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Phosphorylation of the Oncogenic Transcription Factor Interferon Regulatory Factor 2 (IRF2) In Vitro and In Vivo

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Abstract IRF2 is a transcription factor, possessing oncogenic potential, responsible for both the repression of growth-inhibiting genes (interferon) and the activation of cell cycle-regulated genes (histone H4). Surprisingly little is known about the post-translational modification of this factor. In this study, we analyze the phosphorylation of IRF2 both in vivo and in vitro. Immunoprecipitation of HA-tagged IRF2 expressed in ^{32}P-phosphate labelled COS-7 cells demonstrates that IRF2 is phosphorylated in vivo. Amino acid sequence analysis reveals that several potential phosphorylation sites exist for a variety of serine/threonine protein kinases, including those of the mitogen activated protein (MAP) kinase family. Using a battery of these protein kinases we show that recombinant IRF2 is a substrate for protein kinase A (PKA), protein kinase C (PKC), and casein kinase II (CK2) in vitro. However, other serine/threonine protein kinases, including the MAP kinases JNK1, p38, and ERK2, do not phosphorylate IRF2. Two-dimensional phosphopeptide mapping of the sites phosphorylated by PKA, PKC, and CKII in vitro demonstrates that these enzymes are capable of phosphorylating IRF2 at multiple distinct sites. Phosphoaminoacid analysis of HA-tagged IRF2 immunoprecipitated from an asynchronous population of proliferating, metabolically phosphate-labelled cells indicates that this protein is phosphorylated exclusively upon serine residues in vivo. These results suggest that the oncogenic protein IRF2 may be regulated via multiple pathways during cellular growth. J. Cell. Biochem. 66:175–183, 1997.

Key words: phosphorylation; interferon regulatory factor 2; transcription factor; oncogene

Interferon regulatory factors (IRFs) belong to a family of transcription factors originally identified as regulators of interferon (IFN) and interferon-stimulated genes. These proteins recognize and bind to specific DNA elements, typified by the positive regulatory domains (PRD) I and III of the IFN-β gene [Miyamoto et al., 1988]. IRF1 is known to activate the transcription of the IFN-β gene. This factor is barely detectable in unstimulated cells, and its levels of expression rise dramatically upon viral infection or IFN treatment. On the other hand, IRF2, which is also inducible by IFN and virus, is present in higher amounts in unstimulated cells, and is known to repress the transcriptional activation of the IFN-β gene by IRF1 [Harada et al., 1989]. While IRF1 and 2 knockout mice appear normal, more subtle phenotypes, including extremely reduced CD8+ cells (IRF1) and increased susceptibility to some viral infections (IRF2), are observed [Matsuyama et al., 1993; Reis et al., 1994; Kimura et al., 1994]. Overexpression of IRF2 causes the oncogenic transformation of NIH-3T3 cells, which can be blocked upon co-expression with IRF1 [Harada et al., 1993]. These data reveal that IRF1 possesses tumor suppressor activity, whereas IRF2 has oncogenic potential.

Consistent with the finding that IRF2 can be associated with cell growth control, recent reports indicate that IRF2 can function as a transcriptional activator of a human H4 histone gene (FO108) that is functionally coupled to cell

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cycle progression [Vaughan et al., 1995]. This gene, encoding a critical structural and functional component of eukaryotic chromatin, has been used as a model for the transcriptional control of gene expression at the G1/S phase transition point of the cell cycle [reviewed in Osley, 1991; Stein et al., 1996]. The transcription of this histone gene becomes activated three- to fivefold upon entry of cells into S phase, and this activation is dependent upon an 11 base pair sequence found in the proximal region of the promoter [Ramsey-Ewing et al., 1994]. This sequence, termed the cell-cycle element (CCE) is, in fact, an IRF consensus binding sequence [Vaughan et al., 1995]. Overexpression of either IRF1 or IRF2 specifically activates transcription from the H4 histone promoter in transient transfection assays in several mammalian cell lines. These data were the first to demonstrate that wild-type IRF2 can function as a transcriptional activator, and suggest a link between IRF2 and cellular growth control.

