Ligand-dependent and -independent activation of the transcription factor γ RF-1 in a cell-free system

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γ RF-1 is a recently identified transcription factor induced by interferon- γ (IFN- γ) which binds to a unique palindromic enhancer, γ RE-1, in the promoter of the *mig* gene. This paper describes the ligand-dependent and ligand-independent activation of γ RF-1 in a cell-free system. γ RF-1 activity was induced by IFN- γ in a time-dependent manner from 5 to 60 min in lysates prepared from the human monocytic leukaemia line THP-1 and the human epidermoid carcinoma line A431. The activation of γ RF-1 *in vitro* required both ATP and an inhibitor of tyrosine phosphatases (sodium orthovanadate or pervanadate). In the presence of limiting concentrations (micromolar) of ATP, activation was also dependent upon stimulation with IFN- γ , whereas at millimolar concentrations of ATP, γ RF-1 was acti-

vated by either sodium orthovanadate or pervanadate in the absence of ligand. Based on cell fractionation studies, both membrane and cytosol components were essential for activation of γ RF-1 *in vitro*. Consistent with a role for one or more tyrosine kinases in the activation of γ RF-1, its DNA binding activity was blocked by monoclonal anti-phosphotyrosine antibodies and by the tyrosine kinase inhibitors genistein, lavendustin A and herbimycin A. A comparison with recently described pathways of IFN-mediated transcription factor regulation indicates that the *in vitro* activation of γ RF-1 is unique, requiring both membrane and cytosol fractions and inhibition of endogenous tyrosine phosphatase activity.

INTRODUCTION

Interferon- γ (IFN- γ) is a cytokine with diverse functions, including the inhibition of cell growth and viral replication, and the control of a variety of cell functions involved in immune responses [1–3]. The mechanism by which IFN- γ exerts its multiple effects on target cells is not fully understood. Recent studies have demonstrated that, upon binding to its specific cell surface receptor, IFN- γ triggers a series of events which include activation of tyrosine phosphatases and kinases, association of proteins, phosphorylation of transcription factors with their subsequent translocation to the nucleus, and a wave of new mRNA and protein synthesis (reviewed in [4]). To date, several IFN- γ -activated transcription factors have been described, including GAF (IFN- γ -activation factor) [5], ICSPB (IFN consensus sequence binding protein) [6], FcRF γ 1 and 2 [7] and IRFi (IFN regulatory factor) [8], which recognize specific DNA elements in the promoter regions of many IFN- γ -responsive genes (reviewed in [4]).

IFN- γ also results in specific induction of *mig* gene expression [9]. *mig* (which stands for Monokine Induced by Gamma-IFN) encodes a member of the platelet factor 4/interleukin-8 family of cytokines [10]. Previous work from our laboratory has shown that *mig* gene expression is induced by IFN- γ , but not by IFN- α or - β , and its transcription does not require *de novo* protein synthesis [9]. Selective induction of *mig* by IFN- γ is mediated by a *cis* element referred to as γ RE-1 (for IFN- γ Responsive Element) which consists of an imperfect inverted repeat of 27 bp [5'-TTACTATAAACTCCCCGTTTATGTGAA-3'] located at positions -198 to -172 of the *mig* promoter [11]. The transcription factor which binds to this element, designated γ RF-1 (for IFN- γ Responsive Factor), binds to the γ RE-1 element in a symmetrical manner and is present in the nucleus within 1–5 min after stimulation of cells with IFN- γ [11].

During the past 2 years the *in vitro* activation of four IFN-activated transcription factors [IFN-stimulated gene factor 3 (ISGF-3), GAF and FcRF γ 1 and 2] has been reported and the requirements for their activation were identified. The *in vitro* activation of ISGF-3 via the IFN- α receptor required both tyrosine phosphatase(s) and kinase(s) and was inhibited by sodium orthovanadate [12]. Similarly, the IFN- γ -mediated activation of GAF in a cell-free system was inhibited by tyrosine phosphatase inhibitors such as zinc chloride and phenylarsine oxide, suggesting a role for a tyrosine phosphatase in the cascade leading to its activation [13]. In contrast, the activation of FcRF γ 1 and FcRF γ 2 in cell lysates treated with IFN- γ was enhanced by the tyrosine phosphatase inhibitor sodium orthovanadate. In addition, it was found that the membrane fraction of human monocytes was sufficient for activation of the FcRF γ factors, suggesting that activation of FcRF γ 1/2 involved a membrane-associated tyrosine kinase [14].

In this paper the *in vitro* activation of γ RF-1 in lysates from THP-1 cells and A431 cells is presented. Our data indicate that the *in vitro* activation of γ RF-1 can be distinguished from the activation of other IFN-activated transcription factors based on the need for membrane and cytosol components, sensitivity to kinase inhibitors and an absolute requirement for a tyrosine phosphatase inhibitor.

