Resting Lymphocyte Kinase (Rlk/Txk) Targets Lymphoid Adaptor SLP-76 in the Cooperative Activation of Interleukin-2 Transcription in T-cells*

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Helga Schneider‡‡, Benoit Guerette‡‡, Christine Guntermann‡‡, and Christopher E. Rudd‡‡

From the ‡Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute and the Departments of §Medicine and ¶Pathology, Harvard Medical School, Boston, Massachusetts 02115

Rlk/Txk is a T-cell-specific member of the Btk/Tec family of tyrosine kinases, whereas SLP-76 is a lymphoid adaptor that is essential for pre-TcR and mature TcR signaling. Although Rlk deficient T-cells show partial defects in T-cell proliferation, Rlk can complement ITK−/− cells with multiple defects in TcR initiated early events and interleukin (IL)-2 production. A key question is the nature of the target of Rlk responsible for bridging the TcR with the activation of IL-2 transcription. In this study, we identify a pathway in which Rlk phosphorylates SLP-76 leading to the phosphorylation of PLCγ1, activation of ERKs, and the synergistic up-regulation of TcR-driven IL-2 NFAT/AP-1 transcription. Rlk phosphorylated the N-terminal region of SLP-76, a region that has been previously shown to serve as a target for ZAP-70. Loss of N-terminal YESP/YEPP sites of SLP-76 or the Rlk kinase activity attenuated cooperativity between Rlk and SLP-76. These observations support a model where the TcR can utilize Rlk (as well as ZAP-70) in the phosphorylation of key sites in SLP-76 leading to the up-regulation of Th1 preferred cytokine IL-2.

T-cell activation involves the ligation of CD4/CD8-p56lk and the T-cell receptor complex leading to the activation of Src kinases p56lk and p59fyn (1) and the phosphorylation of immunoreceptor tyrosine-based activation motifs of the TcR/CD3 chain (2–4). ZAP-70 catalytic activity is then up-regulated by p56lk phosphorylation (5, 6). p56lk and ZAP-70 play pivotal roles in thymic differentiation (7–9) and TcR-mediated signaling in mature T-cells (10, 11).

In addition to Src and ZAP/SYK-related kinases, the Tec family of tyrosine kinases has recently been implicated in the regulation of immune function. Tec kinases typified by Btk are comprised of an N-terminal phosphotyrosinolinositol phosphate binding pleckstrin homology domain, followed by an SH2 and SH3 domain and a catalytic domain. Btk mutations have been documented to cause severe immunodeficiencies (12–15) and aberrant B-cell development (16). Tec kinases are positioned downstream of Src kinases that regulate their activity by phosphorylating within the activation loop of the kinase domain (17–19). T-cells express three members of the Tec family of tyrosine kinases that include Rlk (resting lymphocyte kinase), ITK (interleukin-2-responsive T-cell specific kinase) (otherwise known as Tsk (T-cell-specific kinase) or EMT), and Tec (20–22). Rlk resembles other Tec kinases except for the absence of a pleckstrin homology domain, which has been replaced with palmitoylated cysteine ring motif that is required for membrane localization (23). Two forms of Rlk exist that include a full-length form and an alternately spliced version that lacks the cysteine ring motif (23). ITK and Rlk appear to complement each other in the regulation of T-cell function. Although ITK−/− mice show mild to moderate impairments of responses to TcR ligation (24), the Rlk−/− phenotype is less pronounced with a slight decrease in numbers of mature T-cells, in particular of the CD4+ subset (25). By contrast, the combined loss of Rlk and ITK shows major defects in TcR signaling events such as Ca2+ mobilization and inositol 1,4,5-trisphosphate and IL-2 production (25).

