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Protein tyrosine kinase Pyk2 mediates the Jak-dependent activation of MAPK and Stat1 in IFN-γ, but not IFN-α, signaling

Akinori Takaoka[1], Nobuyuki Tanaka, Yukiko Mitani, Tadaaki Miyazaki, Hodaka Fujii, Mitsuharu Sato, Pavel Kovarik[2], Thomas Decker[2], Joseph Schlessinger[3] and Tadatsugu Taniguchi

Department of Immunology, Graduate School of Medicine, and Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, [2]Vienna Biocenter, Institute of Microbiology and Genetics, Dr Bohr-Gasse 9, A-1030 Vienna, Austria and [3]Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA

[1]Corresponding author
e-mail: takaoka9@m.u-tokyo.ac.jp

Two distinct types of interferon, IFN-α/β and IFN-γ, commonly exhibit antiviral activities by transmitting signals to the interior of the cell via their homologous receptors. Receptor stimulation results in the activation of distinct combinations of Janus family protein tyrosine kinases (Jak PTKs); Jak1/Tyk2 and Jak1/Jak2 for IFN-α/β and IFN-γ, respectively. Jak PTK activation by these IFNs is commonly followed by tyrosine phosphorylation of the transcription factor Stat1 at Y701, which is essential for dimerization, translocation to the nucleus and DNA-binding activity. To gain full transcriptional activity, Stat1 also requires serine phosphorylation at S727. In this paper we demonstrate that Pyk2, which belongs to another PTK family, is critical for the Jak-mediated MAPK and Stat1 activation by IFN-γ, but not IFN-α. Pyk2 is selectively associated with Jak2 and activated by IFN-γ. Overexpression of PKM, a dominant interfering form of Pyk2, in NIH 3T3 cells results in a strong inhibition of the IFN-γ-induced activation of Erk2, serine phosphorylation of Stat1 and Stat1-dependent gene transcription. Finally, the antiviral action of IFN-γ, but not IFN-α, is severely impaired by PKM overexpression. Thus, the two types of IFN may utilize distinct Jak-mediated Erk2, and possibly other MAPK activation pathways for their antiviral action.

Keywords: interferon-γ/Jak-Stat/MAPK/Pyk2/signal transduction

Introduction

Interferons (IFNs) are a family of multifunctional cytokines, originally identified by their ability to confer cellular resistance against viral infection. Type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) bind to their homologous receptor complexes (IFN-α/βR and IFN-γR), which are expressed in a wide variety of cells (DeMaeyer and DeMaeyer-Guignard, 1988; Vilcek and Sen, 1996). The mechanisms by which signal transmission by these IFNs result in transcriptional activation of the target genes have been extensively studied, and two families of transcription factors, the signal transducers and activators of transcription (Stat) and interferon regulatory factor (IRF) have been shown to play central roles (Darnell *et al*., 1994; Bluyssen *et al*., 1996; Taniguchi *et al*., 1997). Briefly, ligand-induced stimulation of each IFN receptor complex results in the activation of the receptor-associated Janus family protein tyrosine kinases (Jak PTKs); specifically, Jak1 and Tyk2 PTKs for IFN-α/βR, and Jak1 and Jak2 PTKs for IFN-γR (Darnell *et al*., 1994; Ihle and Kerr, 1995). It has been shown that three transcriptional activators, involving those two family members, activate the target genes, namely GAF (IFN-γ activated factor)/AAF (IFN-α activated factor), ISGF3 (interferon stimulated gene factor 3)/Stat1-p48 and IRF-1 (Darnell *et al*., 1994; Bluyssen *et al*., 1996; Taniguchi *et al*., 1997).

As for the IFN-α/βR signaling, the activation of the Jak1/Tyk2 PTKs results in tyrosine phosphorylation of Stat1 (at Y701) and Stat2 (at Y690), and Stat1 then undergoes homodimerization to form GAF/AAF (Darnell *et al*., 1994). Stat1 also forms a heterotrimeric complex ISGF3, together with the tyrosine-phosphorylated Stat2 and an IRF-family member p48 (Darnell *et al*., 1994; Bluyssen *et al*., 1996). Although these events do not require *de novo* synthesis of these factors, IRF-1, which is expressed at low levels in normally growing cells, needs to be induced by IFNs, wherein the *IRF-1* gene is activated by GAF/AAF whose binding site, termed GAS, is found within the *IRF-1* promoter (Harada *et al*., 1994; Pine *et al*., 1994). It has been reported that IRF-1 binds to and activates essentially the same *cis*-elements as those for ISGF3, but these two factors perform non-redundant functions in the antiviral action of both IFNs (Kimura *et al*., 1994, 1996). Despite the utilization of different combinations of the Jak PTK, it has been known that IFN-γR stimulation also results in similar activation or induction of these transcription factors (Darnell *et al*., 1994; Bach *et al*., 1997). Thus, in effect, both types of IFNs can activate an overlapping set of target genes for the induction of the antiviral state, in which Stat1 activation is central to these two IFN–Jak–Stat/IRF pathways (Darnell *et al*., 1994; Bluyssen *et al*., 1996; Taniguchi *et al*., 1997).

In addition to the critical role of the Jak PTKs in the activation of Stat1, it is known that Stat1 is also phosphorylated at a specific serine residue, S727, found in the C-terminal region, and that this phosphorylation is essential for its full functioning as a transcriptional activator (David *et al*., 1995; Wen *et al*., 1995; Zhang *et al*., 1995). This region contains a typical mitogen-activated protein kinase (MAPK) phosphorylation site, PX_nS/TP (P, proline; S/T, serine or threonine;

