Activation of Stat1 and subsequent transcription of inducible nitric oxide synthase gene in C6 glioma cells is independent of interferon-γ-induced MAPK activation that is mediated by p21^{ras}

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Abstract Rat C6 glioma cells have been used to characterize molecular events involved in the regulation of inducible nitric oxide synthase (iNOS) gene expression stimulated by interferon-γ (IFN-γ) plus lipopolysaccharide (LPS). IFNs induce a signaling event which involves activation of Stat1 transcription factor. Previous studies have shown that IFNs also induce extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) activation. However, the mechanisms by which IFNs stimulate MAPK activation remain elusive. Here we show that in C6 glioma cells, transiently expressing the dominant-negative form of c-Ha-Ras (Asn-17) abrogated IFN-γ-induced ERK1 and ERK2 activation. Furthermore, PD98059, a specific MEK1 inhibitor, also blocked this activation. These results indicate that p21^{ras} and MEK1 are required for IFN-γ-induced ERK1 and ERK2 activation. Recent studies have reported that MAPK is responsible for serine phosphorylation of Stat1 which is required for Stat1's DNA binding and maximal transcriptional activity. Thus, we examined the role of the Ras-MAPK pathway in Stat1 activation and subsequent iNOS induction in C6 glioma cells. Further experiments showed that neither Asn-17 Ras expression nor concentrations of PD98059, which completely abrogated IFN-γ-induced ERK1 and ERK2 activation, affected Stat1 DNA binding activity or iNOS induction, indicating that the Ras-MAPK pathway does not appear to be involved in the activation of Stat1 and subsequent iNOS induction in C6 glioma cells.

Key words: Interferon-γ; Ras; ERK/MAPK; Stat1; iNOS; C6 glioma cell

1. Introduction

There are three gene products which are known to act as nitric oxide synthase (NOS). The high-output inducible NOS isoform (iNOS) is expressed in different cell lines, tissues and species in response to various cytokines (such as IFN-γ, IL-1β and TNF-α) and microbial products (including lipopolysaccharide (LPS)) [1]. iNOS plays critical roles in many biological processes, such as host defense, vasodilation, synaptic plasticity and neurotransmission [2]. In contrast, iNOS contributes to the pathogenesis of various diseases and leads to organ destruction in some inflammatory [3] and autoimmune diseases [4]. Therefore, to regulate production and prevent adverse effects of iNOS-produced nitric oxide (NO), it is necessary to understand how expression of iNOS is regulated.

Unfortunately, the signaling events involved in iNOS expression are not well understood. It has been suggested that two positions on the murine macrophage iNOS gene promoter region that contain a κB site and IFN-γ-activated site (GAS) are necessary for iNOS induction in response to IFN-γ/LPS [5,6]. In glial cells, iNOS induction stimulated by IFN-γ/LPS has been reported to require tyrosine kinase activity [specifically, JAK2] [7–9]. IFN-γ activates the Janus kinases JAK1 and JAK2 and subsequent tyrosine phosphorylation, nuclear translocation and binding of Stat1 transcription factor to the GAS element [10]. Stat1 is also phosphorylated on serine as well as tyrosine residue [11]. The substitution of Ser-727 with Ala-727 renders Stat1 unable to induce transcription [11]. The COOH-terminal region of Stat1 (-Pro-Met-^{727}Ser-Pro-) is similar to the MAPK recognition consensus sites [11,12]. David and colleagues [12] have noted that ERK2 constitutively associates with the IFNα/β receptor and enzymatically activated and associated with Stat1 in response to IFN-β. IFN-γ has also been shown to induce the activation of MAPK in several cell types [13,14], although the pathway(s) involved in this event are unclear.

We studied the mechanisms of MAPK activation in response to IFN-γ. Furthermore, we investigated whether or not the Ras-MAPK pathway is involved in IFN-γ-induced DNA binding of Stat1 and IFN-γ/LPS-enhanced iNOS induction in C6 glioma cells. Here we showed that either expression of dominant-negative mutant of c-Ha-Ras (Asn-17) in C6 cells or pretreatment of the C6 cells with PD98059, a specific MEK1 inhibitor, led to a repression of IFN-γ-induced ERK1 and ERK2 activation. This suggests that p21^{ras} and MEK1 are required for IFN-γ-induced ERK1 and ERK2 activation. However, neither Asn-17 Ras expression nor PD98059 affected IFN-γ-induced DNA binding of Stat1 to the GAS element, or on IFN-γ/LPS-induced iNOS induction. Thus, the Ras signaling pathway may not be involved in the activation of Stat1 and subsequent iNOS induction in C6 cells.

2. Materials and methods

2.1. Cell culture and DNA transfection

Rat C6 glioma cells (American Type Culture Collection) were maintained in F-10 medium (Gibco) supplemented with 15% horse serum (Gibco), 2.5% fetal calf serum (Boehringer Mannheim), 50 μg/ml penicillin and 100 μg/ml streptomycin in a humidified incubator containing 5% CO_2.

