Phosphorylation of the c-Fos and c-Jun HOB1 motif stimulates its activation capacity

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ABSTRACT

The c-Fos and c-Jun proteins bind an AP1 site and activate transcription synergistically. These two proteins have a common activation domain which has two co-operating motifs, HOB1 and HOB2. The HOB1 motif of c-Jun includes S₇₃ which is required for Ha-Ras-induced super-activation and phosphorylation by MAP kinase-like enzymes. Since c-Fos HOB1 has a conserved Thr residue (T₂₃₂) analogous to c-Jun S₇₃ we have proposed that c-Fos HOB1 will be regulated in the same way as c-Jun HOB1. Here we show that the HOB1-containing activation domain of c-Fos is stimulated by Ha-Ras *in vivo* and phosphorylated by a MAP kinase family member *in vitro* and that mutating T₂₃₂ to Ala abolishes both functions. Collectively these results suggest that phosphorylation of the HOB1 motif increases its activation capacity. To provide direct evidence for this we change the context of c-Fos T₂₃₂ to a PKA recognition site, and show that HOB1 activity is now stimulated by the catalytic subunit of PKA. This 'PKA specificity' experiment represents a novel and powerful way to analyse phosphorylation events involved in a variety of biological functions.

INTRODUCTION

Intracellular signalling pathways initiated at the cell surface can regulate the activity of nuclear transcription factors such as c-Jun (1) and Elk1 (2). These pathways involve a cascade of phosphorylation events which ultimately lead to the phosphorylation of these factors and subsequent augmentation of their transcriptional activation potential. Phosphorylation of both c-Jun and Elk1 is brought about by proline-directed kinases of the MAP superfamily (2, 3, 4, 5) suggesting that this family of kinases may be dedicated, at least in part, in mediating the transcriptional response to external stimuli.

The Ras protein has been shown to mediate the phosphorylation of the c-Jun protein at two ser residues (S₆₃ and S₇₃) which are within the c-Jun activation domain (6). These two serines can be phosphorylated by MAP kinase (ERK2) (3) and a UV-inducible MAP kinase-like enzyme, JNK1 (5). Mutagenesis of

S₆₃ and S₇₃ affects the activation capacity of c-Jun and destroys the Ras-mediated 'superactivation' of c-Jun (3, 6).

The c-Jun protein is part of the AP1 complex which includes c-Fos. These two proteins share a common DNA binding domain (bZIP) which allows them to form a heterodimer and bind an AP1 site (7, 8, 9). Activation of AP1-containing promoters results from transcriptional activation functions provided by both c-Fos and c-Jun (10). The c-Jun protein contains two characterised activation domains A1 and A2 (11, 12) whereas c-Fos appears to have multiple domains (13, 14). However, c-Fos and c-Jun contain at least one activation domain with common structural and functional characteristics (14). This domain has two adjacent motifs, HOB1 and HOB2, which are situated at exactly the same distance apart in both c-Fos and c-Jun. Structure/function analysis indicates that these motifs activate transcription co-operatively and domain-swap experiments reveal that they are interchangeable between c-Fos and c-Jun (14).

The HOB1 motif of c-Jun falls within the A1 activation domain and encompasses the Ras-regulated and phosphorylated residue S₇₃. Considering this similarity, we have proposed that the homologous residue in c-Fos HOB1, T₂₃₂, may also be regulated by Ras-induced phosphorylation (14). Here we provide evidence which supports this hypothesis. We show that Ha-Ras can augment the activity of the HOB1 containing activation domain of c-Fos (210–308) and that mutating T₂₃₂ to A abolishes this effect. We find that a proline-directed kinase, ERK2, can phosphorylate c-Fos 210–308 *in vitro* and that the integrity of T₂₃₂ is essential for this phosphorylation. Finally, we provide direct evidence that phosphorylation of HOB1 elevates activation potential by making c-Fos HOB1 regulatable by PKA.

MATERIALS AND METHODS

Recombinant DNA

The construction of all GAL4 fusions have been described previously (14). The constitutively active and inactive Ha-Ras expression vectors were gifts from C.Marshall. Plasmid EXVKT expresses the Ha-Ras Val 12 protein whereas plasmid EXVKTS₁₈₆ expresses Ha-Ras Val 12 S₁₈₆ inactive protein.

