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Phosphorylation of Grb2-Associated Binder 2 on Serine 623 by ERK MAPK Regulates Its Association with the Phosphatase SHP-2 and Decreases STAT5 Activation

Mary Arnaud,* Catherine Crouin,* Catherine Deon,† Denis Loyaux,‡ and Jacques Bertoglio²*²

IL-2 stimulation of T lymphocytes induces the tyrosine phosphorylation and adaptor function of the insulin receptor substrate/Grb2-associated binder (Gab) family member, Gab2. In addition, Gab2 undergoes a marked decrease in its mobility in SDS-PAGE, characteristic of migration shifts induced by serine/threonine phosphorylations in many proteins. This migration shift was strongly diminished by treating cells with the MEK inhibitor U0126, indicating a possible role for ERK in Gab2 phosphorylation. Indeed, ERK phosphorylated Gab2 on a consensus phosphorylation site at serine 623, a residue located between tyrosine 614 and tyrosine 643 that are responsible for Gab2/Src homology 2 domain-containing tyrosine phosphatase (SHP)-2 interaction. We report that pretreatment of Kit 225 cells with U0126 increased Gab2/SHP-2 association and tyrosine phosphorylation of SHP-2 in response to IL-2, suggesting that ERK phosphorylation of serine 623 regulates the interaction between Gab2 and SHP-2, and consequently the activity of SHP-2. This hypothesis was confirmed by biochemical analysis of cells expressing Gab2 WT, Gab2 serine 623A or Gab2 tyrosine 614F, a mutant that cannot interact with SHP-2 in response to IL-2. Activation of the ERK pathway was indeed blocked by Gab2 tyrosine 614F and slightly increased by Gab2 serine 623A. In contrast, STAT5 activation was strongly enhanced by Gab2 tyrosine 614F, slightly reduced by Gab2 WT and strongly inhibited by Gab2 serine 623A. Analysis of the rate of proliferation of cells expressing these mutants of Gab2 demonstrated that tyrosine 614F mutation enhanced proliferation whereas serine 623A diminished it. These results demonstrate that ERK-mediated phosphorylation of Gab2 serine 623 is involved in fine tuning the proliferative response of T lymphocytes to IL-2. The Journal of Immunology, 2004, 173: 3962–3971.

The Grb2-associated binder (Gab)³ protein family includes Drosophila daughter of sevenless (Dos), Caenorhabditis elegans suppressor of Ctr (Soc)-1, and mammalian Gab1, Gab2, and Gab3 (1–6). Gab2 was initially cloned as a binding protein and substrate of the Src homology 2 (SH2) domain-containing tyrosine phosphatase (SHP)-2 in an IL-3 responsive cell line (5). Gab proteins behave as scaffolding adaptor proteins that are able to recruit signaling intermediates containing protein-protein interaction domains such as SH2 or SH3 domains. Gab proteins contain an N-terminal pleckstrin homology domain, which is thought to address them to membranes, proline-rich motifs that are binding sites for SH3 domain-containing proteins, and a large number of potential tyrosyl phosphorylation sites, which could recruit SH2 domain-containing proteins. Gab proteins assemble multimeric signaling complexes by binding to various signal relay molecules including SHP-2, p85 PI3K, CrkL, phospholipase C, SHIP, and Grb2 in response to a variety of growth factors and cytokines (reviewed in Refs. 7 and 8).

SHP-2 is a ubiquitously expressed tyrosine phosphatase characterized by two SH2 domains in tandem in its N-terminal region followed by a tyrosine phosphatase domain and a C-terminal tail, which contains two phosphorylable tyrosine residues and a proline-rich region (9). Activation of the phosphatase activity requires interaction of the two SH2 domains with a bisphosphotyrosyl activation motif found either in receptor cytoplasmic domains or in scaffold proteins. This interaction induces conformational changes that release an intramolecular inhibitory bound between the N-terminal SH2 domain and the phosphatase domain, leading to an active conformation (10). SHP-2 is thought to behave either as a positive or as a negative regulatory protein. In many signaling pathways SHP-2 positively regulates MAPK activation either through its adaptor function or through its phosphatase activity (11–18). In response to prolactin, it has been reported that SHP-2-enhanced tyrosine phosphorylation and transcriptional activity of STAT5 probably by dephosphorylating an inhibitory tyrosine residue in Jak2 (19). However, the phosphatase activity of SHP-2 is also involved in termination of signaling, for example by direct dephosphorylation of STAT proteins as demonstrated in various cell types (20–24).

