Identification of Ser-386 of Interferon Regulatory Factor 3 as Critical Target for Inducible Phosphorylation That Determines Activation*

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Interferon regulatory factor (IRF)-3 is a critical transcription factor regulating innate immune responses against viral and bacterial infections. Signals activated by various pathogens are integrated by IRF-3 kinase, resulting in the specific phosphorylation of IRF-3 in the cytoplasm. This phosphorylation induces dimerization and association with the coactivators CREB-binding protein/p300, and the resultant complex activates the target genes in the nucleus. However, the phosphorylation sites that determine the active/inactive status of IRF-3 have been a source of controversy. In this study, we generated an antibody that specifically detects the phosphorylation of Ser-386 and used it as a probe. We found: 1) viral infection specifically induces phosphorylation of the Ser-386; 2) recently identified IRF-3 kinases (IKK-i/ε and TBK-1) phosphorylate Ser-386 and induce its dimerization; 3) phosphorylation of Ser-386 is exclusively observed with the dimer; 4) mutation at Ser-386 abolishes the dimerization potential; 5) a constitutively active 5D mutant designed to mimic phosphorylation of Ser/Thr residues other than Ser-385 and -386 is secondarily phosphorylated at Ser-386, presumably by an irrelevant kinase. These results strongly suggest that Ser-386 is the target of the IRF-3 kinase and critical determinant for the activation of IRF-3.

Immediately after infection by bacteria or viruses, a series of host responses known as innate immunity takes place. Production of type I interferon (IFN-α/β) is induced by these pathogens and plays an important role in primary defense as well as subsequent adaptive immunity (1–3). IFN-α/β genes are normally silent, but their expression is dramatically induced at the transcriptional level following infection. Regulatory transcription factors IRF-3 and IRF-7 play a critical role in this regulation (4). IRF-3 is ubiquitously expressed in its active form in the cytoplasm. Upon signaling triggered by the infection, cellular kinases are activated and result in the phosphorylation of IRF-3 on specific Ser residues (5–9). Recently, IKK-i (also known as IKK-ε) and TBK-1 were shown to function as IRF-3 kinases (10, 11). The phosphorylated IRF-3 undergoes homodimerization (12, 13) and associates with coactivators CBP/p300, and then a holocomplex of IRF-3 results (5, 12). The holocomplex is conferred an ability to specifically recognize the target DNA sequence and accumulate in the nucleus (5, 14, 15). Thus, IRF-3 plays a major role in the primary production of IFN-α/β. In contrast, IRF-7 is normally absent, and its expression is secondarily induced by IFN-α/β (4). Like IRF-3, IRF-7 needs to be specifically phosphorylated to become active, presumably by the same kinases (6, 16, 17). Thus, unlike IRF-3, IRF-7 plays a role in the later amplification phase of the gene expression. The primary structure of IRF-7 shows that it is the closest relative of IRF-3. Notably, the SSL motif, which is indispensable for their activity, is conserved among IRF-3 from different species and IRF-7 (see Fig. 1A) (16, 17).

The exact phosphorylation site of IRF-3 is the subject of some controversy (5, 9, 12, 13, 18). However, extensive mutagenesis has identified critical Ser residues. The mutation of Ser-385 or -386 to Ala totally abolished the activity of IRF-3 (5, 12, 13, 18), whereas the substitution of Ser/Thr on the 5ST site also resulted in an active IRF-3 (19). The initial observation that the deletion of the C-terminal region results in constitutive phosphorylation and the 5ST site is the target for inducible phosphorylation. More recently, they showed that Ser-396 is phosphorylated by virus-activated kinase in vivo (19) and is the target of IKK-i and TBK-1 in vitro (11). Furthermore, they made “phospho-mimetic” mutants, 5D, S396D and S396DS398D, which have substitutions of Ser with Asp at the 5ST site, Ser-396 is phosphorylated by virus-activated kinase in vivo (19) and is the target of IKK-i and TBK-1 in vitro (11). Furthermore, they made “phospho-mimetic” mutants, 5D, S396D and S396DS398D, which have substitutions of Ser with Asp at the 5ST site, Ser-396, and Ser-396/398, respectively. These mutants constitutively dimerize, associate with CBP/p300, translocate to the nucleus, and activate the reporter gene (19). The initial observation that the deletion of the C-terminal region including the 5ST site also resulted in an active IRF-3 (12) led them to propose an “auto-inhibition” model in which the C-terminal region acts as a steric intramolecular inhibitor and blocks DNA binding and dimerization. According to their model, the inhibition is reversed by the phosphorylation of the 5ST site, which confers negative charges to the inhibitory domain, resulting in a dramatic conformational change (12).

