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GRB2-Mediated Recruitment of THEMIS to LAT Is Essential for Thymocyte Development

Wolfgang Paster,* Claudia Brockmeyer,*1 Guo Fu,† Philip C. Simister,* Ben de Wet,* Ana Martinez-Riaño,*2 John A. H. Hoerter,† Stephan M. Feller,‡ Christoph Wülfing,§ Nicholas R. J. Gascoigne,† and Oreste Acuto*

Thymocyte-expressed molecule involved in selection (THEMIS) is a recently identified regulator of thymocyte positive selection. THEMIS’s mechanism of action is unknown, and whether it has a role in TCR-proximal signaling is controversial. In this article, we show that THEMIS and the adapter molecule growth factor receptor–bound protein 2 (GRB2) associate constitutively through binding of a conserved PxR PxK motif within the proline-rich region 1 of THEMIS to the C-terminal SH3-domain of GRB2. This association is indispensable for THEMIS recruitment to the immunological synapse via the transmembrane adapter linker for activation of T cells (LAT) and for THEMIS phosphorylation by Lck and ZAP-70. Two major sites of tyrosine phosphorylation were mapped to a YY-motif close to proline-rich region 1. The YY-motif was crucial for GRB2 binding, suggesting that this region of THEMIS might control local phosphorylation-dependent conformational changes important for THEMIS function. Finally, THEMIS binding to GRB2 was required for thymocyte development. Our data firmly assign THEMIS to the TCR-proximal signaling cascade as a participant in the LAT signalsome and suggest that the THEMIS–GRB2 complex might be involved in shaping the nature of Ras signaling, thereby governing thymic selection. The Journal of Immunology, 2013, 190: 3749–3756.

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1Sir William Dunn School of Pathology, University of Oxford, Oxford, OX1 3RE, United Kingdom; 2Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037; 3Biological Systems Architecture Group, Wehi Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford, OX3 9DS, United Kingdom; and 4School of Cellular and Molecular Medicine, University of Bristol, Bristol, BS8 1TD, United Kingdom

Current address: Department of Molecular and Cell Biology, University of California, Berkeley, CA.

Current address: Departamento de Biologia Celular e Inmunologia, Centro Biologia Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Madrid, Spain.

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Address correspondence and reprint requests to Prof. Oreste Acuto, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, United Kingdom. E-mail address: oreste.acuto@path.ox.ac.uk

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RATIONALE: THEMIS relocates and undergoes posttranslational modifications during TCR triggering. Here, we aim to clarify its molecular function and role in T cell development. In this article, we demonstrate that THEMIS PRR1, an atypical binding motif for the C-terminal SH3 domain (SH3C) of GRB2, mediates the constitutive association of THEMIS to GRB2 and THEMIS recruitment via LAT to the immunological synapse (IS) after Ag stimulation. The Lck and ZAP70 kinases control phosphorylation of two tyrosines located shortly upstream of PRR1, regulating key TCR signaling events, and suggest that in DP thymocytes, THEMIS–GRB2 may compete with son of sevenless (SOS)-GRB2 for LAT binding, thus favoring positive selection.