C6 cells were transfected by lipofectAMINE reagent (Gibco) with the vector P3′C:\TMP\equSS (Stratagene) expressing the LACI re-

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pressor and inhibitory Asn-17 Ras [15] cloned into the LACI repressible pOPRSV1 vector (Stratagene). Stable clones were selected in 400 μg/ml hygromycin B (Wako) and 400 μg/ml G418 (Gibco) and screened for inducible expression of c-Ha-Ras by immunoblotting. Incubation in 5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Wako) for 8–24 h was used to induce Ras expression. Several independent, inducible Asn-17 Ras clones were isolated, and the most expressive one was chosen for further analysis.

2.2. MAP kinase assays

C6 cells (6.2×10^6) were washed with 10 ml of ice-cold PBS, scraped into 1 ml of lysis buffer (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.4) and broken by passage through a 21-gauge needle. The lysates were on ice for 1 h and centrifuged at 15000 rpm for 20 min at 4°C. The supernatants were subjected to ERK1 or ERK2 immunoaffinity column (Santa Cruz). The partially purified ERK1 or ERK2 was boiled with SDS sample buffer for 3 min and resolved in 10% SDS gel in which myelin basic protein (MBP) (Sigma) were precrosslinked. Gels were washed in 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (Hepes), pH 7.4, containing 20% 2-propanol for 1 h (100 ml each, two changes) at room temperature (r.t.) to remove SDS. Gels were then washed in 250 ml of buffer A (50 mM Hepes, pH 7.4, 5 mM 2-mercaptoethanol) for 1 h at r.t.. Next, gels were treated in buffer A containing 6 M guanidine-HCl for 1 h at r.t., then renatured in buffer A containing 0.04% Triton X-100 for 16 h at 4°C (250 ml each, four changes). Gels were incubated in buffer B (25 mM Hepes, pH 7.4, 10 mM $MgCl_2$ and 2 mM dithiothreitol (DTT)) for 1 h at 25°C. The kinase reaction was performed in buffer C (25 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EGTA, 2 μM cAMP-dependent protein kinase inhibitor (Sigma), 40 mM nonradioactive ATP and 10 μCi/ml [γ-^{32}P]ATP (Dupont NEN)) for 1 h at 25°C. Gels were extensively washed with a solution of 5% TCA and 10 mM sodium pyrophosphate for 2 h at r.t. (250 ml each, four changes), dried on Whatman 3MM paper and subjected to autoradiography. Band quantitation was performed using FUJIX BASTATION software (Fuji Photo Film Co.). MAPK electrophoretic shift and its presence were determined by immunoblotting using anti-ERK1 polyclonal antibody (pAb) (Santa Cruz) or anti-ERK2 monoclonal antibody (mAb) (UBI). In this case, polyacrylamide gels with an acrylamide/bisacrylamide ratio of 30:0.2 were used (30:0.8 acrylamide/bisacrylamide gels were used for other experiments). Tyrosine phosphorylation of MAPK was determined by immunoblotting using anti-phosphotyrosine (pY) mAb (4G10) (UBI).

2.3. Phosphoamino acid analysis

The analyses were performed essentially as described by Boyle et al. [16]. Cells were starved for 2 h in phosphate-free DMEM (Sigma) with 15% horse serum and 2.5% fatal calf serum dialyzed against physiological saline. The labeling was carried out with 0.4 mCi/ml of [^{32}P] orthophosphate (Dupont NEN) for 4 h in medium otherwise lacking in phosphate. ERK1 proteins were partially purified as mentioned above and subjected to 11% SDS-PAGE and autoradiography. The ^{32}P-labeled ERK1 proteins were recovered after SDS-PAGE, digested with tosylphenylalanyl chloromethylketone (TPCK) -treated trypsin (0.33 mg/ml; Worthington Biochemical Corporation) in 50 mM NH_4HCO_3 and lyophilized. Phosphopeptides were hydrolyzed in 6 N HCl for 90 min at 110°C. Phosphoamino acids were spotted onto TLC plates (Merck, Art 5716), and separated by electrophoresis at pH 3.5. TLC plates were dried, exposed to imaging plates and visualized on a Fuji BAS 2000 (Fuji Photo Film Co.). Positions of individual phosphoamino acids were determined by comparison with the comigration of commercially available standards (Sigma) after staining with ninhydrin.

