Inhibitors of serine/threonine phosphatases enhance phosphorylation of the interferon- γ receptor while selectively attenuating interferon- γ -induced gene expression in human peripheral-blood monocytes

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Since many events following ligand-induced receptor clustering are controlled by serine and threonine (Ser/Thr) phosphorylation, we initiated an investigation into the role of Ser/Thr phosphatases in both phosphorylation of the interferon- γ (IFN γ) receptor and IFN γ -induced gene expression in human peripheral-blood monocytes. Whereas IFN γ alone did not enhance phosphorylation of the IFN γ receptor, treatment of monocytes with the Ser/Thr phosphatase inhibitors, okadaic acid and calyculin A, resulted in increased phosphorylation of the IFN γ receptor. However, when these cells were analysed for IFN γ -induced IP-10 gene expression, there was profound inhibition. Using three IFN γ -induced early-response genes, IP-10, the Fc γ

receptor type I (Fc γ RI) and ISG-54, we found selective sensitivity to pretreatment with okadaic acid and calyculin A. Whereas IFN γ induction of IP-10 was blocked by both inhibitors, only calyculin A prevented Fc γ RI-gene expression. Neither inhibitor prevented ISG-54 induction by IFN γ . IFN- γ -activated formation of the DNA-binding-protein complex FcRF γ (which binds to the promoter of the Fc γ RI gene) remained unaffected by okadaic acid or calyculin A. Therefore these data suggest that Ser/Thr phosphatases have no major part in IFN γ -initiated signal transduction across the membrane, but selectively control the ultimate transcription of a set of early-response genes.

INTRODUCTION

Interferon- γ (IFN γ), a T-cell-derived cytokine, regulates in monocytes and macrophages the expression of several early-response genes such as IP-10 [1], an interleukin-8/platelet factor-4-like protein, and the high-affinity Fc γ receptor (Fc γ RI) [2]. Both of these gene products play major roles in inflammation [3,4]. These genes are transcriptionally activated within minutes after IFN γ binds to cells, resulting in increased concentrations of steady-state RNA within 0.5–1.0 h. The transcriptional regulation of one of these genes, Fc γ RI, has recently been better defined [5–7]. IFN γ activates by tyrosine phosphorylation a latent protein called p91 transcription factor [8]. This protein, possibly along with other protein components, assemble into a DNA-binding complex that we have termed FcRF γ [5,6]. This complex binds to the γ -response region (GRR) in the promoter of the Fc γ RI gene. p91 has been recently shown to be part of several cytokine- and growth-factor-activated DNA-binding proteins [9–12].

Previous studies have addressed the issue as to whether or not phosphorylation of the IFN γ receptor is a critical step during signal transduction [13,14]. These investigations show in tumour cell lines such as Colo-205 and RAJI enhancement of serine/threonine (Ser/Thr) phosphorylation after the addition of IFN γ . However, the kinetics of phosphorylation (> 10 min) are clearly discordant with the rapid (< 1 min) activation of FcRF γ [7]. It therefore seems unlikely that Ser/Thr phosphorylation of the IFN γ receptor is necessary for immediate signal-transduction events. However, the role of Ser/Thr phosphorylation in IFN γ -induced early-response genes remains to be established. In this report we investigate the ligand-induced phosphorylation of the IFN γ receptor and relate this to IFN γ -induced expression of

early-response genes in normal peripheral-blood monocytes, an important physiological target of IFN γ action. In addition, we describe the differential effects of Ser/Thr phosphatase inhibitors on both IFN γ -receptor phosphorylation and IFN γ -induced gene expression.

MATERIALS AND METHODS

Cells

Human peripheral-blood monocytes were obtained from volunteers by leukapheresis and were purified by Ficoll–Hypaque sedimentation followed by counter-current centrifugal elutriation [15]. Monocytes prepared in this fashion are > 95% pure as determined by non-specific esterase staining and monocyte-specific markers. Cells [in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal-bovine serum] were treated with recombinant *Escherichia coli*-derived human IFN γ (2×10^7 units/mg) (Genentech, S. San Francisco, CA, U.S.A.). Okadaic acid and calyculin A were obtained from Biomol Research Laboratories (Philadelphia, PA, U.S.A.).

