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Revisiting the Timing of Action of the PAG Adaptor Using Quantitative Proteomics Analysis of Primary T Cells

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The protein tyrosine kinase LCK plays a key role in TCR signaling, and its activity is dynamically controlled by the protein tyrosine C-terminal Src kinase (CSK) and the protein tyrosine phosphatase CD45. CSK is brought in contiguity to LCK via binding to a transmembrane adaptor known as phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG). The lack of a blatant phenotype in PAG-deficient mice has impeded our understanding of the mechanisms through which PAG exerts its negative-regulatory role in TCR signaling. We used quantitative mass spectrometry and both thymocytes and CD4+ T cells from mice in which a tag for affinity purification was knocked in the gene coding for PAG to determine the composition and dynamics of the multiprotein complexes that are found around PAG over 5 min of activation. Most of the high-confidence interactions that we observed were previously unknown. Using phosphoproteomic analysis, PAG showed low levels of tyrosine phosphorylation in resting primary mouse CD4+ T cells; the levels of tyrosine phosphorylation increased and reached a maximum 2 min after stimulation. Analysis of the dynamics of association of the protein tyrosine phosphatase PTPN22 and lipid phosphatase SHIP-1 with PAG following T cell activation suggests that both cooperate with CSK to terminate T cell activation. Our findings provide a model of the role for PAG in mouse primary CD4+ T cells that is consistent with recent phosphoproteomic studies of the Jurkat T cell line but difficult to reconcile with former biochemical studies indicating that PAG is constitutively phosphorylated in resting T cells and rapidly dephosphorylated once the TCR is engaged. The Journal of Immunology, 2015, 195: 5472–5481.

The recognition of peptide-MHC (pMHC) ligands by T cells and the ensuing transduction into intracellular signals is accomplished by the TCR-CD3 complex. The cytoplasmic tails of CD3 subunits contain one or several ITAMs. The CD4 and CD8 coreceptors assist TCRs in the recognition of pMHC ligands, and their cytoplasmic segments are associated with the LCK protein tyrosine kinase (PTK). Phosphorylation of CD3 ITAMs by LCK leads to the recruitment and activation of the cytosolic PTK ZAP-70, which, in turn, phosphorylates the adaptor proteins LAT and SLP-76. After their many tyrosines are phosphorylated, LAT and SLP-76 nucleate the assembly of multiprotein complexes that are denoted as signalosomes and are responsible for most of the responses resulting from TCR engagement. Many studies support the view that TCR triggering in response to foreign agonist pMHC does not build up on a blank state (reviewed in Ref. 1). For instance, in response to very weak interactions with self-pMHC, the TCR signaling pathway found in naive T cells, rather than being silent, delivers low-intensity signals that imprint on naive T cells a heightened reactivity to foreign pMHC (2). A network of PTKs, protein tyrosine phosphatases (PTPases), and transmembrane adaptors dynamically control the activity of LCK in the ground state (3–8). In the ground state, the levels of active LCK are set to a point ensuring that maximal CD3 ITAM and ZAP-70 phosphorylation only occurs upon engagement of the TCR with agonist pMHC ligands. Consistent with the existence of such basal PTK–PTPase balance, its alteration in favor of PTK via genetic or pharmacological interventions can increase TCR sensitivity (9) and even trigger T cell activation, irrespective of TCR-pMHC engagement (5, 10).

The catalytic activity of LCK is regulated by phosphorylation of two conserved tyrosines (6). Phosphorylation of the tyrosine residue found at position 505 (Y505) near the C terminus of LCK promotes an autoinhibited conformation of LCK, in which intramolecular binding of its Src homology 2 (SH2) domain with phosphorylated Y505 stabilizes the inactive conformation of the catalytic domain. In contrast, phosphorylation of Y394 stabilizes the activation loop of the catalytic domain and increases LCK activity. Y394 of LCK is autoprophosphorylated in trans, whereas Y505 phosphorylation is catalyzed by the C-terminal Src kinase (CSK) (11, 12). CSK is a cytosolic protein that is recruited in contiguity to plasma membrane–bound LCK via binding of its SH2 domain to phosphorylated transmembrane adaptor proteins, such as...
phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG; official name PHAG1, also known as Csk-binding protein) (13, 14). PAG is ubiquitously expressed and possesses a 429-aa-long cytoplasmic domain that is palmitoylated and has 10 potential tyrosine phosphorylation sites (15). Upon phosphorylation by the FYN PTK, the tyrosines found at positions 165 and 183 of mouse PAG are capable of binding to the SH2 domain of FYN. In turn, FYN phosphorylates Y314 of mouse PAG, leading to the recruitment of CSK via its SH2 domain (15).

