INFECTION AND IMMUNITY, Mar. 1994, p. 948–955
0019-9567/94/$04.00+0

Vol. 62, No. 3

The Signal Transduction Mechanism Responsible for Gamma Interferon-Induced Indoleamine 2,3-Dioxygenase Gene Expression

YUKIO KOIDE* AND ATSUSHI YOSHIDA

Department of Microbiology and Immunology, Hamamatsu University School of Medicine, Hamamatsu 431-31, Japan

Received 26 July 1993/Returned for modification 23 September 1993/Accepted 2 December 1993

We examined the signal transduction mechanism responsible for the gamma interferon-induced indoleamine 2,3-dioxygenase (IDO) gene expression in a human monocytic cell line, THP-1. Our results suggest that gamma interferon-induced activation of protein tyrosine kinase is a prerequisite for gene expression and that activation of protein kinase C and another unknown signal(s), both of which are transduced by the protein tyrosine kinase, synergistically induce IDO gene expression. Neither Ca^{2+} influx nor cyclic nucleotide-dependent kinases were suggested to be involved in the signaling pathway.

Gamma interferon (IFN-γ), a cytokine produced by activated T lymphocytes and large granular lymphocytes, exerts pleiotropic effects. These include inhibition of intracellular replication of viruses, increased expression of major histocompatibility complex antigens, inhibition of division of certain tumor cells, and inhibition of intracellular pathogens (10, 20, 52).

Among these multiple biological effects of IFN-γ, the inhibition of intracellular pathogens is known to be mediated by both oxygen (respiratory burst)-dependent and oxygen-independent mechanisms (31).

Current evidence suggests that indoleamine 2,3-dioxygenase (IDO), which catalyzes oxygenative decyclization of L-tryptophan to form *N*-formylkynurenine, represents one of several potential oxygen-independent mechanisms which contribute directly to inhibition of intracellular pathogens. It has been demonstrated that inhibition of intracellular pathogens such as *Chlamydia psittaci* and *Toxoplasma gondii* by IFN-γ is at least partly due to the depletion of tryptophan because of the induction of IDO activity (4, 6, 36). The inhibitory effect of IFN-γ can be overcome by the addition of tryptophan to the culture at superphysiologic concentrations. Furthermore, IDO is suggested to be involved in the antiproliferative effect of IFN-γ on a spectrum of tumor cells (35, 44). Mutants of ME 180 human cervical carcinoma cells resistant to IFN-γ were revealed to be deficient in the induction of IDO activity by IFN-γ (12). More recently, it has been reported that these mutant cells treated with IFN-γ were unable to suppress the growth of *C. psittaci*, *C. trachomatis*, and *T. gondii* in the cells (45).

Evidence has been presented indicating that IFN-γ induces the transcription of the IDO gene (5) and that there are IFN-γ responsive elements in the upstream sequence of the IDO gene (9). However, little is known about the signal transduction mechanism responsible for the IFN-γ-inducible IDO gene expression. Previously, we investigated the signaling pathway responsible for IFN-γ-inducible human leukocyte antigen DR (HLA-DR) molecule expression on a glioblastoma cell line, T98G (32, 39). We have reported that tyrosine phosphorylation is an early and critical event that most probably precedes phosphatidylinositide (PI) breakdown, leading to activation of protein kinase C (PKC) and elevation of the intracellular Ca^{2+} concentration during the IFN-γ-inducible DR molecule expression.

In the present study we have used a human monocytic cell line, THP-1, to examine the signal transduction mechanism responsible for IFN-γ-inducible IDO gene expression with the use of protein tyrosine kinase (PTK) inhibitors, PKC inhibitors, a PKC activator, and a Ca^{2+} ionophore. We used genistein (1), herbimycin A (48, 49), and tyrphostin (15) as PTK inhibitors; H-7, H-8, and staurosporine as selective PKC inhibitors; phorbol myristate acetate (PMA) as a direct activator of PKC; and A23187 as a Ca^{2+} ionophore. Our results suggest that IFN-γ-induced tyrosine phosphorylation is a prerequisite for the gene expression and that activation of PKC and another unknown signal(s) through the PTK-dependent pathway synergistically induce the gene expression. Neither Ca^{2+} influx nor cyclic nucleotide-dependent kinases were suggested to be involved in the signaling pathway.

MATERIALS AND METHODS

Cells. THP-1, a human acute monocytic leukemia cell line, was provided by the Japanese Cancer Research Bank. The cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 μg/ml), streptomycin (100 U/ml), and 2 mM L-glutamine.

Reagents. PMA, the calcium ionophore A23187, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), actinomycin D, 8-bromo-cyclic AMP (cAMP), and 8-bromo-cGMP were purchased from Sigma Chemical Co., St. Louis, Mo. H-7, H-8, W-7, and HA1004 were purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Staurosporine was purchased from Boehringer GmbH, Mannheim, Germany. The PTK inhibitor genistein (4', 5', 7-trihydroxyisoflavone) was purchased from Extra Synthese, Genay, France; and herbimycin A and tyrphostin were purchased from GIBCO BRL, Gaithersburg, Md. Stock solutions of the following compounds were prepared in dimethyl sulfoxide and stored at −20°C: PMA (5 mg/ml), A23187 (10 mM), staurosporine (1

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan. Phone: (053) 435-2335. Fax: (053) 435-1626.

mM), genistein (50 mg/ml), herbimycin A (1 mg/ml), and tyrphostin (10 mg/ml). H-7 (10 mM), H-8 (10 mM), W-7 (10 mM), and HA1004 (10 mM) were prepared in distilled water and stored at 4°C. Recombinant DNA-produced IFN-γ was kindly supplied by Shionogi Pharmaceutical, Osaka, Japan (16).

Induction of IDO mRNA expression by IFN-γ. THP-1 cells were cultured in 24-well flat-bottom culture plates (Coster, Cambridge, Mass.) at 6×10^5 cells per well at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were pretreated for 30 min with H-7, H-8, W-7, and HA1004, for 1 h with genistein and herbimycin A, and for 12 h with tyrphostin before addition of IFN-γ (10^3 U/ml). After 6 h of incubation at 37°C with IFN-γ, the cells were washed twice and were assayed for mRNA expression. We found that these conditions are appropriate for each drug to express inhibitory activity without having any toxic effects.

Primers. Oligonucleotide primers used for PCR amplification were synthesized on an ABI 381A DNA synthesizer. The primers were chosen to flank an intron so that the amplified product is readily distinguished from contaminating genomic DNA that may be present. These sequences are as follows: IDO forward primer, 5′-CCTGACTTATGAGAGAACATG GACGT-3′; IDO reverse primer, 5′-ATACACCAGACCGT CTGATAGCTG-3′; β-actin forward primer, GCACCACAC CTTCTACAATGAG-3′; β-actin reverse primer, 5′-ATAG CACAGCCTGGATAGCAAC-3′. The sizes of cDNA amplified by PCR are approximately 321 and 150 bp for IDO and β-actin, respectively.

