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Contribution of Ser386 and Ser396 to activation of interferon regulatory factor 3

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Summary

IRF-3, a member of the interferon regulatory factor (IRF) family of transcription factors, functions in innate immune defense against viral infection. Upon infection, host cell IRF-3 is activated by phosphorylation at its 7 C-terminal Ser/Thr residues, ³⁸⁵SSLENTVDLHISNSHPLSLTS⁴⁰⁵. This phosphoactivation triggers IRF-3 to react with the coactivators, CREB-binding protein (CBP)/p300, to form a complex that activates target genes in the nucleus. However, the role of each phosphorylation site for IRF-3 phosphoactivation remains unresolved. To address this issue, we screened all 7 Ser/Thr potential phosphorylation sites by mutational studies, size-exclusion chromatography, and isothermal titration calorimetry. Using purified proteins, we show that CBP (aa 2067–2112) interacts directly with IRF-3 (aa 173–427) and 6 of its single-site mutants to form heterodimers, but when CBP interacts with IRF-3 S396D, oligomerization is evident. CBP also interacts *in vitro* with IRF-3 double-site mutants to form different levels of oligomerization. Among all the single-site mutants, IRF-3 S396D showed the strongest binding to CBP. Although IRF-3 S386D alone did not interact as strongly with CBP as did other mutants, it strengthened the interaction and oligomerization of IRF-3 S396D with CBP. In contrast, IRF-3 S385D weakened the interaction and oligomerization of IRF-3 S396D and S386/396D with CBP. Thus, it appears that Ser 385 and Ser 386 serve antagonistic functions in regulating IRF-3 phosphoactivation. These results indicate that serines 386 and 396 are critical for IRF-3 activation and support a phosphorylation-oligomerization model for IRF-3 activation.

Keywords

interferon regulatory factor-3; CREB-binding protein; phosphomimetic form; phosphorylation; oligomerization

Introduction

Interferons (IFNs) are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation, and immune activation.¹ The pathway leading to expression of IFN- β is guarded by IRF-3, a member of the interferon regulatory factor (IRF) family of transcription factors.^{2,3,4} In the absence of infection, IRF-3 is localized in the cytoplasm in an inactive form. Upon infection, cytoplasmic IRF-3 is phosphorylated and translocates into

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the nucleus, where it interacts with the closely related coactivators, CREB-binding protein (CBP)/p300, to form a nucleoprotein complex at the promoter region of the IFN- β gene. The resultant complex then activates IFN- β transcription.^{5,6,7,8,9}

Virus-induced phosphoactivation of IRF-3, thought to be mediated directly or indirectly by IKK ϵ and/or TBK1,^{10,11,12,13,14} occurs in the C-terminal region of IRF-3 at 7 Ser/Thr residues, ³⁸⁵SSLENTVDLHISNSHPLSLTS⁴⁰⁵_{4,11,15,16} (Figure 1A). Within this region, IRF-3 has two phosphorylation sites: site 1 includes Ser385 and Ser386, whereas site 2 includes Ser 396, Ser 398, Ser 402, Ser 405, and Thr 404. This C-terminal region also forms the bulk of an autoinhibitory domain (aa 380–427) that, along with N-terminal helix (aa 173–200) (Figure 1 B), blocks IRF-3 interactions with molecules such as CBP/p300.^{17,18,19} Phosphorylation of these 7 Ser/Thr residues reduces autoinhibition. Indeed, recent evidence suggests that phosphorylating residues in site 2 alleviates autoinhibition and allows IRF-3 to interact with CBP/p300.²⁰ However, the contribution of each residue for IRF-3 phosphoactivation remains controversial.

Phosphorylation at site 1 (Ser385/Ser386) was found by Fujita and co-workers¹⁶ to be key to activation of IRF-3, with phosphorylation at site 2 (Ser396 to Ser405) having an auxiliary but not essential role. On the other hand, phosphorylation at site 2 (Ser396, Ser398, Ser402, Thr404, and Ser405, particularly Ser396 and Ser398) was identified by Hiscott and colleagues⁵ as the regulatory phosphorylation event for IRF-3. This result was supported by the identification of Ser 396 in the IRF-3 site 2 as a key residue that is phosphorylated *in vivo* upon virus infection¹⁵. A more complex scenario was shown when Harrison and colleagues²⁰ demonstrated *in vitro* that TBK1 can directly phosphorylate full-length IRF-3. Phosphorylation at residues in site 2 (Ser396 to Ser405) alleviated autoinhibition to allow interaction with CBP/p300 and facilitated phosphorylation at site 1 (Ser385 or Ser386). Phosphorylation at site 1 was, in turn, required for IRF-3 dimerization.²⁰ Thus, these results suggest that IRF-3 activation is mediated by TBK1 in a two-step phosphorylation model. Taken together, the results of mutagenesis studies,^{5,9,18,21} kinase analyses,^{10,11,13,14,20,22} phosphopeptide mapping,⁹ and studies with phosphor-specific antibodies,¹⁵ do not reach a consensus on the role of individual phosphorylation sites in the IRF-3 C-terminal region.

