The immuncell adaptore-hesion and degranulation-promoting adpator protein (ADAP) and its binding to T-cell adaptore Src kinase-associated protein of 55 kDa (SKAP-55) play a key role in the modulation of T-cell adhesion. While primary binding occurs via SKAP-55 SH3 domain binding to a proline-rich region in ADAP, a second interaction occurs between the ADAP C-terminal SH3 domain (ADAP-SH3c) and a non-canonical RKXY\(^{294}\)XX\(^{297}\) motif in SKAP-55. Increasing numbers of non-canonical SH3 domain binding motifs have been identified in a number of biological systems. The presence of tyrosine residues in the SKAP-55 RKXY\(^{294}\)XX\(^{297}\) motif suggested that phosphorylation might influence this unusual SH3 domain interaction. Here, we show that the Src kinase p59\(fyn\) can induce the in vivo phosphorylation of the motif, and this event blocks ADAP-SH3c domain binding to the peptide motif. The importance of tyrosine phosphorylation was confirmed by plasmereon resonance interaction analysis showing that phosphorylation of Tyr\(^{297}\) residue plays a central role in mediating dissociation, whereas phosphorylation of the second Tyr\(^{297}\) had no effect. Although loss of this secondary interaction did not result in the disruption of the complex, the Y294F mutation blocked T-cell receptor-induced up-regulation of lymphocyte function-associated antigen-1-mediated adhesion to intercellular adhesion molecule-1 and interleukin-2 promoter activity. Our findings identify a RKXY\(^{294}\) motif in SKAP-55 that mediates unique ADAP-SH3c domain binding and is needed for LFA-1-mediated adhesion and cytokine production.

Modular domains in proteins such as protein-tyrosine kinases, phosphatases and adaptors play central roles in the generation of signals needed for mammalian cell function (1). Of these, Src homology domain 2 (SH2)\(^3\) recognizes phosphotyrosine-based motifs, whereas Src homology domain 3 (SH3) domains recognize proline-based PXXP motifs (2). Since the description of Abelson SH3 domain recognition of the 3BP1 protein (3), numerous SH3 domain-mediated interactions have been documented. Examples include SH3-mediated interactions between the adaptor Grb-2 (growth factor receptor-bound protein-2) and Son-of-Sevenless, Src kinase SH3 domain binding to the p85 subunit of phosphatidylinositol 3-kinase, and Crk SH3 domain binding to Crk SH3 domain-binding guanine nucleotide-releasing factor, among others. SH3 domain binding is involved in subcellular localization, cytokeskeletal organization, and signal transduction (4).

Structurally, SH3 domains are comprised of two anti-parallel \(\beta\) sheets packed at right angles to one other (2, 4, 5). The core \(\alpha\)P\(\alpha\) motif (where \(\alpha\) represents a hydrophobic residue) interacts with SH3 through two defined consensus sequences: Class I (R/KXRXPXXP) and Class II (PXRXPR) (6). Domains can bind ligands in either an N- to C-terminal or C- to N-terminal orientation due to the pseudosymmetrical nature of the proline-proline class II helix that is stabilized primarily by hydrophobic and additional electrostatic interactions. Directionality is conferred by the interaction of the arginine or lysine residues with the charged outer face on the SH3 domain, while the tandem prolines bind to two hydrophobic pockets. Binding depends on the two SH3 variable loops, the RT and n-Src loops, that flank a ligand-binding region.

In addition to the binding to \(\alpha\)P\(\alpha\)-based motifs, an increasing number of studies have documented SH3 domain binding to non-canonical motifs (7). These include a PX(V/I)D(N/R)XXKP motif that is responsible for STAM2 SH3 domain binding to the deubiquitination enzyme ubiquitin isopeptidase Y/Usp8 (8, 9) and Grb-2 related protein (GADS), SH3 domain binding to SH2-domain-containing leukocyte protein of 76 kDa (SLP-76) (10–13). In the latter case, the interaction has a 10–20 times higher affinity than interaction between SH3 domains and their \(\alpha\)P\(\alpha\) motifs. Other examples of non-canonical interactions include amphiphsin SH3 domain binding to dynamin (14), Eps8 SH3 domain binding to the PXDXY motif (15), and HPK1 SH3 domain binding to a RXXK motif (12). We recently identified a novel RKXXXXYY motif that is found in the T-cell adaptor Src kinase-associated protein of 55 kDa (SKAP-55) and that is recognized by the FYN-SH3 and ADAP-SH3c domains (16).

