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Tyrosine phosphorylation is an early and requisite signal induced by interferon-γ in HL-60 cells

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Interferon-γ (IFNγ) is a potent immunomodulatory cytokine. However, the early mechanisms which mediate the pleiotropic effects of IFNγ on different cells are as yet poorly understood. Therefore, we tested the role of tyrosine phosphorylation in signalling induced by IFNγ. IFNγ was found to induce rapid tyrosine phosphorylation of several proteins in HL-60 cells. This effect was detectable by 2 min, reached a maximum by about 4–16 min and thereafter declined. Tyrosine phosphorylation was dependent on receptor occupation and was maximally stimulated by 10 ng/ml IFNγ. Treatment of HL-60 cells with the tyrosine kinase inhibitors, genistein and herbimycin A, inhibited both IFNγ-stimulated tyrosine phosphorylation and IFNγ-induced Fc receptor expression. Thus, increased tyrosine phosphorylation appears to be an obligatory early and proximal signal mediating at least some of the later cellular responses induced by IFNγ in HL-60 cells.

Interferon-γ; Tyrosine phosphorylation; Fc receptor; HL-60 cell.

1. INTRODUCTION

Interferon-γ (IFNγ), a cytokine produced by activated T-cells and natural killer (NK) cells [1], modulates a number of immune functions such as expression of major histocompatibility complex (MHC) antigens and Fc receptors [2], macrophage activation [3] and B-cell function [4]. Purification of the human IFNγ-receptor, cloning of its cDNA and biosynthetic analysis [5–8] revealed that IFNγ receptors are single chain glycoproteins with molecular masses of about 90 kDa and one membrane spanning segment. The receptor cannot be assigned to any known cytokine or growth factor receptor family. The human IFNγ receptor polypeptide by itself displays ligand binding but requires a further as yet undefined, species-specific cofactor, encoded on the human chromosome 21, to be functionally active [9].

Little is known about the early signal transduction mechanisms subsequent to interaction of IFNγ with its receptor. Involvement of protein kinase C [10,11], Ca^{2+}/calmodulin-dependent mechanisms [12,13] and activation of Na^+/H^+ antiporters [14] have been reported.

Many growth factors stimulate protein tyrosine phosphorylation via an intrinsic tyrosine kinase activity of their receptors [15]. Stimulation of tyrosine phosphorylation has also been implicated in signal transduction of several cytokines lacking an intrinsic tyrosine kinase activity [16]. Besides its role in cell proliferation, increasing evidents indicates that tyrosine phosphorylation also plays a role in different cellular activation events. For instance, tyrosine phosphorylation can rapidly be observed after activation of B- and T-cell antigen-receptors as well as of IgG- and IgE-receptors [17–20] and is involved into the cellular activation of platelets, neutrophils and NK cells [21–24].

In this report, we show that IFNγ specifically induces tyrosine phosphorylation in HL-60 cells and that this effect is obligatory for the induction of Fc receptor expression.

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2. EXPERIMENTAL

2.1. *Cell incubation and preparation of cell lysates*

Cells were harvested from culture medium by centrifugation for 10 min at 250 × g and resuspended in a buffer containing 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose and 20 mM HEPES, pH 7.4. About 1×10^6 cells were preincubated for 3 min at 37°C in a volume of 40 μl. Incubation was initiated by the addition of stimuli (20 μl). If not stated otherwise, the reaction was stopped after 10 min by adding 30 μl of lysis buffer (6% (w/v) SDS, 18% (v/v) 2-mercaptoethanol, 30% (v/v) glycerol, 1 mM Na_3VO_4, and a trace amount of bromphenol blue dye in 200 mM Tris-HCl, pH 7.5). The samples were immediately heated for 5 min at 100°C.

2.2 *SDS-PAGE, immunoblotting and antibodies*

SDS-PAGE, transfer of proteins to nitrocellulose filters and detection of phosphotyrosine-containing proteins using anti-phosphotyrosine antibodies and the chemiluminescence (ECL) Western blotting detection system (Amersham, Braunschweig, Germany) has been described [25]. Polyclonal anti-phosphotyrosine antisera were generated by injecting rabbits with a preparation of phosphotyrosine, glycine and alanine coupled to keyhole limpet hemocyanin (Sigma), using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) as described by Kamps and Sefton [26]. Immunglobulins were precipitated with 40% saturated $(NH_4)_2SO_4$, resuspended in a 1/3 vol. of 50 mM Tris/

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HCl, pH 7.3), 150 mM NaCl and dialyzed for 48 h against this buffer (3 changes, 100 vols. each). Affinity purification of the dialysates was performed as described by Kamps and Sefton [26] with the exception that we used phosphotyrosine instead of phosphotyramine in the coupling reaction. Mouse monoclonal anti-human IFNγ receptor IgG1 with receptor-blocking potency was from Genzyme (Cambridge, MA, USA).

