Cooperation of STAT-1 and IRF-1 in Interferon-γ-induced Transcription of the gp91phox Gene*

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Interferon (IFN)-γ induces the expression of the gp91phox gene both during myeloid differentiation and also in mature phagocytes through several cis-elements and their binding proteins. To find new cis-elements for this induction, transient expression assays were performed using a reporter gene driven by serially truncated gp91phox promoters in U937 cells. The results suggest that a critical cis-element for induction exists in the region from bp −115 to −96 of the promoter. Site-directed mutagenesis showed that a γ-activated sequence (GAS) element at bp −100 (−100GAS) of the gp91phox promoter plays a pivotal role for the IFN-γ-dependent activity of the bp −115 to +12 region of the gp91phox promoter. Electrophoretic mobility shift assays using several GAS competitors and specific antibodies indicated that phosphorylated STAT-1a specifically binds to the −100GAS. Site-directed mutagenesis showed that an interferon-stimulated response element (ISRE) at bp −88 (−88ISRE) mediates the induction of the gene by IFN-γ in cooperation with −100GAS. Electrophoretic mobility shift assay showed that IRF-1 dominantly binds to −88ISRE in an IFN-γ-dependent fashion. These results demonstrate a new mechanism for IFN-γ-induced transcription of the gp91phox gene by the cooperation of STAT-1a and IRF-1 binding to −100GAS and −88ISRE, respectively.

Phagocytes, such as macrophages and granulocytes, generate superoxide anions by the phagocyte NADPH oxidase to kill ingested microorganisms (1). The gp91phox gene encodes an essential component of the oxidase, and mutations including a deletion (2, 3) of the gene result in X-linked chronic granulomatous disease characterized by severe and recurrent infections due to lack of superoxide generation (4). The gp91phox gene is expressed almost uniquely in differentiated myeloid cells (5). The human gp91phox promoter from bp −450 to +12 directs the transcription of a reporter gene in a subset of mouse monocytes/macrophages in transgenic mice (6). Eosinophils, in particular, express gp91phox in a patient with chronic granulomatous disease (7). These observations indicate that the expression of the gp91phox gene is both lineage- and differentiation-stage-specific. Transcription of the gp91phox gene is also modulated by inflammatory mediators such as interferon (IFN)-γ, tumor necrosis factor-α, and lipopolysaccharide in myeloid cells (8, 9). Although enhancer elements for the expression of gp91phox gene in mature myeloid cells have been suggested to be in a 50-kb region located upstream of a transcription start site of the gene (10), principal cis-regulatory elements are clustered in a 450-bp proximal promoter region of the gene. Several transcription factors regulate gp91phox gene expression through these cis-elements. A transcriptional repressor, CCAAT displacement protein (CDP/cut), binds to multiple sites in the proximal promoter in immature myeloid cells (11, 12).

Several transcription factors regulate gp91phox gene expression, the binding activity of CDP to these binding sites decreases during differentiation of the cells (11–14). Moreover, the induction of the gp91phox gene is prevented by the constitutive overexpression of CDP in a myeloid cell line (13). Finally, CDP itself disappears in the maturation course to peripheral monocytes and macrophages (14). Although general transcriptional activators such as those with increased binding during differentiation, like (BID)/YY1, CCAAT box-binding factors, CP1, and NF-Y, bind to the promoter, these factors can work only after CDP has been released because their binding sites overlap those for CDP (12, 14–16). Interferon regulatory factor (IRF)-2 also activates the gp91phox promoter through two interferon-stimulated response elements (ISRE) in the absence of CDP binding (15, 17). PU.1 works as a pivotal transcription factor of the gp91phox gene by binding a PU.1/hematopoietic-associated factor (HAF)-1-binding element centered at bp −53 in human neutrophils, monocytes, and B-lymphocytes (18). HAF-1 is a multiprotein complex, and its components are still not defined, but PU.1, IRF-1, ICSBP, and Elf-1 are picked out as candidates (19, 20). GATA-1, GATA-2, and GATA-3 regulate transcription of the gp91phox gene in eosinophilic cells (21, 22).