In the immune system, SH3 domains are found in Src-related kinases and immune specific adaptor proteins (17, 18). Adaptors lack enzymatic activity and transcription binding domains, and instead are comprised of multiple binding domains and sites that facilitate protein-protein aggregation. This family of adaptors in T-cells includes linker for activation of T cells (LAT), lymphocyte cytosolic protein-2 (SLP-76), adaptor adhesion and degranulation promoting adaptor protein (also known as Fyb, FYN-T-binding protein, and SLAP, SLP-76-associated protein); ADAP-SH3c, C-terminal SH3 domain of ADAP; TBS, Tris-buffered saline; SPR, surface plasmon resonance; TcR, T-cell receptor; GST, glutathione S-transferase; GFP, green fluorescent protein.
SKAP-55 Motif Binding to ADAP SH3c

with C-terminal SH3 domains and are selectively phosphorylated by the Src kinase FYN-T (19–22). The SH3 domain of SKAP-55 can bind to a proline-rich region in ADAP (20, 21), whereas the ADAP SH3c domain binds to the RKXY^294-XXY^297 motif in SKAP-55 (16). Essentially all cellular SKAP-55 is complexed to ADAP (21), an interaction that protects free SKAP-55 from rapid degradation by proteolysis (23). Furthermore, both ADAP and SKAP-55 have been implicated in T-cell adhesion. Transfection and knock-out models have shown that ADAP mediates inside-out signaling events that regulate LFA-1 clustering (24–27). Retroviral expression of SKAP-55 also enhances LFA-1 clustering and adhesion (28, 29), whereas the loss of SKAP-55 by small interference RNA blocks these events (30). Disruption of SLP-76-ADAP also interferes with peripheral supramolecular activation complex formation at the immunological synapse (28), whereas ADAP and SLP-76 can cooperatively up-regulate IL-2 transcription (22, 31, 32).

Unlike other non-canonical motifs, the SKAP-55 RKXY^294-XXY^297 sequence incorporates two tyrosine residues that may serve as sites for phosphorylation, which could potentially regulate SH3 domain binding and influence the interaction with ADAP. Here, we show that the Src kinase p59^fyn can induce the in vivo phosphorylation of tyrosines within the SKAP-55 motif, and this event potently blocks ADAP-SH3c domain binding to the ligand. The importance of tyrosine phosphorylation was confirmed by plasmoid resonance interaction analysis showing that phosphorylation of Tyr^294 residue plays a central role in mediating dissociation, whereas phosphorylation of the second Tyr^297 had no effect. Although loss of this secondary interaction did not result in the disruption of the complex and likely plays little role in complex structural integrity, the Y294F mutation blocked TcR-induced up-regulation of LFA-1-mediated adhesion to ICAM-1 and IL-2 promoter activity. Our findings thus identify a RKXY^294 motif in SKAP-55 that interacts with ADAP SH3c domain and is required for LFA-1-mediated adhesion and cytokine production.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—COS-1 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (Interagen), 1% (v/v) penicillin and streptomycin (Invitrogen), and 1% (v/v) L-glutamine (Invitrogen). COS-1 cell transfection was conducted using standard protocols (32). Jurkat cells were maintained in the same medium and expressed SKAP-55 or appropriate mutants following transfection as previously described (28–30). Anti-GST monoclonal antibody was purchased from Santa Cruz Biotechnology, and anti-HA monoclonal antibody was from Roche Biochemicals. Anti-phosphotyrosine monoclonal antibody 4G10 was kindly provided by Dr. Tom Roberts (Dana-Farber Cancer Institute, Boston, MA). Anti-FYN-T, LCK, and ZAP70 monoclonal antibodies were purchased from BD Transduction. Peptides were synthesized and high-performance liquid chromatography-purified by the Molecular Biology Core Facility (Dana-Farber Cancer Institute) with sequences as follows: unphosphorylated peptide TRRKGDY^294 ASV^295-Υ^296 GQ, the same peptide phosphorylated at Tyr^294, phosphorylated at Tyr^295, phosphorylated at Tyr^294 and Tyr^295, and phosphorylated at Tyr^296 (Table 1). TTGVFVKMPPTE served as an irrelevant peptide.

