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Resting Lymphocyte Kinase (Rlk/Txk) Phosphorylates the YVKM Motif and Regulates PI 3-Kinase Binding to T-Cell Antigen CTLA-4

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CTLA-4 and CD28 are differentially expressed on T-cells. They bind to a common ligand B71/2 (CD80/86), however with different avidities. Unlike CD28 which augments the T-cell response, CTLA-4 operates predominately as a negative regulator of T-cell proliferation. The mechanism by which CTLA-4 can generate these intracellular signals is unclear. Little is known regarding the identity of the protein-tyrosine kinase(s) responsible for CTLA-4 phosphorylation and thus creating conditions for the reported binding to PI 3-kinase and the protein tyrosine phosphatase SHP-2. In this study, we demonstrate that Rlk (resting lymphocyte kinase) is capable of phosphorylating CTLA-4 at the YVKM motif. Consistent with this finding, Rlk is capable of providing conditions for the binding of the SH2 domains of PI 3-kinase to the receptor. CTLA-4 is therefore the first known substrate for Rlk suggesting the possibility that this kinase may participate in CTLA-4 function.

CD28 and CTLA-4 determine the destiny of the T-cell response following ligation of TcRζ/CD3 and CD4/CD8-p56^{lck} (1-4). CD28 can both lower the threshold needed for T-cell activation and increase the longevity of the response, an effect that is linked to increased transcription and stability of mRNA for lymphokines, in particular, interleukin -2 and -4 (IL-2 and IL-4) (3, 4). CD28 deficient mice have markedly diminished T-cell responses to certain antigens (5), while CD28 amplifies cytolytic responses in anti-tumor and autoimmune responses (6-9). Co-receptor ligation may also influence Th_1 and Th_2 subset differentiation (4, 10, 11), as well as the expression of the anti-apoptotic factor Bcl-$_{XL}$ (12), and surface antigens such as CTLA-4, the high affinity IL-2 receptor and the CD40 ligand (CD40L). The absence of co-stimulation has also been reported to result in the functional inactivation, or anergy of Th_1 cells (13).

The intracellular signaling pathway(s) responsible for these pleiotropic effects remains still unclear. CD28 binds to phosphatidylinositol 3-kinase (PI 3-kinase) and growth factor receptor-bound protein-2 (GRB-2) by means of a tyrosine based motif YMNM (14-17). Functionally, mutations in the YMNM motif abrogate T-cell co-stimulation involving CD28 and TCR/CD3 ligation (18, 19). The loss of PI 3-kinase binding also interferes with proper CD28 endocytosis (20), thereby providing a potential mechanism for the involvement of the kinase in co-stimulation. By contrast, mutation of asparagine at residue 193 disrupts GRB-2 binding, the phosphorylation of the hematopoietic protein Vav and co-stimulation (19).

With the exception of one report (21), CTLA-4 operates predominately as a negative regulator of T-cell proliferation (22, 23). Although certain studies have demonstrated a potentiating effect of anti-CTLA-4 antibodies, these effects have been interpreted in terms of negative signaling. As with CD28, the mechanism by which CTLA-4 can generate these intracellular signals is unclear. CTLA-4 has been reported to bind to PI3-kinase and the protein tyrosine phosphatase SHP-2 (PTP2C, PTP1D, SH-PTP2) (24-26). CTLA-4 possesses a pYVKM motif which can bind to the SH2 domains of PI 3-kinase, and peptide binding studies have shown the binding avidity of the p85 SH2 domains for the CTLA-4pYVKM motif to equal that observed for the binding motif within CD28 and the platelet-derived growth factor receptor (PDGF-R) (17).

Given the potential significance of the YXXM motif to CD28 and CTLA-4 function, it was important to determine the nature of the kinase that is responsible for its phosphorylation. Co-expression studies showed

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that $p56^{lck}$ and $p59^{fyn}$ specifically phosphorylate CD28 at the YMNM motif. (27). Bradshaw et al. demonstrated that CTLA-4 is phosphorylated by $p56^{lck}$, however primarily at the second YFIP site (28). A remaining question is whether there exist other T-cell kinases that phosphorylate CTLA-4.