Before phosphorylated IRF-3 can bind to DNA and stimulate IFN- β transcription, it must associate with the coactivator protein, CBP/p300.^{23,24,25} The direct interaction of CBP/p300 with DNA was shown to be indispensable for the DNA binding activity of phosphorylated IRF-3.²⁵ Furthermore, IRF-3 was associated with CBP/p300 after virus infection, and this interaction was mediated by the C-terminal domains of both proteins.⁵ Mutating Ser396 and Ser398 in IRF-3 abrogated its binding to CBP/p300. Thus, C-terminal phosphorylation of IRF-3 was suggested to alter protein conformation, permitting nuclear translocation, association with transcriptional partners, and primary activation of IFN genes.⁵ However, the individual roles of the 7 Ser/Thr in IRF-3 binding to CBP/p300 remains unclear.

The objective of this study was to obtain insights into the activation events of IRF-3 by identifying the role of individual phosphorylation sites using various IRF-3 phosphomimetic forms. To that end, size-exclusion chromatography, isothermal titration calorimetry, and mutagenesis were used in *in vitro* interaction assays to assess the binding of purified IRF-3 (aa 173–427) with purified CBP (aa 2067–2112). Here, we present biochemical and calorimetric evidence that serine 386 and serine 396 play critical and complementary functions in IRF-3 phosphorylation.

Results

IRF-3 and its phosphomimetic forms with single-site mutations are monomers

To assess the role of the 7 C-terminal Ser/Thr residues in the phosphorylation events of IRF-3, we examined the role of each residue in IRF-3 oligomerization with CBP. To that end, we first made phosphomimetic forms of IRF-3 by mutating each of the seven implicated Ser/Thr residues in the region of 385 to 405 to Asp (Figure 1). When purified IRF-3 and its single-site serine mutants were analyzed by size-exclusion chromatography, they all eluted as a single peak at fraction numbers 21–23 (Figure 2a), corresponding to an apparent molecular mass of 28 kDa. Since the calculated molecular weight of IRF-3 (173–427) and its mutants is 28 kDa, these results suggest that wild-type IRF-3 and its phosphomimetic forms are all monomers at a loading concentration of 40 μ M.

CBP promotes oligomerization of phosphomimetic IRF-3 mutated at S396D

To analyze the *in vitro* effect of CBP on the oligomerization of IRF-3, IRF-3 and its single-site phosphomimetic forms were incubated with CBP, and the resulting complexes were analyzed by size-exclusion chromatography. Only one complex, IRF-3 S396D/CBP, had a markedly different elution profile from the others (Figure 2b). IRF-3 S396D/CBP eluted as a single peak that was 3.0 fractions earlier relative to the peak of IRF-3/CBP, consistent with a molecular weight of around 65 kDa for the IRF-3 S396D/CBP complex. These results show that CBP can promote oligomerization of IRF-3 S396D at this loading concentration.

To further characterize the interactions between CBP and IRF-3, isothermal titration calorimetry (ITC) was used to analyze the thermodynamics and stoichiometry of heteromeric IRF-3/CBP complex formation. Our results show that CBP titrated into IRF-3 and its phosphomimetic forms at a relative molar ratio of 1:1 (Table 1), meaning that a single molecule of CBP can interact with one molecule of IRF-3 or its mutants. However, IRF-3 and its mutants exhibit markedly different binding affinities with CBP. The apparent dissociation constants (K_d) for CBP binding with IRF-3 and five single phosphomimetic mutants ranged from 0.39 to 7.38, μ M (Table 1), with changes in Gibbs free energy (ΔG) for complex formation with CBP ranging from -6.7 to -8.3 kcal/mol per subunit (Table 1). The results show that, among the single-site mutants, IRF-3 S396D has the strongest binding to CBP. The ITC experiments measure the sum of energies involved in the direct binding of CBP to IRF-3 and also any energy released due to oligomerization induced by CBP. Separating these two effects is not possible and the free energies reported reflect both contributions from direct binding between IRF-3 and CBP and any oligomerization that results.