Despite the fact that IRF1 and IRF2 are believed to play a role in cell growth and oncogenesis, surprisingly little is known about the post-translational modification of these proteins. Phosphorylation of transcription factors often plays a key role in modulating DNA-binding and/or functional activity. IRF1 is known to be phosphorylated in vivo, and the dephosphorylation of this protein in vitro results in a reduction in its DNA-binding activity [Pine et al., 1990]. In addition, treatment of cells with the serein/threonine protein kinase inhibitor staurosporine results in a decrease in IRF1 activated transcription [Watanabe et al., 1991]. However, no data are available concerning the phosphorylation of IRF2 in any system. In this study, we analyze the phosphorylation of the oncogenic factor IRF2 both in vivo and in vitro. Our results show that IRF2 exists as a phosphoprotein in living cells, and suggest that multiple protein kinase pathways may impinge upon the regulation of IRF2 during cellular growth.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Transfection

COS-7 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS). DNA transfection was carried out on six-well plates using the DEAE dextran method according to the protocol of Ausubel et al. [1993].

Plasmid Constructions and Isolation of Recombinant Proteins

cDNA encoding the human IRF2 gene [Harada et al., 1989] was cloned into several different vectors for expression in either *Escherichia coli* or mammalian cells. For expression as fusion proteins in bacteria, IRF2 cDNA was cloned in frame into the *BamH1* site of pGEX 5X-1 (Pharmacia, Gaithersburg, MD) as a glutathione-S-transferase (GST) fusion protein, or into the *BamH1* site of pQE-32 (Qiagen, Chatsworth, CA) as a his-tagged fusion protein. For studies requiring the phosphorylation of IRF2 in vivo, the IRF2 cDNA was modified at both ends with *BamH1* sites using PCR, and cloned in frame into the *BamH1* site of a version of pcDNAI/AMP (Invitrogen, La Jolla, CA) that contained an HA tag (P. Bangs, personal communication). Purification of fusion proteins was carried out using established procedures [Ausubel et al., 1993]. When these recombinant proteins were used as substrates for protein kinases, the final pellets were washed three times in protein kinase buffer (20 mM Tris, pH 7.4, 7.5 mM $MgCl_2$, and 1 mM DTT) prior to use.

Immunoprecipitation and Metabolic Labelling of Cells

Two days following transfection of plasmid DNAs, COS-7 cells in six-well culture plates were labelled with either ^{35}S or ^{32}P. For ^{35}S labelling, cells were grown to 70% confluence, washed twice in methionine-free DMEM (Gibco/BRL, Gaithersburg, MD) containing 5% dialyzed FCS, and cultured in this medium for 30 min. After this period, the medium was removed and replaced with fresh medium containing ^{35}S-methionine (0.2 mCi/ml, ICN, Irvine, CA), and the cells were incubated with CO_2 at 37°C for 3 h, after which time the cells were harvested and lysed for immunoprecipitation as described below. For ^{32}P labelling, cells were transfected and grown as described above, washed twice in phosphate-free DMEM (Gibco/BRL) containing 10% dialyzed FCS, and cultured in this medium for 30 min. This medium was then removed and replaced with fresh medium containing ^{32}P-inorganic phosphate (ICN, 1 mCi/ml), and the cells were incubated with CO_2 at 37°C for 4 h. The cells were then lysed and harvested for immunoprecipitation by the addition (1 ml) of ice cold RIPA buffer (1%

NP40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 0.5 mM PMSF, 0.01 mg/ml TPCK, 0.7 µg/ml Pepstatin, and 0.5 µg/ml Leupeptin). The cells were scraped from the plate and allowed to sit on ice for 20 min. The resulting lysate was collected into Eppendorf tubes and centrifuged at 15,000*g* for 20 min. The supernatant was recovered and used in subsequent immunoprecipitation steps.

All immunoprecipitations were carried out in RIPA buffer according to established procedures [Ausubel et al., 1993]. Briefly, the 1 ml supernatants were pre-cleared for non-specific binding by treating with 1 µg of non-specific antibody and 20 µl of Protein-G Plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA). After a brief centrifugation, specific monoclonal HA antibodies were added to these supernatants and incubated on ice for another hour. Another 20 µl of Protein-G Plus Agarose were added, and the tubes were placed at 4°C for another hour with gentle rotation. After another brief centrifugation, the pellets were recovered and washed three times with RIPA buffer. For immunoprecipitations of HA-tagged IRF2 labelled in vivo, the samples were run immediately on SDS-PAGE [Laemmli, 1970]. These gels were then dried and exposed to X-ray film. For immunoprecipitations of HA-tagged protein kinases, the washed pellets were then washed three more times with protein kinase buffer (see above).

