Roles of Phosphatidylinositol 3-Kinase in Interferon-γ-dependent Phosphorylation of STAT1 on Serine 727 and Activation of Gene Expression*

Hannah Nguyen‡, Chilakamarti V. Ramana, Joshua Bayes, and George R. Stark§

From the Department of Molecular Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Received for publication, June 1, 2001
Published, JBC Papers in Press, July 3, 2001, DOI 10.1074/jbc.M105070200

STAT1 must be phosphorylated on serine 727 to be fully active in transcription. We show that phosphatidylinositol 3-kinase (PI3K) and its effector kinase Akt play an important role in the serine phosphorylation of STAT1 and in the activation of gene expression in response to interferon-γ (IFNγ). IFNγ activates PI3K as well as Akt in a variety of cell lines. Specific inhibition of PI3K abrogates IFNγ-induced, but not interleukin-1 or tumor necrosis factor-α-induced, phosphorylation of STAT1 on serine and reduces STAT1-dependent transcription and gene expression by ~7-fold. Constitutively active forms of PI3K or Akt activate and their dominant-negative derivatives inhibit STAT1-driven transactivation in response to IFNγ. In addition to PI3K and Akt, JAK1, JAK2, and the tyrosine 440 STAT1 docking residue of IFNGR1 are required for STAT1 to be phosphorylated on serine. Taken together, these results suggest that the following events lead to the activation of STAT1 upon IFNγ stimulation: 1) PI3K and Akt are activated by the occupied receptor and Tyr-440 is phosphorylated by the activated JAKs; 2) STAT1 docks to Tyr-440; and 3) Tyr-701 is phosphorylated by the JAKs and Ser-727 is phosphorylated by a kinase downstream of Akt.

Full activation of STAT1 by IFNγ requires two distinct phosphorylation events. Receptor-mediated phosphorylation of STAT1 on tyrosine 701 is required for STAT1 homodimers to form and subsequently to bind to the promoters of IFNγ-responsive genes through γ-activated sequence (GAS) elements (reviewed in Refs. 1 and 2). The mechanistic basis for STAT1 tyrosine phosphorylation has been established for some time. JAK1 and JAK2, constitutively bound to specific cytoplasmic domains of the IFNGR1 and IFNGR2 subunits, phosphorylate each other when IFNγ binds and the receptor subunits aggregate. The activated JAKs then phosphorylate tyrosine 440 of IFNGR1, creating a docking site that recruits STAT1 to the receptor. STAT1 binds to phosphorylated Tyr-440 through its SH2 domain, allowing its Tyr-701 residue to be phosphorylated by the JAKs. STAT1 then dissociates from the receptor, dimerizes through reciprocal SH2-phosphotyrosine interactions, and binds to IFNγ-inducible promoters.

At some point, STAT1 is also phosphorylated on serine 727 independently of tyrosine phosphorylation (3–7). Serine phosphorylation substantially enhances the transcriptional activity of STAT1; the transactivation potential of the S727A mutant of STAT1 is reduced by ~80% (3). STAT1 can also be phosphorylated on serine in response to IFNα (5, 6), UV irradiation (7), bacterial lipopolysaccharide (5, 7), platelet-derived growth factor (3), phorbol esters, interleukin-2 and -12 (8), cross-linking of B-cell or T-cell receptors (9, 10), or tumor necrosis factor-α (7) (reviewed in Ref. 11).

The mechanisms through which STAT1 is phosphorylated on serine are not yet well understood. Serine 727 lies within the mitogen-activated protein kinase (MAPK) consensus motif PXϕX(S/T)ϕP, where S/T is serine or threonine and ϕ is 1 or 2, suggesting that MAPK family members may be STAT1 serine kinases. Through the use of knockout cells and dominant-negative and chemical inhibitors, several kinases have been implicated directly or indirectly (reviewed in Ref. 11). Phosphorylation of STAT1 on serine in response to IFNγ has been shown to involve JAK2 (4), proline-rich tyrosine kinase 2 (Pyk2), potentially through its activation of ERK2 (12), p38 MAPK (6), and double-stranded RNA-activated protein kinase (PKR)(13).

