Phosphorylation Events Modulate the Ability of Interferon Consensus Sequence Binding Protein to Interact with Interferon Regulatory Factors and to Bind DNA*

(Received for publication, September 17, 1996, and in revised form, January 28, 1997)

Rakefet Sharf††, David Meraro‡‡, Aviva Azriel*, Angela M. Thornton*,
Keiko Ozato*, Emanuel F. Petricoin†, Andrew C. Larner†, Fred Schaper**,
Hansjoerg Hauser**, and Ben-Zion Levi***

From the †Department of Food Engineering and Biotechnology, Technion, Haifa 32000, Israel, the **Laboratory of Molecular Growth Regulation, NICHD, National Institutes of Health, Bethesda, Maryland 20892, the ‡Division of Cytokine Biology, Center for Biologies Evaluation, Food and Drug Administration, Bethesda, Maryland 20892-4555, and the ***Genetics of Eukaryotes, GBF-Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Federal Republic of Germany

Two families of transcription factors mediate interferon (IFN) signaling. The first family, signal transducers and activators of transcription (STATs), is activated within minutes of IFN treatment. Specific phosphorylation events lead to their translocation to the nucleus, formation of transcriptional complexes, and the induction of the second family of transcription factors termed interferon regulatory factors (IRFs). Interferon consensus sequence binding protein (ICSBP) is a member of IRF family that is expressed only in cells of the immune system and acts as a transcriptional repressor. ICSBP binds DNA through the association with other transcription factors such as IRF-1 or IRF-2. In this communication, the domain that is involved in protein-protein interactions was mapped to the carboxyl terminus of ICSBP. This domain is also important for mediating ICSBP-repressing activity. In vitro studies demonstrated that direct binding of ICSBP to DNA is prevented by tyrosine (Tyr) phosphorylation. Yet, Tyr-phosphorylated ICSBP can bind target DNA only through the association with IRF-2 and IRF-1. This type of phosphorylation is essential for the formation of heterocomplexes. Tyr-phosphorylated ICSBP and IRF-2 are detected in expressing cells constitutively, and Tyr-phosphorylated IRF-1 is induced by IFN-γ. These results strongly suggest that like the STATs, the IRFs are also modulated by Tyr phosphorylation that affects their biological activities.

The activities of interferons (IFNs)1 is mediated mainly via successive phosphorylation events of IFN receptors through

§ The first two authors made an equal contribution to this work.
** The first two authors made an equal contribution to this work.
†† The first two authors made an equal contribution to this work.
‡‡ The first two authors made an equal contribution to this work.

* This research was supported in part by a grant from the German-Israeli Foundation for Scientific Research and Development (to B. Z. L. and H. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** The first two authors made an equal contribution to this work.
‡‡ The first two authors made an equal contribution to this work.
†† The first two authors made an equal contribution to this work.

1 The abbreviations used are: IFN, interferon; ICSB, interferon consensus sequence; ICSBP, interferon consensus sequence binding protein; ISRE, interferon-stimulated response element; IRF, interferon regulatory factor; ISGF, interferon-stimulated gene factor; PRD, positive regulatory domain; aa, amino acid; RRL, rabbit reticulocyte lysate; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; DBD, DNA binding Domain; EMSA, electrophoretic mobility shift assay; PRDI, positive regulatory domain I; PAGE, polyacrylamide gel electrophoresis.

2 A. Grossman, L. Lantonio, and T. W. Mak, EMBL accession number U51127.
apoptosis of mitogen-activated T lymphocytes (14, 19–26). On the other hand, IRF-2 binds to similar DNA motifs such as IRF-1 and yet acts as a repressor of IFN-stimulated genes and can induce oncogenic transformation (15, 23, 24, 27). Recently, it was demonstrated that IRFs can associate either with other family members (28, 29) or with other transcription factors (11, 30).

We have been working on the characterization of the IFN consensus sequence binding protein (ICSBP) that is expressed exclusively in cells of the immune system such as monocytes, B-cells, and T-cells (12, 13, 31). The expression of this protein is enhanced following exposure of the cells to IFN-γ and to a lesser degree following exposure to IFN-β. ICSBP functions as a repressor on promoters containing either ICS motif or positive regulatory domain 1 (PRD1), a DNA motif located on the promoter of IFN-β (13, 32). The repression mediated by this factor can be alleviated by exposing the cells to IFNs (13, 31, 33). ICSBP has a modular structure that includes the DBD and the repression domain (28, 29). ICSBP can associate with IRF-1, IRF-2 (28, 29), and PU.1 that belongs to the Ets family of transcription factors (11).

