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Communication

Activation of Protein Kinase A Inhibits Interferon Induction of the Jak/Stat Pathway in U266 Cells*

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Michael David, Emanuel Petricoin III, and Andrew C. Larner‡

From the Division of Cytokine Biology, Center for Biologics Evaluation and Research, National Institutes of Health, Bethesda, Maryland 20892

Activation of early response genes by interferons (IFNs) requires tyrosine phosphorylation of the Stat transcription factors and is mediated by the Jak family of tyrosine kinases. Recent evidence suggests that ERK2 serine/threonine kinase modulates the IFN-stimulated Jak/Stat pathway. In this report we show that in the myeloma cell line U266 protein kinase A specifically interacts with the cytoplasmic domain of the IFNα/β receptor. Treatment of cells with the adenylate cyclase activator forskolin inhibits IFNβ-, IFNγ-, and hydrogen peroxide/vanadate-induced formation of complexes that bind to enhancers known to stimulate the expression of IFN-regulated genes. Immunoprecipitations followed by anti-phosphotyrosine immunoblots indicate that tyrosine phosphorylation of the α chain of the IFNα/β receptor, Jak1, Tyk2, as well as Stat1 and Stat2 is reduced as a consequence of incubation of cells with forskolin. In contrast, dideoxyforskolin, which fails to activate adenylate cyclase, has no effect on IFN induction of the Jak/Stat pathway. These results indicate a novel regulatory mechanism by which protein kinase A can modulate the Jak/Stat signaling cascade.

The Janus family of tyrosine kinases (Jak1, Jak2, Jak3, and Tyk2) is an integral component of many cytokine-activated signaling cascades, which regulate tyrosine phosphorylation of the Stat proteins such that they can translocate to the nucleus and bind DNA (1). These kinases are associated with one or more chains of a given cytokine receptor. For instance, Tyk2 interacts with the α subunit of the type I IFN[1] receptor. Along with Tyk2, we have determined that Jak1 and the SH2 domain containing tyrosine phosphatase PTP1C, which acts as a negative regulator of the Jak/Stat pathway, also selectively bind to a GST fusion protein, which consists of the membrane proximal 50 amino acids of the α subunit of the IFNα/β receptor (2). During the course of these experiments we showed that the 42-kDa form of MAPK (ERK2) binds the IFNα/β receptor and is enzymatically activated by IFNβ, probably leading to the phosphorylation of Stat1 on serine 727 (3). This serine phosphorylation has been proven crucial for the transactivation of IFNβ-regulated genes without being necessary for DNA binding of the Stat proteins (4). As it has been previously shown that in some cells the activation of MAPK through various growth factors can be inhibited by increased levels of intracellular cAMP (5–8), we wanted to explore the possible involvement of protein kinase A, one of the main targets of elevated cAMP, in the regulation of the IFNβ-induced Jak/Stat pathway.

MATERIALS AND METHODS

Cells—U266 cells were grown as a suspension culture in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Upstate Biotechnology, Inc.).

Whole Cell Extracts—After treatment, cells were diluted with ice-cold phosphate-buffered saline and centrifuged at 1500 × *g* for 10 min at 4 °C, washed with phosphate-buffered saline, and resuspended in 1 ml of lysis buffer containing 20 mM Hepes pH 7.4, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (all from Sigma). For some experiments, 1% digitonin was substituted for Triton X-100. The lysate was vortexed, incubated on ice for 10 min, and centrifuged at 18,000 × *g* for 10 min at 4 °C.

Electrophoretic Mobility Shift Assay (EMSA) and Measurement of Intracellular Cyclic AMP—EMSAs were performed using whole cell extracts prepared with Triton-containing buffers as described (9). Probes consisting of the GRR found within the promoter of the *FcγR1* gene (IFNγ-induced gene) (5′ AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG 3′) and the ISRE of the *ISG15* gene (5′ GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC 3′) were end-labeled using polynucleotide kinase and [γ-^{32}P]ATP. Intracellular cAMP was assayed by radioimmunoassay (10).