Phosphorylation of the IFN γ receptor

For this, 50×10^6 adherent monocytes in 100 mm tissue-culture dishes were incubated in serum-free phosphate-free RPMI 1640 (GIBCO) for 30 min at 37 °C. The medium was discarded and replaced with 5 ml of fresh phosphate-free medium containing 200 μ Ci of [32 P]P $_i$ /ml (DuPont NEN, Boston, MA, U.S.A.). Monocytes were incubated for 3 h at 37 °C and then washed free of radioactive media with warm DMEM supplemented with 10% fetal-bovine serum. Cells were then exposed to IFN γ (up to 50 ng/ml) for 5–15 min at 37 °C. Cells were then washed quickly

Abbreviations used: IFN γ , interferon- γ ; Fc γ RI, high-affinity receptor for IgG (CD64); FcRF γ , IFN γ -induced DNA-binding protein to the Fc γ RI promoter; ISG54, interferon-stimulated gene 54; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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several times with cold PBS and solubilized in lysis buffer (1% Triton X100, 100 mM KCl, 50 mM Tris, pH 7.4, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM vanadate, 2 mM phenylmethanesulphonyl fluoride, 5 μ g of each of aprotinin, leupeptin and pepstatin, 0.6 mM tosyl-lysylchloromethane, 1 mM tosyl-phenylalanylchloromethane and 1 mM *o*-phenanthroline). The lysate was centrifuged at 13000 *g* for 15 min at 4 °C and then pre-cleared with 25–50 μ l of a 50% suspension of Protein G–Sepharose (Pharmacia) for 1 h at 4 °C. The remaining lysate was then exposed sequentially first to control murine IgG1 antibody for 2 h followed by 25–50 μ l of Protein G–Sepharose beads for 1 h and then to a murine IgG1 monoclonal anti-(IFN γ receptor) antibody [16] (kindly provided by Dr. Gianni Garotta, Hoffmann–La Roche, Basel, Switzerland) for 2 h or overnight with equivalent results. This was followed by a 1 h incubation with 25–50 μ l of Protein G–Sepharose. Both antibody–Protein G–sepharose conjugates were washed six times with lysis buffer containing 0.1% Triton X100. The immunoprecipitates were then boiled in 2% SDS sample buffer and electrophoresed in an SDS/polyacrylamide gel. Equal numbers of cell equivalents were applied to each lane of the SDS/PAGE gels.

IFN γ -induced early-response genes

Monocytes were exposed to IFN γ for 1 h at 37 °C, after which the cells were washed and total cellular RNA was isolated by using RNazol (True-test Inc., Friendswood, TX, U.S.A.) according to the instructions supplied by the manufacturer. Three early-response genes were examined by using a RNAase protection assay. The probe for the gene IP-10 [1] was kindly provided by Dr. Jeffrey Ravetch (Sloan–Kettering Memorial Hospital, New York, NY, U.S.A.). A 875 bp *Pst*I fragment of the cDNA was subcloned into the pGEM1 vector (Promega, Madison, WI, U.S.A.) and linearized with *Pvu*II. The 32 P-labelled antisense RNA probe was transcribed with T7 RNA polymerase (GIBCO/BRL) and had a protected fragment of 365 bp. A 490 bp *Hind*III–*Eco*RI fragment of the Fc γ RI cDNA (F90) [17] (kindly provided by Dr. Brian Seed, Harvard University, Boston, MA, U.S.A.) was sub-cloned into the BlueScript plasmid (Stratagene, La Jolla, CA, U.S.A.), linearized with *Xho*I, and transcribed *in vitro* in the presence of [α - 32 P]UTP, yielding a protected fragment (about 500 bp). ISG-54 (interferon-stimulated gene 54) mRNA was assayed by using a 367 bp *Eco*RI probe corresponding to a portion of the second exon of the ISG-54 gene [18]. The size of the protected fragment was 367 bp. The cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whose gene is unaffected by cytokine treatment, was subcloned into pGEM1 and linearized with *Sau*3A [19]. This was used as an internal control in all samples. The 32 P-labelled RNA antisense probe yielded a protected fragment of 300 bp. Total cellular RNA (5–10 μ g) was hybridized in solution with the radiolabelled probe of interest in 80% formamide for 16 h at 56 °C. The hybridized probes were then subjected to T1 RNAase digestion for 1 h at 37 °C. The RNA was then extracted with phenol/chloroform, precipitated with ethanol, dried, and dissolved in 80% formamide. The protected fragments were then analysed by denaturing gel electrophoresis.