Phosphomimetic forms of IRF-3 with multi-site mutations are monomers

To determine if the C-terminal Ser/Thr residues of IRF-3 influenced each other in IRF-3 phosphoactivation, the effects of multi-site C-terminal serine mutations in phosphorylation events were assessed. Purified phosphomimetic forms of IRF-3 Ser385/396D, Ser386/396D, Ser396/398D, and Ser385/386/396D were analyzed first by size-exclusion chromatography. All mutants eluted with purified IRF-3 as a single peak at fraction numbers 21–23, corresponding to an apparent molecular mass of 28 kDa (Figure 2c), indicating that these phosphomimetic forms of IRF-3 with double- or triple-site mutations are all monomers at loading concentrations of 40 μ M.

CBP promotes oligomerization of phosphomimetic IRF-3 with multi-site mutations at S385, S386, S396, and S398

To analyze the interactions of these phosphomimetic forms with CBP, they were incubated *in vitro* with CBP, and the resulting complexes were analyzed by size-exclusion chromatography.

All 4 complexes eluted as single peaks that were shifted earlier relative to the peak of IRF-3/CBP, with the relative shift increasing from smallest to largest in the following order: IRF-3 S385/396D/CBP, IRF-3 S385/386/396D/CBP, IRF-3 S396/398D/CBP, and IRF-3 S386/396D/CBP (Figure 2d), eluting with apparent molecular masses of 40, 65, 80, and 95 kDa, respectively, all at loading concentrations with 40 μ M. Taken together, these results show that CBP can promote the oligomerization of IRF-3 phosphomimetic forms with multi-site mutations.

To further characterize the interactions between these phosphomimetic forms of IRF-3 and CBP, ITC was used to analyze the thermodynamics and stoichiometry of heteromeric IRF-3/CBP complex formation. CBP titrated into IRF-3 phosphomimetic forms at a relative molar ratio of 1:1 (Table 1), meaning that a single molecule of CBP can interact with one molecule of mutated IRF-3. However, the IRF-3 mutants had different binding affinities for CBP that correlated with the level of oligomerization observed (Fig. 2D). The apparent CBP dissociation constants (K_d) for multi-site IRF-3 mutants ranged from 0.06 to 1.96 μ M (Table 1), with changes in Gibbs free energy (ΔG) for complex formation ranging from -7.5 to -9.5 kcal/mol per subunit (Table 1). Among the double-site mutants, IRF-3 S386/396D had the strongest binding to CBP and the highest propensity to oligomerize.

IRF-3 S386D strengthens the interaction between IRF-3 S396D and CBP

The results above show that IRF-3 S396D was the only single-site C-terminal serine mutant that showed significant oligomerization after binding to CBP (Figure 2b). Interestingly, when another mutation was added to create the double-site mutant, IRF-3 S386/396D, size exclusion chromatography suggested even greater assembly upon binding to CBP (Figure 2d). To more clearly show the effects of the S386D mutation on the interaction between IRF-3 S396D and CBP, four elution peaks were compared: IRF-3/CBP, IRF-3 S386D/CBP, IRF-3 S396D/CBP, and IRF-3 S386/396D/CBP. This comparison shows that the double-site mutant IRF-3/CBP complex eluted as a single peak that was shifted 3.2 fractions earlier relative to the peak of the single-site IRF-3/CBP complex (Fig. 3a). Thus, the S386D mutation in IRF-3 significantly strengthened CBP-promoted oligomerization of IRF-3 S396D.

Likewise, the results of the thermodynamic analyses of heteromeric IRF-3/CBP complex formation are compared in Table 1 and Figure 3 (b–c). Although a single molecule of CBP can interact with one molecule of IRF-3 or its phosphomimetic forms, their binding affinities are quite different. IRF-3 S386D (Figure 3b, left) bound to CBP with 3.6-fold greater affinity than IRF-3 (Figure 3b, right), whereas IRF-3 S386/396D (Figure 3c, left) bound to CBP with 6.5-fold greater affinity than IRF-3 S396D (Figure 3c, right), indicating that the S386D mutation increased the binding affinity between IRF-3 and CBP. However, an even more dramatic effect is seen by comparing the CBP-binding affinities of these phosphomimetic forms and native IRF-3. IRF-3 S396D and IRF-3 S386/396D bound to CBP with 18.9- and 123-fold greater affinities, respectively, than IRF-3 (Table 1). These results clearly show that S386D greatly strengthened the interaction between CBP and IRF-3 mutated at Ser 396.