PAG primarily exerts a negative-regulatory role (16). Upon engagement of receptor tyrosine kinases, immunoreceptors, and integrins found on B cells (17), mast cells (18), erythroid cells (19), epithelial carcinoma cells (20), and embryonic fibroblasts (21), the peak of PAG phosphorylation is reached within 5–15 min of activation and then returns to basal levels. This suggests that, together with FYN and CSK, PAG initiates a negative-regulatory loop contributing to signal termination (22). In contrast, in resting T cells, PAG is constitutively phosphorylated and associated with CSK. Once the TCR is engaged, PAG is rapidly and transiently dephosphorylated by an unknown PTPase controlled by the TCR. As a result, CSK is released from PAG, and the negative-regulatory tyrosine found at position 505 of LCK is dephosphorylated. This increases the activity of LCK, leading to tyrosine phosphorylation of CD3 ITAMs and of other downstream substrates (13, 23). Therefore, T cells appear unique among the studied cell types in that they primarily use PAG to control the initiation of TCR signaling. A recent study in mast cells suggests that PAG can function as a positive or negative regulator, depending on the signaling pathway under study (25).

In addition to FYN, PAG, and CSK, the transmembrane PTPase CD45 contributes to set up the signal threshold that has to be overcome to trigger T cell activation. Although phosphorylated CD3 ITAMs are better substrates for CD45 than phosphorylated LCK (6), CD45 plays a positive role in setting up the pool of active LCK present in the ground state by dephosphorylating Y505 (26). Therefore, the combined action of CD45 and the FYN–PAG–CSK axis establishes the activity of LCK in the ground state. However, PAG is dispensable for the recruitment of CSK to the membrane, suggesting that undiscovered transmembrane adapter proteins compensate for its loss (27). For instance, the transmembrane adapter LIME, another lipid raft–associated CSK-binding phosphoprotein, may contribute to recruit CSK. However, combined genetic deficiencies in PAG and LIME had little effect on TCR signaling (28). As a result, our understanding of the role of PAG during TCR signal initiation and termination remains incomplete (15).

Many of the previous studies conducted on PAG involved transformed T cell lines and used anti-phosphotyrosine mAbs and immunoblots to probe for the PAG phosphorylation status. Affinity purification coupled with mass spectrometry (AP-MS) allows highly sensitive analysis of the dynamics of noncovalent protein complexes. We developed mice that bear a genetic tag that permits AP-MS analysis of protein complexes isolated from primary CD4+ T cells. Furthermore, we combined AP-MS analysis with phosphoproteomic analysis to provide quantitative information on the phosphorylation of the tyrosine residues present on PAG over the course of CD4+ T cell activation.

### Materials and Methods

**Pag1<sup>OST</sup> targeting vector**

A 6.3-kb genomic containing exons 8 and 9 of the Pag1 gene was isolated from a bacterial artificial chromosome clone (clone RP23-84P4; http://www.lifesciences.sourcebioscience) of C57BL/6J origin. Using homologous recombination in *Escherichia coli* (30), a chloramphenicol-resistance gene bracketed by BspEI and SalI sites was inserted at the 3′ end of the Pag1-coding sequence found in exon 9. The chloramphenicol-resistance gene was excised using BspEI and SalI digestion and replaced by a Xmal-Sall fragment corresponding to a one-STEM (ost) (Stop) 2loxP-TACE-CRE-PGK-gb2-neo-loxP cassette (29). Finally, the targeting construct was abutted to a thymidine kinase expression cassette and linearized with FseI.

**Isolation of recombinant embryonic stem cell clones**

JMS.F6 C57BL/6N embryonic stem (ES) cells (31) were electroporated with the *Pag1<sup>OST</sup>* targeting vector. After selection in G418 and ganciclovir, ES cell clones were screened for proper homologous recombination by Southern blot analysis. When tested on HindIII-digested genomic DNA, the 5′ single-copy probe hybridized to a 12.2-kb wild-type (WT) fragment and to a 6.7-kb recombinant fragment, whereas the 3′ single-copy probe hybridized to a 12.2-kb WT fragment and to a 9.2-kb recombinant fragment. A neomycin-specific probe was used to ensure that adventitious nonhomologous recombination events had not occurred in the selected ES clones.

**Production of knock-in mice**

Mutant ES cells were injected into FVB blastocysts. Germline transmission led to the self-excision of the loxp-TACE-CRE-PGK-gb2-neo-loxP cassette in male germinal cells. Screening for proper deletion of the loxp-TACE-CRE-PGK-gb2-neo-loxP cassette and for the presence of the sequence coding for OST was performed by PCR using the following pair of primers: sense 5′-CATGACCTCTTATGCACTGTTGAAA-3′ and antisense 5′- CAGCCACCTCTTTTCCACAG-3′. This pair of primers amplified a 286-bp band for the WT allele and a 476-bp band for the *Pag1<sup>OST</sup>* allele.

**Mice**

WT and *Pag1<sup>OST</sup>* (B6-*Pag1<sup>tm1Mal</sup>*) knock-in mice were maintained in specific pathogen–free conditions, and all experiments were done in accordance with French and European guidelines for animal care.

**Abs**

The following Abs were used for flow cytometry: anti-CD4 (RM4-5), anti-CD8 (53-6-7), anti-TCRα (GL-3), anti-TCRβ (53-7-3), anti-CD19 (6D5), anti-CD20 (AME-14), and anti-CD44 (IM7; all from BD Biosciences). The following mAbs were used for immunoblotting: anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-PAG (PAG-C1; Eshio Pharma), anti-LAT (11B12; Santa Cruz Biotechnology), and anti-NCK1 (610100; BD Biosciences). The following polyclonal rabbit Abs were used: anti-FYN, anti-ZAP-70, anti-VAV1, anti-cCBL, anti–PLC-γ1 (all from Cell Signaling Technology), anti-CSK (Santa Cruz Biotechnology), anti-GRB2, and anti-Dock2 (both from Millipore), and anti-RITPR (32).