In this communication, we characterize the association of ICSBP with IRF-1 and IRF-2. The association domain was mapped to the carboxyl terminus and shows homology to other IRFs. This homology suggests a general role for this domain in mediating protein-protein interaction in this family of transcription factors. Deletion in this domain also affects the repression activity of ICSBP. Phosphorylation on Tyr residues (Tyr(P)) is essential for this association, yet Tyr phosphorylation also modulates the ability of each individual factor to bind target DNA sequences. Thus, in analogy to the STATs, phosphorylation events can modulate interaction of IRFs with other factors and alter DNA binding activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells were obtained from ATCC (Rockville, MD) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Human monocytic U937 cells that are overexpressing ICSBP and U937 cells that were transfected with an empty vector were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 400 μg/ml G418 (34).

**Plasmids**—For transient co-transfection assays, the reporter gene chloramphenicol acetyltransferase (CAT) driven by the basal human β-globin promoter, to which four repeats of PRDI motif were connected, was used (13). To generate expression vectors corresponding to the coding region of ICSBP and the DBD of ICSBP, the full-length human ICSBP and the segment corresponding to the first 121 aa were both cloned into the mamalian expression vector, pcDNA1/Neo (Invitrogen) under the control of the cytomegalovirus promoter (pCMVICSBP and pCMVICSBPDBD, respectively). The expression vectors containing carboxyl-terminal truncations of ICSBP, pCMVICSBP377, and pCMVICSBP363 were generated by polymerase chain reaction (PCR) and cloned into pcDNA1/Neo (see below for details).

For in vitro translation the plasmids containing ICSBP, IRF-1, and IRF-2 under the bacteriophage T7 promoter were as described previously (29).

**In Vitro Translation and Transfection**—The assays were performed as described previously (29). Plasmids containing ICSBP, IRF-1, and IRF-2 under the T7 promoter were linearized downstream to the coding region with the appropriate restriction enzyme. 5 μg of linearized plasmids were transcribed in vitro by T7 RNA polymerase using a commercial kit (Stratagene). Proteins were translated in vitro using rabbit reticulocyte lysate (RRL) system (Promega) according to the manufacturer’s instructions. To block proteins phosphorylated during the translation reaction, genistein (25 μg/ml) and/or staurosporine (75 nM) were added. To dephosphorylate Tyr residues, the translated protein was incubated with 2 units of Yop phosphatase at 30 °C for 30–60 min (New England Biolabs). To monitor translation efficiency, small scale reactions containing [35S]methionine were performed each time, and the labeled protein was separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to autoradiography.

In *In Vitro Generation of ICSBP Truncation and Deletion Mutants*—PCR was employed to generate *in vitro* carboxyl-terminal or amino-terminal truncations of ICSBP as well as internal deletions. For carboxyl-terminal deletions, the 5’ primer contained an engineered restriction site and 17 base pairs corresponding to the T7 RNA polymerase recognition site. Three 5’ primers, 20–31 base pairs long, contained DNA sequences corresponding to various positions on ICSBP as indicated in the text (see Fig. 2A, scheme) and engineered restriction site. A 33-aa amino-terminal deletion was generated using a 5’ primer containing the T7 RNA polymerase recognition site followed by ATG and 20-base pair sequence from amino acid residue 33. To generate internal deletions, segments of ICSBP were amplified separately. Segments corresponding to the first 180 aa (or less) were amplified as above. The other segments corresponding to the carboxyl end of ICSBP were amplified using various 5’ primers corresponding to the desired internal deletions (see Fig. 2B, scheme) and a 3’ primer corresponding to the end of the coding region. Following PCR reactions, the correct size fragments were purified from agarose gels, and 50 ng of each fragment was digested with engineered restriction enzymes, mixed, and subjected to ligation. The primers were designed such that an in frame fusion will occur. Following ligation, 1 μl of the reaction was PCR-amplified with primers corresponding to the beginning and the end of the coding region of ICSBP as above. The correct size DNA fragments were purified from agarose gels, and 1 μg of each fragment was transcribed in *in vitro* and translated in *in vitro* as above. For each set of experiments a autoradiogram of [35S]methionine, the translated proteins were separated on a 10% SDS-PAGE and subjected to autoradiography to ensure that the correct sizes and the expected amount of proteins were achieved. Each set of experiments was repeated at least three times with at least two independent batches of PCR fragments. In all of these reactions we have used Pu (Stratagene) as the source of high fidelity polymerase.

