In Vivo Significance of ITK–SLP-76 Interaction in Cytokine Production

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In vitro data have suggested that activation of the inducible T-cell kinase (ITK) requires an interaction with the adaptor protein SLP-76. One means for this interaction involves binding of the ITK SH3 domain to the polyproline-rich (PR) region of SLP-76. However, the biological significance of this association in live cells and the consequences of its disruption have not been demonstrated. Here, we utilized a polyarginine-rich, cell-permeable peptide that represents the portion of the SLP-76 PR region that interacts with the ITK SH3 domain as a competitive inhibitor to disrupt the association between ITK and SLP-76 in live cells. We demonstrate that treatment of cells with this peptide, by either in vitro incubation or intraperitoneal injection of the peptide in mice, inhibits the T-cell receptor (TCR)-induced association between ITK and SLP-76, recruitment and transphosphorylation of ITK, actin polarization at the T-cell contact site, and expression of Th2 cytokines. The inhibition is specific, as indicated by lack of effects by the polyarginine vehicle alone or a scrambled sequence of the cargo peptide. In view of the role of ITK as a regulator of Th2 cytokine expression, the data underscore the significance of ITK as a target for pharmacological intervention.

The Tec family of tyrosine kinases plays a critical role in lymphocyte development and activation through antigen receptors (4, 40). The inducible T-cell kinase (ITK), a member of the Tec family, regulates selection during thymocyte development and controls the generation of effective Th2 responses (15, 40). Phosphorylation of ITK on Tyr 511 by the Src family kinase LCK occurs early upon the engagement of the T-cell antigen receptor (TCR) and is critical for the enzymatic activation of ITK (18, 44). Upon its activation, ITK phosphorylates phospholipase C-γ1 (PLC-γ1) on tyrosines 775 and 783, an event critical for phospholipase activity (3, 5) and ensuing intracellular and capacitative Ca2+ mobilization (26). In this fashion, ITK regulates downstream signaling events that regulate biological responses, such as cytokine production (4, 40).

ITK is organized in modular domains that play critical roles in its activation (47). Upon T-cell engagement, ITK colocalizes with the TCR, a process dependent on the pleckstrin homology (PH) domain of ITK and its interaction with PIP3 at the plasma membrane (11, 19). Activation of ITK also requires interaction with adaptor proteins, such as SLP-76 and LAT (8, 10). The SH2 domain of ITK appears to be critical for its interaction with LAT, whereas both the SH2 and SH3 domains are required for interaction with SLP-76 (8, 10). In vitro studies have demonstrated that the SH3 domain of ITK interacts with the proline-rich (PR) region of SLP-76, and it has been speculated that this interaction is critical for the activation of ITK (6, 8). However, the biological significance of the interaction has not been demonstrated in live cells. In the present investigation, we used a cell-permeable peptide as a competitive inhibitor of the interaction between ITK and SLP-76. To this end, we synthesized a 12-amino-acid peptide, which represents the PR region of SLP-76 that binds to the ITK-SH3 domain, and rendered it cell permeable by the addition of nine arginines at its N-proximal end. Here, we show that this cell-permeable peptide, henceforth called R9-QQP, is readily taken up by both Jurkat T cells and murine splenocytes and disrupts events that are mediated by the engagement of the TCR. Thus, association of ITK and SLP-76, recruitment of ITK and actin polarization at the T-cell contact site, LCK-mediated transphosphorylation of ITK on tyrosine 511, and production of Th2 cytokines are inhibited by R9-QQP in a dose-dependent and peptide-specific manner. The data presented here are novel and significant because they provide the first demonstration of the biological relevance of the specific interaction between the ITK-SH3 domain and the SLP-76 PR region in live cells. Furthermore, the data underscore the potential of cell-permeable peptides as useful probes for dissecting signal transduction pathways in live cells, and in view of the regulatory role that ITK plays in Th2 cytokine production, they have implications for the pharmacological manipulation of ITK in disease situations.

MATERIALS AND METHODS

Cell lines, mice, antibodies, and other reagents. Wild-type Jurkat T cells (E6.1) were obtained from the American Type Culture Collection (ATCC). The SLP-76-deficient mutant, J14, was a kind gift from Art Weiss (University of California—San Francisco). The cells were cultured as previously described (10). Male C57BL/6 mice were purchased from Harlan Sprague Dawley or Jackson Laboratories and were used between the ages of 6 and 12 weeks. All experimental protocols using animals were approved by the IACUC of San Diego State University.

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Anti-human CD3ε monoclonal antibody OKT3 was prepared in house from a hybridoma (CLB-001) obtained from the ATCC. Isotype control antibody UPC-10 (catalog number M5409) and recombinant protein G-Sepharose (catalog number 10124) were obtained from Sigma. Anti-CD3ε (2C11; catalog number 553058), anti-CD28 (catalog number 553295), Alexa Fluor 647-conjugated anti-CD4 (catalog number 100530), phycoerythrin (PE)-conjugated anti-ITK (p531; catalog number 558129), and isotype control antibodies were obtained from BD Biosciences. Anti-ITK, anti-p531 (catalog number 1685-1) was obtained from Epitomics Inc. Goat anti-Armenian-hamster antibody (catalog number 127-005-160), rabbit anti-murine IgG (catalog number 315-005-044), and normal rabbit IgG were purchased from Jackson ImmunoResearch. Anti-ITK antibody for immunoprecipitation (catalog number 06-546), anti-ITK for immunoblotting (catalog number 05-476), anti-GADS (catalog number 06-983), and anti-pphos-tyrosine (protein (catalog number 05-321, clone 4G10) antibodies were purchased from Millipore; anti-FHPC amide resin (4-(2,4-dimethoxyphenyl)-aminoethyl)-phenoxy using standard Fmoc protocols (29). FITC was linked to RRQQPPVPPQRPMA (R9-QQP), RRRRRRRRRPQMPAPQRPQPV (R9-SCR) according to the manufacturer’s instructions.