2.3. *Fc receptor binding assay*

Quantification of induction of Fc receptors was performed according to Crabtree [27]. Cells exposed for the indicated time periods to IFNγ were collected by centrifugation and washed twice in PBS containing 0.1% (w/v) bovine serum albumin (PBS-BSA). Thereafter, cells were incubated for 30 min at 37°C and pelleted, and 2×10^6 cells were incubated for 2 h at 4°C with 10 nM [^{125}I]IgG1 in a total volume of 40 μl of PBS-BSA. Cells were separated from unbound [^{125}I]IgG1 by rapid washing in 300 μl PBS-BSA. Nonspecific binding of ^{125}I]IgG1 was determined by incubating cells with [^{125}I]IgG1 in the presence of a 1,000-fold molar excess of unlabeled IgG1.

2.4. *Miscellaneous*

HL-60 cells were cultured and differentiated with 1,25-dihydroxyvitamin D_3 and dibuturyl cyclic AMP (Bt_2-cAMP) as described [25]. Differentiation with IFNγ (1 ng/ml) was for 2 days. ^{125}I-labelled IgG1 was synthesized using Iodo-Gen (Pierce, Rodgau, Germany) as oxidizing agent [28] and purified by chromatography on a Sephadex G-25 (PD10) column. IgG1 was from Sigma (Deisenhofen, Germany), Na^{125}I from Amersham Buchler (Braunschweig, Germany). Human recombinant INFv was from Bissendorf Biochemicals (Hannover, Germany), genistein from Sigma (Deisenhofen, Germany), and herbimycin A was from Calbiochem (Bad Soden, Germany). All other reagents were of highest purity available.

3. RESULTS AND DISCUSSION

Immunoblot analysis using anti-phosphotyrosine antibodies showed that IFNv induced protein tyrosine phosphorylation in the human myelomonocytic cell lines HL-60 and U937. In undifferentiated HL-60 cells and U937 cells, most prominently a 91 kDa protein and, to a smaller extent, a 85 kDa protein displayed increased tyrosine phosphorylation in response to IFNγ (Fig. 1). Differentiation of HL-60 cells by several agents [29] led to changes in the extent of tyrosine phosphorylation stimulated in response to IFNγ. Monocytic differentiation of HL-60 cells by 1,25-dihydroxyvitamin D_3 and granulocytic differentiation by Bt_2-cAMP markedly decreased the extent of stimulation, whereas monocytic differentiation by INFv resulted in a pronounced in-

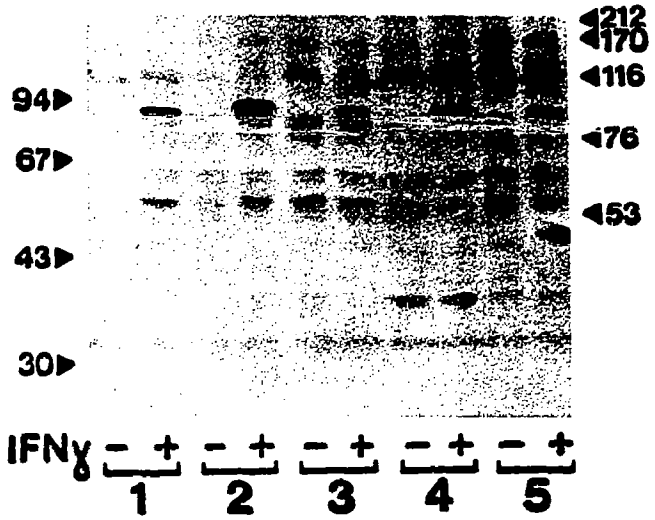

Fig. 1. IFNγ-induced tyrosine phosphorylation in myelomonocytic cells. HL-60 and U937 cells ($\times10^6$ per tube) were incubated in the absence (−) or presence (+) of 10 ng/ml IFNγ for 10 min, and proteins were analyzed for phosphotyrosine content as described in section 2. Cells used were U937 cells (lane 1), undifferentiated HL-60 cells (lane 4) and HL-60 cells differentiated with IFNγ (lane 2), Bt_2-cAMP (lane 3) or vitamin-D_3 (lane 5). The autoluminogram of a blot with molecular masses of marker proteins (kDa) on the left and right is shown.