**Flow cytometry**

Flow cytometry analysis was performed as previously described (33). Before staining, cells were incubated on ice for ≥10 min with 2.4G2 Ab to block FcRs. Cells were subsequently stained, and multiparameter analysis was performed using a FACS LSR II system (BD Biosciences). Data analysis was performed using BD FACSDiva software (BD Biosciences).

**Calcium flux measurement**

Thymocytes or splenocytes were loaded with Indo-1 (Molecular Probes) for 30 min at 37°C and stained with PE-Cy7–coupled anti-CD4 (RM4-5) and allophycocyanin-conjugated anti-CD8 (Ly-2) (both from BD Biosciences). Baseline fluorescence was recorded with an LSR II (Becton Dickinson) after the addition of biotinylated anti-CD3 (2C11; BD Biosciences). Cross-linking of the TCR–CD3 complexes was induced by the addition of avidin. Calcium fluxes were determined for gated CD4+ and CD8+ cells. As a control, cells were stimulated with ionomycin.

**Cell-proliferation assay**

Purified CD4+ T cells were labeled with CellTrace Violet (Life Technologies) and plated at 10⁶ cells/ml on uncoated plates or on plates coated...
with anti-CD3 alone or with anti-CD3 and soluble anti-CD28. Cell proliferation was analyzed by FACS after 3 days of culture.

**CD4+ T cell isolation and short-term expansion**

CD4+ T cells were purified from pooled lymph nodes and spleens with a Dynabeads Untouched Mouse CD4+ T Cell Kit (Life Technologies); cell purity was >95%. Purified CD4+ T cells were briefly expanded with plate-bound anti-CD3 (145-2C11) and soluble anti-CD28 (37-51; both from American Type Culture Collection). After 48 h of culture, CD4+ T cells were harvested and grown in the presence of IL-2 (5–10 U/ml) for 48 h prior to stimulation.

**Stimulation and lysis of thymocytes and T cells**

A total of 100 × 10^6 thymocytes or short-term expanded CD4+ T cells from C57BL/6 and Pagfost mice was left unstimulated or stimulated at 37°C with perevanadate or Abs. Pervanadate stock solution was made as described (29). For stimulation with Abs, CD4+ T cells were incubated with anti-CD3 (0.2 μg/10^6 cells; 145-2C11) and anti-CD4 (0.2 μg/10^6 cells; GK1.5; both from American Type Culture Collection) on ice, followed by one round of washing at 4°C and then cross-linkage with purified Rabbit anti-Rat (0.4 μg/10^6 cells; Jackson ImmunoResearch) at 37°C. Stimulation was performed for 30, 120, and 300 s and stopped by the addition of a twice-concentrated lysis buffer (2% n-dodecyl-β-maltoside, 10% glycerol, 100 mM Tris [pH 7.5], 270 mM NaCl, 1 mM EDTA, [pH 8]) supplemented with protease and phosphatase inhibitors. After 10 min of incubation on ice, cell lysates were centrifuged at 20,000 × g for 15 min at 4°C. Postnuclear lysates were used for affinity purification or immunoblot analysis.

**Affinity purification of protein complexes**

Postnuclear lysates were incubated with prewashed Strep-Tactin Sepharose beads (Iba) for 1.5 h at 4°C on a rotary wheel. Mild salt and pH conditions were used during the whole-complex purification to favor copurification of PAG-interacting proteins. Beads were washed five times with 1 ml lysis buffer in the absence of detergent and protease and with phosphatase inhibitors. Proteins were eluted from the Strep-Tactin Sepharose beads with 2.5% acetic acid. To achieve the t-biotin affinity pull-downs were precipitated with trichloroacetic acid, washed three times with acetone, air-dried, and resolubilized in 50 μl 8 M urea in 50 mM NH4HCO3 (pH 8.8). Cysteine bonds were reduced with DTT and alkylated with chloroacetamide.

**Tandem mass spectrometry analysis**

Protein samples were loaded on an SDS-PAGE gel (0.15 × 8 cm) and subjected to electrophoresis. Migration was stopped as soon as the protein sample entered the gel. The gel was briefly stained with Coomassie blue, and a single slice containing the whole sample was excised. The gel slice was washed twice with 100 mM ammonium bicarbonate and once with 100 mM ammonium bicarbonate-acetoniitrile (1:1). The three extracted fractions were pooled with the solution in which the digestion occurred and air-dried. To identify PAG-binding partners, the tryptic peptides were directly resuspended in 22 μl 5% acetonitrile, 0.05% trifluoroacetic acid and analyzed by mass spectrometry (MS). For mapping of PAG phosphorylation sites, an enzymatic cleavage step was performed using 0.5 μg endoproteinase-GluC (Sigma) overnight at 37°C. The resulting peptides were extracted from the gel by one round of incubation (15 min, 37°C) in 50 mM ammonium bicarbonate overnight at 37°C. The resulting peptides were extracted from the gel by one round of incubation (15 min, 37°C) in 50 mM ammonium bicarbonate and two rounds of incubation (15 min each, 37°C) in 10% formic acid–acetonitrile (1:1). The three extracted fractions were pooled with the solution in which the digestion proceeded and air-dried. To identify PAG-binding partners, the tryptic peptides were directly resuspended in 22 μl 5% acetonitrile, 0.05% trifluoroacetic acid and analyzed by mass spectrometry (MS). For mapping of PAG phosphorylation sites, an enzymatic cleavage step was performed using 0.5 μg endoproteinase-GluC (Sigma) overnight at 37°C. The resulting peptides were air-dried and resuspended, as described above.