Immunoprecipitation and Immunoblotting—Cell lysis, immunoprecipitation, and detection were performed as described previously (19). Briefly, 2 × 10^6 COS-1 or Jurkat cells were transfected with cDNA using DEAE-dextran as described (30). After 2 days, cells were harvested and lysed with 200 μl of lysis buffer (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_4VO_4, 1 mM NaF, 1 mM leupeptin, 1 mM pepstatin, and 1% aprotinin). Immunoprecipitation was carried out by incubation of the lysate with the antibody for 1 h at 4 °C, followed by incubation with 50 μl of glutathione-Sepharose beads (50% w/v) for 1 h at 4 °C. For immunoblotting, the immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose filters (Schleicher and Schuell). Filters were blocked with 5% (w/v) skimmed milk for 1 h in Tris-buffered saline (Tris-buffered saline) pH 8.0 and then probed with the indicated antibody or GST fusion proteins followed by binding with an anti-GST monoclonal antibody. Bound antibody was revealed with horseradish peroxidase-conjugated rabbit anti-mouse antibody using enhanced chemiluminescence (ECL, Amersham Biosciences). For the Far Western technique, ADAP-SH3c was with a T7 tag, and binding to SDS-PAGE-separated and blotted GST fusion proteins was detected with anti-T7 antibody followed by chemiluminescence development.

Expression and Purification of GST Fusion Proteins—Plasmids were transformed into the DH5 strain of Escherichia coli and induced with isopropyl-β-D-thiogalactopyranoside to produce GST fusion proteins as described (16).

Luciferase Assays for IL-2 Promoter Activity—A total of 5 × 10^5 Jurkat cells transfected with the test SKAP-55 constructs, IL-2 promoter reporter vector, and control vector (Promega, Madison, WI) were stimulated with 1 μg/ml anti-CD3 and 2 μg/ml rabbit anti-mouse Ab at 37 °C for 6 h and subsequently assayed for luciferase activity using a luminometer (MicroLumat, EG&G Berthold). Luciferase units of the experimental vector were normalized to the level of the control vector in each sample. Adhesion assays were conducted according to Jo et al. (30).

Measurement of Peptide Binding Using Surface Plasmon Resonance—All interactions were determined using a BIAcore 3000 and a fully upgraded BIAcore 1000 instrument. Various SH3 fusion proteins (10 μg/ml in 100 mM sodium acetate, pH 4.5) were immobilized on CM5 Biosensor chips (BIAcore) through cross-linking of free amine groups to the N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide-HCl-activated flow cell surface, followed by blocking of free succinimidyl ester with 1 M ethanolamine. After extensive washing of the surface with binding buffer (10 mM HEPES, 150 mM NaCl, pH 7.0), peptide binding was assessed by injecting the indicated concentrations in binding buffer over the flow cell surface. When required, the surface was regenerated using 100 mM glycine, pH 2.8. The data were analyzed using the BIAevaluation version 3.2 software and fitted to a 1:1 Langmuir binding model with separate K_d and K_a determination. The association constant (K_a) was determined as k_a/k_d, and the dissociation constant (K_d) was determined as 1/K_d.

RESULTS

Phosphorylation of the RKXY^294-XXY^297 Motif Interferes with ADAP SH3c Domain Binding—Previous studies (16) have demonstrated that the atypical ADAP SH3c domain binds to a non-canonical non-Tyr-phosphorylated RKXY^294-XXY^297 motif in the SK4 region of the SKAP-55 SK region (Fig. 1A). To assess whether the tyrosine residues in the motif could be phosphorylated and whether this affects ADAP SH3c domain binding, cells were co-transfected with GST-tagged peptide SK4, which carries the RKXY^294-XXY^297 motif (residues 290–297) and the protein-tyrosine kinase p59^fyn. Within the SK4 peptide, tyrosines are located at residues 294, 297, and 298. We had previously shown that SKAP-55 is phosphorylated by the Src kinase p59^fyn (19, 22). Following cell lysis, the GST-tagged SK4 was precipitated using glutathione beads, and tyrosine phosphorylation was detected by anti-pTyr blotting. Co-expression of p59^fyn resulted in the phosphorylation of pEBG-expressed SK4 (Fig. 1B, left panel, lane 4 versus 3). As a control for nonspecific effects, co-expression of p59^fyn plus GST alone failed to result in GST phosphorylation (lane 2 versus 1). SK4 SKAP-55 peptide was preferentially phosphorylated by p59^fyn, because co-expression of another T-cell kinase
SKAP-55 Motif Binding to ADAP SH3c