IFN-γ is a cytokine that plays an important role in both innate and adaptive immunities (23). IFN-γ binds to the specific cell surface receptor and activates the receptor-associated Janus family tyrosine kinase (JAK)1 and JAK2. The kinases phosphorylate and activate a latent cytoplasmic transcription factor, STAT-1α, which is then translocated to the nucleus as a transcriptionally active homodimer (γ-interferon activation

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1 The abbreviations used are: IFN, interferon; EMSA, electrophoretic mobility gel shift assay; CDP, CCAAT displacement protein; BID, binding increased during differentiation; ISRE, interferon-stimulated response element; HAF, hematopoietic-associated factor; ICSBP, IFN consensus-binding protein; ICSAT, IFN consensus sequence-binding protein in adult T-cell leukemia; ISGF-3γ, interferon-stimulated gene factor-3γ; STAT, signal transducers and activators of transcription; GAS, the γ-activated sequence; IFP, IFP53/tryptophanyl-tRNA synthetase promoter; GBP, the guanylate-binding protein; SIE, the c-fos cis-inducible element; GRR, the Fcy receptor I promoter; IRF, interferon regulatory factor; DIF, differentiation-induced factor; CBP, CREB-binding protein.
factor) and binds to the γ-activated sequence (GAS) element of IFN-γ-responsive genes resulting in their activation (24). IFN-γ also induces gp91<sub>phox</sub> gene expression, coinciding with monocytic differentiation of committed progenitors (9) and increases gp91<sub>phox</sub> transcription in mature monocytes ex vivo (8). Two positive regulation mechanisms for the IFN-γ-induced transcription of the gp91<sub>phox</sub> gene have been proposed. One mechanism is the binding of BID/Y11 to the four binding sites that are scattered in a region from bp –90 to –355 of the promoter after dissociation of CDP from the gene (15, 16). The other is the binding of an undefined multiprotein complex of HAF-1 to the PU.1/HAF-1-binding element centered at –53 of the promoter (19, 25, 26).

In this study, we show that STAT-1α bound to the GAS element at bp –100 of the gp91<sub>phox</sub> gene has an unequivocal role in IFN-γ-induced transcription of the gene. We further show that IRF-1 participates in the STAT-1-mediated transcription through binding to the ISRE at bp –88 of the gene. These results suggest a new mechanism for IFN-γ-induced transcription of the gp91<sub>phox</sub> gene in which STAT-1α and IRF-1 cooperate with each other.

MATERIALS AND METHODS

**Cell Culture—**U937 cells (Japanese Collection of Research Bioresources, Tokyo) were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum. The cells were either untreated or treated with 100 units/ml human IFN-γ (kindly provided by Shionogi Corp., Osaka, Japan) for the indicated periods.

**Northern Blot Analysis—**Total RNA was prepared from cells with Trizol LS reagent (Invitrogen) according to the manufacturer’s protocol. The RNA (5 μg/lane) was electrophoresed on formaldehyde-containing 0.9% agarose gels, transferred to Hybond TM-N (Amersham Biosciences), and fixed by ultraviolet light. A probe of [32P]dCTP using the Megaprime DNA labeling system (Amersham Biosciences). Messenger RNAs were hybridized with these labeled probes. The activity of each band was estimated on a Molecular Imager FX (Bio-Rad).

