Enhanced Antiviral and Antiproliferative Properties of a STAT1 Mutant Unable to Interact with the Protein Kinase PKR*

Andrew Hoi-Tao Wong§§, Joan E. Durbin§, Suiyang Li‡, Thomas E. Dever**, Thomas Decker‡‡, and Antonis E. Koromilas$$$§§§

From the §§Terry Fox Molecular Oncology Group, Lady Davis Institute, Jewish General Hospital, Montreal H3T 1E2, Canada, the §§Children’s Hospital Research Foundation and Department of Pediatrics, Ohio State University, Columbus, Ohio 43205, the **Laboratory of Eukaryotic Gene Expression, NICHD, National Institutes of Health, Bethesda, Maryland 20892, and the §§Vienna Biocenter, Institute for Microbiology and Genetics, Vienna 1030, Austria

We have previously reported a physical association between STAT1 and the protein kinase double-stranded RNA-activated protein kinase (PKR). PKR inhibited STAT1 function in a manner independent of PKR kinase activity. In this report, we have further characterized the properties of both molecules by mapping the sites of their interaction. A STAT1 mutant unable to interact with PKR displays enhanced interferon γ (IFN-γ)-induced transactivation capacity compared with STAT1. This effect appears to be mediated by the higher capacity of STAT1 mutant to heterodimerize with STAT3. Furthermore, expression of STAT1 mutant in STAT1−/− cells enhances both the antiviral and antiproliferative effects of IFNs as opposed to STAT1. We also provide evidence that STAT1 functions as an inhibitor of PKR in vitro and in vivo. That is, phosphorylation of eIF-2α in STAT1−/− than STAT1+/+ cells in vivo, and this correlates with higher activation capacity of PKR in STAT1−/− cells. Genetic experiments in yeast demonstrate the inhibition of PKR activation and eIF-2α phosphorylation by STAT1 but not by STAT1 mutant. These data substantiate our previous findings on the inhibitory effects of PKR on STAT1 and implicate STAT1 in translational control through the modulation of PKR activation and eIF-2α phosphorylation.

Cytokines and growth factors exert a diverse range of biological activities, from host defense, growth regulation, to immunomodulation. Upon ligand binding to cell-surface receptors, JAK1 kinases are activated and proceed to phosphorylate the receptor on tyrosine residues, which then function as docking sites for cytoplasmic transcription factors of the STAT family (1, 2). STATs are subsequently activated by tyrosine phosphorylation, dimerize by phosphotyrosyl-SH2 interactions, and translocate to the nucleus to induce transcription of cytokine-responsive genes (3). A single tyrosine phosphorylation site in the carboxyl-terminal activation domain is absolutely essential for STAT dimerization and DNA binding (3), whereas phosphorylation of a serine residue in this region is important for transactivation activity (4).

One of the major STATs intimately involved in both the innate and acquired immune responses is STAT1. Upon virus infection or exposure to interferons (IFNs), STAT1 is found in protein complexes that bind specific DNA sequences upstream to genes responsible for host resistance. For instance, IFN-α/β induces formation of the heterodimeric ISGF3, whereas IFN-γ induces binding of homodimeric STAT1 (2, 3). Moreover, dsRNA, an intermediate produced during virus replication, can also activate STAT1 DNA binding (5, 6). The non-redundant role of STAT1 in the antiviral response is further appreciated by findings that STAT1 null mice (STAT1−/−) are highly susceptible to microbial infection (7, 8). IFN signaling leads to the expression of a number of genes, one of which encodes for the dsRNA-dependent protein kinase, PKR (9, 10). PKR is a serine/threonine protein kinase that displays two distinct activities: (i) autophosphorylation upon dsRNA binding (9, 10) and (ii) phosphorylation of the eukaryotic translation initiation factor eIF-2α (9, 10), a modification resulting in inhibition of protein synthesis (11). Several studies with cultured cells provide evidence for antiviral (12, 13), antiproliferative, and tumor suppressor functions of PKR (9, 10). However, pkr null (PKR−/−) mice exhibit a modest susceptibility to viral infection (14–17) and show no signs of tumor formation (14, 17), suggesting that the lack of PKR can be compensated by other PKR-like molecules (9, 10). This hypothesis is supported by the recent identification of the PKR-related genes, PERK/PEK (18) and the mouse homolog of the yeast eIF-2α kinase, GCN2 (19).

We previously described an association between PKR and STAT1 (6). This interaction takes place in unstimulated cells and diminishes upon treatment with IFNs or dsRNA. Increased levels of PKR/STAT1 complex have a negative effect on STAT1 DNA-binding and transactivation capacities. In this report we have mapped the interaction sites between the two

* This work was supported in part by research grants from the Canadian Institutes of Health Research and the Human Frontier Science Program (to A. E. K.). 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.

§§ A member of the Terry Fox Group in Molecular Oncology and recipient of a Canadian Institutes of Health Research Scientist Award. To whom correspondence should be addressed: Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, 3755 Cote-Ste-Catherine Rd., Montreal, Quebec H3T 1E2, Canada. Tel.: 514-340-8260 (ext. 3697); Fax: 514-340-7576; E-mail: akoromin@lidi.jgh.mcgill.ca.

‡‡‡ A recipient of a post-doctoral award from the Cancer Research Society of Canada.