Aside from kinases, recent studies have identified an array of immune cell-specific adaptor proteins in T-cells that act as substrates for upstream kinases and play important roles in T-cell function (26, 27). One of these adaptors, SLP-76, is a relatively hydrophilic protein with an acidic N-terminal region, several tyrosine consensus motifs, a central proline-rich region, and a C-terminal SH2 domain (28). As with Rlk-deficient T-cells, SLP-76−/− T-cells show defects in Ca2+ mobilization and inositol 1,4,5-trisphosphate and IL-2 production (29). To date, the ZAP-70 and SYK kinases have been shown to couple receptors to SLP-76 by virtue of their ability to phosphorylate SLP-76 (30, 31). ZAP-70 phosphorylates residues within an acidic region of SLP-76 that includes several tyrosine motifs: YESP, YESP, and YEPP (31). These N-terminal residues are required for the ability of SLP-76 to potentiate IL-2 production (32).

Despite its importance to T-cell proliferation, the signaling pathway that connects Rlk to the TcR complex and the subsequent up-regulation of ERK activity and IL-2 transcription is unclear. In particular, it has been uncertain whether the Rlk kinase targets any of the newly identified lymphoid adaptors in T-cells. In this study, we report that Rlk phosphorylates SLP-76 at its N-terminal YESP/YEPP sites, a region that previously was found to act as a site for ZAP-70, and further that Rlk and SLP-76 potently and cooperatively up-regulate TcR-driven IL-2 transcription in T-cells. Loss of the N-terminal YESP/YEPP sites of SLP-76 or the Rlk kinase activity attenuated the cooperative effect. These observations support a model where the TcR can utilize the two kinases Rlk and ZAP-70 in the phosphorylation of SLP-76 leading to the up-regulation of IL-2 production in T-cells.

MATERIALS AND METHODS

Cells, Reagents, and Antibodies—COOS cells and Jurkat T-cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine

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† To whom correspondence should be addressed. Tel.: 617-632-3574; Fax: 617-632-5259; E-mail: christopher_rudd@dfci.harvard.edu.

§ The abbreviations used are: IL-2, interleukin-2; mAb, monoclonal antibody; PLC, phospholipase C; HA, hemagglutinin; RoM, rabbit anti-mouse.
FIG. 1. Rlk expression induces SLP-76 phosphorylation in COS and T-cells. A, Rlk transfection of Jurkat T-cells leads to increased tyrosine phosphorylation of SLP-76. Top left panel, Jurkat T-cells transfected with vector (Mock) (lanes 1 and 2), ZAP-70 (lanes 3 and 4), or Rlk (lanes 5 and 6) were either left unstimulated (RoM) (lanes 1, 3, and 5) or stimulated with OKT3 and RoM (lanes 2, 4, and 6) for 10 min, lysed, immunoprecipitated with anti-pTyr mAb, and subjected to immunoblotting with anti-SLP-76 mAb. Similar amounts of cell lysates were subjected to immunoprecipitation as shown in an anti-SLP-76 immunoblot (right panel). Second left panel from top, the same blot was reprobed with anti-PLCγ1 mAb. Similar amounts of cell lysates were subjected to immunoprecipitation as shown in an anti-PLCγ1 immunoblot (right panel). Two bottom left panels, immunoblot with anti-ZAP-70 mAb and anti-murine specific Rlk antiserum shows the expression levels of transfected ZAP-70 and Rlk, respectively. B, Rlk directly phosphorylates SLP-76 in COS cells. Top panel, COS cells were transfected with either SLP-76 alone (lanes 1), SLP-76 plus Rlk (lane 2), SLP-76 plus kinase inactive Rlk (dRlk) (lane 3), or SLP-76 plus ZAP-70 (lane 4), lysed, immunoprecipitated with anti-SLP-76 mAb, and assessed for SLP-76 phosphorylation by immunoblotting with anti-pTyr mAb. Middle panel, the same blot was reprobed with anti-PLCγ1 mAb. Bottom panel, anti-Rlk immunoblotting shows the expression level of transfected murine Rlk.
cells were lysed in 100 μl of lysis buffer (Promega kit), and luciferase activity was determined using the luminometer (MicroLumat, EG7G Berthold).