Phorbol myristate acetate (PMA) (catalog number 524400) and ionomycin (catalog number 2101), and anti-phospho (pY493)-ZAP-70 (catalog number 2704S) antibody were purchased from Biosciences; anti-gamma interferon (anti-IFN-γ) (catalog number 505813), fluorescein isothiocyanate (FITC)-conjugated anti-IL-2 (catalog number 554427), and isotype control antibodies were obtained from BioLegend. PE-conjugated anti-IL-13 (catalog number 12-7133-81) and isotype control antibodies were from eBioscience.

Enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Bioscience (IL-4, catalog number 555232, and IFN-γ, catalog number 555138) and eBioscience (IL-13, catalog number 88-7137-88). The limits of detection of the assays were 8 pg/ml for IL-4, 4 pg/ml for IL-13, and 32 pg/ml for IFN-γ. Phospho-specific antibodies (PA30; catalog number Phs400) and isocyanin (catalog number 407952) were from EMD Chemicals, Inc. Merek.

7-Amino-actinomycin D (7-AAD) was obtained from BioLegend and used according to the manufacturer’s instructions.

Cell-permeable synthetic peptides and treatment. The peptides RRRRRRRRRR ROQQPPVPPQRPMA (R9-OPQ), RRRRRRRRRPOMPQVP (R9-SCR), and RRRRRRRRRR (R9) were synthesized on an Applied Biosystems 433A peptide synthesizer. Anti-FHPC amide resin (4-(2,4-dimethoxyphenyl)-aminoethyl)-phenoxy using standard Fmoc protocols (29). FITC was linked to the N terminus of the peptide through an aminocaproic spacer. Peptides were purified on a C18 preparative reverse-phase–high-performance liquid chromatography (HPLC) column to 95% purity. The mass was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry on a Micro-Tag (HPLC) column to 95% purity. The mass was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry on a Micro-Tag (HPLC) column to 95% purity. The mass was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

For phospho-flow cytometry, Jurkat cells or splenocytes (1 × 10^6 in 100 μl) were stimulated as described above and then processed for flow cytometry analysis as described below. For intracellular cytokine production, the cells were fixed and permeabilized with the appropriate reagents and stained as described above. The cells were then analyzed with a Leica TCS SP2 laser confocal microscope. In some experiments, cells were also analyzed with a Leica TCS SP2 laser confocal microscope. In some experiments, cells were also analyzed with a Leica TCS SP2 laser confocal microscope. In some experiments, cells were also analyzed with a Leica TCS SP2 laser confocal microscope. In some experiments, cells were also analyzed with a Leica TCS SP2 laser confocal microscope.

Cellular localization of ITK and SLP-76. Molecular localization within cells was measured as previously described for ITK (19). Briefly, Jurkat cells were transfected with green fluorescent protein (GFP)-ITK or GFP-SLP-76 by Lipofectamine (Amaxa), following the manufacturer’s protocol. The cells were then fixed with anti-CD3ε- or isotype control-coated polystyrene beads (6-μm diameter; Polysciences Inc.), pelleted (100,000 g for 5 min) and incubated with protein G-conjugated Sepharose beads (1:25 [vol/vol]). Preceding slides were incubated overnight with anti-SLP-76 antibody (5 μg/ml), followed by incubation with protein G-Sepharose beads (as described above) for 1 h. Immunoglobulins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gels) and Western blotted to nitrocellulose. Bands were detected with anti-ITK, anti-GADS, anti-HPK-1, anti-LCK antibodies, followed by stripping (2% SDS, 62 mM Tris, pH 6.7) and reprobing with anti-SLP-76 antibodies. All antibodies were used at 1 μg/ml.

In coinmunoprecipitation experiments, the percent association of various signaling proteins with SLP-76 was determined by densitometric analysis of the immunoblotted bands using NIH Image J software. Under stimulation conditions and in the absence of peptide, the pixel intensities of the various associating molecular bands were divided by the pixel intensity of the SLP-76 band (loading control) and defined as 100% association. Similarly obtained ratios in the presence of peptide were normalized by dividing them by the above ratio and presenting them as percentages of association with SLP-76.

For assessing ITK phosphorylation by immunoblotting, cells were stimulated as described above and preexposed lysates were immunoprecipitated with anti-ITK antibody, resolved by SDS-PAGE, and sequentially blotted with phospho-specific, anti-ITK Tyr 511, and anti-ITK antibodies. The immunoblots were analyzed by densitometry using NIH Image J software to calculate the percent ITK phosphorylation. Under stimulation conditions and in the absence of peptide, the pixel intensity of the phosphorylated ITK band was divided by the pixel intensity of the total ITK band (loading control) and defined as 100% phosphorylation. Similarly obtained ratios in the presence of peptide were normalized by dividing them by the above ratio and presenting them as percentages of ITK phosphorylation

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phospho-flow cytometry, the cells were fixed with 2% paraformaldehyde following stimulation and then permeabilized with 100% methanol, blocked with species-specific normal serum (30 μg/ml in phosphate-buffered saline [PBS]), stained with specific anti-phospho-antibody (anti-ITK pY511 or anti-LCK pY394; 1/5 dilution of commercial stock antibody in PBS), and analyzed with a FACSAria as described above.