2.4. Electrophoretic mobility shift assays (EMSA) and antibody inhibition

Nuclear extracts were prepared using a slight modification of the method described by Schreiber et al. [17]. Briefly, 3.1×10^6 cells were harvested, washed once with 2 ml of ice-cold PBS and resuspended in 400 μl of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After incubation on ice for 15 min, Nonidet P-40 was added to a final concentration of 0.6%, and vigorously vortex-mixed for 10 s. The lysates were then centrifuged at 15000 rpm for 30 s at 4°C. The supernatants were removed and subjected to immunoblotting using anti-Ras mAb. The pellets (crude nuclei) were resuspended in 50 μl of buffer B (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and were vigorously vortex-mixed for 15 min at 4°C. The lysates were centrifuged at 15000 rpm for 5 min at 4°C and the supernatants containing the nuclear proteins were transferred into fresh vials. The protein concentrations of extracts were measured using a Bio-Rad protein assay kit.

EMSA was performed by incubating 5 μg of nuclear extracts with 2 μg of poly(dI-dC) (Pharmacia Biotech) in binding buffer (13 mM Hepes, pH 7.9, 65 mM NaCl, 0.15 mM EDTA, 1 mM DTT, 0.02% Nonidet P-40, 1 mg/ml bovine serum albumin (BSA), and 5% glycerol) (20 μl final volume) for 15 min on ice. Then, an end-labeled, double-stranded oligonucleotide probe (50000 cpm/0.3 ng) was added and the reaction mixture was incubated for 15 min at 25°C. The probe (5′-GTATTTCCCAGAAAAGG-3′) contained a core sequence corresponding to the IFN-γ response region (GRR) of the Fcγ receptor typegene [18]. Protein-DNA complexes were separated by electrophoresis in 0.25×TBE (Tris-Borate-EDTA) buffer through a 3.5% polyacrylamide gel. Gels were dried, exposed to imaging plates and visualized on a FUJI BAS2000. For supershift assay with specific antibody against the Stat1 C-terminal region (Santa Cruz), nuclear extracts were preincubated with 1 μg of antibody or 1 μg of normal rabbit serum for 1 h at 4°C before the probe was added.

2.5. Preparation of crude cytosol and membrane fractions

Cells (1.1×10^6) were washed with 4 ml of ice-cold PBS, added 200 μl of ice-cold buffer A (PBS, pH 7.4, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin), harvested and sonicated for 5 s. The lysates were centrifuged at 15000 rpm for 20 min at 4°C, and the supernatants were removed and used as crude cytosol fractions. The pellets were resuspended in 100 μl of ice-cold buffer A, sonicated for 5 s and used as crude membrane fractions. Crude cytosol and membrane fractions were boiled with SDS sample buffer for 5 min and subjected to immunoblotting using anti-maciNOS mAb and anti-Ras mAb, respectively. Protein concentrations of each fraction were measured using a Bio-Rad protein assay kit.

2.6. Immunoblotting

All immunoblots were blocked in TBST (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20) containing 1% BSA and then incubated with a 1:200 dilution (anti-ERK1 pAb) (Santa Cruz), 1:250 dilution (anti-ERK2 mAb) (UBI), 1:500 dilution (anti-Ras mAb) (Transduction laboratories), 1:1000 dilution (anti-pY mAb) (UBI), or a 1:2500 dilution (anti-macNOS mAb) (Transduction laboratories) in TBST. The blots were washed and incubated with a 1:2000 dilution of anti-mouse or -rabbit Ig-horseradish peroxidase conjugate (Amersham) in TBST. After several washes, antibody-reactive bands were visualized by enhanced chemiluminescence (ECL Western detection kit; Amersham).

2.7. RNA isolation and Northern blot analysis

Total cellular RNA was isolated from 1.2×10^7 cells by acidic guanidinium thiocyanate-phenol-chloroform extraction [19]. A 30 μg amount of total RNA was denatured by heating 65°C for 15 min in 2.2 M formaldehyde, 50% (v/v) formamide, electrophoresed in 1% agarose gels containing 2.2 M formaldehyde and then transferred on nitrocellulose filters by capillary action using 20×SSC (1×SSC is 0.015 M sodium citrate buffer, pH 7.0, containing 0.15 M NaCl). Filters were prehybridized for 7 h at 42°C in a solution containing 50% formamide, 50 mM NaH_2PO_4, 5×SSC, 1×Denhardt's solution and 40 μg/ml salmon sperm DNA, and then incubated for 16 h at 42°C with ^{32}P-labeled, randomly-primed cDNA fragments. After washing the filters twice at r.t. in 2×SSC, 0.1% SDS for 15 min and twice at 68°C in 1×SSC, 0.1% SDS for 15 min, autoradiography was performed by exposing the filter to imaging plates and visualizing then on a FUJI BAS2000. Between hybridizations using different cDNA probes, probes were stripped off of the membrane by boiling for 20 min in distilled water. The DNA probes used were a 700-bp fragment from the 5′ end of the rat liver iNOS cDNA [20], a 700-bp *Hin*dIII-*Pst*I fragment of the human p21^{ras} cDNA, and a 600-bp PCR fragment from the rat β-actin cDNA.