IRF-3 S385D weakens the interactions of CBP with IRF-3 S396D and IRF-3 S386/396D

The effect of IRF-3 S385D is very different from that of IRF-3 S386D, also in site 1, when combined with the phosphomimetic mutant S396D. Size exclusion chromatography provides evidence of oligomerization of S396D upon binding (Figure 2b); whereas the double-site IRF-3 mutant, S385D/S396D elutes near that expected for a simple heterodimer of IRF-3 bound to CBP (Figure 2d). To more clearly show the effects of the S385D mutation on the interaction between IRF-3 S396D and CBP four elution patterns are shown in Fig. 4a: IRF-3/CBP, IRF-3 S385D/CBP, IRF-3 S396D/CBP, and IRF-3 S385/396D/CBP. This comparison shows that IRF-3 S385/396D/CBP eluted as a single peak that was shifted 2.2 fractions later relative to

the peak of IRF-3 S396D/CBP, indicating that mutating Ser 385 to Asp in IRF-3 inhibited CBP-promoted oligomerization of IRF-3 S396D.

Similarly, the effects of the S385D mutation on the interaction between IRF-3 S386/396D and CBP were examined by comparing the elution peaks of IRF-3/CBP, IRF-3 S385D/CBP, IRF-3 S386/396D/CBP, and IRF-3 S385/386/396D/CBP (Figure 4b). The results show that IRF-3 S385/386/396D/CBP eluted as a single peak shifted 3.5 fractions back relative to the peak of IRF-3 S386/396D/CBP, indicating that mutation of Ser 385 to Asp in IRF-3 also inhibited CBP-promoted oligomerization of IRF-3 S386/396D.

As above, the results of the thermodynamic analyses of heteromeric IRF-3/CBP complex formation are compared in Table 1 and Figure 4 (c–d). In isolation, the IRF-3 S385D mutation results in a modest (1.7 fold) increase in binding affinity with CBP over native IRF-3, however in combination with other phosphomimetic mutants, S385D shows an inhibitory effect. IRF-3 S396D (Figure 4c, left) bound to CBP with 5.0-fold greater affinity than IRF-3 S385/396D (Figure 4c, right), whereas IRF-3 S386/396D (Figure 4d, left) bound to CBP with 10.8-fold greater affinity than IRF-3 S385/386/396D (Figure 4d, right), indicating that the S385D mutation decreased the binding affinity between IRF-3 and CBP. These results clearly show that adding the S385D mutation to the single- and double-site mutant IRF-3 phosphomimetic forms, S396D and S386D/S396D, greatly weakens their interactions with CBP.

The IRF-3 C-terminal autoinhibitory domain is critical for oligomerization when phosphorylated, but is dispensable for IRF-3 to interact with CBP

Since IRF-3 phosphoactivation at residues in site 2 has been shown to alleviate autoinhibition, thus allowing interaction with CBP/p300,²⁰ we sought to clarify the role of the C-terminal autoinhibitory domain in IRF-3 activation. The CBP-binding affinities of three purified IRF-3 constructs were examined: a control construct with the autoinhibitory domain, IRF-3 (173–427); a construct without the autoinhibitory domain, IRF-3 (173–394); and a phosphomimetic autoinhibitory-domain construct, IRF-3 (173–427, with two mutations, S396D/S398D) (Figure 5a). All 3 IRF-3 constructs interacted with CBP, but with different binding affinities. IRF-3 (173–394) without the autoinhibitory domain interacted more strongly with CBP than IRF-3 (173–427), but more weakly than the phosphomimetic form of IRF-3 (173–427) (Figure 5b). Size-exclusion chromatography suggests that the complexes of CBP/IRF-3 (173–427) and CBP/IRF-3 (173–394) are simple heterodimers, but the complex of CBP/IRF-3 (173–427) S396/398D is a higher order oligomer (Figure 5c). Thus, phosphorylation of the C-terminal autoinhibitory domain of IRF-3 is clearly necessary for its oligomerization with CBP, but not for its interaction with CBP.

Discussion

The results presented here show that Ser386 and Ser396 play critical roles in IRF-3 phosphoactivation. Thus, IRF-3 activation depends on phosphorylation of serines in both site 1 (residues 385–386) and site 2 (residues 396–405). Furthermore, the phosphomimetic mutations alone did not lead to oligomerization of IRF-3; rather oligomerization required an interaction with CBP. This work provides the first direct detailed thermodynamic evidence for the interaction of CBP with IRF-3 phosphomimetic forms and subsequent oligomerization.