MATERIALS AND METHODS

Cell lines and reagents

THP-1, a human monocytic leukaemia cell line, and A431, a human epidermal carcinoma cell line, were obtained from American Type Culture Collection. Both cell lines were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum.

Abbreviations used: IFN- γ , interferon- γ ; GAF, IFN- γ -activation factor; ISGF-3, IFN-stimulated gene factor 3; PEP, phosphoenolpyruvate; PK, pyruvate kinase; DTT, dithiothreitol; NP-40, Nonidet P40; EMSA, electrophoretic mobility shift assay.

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Recombinant human IFN- γ was purchased from GIBCO/BRL. Genistein, lavendustin A and herbimycin A (GIBCO/BRL) stock solutions were prepared in DMSO. Sodium orthovanadate was obtained from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). Pervanadate was prepared by the reaction of sodium orthovanadate with hydrogen peroxide as described [15], and was used at a final concentration of 0.1 mM sodium orthovanadate/1 mM hydrogen peroxide. Streptavidin-linked magnetic beads (DynaM M280) were purchased from Dynal Inc. (New York, NY, U.S.A.). [α - 32 P]dGTP, [α - 32 P]dATP and [γ - 32 P]ATP (all with a specific radioactivity of 6000 Ci/mmol) were purchased from Dupont/New England Nuclear. Monoclonal anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, U.S.A.) and monoclonal anti-phosphotyrosine antibody PY20 was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA, U.S.A.).

In vitro activation

The preparation of cell lysates and subcellular fractionation were performed at 4 °C. THP-1 (or A431) cells were harvested and washed twice with cold PBS, resuspended in an equal pellet volume of reaction buffer [20 mM MgCl₂, 200 μ M CaCl₂, 100 mM Hepes, pH 7.9, 200 μ M sodium ascorbate, 10 mM phosphoenolpyruvate (PEP), 20 μ g/ml pyruvate kinase (PK), 600 μ M PMSF and ATP as indicated for each experiment], and subjected to Dounce homogenization. Complete homogenization was confirmed by Trypan Blue staining (> 99 % of cells were lysed). Homogenates, referred to as lysates (LYS), were divided into 20 μ l aliquots and incubated under various conditions as described for each experiment. Incubation was terminated by the addition of 10 vol. of ice-cold stop buffer [1 mM MgCl₂, 10 mM KCl, 20 mM NaHepes, pH 7.0, 20 % glycerol, 500 μ M dithiothreitol (DTT), 250 μ M PMSF and 0.1 % Nonidet P40 (NP-40)]. The mixture was vortexed and centrifuged at 17 500 *g* for 5 min at 4 °C. The supernatant was then assayed by electrophoretic mobility shift assay (EMSA; see below). In additional experiments, homogenates were separated into membrane and cytosol fractions. For the preparation of membranes (M), lysates were layered over 12 ml of a 39 % (w/v) sucrose solution and centrifuged at 3000 *g* for 10 min. The upper layer was recovered and washed with 4 $\times$ 1 ml of membrane wash buffer (20 mM MgCl₂, 100 mM Hepes, pH 7.9, 200 μ M sodium ascorbate and 600 μ M PMSF) and resuspended in reaction buffer. For the preparation of cytosol, referred to in the figures as supernatant (S), lysates were centrifuged at 17 500 *g* for 15 min. The supernatant was collected and centrifuged at 100 000 *g* for 1 h. The supernatant obtained from this centrifugation constituted the cytosol fraction. For co-incubation, equal volumes of membrane (M) and cytosol (S) fractions were used. Unless otherwise noted, reactions were stopped by the addition of NP-40 at a final concentration of 0.1 %. Mixtures were vortexed and centrifuged at 17 500 *g* for 5 min at 4 °C. The resulting supernatant was then analysed by EMSA.

EMSA

Complementary oligonucleotides corresponding to the γ RE-1 sequence from -200 to -167 of the *mig* promoter region were annealed and radiolabelled with [α - 32 P]dGTP and [α - 32 P]dATP by a fill-in reaction using the Klenow fragment of DNA polymerase I as described previously [11]. Binding reactions (25 μ l) contained the indicated amounts of nuclear extract, cell lysate or subcellular fractions, 5 fmol (approx. 10⁶ c.p.m.) of

radiolabelled DNA fragment and binding buffer [10 mM Tris, pH 7.5, 25 mM KCl, 0.5 mM EDTA, 1 mM MgCl₂, 2 mM DTT, 2 mM CaCl₂, 0.1 % Triton X-100, 12.5 % (w/v) glycerol, 2 mM spermidine and 0.08 mg/ml poly(dG-dC)]. Binding reactions were incubated for 20 min at room temperature. Immediately prior to loading on a 6 % (w/v) polyacrylamide gel in 0.25 $\times$ TBE buffer [1 $\times$ TBE = 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0], 5 μ l of 0.1 % Bromophenol Blue in binding buffer was added to the binding mixture. Electrophoresis was performed at 180 V for 1.5–2 h. The gel was then dried and autoradiography was performed.

