Regulation of FynT Function by Dual Domain Docking on PAG/Cbp*

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In resting T-cells, the transmembrane adaptor protein PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains) is constitutively tyrosine-phosphorylated, a state maintained by the Src family kinase FynT. PAG has a role in negative regulation of Src family kinases in T-cells by recruitment of Csk (C-terminal Src kinase) to the membrane via binding to PAG phosphotyrosine 317. The interaction between FynT and PAG is essential for FynT function; however, so far the FynT binding mode has been unknown. Here, we demonstrate that the FynT-PAG complex formation is a dual domain docking process, involving SH2 domain binding to PAG phosphotyrosines as well as an SH3 domain interaction with the first proline-rich region of PAG. This binding mode affects FynT kinase activity, PAG phosphorylation, and recruitment of FynT and Csk, demonstrated in Jurkat TAg cells after antibody stimulation of the T cell receptor. Furthermore, we show that TCR-induced tyrosine phosphorylation is regulated by SH3 domain modulation of the FynT-PAG interaction in human primary T-cells. Although FynT SH3 domain association is shown to be crucial for efficiently initiating PAG phosphorylation, we suggest that engagement of the SH2 domain on PAG renders FynT insensitive to Csk negative regulation. Thus, in T-cells, PAG is involved in positive as well as negative regulation of FynT activity.

Protein-tyrosine kinases are key mediators of signaling through cell surface receptors, and specificity and regulation of their activities is critical for creating the correct responses to external stimuli. In T-cells, ligand engagement of the T-cell receptor leads to activation of proximal protein-tyrosine kinases of the Src kinase family (Lck and FynT), which subsequently induce tyrosine phosphorylation of proteins in the T-cell receptor (TCR) complex (1, 2). Recruitment and activation of the kinase Zap-70 to the complex initiates an array of downstream events involving other enzymes and cellular adaptors and scaffold proteins and finally results in nuclear transcription, changes in T-cell morphology, and proliferation.

Protein-tyrosine kinases of the Src kinase family have a conserved modular architecture fundamental to their highly specific function, interactions, and regulation (3). The archetypal Src family kinase (SFK) consists of an N-terminal membrane-targeting region (myristoylated and/or palmitoylated) (4), followed by successive SH3 and SH2 domains, capable of binding to proline-rich and phosphotyrosine-containing motifs, respectively, as well as a catalytic domain. In addition, SFKs contain a regulatory tyrosine residue within the kinase domain and an autoinhibitory tyrosine phosphorylation site in the C-terminal tail (5–7). Regulation of SFKs is mediated by the cytoplasmic kinase Csk (C-terminal Src kinase), which phosphorylates the C-terminal tyrosine of SFKs to initiate the formation of an inactive, closed configuration through intramolecular engagement of the SH2 domain.

Phosphoprotein associated with glycosphingolipid-enriched microdomains (hereafter referred to as PAG) (8, 9), is a transmembrane adaptor protein involved in the negative regulatory mechanism of SFKs by anchoring of Csk to lipid rafts through specific binding of the SH2 domain to PAG-phosphorylated tyrosine 317 (human cells; Tyr314 in mice). The interaction increases Csk activity (10), which subsequently acts by phosphorylating the regulatory C-terminal tyrosine of SFKs. PAG, also known as Cbp (Csk-binding protein), is localized to lipid rafts by palmitoylation and consists of a large cytoplasmic domain containing two proline-rich regions as well as 10 tyrosine residues, nine of which are potential candidates for phosphorylation by SFKs.

In resting T-cells, PAG is constitutively phosphorylated; however, stimulation of the TCR induces a rapid dephosphorylation of PAG, leading to a transient displacement of Csk from lipid rafts (9, 11). The dissociation allows the signaling mediators Lck and FynT to become activated, thus initiating a TCR-induced tyrosine phosphorylation cascade leading to T-cell activation (12). Termination of this activating signaling event occurs by rephosphorylation of PAG at tyrosine 317 and recruitment of Csk back to lipid rafts. An additional role in negative regulation has been suggested for PAG through its association with EBP50, which may mediate a connection between lipid rafts and the actin cytoskeleton (13, 14).

PAG phosphorylation and Csk recruitment are both greatly reduced in FynT-deficient cells (9, 15–17), demonstrating that the FynT-PAG complex formation is essential for the negative regulatory role of PAG. Little structural information is available for the interaction, although it has been suggested that the assoc-
FynT Dual Domain Docking on PAG

ciation is mediated by direct binding of the FynT SH2 domain to phosphorylated tyrosines in PAG (9). Interaction via the FynT SH3 domain has also been discussed, since mutations of PAG tyrosines have been shown not to abolish FynT association completely (18). Interestingly, phosphorylation of PAG in mast cells is dependent on the association with the SFK Lyn (19–21), and this interaction has recently been shown to involve concomitant, dual binding of Lyn SH3 and SH2 domains (22).