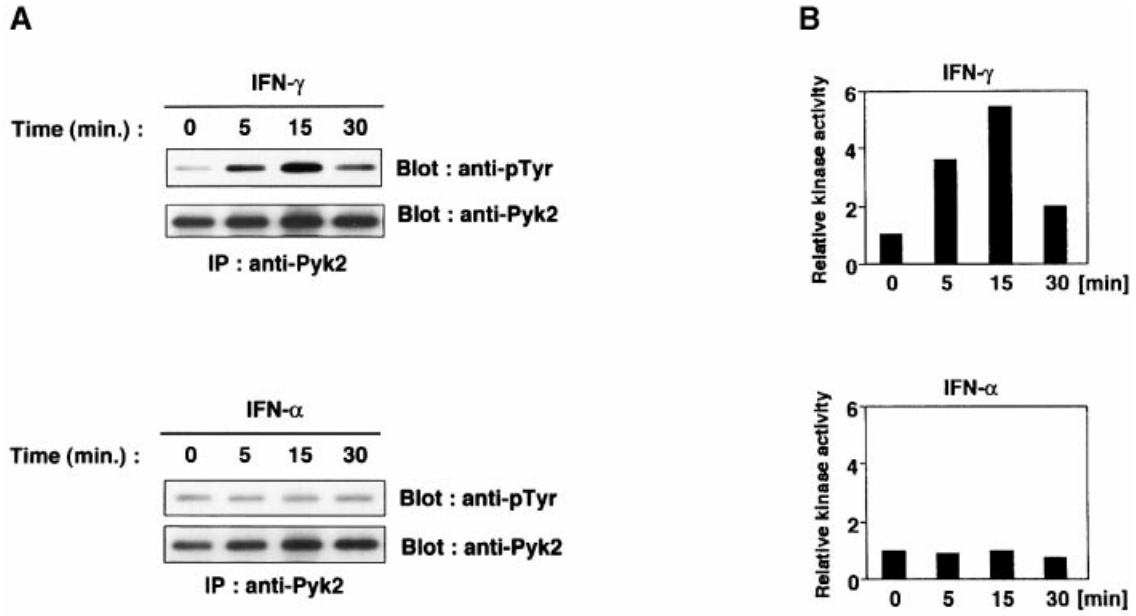

Fig. 1. IFN-γ, but not IFN-α, selectively activates Pyk2 in EFs. (**A**) EFs were stimulated with IFN-γ (250 U/ml; upper) or IFN-α (250 U/ml; lower) for the time indicated and cell lysates were immunoprecipitated (IP) with anti-Pyk2 antibody (anti-Pyk2). Immunoprecipitates were separated by 7.5% SDS–PAGE and immunoblot analysis (Blot) was carried out using anti-phosphotyrosine (anti-pTyr) or anti-Pyk2 antibody. (**B**) Tyrosine phosphorylation of an exogenous substrate by Pyk2. Immunoprecipitated Pyk2 from EFs treated with IFN-γ (upper panel) or IFN-α (lower panel) as described in (A), were subjected to an *in vitro* kinase assay in the presence of poly(Glu-Tyr) (4:1) as an exogenous substrate. The data are presented as kinase activity relative to that of untreated cells.

X, any amino acid; $n = 1$ or 2), and it has been shown that the MAPK member, extracellular signal-regulated protein kinase 2 (Erk2) is activated by IFN-α/β, through interaction with one of the subunits of IFN-α/βR (David *et al*., 1995). As for IFN-γ signaling, it has been reported that the serine phosphorylation of Stat1 occurs via a Jak2-dependent, but Ras-independent, pathway (Zhu *et al*., 1997), and IFN-γ-induced Erk1/Erk2 activation has been demonstrated (Ding *et al*., 1994; Liu *et al*., 1994; Singh *et al*., 1996; Nishiya *et al*., 1997; Zhu *et al*., 1997; Kovarik *et al*., 1998). In addition, Stat1 is phosphorylated, albeit weakly, by MAPK *in vitro* (Chung *et al*., 1997). However, the mechanism(s) leading to the MAPK activation in the IFN–Jak signaling pathways remains unclear.

Proline-rich tyrosine kinase 2 (Pyk2), also known as cellular adhesion kinase β (CAK β), related adhesion focal tyrosine kinase (RAFTK) and calcium-dependent tyrosine kinase (CADTK), has been shown to function in signal transduction through G protein-coupled receptors in neuronal cells (Avraham *et al*., 1995; Lev *et al*., 1995; Sasaki *et al*., 1995; Yu *et al*., 1996), as well as in signaling via the T-cell receptor (Berg and Ostergaard, 1997; Qian *et al*., 1997). Pyk2 is structurally related to focal adhesion kinase (FAK) and contains the canonical binding site (Y881) for the SH2 domain of Grb2 and the interacting site for the SH2 domain of Src (Avraham *et al*., 1995; Lev *et al*., 1995; Sasaki *et al*., 1995; Dikic *et al*., 1996). It has been shown that Pyk2 is one of the signaling mediators critical for the G protein-coupled receptor to the MAPK pathway and that in many cells Pyk2 is activated by signals that elevate the intracellular Ca^{2+} concentration (Lev *et al*., 1995; Dikic *et al*., 1996; Tokiwa *et al*., 1996; Della Rocca *et al*., 1997). More recently, we have shown that Pyk2 is associated with, and tyrosine phosphorylated by, Jak3 in the pathway of interleukin-2 (IL-2) receptor signaling (Miyazaki *et al*., 1998).

In the present study, we demonstrate that Pyk2 is associated with and activated by Jak2. We also provide evidence that Pyk2 is a critical mediator of the IFN-γ-, but not IFN-α-mediated activation of Erk2 to modulate the Stat1 activity. Our findings also suggest that the activation of Erk2, and possibly other MAPKs, by these two types of IFN is mediated by mechanisms distinct from each other, and that Pyk2 is an integral member of the IFN-γ–Jak–Stat/IRF pathway conferring resistance against viruses.