**Electrophoretic Mobility Shift Assays (EMSA)—**Preparation of the double-stranded oligonucleotide probes and EMSAs were performed as described previously (18). In competition assays, a 100-nmol excess of the unlabeled competitor oligonucleotide was added to a mixture of the probe to the mixture, which was then preincubated on ice for 15 min. For the inhibition assay with antibodies, an aliquot of nuclear extracts was incubated on ice with 2–4 μg of the indicated antibody for 1 h before the addition of the probe. Bound and free DNA were separated by electrophoresis in a native 6% polyacrylamide gel at 4 °C. Sequences of the upper strands of double-strand oligonucleotides are as follows. Mutated bases are underlined and names used in the text are indicated in bold: W-107A (107 to –85), 5′-AATTCTTGTAAAGA-AAGGAA-3′; W-107B (107 to –65), 5′-AATTCTTGATAAAGAAGAAAGGGACCTGTC-3′; W-115B (115 to –90), 5′-TATTAGCCAATTTCTGATAAAAGAA-3′; GSmut (115 to –90), 5′-TATTCTGAAATTTCTGATAAAAGAA-3′; W-102A (102 to –65), 5′-CTGATAAAAAGAAAGGACCTGTC-3′; W-102B (102 to –73), 5′-CTGATAAAAAGAAAGGACCTGTC-3′; W-85 (85 to –65), 5′-AGAAAGAAAGGACCTGTC-3′; W-107C (107 to –73), 5′-CTGATAAAAAGAAAGGACCT-3′. The following four oligonucleotides are used as competitors (28): GAS/GRR (GAS site from the Fc γR II promoter), 5′-GATTGCCCCAGGGCTGC-3′; GAS/SIE (GAS/SIE elements in human promonocytic U937 cells after addition of 100 nM of IFN-γ), 5′-AGGATCGCGCGCGACCCATCC-3′; GAS/IRF (GAS site from the IFP53/tryptophanyl-tRNA synthetase promoter), 5′-TCCACGACATTCGAAACACTT-3′; GAS/GGBP (GAS site from the guanylate-binding protein promoter), 5′-ACTGTTATATATCCATATATTCAAT-3′; GAS/SIE (gene in the c-fos cis-inducible element), 5′-AATTTCTGATAAAAGAA-3′; GAS/GRR (gene from the Fcy receptor I promoter), 5′-GATTGCCCCAGGGCTGC-3′; GAS/GGBP (gene from the guanylate-binding protein promoter), 5′-ACTGTTATATATCCATATATTCAAT-3′.

**Western Blot Analysis—**Western blot analysis was carried out using standard methods (30). Nuclear extracts (10 μg of protein) were mixed with an equal volume of 250 mM Tris–HCl (pH 6.8) containing 4.6% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 20 μg/ml bromophenol blue, and heated at 100 °C for 5 min. Samples separated by SDS-PAGE in 10% gel were transferred onto an Immobilon™ polyvinylidene difluoride membrane (Millipore, Bedford, MA) using a semi-dry type electrophoretic apparatus (model BE-300, Bio-Craft, Tokyo, Japan). The protein-blotted polyvinylidene difluoride membrane was stained with Coomassie Brilliant Blue, photographed, and destained with methanol. The membranes were pretreated with Block Ace (Yuki- jirushi Co., Sapporo, Japan) containing 0.1% Tween 20 for 1 h at room temperature followed by washing with TBS (20 mM Tris pH 7.4, 150 mM NaCl) containing 0.1% Tween 20 (TBS/Tween) and then incubated with 1/2500 diluted anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature. The membrane was then washed extensively with TBS/Tween and then rinsed with TBS. The antigen antibody complexes were detected using an Enhanced Chemiluminescence Kit (Amersham Biosciences Inc.).

**Statistical Analyses—**Data are expressed as mean ± S.E. Statistical analysis was done on data from at least three independent experiments by Student’s t test.

