Interferon γ-dependent Induction of Human Intercellular Adhesion Molecule-1 Gene Expression Involves Activation of a Distinct STAT Protein Complex

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In response to interferon γ (IFNγ), intercellular adhesion molecule-1 (ICAM-1) is expressed on human keratinocytes, a cell type that is critically involved in cutaneous inflammation. An ICAM-1 5′ regulatory region palindromic response element, p3γRE, has been shown to confer IFNγ-dependent transcription enhancement. By electrophoretic mobility shift assays (EMSA), p3γRE forms a distinct complex with proteins from IFNγ-treated human keratinocytes, termed γ response factor (GRF). Binding of GRF is tyrosine phosphorylation-dependent, and mutations of p3γRE that disrupt the palindromic sequence or alter its spatial relationship abrogate GRF binding. Supershift EMSAs using antibodies to characterized STAT proteins suggest that GRF contains a Stat1α-like protein; however, non-ICAM-1 IFNγ-responsive elements (REs) known to bind Stat1α homodimers fail to compete for GRF binding in EMSA, and p3γRE does not cross-compete with these REs that complex with homodimeric stat1α. The p3γRE-GRF complex also displays a distinctly different electrophoretic mobility compared to that of IFNγREs complexed to homodimeric Stat1α. These findings indicate that a distinct complex containing a Stat1α-like protein mediates IFNγ-induced ICAM-1 gene transcription and identifies a subset of IFNγ-responsive genes that appear to be regulated by this complex.

The initiation and evolution of localized inflammation is a consequence of homing and extravasation of leukocytes at sites of tissue injury or threat (1). Intercellular adhesion molecule-1 (ICAM-1)1, a cell surface glycoprotein that belongs to the immunoglobulin gene superfamily, serves as a specific ligand for receptors expressed by leukocytes (2). ICAM-1 plays a pivotal role in the adhesion and transmigration of leukocytes at sites of inflammation, and the up-regulation of ICAM-1 cell surface expression during inflammatory responses is essential in facilitating leukocyte migration (3, 4).

IFNγ, a pleiotropic cytokine produced by activated T lymphocytes, plays a critical role in host defenses and inflammation (5). In the skin IFNγ induces de novo expression of ICAM-1 on human keratinocytes (HK), cells that are critically involved in cutaneous inflammatory processes (6). Upon binding to its receptor, IFNγ initiates a signal transduction cascade that involves rapid activation of two members of the Janus tyrosine kinase family, JAK-1 and JAK-2 (7), and consequent tyrosine phosphorylation and activation of a latent cytoplasmic protein, originally referred to as p91 and now known as Stat1α (8, 9). Stat1α is the first described member of a family of proteins known as STATs, or signal transducers and activators of transcription. When activated by IFNγ, activated Stat1α homodimerizes through Src homology domains (10, 11) to form a γ-activated factor (GAF). The Stat1α homodimers, after translocation to the nucleus, bind to the γ-activated site (GAS), first identified in the human guanylate-binding protein (GBP) gene, and initiate gene transcription (8, 9, 12). Stat1β (p84) is an alternatively spliced product of the gene for Stat1 and is a truncated protein that lacks 38 amino acids at the carboxy end of Stat1. Stat1β also homodimerizes and is capable of binding GAS but does not activate transcription (13). It has recently been shown that Stat1α, which obligatorily requires tyrosine phosphorylation to become active, also requires phosphorylation of a serine residue for maximal activation of gene transcription (14).

In addition to formation of the homodimeric GAF, Stat1 also participates in forming the heteromultimeric transcription complex ISGF3, composed of Stat1α, Stat2, and a non-STAT protein, p48 (15–18). ISGF3 binds to the IFN-γ-stimulated response element (ISRE), which is an IFNα/β- and IFNγ-inducible element involved in the regulation of a variety of genes (11, 13, 17). Recently, multiple cytokines and growth factors have been shown to mediate their transcriptional effects through these and additional STAT proteins, of which there are now six characterized members (19). However, Stat1α is frequently activated in response to a wide variety of extracellular signals, including those involved in transcriptional activation of a number of responses.
ber of genes involved in immune responses (19).