Results

Selective stimulation of tyrosine phosphorylation of Pyk2 by IFN-γ

To determine the role of Pyk2 in the IFN-signaling cascades, we tested first whether Pyk2 tyrosine phosphorylation is induced upon stimulation by IFN-α or IFN-γ. Immunoblotting analysis using anti-phosphotyrosine antibody revealed that in mouse embryonic fibroblasts (EFs), Pyk2 was weakly tyrosine phosphorylated in the absence of cytokine stimulation and that this phosphorylation was accelerated markedly by treatment with IFN-γ. As shown in Figure 1A (upper panel), the Pyk2 phosphorylation level increased within 5 min and peaked 15 min after IFN-γ stimulation. In contrast, no such increase was observed in the same cells upon IFN-α stimulation (Figure 1A, lower panel). We further measured *in vitro* kinase activities of Pyk2 using poly(Glu-Tyr) (4:1) as an exogenous substrate (Lev *et al*., 1995; Miyazaki *et al*., 1998). As shown in Figure 1B, stimulation by IFN-γ, but not by IFN-α, induced the kinase activities of

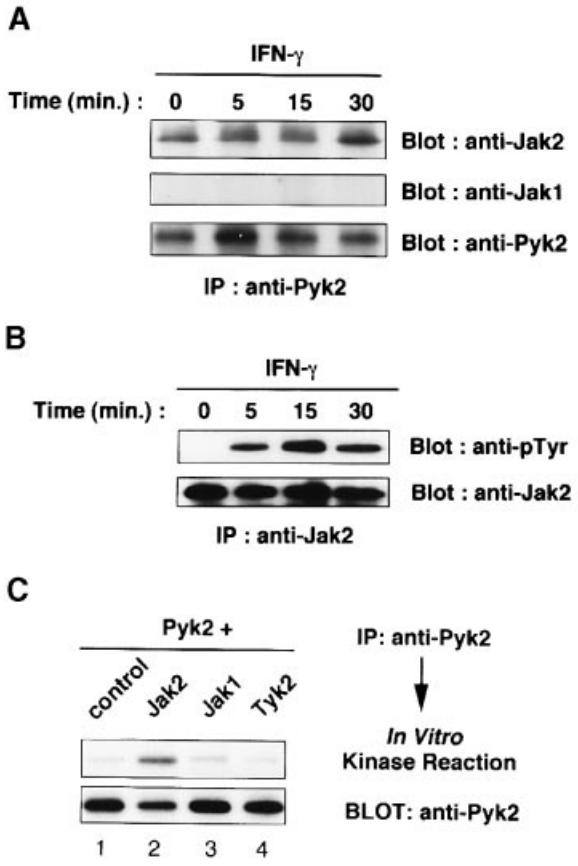

Fig. 2. Pyk2 is constitutively associated with Jak2 and preferentially activated by Jak2 but not by Jak1 or Tyk2. (**A**) Cell extracts were prepared from mouse EFs treated with IFN-γ (250 U/ml) for the indicated times. Cell extracts were then immunoprecipitated (IP) with anti-Pyk2, followed by immunoblot analysis (Blot) using anti-Jak2 (upper panel), anti-Jak1 (middle panel) and anti-Pyk2 (lower panel). (**B**) Mouse EFs were treated with IFN-γ (250 U/ml) as described above. Cell lysates were precipitated with anti-Jak2, resolved by SDS–PAGE and blotted with anti-pTyr (upper) or anti-Jak2 (lower). (**C**) Pyk2 was co-transfected with control vector (lane 1), Jak2 (lane 2), Jak1 (lane 3) or Tyk2 (lane 4) expression vector into COS cells. Forty-eight hours after transfection, cell lysates were prepared, immunoprecipitated with anti-Pyk2 and then subjected to *in vitro* kinase reaction. The expression of Pyk2 in each transfected cell was determined by immunoblotting using whole-cell lysates with anti-Pyk2 (lower panel).

Pyk2. This result is correlated with increased tyrosine phosphorylation of Pyk2 after IFN-γ treatment (Figure 1A). We also observed the IFN-γ-induced activation of Pyk2 in human peripheral T lymphocytes, indicating that the activation of Pyk2 in response to IFN-γ is not restricted to fibroblasts (data not shown).

Selective association of Pyk2 with Jak2

Early events in IFN-α and IFN-γ signaling involve the activation of Jak family kinases; Jak1 and Tyk2 for the former, and Jak1 and Jak2 for the latter (Darnell *et al*., 1994; Ihle and Kerr, 1995). In order to gain insight into the IFN-γ-induced Pyk2 tyrosine phosphorylation, we first examined whether Pyk2 interacts with Jak1 or Jak2 in EFs using co-immunoprecipitation analysis. It was found that Pyk2 is selectively associated with Jak2 before and after IFN-γ stimulation (Figure 2A). As shown in Figure 2B, Jak2 tyrosine phosphorylation was induced within 5 min and peaked 15 min after IFN-γ stimulation; these kinetics are similar to those for Pyk2 phosphorylation (Figure 1A, upper panel), suggesting that Pyk2 is a direct substrate of Jak2. To test this possibility further, a Pyk2 expression vector, pEF-Pyk2, was co-transfected with the Jak1, Jak2 or Tyk2 expression vectors into COS cells, and the effect of these Jak PTKs on the Pyk2 PTK activity was determined *in vitro*. As shown in Figure 2C, accelerated Pyk2 kinase activity was selectively observed in cells co-transfected with Pyk2 and Jak2 expression vectors. These results further suggest that, among the Jak PTKs involved in the IFN-α/β and IFN-γ signaling, Pyk2 association is specific to Jak2 and Pyk2 activation is mediated by Jak2.

Effect of a dominant interfering mutant of Pyk2 on the IFN-induced activation of Stat1

To determine the functional role of Pyk2 in the IFN signal transduction pathway, we established 3T3 PM1, an NIH 3T3-derived cell clone expressing a kinase inactive form of Pyk2 (PKM; Lev *et al*., 1995). It has been reported previously that in NIH 3T3 cells, Pyk2 is also expressed at a level lower than that in EFs, and that PKM functions as the dominant interfering mutant of Pyk2 in these cells (Miyazaki *et al*., 1998). Indeed, the IFN-γ-induced Pyk2 PTK activation, as monitored by the *in vitro* kinase assay, is almost completely inhibited in 3T3 PM1 cells (data not shown). The 3T3 PM1 and control 3T3 cells were treated with IFN-γ for 15 min, and whole-cell lysates were prepared and subjected to electrophoretic mobility shift assay (EMSA) using a DNA probe for Stat1 (the IRF-1-derived GAS element). As shown in Figure 3A, IFN-γ-induced Stat1 DNA-binding activity was detected in the cell extracts from both 3T3 PM1 and 3T3 cells at similar levels. As expected, in EMSA this band was supershifted by pre-incubation with an anti-Stat1 antibody (data not shown). We also examined the IFN-γ-induced the tyrosine phosphorylation of Stat1, and found it to be indistinguishable between 3T3 and 3T3 PM1 cells (Figure 3A, lower). Thus, PKM does not interfere with the IFN-γ-induced DNA-binding activity of Stat1 and the Jak-mediated tyrosine phosphorylation of Stat1.