IL-2 is a major growth factor for T lymphocytes (25). We and others have demonstrated that IL-2-induced tyrosine phosphorylation of Gab2 and its interaction with its SH2 domain-containing partners, SHP-2, p85 PI3K, and CrkL (5, 26, 27). Furthermore, IL-2 stimulation of Kit 225 human T lymphocytes induced a slower mobility of Gab2 in SDS-PAGE. In addition to other possible posttranslational modifications induced by cell stimulation, characteristic migration shifts can be induced in many proteins by...
phosphorylation on serine and/or threonine residues. All Gab pro-
teins contain multiple potential seryl/threonyl phosphorylation
sites whose phosphorylating kinases and resulting functions are
largely unknown. We report that pretreatment of Kit 225 cells with
the MEK inhibitor U0126, strongly decreased the characteristic
shift of Gab2 in response to IL-2 and increased Gab2/SHP-2 asso-
ciation, an effect that could be ascribed to ERK phosphorylation
of serine 623.

Signaling pathways activated by IL-2 have been studied in
depth. IL-2 activates the MAPK pathway, the Jak/STAT pathway
and the PI3K/Akt pathway (28–30). Recently, we have investi-
gated the role of Gab2/SHP-2 interaction in the regulation of the
ERK pathway and demonstrated that this interaction is critical for
ERK activation, Elk-1 transactivation and c-fos expression (see Ref. 55). To analyze the role of serine phosphorylation of Gab2 in
human T lymphocytes, we investigated the effect of expressing a
Gab2 S623A mutant on the ERK and STAT pathways. Our results
suggest that ERK-mediated phosphorylation of Gab2 reduces
SHP-2 recruitment, which down-regulates ERK activation and al-
 lows for sustained activation of STAT5 and subsequent cell
proliferation.

Materials and Methods

Cell lines and culture conditions

The human T cell chronic lymphocytic leukemia-derived, IL-2-dependent
Kit 225 cell line was initially provided by Dr. T. Hori (Kyoto University,
Kyoto, Japan) (31). Cells were maintained in RPMI 1640 culture medium
containing 2 mM l-glutamine, 100 μM streptomycin, 100 U/ml penicil-
in, 2% sodium pyruvate, 10% FCS, supplemented with 0.5 mM recombi-
nant human IL-2 (proleukin), which was a gift from Chiron (Amsterdam,
The Netherlands). For stable transfectants, Kit 225 cells (10^5) were
electroporated with 10 μg of pcDNA-myc Gab2 WT, pcDNA-myc Gab2
Y614F or pcDNA-myc Gab2 S623A at 960 V and 250 V (GenePulser;
Bio-Rad, Hercules, CA), cultured 48 h in culture medium supplemented
with 800 μg/ml Geneticin (Gibco-BRL) before cloning by limiting dilution. Clones
were then screened for their myc expression by ELISA.

Reagents and Abs

Polyclonal antiserum (ref C-18) against SHP-2 protein was purchased from
Santa Cruz Biotechnology (Santa Cruz, CA). mAb against SHP-2 (PTP1D)
was obtained from BD Transduction Laboratories (Lexington, KY), Abs
against STAT5B (ref 06-969) and against phosphotyrosine (4G10) were from
Upstate Biotechnology (Lake Placid, NY), polyclonal antiserum against
hemagglutinin (HA) (12CA5), and anti-


GST-Gab2 fusion proteins were puri
fied by DNA sequencing.

For immunoprecipitation experiments, Kit 225 cells were starved 48 h (or
6 h when transfected cells were used), harvested by centrifugation, and
stained for 30 min at 4°C. Posttransfection Kit 225 cells were lysed with pre-
pared G-Sepharose beads for 30 min at 4°C, then incubated with 2–5 μg of Abs overnight at 4°C. Immune complexes were collected on protein G-Sepharose beads and
3 times with phosphate buffer and incubated with 5 μl of CIAP for 1 h at
37°C. Reaction was terminated by boiling in Laemmli sample buffer and
proteins were resolved by SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences).

For treatment with CIAP, immunoprecipitation of Gab2 was conducted as
described above, then protein-G-Sepharose beads were washed three
times with phosphate buffer and incubated with 5 μl of CIAP for 1 h at
37°C. Reaction was terminated by boiling in Laemmli sample buffer and
proteins were resolved by SDS-PAGE and electrotransferred onto PVDF
membranes (Amersham Biosciences).

G-actin

GST-Gab2 fusion proteins were purified by adsorption onto GSH-Sepha-
rose beads and eluted against 20 mM MgCl_2, 20 mM β-glycerophosphate, 0.1 mM vanadate, 2 mM DTT, 500 ng GST-Gab2 was then mixed with 1.5 unit of active Erk1 or Erk2 in
20 μl kinase buffer containing 20 μM ATP and 20 μM NPP. Reactions
were started by the addition of 10 μl of [γ-32P]ATP (10 μCi/μl; NEN),
incubated 20 min at 30°C and terminated by boiling in Laemmli sample buffer. Proteins were resolved by SDS-PAGE and electrophoretically
transferred onto PVDF membranes (Amersham Biosciences). Signals from 32P-labeled proteins were acquired quantitatively using a STORM 480 phosphor imager
(Amersham Biosciences).