**RESULTS**

**Identification of IFN-γ-responsive Elements in the gp91<sub>phox</sub> Promoter—**We analyzed the time course of gp91<sub>phox</sub>mRNA levels in human promonocytic U937 cells after addition of 100 units/ml IFN-γ by Northern blot analysis. As shown in Fig. 1, the amount of gp91<sub>phox</sub>mRNA increased ~4.5-fold within 5 h and reached its maximal level (~7.5-fold) at 48 h after the
addition of IFN-γ as demonstrated in previous studies (8, 9, 26) with U937 cells and cultured human monocyte-derived macrophages. Similar studies previously done (15, 25, 26) for IFN-γ induction of the gp91phox gene were focused on the later phase of induction (at 24–48 h after the addition of IFN-γ). In this study, we focused on the early phase of IFN-γ induction of the gp91phox gene because the changes in this phase are the most pronounced. The bp −450 to +12 fragment of the gp91phox promoter directs IFN-γ-inducible transcription in a stably transfected PLB-958 myeloid cell line (25). Therefore, we initially prepared a p−487/Luc construct that contains the gp91phox gene fragment from bp −487 to +12 in front of a luciferase reporter gene and transiently transfected it with the reference plasmid pRL-TK into promonocytic U937 cells. The reporter activity of the cells was assessed following treatment with p−100GAS, indicating that the bp −115 to −90 region of the gp91phox with p−115 to −96 of the gp91phox promoter activity in the functional assay (Fig. 2). Therefore, we focused on GAS-binding proteins and did further experiments. We examined the effect of mutations in the −100GAS element for the binding of this protein complex to the W-107A probe using a fragment from bp −115 to −90 (W-115 in Fig. 3) and a −100GAS mutant version of W-115 (GASmut in Fig. 3) as competitors. As shown in Fig. 5, the binding of this protein complex (shown by an arrow) was abolished by the W-115 competitor (lane 4) but not by the GASmut competitor (lane 5), indicating that the −100GAS element is essential for binding of the protein complex to the W-107A probe. Previous studies (32–35) showed that the STAT-1α transcription factor frequently binds to GAS elements in the genes of IFN-γ-induced proteins such as IRF-1, class II transactivator, and CD40. Differentiation-induced factor (DIF) also binds to GAS elements of several IFN-γ-induced genes such as the Fcy receptor I (FcyR) in U937 cells after the addition of IFN-γ (28). To examine whether either one or both factors bind to the −100GAS of the gp91phox promoter in the IFN-γ-treated U937 cells, we performed EMSAs using IFP GAS and GBP GAS as common competitors to both STAT-1α and DIF and FcγRI GAS and c-fos SIE GAS as specific competitors to STAT-1α (28). As shown in Fig. 5, binding of the protein complex to the W-107A probe was abolished by all four GAS competitors (lanes 6–9), suggesting that this protein complex includes STAT-1α but not DIF. To confirm the binding of STAT-1α to the −100GAS, we performed gel shift immunoassays using three different anti-STAT-1 antibodies (Fig. 5) as follows: an anti-STAT-1 antibody specific for both STAT-1α and STAT-1β (lane 13), an anti-STAT-1α antibody specific for STAT-1α (lane 11), and an anti-phosphorylated STAT-1 antibody specific for Tyr-701-phosphorylated STAT-1α and STAT-1β (lane 12). All three antibodies completely abolished the binding of the protein complex to the W-107A probe. In contrast, a control antibody did not inhibit the binding of the complex to the probe (lane 10). These results indicate that phosphorylated STAT-1α binds to the −100GAS element of the gp91phox promoter in the IFN-γ-treated U937 cells. Binding of STAT-1α to the −100GAS was detected within 15 min after IFN-γ treatment and was not inhibited by cycloheximide (data not shown), suggesting the rapid phosphorylation of pre-existing STAT-1α and its translocation from the cytosol into the nucleus by IFN-γ treatment.

Both −100 GAS and an ISRE Are Required for Maximal gp91phox Promoter Activity Induced by IFN-γ—To determine whether −100GAS actually contributed to the IFN-γ-depend-
ent gp91phox promoter activity through the bp −115 to −96 region, we examined the effect of a −100GAS mutation (TTCT-GATAA to TGATGATAA), which abolishes the binding of STAT-1 to −100GAS (Fig. 5), on gp91phox promoter activity of p−115/Luc in IFN-γ-treated U937 cells. As shown in Fig. 6, the −100GAS mutant reduced both the inducibility (from 5.5 ± 0.5 to 1.4 ± 0.1-fold) and IFN-γ-dependent promoter activity (from 5.5 ± 0.5 to 1.7 ± 0.2) to levels comparable with those (1.8 ± 0.2) of the p−95/Luc (compare p−115GASm/Luc and p−95/Luc). This result indicates that −100GAS is pivotal for the IFN-γ-induced gp91phox promoter activity dependent on its bp −115 to −96 region in U937 cells.

