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Biochemical and Biophysical Research Communications 338 (2005) 660–667
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Phosphorylation inhibits DNA-binding of alternatively spliced aryl hydrocarbon receptor nuclear translocator

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Received 15 July 2005
Available online 19 August 2005

Abstract

The basic helix–loop–helix/PER-ARNT-SIM homology (bHLH/PAS) transcription factor ARNT (aryl hydrocarbon receptor nuclear translocator) is a key component of various pathways which induce the transcription of cytochrome P450 and hypoxia response genes. ARNT can be alternatively spliced to express Alt ARNT, containing an additional 15 amino acids immediately N-terminal to the DNA-binding basic region. Here, we show that ARNT and Alt ARNT proteins are differentially phosphorylated by protein kinase CKII in vitro. Phosphorylation had an inhibitory effect on DNA-binding to an E-box probe by Alt ARNT, but not ARNT, homodimers. This inhibitory phosphorylation occurs through Ser77. Moreover, a point mutant, Alt ARNT S77A, shows increased activity on an E-box reporter gene, consistent with Ser77 being a regulatory site in vivo. In contrast, DNA binding by an Alt ARNT/dioxin receptor heterodimer to the xenobiotic response element is not inhibited by phosphorylation with CKII, nor does Alt ARNT S77A behave differently from wild type Alt ARNT in the context of a dioxin receptor heterodimer.

Keywords: Transcription; bHLH/PAS proteins; ARNT; Dioxin receptor

The bHLH/PAS transcription factor ARNT functions as an obligate partner protein for signal regulated bHLH/PAS factors such as the dioxin receptor (DR), hypoxia inducible factors (HIF-1α, HIF-2α, and HIF-3α, or HIFs), and single minded proteins (Sim1 and Sim2) [11]. The DR and HIFs respond to the respective environmental stimuli of xenobiotic exposure or low oxygen tension by forming active heterodimeric complexes with ARNT [22,8]. In addition, ARNT can recognise the CACGTG E-box element as a homodimer in vitro and in vivo, indicating the likelihood of further transcription regulatory roles in the absence of DR ligand or hypoxia [2,21,10].

An alternatively spliced form of the ARNT gene has been identified in both mouse and human cell lines. The sequence of the alternative exon (exon 5) is 45 bp in length and is only present in the larger form of ARNT mRNA, encoding for an additional 15 amino acids just N-terminal to the basic region [9,18]. This larger form of ARNT, termed Alt ARNT by this laboratory, has been reported to behave in a similar manner to ARNT in TCDD-induced signal transduction with the dioxin receptor when transfected into mutant Hepa c4 cells [9]. There have been no further studies investigating the function of Alt ARNT since this initial report, and as such, the role of Alt ARNT has yet to be explored.

Alternative splicing is not uncommon amongst bHLH transcription factors. E2A mRNA, absolutely required for the B lymphocyte lineage, is alternatively spliced to generate E12 and E47 proteins [24,3]. E12 and E47 are both bHLH (bHLH) transcription factors, differing only in sequence within the inhibitory region just N-terminal to the basic DNA-binding domain. The overall highly negative nature of the E12 inhibitory region is thought to prevent a strong interaction with negatively charged DNA, explaining the poor DNA-binding ability of E12 homodimers

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.073

compared to E47 homodimers [23]. In support of this are experiments demonstrating that two serine residues within the E47 inhibitory region can be phosphorylated, preventing DNA-binding by E47 homodimers, but not heterodimers with MyoD [20].

The bHLH/LZ transcription factor Max (p21Max) also has an alternatively spliced form known as p22Max. p21Max and p22Max differ by an insertion of 9 amino acids just N-terminal to the basic DNA-binding domain [5]. Max is a nuclear phosphoprotein with the major in vivo phosphorylation sites determined to be Ser2 and Ser11 in both p21Max and p22Max, within the region N-terminal to the basic region [6]. EMSA experiments found that protein kinase CKII phosphorylation affected the DNA-binding properties of p21Max homodimers, p22Max homodimers, and Max/Myc heterodimers through the phosphorylation of both Ser2 and Ser11 [4,6].

The presence of two consensus protein kinase CKII sites within the additional 15 amino acids in Alt ARNT led us to analyse the possibility of differential phosphorylation between ARNT and Alt ARNT. Given the negative effect of phosphorylation on the DNA-binding abilities of E47 and Max, we also chose to analyse the DNA-binding abilities of ARNT and Alt ARNT. We report here the phosphorylation of a serine residue found within Alt ARNT, but not ARNT. Phosphorylation of Alt ARNT, but not ARNT, inhibits binding of homodimers to the E-box, but phosphorylation does not affect the ability of Alt Arnt to bind DNA as a heterodimer with the dioxin receptor.

Materials and methods

Recombinant plasmids. pARNT (1–358)/pET32α was generated by inserting an *Nco*I/*Hin*cII fragment from full-length human ARNT cDNA [9] into *Nco*I/*Xho*I-digested pET32α (Novagen). pAltARNT (1–358)/pET32α was generated by inserting an *Nco*I/*Spe*I fragment from full-length human Alt ARNT cDNA [9] into *Nco*I/*Spe*I-digested pARNT (1–358)/pET32α. pAltARNTS77A (1–358)/pET32α, pAltARNTS82A (1–358)/pET32α, and pAltARNTS77A/S82A (1–358)/pET32α were generated using the QuikChange site-directed mutagenesis kit (Stratagene).