For nanoscale liquid chromatography (LC)-mass spectroscopy (MS)/MS analysis, GST-Gab2 WT or GST-Gab2 S623A were phosphorylated by Erk2 as previously described except that radioactive ATP was omitted. Following one-dimensional SDS-PAGE and Coomassie blue staining, gel bands were cut using a Spot Cutter (Bio-Rad) then destained, in-gel digested with trypsin and the peptides were extracted using a MassPrep
station (Waters, Milford, MA).

Site of phosphorylation on peptide T34 was identified by LC-MS/MS analysis using a capillary LC system (Waters) coupled to a quadrupole time of flight micro mass spectrometer (Waters). The LC system was configured with a preconcentration column (C18 PepMap, 300 μm ID × 5 mm, LC Pack-
ings; Dionex, Sunnyvale, CA) in series with a nanoscale analytical column (C18 PepMap, 75 μm ID × 25 mm, LC Packings). Peptides mixture was
load onto the precolumn and washed with 0.2% formic acid for 5 min at
20 μl/min then transferred onto the analytical column and eluted at a flow
rate of 200 nM/min using the following gradient: 5% B (0–5 min) and 5–60% B (5–35 min). Solvent A consisted of 0.2% formic acid in H_2O/
CH_3CN (98/2), and solvent B consisted of 0.2% formic acid in CH_3CN/
H_2O (80/20). The column was directly coupled to the MS. The nanospray needle voltage was typically set to 1700 V. The instrument was run in automatic MS/MS mode switching between one full MS mode and six MS/MS scans. The precursor ions were fragmented in the collision cell.
using nitrogen as the collision gas. Data analysis was performed using MassLynx and ProteinLynx software and the resulting MS/MS data set was analyzed using the Mascot search engine (Matrix Science, London, U.K.). Software parameters were set to detect a phosphorylation (+80 Da) on serine, threonine, or tyrosine. The assignment of the phosphopeptide sequence was then manually confirmed by comparing the acquired MS/MS spectra of both T34 and phosphorylated T34 peptides to their theoretical fragmentation patterns.

**DNA affinity precipitation of STAT proteins**

Kit 225 and stably transfected cells were deprived of IL-2 for 6 h and stimulated or not for 10 min with 1 nM IL-2 (Sanofi-Synthélabo). Cells were harvested by centrifugation, washed in PBS and incubated for 30 min on ice in cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM vanadate, and 1 μg/ml each leupeptin, pepstatin, and aprotonin). The double-stranded 5'-biotinylated oligonucleotides (5'-GTATTTCCCAGAAAGGAAAC-3') from the GAS (IFN-γ activated sequence) site of the FcγR promoter were coupled to streptavidin-agarose beads for 1 h at 4°C. Total cell extracts were then incubated for 2 h with the precoated beads. Beads were then washed three times with lysis buffer and bound proteins were recovered by boiling in Laemmli sample buffer, resolved by SDS-PAGE and electrotransferred onto PVDF membranes (Amersham Biosciences). Immunoblot analysis was performed using anti-STAT5Ab Ab.

**Western blotting**

For immunoblotting, membranes were blocked for 2 h at room temperature with either 5% nonfat dry milk or 3% BSA in TBS, 0.5% TBST. Membranes were washed four times in TBST and incubated for 1 h (or overnight at 4°C) with optimal concentrations of primary Abs diluted in TBST. Following four additional washes in TBST, the membranes were further incubated for 45 min with HRP-conjugated secondary Abs. Bound Abs were detected using Amersham ECL reagents and autoradiographic films. Blots were stripped between probings with a 30 min, 50°C incubation in 62.5 mM Tris pH 6.8, 100 mM 2-ME, and 1% SDS. Membranes were then washed and blocked for 2 h with either 5% nonfat dry milk or 3% BSA in TBST.

**Transient transfections and luciferase assay**

Transfections were performed by electroporation using a gene pulser apparatus (Bio-Rad) set at 250 V and 960 μF.

For analysis of Gab2 shift or Gab2/SHP-2 interactions, transiently transfected cells were cultured 24 h without IL-2 after electroporation and then stimulated without (control) or with 1 nM IL-2 for 10 min (or as indicated). Cells were then lysed and processed for immunoprecipitation and immunoblot analysis as previously described.

For ERK phosphorylation analysis, Kit 225 cells were transfected with pcDNA-mycGab2, Gab2 Y614F, or Gab2 S623A, and pcDNA-HA-ERK1, and cultured 12 h without IL-2 before stimulation. Cells were then lysed with hot Laemmli sample buffer and lysates were subjected to SDS-PAGE and electrotransferred onto PVDF membrane.