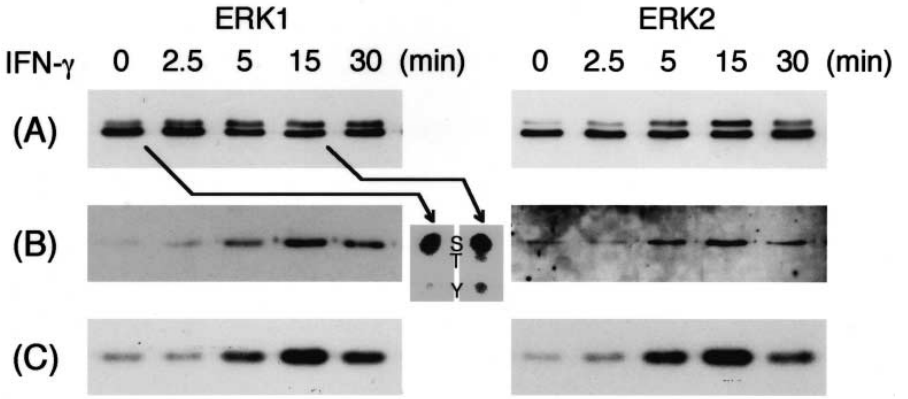

Fig. 1. ERK1 and ERK2 were phosphorylated and activated in response to IFN-γ in C6 cells. (A) MAPK electrophoretic shift by IFN-γ. C6 cells were treated for indicated times with 250 U/ml IFN-γ (Gibco). ERK1 and ERK2 were isolated from cell lysates by ERK1 or ERK2 immunoaffinity column. Partially purified ERK1 or ERK2 was fractionated by SDS-PAGE (acrylamide/bisacrylamide, 30:0.2), transferred to nitrocellulose, and immunoblotted using anti-ERK1 pAb or anti-ERK2 mAb. (B) Tyrosine phosphorylation of ERK1 and ERK2 by IFN-γ. Partially purified ERK1 or ERK2 was fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-pY mAb. Phosphoamino acid analysis of partially purified ERK1 isolated from ^{32}P-labeled C6 cells untreated or treated with 250 U/ml IFN-γ for 15 min was performed as described in Section 2. (C) ERK1 and ERK2 activation by IFN-γ. ERK1 and ERK2 activity were analyzed by in gel MBP kinase assay as described in Section 2.

3. Result

3.1. IFN-γ induces phosphorylation and activation of ERK1 and ERK2 in C6 cells

First, we examined whether or not IFN-γ induces MAPK activation in C6 cells. Three techniques previously established for assaying MAPK activation were simultaneously used: (1) electrophoretic shift of MAPK, (2) tyrosine phosphorylation of MAPK, and (3) in gel kinase assay using MBP as a substrate (see Section 2). Fig. 1A shows the electrophoretic shift of MAPK stained with anti-ERK1 or ERK2 antibody in which the slower migrating form represents phosphorylated MAPK. The slower migrating ERK1 was detected in a quiescent state, while the slower migrating ERK2 was detected in a time-dependent fashion. However, as shown in Fig. 1B, the immunoblot with anti-phosphotyrosine antibody showed that IFN-γ induced tyrosine phosphorylation of both ERK1 and ERK2 in a time-dependent fashion as can be seen in the figure as the ERK2 electrophoretic shift. Phosphoamino acid analysis of partially purified ERK1 from ^{32}P-labeled C6 cells demonstrated that basal phosphorylation occurred on serine. Treatment of the C6 cells with IFN-γ for 15 min resulted in the additional appearance of both phosphothreonine and phosphotyrosine, suggesting that the appearance of the slower migrating ERK1 in a quiescent state is due to phosphorylation of serine residues at the basal level. To directly determine whether MAPK activity was enhanced as a result of IFN-γ treatment of cells, we assayed MAPK activity using MBP as a substrate. As shown in Fig. 1C, IFN-γ stimulation increased phosphorylation of MBP by ERK1 and ERK2 within 5 min, and became maximal at 15 min, decreasing thereafter.

3.2. Expression of Asn-17 Ras inhibits IFN-γ-induced ERK1 and ERK2 activation in C6 cells

It has been shown that a large number of growth factors activate MAPK via p21^{ras}. To study whether or not the Ras