Analysis of *in vitro* γ RF-1 phosphorylation

Membrane (M) and cytosol (S) fractions were prepared from 10⁷ THP-1 cells as described above except that the reaction buffer contained a final concentration of 4 μ M ATP and [γ - 32 P]ATP [1.48 MBq (4 μ l or 40 μ Ci)/reaction]. Mixtures (20 μ l of S + 20 μ l of M) were incubated in the absence or presence of 1 mM sodium orthovanadate with or without 2000 units of IFN- γ per reaction (5 $\times$ 10⁴ units/ml, final) for 30 min at 30 °C. *In vitro* phosphorylation reactions were stopped by the addition of 0.1 % NP-40 as described above. Streptavidin-coupled magnetic beads (25 μ l) were washed with TEN buffer (10 mM Tris/HCl, pH 7.5, 1.0 mM EDTA, 0.1 M NaCl) containing 0.1 % BSA and pretreated with 125 μ l of a buffer consisting of 1 $\times$ binding buffer (see EMSA above), 200 μ g/ml calf thymus DNA and 500 μ g/ml BSA for 5 min at room temperature. The beads were washed three times with 1 $\times$ binding buffer/1 M NaCl and incubated with 1 pmol of a biotinylated DNA fragment containing three repeats of the γ RE-1 sequence for 30 min at room temperature. The beads were washed four times with buffer consisting of 10 mM Tris/HCl, pH 7.5, 1.0 mM EDTA and 1 M NaCl, and resuspended in 10 μ l of TEN buffer. Equal volumes (3 μ l) of the magnetic bead suspension were added to the *in vitro* phosphorylation reactions followed by the addition of an equal volume of 2 $\times$ binding buffer. The mixture was incubated overnight at 4 °C with gentle rocking. The beads were washed three times with 1 $\times$ binding buffer supplemented with 0.1 mM sodium orthovanadate. The bound proteins were eluted for 30 min at 4 °C in elution buffer (1 $\times$ binding buffer with 0.6 M KCl), mixed with SDS sample buffer and resolved by SDS/PAGE on a 10 % (w/v) polyacrylamide gel. After drying, 32 P-containing proteins were visualized by autoradiography.

RESULTS

Activation of γ RF-1 in cell lysates

In the process of establishing a cell-free system for γ RF-1 activation, a variety of conditions were evaluated. A number of the variables examined during this process are summarized in the experiment shown in Figure 1. As indicated, γ RF-1 activity was detected in THP-1 lysates stimulated with IFN- γ in the presence of either sodium orthovanadate or pervanadate, two well described inhibitors of tyrosine phosphatases [15,16]. This effect was specific for tyrosine phosphatase inhibitors, since NaF (100 mM), a serine/threonine phosphatase inhibitor, did not stimulate γ RF-1 activation alone or in combination with IFN- γ (Figure 1, lanes 10 and 11). Also shown in Figure 1 is the finding that pervanadate (also known as vanadyl hydroperoxide) induced γ RF-1 in the absence of IFN- γ (lanes 12 and 17), indicating that the activation of γ RF-1 can occur independently of ligand *in vitro*. This property was not shared by sodium orthovanadate under the same reaction conditions (lanes 6, 8 and 15). Hydrogen peroxide (3 mM), with which sodium orthovanadate was mixed

Figure 1 Effect of temperature and phosphatase inhibitors on the activation of γ RF-1 *in vitro*

Lysates were prepared from unstimulated THP-1 cells in reaction buffer containing 4 μ M ATP. Incubations were performed at either 30 °C or 37 °C for 30 min unless noted otherwise (5, 15 min). As indicated, reactions were supplemented with sodium orthovanadate (VAN), NaF (100 mM) or pervanadate (PV) with or without IFN- γ . Reactions were stopped by the addition of 10 vol. of ice-cold stop buffer and centrifugation for 5 min at 17500 *g*. An aliquot of the supernatant (8 μ l) was assayed by EMSA. 'p' in this and subsequent Figures indicates the position of unbound radiolabelled fragment (probe).

to form pervanadate, failed to activate γ RF-1 (results not shown). The data in Figure 1 also demonstrate that the activation of γ RF-1 in cell lysates occurred more efficiently at 30 °C (lanes 15–18) than at 37 °C (lanes 6–9, 12 and 13). The opposite temperature effect was observed when intact cells were stimulated with IFN- γ and nuclear extracts from these cells were analysed for γ RF-1 activity by EMSA (results not shown). These results indicate that γ RF-1 was activated in a ligand-dependent and -independent manner in a cell-free system and that inhibition of one or more tyrosine phosphatases was essential to this process.