In view of the fact that the transcriptional activity, but not DNA-binding activity, of Stat1 needs serine phosphorylation at S727 (David *et al*., 1995; Wen *et al*., 1995; Zhang *et al*., 1995), we next examined whether PKM affects Stat1-dependent transcription induced by IFN-γ. First, 3T3 PM1 or 3T3 cells were transfected with a luciferase reporter gene containing the GAS element derived from the promoter of the *IRF-1* gene (Harada *et al*., 1994). As shown in Figure 3B, treatment of IFN-γ (15 or 60 U/ml) resulted in ~14-fold or 30-fold increase of the luciferase activity in the 3T3 cells, respectively. Interestingly, this IFN-γ-induced luciferase activity was found to be significantly reduced in 3T3 PM1 cells (Figure 3B).

The effect of PKM on the *IRF-1* gene induction by IFN-γ or IFN-α was then examined using RNA blotting analysis. As shown in Figure 3C (upper panel), induction of IRF-1 mRNA observed in IFN-γ-stimulated 3T3 PM1 was ~60% lower than that of 3T3 cells, whereas the IFN-α-mediated induction of IRF-1 mRNA was essentially the same in both 3T3 PM1 and 3T3 cells (Figure 3C; lower

panel). These results are consistent with those from the luciferase assay and collectively demonstrate that PKM affects IFN-γ-stimulated gene expression. Essentially the same observations were made in 3T3 PM2, an independently established cell line similarly expressing PKM (data not shown).