Phosphatidylinositol 3-kinase (PI3K) is a member of a subfamily of lipid kinases implicated in many physiological processes, including regulation of cell growth, proliferation, survival and differentiation, vesicle trafficking, glucose transport, platelet function, and cytoskeletal remodeling (reviewed in Refs. 14 and 15). PI3K is activated by phosphorylation on tyrosine residues in response to many growth factors and cytokines by receptors with intrinsic tyrosine kinase activity or by receptor-associated tyrosine kinases. A heterodimer comprised of one of three catalytic isoforms and one of seven adapter/regulatory proteins, PI3K, once active, catalyzes the addition of a phosphate moiety specifically to the 3′-OH position of the inositol ring of phosphatidylinositol 3,4,5-trisphosphate (reviewed in Refs. 14 and 15). The resulting 3-phosphorylated phosphatidylinositol 3,4,5-trisphosphate serves as a second messenger to activate many downstream signaling targets, initiating the physiological effects of PI3K. One of the best characterized PI3K effectors is the serine/threonine protein kinase Akt(PKB), whose activation following growth factor or cytokine stimulation is directly dependent on PI3K-derived phosphorylated phosphatidylinositol 3,4,5-trisphosphate (reviewed in Refs. 16–18).

* This work was supported in part by Grant P01 CA 62220 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a Cancer Research Institute Fellowship.

§ To whom correspondence should be addressed; Dept. of Molecular Biology, Lerner Research Inst., The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-444-3900; Fax: 216-444-3279; E-mail: starkg@ccf.org.

1 The abbreviations used are: STAT, signal transducer and activator of transcription; GAS, γ-activated sequence; STAT, signal transducer and activator of transcription; PI3K, phosphatidylinositol 3-kinase; JAK, Janus kinase; IFN, interferon; TNF, tumor necrosis factor-α; IL, interleukin; MAPK, mitogen-activated protein kinase; MEFs, mouse embryonic fibroblasts; PGE2, polyacylamide gel electrophoresis; GBP-1, guanylate-binding protein-1; SH2, Src homology domain 2; ERK, extracellular signal-regulated kinase.
We now find that the phosphorylation of STAT1 on serine in response to IFNγ requires the activation of PI3K and Akt. Our results reveal that PI3K functions in a novel pathway that plays an important role in signaling and in the activation of gene expression in response to IFNγ and defines receptor-associated functions that are additionally required for the phosphorylation of STAT1 on Ser-727.

EXPERIMENTAL PROCEDURES

Biological Reagents and Cell Culture—The following reagents were used at the final concentrations specified: recombinant human IFNγ (Roche Diagnostics), 1000 IU/ml; recombinant human IL-1α (National Cancer Institute), 1 ng/ml; recombinant human TNFα (BD Biosciences), 20 ng/ml; LY294.002 (LY, Sigma), 50 μM. Cells were incubated with LY for 30 min at 37 °C before cytokine treatment. Human fibrosarcoma 2FTGH and 2C4 parental cells and the derivative U4A, y2A, and B9 lines (19), T98G human glioblastoma cells, HeLa cells, primary or immortalized mouse embryo fibroblasts (MEFs), and JAK2−/− MEFs (20) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. The U4A-JAK1 or y2A-JAK2 cells (21) and the SCC16-5-hgr or SCC16-5-Y440F murine fibroblast cell lines (22, 23) were maintained in the same complete medium with the addition of 400 μM of active G418. Before cytokine treatment, 2FTGH and derivative cell lines, as well as the SCC16-5-hgr and Y440F cell lines, were grown in 1% serum for 18 h to minimize constitutive levels of STAT1. Further, the SCC16-5-hgr cell line was maintained in the same complete medium with the addition of 400 mM NaCl, and cellular debris was removed by centrifugation for 10 min. Supernatant solutions from the two lysis steps were then combined. Cell extracts were fractionated by electrophoresis in 8–10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. The following antibodies were used for Western analyses: anti-phospho-Ser-727 STAT1, anti-phospho-Tyr-701 STAT1 (both from Upstate Biotechnology), either anti-C-terminal STAT1 (Santa Cruz Biotechnology) or anti-N-terminal STAT1 (Transduction Laboratories), anti-phospho-Akt (Cell Signaling), anti-Akt (Transduction Laboratories), anti-phospho-p42/p44, anti-p42/44, anti-phospho-p38, and anti-p38 (all from Cell Signaling). Horseradish peroxidase-coupled goat anti-rabbit or goat anti-mouse immunoglobulin was used for visualization, using the enhanced chemiluminescence (ECL) Western detection system (PerkinElmer Life Sciences).

