recombinant virus plaques. Recombinant virus was purified from contaminating wild type virus by two rounds of plaque purification.

Immunoprecipitation and kinase assay. 1.5×10^6 cells were infected with the baculovirus encoding the different proteins. After two days cells were harvested and lysed with 200μl lysis buffer (20mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 1mM sodium vanadate, 1 mM PMSF, 1mM leupeptin. Immunoprecipitation was carried out by incubation of the lysate with the antibody for 1 h at 4°C, followed by incubation with 50μl of Protein-A-Sepharose beads (10% wt/vol) for 1 h, at 4°C. Immunoprecipitates were washed 3 times with ice cold lysis buffer and suspended in 30μl of kinase buffer (10mM HEPES, pH7.2; 3.5mM $MgCl_2$, 3.5mM $MnCl_2$), containing 10–20μCi of [γ-^{32}P]-ATP and incubated at RT for 10 min. The samples were boiled 5 min in SDS sample buffer, subjected to SDS-PAGE analysis and the phosphorylated proteins were visualized by autoradiography.

For re-precipitations, SDS (1%, w/v) and β-mercaptoethanol (0.1%, v/v) were added to the samples, which were then denaturated by boiling for 5 min, diluted to 0.1% SDS with lysis buffer, and subjected to immunoprecipitation, as described above.

Western blotting. Immunoprecipitates from $1–2\times10^6$ cells were separated by SDS-PAGE and transferred onto nitrocellulose filters (Schleicher and Schuell, Keene, NH). For immunoblotting, filters were blocked with 5% (w/v) skim-milk for 1h in Tris-buffered saline (TBS), pH 8.0 and then probed with the indicated antibody. Bound antibody was revealed with horseradish peroxidase-conjugated rabbit anti-mouse or donkey anti-rabbit antibodies using enhanced chemiluminescence (ECL, Amersham).

RESULTS

Given the importance of $p56^{lck}$, $p59^{fyn}$(T) and ZAP-70 in T cell signaling, we initially assessed the relative degree to which ζ chain can be phosphorylated by $p56^{lck}$, $p59^{fyn(T)}$ and ZAP-70. Previous studies have shown that TcRζ can be phosphorylated by $p56^{lck}$ (33,26), which acts to recruit ZAP-70 to the TcRζ chain (34,24). In an attempt to reconstitute this interaction, $p56^{lck}$, $p59^{fyn(T)}$ and ZAP-70 were co-expressed in Sf21 cells with TcRζ, subjected to immunoprecipitation with anti-zeta, anti-fyn or anti-lck antibodies. Phosphorylation was detected by anti-phosphotyrosine blotting. As seen in Fig.1A, the expression of TcRζ along (lane 1) failed to undergo detectable tyrosine phosphorylation, although, significant levels of TcRζ expression could be detected by anti-zeta blotting (lane 10). Co-expression of TcRζ with $p56^{lck}$ or $p59^{fyn}$ showed the presence of phosphotyrosine labelled TcRζ showing a broad band between 18–21 KDa (lane 3 and 6).

Although both $p56^{lck}$ and $p59^{fyn(T)}$ phosphorylated TcRζ, consistent differences were noted in their phosphorylation efficiencies. Zeta was phosphorylated to a greater level by $p56^{lck}$ than