ZAP-70 resulted in only a background phosphorylation (lane 5). Anti-GST blotting of cell lysates confirmed the expression of GST (lanes 1 and 2) and GST-SK4 (lanes 3–5) (Fig. 1B, right upper panel), whereas anti-p59<sup>SH3</sup> and anti-ZAP70 blotting confirmed expression of the kinases (Fig. 1B, right middle and lower panels). These observations indicate that p59<sup>SH3</sup> can phosphorylate the SK4 region of SKAP-55, the region that interacts with the C-terminal SH3 domain of ADAP.

To assess whether phosphorylation might interfere with ADAP SH3c domain binding, the same samples were blotted with ADAP-SH3c-T7 in a Far-Western protocol followed by detection with anti-T7 antibody (Fig. 1C). Under these conditions, the ADAP-SH3c domain could readily bind to GST-SKAP-55-SK4 on nitrocellulose (lane 3). This confirms by another technique the binding of the ADAP-SH3c domain with the SK4 peptide (16). As a control, no binding was observed between ADAP-SH3c and the GST control, verifying the specificity of the interaction between SKAP-55 and ADAP-SH3c. Significantly, in the presence of p59<sup>SH3</sup>, where the SKAP-55 peptide was phosphorylated (Fig. 1B, left panel, lane 4), the ADAP-SH3c domain was greatly impaired in binding to the SKAP-55 peptide (Fig. 1C, left panel, lane 4). As a further control, in the absence of SKAP-55 peptide but the presence of p59<sup>SH3</sup>, no ADAP-SH3c was precipitated (Fig. 1C, left panel, lane 2). Co-expression of ZAP-70 partially inhibited the interaction between SKAP-55 peptide and ADAP-SH3c (Fig. 1C, left panel, lanes 5 versus 3), with little if any phosphorylation (Fig. 1B, left panel, lane 5), but it was significantly less than the phosphorylation and inhibition by p59<sup>SH3</sup> (Fig. 1C, left panel, lanes 4 versus 5). Overall, these observations show that phosphorylation of the SK4 peptide <em>in vivo</em> correlated with a major reduction in the ability of the ADAP-SH3c domain to interact with the RKXXXYYY-containing peptide.

**RKXXXYYY Phosphorylation Interferes with ADAP-SH3 Domain Binding as Confirmed by Surface Plasmon Resonance**—The co-expression studies suggested that the ADAP-SH3c domain interaction with the RKXXXYYY motif can be regulated by a phosphorylation event. Nevertheless, the complexity of the interactions of kinases with sub-
The dissociation constant (K\textsubscript{D}) for the SKAP-55 SK4-ADAP-SH3c interaction was determined by SPR analysis (Fig. 2A). The ADAP-SH3c domain was immobilized on the chip and the SKAP-55 SK4 peptide was injected at various concentrations. The SPR response was then measured and the association/dissociation curves were fitted to the Biacore Evaluation software to calculate the dissociation constant (K\textsubscript{D}). In control experiments, the SKAP-55 SK4 peptide interaction with ADAP-SH3c was revealed by SPR interaction analysis (Fig. 2B). Given that we were examining the effects of inorganic phosphate anion that was absent in the SPR buffer, the influence of inorganic phosphate anion might be of particular concern given the lower avidity and charge-dependent interaction of SK4 with the ADAP SH3c domain. To assess this, we repeated the SPR analysis in the presence of the buffer used in the NMR analysis that included 20 mM phosphate. Whereas the ADAP-SH3c domain-SK4 interaction occurred in HEPES buffer in the absence of phosphate (Fig. 3A), no interaction of SK55 SK4 peptide with ADAP-SH3c was detected in the presence of high levels of free inorganic phosphate anion (Fig. 3B) in agreement with Heuer and co-workers (34). This indicates that the presence of free inorganic phosphate anion can interfere with the binding of ADAP-SH3c domain to the SK4 peptide and offers an explanation for the differing result.