Electrophoretic Mobility Shift Assays—Cells at 80% confluence in 100-mm dishes were pretreated with either solvent (methanol) or LY for 30 min, followed by stimulation with IFNγ for 20 min. Cells were washed once with phosphate-buffered saline, and cell pellets were lysed for 20 min at 4 °C in 100 μl of 0.5% Nonidet P-40 buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 25 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM diithiothreitol, 0.4 mM phenylmethanesulfonyl fluoride, 3 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 μg/ml leupeptin. Cellular and nuclear debris was pelleted by centrifugation at 16,000 × g at 4 °C for 2 min. The high affinity STAT binding site, c-sis inducible element GAS, was end-labeled with polynucleotide kinase (Roche Diagnostics) and [γ-32P]ATP (Amersham Pharmacia Biotech) and used as a probe. The binding reaction was carried out at room temperature for 20 min with 20 μg of whole cell extract, 2 μg of poly(dI-dC) (Amersham Pharmacia Biotech), and ~20,000 cpm of probe in a total volume of 20 μl containing 20 mM HEPES, pH 7.0, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM diithiothreitol, 0.25 mM phenylmethanesulfonyl fluoride, and 10% glycerol. The DNA-STAT1 complexes were separated on 5% polyacrylamide gels by electrophoresis in 0.2× Tris borate/EDTA buffer. The gels were dried, and the labeled complexes were visualized by autoradiography.

Transfection and Reporter Assays—For luciferase reporter assays, cells at 60–70% confluence, seeded the previous day in 150-mm plates,
were transfected by the calcium phosphate method (25) with 10 μg of 4×GBP-GAS-luc (Stratagene) and 2 μg of pSV2-β-gal (to verify transfection efficiency). Eight hours after transfection, the cells were divided into four 100-mm plates. Cells were pretreated with methanol or LY for 30 min then stimulated with IFNγ for 6 h, 40 h after transfection. For experiments involving PI3K expression plasmids, cells at 60–70% confluence, seeded the previous day in 100-mm plates, were cotransfected with 2 μg of 4×GBP-GAS-luc along with 2, 4, or 8 μg of membrane-targeted, constitutively active versions of wild-type PI3K, wild-type Akt, kinase-dead PI3K, or dominant-negative Akt. Use of an additional control plasmid allowed equal amounts of DNA to be transfected. Luciferase and β-galactosidase activities were determined with the Promega luciferase assay and chemiluminesence reagents, respectively. Results are shown for one of at least three independent experiments.

Northern Analyses—Cells were stimulated for the indicated times. Total RNA was isolated with the TRIzol reagent (Invitrogen). Twenty micrograms of total RNA was denatured, separated by electrophoresis in a formaldehyde–1.2% agarose gel, and transferred to Hybond-N nylon membrane (Amerham Pharmacia Biotech). GBP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were detected with cDNAs labeled with [α-32P]dCTP (Amerham Pharmacia Biotech) by nick-translation using the DNA megaprime labeling system (Amerham Pharmacia Biotech) and visualized by autoradiography.

RESULTS

Specific Inhibition of PI3K Abolishes IFNγ-induced Phosphorylation of STAT1 Ser-727 but Not Tyr-701 and Does Not Affect IL-1- or TNF-induced Phosphorylation of STAT1 Ser-727— Pretreatment of T98G (Fig. 1A) or 2fTGH cells (data not shown) with the PI3K inhibitor LY kept serine phosphorylation of STAT1 at basal levels in response to IFNγ. The bands detected by the phosphoserine-specific STAT1 antibody are specific for residue 727, since the antibody did not detect any phosphorylated STAT1 protein in extracts from IFNγ-treated U3A-S727A cells, STAT1-null cells reconstituted with a STAT1 mutant protein containing a serine-to-alanine substitution (data not shown). Pretreatment with LY of T98G (Fig. 1B) or 2fTGH cells (data not shown) did not affect IFNγ-activated STAT1 tyrosine phosphorylation or the ability of STAT1 to bind to DNA (Fig. 1C). These findings indicate a potential role for PI3K in the serine phosphorylation of STAT1 in response to IFNγ. Because IL-1 (this study) and TNF (7) activate both STAT1 Ser-727 phosphorylation as well as PI3K (27), we tested the effect of LY on STAT1 serine phosphorylation in response to these cytokines. Interestingly, the PI3K inhibitor did not affect IL-1- or TNF-induced STAT1 serine phosphorylation (Fig. 1D).