**Electrophoretic Mobility Shift Assay (EMSA)**—*In vitro* gel shift reactions were carried out as described previously (25). A typical reaction contained 1–5 μl of *in vitro* translated proteins that were incubated in binding buffer (10 mM Hepes, pH 8.0, 5 mM MgCl₂, 50 mM KCl, 1 mM DTT, 0.025% bromphenol blue, 0.005% xylene cyanole, 1% Ficoll, 3% glycerol, 1 μg of sonicated poly(dIdC)/I), and 1 μg of sheared salmon sperm DNA) with at least 50,000 cpm of labeled trimer of PRDI motif (AAGTGA), for 10 min on ice. In supershift reactions, 1–2 μl of anti-serum was incubated for 1 h with extracts, and the labeled probe in the binding buffer was added in the last 10 min. The samples were loaded on a pre-run 7% polyacrylamide gel. The dried gels were exposed to x-ray films.

**DNA Transfection and CAT Assays**—HeLa cells were transfected by the DNA coprecipitation assay using the modified calcium phosphate-mediated transfection protocol (35) with 2–4 μg of plasmid(s) DNA, and pUC19 was added as carrier DNA up to 20 μg. CAT assays were performed and normalized for protein concentration and transfection efficiencies as described previously (29). All experiments were repeated at least three times.

**Preparation of Partially Purified bICSBP—ICSBP** was cloned under the T7 promoter in the bacterial expression vector pET-3d, and large scale expression of the recombinant protein was performed according to manufacturer protocols (Novagen). The cells were harvested, and the pellet was resuspended in TE buffer in the presence of protease inhibitors and subjected to intensive sonication at 4 °C. Following centrifugation (10,000 × g for 10 min), the pellet was taken in 7 M guanidine HCl and sonicated again as above. The supernatant was diluted 8 times in EMSA binding buffer in the presence of protease inhibitors mixture. The preparation was dialyzed for 16 h at 4 °C against the same buffer, and the insoluble proteins were removed by centrifugation. This crude preparation was loaded over Sepharose-coupled ICS oligomer column as described (36), and the enriched preparation (~ 75%) was used for the binding experiments.

**In Vitro Modification of bICSBP**—1 μg of bICSBP was incubated in 10 μl of RRL at different temperatures to test the effect of modification on DNA binding activity. During the various incubation periods, phosphorylation inhibitors such as genistein or staurosporine were added as above. The effect of Yop phosphatase (2 units at 30 °C) or alkaline phosphatase (1 unit at 37 °C, Boehringer Mannheim) was tested upon addition of the enzymes to 100 μg (in 50 mM Tris) of bICSBP that were first incubated at 37 °C at various time intervals in RRL.

**Immunoprecipitation and Western Blot Analyses**—U937 cells or U937 cells that are overexpressing ICSBP (34) were either not treated or treated with IFN-γ (100 units/ml) for 5 h. The cells (10²/sample) were harvested, and the pellet was washed once with phosphate-buffered saline and suspended in 200 μl of lysis buffer (150 mM NaCl, 25 mM...
mixed, the band corresponding to IRF-2-ICSBP complex is barely detected (Fig. 1A lane 6). Thus, inhibition of phosphorylation results in a sharp decrease in heterocomplex formation that is more profound than the observed decrease in the intensity of the IRF-2 band. This implies that heterocomplex formation is sensitive to the phosphorylation state of at least one of the interacting factors.

To determine whether the phosphorylation state of both factors is equally important, we have mixed in vitro translated IRF-2 with ICSBP that was translated in the presence of genistein and staurosporine. It is clear that heterocomplex formation under these conditions is comparable with the one achieved in the absence of inhibitors (compare lane 3 to lane 7 in Fig. 1A). Conversely, we have mixed IRF-2 translated in the presence of the inhibitors with ICSBP. In this case, a significant decrease in heterocomplex formation is observed (compare lane 8 to either lane 7 or 3 in Fig. 1A) similar to the one detected when both factors were blocked for phosphorylation (compare lane 8 to lane 6 in Fig. 1A). This result suggests that only phosphorylated IRF-2 can effectively interact with ICSBP.

To test if phosphorylation events on Tyr residues are important for such association, we have incorporated a specific recombinant tyrosine phosphatase, Yersinia phosphatase (Yop), in the assay. It is clear from Fig. 1B that the ability of IRF-2 to bind DNA is only slightly decreased in the presence of the phosphatase (Fig. 1B lanes 2 and 5, respectively), whereas the ability to associate with ICSBP is severely impaired (Fig. 1B lanes 3–6, respectively). Dephosphorylation of IRF-2 on Tyr(P) is the reason for the decreased association of the two factors since dephosphorylation of ICSBP had no effect on heterocomplex formation, whereas dephosphorylation of IRF-2 resulted in marked decrease (Fig. 1B lanes 7 and 8, respectively). Similar results were obtained with IRF-1 indicating that the two factors interact with ICSBP in a similar manner (data not shown).

