c-Jun is phosphorylated by the DNA-dependent protein kinase *in vitro*; definition of the minimal kinase recognition motif

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ABSTRACT

The DNA-dependent protein kinase (DNA-PK) phosphorylates a number of transcription factors. Here, we show that the DNA-PK modifies c-Jun *in vitro* and that serine residue 249 (Ser-249) is required for phosphorylation to occur. This residue corresponds to one of three sites of c-Jun that are phosphorylated *in vivo* and which negatively regulate c-Jun DNA binding *in vitro*. However, we find that phosphorylation of c-Jun by the DNA-PK does not interfere with DNA binding, indicating that phosphorylation at other sites is required for this effect. Mutagenesis of the phosphorylated region of c-Jun reveals that the primary amino acid sequence recognised by the DNA-PK consists of the sequence Ser–Gln, and that adjacent acidic residues potentiate kinase activity. Furthermore, when this site is placed within the context of a second protein, it confers DNA-PK directed phosphorylation upon that protein. Our findings will facilitate identification of DNA-PK phosphorylation sites in other transcription factors.

INTRODUCTION

In recent years, it has become clear that phosphorylation plays a pivotal role in modulating the activity of sequence-specific transcription factors (1, 2). It is therefore of great importance to identify and characterise the kinases that bring about these events. One kinase that appears to be ideally placed to modulate transcription factor activity is the DNA-dependent protein kinase (DNA-PK) that has been shown to phosphorylate a wide variety of transcription factors *in vitro*. These include Sp1, CTF/NF-1, Oct-1, Oct-2, c-Myc, SRF, p53, and the chicken progesterone receptor (3–9). The DNA-PK is a relatively abundant nuclear serine/threonine kinase that displays the interesting property of being itself a DNA-binding protein (4, 6, 9, 10). Indeed, the DNA-PK does not become active until it has bound to DNA. Furthermore, for Sp1, we have shown that the kinase has a strong preference for DNA-bound substrate, due to the fact that efficient phosphorylation only takes place when the kinase and Sp1 are bound *in cis* on the same DNA molecule (4, 11).

To date, the functional consequences of DNA-PK-mediated transcription factor phosphorylation are not known. However, studies have revealed that phosphorylation has no measurable effect on the DNA binding properties of Sp1, suggesting that its function is to regulate the transcriptional activation potential of this factor (4; T.Gottlieb and S.Jackson, unpublished data). Thus, DNA-dependent phosphorylation of Sp1 might be an effective mechanism for generating an active transcription factor only when and where it is needed. Consistent with this possibility, preliminary mapping studies have indicated that the sites of Sp1 phosphorylation lie outside of the DNA binding domain but within the N-terminal portion of the protein that contains the transcriptional activation domains (4).

One obvious approach to elucidate the function of DNA-PK-mediated phosphorylation will be to define sites of phosphorylation on transcription factors and determine whether mutation of these sites affects factor activity. For Sp1, this has proved extremely difficult because multiple, and heterogeneous phosphorylation appears to occur in more than one region of the protein (S.Jackson, unpublished data). A mapping/mutational approach would clearly be much easier if a transcription factor with a more simple DNA-PK phosphorylation pattern was identified. Analysis of known DNA-PK substrates suggests that one such factor is the product of the *c-jun* proto-oncogene, since it is phosphorylated by the DNA-PK less strongly than other factors that are believed to be multiply phosphorylated (9). c-Jun is phosphorylated *in vivo* in two regions. One of these lies towards the N-terminus of the protein. Here, phosphorylation of two serine residues by MAP kinases occurs in response to mitogenic signals and results in potentiation of the transcriptional activation properties of c-Jun (12–14). The other phosphorylated region of c-Jun lies just upstream from the C-terminal bZIP domain that specifies dimerisation and DNA-binding. Here, phosphorylation by casein kinase II (CKII), and/or glycogen synthase kinase 3 (GSK3), interferes with the DNA-binding potential of c-Jun and appears to occur on three residues; Ser-243, Ser-249, and Thr-239 or Thr-231 (15, 16).