Expression of bHLH/PAS proteins. pET32α plasmids in *Escherichia coli* strain BL21(DE3, Rosetta) were grown in LB broth supplemented with 2% glucose at 30 °C and induced in the absence of glucose with 0.1 mM IPTG for 3 h. Harvested cells were resuspended in buffer A (500 mM NaCl, 20 mM Tris (pH 7.9), and 5 mM imidazole) containing 0.1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride, and 0.5 mg/ml lysozyme. Resuspended cells were lysed by sonication. The lysate was centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was desalted using a PD-10 column (Pharmacia), exchanged into loading buffer (10 mM Tris (pH 7.5), 1 mM EDTA, and 10% glycerol), and loaded onto a freshly charged HiTrap Ni–NTA (Pharmacia) column using an FPLC (Pharmacia). The column was washed with buffer A before the proteins were eluted with 250 mM imidazole in buffer A. Eluted protein was concentrated using a Centricon-10 device (Amicon) to a volume of 0.5 ml and loaded onto a 25 ml Superdex 200 column. Protein fractions were eluted with Sephadex buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 5% glycerol, and 0.2 mM DTT). The purity of the isolated proteins was analysed by SDS–PAGE, and the protein concentration was determined by Bradford assay (Bio-Rad).

In vitro kinase assays. 0.5 μg of purified protein was incubated with 85 U of purified protein kinase CKII (New England BioLabs) according to the manufacturer's instructions with the addition of 5 μCi [γ-^{32}P]ATP. Reactions were stopped by the addition of SDS sample buffer (100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 2 mM EDTA, and 1× bromophenol blue), separated by SDS–PAGE, transferred to nitrocellulose, and analysed by autoradiography. For reactions analysed by electrophoretic mobility shift assay, instead of adding SDS sample buffer, the reaction mixtures were immediately incubated at 37 °C for 15 min (with purified DR (1–287) where appropriate) to allow proteins to homo- or heterodimerise, before adding the binding buffer and probe, as described below.

Electrophoretic mobility shift assays. Annealed double-stranded oligonucleotides were end-filled with ^{32}P-labelled dNTPs using Klenow enzyme and used as probes for Electrophoretic mobility shift assays (EMSAs). The AdML probe contains the CACGTG E-box motif from the adenovirus major late promoter [19]. The XRE probe contains a xenobiotic response element from the cytochrome P4501A1 gene [16]. Gel-mobility shift assays were carried out in a 20 μl volume with the bacterially expressed and purified proteins pARNT (1–358), pAltARNT (1–358), pAltARNTS77A (1–358), pAltARNTS82A (1–358), and pAltARNTS77A/S82A (1–358), either alone as homodimers or as heterodimers with DR (1–287). Briefly, protein samples (300 ng/reaction) were allowed to dimerise and interact at 37 °C for 15 min. Binding buffer (E-box, final concentration; 20 mM Hepes (pH 7.3), 3 mM $MgCl_2$, 1 mM EDTA, 8% glycerol, 10 mM DTT, 0.5 mg/ml BSA, and 50 μg/ml ssDNA. XRE, final concentration; 10 mM Hepes (pH 7.9), 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 3 mM $MgCl_2$, 4 mM spermidine, 15 ng/μl poly(dI–dC), and 30 ng/μl dA–dT.) and radiolabelled probe E-box or XRE (>8000 cpm) were then added, and the reaction mixtures were incubated at room temperature for 15 min. Protein-bound DNA complexes were resolved on a 5.5% nondenaturing polyacrylamide gel run in 0.25× TBE (25 mM Tris, 25 mM boric acid, and 0.5 mM EDTA; E-box) or 25 mM Tris/glycine (pH 8.0/0.1 mM EDTA; XRE) at 4 °C. In indicated EMSA experiments, polyclonal antibodies against ARNT [13] or pre-immune serum were added to the binding reactions together with the protein extracts and the radiolabelled probe to assess the specificity of protein–DNA complexes.

Analysis of reporter gene activity in transient transfected cell lines. Cells were seeded onto 24-well plates at 30,000 cells per well (Hepa 1c1c7, or 50,000 cells per well for 293T cells). The following day, cells were transfected with plasmid DNA using Fugene-6 (Roche) as per the manufacturer's protocol. After 36 h, cells were lysed and the luciferase activities were determined by the use of the Dual-Luciferase Reporter Assay System (DLR, Promega) as per the manufacturer's protocol. Results are presented as relative luciferase activity (Firefly: *Renilla*). Where cells were not transfected with the *Renilla* internal control plasmid, firefly luciferase activity was normalised to the protein concentration of cell extracts and presented as normalised luciferase activity. Where applicable, statistical significance was determined using Student's *t* test (two-tailed, two sample, equal variance), with significance determined when $P < 0.05$.