**The Carboxyl-terminal Domain of ICSBP Is Required for the Association with IRF-2**—To map the domain responsible for the ability of ICSBP to associate with IRFs, a series of deletions were made at the carboxyl terminus as well as one deletion at the amino terminus (for details see “Experimental Procedures”). Fig. 2A demonstrates that deletions from position 377 to the carboxyl-terminal end of ICSBP had no effect on its ability to associate with IRF-2 (Fig. 2A lanes 1–9). However, the additional deletion of 14 or more aa from residue 377 toward the amino terminus prevented heterocomplex formation (Fig. 2A, lanes 10–13). This suggests that the carboxyl end of the association domain resides around position 377.

When the first 33 aa of ICSBP, which reside in the DBD, (ICSBPdel33) were deleted, no heterocomplex was observed (Fig. 2A, lane 15). Since ICSBPdel33 does not form heterocomplex with IRF-2, it is likely that the formation of heterocomplexes that bind to the PRDI is dependent upon intact DBDs of both interacting partners. Therefore, to map the amino-terminal end of the association domain, internal deletions were made in ICSBP (Fig. 2B). Deletion of 20 aa from positions 180–200 did not affect the ability of ICSBP to generate a heterocomplex with IRF-2 (Fig. 2B, lanes 1–5). Larger internal deletions of ICSBP (38 and 57 aa) prevented the interactions with IRF-2 (Fig. 2B, lanes 6–10), suggesting an amino-terminal border around aa 200. A deletion from position 200 to position 126 did not affect the ability of ICSBP to interact with IRF-2 (data not shown) indicating that it is not necessary for the formation of heterocomplexes. Similar results were obtained when the various deletion factors were reacted with IRF-1 indicating that the same region of ICSBP is responsible for the association with multiple IRFs (data not shown). The associa-

### RESULTS

**Phosphorylation Events Affect the Ability of ICSBP to Associate with IRF-2**—Previously, we demonstrated that ICSBP can associate with IRF-1 or IRF-2 both in vivo and in vitro (28, 29, 37). Fig. 1A demonstrates the ability of ICSBP to associate with IRF-2 in vitro using RRL. In vitro translated IRF-2 can bind a labeled trimer of PRDI as detected by EMSA, whereas no shifted band is detected for in vitro translated ICSBP (Fig. 1A, lanes 1 and 2, respectively). When both translated factors are mixed, a new band corresponding to the heterocomplex is obvious (Fig. 1A lane 3). To test the effect of phosphorylation on the association between ICSBP and IRF-2, phosphorylation inhibitors (genistein and staurosporine) were included during the translation step. In the presence of the inhibitors some decrease in the DNA binding activity of IRF-2 is observed while the DNA binding activity of ICSBP is still undetectable (Fig. 1A, lanes 4 and 5). However, when the two factors that were translated in the presence of phosphorylation inhibitors are
Effect of Phosphorylation on the Activity of ICSBP

**FIG. 1.** The Interaction of ICSBP with DNA Is Dependent Upon Its Phosphorylation State—ICSBP shares high sequence similarity with IRFs at the DBD. The ability of other IRF family members to bind ISRE/PRDI motifs was demonstrated mainly by EMSA. As shown in Fig. 1, in vitro translated ICSBP does not interact with DNA. In addition, direct binding of ICSBP to DNA was not detected in nuclear extracts prepared from different cell lines (28, 29). The specific interactions of ICSBP with ISRE/PRDI motifs were more readily demonstrated only by Southwestern analyses in which ICSBP was expressed in *Escherichia coli* (12, 13). This suggested that the inability of *in vitro* translated ICSBP or mammalian expressed ICSBP to bind DNA is due to post-translational modifications that do not take place in bacteria. To test this assumption, ICSBP was expressed in *E. coli*, and the ability of the recombinant protein to bind DNA was tested by EMSA. 1–2 μg of partially purified bacterially expressed ICSBP (bICSBP, for details see “Experimental Procedures”) was reacted with a 32P-labeled trimer of PRDI and analyzed by EMSA. Fig. 4A shows that a discrete band is detected with bICSBP that is not competed with 50-fold excess of mutant oligomer corresponding to the ICS of the major histocompatibility complex class I but is competed with the native ICS oligomer (Fig. 4A, lanes 1, 2, and 3, respectively). The same band is still observed when bICSBP was incubated in the presence of preimmune serum; however, this band is supershifted when antisera directed against ICSBP was included in the EMSA (Fig. 4A, lanes 4 and 5, respectively). Extract prepared from *E. coli* cells harboring the empty expression vector did not demonstrate any bands by EMSA (data not shown). These results indicate that bICSBP interacts with the DNA in a specific manner.