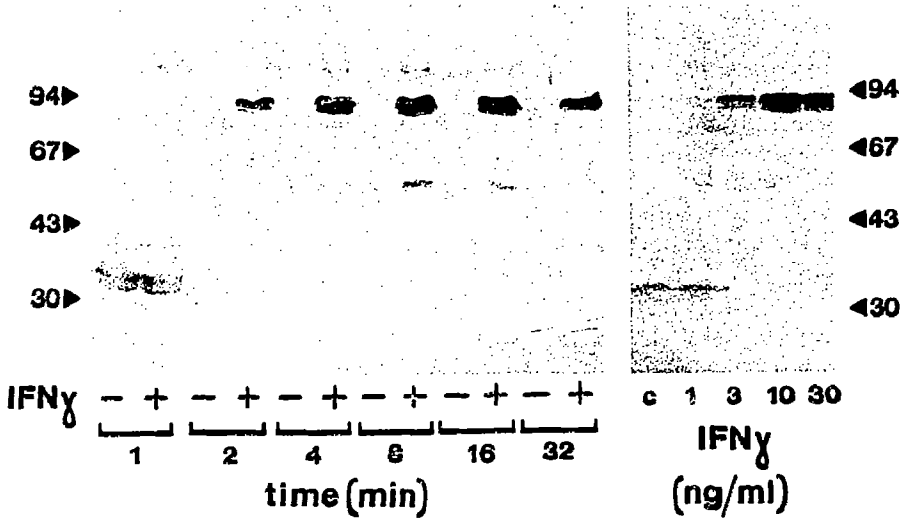

Fig. 2. Time-course and concentration dependence of IFNγ-induced tyrosine phosphorylation in IFNγ-differentiated HL-60 cells. Cells (1×10^6/tube) were incubated with or without 10 ng/ml IFNγ for the indicated time periods (left panel) or were incubated with IFNγ at the indicated concentrations for 10 min (right panel). Samples were processed as described in section 2. The autoluminogram of a blot with the molecular masses of marker proteins (kDa) on the left and right is shown.