Results and discussion

Alignment of the region N-terminal to the basic domain of ARNT, Alt ARNT, p21Max, and p22Max

ARNT and Max proteins are both able to bind the CACGTG E-box element as homodimers and act as the general partner protein for members of their respective transcription factor families. ARNT and the alternatively spliced form, Alt ARNT, differ by the addition of 15 amino acids, while the Max gene is alternatively spliced to generate a form of Max containing an additional 9 amino acids. Given that the alternative exons of Max and ARNT are both located just before their basic regions, a comparison of the amino acid sequence of the region N-terminal to the basic region was made between ARNT, Alt ARNT, p21Max, and p22Max (Fig. 1). The amino

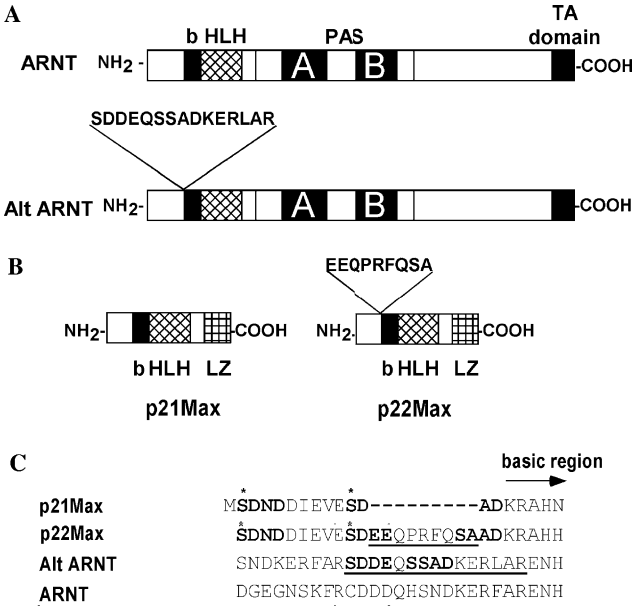

Fig. 1. Alignment of ARNT and Alt ARNT with both spliced forms of Max. A schematic representation of ARNT and Alt ARNT (A), and the two spliced forms of Max, p21Max and p22Max (B), illustrating the position of the alternative exon-encoded additional amino acids present in Alt ARNT and p22Max. (C) A partial amino acid sequence comparison between p21Max, p22Max, Alt ARNT, and ARNT, N-terminal to the start of the basic region. The alternative exon-encoded amino acids are underlined. Protein kinase CKII consensus sites are in bold. Known in vivo phosphorylation sites are marked with an asterisk.

acid sequence of Alt ARNT is more similar to those of p21 and p22Max than ARNT. The sequence of Alt ARNT, like those of p21 and p22Max, contains protein kinase CKII consensus sites (S/T.X.X.D/E [1]), with Ser77 and Ser82 being potential phosphorylation sites. Interestingly, Ser77 in Alt ARNT correlates with Ser11 in p22Max and Ser2 in p21Max, both known in vivo phosphorylation sites of Max. There are no consensus CKII sites in the equivalent region of ARNT.

ARNT and Alt ARNT are differentially phosphorylated by protein kinase CKII

Initial experiments using purified truncated forms of ARNT and Alt ARNT, containing only the bHLH domain, suggested that ARNT and Alt ARNT were differentially phosphorylated (data not shown). However, these truncations were poorly expressed and difficult to purify. Larger fragments of ARNT and Alt ARNT, containing the bHLH/PAS A domains (1–358), were expressed as thioredoxin/His-tag fusion proteins and purified to near homogeneity by nickel affinity chromatography and Sephadex chromatography (Fig. 3A, lower panel). Three mutant forms of Alt ARNT (1–358) were also generated, substituting Ser77, Ser82, or both Ser77 and 82 in alanine residues (Fig. 2A). To ensure that all five ARNT proteins were functional, we performed electrophoretic mobility shift assay (EMSA) experiments using an E-box probe (Fig. 2B). A single band was found when each protein was incubated with the probe (lanes 1–5). The bands were confirmed as ARNT/DNA complexes, as the addition of the ARNT antibodies resulted in a supershift of each complex (lanes 6–10).

In vitro kinase experiments performed with protein kinase CKII showed that Alt ARNT (1–358) is phosphorylated to a higher extent than ARNT (1–358) (Fig. 3A, compare lanes 1 and 2). When Ser77 is mutated to Ala, there is a dramatic reduction in CKII phosphorylation (compare lanes 2 and 3). In contrast, when Ser82 is mutated to Ala, minimal difference in phosphorylation is observed. The double Alt ARNT (1–358) mutant shows similar levels of phosphorylation to ARNT (1–358), also indicating that Ser77 is a predominant site of phosphorylation (compare lanes 1 and 5). The lower panel of Fig. 3A shows that equivalent levels of pure protein were used in these phosphorylation assays.