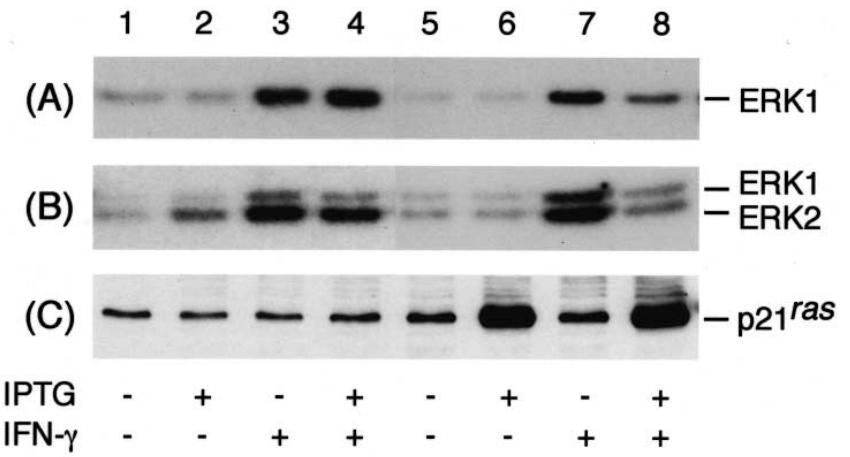

Fig. 2. Expression of dominant-negative Asn-17 Ras blocks IFN-γ-induced ERK1 and ERK2 activation in C6 glioma cells. (A) Inhibition of ERK1 activation by IFN-γ in Asn-17 Ras-expressing C6 cells. LacI (lanes 1–4) or Asn-17 Ras cells (lanes 5–8) were uninduced (−) or induced (+) with 5 mM IPTG for 24 h. Cells were unstimulated (−) or stimulated (+) for 15 min with 250 U/ml IFN-γ. ERK1 activity was assayed as in Fig. 1. (B) Inhibition of ERK2 activation by IFN-γ in Asn-17 Ras-expressing C6 cells. LacI or Asn-17 Ras cells were induced with IPTG, stimulated as indicated, and analyzed for ERK2 activation. (C) Immunoblot of Asn-17 Ras expression. Of each cell lysates, 20 μg were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-Ras mAb.

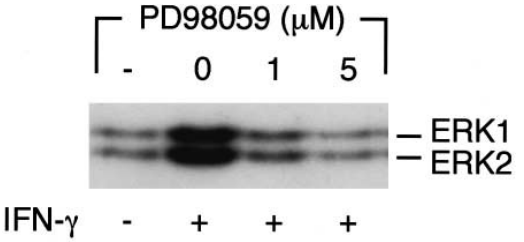

Fig. 3. PD98059 blocks IFN-γ-induced ERK1 and ERK2 activation in C6 glioma cells. C6 cells were pretreated for 1 h with PD98059 (New England Biolabs) at the indicated concentrations followed by incubation with (+) or without (−) 250 U/ml IFN-γ for 15 min. ERK1 and ERK2 were isolated from cell lysates and their activities were assayed as in Fig. 1.

signaling pathway is involved in IFN-γ-induced MAPK activation, the Lac Switch-inducible expression system (see Section 2) was used to control the expression of the dominant-negative mutant of c-Ha-Ras (Asn-17) in C6 cells. Fig. 2 shows the functional consequence of expressing Asn-17 Ras on MAPK activation in response to IFN-γ. IPTG-regulated expression of Asn-17 Ras is shown in Fig. 2C. Expression of Asn-17 Ras inhibited IFN-γ-induced activation of ERK1 (Fig. 2A) and ERK2 (Fig. 2B) (decreased 65% and 85%, respectively). Electrophoretic shift and tyrosine phosphorylation of MAPK in response to IFN-γ were also inhibited by expression of Asn-17 Ras (data not shown), suggesting that IFN-γ-induced MAPK activation is mediated by $p21^{ras}$. Expression of Asn-17 Ras, however, could not completely block the IFN-γ-induced MAPK activation. It is likely that the level of Asn-17 Ras expression was too low to completely block endogenous Ras or that IFN-γ activates MAPK via a Ras-independent pathway. It was surprising that, as shown in Fig. 2, phosphorylation of MBP by ERK1 was also detected. This suggests that different lots of ERK2 immunoaffinity column were used between the experiments shown in Figs. 1 and 2. The ERK2 immunoaffinity column used in Fig. 2 had cross-reacted with ERK1 proteins. Indeed, when purified proteins isolated by the ERK2 immunoaffinity column used in Fig. 2 were subjected to immunoblotting using anti-ERK1 pAb, ERK1 proteins were detected in addition to ERK2 proteins (data not shown).

3.3. *PD98059, a specific MEK1 inhibitor, blocks IFN-γ-induced ERK1 and ERK2 in C6 cells*

It is generally known that MAPK activation in response to growth factors, such as EGF, NGF, or PDGF, requires phosphorylation at both threonine and tyrosine residues by a dual specificity kinase, MEK. Because PD98059 was recently described by Dudley et al. [21] as a synthetic inhibitor of MEK1, specifically activating ERK1 and ERK2, we analyzed whether the addition of PD98059 blocks IFN-γ-induced ERK1 and ERK2 activation. As shown in Fig. 3, PD98059 blocked ERK1 and ERK2 activity toward MBP in a concentration-