Quantification of IDO mRNA by reverse transcriptase-PCR. Total RNA was isolated from THP-1 cells by the acid guanidium thiocyanate-phenol-chloroform extraction method (8). Total RNA (2 μg) was used for first-strand cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (RT; 200 U) and random hexonucleotides (100 nM). After the reaction had been terminated by heating for 5 min at 65°C, we added distilled water to 100 μl before proceeding to PCR amplification. PCR amplification was performed by adding a 5-μl aliquot of each cDNA sample to 50 μl of reaction mixture (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 2 mM $MgCl_2$, 0.4 mM each deoxynucleoside triphosphate, 0.2 μM forward and reverse primers) containing 1.25 U of *Taq* DNA polymerase. For quantification of PCR products, the 5′ end of each reverse primer was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. The following amplification program was used: denaturation at 95°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The PCR products (10 μl each) were electrophoresed in 8% polyacrylamide gels in Tris borate–EDTA buffer. After autoradiography, appropriate PCR bands were cut out from the gel, and the radioactivity in each band was determined by liquid scintillation counting. For each sample the number of counts per minute in the IDO band was normalized to that found in the β-actin, an internal standard, and the results were expressed as percent inhibition or fold enhancement (FE), given by the following formulas: percent inhibition = [1 − (IDO cpm/β-actin cpm test)/(IDO cpm/β-actin cpm control)] × 100; FE = (IDO cpm/β-actin cpm test)/(IDO cpm/β-actin cpm control).

Assay for PTK activity. Cell fractions and extracts were prepared as previously described (46). Briefly, after incubation with IFN-γ (10^3 U/ml) for the time indicated, cells were washed and resuspended at 10^7 cells per ml in ice-cold extraction buffer A (50 mM Tris-HCl [pH 7.4], 1 mM EGTA, 1.5 mM $MgCl_2$, 100 mM NaF, 5 mM dithiothreitol, 250 mM sucrose, 10 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml). The cells were disrupted by sonication for 30 s and centrifuged at 1,000 × *g* for 5 min to remove nuclei. The supernatant was removed and centrifuged at 100,000 × *g* for 60 min at 4°C. The 100,000 × *g* supernatant (cytosolic fraction) and the 100,000 × *g* pellet (membrane fraction) was assayed for PTK activity by using the BRL PTK assay system. The protein fraction to be assayed was incubated with the PTK-specific synthetic substrate peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) in the presence of [γ-^{32}P]ATP, and the PTK activity was determined by measuring the incorporation of [γ-^{32}P]ATP into the substrate (37). The protein concentration of each fraction was determined by the method of Lowry et al. (27).

Assay for PKC activity. Cell disruption and fractionation were performed as previously described (53). Briefly, after incubation with IFN-γ (10^3 U/ml) or PMA (100 ng/ml) for 10 min, cells were washed and resuspended at 10^7 cells per ml in ice-cold extraction buffer B (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 10 mM EGTA, 0.3% [wt/vol] β-mercaptoethanol, 10 mM benzamide, 1 mM phenylmethylsulfonyl fluoride), sonicated for 30 s, and then centrifuged at 100,000 × *g* for 60 min at 4°C. The pellet was resonicated for 20 s in buffer B containing 1% Triton X-100, rocked gently for 60 min at 4°C, and then centrifuged at 13,000 × *g* for 10 min to remove nuclei. Aliquots from the 100,000 × *g* supernatant (cytosolic fraction) and the 100,000 × *g* pellet (membrane fraction) were assayed for PKC activity by using the Amersham PKC enzyme assay kit. This system is designated to detect PKC activity by measuring the incorporation of [γ-^{32}P]ATP into PKC-specific synthetic substrate peptide (Arg-Lys-Arg-Thr-Leu-Arg-Leu-OH) (19). Data were expressed as PKC specific activity (picomoles of transferred phosphate per minute per milligram of protein).

RESULTS

Analysis of the IDO gene expression by semiquantitative RT-PCR. Semiquantitative RT-PCR was used to analyze IDO mRNA expression. THP-1 cells were incubated with IFN-γ (10^3 U/ml) for 16 h, and total RNA was extracted and reverse transcribed into cDNA. Aliquots of resulting first-strand cDNA were amplified with the ^{32}P-labeled reverse primers in combination with forward primers, as described in Materials and Methods. The amplified products were separated on an acrylamide gel, and the amounts of radioactivity recovered from the excised PCR bands were plotted against the number of PCR cycles. As shown in Fig. 1, the rate of amplification with the IDO primer set and that with the β-actin primer set were exponential for 30 and 26 cycles, respectively. The pattern of amplification remained constant in different runs. Therefore, amplification was performed with 26 cycles, and the amount of IDO PCR product was corrected to that of β-actin PCR product.

Induction of IDO mRNA expression by IFN-γ. Figure 2 shows the time course of accumulation of the IDO mRNA in THP-1 cells treated with IFN-γ for various periods. The IDO mRNA appeared within 3 h and continued to accumulate by about 24 h. This level of IDO mRNA was maintained for long periods (>48 h) in the presence of IFN-γ (data not shown). In contrast, the amount of β-actin mRNA was not influenced by IFN-γ as expected. The specificity of amplified products was established (i) by the size of the fragment (Fig. 3), (ii) by using *Pvu*II, which creates two bands, of 94 and 227 bp, from the 321-bp PCR product of IDO (Fig. 3), and (iii) by the fact that the PCR product of IDO was detected only from cells treated with IFN-γ.

As shown in Fig. 4, an RNA polymerase II inhibitor,

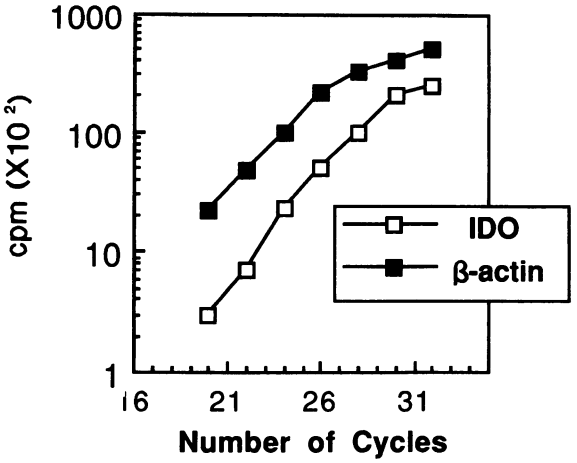

FIG. 1. Semilogarithmic representation of PCR amplification of IDO and β-actin cDNA. THP-1 cells were treated with IFN-γ (10^3 U/ml) for 16 h. Total RNA was isolated, and then first-strand cDNA was synthesized as described in Materials and Methods. Aliquots of cDNA sample were subjected to PCR amplification, and the radioactivity of the PCR bands excised from the gel was plotted against the number of amplification cycles.