In this paper, we attempt a dissection of the FynT-PAG interaction at a higher resolution using peptide array technology in combination with mutational analyses. Our results identify the complex formation as a dual domain binding process, involving both binding of the FynT SH3 domain to the first proline-rich motif in PAG and a FynT SH2-mediated interaction with PAG phosphorylated tyrosines. Complexed to PAG, we postulate that the activated FynT kinase is still accessible for Csk C-terminal phosphorylation; however, inhibition of FynT activity is not effectuated until phosphorylated FynT dissociates from PAG. Thus, binding to PAG is important for positive as well as negative regulation of FynT activity. Our results are verified in a physiological setting by temporal analyses performed in Jurkat TAg cells, showing FynT and Csk interactions with PAG and the impact of TCR stimulation on these interactions as well as on PAG phosphorylation. In human primary T-cells, modulation of the FynT-PAG interaction is shown to regulate TCR-induced tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies against FynT, Csk, phospholipase C-γ, and glutathione S-transferase (GST) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-CD3ε (OKT3) monoclonal antibody was affinity-purified by Diatiec (Oslo, Norway) from supernatants of a hybridoma cell line purchased from ATCC (Manassas, VA), whereas the F(ab')2 fragment was from Jackson ImmunoResearch Europe Ltd. (Newmarket, UK). Antibodies against tyrosine-phosphorylated proteins (4G10 and 4G10-horseradish peroxidase) and the full-length, active Fyn kinase were purchased from Upstate Biotechnology, Inc. (Charlottesville, VA). The FLAG tag antibody was from Cell Signaling Technology Inc. (Beverly, MA), and the GST-horseradish peroxidase conjugate was from GE Healthcare (UK). Glutathione and FLAG beads were from Sigma. The phosphotyrosine-specific antibody against Zap-70 (pY319) was from Jackson ImmunoResearch Europe Ltd. (Newmarket, UK). Cells and Transfections—Human embryonic kidney (HEK 293T) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, nonessential amino acids and sodium pyruvate, and 10% fetal calf serum in a humidified atmosphere of 5% CO2. Transfections were carried out using FuGENE® 6 transfection reagent (Roche Applied Science). Jurkat TAg cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and nonessential amino acids and sodium pyruvate. Transfections were carried out by electroporation as described (11), and the electroporate was expanded in complete medium and incubated for 18 h before harvesting. Human CD3+ T cells were purified from normal blood donors by negative selection as described previously (11). Transfections were performed using the Amaxa Nuclefect™ kit (Gaithersburg, MD) according to the manufacturer’s instructions. Cells were incubated in complete medium for 18 h before harvesting and stimulation.

Cell Stimulation, Lysis, and Immunoprecipitations—Prior to all stimulations, cells were preincubated at 37 °C for 5 min. Jurkat TAg cells (1.5 × 10⁶ cells/ml) were stimulated with anti-CD3ε antibodies (OKT3 at 1 μg/ml) for the indicated time at 37 °C. Human primary T cells (1.0 × 10⁶ cells/ml) were stimulated with anti-CD3 (OKT3 at 0.3 μg/ml) followed by the addition of F(ab')2 fragment (10 μg/ml) after 2 min for cross-ligation. Stimulation was continued for the indicated time before lysis as described above.

Cells were lysed in ice-cold lysis buffer (50 mm HEPES, pH 7.4, 100 mm NaCl, 5 mm NaF, 10 mm NaPO4, 5 mm EDTA, 1% Triton X-100, 50 mm n-β-octyl-d-glucoside, 1 mm NaVO₄, and 1 mm phenylmethylsulfonyl fluoride). When specified, sodium pervanadate treatment was performed at 37 °C by adding a mixture of 0.01% H₂O₂ and 100 μM NaVO₄ for 4 min before cell lysis. For immunoprecipitation of FLAG-PAG, transfected cells were disrupted in the lysis buffer and incubated with anti-FLAG beads for 3 h at 4 °C. Immune complexes were subsequently washed in lysis buffer and subjected to Western blot analysis.

SPOT Synthesis of Peptide Arrays and Array Interaction Detection—Peptide arrays were synthesized on nitrocellulose membranes using a MultiPep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG) as described (25). Peptide interactions with GST and GST fusion proteins were determined by overlaying the cellulose membranes with 1 μg/ml protein. Bound proteins were detected with horseradish peroxidase-conjugated anti-GST antibodies and visualized by ECL.

In Vitro Phosphorylation Assay—Pulse-chase experiments were carried out as described (26) with minor modifications. Briefly, full-length, active Fyn (15 nM) and purified recombinant
PAG (0.3 μM, if not stated otherwise) were incubated in the presence of 80 nM \( [γ-32P]ATP \), 10 mM MgCl2, and 100 mM Tris·HCl, pH 7.5, for 10 min on ice. This was followed by a chase reaction containing 0.5 mM unlabeled ATP at 30 °C, and aliquots were withdrawn at the specified times. Reactions were terminated by boiling in SDS sample buffer. The samples were analyzed by SDS-PAGE, and phosphorylated PAG was detected using a phospho-screen (PhosphorImager, Amersham Biosciences).

**RESULTS**

**Interaction of FynT with PAG in Mammalian Cells**—Previous reports have demonstrated that FynT is critically important for PAG phosphorylation and Csk binding by analysis of T-cells from Fyn \(^{-/-} \) mice (16). Despite these observations, few attempts have been made to elucidate the molecular basis of the interaction between FynT and PAG. Involvement of either the FynT SH2 or SH3 domains has been considered, following studies using mutational analysis and immunoprecipitations of recombinant proteins (9, 15, 18).