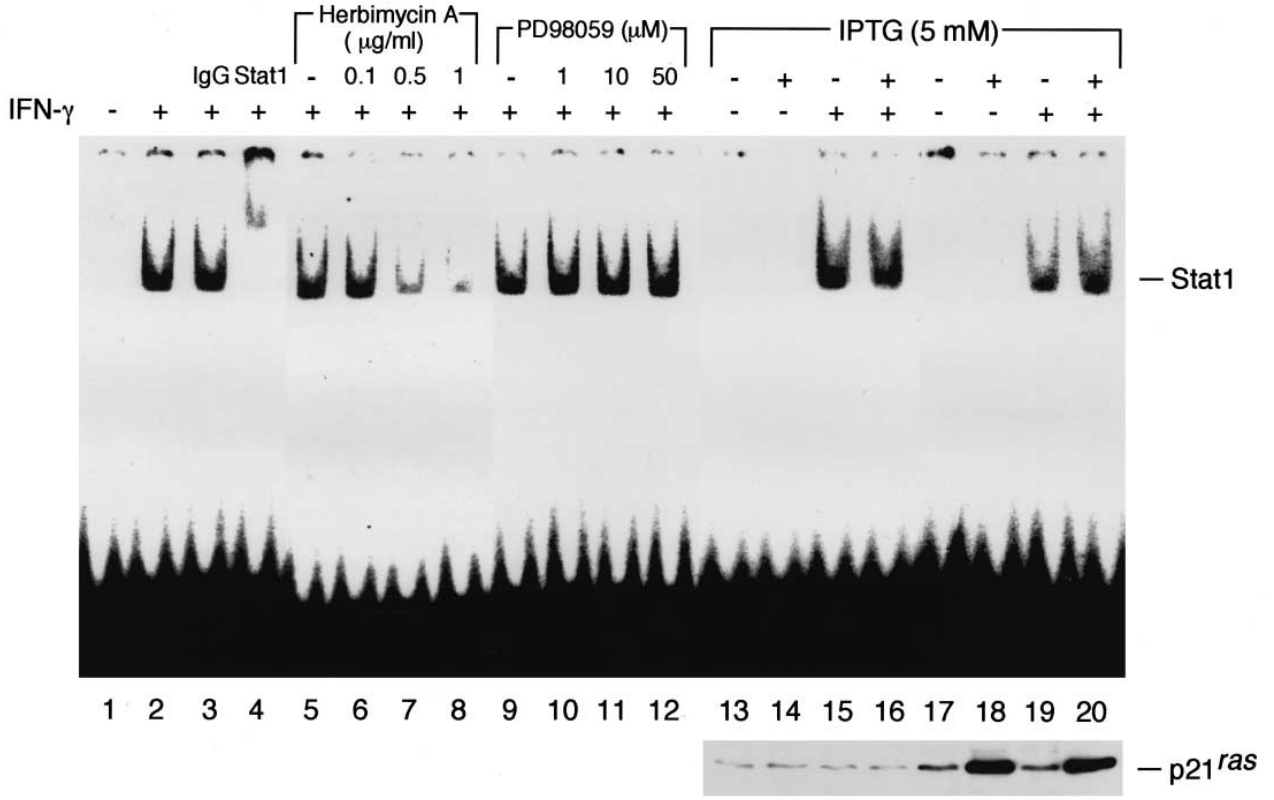

Fig. 4. Effects of herbimycin A, PD98059 and Asn-17 Ras expression on DNA binding of Stat1 in response to IFN-γ in C6 glioma cells. C6 cells were pretreated for 12 h with herbimycin A (lanes 5–8) or for 1 h with PD98059 (lanes 9–12) at the indicated concentrations followed by incubation with 250 U/ml IFN-γ for 30 min before preparing nuclear extracts for analysis by EMSA. LacI (lanes 13–16) or Asn-17 Ras cells (lanes 17–20) were pretreated for 24 h with 5 mM IPTG followed by incubation with 250 U/ml IFN-γ for 30 min. Then, nuclear extracts and whole cell lysates were prepared and subjected to EMSA and immunoblotting using anti-Ras mAb, respectively.