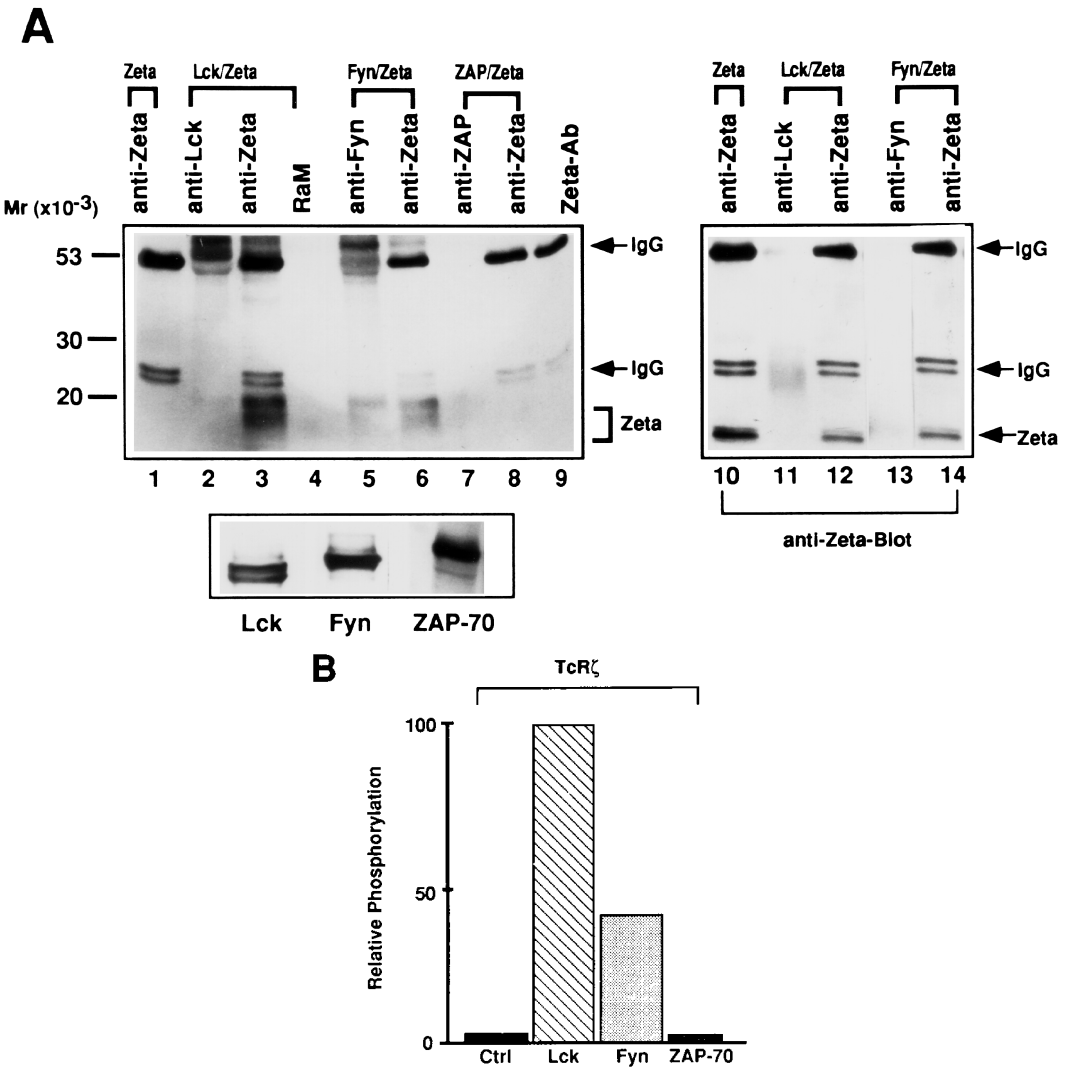

FIG. 1. Phosphorylation of TcRζ by $p56^{lck}$ and $p59^{fyn}$(T). (A) (Upper panel) TcRζ was expressed in insect cells alone (lanes 1,10) or together with $p56^{lck}$ (lanes 2–4,11,12), p59fyn(T) (lanes 5,6,13,14) or ZAP-70 (lanes 7,8). Precipitates were performed using the indicated antibody and then subjected to anti-phosphotyrosine (lanes 1–90) or anti-TcRζ blotting (lanes 10–14). Lanes 1,3,6,8,10 and 12: anti-zeta; lanes 2 and 11: anti-lck; lane 5: anti-fyn, Lane 7: anti-zap-70, lane 4: rabbit anti-mouse; lane 9: zeta MAb control. (Lower panel) Detection of expression of Lck (lane 1), Fyn (lane 2) and ZAP-70 (lane 3) in cell lysates from Sf21 cells infected with the virus containing the cDNAs encoding for the indicated proteins. The lysates were blotted on nitrocellulose and developed with the respective antibodies. (B) Histogram depiction of the relative levels of phosphorylation of TcRζ by $p56^{lck}$, $p59^{fyn}$ and ZAP-70. The level of $p56^{lck}$ phosphorylation of TcRζ served as 100 percent control.