In this report, we describe a GST-protein fusion strategy that can be used to define sites of protein phosphorylation by the DNA-PK and, potentially, by other kinases. Using this approach,

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we show that the DNA-PK specifically phosphorylates the C-terminal region of c-Jun. Furthermore, by point-mutagenesis, we define the DNA-PK recognition site to be centred around Ser-249. This is one of a cluster of three phosphorylated residues that, together, negatively regulate the DNA-binding potential of c-Jun *in vivo*. We therefore investigated the effect of DNA-PK mediated phosphorylation on c-Jun DNA binding. In addition, by mutagenesis of the residues surrounding the site of phosphorylation, we define a minimal primary amino acid sequence motif that serves as a DNA-PK recognition signal, and which directs phosphorylation by the DNA-PK when introduced into another protein. These findings should greatly facilitate the identification of DNA-PK recognition sites on other proteins. The possible implications for DNA-PK-mediated c-Jun phosphorylation are discussed.

MATERIALS AND METHODS

Bacterial expression vectors

Fusion of test proteins to the GST protein coding region were generated using the pGEX vector system described by Smith and Johnson (17). The Jun-Core domain (residues 239–322 of Jun P; ref. 18) was cloned into pGEX-2T (a gift from E.Laue). A BglII fragment containing the Fos-Core 7 domain (19) was cloned into the BamHI site of pGEX-2T. A SmaI/EcoRI fragment (encoding c-Jun amino acid residues 1–193; J.Sutherland and T.K., unpublished) was cloned into pGEX-2T. An NcoI/SacI fragment encoding Fos-Core (amino acids 127–223; ref 19) was cloned into pGEX-KG (20). A SmaI/EcoRI DNA fragment encoding GAL4 (amino acid residues 1–147) was cloned into pGEX-3X to generate pGEX-3XGAL4. An EcoRI fragment encoding c-Jun (amino acids 1–193) was cloned into pGEX-3XGAL4 to generate pGEX-3XGAL4Jun1–193.

Mutagenesis

To carry out mutagenesis, fragments were cloned into pING vectors, which are derivatives of pSP64T (21), containing a polylinker and an F1 origin of replication (S.Inglis and I.Brierley, unpublished). To generate the Jun-Core series of mutants, and the Fos-Core-ESQE mutant, the coding regions were subcloned into pING14. For mutagenesis, the relevant pING clone was transformed into *E.coli* RZ1032 and single stranded 'uracilated' templates were prepared after rescue with phage R408 (22). Single-stranded oligonucleotides describing the desired changes were synthesised and used in a mutagenesis protocol described by Kunkel (23). The mutants were identified by sequencing and subcloned into the relevant pGEX vector.

Jun DNA binding assays

Gel retardation assays were performed as described previously (24). The c-Jun DNA binding site used was a 27 bp double stranded oligonucleotide containing the 'fat specific element' (FSE; refs 24 and 25). To determine the effect of c-Jun DNA binding sites on c-Jun phosphorylation by the DNA-PK, two FSE sites were cloned into the plasmid pLSO.CAT (26). The recombinant and parental pLSO vectors were then used as source DNA in an *in vitro* kinase assay.

E.coli expression and purification

The procedure used was an adaptation of the method of Smith and Johnson (17), and has been described previously (27). Before kinasing, the fusion proteins were cleaved with thrombin and

purified away from GST (27), except for the GST-GAL4 fusions, which were not cleaved.