Peptides were analyzed by nano–liquid chromatography (LC) coupled to tandem MS using an UltiMate 3000 system (Dionex, Amsterdam, The Netherlands) coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Five microliters of each sample was loaded on a C-18 precolumn (300 μm inner diameter × 5 mm; Dionex) in a solvent mode of 0.5% aceto-nitrile and 0.1% formic acid and at a flow rate of 20 μl/min. After 5 min of desalting, the pre-column was switched online with the analytical C-18 column (75 μm inner diameter × 15 cm; Reprosil C18) equilibrated in 95% solvent A (5% acetonitrile, 0.2% formic acid) and 5% solvent B (90% acetonitrile, 0.2% formic acid). Peptides were eluted using a 5–50% gradient of solvent B over 105 min at a flow rate of 300 nM/min. The LTQ Orbitrap Velos was operated in a data-dependent acquisition mode with Xcalibur software. Survey scan MS was acquired in the Orbitrap on the 350–2000 m/z range, with the resolution set to a value of 60,000. The 20 most intense ions/scan were selected for fragmentation by collision-induced dissociation, and the resulting fragments were analyzed in the linear trap. Dynamic exclusion was used within 60 s to prevent repetitive selection of the same peptide. Triple-precursor LC-MS measurements were performed for each sample.

**Protein identification and quantification**

Raw MS files were processed with MaxQuant software (version 1.5.0) for database search with the Andromeda search engine and quantitative analysis. Data were searched against Mus musculus entries in the Uniprot protein database (release UniprotKB/Swiss-Prot 2014_09; 16699 entries). Carbanidithiolemonium of cysteines was set as a fixed modification, whereas oxidation of methionine, protein N-terminal acetylation, and phosphorylation of serine, threonine, and tyrosine were set as variable modifications. Specificity of trypsin digestion was set for cleavage after K or R, and two missed trypsin cleavage sites were allowed. The precursor mass tolerance was set to 20 ppm for the first search and 4.5 ppm for the main Andromeda database search. The mass tolerance in tandem MS mode was set to 0.5 Da. Minimum peptide length was set to 7 aa, and minimum number of unique peptides was set to one. Andromeda results were validated by the target-decoy approach using a reverse database at both a peptide and protein false-discovery rate of 1%. For label-free relative quantification of the samples, the match between runs option of MaxQuant was enabled with a time window of 0.5 min, to allow cross-assignment of MS features detected in the different runs.

**Filtering of unspecific binding proteins**

To identify the most specific PAG-binding partners, the intensity metric from the MaxQuant protein group.txt output (sum of peptide intensity values for each protein) was used to compare proteins identified in samples purified from CD4+ T cells or thymocytes from Pagfost and from WT (control) mice. Normalization across the compared samples was performed by adjusting the median of the intensity values for all of the proteins identified and quantified in each analytical run. Missing protein intensity values were replaced by a constant value calculated independently for each sample as the lowest one percentile value of the total population. For each protein, a mean intensity value was computed from technical LC-MS replicate runs of each sample, and then a total summed intensity value was calculated over the different times of stimulation. Only proteins that were enriched 2-fold compared with the control experiments and present in at least three out of the four independent biological experiments were considered PAG-interacting partner candidates.

**Kinetics of the binding of proteins to PAG**

Prior to analyzing the kinetics of assembly and disassembly of the proteins interacting with the PAG bait over 300 s of stimulation, the variability that may occur during affinity purification (yielding slightly distinct amounts of bait per sample) and MS analytical processes was corrected in each sample by normalizing the protein intensity values to that of the PAG bait. The abundance of PAG present in each sample was estimated from the sum of the intensity values of PAG phosphorylated peptide ions. To validate the results from PAG phosphorylation during stimulation, all of the PAG peptide ions containing a residue susceptible to phosphorylation were eliminated from the calculation. Following normalization, mean intensity values were calculated from triplicate MS measurements. Low SD values (mean SD across the four time stimulation time points ≤ 0.3) were used to select the PAG-interacting proteins showing reproducible kinetics of interaction. To facilitate the comparison of the kinetics of binding of different proteins with PAG, protein-intensity values were standardized on a 0 to 1 scale, with 1 corresponding to the highest value reached over the 300 s of stimulation.