Intracellular cytokine staining and flow cytometric analysis of splenocytes were performed in a similar fashion, with the addition of blocking with species-specific normal serum (30 μg/ml in PBS) in a 0.1% saponin-containing buffer (PBS-0.5% BSA) before staining with specific anti-cytokine or isotype control antibodies (5 μg/ml). In all experiments with splenocytes, the cells were first stained with Alexa Fluor 647-conjugated anti-murine CD4 antibody (5 μg/ml) before fixation and intracellular staining. The data were analyzed using either FACSDiva (BD Biosciences) or FlowJo (Tree Star, Inc.) software.

Percent phosphorylation or cytokine production, as determined by flow cytometric analysis, in the absence of peptide was calculated by dividing the number of gated positive events under stimulation conditions by those under nonstimulation conditions and defining this as 100% phosphorylation/cytokine production. In the samples treated with peptide, the values were calculated similarly and were normalized by dividing them by the above ratio and presenting them as percent phosphorylation/cytokine production.

The supernatants from cultures established as described above, without brefeldin A, were assessed for the content of cytokines using commercial ELISA kits, and the cytokine content was quantified based on standard curves following the manufacturer’s directions.

Data analysis. Statistical analysis and P value determinations were performed by Student’s t test.

RESULTS

Internalization of R9-QQP peptide. We synthesized a poly-arginine-rich, cell-permeable peptide consisting of 9 arginine residues followed by 12 residues representing amino acids 184 to 195 of the SLP-76 PR region that interact with the SH3 domain of ITK (8). We have named this peptide R9-QQP (corresponding to the arginine tag and the first 3 amino acids of the peptide). To assess the ability of R9-QQP to enter cells, we incubated Jurkat cells and murine splenocytes with a fluorescein conjugate of the peptide and analyzed the cells for the presence of fluorescence by flow cytometry. R9-QQP was taken up by both cell types in a dose-dependent fashion with peptide internalized by most cells when incubated with concentrations of 5 μM and above (Fig. 1A and B, top and middle). Internalization of the peptide was further confirmed by analysis of confocal midsection images of Jurkat cells and splenocytes. Under our experimental conditions, the peptide is internalized in a punctate pattern of fluorescence displayed by the majority (~90%) of the cells (Fig. 1A and B, bottom). To eliminate the possible extracellular association of the peptide with the cell surface, we repeated the experiment by treating cells with trypsin under conditions where the presence of cell surface proteins (e.g., CD71) was significantly reduced, as assessed by flow cytometry (data not shown). Even though incubation with trypsin reduced the brightness (mean fluorescence intensity [MFI]) of the FITC–R9-QQP-treated cells, it did not alter the percentage of fluorescence-positive cells (Fig. 1C). Thus, there exists an intracellular, trypsin-resistant peptide pool that is visualized by analysis of midsection confocal images (Fig. 1C, bottom). The cells in these experiments were pretreated with Cy5 succinimidyl ester to accentuate the membrane outline. The peptide was internalized rapidly, as indicated by the fact that about 80% of the cells displayed peptide-associated fluorescence within 5 min of incubation (Fig. 1D).

The cells continued internalizing peptide, with maximal uptake at 30 min of incubation at 37°C (Fig. 1D). The peptide was not toxic to either resting or TCR-activated splenic T cells, as the percentage of viable cells incubated with peptide for up to 30 h was not significantly different from that of non-peptide-treated cells (Fig. 1E).

R9-QQP specifically disrupts the association between ITK and SLP-76. Upon TCR engagement, ITK associates with the adaptor protein SLP-76 (5, 43). We assessed whether the presence of R9-QQP has any effects on this association. Jurkat T cells incubated with R9-QQP or control peptides were stimulated with antibodies to the TCR complex (anti-CD3ε), and the association between ITK and SLP-76 was assessed by coimmunoprecipitation. The data in Fig. 2A (top row) demonstrate that R9-QQP inhibits the association between ITK and SLP-76. Quantification of the gel bands from three replicate experiments indicated that 50% inhibition of ITK and SLP-76 association was affected by approximately 1 μM peptide treatment (Fig. 2B). Even though treatment with 0.5 μM R9-QQP did not result in a statistically significant difference from untreated controls (P > 0.05) (Fig. 2B), inhibition was dose dependent, as treatment with higher doses of peptide resulted in significant inhibition of the inducible ITK–SLP-76 interaction (P < 0.05) (Fig. 2B). Importantly, two control peptides, R9-SCR, a QQP scrambled sequence with intact R9 sequence (see Materials and Methods), and R9 (nine arginines in tandem alone), did not have any significant effect on the association between ITK and SLP-76 at the highest concentration tested (Fig. 2A and B). To further establish the specificity of the inhibitory effect of R9-QQP, we assessed its effects on the association of SLP-76 with other signaling partners whose interaction with the adaptor is either inducible (LCK and HPK-1 [37, 38]) or constitutive (GADS [8, 28]). The peptide displayed no significant effect on any of these interactions (Fig. 2A and B). The slight inhibitory effect seen with LCK (Fig. 2B) had marginal statistical significance (P = 0.08 compared to no-peptide control), and it was not reproducibly observed (see Fig. 7A and B). Collectively, the above data indicate that R9-QQP specifically disrupts the ITK–SLP-76 association.