To confirm that ARNT and Alt ARNT are differentially phosphorylated at Ser77, we analysed further C-terminally truncated forms of Trx-H_6-tagged ARNT and Alt ARNT, corresponding to amino acids 1–102 as determined by mass spectrometry, which were obtained as cleanly cleaved by-products during the purification of ARNT and Alt ARNT (1–358). In vitro kinase experiments were performed with these truncated proteins, containing the N-terminus of ARNT up to the end of the DNA-binding basic region (Fig. 3B). Similar to ARNT and Alt ARNT (1–358), ARNT and Alt ARNT (1–102) are differentially phosphorylated, predominantly at Ser77.

CKII phosphorylation has an inhibitory effect on Alt ARNT DNA-binding to an E-box probe

The data of Figs. 3A and B show that Ser77 is a phosphorylation site in the alternative exon of ARNT, with the context of this serine (SDEE) being strikingly similar to the context of two serines in Max (SDEE and SDND for p22Max, SDND and SDAD for p21Max) which are phosphorylated by CKII. In the case of the Max proteins, phosphorylation at these sites influences DNA binding, so we therefore investigated the effects of CKII phosphorylation on DNA binding by Alt ARNT and Alt ARNT S77A. Kinase reactions were performed either in the absence or presence of protein kinase CKII enzyme, followed by an EMSA using an E-box probe (Fig. 4). As reported [4], p22Max shows a decrease in E-box binding when phosphorylated (compare lanes 2 and 3). Alt ARNT similarly exhibited a distinct decrease in DNA-binding when phosphorylated by CKII (compare lanes 6 and 7). In stark contrast, however, ARNT showed no difference in DNA-binding following treatment with CKII (compare lanes 4 and 5). Likewise, DNA-binding of Alt ARNT S77A mutant was not affected by CKII phosphorylation, consistent with phosphorylation of Ser77 inhibiting Alt ARNT DNA-binding activity and establishing the importance of Ser77 in regulation of DNA binding.

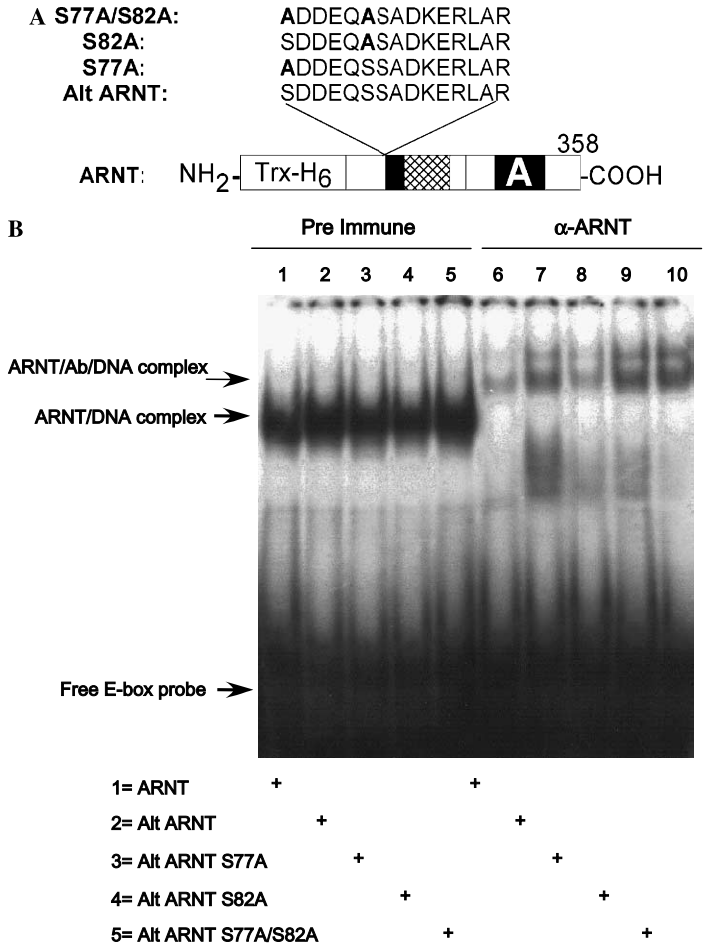

Fig. 2. ARNT, Alt ARNT, and mutant forms of Alt ARNT bind the CACGTG E-box element in vitro. (A) N-terminal regions (aa 1–358) containing the bHLH domain and PAS A of ARNT and Alt ARNT were expressed in bacteria as Trx/His-tag fusions and purified to near homogeneity. Mutant forms of Alt ARNT were generated (S77A, S82A, and S77A/S82A) with serine 77 or 82 or both 77 and 82 replaced by an alanine residue, removing one or two potential protein kinase CKII phosphorylation sites. Alt ARNT mutant proteins were similarly expressed and purified from bacteria. (B) Hundred nanograms of ARNT and Alt ARNT 1–358 proteins was assayed for DNA-binding activity in a gel mobility shift assay using the ^{32}P-labelled E-box probe in the presence of preimmune serum (PI) or αARNT antibodies. The arrows indicate the positions of the ARNT-retarded complexes and the antibody-supershifted complexes.