RESULTS

T-cell activation is accompanied by an increase in phosphorylation of the lymphoid adaptor protein SLP-76 (28, 31, 34, 35). Recent studies have shown that SLP-76+/− and Rlk−/− T-cells show similar defects in Ca2+ mobilization and inositol 1,4,5-trisphosphate and IL-2 production (29). To assess the role of Rlk in TcRζ/CD3 signaling and in particular whether the kinase might target SLP-76, Rlk was transfected into Jurkat T-cells and assessed for an effect on tyrosine phosphorylation of SLP-76. As shown in Fig. 1A (top left panel), anti-CD3 stimulation induced an increase in SLP-76 phosphorylation (lane 2 versus lane 1), whereas overexpression of Rlk caused a further increase (lane 6). Significantly, the increase was comparable with that observed with the overexpression of ZAP-70 (lane 4), a kinase previously shown to phosphorylate SLP-76 (31). Further, the levels of ZAP-70 and Rlk expression were roughly equivalent in the transfectants (lower panels). The anti-Rlk antiserum is specific for the transfected murine Rlk protein without cross-reaction with the endogenous human kinase (Txk). As a further control, similar levels of SLP-76 were found expressed in lysates from the different cells (right upper panel). These data indicate that Rlk can cooperate with the TcR complex in the generation of signals leading to SLP-76 phosphorylation.

A previous study using SLP-76-deficient cells have dem-
together, these observations are consistent with a role for Rlk in events leading to SLP-76 phosphorylation and its connection with PLCγ1. No effect was observed on TcR phosphorylation or the recruitment of ZAP-70 (data not shown). Rlk-induced phosphorylation of SLP-76 could be either direct or indirect. We and others have previously shown that SLP-76 acts as a substrate for the tyrosine kinase ZAP-70 (30, 31). To assess further whether SLP-76 acts as a direct substrate for Rlk, the kinase and SLP-76 were co-expressed in COS cells and analyzed for changes in tyrosine phosphorylation. Transfection of heterologous cells such as COS cells allows for an analysis of lymphoid kinases and substrates in the absence of other lymphoid proteins. As observed in T-cells, the expression of Rlk resulted in the marked phosphorylation of SLP-76 (Fig. 1B, top panel, lane 2 versus lane 1). By contrast, as a control, kinase inactive Rlk (dRlk) failed to induce phosphorylation (lane 3). Significantly, Rlk phosphorylated SLP-76 at levels that were roughly comparable with that mediated by ZAP-70 (lane 4). Similar amounts of SLP-76 were immunoprecipitated from the different transfectants as shown in an anti-SLP-76 immunoblot (middle panel). Immunoblotting with anti-Rlk antiserum was used as a control for the expression of Rlk (lower panel).

SLP-76 possesses three key tyrosines (YESP, YESP, and YEPP) in an acidic N-terminal region that are important for the ability of the adaptor to augment IL-2 transcription in T-cells (32). ZAP-70 and SYK kinases phosphorylate SLP-76 at these tyrosines (30, 31, 36). To assess the specificity of Rlk phosphorylation and in particular whether Rlk targeted the same region as ZAP-70, Rlk was co-expressed in T-cells (Fig. 2A, top panel) and in COS cells (Fig. 2B, top panel) with a mutant form of SLP-76 in which the three key tyrosines have been mutated to phenylalanines. Significantly, the mutation of the key tyrosines resulted in a complete loss of Rlk-mediated phosphorylation in T-cells (Fig. 2A, top panel, lane 6). An anti-SLP-76 immunoblot showed the same amounts of SLP-76 in the different transfectants (middle panel). Anti-CD3-mediated phosphorylation of SLP-76 in cells transfected with mutant SLP-76Y3F3 alone was also abolished (lane 4 versus lane 2). In COS cells, a 50–60% loss in SLP-76 phosphorylation was observed when the mutant was co-transfected with Rlk (Fig. 2B, upper panel, lane 4 versus lane 3). As a control, phosphorylation of SLP-76 by ZAP-70 was no longer detectable with the loss of the tyrosines (lane 2 versus lane 1). The same amounts of SLP-76 were immunoprecipitated from the different COS cell transfectants as shown in an anti-SLP-76 immunoblot (middle panel). Immunoblotting with anti-Rlk antiserum was used as a control for the expression of Rlk (lower panel). These data indicate that Rlk and ZAP-70 share an ability to phosphorylate SLP-76 within its acidic N-terminal region, a region that is crucial to the function of the SLP-76 adaptor. The residual phosphorylation of SLP-76Y3F3 in COS cells could be due to the phosphorylation of tyrosines other than the YESP/YEPP sites. In T-cells, overexpressed Rlk mainly phosphorylated the
three key tyrosines. The affinity for other tyrosines might be very low, which resulted in no detectable phosphorylation of transfected SLP-76.Y3F3.