Specific Inhibition of PI3K Reduces IFNγ-induced, STAT1-dependent Activation of Transcription and Endogenous Gene Expression—Because the phosphorylation of STAT1 on serine contributes substantially to its activity as a transcription factor, we investigated the effect of inhibiting PI3K on STAT1-driven reporter activity. Pretreatment with LY of T98G cells transfected transiently with the reporter construct 4×GBP-GAS-luc resulted in a −7-fold reduction of IFNγ-induced luciferase activity compared with control cells (Fig. 2A). This decrease did not reflect toxicity because pretreatment with LY did not increase the fraction of cells stained with trypan blue (data not shown).

The involvement of PI3K in STAT1-dependent transactivation indicates that PI3K is likely to play a role in IFNγ-mediated, STAT1-dependent gene expression. Expression of the human guanylate-binding protein-1 (GBP-1) gene is induced by IFNγ. The gene contains overlapping GAS and ISRE sites that bind to STAT1 in association with IRF-9 (28). Consistent with an independent study (29), we find that GBP-1 is not induced in STAT1-deficient U3A cells in response to IFNγ and that U3A-S727A cells suffer a 5-fold loss in IFNγ-induced GBP-1 expression. Similar to the result with U3A-S727A cells, pretreatment of T98G cells with LY decreased IFNγ-induced activation of GBP-1 by −7-fold compared with controls (Fig. 2B). Similar observations were made in 2fTGH cells (data not shown). These results confirm that PI3K plays an important role in IFNγ-mediated, STAT1-dependent gene expression.

IFNγ Activates the PI3K Pathway—Assays with phosphatidylinositol diphosphate as a substrate revealed that the lipid kinase activity of PI3K was rapidly and transiently induced (−3-fold) upon IFNγ treatment of T98G cells, peaking after about 3 min (Fig. 3A), a pattern of activation typical of other PI3K inducers (27, 30, 31). Similar results were observed in 2fTGH cells (data not shown). The constitutive activity of PI3K observed in untreated cells is most likely due to low levels of

2 H. Nguyen and G. R. Stark, unpublished observations.
Fig. 3. IFNγ stimulates PI3K activity. A, T98G cells were serum-starved for 18–24 h before treatment to reduce constitutive levels of PI3K. Cells lysates were immunoprecipitated with anti-phosphotyrosine, and the immunoprecipitates were assayed for PI3K activity. PI3K phosphorylates phosphatidylinositol (4,5)P2 to phosphatidylinositol (3,4,5)P3 (4,5P3/4,5P3). The sample labeled −Ab was processed in the absence of anti-phosphotyrosine. The sample labeled +LY was preincubated with 10% SDS-PAGE, and Western analysis was performed sequentially using anti-phospho-Ser-473 or anti-Akt, respectively.

The PI3K Pathway Regulates STAT1-driven Transcription—To corroborate our observations involving the PI3K inhibitor, we also analyzed the effects of cotransfected constitutively active, membrane-targeted forms of PI3K or Akt (kind gifts of N. Grammatikakis; described in Refs. 32 and 33) on the STAT1-dependent activation of 4×GBPGAS-luc. Increasing concentrations of constitutively active PI3K enhanced IFNγ-induced activation by ~4-fold (Fig. 4A). Constitutively active Akt also augmented activity by ~5-fold in response to IFNγ (Fig. 4B). Conversely, cotransfection of increasing amounts of a membrane-targeted, kinase-dead form of PI3K reduced the activation of 4×GBPGAS-luc in response to IFNγ by ~2-fold (Fig. 4C). Similarly, a dominant-negative derivative of Akt (Akt-CαAX; where A is an aliphatic residue) reduced IFNγ-induced reporter activity by ~7-fold (Fig. 4D). These findings substantiate the role of the PI3K pathway in STAT1-dependent transcription in response to IFNγ.