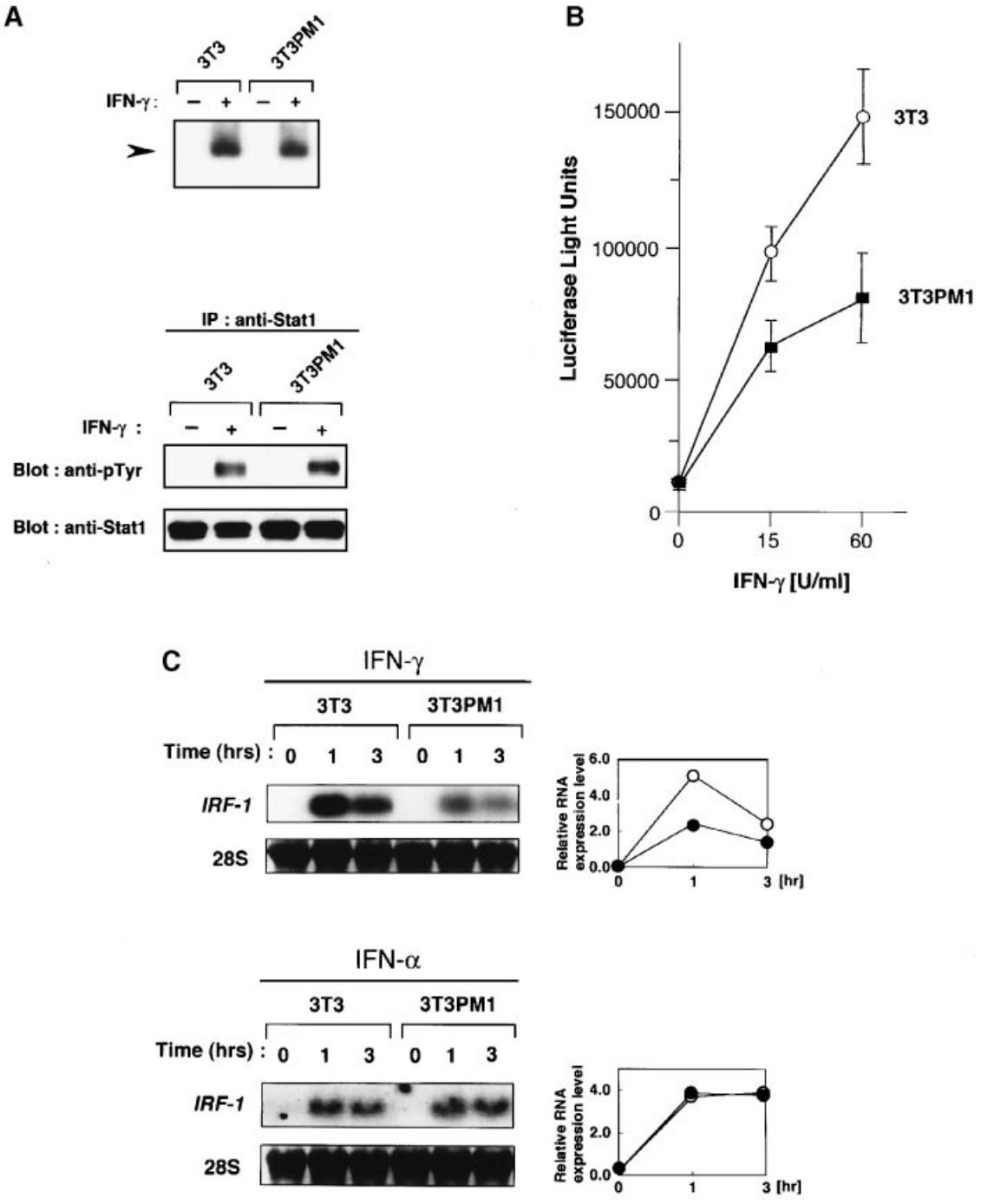

Fig. 3. Dominant interfering mutant PKM did not affect IFN-γ-induced DNA-binding activity of Stat1, but does affect the IFN-γ-stimulated gene induction. (**A**) The IFN-γ-induced DNA-binding activities (upper panel) and tyrosine phosphorylation (lower panel) of Stat1 in 3T3 and 3T3 PM1 cells. Cells were untreated (–) or treated (+) with IFN-γ (250 U/ml) for 15 min. Whole-cell extracts were incubated with ^{32}P-labeled oligonucleotide probe comprising the IRF-1-GAS element and subjected to EMSA (upper panel). The position of IFN-γ-induced DNA-binding complex is shown by the arrow. Cell lysates were also immunoprecipitated (IP) with anti-Stat1, followed by immunoblotting analysis (Blot) with anti-pTyr or anti-Stat1 antibody (lower panel). (**B**) 3T3 PM1 or 3T3 cells were transfected with pIRF1-GAS-luc. Forty-eight hours after transfection, cells were treated with IFN-γ for 6 h, cell extracts were prepared and subjected to luciferase assay. The averages and standard deviations (SD, error bars) of the values of luciferase activities from triplicates of a representative experiment are shown. (**C**) Total RNA was isolated from 3T3 PM1 or 3T3 cells, untreated or treated with IFN-γ (250 U/ml) or IFN-α (250 U/ml) for the indicated times, and then subjected to RNA blotting analysis using ^{32}P-labeled IRF-1 cDNA as a probe. An equal amount of RNA in each slot was confirmed by visualizing 28S ribosomal RNA (28S) using methylene blue staining. The relative band intensities of IRF-1 mRNA quantified by densitometer were normalized to that of 28S rRNA and depicted in graphs beside the autograms. Open circles, 3T3 cells; closed circles, 3T3 PM1 cells.

Inhibition of S727 phosphorylation of Stat1 protein by PKM

It has been reported that maximal transcriptional activation of Stat1 requires serine phosphorylation at 727 (S727; David *et al*., 1995; Wen *et al*., 1995). We then examined whether the kinase-inactive PKM affects IFN-γ-induced S727 phosphorylation of Stat1, using the antiserum against phospho-S727-Stat1 (anti-pS727; Kovarik *et al*., 1998). As shown in Figure 4, a low constitutive S727 phosphorylation of Stat1 was observed in both 3T3 cells and 3T3 PM1 cells. The phosphorylation level was enhanced further (~10-fold) following IFN-γ stimulation in 3T3 cells; however, such an enhancement was not detected in 3T3 PM1 cells (Figure 4). These results are congruent with the above results, suggesting *in toto* that Jak2-associated Pyk2 is involved in the S727 phosphorylation of Stat1 in the IFN-γ signaling.

Effect of PKM on IFN-γ-induced activation of the Erk2 MAP kinase

The above results demonstrate that the IFN-γ-induced transcriptional activity, but not the DNA-binding activity, of Stat1 is regulated by the Jak2-associated Pyk2. What is the mechanism(s) by which the Stat1 serine phosphorylation mediated through Pyk2? Previously, it has been shown that the transcriptional activity of Stat1, induced by IFN-α/β, is regulated through serine phosphorylation by the MAPK, Erk2 (David *et al*., 1995). In addition, it has been reported that both IFN-α/β and IFN-γ induce tyrosine phosphorylation and activation of Erk2 in several cell types (David *et al*., 1995; Singh *et al*., 1996; Nishiya *et al*., 1997; Kovarik *et al*., 1998), and that Pyk2 is involved in the linkage of G-protein-coupled receptor-mediated signals with the MAPK signaling pathway (Lev *et al*., 1995; Dikic *et al*., 1996; Della Rocca *et al*., 1997). To this end, we next examined the IFN-induced Erk1/Erk2 activation. Immunoblotting analysis using the polyclonal antibody against activated (tyrosine- and threonine-phosphorylated) Erk1/Erk2 (Chaudhary and Avioli, 1997; Sgambato *et al*., 1998) was performed using cell extracts from 3T3 PM1 and 3T3 cells stimulated by IFN-α or IFN-γ. As shown in Figure 5A, activation of Erk1 and Erk2 by IFN-α was observed in both 3T3 PM1 and 3T3 cells at similar levels, whereas activation by IFN-γ was significantly reduced in 3T3 PM1 cells, particularly for Erk2. Furthermore, an *in vitro* kinase assay revealed that the IFN-γ-induced Erk2 activity was also reduced in response to IFN-γ treatment in 3T3 PM1 cells, compared with that in 3T3 cells (a reduction from ~12-fold induction to 6-fold induction at 10 min; Figure 5B). In contrast, the IFN-α-induced activation of Erk2 was not affected in 3T3 PM1 cells, a result expected from data in Figure 5A. These results suggest that Pyk2 is involved in the regulation of Erk2 activity in the IFN-γ-induced Jak–Stat signaling cascade.

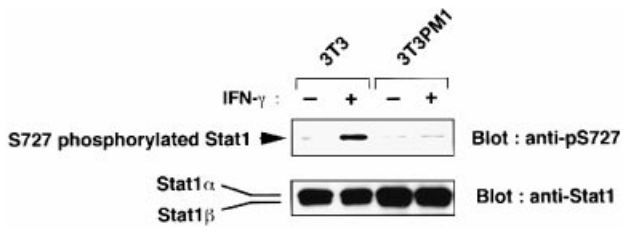

Fig. 4. Inhibition of Stat1–S727 phosphorylation in PKM-expressing cells. Cells were untreated (–) or treated (+) for 20 min with IFN-γ (250 U/ml), and cell extracts were subjected to immunoprecipitation with anti-Stat1 antibody and separated by 10% SDS–PAGE, and immunoblotting was carried out with anti-pS727 serum or anti-Stat1 antibody.