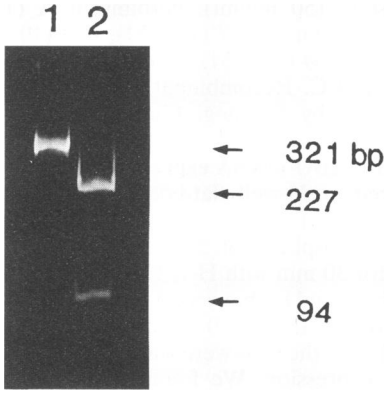

FIG. 3. Specificity of PCR products amplified by the IDO primer set. THP-1 cells were treated with IFN-γ (10^3 U/ml) for 16 h. Total RNA was isolated for first-strand cDNA synthesis, and RT-PCR was performed as described in Materials and Methods. Lanes: 1, PCR product (10 μl) was electrophoresed and stained with ethidium bromide; 2, PCR product (10 μl) was digested with *Pvu*II, electrophoresed, and stained with ethidium bromide.

actinomycin D, inhibited the IFN-γ-induced IDO mRNA expression between 3 and 9 h, suggesting that the mRNA expression is regulated mainly at the transcriptional level.

Effect of PTK inhibitors on IFN-γ-induced IDO gene expression. To examine the possible role of PTK in the signal transduction responsible for IFN-γ-induced IDO mRNA expression, we analyzed the effects of three different PTK inhibitors, genistein, herbimycin A, and tyrphostin, on gene expression. To circumvent unnecessary exposure of these inhibitors, we stimulated the cells with IFN-γ for 6 h in the presence of the inhibitors. The results of a representative experiment are shown in Fig. 5. All three PTK inhibitors, each of which has a different mechanism of action, inhibit IFN-γ-induced IDO mRNA expression in a dose-dependent manner. β-Actin gene expression and viability of the cells were not affected by these PTK inhibitors, and vehicle dimethyl sulfoxide at the concentration used in the solution of the PTK inhibitors showed no apparent effect on the IDO mRNA expression (data not shown). Herbimycin A did not show complete inhibition of the mRNA expression at 2 μg/ml. The toxic effect appeared with more than 2 μg of the drug per ml.

These results suggest that PTK is involved in the induction of IDO mRNA expression by IFN-γ.

IFN-γ induces activation of PTK in THP-1 cells. Having proved the involvement of PTK in IFN-γ-induced IDO mRNA expression, we investigated whether IFN-γ induces activation of PTK in THP-1 cells. PTK activity was measured by the incorporation of [γ-^{32}P]ATP into the PTK-specific substrate peptide. As shown in Fig. 6, membrane-associated PTK activity increased as early as 1 min after IFN-γ stimulation, reaching a plateau level by about 5 min, and began declining within 10 min. It was, however, evident that PTK activities for the cytosol fraction showed no significant change with the IFN-γ stimulation. As expected, the PTK activation induced by IFN-γ was inhibited by the PTK inhibitors used in this study (data not shown).

PKC is involved in IFN-γ-induced IDO gene expression. We have previously demonstrated that the IFN-γ-induced PTK activation precedes PI breakdown, leading to activation of PKC and elevation of [Ca^{2+}] during the HLA class II molecule expression in T98G cells (39). We therefore examined the effects of three PKC inhibitors, H-7, H-8, and staurosporine,

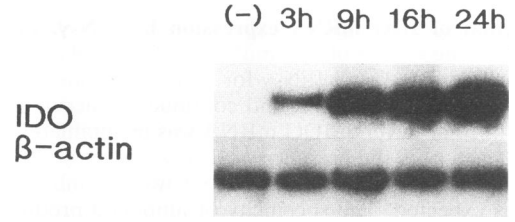

FIG. 2. Time course of IDO mRNA expression induced by IFN-γ. THP-1 cells were incubated with IFN-γ (10^3 U/ml) for the times indicated. Total RNA was isolated for first-strand cDNA synthesis, and RT-PCR was performed as described in Materials and Methods. PCR product (10 μl) was electrophoresed in each lane and analyzed by autoradiography.

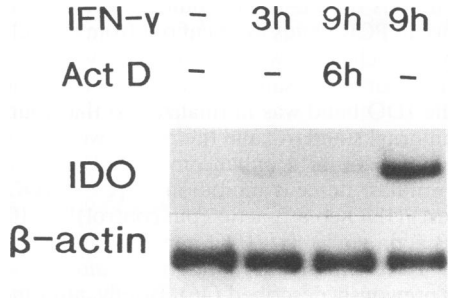

FIG. 4. Effect of actinomycin D on the induction of IDO mRNA by IFN-γ. THP-1 cells were incubated with IFN-γ (10^3 U/ml) for 3 h, and additional culture was performed for 6 h with actinomycin D (5 μg/ml) in the presence of IFN-γ. Expression of IDO and β-actin mRNA was analyzed by RT-PCR. PCR product (10 μl) was electrophoresed in each lane and analyzed by autoradiography. Act D, actinomycin D.

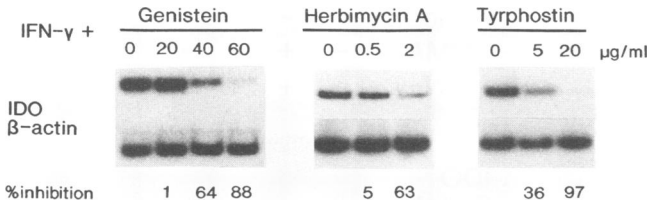

FIG. 5. Effects of PTK inhibitors on the IFN-γ-induced IDO mRNA expression. THP-1 cells were pretreated for 1 h with the PTK inhibitors genistein and herbimycin A and for 12 h with tyrphostin at the indicated concentration. After incubation with IFN-γ (10^3 U/ml), cells were harvested and assayed for mRNA expression by RT-PCR as described in Materials and Methods. PCR products (10 μl) was electrophoresed in each lane and analyzed by autoradiography. Percent inhibition was determined as described in Materials and Methods and is shown under each lane. The experiment shown is representative of three independent experiments.

on IFN-γ-inducible IDO mRNA expression. The results are shown in Fig. 7. All three PKC inhibitors were able to inhibit the IFN-γ-induced IDO mRNA expression in a dose-dependent manner, suggesting that PKC is also involved in the induction of the IDO mRNA expression by IFN-γ. Staurosporine showed only partial inhibition of mRNA expression. Because of the toxic effect, we could not use more than 30 nM staurosporine. W-7, which is an antagonist of the Ca^{2+}-dependent regulatory protein calmodulin, did not inhibit gene expression (data not shown). However, the isoquinolinesulfonamide derivatives, H-7 and H-8, inhibit not only PKC but also cyclic nucleotide-dependent kinases (17). Furthermore, staurosporine is known to inhibit tyrosine phosphorylation of phospholipase C (PLC)-γ1 and inositol phospholipid hydrolysis in addition to PKC (43). In this context, we used another sulfonamide derivative, HA1004, which inhibits cyclic nucleotide-dependent kinases with almost same inhibition constant (K_i) as H-7 and H-8 but inhibits PKC with a substantially higher K_i (2). Although both H-7 and H-8 inhibited the IFN-γ-induced IDO mRNA expression in a dose-dependent manner, HA1004 scarcely inhibited it (Fig. 7). The data obtained with these inhibitors strongly suggest that PKC is involved in the signal transduction pathway responsible for gene expression. This interpretation is consistent with our observation that IFN-γ was capable of activating PKC in THP-1 cells as described below (see Fig. 10). To confirm the