IFNγ-dependent Induction of STAT1 Ser-727 Phosphorylation, but Not Activation of PI3K or ERK, Is Abrogated in JAK2-deficient Cells—As expected, STAT1 was not phosphorylated on Tyr-701 in JAK2-deficient γ2A cells in response to IFNγ (Fig. 6A). Consistent with previous findings (4), STAT1 serine phosphorylation in response to IFNγ was also not observed in γ2A cells, and this defect was reversed upon reconstitution with JAK2 (Fig. 6B). Similar to observations in JAK1-null U4A cells, the activation of Akt in response to IFNγ was not affected by the absence of JAK2 (Fig. 6C). The phosphorylation of ERK appeared to be deficient in γ2A cells compared with parental 2C4 cells; however, ERK activation was not restored upon reconstitution of the cells with JAK2 (Fig. 6D). ERK was activated by IFNγ in an independent JAK2-deficient clone (B9; Fig. 6E) as well as in JAK2-null MEFs (Fig. 6F). Therefore, the defect in ERK activation is peculiar to the γ2A clone, and ERK activation in response to IFNγ does occur in the absence of JAK2 in other cells. These findings show that JAK2 is necessary, in addition to and independently of PI3K, for the induction of STAT1 serine phosphorylation in response to IFNγ.

The Y440F Mutation of IFNGR1 Prevents STAT1 Ser-727 Phosphorylation but Not Activation of PI3K or MAPK Activation in Response to IFNγ—JAK1 and JAK2 phosphorylate IFNGR1 Tyr-440 to provide a docking site for STAT1, facilitating its subsequent tyrosine phosphorylation in response to IFNγ. Because JAK1 and JAK2 are both required for STAT1 serine phosphorylation, we tested the role of IFNGR1 Tyr-440. Activation of STAT1 and other responses to human (h)IFNγ were analyzed in SCC16-5 murine fibroblast cells stably expressing either wild-type human IFNGR1 (hGR) or the Y440F mutant form (Fig. 7). STAT1 was not phosphorylated on Tyr-701 in Y440F cells in response to hIFNγ (Fig. 7A). Interestingly, the induction of serine phosphorylation of STAT1 in Y440F cells was attenuated significantly (~3-fold) in response to hIFNγ (Fig. 7B) but not in
The regulation of STAT1 by a PI3K effector has been found recently in Dictyostelium, DdSTATa, the Dictyostelium homolog of mammalian STATs, is phosphorylated on serine by GskA, the Dictyostelium homolog of glycogen synthase kinase-3 (43), a protein kinase that is phosphorylated and inactivated by Akt following treatment of mammalian cells with insulin (44). Extracellular cAMP induces the tyrosine phosphorylation and subsequent import of DdSTATa into the nucleus as a prerequisite for the transition from a unicellular amoeba to a multicellular mound. GskA, required for establishing the correct cell-type pattern in the mound, is concomitantly activated by cAMP and then phosphorylates DdSTATa on serine, enhancing its nuclear export (43). There are several Dictyostelium homologues of Akt, some of which are activated by cAMP through a G-protein or PI3K-dependent pathway and one of which plays a substantial role in multicellular development (45), although no association between Dictyostelium Akt and DdSTATa has yet been reported. These findings suggest that the involvement of PI3K signaling in the regulation of STAT1 activity may be evolutionarily conserved.

Is Akt the STAT1 serine kinase? Peptide substrates that are effectively phosphorylated by Akt contain the minimum amino acid sequence RXRXXS/T*y, where X is any amino acid and * is a bulky hydrophobic residue (Phe or Leu) (46). This motif is found in most of the sequences surrounding the phosphorylation sites in proposed Akt substrates so far (reviewed in Ref. 47). The residues near STAT1 Ser-727 do not conform to this motif, suggesting that Akt probably does not phosphorylate STAT1 directly on serine, although the possibility cannot be ruled out without a direct experiment. Alternatively, the STAT1 serine kinase may be an Akt target, such as the mammalian target of rapamycin, mTOR/FRAP, a serine kinase involved in regulating protein synthesis and the cell cycle (48, 49).

Another scenario is that PI3K and Akt lie upstream of either ERK, p38, or both, because both were proposed previously to be involved in STAT1 serine phosphorylation in response to IFNγ (6, 12). Two unpublished observations make this possibility unlikely. First, although p38 was activated in response to IL-1 or TNF, phosphorylation of p38 was undetectable in response to IFNγ in T98G and 2fTGH cells, diminishing the possibility that this kinase could phosphorylate STAT1 on Ser-727 in response to IFNγ in these cells. This finding does not necessarily eliminate the possibility that p38 is involved in IFNγ-me-
diated phosphorylation of STAT1 on serine in HeLa S3 cells, in which the role of p38 in the serine phosphorylation of STAT1 was originally demonstrated (6). Secondly, peak activation of ERK preceded peak Akt phosphorylation and was not inhibited by LY in T98G cells. Moreover, pretreatment of T98G cells with the MEK1 inhibitor PD98059 prevented the activation of ERK but did not inhibit the serine phosphorylation of STAT1 in response to IFNγ, indicating that ERK cannot be the PI3K-activated STAT1 serine kinase. These observations are consistent with other studies that present evidence against the role of either p38 or ERK in STAT1 serine phosphorylation in response to IFNγ in certain cell lines (4, 7, 29, 50).