IFN-γ-dependent induction of ICAM-1 gene expression is reg-
ulated at the transcriptional level (20, 21). The 5′ flanking region of ICAM-1 gene contains an 11-base pair (bp) element, which we refer to as palindromic IFN-γ response element (RE), or p1RE, located upstream of the ICAM-1 transcription initiation site between nucleotides −76 and −66. p1RE is composed of the sequence 5′-TTTCCGGGAAA-3′. Several labora-
tories have demonstrated that p1RE is both necessary and sufficient for IFN-γ-dependent gene transcription (20, 22).

The present studies were designed to characterize the molecular events and trans-acting factors involved in the IFN-γ-induced regulation of ICAM-1 gene transcription. The data presented show that the protein complex activated by IFN-γ, which trans-activates ICAM-1 gene expression by binding to p1RE, shares both similarities and distinct differences with previously characterized IFN-γ-activated STAT complexes. From these data we propose that the protein complex mediating IFN-γ-dependent ICAM-1 gene transcription, which we refer to as the γ response factor, or GRF, represents a distinct form of IFN-γ-induced transcription trans-activator and likely mediates trans-activation of a subset of IFN-γ-inducible early response genes.

MATERIALS AND METHODS

Cell Culture—As described previously (22), HK were isolated from neonatal foreskins at the Emory Skin Diseases Research Center. HK were cultured in KGM supplemented with bovine pituitary extract (Clonetics Corp., San Diego, CA). Cultures were maintained at 37 °C in humidified 5% CO2 and passed at 60–70% confluence using subculturing.

Cytoplasmic and Nuclear Extract Preparation—Cytoplasmic and nuclear extracts were prepared as described previously (23): 200 μg of nuclear or cytoplasmic protein extract, 2.5 μg of nuclear or cytoplasmic protein extract, 20 μg of nuclear or cytoplasmic protein extract, 10 μg of nuclear or cytoplasmic protein extract, 10 μg of nuclear or cytoplasmic protein extract, 1 μg of nuclear or cytoplasmic protein extract, 1 μg of nuclear or cytoplasmic protein extract, 0.5 μg of nuclear or cytoplasmic protein extract, and 0.1 μg of nuclear or cytoplasmic protein extract were prepared from cells that were either left untreated or treated with 250 units/ml recombinant human IFN-γ (R&D Systems, Minneapolis, MN). Cells were washed twice in ice-cold phosphate-buffered saline (Life Technologies, Inc.) and then quickly washed in buffer A (10 mM Hepes, pH 7.4, 1.5 mM MgCl2, 10 μM KCl, 0.5 mM dithiothreitol (DTT), 50 μM phenylmethylsulfonyl fluoride (PMSF); all from Sigma). After centrifugation at 2,000 rpm (4°C for 5 min), the cell pellet was resuspended in buffer A containing 0.1% Nonidet P-40 (U.S. Biochemical Corp.) and incubated on ice for 10 min. The nuclei were collected by centrifugation at 4,000 rpm for 2 min at 4 °C. The supernatant (cytosolic fraction) was removed and saved, and the pellet was resuspended in buffer B (20 mM Hepes, pH 7.4, 1.5 mM MgCl2, 420 mM NaCl, 1 mM EDTA, 50 μM PMSF, 0.2 mM EDTA, 25% glycerol). The nuclear pellet was incubated on ice for 30 min, followed by centrifugation at 14,000 rpm for 15 min. The protein concentrations of the cytosolic and nuclear fractions were determined using UV absorbance at 280 nm as described (24). Proteins were used immediately in a binding reaction or aliquoted and stored at −70 °C.

