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Protein-Protein and DNA-Protein Interactions Affect the Activity of Lymphoid-Specific IFN Regulatory Factors

David Meraro,* Sharon Hashmueli,* Belly Koren,* Aviva Azriel,* André Oumard,† Sabine Kirchhoff,‡ Hansjörg Hauser,‡ Sujatha Nagulapalli,‡ Michael L. Atchison,‡ and Ben-Zion Levi‡*

IFN regulatory factors (IRFs) constitute a family of transcription factors that are involved in IFN signaling and the development and differentiation of the immune system. Targeted gene disruption studies in mice assigned their primary role to the immune system. Two lymphoid-specific IRF members, IFN consensus sequence binding protein (ICSBP) and IRF-4, bind target DNA with greater efficiency following interaction with two transcription factors, PU.1 and E47, leading to transcriptional synergy. PU.1 and E47 are essential for proper differentiation and maturation of lymphoid cells. In addition, ICSBP interacts with two IRF members, IRF-1 and IRF-2, which also have central roles in the regulation of cell-mediated immunity. Previously, we identified a region in ICSBP, termed the IRF association domain (IAD), that is conserved in all IRFs (excluding IRF-1 and IRF-2) and is essential for its interactions with other IRF proteins. Here we show that the IAD is an independent module used by ICSBP and IRF-4 for protein-protein interactions. In addition, an IAD of IRF-2 (IAD2), necessary for interaction with ICSBP, was identified and found to be conserved in IRF-1. The IAD2 shares similar characteristics with the PEST domain that is essential for the interaction of PU.1 with IRF-4. We also show that the ICSBP DNA binding domain is indispensable for the formation of DNA binding heterocomplexes and transcriptional activity. Therefore, our results shed light on the molecular mechanisms that affect IRF activities in the immune system via discrete functional domains. The Journal of Immunology, 1999, 163: 6468–6478.

Interferon regulatory factors (IRFs) constitute a family of transcription factors that mediate in part IFN signaling, confer an antiviral state, and modulate the immune system. Nine cellular members and several virus-encoded genes have been identified to date. All IRFs share significant homology within the N-terminal DNA binding domain (DBD), which is characterized by a winged type helix-loop-helix motif with five tryptophan repeats. Although some of the characterized IRFs are lymphoid specific, others are ubiquitously expressed. Targeted gene disruption studies in mice assigned their primary role to the immune system (for review, see Refs. 1). IRF transcriptional activities are varied, resulting in either activation or repression. However, some have dual functions, which are attributed in part to the fact that IRFs are found in protein complexes (1). These protein complexes enhance the ability of IRFs to bind target DNA sequences known as IFN-stimulated response elements (ISREs). For example, ISGF3γ/IRF-9 is the DNA binding subunit that associates with Stat1 and Stat2 to form the ISGF3 complex in response to IFN type I signaling (2). ICSBP/IRF-8 forms multiple protein complexes with both IRF-1 and IRF-2 (3, 4). IRF-4/Pip/LSIRF/ICSAT was identified as a component of a protein complex with PU.1 on Ig light chain enhancers (5–7). IRF-3 and IRF-7 have been recently reported to be part of large protein complexes that also include CBP/p300 proteins (8, 9). Similar complexes were reported for IRF-1 and IRF-2 (10). All these observations strengthen the idea that protein-protein complexes play central roles in the ability of IRFs to bind target DNA sequences and affect their biological activities.

The ICSBP is a lymphoid-specific IRF member that acts mainly as a transcriptional repressor (11–13). Its role in Th1-mediated immune responses and in normal myelocytic cell differentiation was revealed by gene disruption studies in mice (14). The ICSBP binds to ISREs only following interaction with other transcription factors (3, 4). These interactions are dependent upon the milieu of transcription factors in different immune cells (15). Previously, we identified a stretch of 177 aa toward the carboxyl terminus of ICSBP that is essential for its association with IRF-1 and IRF-2 (4). Interestingly, this region shows significant homology in all IRF members except IRF-1 and IRF-2 (see homology in Fig. 3B). The same region was shown to be essential for interaction of ISGF3γ with the Stat proteins (2). Therefore, we termed this domain an IRF association domain (IAD). The ICSBP also interacts with the lymphoid essential factors PU.1 and E47 (6, 16). These interactions were first described for IRF-4, which demonstrates the highest homology to ICSBP and is also lymphoid specific. IRF-4 knockout mice produce normal numbers of B cells that express IgM with κ or λ light chains, but exhibit dramatic reductions in serum Ig levels (17). Recent studies suggest that IRF-4 is essential for enhanced expression of Ig light chain genes during B cell activation through interaction with PU.1 and E47 (6, 18). The ability of various transcription factors to form combinatorial complexes with different biological activities is a hallmark of
eukaryotic gene regulation. Protein-protein interactions play crucial roles in the activity of IRFs, and different protein complexes were reported in various immune cells (15, 19, 20). The characterization of the different protein modules essential for these interactions should provide insight into the organization of these proteins in ternary complexes. In this presentation we have further characterized the interactions of ICSBP with IRF-1 and IRF-2. We show that the DBD and the IAD of ICSBP are independent modules that are essential for the formation of DNA binding heterocomplexes. The IAD also mediates interaction and transcriptional synergy of ICSBP with non-IRF members such as PU.1 and E47. Moreover, the same conserved IAD in IRF-4 is also essential for its transcriptional synergy with these two factors. We have also identified the association domain of IRF-2 necessary for association with ICSBP. This IAD2 is also conserved in IRF-1, and constitutes a PEST domain with similar characteristics as the PEST domain of PU.1 that enables PU.1 association with IRF-4 and ICSBP. Our results allow us to formulate a model of protein-protein and DNA-protein interactions among some IRF members that affect their biological activities in the immune system.