Protein Kinase Assays

Bacterially expressed GST-IRF2 or his-IRF2 were used as substrates to challenge several different protein kinase preparations. These protein kinases were obtained either in purified form commercially, or as crude immunoprecipitated proteins (see above). For experiments involving PKA, 3 µg of recombinant IRF2 was incubated in a final volume of 25 µl containing 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 50 µM ATP, 10 µCi of ^{32}P-ATP, and 5 U of purified PKA catalytic subunit (Sigma, St. Louis, MO). For experiments with CK2, 3 µg of recombinant IRF2 was incubated in the same volume containing 25 mM Tris (pH 7.4), 10 mM $MgCl_2$, 100 µM ATP, 10 µCi of ^{32}P-ATP, and 20 ng of purified CK2 (Promega, Madison, WI). PKC experiments were carried out in the same volume containing 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mg/ml phosphatidyl serine, 0.02 mg/ml diacylglycerol, 25 µM ATP, 10 µCi ^{32}P-ATP, and 0.25 µU/µl purified PKC (Boehringer, Indianapolis, IN). For experiments requiring HA-tagged ERK2, JNK1, or p38, the immunoprecipitated pellets and the GST-IRF2 pellets were washed in protein kinase buffer, and assayed according to previous methods [Whitmarsh et al., 1995]. All reactions were run at 37°C for 15 min and stopped by the addition of SDS-PAGE loading buffer. The samples were then subjected to SDS-PAGE and autoradiography.

Two-Dimensional Phosphopeptide Mapping

Phosphopeptide mapping of in vitro phosphorylated IRF2 was accomplished using a modification of methods described previously [Boyle et al., 1991]. Briefly, proteins phosphorylated in vitro were run on 10% SDS-PAGE gels, and the phosphorylated IRF2 protein was excised from the gel following autoradiography. The gel slice was washed twice with distilled H_2O, and then incubated at room temperature for 30 min in N-ethylmorpholine (NEM, 25 mM, pH 8.0). This solution was removed and replaced with fresh NEM containing 2 µg/ml trypsin and incubated overnight at 37°C. The next day, the solution was removed from the gel slice, stored at 4°C, and another ml of trypsin/NEM was added to the gel slice. This process was repeated 2 more times, and the recovered trypsin/NEM solutions were pooled and evaporated using a speed-vac. The dried samples were re-dissolved in 3 µl of 30% formic acid, spotted onto a TLC-cellulose plate, and electrophoresed using low pH buffer (8% acetic acid, 2.2% formic acid). Following electrophoresis, the plates were allowed to air dry overnight, and were then subjected to chromatography for 2 h in Buffer A (37.5% n-Butanol, 7.5% acetic acid, and 25% pyridine). Following chromatography, the plates were again allowed to air dry, and were subjected to autoradiography.

Phosphoamino Acid Analysis

Phosphoamino acid analysis of in vivo phosphorylated, HA-tagged, and immunoprecipitated IRF2 was carried out as described previously [Boyle et al., 1991] with some modifications. Briefly, immunoprecipitated IRF2 was recovered from ^{32}P-labelled COS-7 cells as described above, and subjected to SDS-PAGE and autoradiography. The region of the gel con-