IRFs reportedly mediate IFN-γ-induced expression of genes such as guanylate-binding protein, major histocompatibility complex class I, and inducible nitric-oxide synthase through ISRE (36). A previous study (17) showed that both IRF-1 and IRF-2 bound to the bp −102 to −65 fragment of the gp91phox promoter including an ISRE centered at bp −88 (−88ISRE in Fig. 3). These findings suggested a role for the IRF-1 and/or IRF-2 in IFN-γ-induced gp91phox promoter activity. As shown in Fig. 6, p−95/Luc including the −88ISRE failed to respond to IFN-γ, suggesting that −88ISRE contributes nothing by itself to the IFN-γ-dependent activation of gp91phox gene. This result, however, did not eliminate the possibility of a cooperative contribution of the element with other elements lying upstream of bp −95. To test this possibility, we examined the effect of a
−88ISRE mutation (5′-AAGAAAAGGAACCC-3′ to 5′-AA-
GAAAAGTCCTCCC-3′ as IRFmut in Fig. 3), which abolishes the
binding of IRF-1 and IRF-2 to −88ISRE, but not that of BID
(17), on the IFN-γ-inducible promoter activity of p−115/Luc. As
shown in Fig. 6, the −88ISRE mutation abolished the IFN-γ-
dependent activity of p−115/Luc (compare p−115IRFm/Luc-I
with p−115/Luc-I), suggesting that −88ISRE is also required for
the maximal gp91phox promoter activity induced by IFN-γ.

The activity of p−115IRFm/Luc after the addition of IFN-γ was
equal to that of p−115/Luc (compare p−115IRFm/Luc-I with
p−115/Luc-N). Its fold induction, however, was significantly
higher (p < 0.05) than that of pXP2N (compare p−115IRFm/
Luc with pXP2N), because of the low constitutive activity of
p−115IRFm/Luc. This significant inducibility might show a
−88ISRE-independent mechanism for the activation of the
gp91phox gene through −100GAS by IFN-γ. It should be noted
that the −88ISRE mutation significantly (p < 0.001) reduced
the constitutive activity of the p−115/Luc (compare
p−115IRFm/Luc-N with p−115/Luc-N), suggesting that the
element also contributes to the constitutive expression of the
gp91phox gene.

**FIG. 6.** Functional analysis of −100GAS and −88ISRE cis-regulatory elements for IFN-γ-induced activity of the gp91phox promoter
in U937 cells. Three wild-type constructs (p−115/Luc, p−95/Luc, and p−84/Luc), two mutants of p−115/Luc (p−115GASm/Luc and
p−115IRFm/Luc), and a promoter-less construct (pXP2N) were transfected into U937 cells incubated with (I) or without (N) IFN-γ (100 units/ml) for
5 h. Firefly luciferase activities were normalized using the accompanied activities of the co-transfected Renilla luciferase reporter gene (see
“Materials and Methods”). Each column and bar in the graph are the relative means of three independent data sets and the S.E., respectively. The
activity of p−115/Luc N was set to 1. Fold induction was calculated as the ratio of relative luciferase activity of IFN-γ-treated samples to that of
the untreated one and is shown as the mean ± S.E. Double and single asterisks indicate statistically significant differences with p < 0.001 and p < 0.05, respectively.
bodies (lanes 8–11). None of antibodies inhibited the binding of C1 and C2 complexes (Fig. 7B, lanes 6–11). These results indicate that the C3 complex, but not other complexes, contains IRF-1. The C1 and C2 complexes may contain other members of IRF family or other transcription factors whose binding sites overlap with −88ISRE. We also confirmed binding of IRF-1 to −88ISRE of p−95/Luc by using the fragment from bp −95 to −65 (W-95 in Fig. 3) of the gp91-phox promoter as a probe (Fig. 7C). Next we analyzed the expression level of the IRF-1 protein in the nucleus of IFN-γ-treated U937 cells by Western blot analysis (Fig. 8). Consistent with the results of EMSAs, IRF-1 could be detected in the nucleus of U937 cells from 2 h (Experiment 1), and its amount increased until 48 h after addition of IFN-γ (Experiment 2). Binding of IRF-1 in U937 cells treated with IFN-γ for 48 h to −88ISRE was confirmed (data not shown). The expression of IRF-1 appears to be linked kinetically to that of gp91-phox mRNA in IFN-γ-treated U937 cells (Fig. 1), suggesting the cooperation of STAT-1α with concomitantly induced IRF-1 efficiently activates the gp91-phox gene.