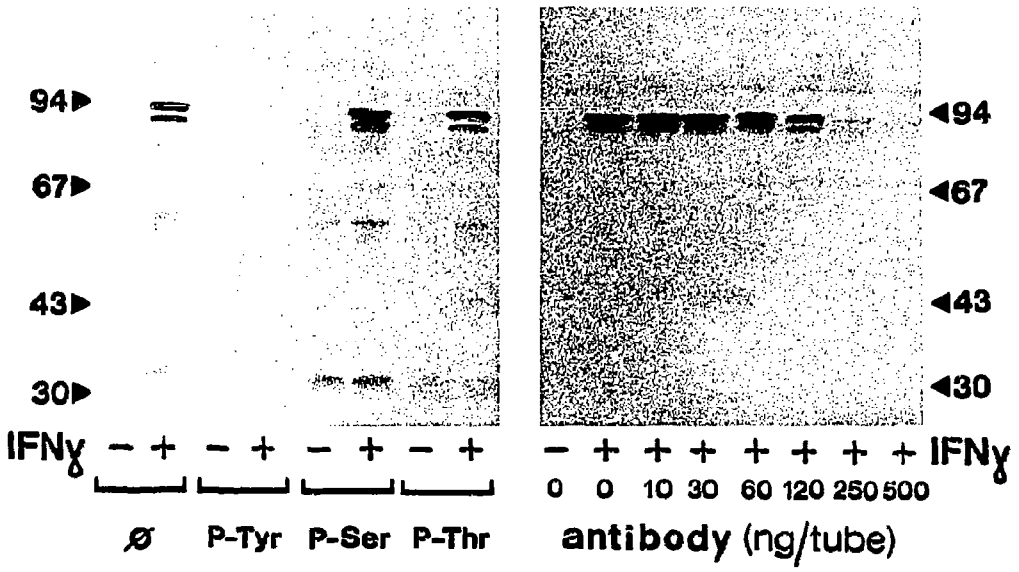

Fig. 3. Specificity of anti-phosphotyrosine antibodies and receptor dependence of IFNγ-induced tyrosine phosphorylation in IFNγ-differentiated HL-60 cells. (Left panel) HL-60 cells (1×10^6/tube) were incubated with 10 ng/ml IFNγ for 10 min and processed as described. Filters were cut and incubated with anti-phosphotyrosine antibodies alone (ϕ) or with antibodies preincubated overnight at 4°C with 0.5 mM phosphotyrosine (P-Tyr), 1 mM phosphothreonine (P-Thr) or 1 mM phosphoserine (P-Ser). (Right panel) Cells (1×10^6/tube) were incubated with the indicated concentrations of blocking anti-human IFNγ receptor antibodies. After 5 min 5 ng/ml IFNγ (+) or carrier (−) were added, and incubation proceeded for further 10 min. Proteins were visualized as described. The autoluminograms of blots with the molecular masses of marker proteins (kDa) on the left and right are shown.

crease of IFNγ-stimulated tyrosine phosphorylation of 85 and 91 kDa proteins. In some experiments, IFNγ-stimulated tyrosine phosphorylation of a 56 kDa protein was observed.

For further characterization of the IFNγ-stimulated tyrosine phosphorylation, we used IFNγ-differentiated HL-60 cells. Qualitatively similar but less pronounced effects were observed in undifferentiated HL-60 cells. Stimulated tyrosine phosphorylation was detectable after 2 min, reached a maximum between 4 and 16 min and thereafter decreased (Fig. 2). The effect of IFNγ was concentration dependent (Fig. 2) with a maximal effect at 10 ng/ml IFNγ. To confirm the specificity of immunoblotting, we demonstrated that binding of the anti-phosphotyrosine antibodies to the immunoblot can be prevented by preincubation with phosphotyrosine but not with phosphothreonine and phosphoserine (Fig. 3). As shown in Fig. 3, anti-human IFNγ receptor antibodies concentration-dependently inhibited the IFNγ-stimulated tyrosine phosphorylation, indicating that the effect of IFNγ was receptor-dependent. Neither loading of cells with the intracellular Ca^{2+} chelator bis-(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) or pretreatment with the calmodulin antagonist, W-7, nor inhibition or down-regulation of protein kinase C, influenced the effect of IFNγ on tyrosine phosphorylation (not shown). Thus, Ca^{2+}/calmodulin-dependent mechanisms or protein kinase C are obviously not involved in the effect of IFNγ on tyrosine phosphorylation.

The identity of the proteins phosphorylated on tyrosine in response to IFNγ is currently not known. Most recently, it was suggested that the activated IFNα receptor, which is distinct from the IFNγ receptor [30], associates with the protein tyrosine kinase *tyk2* [31] and that the IFNα receptor leads to the activation of the interferon-stimulated gene factor 3 (ISGF3) via tyrosine

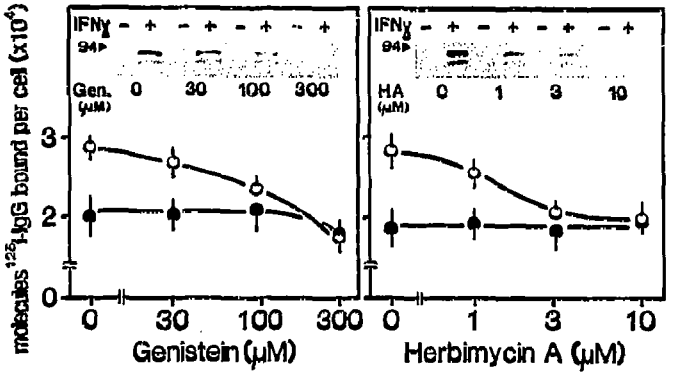

Fig. 4. Effect of genistein and herbimycin A on IFNγ-induced tyrosine phosphorylation and expression of Fc receptors in undifferentiated HL-60 cells. Cells (1×10^6/ml) were cultured for 30 min in the presence of increasing concentrations of genistein and herbimycin A as indicated. Thereafter, cells were either collected, exposed to 5 ng/ml IFNγ for 10 min and analyzed for phosphotyrosine content as described (shown in the inset) or were cultured for an additional 5 h in the absence (●) or presence (○) of 5 ng/ml IFNγ. Thereafter, induction of Fc receptors was quantitated as described in section 2. Each data point represents the mean of triplicate cultures ± S.E.M. The insets show the 80–100 kDa region of representative autoluminograms with the position of the 94 kDa standard indicated on the left.

phosphorylation of ISGF3α proteins with molecular masses of 113, 91 and 84 kDa [32]. Whether a similar mechanism, involving tyrosine phosphorylation of specific transcription factors, also mediates the effects of IFNγ remains to be elucidated.