In vitro kinase assays

In vitro kinase assays were performed in a 30 μ l reaction which contained the following; 10 μ g HeLa nuclear extract (28) or 200 ng of purified DNA-PK (a gift from C.Anderson), 250 ng substrate protein, 25 mM Hepes pH 7.5, 12.5 mM MgCl₂, 20% glycerol, 0.1% NP-40, 100 mM KCl, 50 μ M ATP (containing 10 μ Ci [γ -³²P]ATP; 6000 Ci/mmol) and 1 mM DTT. 200 ng of linearised DNA template was added to the appropriate reaction as indicated in the text. Reactions were incubated at 30°C for 10 minutes after which they were mixed with 10 μ l 4 $\times$ SDS sample buffer, boiled for 5 minutes and resolved by SDS–PAGE. The gel was then subjected to autoradiography. For large proteins (GST.GAL4 or GST.GAL4.Jun1–193), the GST fusion was left 'on the bead'. After the kinase reaction the beads were washed 2 $\times$ with kinase buffer and the products resolved by SDS–PAGE. Purified human Sp1 protein was produced by over-expression in HeLa cells using a recombinant vaccinia virus vector (4). When Jun-Core mutants were assayed as DNA-PK substrates, low substrate concentrations were used (approximately 30 ng per reaction). This enabled differences in phosphorylation, due to differing K_m values, to be observed.

RESULTS

A DNA-dependent kinase phosphorylates c-Jun

It has been reported that the c-Jun oncoprotein is a substrate for the DNA-PK (9). We set out to establish which region(s) of c-Jun is phosphorylated by this kinase. To address this question, we expressed in bacteria the biologically important regions of the human c-Jun protein for use in *in vitro* kinase reactions. We chose the pGEX GST-fusion expression system (17) because it allows the large-scale production of highly purified, soluble fusion proteins.

Two regions of c-Jun were expressed, which together encompass the majority of the c-Jun protein (Figure 1A). The first region consists of amino acid residues 1 to 193 and corresponds to the A1 activation domain (29). Deletion and mutagenesis of c-Jun has identified two motifs within the A1 activation domain (HOB1 and HOB2; Figure 1A) which are essential for transcriptional activation (30). Moreover, HOB1 contains MAP kinase phosphorylation sites that are important for transcriptional activation (13, 14). The second region of c-Jun that we expressed, Jun-Core (amino acid residues 239 to 322), contains the A2 activation domain and the bZIP region that specifies DNA binding and dimerisation (29).

The two c-Jun derivatives were then tested for an ability to be phosphorylated by kinases present in a crude HeLa cell nuclear extract. To do this, and to ascertain whether any phosphorylation might be due to the DNA-PK, Jun 1–193 and Jun-Core were incubated with nuclear extract and [γ -³²P]ATP either in the presence or absence of DNA. Samples were then analysed by SDS–PAGE followed by autoradiography (Figure 1B). These studies revealed that Jun-Core is strongly phosphorylated only in the presence of DNA (compare lanes 3 and 4). By contrast, Jun 1–193 is not detectably phosphorylated either in the absence or presence of DNA (compare lanes 1 and 2). As a control, the bZIP region of the c-Fos protein (which contains homology to the c-Jun bZIP region) was also analysed. This protein did not display DNA-dependent phosphorylation (lanes 5 and 6). Taken

together, these data indicate that a DNA-dependent kinase present in crude HeLa nuclear extracts specifically phosphorylates the bZIP region of c-Jun.

Figure 1. Jun-Core is phosphorylated by a DNA-dependent kinase in HeLa nuclear extracts. **A.** Schematic representation of the c-Jun protein. The previously defined *in vivo* activation regions of c-Jun are indicated. These consist of the HOB1 and HOB2 motifs (30) and regions A1 and A2 (29). Also shown are the regions of the c-Jun protein which were expressed in *E. coli* and assayed in this report. **B.** Phosphorylation of bacterially-synthesised proteins. The bacterially-synthesised proteins, as indicated, were incubated with HeLa cell nuclear extract and $\gamma^{32}\text{P}$ -[ATP] in the presence (+) or absence (-) of DNA, then subjected to SDS-PAGE followed by autoradiography. The position and sizes of molecular weight standards are indicated. The apparent molecular weight of the bacterially produced proteins in SDS gels are as follows; Jun1-193, 17 kDa; Jun-Core, 14 kDa; Fos-Core7 (Fos, amino acids 124-200) 12 kDa. Equal amounts of each protein (250 ng), as judged by Coomassie Blue staining, were used in each reaction. The high molecular weight proteins phosphorylated in this experiment are proteins endogenous to the nuclear extract (lanes 7 and 8). The low level of phosphorylation observed in the absence of DNA in lane 3 could either be due to the presence of trace DNA in the extract or Jun protein preparation, or may be due to non DNA-dependent kinases in the extract. **C.** Jun residues 1-193 are phosphorylated inefficiently even when tethered to DNA through a heterologous DNA binding domain. Bacterially-synthesised proteins GST-GAL4[1-147] (lanes 1 and 2) and GST-GAL4[1-147]-Jun [1-193] (lanes 3 and 4) were incubated with HeLa cell nuclear extract and $\gamma^{32}\text{P}$ -[ATP] in the presence (+) or absence (-) of DNA, then subjected to SDS-PAGE followed by autoradiography. The fact that the GAL4-Jun derivative is phosphorylated more efficiently than the GAL4 derivative is presumably due to the presence in the extract of kinases, such as MAP kinases, that phosphorylate the N-terminal region of Jun.