PERK, PKR-like endoplasmic reticulum kinase; PEK, pancreatic eIF-2α kinase; GCN2, general control non-derepressible-2; ISGF3, interferon-stimulated gene factor 3; HA, hemagglutinin; GST, glutathione S-transferase; GAS, γ-interferon-activating sequence; VSV, vesicular stomatitis virus; CPE, cytopathic effect; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TM, triple-mutant.
proteins and have identified a novel function of STAT1. Specifically, we demonstrate that STAT1 functions as an inhibitor of PKR activation and eIF-2α phosphorylation in vitro and in vivo. A mutant of STAT1, which was unable to interact with PKR, could not inhibit PKR function in yeast and was better able to mediate the transcriptional, antiviral, and antiproliferative responses of IFNs compared with STAT1. Taken together, these findings not only support our previous observations, but they also provide strong evidence for tight regulation of PKR and STAT1 functions by virtue of their interaction. In addition, our data suggest that STAT1 has a dual role in regulation of gene expression by functioning as a transcriptional factor and possibly as translational regulator through PKR activation and eIF-2α phosphorylation.

MATERIALS AND METHODS

Cell Culture and Transfections—HeLa S3, U3A, STAT1−/−, PKR−/−, and PKR−/− cells were maintained in Dulbecco’s modification of Eagle’s medium supplemented with 10% calf serum, 2 mM l-glutamine, and 100 units/ml penicillin/streptomycin (Life Technologies). For IFN treatment, cells were incubated with 1000 IU/ml of recombinant murine IFN-α/β (Lee Biomolecules) or 100 IU/ml of IFN-γ (PharMingen). Double-stranded RNA transfactions were performed as previously described (6). Transient transfections were performed with LipofectAMINE Plus reagent (Life Technologies). T7 vaccinia virus was replated and treated with IFN-γ.

Extraction Preparation, Immunoprecipitation, and Immunoblot Analysis—Cell extract preparation, immunoprecipitation, and immunoblotting were performed as previously described (6). The following antibodies were used: STAT1α (Santa Cruz Biotechnology); STAT2 (Upstate Biotechnology Inc.); STAT3 (Santa Cruz; PKR; GST (Amersham Pharmacia Biotech); Myc (9E10, Roche Molecular Biochemicals); eIF-2α, phosphosine 51 of eIF-2α (Upstate Biotechnology Inc.); Flag (2M2, Cell Signaling Technology); and the pGEX reverse primer (CAGCTCTTGCAATTTCACGTCGTCTG-3') were ligated into the mammalian expression vector, pCDNA3.1/zeo, and confirmed by sequencing. Site-directed mutagenesis was carried out using the following primer pairs: R346A, 5'-GGGGCGGCCGCCTACTTCCTAAATCCTTTTACTGTATTTC-3'; 5'-d[GGGGCGGCCGCCTACTTCCTAAATCCTTTTACTGTATTTC]-3'; R346A/L347D/L348D, 5'-d[GGGGCGGCCGCCTACTTCCTAAATCCTTTTACTGTATTTC]-3'; 5'-d[GGGGCGGCCGCCTACTTCCTAAATCCTTTTACTGTATTTC]-3'.

RESULTS

The catalytic domain of PKR specifically associates with the DNA-binding domain of STAT1. To map the PKR-STAT1 interaction we performed a series of binding assays using full-length GST-PKR2926R mutant (GST-PKR N) and GST-PKR 2926C-551 (PKR C) were generated as previously described (25). For the GST-pPKRL529 (PKR C) were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) with the following primer pairs (Sheldon Biotechnology): 5'-d[CCAGAGGGTGAAGTGGACAGGTAGAAGGAAAAGAACCCGGCC]-3' and 5'-d[GCGCCATTTTTGTCCTCTCACTTGCACCATCTCACCCTTGGTCAGT]-3'.

Cell Extract Preparation, Immunoprecipitation, and Immunoblot Analysis—Cell extract preparation, immunoprecipitation, and immunoblotting were performed as previously described (6). The following antibodies were used: STAT1α (Santa Cruz Biotechnology); HA horseradish peroxidase antibody (3F10, Roche Molecular Biochemicals); phosphotyrosine (4G10/PY20, UBI and Transduction Laboratories); and phospho-eIF-2α (Research Genetics Inc.); FLAG (M2, Kodak); HA horseradish peroxidase antibody (3F10, Roche Molecular Biochemicals); phosphotyrosine (4G10/PY20, UBI and Transduction Laboratories); and phospho-eIF-2α (Research Genetics Inc.).

DNA Binding and Transactivation Assays—Electrophoretic mobility shift analysis was performed using the dsDNA c-Fos c-sis-inducible element (5'-GATCATAGTCTCGGTCGAAAATTCTCTCCGATGGATTCTTTTTTGGCATTTTCTAAATTTCACGTCGTCTG-3') and the pGEX reverse primer (CAGCTCTTGCAATTTCACGTCGTCTG-3') according to protocols previously described (6). The Dual Luciferase system (Promega) was used to assess the transactivation potential of STAT1. Briefly, STAT1−/− cells or cells expressing STAT1 WT or TM were transfected with Renilla luciferase (pRL-TK) and pGL-2XP53 GAS luciferase. Twenty-four hours after transfection, cells were replated and treated with IFN-γ for 18 h before harvesting. The results presented represent quadruplicate experiments where GAS luciferase activity was normalized to Renilla luciferase activity.