The observed differences in the ability of bICSBP to bind DNA in comparison to *in vitro* translated ICSBP implies that
post-translational modification can affect its interaction with the DNA. To further address this question, we incubated bICSBP in RRL at 37 °C for 30 or 60 min, and its ability to bind to the PRDI motif was tested by EMSA. After 30 min of incubation the intensity of binding was markedly decreased, and after 60 min of incubation the binding was essentially undetectable when compared with the same sample that was incubated at 4 °C (Fig. 4B, lanes 2–4). To half of each sample 1 unit of alkaline phosphatase (AP) was added at 37 °C for an additional 30 min (lanes 5–7). The sample were analyzed by EMSA as in Fig. 1A, C, bICSBP was incubated in RRL for 30 min at 37 °C as in B either in the absence (lane 1) or in the presence of either genistein (lane 3, GEN, 25 μg/ml) or staurosporine (lane 4, STAU, 75 nM) or both (lane 5) (see “Experimental Procedures” for details). Treatment with alkaline phosphatase (lane 2, AP) was as in B. All samples were reacted in EMSA as in Fig. 1A.

**Fig. 4.** Modulation of bICSBP binding in RRL as determined by EMSA. A, the ability of bICSBP to bind 32P-labeled trimer of PRDI was analyzed by EMSA as under Fig. 1A. The binding was competed with 50 μ excess of either ICS or mutant ICS (Mu ICS) oligomers. The specificity was determined by supershifted analysis with polyclonal antibodies against ICSBP (α-ICSBP) in comparison to preimmune serum (Preimmune). B, bICSBP was incubated in the absence (lane 1) or the presence of RRL at either 4 °C (0 min) or 37 °C at indicated times (lanes 2–4). To half of each sample 1 unit of ALP was added at 37 °C for an additional 30 min (lanes 5–7). The sample were analyzed by EMSA as in Fig. 1A. C, bICSBP was incubated in RRL for 30 min at 37 °C as in B either in the absence (lane 1) or in the presence of either genistein (lane 3, GEN, 25 μg/ml) or staurosporine (lane 4, STAU, 75 nM) or both (lane 5) (see “Experimental Procedures” for details). Treatment with alkaline phosphatase (lane 2, AP) was as in B. All samples were reacted in EMSA as in Fig. 1A.

Effect of Phosphorylation on the Activity of ICSBP

phosphatase, robust DNA binding was observed in all three samples (Fig. 4B, lanes 5–7). This binding of bICSBP was even more intense than the DNA binding observed for the control (Fig. 4B, compare lanes 5–7 with lane 2). These results imply that phosphorylation of bICSBP occurs during incubation in RRL. These phosphorylations are probably preventing bICSBP from binding to the DNA. Treatment with alkaline phosphatase either removes all or some of the phosphate groups thus enabling the dephosphorylated factor to bind DNA even better than untreated bICSBP implying that some phosphorylation events may occur in bacterial cells.

To further characterize these phosphorylation events that are taking place in RRL, phosphorylation inhibitors were added as follows: genistein, which primarily blocks tyrosine (Tyr) phosphorylation at a concentration of 25 μg/ml, and staurosporine, which primarily blocks serine/threonine (Ser/Thr)
Effect of Phosphorylation on the Activity of ICSBP

Phosphorylation at concentration of 75 nM. Fig. 4C shows the effect of these inhibitors on the DNA binding capability of bICSBP. The DNA binding of bICSBP following 30 min of incubation at 37 °C in RRL without the inhibitors is shown in Fig. 4C, lane 1. Under this condition bICSBP lost at least 50% of its DNA binding activity as shown in Fig. 4B, lanes 3 and 2, respectively. It is clear from Fig. 4C that when bICSBP is incubated in RRL in the presence of genistein, which blocks tyrosine phosphorylation, its DNA binding activity is enhanced as in the case of treatment with alkaline phosphatase (Fig. 4C compare lanes 2 and 3 with lane 1). In the presence of staurosporine, which blocks mainly serine/threonine phosphorylation, the DNA binding pattern of bICSBP resembled that of the untreated control sample that was incubated just with RRL (Fig. 4C, lanes 4 and 1, respectively). When both inhibitors were included, DNA binding was even weaker than that of the untreated control sample (Fig. 4C, compare lanes 1 and 5). The results suggest that RRL can support both tyrosine and serine/threonine phosphorylation and that bICSBP phosphorylation on Tyr residues inhibits DNA binding.