Rlk (resting lymphocyte kinase) (also termed Txk) is a member of the Btk/Tec family of protein-tyrosine kinases, being restricted in expression to T-cells (29-31). Rlk possesses an unique N-terminus, a SH3 domain, a SH2 domain and a kinase catalytic domain. Rlk encodes two proteins of sizes 58 and 52 KDa. However, unlike other Btk family members, Rlk lacks a region of pleckstrin homology (PH) in its amino-terminal region. To date, no functions and target substrates of Rlk have been identified.

In this study, we demonstrate that Rlk is capable of phosphorylating CTLA-4, and most significantly, despite the presence of two phosphorylation sites, the phosphorylation occurs specifically at the CTLA-4pYVKM motif. Consistent with this finding, Rlk is capable of providing conditions for the binding of the SH2 domains of PI 3-kinase to the receptor. CTLA-4 is therefore the first known substrate for Rlk suggesting the possibility that this kinase may participate in CTLA-4 function.

MATERIALS AND METHODS

Cells and antibodies. COS-1 cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum (FBS, Intergen, New York), 100U/ml penicillin,100 mg/ml streptomycin (Gibco, Grand Island, NY), 2mM L-glutamine (Gibco) and 50μM 2-mercaptoethanol. Anti-CTLA-4 (11D4) has been previously described (32) (from Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). The anti-phosphotyrosine monoclonal antibody (anti-Tyr (P); 4G10) was kindly provided by Drs. B. Druker and T. Roberts, Dana Farber Cancer Institute, Boston. Monoclonal antibodies to ZAP-70 were purchased from Transduction Laboratories (Lexington, KY). MAb to SLP-76 and cDNA encoding SLP-76 were provided by Dr. P. R. Findell (Syntex, Palo Alto, CA). Anti-p85 of PI3-kinase was provided by Dr. M. White (Joslin Diabetes Center, Boston). The Rlk antiserum and cDNA was a kind gift from Dr. Pamela Schwartzberg (NIH, Bethesda).

Transient transfection. Human CTLA-4 was obtained by PCR using pCDM8-CTLA-4 (kindly provided by Dr. G. Freeman, Dana-Farber Cancer Institute, Boston). Amino acid residues at positions Y201 and Y218 were subjected to site-directed mutagenesis based on the protocol provided by Promega (Madison, WI). The mutants Y201F and Y218F plus the wild-type CTLA-4 were co-transfected with either Rlk inactive kinase (lysine at position 299 was mutated to arginine), Rlk, ZAP-70 or p85 into COS cells mediated by DEAE-dextran as described (33). The cDNAs were cloned into the pSRα2 expression vector (gift of M. Streuli, Dana-Farber Cancer Institute, Boston).

Immunoprecipitation and immunoblotting. For immunoprecipitations, COS cells were lysed in ice cold lysis buffer containing 1% NP-40 (v/v) in 20 mM Tris-HCl pH 8.3, 150 mM NaCl. The lysis buffer contained 1mM PMSF, 1mM Na_4VO_3, 10mM NaF and 1mM $Na_4P_2O_7$. Lysates were incubated for 20min on ice before centrifugation at 15000 x g for 15 min at 4°C. Aliquots of 1 ml of clear postnuclear lysates were incubated for 1 hour with agitation at 4°C with the indicated monoclonal antibody. Protein A-Sepharose beads (40μl, Pharmacia), swollen and washed in lysis buffer were added and incubated for 1 hour at 4°C. The beads were washed three times in cold lysis buffer and proteins were eluted by boiling for 5 min in SDS sample buffer, separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. The membranes were blocked with 5% milk in TBS (10mM Tris-HCl pH 7.6, 150mM NaCl) and incubated with the indicated antibodies. Bound antibody was revealed with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse or donkey anti-rabbit antibodies using enhanced chemiluminescence (ECL, Amersham).