Selective suppression of antiviral activity induced by IFN-γ in cells expressing PKM

Activation of Stat1 is known to be a critical transcriptional factor for the establishment of the antiviral state by IFNs (Durbin *et al*., 1996; Meraz *et al*., 1996). The above results prompted us to examine whether this IFN action is affected in 3T3 PM1 cells, using cytopathic effect (CPE) assay with encephalomyocarditis virus (EMCV). As shown in Figure 6, ~10-fold concentration of IFN-γ is required to reach 50% protection of CPE (left panel) when 3T3 PM1 and 3T3 PM2 cells were pretreated with IFN-γ, compared with the 3T3 cells, whereas the response of the 3T3 PM1 and 3T3 PM2 cells to IFN-α was indistinguishable from that of the 3T3 cells (right panel). These results indicate that Pyk2 selectively participates in the antiviral response by IFN-γ.

Discussion

Both type I and type II IFNs induce cellular resistance against viral infection by induction of an overlapping set of target genes (DeMaeyer and DeMaeyer-Guignard, 1988; Darnell *et al*., 1994; Vilcek and Sen, 1996). However, these two types of IFNs utilize distinct receptor molecules and different combinations of Jak PTKs, suggesting that each type of IFN might employ distinct regulatory mechanisms to control the Jak–Stat signaling pathway. In the current study, we present results indicating that Pyk2 is a selective mediator of the IFN-γ-dependent activation of Stat1, thereby playing an essential role in the antiviral action of IFN-γ.

Pyk2 shares structural similarity with FAK (~65% similarity at the amino acid sequence level). However, accumulating reports have shown that Pyk2 mediates various signals via distinct pathways from FAK, such as activation by signals that elevate intracellular Ca^{2+} concentration, by G-protein-coupled receptors, tumor necrosis factor-α (TNF-α) and stress signals (Earp *et al*., 1995; Lev *et al*., 1995; Dikic *et al*., 1996; Tokiwa *et al*., 1996; Yu *et al*., 1996). Recently, we demonstrated that IL-2 stimulation resulted in the activation of Pyk2, and that Pyk2 physically associates with and is activated by Jak3 (Miyazaki *et al*., 1998). Previously, it has been reported that Jak2 is required for both tyrosine and serine phosphorylation of Stat1 (Zhu *et al*., 1997). In the present study, we found that Pyk2 is selectively activated by IFN-γ, but not by IFN-α, via its association with and activation by Jak2. Furthermore, the expression of PKM was found to inhibit the IFN-γ signaling pathway targeting the Stat1 activation. In fact, this inhibition was found to be at the specific serine phosphorylation of Stat1 at S727 (Figure 4), which is critical for its transcriptional activity (Figure 3; Wen *et al*., 1995). Finally, expression of PKM in 3T3 cells was found to result in an impaired antiviral response to IFN-γ but not to IFN-α (Figure 6). We also found that PKM did not affect the Jak2 activation by