Electrophoretic Mobility Shift Assay (EMSA)—The DNA binding reaction was performed for 30 min at room temperature in a volume of 20 μl, containing 5 μg of nuclear or cytoplasmic protein extract, 2.5 μg of bovine serum albumin (Life Technologies, Inc.), 2 μg of poly(dI-dC) (Sigma) 5 μl of 4 × binding buffer (1 × binding buffer: 12 mM Hepes, pH 7.8, 4 mM Tris, 60 mM KCl, 1 mM EDTA, 12% v/v glycerol, 1 mM DTT, 1 mM PMSF) with or without 10–100-fold molar excess of cold competitor DNA. Radiolabeled probe (1 × 107 cpm) was added for an additional incubation period of 20 min. In supershift EMSA, cytoplasmic or nuclear extracts were incubated with experimental or isotype control antibody, at supplier’s recommended concentrations, prior to the addition of the 32P-labeled probe. Polyclonal antibodies to STAT1 and monoclonal antibodies to STAT1 through STAT6 and to p48 were obtained from Transduction Laboratories, Lexington, KY. Polyclonal antibodies to both the amino and carboxyl terminus of STAT1 were kindly provided by J. E. Darnell, Rockefeller University, New York, NY. DNA binding reactions were separated on 4% native polyacrylamide gels. Gels were subsequently dried and autoradiography performed. The autoradiographs were scanned on a La Cie flat bed scanner (La Cie Ltd., Beaver, OR) utilizing Adobe Photoshop software (Adobe Systems, Inc., Mountain View, CA). Subsequently the digitized image was labeled in Microsoft Power Point (Microsoft Corp., Redmond, WA) and printed on a high resolution laser printer. Each figure represents a computer-generated image of the autoradiograph, and each is typical of the autoradiograph in the context of relative band and background densities.

To test the requirement of tyrosine phosphorylation for DNA-protein complex formation, nuclear protein extracts were exposed to 10 units of protein-tyrosine phosphatase (PTPase) 1-B from Yersinia pestis (Upstate Biotechnology, Inc., Lake Placid, NY) in the presence or absence of the PTPase inhibitor sodium orthovanadate (1 mM). These reactions were carried out at 30 °C for 30 min in a 15-μl reaction containing 20 mM Tris·HCl, pH 7.4, 0.5 mM DTT, 0.1 mM EDTA. Treated extracts were then incubated with 32P-labeled p1RE as probe and analyzed by EMSA.

All oligonucleotides used for probes or as cold competitors were synthesized at the Emory University Microchemical Facility. Double-stranded oligonucleotides were prepared by annealing complementary strands as described (25). The p1RE oligonucleotide was synthesized to include the IFN-γ-responsive site (in bold letters) found in the ICAM-1 gene promoter (5′-CGAAGCTTTTCCGGGAAAAGATCC-3′). The underlined sequences in the p1RE oligonucleotide represent restriction sites that were used to create overhangs for labeling with 50 μCi of [α-32P]dCTP (DuPont NEN) by fill-in reaction using Klenow (Stratagene, La Jolla, CA) as described (25). Alternatively, a short primer, GCAAGCTTTCC, complementarity to the 3′ end of the above sequence was used to label p1RE by a primer-extension fill-in reaction using Klenow and 50 μCi of [α-32P]dCTP (25). Unincorporated nucleotides were removed by column chromatography over G25 Sephadex columns (Boehringer Mannheim). The [α-32P]dCTP-labeled probe was used in analysis of the phosphatase-treated or untreated proteins in EMSA. In all other EMSA, 5′-end-labeled probes were prepared with 100 μCi of γ[32P]ATP (Amersham Corp.) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and were gel-purified as described (25).

Oligonucleotides corresponding to IFNREs (written in bold letters) of the genes indicated were used as radiolabeled probes or as cold competitors. For all oligonucleotides, non-wild type restriction sites (underlined) were incorporated flanking the identified REs for cloning and subcloning purposes. Oligonucleotides used were as follows: GAS of the GBP gene: CGAAGCTTATTTGATCTTACACATTTGAGATCC (26); ISRE of the GBP gene: CGAAGCTTTCAAGAATGTACATTTGAGATCC (27); GRR of the 6–16 gene: CGAAGCTTTTCTGGGAAGATCC (28); ISRE of the ICSBP gene: CGAAAGCTTTTCCGGGAAAAGATCC (29); IFRE of the IFN-1 gene: CGAACGCTTTTCGGGAAAAGATCC (30).