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Mutagenesis

All GAL4 fusions were expressed from plasmid pHKG3 (Kouzarides and Hagemeyer, unpublished). This vector contains an F1 origin of replication. To generate mutants, the relevant clone was transformed into *E. coli* RZ1032 and single-stranded 'uracilated' templates were prepared after rescue with phage R408. Single-stranded oligonucleotide, describing the desired changes were synthesised and used in a mutagenesis protocol as described previously (14). Mutants were identified by DNA sequencing.

Cell lines and DNA transfection

The growth and transfection of 1BR cells has been described previously (14). F9 cells were grown at 37°C, 5% CO₂ in Dulbecco's modified Eagle Medium supplemented with 10% foetal calf serum and 10⁻⁴M β-mercaptoethanol. Cells were split 1:10 every three days and seeded into flasks previously coated with 0.1% gelatin. For transfection, F9 cells were seeded to ~30% confluency. Four hours prior to DNA addition, Hepes pH 7.12 was added to 50 mM final concentration. A total of 10 μg of DNA was transfected using the calcium-phosphate technique. The precipitate was washed 16 h after transfection and Hepes pH 7.12 was added to 15 mM final concentration. Cells were harvested 24h later and extracts used for CAT assays. The CAT assays were quantitated on a phosphorimager. Each transfection was repeated a minimum of two times.

E. coli expression and purification

The procedure used was an adaptation of the method of Smith and Johnson (21), and has been described previously (22).

In vitro kinase assay

In vitro kinase assays were performed in a 30 μl reaction which contained the following: 200 ng of substrate protein, 100 ng of purified MAP kinase pp42 (a gift from T. Sturgill), 70 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 2 mM DTT, 1 mM sodium orthovanadate, 500 nM Okadaic acid, 100 μM ATP (containing ³²P-ATP at approximately 10,000 c.p.m./pmol).

Reactions were incubated at 30°C for ten minutes after which they were mixed with 10 μl 4×SDS sample buffer, boiled for five minutes and resolved by SDS-PAGE. The gel was then subjected to autoradiography.

RESULTS

HOB1-containing domains are regulated by Ras

Two serine residues (S₆₃ and S₇₃) in c-Jun have been shown to be phosphorylated in response to Ha-Ras (6). One of these Ser residues (S₇₃) is within c-Jun HOB1 and is conserved as a Thr (T₂₃₂) in c-Fos HOB1 (Figure 1). The residues around c-Jun S₇₃ and c-Fos T₂₃₂ are conserved (including Pro at +1), suggesting that c-Fos, like c-Jun may be phosphorylated by a proline-directed kinase and superactivated by a Ras-responsive pathway (14). To test this possibility we linked the HOB1-containing activation domains of c-Fos (210–308) and c-Jun (1–193) onto the DNA binding domain (DBD) of GAL4 (1–147) and asked whether the activity of these domains is regulated by oncogenic Ha-Ras protein. As shown in Figure 2, Ha-Ras elevated the activity of the c-Fos activation domain (GAL4-Fos 210–308), whereas a Ha-Ras (mut), which expresses a defective Ras protein, does not. This effect mirrors that seen with the c-Jun activation domain

Figure 1. Alignment of the HOB1 motif of c-Fos and c-Jun. Diagrammatic representation of the Fos and Jun proteins shows the position and alignment of HOB1 (14). The position of c-Fos T₂₃₂ corresponds to c-Jun S₇₃ which has been shown to be required for Ras superactivation (6) and phosphorylation by the MAP kinase family (3, 5).

Figure 2. Activation domains containing HOB1 can be superactivated by Ha-Ras. SV40 promoter bearing plasmids expressing the indicated constructs (1 μg each) were transfected into 1BR cells along with the GAL4 site bearing reporter (containing five GAL4 sites next to the TK promoter) pUAS10CAT (4 μg), as described previously (14). The activity of the GAL4-fusions was dependent on the presence of the GAL4 binding sites (data not shown). Ha-Ras is a constitutively active mutant (Val 12) whereas the Ha-Ras (mut) is an inactive version (Val 12 Ser 186) expressed from an SV40 promoter (EXV3). Following the removal of the DNA precipitate the cells were serum-starved for 16h prior to CAT extract preparation. A CAT assay was performed and the result quantitated using a phosphorimager. Results are expressed relative to the GAL4 DBD alone.