Because SLP-76 expression is needed for TcR-mediated activation of ERKs (29), the next question was whether the TcR/CD3 complex employed Rlk-SLP-76 in the regulation of ERK activity. For this purpose, the kinase and substrate were individually and jointly transfected into Jurkat T-cells, stimulated for 15 min with CD3 mAb, and subjected to immunoprecipitation with ERK1/2 antibodies. Myelin basic protein was then used as a substrate in an in vitro kinase assay for the assessment of ERK activity. As shown in Fig. 3, anti-CD3 stimulation induced an increase in ERK activity in vector transfected cells (lane 2 versus lane 1) that was further augmented by expression of SLP-76 (lane 4) or Rlk (lane 6). The combined expression of SLP-76 and Rlk led to a further increase in ERK activity in response to TcR/CD3 ligation (lane 8). Similar amounts of cell lysates were subjected to the in vitro kinase assay as shown in an anti-ERK1/2 immunoblot (lower panel). These data therefore demonstrate that both Rlk and SLP-76 operate in response to TcR/CD3 ligation to up-regulate ERK activity.

Given these stimulatory effects, a key question was whether the TcR complex also uses Rlk and its phosphorylation of SLP-76 in the regulation of IL-2 transcription. As seen in Fig. 4, Rlk or SLP-76 itself led to a slight increase in anti-CD3 induced NFAT/AP-1 transcriptional activity when compared with cells transfected with vector alone. The effect of SLP-76 on NFAT/AP-1 transcription is comparable with that reported by others (32). Interestingly, the combined expression of Rlk and SLP-76 resulted in a marked synergy in the potentiation of IL-2 transcription. In fact, the level of transcription was some 100-fold greater than in cells that were individually transfected with Rlk or SLP-76 (Fig. 4A). Levels of SLP-76 and Rlk expression were monitored by anti-SLP-76 and anti-Rlk immunoblotting, respectively (right panels). Significantly, the importance of Rlk-mediated phosphorylation was evident with the attenuation of SLP-76/Rlk cooperativity with the kinase inactive Rlk (dRlk) (Fig. 4A). These data indicated that Rlk kinase activity was needed for the potentiation of IL-2 gene activation. To further connect this phosphorylation with the N-terminal region of SLP-76, the mutant form of SLP-76 (lacking the key tyrosine residues) was also analyzed. In this case, it was also defective in supporting increased IL-2 transcription (Fig. 4B).

Levels of SLP-76 and Rlk expression were monitored by immunoblotting with anti-HA and anti-Rlk antibodies, respectively (right panels). Taken together, these results demonstrate that Rlk-mediated phosphorylation of SLP-76 cooperatively up-regulates TcR/CD3-induced IL-2 transcription in T-cells and that the kinase activity of Rlk and its phosphorylation site on SLP-76 are needed for this potentiation.