**Mass spectrometric analysis of PAG phosphorylation**

PAG phosphorylation sites were mapped and quantified over the 300 s of stimulation by MS analysis of PAG peptides obtained after cleavage of the affinity-purified samples with trypsin alone or with trypsin and endopro-teinase-GluC (Sigma). Phosphorylated peptide ions and their nonphosphorylated counterparts were quantified by retrieving their intensity values from the MaxQuant evidence.txt quantitative output that contains quantitative data for all peptide ions. Run-to-run variation in intensity values was normalized according to the abundance of the PAG bait, as described above. Mean intensity values were then calculated from technical LC-MS replicates. To evaluate the relative abundance of phosphorylation at a given site, total areas of tryptic peptides encompassing the site were calculated for both the unphosphorylated and phosphorylated forms by aggregating data corresponding to peptide ions charge states (2+ and 3+), modification other than phosphorylation (oxidized methionine), and trypsic miscleavages.
Results

Generation and validation of Pag$^{OST}$ mice

To identify the composition and dynamics of the signalosome that formed around PAG after TCR activation of thymocytes and CD4$^+$ T cells, we generated a line of gene-targeted mice that express an OST for affinity purification (35) at the C terminus of endogenous PAG. Mice homozygous for alleles encoding OST-tagged PAG (Pag$^{OST}$; also known as B6-Pag$^{m1Mal}$) were established from an C57BL/6-derived ES cell line (Supplemental Fig. 1A, 1B). Immunoblot analysis of thymocytes from WT and Pag$^{OST}$ mice showed that addition of the 29-aa OST sequence resulted in PAG-OST protein with a higher m.w. than that of WT PAG, and it had no effect on PAG expression (Supplemental Fig. 1C). Analysis of the thymus of Pag$^{OST}$ mice showed a normal sequence of T cell development, and Pag$^{OST}$ spleen contained normal numbers of CD4$^+$ and CD8$^+$ T cells with a normal ratio of naive (CD44$^+$CD62L$^{hi/p}$)/memory (CD44$^{hi/p}$CD62L$^{lo}$$^m$) cells (Fig. 1A). Comparison of CD4$^+$ and CD8$^+$ T cells purified from WT and Pag$^{OST}$ mice and stimulated with Ab to CD3 in the presence or absence of anti-CD28 showed that PAG-OST molecules had no detrimental effect on their proliferation (Fig. 1B) or production of intracellular calcium mobilization (Fig. 1C). Therefore, thymocytes and T cells of Pag$^{OST}$ knock-in mice were normal.

PAG can be affinity purified from thymocytes and CD4$^+$ T cells from Pag$^{OST}$ mice

Thymocytes from WT and Pag$^{OST}$ mice were stimulated with anti-CD3 and anti-CD4 Abs. Cell lysates were prepared before

![FIGURE 1. Normal development and function of T cells in Pag$^{OST}$ mice.](http://www.jimmunol.org/)

(A) Flow cytometry of WT and Pag$^{OST}$ thymocytes and splenocytes. Thymocytes were analyzed for the expression of CD4 and CD8; numbers in quadrants indicate percentages of CD4$^+$CD8$^+$ double-positive, CD4$^+$ single-positive, CD8$^+$ single-positive, and CD4$^+$CD8$^+$ double-negative thymocytes. Splenocytes were analyzed for the expression of the T cell–specific marker CD5 and the B cell–specific marker CD19. Splenic CD19$^+$CD5$^+$ T cells were analyzed for CD4 and CD8 expression, and CD4$^+$ T cells were further analyzed for the expression of CD44 and CD62L. (B) CellTrace Violet (CTV) dilution by WT and Pag$^{OST}$ CD4$^+$ T cells that were left unstimulated (None) or activated for 72 h with plate-bound anti-CD3 in the presence or absence of soluble anti-CD28. (C) Changes in intracellular calcium in WT and Pag$^{OST}$ CD4$^+$ T cells stimulated with biotinylated anti-CD3 and avidin (arrow indicates time of avidin addition), assessed with the fluorescent Ca$^{2+}$ indicator dye Indo-1 AM. All data are representative of at least two experiments.
and after activation and incubated with Sepharose beads coupled to Strep-Tactin, a streptavidin derivative that binds with high affinity to the OST sequence. Bound proteins were eluted with D-biotin (a competitive ligand that binds to Strep-Tactin with a higher affinity than the OST sequence) and subjected to immunoblot analysis with anti-PAG Ab prior to and after stimulation. Eluted proteins were analyzed by immunoblots and probed with Ab to p-tyrosine (Anti–P-tyr), anti-PAG, or anti-CSK. Data are representative of at least three experiments.

**FIGURE 2.** Analysis of the kinetics of PAG tyrosine phosphorylation and CSK recruitment by PAG molecules in thymocytes. Thymocytes from WT and Pag1OST mice were left unstimulated (0) or stimulated for 30, 120, or 300 s with anti-CD3 and anti-CD4 (A) or with pervanadate (B). Equal amounts of proteins from total lysates were subjected to affinity purification (AP) on Strep-Tactin Sepharose beads, followed by elution of proteins with D-biotin. Eluted proteins were analyzed by immunoblots and probed with Ab to p-tyrosine (Anti–P-tyr), anti-PAG, or anti-CSK. Also shown are loading control corresponding to total lysates probed with anti-PAG and molecular mass (kDa). Data are representative of at least two experiments.