Mutation of Tyr\textsuperscript{294} Does Not Disrupt SKAP-55/ADAP Complex Formation—In addition to ADAP-SH3c domain binding to SKAP-55, we and others have shown that a second interaction occurs between the SKAP-55 SH3 domain and proline residues in ADAP (21, 22), where this latter interaction is generally accepted to be the major basis of binding between the two proteins.

Nevertheless, to assess whether the ADAP-SH3c-mediated interaction is necessary for complex formation, or whether it constitutes a secondary interaction, the Tyr\textsuperscript{294} residue was mutated, tagged with GFP, and assessed for binding to ADAP via co-precipitation analysis (Fig. 4). Independent of whether the transfected cells were resting or activated by anti-CD3 cross-linking, both wild-type SKAP-55-GFP and mutant SKAP-55-Y294F-GFP could be detected as an 80-kDa band following ADAP immunoprecipitation (Fig. 4C). Detection of phosphotyrosine in the same co-precipitates revealed that SKAP-55 was phosphorylated in all instances (Fig. 4D). The lack of SKAP-55 in appropriate controls (Fig. 4, A and B) confirmed the specificity of the ADAP-mediated co-precipitation. These data indicate that the loss of the Tyr\textsuperscript{294} site has no effect on ADAP-SKAP-55 complex formation in T-cells. The interaction must therefore be considered as a secondary interaction to SKAP-55 SH3 domain binding to ADAP (21, 22).

Mutation of Tyr\textsuperscript{294} Disrupts SKAP-55 Regulation of LFA-1-mediated Adhesion—We have shown previously that ADAP and SKAP-55 cooperate to provide the inside-out signals that induce LFA-1-mediated T cell adhesion to ICAM-1 (28–30). Despite its secondary role in complex formation, the ADAP-SH3c domain interaction with SKAP-55 could still influence the conformation and functionality of the ADAP-SKAP-55 complex. We therefore tested the effects of mutations at Tyr\textsuperscript{294} and Tyr\textsuperscript{294}/Tyr\textsuperscript{297} (SKAP-55-Y294F and SKAP-55-Y294F/Y297F, respectively) on TCR induction of adhesion and IL-2 transcription. Adhesion to ICAM-1 of cells expressing native or mutant SKAP-55 was assessed by binding of anti-CD3-stimulated Jurkat T cells to recombinant ICAM-1-coated plates as described previously (28–30). As shown in Fig. 5, stimulation through CD3 of control vector-transfected cells increased adhesion 2-fold, and this could be increased a further 50% by introduction of exogenous full-length SKAP-55. Transfection with either SKAP-55-Y294F or SKAP-55-Y294F/Y297F abrogated completely the adhesion response, implying that the mutant SKAP-55 has a dominant negative effect in blocking the function of endogenous SKAP-55. The lower panels in Fig. 5 show representative light microscopy images of each condition, indicating the effects of each SKAP-55 variant and confirming that the SKAP-55 Tyr\textsuperscript{294} residue is needed for TCR up-regulation of LFA-1 adhesion.

Mutation of Tyr\textsuperscript{294} Disrupts SKAP-55 Potentiation of Interleukin-2 (IL-2) Promoter Activity—ADAP can also potentiate TCR-driven IL-2 promoter activity (22, 31, 32). Jurkat cells were transfected with wild-type SK55, SKAP-55-Y294F, or SKAP-55-Y294F/Y297F together with a vector carrying a nuclear factor of activated T cells/AP-1-dependent IL-2 promoter and activated by cross-linking with anti-CD3.
As shown in Fig. 6, transfection of wild-type SKAP-55 resulted in a 3-fold increase in IL-2 promoter activity over non-transfected control. In contrast, expression of the SKAP-55-Y294F mutant blocked up-regulation of IL-2 promoter activity, as did expression of the SKAP-55-Y294F/Y297F double mutant. Overall, these data underscore the importance of the ADAP-SH3c binding motif in the TcR up-regulation of LFA-1 adhesion and IL-2 promoter activity.