Precipitation with Antibodies or GST Fusion Proteins—Lysates were incubated with the indicated antibodies for 2 h prior to the addition of Protein G-Sepharose beads (Pharmacia Biotech Inc.) and incubation for an additional hour. All antibodies used for immunoprecipitation have been described (3). GST fusion proteins representing the cytoplasmic domain of the IFNα/β receptor (11) bound to agarose beads were incubated with the extracts at 4 °C for 2–12 h. In either case the beads were pelleted at 15,000 × *g* for 2 min and washed three times with ice-cold lysis buffer (1 ml).

In Vitro Kinase Assays—Cellular extracts were prepared as described above and incubated with GST fusion proteins. The beads were washed and resuspended in a buffer containing 1.4 mg/ml myelin basic protein (MBP), 20 μM ATP, 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 1 mM vanadate, 5 mM EGTA (all from Sigma), and 1 μCi of [γ-^{32}P]ATP (Amersham Corp.). The samples were incubated at 30 °C for 20 min, SDS sample buffer was added to terminate the reaction, the proteins were separated by SDS-PAGE and transferred to Immobilon, and autoradiography was done.

Western Blotting—Proteins were separated on 4–16% SDS-PAGE gels (Novex) and transferred to Immobilon (Millipore). Membranes were probed with monoclonal antibodies against Tyk2, Jak1, Stat1, Stat2, pan-MAPK, the R1 subunit of protein kinase A (all from Transduction Laboratories), or the α chain of the IFNα/β receptor (Biogen) or phosphotyrosine (PY20, ICN) using concentrations and conditions recommended by the manufacturer. Immunoblots were developed using appropriate secondary antibodies and enhanced chemiluminescence (ECL, Amersham Corp.).

RESULTS AND DISCUSSION

The antagonistic actions of cAMP on growth factor activation of MAPK have been shown to be cell-specific (12). We therefore

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‡ To whom correspondence should be addressed: Division of Cytokine Biology, Center for Biologics Evaluation and Research, Bldg. 29A, Rm. 2B01, NIH, 8800 Rockville Pike, Bethesda, MD 20892. Tel.: 301-402-0361; Fax: 301-402-1659; E-mail: larner@a1.cber.fda.gov.

[1] The abbreviations used are: IFN, interferon; GST, glutathione *S*-transferase; ISRE, interferon-stimulated response element; GRR, IFNγ response region; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinase (also termed ERK or extracellular signal-regulated kinase); MBP, myelin basic protein; α subunit of IFNα/β receptor, the cDNA clone of the human IFNα/β receptor described by Uze *et al.* (20).

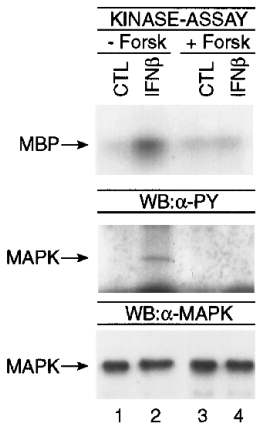

FIG. 1. **Forskolin inhibits IFNβ-stimulated MAPK activity in U266 cells.** U266 cells were incubated without (*lanes 1* and *3*) or with 10^3 units/ml recombinant human IFNβ (*lanes 2* and *4*) for 10 min at 37 °C. Forskolin (*Forsk*, 30 μM) was added to cells for 20 min at 37 °C prior to the addition of IFNβ (*lanes 3* and *4*). MAPK activity was visualized by autoradiography of ^{32}P incorporated into MBP (*top panel*), and the blot was then probed either with anti-phosphotyrosine (*middle panel*) or pan-MAPK antibody (*lower panel*). The membranes were developed using enhanced chemiluminescence (ECL). All monoclonal antibodies except PY20 (ICN) were obtained from Transduction Laboratories. *WB*, Western blot; *CTL*, control.