In order to ascertain involvement of individual FynT domains in the association with PAG, we created two mutants of FynT, W119A and R176K, to isolate contributions from SH2 and SH3 domain binding, respectively. Tryptophan 119 is a highly conserved amino acid in various Src SH3 domains, and alanine substitution of this residue is known to abolish SH3 domain function (23). The FynT R176K mutant contained a point mutation of a critical arginine residue in the SH2 domain, which has been shown to reduce phosphoryosine binding of Src family kinases (24). Domain structure and positioning of the mutations are shown schematically in Fig. 1A. Using these constructs and WT FynT, we performed immunoprecipitations from HEK 293T cells stimulated with sodium pervanadate to increase levels of tyrosine phosphorylation, subsequently securing a potential SH2 domain interaction with more homogeneously phosphorylated PAG species. In addition, cells left untreated with sodium pervanadate were included in experiments to assess levels of PAG phosphorylation in HEK 293T cells before and after transfection with WT FynT. Western blot analysis showed that PAG phosphorylation in these cells is restored after introduction of WT FynT, whereas pervanadate-treated cells have constitutively phosphorylated PAG molecules (Fig. 1B). We assume that hyperphosphorylation of PAG in cells without transfection of WT FynT is caused by endogenous Fyn, detected in the HEK 293T cells lysates at very low levels.

Further analysis showed a strong interaction between WT FynT and FLAG-tagged PAG and reduced binding for both mutants FynT W119A and FynT R176K. Association via the SH2 domain only (FynT W119A) appeared markedly stronger than the interaction mediated by the SH3 domain (FynT R176K). The observation conforms to the general binding pattern for these domains, and SH2 domains are known to interact with submicromolar affinities with sequences containing phosphotyrosines (27), whereas SH3 domains bind with somewhat lower affinities to proline-rich ligands (28). Binding to PAG was virtually abolished using a double mutant FynT (W119A/R176K) incapable of interacting via either domain and confirmed that the FynT-PAG interaction involved more than one domain.

**Identification of the FynT SH3 Binding Motif**—The cytoplasmic tail of PAG contains two proline-rich sequences with the minimal SH3-binding motif PXXP, located at amino acid residues 134–137 and 257–260, respectively. In erythroid cells, phosphorylation of PAG is dependent on the kinase Lyn, and the second proline-rich region has already been identified as a binding site for Lyn in a yeast two-hybrid screen (22). To locate the FynT SH3 domain-interacting site on PAG, we performed a peptide walk by synthesizing a SPOT peptide array on nitrocellulose membranes consisting of overlapping 20-mer peptides at 3-amino acid intervals. This array, which encompassed the full-length PAG sequence, was probed with purified recombinant FynT-SH3-GST fusion proteins. Binding was assessed immunologically with positive interactions identified as dark spots. Strong interactions were detected in five spots exclusively, which all spanned the first proline-rich region as marked in the PAG amino acid sequence (Fig. 2A). Probing the membrane with GST protein alone produced virtually no interaction (data not shown).

The FynT SH3 interaction sequence was analyzed subsequently by N- and C-terminal deletions to reveal the boundaries of the interaction site (Fig. 2B). These truncations defined clearly residues Arg131 and Pro137 as the N- and C-terminal boundaries for binding, respectively. Furthermore, replacement walk by sequential substitution of alanine identified amino acids Arg131, Leu133, Pro134, and Pro137 as essential for interaction between PAG and the FynT SH3 domain (Fig. 2C). Thus, the core sequence of the detected binding site reads RXLPXXP, which is identical to the consensus motif for class I domain.
SH3 ligands (29). The critical arginine residue (Arg131) defines the orientation of the FynT SH3 domain binding to PAG by forming a salt bridge with a conserved residue within the SH3 domain (30).

Further characterization of the FynT SH3 domain interaction was performed using a two-dimensional peptide array, generated by systematically substituting the 14-mer peptide containing the interaction site by all natural amino acids (Fig. 3A). Again, this array identified the Arg, Leu, and Pro residues of the consensus sequence as essential for interaction with the FynT SH3 domain. Additionally, we identified residues outside this sequence, histidine 128, glutamine 129, and glutamate 139, which displayed some reduction in binding when substituted with certain amino acids. Flanking residues both N- and C-terminal to the core binding sequence have been shown to contribute toward SH3 domain binding and may serve as determinants of SH3 affinity and selectivity; however, these additional interactions have been shown to require an intact core motif (29, 31).

Combining the interaction data created by the two-dimensional array, we were also capable of creating an optimal binding sequence for SH3 binding. All possible combinations of single substitutions giving stronger binding than the native sequence were generated, and their relative affinities for the FynT SH3 domain were tested (data not shown). The optimal binding sequence contained the core binding motif with two amino acid substitutions, E132P and P138R, marked by white circles in Fig. 3A, and was introduced into full-length PAG to produce a high affinity ligand for FynT. This construct, referred to as superPAG (Fig. 3B), was used in further experiments as a functional tool for the analysis of kinase activity and effects of FynT binding.