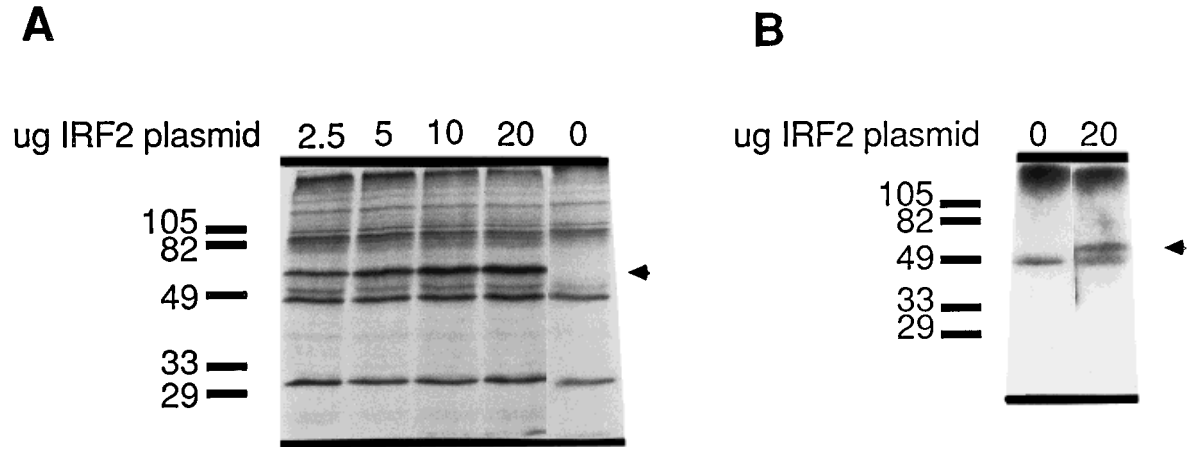

Fig. 1. IRF2 is phosphorylated in vivo. HA-IRF2 was immunoprecipitated from metabolically labelled COS-7 cells and was subjected to SDS-PAGE followed by autoradiography. **A:** Cells were transfected with a control plasmid (**lane 0**) or with increasing quantities of the plasmid overexpression HA-IRF2 before labelling with ^{35}S-methionine. **B:** Cells were transfected with a control plasmid (**lane 0**) or with 20 µg of the plasmid overexpressing HA-IRF2 before labelling with ^{32}P. The arrowheads to the right identify the specific labelled HA-IRF2 protein. The numbers to the left represent MW (kDa).

taining the phosphorylated IRF2 was excised and boiled for 1 h in 400 µl of 6M HCl. The tube was centrifuged briefly, and 500 µl of distilled water was added to the recovered supernatant. This radioactive mixture was dried in a speed vac, resuspended in 1 ml of distilled water, and dried again. The dried pellet was resuspended in 10 µl of buffer B (5% acetic acid, 0.5% pyridine, pH 3.5) containing unlabelled phosphoserine, phosphothreonine, and phosphotyrosine. The sample was applied to a TLC plate, and electrophoresed for 1.5 h in buffer B without added amino acids. The plates were air dried, sprayed with ninhydrin to visualize the unlabelled phosphoamino acid, and subjected to autoradiography.

RESULTS

IRF2 Is Phosphorylated In Vivo

To determine whether human IRF2 is phosphorylated in living cells, we carried out immunoprecipitation experiments on metabolically labelled cells. Because the endogenous IRF2 signal is subthreshold in non-IFN stimulated cells using conventional detection (data not shown), we expressed recombinant IRF2 as an epitope (HA)-tagged fusion protein in cultured cells, and utilized the HA antibody to precipitate the expressed protein. Figure 1A shows anti-HA immunoprecipates from transfected cells metabolically labelled with ^{35}S-methionine. Cells transfected with a control plasmid (Fig. 1A, lane 0) displayed no immunoprecipitated band in the predicted molecular weight range (~55 kDa) for the HA-IRF2 fusion protein. However, titration of increasing quantities of the HA-IRF2 plasmid during the transfection resulted in the appearance of increasing quantities of the 55 kDa labelled protein (Fig. 1A, lanes 2.5–20). These results confirmed the expression HA-IRF2 in COS cells. We therefore carried out a similar experiment using ^{32}P labelling (Fig. 1B). In the presence of a transfected control plasmid, only a non-specific band was observed (Fig. 1B, lane 0). However, in the presence of the HA-IRF2 transfected plasmid, we observed a new phophoprotein in the predicted size range (Fig. 1B, lane 20). These results demonstrate that recombinant, overexpressed IRF2 is phosphorylated in living cells.

Recombinant IRF2 Is Phosphorylated by Multiple Protein Kinases In Vitro

Computer analysis of the IRF2 amino acid sequence revealed multiple potential phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), casein kinase II (CK2), and the MAP kinases, and a single potential sites for cdc2 (data not shown). We therefore challenged this battery of protein kinases in vitro with recombinant IRF2 under optimal conditions for each enzyme. Human IRF2 cDNA was cloned into vectors enabling expression in *E. coli* as either a glutathione-S-transferase (GST)-fusion protein or as an HIS-fusion protein (see