wanted to determine whether forskolin, a direct stimulator of adenylate cyclase, could inhibit IFNβ activation of MAPK in the myeloma cell line U266. Cells were pretreated with forskolin for 20 min prior to the addition of IFNβ for 5 min. Extracts were prepared and incubated with the IFNα/β receptor GST fusion protein as described (2, 3). MAPK activity was then assayed with myelin basic protein and [γ-^{32}P]ATP to GST fusion protein beads that had been incubated with the extracts. After SDS-PAGE, the proteins were blotted to Immobilon and either exposed for ^{32}P incorporation into MBP (Fig. 1, *upper panel*), probed with anti-phosphotyrosine antibody (*middle panel*) or with anti-MAPK antibody (*lower panel*). Incorporation of ^{32}P into MBP was enhanced about 5-fold when cells were treated with IFNβ (*lanes 1* and *2*). Prior treatment of U266 cells with forskolin inhibited activation of MAPK bound to the GST fusion protein by IFNβ, as well as IFNβ-stimulated tyrosine phosphorylation of MAPK (compare *lanes 2* and *4* of the *upper* and *middle panels*). The amount of MAPK bound to the beads was not altered by either forskolin or IFNβ treatment of cells (*lower panel*).

MAPK activity is presumably only required for Stat1α serine phosphorylation, which augments the transactivation of IFNβ-induced genes, but not for the tyrosine phosphorylation that mediates DNA binding. In this context it also has been shown that the serine/threonine kinase inhibitor H7 can block the transcription of IFNβ-regulated genes without affecting the formation of the ISGF3 complex (13, 14). To determine whether the inhibitory actions of forskolin were limited to MAPK, activation of Stat proteins was examined by their ability to bind known enhancers using EMSA. IFN-activated Stat1α binds specifically to the GRR, an enhancer present in promoter of the high affinity *FcγR1* gene (15). Stat1α and Stat2 together with the DNA binding component ISGF3γ form the multiprotein complex ISGF3, which interacts with the ISRE present in many IFNα/β-activated early response genes (1).

Surprisingly, forskolin treatment of U266 cells abrogated IFNβ-stimulated formation of DNA binding complexes interacting with both the GRR (Fig. 2*A*, *lanes 2* and *3*) and the ISRE enhancers (*lanes 10* and *11*) by 70–90%. Furthermore, forskolin also prevented the activation of GRR binding complexes by treatment of cells with either IFNγ (*lanes 5* and *6*) or with H_2O_2 and vanadate (*lanes 7* and *8*). In order to exclude other possible effects of forskolin besides activation of adenylate cyclase as the mechanism of inhibition of the Jak/Stat pathway, U266 cells were also exposed to dideoxyforskolin, a derivative of forskolin that fails to activate adenylate cyclase. As expected, this compound had no effect on the activation of the Jak/Stat pathway (compare *lanes 2*, *3*, and *4*).

To determine whether there was a correlation between forskolin-stimulated accumulation of intracellular cAMP and inhibition of IFNβ-stimulated GRR binding activity, cells were incubated with increasing concentrations of forskolin prior to treatment with IFNβ. Cell extracts were prepared and intracellular cAMP concentrations were measured, and IFNβ-stimulated GRR binding complexes were assayed by EMSA and subsequently quantitated on the PhosphorImager (Fig. 2*B*). Inhibition in IFNβ-stimulated GRR binding correlated with dose-dependent forskolin-induced increases in intracellular cAMP, confirming that increases in cAMP in these cells could be inhibiting IFNβ activation of the Jak/Stat pathway.

To determine directly whether IFNβ-stimulated tyrosine phosphorylation of Stat1α and Stat2 was affected by forskolin treatment of U266 cells, cellular extracts were subjected to immunoprecipitation with anti-Stat1α and anti-Stat2 antisera, and immunoprecipitates were analyzed using anti-phosphotyrosine antibodies (Fig. 2, *C* and *D*, *upper panels*). These results clearly demonstrated that IFNβ-stimulated tyrosine phosphorylation of these proteins is drastically decreased in U266 cells incubated with forskolin (compare *lanes 2* and *3*). Reprobing of the blots with Stat1α or Stat2 antisera indicated that equal amounts of protein were immunoprecipitated from all samples (*lower panels*).