Materials and Methods

Cell culture

The NIH-3T3 cell line was obtained from American Type Culture Collection (Manassas, VA), and the mouse fibroblastoid C243 cell line was described previously (21). All cells were maintained in DMEM supplemented with gentamicin, tunicamycin, and 10% FCS.

Plasmids

To generate the chimeric proteins IRF2/ICSBP and ICSBP/IRF-2 illustrated in Fig. 1, the DNA binding domains (DBDs) and the C-termini lacking the DBDs were amplified separately by PCR with proper primers, cloned in pGEM-T vector (Promega, Madison, WI), and sequences were verified. The chimeric segments containing engineered restriction sites were then ligated and cloned into pTarget mammalian expression vector pTarget (Promega). The NIH-3T3 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The GST fusion constructs were generated using the plasmid pGEX-KG (25).

The GST-ICSBP was generated by cloning NcoI and HindIII fragments of ICSBP cloned in mammalian expression vector pTarget. The IAD of ICSBP from aa 200–377 was amplified by PCR with the 5' primer IAD377 (5'-GCTCTAGACTTCTGCCAGTTGCCG-3') and the 3' primer IAD377 (5'-TCCCTGAGGTTCTCTGGAGTG-3'), and the amplified fragment was digested with the engineered restriction sites XhoI and SalI and cloned into the corresponding sites in pGEX-KG. To fuse the IAD of IRF-2 (IAD2) to GST, PCR amplification of IRF-2 from aa 210–255 was performed. The forward primer was IAD2-5' (5'-CGAGGATCCCGGAGACAGCCAAGGAGGTTCTCTGGAGTG-3') and the reverse primer was IAD2-3' (5'-CGAGGATCCCGGAGACAGCCAAGGAGGTTCTCTGGAGTG-3'), and the amplified fragment was digested with the engineered restriction sites NcoI and HindIII and subcloned to the corresponding sites in pGEX-KG, generating the plasmid pGEX-IAD2.

The plasmids GAL-4-ICSBP-IAD and GAL-4-mutICSBP-IAD, in which the IAD and the point-mutated IAD of ICSBP were fused to the DBD of GAL-4, were generated for mammalian two-hybrid assays by PCR amplification. The IADs were PCR amplified as described above and cloned in-frame with the corresponding restriction enzymes to the GAL-4 DBD in the plasmid pSG424 (26). Similarly, the IAD2 of IRF-2 was amplified by PCR as described above and was fused to the GAL-4 DBD in the plasmid pSG424, generating the plasmid pGAL-4-IRF2-IAD.

In vitro transcription and translation (IVT)

The assays were performed as described previously (15). Plasmids containing the gene of interest under the T7 promoter were linearized down-stream of the coding region with the appropriate restriction enzyme. Five micrograms of linearized plasmids were in vitro transcribed by T7 RNA polymerase using a commercial kit (Stratagene, La Jolla, CA). Proteins were translated in vitro using the rabbit reticulocyte lysate system (Promega) according to the manufacturer’s instructions. To monitor translation efficiency, small scale reactions containing [35S]methionine were performed each time, and the labeled proteins were separated on 10% SDS-PAGE and subjected to autoradiography.

Electromobility shift assay

Gel-shift reactions were conducted as previously described (4). A typical reaction contained 1–5 μl of IVT proteins that were incubated in binding buffer (10 mM HEPES (pH 8.0), 5 mM MgCl2, 50 mM KCl, 0.025% bromophenol blue, 0.005% xylene cyanole, 10% Ficoll, 3% glycerol, 1 μg of sonicated poly(dI-dC), and 1 μg of sheared salmon sperm DNA) with at least 50,000 cpm of labeled trimer of the PRDI motif (AAGTGA)3 (to which IRFs bind) (5) for 10 min on ice. The samples were loaded on a pre-run 7% polyacrylamide gel. The dried gels were exposed to x-ray film.