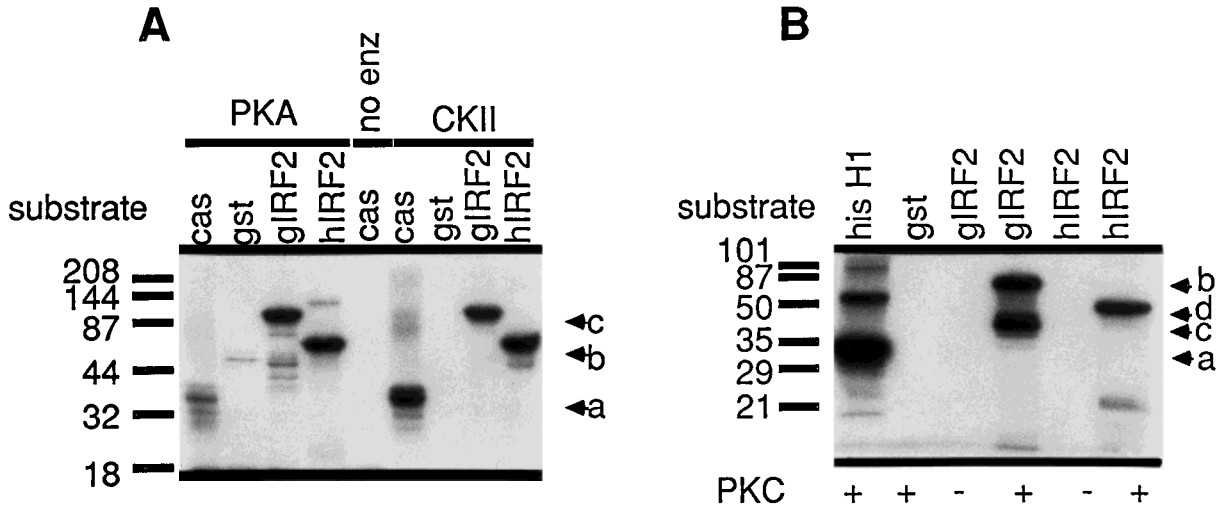

Fig. 2. Phosphorylation of recombinant IRF2 by PKA, CK2, and PKC in vitro. Bacterially expressed IRF2 was purified and used as a substrate for PKA (A), CK2 (A), and PKC (B) in vitro. ^{32}P-labelled ATP was included in the reactions, which were analyzed using SDS-PAGE followed by autoradiography. **A:** Casein (cas), GST protein alone (gst), GST-IRF2 (gIRF2), or his-IRF2 (hIRF2) were incubated under phosphorylating conditions with either PKA, CK2, or without enzyme. The arrowheads to the right of the figure show the location of phosphorylated casein (a), phosphorylated his-IRF (b), or phosphorylated GST-IRF2 (c). **B:** Histone H1 (his H1), GST protein alone (gst), GST-IRF2 (gIRF2), or his-IRF2 (hIRF2) were incubated under phosphorylating conditions with (+) or without (−) PKC. The arrowheads to the right show the location of phosphorylated histone H1 (a), phosphorylated GST-IRF2 in its intact (b) and degraded (c) forms, and phosphorylated his-IRF2 (d). The numbers to the left represent MW (kDa).

Materials and Methods). These plasmids yielded quantities of purified IRF2-fusion proteins sufficient for these studies (data not shown).

Recombinant IRF2 proved to be an effective substrate for purified PKA, PKC, and CK2 in vitro (Fig. 2A, left half). While purified GST was a very poor substrate for PKA (PKA/gst lane), purified and partially dephosphorylated casein (a positive control, PKA/cas lane, arrowhead a), and especially GST-IRF2 (PKA/gIRF2 lane, arrowhead c) and his-IRF2 (PKA/hIRF2 lane, arrowhead b), were suitable substrates for this enzyme. No phosphorylation of casein was observed in the absence of enzyme (no enz). Recombinant IRF2 was also an effective substrate for purified CK2 in vitro (Fig. 2A, right half). While purified GST was not phosphorylated by CK2 (CKII/gst lane), casein (CKII/cas lane, arrowhead a), GST-IRF2 (CKII/gIRF2 lane, arrowhead c), and his-IRF2 (CKII/hIRF2 lane, arrowhead b) were suitable substrates for CK2. Lastly, the recombinant proteins were also good substrates for purified PKC in vitro (Fig. 2B). While histone H1 was a suitable substrate for purified PKC (his H1 lane, arrowhead a), purified GST was not (gst lane). On the other hand, GST-IRF2 (gIRF2 lane, arrowhead b) and his-IRF2 (hIRF2 lane, arrowhead d) were excellent substrates for this protein kinase. The faster migrating form of recombinant, phosphorylated GST-IRF2 (arrowhead c) may be attributable to proteolytic degradation. Neither the GST-IRF2 or the his-IRF2 recombinant proteins were phosphorylated in our reactions in the absence of PKC [lanes gIRF2 (−) and hIRF2 (−), respectively]. Taken together, these data show that IRF2 is a substrate for PKA, CK2, and PKC in vitro.