Tyk2 and Jak1 are required for IFNα/β-stimulated tyrosine phosphorylation of Stat1α and Stat2 and become activated and tyrosine-phosphorylated as a result of incubation of cells with IFNα/β. In order to determine whether forskolin was exerting its inhibitory effects on activation of Tyk2 and Jak1, U266 cells were incubated with IFNβ in the presence or absence of forskolin, and Tyk2 and Jak1 were immunoprecipitated from cell lysates. The immunoblots from this experiment were probed with anti-phosphotyrosine antibody (Fig. 2, *E* and *F*). IFNβ-stimulated tyrosine phosphorylation of both Tyk2 and Jak1 was also significantly inhibited with forskolin treatment of U266 cells (compare *lanes 2* and *3*) while the total amount of these proteins in the immunoprecipitates is not altered (*lower panels*).

It has been previously shown that the α chain of the IFNα/β receptor becomes tyrosine-phosphorylated in response to IFN treatment (16). In order to determine whether the IFNα/β receptor itself was also a target for the inhibitory effects of elevated cAMP levels, monoclonal antibodies were used to immunoprecipitate the α chain of the IFNα/β receptor from cells that had been exposed to IFNβ with or without prior treatment with forskolin (2, 3). Proteins were resolved on SDS-PAGE and transferred to Immobilon, and the blots were probed with anti-phosphotyrosine antibody. The results shown in Fig. 2*G* clearly demonstrate that IFN-stimulated tyrosine phosphorylation of the IFNα/β receptor is decreased in U266 cells incubated with forskolin and IFNβ (compare *lanes 2* and *3*) compared with cells treated with IFNβ alone.

It has been previously shown that many of the key signaling components required for IFNα/β stimulation of the Jak/Stat pathway constitutively associate with a GST fusion protein containing the membrane-proximal 50 amino acids of the α chain of the IFNα/β receptor (3, 11). In order to explore a possible association of protein kinase A with the receptor, GST fusion proteins were expressed, which contained either the