$p59^{fyn(T)}$ (lane 3 vs 6). $p59^{lck}$ caused three to four fold greater TcRζ phosphorylation when compared with $p59^{fyn(T)}$ (Fig.1B). Immunoblotting against cell lysates showed that similar amounts of $p56^{lck}$ and $p59^{fyn(T)}$ were expressed in these cells (Fig.1A, lower panel). ZAP-70 did not phosphorylate TcRζ (lane 8), although expressed at levels similar to $p56^{lck}$ and $p59^{fyn(T)}$ (Fig.1A, lower panel). The difference in phosphorylation was observed in several experiments and indicates that $p56^{lck}$ and $p59^{fyn(T)}$ differentially phosphorylate TcRζ.

Previous studies have shown that $p56^{lck}$ can co-precipitate with the TcR/CD3 complex (27,35).

Although lck can bind to ZAP-70 (28), to our knowledge, direct binding to the CD3 chains has not been assessed. Co-expression of $p56^{lck}$ with TcRζ resulted in little if any detectable association between these proteins (lanes 2,3 and lanes 5,6). Further, co-expression of $p56^{lck}$ and TcRζ followed by anti-zeta immunoblotting of anti-lck precipitates also failed to detect co-precipitated zeta (lane 11). By contrast, a faint amount of tyrosine phosphorylated zeta was found to co-precipitate with anti-fyn (lane 5 and 13), a result likely related to the ability of the N-terminus of $p59^{fyn}$ to bind to TcRζ (36). To further exclude the possibility that the Lck SH2 domain might bind to TcRζ, *in vitro* phosphorylated TcRζ was eluted from gels and subjected to reprecipitation with GST-lck fusion proteins. Although phosphorylated TcRζ could be re-precipitated with an anti-TcRζ antibody (Fig. 2, lane 3), there was no detectable recognition by the GST-SH2lck fusion protein (lane 2). GST-lckSH2 also failed to re-precipitate *in vivo* labelled TcRζ as detected by anti-phosphotyrosine blotting (data not shown). Hence, phosphorylated TcRζ failed to be recognised at detectable levels by the SH2 domains. We next reconstituted ZAP-70 binding to TcRζ in the baculoviral expression system. Co-expression of $p56^{lck}$, ZAP-70 and TcRζ resulted in association of ZAP-70 with TcRζ as seen in anti-zeta precipitates (Fig.3, lane 7). This was not observed in anti-zeta precipitates from cells expressing only ZAP-70 and TcRζ (Fig.1A, lane 8). This result clearly shows that we can reconstitute $p56^{lck}$ dependent ZAP-70 binding to TcRζ in Sf21 cells.