The Activation of PI3K Is a Novel Signal in Response to IFNγ; Similarities between the IFNγ Receptor and Growth Factor Receptors—This report has shown that the PI3K pathway is activated by IFNγ, and PI3K is known to be activated by IFNα/β as well (51, 52). Activation of PI3K is required for induction of STAT1 serine phosphorylation in response to IFNγ and for full activation of gene expression. We found that STAT1 was phosphorylated very weakly on Ser-727 in response to IFNγ and that induced levels were insignificantly affected by LY in T98G cells. These results complement previous studies that demonstrate that inhibition of PI3K by wortmannin does not affect the IFNα-induced formation of ISGF3, a complex composed of STAT1, STAT2, and p48 transcription factors, or IFNα-mediated gene transcription driven by ISRE, the DNA element to which ISGF3 binds (53). These findings are not surprising, since the transactivation domain of STAT1 is dispensable for ISGF3 transcriptional activity, which is provided primarily by STAT2 (54–56) and is very different from the situation with IFNγ-induced, STAT1-dependent gene expression, which depends entirely on the ability of STAT1 dimers to activate transcription (54).

The activation of PI3K by IFNs presents a new perspective in understanding the full function of the IFN receptors. Evidence is accumulating that reveals the activation in response to IFNs of many proteins that are involved in growth factor-dependent signaling. MAPK and PI3K are activated by growth factors such as platelet-derived growth factor (reviewed in Ref. 57). In addition, both IFNα and IFNγ activate vav- and Crk-dependent signaling pathways in hematopoietic cells (reviewed in Ref. 58), and these proteins are also activated in response to epidermal growth factor, colony-stimulating factor-1, and B-cell or T-cell activation (59–68). Participants in pathways that are unique...
in comparison to JAK-STAT signaling have now assumed increased importance with the discovery of substantial IFNγ-induced, STAT1-independent gene expression, the mechanism of which remains to be elucidated. In the absence of STAT1, IFNγ retains the ability to confer resistance to viral infection and to regulate the growth of primary bone marrow-derived macrophages. Furthermore, IFNγ activates a surprisingly large number of genes in STAT1-deficient MEFs or bone marrow-derived macrophages, some of which exert important roles in immunomodulation and cell growth regulation. Interestingly, in the presence of STAT1, expression of some of these genes is actually suppressed (13, 69, 70). Expression of the c-myc gene by the novel pathway requires Raf1, since it is inhibited by the HSP90-specific inhibitor geldanamycin and by expression of a mutant form of p50dc37 that is unable to recruit HSP90 to the Raf1 complex (13). Our finding that PI3K is activated by IFNγ makes it an attractive candidate for participation in the novel pathway. Interestingly, Akt interacts with the cdc37/HSP90 complex (18), an association that positively regulates Akt kinase activity in response to various stimuli (71). It is plausible that PI3K can activate signaling components independently of STAT1 to activate the novel pathway and subsequent gene expression and concurrently stimulate STAT1 serine phosphorylation to suppress induction of some of the same genes.

**STAT1 Tyrosine and Serine Phosphorylation in Response to IFNγ Requires the Same Receptor-associated Functions**—It is interesting that the activation of PI3K and ERK in response to IFNγ was not reduced in cells lacking either JAK1 or JAK2. Although we can conclude that STAT1 serine phosphorylation requires both JAK1 and JAK2, we cannot state that both JAKs are dispensable for the activation of PI3K or ERK. It is possible that either one of the JAKs is capable of catalyzing activation and that they can substitute for each other when one of them is missing. Analysis of a cell line in which the function of both JAKs are lacking would truly determine whether they play a role in the activation of PI3K or ERK.