Previous studies have shown that the DNA-PK recognises preferentially DNA-bound substrates (4, 11). Therefore, the lack of Jun 1-193 phosphorylation in Figure 1B might not be due to lack of DNA-PK recognition sites *per se* but, instead, might reflect the fact that this protein does not bind DNA. To address this issue, we expressed in bacteria a GST fusion protein consisting of Jun residues 1-193 attached to the DNA binding domain of yeast GAL4. This protein was then purified and tested for an ability to be phosphorylated in a crude HeLa cell nuclear extract (Figure 1C). The recombinant protein was phosphorylated extremely inefficiently as compared to the c-Jun bZIP region (the exposure time in Figure 1C is over 20-fold longer than in Figure 1B). Furthermore, no stimulation of phosphorylation was observed by the addition of DNA. From this, we conclude that residues 1-193 of c-Jun are not recognised effectively by the DNA-PK even when tethered to DNA.

DNA-PK phosphorylates c-Jun

We have shown previously that DNA-dependent phosphorylation of transcription factor Sp1 in HeLa nuclear extracts is catalysed by the DNA-PK (4). This kinase has been purified and shown to be a multicomponent complex consisting of an approximately 350 kDa catalytic subunit and a heterodimeric DNA-binding subunit (6, 10, 11). Given the DNA-dependent phosphorylation of Jun-Core in crude nuclear extracts, we were interested to determine whether it could serve as a substrate for the DNA-PK. When Jun-Core was incubated with a highly-purified DNA-PK preparation, it was found to be phosphorylated strongly, in a DNA-dependent manner (Figure 2, compare lanes 1 and 2). Indeed, the level of Jun-Core phosphorylation compared favourably with that of Sp1 (lanes 3 and 4). In contrast, the bZIP region of the Fos protein was not phosphorylated (lanes 5 and 6).

Phosphorylation often causes proteins to migrate more slowly on SDS-polyacrylamide gels. For Sp1, it has been found that multiple phosphorylation events generate a large number of electrophoretic variants of the protein (4). Following phosphorylation by the DNA-PK, Jun-Core also migrates more slowly in SDS gels as judged by Coomassie Blue staining (data not shown). However, in this case, only one slower-migrating species is observed, suggesting that a single phosphorylation event might be taking place.

Figure 2. Purified DNA-PK phosphorylates Jun-Core. Highly purified DNA-PK was used in the standard kinase assay with the following substrates; Bacterially-expressed Jun-Core (lanes 1 and 2), Sp1 (lanes 3 and 4; ref. 4) and Fos-Core (lanes 5 and 6). The reactions were performed in the absence (-) or presence (+) of DNA and then subjected to SDS-PAGE with subsequent autoradiography. The expected position of each protein is indicated.

Figure 3. Ser-249 of c-Jun is required for phosphorylation by the DNA-PK. **A.** Amino acid sequence comparison of wild-type Jun-Core and the mutant Jun-Core_{S→A}. The open box indicates the Ser-Gln motif. The underlined amino acid corresponds to the change introduced into Jun-Core. **B.** Mutation of Ser-249 severely reduces the DNA-PK phosphorylation of Jun-Core. Bacterially-expressed Jun-Core and Jun-Core_{S→A} were used in a kinase assay with purified DNA-PK. Both reactions were performed in the presence of DNA and then subjected to SDS-PAGE with subsequent autoradiography.