It was next of interest to assess whether these events could be regulated by the tyrosine phosphatase HCP. Co-expression of $p56^{lck}$ or ZAP-70 with HCP resulted in dephosphorylation of both kinases. (Fig.3, lane 2 and 6, respectively) and reduced $p56^{lck}$ activity towards the exogenous substrate enolase (data not shown). We then co-expressed $p56^{lck}$, TcRζ and ZAP-70 with HCP and performed anti-zeta immunoprecipitations and anti-phosphotyrosine immunoblotting. Under these conditions, co-expression with HCP resulted in a complete loss of detectable zeta phosphorylation in Lck/Zeta expressing cells (lanes 3 vs 4). The expression of all constructs showed partial dephosphorylated TcRζ (lane 8). This allowed densitometric reading of phosphorylated TcRζ which show that the phosphorylation of the 18 kDa as well as the 20–21 kDa form of TcRζ are equally reduced when co-expressed with HCP (Fig.3B). The phosphatase therefore dephosphorylates the differentially phosphorylated forms of TcRζ. We next assessed whether HCP could disrupt the binding of ZAP-70 to TcRζ. Significantly, the anti-zeta blot of anti-zap-70 immunoprecipitations showed a loss of Zeta/ZAP-70 association (Fig.4B, lanes 1 and 2). These results suggest that

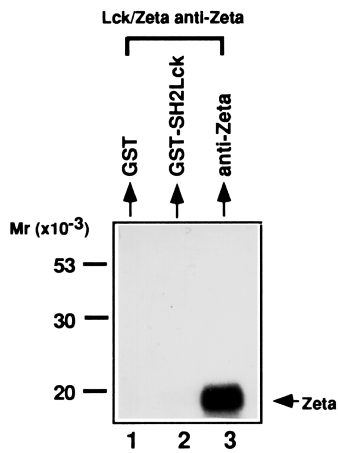

FIG. 2. Re-precipitation analysis of TcRζ by using GST-SH2 fusion proteins. In vitro labelled TcRζ from cells expressing Lck and Zeta was eluted from gels, re-precipitated with GST (lane 1), SH2lck fusion protein (lane 2) or anti-zeta antibodies (lane 3). The precipitates were separated on an SDS-gel and analyzed by autoradiography.