As outlined in the Introduction, previous studies of T cells showed that PAG was constitutively phosphorylated in resting T cells, rapidly dephosphorylated upon TCR stimulation, and rephosphorylated at later time points. In contrast, we showed that stimulation with anti-CD3 plus anti-CD4 induced a transient increase in PAG-OST tyrosine phosphorylation (Fig. 2A). PAG molecules contain a C-terminal motif that interacts with the cytoskeletal linker ezrin-radixin-moesin binding protein of 50 kDa (37, 38). Although the functional consequence of this last interaction remains to be defined, introduction of the OST sequence at the C-terminal end of PAG may have disrupted such interaction and accounted for the unexpected kinetics of tyrosine phosphorylation observed with PAG-OST molecules. To determine whether PAG-OST molecules behaved differently from WT PAG molecules, CD4+ T cells from WT mice were stimulated with anti-CD3 and anti-CD4 or with pervanadate, and PAG molecules were immunoprecipitated with anti-PAG Ab prior to and after stimulation. Anti-CD3 plus anti-CD4 stimulation induced a transient increase in the tyrosine phosphorylation of WT PAG molecules, whereas pervanadate stimulation induced a sustained tyrosine phosphorylation of WT PAG molecules (Fig. 3C). Therefore, upon T cell activation, the intensity of PAG tyrosine phosphorylation was commensurable to the intensity of PAG tyrosine phosphorylation. Tyrosine-phosphorylated PAG molecules migrate with a lower mobility than unmodified PAG molecules (24); immunoblotting with anti-PAG Ab revealed that, upon pervanadate stimulation, all of the PAG-OST molecules had their mobility shifted toward higher m.w. (Fig. 2B). Consistent with the view that T cell responses induced with pervanadate are of higher magnitude than those resulting from anti-CD3 and anti-CD4 treatment, such a global shift in the migration of PAG-OST molecules was not observed in the case of anti-CD3 plus anti-CD4 treatment (Fig. 2A). Comparable results were observed with PAG-OST molecules isolated from peripheral Pag1OST CD4+ cells prior to and after activation with anti-CD3 and anti-CD4 or with pervanadate (Fig. 3A, 3B). Therefore, analysis of thymocytes and peripheral T cells from Pag1OST mice showed that stimulation with anti-CD3 plus anti-CD4 triggered a transient increase in PAG-OST tyrosine phosphorylation that is accompanied by a transient increase in associated CSK molecules. In contrast, stimulation with pervanadate led to a sustained increased in PAG-OST tyrosine phosphorylation and CSK association.

**Comparison of WT and OST-tagged PAG molecules**

**FIGURE 3.** Analysis of the kinetics of tyrosine phosphorylation and CSK recruitment by PAG molecules in CD4+ T cells. CD4+ T cells from WT and Pag1OST mice were left unstimulated (0) or stimulated for 30, 120, or 300 s with anti-CD3 and anti-CD4 (A) or with pervanadate (B). Equal amounts of proteins from total lysates were subjected to affinity purification (AP) on Strep-Tactin Sepharose beads, followed by elution of proteins with D-biotin. Eluted proteins were analyzed by immunoblots and probed with Ab to p-tyrosine (Anti–P-tyr), anti-PAG, or anti-CSK. Also shown are loading control corresponding to total lysates probed with anti-PAG and molecular mass (kDa). (C) Equal amounts of proteins from total lysates of WT CD4+ T cells were immunoprecipitated (IP) using anti-PAG Abs, analyzed by immunoblots, and probed with Ab to p-tyrosine (Anti–P-tyr), anti-PAG, or anti-CSK. Also shown is loading control corresponding to total lysates probed with anti-PAG. Data are representative of at least two experiments.

As outlined in the Introduction, previous studies of T cells showed
FIGURE 4. PAG protein–protein interaction network in resting and activated thymocytes and CD4⁺ T cells. Affinity purification and MS analysis of protein–protein interactions in thymocytes (A) and CD4⁺ T cells (B) before and at 30, 120, and 300 s after pervanadate stimulation using PAG as a bait (all proteins are identified by UniProt designations; additional details on the high-confidence interacting proteins identified are given in Supplemental Tables I, II). The key shows the classification according to function. Interactions between the PAG bait and proteins are represented by lines. Interactions documented in four out of four and three out of four independent biological experiments are shown in dark or pale blue lines, respectively. It should be noted that AP-MS analysis does not permit a conclusion about whether such interactions are direct or indirect. E3 ligase, ubiquitin ligase, transporter, adaptor, and transmembrane receptor; GAP, GTPase-activating protein; GEF, guanine-exchange factor; Kinase, tyrosine or serine-threonine kinase; Phosphatase, phospholipid or tyrosine phosphatase.
activation, PAG-OST proteins showed kinetics of inducible tyrosyl-phosphorylation and an ability to recruit CSK similar to that of WT PAG molecules, suggesting that they can be used in AP-MS analysis.