For STAT5 luciferase assay, exponentially growing Kit 225 cells or cells stably expressing Gab2 WT, Gab2 Y614F, or Gab2 S623A (104) were washed in RPMI 1640 medium, resuspended in 150 μl of RPMI 1640 and electroporated with 5 μg of 3xGAS-luc and 0.05 μg of pSRα-Renilla reporter, included as an internal transfection control to normalize luciferase reporter activity. Following electroporation, cells were left 24 h without IL-2, then half of the cells were stimulated with IL-2 for 12 h and cells were lysed to proceed to a dual luciferase assay according to manufacturer’s instructions (Promega). Luciferase activity was measured on a MultiLumat Plus LB 96 V luminometer (Berthold Technologies, Bad Wildbad, Germany). Results were analyzed by dividing firefly signals by Renilla signals and expressed as fold increase relative to the basal activity bad, Germany). Results were analyzed by dividing firefly signals by Renilla signals and expressed as fold increase relative to the basal activity bad, Germany). Results were analyzed by dividing firefly signals by Renilla signals and expressed as fold increase relative to the basal activity.

**Results**

**MEK-dependent phosphorylation of Gab2 and STAT5 regulation**

In cells stimulated with cytokines or growth factors, Gab2 becomes tyrosine phosphorylated and we, as well as others, have observed that Gab2 undergoes a characteristic shift in its migration pattern in SDS-PAGE. This migration shift is detectable as soon as 30 s following stimulation and persists for at least 30 min. To investigate whether this shift depended upon phosphorylation of Gab2, rather than upon other modifications that could be induced by IL-2, Gab2 immunoprecipitates were treated in vitro with alkaline phosphatase. This treatment, whose efficacy is demonstrated by extinction of the anti-phosphotyrosine signal, resulted in complete abrogation of the migration shift (Fig. 1A). Thus, the shift of Gab2 is related to its phosphorylation, and likely due to phosphorylation on serine or threonine residues because tyrosine phosphorylation rarely results in such dramatic migration changes. To investigate the pathways responsible for serine/threonine phosphorylation of Gab2, Kit 225 cells were stimulated with IL-2 in the presence of various kinase inhibitors. As shown in Fig. 1, B and C, inhibition of MEK with either

**FIGURE 1.** The IL-2-induced migration shift of Gab2 is related to its phosphorylation and blocked by MEK inhibitors. A. Kit 225 cells were stimulated with IL-2 (1 nM) for 10 min (+) or not (−). Cells were then lysed and Gab2 was immunoprecipitated with anti-Gab2 (IP: α-Gab2). Half of the immunoprecipitates (IP) were incubated with 5 U of CIAP in phosphatase buffer before immunoblot (IB) analysis. B. Kit 225 cells were stimulated with IL-2 (1 nM) for 10 min (+) or not (−), ± pretreatment with U0126 (25 μM) or Ly294002 (100 μM) for 30 min. Cells were then lysed and Gab2 was immunoprecipitated with anti-Gab2 (IP: α-Gab2). Proteins were resolved by SDS-PAGE and immunoblotted with anti-Gab2 (IB: α-Gab2). C. Kit 225 cells were stimulated with IL-2 (1 nM) for the indicated times ± pretreatment with PD98059 (50 μM), U0126 (25 μM), or SB203580 (50 μM) for 30 min. Cells were then treated as in A.
PD98059 or U0126 induced a clear reduction in the migration shift of Gab2, whereas inhibition of PI3K with LY294002 or inhibition of p38-MAPK with SB203580 had no effect. It should be noted that as Gab2 spreads toward higher m.w., signal detection decreases significantly, whether using commercially available anti-Gab2 Abs, or the mAb raised in our laboratory; the converse is also true and MEK inhibition, or in vitro treatment with CIAP, result in a more focused and higher intensity signal.

**ERK phosphorylates Gab2 on residue serine 623**

Gab2 contains a number of serine/threonine residues that could represent potential targets of phosphorylation by various serine/threonine kinases. One of these residues, S623, is located within a consensus (PxSP) phosphorylation site by ERK MAPK. Of interest this site is conserved in human, mouse and rat Gab2, but is absent in the highly homologous Gab1 and Gab3 proteins (Fig. 2A). Identification of in vivo phosphorylation sites in large proteins such as Gab, which are targets of both tyrosine and serine/threonine kinases is not a straightforward experiment, and we resorted to in vitro phosphorylation of GST-Gab2 fusion proteins to confirm that S623 was a target of ERK. For this purpose the C-terminal end of human Gab2 (aa 601–676), either wild type (GST-Gab2) or mutated on residue 623 (GST-Gab2 S623A), was subjected to an in vitro kinase assay with activated ERK1 or ERK2. Under these conditions, GST-Gab2, but not GST-0 was phosphorylated by both activated ERK1 and ERK2. Mutation of S623 in Gab2 almost completely abrogated its ability to behave as a substrate for ERK (Fig. 2B). To further establish that S623 is a direct site for ERK, in vitro phosphorylated GST-Gab2 WT and GST-Gab2 S623A were subjected to MS analysis. A peptide (T34) corresponding to aa 608–628 was identified in both Gab2 WT and Gab2 S623A, and a monophosphorylated form of the T34 peptide was detected.