We extended these studies to examine the kinetics of γ RF-1 formation in lysates prepared from THP-1 cells (Figure 2a) and the human epidermoid carcinoma line A431 (Figure 2b). The level of γ RF-1 activity increased in a time-dependent manner from 5 to 30 min when both sodium orthovanadate and IFN- γ were present (Figures 2a and 2b, lanes 1–4). In contrast to its effect on cell lysates, sodium orthovanadate had no effect on the activation of γ RF-1 in intact cells over this time period (results not shown). The addition of exogenous ATP during the incubation enhanced both ligand-dependent and -independent γ RF-1 activation (Figure 2, lanes 4 and 7 compared with lanes 5 and 6). Similar to the data presented above (Figure 1), γ RF-1 activation was not observed in the absence of a tyrosine phosphatase inhibitor. In contrast to the results in Figure 1, γ RF-1 was weakly activated in samples containing sodium orthovanadate (1 mM) alone (lane 5), which may have been due to higher levels of endogenous ATP in these cell lysate preparations. Although both sodium orthovanadate and pervanadate activated γ RF-1 independently of ligand, the effect was enhanced by the addition of IFN- γ . This effect was most pronounced at limiting ATP concentrations (Figures 2a and 2b, lanes 5 and 6). As in Figure 1, pervanadate alone or in combination with IFN-

Figure 2 *In vitro* activation of γ RF-1 in THP-1 and A431 cell lysates

Lysates were prepared from unstimulated cells in reaction buffer. Reactions contained THP-1 lysates (a) or A431 lysates (b) and, as indicated, sodium orthovanadate (VAN, 1 mM), pervanadate (PV), ATP (4 mM) and IFN- γ (2000 units). Reactions were incubated at 30 °C for the indicated times and analysed by EMSA as described in the legend to Figure 1.

γ (lanes 10 and 11) resulted in the highest levels of γ RF-1 in cell lysates.

Activation of γ RF-1 in membrane and cytosol fractions

In the next series of experiments, the need for membrane (M) and cytosol (supernatant; S) components in the *in vitro* activation of γ RF-1 was examined. In these experiments THP-1 cells were lysed by Dounce homogenization, and cytosol and membrane fractions were prepared as detailed in the Materials and methods section. The resulting subcellular fractions were incubated alone or in combination, and as shown in Figure 3(a), γ RF-1 activity

Figure 3 Activation of γ RF-1 in membrane and cytosol fractions

(a) Lysates (LYS) and subcellular fractions [membranes (M) and cytosol (S)] were prepared from unstimulated THP-1 cells in reaction buffer containing 40 μ M ATP. Reactions were incubated for 30 min at 30 $^{\circ}$ C and contained the indicated cellular fraction(s) supplemented with sodium orthovanadate (VAN; 1 mM) and IFN- γ (2000 units), as noted. Reactions were terminated by the addition of 0.1% NP-40 and centrifugation. Supernatants (8 μ l for LYS, 2 μ l

was present in samples containing combined membrane and cytosol fractions incubated with sodium orthovanadate in the presence or absence of IFN- γ (lanes 11 and 12). In contrast, γ RF-1 activation did not occur in membrane or cytosol fractions incubated separately with sodium orthovanadate and/or IFN- γ (lanes 5–9). Thus both cytosol and membrane components were essential for γ RF-1 activation. The time courses of γ RF-1 activation in reconstituted membrane and cytosol fractions incubated with sodium orthovanadate plus IFN- γ (Figure 3b) or pervanadate (Figure 3c) demonstrated kinetics similar to those of γ RF-1 activation in lysates stimulated with sodium orthovanadate plus IFN- γ (Figures 2a and 2b) or pervanadate alone (results not shown).

Requirement for ATP in γ RF-1 activation

Although previous experiments (Figures 2a and 2b) indicated that γ RF-1 activity was augmented in samples containing exogenously added ATP, they did not determine the requirement for ATP in γ RF-1 activation. In order to examine this further, cytosol and membrane fractions were prepared from THP-1 cells and aliquots of these fractions were maintained on ice. An aliquot of the membrane fraction was washed four times and an aliquot of the cytosol fraction was dialysed in order to remove any endogenous ATP. These fractions were then combined and evaluated for sodium orthovanadate- or pervanadate-induced γ RF-1 activation. As shown in Figure 4, there was adequate ATP in untreated membrane and cytosol fractions to result in γ RF-1 activation in response to pervanadate (lane 4), which was further enhanced by the addition of 40 μ M ATP (lane 3). The addition of ATP alone did not result in γ RF-1 activation (lane 2). When ATP was removed by washing the membranes and dialysing the cytosol, no γ RF-1 was detected in response to pervanadate (lane 6). However, γ RF-1 activation was restored when ATP (40 μ M) was replenished prior to the addition of pervanadate (lane 7) or sodium orthovanadate (lane 8). The results indicate an absolute requirement for ATP in the activation of γ RF-1 and further support the role of phosphorylation in this process. Similar results were obtained when IFN- γ was added to the reactions in the presence of sodium orthovanadate or pervanadate (results not shown).