**SH3 Interaction Analysis in Vivo**—Immunoprecipitations of full-length PAG containing single, double, and triple mutations at the critical positions defined by the two-dimensional peptide arrays were performed to confirm ablation of SH3 domain binding (data not shown). Experiments were carried out using the FynT mutant R176K, which displays greatly reduced association with phosphotyrosines. Surprisingly, we were not capable of abrogating binding via the SH3 domain completely, not even with a PAG construct containing a triple alanine substitution at residues Arg131, Leu133, and Pro134 (Fig. 3C).
Pro134 was performed to create PAG ciating with all peptide spots containing a phosphorylated tyro-
sation, we found that the SH3-SH2 proteins were capable of asso-
with the first proline-rich region of PAG was detected; in addi-
SH3-SH2-GST fusion proteins (Fig. 4
struct, named RLP*, produced a ~70% reduction in binding. It
is likely that the residual binding is a result of incomplete
removal of SH2 domain contribution through mutation of
R176K or, alternatively, that amino acid residues outside the
core SH3-binding motif are capable of stabilizing the interac-
tion with PAG. Identification of alternative interaction modes
was not pursued. Immunoprecipitations were also performed
with the high affinity binder superPAG, which displayed a rel-
avite increase in FynT SH3 domain binding of ~100%.
Identification of the FynT SH2 Binding Motif—Peptide array
synthesis of full-length PAG was repeated using phosphoty-
rosine at all tyrosine residues to assess interaction with FynT
SH3-SH2-GST fusion proteins (Fig. 4A). Again, interaction
with the first proline-rich region of PAG was detected; in addi-
tion, we found that the SH3-SH2 proteins were capable of asso-
ciating with all peptide spots containing a phosphorylated tyro-
sine residue, barring Tyr(P)299. It has previously been observed
that the Fyn SH2 domain binds promiscuously to many unre-
lated phosphotyrosine-containing peptides (32); thus, due to
the low level of discrimination among phosphotyrosine-con-
taining peptides on array membranes, this approach was not
pursued further.

To reveal the position of the PAG phosphotyrosine involved
in FynT SH2 domain binding, we created PAG constructs con-
taining phenylalanine substitutions at all tyrosine residues but
one to isolate the contribution from single phosphotyrosine
residues. A PAG construct having all tyrosine residues removed
by phenylalanine substitution was also made (PAG 10YF). Immunoprecipitations from HEK 293T cells transiently

co-transfected with FynT and the various full-length PAG
mutants were performed to analyze the SH2 domain interac-
tion in vivo (Fig. 4B). In addition, we performed repeat exper-
iments using FynT W119A and cells stimulated with sodium
pervanadate to increase tyrosine phosphorylation and maxi-
mize SH2 domain interaction. Note that residue Tyr299, which
did not interact with FynT SH3-SH2 when included in the pep-
tide array, was not a target for tyrosine kinases in these cells as
measured by 4G10 antibody detection of tyrosine phosphory-
lation. The low amount of WT FynT and FynT W119A binding
to PAG pY299 was assumed to be a result of residual interac-
tions with the FynT SH3 domain. Western blot analysis defined
the N-terminal tyrosine residues Tyr295, Tyr296, and Tyr298
as preferred interaction sites for the FynT SH2 domain in both
sets of experiments; however, since the SH3 domain interaction
is directional, defined by residue Arg131 upstream of the core
binding motif, we considered interaction only with residues
Tyr296 and Tyr298 plausible.

These results were confirmed by further immunoprecipita-
tions from HEK 293T cells transiently co-transfected with
FynT W119A and PAG constructs containing mutations at
either or both of these tyrosines (Fig. 4C). Single phenylalanine
substitutions at either site resulted in a small decrease in bind-
ing, consistent with previously published studies showing that
single tyrosine substitutions are not sufficient to abolish SH2
domain binding (9). However, introduction of a double
mutated PAG Y163F/Y181F resulted in a significant loss of
FynT SH2 domain binding. Based on these results, we conclude
that FynT is capable of binding alternative phosphotyrosine
sites, 163 and 181, and that loss of one of these tyrosines can
be compensated for through binding of the other site.

Finally, using WT FynT and WT PAG as well as PAG con-
structs containing mutations in the SH3 and/or SH2 domain-
interacting sites, we performed immunoprecipitations from
HEK 293T cells left untreated with sodium pervanadate (Fig.
4D). FynT and PAG interacted strongly as observed earlier
using pervanadate-stimulated cells. Removal of the SH2
domain interaction by substitutions of PAG tyrosines 163 and
181 reduced the WT FynT binding and was also followed by a
slight reduction in phosphorylation levels and Csk recruit-
ment. Using the RLP* mutant to eliminate FynT SH3-mediated bind-
ing also produced an interaction reduction, again reflected by a
further decrease in tyrosine phosphorylation and Csk recruit-
ment. Significantly reduced binding was also observed using a
combination of the two interaction mutants, with markedly
lower levels of tyrosine phosphorylation and concomitant
recruitment of Csk.