**FIGURE 2.** Gab2 is phosphorylated on Ser 623 by ERK. A. Comparison of human and mouse C-terminal sequences of Gab2, Gab1, and Gab3. Binding sites for SHP-2 and consensus sequences for ERK are shown in bold, S623 consensus sequence is underlined. The first row represents the numbering of amino acid residues for human Gab2. B. ERK phosphorylates Gab2 S623 in an in vitro kinase assay, GST-Gab2 (aa 601–676) wild-type or mutated (S623A), GST-0 or MBP were subjected to an in vitro kinase assay with recombinant ERK1 or ERK2 as described in Materials and Methods. An autoradiograph (top) of the reaction products following SDS-PAGE and transfect to a PVDF membrane. Ponceau stain of the membrane is shown (bottom). C. GST-Gab2 WT and GST-Gab2 S623A (500 ng) were phosphorylated in vitro with 1.5 U of recombinant active ERK2, digested with trypsin and analyzed by mass spectrometry. A region of the spectra showing phosphorylation of a peptide (T34) from aa 608–628 of Gab2 is shown. Comparison of the two spectra and fragmentation of this peptide show that S623 is phosphorylated by ERK in vitro.
in Gab2 WT (Fig. 2C). Further MS/MS analysis of the phosphorylated T34 peptide (at m/z 781.01) clearly identified S623 as the phosphorylated residue. The absence of other phosphopeptides and the lack of detection of a phosphorylated T34 in Gab2 S623A confirmed that S623 is the only target for ERK2 in this sequence.

To confirm that phosphorylation of Gab2 on S623 played a role in the observed shift in vivo, myc-tagged Gab2 WT or S623A were transiently expressed in Kit 225 with or without active MEK and cells were stimulated with IL-2. Although detection of Gab2 is sometimes difficult in transiently transfected cells, a difficulty that is increased, as previously discussed, on phosphorylated Gab2, these experiments reproducibly indicated that expression of MEKDD induced a strong migration shift of Gab2 WT, even in the absence of IL-2 (Fig. 3, lane 3). Gab2 S623A clearly shifted less significantly than wild type both in the presence of MEKDD and in response to IL-2 (Fig. 3, lanes 6–8). That the migration shift of Gab2 S623A was not completely abolished is consistent with the presence of additional phosphorylation sites by ERK and/or other serine/threonine kinases activated in response to IL-2. Thus, serine 623 of human Gab2 is a target for phosphorylation by ERK MAPK.

**Phosphorylation of S623 modulates Gab2 interaction with SHP-2**

It is of interest that S623 is located between Y614 and Y643, the binding sites for SHP-2 on Gab2. Because Gab2/SHP-2 interaction has been shown to regulate ERK activation (see Ref. 55), we hypothesized that Gab2 phosphorylation by ERK might provide feed back regulation of the pathway. To test this hypothesis, Gab2 was immunoprecipitated from IL-2-stimulated cells treated with U0126 and its association with SHP-2 assessed by Western blot analysis. Treatment of cells with U0126 reproducibly induced a slight increase in the amount of SHP-2 associated with Gab2 (Fig. 4). This was best evidenced at 10 min following IL-2 stimulation and remained detectable at least up to 60 min. Thus ERK inhibition results in increased binding of SHP-2 to Gab2. The time course of the observed effect suggested that ERK-dependent phosphorylation of Gab2 may not affect the initial interaction of Gab2 with SHP-2, but might be involved in down-regulation of this association as a putative negative feedback loop.

We then established subclones of Kit 225 cells that stably express myc-tagged Gab2, either wild type or S623A as described in Materials and Methods. In preliminary experiments, five to eight clones of each type were characterized for myc-Gab2 expression and IL-2 responsiveness, and representative clones were selected for further studies, based upon an expression of tagged Gab2 that was estimated to be roughly similar to that of endogenous Gab2 (Fig. 5A).

These clones were then used, in parallel to Kit 225, to further analyze the role of S623 phosphorylation on Gab2/SHP-2 interaction by means of SHP-2 immunoprecipitation. As shown in Fig. 5B, more Gab2 associated with SHP-2 in clone B29, and the average migration of the Gab2 band appears less shifted than in Gab2 WT-expressing cells, indicating that indeed SHP-2-associated Gab2 S623A is less phosphorylated on serine than Gab2 WT. Given that Gab2 S623A undergoes a smaller migration shift than Gab2 WT, U0126 inhibition of ERK activation has a smaller effect at reducing this shift than on endogenous Gab2 in Kit 225 cells or on transfected Gab2 WT in clone A20. In addition, analysis of tyrosine phosphorylation of SHP-2 itself by Western blotting with the 4G10 anti-phosphotyrosine Abs revealed very interesting variations. Conversely, in both Kit 225 and A20 cells, treatment with

**FIGURE 3.** Expression of active MEK induces phosphorylation of Gab2 in Kit 225 cells. Kit 225 cells were transiently transfected with myc-tagged Gab2 or Gab2 S623A and with or without constitutive active MEK (MEKDD), cultured 24 h without IL-2 and then stimulated without (−) or with 1 nM IL-2 (+) for 10 min. Cells were then lysed and processed for immunoprecipitation with anti-myc (IP: α-Myc) and anti-myc immunoblotting (IB: α-Myc).