Previous studies have identified sites of phosphorylation by the DNA-PK in hsp90 and SV40 large T antigen (9, 31, 32). Comparison of the sequences surrounding these phosphorylation sites suggests that the DNA-PK recognises serine or threonine residues that are flanked on their C-terminal side by a glutamine residue. Interestingly, examination of the amino acid sequence of Jun-Core identified a single site, Ser-249, that conformed to this consensus (ESQE motif in Figure 3A). To determine whether this site might correspond to the site of phosphorylation by the DNA-PK, we mutated Ser-249 to an alanine residue to generate Jun-Core_{S→A} (Figure 3A). This mutant protein was then expressed and compared as a DNA-PK substrate with wild-type Jun-Core protein (Figure 3B). This revealed that, in marked contrast to Jun-Core, the Jun-Core_{S→A} protein is an inefficient target for the purified DNA-PK (compare lanes 1 and 2). The Jun-Core_{S→A} protein also failed to display DNA-dependent phosphorylation when assayed in nuclear extracts (data not shown). These results indicate that Ser-249 forms part of the DNA-PK recognition sequence.

Determination of a minimal DNA-PK recognition site

Next, we determined which residues flanking Ser-249 in the Jun-Core protein are required to direct phosphorylation by the DNA-PK. To do this, single mutations (to alanine) were introduced into Jun-Core at the following positions: Glu-248, Gln-250 and Glu-251. The resulting proteins were then expressed and tested for an ability to serve as DNA-PK substrates (Figure 4). The most severe effect was obtained with the Gln-250 mutation, which dramatically impaired DNA-PK-mediated phosphorylation relative to the wild-type Jun-Core protein (compare Q₂₅₀→A and WT). Less severe was the Glu-251 mutation (E₂₅₁→A) which caused a lesser reduction in the amount of phosphorylation by the DNA-PK. In contrast, the Glu-248 mutation (E₂₄₈→A)

Figure 4. Characterisation of the DNA-PK recognition motif. Bacterially-expressed Jun-Core and Jun-Core mutants (mutated to alanine at the positions indicated) were used in a kinase assay with purified DNA-PK. All reactions were performed in the presence of DNA and then subjected to SDS-PAGE. The positions of the proteins were determined by autoradiography, the bands excised from the gel, and ³²P incorporation determined by liquid scintillation counting.

had only a very small effect on phosphorylation. Since the core Ser-Gln sequence is flanked on both sides by acidic residues it seemed possible that there may be some degeneracy in that one Glu residue may be sufficient to maintain recognition by the DNA-PK. To test this, we made a mutant in which both Glu residues were mutated to Ala. Strikingly, this mutant was severely deficient as a substrate for the DNA-PK (Figure 4, compare E₂₄₈, E₂₅₁→A to WT). Taken together, these results demonstrate that the optimal DNA-PK recognition motif in c-Jun is the sequence Glu-Ser-Gln-Glu.

The results described above strongly suggested to us that Jun-Core is phosphorylated by the DNA-PK on Ser-249. At this stage, however, it remained a possibility that, although this residue formed an essential part of the DNA-PK recognition motif, phosphorylation might actually be occurring elsewhere. If Glu-Ser-Gln-Glu did indeed correspond to the minimal DNA-PK phosphorylation site, we reasoned that this region of c-Jun might serve as a phosphorylation site when placed in the context of another protein. Since we had shown previously that the bZIP region of the Fos protein is not phosphorylated by the DNA-PK (Figure 2, lanes 5 and 6), we introduced into it the Glu-Ser-Gln-Glu motif of c-Jun that contains Ser-249 (Fos-Core-ESQE; Figure 5A). Strikingly, when the Fos-Core-ESQE protein was incubated with purified DNA-PK, it was found to be strongly phosphorylated (Figure 5B). Taken together with the fact that this region is required for DNA-PK directed phosphorylation of c-Jun and, since Ser-249 of c-Jun was initially identified because it contains homology to sites of DNA-PK phosphorylation in other proteins, these data strongly suggest that the DNA-PK phosphorylates c-Jun at Ser-249.