The GST pull-down assay

Escherichia coli BL21cells harboring the various GST fusion constructs were grown to 0.7OD600 at 37°C and were induced with 0.1 m isopropyl β-D-thiogalactoside (IPTG) for an additional 3 h. Cells were harvested in PBS and lysed by sonication, and the lysate was cleared by 10 min of centrifugation at 10,000 × g. Cell lysates containing 3–5 μl of fusion protein were incubated with 20 μl of 50% glutathione-Sepharose beads (Pharmacia, Uppsala, Sweden) in a final volume of 500 μl for 1 h at 4°C with gentle rotation. The beads were then washed three times with binding buffer (50 mM Tris-HCl (pH 8.0), 250 mM KCl, 10 mM MgCl2, 0.5% Triton X-100, and 1% Nonidet P-40), blocked with 500 μl of binding buffer containing 15 μl of IVT luciferase for 30 min as described above, and subsequently, 5 μl of IVT [35S]methionine-labeled target protein was added for an additional 60 min. In experiments testing the effect of a target DNA, 1 pmol of DNA was added following 30 min of incubation with IVT [35S]methionine-labeled interacting protein and incubated for an additional 30 min. The beads were washed five times with 500 μl of binding buffer.
and the bound proteins were eluted with 20 \mu l of 15 mM reduced glutathione and separated by 12\% SDS-PAGE. The gel was fixed, dried, and exposed to a x-ray film.

DNA transfections and reporter gene analyses

NIH-3T3 cells were transfected by the calcium phosphate-DNA coprecipitation method as described previously (28). Cells were transfected with 3–5 \mu g of the various reporter plasmids, 1 \mu g of pRSV\betaGal (to monitor transfection efficiencies), and pUC19 serving as carrier DNA up to a total of 15–20 \mu g. The amounts of transfected plasmid DNA corresponding to the various expression plasmids coding for the transcription factors are indicated in the text. The cells were harvested 48 h later and lysed using the lysis buffer of the luciferase assay kit (Promega), and luciferase activities were determined according to the manufacturer’s instructions using a TD-20/20 luminometer (Turner Design, Promega). Reporter gene activities were normalized for protein concentration and transfection efficiencies as described previously (4). Each set of transfection experiments was repeated at least three times, generating similar results. In some of the transfection assays the Oligo7LBKCAT reporter plasmid was used, and relative fold synergy was calculated as previously described (6).

Mammalian two-hybrid assays were performed in C243 cells as described previously (29). Cells were transfected with 1 \mu g of GAL-4-driven CAT gene, 0.5 \mu g of GAL-4 fusion plasmids (described above and in Tables I and II), and 1 \mu g of full-length IRF-1 fused to VP16, the herpes simplex VP16 activation domain, (29), or full-length ICSBP fused to VP16 (15). One microgram of luciferase expression plasmid was also transfected to account for transfection efficiency. The CAT analyses were performed using CAT-ELISA (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. The CAT levels were normalized to protein concentration and corrected for transfection efficiency.

Results

The DBD is essential for the formation of a DNA binding heterocomplex between ICSBP and IRF-2

The IRFs are characterized by significant homology in the DBD, yet not all IRFs bind directly to target DNA. We have shown that ICSBP can bind to DNA following association with IRF-1 or IRF-2 and that the IAD of ICSBP is involved in these interactions (4). However, the role of the DBD in these interactions has not been determined. Studies with IRF-4, which demonstrates the highest homology to ICSBP, pointed to the existence of an autoinhibitory domain at the very carboxyl-terminal end of the protein, which interferes with the ability to bind DNA (16). Our previous studies, using successive deletions from the carboxyl terminus of ICSBP, did not reveal such an autoinhibitory domain at the C-terminus of ICSBP but, rather, pointed to the DBD (4).

To demonstrate that the inability of ICSBP to bind DNA resides within its DBD, we have swapped the DBD of ICSBP with that of IRF-2, generating two chimeric proteins, ICSBP/IRF2 and IRF2/ICSBP (see Fig. 1). The wild-type (wt) proteins and the chimeric proteins were IVT and tested by EMSA for the ability to form DNA binding heterocomplexes. It is clear from Fig. 1 that neither ICSBP nor ICSBP/IRF2 proteins bound to the DNA (Fig. 1, lanes 2 and 4, respectively). On the other hand, both IRF-2 and IRF2/ICSBP proteins bound to the DNA very well. As expected, IRF-2 and ICSBP formed a DNA binding heterocomplex (Fig. 1, lane 3). The same was observed when IRF-2 was mixed with IRF2/ICSBP (Fig. 1, lane 8) or when the two chimeric proteins, IRF2/ICSBP and ICSBP/IRF2, were mixed together (Fig. 1, lane 10). No DNA binding heterocomplexes were observed when ICSBP was mixed with IRF2/ICSBP (Fig. 1, lane 9) or ICSBP/IRF-2 (Fig. 1, lane 6). Similarly, IRF-2 and ICSBP/IRF2 did not produce a DNA binding heterocomplex (Fig. 1, lane 5). The specificity of the various heterocomplexes was identified with Abs directed against either ICSBP or IRF-2 (data not shown). The results suggest that to form a DNA binding heterocomplex, the carboxyl termini of both IRF-2 and ICSBP must be present. Here we show that the IRF-2 DBD can replace that of ICSBP, but not the opposite. Although the ICSBP-DBD is homologous to the DBD of IRF-2, it binds to DNA effectively only when IRF-2 DBD is present.