**FIGURE 4.** MEK inhibition increases Gab2/SHP-2 interaction. A, Kit 225 cells were starved 48 h then stimulated with 1 nM IL-2 for indicated times with or without pretreatment with U0126 (25 μM) for 30 min. Cells were then lysed and subjected to immunoprecipitation with anti-Gab2 (IP: α-Gab2). Proteins were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine (IB: α-pTyr), anti Gab2 (IB: α-Gab2) and polyclonal anti-SHP-2 (IB: α-SHP-2). B, Densitometry scanning of the blots in A was performed on several exposures to determine the limits of the linear response of the films. Data acquired within these limits were used to calculate the ratio of SHP-2 over tyrosine phosphorylated Gab2 (α-pTyr blot).
phosphotyrosine (IB: -P-Tyr), anti Gab2 (IB: -Myc), and monoclonal anti-SHP-2 (IB: -SHP-2).

U0126 significantly increased the level of tyrosine phosphorylation of SHP-2 (Fig. 5B, lanes 4 and 8 as compared with lanes 2 and 6). Conversely, the level of tyrosine phosphorylation of SHP-2 in clone B29 was much higher in response to IL-2 than in the other two cell types (Fig. 5B, lane 10 as compared with lanes 2 and 6). Consequently, treatment of B29 cells with U0126 showed no additional increase in SHP-2 phosphotyrosine content.

In a separate report, using a Gab2 Y614F mutant, which is defective in interacting with SHP-2, we have shown that SHP-2 association with Gab2 is required for tyrosine phosphorylation and activation of SHP-2 (see Ref. 55). Thus the increased association of SHP-2 with Gab2, induced by ERK inhibition in Kit 225 cells, or through expression of Gab2 S623A, correlating with increased SHP-2 tyrosine phosphorylation agrees with our previous observation.

Signaling consequences of Gab2 phosphorylation on S623

SHP-2 has been reported to be critical for, or to regulate several growth factor-induced events including the ERK-MAPK and the JAK-STAT pathways. To investigate whether this putative feedback regulatory loop influenced ERK activation induced by IL-2, Kit 225 cells were cotransfected with Gab2 and HA-tagged ERK expression plasmids, and stimulated with IL-2. Indeed the relatively low transfection efficiency in Kit 225 cells has generally prevented interpretation of effects on endogenous proteins whereas analysis of HA-ERK allowed to focus on cells that effectively co-express Gab2 constructs. Western blot analysis with anti-phospho ERK Abs clearly evidenced increased phosphorylation of transfected HA-ERK when coexpressed with Gab2 WT, whereas Gab2 Y614F prevented it. When cells were transfected to express Gab2 S623A, a slight increase in HA-ERK phosphorylation was detectable at the 15 min time point, which however did not seem to persist thereafter (Fig. 6A). Similar results were observed when IL-2-induced ERK phosphorylation was assessed in several stable clones expressing Gab2 WT or Gab2 S623A as illustrated for clones A20 and B29 (Fig. 6B).

Because it has been shown that SHP-2 could dephosphorylate STAT5, we wondered whether Gab2-mediated regulation of SHP-2 might also influence STAT5 activation in response to IL-2. STAT5 activation was first assessed using a 3xGAS-luc reporter assay in Kit 225 cells and in clones stably expressing Gab2 or its Y614F or S623A mutants. As seen in Fig. 7A, IL-2 stimulation of Kit 225 induced a 35-fold increase in luciferase expression. In cells overexpressing Gab2 WT, the IL-2 effect was reduced to a 19-fold increase whereas Gab2 Y614F expression resulted in an enhanced IL-2 response that reached an average 44-fold increase in luciferase induction. Because the only difference between Gab2 WT and Gab2 Y614F is its ability to interact with and activate SHP-2, the observed 3-fold difference in 3xGAS-luc reporter assay can probably be ascribed to SHP-2. Expression of Gab2 S623A further reduced STAT5 activation ~2-fold as compared with Gab2 WT. Similar results were observed in transient transfection experiments...
thus ruling out that these results depended upon unidentified clonal effects. In parallel experiments, we then assessed the ability of STAT5 to associate to its target DNA sequences in an affinity binding assay using GAS oligonucleotides coupled to streptavidin-agarose beads. Protein extracts from the indicated clones were incubated with immobilized oligonucleotides and analyzed in western blot with anti-STAT5 Abs. Consistent with results from the 3xGAS-luc reporter assay, Gab2 WT reduced and Gab2 Y614F increased STAT5 binding to the GAS oligonucleotide. In cells from clone B29 expressing Gab2 S623A, the amount of activated STAT5 detectable by this assay was even further reduced as compared with Gab2 WT (Fig. 7B). Expression of myc-Gab2 in clones was confirmed by immunoblot analysis (Fig. 7C).