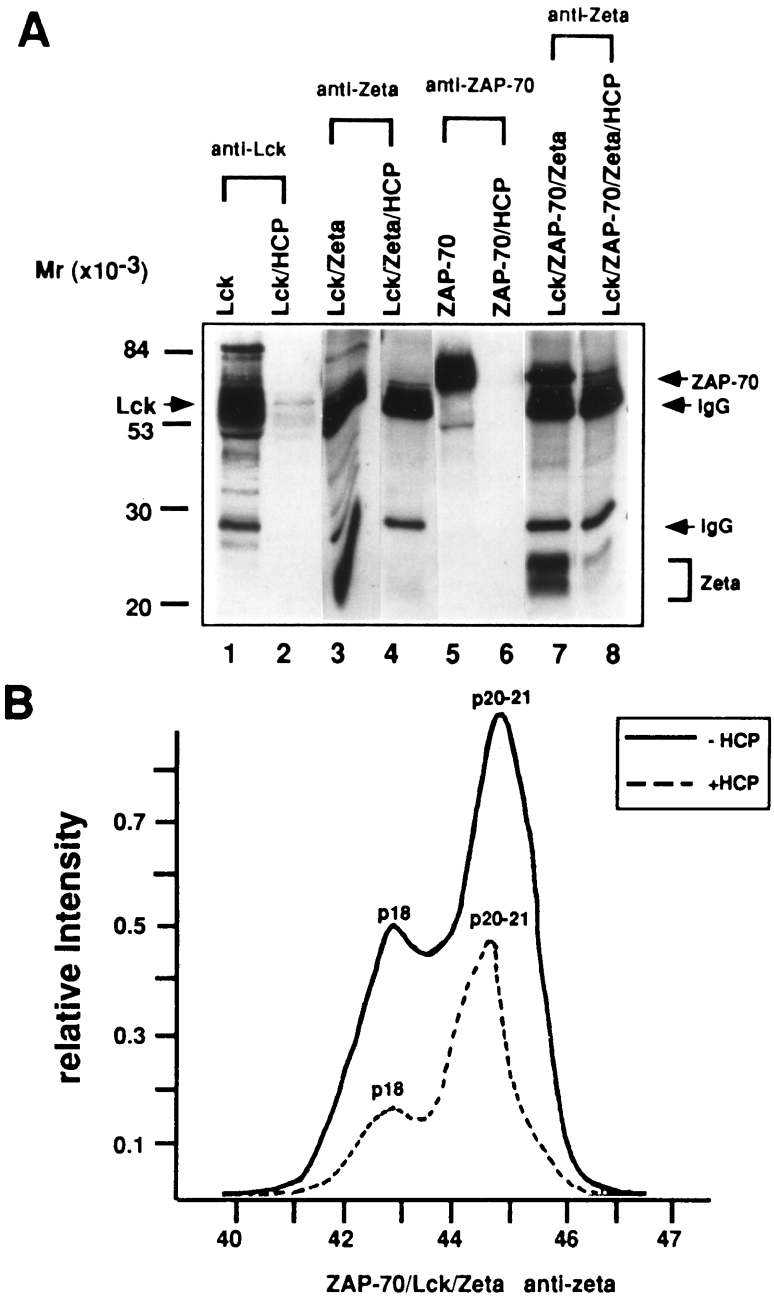

FIG. 3. Dephosphorylation of $p56^{lck}$, ZAP-70 and TcRζ by HCP. (A) Lysates of virus-infected Sf21 cells were prepared by using 1% Triton X-100 lysis buffer, immunoprecipitated with the indicated antibodies, separated on an SDS-10% polyacrylamide gel and subjected to anti-phosphotyrosine blotting. Lane 1: Lck anti-lck; lane 2: Lck/HCP, anti-lck; lane 3: Lck/Zeta, anti-zeta; lane 4: Lck/Zeta/HCP, anti-zeta; lane 5: ZAP-70, anti-zap-70; lane 6: ZAP-70/HCP, anti-zap-70; lane 7: Lck/ZAP-70/Zeta, anti-zeta; lane 8: Lck/ZAP-70/Zeta/HCP, anti-zeta. (B) Densitometric profile of TcRζ from ZAP-70/Lck/Zeta infected Sf21 cells immunoprecipitated with anti-zeta antibody using the Scantjet laser scanner (Hewlett Packard Co.).

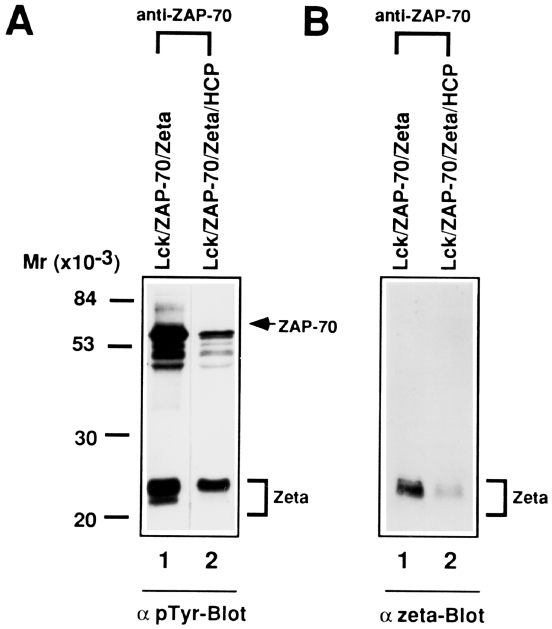

FIG. 4. Dephosphorylation of TcRζ by HCP and disruption of ZAP-70-Zeta binding. Lck, ZAP-70, Zeta and HCP were expressed in Sf21 cells, precipitated with anti-zap-70 antibody and subjected to blotting using anti-phosphotyrosine antibody (A) or anti-zeta antibody (B). Cells were infected with Lck/ZAP-70/Zeta (lane 1) or Lck/ZAP-70/Zeta/HCP (lane 2).

HCP could negatively regulate cell growth in T cells by dephosphorylating different steps in the T cell activation cascade and disruption of ZAP-70 binding to TcRζ.