IFN γ -induced transcription factors

Nuclear extracts were prepared as previously described [7] from monocytes that were treated with the phosphatase inhibitors for

2–3 h before a 15 min exposure to IFN γ . Electrophoretic-mobility-shift assays were performed in native 6% polyacrylamide gels in 0.25 $\times$ Tris/borate/EDTA buffer, pH 8.3. The 32 P-labelled double-stranded oligonucleotide probe consisted of the 39 bp IFN γ response region of the Fc γ RI gene: 5'-AGCATGTTTCAAGGATTTGAGATGTATTTCCAGAAAAG-3' [2]. Briefly, the binding assay was performed by incubating 10 μ g of nuclear extract prepared as described previously with 2 ng of end-labelled probe in the presence of 4 μ g of poly(dI-dC) (Pharmacia, Piscataway, NJ, U.S.A.) for 30 min at room temperature. Separation of free and complexed probe was performed by electrophoresis followed by autoradiography.

RESULTS

Phosphorylation of the IFN γ receptor

Monocytes were labelled with [32 P]P $_i$, exposed to IFN γ , and subjected to immunoprecipitation with anti-(IFN γ receptor) monoclonal antibody. Non-specific antibody precipitated no detectable bands. There was minimal or no constitutive phosphorylation of the IFN γ receptor (Figures 1a and 1b, control lane), and the addition of IFN γ for 15 min resulted in no enhanced incorporation of 32 P into the receptor. Treatment of cells with IFN γ for as little as 5 min revealed no increased phosphorylation of the IFN γ receptor (results not shown). We therefore used the Ser/Thr phosphatase inhibitors, okadaic acid and calyculin A, during the 32 P incorporation to minimize any enzymic loss of 32 P from the IFN γ receptor. The inhibitors had no effect on cell viability as determined by Trypan Blue exclusion. Cells pretreated with okadaic acid and calyculin A readily incorporated 32 P into the receptor (Figure 1b). The extent of 32 P incorporation into the IFN γ receptor varied from donor to donor (cf. Figures 1 and 2). The addition of IFN γ resulted in

Figure 1 Phosphorylation of IFN γ receptor in response to IFN γ and Ser/Thr phosphatase inhibitors

For this, 50×10^6 monocytes were labelled with 400 μ Ci of [32 P]P $_i$ for 3 h at 37 °C, solubilized in 1% Triton X-100, and immunoprecipitated with anti-(IFN γ receptor) antibody as described in the Materials and methods section. Two separate experiments are shown. In (a), lane 1 represents the immunoprecipitation with isotope control antibody (mAb). In lanes 2 and 3, anti-(IFN γ receptor) antibody is used. In lane 3, cells were treated with IFN γ at 15 ng/ml (receptor saturating dose) for 15 min at the end of the 32 P labelling. In (b), all lanes represent immunoprecipitation with the anti-(IFN γ receptor) antibody. Lane 1 represents untreated cells. In lanes 2 and 4 cells were treated with IFN γ at 15 ng/ml for 15 min at the end of the labelling. In lanes 3 and 4, cells were treated with the combination of 10 nM okadaic acid (Oka) and 10 nM calyculin A (CyA) for the last 1 h of 32 P labelling and just before the addition of IFN γ . Abbreviation: IFN γ R, IFN γ receptor. Molecular mass markers (kDa) are shown.