**Effect of Gab2 mutants on Kit 225 cell proliferation**

Because ERK and STAT5 activation are known to be involved in the antiapoptotic and the proliferative effects of IL-2 on T cells, we investigated whether expressing Gab2 had any effects on cell growth. In initial experiments, we observed that the rate of apoptosis induced upon IL-2 withdrawal was comparable between Kit 225 and Gab2-expressing clones regardless of whether Gab2 was wild type or mutated (data not shown). In addition, we observed no difference with parental Kit 225 cells in clones that express myc-Gab2 WT, illustrated by clone A20 in Fig. 8. With regards to clones expressing mutated Gab2, maximum thymidine incorporation achieved at saturating doses of IL-2 was significantly higher than control for Y614F clones (Fig. 8, left panel) and slightly lower for at least one S623A clone (B29, Fig. 8, right panel). As calculated from these dose response experiments, cells expressing Gab2 S623A required an average 3.5 times as much IL-2 as cells expressing Gab2 Y614F to achieve 50% proliferation (Fig. 8). Although these effects were less than dramatic, they were confirmed using a different assay for cell proliferation, namely the MTT colorimetric assay (data not shown) and are in agreement with the observation that Y614F clones have a tendency to grow better than parental Kit 225 under our routine culture conditions, whereas most S623A clones behave as slow growers. Given the role of STAT5 in IL-2-induced up-regulation of the IL-2Rα chain, cells were studied by FACS analysis for expression of all three chains of the IL-2R. However, no significant difference in IL-2Rα, IL-2Rβ, or IL-2Rγ expression was observed (data not shown) that could provide a simple explanation for the difference in IL-2 sensitivity of these clones.
Discussion

Proteins of the Gab family act as scaffolding adaptors to regulate signaling pathways in response to cytokines and growth factors (7, 8). They do so essentially by becoming tyrosine phosphorylated, which in turn provides binding sites for SH2 domain-containing partners. They are therefore targets for a number of receptor or cytosolic tyrosine kinases. For instance Gab1 is phosphorylated by EGF-R in vitro and phosphopeptide analysis in vitro indicated that Y657 (also referred to as Y627 in most publications), which binds SHP-2, is the major site of tyrosine phosphorylation by recombinant EGF-R (34). Gab2 is also involved in EGF signaling. In contrast to Gab1, Gab2 is not phosphorylated by EGF-R but by the c-Src tyrosine kinase, which is constitutively bound to Gab2 in rat hepatocytes (35). Other kinases have been shown to phosphorylate Gab2, for example p59Fyn in mast cells in response to FceRI ligation by IgE, or p72Syk after integrin β1 activation (36, 37). In response to CSF-1, both c-Src and the CSF-1 receptor, c-Fms, appear to phosphorylate Gab2, and c-Fms also phosphorylates Gab3 (38–40). Gab2 is also a substrate for ZAP70 in response to TCR activation (41, 42).

Most of the signaling pathways that are initiated following tyrosine phosphorylation of Gab proteins lead to activation of serine/threonine kinases, such as protein kinase B/Akt, a critical actor of the PI3K pathway, or the ERK1/2 members of the MAPK family. These kinases are not only activated to transmit downstream signals, but they have also been shown to mediate either positive or negative feedback regulation by phosphorylating the scaffolding adaptors themselves. It is of interest that Gab proteins contain a large number of serine/threonine consensus sequences whose role and phosphorylating kinases are almost unknown. Protein kinase C has been reported to phosphorylate Gab1 resulting in negative regulation of hepatocyte growth factor signaling, whereas ERK phosphorylation of Gab1 in this system results in increased stimulation (43, 44). In contrast, ERK-mediated phosphorylation of Gab1 on threonine 476 in response to epidermal growth factor down-regulates PI3K activation (45). Another negative feedback mechanism has been proposed as a consequence of protein kinase B phosphorylation of serine 159 in Gab2 in heregulin-stimulated MCF7 epithelial cells (46). However, as shown in Fig. 1B, treatment with the PI3K inhibitor Ly 294002 did not affect the migration of Gab2 in response to IL-2. Therefore, we found no evidence that PI3K-activated PKB could phosphorylate Gab2 in IL-2-dependent T lymphocytes.