In experiments comparing complexes and mobility of p1RE versus the GAS element, a short primer (GGAAGCCGTTTCAGATTAGTTTGAGATCC) complementary to the 3′ end of the GAS sequence of the GBP gene, was used to label GAS by a primer-extension fill-in reaction using Klenow and 50 μCi of [α-32P]dCTP. Unincorporated nucleotides were removed by column chromatography over G25 Sephadex columns. In addition, four oligonucleotides incorporating distinct mutations, insertions, or deletions in the wild type p1RE sequence were also used as competitors or probes. These p1RE mutants, with mutated nucleotides shown in italics, were: MUT 1: CGAAGCTTCCCGGGAAGATCC; MUT 2: CGAAGCTTTTCCCCGAGGAGATCC; MUT 3: CGAAGCTTTTCCATGCTACCTGGAAGGAGATCC; MUT 4: CGAAGCTTTTCCXGGAAGGAGATCC. Each mutant incorporates a distinct class of change; MUT 1 has a two-nucleotide mutation in the 5′ side of the of p1RE; MUT 2 has a one-nucleotide mutation in the 3′ side of p1RE, MUT 3 has a 10-bp insertion separating the 5′ and 3′ sides of the palindromic, and MUT 4 deletes the central G-C hinge in the palindromic. In addition, two different 30-bp sequences from regions of ICAM-1 located upstream from the p1RE were used as irrelevant competitor DNA fragments.

RESULTS

IFN-γ Induces Activation of a Specific p1RE-binding Factor (GRF) in a Time-dependent Manner—We examined by EMSA whether specific factors that can bind to p1RE are activated in HK by IFN-γ treatment. As seen in Fig. 1A, stimulation of cells with IFN-γ led to the induction of a distinct DNA-binding protein complex (GRF), resulting in retarded mobility of the labeled p1RE probe. However, no p1RE binding activity was observed in nuclear extracts isolated from untreated HK. Bind-
ICAM-1 Gene Induction by IFNγ via a Distinct STAT Complex

10-fold molar excess of unlabeled competitor oligonucleotide was added to display any retarded complexes upon incubation with lysates and reactions with extracts from cells treated with IFN.

1000-fold molar excess was used in a competition EMSA. As we investigated whether these mutants displayed a concentration-dependent competition for GRF at higher molar ratios.

We investigated whether tyrosine phosphorylation is necessary for formation of the pIyRE-GRF complex, a property consistent with GRF containing a STAT-like protein (13).

Anti-Stat1α Antibodies Interact with the GRF—Since IFNγ activation of Stat1α is known to involve tyrosine phosphorylation of quiescent cytoplasmic proteins, we investigated the possibility of Stat1α involvement in GRF using anti-Stat1α antibodies in supershift EMSAs. As shown in Fig. 4, addition of polyclonal anti-Stat1α antibodies directed against either the amino-terminal or the carboxyl-terminal portions of Stat1α and thus do not supershift the pIyRE-GRF complex.

Since recent reports have shown that numerous STATs and non-STAT proteins interact with Stat1 in nuclear transcription complex formation (19), we investigated whether GRF contained any other known STAT proteins by supershift EMSA. As shown in Fig. 4, both polyclonal and
monoclonal antibodies to Stat1α were able to supershift the plγRE-GRF complex. However, antibodies to Stat2, Stat3, Stat4, Stat5, and Stat6 did not interact with this complex (Fig. 5). Antibodies to p48, a component of the ISGF3 complex, also did not react with the GRF (data not shown).