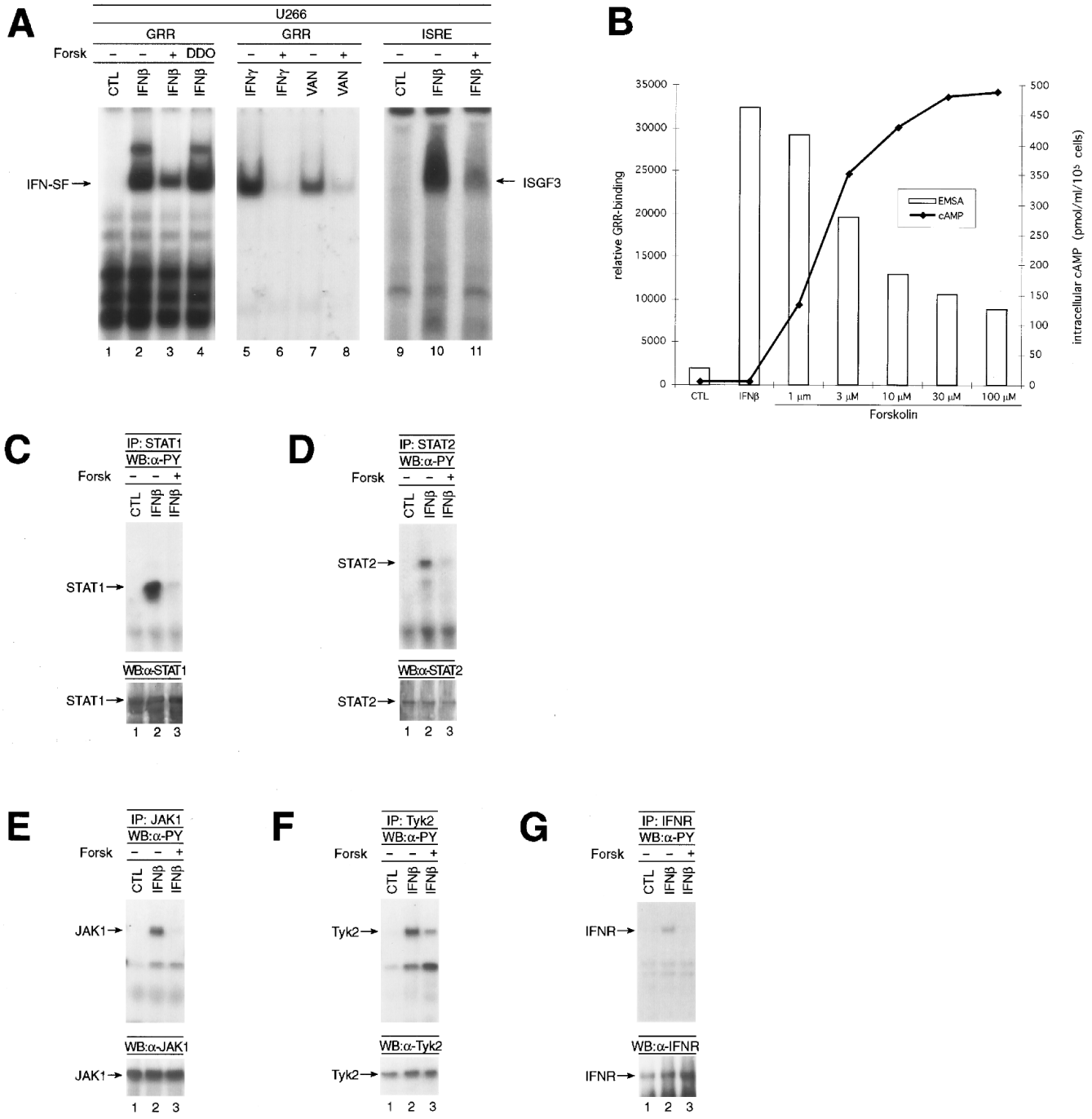

FIG. 2. **Forskolin treatment of U266 cells inhibits IFNβ activation of the Jak/Stat pathway.** *A*, extracts were prepared from U266 cells incubated with IFNβ or with combinations of forskolin (*Forsk*) and IFNβ. Electrophoretic mobility shift assays were performed using equal amounts of protein and a ^{32}P-labeled oligonucleotide probe corresponding to the γ response region in the high affinity *FcγR1* gene (*lanes 1–8*) or the ISRE (*lanes 9–11*). The IFNβ-induced complex which binds to the GRR is labeled *IFN-SF* and that which binds to the ISRE is termed *ISGF3*. *CTL*, control; *VAN*, vanadate; *DDO*, dideoxyforskolin. *B*, forskolin-induced intracellular cAMP correlates with inhibition of IFN-induced IFN-SF. U266 cells were either untreated or incubated with 0, 1, 3, 30, or 100 μM forskolin for 15 min prior to the addition of IFNβ for 15 min. Cell extracts were prepared, and cAMP was assayed (*right abscissa*) or IFN-SF formation was quantitated using a PhosphorImager (Molecular Dynamics) (*left abscissa*). *C* and *D*, forskolin inhibits IFNβ-stimulated tyrosine phosphorylation of Stat1α and Stat2. Lysates from U266 cells were incubated with anti-Stat1α or anti-Stat2 antiserum for 2 h. Immune complexes were collected on protein G beads, subjected to SDS-PAGE, and immunoblotted with PY20 anti-phosphotyrosine antibody and detected with ECL. Membranes were reprobed with anti-Stat1α or anti-Stat2 antibodies (*lower panel*). *WB*, Western blot; *IP*, immunoprecipitate; *α-PY*, anti-phosphotyrosine. *E, F,* and *G*, forskolin inhibits IFNβ-stimulated tyrosine phosphorylation of Jak1, Tyk2, and the IFNα/β receptor. Treatment of U266 cells, preparation of cell lysates, immunoprecipitations, and Western blots with PY20 were identical to *B* and *C*. Membranes were reprobed with anti-Tyk2, anti-Jak1, or anti-IFNα/β receptor monoclonal antibodies (*lower panels*). The antibodies used to immunoprecipitate Tyk2, Jak1, and the IFNα/β receptor have been previously described (2).