Binding of STAT-1α and IRF-1 to the Element Containing Both −100GAS and −88ISRE—Because binding of STAT-1α and IRF-1 to their isolated binding elements (−100GAS in W-107A and −88ISRE in W-102A) might not reflect binding to the native element containing both −100GAS and −88ISRE, we next analyzed whether STAT-1α and IRF-1 would also bind to the oligonucleotide containing the entire −100GAS and −88ISRE (W-107B in Fig. 3). As shown in Fig. 9, both STAT-1α and IRF-1 clearly bound to the combination −100GAS/−88ISRE element of W-107B, and the binding was abolished by a homologous sequence competitor (W-107B) but not by a
Fig. 8. Immunoblot analysis of IRF-1 expression in U937 cells after addition of IFN-γ. The results of two independent experiments are shown. Cells were harvested at the indicated times of culture with IFN-γ (100 units/ml). Proteins of nuclear extracts from the cells were separated by SDS-PAGE and subjected to immunoblotting as described under "Materials and Methods" using anti-IRF-1 polyclonal antibody (Anti IRF-1) and a nonimmunized rabbit IgG (Control). An arrow indicates the position of IRF-1. Other bands are nonspecific because they were also observed using a control antibody.

Fig. 9. Binding of STAT-1α and IRF-1 to the element containing both −100GAS and −88ISRE. EMSA was performed as described under "Materials and Methods" except using 2 μg/ml heat-denaturated salmon sperm DNA instead of poly(dI-dC)/poly(dI-dC). The W-107B probe containing both −100GAS and −88ISRE was incubated with nuclear extracts isolated from U937 cells untreated or treated with 100 units/ml IFN-γ for 5 h. The binding was competed with a 100-fold molar excess of one of the following oligonucleotides: unlabeled probe (W-107B); heterogeneous oligonucleotide (Hetero); oligonucleotide from bp −107 to −85 of the promoter (W-107A) containing −100GAS; oligonucleotide from bp −102 to −65 of the promoter (W-102A) containing −88ISRE.

heterogeneous one (Hetero). Binding of STAT-1α to the combination −100GAS/−88ISRE element could only be competed by cold W-107A containing −100GAS but not by cold W-102A containing −88ISRE. On the contrary, binding of IRF-1 to the combination element could only be competed by cold W-102A but not by cold W-107A, suggesting that both factors can bind independently to their binding sites in the gp91phox promoter.

Low molecular mass polypeptide 2 (LMP2) has the overlapped interferon consensus sequence 2′γ-interferon-activated sequence (ICS-2/GAS). A recent study showed that a complex of STAT-1 and IRF-1 binds to the ICS-2/GAS (37), suggesting simultaneous binding of STAT-1α and IRF-1 to the gp91phox promoter.