**GRF Contains a Distinctly Different DNA-Protein Complex**—In order to investigate further whether the anti-Stat1α antibody supershifted GRF complex contained a form of Stat1 that could be competed by GBP-GAR or GBP-ISRE, we performed competition supershift EMSA. As seen in Fig. 6, the supershifted GRF when bound to plγRE was not competed by excess unlabeled double-stranded oligonucleotide corresponding to either the GAS or ISRE elements of the GBP gene. However, this complex was competed by excess of unlabeled plγRE and was also competed by double-stranded DNA corre-
The \( płyRE-GRF \) Complex Displays a Distinct Mobility in EMSA, and \( płyRE \) Does Not Compete with the GAS-GAF Complex—Our data from the supershift EMSA using anti-Stat1 \( \alpha \) antibodies suggests that Stat1\( \alpha \), or a Stat1\( \alpha \)-like protein, is part of the GRF that binds to the ICAM-1 \( płyRE \), but both the GAS and ISRE elements failed to compete with \( płyRE \) for complex formation. Therefore, we investigated whether \( płyRE \) could cross-compete with GAS. In addition, because Stat1\( \alpha \) had previously been shown to bind to the GAS element of several genes as a homodimer referred to as GAS (8), we also investigated whether the complexes formed with \( płyRE \) and GAS displayed any differences in EMSA. Using \( płyRE \) or GAS as probes with IFN\( \gamma \)-treated HK cell lysates, we observed striking differences in the mobility of complexes formed with \( płyRE \) and GAS when run in the same gel (Fig. 7). GRF displayed a distinctly slower mobility compared to the GAS complex. Furthermore, unlabeled \( płyRE \) at a 1000-fold molar excess failed to compete with GAS for binding to GAS complex. However, a 1 to 1 molar ratio of unlabeled ISRE displayed significant competition with labeled GAS for binding of GAS complex, but a 10-fold molar excess of unlabeled ISRE completely competed for GAS-GAF complex formation. More importantly, unlabeled GAS displayed a concentration-dependent competition for binding of GAS with a significant diminution of the labeled GAS-GAF complex when unlabeled GAS was added at only a 0.1 to 1 molar ratio. However, neither unlabeled GAS or ISRE, when added to labeled \( płyRE \) reactions, displayed any ability to compete for GRF complex formation even when added at a 1000-fold molar excess (Fig. 7). Interestingly, excess unlabeled IFNRE of the IRF-1 gene displayed a similar concentration-dependent competition for GRF complex formation to that displayed by unlabeled \( płyRE \), suggesting similar binding affinities of GRF to these two IFN\( \gamma \)-responsive elements, as was the case using the FcyR1-GRR element as competitor for GRF binding in earlier experiments (Fig. 2C). These results indicate that GRF, which complexes with \( płyRE \) of ICAM-1, IFNRE of IRF-1, and GRF of FcyR1, is distinct from the classic GAF complex that binds to GAS.

GRF Displays Similarity with the DNA-Protein Complex Formed with the IFN\( \gamma \)RE of the FcyR1 Gene—In addition to the ability of IFNRE of the IRF-1 gene to compete for GRF binding to \( płyRE \) (Fig. 6 and 7), studies in our laboratory revealed that the IFN\( \gamma \) response element pIRE of the ICSBP gene (29) also functioned well as a competitor for GRF binding (data not shown). We therefore investigated whether the complexes formed with \( płyRE \) and those formed with a representative element of those genes that contain IFN\( \gamma \)REs with sequences very similar to \( płyRE \) (see Table I and below) displayed similar or different mobilities in EMSA. Using labeled oligonucleotides of equal size (25 bp) containing either \( płyRE \) or the GRF of the FcyR1 gene (28) as probes, we compared EMSA mobilities of the DNA-protein complexes formed when these probes were

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**TABLE I**

Comparison of the sequences and GRF binding capacities of wild type and mutated \( \text{IFN} \gamma \)-REs

| Gene        | IFNRE  | Reference | Sequence                        | GRF binding capacity |
|-------------|--------|-----------|---------------------------------|----------------------|
| FcyR1       | GRR    | (28)      | TTTCCGGAAAA                     | +                    |
| ICSBP       | pIRE   | (29)      | TTTCGCAGAAAA                    | +                    |
| IRF-1       | IFNRE  | (30)      | TTTCGCGAAAA                     | +                    |
| ICAM-1      | pIRE   | (20, 22, Footnote 2) | TTTCGGCGAAAA                  | +                    |
| ICAM-1      | MUT1   | This study | TCACCGGAAAA                     | −                    |
| ICAM-1      | MUT2   | This study | TTTCCGGAGA                      | −                    |
| ICAM-1      | MUT3   | This study | TTCC (10 bases) GAAAA           | −                    |
| ICAM-1      | MUT4   | This study | TTCC (X) GAAAA                  | −                    |
| GBP         | GAS    | (26)      | AGTTTTTATCTTCTCTAAATC           | −                    |
| GBP         | ISRE   | (9)       | CGGAGCTTCTCCATATTT             | −                    |
| 6–16        | ISRE   | (27)      | CCCAAAATGAAAAA                  | −                    |
| Ly6A/E      | GAS    | (41)      | TTTCCLGAAAA                     | −                    |
incubated with IFN-γ-treated HK cell lysates. As seen in Fig. 8, the complexes that formed with pI-RE and GRR displayed similar mobility. GRR also competed for GRF binding to pI-RE, as seen in Fig. 2C, and unlabeled pI-RE displayed a similar competition for GRF binding to GRR (data not shown). In addition, both complexes were supershifted in a similar manner with monoclonal antibodies to Stat1α.