Isoelectric Focusing and PKR in Vitro Kinase Assays—Isoelectric focusing and immunoblot analysis of yeast eIF-2α were performed as previously described (29). PKR in vitro kinase assays were carried as previously described (6). GST Pull-down Assays—Protein production and extraction were performed according to previously described protocols (25, 30). Normalized PKR expression proteins were captured with GST (PharMingen). HeLa whole cell lysates or [35S]methionine-expressed GST-PKR fusion proteins, subjected to SDS-PAGE, and visualized by fluorography (25).

Yeast Plasmids, Transformations, Growth Protocols, and Protein Extractions—Wild-type and mutants of HA-STAT1 1–413 were subcloned by restriction digest of BamHI site in the mammalian expression vector, pEBMILex4 (29), containing a NolI site in the multiple cloning site. Transformation of yeast strains H2544 and J100 and growth analyses were performed as described previously (31).

RESULTS

The catastrophic domain of PKR specifically associates with the DNA-binding domain of STAT1. To map the PKR-STAT1 interaction we performed a series of binding assays using full-length GST-PKR2926R mutant (GST-PKR WT could not be overexpressed in bacteria (32)) or truncations of PKR bearing either the dsRNA-binding (GST-PKR N, amino acids 1–262) or catalytic (GST-PKR C, amino acids 263–551) domain (Fig. 1A). HeLa S3 extracts expressing human HA-STAT1α were incubated with GST-PKR fusion proteins, subjected to SDS-PAGE, and immunoblotted with an anti-HA antibody. As shown in Fig. 1B (top panel), both full-length GST-PKR (lane 5) and the carboxyl terminus of PKR (lane 4) interacted with STAT1 whereas binding to the amino terminus was not detectable (lane 3). Furthermore, this interaction is specific for STAT1,
because we could not detect binding of GST-PKR proteins with other STATs (Fig. 1B).

We previously reported that binding of PKR to STAT1 is independent of RNA but requires an intact RNA-binding domain, because the dsRNA-binding-defective mutant PKR645 (Arg58-Ser59-Lys60 to Gly58-Ala59-Leu60) (33) does not interact with mouse STAT1 in NIH3T3 cell extracts stably expressing this PKR mutant (6). Based on this, we proposed that the integrity of the dsRNA-binding domain of PKR plays a role in PKR-STAT1 interaction in vivo (6). The observation, however, that the carboxyl terminus of PKR is required for binding to STAT1 in vitro (Fig. 1B) prompted us to examine the interaction of STAT1 with GST-PKR bearing either the LS4 or the LS9 mutation (Ala66-Ala68 to Gly66-Pro68), which also abolishes RNA binding (33) (Fig. 1C). The RNA-binding-defective mutants were expressed and purified in the GST-PKR WT background, because expression of LS4 and LS9 in the GST-PKR background could not be achieved (data not shown). HeLa S3 extracts containing HA-STAT1a were incubated with GST-PKR296R, GST-PKR296RL54, or GST-PKR296RSL9, and GST-PKR bound proteins were subjected to immunoblotting with anti-HA antibody. All GST-PKR mutants interacted with STAT1 equally well (Fig. 1C, top panel), suggesting that mutations within the dsRNA-binding domain do not interfere with PKR binding to STAT1 in vitro.

To map the region on STAT1 that facilitates its interaction with PKR, we used truncated STAT1 proteins corresponding to its amino-terminal, DNA-binding, linker, SH2, or transactivation domains (Fig. 2A). The pull-down assays were performed with GST-PKR C, because this protein binds to STAT1 (Fig. 1A) and is more stable than GST-PKR296R (data not shown). We observed that GST-PKR C specifically interacted with the DNA-binding domain of STAT1 (Fig. 2B, lane 14). Upon truncation of the STAT1 DNA-binding domain from the carboxy-terminal end, a critical junction is reached when binding to PKR is retained (middle panel, HA-STAT1 1–342, lane 12) and when binding is abolished (HA-STAT1 1–342, lane 12). Further truncation of STAT1 from the amino terminus also presented a similar junction between amino acids 343–365 (Fig. 2C, lower panel, compare lanes 25 and 26). Moreover, truncation of the carboxyl terminus of the DNA-binding domain of STAT1 at positions 348 and 357 still retained binding to GST-PKR (Fig. 2D, lanes 10 and 11), suggesting that the region of interaction lies between amino acids 343 and 348 (b-sheet 3) on the DNA-binding domain of STAT1 (33, 34).

Mutations of STAT1 That Affect Binding to PKR—To identify amino acids in STAT1 that form contacts with PKR, we next constructed mutations within amino acids 343–348 of HA-STAT1a that abolished binding to PKR. Alanine-scan mutagenesis of each of the six amino acids did not yield a point mutant of HA-STAT1a that disrupted interaction with PKR (data not shown). However, a three-amino acid substitution (TM; Arg346-Leu347-Leu348 to Ala346-Asp347-Asp348) within this region disrupted the ability of STAT1 to interact with GST-PKR C (Fig. 2E, lane 6). Interestingly, HA-STAT1 1–413 TM possessed faster mobility on SDS-PAGE gels compared
with WT most likely through changes in the overall charge of the molecule.

To further characterize the interaction of full-length HA-STAT1α TM with PKR, we utilized the human fibrosarcoma, U3A cell line, which lacks endogenous STAT1 (35). HA-STAT1α WT or TM were co-transfected with PKRK296R into U3A cells, after which, the protein extracts were immunoprecipitated against PKR and immunoblotted with HA antibodies.