CKII phosphorylation has no effect on DNA-binding by ARNT or Alt ARNT-containing heterodimers

CKII phosphorylation has a greater inhibitory effect on DNA-binding by Max homodimer than Max-containing heterodimers [4]. Similarly, phosphorylated E47 homodimers, but not phosphorylated E47-containing heterodimers, are inhibited from binding DNA [20]. We sought to investigate whether ARNT heterodimers were affected by CKII phosphorylation. In vitro kinase assays of ARNT, Alt ARNT or mutant Alt ARNT protein in the absence or presence of CKII were performed, followed by the addition of purified GST-tagged DR (1–287) (a truncated form of the DR able to dimerise with ARNT but lacking PASB and the C-terminal transactivation domain). Following the formation of an ARNT/DR heterodimer, an EMSA was performed with a radiolabelled xenobiotic response element (XRE) probe (Fig. 5). In contrast to E-box binding by Alt ARNT homodimers, phosphorylation by CKII did not have an inhibitory effect on DNA-binding by the DR/Alt ARNT heterodimer. As expected, CKII phosphorylation also had no inhibitory effect on ARNT/DR and Alt ARNT S77A/DR heterodimers.

Activation of reporter genes by ARNT and Alt ARNT

The differences in Alt ARNT and ARNT DNA-binding activity in vitro led us to investigate the potential differences of these forms of ARNT on reporter genes. Initially, we examined the activity on an XRE-driven reporter gene. Full-length ARNT, Alt ARNT, and Alt ARNT S77A were transiently expressed in Hepa 1c1c7 cells together with a constitutively active DR (DRΔPASB, which lacks the PASB domain and does not require any ligand for dimerisation or DNA-binding [14]). Consistent with the in vitro DNA-binding assays, there was little difference in activity between ARNT and Alt ARNT proteins on an XRE reporter when complexed as a heterodimer with the DR

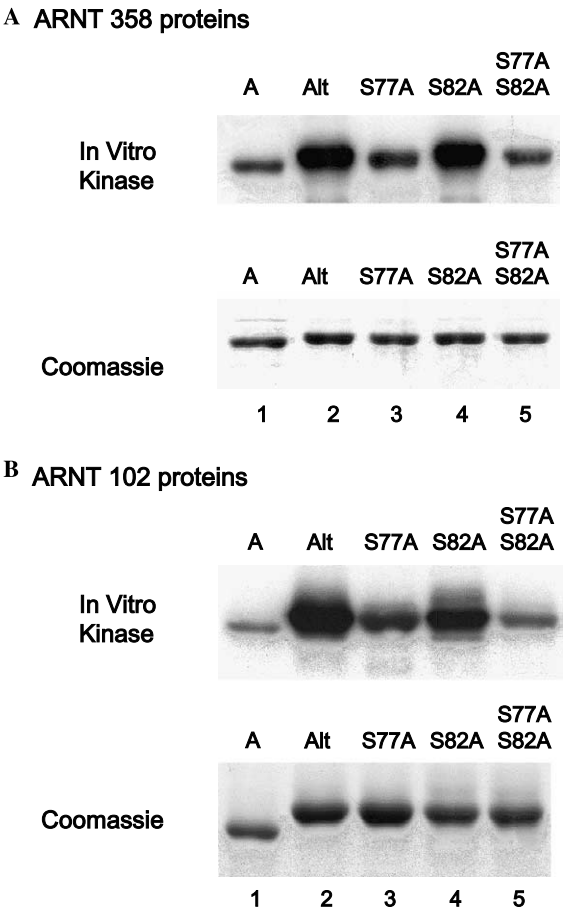

Fig. 3. ARNT and Alt ARNT are differentially phosphorylated by protein kinase CKII. In vitro kinase reactions were performed with the following proteins using protein kinase CKII and [γ-^{32}P]ATP, and analysed by gel electrophoresis and autoradiography (IVK), (A) ARNT, Alt ARNT, S77A, S82A, and S77AS82A 1–358 proteins, (B) ARNT, Alt ARNT, S77A, S82A, and S77A/S82A 1–102 proteins. The second panel in (A) and (B) is a Coomassie-stained gel (Coom) containing four times as much protein as was used in the corresponding in vitro kinase assay (IVK).

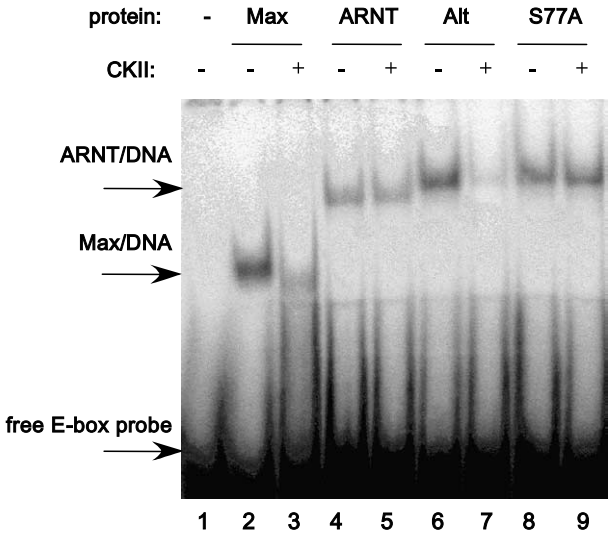

Fig. 4. Protein kinase CKII phosphorylation inhibits E-box binding by Alt ARNT, but not ARNT homodimers. To assess the effect of protein kinase CKII phosphorylation on DNA-binding as homodimers, in vitro kinase reactions were performed with Max (lanes 2 and 3), ARNT (lanes 4 and 5), and Alt ARNT 1–358 proteins (Alt ARNT, lanes 6 and 7; S77A, lanes 8 and 9), in the absence (−) or presence (+) of protein kinase CKII, and immediately used in a gel mobility shift assay with an E-box probe. Lane 1, probe alone.