RESULTS

Previous studies by ourselves and others have shown that CTLA-4 is capable of binding to the SH2 domains of the lipid kinase PI 3-kinase (24, 27). However, little is known regarding the identity of the protein-tyrosine kinase(s) responsible for CTLA-4 phosphorylation, and specifically at the YVKM motif. Likewise, to date no substrates of the hematopoietic tyrosine kinase Rlk have been identified (28-31). To test whether Rlk is capable of phosphorylating CTLA-4, the kinase was co-expressed in COS cells and examined for an ability to phosphorylate the receptor *in vivo* as detected by anti-phosphotyrosine immunoblotting (Fig. 1B). Under these conditions, CTLA-4 was readily phosphorylated by the kinase (Fig. 1B, upper panel, lane 2; also see histogram). As a negative control, an inactive form of the kinase (dRlk) failed to cause phosphorylation (lane 1), despite being expressed at similar levels in cells (lower panel). As an additional negative control, we also examined another lymphoid kinase ZAP-70 for its ability to phosphorylate the receptor (Fig. 2). In this case, no phosphorylation of CTLA-4 was evident (Fig. 2A, upper panel, lane 2) despite significant levels of protein expression (Fig. 2A, lower panel). As a control for active ZAP-70 kinase, the same kinase was co-expressed with SLP-76, a documented substrate of the kinase (34). Under these conditions, ZAP-70 was found to readily phosphorylate SLP-76 (Fig. 2B, lane 2). Together, these data indicate that Rlk can phosphorylate CTLA-4 in a specific manner, an effect not mediated by other kinases such as ZAP-70.

In order to map the site of phosphorylation by Rlk in CTLA-4, we mutated residues in the cytoplasmic tail and assessed changes in phosphorylation. Two tyrosine residues at Y201 and Y218 are located within the motifs YVKM and YFIP, respectively (Fig. 1A). The YVKM motif has been reported to bind to the SH2 domains of PI 3-kinase (24, 25, 27). Under these conditions, mutation of the tyrosine at residue Y201 caused an 80 percent reduction in phosphorylation by Rlk (Fig. 1, lane 3; also see histogram). By contrast, mutation of Y218 had little if any effect on phosphorylation (lane 4). As expected, mutation of both tyrosines also prevented phosphorylation (lane 5). Similar levels of expression of the various mutants were obtained as measured by [^{35}S] methionine labeling