Jun DNA binding sites enhance DNA-PK activity but phosphorylation does not affect c-Jun DNA binding

Efficient phosphorylation of Sp1 by the DNA-PK requires both a functional DNA binding domain within the Sp1 protein and Sp1-binding sites on the DNA (4). If the DNA-dependent phosphorylation of c-Jun is of physiological significance then one might expect that the presence of Jun binding sites (FSEs; refs. 24 and 25) within the activating DNA would increase kinase efficiency. To test this, we introduced two FSE elements into

Figure 5. The DNA-PK recognition motif in c-Jun functions in the context of a different protein. **A.** Amino acid sequence comparison of wild type Fos-Core and the mutant Fos-Core-ESQE. The underlined amino acids correspond to the changes introduced into Fos-Core. **B.** Introduction of a Glu-Ser-Gln-Glu motif into Fos-Core converts the protein into an efficient DNA-PK substrate. Bacterially-expressed Fos-Core and Fos-Core-ESQE were used in a kinase assay with purified DNA-PK. Both reactions were performed in the presence of DNA and then subjected to SDS-PAGE with subsequent autoradiography. Although previous studies indicate that DNA binding by the substrate potentiates DNA-PK mediated phosphorylation (4), it is clearly not essential because the non DNA binding proteins hsp90 and casein are DNA-PK substrates *in vitro* (4, 6, 9–11). Furthermore, though the Fos-Core region does not bind DNA effectively alone, it does contain a basic region and has been shown to bind DNA non-specifically. These considerations may explain the relatively efficient phosphorylation of Fos-Core-ESQE by the DNA-PK.

a plasmid. Both the parent and recombinant plasmid were then used as cofactors for the DNA-PK in *in vitro* kinase assays (Figure 6A). By comparing lanes 2 and 3, it is clear that the presence of the FSE elements in the plasmid DNA dramatically increases the level of c-Jun phosphorylation. (The low level activation by the parent plasmid alone may be due to cryptic weak Jun-binding sites in this DNA). From these results, we conclude that DNA-bound Jun-Core protein is the preferred substrate for the DNA-PK.

Previous studies have implicated Ser-249 in regulating the DNA binding potential of full-length c-Jun and Jun-Core proteins (15, 16). The conclusion from these studies was that DNA binding by c-Jun is severely inhibited when this protein is phosphorylated at Ser-243, Ser-249, and either Thr-239 or Thr-231. However, for practical reasons, these workers were unable to assess the individual contributions of the three phosphorylation sites towards the inhibition of c-Jun DNA binding. Our results strongly suggest that the DNA-PK specifically phosphorylates Ser-249 of c-Jun, and therefore we asked whether this phosphorylation could inhibit c-Jun binding. In the DNA binding assay shown in Figure 6B, Jun-Core protein phosphorylated by the DNA-PK demonstrates identical DNA binding properties to the unphosphorylated Jun-Core protein. Thus, we conclude that, in isolation, phosphorylation of c-Jun by the DNA-PK is unable to inhibit DNA binding under our assay conditions. This suggests that other phosphorylation events, either exclusively or in combination with phosphorylation of Ser-249, are required for the inhibition of DNA binding.