Our observation that STAT1 serine phosphorylation requires the JAK-mediated phosphorylation of IFNγR1 Tyr-440 suggests that STAT1 must dock on the receptor in order to be phosphorylated not only on tyrosine but on serine as well. A relevant consideration is that Akt is activated at the plasma membrane (72–74), where it can act on its target substrates. To test this hypothesis, we analyzed IFNγ-mediated induction of STAT1 serine phosphorylation in STAT1-deficient cells reconstituted with STAT1 containing an arginine—lysine substitution at residue 602, which is critical for STAT1 interaction with the receptor via its SH2 domain (U3A-SH2), as well as in STAT1-deficient cells reconstituted with a STAT1 variant containing a tyrosine—phenylalanine substitution at residue 701, which is also critical for STAT1 interaction with the receptor, as well as for STAT1 dimerization (U3A-Y701F). Although STAT1 serine phosphorylation was not induced by IFNγ in both of these cell lines, it was difficult to make a definite conclusion since the basal levels of serine-phosphorylated STAT1 were high. However, a recent study also reported that in STAT1-deficient murine cells expressing the same STAT1 mutants, STAT1 was not phosphorylated on serine in response to IFNγ (29). Several conclusions can be drawn from these observations. First, STAT1 may have to dock on the IFNγ receptor to be phosphorylated on serine. Second, phosphorylation of IFNγR1 Tyr-440 may signal to a yet unidentified component that is required in addition to PI3K for STAT1 serine phosphorylation. Third, STAT1 may have to be dimeric in order to be recognized by its serine kinase, or dimerization and subsequent nuclear translocation of STAT1 may be required to move it to its serine kinase. In any case, our work reveals a two-step process leading to STAT1 serine phosphorylation in response to IFNγ: 1) PI3K and Akt are activated, leading to the activation of the STAT1 serine kinase; and 2) in order to be phosphorylated on serine, STAT1 has to dock to the receptor and perhaps also dimerize and translocate to the nucleus; a process initiated by JAK-mediated phosphorylation of IFNγR1 Tyr-440.

**Acknowledgments**—We thank Nywana Sizemore for helpful discussions and Nicholas Grammatikakis for the PI3K constructs.

**REFERENCES**

1. Bach, E. A., Aguet, M., and Schreiber, R. D. (1997) Annu. Rev. Immunol. 15, 563–591
2. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) Annu. Rev. Biochem. 67, 237–264
3. Wu, Z., Zhong, Z., and Darnell, J. E., Jr. (1995) Cell 82, 241–250
4. Zhu, X., Wu, Z., Xu, L. Z., and Darnell, J. E., Jr. (1997) Mol. Cell. Biol. 17, 6618–6623
5. Kovarik, P., Steiber, D., Nevy, M., and Decker, T. (1998) EMBO J. 17, 3660–3668
6. Geh, K. C., Hauge, S. J., and Williams, B. R. (1999) EMBO J. 18, 5601–5608
7. Kopp, P., Steiber, D., Eygen, A., Menghini, R., Neusinger, A., Gaestel, M., Cohen, P., and Decker, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13956–13961
8. Gollob, J. A., Schnipper, C. P., Murphy, E. A., Bita, J., and Frank, J. A. (1996) Immunity 6, 447–452
9. Su, L., Rickert, R. C., and David, M. (1999) J. Biol. Chem. 274, 31770–31774
10. Camero, A. M., and Larner, A. C. (2000) J. Biol. Chem. 275, 16574–16578
11. Staerk, T., and Kovarik, P. (2000) Oncogene 19, 262–2637
12. Takasao, A., Tanaka, N., Mitani, Y., Miyazaki, T., Fujii, H., Sato, M., Kovarik, P., Decker, T., Schlessinger, J., and Taniguchi, T. (1999) EMBO J. 18, 2480–2486
13. Rasmussen, C. M., Grammatikakis, N., Chernov, M., Nguyen, H., Geh, K. C., Williams, B. R., and Stark, G. R. (2000) EMBO J. 19, 263–272
14. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) Annu. Rev. Biochem. 67, 581–607
15. Wynn, M. P., and Pirolo, L. (1998) Biochem. Biophys. Acta 1436, 127–150
16. Vanhaesebroeck, B., Levers, S. J., Panayotou, G., and Waterfield, M. D. (1997) Trends Biochem. Sci. 22, 257–272
17. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) Biochim. Biophys. Acta 1355, 1–13
18. Chan, T. O., Rittenhouse, S. E., and Tischkis, P. N. (1999) Annu. Rev. Biochem. 68, 965–1014
19. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421
20. Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J. C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deursen, J. M., Grosveld, G., and Eble, J. N. (1999) Cell 93, 385–395
21. Gueschin, D., Rogers, N., Briscoe, J., Wittbusch, B., Watling, D., Horn, F., Pellegrini, S., Yasukawa, K., Heinrich, P., and Stark, G. R. (1995) EMBO J. 14, 1421–1429
22. Farrar, M. A., Campbell, D. J., and Decker, T. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11706–11710
23. Greenlund, A. C., Farrar, M. A., Viviano, B. L., and Schreiber, R. D. (1994) EMBO J. 13, 1591–1600
24. Vignais, M. L., Sadowski, H. B., Watling, D., Rogers, N. C., and Gilman, G. (1996) Mol. Cell. Biol. 16, 1759–1769
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual 2nd Ed, pp. 16.33–16.36, Cold Spring Harbor University Press, Cold Spring Harbor, N. Y.
26. Royal, I., and Park, M. (1995) J. Biol. Chem. 270, 27780–27787
27. Sizemore, N. Leung, S., and Stark, G. R. (1999) Mol. Cell. Biol. 19, 4798–4805
28. Seegert, D., Streihol, I., Klose, B., Levy, D. E., Schindler, C., and Decker, T. (1994) J. Biol. Chem. 269, 8590–8599
29. Kruglik, P., Mangold, M., Rasagre, H., Heidari, H., Steinborn, H., Zetter, A., Levy, D. E., Müller, M., and Decker, T. (2001) EMBO J. 20, 91–100
30. Ruderman, N. B., Kapeller, R., White, M. F., and Cantley, L. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1411–1415
31. Burridge, K., and Spierer, L. J. (2001) Cell 104, 127–138
32....)
Role of PI3K in STAT1 Ser-727 Phosphorylation by IFN-γ