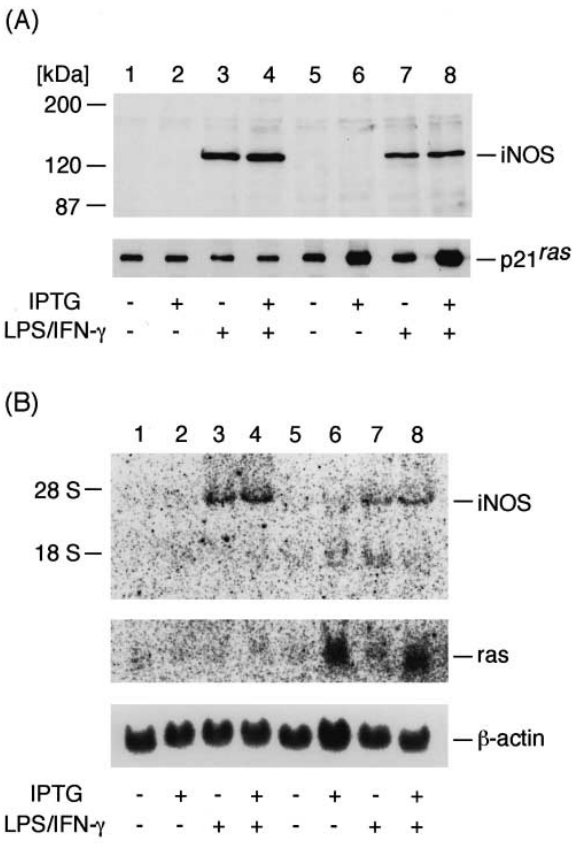

Fig. 5. Asn-17 Ras expression in C6 cells does not alter iNOS induction stimulated by IFN-γ/LPS in C6 glioma cells. (A) Crude cytosol and membrane fractions were prepared from LacI (lanes 1–4) or Asn-17 Ras cells (lanes 5–8) pretreated for 24 h with 5 mM IPTG followed by stimulation with 250 U/ml IFN-γ/10 μg/ml LPS (from *Escherichia coli* Serotype 055:B5, Sigma, L-2637) for 24 h. Crude cytosol fractions (30 μg) were subjected to immunoblotting using anti-maciNOS mAb, and crude membrane fractions (10 μg) were subjected to immunoblotting using anti-Ras mAb. (B) Total RNAs were isolated from LacI (lanes 1–4) or Asn-17 Ras cells (lanes 5–8) pretreated for 24 h with 5 mM IPTG followed by stimulation with 250 U/ml IFN-γ/10 μg/ml LPS for 24 h and subjected to Northern blot analysis using ^{32}P-labeled cDNA derived from rat iNOS and human Ras as described in Section 2. ^{32}P-labeled cDNA derived from rat β-actin was used as a control for the amounts of RNA used in each lane. Exposure times, 12 to 48 h.

dependent fashion. It appears, therefore, that IFN-γ-induced ERK1 and ERK2 activation is mediated by the classical MAPK cascade.

3.4. IFN-γ-induced DNA binding of Stat1 and IFN-γ/LPS-stimulated iNOS induction are independent of the Ras-MAPK pathway in C6 cells

According to functional analysis using deletion mutants of a 1753-bp fragment of the promoter region of iNOS cloned from the murine RAW 264.7 macrophage cell line, positions 50 to 250 and 900 to 1030 are important regulatory regions for iNOS induction stimulated by IFN-γ/LPS, each containing a κB site for NF-κB and an IFN-γ-activated site (GAS) for Stat1 homodimer [5,6]. We have previously demonstrated that IFN-γ-induced JAK2 activation can be involved in iNOS induction stimulated by IFN-γ/LPS in C6 cells [8]. JAK2 induces tyrosine phosphorylation and nuclear translocation of Stat1 transcription factor [10]. It has recently been suggested that H7, a serine/threonine kinase inhibitor, blocks IL-6-induced nuclear translocation and DNA binding of Stat3 [22]. Furthermore, the mutants of Ser-727 in Stat1 and Stat3 greatly reduced the ability of Stat1 and Stat3 to induce transcription. These mutants are present in the highly-conserved sequence (-Pro-Met-Ser-Pro-) in the COOH-terminal region of Stat1, Stat3, and Stat4 and in the sequence (-Pro-Leu-Ser-Pro-) in Stat5, which are similar to MAPK recognition consensus sites [23,24]. Therefore, we determined whether Asn-17 Ras expression or PD98059 inhibits DNA binding of Stat1 in response to IFN-γ by EMSA using ^{32}P-labeled GRR GAS. According to the supershift analysis by specific antibody against the Stat1 C-terminal region, it was confirmed that Stat1 is the protein which translocates to the nucleus and binds to GRR GAS in response to IFN-γ (Fig. 4, lanes 1–4). Herbimycin A, a specific tyrosine kinase inhibitor [25] which potently inhibits iNOS induction stimulated by IFN-γ/LPS in C6 cells [8], abolished IFN-γ-induced DNA binding of Stat1 to GRR GAS in a dose-dependent fashion (Fig. 4, lanes 5–8). However, addition of up to 50 μM PD98059 to cells had no effect on Stat1 DNA binding activity (Fig. 4, lanes 9–12). Similarly, no effect was observed on this event with expression of Asn-17 Ras (Fig. 4, lanes 19 and 20).

Wen et al. [11] have suggested that phosphorylation of Ser-727 in Stat1 and Stat3 has no effect on their DNA binding activity, but greatly reduces their ability to induce transcription. We then determined the effect of Asn-17 Ras expression on iNOS induction stimulated by IFN-γ/LPS in C6 cells by immunoblot and Northern blot analysis. We found that expression of Asn-17 Ras has no effect on this iNOS induction at either the transcription (Fig. 5B, lanes 7 and 8) or translation stage (Fig. 5A, lanes 7 and 8). PD98059 also appears to have no effect on the accumulation of NO_2, a breakdown product of NO produced by the iNOS induction (data not shown). We have therefore concluded that the Ras-MAPK pathway does not contribute to the activation of Stat1 and subsequent iNOS induction in C6 cells.