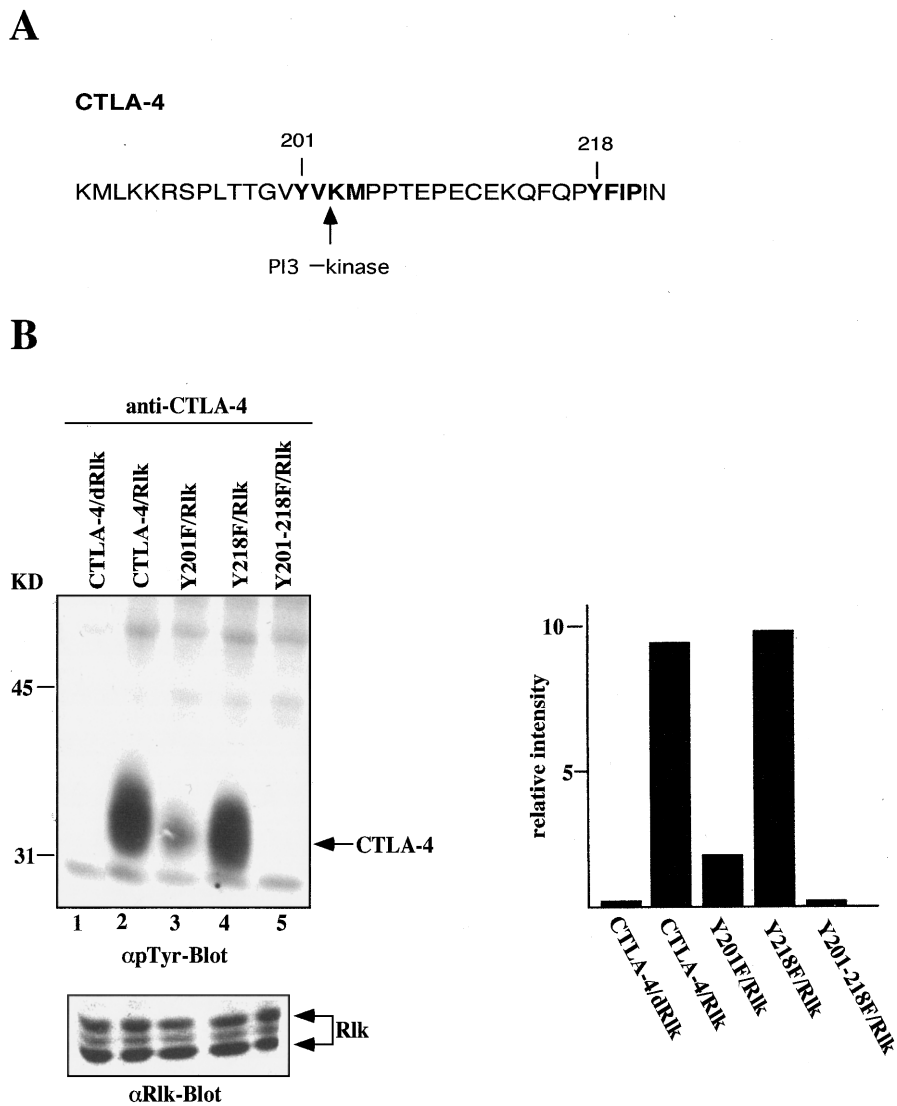

FIG. 1. In vivo phosphorylation of CTLA-4 by Rlk. (A) CTLA-4 possesses two tyrosine residues at Y201 and Y218. Y201 resides within the pYVKM motif, a potential site of PI 3-kinase binding. (B) (upper panel) COS cells co-transfected with dRlk or Rlk and wild-type CTLA-4 (lanes 1 and 2), the mutants Y201F (lane 3), Y218F (lane 4) and Y209-218F (lane 5) were lysed, immunoprecipitated with anti-CTLA-4 mAb and immunoblotted with anti-Tyr (P) mAb. (lower panel) Equivalent levels of Rlk were expressed as demonstrated by immunoblotting of the cell lysate with anti-Rlk antiserum.

(data not shown). The expression level of Rlk was comparable in each transfectant (lower panel). These data further demonstrate the relative specificity of phosphorylation of the cytoplasmic pYVKM motif by Rlk.

The basis for the ability of mutations at the YVKM and YFIP motifs to disrupt phosphorylation, when the loss of the single YFIP motif had no effect is unclear, but could indicate cooperativity between tyrosine motifs, as described for CD28 (35).

We were next interested whether Rlk could associate with CTLA-4. Cells transfected either with CTLA-4 alone (Fig. 3A, lane 1), or with a combination of CTLA-4 and Rlk (lane 2) were analyzed for binding. Transfectants were lysed, immunoprecipitated with CTLA-4 mAb and immunoblotted with an anti-Rlk antiserum. As shown in Figure 3A (upper panel), CTLA-4 did not co-precipitate the kinase (lane 2), although transfected Rlk could be detected by immunoblotting (lower panel, lane 2). Lysates from COS cells transfected with Rlk served as a positive control (lane 3).

Since CTLA-4 can associate with the lipid kinase PI 3-kinase in T-cells (24), we were next interested in whether Rlk phosphorylation of CTLA-4 leads to productive binding between receptor and PI 3-kinase. Indeed, for Rlk phosphorylation to be of functional relevance, we would expect that it would create conditions for PI 3-kinase binding. COS cells transfected with a combination of CTLA-4 and Rlk inactive kinase (dRlk)