Phosphorylation on Tyrosine Residues Prevents ICSBP from Binding to DNA—We next wanted to demonstrate that phosphorylation on Tyr residues accounts for the inability of ICSBP to bind DNA. For that purpose, bICSBP was incubated in RRL as described above, and the phosphorylated protein was then reacted with recombinant *Yersinia* phosphatase that acts only on phosphorylated tyrosine residues (for details see “Experimental Procedures”) (39). Fig. 5 clearly shows that DNA binding activity of bICSBP is almost abolished following incubation in RRL (lanes 1 and 2, respectively). However, bICSBP did not lose its binding activity when incubated further (30 °C for 60 min) in the presence of Yop phosphatase (lane 3). Similar results were also obtained with in vitro translated ICSBP (data not shown); however, the amounts of translated product needed were much larger than those used to demonstrate heterocomplex with IRF-2 as in Fig. 1. Thus, these results confirmed that in RRL bICSBP is phosphorylated on tyrosine residues, and this modification blocks its ability to bind DNA.

**ICSBP, IRF-1, and IRF-2 Are Tyrosine-phosphorylated in Vivo**—The data presented above suggest that phosphorylation of ICSBP might have a role in modulating its DNA binding ability. Thus, we tested whether the factor is phosphorylated in *vivo*. For that purpose we have used the promonocytic cell line, U937, in which ICSBP was stably transfected and expressed at high levels (34, 37). Control cells or cells overexpressing the factor were treated for 5 h with IFN-γ, and cell extracts were immunoprecipitated with α-Tyr(P) antibodies. The precipitated protein were separated by 10% SDS-PAGE. Western blot analysis was performed with the various antibodies as indicated (see details under “Experimental Procedures”).

To determine if other members of the IRF family of proteins are also subjected to phosphorylation on Tyr residues, the same blot was reacted with antibodies directed against IRF-1 and IRF-2. It is apparent from Fig. 6 that in response to IFN treatment, a specific phosphorylated IRF-1 band was induced in both cell lines tested. This induction correlates with the reported induction of IRF-1 following treatment with IFN-γ (31, 40). When the same blot was also reacted with antibodies directed against IRF-2, a band with the expected molecular mass appeared and its intensity was not affected by IFN treatment in both cell lines. As a control to our combined immunoprecipitation-Western blot analysis, the membrane was also reacted with anti-STAT 1α (p91) antibodies (41). It is evident that indeed p91 phosphorylation was induced in both cell lines following treatment with the cytokine as previously reported (6, 42). Our results suggest that members of the IRF family of proteins are phosphorylated on Tyr residues. ICSBP and IRF-2 are constitutively phosphorylated, although some increase in the phosphorylation state of ICSBP was observed following treatment with IFN-γ. The induction of IRF-1 expression following exposure to the cytokine was accompanied by phosphorylation on Tyr residues.

**DISCUSSION**

IRFs are a family of transcription factors that, like the STATs, mediate IFN signaling. Unlike the STATs that are activated within minutes following the binding of the ligand to the receptor, IRFs represent a secondary wave of response to the IFN signal. While STATs are activated through specific phosphorylation events (1–3), the role of phosphorylation in the modulation of the biological activity of IRFs has not been elucidated yet. Previously, we have demonstrated that ICSBP can interact with other members of the IRF family such as IRF-1 and IRF-2. Here we demonstrate that this interaction is de-
dependent upon the phosphorylation state of the factor interacting with ICSBP. A 177-aa long domain on ICSBP is essential for this association and a deletion in that region affects the repressor activity of ICSBP. It is also demonstrated that the ability of ICSBP to bind to DNA is blocked through tyrosine phosphorylation. Finally we show that these members of the IRF family are phosphorylated in vivo on Tyr residues.

The association domain of ICSBP with IRF-1 and IRF-2 is located near the carboxyl terminus between residues 200 and 377. Interestingly, this 177-aa segment shares significant homology with ISGF3γ as first noted by Veals et al. (16). Moreover, this homology spans over a region that is necessary for the interaction with ISGF3α subunit (i.e. STAT 1 and STAT 2). This lead to our initial search for interacting partners with ICSBP. As shown in Fig. 7, this homology is also found in ICSAT/Pip, IRF-3, and IRF-5 suggesting that this conserved motif is probably essential for the association of IRFs with factors. Based on the functionality of this domain in both ISGF3γ and ICSBP, we propose to define it as IRFs Association Domain (IAD). This is in agreement with the finding that ICSAT/Pip can associate with PU.1. This results in a transcriptional activation complex on the enhancer of the immunoglobulin light chain. Similarly, ICSBP can associate with this factor, but the nature of this complex has not been reported (11). Since IRF-3 also shares similarity at this domain, it was postulated that it might exert its activity via the formation of complexes that are similar to those of ISGF3 (43).