R9-QQP specifically inhibits the TCR-induced recruitment of ITK. TCR engagement induces the redistribution of ITK from a diffuse cytoplasmic pattern to that of a distinct aggregation at the T-cell contact site and association with the TCR complex (11, 19). The role of SLP-76 in this process has not been determined. To assess this, a previously published technique (19) was employed in which polystyrene beads coated with either stimulatory anti-CD3ε or control antibodies were incubated with Jurkat T cells that had been transfected with GFP-ITK, and the distribution of ITK was assessed by confocal microscopy. Compared to control-coated beads, wild-type Jurkat T cells (E6.1) incubated with anti-CD3ε-coated beads displayed a significant increase in ITK accumulation at the contact site (Fig. 3D) (P < 0.001). In contrast, Jurkat cells deficient in SLP-76 (J14) did not display such an increase (Fig. 3D) (P = 0.18). To determine whether R9-QQP had any effect on ITK localization, the experiment was repeated with Jurkat cells (E6.1) that had been pretreated with either 10 or 20 μM peptide. The presence of peptide mediated a significant dose-dependent inhibition of ITK localization (Fig. 3E) (significant levels between stimulated cells without peptide and those with 10 and 20 μM peptide were P = 0.007 and P < 0.001, respectively). This inhibition was specific, because R9-
SCR did not cause a significant decrease in ITK localization (Fig. 3E) \( (P = 0.20) \). ITK localization in nonstimulated cells was not affected by the presence of peptide (Fig. 3E). Examples of confocal images from which the data were derived are shown in Fig. 3A to C. To further establish the specificity of the R9-QQP-mediated inhibition, we tested its effects on the TCR-induced localization of SLP-76. It was observed that the localization of GFP–SLP-76 transfected into J14 (SLP-76-deficient) Jurkat cells was not affected by the presence of the peptide (Fig. 3F). Also, the presence of peptide did not alter the localization of SLP-76 in nonstimulated cells (Fig. 3F). Thus, the inhibitory effect of R9-QQP is specific for the TCR-induced localization of ITK, presumably reflecting the inhibition of the ITK–SLP-76 interaction.

R9-QQP specifically disrupts TCR-induced actin polarization. ITK and SLP-76 are part of a multimolecular complex that regulates the TCR-induced reorganization of the actin cytoskeleton at the T-cell–APC contact site \((7, 24)\). The importance of the association between ITK and SLP-76 in this process has not been previously addressed. To assess it, Jurkat cells that had been treated with R9-QQP, R9-SCR, or medium were incubated with polystyrene beads that were coated with anti-CD3ε antibody (stimulatory) or BSA (nonstimulatory). Actin polarization was assessed by staining with Texas Red-phalloidin and visualized by confocal microscopy as described in Materials and Methods. The data shown in panels A to E are from single experiments.
Treatment of cells with R9-QQP caused a dose-dependent inhibition of actin polarization (Fig. 4C, confocal image, and D). Even though treatment with 10 μM R9-QQP caused marginally significant inhibition (P = 0.09), the effect was dose dependent, as treatment with 20 μM R9-QQP caused statistically significant (P = 0.0012) inhibition of actin polarization (Fig. 4D). Furthermore, the effect was peptide specific, as treatment with the R9-SCR peptide had no significant effect (Fig. 4D).

**R9-QQP specifically inhibits the TCR-mediated phosphorylation of ITK on Tyr 511.** The TCR-mediated activation of ITK

![Graph showing the relationship between peptide concentration and actin polarization inhibition](image1)

![Graph showing the association between ITK and SLP-76](image2)

**FIG. 2.** R9-QQP specifically disrupts the association between ITK and SLP-76. (A) Jurkat cells treated with various peptide concentrations, as indicated, were stimulated with anti-CD3ε (+) or isotype control (−) antibodies, the cells were lysed, the lysates were pre-cleared with rabbit IgG and immunoprecipitated (IP) with anti-SLP-76 antibody, and immune complexes were resolved by SDS-PAGE. The membranes were sequentially immunoblotted (WB) with anti-ITK, anti-Lck, anti-HPK-1, anti-GADS, and anti-SLP-76 antibodies. (B) Graphic representation of SLP-76 association with the indicated signaling partners displayed as percent association with SLP-76. The results are the averages (± standard errors of the mean [SEM]) of three replicate experiments performed as in panel A. The percent association with SLP-76 was calculated by densitometric analysis of immunoblotted bands as detailed in Materials and Methods.