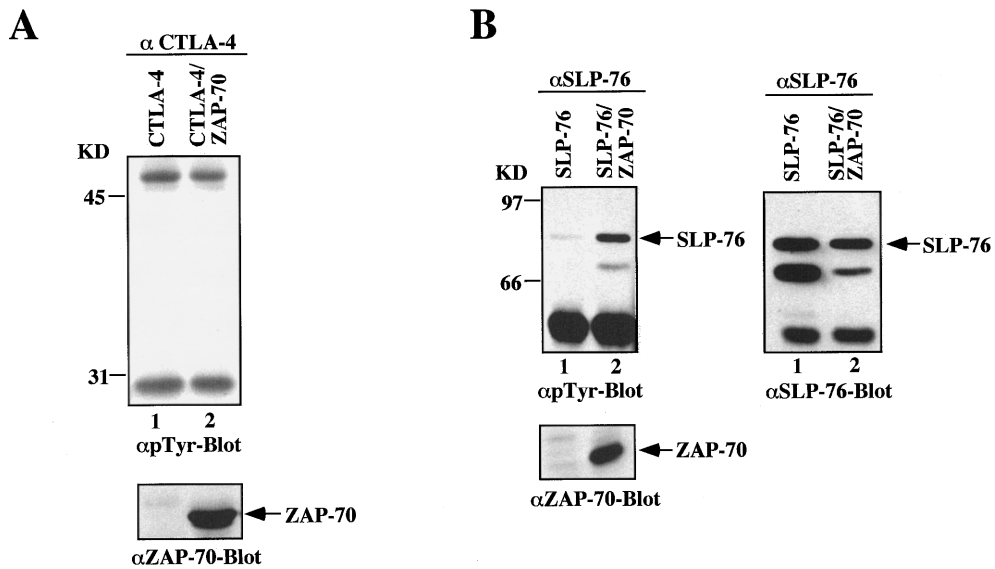

FIG. 2. ZAP-70 failed to phosphorylate CTLA-4. (A) COS cells transfected with CTLA-4 alone (lane 1) or together with the kinase ZAP-70 (lane 2) served as a control for the specificity of Rlk in CTLA-4 phosphorylation. (B) COS cells transfected with SLP-76 alone (lane 1) or together with ZAP-70 (lane 2) were lysed, immunoprecipitated with anti-SLP-76 mAb and immunoblotted with anti-Tyr(P) mAb (left panel) or anti-SLP-76 mAb (right panel).

or Rlk were lysed, mixed with lysates expressing the p85 subunit of PI3-kinase and assessed for binding. As shown in Figure 3B, co-transfection of Rlk and CTLA-4 led to phosphorylation of CTLA-4 (upper panel, lane 2) and recruitment of p85 (lane 2, lower panel). As expected, no p85 binding could be detected to non-phosphorylated CTLA-4 (lane 1). The position of p85 is shown in an anti-p85 immunoprecipitate (lower panel, lane 3). These data confirm that Rlk phosphorylation of CTLA-4 leads to the association of PI-3 kinase with the CTLA-4 receptor.

DISCUSSION

In this study, we demonstrate that T-cell specific kinase Rlk is capable of phosphorylating CTLA-4 at the YVKM motif, and mediating the binding of the receptor to PI 3-kinase. CTLA-4 and Rlk are both restricted in expression to T-cells. There is little information about the identity of the protein-tyrosine kinase(s) responsible for CTLA-4 phosphorylation, and the target substrates of Rlk are not identified. Previous studies have shown that CTLA-4 undergoes tyrosine phosphorylation in a manner that allows for PI 3-kinase and SHP-2 phosphatase binding (24-26). However, the nature of the kinase responsible for this event has been elusive. Specificity in phosphorylation was demonstrated by the fact that mutation of Y201 reduced phosphorylation over 80 percent, and further, the Rlk inactive kinase as well as ZAP-70 kinase failed to phosphorylate the receptor (Figs. 1,2). Interestingly, the specificity of Rlk for this motif contrasts with a recent report that Lck can phosphorylate the second YFIP site (27).