Based on the evidence presented here, Ser396 is the most important of the 7 C-terminal Ser/Thr residues long known to play a role in IRF-3 activation.^{4,5,9,10,15,16,20} The phosphomimetic IRF-3 mutated at S396D binds to CBP with the strongest affinity among the 7 single-site IRF-3 mutants and shows the clearest evidence of oligomerization with CBP. This result is consistent with the previous identification of Ser396 as a key IRF-3 site 2-residue that is phosphorylated *in vivo* upon virus infection.¹⁵

S386 is also important for IRF-3 activation. IRF-3 mutated at both S386 and S396 (IRF-3 S386/396D) not only produces a higher MW complex with CBP, but its binding to CBP is also strengthened by 6-fold. This result is consistent with several previous reports that phosphorylation at Ser385/386 (in site 1) is key to activation of IRF-3.^{9,16,18,26} Therefore, these findings suggest that IRF-3 activation depends on phosphorylation at both sites 1 and 2, with phosphorylation at site 2 (containing Ser396) playing an essential role, and phosphorylation at site 1 (containing Ser386) playing a complementary role. This finding is consistent with results indicating that site 1 (including Ser 385/386) and site 2 (Ser/Thr 396 to 405) are two classes of regulatory phosphorylation sites with differing effects on IRF-3 activity.^{18,20}

The phosphorylation site 1 in IRF-3 comprises two residues, Ser385 and Ser386. Despite their close proximity, phosphorylation of these two residues has opposite effects on IRF-3 activation. Single-site phosphomimetic mutants S385D and S386D do not bind strongly to CBP and do not subsequently oligomerize. When combined with S396D, S386D strengthens binding to CBP and subsequent oligomerization. In contrast, when combined with S396D, S385D inhibits binding to CBP and subsequent oligomerization (Table 1 and Figure 4). Thus, phosphorylation of Ser 385 appears to be antagonistic with the activation of IRF-3 through phosphorylation of Ser 396 and Ser 386.

CBP/p300 has been shown to be indispensable for this DNA-binding activity.²⁵ Therefore, it is very important to assess IRF-3 activation by determining its binding affinity for CBP/p300. Here, we demonstrate that CBP not only promotes oligomerization of the phosphomimetic form of IRF-3 S386/S396D, but also binds with 123-fold greater affinity to IRF-3 S386/S396 than to IRF-3. Taken together with previous results,^{5,17,20} this finding suggests that phosphorylation at Ser 386 and Ser 396 of IRF-3 alters its protein conformation to permit stronger binding to CBP/p300. It is worth noting that increasing the negative charge of IRF-3 by mutating Ser 396 to Asp or by mutating Ser 386 and Ser 396 to Asp did not result in IRF-3 oligomerization, but after interacting with CBP/p300, IRF-3 S396D and IRF-3 S386/396D oligomerized to form high level oligomers. However, studies on IRF-3 in which all five Ser/Thr residues in site 2 are mutated to the phosphomimetic Glu, and a residue from site 1 was constitutively phosphorylated during expression, did show evidence of dimerization in the absence of CBP/p300²⁰. Thus, a greater level of phosphorylation or phosphomimetic substitution than studied here may be required for dimerization in the absence of CBP/p300.

Our results show that the phosphorylated C-terminal region of IRF-3 plays a primary role in its oligomerization. As mentioned before, phosphorylation sites in C-terminus of IRF-3 are also located in the autoinhibitory region. Pseudophosphorylation of only 2 C-terminal serines, S386 and S396, in IRF-3 greatly changes its behavior from that of the unphosphorylated state. Unlike unphosphorylated IRF-3, its phosphomimetic form, IRF-3 S386/396D, forms a complex with CBP, and this complex has a strong propensity to oligomerize. This difference in behavior indicates that the phosphorylated C-terminal region of IRF-3 plays a primary role in its oligomerization, potentially by bridging subunit-subunit contacts. Phosphorylation at IRF-3 residues in site 2 has been suggested to alleviate inhibition by the C-terminal autoinhibitory domain (aa 395–427), thus allowing IRF-3 to interact with CBP/p300.^{18,20} Our results indicate that the presence of the autoinhibitory domain doesn't prevent interaction of IRF-3 with CBP/p300, but greatly reduces its binding affinity for CBP/p300. These results suggest that phosphorylation promotes release of the autoinhibitory region, and once released, CBP/p300 can bind more readily. The lower affinity for binding CBP/p300 to wild-type IRF-3 suggests that it does so by competing with the autoinhibitory region.