Figure 2 Effects of Ser/Thr phosphatase inhibition on both phosphorylation of the IFN γ receptor and induction of the IP-10 gene

In (a) (lanes 1–4), 50×10^6 cells were labelled with 400 μ Ci of [³²P]P_i for 3 h, solubilized in 1% Triton X-100, and then immunoprecipitated with anti-(IFN γ receptor) antibody. For the final 1 h of labelling, okadaic acid (Oka; 10 nM) and calyculin A (CyA; 10 nM) were added. In lanes 2 and 4 cells were treated with IFN γ at 15 ng/ml (a receptor saturating dose) for 10 min at 37 °C at the end of the 3 h of labelling and just before solubilization in 1% Triton X-100. In (b) the cells were treated similarly, except that no [³²P]P_i was added and the cells were incubated with IFN γ for 60 min. Total cellular RNA was then isolated and assayed for IP-10 expression by RNAase protection assay. Abbreviation: IFN γ R, IFN γ receptor.

Figure 3 Effect of okadaic acid or calyculin A on IFN γ -induced IP-10 gene expression

In (a) increasing doses of okadaic acid (Oka) were added for 120 min before stimulation with IFN γ at 2 ng/ml for 60 min. In lane 6 okadaic acid alone was added. In (b) calyculin A (CyA) was added 120 min before addition of IFN γ at 2 ng/ml for 1 h; in lane 3 calyculin A alone was added. At the end of the incubation, total cellular RNA was isolated and analysed for IP-10 mRNA by RNAase protection assay.

essentially no change, or even a slight decrease, in the extent of phosphorylation of the receptor.

IFN γ -induced early-response genes

Since okadaic acid and calyculin A were able to enhance IFN γ -receptor phosphorylation, we examined whether this treatment

Figure 4 Effect of okadaic acid (Oka) or calyculin A (CyA) on IFN γ -induced Fc γ RI gene expression

Cells were treated as described in the legend to Figure 3. After the incubation with IFN γ , total cellular RNA was prepared and analysed for Fc γ RI gene expression by RNAase protection assay.

Figure 5 Effect of okadaic acid (Oka) or calyculin A (CyA) on IFN γ -induced ISG54 gene expression

Cells were treated as in the legend to Figure 3. After the incubation with IFN γ , total cellular RNA was prepared and analysed for ISG54 gene expression by RNAase protection assay.

resulted in the activation of IFN γ -induced genes. Parallel sets of cells were evaluated both for IFN γ -receptor phosphorylation and IP-10 expression (Figure 2). Pretreatment of monocytes with okadaic acid and calyculin A again resulted in a moderate incorporation of ³²P into the IFN γ receptor (Figure 2a, lane 3), with a slight but perceptible decrease in receptor phosphorylation in response to IFN γ treatment (Figure 2a, lane 4). Whereas IFN γ treatment resulted in a marked increase in IP-10 gene expression, okadaic acid and calyculin A alone had no effect on IP-10 expression (Figure 2b, lane 3). However, pretreatment of monocytes with okadaic acid and calyculin A resulted in nearly complete suppression of IFN γ -induced IP-10 expression (Figure 2b, lane 4). In order to ensure that okadaic acid and calyculin A treatment was not simply down-modulating IFN γ -receptor expression, we measured IFN γ receptor number on control and treated cells, using Bolton–Hunter-labelled IFN γ [20]. There was

Figure 6 Effect of okadaic acid or calyculin A on IFN γ activation of FcRF γ

Monocytes were pretreated with either 240 nM okadaic acid (Oka) or 10 nM calyculin A (CyA) for 90 min before stimulation with IFN γ at 10 ng/ml for 30 min. Nuclear extracts were prepared and analysed by gel shift assay as described in the Materials and methods section.

no difference in the extent of IFN γ -receptor expression between control and okadaic acid/calyculin A-treated cells (results not shown).

We next examined whether inhibition by okadaic acid and calyculin A was specific for IP-10 or was more global in their effect on the IFN γ -induced early-response genes, IP-10, Fc γ RI and ISG54. All three genes are transcriptionally regulated by IFN γ in peripheral-blood monocytes (D. S. Finbloom, unpublished work). For IFN γ -induced IP-10 expression, there was a dose-dependent inhibition by okadaic acid (Figure 3a), such that at 200 nM gene expression was inhibited by 82% as determined by laser scan densitometry. At doses of calyculin A of 10 nM and above, IP-10 induction by IFN γ was markedly suppressed (98% inhibition) (Figure 3b). For the Fc γ RI gene, a different pattern emerged. Since monocytes normally express Fc receptors when freshly isolated, there is usually a small amount of constitutive expression of this gene. Okadaic acid had essentially no effect on IFN γ induction of the gene (Figure 4a) (10% inhibition, as determined by laser scan densitometry) (Figure 4a). However, calyculin A at 10 nM resulted in 94% inhibition of expression of the gene (Figure 4). Finally, ISG54, a gene predominantly

induced by IFN α but which is also inducible in monocytes by IFN γ , showed essentially no sensitivity to inhibition of IFN γ -mediated induction by either okadaic acid or calyculin A (Figure 5).