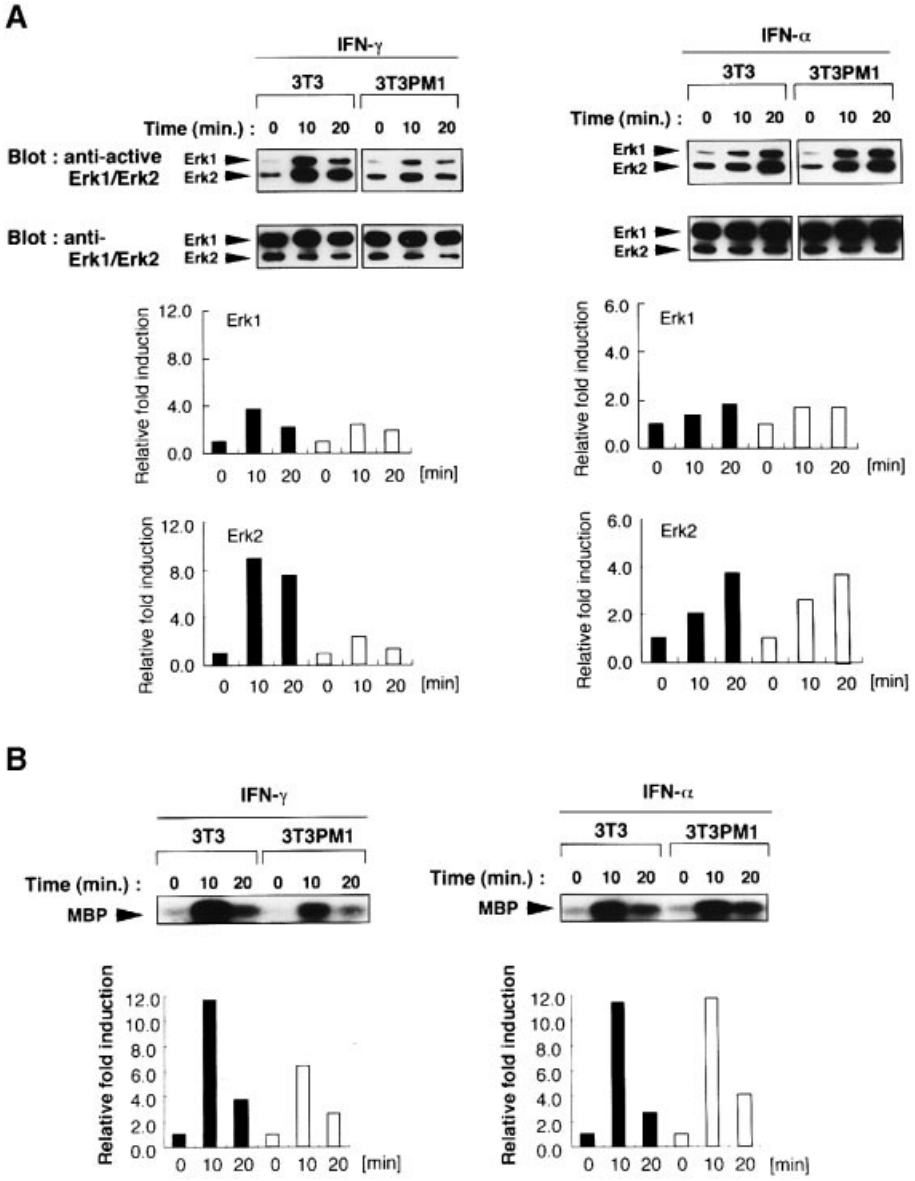

Fig. 5. Erk2 activation by IFN-γ treatment was selectively inhibited in cells expressing PKM. (**A**) After serum starvation for 48 h, cell lysates were prepared from either untreated cells or cells incubated for the indicated times with IFN-γ or IFN-α, separated by SDS–PAGE and subjected to the immunoblotting analysis, the polyclonal antibody against the active form of Erk1/Erk2 or anti-Erk1/Erk2 antibody. The lower panels represent the quantitative display for intensities of the corresponding bands normalized to that of blot by anti-Erk1/Erk2 as determined by densitometer. (**B**) Cell extracts were prepared from 3T3 or 3T3 PM1 cells treated with IFNs for the indicated times. Following cell lysis, Erk2 protein was immunoprecipitated as described in Materials and methods, and immunocomplexes were incubated at 30°C for 20 min with MBP. Equal volumes of the proteins were separated by 12% SDS–PAGE. (Upper panel) autographs of phosphorylated MBP. (Lower panel) the relative radioactivity of the corresponding bands as measured by liquid scintillation counter.

IFN-γ (data not shown), indicating that Pyk2 is downstream of Jak2. Collectively, our results suggest that Pyk2 acts downstream of Jak2 to modulate Stat1 activation in IFN-γ signaling to confer cellular resistance against viruses. At this stage we cannot rule out the possibility that PKM also affects other PTKs that are essential for MAPK activation, thereby exerting its dominant interfering function, on signaling via IFN-γ.

The detailed mechanism by which IFN-γ-mediated Pyk2 activation leads to the serine phosphorylation of Stat1 remains unclear. In this regard, it has been shown that in the case of IFN-α/β signaling, a MAPK member Erk2 is constitutively associated with IFN-α/βR subunit 1 and activated by IFN-β, resulting in the association of Erk2 with Stat1 (David *et al*., 1995). It has also been demonstrated that IFN-γ induces the activation of the MAPKs Erk1 and Erk2 in several cell types (Liu *et al.*, 1994; David *et al*., 1995; Singh *et al*., 1996; Nishiya *et al*., 1997; Kovarik *et al*., 1998). In the context of Pyk2-mediated signal transduction, it has been shown that Pyk2 activates the MAPK pathway through Ras (Lev *et al*., 1995). Taken together, a probable scenario is that the Jak2-dependent Pyk2 activation by IFN-γ is coupled to the Ras–Raf–MEK–MAPK pathway.

In contrast, the above scenario is at odds with previous reports (Zhu *et al*., 1997; Sakatsume *et al*., 1998; see below). We have shown a strong inhibition of Stat1 phosphorylation at S727 and Erk2 activation by IFN-γ in

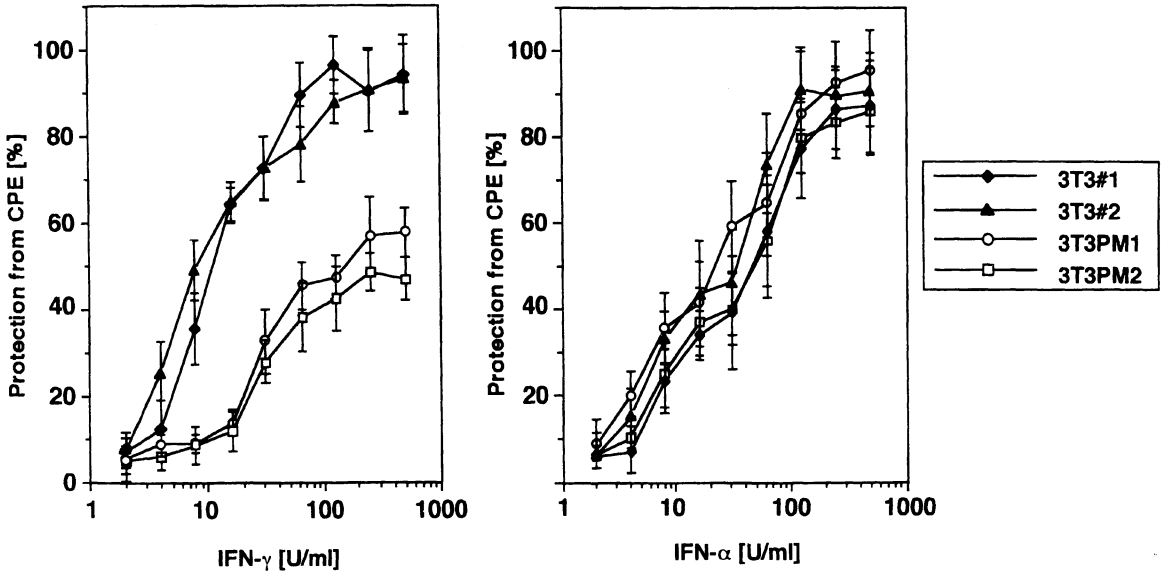

Fig. 6. Selective involvement of Pyk2 in the antiviral activity of IFN-γ. Cells in 96-well plates were pretreated with the appropriate IFN dose for 18 h, and subsequently infected with EMCV (m.o.i. = 1). After 24 h, the cytopathic effect was quantitated. Indicated values are means and SD (error bars) of triplicates in a representative experiment.

cells expressing PKM, supporting the notion that, as in IFN-α signaling, Erk2 is mainly responsible for Stat1 activation (David *et al*., 1995). However, it has been reported that in NIH 3T3 cells, in which the Ras-mediated Erk2 activation is inhibited, Stat1 serine phosphorylation can still take place, even in the absence of other MAPKs, i.e. JNK and p38 (Zhu *et al*., 1997). Therefore, it is possible that another, as yet unidentified serine kinase(s) controlled by Pyk2 may also be involved in the serine phosphorylation of Stat1 (Zhu *et al*., 1997). Further work will be required to clarify at these points.

In summary, Pyk2 appears to function as a selective effector mediating the IFN-γ-induced signaling pathway to establish an antiviral state. Further investigation, including the characterization of the interaction between Pyk2 and Jak2, elucidation of the mechanism of the Pyk2-linked MAPK pathway, and identification of other kinases will be required to fully elucidate the IFN-γ-induced intracellular signaling mechanism for the Stat1 activation.