We have shown previously (15) that deletion of 35 N-terminal amino acids for the DBD of ICSBP resulted in an inability to form a heterocomplex with IRF-2, indicating that two intact DBDs are essential. The results in Fig. 1 show that two IRF-2 DBDs can promote heterocomplex formation if the C-termini are compatible (Fig. 1, lane 8). Although IRF-2 binds DNA by itself, a DNA binding heterocomplex requires two intact DBDs. This is supported by the fact that an IRF-2 mutant that contains a 39-aa deletion from the DBD (IRF2del39) did not bind to the DNA (Fig. 1, lane 12) and did not form a DNA binding heterocomplex with the IRF2/ICSBP chimeric protein (Fig. 1, lane 11). Thus, interactions between these two factors are dependent upon the availability of
two compatible carboxyl termini and two compatible DBDs. Interestingly, although IRF-2/ICSBP is a bigger protein (425aa) than IRF-2 (349 aa), it migrates faster on the gel (Fig. 1, *lanes 1* and 7, respectively). Because this is a native gel, it can imply that either the chimeric protein is more negatively charged or it maintains a compacted tertiary structure. Alternatively, Furui et al. (30) demonstrated that IRF-2 forms homodimers in solution via DBD interactions. This might suggest that IRF-2 binds to DNA as a homodimer, while the IRF-2/ICSBP chimeric protein binds to the DNA as a single chain and therefore migrates faster.

**The IAD of ICSBP alone mediates protein-protein interactions with IRF-1 and IRF-2**

We have demonstrated that two DBDs are essential for the formation of a DNA binding heterocomplex between ICSBP and IRF-2. However, our experiments did not distinguish between protein-protein and DNA-protein interactions. To show that the IAD alone can support protein-protein interactions with IRF-1 and IRF-2, GST pull-down assays were performed. The ICSBP or its IAD (residues 200–377) were fused to GST, and the ability of these fusion peptides, bound to glutathione-Sepharose beads, to retain $^{35}$S-labeled IRF-1 (*Fig. 2A*) or IRF-2 (*Fig. 2B*) prepared by IVT was analyzed. The IAD alone was sufficient to confer effective association with both IRF-1 and IRF-2 in the absence or the presence of target DNA sequence (*Fig. 2, A and B, *lanes 3* and 4, respectively). The full-length ICSBP interacted with IRF-1 with almost similar efficiency in the absence and the presence of the target DNA sequence (*Fig. 2A, *lanes 1* and 2, respectively*). However, the interaction of IRF-2 with ICSBP was significantly enhanced when target DNA was present (*Fig. 2B, *lanes 1* and 2, respectively*). These data suggest that the interaction of IRF-2 with ICSBP may be different from that of IRF-1, implying that structural changes due to the binding of IRF-2 to the DNA may mediate efficient interaction with ICSBP. The GST alone did not bind either IRF-1 or IRF-2 (*Fig. 2, A and B, *lanes 5* and 6, respectively*). Western blot analysis with Abs directed against GST was performed to ensure that equal amounts of GST fusion proteins were loaded on the gel (data not shown).

To further test the importance of the ICSBP-IAD for protein-protein interactions, point mutations in two conserved residues within this domain were generated by PCR mutagenesis. Leu$^{331}$ (L) was mutated to Pro (P) and Gly$^{351}$ (G) to Gln (E) to produce mutICSBP (see locations of point mutations in the IAD homology in *Fig. 3*). The EMSA was performed with IVT proteins using either wt ICSBP or mutated ICSBP (mutICSBP) and IRF-1 or IRF-2. As expected, both IRF-2 and IRF-1 bound to the PRDI probe (*Fig. 3*). The ability to interact with ICSBP (Fig. 4A, *lanes 1* and 6, respectively), while ICSBP binding was observed only following interaction with IRF-2 or IRF-1 (*Fig. 3, compare *lanes 2* and 3 and *lanes 2*–7, respectively). However, the formation of heterocomplexes with mutICSBP were not detected (*Fig. 3, *lanes 5* and 8, respectively*). This suggests that certain residues and possibly structural motifs within the IAD are essential for interactions with IRF-1 and IRF-2. Interestingly, Leu$^{331}$ is conserved in all IRF-IADs, while Gly$^{351}$ is less well conserved (see IAD homology in *Fig. 3*).