As seen in Fig. 2F, STAT1α WT associated with both transfected and endogenous PKR (upper panel, lanes 2 and 5). Conversely, STAT1 TM binding with endogenous PKR was completely abolished (lane 3) and displayed very marginal binding to transfected PKR, which was detectable only after long exposures (lane 6). In contrast to HA-STAT1 1–413 TM, full-length STAT1α TM did not display any difference in its migration pattern compared with STAT1α WT. These in vitro findings
were also verified in vivo by the yeast two-hybrid assay (data not shown). Taken together, it appears that the DNA-binding domain of STAT1 interacts with PKR in vitro and in vivo.

DNA Binding and Transcriptional Properties of STAT1 TM—To test the ability of STAT1 TM to respond to IFN-γ treatment, we performed transient transfection assays in STAT1−/− cells using STAT1 WT or STAT1 TM and a luciferase reporter construct driven by two copies of the GAS element from the IFP53 gene (28). As shown in Fig. 3A, we observed that luciferase expression in cells transfected with STAT1 WT

Regulation of PKR Activity by STAT1

FIG. 3. STAT1 TM displays elevated transcriptional properties. A, STAT1−/− cells were transiently transfected with pRL-TK, pGL-2xIFP53-GAS, and control vector, HA-STAT1α WT, or HA-STAT1α TM. Transfected cells were left untreated (white bars) or stimulated with IFN-γ for 18 h (black bars) and harvested. B, STAT1−/− cells stably transfected with control vector, HA-STAT1α WT, or HA-STAT1α TM were transfected with pRL-TK and pGL-2xIFP53-GAS. Transfected cells were left untreated (white bars) or stimulated with IFN-γ for 18 h (black bars) and harvested. Luciferase activity was measured and normalized to Renilla luciferase activity. The data represent the average of quadruplicate experiments. The relative protein levels of STAT1 WT and STAT1 TM were compared by immunoblotting with antibodies against HA. C, control, WT, and TM cells were treated with IFN-γ for 30 min or left untreated. Protein extracts were incubated with 32P-radiolabeled c-sis-inducible element from the c-Fos promoter and subjected to DNA binding assays. Competition reactions were performed using a 200-fold excess of unlabeled oligonucleotide. Supershift experiments were performed using non-reactive mouse IgG1, STAT1α, or STAT3 antibodies. D, control, STAT1 WT, and STAT1 TM cells were left untreated, or stimulated with IFN-α/β or IFN-γ for 30 min. STAT1 WT and TM protein levels were normalized with the appropriately stimulated or unstimulated control cell extracts (STAT1−/−) and subsequently immunoprecipitated with STAT1α antibody and immunoblotted against phosphotyrosine (top panel), STAT1α phosphoserine 727 (middle panel), and HA (bottom panel) antibodies. E, normalized protein extracts were immunoprecipitated with antibodies against STAT1 and immunoblotted with STAT3 (top panel) or HA (bottom panel) antibodies. F, the above extracts were immunoprecipitated with antibodies against STAT3 and immunoblotted against phosphotyrosine (top panel) or STAT3 (bottom panel).
was induced by IFN-γ treatment. However, in cells transfected with STAT1 TM, we observed, after normalization to Renilla luciferase, a much higher basal luciferase activity (~5-fold) that could be slightly induced by IFN-γ stimulation.

To better characterize STAT1 TM, we infected STAT1−/− fibroblasts with retroviruses harboring the puromycin-resistant gene and HA-STAT1a WT or HA-STAT1a TM. As a control, retroviruses containing only the puromycin-resistant gene were used. After puromycin selection, polyclonal populations of STAT1 WT-expressing cells showed ~5-fold greater expression over STAT1 TM pools (Fig. 3B, compare lanes 2 and 3). Transactivation assays using the 2XIFP53-GAS luciferase reporter correlated with our findings in transient transfection experiments that STAT1 TM confers higher basal reporter activity, which can be induced by IFN-γ treatment (Fig. 3B). We next tested whether STAT1 TM could bind DNA after IFN treatment. Although we could not detect ISGF3 formation in STAT1 TM cells in response to IFN-α/β (data not shown), IFN-γ stimulation resulted in the formation of DNA-binding complexes consisting of either STAT1-STAT3 heterodimers or STAT3 homodimers, but not that of STAT1 homodimers (Fig. 3C, compare lanes 7–16). This finding is consistent with previous reports that STAT3 is activated following IFN treatment (3). Moreover, the intensity of the STAT3 homodimer appears to be higher compared with control or STAT1 WT cells (compare lanes 2, 4, and 6).