In vitro activation of γ RF-1 involves tyrosine phosphorylation

The observation that γ RF-1 activation required the presence of ATP and a tyrosine phosphatase inhibitor (sodium orthovanadate or pervanadate) strongly suggested that phosphorylation of γ RF-1 on one or more tyrosine residues was an important step in the regulation of its DNA-binding activity. To investigate the role of tyrosine kinase(s) in this process, the effect of tyrosine kinase inhibitors was examined. As shown in Figure 5, pre-incubation of THP-1 membrane and cytosol fractions with the protein tyrosine kinase inhibitors genistein, herbimycin A or lavendustin A for 10 min at 30 $^{\circ}$ C resulted in complete inhibition of γ RF-1 activation in response to sodium orthovanadate plus IFN- γ . However, simultaneous addition of the indicated tyrosine

for M or S and 4 μ l for M+S) were assayed by EMSA. (b) and (c) Reactions contained combined membrane and cytosol fractions prepared from unstimulated THP-1 cells with the addition of 1 mM sodium orthovanadate in the presence or absence of 2000 units of IFN- γ (b) or pervanadate (c) and were incubated at 30 $^{\circ}$ C for the indicated times. They were terminated by the addition of 0.1% NP-40 and, after centrifugation, an aliquot (2 μ l) of each reaction was analysed by EMSA.

Figure 4 Activation of γ RF-1 *in vitro* requires ATP

Membrane and cytosol fractions were prepared from unstimulated THP-1 cells. An aliquot of the cytosol fraction was dialysed against reaction buffer lacking ATP, PEP or PK (DS). Another aliquot was supplemented with 40 μ M ATP, 10 mM PEP and 20 μ g/ml PK (S). Membrane fractions were washed four times and resuspended in reaction buffer lacking ATP, PEP or PK (lanes 4 and 6) or in reaction buffer containing 40 μ M ATP, 10 mM PEP and 20 μ g/ml PK (lanes 2, 3, 5, 7 and 8). Samples contained the indicated cell fractions with or without the addition of sodium orthovanadate (VAN; 1 mM) or pervanadate (PV) and were incubated for 30 min at 30 °C. Reactions were terminated by the addition of 0.1% NP-40, vortexed and centrifuged. Supernatants (2 μ l aliquots) were analysed by EMSA.

kinase inhibitor, sodium orthovanadate and IFN- γ (lanes designated with an asterisk) did not block γ RF-1 activation. The results indicate that tyrosine kinase-dependent phosphorylation is an early step in the process of γ RF-1 activation.

Having implicated tyrosine phosphorylation in the activation of γ RF-1, it was important to determine whether the transcription factor itself was the target of this modification or whether activation might result from phosphorylation of other proteins (e.g. inhibitors or modifying enzymes). To evaluate this further, we examined the effects of anti-phosphotyrosine antibodies on the formation and mobility of the γ RF-1 complex. Aliquots of a sample containing membrane and cytosolic fractions activated *in vitro* (sodium orthovanadate plus IFN- γ , 30 °C, 30 min) were incubated for 3 h at 4 °C with various amounts (1–6 μ g) of one of two anti-phosphotyrosine monoclonal antibodies (4G10 and PY20), an IgG2b isotype control antibody (2–4 μ g) or no addition, followed by analysis of γ RF-1 binding by EMSA. As shown in Figure 6, the anti-phosphotyrosine monoclonal antibody PY20 inhibited binding of γ RF-1 to γ RE-1 in a dose-dependent manner. In contrast, the use of a different anti-phosphotyrosine monoclonal antibody (4G10) resulted in a reduction in the mobility of the γ RF-1 complex ('super-shift') upon gel electrophoresis, while an isotype control monoclonal antibody had no effect. These results demonstrate that γ RF-1 contains one or more phosphotyrosine residues that may be important in the assembly of the transcription factor complex and/or that may be in proximity to the DNA-binding domain.