Our transfection studies also demonstrate that truncation of the association domain of ICSBP results in a reduced repression activity. Using domain swap analysis, we demonstrated that ICSBP has a modular structure that is comprised of two modules, a DBD and a repression domain (29). Here we show that a third functional module, the association module, overlaps at least in part with that of the repression domain. It is not clear if heterodimerization is a prerequisite for the repression activity of ICSBP. However, testing the IAD of either ICSBP or IRF-2 in a dominant negative assay will enable us to answer this question.

We show that in vitro phosphorylation of ICSBP on Tyr residues prevents its ability to bind DNA. All IRF members share significant homology at the DBD and can readily bind DNA with the exception of ICSBP and the murine homologue of ICSAT, Pip (11, 29). This implies that the non-binding factors (ICSBP and Pip) may have unique Tyr residues that are not conserved in the other IRF members. ICSBP has 4 Tyr residues in the first amino-terminal 115 aa at positions 23, 48, 107, and 110. The Tyr at position 110 is conserved among all known IRFs, whereas Tyr at position 107 is conserved only in ICSAT, ISGF3γ, and IRF-5. Since ISGF3γ can bind DNA it is not probable that this residue affects binding. However, Tyr at position 23 is shared only with ICSBP and ICSAT, whereas Tyr at position 48 is shared with only ICSAT. Since no information is available with respect to the DNA binding ability of IRF-5, it is most probable that either both residues or just one of them can prevent the DNA binding of at least ICSBP and ICSAT upon phosphorylation. This does not exclude the possibility that phosphorylation of Tyr residues outside the DBD might also be involved in blocking ICSBP from binding to DNA. In an attempt to identify these Tyr residues, site-directed mutagenesis and domain swap analysis with IRF-2 are being performed.

The data presented in this communication show that IRF-1, IRF-2, and ICSBP can be phosphorylated in vivo. In U937 cells that are overexpressing ICSBP, the protein is constitutively phosphorylated on Tyr residues. Phosphorylated ICSBP was not detected in parental U937 cells although the protein can be detected by Western blot analysis (data not shown) (18, 34). It is probable that our detection technique that combines immunoprecipitation with Western blot analysis is not sensitive enough. IRF-1 expression is induced following treatment with IFN-γ (data not shown) (13, 41). It is demonstrated here that this induction of IRF-1 is accompanied by Tyr phosphorylation of the protein. IRF-2, on the other hand, is constitutively phosphorylated. The pattern of Tyr phosphorylation of the three IRFs tested implies that phosphorylation events might play an important role in the transcriptional activity of these factors.

In conclusion, we have demonstrated in this work that ICSBP can associate with IRF-1 or IRF-2 through a specific domain, IAD, which is conserved among some of the IRF members. This suggests a novel model mechanism for the association of IRFs with other transcription factors. In addition, it is demonstrated that the association between ICSBP and IRFs is dependent upon phosphorylation of the interacting factor. Phosphorylation events can also modulate the ability of ICSBP to bind DNA; ICSBP can bind DNA either through association with other factors or directly when not phosphorylated on Tyr residues. In response to specific inducers, such as interferon, a signaling cascade is initiated that also results in the induction of pathway-specific function.?
of IRFs. These factors (also phosphorylated on Tyr residues) influence gene expression and also associate with ICSSBP. This interaction in turn might affect their biological activity. A further delayed response to IFN stimulation involves the induction of specific phosphatases (7–9) which, for example, dephosphorylate STAT1 and down-regulates IFN-stimulated gene expression. It is feasible that these phosphatases also act on ICSBP and IRF-1 or IRF-2 leading to dissociation of the heterocomplexes from the DNA. Dephosphorylated IRF-1 and IRF-2 bind DNA less effectively, whereas dephosphorylated ICSSBP becomes an active repressor that binds directly to the DNA and down-regulates IFN-stimulated gene expression. Thus, phosphorylation events might be responsible for the modulation of IRFs activities either by promoting interaction with other transcription factors or by enhancing or preventing binding to target DNA sequences.

Acknowledgments—We thank Drs. P. Wade and S. Kass for critical reading of the manuscript, with special thanks to Dr. A. Wolfle for his help and encouragement.