**FIG. 3.** R9-QQP specifically inhibits the TCR-induced recruitment of ITK, but not SLP-76. Shown are representative confocal images of conjugates between GFP-ITK-nucleofected Jurkat (E6.1) cells (treated or not with 20 μM R9-QQP) and control (nonstimulated) (A) or anti-CD3ε antibody-coated (stimulated) (B and C) beads. (Left) Confocal fluorescence images. The asterisks indicate the positions of the beads. (Right) Differential interference contrast images. (D) Wild-type (E6.1) or SLP-76-deficient (J14) Jurkat cells that had been transfected with GFP-ITK by nucleofection were incubated with control (− stimulation) or anti-CD3ε (+ stimulation) antibody-coated beads, and the ITK localization index was determined as described in Materials and Methods. The results are displayed as the average ITK localization index (±SEM) of 20 to 40 conjugates from two replicate experiments. (E) Wild-type Jurkat cells (E6.1) transfected with GFP-ITK as described above were pretreated with R9-QQP or R9-SCR peptide and incubated with either control (− stimulation) or anti-CD3ε antibody-coated (+ stimulation) beads, as indicated. The ITK localization index was determined as described in Materials and Methods and is expressed as the average (±SEM) of 25 to 46 conjugates from two replicate experiments. (F) SLP-76-deficient Jurkat cells (J14) transfected with GFP-SLP-76 were treated as for panel E, and the SLP-76 localization index was determined as described above. The results are the average SLP-76 localization index (±SEM) of 17 to 32 conjugates from two replicate experiments.
depends on the transphosphorylation of its Tyr 511 by LCK (18, 44). We wanted to determine whether treatment with R9-QQP is able to disrupt this event. Jurkat cells were treated with various concentrations of peptide and stimulated with anti-CD3ε or control antibodies, and phosphorylation of ITK Tyr 511 was assessed by flow cytometry using anti-phosphotyrosine 511-specific antibodies. Treatment with R9-QQP inhibited the anti-CD3ε-induced phosphorylation of tyrosine 511 (Fig. 5A and B). In four replicate experiments, the average amount of peptide causing 50% inhibition of Tyr 511 phosphorylation was approximately 0.8 μM (Fig. 5B). The percentages of phosphotyrosine 511 values in the 1 to 27 μM R9-QQP-treated groups are statistically significant versus the no-peptide control values at a P value of <0.05. The inhibition was specific, as treatment with control R9-SCR or R9 peptide did not cause significant inhibition at the highest concentrations tested (Fig. 5A and B). In these experiments, there was an average of about 30% inhibition with R9-SCR that probably reflects nonspecific effects, since equivalent amounts of R9-QQP caused 95% inhibition (Fig. 5B) (P < 0.001 compared to no-peptide control). To further establish the significance of the R9-QQP inhibitory effects, we repeated this experiment using primary murine splenic lymphoid cells. The results (Fig. 5C and D) indicate that, similar to Jurkat cells, R9-QQP inhibited the TCR-induced Tyr 511 phosphorylation of ITK in a dose-dependent manner (50% inhibition at approximately 0.85 μM peptide treatment). The percentage of phosphotyrosine 511 values of all R9-QQP-treated groups was statistically significant versus the no-peptide control (P < 0.05). In these experiments, pY511 phosphorylation was assessed by gating on CD4+ cells. The effects of R9-QQP were specific, as indicated by lack of significant effects by the two control peptides, R9-SCR and R9 (Fig. 5C and D). To further confirm the effects of R9-QQP on ITK phosphorylation in mouse splenocytes, we repeated the experiment by measuring ITK Tyr 511 phosphorylation by immunoblotting. The results of these experiments reproduced those obtained by phospho-flow cytometry, again demonstrating a specific reduction of ITK phosphorylation by R9-QQP (Fig. 5E and F). The inhibition in the presence of peptide compared to no-peptide control was significant (P < 0.05) for all peptide concentrations tested. The specificity of inhibition of ITK phosphorylation was further confirmed by the fact that R9-QQP had no detectable inhibitory effects on global tyrosine phosphorylation (Fig. 5G).

R9-QQP causes specific inhibition of Th2 cytokines induced by the engagement of the TCR. ITK regulates the expression of Th2 cytokines (1, 15). Therefore, we wanted to test the effects of R9-QQP on cytokine expression. Murine splenocytes treated with R9-QQP or control peptides were stimulated with anti-CD3ε plus anti-CD28 antibodies, and the intracellular expression of IL-4, IL-13, IL-2, and IFN-γ was assessed by intracytoplasmic cytokine staining and flow cytometric analysis of gated CD4+ cells. Treatment with R9-QQP inhibited the expression of IL-4 (Fig. 6A and B) with 50% inhibition effected at about 5 μM peptide (Fig. 6B). The expression of IL-13 was similarly inhibited (Fig. 6A and B). Inhibition in all peptide-treated groups was significant (P < 0.05) and displayed specificity, as the control peptides did not cause any significant inhibition (Fig. 6B). Treatment with R9-QQP had a marginal effect (P = 0.014) on IL-2 expression (Fig. 6A and C). Interestingly, R9-QQP had no significant inhibitory effect (P > 0.05) on the expression of the signature Th1 cytokine, IFN-γ (Fig. 6A and D). Also, intracellular expression of cytokines induced by PMA and ionomycin was not affected by R9-QQP (data not shown).

To examine whether the inhibitory effect of R9-QQP on cytokines is also reflected in their secretion and to further confirm the flow cytometric data, we tested the supernatants of parallel cultures (produced in the absence of brefeldin A) for IL-4, IL-13, and IFN-γ using commercial cytokine assay kits. Similar to the effects described above, R9-QQP selectively inhibits secretion of both IL-4 and IL-13 but has no effect on the secretion of IFN-γ (Fig. 6E to G). Inhibition is specific, as the two control peptides R9 and R9-SCR had no significant effects (Fig. 6E and F). R9-QQP had no effect on cytokine secretion induced by PMA and ionomycin, a mode of stimulation that bypasses TCR-proximal events (Fig. 6E and F).