Figure 6. Jun DNA binding sites enhance DNA-PK activity but phosphorylation does not affect c-Jun DNA binding. **A.** The presence of c-Jun DNA binding sites stimulates DNA-PK activity. Bacterially-synthesised Jun-Core was used in a kinase assay with purified DNA-PK. The kinase reactions were performed in the absence or presence of linearised DNA as indicated. Plasmid pLSO.CAT has been described previously (26). Plasmid pLSO.CAT:2FSE is pLSO.CAT containing two copies of the Fat-Specific Element to which c-Jun binds (24, 25). Following incubation, the reactions were subjected to SDS-PAGE with subsequent autoradiography. **B.** Phosphorylation of Jun-Core by the DNA-PK does not inhibit DNA binding. Bacterially-synthesised Jun-Core was mixed with kinase buffer containing 200 ng of linear DNA. Reactions were incubated in the presence or absence of the DNA-PK and/or ATP. After incubation a ³²P-labelled double-stranded oligonucleotide containing an FSE site was added and the reactions incubated further. Samples were then subjected to an electrophoretic mobility shift assay as described previously (24). The specific Jun/DNA complex (SC) and a non-specific complex (NSC) are indicated. Kinase reactions were performed such that the DNA-PK phosphorylated over 50% of the Jun-Core protein as judged by a kinase-induced mobility-shift of phosphorylated Jun-Core as observed by SDS-PAGE and Coomassie Blue staining.

DISCUSSION

In this report, we have shown that a DNA-dependent kinase in HeLa nuclear extracts phosphorylates human c-Jun in the vicinity of its DNA binding and dimerization domain. A highly-purified preparation of the DNA-PK derived from HeLa cells (6, 10, 11), and free of other kinase activities, also phosphorylates c-Jun in a DNA-dependent manner. Since previous fractionation studies have indicated that only a single DNA-activated kinase exists in HeLa cells, we are led to the conclusion that it is the DNA-PK that phosphorylates the c-Jun bZIP region in crude cell extracts. Through primary amino acid sequence analysis we have identified an evolutionary conserved serine residue (Ser-249) in Jun-Core which contains homology to known sites of DNA-PK phosphorylation in other proteins. By mutagenesis we have shown that this residue constitutes part of the DNA-PK recognition site and is required for DNA-PK phosphorylation of the Jun-Core protein. Furthermore, when a tetrapeptide containing this

sequence is placed in the context of another protein, it confers DNA-PK phosphorylation upon that protein. Taken together, these results strongly suggest that the DNA-PK phosphorylates Jun-Core at Ser-249, although at this point, we can not rule out the possibility that the kinase phosphorylates a flanking residue(s).

Though the physiological relevance of the DNA-PK phosphorylation of Jun-Core is still unproven, several lines of evidence suggest that DNA-PK catalyses c-Jun phosphorylation *in vivo*. First, the site on c-Jun which appears to be phosphorylated by the DNA-PK *in vitro* is known to be phosphorylated *in vivo*. Second, both c-Jun and the DNA-PK are nuclear DNA-binding proteins, and therefore are likely to have access to one another inside the cell. Third, DNA-PK-mediated phosphorylation of c-Jun is potentiated by c-Jun binding to its cognate DNA recognition sequence. Thus, it is likely that c-Jun protein engaged in active transcription complexes within the cell can be recognised by the DNA-PK. Fourth, under our assay conditions in the presence of DNA, the dominant c-Jun kinase in unfractionated crude nuclear extracts is the DNA-PK.

Two other kinases have been shown to phosphorylate Ser-249 of c-Jun. Glycogen synthase kinase 3 (GSK3) can phosphorylate c-Jun at Thr-239, Ser-243 and Ser-249 and thereby inhibit c-Jun DNA binding *in vitro* (15). However, this enzyme is predominantly cytoplasmic and thus is thought unlikely to phosphorylate c-Jun *in vivo*. More recently, it has been demonstrated that casein kinase II (CKII) phosphorylates c-Jun at Thr-231 and Ser-249 both *in vitro* and *in vivo* (16). As with GSK3, phosphorylation by CKII also inhibits c-Jun DNA binding *in vitro* and it is thought that this is a mechanism by which the cell can maintain c-Jun in an inactive state until it is activated by dephosphorylation. We have shown that the DNA-PK phosphorylates c-Jun more efficiently when both proteins are bound to DNA. For c-Jun to be accessible to the DNA-PK, therefore, it must be dephosphorylated at Thr-231 and Ser-249. Thus, it seems unlikely that the DNA-PK and CKII will compete for phosphorylation of Ser-249 *in vivo*. Instead, we speculate that the DNA-PK and CKII both phosphorylate Ser-249 *in vivo*, but do so when c-Jun is present in different subcellular locations.