Since ISG54-gene induction occurred in the presence of the Ser/Thr phosphatase inhibitors, inhibition by these agents appeared to be occurring at a point distal to the receptor itself. This was corroborated by studying the activation of FcRF γ . This IFN γ -induced DNA-binding protein is activated near the cell membrane and is an indicator of early signalling events. Since the IFN γ response elements have not been precisely defined for ISG54 or human IP-10, only the formation of FcRF γ was studied as an indicator of early transmembrane signalling. Since there was no inhibition of FcRF γ assembly by pretreatment of cells with either calyculin A (10 nM) or okadaic acid (240 nM) (Figure 6), the immediate events surrounding receptor occupancy appear unaffected.

DISCUSSION

IFN γ transcriptionally activates several immediate-early-response genes in monocytes that initiate a cascade of events that ultimately results in the phenotype of the activated macrophage. Data from our laboratory and others have demonstrated that tyrosine phosphorylation of the transcription factor p91 occurs within minutes after binding of IFN γ to the cell surface [6–8] and can occur entirely in a membrane preparation [5]. Although serine/threonine phosphorylation of the IFN γ receptor has been observed in tumour cell lines, its precise role in the cascade of early events remains uncertain, especially since maximal phosphorylation is observed at 30 min after exposure to IFN γ [13], well past the time of maximal p91 phosphorylation (3 min) [7]. In this paper, we show not only that treatment of monocytes with IFN γ results in no detectable increase in phosphorylation of the IFN γ receptor, but also that okadaic acid and calyculin A, two well-defined Ser/Thr phosphatase inhibitors, enhance phosphorylation of the IFN γ receptor, with no concomitant induction of IFN γ responsive genes. Taken together, these data suggest that Ser/Thr phosphorylation of the IFN γ receptor is not a critical event in immediate early signal transduction.

Ser/Thr phosphatases, however, have a major role in the downstream events leading to IFN γ -induced gene expression. Inhibition of these phosphatases results in a selective suppression of IFN γ -induced gene expression. Whereas okadaic acid is only capable of preventing IFN γ induction of IP-10, calyculin A can block induction of both IP-10 and Fc γ RI. Neither okadaic acid nor calyculin A blocks ISG54 induction by IFN γ . Since neither calyculin A nor okadaic acid blocks the formation of IFN γ -activated FcRF γ , these agents probably affect other factors that are ultimately necessary for both Fc γ RI and IP-10 gene transcription. Since the induction of Fc γ RI and IP-10, unlike that of ISG54, is restricted to cells of the myeloid lineage [2,21], other factors probably play a critical role in the ultimate transcription of these genes. This has recently been shown to be the case for the Fc γ RI gene [22].

The effects of okadaic acid and calyculin A on gene transcription may be dependent on cell type and tissue. In hepatocytes, okadaic acid inhibits the insulin-induced transcription of the glucokinase gene [23], but in fibroblasts, okadaic acid mimics the effects of tumour-necrosis-factor- and interleukin-1-induced gene expression and protein phosphorylation [24]. In monocytes, okadaic acid induces tumour-necrosis-factor gene expression itself [25]. It is clear from the data presented herein that Ser/Thr phosphatase activity is important for the IFN γ induction of IP-10 and Fc γ RI. Since these two genes exhibited a differential

sensitivity to the phosphatase inhibitors, it is feasible that more than one Ser/Thr phosphatase may be involved in their regulation. Since IFN γ induction of ISG54 is not suppressed, the effects of okadaic acid and calyculin A are not global. Therefore, multiple pathways downstream of immediate signal-transduction events are likely to occur in order to control adequately the regulation of various genes induced by IFN γ .

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