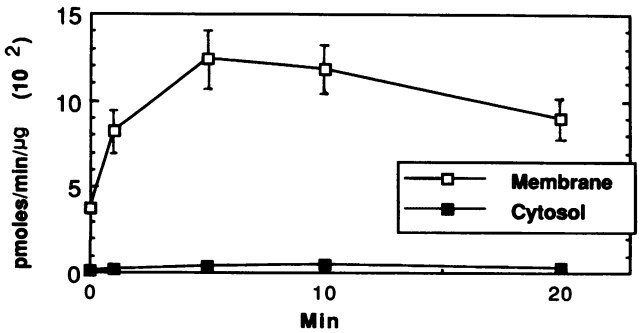

FIG. 6. IFN-γ-induced PTK activation in THP-1 cells. THP-1 cells were treated with IFN-γ (10^3 U/ml) for the times indicated. The cells were disrupted and fractionated into cytosol and membrane components, and then PTK activities were determined as described in Materials and Methods. The data are expressed as the mean ± standard deviation of three independent experiments.

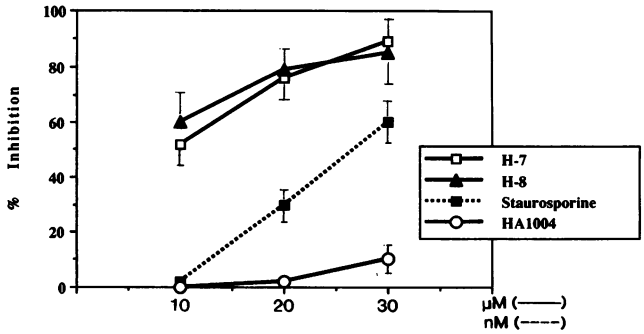

FIG. 7. Effects of the isoquinolinesulfonamide derivatives H-7, H-8, and HA1004 and of staurosporine on IFN-γ-induced IDO mRNA expression. Cells were pretreated for 30 min with the indicated concentrations of H-7, H-8, HA1004, or staurosporine before the addition of IFN-γ (10^3 U/ml). After 6 h of incubation with IFN-γ, cells were harvested and assayed for IDO and β-actin mRNA expressions by RT-PCR as described in Materials and Methods. PCR products were electrophoresed and analyzed by autoradiography. The percent inhibition obtained with each inhibitor was calculated (see Materials and Methods). The data are expressed as the mean ± standard deviation of three independent experiments.

involvement of PKC in gene expression, we examined the effect of PMA, a direct activator of PKC. However, PMA (100 ng/ml) failed to induce the IDO mRNA expression even when the Ca^{2+} ionophore A23187 (1 μM) was added to the culture (Fig. 8). Under the same conditions, however, PMA with A23187 could cooperatively induce HLA-DR expression in THP-1 cells (data not shown) and T98G cells (32, 39).

Nevertheless, it is worth noting that PMA was able to enhance the IDO mRNA expression induced by IFN-γ. Addition of 0.1 through 100 ng of PMA per ml to IFN-γ (10^3 U/ml) led THP-1 cells to express the enhanced level of IDO mRNA, which was 2.3- to 4.1-fold higher than the level expressed by the cells treated with IFN-γ alone (Fig. 9). Taken together, these results suggest that PKC cooperates with another signal(s) to induce IDO gene expression.

A PTK inhibitor, tyrphostin, inhibits IFN-γ-induced but not

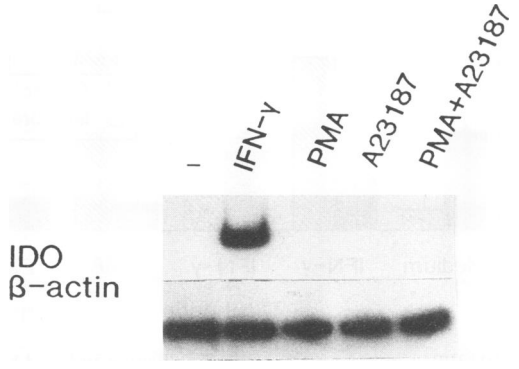

FIG. 8. Effect of PMA and A23187 on IDO mRNA expression. Cells were incubated for 16 h with IFN-γ (10^3 U/ml), PMA (100 ng/ml), A23187 (1 μM), or PMA plus A23187. Expression of IDO and β-actin mRNA were analyzed by RT-PCR as described in Materials and Methods. PCR products were electrophoresed and analyzed by autoradiography.

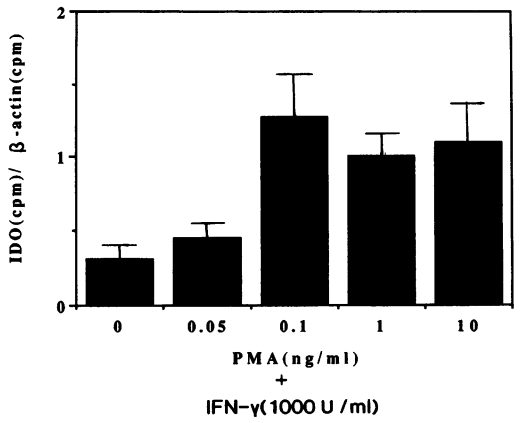

FIG. 9. Effect of PMA on IFN-γ-inducible IDO mRNA expression. Cells were incubated for 16 h with IFN-γ (10^3 U/ml) in the absence or presence of the indicated concentrations of PMA. Expression of IDO and of β-actin mRNA was analyzed by RT-PCR. The data are expressed as the mean ± standard deviation of four independent experiments.

PMA-induced PKC activation. To clarify the relationship between PTK and PKC in this signaling pathway, we examined the effect of a PTK inhibitor, tyrphostin, which seems to be the most specific PTK inhibitor (15, 42), in IFN-γ- and PMA-induced PKC activation. As shown in Fig. 10, stimulation of THP-1 cells with IFN-γ induced activation of PKC in the membrane fraction in 10 min, indicating that PKC was activated by IFN-γ. PMA also induced the activation of PKC as expected. Pretreatment with tyrphostin (20 μg/ml) canceled the translocation of PKC activity induced by IFN-γ. However, PMA-induced activation of PKC was not inhibited by tyrphostin at the concentration at which IFN-γ-induced IDO gene expression was abolished (Fig. 5). These observations imply that PTK activation is a prerequisite for PKC activation in IFN-γ-stimulated THP-1 cells.