Finally, a mammalian two-hybrid assay (*Fig. 2*) was employed to demonstrate in vivo interactions between ICSBP and IRF-1 and to show that IAD alone is sufficient for such interactions. The wt IAD or mutIAD (Leu$^{331}$ and Gly$^{351}$) of ICSBP were fused to the DBD of the yeast transcription factor GAL-4. These constructs were transfected with a CAT reporter gene driven by GAL-4 binding sites (for details, see Materials and Methods). As shown in Table I, neither of these GAL-4 fusion constructs alone nor IRF-1 fused to the strong herpes simplex VP16 activation domain (IRF-1-VP16) were capable of promoting CAT activity. However, when both GAL-4-IAD and IRF-1-VP16 plasmids were cotransfected with the reporter construct, a significant increase in CAT activity was observed. On the other hand, the GAL-4-mutIAD construct did not enhance reporter gene activity. These results demonstrate that interaction between the IAD of ICSBP and IRF-1 occurs in vivo, and that the two point mutations within the IAD were sufficient to abrogate this interaction.

**Identification of the association domain of IRF-2**

The IRF-1 and IRF-2 are the only IRFs that identified that do not exhibit homology at the IAD. To identify the association domain of IRF-2, we generated carboxyl-terminal (illustrated in *Fig. 4A*) and internal deletion mutants (illustrated in *Fig. 4B*). The ability of the mutant proteins to associate with ICSBP was tested by EMSA. Successive deletions from the carboxyl end of IRF-2 up to aa residue 265 led to the formation of heterocomplexes with ICSBP with diminishing intensity (*Fig. 4A, *lanes 1*–10*). A further deletion up to position 233 resulted in a carboxyl-truncated IRF-2 that did not interact with ICSBP (*Fig. 4A, *lanes 11* and 12*). Similarly, successive internal deletions from positions 125–210 resulted in IRF-2 mutant proteins that were capable of interacting with ICSBP. A further deletion toward the C-terminus resulted in an IRF-2 mutant, Del101, which was incapable of interacting with ICSBP (*Fig. 4B*). These data suggest that a region between residues 210–265 is essential for the interaction with ICSBP. These results were supported by GST pull-down assays, shown in *Fig. 4C*. The IRF-2 265 C-terminus-deleted mutant interacted with diminished intensity with ICSBP compared with IRF-2 (*Fig. 4C, *lanes 2* and 1, respectively*), while IRF-2 233 lost all interaction ability (*Fig. 4C, *lane 3*). Similarly, IRF-2 del85, which is not deleted within the IRF-2 IAD, interacted with ICSBP in the same way as the wt protein, while IRF-2 del101 reacted very weakly with ICSBP (*Fig. 4C, *lanes 4* and 5, respectively*). In addition, IRF2-IAD alone was sufficient for the interaction with ICSBP as shown by a GST pull-down assay with just the IRF-2-IAD fusion construct (*Fig. 4D, *lane 1*).

A mammalian two-hybrid assay was performed to show that the IRF-2-IAD interacts with ICSBP in vivo (Table II). The IRF-2-IAD was fused to GAL-4 DBD, and the expression plasmid was prepared by GST pull-down assay to test the ability of the ICSBP-IAD and full-length ICSBP to bind IRF1 and IRF2. Lysates of bacterially expressed GST or GST fused to the IAD of ICSBP (GST-IAD) or GST fused to full-length ICSBP (GST-ICSBP) were bound to glutathione-Sepharose beads. The bound proteins were incubated with $[^{35}]$Smethionine-labeled IVT IRF-1 (A) or $[^{35}]$Smethionine-labeled IVT IRF2 (B) in the absence (−) or presence (+) of 1 pmol target DNA-PRDI. The retained proteins (indicated by arrowheads) were eluted with reduced glutathione, separated by SDS-PAGE, and exposed to x-ray film.
cotransfected with an ICSBP-VP16 construct (15). It is clear that the interaction between IRF-2-IAD and ICSBP-VP16 led to significant activation of the reporter gene (Table II). Computer analyses demonstrated that the IRF-2-IAD is highly conserved in IRF-1 from different species (Fig. 4E), consistent with its functionality for interaction with ICSBP. Our results suggest that the IRF family of transcription factors contains two types of association modules: IAD1, which is homologous to the ICSBP-IAD, and IAD2, which is shared only by IRF-1 and IRF-2.

The interactions of both ICSBP and IRF-4 with PU.1, an ets member, are also mediated through the IAD