4. Discussion

The present data show that in C6 glioma cells, both expression of dominant-negative Asn-17 Ras and PD98059 attenuates IFN-γ-induced ERK1 and ERK2 activation (Figs. 2 and 3). These findings indicate that IFN-γ-induced ERK1 and ERK2 activation appears to be mediated by the classical MAPK cascade via $p21^{ras}$ in C6 cells. However, the IFN-γ signaling events in C6 cells upstream of the Ras-MAPK pathway remain unclear. Winston and Hunter [26] have recently reported that in human embryonic kidney 293 cells, growth hormone (GH)-stimulated ERK2 activation requires transient expression of JAK2 and that this activation is inhibited both by dominant-negative Ras and dominant-negative Raf-1. In addition, in 3T3L1 cells JAK2 has been reported to co-immunoprecipitate with Grb2 or Fyn by IL-11 [27]. Both GH [28–30] and IL-11 [31] activate JAKs and STATs as well as the MAPK cascade. The present findings show that MAPK activity is maximal at 15 min after IFN-γ stimulation, which is markedly slower than growth factors. Surprisingly, the time course of this activation coincided with that of tyrosine phosphorylation of Stat1 in C6 cells (data not shown). Therefore, it is reasonable to presume that JAK2 is a common component in the JAK-STAT and Ras-MAPK pathways activated

by various growth factors or cytokines, both of which activate two different pathways. It seems that the signal caused by these stimuli branches off from JAK2 into these two signaling pathways.

Recently, the signal interaction between the JAK-STAT and Ras-MAPK pathways has attracted considerable attention. It is well known that tyrosine phosphorylation is necessary for dimerization, nuclear translocation and DNA binding of STATs [10]. Recently, it has been reported that serine phosphorylation of Stat1 and Stat3 is required for DNA binding activity [22] and maximal transcriptional activity [11]. It has been suggested that the activation of STATs might be regulated by MAPK for a number of reasons. The serine phosphorylation site in Stat1 is present in a consensus sequence recognized by MAPK, and Stat1 is a substrate for MAPK in vitro [11]. In addition, Stat1 co-immunoprecipitates with ERK2 in IFN-β-treated cells, and MAPK activity is required for Stat-dependent transactivation induced by IFN-β [12]. However, in HC11 mammary epithelial cells, concentrations of PD98059 which completely abrogated lactogenic hormone-induced MAPK activation did not affect either the phosphorylation state of Stat5, its DNA binding activity or transcriptional activation of the β-casein gene promoter [32]. In Kit 225 human T cells, H7 did not prevent ERK2 activation, but did suppress the phosphorylation of Stat5 [33]. Finally, in human hepatoma HepG2 cells, the transcriptional activity of Stat3 induced by IL-6 was not inhibited by a dominant-negative Ras protein [34]. Here we showed that inhibition of IFN-γ-induced MAPK activation with dominant- negative Ras or PD98059 does not affect the DNA binding of Stat1 by IFN-γ (Fig. 4). These findings suggest that MAPK activity does not appear to be involved in Stat1 activation by IFN-γ.

It is clear that the GAS element is present in the promoter region of the murine macrophage iNOS gene and is important in promoter activity [5,6]. It has been suggested that tyrosine kinase activity is required for iNOS induction stimulated by IFN-γ/LPS in glial cells [7–9]. We have previously demonstrated that JAK2 is involved in iNOS induction stimulated by IFN-γ/LPS in C6 cells [8]. In addition, we report here that DNA binding of Stat1 is affected in herbimycin A-treated C6 cells (Fig. 4), which inhibits iNOS induction stimulated by IFN-γ/LPS [8]. However, neither dominant-negative Ras nor PD98059 has any effect on iNOS induction stimulated by IFN-γ/LPS in C6 cells (Fig. 5). Therefore, these findings indicate that IFN-γ-induced Stat1 activation, but not MAPK activation, is important for iNOS induction stimulated by IFN-γ/LPS. This supports the hypothesis that MAPK is not responsible for Stat1's ability to induce transcription.

In summary, our data demonstrate that IFN-γ activates the dual intracellular signaling pathways of JAK-STAT and Ras-MAPK, suggesting that these two pathways do not appear to converge with MAPK modification of Stat1. It is possible that IFN-γ plays essential roles in various biological systems by activating two different signaling pathways at the same time in C6 cells. Further investigations are required to identify the upstream of Ras which is involved in IFN-γ-induced MAPK activation and to understand the physiological functions of IFN-γ induced activation of MAPK in C6 cells.

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