Identification of the phosphorylated component(s) of γ RF-1

In order to identify the number and molecular mass of the phosphorylated protein(s) which constitute(s) γ RF-1, *in vitro*

Figure 5 Pretreatment with tyrosine kinase inhibitors blocks γ RF-1 activation *in vitro*

Membrane and cytosol fractions were prepared from unstimulated THP-1 cells in reaction buffer containing 4 μ M ATP. Fractions were preincubated for 10 min at 30 °C with the indicated concentration of tyrosine kinase inhibitor prior to activation with sodium orthovanadate and IFN- γ for 30 min at 30 °C, or were treated with the inhibitor concomitantly with sodium orthovanadate and IFN- γ (*) for 30 min at 30 °C. Reactions were stopped by the addition of 0.1% NP-40, vortexed and centrifuged. The supernatants (2 μ l aliquots) were assayed by EMSA.

Figure 6 Effect of anti-phosphotyrosine antibodies on γ RF-1

Membrane and cytosolic fractions were prepared from THP-1 cells and were stimulated *in vitro* with 1 mM sodium orthovanadate and 2000 units of IFN- γ for 30 min as detailed in the Materials and methods section. Aliquots (1 μ l) of the supernatant from this preparation were incubated without addition (—), with the indicated amounts (μ g) of monoclonal anti-phosphotyrosine antibodies (PY20 or 4G10) or with an IgG2b isotype control antibody for 3 h at 4 °C. The samples were then analysed by EMSA using a 4.5% polyacrylamide gel. The 'super-shifted' complex is indicated by arrows. The first lane contained radiolabelled DNA only.

activation of γ RF-1 was performed in the presence of [γ - 32 P]ATP. Membrane and cytosol fractions were prepared from THP-1 homogenates and were co-incubated in the absence or presence of sodium orthovanadate or sodium orthovanadate plus IFN- γ

Figure 7 *In vitro* phosphorylation of γ RF-1 components

Membrane and cytosol fractions prepared from unstimulated THP-1 cells were incubated in the presence or absence of sodium orthovanadate (VAN; 1 mM) and IFN- γ (2000 units) in reaction buffer containing [32 P]ATP for 30 min at 30 °C. Reactions were stopped by the addition of 0.1% NP-40, vortexing and centrifugation. The resulting supernatants were then subjected to partial γ RF-1 purification using streptavidin-coupled magnetic beads to which were bound biotinylated DNA fragments containing three tandem repeats of the γ RE-1 sequence. Bound proteins were eluted and resolved by SDS/PAGE on a 10% (w/v) polyacrylamide gel. 32 P-containing proteins were visualized by autoradiography.

in reaction buffer containing [γ - 32 P]ATP (6000 Ci/mmol; 40 μ Ci/reaction). After *in vitro* activation, partially purified γ RF-1 was obtained using biotinylated DNA fragments containing three tandem copies of the γ RE-1 sequence coupled to magnetic streptavidin-coated beads. Proteins eluted from the DNA-affinity beads were resolved by SDS/PAGE and visualized by autoradiography. As shown in Figure 7, proteins of approx. 70–80 and 90 kDa were detected when membrane plus cytosol fractions were incubated with sodium orthovanadate or sodium orthovanadate plus IFN- γ . In addition, a factor of 120–130 kDa was detected when samples were incubated with sodium orthovanadate plus IFN- γ , but was not detected in reactions containing sodium orthovanadate alone.

DISCUSSION

Our studies indicate that the cell-free activation of γ RF-1 by IFN- γ has an absolute requirement for sodium orthovanadate or pervanadate, two known tyrosine phosphatase inhibitors [15,16]. This requirement most likely reflects a need for the inhibition of constitutively active tyrosine phosphatase, although it is also possible that tyrosine phosphatases are activated *in vitro* as a result of IFN- γ receptor binding by ligand. Furthermore, these results show that a tyrosine phosphatase is not required for, but rather inhibits, the cell-free activation of γ RF-1. This is in contrast to the *in vitro* activation of GAF (p91 or Stat1, for Signal Transducer and Activator of Transcription), a transcription factor activated by both IFN- α and IFN- γ which binds a *cis*-element (GAS) present in the promoter of the guanylate binding protein gene [17], among others (reviewed in [18]). Unlike γ RF-1, the *in vitro* activation of GAF was inhibited by tyrosine phosphatase inhibitors including zinc chloride and phenylarsine oxide [13]. Another distinction between the activation of γ RF-1 and GAF is our finding that γ RF-1 activation is inhibited by genistein only if added prior to IFN- γ /sodium orthovanadate treatment, while GAF activation *in vitro* was blocked by genistein whether added before or after IFN- γ [13].

The *in vitro* activation of γ RF-1 can also be distinguished from that of ISGF-3, a multimeric transcription factor (p91/p84, p113 and p48) that binds to the ISRE element found in the promoters of several interferon-responsive genes [19,20]. It was reported that ISGF-3 activation, similar to that of GAF, required a tyrosine phosphatase activity and was inhibited by sodium orthovanadate *in vitro* [12]. Furthermore, the cell-free activation of ISGF-3 occurred readily in HeLa lysates [13,21], whereas γ RF-1 could not be activated *in vitro* in HeLa lysates under conditions suitable for both A431 and THP-1 cells (results not shown).