Taken together, the data presented in these experiments indicate that pI-RE of the ICAM-1 gene, GRR of the FcγR1 gene, IFNRE of the IRF-1 gene, and pIRE of the ICSBP gene form a subset of IFN-γ response elements that bind to a trans-activating complex, γ response factor, that contains a Stat1α-like protein. This GRF complex clearly appears to be distinct from the homodimeric Stat1α complex that forms GAF and binds to the GAS element of other IFN-γ-responsive genes, such as that characterized in the guanylate-binding protein gene. Comparison of the identified critical sequences of these GRF-binding and non-GRF-binding elements and their distinct differences, as well as the mutations used in the above studies, are shown in Table I. These data and comparisons indicate that a potential consensus sequence, TTTCNGNGAAA, is required for binding of GRF. They also indicate that elements such as GAS and ISRE, which do not contain the characteristic 11-bp palindrome sequence displayed by pI-RE, bind to Stat1α containing complexes but not to the complex typified by the γ response factor, which binds to pI-RE and similar elements identified in a subset of IFN-γ-responsive genes.

**DISCUSSION**

We have characterized a specific DNA-binding complex, which we have termed GRF, that binds to the minimal IFN-γ-responsive element of the ICAM-1 gene, pI-RE. Formation and binding of GRF is dependent on IFN-γ-induced activation of pre-existing proteins, as demonstrated by the rapid activation and binding of GRF and by the activation of this complex in the presence of cycloheximide. In addition, we have demonstrated that the pI-RE sequence is necessary for binding GRF and that mutant pI-RE sequences neither bind GRF nor compete with pI-RE for the pI-RE-GRF complex even when used at high molar ratios. Moreover, pI-RE displays a high and specific binding affinity for GRF.

IFN-γ signaling involves activation of Stat1 by phosphorylation of a tyrosine residue in order to assemble active transcription-stimulating complexes (33, 34). Treatment with PTPase 1-B, which specifically dephosphorylates tyrosine residues, has been shown to abrogate binding of these transcription complexes as shown by EMSA (34, 35). Our data indicate that IFN-γ-induced activation of GRF is tyrosine phosphorylation-dependent as well.

It has been shown that Stat1α is activated by a number of

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3 L.-J. Li, and S. W. Caughman, unpublished results.
other agonists, in addition to IFNγ and IFNa, based largely on reactivity of binding complexes with anti-Stat1α antibodies (19, 36–38). Our supershift data using antibodies to the known STAT proteins suggest that Stat1α, or a protein with antigenic similarities to Stat1α, is a component of GRF. In addition, studies using polyclonal antibodies to either the amino-terminal or the carboxyl-terminal portions of Stat1α appear to exclude Stat1β as a possible component of the GRF complex. These data are in agreement with the recently reported GAF-like factor binding to the IFNγRE of ICAM-1 gene (39) and the recent finding of Stat1α involvement in the complex by immuno blotting using anti-Stat1 antibodies (40). Previous reports indicate that Stat1α is a common component of the IFN-activated trans-acting complexes GAF and ISGF3. Stat1α binds to GAS as the homodimer GAF, and the multimeric ISGF3 complex, which binds to ISRE, contains Stat1α in addition to Stat2 and p48 (19). However, in the present studies, neither a double-stranded oligonucleotide corresponding to GBP-GAS nor GBP-ISRE used as unlabeled competitors competed with p1yRE for the binding of GRF. Further, we have observed that double-stranded oligonucleotides corresponding to the IFNγREs of the ISRE from the 6–16 gene (27) and the GAS sequence (GGCGATTCGTCCTCTGATTAAGCTG) from the Ly6A/E gene (41) also fail to compete with p1yRE for GRF complex formation.4

Our studies demonstrate a lack of cross-competition between GAS and p1yRE for formation of their respective DNA-protein complexes. However, ISRE from the GBP gene, which binds Stat1α as part of the heterotramer in the ISGF3 complex and does not compete with p1yRE for complex formation, clearly competes with GAS for the GAS-GAF complex. We have observed that both GAS and ISRE do not compete with p1yRE even at a 1000-fold excess in our experiments using HK cell lysates. Our results are in agreement with the report that shows GBP-GAS does not compete with the ICAM-1 IFNγRE in airway epithelial cells (20) and are in contrast to those obtained by others in MeL JuSo cells (42).