In vivo delivery of R9-QQP affects the TCR-induced activation of ITK. In the next series of studies, we wished to deter-
FIG. 5. R9-QQP specifically inhibits the TCR-mediated phosphorylation of ITK. (A) Jurkat cells pretreated with 27 μM R9-QQP, R9-SCR, or no peptide were incubated with anti-CD3ε (stimulated) or isotype control antibodies (nonstimulated) and analyzed by flow cytometry using anti-ITK phosphotyrosine 511 antibodies. The data are from one representative experiment presented as the total cell count versus the Itk-pY511.
mine whether R9-QQP would have any effects on ITK activation if administered in vivo. Mice were injected intraperitoneally with 20 mg of R9-QQP per kg of mouse weight 24 h and again 30 min before collection of splenocytes and stimulation with anti-CD3ε plus anti-CD28 antibodies. Similar to the effects seen with in vitro peptide treatment (Fig. 2), R9-QQP significantly (P < 0.05) inhibited the activation-induced association between ITK and SLP-76 in a peptide-specific manner; neither R9-SCR nor R9 control peptide had significant effects (Fig. 7A and B). Similar to the results seen with Jurkat cells (Fig. 2), R9-QQP did not affect the interaction of SLP-76 with other signaling partners (LCK, HPK-1, and GADS) (Fig. 7A and B). Furthermore, in CD4+ T cells, R9-QQP caused a significant (P < 0.05) and specific reduction in ITK Tyr 511 phosphorylation without significantly affecting the upstream phosphorylation of LCK on Tyr 394 (Fig. 7C and D). The specific effect of R9-QQP on ITK phosphorylation was also demonstrated by Western blotting, in which the peptide inhibited the TCR-induced phosphorylation of ITK on Tyr 511, but not phosphorylation of the upstream targets LCK and ZAP-70 on their relevant phosphotyrosine residues (Fig. 7E).

Similar to the in vitro treatment, in vivo-administered R9-QQP selectively affected the TCR-induced expression of IL-4 and IL-13 but had no effect on IFN-γ. This was demonstrated by both intracytoplasmic cytokine staining (Fig. 7F and G) and analysis of secreted cytokines (Fig. 7H). All the effects described above are specific, as indicated by the lack of significant inhibition by injection of R9-SCR or R9 control peptide (Fig. 7).

**DISCUSSION**

TCR-mediated activation induces the formation of a dynamically regulated assembly of signaling molecules that includes the adaptors LAT and SLP-76 and tyrosine kinases, such as ZAP-70 and ITK (5, 9, 11, 43). ITK is a critical T-cell regulator, as demonstrated by the defects in T-cell development, TCR-mediated activation, and Th2 cytokine production displayed by mice deficient in its expression (1, 4, 15, 25, 39). The recruitment of ITK to the TCR signaling complex positions ITK as a target of transphosphorylation on Tyr 511 by the Src kinase LCK, an event critical for the enzymatic activation of ITK (18, 44). In vitro experiments utilizing GST-ITK domain fusion proteins have demonstrated that SLP-76 interacts with the ITK-SH2 and -SH3 domains in a cooperative fashion (8). Bunnell et al. screened peptide libraries and identified a specific peptide representing amino acids 184 to 195 of the SLP-76 PR region in live cells impedes the inducible activation of ITK and selectively inhibits the expression of Th2 cytokines, thus providing direct evidence for the biological significance of this interaction.

Cell-permeable peptides encompass cationic sequences, such as polyarginines, which can act as transporters of nonpermeable cargos into the intracellular environment (13, 22). The addition of nine arginine residues to the N-terminal end of the polyproline peptide representing amino acids 184 to 195 of the SLP-76 PR region (R9-QQP) rendered the peptide permeable into both Jurkat cells and murine splenocytes (Fig. 1A to C). Cells took up the peptide rapidly and continued internalizing it, with maximal uptake at 30 min (Fig. 1D). Furthermore, the peptide was not toxic to either resting or TCR-activated T cells for up to 30 h (Fig. 1E). The internalized peptide revealed a distinct punctate pattern of fluorescence that was displayed by the vast majority of cells (Fig. 1A to C, bottom). The rest of the cells displayed a more diffuse distribution of fluorescence. These differences may be related to the mechanism of peptide uptake, which is not clearly understood and is, in fact, controversial (13). The available data suggest two possible pathways of entry, an endocytic pathway and direct translocation through the plasma membrane (42). The mechanism of uptake could also be dependent on the cell type and/or the peptide cargo sequence (22). In particular, cargos rich in proline residues, such as the one used in the present study, have the propensity for more efficient intracellular penetration (35).

The intracellular presence of R9-QQP in both Jurkat cells and splenocytes has profound effects on the inducible association between ITK and SLP-76 (Fig. 2 and 7A and B). This effect is specific for R9-QQP, as both control peptides, R9-SCR and R9, had no significant effects on the ITK–SLP-76 interaction or the protein cargo sequence (22). In particular, cargos rich in proline residues, such as the one used in the present study, have the propensity for more efficient intracellular penetration (35).

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the association between SLP-76 and LCK, HPK-1, and GADS is not affected by R9-QQP (Fig. 2A and B and 7A and B). Previous evidence indicated that the SH3 domain of LCK associates inducibly with the PR region of SLP-76 (confirmed in Fig. 2A and 7A) and that the site of this interaction seemingly overlaps with the site of interaction between ITK and SLP-76 (37). However, we could not demonstrate any significant effects of R9-QQP on this interaction (Fig. 2A and B and 7A and B). Furthermore, R9-QQP did not disrupt the constitutive association between SLP-76 and GADS (Fig. 2A and B and 7A and B), an interaction mediated between the SH3 domain of GADS and a part of the SLP-76 PR region distinct from the one interacting with the SH3 domain of ITK (8, 28). The binding site of the ITK-SH3 domain encompasses amino acids 184 to 208 of the SLP-76 PR region (45), whereas binding of GADS-SH3 involves amino acids 224 to 244 of the SLP-76 PR (27). Interestingly, the inducible association between HPK-1 and SLP-76, not mediated via an SH3-PR interaction, is also not affected by R9-QQP (Fig. 2 and 7A and B). Collectively, these data strongly support the specificity of the effects of R9-QQP on the inducible ITK–SLP-76 association.