Recently the cell-free activation of two transcription factors, FcRF γ 1 and FcRF γ 2 [14], which bind to the IFN- γ response region of the human high-affinity Fc γ receptor gene [22] was reported. Similar to our studies, the activation of FcRF γ 1 and FcRF γ 2 occurred in cell lysates treated with sodium orthovanadate in the presence or absence of IFN- γ [14]. However, the activation of FcRF γ 1 and FcRF γ 2 differed from that of γ RF-1 in many respects. Firstly, sodium orthovanadate was not necessary for the activation of FcRF γ 1/2, whereas we found an absolute requirement for sodium orthovanadate (or pervanadate) to activate γ RF-1 *in vitro*. Secondly, the membrane fraction of human monocytes was sufficient for activation of FcRF γ 1/2; however, activation of γ RF-1 in THP-1 cells, a human monocytic leukaemia line, required both membrane and cytosol components (Figure 3a). Thirdly, the activation of FcRF γ 1/2 was only partially inhibited by genistein pretreatment, whereas that of γ RF-1 was completely blocked (Figure 5). Finally, it was proposed that activation of FcRF γ 1/2 involved a membrane-associated tyrosine kinase, whereas our preliminary data suggest that the activation of γ RF-1 may involve a cytosolic tyrosine kinase (results not shown).

Our results indicate that sodium orthovanadate did not mimic the effect of IFN- γ on the activation of γ RF-1 in the presence of low micromolar concentrations of ATP. Rather, under these conditions the cell-free activation of γ RF-1 required receptor engagement by IFN- γ . Since the IFN- γ receptor has been found to have no homology with known kinases and to have no intrinsic kinase activity [2], ligand binding must be associated with recruitment and/or activation of a tyrosine kinase by the receptor complex. The involvement of members of the Janus family of cytosolic tyrosine kinases has been well described in the IFN- α and IFN- γ signalling pathways (reviewed in [18,23]). The tyrosine kinases Tyk2 and Jak1 have been shown to mediate the effects of IFN- α , whereas Jak1 and Jak2 have been shown to be important in transducing signals via the IFN- γ receptor [18,23–27]. In addition, these kinases have been shown to be involved in the signalling pathways of several other cytokines, including interleukin-3, interleukin-6, ciliary neurotrophic factor, leukaemia inhibitory factor, oncostatin M and certain growth factors such as epidermal growth factor, erythropoietin, granulocyte/macrophage colony-stimulating factor, granulocyte colony-stimulating factor, prolactin and growth hormone [28–33]. A series of recent reports has defined the importance of Jak1 and Jak2 in the activation of GAF by IFN- γ [24–27]. However, as noted above, our studies indicate major differences in the modes of activation of GAF and γ RF-1 *in vitro*, and whether Jak1 or Jak2 is responsible for the activation of γ RF-1 by IFN- γ remains to be determined.

We previously reported that γ RF-1 activation proceeds rapidly in intact cells, with activity detectable in nuclear extracts within 1 min of stimulation with IFN- γ in the absence of tyrosine phosphatase inhibitors [11]. In intact cells, therefore, there must be other mechanisms for regulating phosphatase activity so as to permit the accumulation of phosphorylated γ RF-1. One possi-

bility is that the stimulation of the IFN- γ -receptor-coupled tyrosine kinase(s) results in inhibition of phosphatase activity (either directly or indirectly), but this effect is disrupted *in vitro*. Another possibility is that a different receptor-coupled pathway (e.g. phospholipid metabolism, ion flux) that is not active *in vitro* is responsible for phosphatase inhibition in intact cells. It is also possible that the tyrosine phosphatase activity which dominates the *in vitro* system is compartmentalized or inhibited in intact cells, but is artefactually released or enhanced by the preparation of cell lysates or by the buffer conditions used in these experiments. Additional experiments are needed to further define the regulation of tyrosine phosphatase(s) in modulating the activation of γ RF-1 *in vitro* and *in vivo*.

In contrast to the minimal γ RF-1 activation by sodium orthovanadate alone in samples containing limiting ATP concentrations, pervanadate was effective in activating γ RF-1 under the same conditions. Furthermore, when high concentrations of exogenous ATP were added, both sodium orthovanadate and pervanadate stimulated γ RF-1 activation. This ligand-independent activation was also observed in co-incubated membrane and cytosol fractions. These results indicate that pervanadate or sodium orthovanadate plus high concentrations of ATP can obviate the need for receptor engagement in γ RF-1 activation. Although the mechanism for this ligand-independent activation is not known, both membrane and cytosol fractions are still required (Figure 3a). It is possible that these conditions which lead to ligand-independent stimulation of γ RF-1 result not only in effective tyrosine phosphatase inhibition but also in tyrosine kinase (auto)activation.