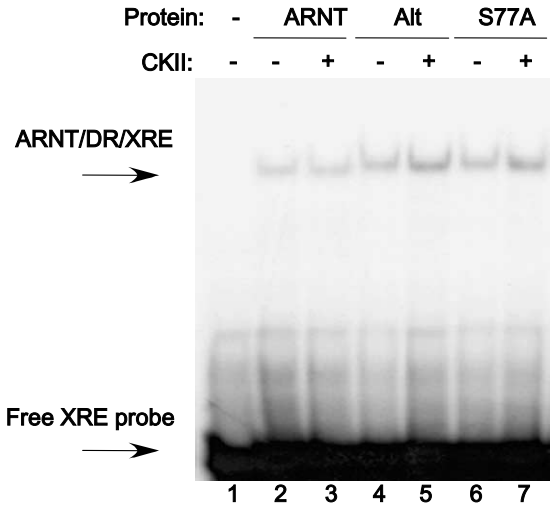

Fig. 5. Protein kinase CKII phosphorylation has no effect on ARNT or Alt ARNT DNA-binding to the XRE as a heterodimer with the dioxin receptor. To assess whether protein kinase CKII phosphorylation had any effect on DNA-binding by an ARNT or Alt ARNT-containing heterodimer, in vitro kinase reactions were performed with ARNT 1–358 (lanes 2 and 3), Alt ARNT (1–358) (lanes 4 and 5), and S77A (1–358) (lanes 6 and 7), in the absence (−) or presence (+) of protein kinase CKII, before being incubated with the dioxin receptor and used immediately in a gel mobility shift assay with the XRE probe. Lane 1, probe alone.

(Fig. 6). In transient transfection assays in 293T cells with an E-box-driven reporter gene, Alt ARNT activity was slightly, but significantly ($P < 0.05$), higher than that of ARNT (Fig. 7A).

Interestingly, the Alt ARNT S77A mutant consistently exhibited a higher activity on an E-box reporter gene than both ARNT and Alt ARNT. Western blot analysis of 293T cells transiently transfected with ARNT, Alt ARNT, and Alt ARNT S77A expression plasmids indicates that these proteins are equally expressed. Thus, while the Alt ARNT S77A mutant does not appear to affect DNA-binding affinity in vitro (Fig. 4, compare lanes 8 and 9 with 6) or protein stability (Fig. 7B, compare lanes 2 and 4) it appears to increase the transactivation potential of Alt ARNT on an E-box. Notably, this increase is not observed in Fig. 6 when Alt ARNT S77A forms a dimer with the DR to activate the XRE reporter. It is possible that the difference in the E-box reporter gene is due to endogenous kinase activity dampening the DNA binding activity of Alt ARNT homodimers, a mechanism to which Alt ARNT S77A would be refractory. However, as Alt ARNT seems to be stronger than ARNT on the E-box reporter, it is also possible that differences in conformation created by the alternative exon may exist, perhaps allowing better interaction with transcription coactivators.

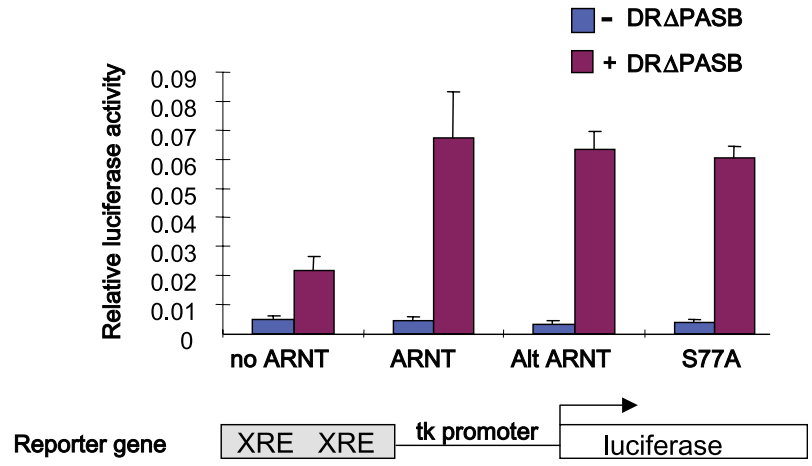

Fig. 6. ARNT and Alt ARNT exhibit similar activity on an XRE reporter gene as a heterodimer with the DR. Two hundred nanogram expression plasmids for ARNT, Alt ARNT, and the mutant form of Alt ARNT (S77A) were transiently transfected into Hepa 1c1c7 in triplicate wells in a 24-well tray format using the Fugene 6 method with the XRE-luciferase reporter (pX1X1) (100 ng), *Renilla* internal control luciferase plasmid (RLTK, 100 ng), and either a constitutively active mDR plasmid (DRΔPASB) or blank expression plasmid. Forty-eight hours following transfection, cells were lysed and assayed for firefly and *Renilla* luciferase activity. Relative luciferase activity results presented are a representative of three separate experiments. Data shown are an average of the triplicate wells of a single experiment ±SD.