The site we have defined as the recognition site for the DNA-PK in human c-Jun is conserved in c-Jun proteins of other species and is also present in murine JunD (15). This suggests that this site plays an important role in regulating c-Jun activity. Interestingly, previous studies have shown that site-specific dephosphorylation in the C-terminal region of c-Jun in response to mitogenic stimuli converts the protein from a form that cannot bind DNA to a form that can (15, 16). Our studies reveal that phosphorylation of c-Jun by the DNA-PK is not sufficient to inhibit DNA binding. This suggests that phosphorylation of c-Jun by the DNA-PK may instead regulate the transcriptional activation potential of c-Jun. Indeed, such an activation mechanism has been suggested previously for DNA-PK-mediated phosphorylation of Sp1 (4). It is known that phosphorylation of the c-Jun A1 activation domain increases transcriptional activity of the protein (13, 14); perhaps phosphorylation of Ser-249 has a similar effect on the A2 domain (amino acid residues 238–257; ref. 29). However, recent studies have shown that mutation of Ser-249 of c-Jun to Ala does not inhibit transcriptional activation by this protein *in vitro* (33). Although it remains possible that phosphorylation of c-Jun at Ser-249 by the DNA-PK activates its transcriptional potential *in vivo*, the body of evidence therefore suggests that it is involved in regulating some other aspect of

c-Jun function. Besides affecting transcription, transcription factors are also known to influence other events such as DNA replication and repair (34, 35, 36). In light of this, it is perhaps worthwhile noting that we have demonstrated recently that the DNA-PK requires DNA breaks in order to become activated efficiently *in vitro*, suggesting that it might be involved in mediating a response to DNA damage (11).

In this report, we have used site-directed mutagenesis to establish the sequence requirements for DNA-PK mediated phosphorylation. We demonstrate that the main determinant for a DNA-PK recognition site is the sequence Ser–Gln. Recently, *in vitro* peptide phosphorylation assays have suggested that basic residues close to potential DNA-PK sites can inhibit the activity of the enzyme (9). However, in c-Jun, Ser-249 lies immediately N-terminal to the basic DNA binding region but it is still an excellent substrate for the DNA-PK. In this regard, it is intriguing to note that both Glu-248 and Glu-251 of c-Jun are required for optimal phosphorylation efficiency by the DNA-PK. Perhaps the acidic nature of these residues overcomes the negative effects of the adjacent basic region. Interestingly, we have noted that many known and potential DNA-PK recognition sites in other factors are also flanked by acidic residues. Although we have identified a recognition sequence for the DNA-PK, this does not necessarily mean that the kinase is unable to recognise other types of sequence. Indeed, it has been shown recently that the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II lacks Ser/Thr–Gln motifs and yet can be phosphorylated by DNA-PK *in vitro* (37).

A number of other transcription factors are known substrates for the DNA-PK, including Sp1, Oct-1, Oct-2, c-Myc, SRF, c-Fos, p53 and the chicken progesterone receptor (3–9). Our results now make it relatively straightforward to predict where phosphorylation might be occurring in these proteins. Indeed, each of these proteins contains one or more Ser–Gln motifs. In addition, our findings should help identify other DNA-PK substrates. For example, we have noted that many other transcription factors contain Ser–Gln sequences, including E2F, BZLF1, MyoD, TFE3 and Oct-4 (38–42). Although we predict that many of these sites are DNA-PK phosphorylation sites, we do not expect all Ser–Gln motifs to serve as DNA-PK recognition elements. For instance, since the DNA-PK appears to be restricted to DNA-bound substrates, non-DNA-binding proteins that bear Ser–Gln motifs are unlikely to be recognised by the DNA-PK *in vivo*. Furthermore, as discussed above, basic residues in close proximity to Ser–Gln sequences may sometimes render these sites inaccessible to the DNA-PK. In addition, protein structure, post-translational modification, or interaction with other proteins may affect site recognition. Indeed, such mechanisms might actually be employed by the cell in some cases to regulate the accessibility of sites to the DNA-PK.

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