Materials and Methods

Plasmids and Abs

Full-length CDNA encoding human THEMIS was obtained from Open Biosystems (NM_00101923.2; giving rise to a 641-aa protein: Uniprot Q8N1K5-1) and used as the PCR template to generate THEMIS-Strep, carrying a C-terminal One-STrEP-Tag (IBA Biotechnology, Göttingen, Germany). THEMIS-Strep was cloned into the lentiviral expression vector pHR-SIN-BX-IRES-Emerald (kindly provided by Prof. V. Cerundolo, Weatherall Institute of Molecular Medicine, Oxford, U.K.) to give rise to pHR-THEMIS-Strep. All mutants described were based on pHR-THEMIS-Strep and derived by site-directed mutagenesis (QuickChange II Kit; Agilent Technologies). The lentiviral helper plasmids psPAX2 (Addgene 10703) and pMD2.G (Addgene 12259) were provided by Dr. Didier Trono (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) via Addgene. Myc-tagged human GRB2-49L, GRB2-203R, and GRB2–49L/203R constructs (kind gift of Dr. R.A. Weinberg, Massachusetts Institute of Technology Ludwig Center for Cancer Research, Cambridge, MA) were cloned into pHRI-SIN-BX-IRES-Emerald by PCR. Plasmids pCEF-LAT-wt-type (wt)-Myc and pCEF-LAT-3YF-Myc were kindly provided by Dr. Lawrence N. Samelson (National Institutes of Health, Bethesda, MD) and used as PCR templates to generate LAT-Strep and LAT-3YF-Strep in pHRI-SIN-BX-IRES-Emerald. For BM reconstitution experiments, murine THEMIS was cloned into the retroviral expression vector pCMV-IRES-GFP to give rise to pCMV-mUTHEMIS-IRES-GFP. Mutants of murine THEMIS described in this study were derived by site-directed mutagenesis. For expression in 2C.C7 T cells, GFP-tagged versions of the above constructs were expressed from the retroviral pOC vector. All constructs were verified by sequencing. Human wt Lck and ZAP70 were cloned into pEP-BOs expression vector. Mouse Mabs used included anti-phosphotyrosine (clone 4G10; Millipore); anti-LCK (clone 3A5; Santa Cruz); anti-ZAP70 (2F3.2); anti-LAT (3A5); and anti–phospho-ZAP70 (Roche). Lysates were cleared by centrifugation at 14,000 × g for 10 min. THEMIS–Strep pull-downs were carried out on cleared lysates for 15–20 min at 4°C with Streptactin-Sepharose beads (IBA Biotechnology, Göttingen, Germany). After washing with 10-column volumes of lysis buffer, and bound proteins were eluted with 5 mM biotin. For GFP immunoprecipitations, anti–GFP-agarose (rat monoclonal, clone RQ2; MBL International) was used.

Production of recombinant Themis

HEK293 cells were transfected with the lentiviral expression construct pHR-THEMIS-Strep. GFP expression from the IRES-Emerald cassette was used to sort for highly expressing cells by FACS. Cells were harvested with PBS/EDTA and lysed in standard lysis buffer (see above). Lysates were cleared by centrifugation at 14,000 × g for 10 min and loaded onto a gravity-flow Streptactin-Sepharose column (IBA Biotechnology, Göttingen, Germany). After washing with 10-column volumes of lysis buffer, bound protein was eluted with elution buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM detsbiotin).

In vitro phosphorylation assay for mass spectrometry

A total of 50 ng recombinant Themis was incubated with 50 ng recombinant Lck (Millipore, Billerica, MA) in kinase buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM MnCl2, 1 mM ATP) for 30 min at 30°C. Reactions were stopped by adding reducing SDS NuPAGE sample buffer (Invitrogen) and incubation for 5 min at 95°C, followed by alkylation with 55 mM iodoacetamide (Sigma). Proteins were separated on a 4–12% gradient Bis-Tris NuPAGE gels (Invitrogen) and used as the PCR template to generate THEMIS-Strep, a uropod as long as an inversion of curvature of the plasma membrane accumulation was scored independently. A T cell was scored to have a uropod when 135% of the background cellular fluorescence. To classify spatial accumulation of when, where, and how THEMIS relocates and undergoes posttranslational modifications during TCR triggering can help clarify its molecular function and role in T cell development.

Cells, transfections, and lentiviral transductions

CD4+ Jurkat subclone 20 and LAT-deficient Jurkat cells (JCaM2.5) were maintained in RPMI 1640 (PAA Laboratories) medium supplemented with 10% FBS (Perbio). Human embryonic kidney epithelial cells (HEK293) were maintained in DMEM, 10% FBS. HEK293 cells were transfected with standard calcium phosphate precipitation. Lentiviral particles were produced in HEK293 cells by cotransfection of the pHRI-SIN-BX-IRES-Emerald vector with the packaging plasmids pSPAX2 and pMD2.G. Forty-eight hours after transfection, viral supernatants were harvested, filtered, and used for the transduction of the cells in the presence of 5 μg/ml Polybrene. Purumycin selection was applied 48 h after transduction where appropriate at 1 μg/ml. Immunoprecipitations and pull-down assays

Resting or anti-CD3–activated wt Jurkat cells or Jurkat cells expressing THEMIS–OST were lysed in ice-cold lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5% dodecyl-b-n-maltoside [Calbiochem], 1 mM Na2VO4, protease inhibitor mixture [Roche]). Lysates were cleared by centrifugation at 14,000 × g for 10 min. THEMIS–Strep pull-downs were carried out on cleared lysates for 15–20 min at 4°C with Streptactin-Sepharose beads (IBA Biotechnology). After pull down, beads were washed three times with lysis buffer, and bound proteins were eluted with 5 mM biotin. For GFP immunoprecipitations, anti–GFP-agarose (rat monoclonal, clone RQ2; MBL International) was used.