Characterization of PAG complexes in thymocytes and CD4+ T cells

To identify the interacting partners of PAG (the PAG interactome) in thymocytes and peripheral CD4+ T cells, both cell types were isolated from Pag1OST or WT mice and lysed with n-dodecyl-β-maltoside before or at various times after activation with pervanadate, and the protein complexes containing PAG-OST were isolated using Strep-Tactin-Sepharose beads. After eluting samples with D-biotin, protein complexes were analyzed by LC coupled with tandem MS. We identified proteins with a final false-discovery rate, 1%. Proteins were quantified based on MS signal-intensity analysis. A protein was considered to interact with PAG when it was enriched 2-fold in CD4+ cells derived from Pag1OST mice over CD4+ cells derived from WT mice and was found to be enriched in at least three of the four independent biological experiments (Supplemental Tables I, II).

Using those high-confidence interaction data sets, 93 and 39 proteins were found to interact specifically with PAG in thymocytes and CD4+ peripheral T cells, respectively (Fig. 4). The proteins identified were functionally diverse and included adaptor proteins, PTPases, kinases, transporters, guanine exchange factor, GTPase activating protein, transmembrane receptor proteins, E3 ubiquitin ligase, and proteins involved in regulation of transcription (Fig. 4). Twelve proteins were associated with PAG in both thymocytes and CD4+ peripheral T cells (Table I), including CSK (13, 14, 23, 24, 39) and FYN (39), two of the best-characterized PAG-interacting proteins. ZAP-70 and the GRB2 adaptor were also present among those shared interactors, consistent with observations made in Jurkat T cells (13). Shared interactors also include the adaptors NCK1 and CRKL, the PTPase PTPN22 (also known as PEP), the phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase SHIP1, and the guanine nucleotide-exchange factor DOCK2. Although these molecules were implicated in T cell signaling, they were not shown previously to participate in PAG signaling in published analyses of human or mouse T cells. Others PAG-interacting proteins, such as the formin-like protein FMNL1, the glutathione peroxidase GPX1, and FAM49b, a protein of unknown function, have not been observed in the context of TCR signaling, to our knowledge. Several identified interactions were confirmed using immunoblot analysis of thymocytes (Fig. 5A) and CD4+ T cells (Fig. 5B). Therefore, our analysis identified many proteins not associated previously with PAG, providing cues for future functional experiments.

Dynamics of the PAG interactome

Among the proteins that constituted the PAG interactome in CD4+ T cells, some qualified for label-free quantitative analysis and showed consistent kinetics of assembly with, and disassembly from, the PAG bait after stimulation of CD4+ T cells with pervanadate.

<table>
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FIGURE 5. Validation of the interaction of PAG with selected interactors. Thymocytes (A) and CD4+ T cells (B) from WT and Pag1OST mice were left unstimulated (−) or stimulated for 30 s with pervanadate (+). Immunoblot of equal amounts of proteins from total lysates were subjected to affinity purification on Strep-Tactin Sepharose beads, followed by elution of proteins with n-biotin. Blots were probed with the Abs specified, and molecular mass is shown (kDa). Data are representative of at least two experiments.
between independent biological replicates. Those proteins were clustered on the basis of similarities in their binding dynamics (Fig. 6A). Over the first min of stimulation, protein assemblages of increasing complexity form around PAG. For instance, a subset of proteins involving ZAP-70, PTPN22, SHIP1, GRB2, NCK1, GPX1, the cytosolic nonspecific dipeptidase CNDP2, the cullin-associated NEDD8-dissociated protein 1 CAND1, and the CD2 transmembrane receptor showed maximal association with PAG-OST ∼1 min after pervanadate stimulation and then disassembled from PAG-OST with different kinetics. Consistent with immunoblot analysis (Fig. 2B), kinetics data acquired from MS analysis showed that the binding of CSK to PAG-OST reached a plateau that persists over the 300 s of observation. Together, these quantitative data provide the first systematic view of the temporal reorganization of the PAG interactorome after the stimulation of primary CD4⁺ T cells.

**Kinetics of phosphorylation of tyrosine residues found in PAG**

To support our biochemical observation that an overall increase in PAG tyrosine phosphorylation occurred following stimulation with pervanadate, we used MS analysis to determine how the degree of phosphorylation of a particular PAG tyrosine residue changes over time following T cell activation. CD4⁺ T cells from PaglOST mice were left unstimulated or stimulated for 30, 120, or 300 s with pervanadate. CD4⁺ T cells from PaglOST mice were left unstimulated (0 s) or stimulated for 30, 120, or 300 s with pervanadate. PAG-OST molecules were affinity purified using Strep-Tactin, eluted with β-biotin, and subjected to tandem MS. Label-free quantitative analysis of phosphorylation of the specified tyrosine (right margin) was performed as specified in Materials and Methods. The percentage of the phosphorylated form is shown for each specified tyrosine. Data are representative of at least two experiments.