Since our sequence analysis also indicated that IRF2 might be a substrate for the MAP kinases, we also challenged these protein kinases in vitro against recombinant GST-IRF2 (Fig. 3). COS-7 cells were transfected with plasmids expressing HA-tagged versions of the MAP kinases. These protein kinases were then activated by treating the cells with either UV light, or with the phorbol ester TPA, and the activated enzymes were immunoprecipitated from cell lysates using anti-HA antibodies. These immunoprecipitates were then challenged with several different recombinant proteins (Fig. 3). While GST alone (GST lane), or GST-IRF2, either bound to sepharose beads (IRF2/B lane) or eluted from these beads (IRF2/F lane), were very poor substrates for activated, immunoprecipitated ERK2, this protein kinase could phosphorylate recombinant GST-ELK-C (a known efficient substrate for ERK2) effectively (ELKC

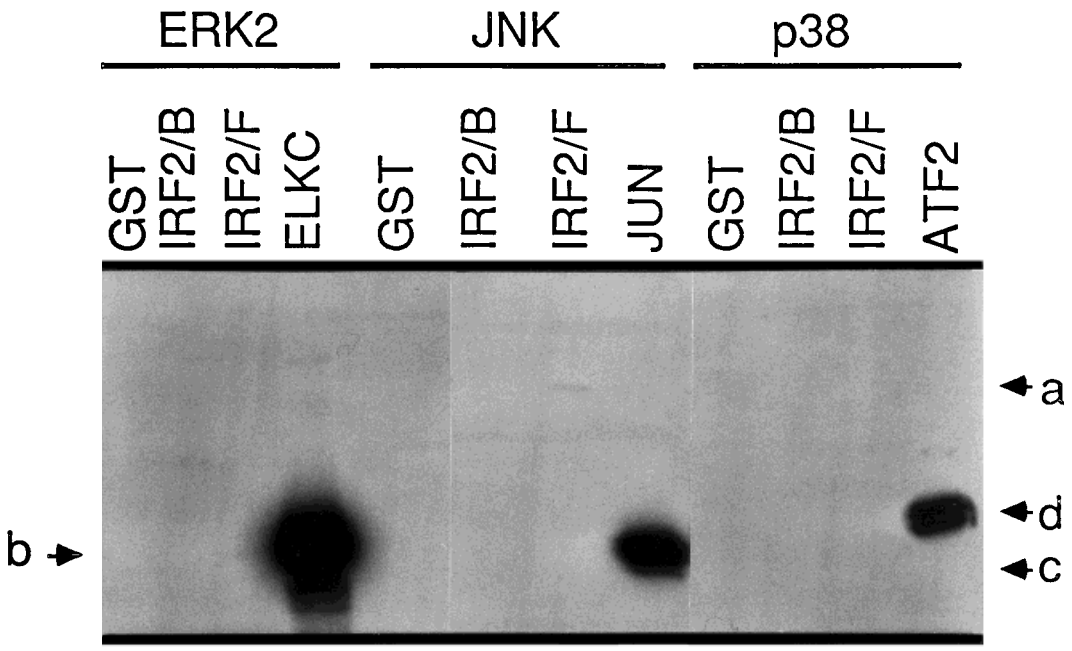

Fig. 3. Recombinant IRF2 is not a good substrate for the MAP kinases ERK2, JNK1, or p38. GST-IRF2, either bound to sepharose beads, or eluted from these beads using glutathione (see Materials and Methods), was used as a substrate for immunoprecipitated ERK2, JNK1, or p38 in vitro and analyzed using SDS-PAGE followed by autoradiography. The substrates used included GST alone (GST), GST-IRF2 bound to sepharose (IRF2/B), free GST-IRF2 (IRF2/F), ELK-C, JUN, or ATF2. The arrowheads to the left and the right show the location of GST-IRF2 (a), phosphorylated ELK-C (b), phosphorylated JUN (c), and phosphorylated ATF2 (d).

lane). Further, while activated, immunoprecipitated JNK1 could not phosphorylate GST, sepharose-bound GST-IRF2, or eluted GST-IRF2, it could phosphorylate GST-JUN (a known substrate for JNK1, JUN lane). Lastly, the activated and immunoprecipitated p38 could not phosphorylate GST, sepharose-bound GST-IRF2, or eluted GST-IRF2, but could phosphorylate GST-ATF2 (a known substrate for p38, ATF2 lane). Taken together, these data indicate that while potential sites along the IRF2 sequence for MAP kinases can be found, there is no evidence that these enzymes are capable of phosphorylating IRF2 in vitro. Therefore, we can eliminate these protein kinases as direct regulators of IRF2 in vivo.