Tyrphostin inhibits the signal(s) acting synergistically with PKC to induce IDO gene expression. In an attempt to clarify

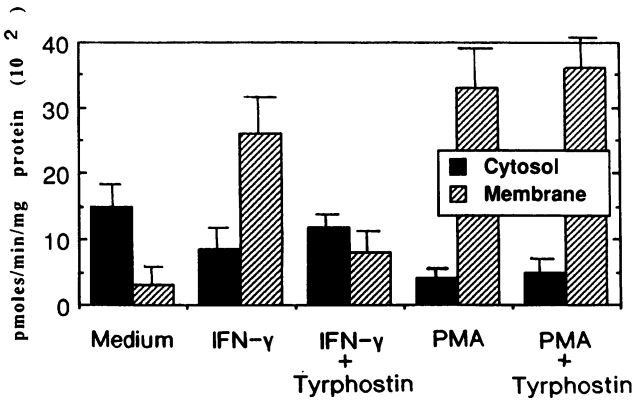

FIG. 10. Effect of tyrphostin on PKC activation induced by IFN-γ or PMA. THP-1 cells were pretreated with or without tyrphostin (20 μg/ml) for 12 h. Then IFN-γ (10^3 U/ml), or PMA was added, and the mixture was further incubated for 10 min at 37°C. PKC activity was measured as described in Materials and Methods. The data are expressed as the mean ± standard deviation of four independent experiments.

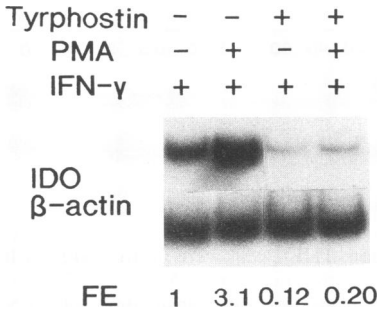

FIG. 11. Effects of tyrphostin on the synergistic interaction of IFN-γ and PMA to induce IDO mRNA. Cells were pretreated with or without tyrphostin (20 μg/ml) for 12 h. Then IFN-γ (10^3 U/ml) was added, and the mixture was further incubated for 6 h in the presence or absence of PMA (100 ng/ml). Expression of IDO and of β-actin mRNA was analyzed by RT-PCR. Fold enhancement (FE) was calculated as described in Materials and Methods and is shown under each lane.

whether the signal(s), which cooperates with PKC to induce IDO gene expression, is transduced via the PTK pathway, we pretreated THP-1 cells with 20 μg of tyrphostin per ml and examined the effect of PMA on IFN-γ-inducible IDO mRNA expression. As shown in Fig. 11, when IFN-γ-induced IDO mRNA expression was impaired by inhibition of PTK by tyrphostin, PMA was unable to restore the IDO mRNA expression. It is therefore conceivable that the signal(s) acting synergistically with PKC may be transduced via the PTK pathway. Tyrphostin did not show any obvious effect on PMA-induced PKC activity (Fig. 10).

Neither cyclic nucleotides nor Ca^{2+} influx is involved in the signaling pathway. As described above (Fig. 7), the results obtained with the sulfonamide derivatives H-7, H-8, and HA1004 suggest that cyclic nucleotide-dependent kinases are not involved in the signaling pathway. We therefore tested whether the cyclic nucleotide pathways are actually not required for IDO mRNA expression induced by IFN-γ. For this, we used cAMP and cGMP analogs (8-bromo-cAMP and 8-bromo-cGMP, respectively) that can enter the living cells. We found that a 16-h exposure of the cAMP or cGMP analog at various concentrations failed to induce IDO mRNA and that neither one was able to enhance IFN-γ-induced IDO mRNA (Fig. 12). Furthermore, neither the cAMP analog nor the cGMP analog was capable of acting synergistically with

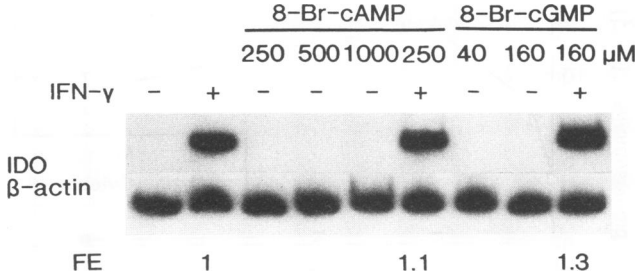

FIG. 12. Effects of cyclic nucleotide analogs on induction of IDO mRNA. THP-1 cells were cultured with 8-bromo-cAMP or 8-bromo-cGMP at the indicated concentrations for 9 h in the presence or absence of IFN-γ (10^3 U/ml). Expression of IDO and of β-actin mRNA was analyzed by RT-PCR. Fold enhancement (FE) was calculated as described in Materials and Methods and is shown under each lane.

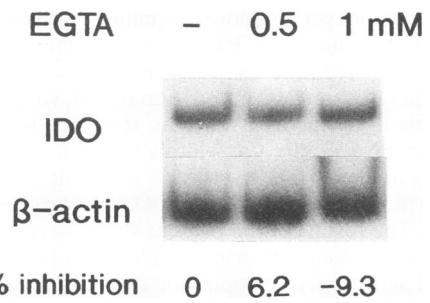

FIG. 13. Effect of EGTA on IFN-γ-induced IDO mRNA expression. THP-1 cells were cultured in the EGTA-containing medium 30 min before addition of IFN-γ (10^3 U/ml). The cells were cultured for an additional 6 h in the presence of IFN-γ and EGTA. Expression of IDO and of β-actin mRNA was analyzed by RT-PCR. Percent inhibition was calculated as described in Materials and Methods and is indicated under each lane.

PMA and A23187 to induce IDO mRNA expression (data not shown). These concentrations of cAMP and cGMP analogs are similar to those used in other experiments; 0.1 mM 8-bromo-cAMP and 0.1 mM 8-bromo-cGMP are sufficient to reduce the extent of total protein synthesis in lactating mouse mammary epithelial cells (47), and 1 mM 8-bromo-cAMP has a strong synergistic effect on neural induction if neuroectoderm is first incubated with PMA (34). As shown in Fig. 13, deletion of extracellular calcium by 1 mM EGTA failed to inhibit the IFN-γ-induced IDO mRNA expression, but the same treatment inhibited IFN-γ-induced Ca^{2+} influx and HLA class II molecule expression in THP-1 cells (data not shown) as has been observed in T98G cells (32). This does not conflict with the data shown in Fig. 8, indicating that the calcium ionophore A23187, either alone or in combination with PMA, was not able to induce IDO mRNA expression. These results suggest that Ca^{2+} influx is not essential for IFN-γ-induced IDO gene expression.

DISCUSSION

From the data described above, we were able to draw the following conclusions about the signal transduction mechanism for the IFN-γ-inducible IDO gene expression: (i) both PTK and PKC activations are involved in gene expression; (ii) the activation of PTK precedes, and is a prerequisite for that of PKC in the signaling pathway; (iii) activation of PKC alone is not enough to induce IDO gene expression but requires another signal(s) transduced by PTK for gene expression; and (iv) neither Ca^{2+} influx nor cyclic nucleotide-dependent kinases are essential for gene expression.