In vitro phosphorylation assay for mass spectrometry

Samples were analyzed on a Q Exactive (Thermo Scientific) coupled to an Ultimate 3000 RSLCnano system ( Dionex). Samples were resolved on a 25-cm-long by 75-μm internal diameter home-packed PicoTips emitter (New Objective) at a flow rate of 300 nL min⁻¹ using a 120-min gradient. The mass spectrometer was operated in a "top 10" data-dependent mode in which the 10 strongest precursors were selected for fragmentation by HCD. +1 charged ions were excluded from isolation. Data were converted to .mzXML format using MSconvert (Proteowizard) and uploaded into the central proteomics facility pipeline (CPFP) (8) for analysis. Enzyme was set to trypsin allowing for up to two missed cleavages. Carbamidomethyl cysteine was set as a fixed modification and oxidation (methionine), deamidation (NQ), acetylation (Protein-N), and phosphorytose as variable modifications. Mass tolerances for MS and MS/MS peak identifications were 20 ppm and 0.1 Da, respectively. Assignment of a tyrosine phosphorylation site required identification by searches in the CPFP at 1% false discovery rate. InterProphet probability is derived by the combination of results from multiple search engines within CPFP, and improves coverage and confidence over use of a single search engine. Modification localization scoring was performed using the ModIDS algorithm (9), a method similar to the AScope algorithm (10). ModIDS expands the AScope method to incorporate automatic specificity expansion. For each variable modification chosen for the database search, all amino acid specificities defined in the Unimod database are considered during localization.

Image acquisition and image analysis

Image acquisition and analysis were performed as described in detail elsewhere (11). In brief, T cell–APC interactions were imaged at 37°C. Every 20 s, 1 differential interference contrast and 21 fluorescence images that spanned 20 μm in the z-plane at 1-μm intervals were acquired. The acquisition and analysis software was MetaMorph (Molecular Devices). The formation of a tight cell couple, time 0 in our analysis, was defined as the first time point with a fully spread T cell–APC interface or 40 s after first membrane contact (whichever occurred first). A region of sensor accumulation was defined by an average fluorescence intensity of >135% of the background cellular fluorescence. To classify spatial accumulation features, we used six mutually exclusive interface patterns: central, invagination, diffuse, lamellal, asymmetric, and peripheral, as defined by strict geometrical constraints (for details see Supplemental Fig. 2). Distal accumulation was scored independently. A T cell was scored to have a uropod as long as an inversion of curvature of the plasma membrane could be detected at the distal pole in the differential interference contrast images. Data were routinely analyzed by two investigators to ensure the reliability of this analysis.
Bone marrow reconstitution experiments

Bone marrow cells were isolated from donor Themis1/−/− mice (B6.129S-Themis1mGlas; Jackson Laboratory Stock no. 010919) (1), which were pretreated with 5-fluorouracil 5 d before isolation. A total of 2 × 10^6 bone marrow cells were cultured in 1 ml DMEM supplemented with 10% FBS, Pen/Strep/Glut, 2-ME, and nonessential amino acids in one well of a 24-well plate. Cytokines (PeproTech) were also added to the media as 2×-3 (20 ng/ml), IL-6 (25 ng/ml), and stem cell factor (100 ng/ml). For virus generation, retroviral vectors (i.e., empty vector, WT-Themis vector, or mutated Themis vector) were transduced into Plat-E packaging cells that were preseeded 1 d before (as 5 × 10^5 cells per 10-cm petri dish). Twenty-four hours posttransfection, spent media were aspirated and replaced with 5 ml fresh media. At 48 and 72 h posttransfection, the derived viral supernatants were collected and concentrated by centrifugation with Amicon Ultra4 Centrifugal Filter Unit (UFC810024). Concentrated viral supernatants were used to spin-infect cultured bone marrow cells in the presence of 8 mg/ml Polybrene. The spin infection was carried out 2500 rpm, 32°C for 2 h. The infection efficiency of bone marrow cells was determined by analyzing the percentage of GFP+ cells with flow cytometry. A typical infection efficiency falls between 30 and 70%. Infected bone marrow cells were i.v. injected into lethally irradiated (i.e., 1100 rads in 30 min) B6.SJL recipient mice (CD45.1+ versus CD45.2+) four days after irradiation and the adoptive transfer efficiency was assessed 7 d later. Typically, 10^6 bone marrow cells were injected before storage at −80°C.