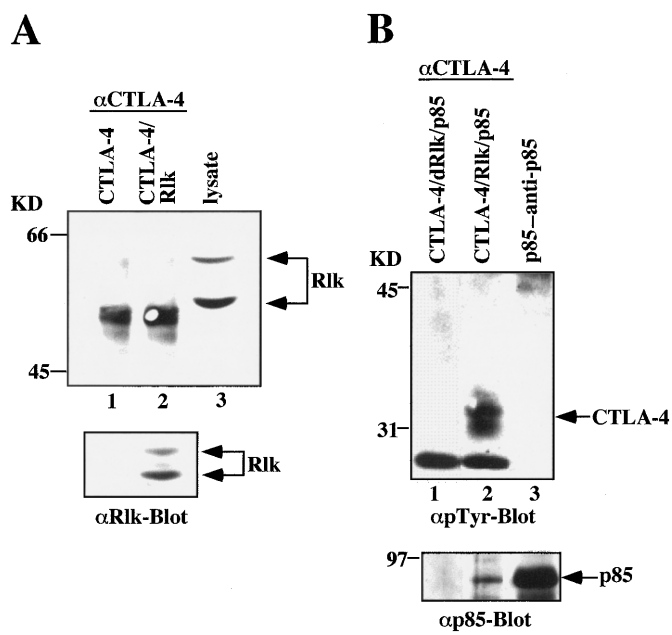

FIG. 3. (A) Rlk does not associate with CTLA-4. (Upper panel) COS cells transfected with wild-type CTLA-4 alone (lane 1) or together with Rlk (lane 2) were lysed, immunoprecipitated with CTLA-4 mAb (lanes 1 and 2) and immunoblotted with anti-Rlk antisera. Lysate from COS cells transfected with Rlk served as a positive control (lane 3). (Lower panel) Equal amounts of lysates from COS cells transfected with CTLA-4 alone (lane 1) or together with Rlk (lane 2) were subjected to immunoblotting with anti-Rlk antisera. (B) Phosphorylation of CTLA-4 by Rlk allows PI3-kinase binding. COS cells co-transfected with CTLA-4 and dRlk (lane 1) or Rlk (lane 2) were lysed, mixed with lysates expressing the p85 subunit of PI 3-kinase, immunoprecipitated with CTLA-4 mAb (lanes 1, 2) and subjected to immunoblotting with anti-Tyr (P) mAb (upper panel) or anti-p85 antiserum (lower panel). Lane 3 shows an anti-p85 immunoprecipitate.

In the case of Rlk phosphorylation, data on the question of whether the second YFIP motif could become phosphorylated at low levels is somewhat unclear. On one hand, mutation of the tyrosine of the second site failed to show a consistent effect in reducing phosphorylation. However, the Y201 mutant showed a low level of residual phosphorylation (about 20 percent of wild-type control) in several experiments. In either case, the results point to the predominant role of the YVKM motif as a target for Rlk. It is therefore possible that the Rlk and Lck kinases might target different sites in the CTLA-4 cytoplasmic tail.

Rlk is a member of the Bruton's tyrosine kinase (Btk) family of kinases, and expressed at high levels in T-cells (29-31). However, neither a function nor a target of the kinase has been elucidated. By contrast, Btk has been linked to the immunodeficiency disorder human X-linked agammaglobulinemia (XLA) and murine X-linked immunodeficiency (xid) (36-39). Btk activity is activated by phosphorylation via src kinases such as Fyn (40, 41). However, Btk and Rlk differ in the absence of a PH domain in Rlk suggesting that they may localise to different regions of the cell. In this context, Rlk has been reported to concentrate in the intracellular compartments of T-cells (42), as has been observed for CTLA-4 (43, 44). CTLA-4 is the first reported substrate for Rlk. Preliminary data have found that Rlk is also capable of phosphorylating CD28 in an overexpression system (data not shown). However, an involvement for members of the Btk/Tec kinases in costimulatory function is not unprecedented. CD28-mediated costimulation has been reported to be increased in Itk−/− mice (45). Further studies will be required to determine the relevance for CTLA-4 phosphorylation by Rlk in CTLA-4 mediated negative signaling and intracellular protein trafficking.

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