DISCUSSION

The present study builds on our initial observation that the SH3c domain of ADAP (as well as Fyn and Lck SH3 domains) can bind to a novel motif in SKAP-55 by showing that phosphorylation of a core Tyr294 in the motif disrupts binding, and that, although the interaction is not required for ADAP-SKAP-55 complex formation, loss of the key tyrosine abrogates TcR-driven LFA-1 adhesion and IL-2 gene transcription. The interaction must therefore be secondary to the interaction to SKAP-55 SH3 domain binding to ADAP (21, 22), but is nevertheless important for functionality of the ADAP-SKAP-55 complex.

SH3 domains can recognize classic proline-based øP øP motifs as well as non-canonical motifs (35). Atypical motifs include PX(IV)(D/N)RXKP binding to the STAM2 and GADS SH3 domains (8–13) and Eps8 SH3 domain binding to the PXDY motif (15). The RKXYXXY motif...
motif of ADAP-SH3c domain binding to SKAP-55 is unique in that it possesses bipartite tyrosine residues that could serve as sites for phosphorylation (16). This possibility was underscored by p59<sub>fyn</sub> binding to ADAP and its phosphorylation of SKAP-55 (19, 33). In agreement with this model, we showed that p59<sub>fyn</sub> phosphorylation of RK<sub>XX</sub>Y<sub>294</sub>XX<sub>Y<sub>297</sub></sub> blocked the binding to the ADAP SH3c domain (Fig. 1). Furthermore, plasmon resonance using peptides phosphorylated at Tyr294, Tyr297, or Tyr294/297 confirmed this finding and identified Tyr294 as the major residue involved in ADAP SH3c domain binding (Fig. 2). Phosphorylation of this site completely prevented SH3c domain binding, a finding in keeping with the physical proximity of the Tyr<sup>294</sup> residue to the Arg<sup>290</sup>/Lys<sup>291</sup> basic residues we had previously shown to be essential for binding (16). By contrast, phosphorylation of residue Tyr<sup>297</sup> or the adjacent Tyr<sup>298</sup> residue had only a nominal effect on binding. Our findings therefore identify a role for protein-tyrosine phosphorylation in the negative regulation of an SH3 domain binding to ligand. Another example involves platelet-derived growth factor-stimulated phosphorylation of c-Src, where phosphorylation of Tyr<sup>278</sup> on the SH3 peptide-binding surface diminished ligand-binding ability (36). These cases may serve as prototypes for phosphoregulation of other SH3 domain interactions, including Eps8 SH3 domain binding to the PXXDY motif (15).

Other examples of non-canonical interactions include STAM2 and GADS SH3 domain binding to a PX(V/I)L(D/N)RXXKP motif in ubiquitin isopeptidase Y/Usp8 and SLP-76, respectively (8–13). In the latter case, the interaction has a 10–20 times higher affinity (K<sub>d</sub> = 0.24 μM) than SH3 domain binding to oPxoP motifs. Another example is HPK1 SH3 domain binding to RXXK and a cluster of N-terminal prolines (12). In each of these cases, charged interactions between RXX and the negatively charged surface of the SH3 domain play a central role in binding. In the case of the RK<sup>XX</sup>Y<sup>294</sup>XX<sup>Y<sup>297</sup></sub> motif, we previously documented the key role played by electrostatic interactions between the RK residues and the negatively charged surface of the FYN SH3 domain (16). The binding site overlapped with that of the classic oPxoP motif and induced a chemical shift perturbation of the negatively charged
Glu^98 and Asp^100 in the RT loop and Glu^116 in the n-Src loop. The K_d of the ADAP SH3c domain-RKXXX^294-XXY^297 interaction was found here to be in the moderate range (i.e. 30–40 μM), in agreement with our previous report of the range 20–60 μM (16).