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1 The abbreviations used are: IFN, interferon; IRF, interferon regulatory factor; IKK, IκB kinase; TBK, TANK binding kinase; CBP, CREB binding protein; CREB, cAMP-response element-binding protein; NDV, Newcastle disease virus; LPS, lipopolysaccharide; TRIF, TIR domain-containing adapter-inducing IFN-β; TICAM-1, TIR-containing adapter molecule.
RESULTS

Although mutagenesis studies show the importance of the 2S site (13), its phosphorylation status was not rigorously investigated so far. To investigate this issue, we immunized a rabbit with a synthetic peptide corresponding to amino acids 380–389 of human IRF-3 in which Ser-386 was phosphorylated (Fig. 1A). To assess the specificity of the antibody (anti-p-386), a tagged IRF-3 was expressed in 293T cells and activated by infection with NDV. NDV infection resulted in the formation of IRF-3 dimer as revealed by native PAGE analysis (Fig. 1B, anti-p50 blot). Anti-p-386 selectively detected the induced dimer, and the reactivity was completely blocked by the immunogen peptide but not by unphosphorylated peptide or peptide phosphorylated at Ser-385 or at Ser-385/Ser-386, showing that anti-p-386 is specific to phosphorylation at Ser-386 (Fig. 1B, anti-p-386). We next examined the phosphorylation of endogenous IRF-3 using anti-p-386. Human glioma cell line U373/CD14 (23) was induced by NDV infection (Fig. 1C, Exp. 1) or with poly(I:C) or LPS (Fig. 1C, Exp. 2); the latter two stimulate TRIF and -4, respectively. Anti-IRF-3 blot detected dimerization of endogenous IRF-3 (anti-NES) after these stimuli. Anti-p-386 selectively detected the induced dimer (anti-p-386). These results show that irrespective of different stimuli, the same residue, Ser-386, is phosphorylated in correlation with the dimerization. Next, wild type and various mutant human IRF-3 were transiently expressed in mouse L929 cells, and their virus-induced dimerization and phosphorylation at Ser-386 was examined. Wild type IRF-3 was strongly phosphorylated at Ser-386 and dimerized after virus infection. Mutants S396A and 3A behaved virtually as wild type, indicating that these residues are not critical for IRF-3 activation, whereas the mutation at 2S site totally abolished the dimerization potential of IRF-3 mutants (2A and 2D). Although the 5A mutant dimerized less efficiently than the wild type, a significant portion of this mutant was dimerized by viral induction with concomitant phosphorylation at Ser-386 (5A). We do not know the reason for the less efficient phosphorylation of the mutant. Either conformational change due to the substitution of multiple residues or involvement of these residues in phosphorylation (see “Discussion”) is a possibility. Although the 2D mutation did not result in constitutive formation of the dimer, a fraction of 5D is expressed as dimer without induction, and further induction by virus increased the dimer fraction. Interestingly, 5D was constitutively phosphorylated at Ser-386, and the phosphorylation and the dimerization were further enhanced by viral induction. The additional mutation at the 2S site abolished the constitutive and induced dimerization (2A/5D). In summary, these results indicate that phosphorylation of Ser-386 is the key determinant for subsequent dimer formation.

Recently Servant et al. (19) identified Ser-396 and -398 as minimal phosphorylation sites critical for activation, based on their observation that S396D and S396D,S398D are constitutively active. These mutants along with 5D were expressed in 293T cells and analyzed by native PAGE (Fig. 2). As expected, a majority of 5D was expressed as a dimer in unstimulated cells (anti-p50 tag). S396D, S398D and S396D, which are partially substituted at the 5S site with Asp, resulted in the formation of a dimer, albeit less efficiently. Probing the same blot with anti-p-386 revealed that specific phosphorylation was observed exclusively on the dimer, indicating a strong correlation between the phosphorylation at Ser-386 and the dimer formation.

Recently, it was reported that the IKK family of protein kinases, IKK-1 and TBK-1, function as IRF-3 kinases and are responsible for the activation (10, 11). Furthermore, it was demonstrated that a signaling adapter molecule TICAM-1/TRIF plays a critical role in the activation of IRF-3 induced by...
Fig. 1. Phospho-specific antibody for the Ser-386 of IRF-3. A, sequence alignment of IRF-3 from various species and human IRF-7. The 2S and 5ST sites are shown as bold and outlined letters, respectively. B, specificity of anti-p-386 antibody. Human IRF-3 with a p50 tag was transiently expressed in 293T cells and mock-treated (−) or infected with NDV (+) for 12 h. The cell extract was subjected to native PAGE and transferred to Immobilon membranes. Probes were as follows: anti-p50 tag (lanes 1 and 2); anti-p-386 (lanes 2 and 3); anti-p-386 preincubated with Critical Phosphorylation Site of IRF-3
stimuli through TLR-3 and 4 (22, 24). When tagged wild type IRF-3 was co-expressed with IKK-i or TBK-1 or TICAM-1/TRIF in 293T cells, an efficient dimerization of IRF-3 was observed, confirming the published results (Fig. 3A). Probing the blot with anti-p-386 showed that the kinases and the adapter induced phosphorylation at Ser-386 with concomitant formation of the dimer. These results are consistent with the result of Fig. 1B that irrespective of different trigger, the signal is integrated at IRF-3 as phosphorylation at the critical Ser-386. Furthermore, 5A is phosphorylated at Ser-386 by TBK-1 and dimerized (Fig. 3B), excluding the possibility that the induced dimerization is due to phosphorylation at the 5ST site. These results strongly suggest that TBK-1 phosphorylates the critical Ser-386, then resulting in the active dimer.