We observed in this study that IFN-γ induced a rapid increase of membrane-associated PTK activity in THP-1 cells. Participation of PTK in IDO gene expression was demonstrated by using three PTK inhibitors, genistein, herbimycin A, and tyrphostin. Genistein, an isoflavone compound, is known to inhibit the activity of PTK such as the epidermal growth factor receptor, $pp60^{v\text{-}src}$, and $pp110^{gag\text{-}fes}$ PTK, whereas it has marginal effects on serine/threonine kinases (1). Herbimycin A was found to induce inactivation of PTK such as $pp60^{v\text{-}src}$ through the interaction of protein sulfhydryl groups with the benzoquinone group of the drug (48, 49). Tyrphostin is a synthetic compound that is a competitive inhibitor of substrate binding to PTK and does not inhibit the binding of ATP (15). Since genistein may not be as specific an inhibitor of PTK as originally believed (28), we used these PTK inhibitors, each of which has a different mechanism of action, to investigate whether PTK is involved in the signaling pathway. We found that all these PTK inhibitors could inhibit IFN-γ-induced IDO mRNA expression in a dose-dependent manner, suggesting that the IFN-γ-induced increase in PTK activity is essential for gene expression.

Three lines of evidence suggest that the inhibitory effects of PTK inhibitors used here resulted from neither nonspecific interference nor drug toxicity. (i) The viability of the cells was not affected by culture in the presence of genistein (60 μg/ml), herbimycin A (2 μg/ml), or tyrphostin (20 μg/ml) for 18 h (data not shown). (ii) Treatment with these PTK inhibitors showed no effect on the expression of β-actin mRNA (Fig. 5). (iii) These PTK inhibitors could not prevent the PKC activation induced by PMA (Fig. 10; data not shown). It seems unlikely that the inhibition observed was caused by instability of mRNA induced by the inhibitors since the level of β-actin was not affected by treatment with the PTK inhibitors.

In view of accumulating evidence for the critical role of PKC in IFN-γ-induced transcriptional activation (11, 29, 32, 39), we addressed the question of whether PKC plays a pivotal role in the IFN-γ-inducible IDO gene expression. It is evident that activation of PKC is induced by IFN-γ in THP-1 cells. In addition, the PKC inhibitors H-7, H-8, and staurosporine inhibited IDO gene expression, thereby suggesting that PKC is also involved in the signaling pathway. However, the isoquinolinesulfonamide derivatives H-7 and H-8 inhibit not only PKC but also cyclic nucleotide-dependent kinases (17). Furthermore, staurosporine is known to inhibit tyrosine phosphorylation of PLC-γ1 and inositol phospholipid hydrolysis in addition to PKC (43). Therefore we used another sulfonamide derivative, HA1004, which inhibits cyclic nucleotide-dependent kinases with almost same K_i value as that of H-7 and H-8 but inhibits PKC with a substantially higher K_i value (2). HA1004 appeared to scarcely inhibit gene expression. Taken together, these results strongly suggest that PKC is also involved in the signaling pathway. Our preliminary experiments with T98G cells have shown that neither 10 μM H-7 nor 10 nM staurosporine inhibits the expression of IDO mRNA (24). Further analyses revealed that mRNA expression can be inhibited by either more than 30 μM H-7 or more than 20 nM staurosporine (data not shown). The toxic effect of the PKC inhibitors can be excluded by the observations that the PKC inhibitors had no effect on viability of the cells. However, the activation of PKC with PMA failed to induce IDO gene expression even in the presence of A23187. Nevertheless, we found that PMA was capable of enhancing the IFN-γ-induced IDO gene expression. The possibility exists that the enhancing effect of PMA is artificial and has nothing to do with the signaling pathway. However, our findings obtained with isoquinolinesulfonamide derivatives provided evidence suggesting that PKC is indeed involved in the signal transduction responsible for IFN-γ-inducible IDO mRNA expression. Taken together, these findings imply that PKC may act synergistically with another signal(s) to induce IDO gene expression. From our data, it seems unlikely that PTK cooperates with PKC to induce gene expression.

Activation of PTK is suggested to be a prerequisite for PKC activation. Evidence has been accumulated to demonstrate a link between PTK activation and PKC activation. The epidermal growth factor and platelet-derived growth factor receptors, whose cytoplasmic domains contain a PTK receptor-type kinase, phosphorylate tyrosine residues on PLC-γ1 after stimulation with appropriate ligands (26, 33). The phosphorylation activates its catalytic activity, resulting in hydrolysis of phosphatidylinositol 4,5-biphosphate. This gives rise to two second

messengers, inositol-1,4,5-triphosphate and diacylglycerol, which increase the intracellular [Ca^{2+}] and activate PKC (18, 25, 26, 33, 50). On the other hand, T cells possess nonreceptor-type PTKs, *lck*, *fyn*, and ZAP-70. *lck* is coupled with CD4 or CD8, and *fyn* and ZAP-70 are coupled with T-cell receptor (ζ chains) (7, 41, 51). In B cells, nonreceptor-type PTKs, *lyn*, *blk*, and *fyn* can associate with B-cell antigen receptor complex (3, 30, 54). These PTKs phosphorylate several intracellular substrates, leading to PKC activation. Similar relationship between PTK and PKC seems to exist in the IFN-γ-induced signaling pathway (39).

We have investigated the signal transduction mechanism responsible for IFN-γ-inducible HLA class II molecule expression. Our results indicate that IFN-γ induces the activation of PTK and that the resultant tyrosine phosphorylation may precede PI breakdown, leading to PKC activation and Ca^{2+} influx, both of which synergistically induce HLA class II molecule expression (32, 39). The PTK-PI breakdown pathway seems to elicit Ca^{2+} influx, because we observed that inhibition of PTK activity prevents IFN-γ-induced Ca^{2+}-influx in T98G cells (39) and because inositol-1,4,5-triphosphate receptors localize to plasma membrane in addition to endoplasmic reticulum membrane in hematopoietic cells (21). Our results obtained with EGTA and A23187 indicate that Ca^{2+} influx is not essential for IFN-γ-inducible IDO gene expression, although the Ca^{2+} influx acts synergistically with PKC to induce HLA class II molecule expression after stimulation with IFN-γ (32). However, our results by no means excluded the possibility that the elevation of intracellular [Ca^{2+}] made by release from intracellular store is involved in IDO mRNA expression. In addition, it was revealed that neither the cAMP pathway nor the cGMP pathway is involved in gene expression by using cAMP and cGMP analogs (Fig. 12) and the sulfonamide derivatives H-7, H-8, and HA1004 (Fig. 7).