(GAL4-Jun [1–193] Figure 2) as previously reported (1). This augmentation is directed via the c-Fos and c-Jun activation domains since the activity of the GAL4 DBD is not affected by Ha-Ras (data not shown).

We next wanted to establish if Ha-Ras-induced superactivation of c-Fos is abolished by mutagenesis of c-Fos T₂₃₂. Mutagenesis

Figure 3. Thr-232 of c-Fos HOB1 is required for superactivation by Ha-Ras. SV40 promoter-bearing plasmids expressing GAL Fos (210–308) (1 μ g) or the same with a mutation to alanine at Thr-232 (1 μ g) were transfected into 1BR cells and a CAT assay was performed as described in Figure 2. A minus (–) indicates that 1 μ g an inactive version of Ha-Ras (V12/S186) was co-transfected along with the GAL Fos. A plus (+) indicates that 1 μ g of constitutively active version of Ha-Ras (V12) was co-transfected. Results are expressed relative to the GAL4 DBD alone.

Figure 4. Thr-232 of c-Fos is required for phosphorylation by ERK2. Bacterially expressed and purified GST-fusions as indicated were used in a kinase assay with purified ERK2. The second phosphorylated band in lane 2 is due to a degradation product of GST-Fos (210–308) being phosphorylated. The reactions were then subjected to SDS–PAGE with subsequent autoradiography.

of the analogous residue in c-Jun (S_{73}) abolishes superactivation of c-Jun (6). Figure 3 shows that mutagenesis of T_{232} to A reduces but does not abolish the activity of c-Fos 210–308. However this point mutated activation domain now becomes completely unresponsive to superactivation by Ha-Ras suggesting that the integrity of c-Fos T_{232} is essential for the Ras-mediated response. Since the mutated activation domain still retains some activation capacity, these data also demonstrate that Ha-Ras does not simply augment all activation domains.

HOB1 is a target of MAP kinase-related enzymes

Ras induces phosphorylation of c-Jun S_{73} present within the HOB1 motif. This phosphorylation can be brought about by

Figure 5. Phosphorylation of HOB1/PKA by PKA leads to increased activation capacity. **A.** Sequence of HOB1 and HOB1-PKA. **B.** The activity of GAL4-Fos (210–308) and GAL4-Fos (210–308) PKA was assayed on a G5-E1B-CAT promoter, which contains five GAL4 sites upstream of the adenovirus E1B promoter. The activity of these fusions is dependent on the presence of the GAL4 binding sites (data not shown). The fold activation is relative to the GAL4 DBD alone. **C.** The response of GAL4-Fos (210–308) and GAL4-Fos (210–308) PKA to protein kinase A is shown. The activity of these two chimeras has been normalised to a value of 1 and their relative activity in the presence of a plasmid expressing the catalytic subunit of PKA is shown. **D.** Bacterially expressed GST-Fos 210–316 (WT HOB1) and GST-Fos 210–308/PKA (HOB1/PKA) were incubated with purified PKA or ERK2 in a kinase assay. The reactions were then subjected to SDS–PAGE and subsequent autoradiography.

proline-directed kinases of the MAP kinase superfamily. We therefore asked whether a kinase of the MAP kinase family, ERK2, would phosphorylate c-Fos in a T_{232} dependent manner. In Figure 4 we can see that the HOB1-containing activation domain of c-Fos (210–308) is indeed phosphorylated by ERK2 *in vitro*, whereas a point mutant which has an Ala substitution at T_{232} is not. In contrast c-Fos 210–308 was not recognised by CDC2, a member of a distinct family of proline-directed kinases (data not shown), which strongly supports the prediction that a MAP kinase-related enzyme is likely to phosphorylate c-Fos T_{232} *in vivo*.