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REFERENCES

- Epstein, L. B. (1984) Interferon **2**, 186–219
- Farrar, M. A. and Schreiber, R. D. (1993) Annu. Rev. Immunol. **11**, 571–611
- Halloran, P. F. (1993) Transplant. Proc. **25**, 10–15
- Pellegrini, S. and Schindler, C. (1993) Trends Biochem. Sci. **18**, 338–342
- Lew, D. J., Decker, T., Strehlow, I. and Darnell, J. E. (1991) Mol. Cell. Biol. **11**, 182–191
- Driggers, P. H., Ennist, D. L., Gleason, S. L., Mak, W.-H., Marks, M. S., Levi, B.-Z., Flanagan, J. R., Appella, E. and Ozato, K. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 3743–3747
- Wilson, K. C. and Finbloom, D. S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 11964–11968
- Sims, S. H., Cha, Y., Romine, M. F., Gao, P.-Q., Gottlieb, K. and Deisseroth, A. B. (1993) Mol. Cell. Biol. **13**, 690–702
- Wright, T. M. and Farber, J. M. (1991) J. Exp. Med. **173**, 417–422
- Farber, J. M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5238–5242
- Wong, P., Severns, C. W., Guyer, N. B. and Wright, T. M. (1994) Mol. Cell. Biol. **14**, 914–922
- David, M., Romero, G., Zhang, Z.-Y., Dixon, J. E. and Larner, A. C. (1993) J. Biol. Chem. **268**, 6593–6599
- Igarashi, K., David, M., Finbloom, D. S. and Larner, A. C. (1993) Mol. Cell. Biol. **13**, 1634–1640
- Igarashi, K., David, M., Larner, A. C. and Finbloom, D. S. (1993) Mol. Cell. Biol. **13**, 3984–3989
- Pumiglia, K. M., Lau, L.-F., Huang, C.-K., Burroughs, S. and Feinstein, M. B. (1992) Biochem. J. **286**, 441–449
- Swarup, G., Cohen, S. and Garbers, D. L. (1982) Biochem. Biophys. Res. Commun. **107**, 1104–1109
- Decker, T., Lew, D. J., Mirkovitch, J. and Darnell, J. E., Jr. (1991) EMBO J. **10**, 927–932
- Darnell, J. E., Jr., Kerr, I. M. and Stark, G. R. (1994) Science **264**, 1415–1421
- Fu, X.-Y., Kessler, D. S., Veals, S. A., Levy, D. E. and Darnell, J. E., Jr. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 8555–8559
- Kessler, D. S., Veals, S. A., Fu, X.-Y. and Levy, D. E. (1990) Genes Dev. **4**, 1753–1765
- David, M. and Larner, A. C. (1992) Science **257**, 813–815
- Pearse, R. N., Feinman, R. and Ravetch, J. V. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 11305–11309
- Ihle, J. N., Witthuhn, B. A., Quelle, F. W., Yamamoto, K., Thierfelder, W. E., Kreider, B. and Silvennoinen, O. (1994) Trends Biochem. Sci. **19**, 222–227
- Müller, M., Briscoe, J., Laxton, C., et al. (1993) Nature (London) **366**, 129–135
- Shuai, K., Ziemiecki, A., Wilks, A. F., Harpur, A. G., Sadowski, H. B., Gilman, M. Z. and Darnell, J. E. (1993) Nature (London) **366**, 580–583
- Silvennoinen, O., Ihle, J. N., Schlessinger, J. and Levy, D. E. (1993) Nature (London) **366**, 583–585
- Watling, D., Guschin, D., Müller, M., et al. (1993) Nature (London) **366**, 166–170
- Argetsinger, L. S., Campbell, G. S., Yang, X., Witthuhn, B. A., Silvennoinen, O., Ihle, J. N. and Carter-Su, C. (1993) Cell **74**, 237–244
- Silvennoinen, O., Witthuhn, B. A., Quelle, F. W., Cleveland, J. L., Yi, T. and Ihle, J. N. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8429–8433
- Stahl, N., Boulton, T. G., Farruggella, T., et al. (1994) Science **263**, 92–95
- Stahl, N. and Yancopoulos, G. D. (1993) Cell **74**, 587–590
- Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O. and Ihle, J. N. (1993) Cell **74**, 227–236
- Feldman, G. M., Petricoin, E. F., III, David, M., Larner, A. C. and Finbloom, D. S. (1994) J. Biol. Chem. **269**, 10747–10752