PAG Phosphorylation and Effects of FynT Interaction
Modulation—The effects of PAG mutations on PAG phosho-
rylation were explored by in vitro phosphorylation assays using
purified recombinant proteins. In a pulse-chase assay, active
FynT and recombinant GST-PAG were preincubated with a
low concentration of radioactive ATP. Next, a large excess of
ATP was added, and phosphorylation progress was followed
over time by the withdrawal of aliquots. Assays performed with
WT PAG demonstrated a rapid phosphorylation process (Fig.
5A), and discrete populations of phosphorylated PAG species
were seen after only 30 s. At a time point of 2 min, higher
molecular weight species had accumulated corresponding to PAG molecules with an increasing number of phosphoryrosine residues. The accumulation process continued throughout the time course; however, discrete bands were still seen at the end of the course (10 min).

The effect of FynT SH3 domain binding modulation was investigated through phosphorylation assays performed with PAG RLP*, a mutant with significantly reduced affinity for the FynT SH3 domain (Fig. 5B). Here, phosphorylation progress was slow, and no discrete PAG species were seen, indicating a random phosphorylation mechanism. In such a process, random collisions between kinase and substrate would proceed until the SH2 domain binding sites are phosphorylated, allowing the enzyme to dock onto its substrate via SH2 domain binding. PAG is then rapidly phosphorylated; however, overall, this process is considerably slower (~5 min) than that of WT. In contrast, the high affinity SH3 binder superPAG was rapidly phosphorylated, showing a concomitant accumulation of high molecular weight species. These experiments clearly demonstrated the importance of an intact SH3 domain binding site for functional engagement of the SH3 domain, and the interaction was not only critical for FynT kinase activity but also for positioning of the kinase and efficient phosphorylation of its substrate.

Modulation of the SH2 domain binding was performed by phenylalanine substitution of the tyrosines 163 and 181, identified as alternative binding sites for the FynT SH2 domain in previous experiments. We also created a PAG mutant Y387F/Y417F as a comparative substrate containing an identical number of tyrosine sites (seven) available for phosphorylation. Removal of the SH2 domain interaction using PAG Y163F/Y181F resulted in elimination of the band containing the highest molecular weight species, whereas the general pattern of discrete bands of phosphorylation products was retained. The pattern of discrete bands was also found using PAG Y387F/Y417F as a substrate additionally; the highest molecular weight species were again present. Thus, tyrosines 163 and 181, residues immediately downstream of the SH3 domain interaction site, appear to be preferred substrates over tyrosines 387 and 417.

Ordered phosphorylation as shown above is one characteristic of a processive phosphorylation mechanism, and accumulation of phosphorylated products is another (26), and to analyze this further we performed phosphorylation assays with increasing substrate concentrations. A processive phosphorylation mechanism is one where phosphorylation kinetics are not affected by substrate concentration, since only the substrate population already associated with enzyme is substrate for further phosphorylation. In contrast, phosphorylation in a non-processive (distributive) manner is a process involving multiple independent collisions between enzyme and substrate. As a consequence, increasing the substrate concentration in a distributive phosphorylation process would result in accumulation of intermediate species, not discrete populations (33, 34). Using three different substrate concentrations (0.03, 0.3, and 3
Removal of the two tyrosines reduced phosphorylation levels as well as decreasing the association with FynT and Csk. This may imply that FynT SH2 domain binding is required for efficient phosphorylation of Tyr\(^{117}\). As predicted by the in vitro assays, phosphorylation of PAG constructs containing disrupted SH3 domain interaction sites (RLP\(^{117}\) and RLP\(^{117}/Y163F/Y181F\)) was dramatically reduced. Using superPAG, the high affinity SH3 binder, PAG phosphorylation levels were significantly raised in Jurkat TAg cells. Augmenting the level of FynT expression by WT FynT co-transfection proved to mirror these results with more pronounced effects, and recruitment of both Csk and FynT to the various PAG constructs in these settings was found to follow the level of PAG phosphorylation.

We next performed a temporal analysis after TCR stimulation by anti-CD3e antibodies and monitored PAG phosphorylation by endogenous FynT as well as recruitment of Csk and FynT to PAG in Jurkat TAg cells over a time course of 10 min (Fig. 6B). PAG phosphorylation was found to decrease throughout the time course with no apparent change in dephosphorylation kinetics between WT PAG and the superPAG construct despite the markedly raised phosphorylation levels of superPAG. Removal of tyrosines Tyr\(^{163}\) and Tyr\(^{181}\) resulted in overall reduced PAG phosphorylation, and a substantial further reduction in phosphotyrosines was observed for constructs carrying the RLP\(^{117}\) mutation, again results that were in keeping with observation made in the in vitro phosphorylation assays.

Dissociation of both FynT and Csk from the PAG complex occurred in response to antibody TCR stimulation and PAG dephosphorylation. The dissociation was rapid for both kinases, with significant decrease in association after only 1 min. Interestingly, loss of the PAG-Csk complex appeared comparable for WT PAG and superPAG despite the pronounced difference in total phosphotyrosine content. In contrast, the FynT association appeared prolonged for the superPAG construct, reaching the lowest level at 10 min. Together, these results demonstrate that the FynT-PAG complex formation is mediated by a dual domain binding and that modulation of the individual binding sites affects FynT recruitment and activity as well as PAG phosphorylation and Csk recruitment in Jurkat TAg cells after TCR stimulation.