Protein expression and purification

GST and GST-Grb2 fusion proteins (both full-length and SH3C) were expressed in Escherichia coli growing in terrific broth medium at 18°C after induction with 50 μM IPTG. Cells were lysed by sonication in TPE lysis buffer (1% Triton X-100, PBS, and 100 mM EDTA) with a protease inhibitor mixture added. The mixture was centrifuged at 48,000 rpm, 32°C for 2 h. The infection efficiency of bone marrow cells was determined by analyzing the percentage of GFP+ cells with flow cytometry. A typical infection efficiency falls between 30 and 70%. Infected bone marrow cells were i.v. injected into lethally irradiated (i.e., 1100 rads in two equally split doses) B6.SJL recipient mice (CD45.1+ versus CD45.2+ marrow cells were i.v. injected into lethally irradiated (i.e., 1100 rads in two equally split doses) B6.SJL recipient mice (CD45.1+ versus CD45.2+). A typical infection efficiency falls between 30 and 70%. Infected bone marrow cells were i.v. injected into lethally irradiated (i.e., 1100 rads in two equally split doses) B6.SJL recipient mice (CD45.1+ versus CD45.2+ marrow cells were i.v. injected into lethally irradiated (i.e., 1100 rads in two equally split doses) B6.SJL recipient mice (CD45.1+ versus CD45.2+). A typical infection efficiency falls between 30 and 70%.

Peptide arrays

The full amino acid sequence of human THEMIS (Uniprot code: Q8N1K5, isoform 1) was chemically synthesized as an array of spots of overlapping peptides (Multipet synthesizer; Intavis) with a peptide length of 29 amino acids, sliding 3 residues along the sequence with each consecutive peptide spot. Membranes were incubated for 4 h in blocking buffer (5% OVA, 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20) before overnight incubation with elution buffer (100 mM reduced glutathione brought to pH 8.0 with a concentrated Tris buffer stock, pH 8.8). The eluted GST fusion proteins were further purified by gel filtration chromatography on a Superdex 75 column (GE Healthcare) in running buffer (20 mM Tris pH 7.5, 150 mM NaCl). Finally, purified proteins were dialyzed extensively against 5 mM Tris pH 7.5 and concentrated to >10 mg/ml before storage at −80°C.

Results

GRB2 binding is required for THEMIS recruitment via LAT to the IS

The highly conserved proline-rich sequence PPPPPKKHP (residues 553–561, Supplemental Fig. 1A, 1B) of THEMIS (here designated PRR1) resembles consensus binding sites for the SH3C of GRB2 (GRB2-SH3C) on Gab proteins and SLP76 lacking the typical PxxP core motif (12–14). To directly verify this prediction, we assessed binding of recombinant GST-fusion proteins of GRB2-SH3C and full-length GRB2 to an array of 29-mer peptides scanning the entire THEMIS sequence (Fig. 1A). Binding of full-length GRB2 and GRB2-SH3C was observed only to 29-mers containing the intact PRR1 sequence. A core-binding motif of PxRxPK was defined by alanine-scanning substitution and successive truncations (Fig. 1B, 1C). Pull-down experiments of Streptagged THEMIS from transfected HEK293 cells confirmed that binding of GRB2 to THEMIS was mainly mediated via GRB2-SH3C, as mutation of the N-terminal SH3 domain (SH3N) had only a minimal effect (Fig. 1D). This agrees with RxxK motifs having considerably higher affinity for GRB2-SH3C than for SH3N (12, 15). In agreement with a single GRB2-binding site, THEMIS carrying alanine substitutions at P555 and P558 of PRR1 (THEMIS-PRR1) expressed in Jurkat cells showed defective binding to GRB2, whereas mutating PRR2 had no effect (Fig. 2A). THEMIS-PRR1 showed a loss of TCR-associated interaction with LAT, was not tyrosine phosphorylated, and exhibited decreased interaction with PLCγ1 (Fig. 2B), a key component of the LAT–SLP76 complex (16). The N-terminal moiety of LAT contains three (two YVNV and one YENL) binding motifs for the SH2 domain of GRB2 and may thus recruit the THEMIS–GRB2 complex. When a LAT construct with all three tyrosines at these sites mutated to phenylalanine (Y171F, Y191F, and Y226F, i.e., LAT-3YF) was expressed in the LAT-deficient Jurkat line J.CaM2.5, the TCR-induced LAT association to GRB2 and THEMIS was lost (Fig. 2C), as was THEMIS phosphorylation on tyrosine (Fig. 2D). Taken together, these data strongly suggested that constitutive binding of GRB2-SH3C to THEMIS serves to allow THEMIS recruitment onto the LAT signalsome after TCR stimulation.