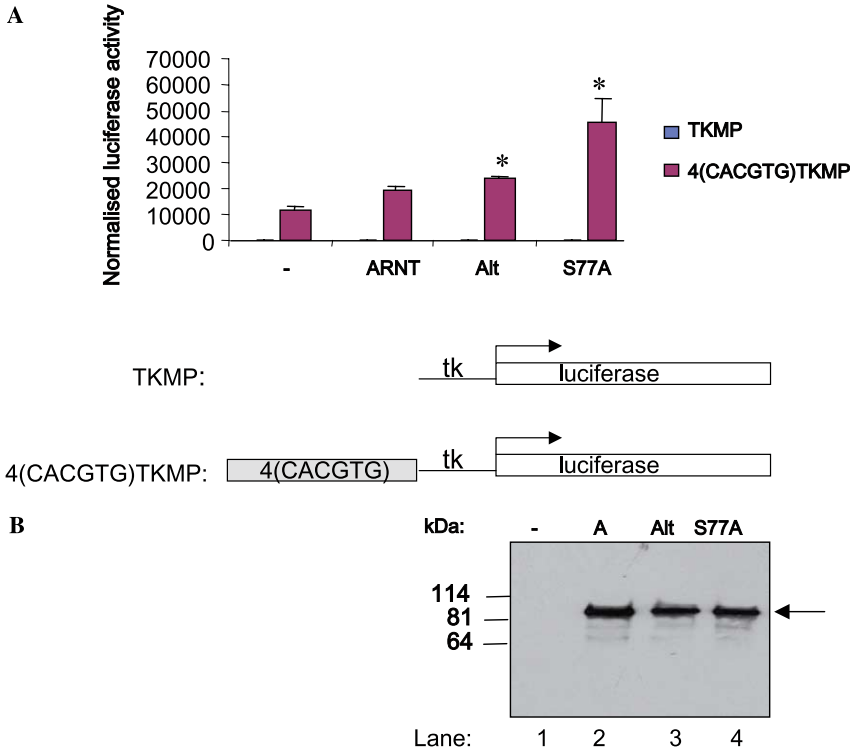

Fig. 7. ARNT and Alt ARNT activity on an E-box reporter. (A) Two hundred nanograms of mammalian expression plasmids containing full-length ARNT—A, Alt ARNT—Alt or the Alt ARNT mutant—S77A was transiently transfected into 293T cells in triplicate wells in a 24-well tray format using the Fugene 6 method with either the TKMP blank luciferase reporter or the 4(CACGTG)-driven luciferase reporter (200 ng). Forty-eight hours following transfection, cells were lysed and assayed for firefly luciferase activity and protein concentration. Normalised luciferase activity results are presented. Data shown are an average of the triplicate wells of a single experiment ±SD. Student's *t* tests were used to determine the statistical significance of E-box reporter activity driven by Alt ARNT compared to ARNT, and the Alt ARNT mutant (S77A) compared to Alt ARNT ($^{*}P < 0.05$). (B) Twenty-five microgram whole cell extracts from cells transiently transfected with 4(CACGTG)TKMP reporter and the indicated ARNT expression plasmids were subjected to SDS–PAGE and analysed for levels of ARNT protein in a Western blot using an α-ARNT primary antibody. The arrow indicates the ARNT protein.

We have begun to characterise differences between two alternatively spliced forms of the ARNT gene, ARNT and Alt ARNT. Previous RT-PCR analysis found a similar expression of both ARNT and Alt ARNT in Hepa c4 cells and in all other cell lines tested within our laboratory ([15]; Kewley and Whitelaw, unpublished results), so we sought

to identify putative means of differentially regulating the two ARNT isoforms.