We proceeded with these results by providing a physiological setting for the interaction and in vitro phosphorylation data through introduction of the various PAG constructs in Jurkat TAg cells. In these cells, endogenous FynT was capable of phosphorylating WT PAG as well as a mutant PAG construct lacking the major SH2 domain-interacting tyrosines (Tyr\(^{163}/
}\)Tyr\(^{181}\)) through replacement by phenylalanines (Fig. 6A).
**FynT Dual Domain Docking on PAG**

**A**

![Image](https://via.placeholder.com/150)

**FIGURE 6. Modulation of PAG interactions with FynT and Csk in Jurkat TAg cells.** A, Jurkat TAg cells were transfected with various FLAG-tagged PAG constructs alone or with WT FynT and subjected to immunoprecipitations (IP) using FLAG antibodies 18 h post-transfection. Western blots were probed with anti-Fyn, anti-Csk, anti-phosphotyrosine (4G10), and anti-FLAG antibodies. Whole cell lysates were also tested for equal FynT expression using anti-Fyn antibodies. B, effects of domain-binding modulations were followed over time using Jurkat TAg cells transfected with various FLAG-PAG constructs. 18 h post-transfection, cells were activated via the TCR complex through antibody stimulation (OKT3) for the indicated time prior to anti-FLAG immunoprecipitations. Associations of FynT and Csk with PAG were examined by immunoblotting using anti-Fyn and anti-Csk antibodies, respectively, whereas PAG phosphorylation was revealed by anti-Tyr(P) (4G10) antibodies.

The RLP* and superPAG constructs, shown to offer the most pronounced effects on PAG tyrosine phosphorylation, were selected to further assess the functional effects of PAG mutants on TCR-induced signaling. Human primary T-cells were co-transfected with FynT and these constructs as well as with WT PAG and stimulated with anti-CD3ε antibodies (OKT3) followed by cross-linking using F(ab')2 fragments, and protein tyrosine phosphorylation in total cell lysates was analyzed using various anti-Tyr(P) antibodies (Fig. 7). Transient transfections with low amounts of FynT alone did not influence protein tyrosine phosphorylation as measured by phosphospecific antibodies 4G10, LAT (Tyr(P)191), and Zap-70 (Tyr(P)317) but was used to augment the levels of PAG phosphorylation as shown in Fig. 6A. The varying degree of tyrosine phosphorylation was clearly detected at ~80 kDa, reflecting the pattern of SH3 domain-mediated PAG phosphorylation established in the previous figures. TCR stimulation resulted in dephosphorylation of PAG in accordance with earlier reports by Davidson et al. (35). Introduction of WT PAG did not affect tyrosine phosphorylation significantly; however, transient transfections with PAG RLP resulted in increased phosphorylation of Zap-70 (Tyr(P)317) as well as pLAT/p36. In contrast, tyrosine phosphorylation of these species was markedly reduced by the introduction of the heavily tyrosine-phosphorylated superPAG mutant, demonstrating the potential of PAG as a negative regulator of TCR signaling mediated via PAG phosphorylation and Csk recruitment.

This region of PAG with binding motif RXLPXXP matches the consensus sequence of the core-binding motif of class I SH3 ligands (29, 37). Substitutions by alanine of critical residues in this motif, however, failed to disrupt the PAG-FynT SH3 domain interaction completely. The residual binding observed may be caused by incomplete removal of SH2 domain interactions in the FynT R176K mutant or by transient contacts made between FynT and PAG outside the core binding sequence, a consequence of SH3 domain binding reported in other systems (29). Alternatively, the complex may be stabilized either by additional contacts with Csk bound to PAG at Tyr(P)317 or by contacts with other proteins yet to be identified. This scenario is likely, since PAG has a large number of potential interaction sites, and the formation of such multiprotein complexes involving PAG has recently been confirmed (38). However, although binding of the SH3 domain to mutated ligand sequences was not abolished completely, FynT kinase activity was greatly reduced in *in vitro* phosphorylation assays using these ligands. Thus, although additional interactions may be sufficient to maintain complex formation via the SH3 domain, an intact motif is required to induce the conformational changes necessary to confer FynT activity and efficient phosphorylation of PAG. These results are in keeping with a suggested general mechanism for SH3 domain-mediated regulation of enzyme activity of Src family kinases via displacement of intramolecular inhibiting interactions and reduction of the substrate $K_m$ (26, 32).

**DISCUSSION**

Resolving the regulation and function of FynT, a central mediator in proximal T-cell signaling, and of kinases and adaptor proteins that act as substrates for FynT is critical to our understanding of the role of this kinase in T-cell activation and development. FynT is associated with PAG in resting T-cells; however, so far, key data concerning the complex formation have been inconclusive, suggesting both phosphotyrosine-dependent and -independent mechanisms (9, 15, 18, 36). Taken together, these observations clearly open for a binding scenario involving more than one interaction domain. In this paper, we show that complex formation between FynT and PAG is mediated by a dual domain docking mechanism involving both SH3 and SH2 domains of FynT, which work cooperatively to facilitate binding specificity and kinase activity.