The dominant effect of Tyr^294, but not Tyr^297 phosphorylation on binding supports the notion that the bipartite YXXY residues do not operate in the same manner as a bipartite PXXP motif. The tyrosine-based motif is unlikely to form a pseudosymmetric polyproline class II helix that has been observed with proline-based motifs (7). Furthermore, the ADAP SH3c domain is unusual in that it lacks the key residue Trp^119 that has been substituted by a lysine or tyrosine (16, 19). Furthermore, the RKXXX^294-XXY^297 motif failed to induce a shift perturbation for the NH groups of Trp^119 and Tyr^137 in the FYN SH3 domain indicating that these residues are unlikely to contribute to the binding of the tyrosine-based motif (16).

Our findings also indicate that the Tyr^294 site is not needed for complex formation between ADAP and SKAP-55 (Fig. 4). This is consistent with previous observations by ourselves and others that the major interaction between ADAP and SKAP-55 relies on SKAP-55 SH3 domain binding to proline residues in ADAP (20, 21). In this way, the ADAP-SH3c domain interaction is likely to act in a supplemental fashion to reinforce or alter the orientation of components within the ADAP-SKAP-55 complex. The slight increase in tyrosine phosphorylation of SKAP-55 in response to anti-CD3 is consistent with previous data showing that both ADAP and SKAP-55 undergo weak but extended levels of phosphorylation (19, 22, 32). Nevertheless, the loss of Tyr^294 had a major inhibitory effect on TCR-driven LFA-1 clustering and extended levels of phosphorylation (19, 22, 32). Nevertheless, the loss of Tyr^294 by SPR analysis underscored the specificity of the interaction (Fig. 2).

To address the reported discrepancy, we noticed that the buffer conditions used in the studies by Heuer and coworkers (37) contained 20 mM free inorganic phosphate. Given the moderate affinity of the ADAP SH3c-SKAP-55 peptide interaction when isolated in vitro, high conductivity buffers incorporating phosphate may well mask interactions that can be detected by NMR with greater sensitivity in low conductivity buffers (38). Phosphate in NMR buffer can interfere with SH2/SH3 domain-ligand interactions. Consistent with this, SPR analysis using the NMR buffer containing 20 mM phosphate as reported failed to reveal significant ADAP-SH3c domain-RKXXX^294-XXY^297 binding (Fig. 3). From this, we conclude that the presence of inorganic phosphate interferes with ADAP-SH3c binding. Although the intracellular concentration of phosphate can rise to almost ~100 mM in resting cells, the majority is complexed to cellular intermediates and only a smaller fraction is available as free anion, predominantly in the form of inorganic pyrophosphate with a concentration in mammalian cells of ~1.5 mM, significantly less than the 20 mM representation in the NMR buffer (39). Whether FYN SH3 domain binding to the motif is also partially affected by the inorganic phosphate remains to be determined.

Initial attempts to purify the complex have been complicated by protein solubility issues. The only ADAP-SH3c domain purified for structural analysis to date failed to bind to any protein or peptide, raising the issues of correct folding or appropriateness of the conditions for measuring peptide binding (34). The absence of binding was surprising given our previous work showing binding by a combination of in vivo co-expression, protein–protein precipitation, peptide precipitation, alnane-scanning mutagenesis, and surface plasmon resonance (16). Instead, non-selective binding to an array of polyvalent phosphoinositides was reported (37).

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Increasing evidence points to the importance of multiple modular interactions in the formation of protein–protein aggregates in the generation of signals that regulate cell function (17, 18). Examples include tandem SH2 domain binding for ZAP-70 and phosphatases such as SHP-2. Preliminary studies using gel filtration have indicated that the ADAP complex is greater than 10^7 Da (unpublished data). Biphasic high affinity and moderate affinity modes for ADAP-SKAP-55 binding could act to reinforce complex formation or alter the orientation of components within the ADAP-SKAP-55 complex. This may be of particular relevance given the importance of ADAP in stabilizing SKAP-55 expression in cells. In the absence of ADAP, the intracellular t_1/2 of SKAP-55 is only 18 min, whereas complexing with ADAP enhances the t_1/2 to 90 min (23). These differences in the rate of SKAP-55 degradation could have profound effects on the requirement for SKAP-55 in the transmission of inside-out signals needed for integrin-mediated T cell adhesion and up-regulation of IL-2 promoter activity (28–30).

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