The differential effect of phosphorylation at each of these residues must reflect their different roles in the three-dimensional structure of IRF3 (Figure 1 B). Noteworthy is the position of

Ser 396, shown here to play a central role in activation. Ser 396 is located at the tip of the L6 loop of the autoinhibitory c-terminal region involved in a potentially favorable hydrogen bond with the main-chain amide nitrogen atom of Glu 201. It is tempting to suggest that Ser 396 acts to lock down this loop, which is required for masking the CBP/p300 binding site. Its location near two negatively charged residues (Glu 200 and Glu 201) suggests that acquisition of a negative charge by residue 396 would lead to charge repulsion that could release the L6 loop from its autoinhibitory position permitting CBP/p300 binding and oligomerization. The distinct effects of pseudo-phosphorylation of Ser 386 and 385 are more difficult to rationalize. These residues are located at the beginning of the autoinhibitory region and thus could be part of a hinge for unfolding the autoinhibitory region to expose the CBP/p300 binding surface. Both serines are largely exposed in the IRF3 structure and it is unclear how phosphorylation would lead to drastic structural changes. Perhaps, the differential effects for pseudo-phosphorylation of these residues result because of contacts within an oligomer that are favorable in the case of Asp 386 but unfavorable in the case of Asp 385.

In conclusion, the studies described here reveal the biochemical and calorimetric basis for the interaction between IRF-3 and its transcriptional coactivator CBP/p300 and shed light on the mechanism of IRF-3 activation, particularly the distinct contributions of individual residues to phosphoactivation. IRF-3 is constitutively expressed in all cell types, remaining in the cytosol in a latent, autoinhibited form until activated. Upon viral infection, IRF-3 is phosphorylated by two serine/threonine kinases known as TBK and IKK ϵ (references 10 and 11). Activation of IRF-3 depends on phosphorylation at both site 1 (including Ser386) and site 2 (including Ser396). Phosphorylation at Ser396 is essential for IRF-3 activation, and phosphorylation at Ser386 complements phosphorylation at site 2 and association with CBP/p300. After cytoplasmic IRF-3 is phosphorylated at Ser386 and Ser396, phosphorylated IRF-3 translocates into the nucleus where it binds to CBP/p300. This phosphorylated IRF-3/CBP/p300 complex then activates IFN and IFN-responsive genes.

Materials and methods

Protein Expression, Purification and Mutagenesis

The cDNA fragment of human IRF-3 (residues 173–427) was generated by PCR and subcloned into the pGEX-6p-1 vector (Amersham). The IRF-3 protein was expressed with a glutathione S-transferase (GST) tag, extracted by glutathione sepharose, and released by PreScission Protease (Pharmacia Biotech). Eluted protein was dialyzed in DEAE buffer (20 mM Tris, pH 7.3, 10 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], and 1 mM phenylmethanesulfonyl fluoride). Protein was purified using a DEAE-sepharose column equilibrated with DEAE buffer and eluted by a gradient of 10 to 300 mM NaCl. Site-directed mutants of IRF-3 (173–427) were generated using the Quik-Change kit (Stratagene) and confirmed by sequencing.

The cDNA fragment of CBP (residues 2067–2112) was generated by PCR and subcloned into the pGEX-6p-1 vector (Amersham). The GST-CBP fusion protein was expressed with a GST tag and extracted by glutathione sepharose. Eluted proteins were dialyzed in DEAE buffer. Protein was purified using an SP-sepharose column equilibrated with DEAE buffer and eluted by a gradient of 10 to 300 mM NaCl.

Isothermal Titration Calorimetry

To measure the binding of purified CBP (2067–2112) with IRF-3 and its mutants, isothermal titration calorimetry (ITC) was used²⁸ with a VP-ITC calorimeter (MicroCal, Northampton, MA). All samples were dialyzed against ITC buffer (20 mM HEPES, pH 7.3, 100 mM NaCl, 0.1 mM EDTA, 1 mM Tris (2-carboxyethyl) phosphine [TCEP]) and degassed prior to titration.

Experiments were performed at 25°C. Isothermal interactions between proteins were measured by titrating over 55 injections using 1.4 ml of 40 μ M protein against 300 μ l of 400 μ M CBP (2067–2112). The heats of dilution for CBP (2067–2112) were measured by titrating CBP (2067–2112) into ITC buffer; these values were subtracted for data analysis. Data were analyzed with Origin 7.0 software (MicroCal), using a single-site binding model. ΔH , ΔS , and K_a values were experimentally determined, and ΔG was calculated from the equation, $\Delta G = -RT \ln K_a$.