Phosphorylation of HOB1 augments activation

These data suggest that the HOB1 motif of c-Fos, [like that of c-Jun (3, 5)] is regulated by phosphorylation mediated by a Ras-induced pathway. There is therefore a correlation between

phosphorylation of HOB1 and augmentation of transcriptional activation capacity. To provide more direct evidence for this we took the following approach. We replaced the sequence at c-Fos T₂₃₂ with the consensus recognition site for protein kinase A (PKA) (Figure 5A). We reasoned that if phosphorylation at residue 232 is required for activation, then PKA should augment the activation potential of this HOB1-PKA mutant. In Figure 5B we can see that introducing the PKA site into the HOB1 motif of the c-Fos activation domain (GAL4-Fos [210–308] PKA) reduces its activation capacity. This drop in activation is consistent with the inability of this HOB1-PKA mutant to be phosphorylated by ERK2 (Figure 5D lanes 3 and 4). However, this HOB1-PKA mutant is now able to be phosphorylated *in vitro* by PKA, whereas the unmutated HOB1 motif is not (Figure 5D, lanes 1 and 2). We therefore asked whether the activity of the HOB1-PKA mutant can be regulated by protein kinase A *in vivo*. Figure 5C shows that the activity of the GAL4-Fos (210–308) PKA mutant is considerably enhanced in the presence of the catalytic subunit of PKA when compared with the unmutated c-Fos activation domain, GAL4-Fos (210–308). These results clearly show that altering the enzyme specificity for c-Fos residue 232 from ERK2 to PKA, results in an activation domain that can be positively stimulated by the catalytic subunit of PKA.

DISCUSSION

We present evidence that the homologous activation domain of c-Fos and c-Jun is regulated in a similar manner by Ras. Our data on c-Fos, along with those previously reported on c-Jun, show that a conserved S/T residue in c-Fos and c-Jun HOB1 is required for Ras-mediated augmentation of activity. This residue (T₂₃₂ in c-Fos and S₇₃ in c-Jun) is within a consensus for proline-directed kinases (S/TP). We show that c-Fos T₂₃₂ is recognised by ERK2 and not CDC2 suggesting that this site is likely to be a target for a proline-directed kinase of the MAP kinase family. During the preparation of this manuscript Deng and Karin (15) reported the identification of a novel kinase which phosphorylates T₂₃₂ of c-Fos. Although the identity of this kinase still remains obscure, the results from c-Jun (3, 5) and our results on c-Fos (this paper), strongly suggest that this novel kinase is of the MAP kinase family.

It is still unclear how phosphorylation can influence activation. One possibility is that phosphorylation of HOB1 mediates a conformational change which allows HOB2 to function. This would explain the co-operative behaviour of HOB1 and HOB2 (14). However we do not favour this model because we find that the co-operative behaviour of these two motifs is not distance-dependent (JAS and TK unpublished).

An alternative role for phosphorylation would be to mediate the binding of a protein which transmits or augments c-Fos and c-Jun activity. The TATA box binding protein TBP is a protein with such characteristics. TBP binds c-Fos *in vivo* and mediates activation of TATA-box bearing promoters (17). Although the binding of c-Fos to TBP is augmented by domains containing HOB1 and HOB2, we have no evidence that this interaction requires phosphorylation by HOB1.

Recently a protein has been identified which binds the phosphorylated activation domain of CREB and mediates transactivation (18). This protein, CBP can also contact the c-Jun protein when phosphorylated on S₆₃ and S₇₃ by JNK (19). The CBP protein may therefore be an 'adaptor' protein which

recognises phosphorylated activation domains. It would be interesting to establish whether CBP can mediate c-Fos and c-Jun activity by binding to a phosphorylated HOB1 motif.

Since both c-Fos/c-Jun and c-Jun/c-Jun complexes have two copies of HOB1, but only one copy of other activation domains, the possibility is raised that this motif only functions as a dimer. The absence of HOB1 in FosB and Fra-1 may therefore reflect a mechanism for functionally discriminating between different heterodimers of the Fos-Jun family. The single HOB1 motif in c-Jun may not be sufficient to carry out HOB1-specific activation within the context of the c-Jun/FosB and c-Jun/Fra-1 heterodimer. However, these complexes may still be able to activate transcription using other domains, such as the C-terminal TBP binding domain of FosB (20). Further characterisation of HOB1 mediated activation, in terms of protein target and promoter specificity, should allow us to answer some of these questions.

We have presented a novel way of analysing the function and regulation of potential phosphorylation sites. This 'PKA specificity' experiment was used here to show that phosphorylation augments the potency of an activation domain. However, the principle of the PKA-specificity experiment can be applied to a variety of biological questions where phosphorylation sites are implicated. Phosphorylation may regulate numerous events including DNA binding, cell transformation and DNA replication. Placing the relevant phosphorylation sites under the control of PKA should aid in defining the role of phosphorylation in these processes.

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