DISCUSSION

The gp91phox gene is a major gene regulated by IFN-γ (23). In the present study, deletion and mutation analyses show that a GAS element centered at bp −100 of the gp91phox promoter (−100GAS) is critical for the IFN-γ-induced transcriptional activation of the gene. EMSA analysis shows that IFN-γ stimulation causes the dominant binding of phosphorylated STAT-1α to this −100GAS. These results indicate that STAT-1α activated by IFN-γ directly enhances the gp91phox promoter activity through binding to −100GAS. Consistent with our results, the gp91phox promoter from bp +12 to −100, which is the center position of the −100GAS, was not activated by IFN-γ in stable transfectants of human promyelocytic PLB985 cells carrying a reporter construct with this promoter (15). The authors also showed that at least one of the BID-binding sites centered at bp −225 (BID-225) or bp −145 (BID-145) of the gp91phox promoter must be intact for IFN-γ induction of the promoter (15). In contrast, the bp −115 to +12 fragment of the gp91phox promoter lacking these two BID sites is strongly activated by IFN-γ treatment in our transient expression system. This discrepancy may be explained by different localization of the reporter constructs in these cells. In the stably transfected cells, the reporter gene is integrated into the genome where chromatin structure and regulatory sequences surrounding the integration site can affect reporter gene expression. On the other hand, the transiently transfected reporter gene is exempt from these regulations because of its existence in the nucleus as an episome. CDP binds to multiple sites within the bp −450 to +12 of the gp91phox promoter (38). It excludes the binding of transcriptional activators by occupying binding sites and represses the expression of the gp91phox gene (12). A recent study showed that the binding of BID/YY1 to elements BID-225 and BID-145, increased by various differentiation-inducing agents including IFN-γ (15), is caused by the decreased binding of CDP to overlapping binding sites but not by active binding of BID factors to their sites (16). The binding of BID/YY1 to the gp91phox promoter did not increase until 12 h after the addition of IFN-γ in PLB-985 cells (15), suggesting that BID/YY1 is not associated with the early phase of the IFN-γ induction. A previous study (17) suggested that the ISRE centered at bp −88 of the gp91phox promoter (−88ISRE) is necessary for constitutive activity of the gp91phox promoter in K562 and HEL erythroleukemia cells expressing no endogenous gp91phox. In this study, we confirm the necessity of −88ISRE for the constitutive activity of the gp91phox promoter. Moreover, we show that −88ISRE is not sufficient for IFN-γ-dependent gp91phox promoter activation but is essential for its maximal induction. We also show that IRF-1 dominantly binds to −88ISRE after IFN-γ treatment. These results indicate that IRF-1 mediates the IFN-γ-induced maximal activation of the gp91phox promoter through −88ISRE in U937 cells. Consistently with our results, IRF-1 binds to −88ISRE in IFN-γ-treated HeLa cells (17). IRF-2 also binds to −88ISRE in phorbol 12-myristate 13-acetate-treated PLB-985 cells, HEL cells, and K562 cells (17). However, we could not detect IRF-2 binding to the site in this study. Taken together, our results indicate that the cooperation of STAT-1α and IRF-1 through −100GAS and −88ISRE, respectively, plays a critical role at least in the early phase of the IFN-γ-induced transcription of the gp91phox gene (Fig. 10). Continuous binding of STAT-1α
and IRF-1 to the gp91phox promoter for 48 h after the addition of IFN-γ in U937 cells suggests that the cooperative mechanism works in a late phase of induction. Phosphorylated STAT-1α and IRF-1 are degraded by the ubiquitin-proteasome pathway, decreasing to low levels between 1 and 2 h (39). In contrast, sustained binding of STAT-1α and IRF-1 to the class II transactivator promoter was observed over 24 h by continuous treatment of 400 units/ml IFN-γ transactivator promoter was observed over 24 h by continuous treatment of 400 units/ml IFN-γ-activated as if the cells had been stimulated by IFN-γ. Elf-1 is also a component of HAF-1 (20).

A recent study showed the recruitment of CBP by PU.1, IRF-1, and ICSBP to increase gp91phox transcription (26). STAT-1α has been shown to cooperate with other transcription factors such as upstream stimulatory factor-1, glucocorticoid receptor, and PU.1 to induce transcription of genes (45, 46). Cell type-specific induction of gp91phox transcription by IFN-γ may be accomplished by cooperation between STAT-1α and PU.1. Indirect physical interaction of both transcription factors through a co-activator CBP is possible because two sites of CBP between amino acids 566 and 664 and amino acids 1283 and 1915 independently can interact with the amino-terminal region of active STAT-1α and the transactivating domain of PU.1, respectively (47, 48). Based on our results and these previous findings, we propose the formation of a large complex composed of STAT-1α, IRF-1, PU.1, ICSBP, and CBP on the proximal promoter of gp91phox after IFN-γ-stimulation (Fig. 10). Further experiments are required to prove this hypothesis.

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