The ability of STAT1 to be phosphorylated upon IFN stimulation was also examined (Fig. 3D). To compare STAT1 phosphorylation per equal amounts of STAT1 protein, we used a 5-fold higher amount of STAT1 TM extracts versus STAT1 WT before and after IFN stimulation. These reactions were also normalized to total protein concentration by the addition of treated or untreated STAT1 WT control extracts. Although STAT1 WT was tyrosine-phosphorylated following IFN-α/β or IFN-γ treatment (top panel, lanes 5 and 6), we failed to detect STAT1 TM tyrosine phosphorylation (lanes 8 and 9). In contrast, phosphorylation of serine 727 did not significantly differ between STAT1 WT and STAT1 TM after IFN treatment (mid-}

**Fig. 4.** STAT1 TM has enhanced antiproliferative and antiviral properties. A, polyclonal STAT1−/− cell lines transfected with control vector, HA-STAT1α WT, or HA-STAT1α TM were synchronized by serum starvation for 48 h and stimulated with IFN-α/β or IFN-γ for 12 and 24 h. Cells were fixed, stained with propidium iodide, and subjected to cell cycle analysis. The data were processed using the WINMDI v2.8 application. The relative cell-cycle distributions presented in the table represent two averaged experiments. B, cells were pretreated with IFN-α/β or IFN-γ for 18 h before being infected with serially diluted VSV for 24 h, after which viable cells were stained with crystal violet (top panel). Whole-cell extracts from the cell lines were immunoblotted with antibodies against STAT1α and immunoblotted with an antibody against the HA epitope (bottom panel).
of STAT3 co-precipitated with STAT1 TM before and after IFN treatment (Fig. 3E, top panel, lanes 7–9), although STAT1 protein levels were approximately equal (bottom panel). We also analyzed expression and activation of STAT3 in the same protein extracts used for the STAT1/STAT3 co-immunoprecipitation. STAT3 phosphorylation was slightly elevated (~50%) in cells expressing STAT1 TM before or after treatment with either IFN-α/β or IFN-γ (Fig. 3F, lanes 7–9). This increase in

Regulation of PKR Activity by STAT1
STAT3 activity may account for increased STAT3 DNA binding in STAT1 TM cells.

**STAT1 TM Enhances the Antiviral and Antiproliferative Effects of IFNs**—The biological effects of STAT1 TM activation were examined by cell cycle analysis after treatment with IFN-α/β or IFN-γ (Fig. 4A). A greater proportion of STAT1 TM-expressing cells (IFN-α/β, 6–8%; IFN-γ, 10–11%) were arrested in G0/G1 phase compared with control (left panel) or STAT1 WT-expressing (middle panel) cells. In addition, the ability of STAT1 TM cells to resist virus infection was also investigated. Control, STAT1 WT, and STAT1 TM cells were primed with IFNs and subsequently infected with serially diluted VSV. The amount of virus needed to induce CPE was qualitatively measured. As shown in Fig. 4B (upper panel), STAT1 TM cells that were treated with IFN-γ were ~50-fold more resistant to VSV infection compared with STAT1 WT cells, and ~10^4-fold more resistant versus control STAT1^{-/-} cells. In contrast, IFN-α/β-treated STAT1 TM cells were 10-fold more susceptible to VSV CPE compared with control, STAT1 WT, and STAT1^{-/-} cells. Interestingly, even untreated STAT1 TM cells provided a greater degree of protection compared with STAT1 WT and STAT1^{-/-} cells. This enhanced ability of STAT1 TM cells to resist virus infection was also observed after encephalomyocarditis virus infection (data not shown). Western blotting against STAT1α revealed that STAT1 TM is expressed at much lower levels than STAT1 WT and endogenous STAT1 from STAT1^{-/-} cells (bottom panel). Taken together, these data suggest that STAT1 TM enhances the antiproliferative and antiviral effects of IFNs on a per molecule basis.

**STAT1 Functions as a PKR Inhibitor in Vitro**—To gain better insight into the PKR-STAT1 interaction, we next mapped

**Fig. 6. STAT1 inhibits PKR activity in yeast.** A, strains J110 (bottom half of both plates) and H2544 (top half of both plates) were transformed with control vector (labeled C), K3L (labeled K3L), HA-STAT1 1–413 WT (labeled WT), or HA-STAT1 1–413 TM (labeled TM). Transformants were streaked on control 10% glucose (upper plate) or 10% galactose agar plates and monitored for slow growth phenotype. B, transformants were grown in galactose medium, and relative growth rates were monitored at the indicated times by trypan blue cell counting. The upper graph represents the growth curves of control J110 transformants whereas the bottom graph shows the growth curves of H2544 transformants. C, J110 and H2544 were grown in galactose medium to induce HA-STAT1 1–413 WT and TM expression. 2 mg of protein extracts were immunoprecipitated with HA antibodies and immunoblotted with HA antibodies. D, J110 and H2544 were grown in galactose medium to induce PKR expression. Total protein extracts were subjected to immunoblot analysis with rabbit antisera to human PKR. The upper band, which is present in J110 extracts lacking PKR, is nonspecific (NS). E, extracts from J110 control and H2544 strains transformed with vector alone, K3L, HA-STAT1 1–413 WT, or HA-STAT1 1–413 TM were subjected to isoelectric focusing after induction with galactose and probed with antibodies against yeast eIF-2α.
were performed in the presence of activator reovirus dsRNA and immunoprecipitated against mouse PKR, and a rabbit anti-mouse PKR polyclonal antibody, subjected to SDS-PAGE, blotting was performed with antibodies against phosphoserine 51 of PKR i.e. in STAT1

the STAT1-binding site on PKR (Fig. 5A). In agreement with earlier results, the full-length kinase domain (lane 4), but not the dsRNA-binding domain (lane 3), bound PKR. Truncation of the kinase domain from either the amino or carboxyl terminus (Fig. 5A) defined a region critical for STAT1 binding: amino acids 367–415 (lanes 5–9). Interestingly, this corresponds to the same region in the large lobe of the PKR kinase domain, to which the vaccinia virus K3L protein binds to block PKR activation (36, 37). In view of this fact, we tested whether the two proteins could compete for binding to PKR. Bacterially expressed FLAG-K3L was co-incubated with GST-PKR C and extracts from HeLa cells expressing increasing amounts of HA-STAT1a. Immunoblotting was performed to detect either binding of HA-STAT1a or FLAG-K3L to GST-PKR C. As seen in Fig. 5B, STAT1 displaced K3L from PKR in a dose-dependent manner (bottom panel, lanes 10–13), indicating that K3L and STAT1 bind to the same region on PKR.