The question arises of which signal(s) acts synergistically with PKC to induce IDO gene expression. The results obtained with the PTK-specific inhibitor tyrphostin suggest that the signal(s) is also transduced by PTK (Fig. 11). Thus, it is conceivable that PTK is upstream of signals (including PKC) responsible for the IFN-γ-inducible IDO gene expression. This is consistent with our speculation that the IFN-γ receptor is coupled with nonreceptor-type PTK (39). Indeed, recent data have indicated that the IFN-γ receptor on human monocytes consists of a complex of at least three distinct protein subunits (13). We infer that molecules transducing the signals responsible for IFN-γ-inducible IDO gene expression may bear *src* homology region SH2 domains. It has been proposed that an autophosphorylated PTK could create a high-affinity binding site for the SH2 domain of a substrate and that the bound substrate could be phosphorylated by the kinase (23). We have observed that IFN-γ induced the increase in tyrosine phosphorylation of several intracellular substrates (39). It is most likely that one of the substrates is PLC-γ1, because phosphorylation on tyrosine residues of PLC-γ1 leads to PI breakdown necessary for PKC activation observed in THP-1 cells and T98G cells (32, 39). The PLC-γ1 is shown to contain the SH2 domain (22). It is conceivable that a molecule(s), which transduces a signal(s) acting synergistically with PKC to induce IDO gene expression, may also bear SH2 domains. Recently, Fu (14) demonstrated that IFN-α activates PTK associated with the IFN-α receptor by autophosphorylation and that the binding of the 113- and 91/84-kDa proteins to the activated PTK via their SH2 domains might further result in tyrosine phosphorylation of these proteins. The findings in this report suggest that IFN-γ-inducible IDO gene expression is mediated by synergistic interaction of the PKC pathway in cooperation with another, as yet unknown signaling pathway(s), both of which are transduced by PTK. It is possible that the latter pathway(s) is mediated by p91, since treatment with IFN-γ induces the tyrosine phosphorylation of p91, which is necessary and sufficient to mediate nuclear translocation and DNA-binding activity (38, 40). Ca^{2+} influx (32, 39), which is essential for IFN-γ-inducible HLA class II molecule expression, appeared to play no pivotal role in IDO gene expression induced by IFN-γ. The results of the present study, in conjunction with the previous evidence, provides evidence indicating that IFN-γ-induced activation of cytoplasmic PTK can transduce several signaling pathways whose different combinations may regulate the transcription of each gene affected.

REFERENCES

1. **Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, and Y. Fukami.** 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J. Biol. Chem. **262:** 5592–5595.
2. **Asano, T., and H. Hidaka.** 1984. Vasodilatory action of HA1004 [N-(2-guanidinoethyl)-5-isoquinolinesulfonamide], a novel calcium antagonist with no effect on cardiac function. J. Pharmacol. Exp. Ther. **231:**141–145.
3. **Burkhard, A. L., M. Brunswick, J. B. Bolen, and J. J. Mond.** 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. Proc. Natl. Acad. Sci. USA **88:**7410–7414.
4. **Byrne, G. I., L. K. Lehmann, and G. J. Landry.** 1986. Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. Infect. Immun. **53:**347–351.
5. **Caplen, H. S., and S. L. Gupta.** 1988. Differential regulation of a cellular gene by human interferon-γ and interferon-α. J. Biol. Chem. **263:**332–339.
6. **Carlin, J. M., E. C. Borden, and G. I. Byrne.** 1989. Interferon-induced indoleamine 2,3-dioxygenase activity inhibits *Chlamydia psittaci* replication in human macrophages. J. Interferon Res. **9:**329–337.
7. **Chan, A., M. Iwashima, C. W. Turck, and A. Weiss.** 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with TCR ζ chain. Cell **71:**649–662.
8. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
9. **Dai, W., and S. L. Gupta.** 1990. Regulation of indoleamine 2,3-dioxygenase gene expression in human fibroblasts by interferon-γ. J. Biol. Chem. **265:**19871–19877.
10. **Dalton, D. K., S. Pitts-Meek, S. Keshav, I. Figari, A. Bradley, and T. A. Stewart.** 1993. Multiple defects of immune cell function in mice with disrupted interferon-γ genes. Science **259:**1739–1742.
11. **Fan, X.-D., M. Goldberg, and B. R. Bloom.** 1988. Interferon-γ-induced transcriptional activation is mediated by protein kinase C. Proc. Natl. Acad. Sci. USA **85:**5122–5125.
12. **Feng, G.-S., and M. W. Taylor.** 1989. Interferon γ-resistant mutants are defective in the induction of indoleamine 2,3-dioxygenase. Proc. Natl. Acad. Sci. USA **86:**7144–7148.
13. **Finbloom, D. S., L. M. Wahland, K. D. Winestock.** 1991. The receptor for interferon-γ on human peripheral blood monocytes consists of multiple distinct subunits. J. Biol. Chem. **266:**22545–22548.
14. **Fu, X.-Y.** 1992. A transcription factor with SH2 and SH3 domains is directly activated by an interferon α-induced cytoplasmic protein kinase(s). Cell **70:**323–335.
15. **Gazit, A., P. Yaish, C. Gilon, and A. Levitzki.** 1989. Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors. J. Med. Chem. **32:**2344–2352.
16. **Gray, P. W., D. W. Leung, D. Pennica, E. Yelverton, R. Najarian, C. C. Simonsen, R. Derynck, P. J. Sherwood, D. W. Wallace, S. L. Bergen, A. D. Levinson, and D. V. Goeddel.** 1982. Expression of human immune interferon cDNA in *E. coli* and monkey cells. Nature (London) **295:**503–508.
17. **Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki.** 1984.

Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent kinase and protein kinase C. Biochemistry **23:**5036–5041.