Consistent with our results, GRF that is supershifted by anti-Stat1α antibodies is not competed away by excess unlabeled GAS or ISRE from the GBP gene. On the other hand, excess unlabeled IFNRE from the IRF-1 gene displayed competition for the supershifted GRF. In fact, semi-palindromic IFNγREs from other IFNγ-responsive genes, such as the IFNRE of the IRF-1, pIRE of the ICSBP gene and GRR of FcyR1 gene, show somewhat closer sequence homology with that of p1yRE than do the sequences of various GAS and ISRE elements as shown in Table I. It is precisely these elements that share greater sequence homology with p1yRE that can function as competitors in EMSAs with p1yRE for complex formation in IFNγ-induced HK cell lysates. Interestingly, these REs show differences in their sequences in nucleotides immediately flanking the G-C hinge of the p1yRE. The mutations that rendered p1yRE completely nonfunctional in vitro and in vivo in our study are either in the 5’ or 3’ end of the palindrome or the G-C hinge of the palindrome, and suggest binding specificity of GRF for these specific sequences. Comparison of the sequences of IFNγREs of the IRF-1, ICSBP, and FcyR1 genes with the p1yRE sequence of ICAM-1 and mutations of p1yRE sequence reveals a potential consensus sequence, TTTCNGNGAAA, that is required for binding of GRF. Among the GAS and ISRE sequences of genes such as GBP, 6–16, and Ly6A/E, the GAS sequence of Ly6A/E displays closest homology to this GRF-binding consensus sequence. However, the sequence of Ly6A/E diverges from the GRF-binding consensus sequence at two sites, the central G-C hinge and in the 3’ half of the palindrome. We have been unable to demonstrate any competition for GRF binding with the Ly6A/E GAS. Whether this inability to bind GRF results from divergence of the nucleotide in the hinge or the 3’ half of the palindrome has not yet been determined.

Finally, the significantly slower mobility of the p1yRE-GRF complex compared to that of the GAS-GAF complex, using identical IFNγ-treated HK cell lysates with GAS and p1yRE as separate probes in the same EMSA, cannot be accounted for by differences in the sizes of the radiolabeled oligonucleotides. In fact, the smaller p1yRE probe (25 bp) formed a more slowly migrating DNA-protein complex when compared to the GAS-GAF complex bound by the larger (36 bp) GAS probe. In contrast, the IFNγ-activated DNA-protein complexes formed with either the FcyR1-GRR or the ICAM-1-p1yRE clearly display similar mobility on EMSA, and both complexes supershift in a similar fashion with anti-Stat1α antibodies. The IFNRE of the IRF-1 gene and the pIRE of the ICSBP gene form a DNA-protein complex with IFNγ-treated HK cell lysates, which also displays a mobility similar to that of the p1yRE-GRF complex in EMSA (data not shown).

Other investigators have recently shown that FcyR1-GRR does not compete with GBP-GAS in competition EMSA (43). Further, FcyR1-GRR was also shown to bind a Stat1α-like protein that interacted with an additional 43-kDa protein in response to IFNγ-stimulation (35, 44). The semi-palindromic IFNγRE of the MIG gene, which displays significant sequence homology to ICAM-1-p1yRE, has been reported to bind an IFNγ-activated trans-activating factor (γRF-1) that is composed of at least two proteins of 95 and 130 kDa (45). Furthermore, γRF-1 was shown to exhibit differences in electrophoretic mobility distinct from GAF and to contain one or more subunits antigenically related to Stat1α (45).

These data thus indicate that p1yRE represents a distinct subset of IFNγREs found in a number of early response genes that mediate trans-activation in response to IFNγ signaling through a DNA-binding protein complex (GRF) that is distinctly different from the previously characterized IFNγ-activated complex, GAF. While GRF certainly appears to contain a Stat1α-like protein, identification and characterization of all of the components of this distinct trans-activating complex, their relationship to other STAT and non-STAT proteins, and the specific biochemical pathways involved in their activation will further elucidate the molecular mechanisms by which IFNγ initiates differential responses at sites of localized inflammation.

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ICAM-1 Gene Induction by IFNγ via a Distinct STAT Complex