Size-Exclusion Chromatography

To investigate the stoichiometry of CBP binding to IRF-3 and its mutants, complexes were analyzed by size-exclusion chromatography on a Superdex 200 HR column using the Äkta Explorer 10 FPLC (fast protein liquid chromatography) system (Pharmacia Biotech, Superdex™ 200, 10/30). All runs were performed at room temperature in FPLC buffer (20 mM HEPES pH 7.3, 0.1 mM EDTA, 100 mM NaCl, and 1 mM DTT). 40 μ M IRF-3 or its mutants alone and the mixture of 40 μ M IRF-3 or its mutants with 120 μ M CBP were incubated overnight at 4 °C. Prior to loading onto the column, the samples were incubated in 1 mM TCEP for 60 min at room temperature. FPLC was operated and data were analyzed with UNICORN software. The column was calibrated with blue dextran (to determine void volume) and molecular weight standards ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa). Elution fractions (0.5 ml) were collected at room temperature at a flow rate of 0.5 ml/min.

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The abbreviations used are

CBP	CREB binding protein
FPLC	fast protein liquid chromatography
IRF	interferon regulatory factor
TCEP	Tris (2-carboxyethyl) phosphine
ITC	isothermal titration calorimetry

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Figure 1. Arrangement of the IRF-3 domains (A) and IRF-3 crystal structure¹⁷ (B)

(A) Arrangement of the IRF-3 domains. Regions known to participate in protein-protein interactions are indicated by colored boxes. The IRF-3 homology region studied in interaction analyses is indicated in the lower box. Seven Ser/Thr residues between 385 and 405 in the C-terminal region are highlighted in yellow. Site1 includes Ser385 and Ser386, Site2 includes Ser396, Ser398, Ser402, Thr 404, and Ser405. (DBD: DAN-binding domain; IAD: IRF-association domain; NLS: nuclear localization sequence; NES: nuclear export signal; AUD: autoinhibition domain). (B) The IRF-3 crystal structure. Potential phosphorylation sites of 7 Ser and Thr residues are in blue and red, residues in autoinhibitory domain (aa 380–427) are in purple.

Figure 2. CBP promotes oligomerization of phosphomimetic IRF-3 mutated at S396D, S386/S396D, S396/S398D, and S385/S386/S396D

Mixtures of IRF-3 or its C-terminal serine mutants and CBP (1:3 molar ratio) were loaded onto a size-exclusion column. **(a) Uncomplexed IRF-3 and its single-site mutants are monomers.** The elution peaks of IRF-3 and its single-site mutations are in fractions 22 to 24. **(b) CBP promotes oligomerization of IFR-3 mutated at S396D.** After incubation with CBP, the elution peak of IRF-3 S396D shifted 3.0 fractions forward relative to the peaks of IRF-3 and its single-site mutations. **(c) Uncomplexed IRF-3 multi-site mutants are monomers.** The elution peaks of IRF-3 with double- and triple-site mutations are in fractions 22 to 24. **(d) CBP promotes oligomerization of IFR-3 mutated at S386/396D, S396/398D, and S385/386/396D.** After incubation with CBP, the elution peaks of IRF-3 S386/396D, IRF-3 S396/398D, and ITF-3 S385/386/396D are shifted 6.0, 4.5, and 2.2 fractions forward (larger size), respectively, relative to the peaks of IRF-3 and other double-site mutations.

Figure 3. IRF-3 S386D strengthens the oligomerization and interaction of IRF-3 S396D with CBP
(a) IRF-3 S386D strengthens the oligomerization of IRF-3 S396D with CBP. Mixtures of IRF-3 or its C-terminal serine mutants and CBP (1:3 molar ratio) were loaded onto a size-exclusion column. After incubation with CBP, the elution peak of IRF-3 S386/396D shifted 3.0 fractions forward relative to the peak of IRF-3 S396D. **(b–c)**. The binding affinities of IRF-3 and its mutants for CBP were determined by isothermal titration calorimetry. CBP titrated into IRF-3 and its mutants at a relative molar ratio of 1:1. **(b)** IRF-3 S386D shows weak interaction with CBP. IRF-3 S386D has a 3- to 4-fold stronger binding affinity for CBP (right) than IRF-3 (left). **(c)** IRF-3 S386 strengthens the interaction of IRF-3 S396D with CBP. IRF-3 S386/S396D has a 6-fold stronger binding affinity for CBP (right) than IRF-3 S396D

(left). The thermodynamic parameters derived from data-fit analysis (MicroCal) are shown in Table 1.