DISCUSSION

Although HCP has been implicated in the negative regulation of hematopoietic cell growth, no reports have shown the role of HCP in the regulation of early T cell signaling events. Activation of $p56^{lck}$ and $p59^{fyn(T)}$ after receptor ligation (20,21,22,23) results in the phosphorylation of TcRζ and the recruitment of ZAP-70 (24,25). The baculovirus expression system has proven to be a powerful tool in dissecting signaling pathways (37,38). Recent studies showing HCP dephosphorylation of JAK2 were conducted in insect cells (17). In this study, we have shown that HCP can act on multiple steps in the T cell activation cascade. Firstly, we showed that HCP dephosphorylated TcRζ, $p56^{lck}$ and ZAP-70 *in vivo* when individually expressed with HCP (Fig.3). Therefore, HCP can act on both $p56^{lck}$ and ZAP-70 and could counteract effects of PTK activity on the receptor components. Secondly, we showed that TcRζ dephosphorylation induced the disruption of ZAP-70 binding to the TcRζ chain (Fig.4). ZAP-70 binding is required for the successful progression of T cell activation (27). Disruption of ZAP-70 interaction with the zeta chain by HCP could therefore dampen or terminate the activation signal in T cells. This effect may explain the refractory response of T cells from motheaten mice to mitogens (10,11). Likewise, the dephosphorylation of $p56^{lck}$ is consistent with the observation of prolonged activation of $p56^{lck}$ from motheaten mice in thymocytes (17). An interaction between HCP and $p56^{lck}$ has also been suggested by the observation that HCP becomes tyrosine phosphorylated in response to CD4 -or CD8-mediated $p56^{lck}$ activation (12).

Conflicting results have been obtained on roles played by $p56^{lck}$ relative to $p59^{fyn}$ in the phosphorylation cascade. $p59^{fyn(T)}$ has been reported to phosphorylate TcRζ poorly relative to $p56^{lck}$ (24), while another report claims that only $p59^{fyn(T)}$ is capable of phosphorylating the zeta chain (39). Our data confirms that $p56^{lck}$ and $p59^{fyn}$ can both phosphorylate TcRζ (Fig.1B). $p56^{lck}$

phosphorylated TcRζ some four to five fold more than $p59^{fyn(T)}$, while ZAP-70 failed to phosphorylate the chain (Fig. 1). All the kinases were expressed at similar levels (Fig. 1A). Further, protein phosphorylation was detected at various stages (24 to 70h) of the infection cycle and was not dependent on levels of protein overexpression (data not shown). Interestingly, $p56^{lck}$ also phosphorylates the co-receptor CD28 with greater efficiency than fyn (38), while p59fyn preferentially phosphorylates the intracellular protein p120/130 (da Silva and Rudd, in preparation). Taken together, our data indicate that the src-related kinases $p56^{lck}$ and $p59^{fyn}$ perform overlapping functions in the T-cell activation cascade with different relative degrees of efficiency.

Lastly, a third issue concerned whether src kinases might interact directly with the zeta chain. Work by Xu and Littman (40) suggested $p56^{lck}$ SH2 binding to TcRζ. Although Lck SH2 binding to ZAP-70 has been demonstrated (28), src kinase-TcRζ binding has never been directly addressed in the literature. Our data demonstrated that $p56^{lck}$ co-precipitated little if any TcRζ and the Lck SH2 domain failed to precipitate phosphorylated TcRζ, under conditions designed to bias the sensitivity of detection (Fig. 2). The N-terminal region of $p59^{fyn}$ (T) can interact with TcRζ and CD3 subunits (37), although the Fyn-SH2 domain also failed to precipitate TcRζ (data not shown). $p56^{lck}$ SH2 binding to ZAP-70 is therefore likely to predominate in the formation of intracellular complexes and provide an alternate mechanism by which $p56^{lck}$ and $p59^{fyn(T)}$ may become physically coupled to the antigen-receptor complex.

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