The PKR pseudosubstrate, K3L, has been shown to bind PKR and block access of eIF-2α to the catalytic pocket of PKR (29, 37). To address the importance of STAT1 binding to PKR, we investigated the ability of STAT1 to act as a cellular inhibitor of PKR. A series of in vitro assays were performed to assess the effect of STAT1 on PKR activation. Human PKR was activated by reovirus dsRNA in vitro from HeLa S3 cells in the presence of increasing amounts of recombinant full-length STAT1 (Fig. 5C, middle panel). We observed that PKR auto-phosphorylation diminished in a dose-dependent manner by the addition of STAT1 (top panel), although PKR protein levels were equal (bottom panel).

**STAT1 Inhibits the Antiproliferative Properties of PKR in Yeast**—To date, the best approach to test for the translational function of PKR is in Saccharomyces cerevisiae. It has been shown that high levels of PKR expression in yeast are toxic due to inhibition of general translation (38). However, at lower levels of expression PKR can substitute the function of GCN2 (39), the only eIF-2α kinase known to exist in S. cerevisiae (40), by phosphorylating eIF-2α on serine 51 to inhibit protein synthesis. Through this approach, a number of PKR inhibitors have been identified and characterized (31).

Given that PKR expression in yeast results in inhibition of cell growth (38, 39), we wanted to analyze whether STAT1 would block this effect when co-expressed with PKR. Yeast strain H2544, which lacks the yeast eIF-2α kinase GCN2, contains a stable integration of human PKR WT cDNA downstream to a galactose-inducible promoter, whereas the isogenic strain J110 is identical except that the PKR cDNA was not inserted. Previous studies have shown that induction of PKR expression in H2544 results in inhibition of cell growth through phosphorylation of eIF-2α (39). Conversely, co-expression of a PKR inhibitor, such as K3L, leads to rescue of PKR-mediated growth inhibition (29). To test the inhibitory activity of STAT1 on PKR, strains J110 and H2544 were transformed with vector alone, K3L, HA-STAT1 1–413 WT, or HA-STAT1 1–413 TM. Transformants were streaked onto minimal media plates containing either glucose or galactose as a carbon source, and the effect of each of these proteins on PKR-mediated growth inhibition was monitored. All transformants of the isogenic J110 strain grew well in either glucose or galactose indicating that expression of these exogenous proteins did not perturb normal yeast growth characteristics (Fig. 6A, upper plate, and Fig. 6B, upper graph). H2544 transformants containing only empty plasmid DNA demonstrated a slow-growth phenotype after PKR induction (Fig. 6A, lower plate, labeled C). However, expression of K3L reversed this growth inhibitory phenotype (lower plate, labeled K3L). Likewise, expression of STAT1 1–413 WT also rescued yeast growth (lower plate, labeled WT), although in contrast, the interaction mutant of STAT1 was unable to counteract the growth inhibitory effects of PKR (lower plate, labeled TM). Growth curves to assess the degree of rescue showed that the ability of HA-STAT1 1–413 WT transformants to rescue growth was half as potent relative to K3L (Fig. 6B, lower graph). Because it was not possible to quantify the relative levels of K3L and STAT1 1–413 WT expression, the degree of rescue may be dependent on their different levels of expression. Efforts to rescue growth by full-length HA-STAT1α were unsuccessful, because we could not detect HA-STAT1α expression in yeast (data not shown). However, the truncated HA-STAT1 1–413 proteins were readily detectable in yeast protein extracts (Fig. 6C) as was the expression of human PKR in H2544 transformants (Fig. 6D, lanes 5–8). In correlation with the growth curves, the extent of eIF-2α phosphorylation, as assessed by isoelectric focusing experiments (29), was diminished in H2544 transformants expressing either K3L or HA-STAT1 1–413 WT (Fig. 6E, lanes 3 and 4). The induction of PKR levels in Fig. 6D, lane 6, was probably translational in nature as a result of inhibition of eIF-2α phosphorylation and up-regulation of PKR protein synthesis by K3L (Fig. 6E, lane 3). This PKR up-regulation was not evident in lane 7 most likely due to the weaker inhibitory effect of STAT1 1–413 WT on eIF-2α phosphorylation compared with K3L (Fig. 6E, compare lanes 3 and 4).