PAG–FynT SH3 Interaction—We identified the first proline-rich region of PAG (amino acids 131–137) as a ligand for the FynT-SH3 domain by peptide array mapping.
In this study, direct regulation of FynT kinase activity via SH3 domain interactions was further demonstrated by the introduction of a high affinity SH3 domain ligand, superPAG, which significantly increased PAG phosphorylation levels. The superPAG ligand sequence may have potential as a tool to interfere with cell functions of FynT SH3-mediated processes.

**PAG-FynT SH2 Interaction**—Mutational analysis revealed that PAG has two main FynT SH2 domain-interacting phosphotyrosines in residues Tyr<sup>163</sup> and Tyr<sup>181</sup>, respectively. Both sites include glutamic acid at the +1-position and a hydrophobic residue (leucine or valine) at the +3-position, features considered important for SH2 domain interaction (3, 40). We were not able to distinguish between the two sites using single substitutions with phenylalanine, and complete removal of SH2 domain binding was only achieved using a double mutated PAG substitutions with phenylalanine, and complete removal of SH2 domain binding was only achieved using a double mutated PAG Y163F/Y181F. Consequently, we conclude that Tyr<sup>163</sup> and Tyr<sup>181</sup> are equal alternatives for the FynT SH2 domain. This characteristic of the FynT SH2 domain binding may explain why the binding mode to PAG has not been resolved previously, since other studies have relied on single tyrosine substitutions.

**Consequences of Dual Domain Docking**—Several substrates for Src family kinases (e.g. p130CAS (41), AFAP110 (42), and focal adhesion kinase (6, 43)) have been shown to interact via similar dual domain docking processes, and as described earlier, a dual domain mode of binding has already been demonstrated for PAG as a ligand for the Src kinase member Lyn (22). Other examples included here (Table 1) all show ligand sequences of dual domain binders with SH2 domain-interacting sites following successively C-terminal to the proline-rich motifs. Speculations that the region spanning the two sites could be used as an interface for additional complex-building contacts have been put forward; however, investigations of the Fyn-focal adhesion kinase complex have provided no evidence of such interactions (6). The region is poorly conserved with the SH3-SH2 spacer length varying from 21 (CAKα) to 43 amino acids (PAG Tyr<sup>181</sup>), and our observation of an alternative two binding sites (Tyr<sup>163</sup> and Tyr<sup>181</sup>) for PAG also point to an interaction with a certain degree of flexibility and a binding region that does not adapt a specific structural configuration.

It is interesting to note that binding of Lyn to PAG in erythroid cells, recently resolved by Ingely et al. (22), involves an SH3 domain interaction with the second, not the first, proline-rich site of PAG (amino acids 246–266). The SH3 domains of Fyn and Lyn have complete identity in 30 of 55 amino acids; however, of the 12 residues making up the variable RT and n-Src loops of the SH3 domain, only two residues are identical. The two loops have been shown to play a role in ligand specificity (29), and it is likely that variability in these regions is the origin of the displayed differences in selectivity toward PAG SH3-ligand sequences. Combined, the results presented here open the possibility of a scenario in other cell systems where PAG serves as a docking station for both Fyn and Lyn. A differential regulation of these kinases binding to the same adaptor could potentially be carried out through two independent phosphatases.

We also wanted to study effects of modulations of the FynT binding sites on PAG phosphorylation and recruitment of Csk and FynT in a physiological setting, and time course analyses were performed in stimulated Jurkat TAg cells. Our results showed clearly the implications of removing the PAG SH3-binding site (RLP<sup>∗</sup>) by significant reduction of PAG phosphorylation as well as minimal FynT and Csk recruitment. In contrast, increasing the SH3 domain interaction via superPAG augmented phosphorylation levels and FynT recruitment.

Removal of the major SH2 domain-interacting phosphotyrosines (Tyr<sup>163</sup> and Tyr<sup>181</sup>) appeared to reduce PAG phosphorylation levels as well as FynT and Csk recruitment. The former was expected following results from our *in vitro* phosphorylation assays, and these constructs were found to migrate further, accordingly. However, reduction in kinase recruitment could also indicate that an intact SH2 domain interaction is essential for achieving efficient phosphorylation of Tyr<sup>317</sup> as well as securing the FynT association. This is in keeping with results presented by Davidson et al. (9, 15, 18), showing that mutations of all tyrosines other than Tyr<sup>317</sup> (mouse) abolished PAG-Csk interaction.