**Discussion**

In this study, mice were developed that bear a genetic tag that permits the use of AP-MS analysis to determine the protein complexes that assemble around PAG molecules isolated from resting thymocytes and CD4⁺ T cells and over 300 s of activation. Proteins that had not been observed previously in the context of PAG signaling were identified, including, among others, the stimulator of IFN genes (also known as Tmem73), a protein that triggers the production of type I IFNs in response to the presence of DNA in the cytosol (41) and also interacts with LAT (29); CAND1, which contributes to the assembly of SFC E3 ubiquitin ligase complexes; the peptidyl-prolyl cis-trans isomerase FKBP11; the NF-κB p105 subunit NFKB1; and a series of proteins that is involved in cytoskeletal organization (COTL1, CAGP, FMNL1). These interactions are of unknown functional relevance. The set of novel PAG interactors includes several transmembrane receptors that are expressed at the surface of CD4⁺ T cells: the transferrin receptor (TFR1, also known as CD71), which is also capable of associating with LAT and ZAP-70 (29); CD2, which binds CD58 in humans (or CD48 in mice) at the surface of APCs and interacts with signaling molecules, such as CD3ζ, Lck, and LAT (42); and 4F2 (also known as CD98), a subunit of a transmembrane receptor that belongs to the solute carrier family of transporters (43). Other PAG interactors were shown to participate in the TCR signaling cascade, and their role with regard to PAG remains to be determined. They include ZAP-70, the guanine nucleotide-exchange factor DOCK2, and the adaptor proteins GRB2, CRKL, and NCK1.

FYN and CSK, two of the best-characterized PAG-interacting proteins (15) were also found in the PAG interactorome in thymocytes and CD4⁺ T cells. FYN binds to PAG through its SH3 and SH2 domains, and this is essential for tyrosine phosphorylation of PAG and the subsequent recruitment of CSK. It was proposed that the negative-regulatory role played by PAG-CSK on LCK activity is enhanced by PTPN22 and PTPN12 (also known as PTP-PEST) (44, 45), two PTPases that are thought to associate with the SH3 domain of CSK and negatively regulate LCK activity by dephosphorylating Y394. However, limited experimental evidence supports the formation of inhibitory PAG-CSK-PTPN22-PTPN12 complexes in vivo (15, 46). Interestingly, our AP-MS analysis showed that PTPN22 associated with PAG in thymocytes and CD4⁺ T cells. SHIP-1, which negatively regulates the PI3K pathway, also associated with PAG in thymocytes and CD4⁺ T cells.
Therefore, based on their kinetics of assembly with, and disassembly from, PAG following T cell activation, SHIP-1 and PTPN22 likely cooperate with CSK to terminate T cell activation.

Using phosphoproteomic analysis, we derived quantitative information on the degree of phosphorylation of eight of the nine tyrosines present in mouse PAG and how the degree of phosphorylation of a particular tyrosine changed over time in response to T cell activation. Analysis of the phosphorylation kinetics of tyrosines found at positions 107, 165, 183, 224, 314, 356, 386, and 414 of mouse PAG for up to 5 min after stimulating CD4+ T cells with pervanadate showed that their phosphorylation steadily increased over the first 2 min of stimulation and then reached a plateau or slowly decreased. Those phosphorylated tyrosines include the binding site of CSK and FYN. Analysis of the phosphorylation kinetics of Y181, Y227, Y341, Y359, and Y417 in human PAG for up to 10 min after stimulating Jurkat T cells with anti-CD3 and anti-CD4 Abs showed that their phosphorylation steadily increased over the first 3 min of stimulation and then returned to ground state levels 10 min after stimulation (8, 47). Therefore, the sustained tyrosine phosphorylation of PAG observed with pervanadate contrasts with the more transient tyrosine phosphorylation of PAG occurring in response to stimulation via the TCR and CD4, a difference that likely reflects the capacity of pervanadate to induce a potent and long-lasting inhibition of PT-Pases and that might alter the dynamics of the PAG interaction. Thus, introduction of a transgenic TCR into Pag−/− mice and the use of pMHC tetramers to stimulate the resulting T cells should better approximate the physiological conditions of stimulation. Regardless of the differences likely resulting from stimulation with pervanadate or via the TCR and CD4, PAG showed low levels of tyrosine phosphorylation in both resting Jurkat cells and primary mouse CD4+ T cells. Moreover, following stimulation with both anti-TCR and anti-CD4 Abs and with pervanadate, PAG showed a dramatic increase in the level of tyrosine phosphorylation, which reached a maximum 2 min after stimulation (our study) (8, 47). The consistent results obtained in these three independent phosphoproteomic studies are congruent with our biochemical analysis of the kinetics of PAG tyrosine phosphorylation following activation of CD4+ T cells with anti-TCR and anti-CD4 Abs, as well as with the kinetics of phosphorylation of PAG observed in many cell types in response to engagement of receptor tyrosine kinases, immunoreceptors, and integrins (15). However, they are difficult to reconcile with previous studies performed on mouse and human T cells that showed that PAG is constitutively phosphorylated in resting T cells and rapidly dephosphorylated once the TCR is engaged. The reason for such a discrepancy remains to be determined.

In conclusion, our study illustrated the power of AP-MS in providing unbiased information on the protein complexes that associate with, or disassemble from, PAG in thymocytes and CD4+ T cells upon T cell activation. A total of 93 and 39 proteins associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell suppression. J. Exp. Med. 191: 191–204.

References