Protein–protein interactions of ICSBP in cells of the immune system are not limited to IRF-1 and IRF-2. The ICSBP is recruited to the Ig light chain composite element by PU.1, an ets member essential for the formation of multiple hemopoietic lineages (16, 31). Like the interactions with IRF-1 and IRF-2, this interaction requires the IAD, as shown in Fig. 5A. Both the full-length ICSBP and deletion mutant ICSBP proteins at segments outside the IAD,
ICSBP-377 and ICSBP-del20, interacted with PU.1. This is demonstrated by EMSA using the Ig light chain enhancer element as a probe (Fig. 5A, lanes 3, 5, and 9). On the other hand, deletions within the IAD resulted in mutant proteins (ICSBP-365 and ICSBP-del38) that were incapable of interacting with PU.1 (Fig. 5A, lanes 7 and 11). The ICSBP missing the first 33 aa within the DBD did not interact with PU.1, indicating the importance of the DBD for the formation of DNA binding heterocomplexes (Fig. 5A, lane 13). Yet, the IAD alone was sufficient to confer protein-protein interaction, as determined by GST pull-down assays (Fig. 5B, lanes 3 and 4). As noted for IRF-2, the interaction of full-length ICSBP with PU.1 was enhanced in the presence of target DNA (Fig. 5B, compare lanes 1 and 2), implying that it is important for heterocomplex formation. Western blot analysis with Abs directed against GST was performed to ensure that equal amounts of GST fusion proteins were loaded on the gel (data not shown). Point mutations within the ICSBP-IAD (Leu331 and Gly351) were sufficient to eliminate the interaction with PU.1, as demonstrated above for IRF-1 and IRF-2 (Fig. 5C, lanes 2 and 3). These results demonstrate that the IAD of ICSBP is essential for interaction with PU.1.

Because both lymphoid-specific ICSBP and IRF-4 interact with PU.1 and share homology within the IAD, we tested whether the same point mutation within IRF-4 would eliminate the interaction of IRF-4 with PU.1. Homology among IRFs in the IAD (Fig. 3) shows that Leu331 in the IAD of ICSBP is conserved in all IRFs, while Gly351 is not. Therefore, we mutated the corresponding Leu368 in IRF-4 to Pro and tested the ability of this point mutant IRF-4 to interact with PU.1 in EMSA (see homology in Fig. 5D). Our results show that this point mutation ablated the interaction of IRF-4 with PU.1 (Fig. 5D, compare lanes 4 and 6). These observations indicate that conserved residues within the IAD are essential for the ability of IRFs to interact with various proteins.

We next tested whether the IADs of IRF-4 and ICSBP are essential for in vivo interactions. The IRF-4 or ICSBP and the corresponding point mutation constructs were cotransfected with a
PU.1 expression construct into NIH-3T3 cells, and the ability to coactivate a \( l \)B-driven reporter gene construct was tested. None of the constructs alone significantly activated the reporter gene (Fig. 6, lanes 1–6). As previously reported (5), cotransfection of PU.1 with IRF-4 resulted in a 27-fold induction of the reporter gene, while the activation observed with IRF-4 mutant L368P was markedly reduced (Fig. 6, compare lanes 7 and 8). Cotransfection of ICSBP with PU.1 resulted in a milder transcriptional activation of the reporter gene (6-fold) compared with the transcriptional activation of IRF-4. However, cotransfection with mutated ICSBP negated this activity to a level that was even lower than the basal level of the reporter gene alone. Western blotting showed that ICSBP, mutICSBP, IRF-4, and IRF-4L368P were expressed in transfected cells at equivalent levels (data not shown). The ICSBP defective in its DBD (ICSBPres135) did not show any transcriptional synergy with PU.1 (data not shown), demonstrating the necessity of the DBD for the interaction between these two factors. Lack of transcriptional synergy was also noted when ICSBP was cotransfected with plasmids expressing PU.1 mutant S148A (which cannot be phosphorylated on serine 148) or a PU.1 construct with the PEST domain deleted (data not shown). These results provide strong evidence that in vivo interaction of both IRF-4 and ICSBP with PU.1 is mediated by both the IAD and the DBD, and that the interaction of PU.1 with ICSBP is facilitated through the PU.1 PEST domain, as has been shown with IRF-4.

The IAD is also essential for transcriptional synergy between IRF-4 or ICSBP and E47

Recently, we demonstrated that both IRF-4 and ICSBP can synergize with E47, a member of the E2A family of transcription factors.

Table II. Mammalian two-hybrid assay demonstrating the formation of heterocomplexes between the IAD of IRF-2 and ICSBP

<table>
<thead>
<tr>
<th>Constructs</th>
<th>CAT Activity (relative units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL-4</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>ICSBP-VP16</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>GAL-4-IRF2-IAD</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>GAL-4 + ICSBP-VP16</td>
<td>1.0</td>
</tr>
<tr>
<td>GAL-4-IRF2-IAD + ICSBP-VP16</td>
<td>62.31 ± 12.77</td>
</tr>
</tbody>
</table>

*Expression vectors coding for a fusion protein containing the DBD of GAL-4 fused to the IAD of IRF-2 (GAL-4-IRF2-IAD), or the DBD alone (GAL-4), were cotransfected with vectors encoding for ICSBP fused to the IRV VP16 transactivation domain (ICSBP-VP16) and GAL-driven CAT-gene reporter construct. The induced CAT activities were normalized to the corresponding control with the GAL-4 DBD alone. Data are given as means ± SEM of at least three independent experiments.