An earlier study failed to detect changes in the distribution of THEMIS upon TCR triggering (5). In light of the data presented earlier, we re-examined this aspect by live-cell imaging using GFP reporters in primary T cells. We used primed T cells from 5C.C7 TCR transgenic mice recognizing a peptide from moth cytochrome c (MCC; aa 82–103) in the context of I-Ek (11). Activated 5C.C7 T cells were transduced with retroviral vectors expressing GFP fusion constructs for murine THEMIS-wt or THEMIS-PRR1 (P557A/P560A), the latter showing no detectable binding to GRB2 (Fig. 3A). Upon conjugate formation with MCC peptide-pulsed murine CH27 B cell lymphoma cells, THEMIS-wt-GFP exhibited a rapid recruitment to the T cell–APC interface, mostly in a diffuse and lamellae pattern (Fig. 3B, see Supplemental Fig. 2 for description of the individual patterns.). By contrast, interface recruitment of THEMIS-PRR1-GFP was significantly reduced (Fig. 3C). The distribution of THEMIS at the T–APC interface was less central than LAT (11), suggesting THEMIS localization with peripheral signaling TCRs. These data demonstrated that upon TCR stimulation, THEMIS is readily recruited from an intracellular pool to the T cell–APC interface in a GRB2-dependent manner, likely via LAT, supporting the idea that THEMIS has a role in regulating TCR signaling.

THEMIS is a target of Lck and ZAP70-mediated phosphorylation

Tyrosine phosphorylation of THEMIS may generate binding sites for interacting partners and/or help induce conformational changes required for protein activation. Three major protein tyrosine kinases, Lck, ZAP70, and IL-2–inducible T cell kinase (Itk), control tyrosine phosphorylation during the earliest phases of TCR signaling. In Jurkat T cells, THEMIS was rapidly tyrosine phosphorylated after CD3 stimulation or sodium pervanadate (NaPV) addition (Fig. 4A). An involvement of Itk could be ruled out by...
using the specific inhibitor BMS-509744 (17), which, in contrast to the Src family kinase inhibitor PP2, had no effect on THEMIS phosphorylation (Fig. 4B). To investigate whether Lck and/or ZAP70 phosphorylated THEMIS, we performed coexpression experiments in HEK293 cells. The data showed that Lck and ZAP70 together induced stronger phosphorylation of THEMIS than either kinase alone (Fig. 4C, 4D), an effect likely caused by Lck-mediated activation of ZAP70 (18), as evidenced by a strong ZAP70-pY493 signal only when Lck was coexpressed (Fig. 4E).

**FIGURE 1.** GRB2-SH3C binds an RxxK motif on THEMIS. (A) A peptide scanning array covering full-length human THEMIS (Uniprot Q8N1K5 isoform 1) was spot synthesized as 29 aa peptides, sliding 3 aa with each step. (Top panel) Membrane probed with 0.1 mM GST-GRB2 (full length); (bottom panel) 0.1 mM GST-GRB2 SH3C domain. Peptides surrounding the single binding region are displayed to the right with the probable binding region emphasized in bold type. (B) Alanine scanning peptide array through the identified binding region. wt residues were sequentially mutated to alanine (or glycine, when alanine present). Peptides were spotted in duplicates and the array was probed as in (A). Key GRB2 binding residues are indicated in the box. (C) Stepwise N- and C-terminal truncations of the THEMIS peptide used for alanine-scanning substitution as seen in (B). (D) HEK293 cells were cotransfected with a human THEMIS construct carrying a C-terminal OneStrepTag (THEMIS-Strep) and either empty vector, GRB2-wt-Myc, GRB2-49L-Myc (SH3N mutant), GRB2-203R-Myc (SH3C mutant), or GRB2-49L-203R-Myc (SH3C and N mutant). THEMIS was precipitated by Streptactin pull down and analyzed for bound GRB2 by immunoblotting. Data are representative of three independent experiments.