18. **Hill, T. D., N. M. Dean, L. J. Mordan, A. F. Lau, M. Y. Kanemitsu, and A. L. Boynton.** 1990. PDGF-induced activation of phospholipase C is not required for induction of DNA synthesis. Science **248:**1660–1663.
19. **House, C., R. E. H. Wettenhall, and B. E. Kemp.** 1987. The influence of basic residues on the substrate specificity of protein kinase C. J. Biol. Chem. **262:**772–777.
20. **Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet.** 1993. Immune response in mice that lack the interferon-γ receptor. Science **259:**1742–1745.
21. **Khan, A. A., J. P. Steiner, M. G. Klein, M. F. Schneider, and S. H. Snyder.** 1992. IP3 receptor: localization to plasma membrane of T cells and cocapping with the T cell receptor. Science **257:**815–818.
22. **Kim, H. K., J. W. Kim, A. Zilberstein, B. Margolis, J. G. Kim, J. Schlessinger, and S. G. Rhee.** 1991. PDGF stimulation of inositol phospholipid hydrolysis requires PLC-γ1 phosphorylation on tyrosine residues 783 and 1254. Cell **65:**435–441.
23. **Koch, C. A., M. Moran, I. Sadowski, and T. Paeson.** 1989. The common src homology region 2 domain of cytoplasmic signaling proteins is a positive effector of v-*fps* tyrosine kinase function. Mol. Cell. Biol. **9:**4131–4140.
24. **Koide, Y., K. Ryu, and T. O. Yoshida.** 1992. The signal transduction mechanism responsible for interferon-g-inducible indoleamine 2,3-dioxygenase (IDO) gene expression in T98G cells. Jpn. J. Bacteriol. **47:**689–694.
25. **Kypta, R. M., Y. Goldberg, E. T. Ulung, and S. A. Courtneidge.** 1990. Association between the PDGF receptor and members of the *src* family of tyrosine kinase. Cell **62:**481–492.
26. **Linassier, C., M. Pierre, J.-B. Le Pecq, and J. Pierre.** 1990. Mechanism of action in NIH-3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity. Biochem. Pharmacol. **39:** 187–193.
27. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
28. **Markovits, J., C. Linassier, P. Fosse, J. Couprie, J. Pierre, A. Jacquemin-Sablon, J. M. Saucier, J. B. LePecq, and A. K. Larsen.** 1989. Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Res. **49:**5111–5117.
29. **Mattila, P., P. Häyry, and R. Renkonen.** 1989. Protein kinase C is crucial in signal transduction during IFN-γ induction in endothelial cells. FEBS Lett. **250:**362–366.
30. **Mizuguchi, J., Y. Yamanashi, K. Ehara, T. Tamura, H. Nariuchi, Y. Gyotoku, H. Fukazawa, Y. Uehara, and T. Yamamoto.** 1992. Tyrosine protein kinase is involved in anti-IgM-mediated signaling in BAL17 B lymphoma cells. J. Immunol. **148:**689–694.
31. **Murray, H. W.** 1988. Interferon-γ, the activated macrophage, and host defense against microbial challenge. Ann. Intern. Med. **108:**595–608.
32. **Nezu, N., K. Ryu, Y. Koide, and T. O. Yoshida.** 1990. Regulation of HLA class II molecule expressions by IFN-γ. The signal transduction mechanism in glioblastoma cell lines. J. Immunol. **145:**3126–3135.
33. **Nishibe, S., M. I. Wahl, S. M. T. Hernández-Sotomayor, N. K. Tonks, S. G. Rhee, and G. Carpenter.** 1990. Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. Science **250:**1253–1256.
34. **Otte, A. P., P. van Run, M. Heideveld, R. van Driel, and A. J. Durston.** 1989. Neural induction is mediated by cross-talk between the protein kinase C and cyclic AMP pathway. Cell **58:**641–648.
35. **Ozaki, Y., M. P. Edelstein, and D. S. Duch.** 1988. Induction of indoleamine 2,3-dioxygenase: a mechanism of the antitumor activity of interferon-γ. Proc. Natl. Acad. Sci. USA **85:**1242–1246.
36. **Pfefferkorn, E. R.** 1984. Interferon γ blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. Proc. Natl. Acad. Sci. USA **81:**908–912.
37. **Pike, L. J., B. Gallis, J. E. Casnellie, P. Bornstein, and E. G. Krebs.** 1982. Epidermal growth factor stimulates the phosphorylation of synthetic tyrosine-containing peptides by A431 cell membrane. Proc. Natl. Acad. Sci. USA **79:**1443–1447.
38. **Ruff-Jamison, S., C. Katherine, and S. Cohen.** 1993. Induction by EGF and interferon-γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. Science **261:**1733–1736.
39. **Ryu, K., Y. Koide, Y. Yamashita, and T. O. Yoshida.** 1993. Inhibition of tyrosine phosphorylation prevents IFN-γ-induced HLA-DR molecule expression. J. Immunol. **150:**1253–1262.
40. **Sadowski, H. B., K. Shuai, J. E. Darnell, Jr., and M. Z. Gilman.** 1993. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science **261:**1739–1744.
41. **Samelson, L. E., A. F. Phillips, E. T. Luong, and R. D. Klausner.** 1990. Association of the *fyn* protein-tyrosine kinase with the T cell antigen receptor. Proc. Natl. Acad. Sci. USA **87:**4358–4362.
42. **Scholl, P. R., N. Trede, T. A. Chatila, and R. S. Geha.** 1992. Role of protein tyrosine phosphorylation in monokine induction by the staphylococcal superantigen toxic shock syndrome toxin-1. J. Immunol. **148:**2237–2241.
43. **Secrist, J. P., I. Sehgal, G. Powis, and R. T. Abraham.** 1990. Preferential inhibition of the platelet-derived growth factor receptor tyrosine kinase by staurosporine. J. Biol. Chem. **265:**20394–20400.
44. **Takikawa, O., T. Kuroiwa, F. Yamazaki, and R. Kido.** 1988. Mechanism of interferon-γ action. Characterization of indoleamine 2,3-dioxygenase in cultured human cells induced by interferon-γ and elevation of the enzyme-mediated tryptophan degradation in anticellular activity. J. Biol. Chem. **263:**2041–2048.
45. **Thomas, S. M., L. F. Garrity, C. R. Brandt, C. S. Schobert, G.-S. Feng, M. W. Taylor, J. M. Carlin, and G. I. Byrne.** 1993. IFN-γ-mediated antimicrobial response. Indoleamine 2,3-dioxygenase-dependent mutant host cells no longer inhibit intracellular *Chlamydia* spp. or *Toxoplasma* growth. J. Immunol. **150:**5529–5534.
46. **Trevillyan, J. M., A. Nordstrom, and T. J. Linna.** 1985. High tyrosine-specific protein kinase activity in normal human peripheral blood lymphocytes. Biochim. Biophys. Acta **845:**1–9.
47. **Turner, M. D., M. E. Rennison, S. E. Handel, C. J. Wilde, and R. D. Burgoyne.** 1992. Proteins are secreted by both constitutive and regulated secretory pathways in lactating mouse mammary epithelial cells. J. Cell Biol. **117:**269–278.
48. **Uehara, Y., H. Fukazawa, Y. Murakami, and S. Mizuno.** 1989. Irreversible inhibition of v-src tyrosine kinase activity by herbimycin A and its abrogation by sulfhydryl compounds. Biochem. Biophys. Res. Commun. **163:**803–809.
49. **Uehara, Y., Y. Murakami, Y. Sugimoto, and S. Mizuno.** 1989. Mechanism of reversion of Rous sarcoma virus transformation by herbimycin A: reduction of total phosphotyrosine levels due to reduced kinase activity and increased turnover of $p60^{v\text{-}src}$. Cancer Res. **49:**780–785.
50. **Ullrich, A., and J. Schlessinger.** 1990. Signal transduction by receptors with tyrosine kinase activity. Cell **61:**203–212.
51. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell **55:**301–308.
52. **Vilcek, J., P. W. Gray, E. Rinderknecht, and C. G. Sevastopolos.** 1985. Interferon-γ: a lymphokine for all seasons. Lymphokines **11:**1–32.
53. **Wada, S., Y. Yasumoto, H. Kosano, N. Kugai, and N. Nagata.** 1991. The effect of PDGF2α on parathyroid hormone-stimulated cyclic AMP production in mouse osteoblastic cell, MC3T3E1. Biochim. Biophys. Acta **1074:**182–188.
54. **Yamanashi, Y., T. Kakiuchi, J. Mizuguchi, T. Yamamoto, and K. Toyoshima.** 1991. Association of B cell antigen receptor with protein kinase *Lyn*. Science **251:**192–194.