**Increased PKR Activation and eIF-2α Phosphorylation in STAT1** Cells—Next we investigated whether the loss of STAT1 would augment PKR activity. To do so, protein extracts from untreated or IFN-treated STAT1+/+ and STAT1−/− cells were used to assess PKR activity in vitro. We noticed that the
basal activity of PKR was 5-fold higher in STAT1−/− cells compared with STAT1+/+ cells (Fig. 7A, upper panel, compare lanes 1 and 3), although PKR protein levels were equivalent, as assessed by in vivo [35S]methionine labeling (lower panel, compare lanes 1 and 2). The increase in PKR activity after IFN treatment in STAT1+/+ cells (top panel) reflects increased PKR expression, whereas no such increase in PKR activity/protein was observed in STAT1−−/− cells. This is consistent with the notion that transcriptional up-regulation of PKR after IFN treatment is dependent on the JAK/STAT pathway (upper panel, compare lanes 2 and 4). To further substantiate our findings that PKR activity is elevated in STAT1−−/− cells, we compared the levels of eIF-2a phosphorylation in STAT1+/+ and STAT1−−/− cells in vivo. The effect of STAT1 on eIF-2a phosphorylation in STAT1−−/− and STAT1−+/+ cells was examined by treatment with dsRNA and immunoblotting with a phosphospecific antibody to phosphoserine 51 of eIF-2a (Fig. 7B, top panel). These experiments showed that a higher amount of eIF-2a was phosphorylated in STAT1−−/− cells compared with STAT1+/+ cells (top panel, compare lanes 1 and 3) and that this phosphorylation was more highly induced after dsRNA treatment (compare lanes 2 and 4). Taken together, the inhibition of PKR activity by STAT1 in mammalian and yeast cells supports our findings that STAT1 can inhibit PKR activity in vitro and in vivo.

**DISCUSSION**

We have mapped the sites of interaction between PKR and STAT1 in vitro using GST pull-down assays and also in vivo. We found that STAT1 interacts with PKR on amino acids 367–415, an area located within the large lobe of the kinase domain of PKR that is also bound by the vaccinia virus encoded PKR inhibitor, K3L (36, 37). Sequence comparison with other eIF-2a family members, GCN2, HRI, and the newly discovered PERK revealed that this part of the kinase domain is highly conserved and suggests that these members might be capable of interacting with STAT1. Our previous observations that the dsRNA-binding mutant PKRLS4 was unable to associate with STAT1 in vivo (6), at first, appear to be challenged by our in vitro studies herein, which show that the carboxyl terminus of PKR is required for binding to STAT1 and that GST-PKR.K96RLS4 can interact with STAT1 (Fig. 1). One possible explanation for this difference may be inferred, based on structural data of the amino terminus of PKR (41) and also on previous mutational studies (33), that the L54 and L59 mutations could introduce conformational changes that would affect its interaction with STAT1 in vivo. Such conformational changes may not take place when GST-PKR.K96RLS4 and GST-PKR.K96RLS9 are purified from bacteria. Another conceivable explanation is that the association of PKR with STAT1 in vivo is facilitated by the presence of other protein(s) whose action is modulated by the amino terminus of PKR and may become limited when the interaction is tested in vitro.

In the case of STAT1, amino acids 343–348 provide a major site of interaction with PKR. This region of STAT1 corresponds to β-sheet 3, a structure located on the back side of the DNA-binding domain of STAT1 (33, 34). Mutation of three amino acids within this region (STAT1 TM; Arg346→Leu347→Leu348 to Ala346→Asp347→Asp348) abolished binding to PKR in vitro and in vivo. Paradoxically, sequence alignment of this three-residue stretch of wild-type STAT1 with other STAT members showed almost complete conservation. Similarly, residues in β-sheet 3 of the DNA-binding domain of STAT3 responsible for specific interaction with c-Jun are also nearly identical between STAT members; however, c-Jun specifically interacts with STAT3 (42). Thus, our observation that PKR specifically interacts with STAT1 can probably only be explained in the context of the presentation of β-sheet 3 in STAT1 compared with other STAT molecules. In the end, structural analysis might be necessary to determine the exact points of contact between PKR and STAT1.

In light of our previous observations that the ability of PKR to interact with STAT1 can block STAT1 DNA binding (6), we examined whether release of STAT1 from this inhibitory mechanism might augment its transcriptional activity. In contrast to STAT1 WT, we were unable to observe tyrosine phosphorylation of STAT1 TM following IFN stimulation, and as a result, ISGF3 and STAT1 homodimer formation could not be detected in DNA binding experiments. We believe that the inability of STAT1 TM to be phosphorylated cannot be the result of a misfolded protein, because this mutant is phosphorylated on serine 727 after IFN treatment. Rather, it appears that some undefined negative regulatory mechanism is responsible for this phenomenon. The protein kinase that directly phosphorylates serine 727 of STAT1 in vivo is not as yet known (4). It is unlikely to be PKR because STAT1 is not phosphorylated by PKR in vitro (6). Interestingly, a recent report shows a defective serine 727 phosphorylation of STAT1 in PKR−−/− fibroblasts after IFN stimulation providing evidence for an indirect role of the kinase in this process (43). Although unable to bind DNA as a homodimer, STAT1 TM was competent and more capable in forming DNA-binding complexes with STAT3 relative to STAT1 WT cells. Interestingly, tyrosine phosphorylation of STAT3 was elevated by 50% before and after IFN treatment in polyclonal cell populations expressing STAT1 TM. The mechanism behind this finding is not clear at this time, but it might be possible that recruitment of STAT3 to the JAK-receptor complex is more efficient in STAT1 TM cells compared with STAT1 WT cells. Nevertheless, the net effect of increased STAT1:STAT3 heterodimer and STAT3 homodimer formation probably contributes to up-regulation of STAT1:STAT3 DNA-binding and GAS-dependent transactivation. Because STAT3 homodimers appear to play a minimal role in the transactivation of IFN-dependent genes by IFN-γ (44), it is likely that the transcriptional activity observed in our reporter assays is contributed by the STAT1:STAT3 heterodimer. This is the second instance where a transcriptional role of STAT1 has been shown to require serine 727 but not tyrosine 701 phosphorylation. An earlier study demonstrated that mutation of serine 727 to alanine, but not tyrosine 701 to phenylalanine, on STAT1 significantly ablated the TNF-α-dependent induction of caspase genes (45). It was speculated that STAT1 could potentially participate in signaling pathways independent of its tyrosine phosphorylation state (46); such a hypothesis is supported by our findings in here.