Activation of ITK requires its transphosphorylation on its Tyr 511 by LCK (18, 44). The importance of SLP-76 in the activation of ITK has been previously demonstrated (8, 21), and inhibition of this event by R9-QQP (Fig. 5 and 7) extended these observations by demonstrating the importance of the ITK-SH3 domain and its interaction with the PR region of SLP-76 in this process.

Previous studies, in both Jurkat and murine lymphocytes, have demonstrated the biological significance of the ITK-SH2 domain in the TCR-induced interaction with SLP-76 (5, 21). Bogen et al., using Jurkat cells, have shown that the N-terminal tyrosines of SLP-76 are critical for the activation of ITK (5). Jordan and colleagues, utilizing T cells from knockin mice bearing a mutation at one of the N-terminal tyrosines (Y145F) of SLP-76, demonstrated that, even though this mutation had no impact on the stability of the interaction between ITK and SLP-76, it had a significant effect on ITK activation (21). These observations, along with the present demonstration of the role of the ITK-SH3 domain in the interaction with SLP-76, support the hypothesis that the first step in the interaction between these two signaling molecules is mediated by ITK-SH3 binding to the PR region of SLP-76, which is a prerequisite for the subsequent binding of the ITK-SH2 domain to the phosphorylated Tyr 145 required for full activation of ITK. Thus, disrupting the binding of ITK-SH2 to pY145 of SLP-76 (as accomplished by the experiments of Jordan et al.) would not have any effect on the interaction between ITK and SLP-76 but would affect ITK activation. In contrast, disrupting the binding of ITK-SH3 to the PR region of SLP-76 (this study) would affect both the association between these two molecules and the subsequent activation of ITK.

Even though the importance of SLP-76 in the activation of ITK has been established (8, 21), its role in the physical recruitment of ITK to the TCR molecular complex has not been previously demonstrated. The results in Fig. 3D demonstrate that its presence is critical for the inducible localization of ITK to the T-cell contact site and that this event is at least partly dependent on the interaction between the PR region of SLP-76 and the SH3 domain of ITK (Fig. 3E). Interestingly, recruitment of SLP-76 was not affected by R9-QQP (Fig. 3F), thus demonstrating the specificity of the peptide inhibitory effect for ITK recruitment.

The TCR-mediated reorganization of the actin cytoskeleton at the T-cell–APC contact site is regulated by a multimolecular signaling complex that includes ITK, SLP-76, the GTPase Cdc42, and the nucleotide exchange factor Vav, as well as other molecules (2, 7, 12, 17, 24). Evidence from our laboratory, as well as those of others, has demonstrated that cells deficient in ITK expression or cells expressing dominant-negative mutants of ITK display defective TCR-induced actin polarization (12, 17, 24). The significance of the interaction between ITK and SLP-76 in this process has not been previously assessed. The data in Fig. 4 demonstrate that R9-QQP causes a dose-dependent inhibition of TCR-induced actin polarization, suggesting that the interaction of ITK with SLP-76 is important in this process. Interestingly, the effects of ITK on the actin cytoskeleton are independent of the enzymatic activity of ITK, and they appear to be due to a putative adaptor role (12, 17). Furthermore, this function appears to be unique to ITK, as it cannot be compensated for by other members of the Tec family (12, 24).

The data in the present report also provide the first demonstration of the importance of the ITK-SH3 domain-mediated interaction with the PR region of SLP-76 in cytokine production (Fig. 6 and 7). We observed selective inhibition of TCR-induced production of Th2 cytokines. R9-QQP had a profound effect on IL-4 and IL-13 production, compared to the lack of effects on the signature Th1 cytokine, IFN-γ. Furthermore, there was less effect on IL-2 production. Importantly, the effects on cytokines are specific, as indicated by the lack of inhibition with control peptides. The inhibition of ITK phosphorylation (i.e., activation) by R9-QQP and the subsequent effects on cytokines are consistent with previous observations using ITK-deficient mice (15, 40). When R9-QQP was injected intraperitoneally, it displayed effects similar to those seen when...
cells were treated in vitro; the ITK–SLP-76 interaction was disrupted (Fig. 7A and B), the phosphorylation of ITK on Tyr 511 was inhibited (Fig. 7C to E), and there was selective reduction of Th2 cytokine expression (Fig. 7F to H). The significance of the inhibitory effects of R9-QQP upon in vivo administration points to the potential use of this peptide inhibitor in the study of animal disease models in which ITK might be involved.

Another signaling molecule that interacts with the PR region of SLP-76 via its SH3 domain is PLC-γ1 (20). In a genetic analysis, using SLP-76 truncation mutants of Jurkat cells, Yablonski et al. found that an N-terminus-proximal portion of the SLP-76 PR region, which they called the P-1 domain, is important for the activation of PLC-γ1 (46). In a subsequent study, however, the same group found that, even though elimination of the P-1 domain abrogated PLC-γ1 function, truncation of portions of P-1 had no effect (16). Furthermore, truncation of most, but not all, of the P-1 domain still retained PLC-γ1 functionality (16). These investigators interpreted these puzzling data by proposing that the PR region of SLP-76 serves a structural role that is sequence independent and that it is not functionally related to the interaction of SLP-76 with other signaling partners. This interpretation seemingly disagrees with the present data, based on which we argue for a biological significance of the interaction of the SLP-76 PR region and its signaling partner, ITK. Possible explanations that would reconcile these differences in results are that the two studies were done using different experimental systems (cells transfected with truncation constructs in the Gonen et al. study [16] versus cells loaded with peptide in the current study); the possibility that loading cells with inhibitory peptide (present study) may block binding of ITK to other possible target sites on SLP-76, whereas deletion of the site (16) may allow binding to these other sites; and the fact that different parameters were measured in the two studies, which might be differently dependent on ITK–SLP-76 interaction.