Materials and methods

Cells and cell cultures

EFs were prepared using a standard procedure (Kimura *et al*., 1994). EFs and the NIH 3T3-derived cell line 3T3αβγ cells (Minami *et al*., 1994) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, MD) supplemented with 10% fetal calf serum (FCS). COS cells were grown in Iscove's modified Dulbecco's medium (Gibco-BRL) with 10% FCS.

Immunoprecipitation and immunoblotting

Cell lysis, immunoprecipitation and immunoblotting were carried out following methods described previously (Miyazaki *et al*., 1994). After washing three times with phosphate-buffered saline (PBS), the cells were cultured in DMEM with 0.1% (v/v) FCS for 12 h and stimulated with recombinant murine IFN-γ (250 U/ml; Genzyme, Cambridge, MA) or immunoaffinity-purified natural murine IFN-α (250 U/ml; kindly provided by Sumitomo Pharmaceuticals Co. Ltd). For immunoprecipitation and immunoblotting, rabbit anti-sera against Pyk2 was used as described (no. 600; Dikic *et al*., 1996). Antibodies specific to phosphotyrosine (4G10; Upstate Biotechnology, NY) and Jak2 (Upstate Biotechnology) were used as described previously (Miyazaki *et al*., 1994). Rabbit antiserum specific for the serine-phosphorylated Stat1 was used (Kovarik *et al*., 1998). Rabbit polyclonal Stat1 p84/p91 (E-23) antibody, directed against a C-terminal region common to Stat1α p91 and Stat1β p84 (Santa Cruz Biotechnology Inc., CA) was used for immunoprecipitation at a concentration of 2 μg/ml and for immunoblotting at 0.4 μg/ml. For these experiments, bound antibody was visualized using the chemiluminescent ECL kit (Amersham, UK) following the manufacturer's instructions.

Analysis of Pyk2 activation

For exogenous substrate phosphorylation by Pyk2, *in vitro* kinase assays were carried out as described previously (Lev *et al*., 1995; Miyazaki *et al*., 1998). The phosphorylated products were resolved by SDS–PAGE and quantified by imaging analysis of the dried gel with Bioimaging Analyzer (BAS 5000, Fuji Photo Films, Tokyo). For COS cell transfection experiments, the cells were co-transfected with cDNA expression vector, pEF-Pyk2 (Miyazaki *et al*., 1998), together with pEF–Jak1, pEF–Jak2 (Miyazaki *et al*., 1998) or pEF–Tyk2 (pEF vector containing Tyk2 cDNA; Gauzzi *et al*., 1996) using the calcium phosphate method. The cell lysates were immunoprecipitated with anti-Pyk2 antibody and the immunoprecipitates were subjected to an *in vitro* kinase assay or immunoblotting with anti-Pyk2 antibody as described previously (Miyazaki *et al*., 1998).

DNA transfection

The dominant-negative Pyk2 mutant expression vector, pEF-PKM (Miyazaki *et al*., 1998) or the control vector pEF were co-transfected into 3T3αβγ cells with the blasticidin-resistance gene (pSV2bsr; Izumi *et al*., 1991) using the calcium phosphate method (Miyazaki *et al*., 1991). Forty-eight hours after DNA transfection, selection was initiated using blasticidin (0.5 μg/ml), and the transformant clones, designated as 3T3 PM1, 3T3 PM2 (PKM-expressing cell clones) and 3T3 (control vector-transfected clone) were established.

EMSA and luciferase assay

Whole-cell extracts (50 μg of protein) were prepared and subjected to EMSA, using a ^{32}P-labeled probe DNA containing GAS element of the mouse IRF-1 promoter according to the method described previously (Fujii *et al*., 1995). pIRF1-GAS-luc was constructed by the insertion of a *Sac*I fragment from p-3500IRF1TK (Harada *et al*., 1994) into the PicaGene basic Vector 2, a luciferase reporter plasmid (Nippon Gene, Japan). DNA transfection and luciferase assay were performed as described previously (Shibuya *et al*., 1994).

RNA preparation and RNA blotting analysis

Total cellular RNA was extracted by guanidinium thiocyanate/CsCl-centrifugation and subjected to RNA blotting analysis using the probe for IRF-1 (Kimura *et al*., 1994).

Analysis of MAPK activation

The serum-starved 3T3 or 3T3 PM cells were treated with IFN-γ (250 U/ml) or IFN-α (250 U/ml). Cell lysates were separated by 10% SDS–PAGE and subjected to immunoblotting analysis using antisera specific for the active forms of Erk1 and Erk2 kinases (Promega, WI) or anti-Erk1/Erk2 antibody (Upstate Biotechnology). For the MAP kinase assay, the same cells were lysed with lysis buffer (Miyazaki *et al*., 1994), and the lysates were subjected to immunoprecipitation with anti-Erk2 antibody (Upstate Biotechnology) as described (Miyazaki *et al*., 1994). The Erk2 immunocomplexes were washed and then incubated in a kinase assay buffer composed of 50 mM Tris–HCl pH 8.0, 25 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM EGTA, 10% glycerol, 20 mM ATP, 1 μCi of [γ-^{32}P]ATP and 0.5 mg/ml myelin basic protein (Sigma Chemical Co., St Louis, MO). After 20 min at 30°C, equal volumes of the proteins were separated by 10% SDS–PAGE. The radioactivity of the phosphorylated MBP band running at 20 kDa was quantified with a liquid scintillation counter (Beckman).

CPE assay

The 3T3, 3T3 PM1 and 3T3 PM2 cells were plated at 1×10^4 cells/well with 96-well plates. After 12 h culture, cells were treated with the appropriate dilutions of IFN-α or -γ for 18 h. After washing, the cells were infected with EMCV (m.o.i. = 1). The CPE was quantified by staining with crystal violet and determining the absorbance at 565 nm. Full protection (100%) from the CPE corresponds to the difference between the absorbance of untreated, uninfected cells and the untreated EMCV-infected cells (Fish *et al*., 1988; Muller *et al*., 1994).

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