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**FIGURE 5.** Characterization of in vitro interactions between ICSBP or IRF-4 and PU.1. A. The abilities of various carboxyl and internal deletion ICSBP mutants (illustrated in the figure) to interact with PU.1 was tested by EMSA using the \( l \)B enhancer element as a probe. Arrows indicate the binding of PU.1 and the binding of heterocomplexes between PU.1 and the various ICSBP mutants (Hetero). B. GST pull-down assays with GST-IAD and GST-ICSBP (described in Fig. 2) were performed with IVT PU.1 in the absence or the presence of \( l \)B target DNA sequence as described in Fig. 2. Arrows indicate the position of the retained PU.1. C. IVT ICSBP and mutICSBP were tested for their ability to form heterocomplexes with IVT PU.1 by EMSA using the \( l \)B sequence as probe. Arrows indicate the binding of PU.1 and the heterocomplexes between PU.1 and ICSBP (Hetero). D. The wt IRF-4 and mutant IRF-4L368P were tested for their ability to form heterocomplexes with PU.1 by EMSA as described above. Arrows indicate the binding of PU.1 and the heterocomplexes between PU.1 and IRF-4 (Hetero). Homology between IRF-4 and ICSBP in the segment of IRF-4 IAD containing the point mutation is shown. Identity is shown in white letters; similarity is shown in gray letters. The point mutation within IRF-4-IAD, L368 to P, is indicated.
interactions, respectively. These modules play crucial roles in the transcription factors present in the cells (15, 19, 20, 32). This study shows that cells of the immune system and vary depending upon the milieu of the immune responses.

**Protein complexes are mediated through various functional domains**

Complexes that enable regulated expression of genes essential for immune responses.

**Necessity of the DBD for the formation of a DNA binding heterocomplex between ICSBP and IRF-2**

The IRF family exhibits significant homology within their DBDs. Yet, some IRFs bind to DNA effectively only following interaction with other transcription factors. This was reported not only for the interaction of ICSBP with IRF-1 and IRF-2 (3, 4), but also for the interaction of ISGF3 with the Stats (2) and the interaction of IRF-4 with PU.1 (16, 33). A short region at the C-terminus of IRF-4 was reported to have an inhibitory effect on the ability of IRF-4 to bind DNA, perhaps through an interaction with its DBD (16). We show that this is not the case for ICSBP, which demonstrates the highest homology to IRF-4. The inability of ICSBP to directly bind to DNA resides within its DBD. Swapping the DBDs of IRF-2 and ICSBP did not render the ICSBP/IRF-2 fusion protein the ability to bind to DNA. A peptide corresponding to the IRF-2 DBD binds target DNA very effectively, while no binding is detected with the DBD of IRF-4 (16). 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Thus, ICSBP is recruited to target promoters such as Ig light chain enhancers (18) or gp91phox (19) only following interaction with other transcription factors that facilitate its binding to a composite DNA binding site.

The role of IADs in mediating protein-protein interaction

**IAD2, the association domain of IRF-2 and possibly IRF-1.** Previously, we identified the IAD of ICSBP and demonstrated that it is conserved among all IRFs, excluding IRF-1 and IRF-2 (4). In this work we have identified a stretch of 55 aa within IRF-2 that is sufficient for interaction with ICSBP and is conserved in IRF-1. Mammalian two-hybrid assays demonstrated that the IAD2 of IRF-2 was sufficient for in vivo interactions with ICSBP. The same region was demonstrated as essential for heterodimerization of IRF-1 with ICSBP (29). The IRF-1 IAD2 was also tested using a
mammalian two-hybrid assay; however, this region alone demonstrated significant transcriptional activity. Nevertheless, when co-transfected with the ICSBP-VP16 fusion construct, further enhancement of reporter gene activity was observed (data not shown). We suggest that these two closely related proteins use the same module for association with ICSBP.

The PEST domain of PU.1 mediates interactions with IRF-4 and ICSBP. There is no obvious homology between this PEST domain and the IADs of IRF-1 and IRF-2. However, these IADs are predicted PEST domains, because, like the PU.1 PEST domain, they are rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues. Using the algorithm for searching potential PEST sequences (PESTfind) (34), these domains scored much higher then the PU.1 PEST domain (+7.71 for IRF-1, +9.44 for IRF-2, and only +2.15 for PU.1). These PEST domains are of similar size, i.e., 42 aa in PU.1 and about 55 aa in IRF-1 and IRF-2. PEST domains were originally believed to be involved in controlling protein instability and in targeting proteins for proteolytic degradation (35). However, some PEST domains can mediate protein-protein interactions, as shown for the formation of the DNA binding heterocomplex between PU.1 and IRF-4 (24).

IRF-1 is a short-lived protein with a half-life of only 30 min, while IRF-2 is more stable, with a half-life >8 h (36). Unlike IRF-2, IRF-1 contains an additional predicted PEST domain between residues 138–161 with a very promising PESTfind score of +12.87. It is possible that IRF-1 instability is determined by this PEST domain, while the other domain is mainly engaged in protein-protein interactions.