Polyarginine cell-permeable peptides have been used by several investigators as competitive inhibitors to study signaling pathways. Matsushita et al. were able to deliver a protein kinase A (PKA)-inhibitory peptide fused to 11 arginines and a nuclear localization signal to the nuclei of neurons in brain slices and to inhibit the phosphorylation of cyclic AMP (cAMP)-responsive element-binding protein and long-term potentiation (30). In another study, Rothbard and colleagues were able to deliver cyclosporine through the skin by using a conjugate of nine arginine residues and cyclosporine (34). In contrast to unmodified cyclosporine, the arginine conjugate penetrated the skin efficiently and was transported into cells in both murine and human skin (34). Significantly, the R9-cyclosporine conjugate entered dermal T lymphocytes and inhibited cutaneous inflammation in a murine model of contact dermatitis (34). In a more recent study, Noguchi et al. were successful in delivering an NFAT-inhibitory cell-permeable peptide into the intracellular environment of both Jurkat and 293 cells (33). This peptide was composed of 11 arginine residues followed by a specific peptide sequence that competitively inhibited the interaction between calcineurin and NFAT (33). Incubation of Jurkat cells with peptide resulted in the inhibition of inducible translocation of NFAT to the nucleus, its transcriptional activity, and the production of IL-2 (33). Even more significant was the fact that intraperitoneal injection of this chimeric peptide significantly prolonged the survival of fully mismatched pancreatic islet allografts in mice (33). Thus, the present report, along with the studies cited above, clearly demonstrates the utility of cell-permeable peptides as powerful probes for the dissection of cell-signaling pathways in live cells.

ITK is critical for the expression of Th2 cell effector function, as evidenced by defective expression of Th2 cytokines and the transcription factor GATA3 in ITK-deficient animals (1). Using a murine model, Mueller and August were the first to demonstrate the involvement of ITK in the development of lung allergy symptoms (32). Mice lacking ITK expression displayed significantly reduced lung inflammation, recruitment of eosinophils, and production of mucus, as well as reduced airway hyperresponsiveness, upon allergen challenge (14, 32). Furthermore, the kinase activity of ITK affected the production of Th2 cytokines and chemokine-mediated migration in allergic asthma differently (36). In view of these findings and the inhibitory effects of R9-QQP when delivered intraperitoneally, we believe that our results lend support to the use of cell-permeable peptides as potential antiallergy therapeutics. Related to this are the recent studies by McCusker et al., who reported on a cell-permeable dominant-negative STAT-6 peptide that displayed selective inhibition of Th2 cytokine expression similar to that of R9-QQP (31). Moreover, the STAT-6 peptide could ameliorate the symptoms of bronchial asthma when delivered intranasally in an experimental animal model (31). Given the similar effects of R9-QQP using in vivo administration (Fig. 7), studies to determine the effects of this peptide inhibitor to inducible allergic responses are under way.

In summary, the present report demonstrates for the first time the biological significance of the specific interaction be-

**FIG. 7.** R9-QQP specifically inhibits ITK activation and function when delivered in vivo. Mice were injected intraperitoneally with 20 mg of the indicated peptides per kg weight or received no peptide treatment, as described in Materials and Methods. (A) Harvested splenocytes were stimulated with anti-CD3ε or control antibodies, and the association of SLP-76 with the indicated signaling partners was assessed as for Fig. 2. (B) Graphic representation of SLP-76 association with various signaling partners: ITK (three replicate experiments) and LCK, HPK-1, and GADS (one experiment each, performed as for panel A). The results were derived and displayed as for Fig. 2B. (C) Splenocytes were stimulated with anti-CD3ε or control antibodies, and ITK pY511 phosphorylation was assessed and presented as for Fig. 5C. (D) Averages (±SEM) of four replicate experiments performed and presented as for Fig. 5D. LCK phospho-flow cytometry was performed using anti-LCK pTyr 394 antibody. (E) Lysates of mouse splenocytes that had been stimulated as for Fig. 2 were immunoprecipitated with anti-ITK antibody and Western blotted serially with anti-ITK pY511, anti-LCK pY394, anti-ZAP-70 pY493, and anti-ITK antibodies. The results are from a single experiment. (F) Splenocytes were stimulated with anti-CD3ε plus anti-CD28 antibodies, and the intracellular expression of cytokines was assessed and presented as for Fig. 6A. The data are from one representative experiment. (G) Averages (±SEM) of three replicate experiments performed and presented as for Fig. 6B to D. (H) Analysis of secreted cytokines from cultures established and tested as described for Fig. 6E to G. The results are from a single experiment, with each sample tested in triplicate.
between ITK-SH3 and SLP-76 PR domains in live cells. Previously, the importance of the ITK-SH3 domain in this interaction had not been directly assessed and it had been inferred from in vitro studies. Our findings may pave the way for the development of novel lung allergy therapies that target early signaling events (i.e., disruption of ITK–SLP-76 interaction) in the production of inflammatory mediators. Such drugs, in combination with other peptide inhibitors that interfere with later signaling events, such as the one reported by McCusker and colleagues (31), could provide effective combination interventions for the treatment of allergic diseases. Such an approach is quite distinct from the actions of current antiallergy therapies that target the already produced effectors that cause the disease symptoms (e.g., corticosteroids, β-adrenoceptor agonists, antihistamines, and leukotriene inhibitors). Finally, the proposed use of cell-permeable peptides to disrupt a specific signaling event may provide the impetus for extending the use of this technology in dissecting additional immunological signaling pathways in live cells.

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