Figure 4. IRF-3 S385D weakens the oligomerization and interaction of IRF-3 S396D and IRF-3 S386/S396D with CBP

(a – b). Mixtures of IRF-3 or its C-terminal serine mutants and CBP (1:3 molar ratio) were loaded onto a size-exclusion column. **(a) IRF-3 S385D inhibits oligomerization of IRF-3 S396D with CBP.** After incubation with CBP, the elution peak of IRF-3 S396D shifted 2.7 fractions forward relative to the peak of IRF-3 S385/S396D. **(b) IRF-3 S385D inhibits oligomerization of IRF-3 S386/S396D with CBP.** After incubation with CBP, the elution peak of IRF-3 S386/S396D shifted 4 fractions forward relative to the peak of IRF-3 S385/S386/S396. **(c–d).** The binding affinities of IRF-3 and its mutants for CBP were determined by isothermal titration calorimetry. CBP titrated into IRF-3 and its mutants at a relative molar ratio of 1:1. **(c) IRF-3 S385D decreases the affinity of IRF-3 S396D binding to CBP by 5-fold.** IRF-3 S396D binds to CBP with a 5-fold stronger affinity (left) than IRF-3 S385/S396D (right). **(d) IRF-3 S385 decreases the affinity of IRF-3 S386/S396D binding to CBP by 11-fold.** IRF-3 S386/S396D binds to CBP with an 11-fold stronger affinity (left) than IRF-3 S385/S386/S396D (right). The thermodynamic parameters derived from data-fit analysis (MicroCal) are shown in Table 1.

Figure 5. Phosphorylation of the C-terminal autoinhibitory domain of IRF-3 is necessary for its oligomerization with CBP, but not for IRF-3 to bind with CBP

a) Three constructs of IRF-3. IRF-3 (173–427) with autoinhibitory domain, *top*; IRF-3 (173–394) without autoinhibitory domain, *middle*; phosphomimetic form of IRF-3 (173–427, with mutations S396D/S398D), *bottom*. **(b) Three constructs of IRF-3 interact with CBP, but with different affinities.** The estimates for binding affinity of CBP to IRF-3 (173–427) and IRF-3 S396/398D are from ITC data; the estimate for IRF-3 (173–394) is based on the level of expression from bands on an SDS gel when IRF-3 (173–427) or IRF-3 (173–394) was co-expressed with CBP. **(c) CBP promotes oligomerization of phosphomimetic IRF-3, but not of the control autoinhibitory domain.** Complexes of 3 IRF-3 constructs with CBP were injected into a size-exclusion column, and eluted fractions were analyzed by SDS-PAGE. Molecular-weight standards are shown at *top*; fraction numbers are shown at *bottom*. Gels were stained with Coomassie blue.

Table 1
Summary of thermodynamic results for interactions of CBP with IRF-3 and its mutants

Complex	<i>n</i>	ΔH <i>kcal/mol</i>	ΔG <i>kcal/mol per subunit</i>	$-T\Delta S$ <i>kcal/mol</i>	K_d <i>apparent</i> μM	<i>-fold</i>
CBP-IRF-3	1.05 ± 0.09	-5.4 ± 1.1	-7.0 ± 0.2	-1.6	7.38 ± 0.84	1.0
CBP-IRF-3 (S385D)	1.06 ± 0.15	-2.8 ± 0.3	-7.3 ± 0.5	-4.5	4.22 ± 0.35	1.7
CBP-IRF-3 (S386D)	1.04 ± 0.06	-8.5 ± 1.3	-7.8 ± 0.2	0.7	2.07 ± 0.71	3.6
CBP-IRF-3 (T402D)	0.96 ± 0.12	-4.8 ± 1.2	-8.3 ± 0.3	-3.5	0.87 ± 0.06	8.5
CBP-IRF-3 (T404D)	1.02 ± 0.09	-6.6 ± 1.2	-8.3 ± 0.1	-1.7	0.80 ± 0.04	9.2
CBP-IRF-3 (S396D)	1.02 ± 0.01	-16.1 ± 1.4	-8.8 ± 0.1	7.3	0.39 ± 0.08	18.9
CBP-IRF-3 (S385/396D)	0.98 ± 0.06	-9.4 ± 0.4	-7.8 ± 0.2	1.6	1.96 ± 0.34	3.7
CBP-IRF-3 (S396/398D)	1.08 ± 0.12	-8.6 ± 0.9	-8.7 ± 0.1	-0.1	0.44 ± 0.05	16.8
CBP-IRF-3 (S386/396D)	1.02 ± 0.05	-13.9 ± 1.0	-9.9 ± 0.1	4.0	0.06 ± 0.02	123.0
CBP-IRF-3 (S385/386/396D)	1.07 ± 0.21	-12.9 ± 1.5	-8.5 ± 0.8	4.4	0.65 ± 0.09	11.4