PKA, PKC, and CKII Phosphorylate IRF2 at Different, Multiple Sites

To begin to assess the number and location of IRF2 sites phosphorylated by PKA, PKC, and CK2, we generated two-dimensional phosphopeptide maps of recombinant IRF2 phosphorylated in vitro (Fig. 4). PKA-phosphorylated GST-IRF2 was isolated using SDS-PAGE, treated with trypsin, and subjected to two-dimensional separation on thin-layer cellulose plates [Boyle et al., 1991], (see Materials and Methods). Figure 4A shows that IRF2 was phosphorylated by PKA at least at one major site (arrowhead a), and to a somewhat lesser extent, at three other sites (arrows b–d). On the other hand, PKC phosphorylated GST-IRF2 at 3 major sites (Fig. 4B, arrowheads a–c), and several minor sites (arrowheads d–e). CK2 generated a third, distinct pattern of multiple phosphopeptides (data not shown). These patterns indicate that these three protein kinases are capable of phosphorylating IRF2 at multiple sites, and that each of these enzymes is active upon different sites within the IRF2 molecule.

IRF2 Is Phosphorylated Exclusively Upon Serine Residues In Vivo

We next carried out phosphoaminoacid analysis of overexpressed, HA-tagged IRF2 immunoprecipitated from transfected, metabollically labelled (^{32}P) cells in order to determine which amino acid(s) (serine, threonine, or tyrosine) are phosphorylated in vivo. The results shown in Figure 5 indicate that IRF2 is exclusively phosphorylated upon serine in logarithmically growing COS-7 cells. While some minor phosphorylated spots were also detected, these did not co-migrate with either phosphothreonine or

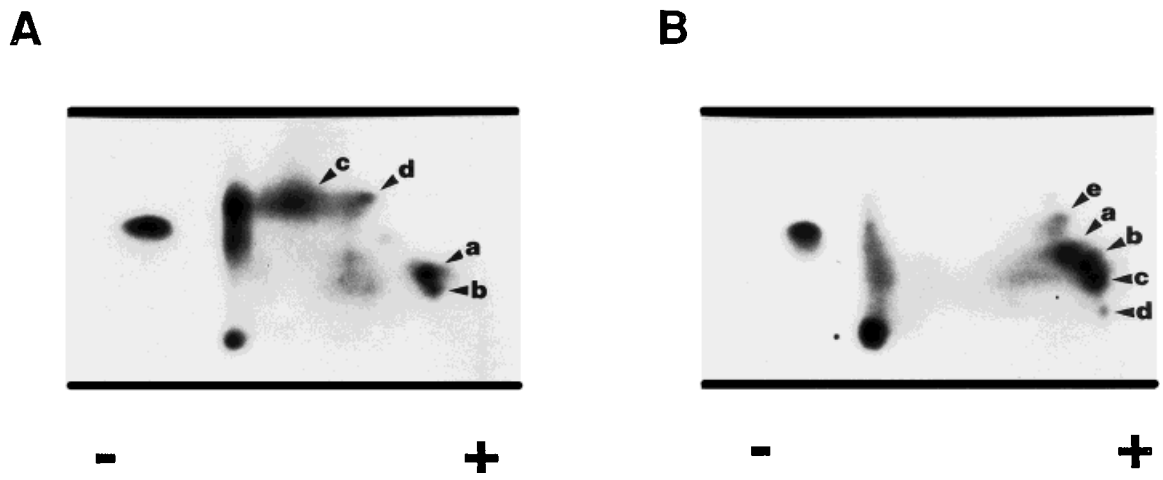

Fig. 4. PKA and PKC phosphorylate IRF2 each at different, multiple sites in vitro. PKA (A) and PKC (B) phosphorylated GST-IRF2 were isolated using SDS-PAGE, treated with trypsin, and subjected to two-dimensional separation on thin-layer cellulose plates followed by autoradiography (see Materials and Methods). **A:** Arrowheads show the location of one major PKA site (a), and to a somewhat lesser extent, four other PKA sites (arrowheads b–d). **B:** Arrowheads show the location of three major PKC sites (a–c), and several lesser sites (d–e). The + and − denote the direction of electrophoresis.