**FIGURE 2.** GRB2 binding is required for THEMIS recruitment to the LAT signalosome. (A) Themis-Strep mutants of PRR1 and 2 (P555/558A and P582/585A, respectively) expressed in Jurkat cells were pulled down with Streptactin-Sepharose and probed for GRB2 association by immunoblotting. (B) As in (A), but from resting and CD3 mAb stimulated cells and analyzed for tyrosine phosphorylation, GRB2, LAT, and PLCγ1 association. (C) LAT-deficient Jurkat J.CaM2.5 were reconstituted with LAT-Strep wt or 3YF (Y171/191/226F) mutant. LAT pull downs of CD3 Ab or NaPV-treated cells were probed for GRB2 and THEMIS binding. Relative amounts of THEMIS pulled down and normalized to LAT are shown. (D) LAT-deficient Jurkat J.CaM2.5 were transduced with THEMIS-Strep and either LAT wt or 3YF (Y171F, Y191F, and Y226F) mutant. THEMIS was pulled down from resting and stimulated cells, and assessed for tyrosine phosphorylation by immunoblotting. Data are representative of three independent experiments.
We deduce that THEMIS can be a substrate of both Lck and ZAP70. Y34, Y95, Y174, Y540, and Y541 were predicted as possible target sites by Netphos 2.0 (score > 0.8) (19). Recombinant THEMIS was phosphorylated in vitro by recombinant Lck or ZAP70, and phospho-sites Y95, Y174, Y220, Y535, Y429, Y540, and Y541 were detected with high confidence by MS (Supplemental Table I). In agreement with this, Y540 and Y541 were predicted as potential Src phosphorylation sites by the NetphosK1.0 tool and as potential Lck and ZAP70 target sites by the group-based phosphorylation scoring system (19, 20). These data were corroborated by chimeric swap constructs between wt and an all-YF mutant (all 19 tyrosines of THEMIS mutated to phenylalanine) cotransfected with Lck in HEK293 cells (Fig. 5A). An all-YF mutant except for Y540/541/S46 was still efficiently tyrosine phosphorylated. In addition, considering conservation of tyrosines both on the interspecies and intraspecies level (i.e., between THEMIS and THEMIS2 [21]; Supplemental Table I) led us to conclude that Y540 and Y541 are major TCR-induced phosphorylation sites. Attempts to detect pY540/pY541-containing tryptic peptide(s) by MS from THEMIS isolated from TCR-stimulated Jurkat cells failed maybe because of inefficient trypsin digestion as previously noted at other phosphorylation sites (22). TCR-induced tyrosine phosphorylation of a double Y540/541F THEMIS mutant expressed in Jurkat cells was profoundly affected (Fig. 5B). However, surprisingly, THEMIS-Y540/541F lost constitutive association with GRB2 and could no longer bind to LAT. Substitutions, deletions, or phosphorylation of Y540 or Y541 in peptide-array analysis did not affect GRB2 binding (Fig. 1B, 1C, 5C), nor could we detect any obvious changes in GRB2 associated to increasingly phosphorylated THEMIS in coexpression studies with Lck in HEK293 cells (Fig. 5D). It seems therefore unlikely that tyrosine phosphorylation directly modulates THEMIS–GRB2 complex formation. Nevertheless, our data suggested that a secondary/tertiary structure connecting Y540/Y541 and PRR1 makes them mutually sensitive to mutations. Indeed, tyrosine phosphorylation of THEMIS-PRR1 by Lck in HEK293 was substantially reduced (Fig. 5D). Moreover, recombinant THEMIS purified as a monomer without GRB2 was poorly phosphorylated by Lck in vitro, whereas addition of recombinant GRB2 reconstituted THEMIS tyrosine phosphorylation (Fig. 5E). In this context, it is of interest that a soluble fragment of THEMIS lacking the CABBIT-I domain did not bind GRB2 (Fig. 5F). This suggests that the PRR1 motif and/or its surrounding region are somehow connected to a distal region of THEMIS, hinting at a global compact structure of the entire protein. Thus, GRB2 association might keep THEMIS accessible to protein tyrosine kinases.