REFERENCES

1. Ihle, J. N., and Kerr, I. M. (1995) Trends Genet. 11, 69–74
2. Darnell, J. E., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421
3. Finbloom, D. S., and Larner, A. C. (1995) Arthritis & Rheum. 38, 877–889
4. Larner, A. C., and Finbloom, D. S. (1995) Biochim. Biophys. Acta 1266, 278–287
5. David, M., Petricoin, E., III, Benjamin, C., Pine, B., Weber, M. J., and Larner, A. C. (1995) Science 269, 1721–1723
6. Wen, Z., Zhong, Z., and Darnell, J. E., Jr. (1995) Cell 82, 241–250
7. Igarashi, K., David, M., Larner, A. C., and Finbloom, D. S. (1995) Mol. Cell. Biol. 15, 3984–3989
8. David, M., Grimley, P. M., Finbloom, D. S., and Larner, A. C. (1995) Mol. Cell. Biol. 15, 7515–7521
9. Igarashi, K., David, M., Finbloom, D. S., and Larner, A. C. (1995) Mol. Cell. Biol. 15, 1634–1640
10. Matsuyama, T., Grossman, A., Mittreuecker, H. W., Siderovski, D. P., Kiefer, F., Kawakami, T., Richardson, C. B., Taniguchi, T., Yosinaga, S. K., and Mak, T. W. (1995) Nucleic Acids Res. 23, 2127–2136
11. Eisenbeis, C. F., Singh, H., and Storb, U. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4776–4780
12. Imamura, T., Mak, T., Waki, K., and Taniguchi, T. (1994) Oncogene 13, 596–599
13. Kirchhoff, S., Schaper, F., and Hauser, H. (1993) Nucleic Acids Res. 21, 2861–2869
14. Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, K., Ishiihara, M., and Taniguchi, T. (1993) Science 259, 971–974
15. Harada, H., Kitagawa, M., Tanaka, N., Yamamoto, H., Harada, K., Ishiihara, M., and Taniguchi, T. (1993) Science 259, 971–974
16. Veals, S. A., Schindler, C., Leonard, D., Fu, X.-Y., Aebersold, R., Darnell, J. E., Jr., and Levy, D. E. (1992) Mol. Cell. Biol. 12, 3315–3324
17. Yamagata, T., Nishida, J., Tanaka, T., Sakai, R., Mitani, K., Yohida, M., Taniguchi, T., Yazaki, Y., and Hirai, H. (1996) Mol. Biol. Cell 16, 1283–1294
18. Politis, A. D., Ozato, K., Coligan, J. E., and Vogel, S. N. (1994) J. Immunol. 153, 2270–2278
19. Kirchhoff, S., Kromilas, A. E., Schaper, F., Grashoff, M., Sonenberg, N., and Hauser, H. (1995) Oncogene 11, 439–445
20. Tamura, T., Ishihara, M., Lampirhi, M. S., Tanaka, N., Oishi, I., Aizawa, S., Matsuyama, T., Mak, T. W., Taki, S., and Taniguchi, T. (1995) Nature 376, 7515–7521
21. Kimura, T., Nakayama, K., Penninger, J., Kitagawa, M., Harada, H., Matsuyama, T., Tanaka, N., Kamijo, R., Vilcek, J., Mak, T. W., and Taniguchi, T. (1994) Science 264, 1921–1924
22. Tanaka, N., Ishihara, M., Kitagawa, M., Harada, H., Kimura, T., Matsuyama, T., Lampirhi, M. S., Aizawa, S., Mak, T. W., and Taniguchi, T. (1994) Cell 77, 829–839
23. Harada, H., Kitagawa, M., Tanaka, N., Yamamoto, H., Harada, K., Ishiihara, M., and Taniguchi, T. (1993) Science 259, 971–974
24. Matsuyama, T., Kimura, Y., Suda, Y., Kishibara, K., Wakeham, A., Potter, J., Purlongo, C. L., Narendran, A., Suzuki, H., Ohashi, P. S., Paige, C. J., Taniguchi, T., and Mak, T. W. (1995) Cell 83, 83–97
Phosphorylation Events Modulate the Ability of Interferon Consensus Sequence Binding Protein to Interact with Interferon Regulatory Factors and to Bind DNA
Rakefet Sharf, David Meraro, Aviva Azriel, Angela M. Thornton, Keiko Ozato, Emanuel F. Petricoin, Andrew C. Larner, Fred Schaper, Hansjoerg Hauser and Ben-Zion Levi

J. Biol. Chem. 1997, 272:9785-9792.
doi: 10.1074/jbc.272.15.9785

Access the most updated version of this article at http://www.jbc.org/content/272/15/9785

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 26 of which can be accessed free at http://www.jbc.org/content/272/15/9785.full.html#ref-list-1