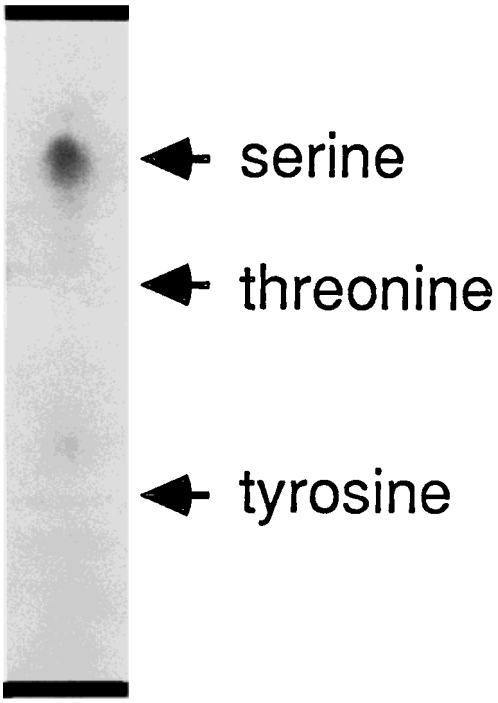

Fig. 5. IRF2 is phosphorylated exclusively upon serine residues in vivo. Phosphoamino acid analysis of in vivo phosphorylated, HA-tagged IRF2 was carried out as described (see Materials and Methods). The arrows to the right indicate the migration of unlabelled phosphoserine, phosphothreonine, and phosphotyrosine detected using ninhydrin stain.

phosphotyrosine, suggesting that these are nonspecific contaminants of the preparation. These results corroborate our findings obtained in vitro (see above) in that they indicate that IRF2 is phosphorylated via serine protein kinases.

DISCUSSION

Many oncogenic proteins, including factors that control transcription, are regulated by phosphorylation and dephosphorylation. In the present study we investigate the phosphorylation of the oncogenic transcription factor IRF2 both in vivo and in vitro. We report that IRF2, isolated from metabolically labelled cells, is a phosphoprotein (Fig. 1). To determine which protein kinases might be responsible for the phosphorylation of IRF2 in vivo, we analyzed the ability of recombinant IRF2 to serve as a substrate for a battery of protein kinases in vitro. We find that while recombinant IRF2 is an efficient substrate for PKA, PKC, and CK2 (Fig. 2), it is not a substrate for the MAP kinases JNK1, p38, and ERK2 (Fig. 3), or cdc2 and cdk2 (data not shown). Therefore, IRF2 may be regulated via multiple pathways during cellular growth.

The concept that PKA, PKC, or CK2 might be involved in the phosphorylation of IRF2 in vivo is supported by several lines of evidence. First, all three of these protein kinases are known to be found within the nucleus. Therefore, they co-localize to the same sub-cellular compartment as IRF2. In addition, phosphoamino acid analysis of IRF2 immunoprecipitated from metabolically labelled cells shows that IRF2 is phosphorylated exclusively upon serine residues in vivo (Fig. 5). PKA, PKC, and CK2 phosphorylate a wide variety of physiological substrates on serine or threonine residues. Taken together, these data suggest that, in

vivo, IRF2 might potentially be regulated by signals influencing adenylate cyclase or Ca2+/phospholipid metabolism. Moreover, since there is also evidence demonstrating that the polyamines spermine and spermidine promote the phosphorylation of proteins by CK2 in vitro [Ahmed et al., 1985; Birnbaum et al., 1987], we speculate that signals affecting changes in polyamine metabolism during cellular growth might also influence the phosphorylation of IRF2 by CK2. Our data generated by two-dimensional phosphopeptide mapping of IRF2 phosphorylated in vitro show that these enzymes are capable of phosphorylating IRF2 at multiple distinct sites (Fig. 4). These data increase the probability that IRF2 might serve as a substrate for these enzymes in different cell types under different environmental conditions.

While no information is available, excluding the current study, concerning the phosphorylation of IRF2, several studies have addressed the question of IRF1 phosphorylation. The tumor suppressor IRF1 is known to be phosphorylated in vivo [Pine et al., 1990]. It is interesting that the dephosphorylation of IRF1 in vitro results in a reduction in its DNA-binding activity [Pine et al., 1990]. The same might also be true for IRF2. However, because conditions that altered the overall amount of IRF1 had no effect upon the different phosphorylated isoforms observed in vivo, Pine et al. [1990] conclude that IRF1 function is most likely regulated by the abundance of the protein, rather than by phosphorylation. While three potential CK2 phosphorylation sites exist within the trans-activation domain of the carboxy terminal region of IRF1, mutation of these sites failed to identify any of them as essential to the activation potential of IRF1 [Lin et al., 1994]. In contrast, Watanabe et al. [1991] argue that the staurosporin-induced reduction in IRF1-activated transcription they observe implies that phosphorylation of IRF1 is important for maximal function in vivo. Therefore, the functional significance of IRF1 phosphorylation in living cells, like IRF2, remains to be investigated.

In summary, we show that IRF2 is phosphorylated in vivo and in vitro by serine protein kinases. Our data implicate several different signal transduction pathways in the regulation of IRF2. Studies are currently underway to identify the sites of IRF2 phosphorylation that occur in vivo, to mutate these sites, and to assay these mutants for their ability to promote oncogenesis, or as regulators of IFN and/or histone H4 gene transcription.

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