The antiproliferative and antiviral effects of IFNs were also investigated, because a number of cell cycle regulatory proteins are regulated at the transcriptional level through the JAK/ STAT signaling pathway (44). IFN-dependent cell cycle arrest, as measured by FACS analysis, was significantly higher in cells expressing STAT1α TM versus control cells or cells expressing STAT1α WT. In addition, the ability of STAT1 TM cells to resist VSV infection was 10-fold greater than control or STAT1 WT cells following IFN-γ stimulation. These differences become more significant when the variation in the expression levels between STAT1 WT and TM is considered (i.e. 5-fold higher expression of WT than STAT1 TM; Fig. 3B, lanes 2 and 3; Fig. 4B, bottom panel). Interestingly, even untreated STAT1 WT cells provided greater protection relative to control and STAT1 WT cells, suggesting that the anti-viral responses available within STAT1 TM cells are already enhanced prior to IFN pretreatment.

Given the fact that STAT1 and K3L, a pseudosubstrate in-
hibitor of PKR, can compete for binding with PKR, we next analyzed whether STAT1 could inhibit PKR activation. We observed that recombinant STAT1 inhibited PKR activation in vitro in a dose-dependent manner. However, it is unlikely that STAT1 is a substrate or pseudosubstrate for PKR, because sequence alignment of STAT1 with K3L and eIF-2α did not reveal any significant similarities, findings that coincide with our earlier observations that PKR does not phosphorylate STAT1 in vitro or in vivo (6). Instead, the interaction of the two proteins might prevent opening of the cleft that separates the two lobes of PKR’s kinase domain, thus inhibiting both PKR activation and eIF-2α phosphorylation.

The capacity of STAT1 to neutralize PKR activity is further appreciated from in vivo studies. PKR exhibits the same substrate specificity as the yeast GCN2 kinase that regulates protein synthesis by phosphorylating eIF-2α to inhibit cell growth and replacement of GCN2 with PKR leads to inhibition of translation and yeast growth (31). As such, this system has been consistently used as a means to probe for PKR activation, as well as for characterizing inhibitors of PKR, like K3L (29). The observation that HA-STAT1 1–413 WT, but not the inter- 

REFERENCES

1. Leonard, W. J., and O’Shea, J. J. (1998) Annu. Rev. Immunol. 16, 283–322
2. Sheng, B. R., Kerr, I. M., Williams, M. H., Silverman, R. H., and Schreiber, S. D. (1998) Annu. Rev. Biochem. 67, 227–264
3. Darnell, J. E., Jr. (1997) Science 277, 1630–1635
4. Decker, T., and Koromilas, A. E. (2000) Oncogene 19, 2938–3037
5. Bandopadhyay, S. K., Leonardi, G. T. J., Bandopadhyay, T., Stark, G. R., and Sen, G. C. (1995) J. Biol. Chem. 270, 16924–16929
6. Wong, A. H., Tam, N. W., Yang, Y. L., Cuddihy, A. R., Li, S., Kirchhoff, S., Hauska, G., Decker, T., Koromilas, A. E. (1997) EMBO J. 16, 1291–1300
7. Merz, A. M., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., Dubois, R. N., Clark, R. A., Aguet, M., and Scherer, R. D. (1996) Cell 84, 431–442
8. Durbin, J. E., Hackenmiller, R., Simon, M. C., and Levy, D. E. (1996) Cell 84, 443–450
9. Kaufman, R. J. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Matthews, M. B., eds) pp. 503–507, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Cremona, M. J., and Elkon, R. G. (1993) J. Biol. Chem. 268, 14382–14388
11. Beaufach, for the 82 retroviral packaging cell line; and K. McDonald for assistance on flow cytometry. We thank J. M. Bergeron and members of our laboratory and of the Molecular Oncology Group for helpful discussions.

Acknowledgments—We thank T. Pannunzio and N. Tam for assistance in some of the experiments; C. Schindler for STAT1a and STAT2 cDNAs, M. Mathews for PKR-L54 and PKR-L59 cDNAs; A. Darveau, G. N. Barber, and J. C. Bell for PKR antibodies; M. Clemens for eIF-2α antibodies; C. Weissmann for PKR-α and PKR-β cells; G. Stark for U3α cells; Y. Yoneda for the pGEX-5X-3-HA-STAT1α; R. Schreiber for purified STAT1α protein; N. Sonenberg for pGEX2TK-FLAG-K3L; J. E. Darnell, Jr. for pRC/CMV STAT1α; A. Veillette for pBABE-puro; N. Beckman for the 82 retroviral packaging cell line; and K. McDonald for assistance on flow cytometry. We thank J. M. Bergeron and members of our laboratory and of the Molecular Oncology Group for helpful discussions.