**PRR1 is crucial for THEMIS function in vivo**

Finally, we determined whether the constitutive THEMIS–GRB2 complex was relevant in thymocyte development. PRR1 in murine THEMIS was disrupted by point mutations (muTHEMIS-PRR1; see Fig. 3A). Lethally irradiated B6.5JL (CD45.1+) recipient mice were reconstituted with Themis−/− bone marrow cells (CD45.2+) retrovirally transduced either with empty vector, muTHEMIS-wt, or muTHEMIS-PRR1. GFP expression driven by an IRES-GFP cassette on the retroviral vector allowed gating on “truly” transduced cells. THEMIS-wt–transduced donor bone marrow cells reconstituted SP thymocyte development (Fig. 6A) and the peripheral T cell compartment (Fig. 6B). By contrast, perturbations of THEMIS-PRR1 led to a severe reduction in CD4 SP thymocytes and peripheral T cells. In line with the critical role of Y540/41 (Y542 and Y543 in mouse) in THEMIS phosphorylation and GRB2 binding (Fig. 6B), muTHEMIS-Y542/43F failed to reconstitute normal T cell development. Taken together, our findings demonstrate that TCR-proximal positioning of THEMIS via GRB2-LAT is physiologically important for thymocyte development.

**Discussion**

Although the essential role of THEMIS in conventional T cell development is well established, its molecular function remains elusive. Protein components that channel and tune TCR-proximal signaling show stereotypical molecular signatures. Thus, soon after TCR engagement, they are recruited directly to the TCR or LAT and are phosphorylated on tyrosine residues (23), inevitably localizing at the IS. Our study shows that these three properties are satisfied by THEMIS, conclusively defining it as an element of the...
TCR-proximal signaling machinery. We demonstrate that THEMIS requires constitutive association with the adaptor GRB2, which permits recruitment onto LAT, followed by THEMIS tyrosine phosphorylation by active Lck and ZAP70. Consistently, mutations affecting GRB2 binding strongly affected THEMIS accumulation at the IS and T cell development in THEMIS-deficient mice.

We defined GRB2-SH3C binding to THEMIS at the conserved PRR1 site, mediated by the core-binding motif of PxRPxK. Our data contradict recent reports suggesting that GRB2-SH3N mediates binding to THEMIS (5, 21). Although the reason for this discrepancy is unclear, our data do agree with published reports indicating that GRB2-SH3N preferentially binds to motifs conforming to the consensus PxxPxR (15), such as the type II polyproline helix in SOS (24), and displays only negligible affinities toward RxxK motifs (12, 15). This leaves open the possibility that GRB2, via SH3N, helps in bridging LAT and THEMIS to an unknown partner. Live-cell imaging of THEMIS-GFP showed GRB2-dependent dynamic recruitment not exclusively at the center of the IS but also into lamellar structures that transiently cover the entire interface. Such structures appear to be active sites of membrane signaling as defined by the presence of LAT and active signaling proteins.

Our data suggest that when bound to LAT, THEMIS becomes a substrate for Lck and ZAP70, which prominently phosphorylate Y540 and Y541. Database searches found no apparent consensus binding motifs surrounding pY540 and/or Y541, suggesting that these pTyr may serve another function. Enhanced tyrosine phosphorylation of THEMIS when in complex with GRB2 was somewhat surprising, as was the dependency of GRB2 binding on