To test the physiological relevance of IFNγ-induced tyrosine phosphorylation, we examined whether the two protein tyrosine kinase inhibitors, genistein [33] and herbimycin A [34], could inhibit IFNγ-induced expression of Fc receptors in undifferentiated HL-60 cells. Increased Fc receptor expression on HL-60 cells in response to IFNγ can be found within a few hours [35,36]. Genistein and herbimycin A inhibited both IFNγ-induced tyrosine phosphorylation of the 85 and 91 kDa proteins and IFNγ-induced Fc receptor expression in undifferentiated HL-60 cells (Fig. 4). Inhibition of both responses was concentration dependent, with detectable inhibition occurring at 1 μM herbimycin A and 30 μM genistein. Complete inhibition of both responses occurred at 10 and 300 μM of herbimycin A and genistein, respectively. There was good correlation between inhibition of tyrosine phosphorylation and inhibition of Fc receptor expression, consistent with the hypothesis that IFNγ-induced tyrosine phosphorylation is involved in the regulation of Fc receptor expression in HL-60 cells.

Tyrosine phosphorylation of the 91 and 85 kDa proteins by IFNγ differed from the induction of Fc receptor expression in HL-60 cells as shown in Fig. 4 in that the latter effect occurred after a constant exposure of cells for at least 3–4 h to IFNγ. Since the tyrosine phosphorylation in response to IFNγ was maximal at about 15 min, we tested whether exposure of cells to IFNγ for 25 min is sufficient to induce Fc receptor expression. Fig. 5 shows that cells exposed to increasing concentrations of IFNγ for only 25 min, then washed three times and cultured for additional 6 h expressed Fc receptors to a comparable amount as cells continuously exposed to IFNγ for 6.5 h. Exposure of cells to the final wash for 6.5 h did not induce Fc receptor expression (not shown), indicating that no free IFNγ remained in the culture medium after the washing procedure. Thus, a 25 min exposure of cells to IFNγ was sufficient to induce Fc receptor expression on HL-60 cells. Maximal expression after a 6.5 h exposure to IFNγ was achieved at a ligand concentration of about 10 ng/ml, comparable to the maximally effective IFNγ concentration leading to tyrosine phosphorylation in HL-60 cells (see Fig. 2).

Our data show that tyrosine phosphorylation is one of the earliest obligatory effects in response to IFNγ in HL-60 cells and provide insight into the poorly defined molecular mechanisms by which IFNγ induces cellular changes. During the writing of this article, Schindler et al. [37] reported that IFNγ caused tyrosine phosphorylation of a 91 kDa ISGFα protein in fibroblasts. Although, we were not able to identify the 91 kDa protein tyrosine phosphorylated in response to IFNγ in the promyelocytic cell line, HL-60, this protein is likely to

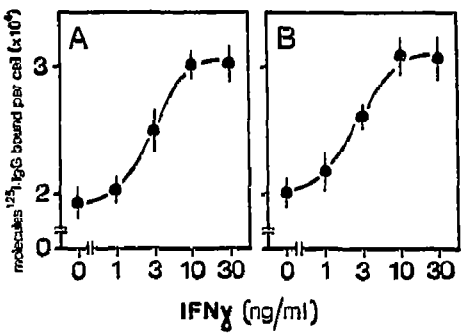

Fig. 5. Effect of time of exposure to IFNγ on induction of Fc receptor expression in undifferentiated HL-60 cells. (Panel A) Cells (1×10⁶/ml) were cultured for 25 min in medium containing the indicated concentrations of IFNγ. Cells were then pelleted and washed three times to remove IFNγ. Thereafter cells were cultured for an additional 6 h in the absence of IFNγ. (Panel B) Cells (1×10⁶/ml) were continuously cultured for 6.5 h in the presence of the indicated concentration of IFNγ. Thereafter, cells from both experiments were assayed for Fc receptor expression as described in section 2. Mean values of triplicate experiments ± S.E.M. are shown.

be identical with the 91 kDa ISGFα protein tyrosine phosphorylated after addition of IFNγ to fibroblasts, suggesting that the proteins tyrosine phosphorylated in response to IFNγ in HL-60 cells serve as transcription factors for the genes induced by IFNγ.

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