Finally, we tested phosphorylation of Ser-396 using phospho-specific antibody used in recent reports (11, 19). Tagged wild type IRF-3 was expressed in 293T cells and stimulated by NDV infection or co-expression of TBK-1 (Fig. 4). Anti-tag antibody clearly detected the generation of the dimer in response to the stimuli (lanes 1–4). The Ser396P antibody exhibited a strong reactivity to the IRF-3 monomer as well as to the dimer (lanes 5–8). Because the antibody exhibited a similar reactivity to S396A (data not shown), it is likely that the antibody preparation contains a high level of antibody reactive to unphosphorylated IRF-3. Due to this undesirable reactivity, we were unable to detect the phosphorylation at Ser-396 with this antibody. Conversely, another antibody prepared by immunizing peptide phosphorylated Ser-385/386, independently prepared by Hiscott and Servant, exclusively reacted with the dimer (lanes 9–12). A blocking experiment similar to the Fig. 1B showed that this antibody is specific to phosphorylation at Ser-386 and Ser-385/Ser-386 but not Ser-385 (data not shown). Furthermore, the antibody detected constitutive phosphorylation of the dimer in the context of 5D (data not shown).

**DISCUSSION**

We found that the Ser-386 is phosphorylated dependent on stimulation by virus infection or treatment with double-stranded RNA or LPS, using a novel phospho-specific antibody. The phosphorylation is also induced by the co-expression of either of two recently identified IRF-3 kinases, IKK-i and TBK-1. A strong correlation between the phosphorylation of Ser-386 and dimerization was observed. Moreover, the 5A mutant, in which candidate phospho-acceptor sites other than 2S are substituted with Ala, is strongly phosphorylated at Ser-386 by TBK-1 and dimerized (Fig. 3). We conclude therefore that IRF-3 is phosphorylated by signal-activated IRF-3 kinases at Ser-386, and this phosphorylation directly determines whether a dimer and subsequently a holocomplex forms. This is consistent with the results of mutagenesis (Fig. 1D) (13, 18). Substitution of Ser-385 to Ala abolishes virus-induced activation of IRF-3 (5, 13) raising a possibility that Ser-385 is necessary for the recognition by IRF-3 kinases, rather than being a phospho-acceptor site.

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Servant et al. (19) proposed Ser-396 as the critical phospho-acceptor for IRF-3 activation. The bases of their conclusion are: 1) Ser-396 is phosphorylated in a viral induction-dependent manner as shown by the use of anti-IRF-3 Ser396P and 2) a phospho-mimetic mutant S396D is constitutively active. During the course of the investigation, we found that the mutants 5D, S396D, and S396D,S389D are constitutively phosphorylated at Ser-386 in 293T cells. Thus, the observed activity is not due to phospho-mimicry of the Asp residues but a peculiar secondary effect of phosphorylation of the critical 2S site by an unknown kinase. Furthermore, the observation that the anti-IRF-3 Ser396P exhibits a strong reactivity to unphosphorylated IRF-3 (Fig. 4) poses a question on the results obtained by the antibody. In sum, the issue of Ser-396 phosphorylation remains for further investigation.

An interesting possibility is that the 5ST site constitutes the primary site of phosphorylation by the activated kinase, and a constitutively active kinase recognizes the 5ST-phosphorylated IRF-3 as substrate, resulting in the phosphorylation of the 2S site. In this case, the 5D mutant may mimic the primary phosphorylation. However, this multistep phosphorylation model is inconsistent with the observation that the 5A mutant, which should not be the substrate for the primary kinase, dimerized and associated with CBP/p300 and activated transcription after viral stimulation (Fig. 1D) (13, 18). Alternatively, phosphorylation within the 5ST site may induce a subtle conformational change, resulting in a better substrate for the induced IRF-3 kinase.

Recently, we determined the crystal structure of IRF-3(175–427), which retains the minimum structure needed for virus-induced phosphorylation and dimerization (25). Although the crystallized IRF-3(175–427) was not phosphorylated, it formed a dimer. Dimerization is an intrinsic property of IRF-3(175–427) since IRF-3 exhibited monomer-dimer equilibrium in solution with $K_d = 300–400 \mu M$. The two subunits were connected by the interaction of a loop that contains Ser-386 with a hydrophobic pocket encircled by basic residues. These results prompted us to propose a novel model, that specific phosphorylation of the 2S site of IRF-3 induces dimerization by increasing the interaction between the loop and the pocket rather than reversing intramolecular autoinhibition. This model is consistent with the present results and the observation that the pocket structure is indispensable for the activation (25).

Furthermore, the model is evolutionarily sound since the overall structure of IRF-3(175–427) is highly similar to the MH2 (MAD homology domain 2) domain of Smad, whose regulation involves the interaction of phosphorylated residues with its acceptor pocket.

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