The role of IAD1. Our results show that the ICSBP-IAD is an independent module that is sufficient to confer protein-protein interactions. This suggests that the formation of a DNA binding heterocomplex is a two-step process. The presence of a target DNA sequence enhances the interaction of ICSBP with either IRF-2 or PU.1 in GST pull-down assays. Furthermore, to form DNA binding heterocomplexes the two DBDs of the interacting factors are essential. When the ICSBP-DBD is defective, protein-protein complexes can still occur; however, they lack transcriptional activity (15). The fact that both ICSBP and IRF-4 do not bind DNA alone suggests that protein-protein interaction is accompanied by conformational changes that confer upon the interacting proteins the ability to bind DNA. We speculate that protein-protein interaction is the first step, because it occurs spontaneously in vitro, and it is independent of the DBD, as shown by mammalian two-hybrid and GST pull-down assays. Similar predictions were made for the mode of interaction between IRF-4 and PU.1 (16, 24).

Among IRF proteins, the ICSBP IAD shows the highest homology with IRF4. Although both proteins interact with PU.1, IRF-4 does not interact with IRF-1 or IRF-2. This suggests that the ICSBP and IRF4 IAD modules are spatially organized like a key-lock mechanism, i.e., the overall structure is similar and is probably determined by conserved residues, while the specificity of interactions is dictated by nonconserved residues. Accordingly, point mutation in conserved Leu331 was able to ablate the interactions of both ICSBP and IRF-4 with PU.1 and the interaction of ICSBP with IRF-1 and IRF-2.

A predicted α-helix structure at the C-terminus of IRF-4 (aa 399–413) was demonstrated to be important for ternary complex formation with PU.1 (16, 18). Our studies extend the region that is essential for protein-protein interaction to the identified IAD.

The transcriptional synergy between ICSBP or IRF-4 with PU.1 or E47 is mediated via DNA-protein and protein-protein interactions

Previously it was demonstrated that ICSBP and IRF-4 can interact with the transcription factors PU.1 and E47, leading to transcriptional synergy within Ig light chain enhancers (6, 16, 33). In this communication we demonstrate that the interactions between IRFBP and IRF-4 with PU.1 and E47 are mediated mainly through the IAD. Unlike the interaction of ICSBP with IRF1 and IRF-2, which results in transcriptional repression (11–13), interaction of ICSBP with PU.1 and E47 leads to transcriptional synergy. The synergism observed between ICSBP and E47 or PU.1 is also dependent upon interaction with target DNA sequences, because a

![FIGURE 8. Model for the recruitment of ICSBP by IRF-2, PU.1, or E47.](http://www.jimmunol.org)
deletion of the first 35 aa from the DBD of ICSBP abolished transcrip-
tional synergy. The mode of interaction between E47 ho-
modimers and IRFs is not yet characterized. Previous studies showed that IRF-4 potentiates the binding of E47 homodimers to DNA through conformational changes (6). Our results support this observation and strongly suggest that physical interaction occurs through the IAD in the context of target DNA only when the DBD is intact. Although the interaction of ICSBP with PU.1 and E47 leads to transcriptional synergy, it is significantly less potent than the synergy observed with IRF-4, suggesting that interplay between these two factors can modulate the transcriptional magnitude of Ig light chain gene expression during B cell maturation. Based on our results described here and increasing evidence from the literature, it is possible to draw some common characteristics for the mode of interaction between ICSBP and IRF-2/IRF-1, PU.1, or E47 (see illustration in Fig. 8).

First, intact DBD and IAD domains of ICSBP are essential for functional interactions with IRF-1, IRF-2, and PU.1. Second, PEST domains in IRF-1, IRF-2, and PU.1 facilitate interactions with ICSBP. The association module of E47 that interacts with IRFs has not been identified yet. However, two predicted PEST domains exist in E47 between aa residues 53–73 and 527–543. Third, phosphorylation is probably a key factor in the above interactions. Tyr residues are essential for efficient interaction of IRF-2 with ICSBP, which fits previous observations that IRF-1 and IRF-2 are phosphorylated on Tyr residues in promonocytic cells (4). Mutation of Tyr220 and Tyr262 to Phe within the IAD of IRF-2 leads to reduced interaction with ICSBP (D. Meraro et al., unpublished observations). The fact that Ser residues are abundant in the IAD2 imply that they might also contribute to the interaction of IRF-1 and IRF-2 with ICSBP. Serine phosphorylation is known to be required for PU.1 interaction with IRF4 (7). Possible modulation of phosphorylation in vivo may affect the affinity and variety of interactions.

IRF-protein interactions are crucial for regulation of genes within the immune system. For instance, interaction between IRF-4 and PU.1 is important for the regulated expression of Ig light chains (6, 18). Similarly, interactions of ICSBP with PU.1 in macrophage cells and with either IRF-1 or IRF-2 in B cells or monocyctic cells have been described (15, 19, 20). ICSBP and IRF-1 knockout mice are both defective in Th1-mediated immune cell function.

IRF protein function is a lymphoid-specific, PU.1-dependent transcriptional activator.

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