59. Bowtell, D. D., and Langdon, W. Y. (1995) Oncogene 11, 1561–1567
60. Donovon, J. A., Wange, R. L., Langdon, W. Y., and Samelson, L. E. (1994) J. Biol. Chem. 269, 22921–22924
61. Fukazawa, T., Miyake, S., Band, V., and Band, H. (1996) J. Biol. Chem. 271, 14554–14559
62. Galisteo, M. L., Dikie, I., Batzer, A. G., Langdon, W. Y., and Schlessinger, J. (1995) J. Biol. Chem. 270, 20242–20245
63. Marcilla, A., Rivero-Lezcano, O. M., Agarwal, A., and Robbins, K. C. (1995) J. Biol. Chem. 270, 9115–9120
64. Meisner, H., and Czech, M. P. (1995) J. Biol. Chem. 270, 25332–25335
65. Meisner, H., Conway, B. R., Hartley, D., and Czech, M. P. (1995) Mol. Cell. Biol. 15, 3571–3578
66. Soltsoff, S. P., and Cantley, L. C. (1996) J. Biol. Chem. 271, 563–567
67. Tonaka, S., Neff, L., Baron, R., and Levy, J. B. (1996) J. Biol. Chem. 270, 14347–14351
68. Wang, Y., Yeung, Y. G., Langdon, W. Y., and Stanley, E. R. (1996) J. Biol. Chem. 271, 17–20
69. Gil, M. P., Bohn, E., O’Grain, A. K., Ramana, C. V., Levine, B., Stark, G. R., Virgin, H. W., and Schreiber, R. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6680–6685
70. Ramana, C. V., Gil, M. P., Han, Y., Ransohoff, R. M., Schreiber, R. H., and Stark, G. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 97, 10832–10837
71. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cren, P., Cohen, P., Luceoq, J. M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 31515–31524
72. Meier, R., Alessi, D. R., Crön, P., Andjelkovic, M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 30491–30497
73. Welch, H., Eginton, A., Stephens, L. R., and Hawkins, P. T. (1998) J. Biol. Chem. 273, 11248–11256
Roles of Phosphatidylinositol 3-Kinase in Interferon-γ-dependent Phosphorylation of STAT1 on Serine 727 and Activation of Gene Expression

Hannah Nguyen, Chilakamarti V. Ramana, Joshua Bayes and George R. Stark

J. Biol. Chem. 2001, 276:33361-33368.
doi: 10.1074/jbc.M105070200 originally published online July 3, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105070200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 73 references, 49 of which can be accessed free at http://www.jbc.org/content/276/36/33361.full.html#ref-list-1