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** THEMIS is a target of Lck and ZAP70-mediated phosphorylation. (A) THEMIS-Strep was pulled down from Jurkat cells treated as indicated and tyrosine phosphorylation was probed by immunoblotting. (B) Jurkat cells were left untreated or pretreated as indicated with either BMS-509744 (10 μM), PP2 (50 μM), or vehicle control (DMSO) for 30 min before CD3 mAb stimulation and THEMIS immunoprecipitation. THEMIS tyrosine phosphorylation was assessed by immunoblotting. Compiled data of two independent experiments can be found in Supplemental Fig. 3. (C) HEK293 cells were transfected with THEMIS-Strep alone or in combination with Lck and/or ZAP70. Tyrosine phosphorylation of pulled-down THEMIS was assessed by immunoblotting. (D) Quantification of THEMIS tyrosine phosphorylation by ZAP70 alone or in combination with Lck across multiple experiments expressed as fold increase over Lck-mediated phosphorylation (n = 4; *p < 0.05, Student t test). As in (C), but probed for ZAP70-pY493. Data are representative of at least three independent experiments, except for (B), which has been repeated twice.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Interdependence of Y540 and Y541 phosphorylation and PRR1 function. (A) HEK293 cells were cotransfected with Lck and either THEMIS-Strep wt, an all tyrosine to Phe mutant (19YF) and swap mutants between wt and 19YF (residue 220 as swapping boundary). Tyrosine phosphorylation of THEMIS pull-downs was assessed by immunoblotting. (B) THEMIS-Strep wt or Y540 and Y541 to Phe mutant were isolated from Jurkat cells treated as indicated and probed for tyrosine phosphorylation, GRB2, and LAT association. (C) Peptide scanning array as in Fig. 1A with phosphorylation and substitutions at Y540 and Y541. (D) HEK293 cells were cotransfected with THEMIS-Strep wt or PRR1 mutant and increasing amounts of Lck. Precipitated THEMIS was probed for tyrosine phosphorylation and GRB2 association. (E) A total of 500 ng recombinant THEMIS-Strep was phosphorylated in vitro with 25 ng recombinant Lck. Where indicated, THEMIS-Strep was preincubated with recombinant GST-GRB2 (10 μM) for 1 h. THEMIS tyrosine phosphorylation was quantified by immunoblotting. (F) HEK293 cells were transduced with C-terminally Strep-tagged lentiviral constructs of either full-length wt and Y540/541F mutant THEMIS or truncation constructs consisting of CABB domain 1 (aa 1–260) and CABB domain 2 including the tail sequence (aa 261–641). After Streptactin pull downs, THEMIS constructs were assessed for GRB2 binding by immunoblotting. Data are representative of three independent experiments.
Y540 and Y541. These data evoke similarity to GAREM, a protein regulating EGFR-proximal signaling that contains a single CABIT domain and constitutively associates to GRB2. Mutation of a tyrosine proximal to the GRB2 binding site also abolished GAREM–GRB2 association (25). These perturbations at the GRB2 binding site (a short, supposedly poorly structured sequence), induced by medium- and long-range distortional changes in THEMIS structure, indirectly suggest the existence of a complex network of intramolecular interactions. Thus, Y540/541 may be gatekeepers at a CABIT domain–proximal region undergoing conformational changes upon their phosphorylation. This may be indicative of a “closed” structure that could be unleashed for functional activation, similar to tyrosine phosphorylation–dependent mechanism that releases autoinhibition in Vav proteins (26). Further studies using recombinant THEMIS should allow testing of this model.

Notably, the THEMIS PRR1 mutant construct tested in this study gave an intermediate phenotype between wt and empty vector in thymocytes. This is in apparent contradiction to the biochemical data where we observed a complete loss of GRB2 binding. However, using a more sensitive imaging approach (Fig. 3B, 3C), we saw severely reduced and delayed, but still detectable, IS recruitment of THEMIS–PRR1 when compared with THEMIS-wt. A residual binding affinity for GRB2 of the disturbed, but not deleted, RXXK region might yield the intermediate phenotype of either favoring a LAT-SLP76 signalosome configuration triggering the weak but sustained ERK and calcium signaling required for positive selection (28). Agonist-driven negative selection (that elicits strong and transient ERK activation) (28) might instead favor SOS1-GRB2/LAT-type signalosomes during the late phases of (or post) positive selection, when THEMIS levels decrease, thus favoring cell death. The observation that positive section requires THEMIS (1–3), but not SOS proteins (27), and recent data that SOS1 is instead required for negative selection (29) agree with this model. A functional role of constitutive THEMIS–GRB2 association in thymocyte positive selection agrees with data on conditional ablation of GRB2 in thymocytes (30), which showed that GRB2–SH2 and –SH3C (required for association to THEMIS) were found to be indispensable for thymocyte development (30).

In conclusion, our data firmly establish that thymocyte development crucially depends on THEMIS as a component of the TCR-proximal signaling machinery. It will be important to identify other THEMIS interacting partners, and the molecular basis by which it regulates the TCR signaling cascade.

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Disclosures
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References
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