membrane-proximal 50 amino acids of the cytoplasmic domain of the α subunit of the IFNα/β receptor or GST alone. U266 cells were either left untreated or treated with IFNβ for 5 min, and cell lysates were prepared and incubated with the GST or the GST fusion proteins coupled to glutathione-agarose. Complexed proteins were resolved on SDS-PAGE and transferred to Immobilon, and the blots were probed with a monoclonal antibody to the type 1 regulatory subunit of protein kinase A.

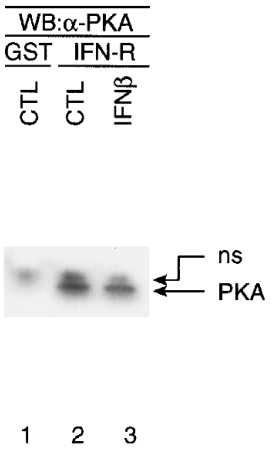

FIG. 3. **Interaction of protein kinase A (*PKA*) with the cytoplasmic domain of the α chain of the IFNα/β receptor.** Cellular extracts were prepared as described above and incubated with either GST alone (*lane 1*) or a GST fusion protein containing the membrane-proximal 50 amino acids of the cytoplasmic domain of the α subunit of the IFNα/β receptor (465) (*lanes 2* and *3*). Expression of the fusion proteins has been previously described (3). *WB*, Western blot; *CTL*, control.

Protein kinase A was found to associate specifically but in a ligand-independent manner with the GST fusion protein representing the membrane-proximal 50 amino acids of the cytoplasmic tail of the IFNα/β receptor (Fig. 3, *lanes 2* and *3*) but not with GST alone (*lane 1*).

Although cross-talk between signaling networks is a well described phenomenon, until recently modulation of the Jak/Stat pathway has been restricted to covalent modification of its components by tyrosine phosphorylation. Recent reports indicate that serine phosphorylation of Stat1 and Stat3 presumably by activation of MAPK can enhance IFNα/β and IFNγ induction of early response genes (3, 4). The results presented here implicate the serine/threonine kinase protein kinase A as a regulator of Jak/Stat activation. The effects of forskolin described here are also cell-specific with regard to inhibition of tyrosine phosphorylation of the Jak/Stat proteins (data not shown). Protein kinase A may directly or indirectly be responsible for modification of one or several of the Jak tyrosine kinases such that they cannot become tyrosine-phosphorylated (and presumably activated). Alternatively, the target for the actions of protein kinase A is upstream of Tyk2 and Jak1. The fact that forskolin also inhibits both IFNγ- and vanadate-induced GRR binding complexes in U266 cells suggests that the IFNα/β receptor is not the primary target for the inhibitory actions of forskolin. There are other key control points in the Jak/Stat pathway though, which could also be regulated by protein kinase A, such as the protein tyrosine phosphatase activity, which appears to be required to initiate tyrosine phosphorylation of Stat1 and Stat2 by IFNα/β but not to maintain the activation cascade (17). Other signaling proteins such as insulin substrate 1 or protein kinase C, which are known to be regulated by IFN treatment of cells but not at present implicated in IFN stimulation of the Jak/Stat pathway, must also be considered targets for the actions of protein kinase A (18, 19). Definition of the cell-specific substrates modified by protein kinase A, which inhibit Jak/Stat activation, is clearly an important piece of information to understand the mechanisms by which these distinct signaling networks influence each other's activities.

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