### Table 1

**Ligand sequences of known Src family kinase dual SH3/SH2 domain binders**

| Complex         | Sequence                                                                 | References |
|-----------------|--------------------------------------------------------------------------|------------|
| PAG-FynT        | KPKCHQ<sup>∗</sup>RLELP<sup>∗</sup>PRLPESVADTLM/TARSDVDOGQLGMEGPPYVLKDSQSQENMVEDCLYETVEIKF | 7, 43      |
| FAK-Fyn/Src     | RPKQKEGER<sup>∗</sup>RPA<sup>∗</sup>S1KLANSEKQMRTHAVSVSETEDYAEI1DEDEKTVAPPFRYIQERIELGR | 41         |
| pC3130-Src      | RTSIQ1QR<sup>∗</sup>RLELP<sup>∗</sup>PRLPESVADTLM/TARSDVDOGQLGMEGPPYVLKDSQSQENMVEDCLYETVEIKF | 41         |
| AFAP110-Src     | SLAPKPPQMLPS1QOPWLPESRQQPLP<sup>∗</sup>SSLQYQEEYAAFLPQ<sup>∗</sup>PAPKAPETVYGYVYGDAM<sup>∗</sup>SYSY<sup>∗</sup> | 42         |
| Sam68-Fyn       | R7AG1QR<sup>∗</sup>RLELP<sup>∗</sup>PRLPESVADTLM/TARSDVDOGQLGMEGPPYVLKDSQSQENMVEDCLYETVEIKF | 44, 45     |
| SIN-Src         | R7GST1QR<sup>∗</sup>RLELP<sup>∗</sup>PRLPESVADTLM/TARSDVDOGQLGMEGPPYVLKDSQSQENMVEDCLYETVEIKF | 47         |

<sup>∗</sup> Binding site not reported, predicted by Scansite.
Antibody stimulation of the Jurkat TAg cells caused a rapid and pronounced dissociation of both Csk and FynT, and interestingly, our results indicated a loss of the Csk-PAG complex prior to that of FynT and PAG with a more pronounced effect observed for the superPAG complex. This opposes observations by Davidsson et al. (18); however, a similar conclusion may be drawn from these two results. The precise mechanism for dephosphorylation of PAG is unknown, and it could involve two phosphatases that may be regulated differently in different settings, as suggested previously (35). Two recent studies have focused on a role for PAG in anergy induction in T-cells (18, 38). Anergic T-cells have enhanced FynT activity as well as increased levels of PAG-FynT complexes but not PAG-Csk levels. Thus, it is interesting to speculate that the superPAG construct may have potential as an experimental tool for anergy induction by providing conditions as observed in this study.

The inhibitory function of PAG is mediated through its tyrosine phosphorylation, and to analyze the regulatory effects of PAG mutants on TCR-induced tyrosine phosphorylation, we used PAG mutants superPAG and RLP*, which produced PAG species with markedly raised and lowered phosphorylation levels, respectively. Introduction of the two PAG mutants in human primary T-cells produced opposite effects on tyrosine phosphorylation of LAT and Zap-70, with superPAG clearly augmenting a negative signal in lipid rafts through significantly diminished protein tyrosine phosphorylation.

Results reported in this paper show that engagement of both SH3 and SH2 domains affects not only PAG phosphorylation but also the regulation of FynT activity. Recruitment of Csk to PAG pY317 has been shown to enhance C-terminal phosphorylation of FynT (Tyr(P)528) as observed by Smida et al. (38). Taken together, we propose the following binding model for formation of the FynT-PAG-Csk complex in resting T-cells (Fig. 8); the FynT SH3 domain provides a likely first contact with PAG, which raises the activity of FynT and thus enables PAG tyrosine phosphorylation. A comparable initial contact has been put forward for p130Cas (41) and for the complex formation between Src family kinases and Sam68 (44, 45), suggesting a role for the SH3 domain in substrate recognition prior to tyrosine phosphorylation. This interaction could be transient and would result in kinase activation, tyrosine phosphorylation of PAG, and subsequent recruitment of other SH2 domain-binding proteins, such as Csk. As a consequence of binding, Csk is brought in close proximity to FynT, which allows for phosphorylation of the inhibitory C-terminal tyrosine residue (Y528). Engagement of the FynT-SH2 domain on PAG, however, ensures that FynT remains enzymatically active and that the inhibition is not effectuated until FynT dissociates from PAG and an inactive “closed” conformation can be formed. A role for the SH2 domain in “priming for inhibition” by Csk has been considered for Lck (46) and recently also for FynT (38).

In conclusion, our results show that FynT binding to PAG is a multistep mechanism, involving both SH3 and SH2 domains. The presence of both binding sites in PAG is likely to potentiate specificity and affinity of this interaction by providing an extended recognition site, with engagement of the FynT SH2 domain following after tyrosine phosphorylation of PAG. The dual domain binding stabilizes the FynT-PAG complex without excessive affinity, thus maintaining the fidelity, reversibility, and rapid dissociation necessary in cell signal transduction.

Acknowledgments—We thank Guri Opsahl, Jorun Solheim, and Gladys Tjørholm for excellent technical assistance.

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FIGURE 8. Model mechanism of Csk regulation of FynT activity. A, a dual domain docking of FynT on PAG activates the kinase, which subsequently phosphorylates PAG tyrosine residues effectively by a processive mechanism. Csk is recruited from cytosol to lipid membrane through SH2 domain binding to PAG phosphotyrosine 317. B, Csk phosphorylates FynT C-terminal tyrosine (Tyr(P)528); however, due to engagement of the FynT SH2 domain on PAG, FynT remains active enzymatically. An inactive, “closed” conformation is adopted by FynT once the kinase dissociates from the PAG complex.
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