DISCUSSION

Previous studies have demonstrated the importance of SLP-76 and the Tec kinases Rlk and ITK in the control of T-cell proliferation (25, 29, 37). However, despite the importance of SLP-76, the identity of kinases that connect the TcR complex to the adaptor and the subsequent up-regulation of ERK activity and IL-2 transcription has been unclear. In this study, we provide the first evidence of a link between Rlk and the SLP-76 adaptor. We found that Rlk phosphorylates SLP-76 at its N-terminal YESP/YEPP sites, a region that previously was found to act as a site for Zap-70 phosphorylation (31). These N-terminal residues are required for the ability of SLP-76 to up-regulate IL-2 transcription (32). Further, Rlk and SLP-76 potently and cooperatively up-regulate TcR-driven IL-2 transcription in T-cells. This synergy depended on Rlk phosphorylation of SLP-76 tyrosine sites. Loss of the N-terminal YESP/YEPP sites of SLP-76 attenuated the cooperative effect, as did the loss of Rlk kinase activity. These observations support a model where the TcR can utilize the two kinases Rlk and Zap-70 in the phosphorylation of SLP-76 leading to the up-regulation of IL-2 production in T-cells (Fig. 5). Whether these two kinases operate in synergy at some other juncture in the cascade (i.e. PLCγ1 phosphorylation) awaits further studies.

In addition to placing Rlk in a TcR-mediated signaling network, our data emphasize the central role of SLP-76 as an integrator of signals from both the Zap-70 and Rlk kinases. It is also consistent with the observation that T-cells from Rlk−/− ITK−/− and SLP-76−/− mice show a similar phenotype with defects in Ca²⁺ mobilization and inositol 1,4,5-trisphosphate and IL-2 production (25, 29). The ability of co-transfected SLP-76 and Rlk to up-regulate ERK activity is also in agreement with the delayed onset of ERK activation in T-cells from Rlk−/− ITK−/− mice (25). In addition, the knock-out mice showed alterations in Ca²⁺ mobilization, which was also observed in our transfection studies (data not shown). Our observation that Rlk transfection led to increased PLCγ1 phosphorylation suggests that Rlk could be one of the bridging protein tyrosine kinases between SLP-76 and PLCγ1. Taken together, our results provide an alternate pathway from the TcR that phosphorylates SLP-76 and potentiates PLCγ1 phosphorylation.

The overlapping roles of Rlk and Zap-70 in the phosphorylation of SLP-76 do not exclude additional functions of Zap-70 and SLP-76 unconnected with Rlk. This is suggested by the difference in the phenotypes of the Rlk−/− ITK−/− and Zap-70−/− mice, where Zap-70−/− mice show a block in thymic development that is not evident in Rlk−/− ITK−/− mice (38). This suggests that Zap-70 mediates additional functions other than SLP-76 phosphorylation that are essential for thymic development. The phosphorylation of the adaptor LAT is one possibility, an adaptor needed for differentiation (39). Similarly, although Rlk−/− X ITK−/− and SLP-76−/− T-cells show similar defects in TcR-mediated signaling (25, 29), the SLP-76-deficient mice show an additional block at the double negative stage of differentiation because of defective pre-TcR signaling (40, 41). This indicates that SLP-76 mediates a central function in thymic differentiation other than that mediated by Rlk phosphorylation.

Nevertheless, Rlk phosphorylation of SLP-76 is likely to play an important role in ensuring optimal T-cell activation. This could be particularly relevant in the stimulation of primary T-cells where Rlk is expressed at its highest levels (42). Following activation, levels of Rlk expression decrease. Further, our observations are unique in pointing to an alternate pathway by which SLP-76 could be phosphorylated as a consequence of TcR ligation. Unlike Zap-70 and its binding to TcR/CD3 immunoreceptor tyrosine-based activation motifs, Rlk operates independently of immunoreceptor tyrosine-based activation motif binding, thus increasing the flexibility of the TcR to respond to antigen, in particular during the earlier phase of TcR signaling. Nevertheless, Rlk involvement would still occur downstream of Lck and Fyn-T because its activation depends on Src kinase (i.e. CD4-p56(c)) phosphorylation within its catalytic activation loop (23). We previously showed that Rlk (Tkx) can phosphorylate CTLA-4, suggesting that this kinase may also be involved in CTLA-4 signalling (43). Further experiments will be needed to establish whether ITK or Tec mediate SLP-76 phosphorylation and whether the TcR-Rlk-SLP-76 pathway is preferentially used under distinct conditions of antigen presentation.
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