Sequence comparison between Gab1 and Gab2 proteins reveals the existence of two potential ERK phosphorylation sites in Gab2. Serine 480 (YPMS480P) would be equivalent to threonine 476 (YVPMT476P) of Gab1 and lies within the second binding sites for PI3K SH2 domains. Although this remains to be investigated, it is conceivable that serine 480 of Gab2 might be phosphorylated by ERK with inhibitory effects on the PI3K pathway similar to those observed with Gab1. In this study, we report for the first time that ERK phosphorylates Gab2 on S623, however we found no evidence for a direct interaction between ERK and Gab2 (data not shown). It should be noted that ERK2 associates with Gab1 via its Met-binding domain (47) and that Gab2 does not have such a Met-binding domain. Although we cannot completely rule out that such an interaction may exist between Gab2 and ERK, if it does, it should then be through a different domain of Gab2. The ERK phosphorylation site at position S623 (PS623P) is unique to Gab2, as opposed to Gab1 and Gab3, and lies only three amino acid residues from the first SHP-2 binding site (Y614LAL), toward the C-terminal end of Gab2. We hypothesized that ERK phosphorylation of S623 could affect SHP-2 binding to Gab2 and downstream signaling events in human T lymphocytes stimulated with IL-2. The results reported indeed demonstrate that ERK inhibition or S623 to A mutation in Gab2 lead to increased association of Gab2 with SHP-2. It can be inferred from this that serine phosphorylation of S623 results in decreased association. Despite several attempts, using synthetic peptides phosphorylated on both tyrosine and serine residues, or using in vitro phosphorylated GST fusion proteins, we have been unable to demonstrate any significant change in binding affinity of isolated SHP-2 SH2 domains. The molecular mechanisms resulting from S623 phosphorylation and underlying the modification in Gab2/SHP-2 interaction therefore remain elusive. It could be speculated, given the position of S623 between the two binding sites for SHP-2, that its phosphorylation induces conformational changes in the C-terminal end of Gab2 that may be detrimental to recognition by the SHP-2 tandem SH2 domains.

Concomitantly to increased association of SHP-2 with Gab2, induced by ERK inhibition or by Gab2 S623A expression, SHP-2 undergoes increased tyrosine phosphorylation. Phosphorylation of SHP-2 on tyrosine residues 542 and 580 has been reported to enhance SHP-2 signaling either by maintaining its activated conformation, and therefore its phosphatase activity (48, 49) and/or by providing binding sites for Grb2, that participate in its adaptor function, as it has been reported in response to several growth factors and cytokines (16, 17, 50–52). In IL-2-stimulated cells, SHP-2 is involved in activation of ERK (see Ref. 55) and regulation of STAT5 transcriptional activity (18, 21, 22, 53). In contrast to the well-established positive role of SHP-2 on ERK activation,
regulation of the STAT5 pathway by SHP-2 still remains controversial. Indeed, both positive and negative effects have been described. Using a catalytically inactive form of SHP-2 in NIH 3T3 containing the three chains α, β, and γ of IL-2R and Jak3, this tyrosine phosphatase was first proposed as a potential positive regulator of STAT5 transcriptional activity in response to IL-2 (54). However, several recent studies further agreed for a negative regulatory role, as SHP-2 was shown to associate with and dephosphorylate STAT5, both in vitro, and in response to IL-2 and IL-3 (21, 22). We report that increased recruitment of SHP-2 by Gab2 correlating with a strong increase of the tyrosine phosphorylation of STAT5 in SHP-2 Gab2 S623A expressing cells, lead to a small and transient, but reproducible, increase in ERK phosphorylation and to a drastic diminution of STAT5 transcriptional activity. Taken together with our observation that expression of the SHP-2 binding-deficient Gab2 Y614F abrogates the inhibitory effect of Gab2 on STAT5 activation, these results led to the conclusion that recruitment of SHP-2 by Gab2 is an essential step in regulating STAT5 activity. Thus SHP-2 plays a dual role in Gab2-mediated signaling from the IL-2R: 1) at the initiation of the response, Gab2-activated SHP-2 is required for ERK activation; 2) at later time points, secondary to ERK-mediated phosphorylation of Gab2, decreased SHP-2 activity helps down-regulating ERK, and allows for STAT5 activation to persist (Fig. 9). Precise kinetics of these fine regulatory events remain to be determined.

Our work thus clearly establishes that serine 623 of Gab2 is a phosphorylation target for ERK, with demonstrated biochemical consequences on activation of ERK itself and on activation of STAT5. Our observation that expression of Gab2 S623A in Kit 225 cells only shows minute effects on cell proliferation suggest that S623 phosphorylation is not critical but rather may be involved in fine tuning the proliferative response of IL-2-dependent cells. It remains to be investigated whether other cell types or other cytokine responses may be more drastically susceptible to such back regulatory mechanisms.

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