Through amino acid sequence analysis and comparisons with the Max proteins, we initially identified putative CKII phosphorylation sites within the additional 15 amino acids in Alt ARNT. We subsequently demonstrated that bacterially expressed fragments of ARNT and Alt ARNT are differentially phosphorylated by protein kinase CKII. Site-directed mutagenesis revealed the major site of phosphorylation to be Ser77, found exclusively in Alt ARNT. Furthermore, CKII phosphorylation inhibits DNA-binding to an E-box probe by Alt ARNT, but not ARNT. DNA-binding in EMSAs by the S77A mutant of Alt ARNT is not affected by CKII phosphorylation, suggesting that Ser77 phosphorylation is the key to the inhibition of DNA-binding by Alt ARNT. Interestingly, phosphorylation by CKII does not inhibit ARNT or Alt ARNT binding to a xenobiotic response element as a heterodimer with the dioxin receptor. In reporter gene assays, consistent with EMSAs, we observed that ARNT and Alt ARNT produced similar activity on an XRE reporter gene when complexed with the DR. In contrast, the Alt ARNT S77A mutant exhibited a higher E-box reporter activity than ARNT or Alt ARNT. Increased activity of the S77A mutant suggests that Alt ARNT might be phosphorylated in 293T cells at Ser77, thus binding to the E-box with reduced affinity and consequently not fully activating transcription of the reporter gene, though Alt ARNT remains more active than ARNT. When Ser77 is mutated to an alanine residue, this inhibition does not take place, and maximal transcriptional activity can occur. Alternatively, mutating Ser77 may alter the conformation of the Alt ARNT protein such that it can interact with, or interact more strongly with, a coactivator or chaperone protein. Both coactivators and chaperones have been found to interact with the bHLH regions of other proteins and it is possible that mutations in the N-terminal region are sufficient to alter this binding. The interaction between coactivator CBP/p300 and the bHLH regions of E-box binding proteins MyoD, myogenin, and E47 increased the transactivation potential of the proteins and has been demonstrated to play an essential role in muscle and B-cell differentiation programs [7,17].

The in vivo role for differential CKII phosphorylation of ARNT and Alt ARNT remains to be investigated. Other transcription factors such as Max, Myb, and c-Jun are phosphorylated by CKII, similarly having a negative affect on DNA-binding. The factors regulating phosphorylation, however, are unknown. CKII is a ubiquitously expressed kinase, with conflicting data in the literature as to whether the activity of CKII can be modified in the cell [12]. Nevertheless, phosphorylation of Ser77 within the alternative exon of ARNT has a major influence on the activity of Alt ARNT homodimers, but not an Alt ARNT heterodimer, providing a further mechanism to diversify the function of the broadly used ARNT transcription factor.

Acknowledgments

We thank Dr. Oliver Hankinson (UCLA) for providing ARNT and Alt ARNT cDNA, Dr. Michael Cole (Princeton University, NJ) for the His-tagged p22Max expression construct (His-Max), and Anne Chapman-Smith for assistance with protein purification and supply of purified GST-tagged DR (1–287). This work was generously supported by the Australian Research Council Special Centre for the Molecular Genetics of Development.

References

[1] J.E. Allende, C.C. Allende, Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation, FASEB J. 9 (5) (1995) 313–323.
[2] C. Antonsson, V. Arulampalam, M.L. Whitelaw, S. Pettersson, L. Poellinger, Constitutive function of the basic helix–loop–helix/PAS factor Arnt, J. Biol. Chem. 270 (1995) 13968–13972.
[3] G. Bain, E.C.R. Maandag, D.J. Izon, D. Amsen, A.M. Kruisbeek, B.C. Weintrub, I. Krop, M.S. Schlissel, A.J. Feeney, M. van Roon, M. van der Valk, H.P.J. te Riele, A. Berns, C. Murre, E2A proteins are required for proper B cell developments and initiation of immunoglobulin gene rearrangements, Cell 79 (1994) 885–892.
[4] S.J. Berberich, M.D. Cole, Casein kinase II inhibits the DNA-binding activity of Max homodimers but not Myc/Max heterodimers, Genes Dev. 6 (1992) 166–176.
[5] E.M. Blackwood, R.N. Eisenman, Max: a helix–loop–helix zipper protein that forms a sequence-specific DNA-binding complex with Myc, Science 251 (1991) 1211–1217.
[6] K. Bousset, M. Henriksson, J.M. Lüscher-Firzlaff, D.W. Litchfield, B. Lüscher, Identification of casein kinase II phosphorylation sites in Max: effects on DNA-binding kinetics of Max homo- and Myc/Max heterodimers, Oncogene 9 (1993) 3211–3220.
[7] R. Eckner, T.P. Yao, E. Oldread, D.M. Livingston, Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation, Genes Dev. 10 (1996) 2478–2490.
[8] A.O. Fedele, M.L. Whitelaw, D.J. Peet, Regulation of gene expression by the hypoxia-inducible factors, Mol. Interv. 4 (2002) 229–243.
[9] E.C. Hoffman, H. Reyes, F.-F. Chu, F. Sander, L.H. Conley, B.A. Brooks, O. Hankinson, Cloning of a factor required for activity of the Ah (dioxin) receptor, Science 252 (1991) 954–958.
[10] J.L. Huffman, A. Mokashi, H.P. Bächinger, R.G. Brennan, The basic helix–loop–helix domain of the aryl hydrocarbon receptor nuclear transporter (ARNT) can oligomerize and bind E-Box DNA specifically, J. Biol. Chem. 276 (2001) 40537–40544.
[11] R.J. Kewley, M.L. Whitelaw, A. Chapman-Smith, The mammalian basic helix–loop–helix/PAS family of transcriptional regulators, Int. J. Biochem. Cell Biol. 36 (2004) 189–204.
[12] D.W. Litchfield, B. Lüscher, Casein kinase II in signal transduction and cell cycle regulation, Mol. Cell. Biochem. 127–128 (1993) 189–199.
[13] G.G. Mason, A.M. Witte, M.L. Whitelaw, C. Antonsson, J. McGuire, A. Wilhelmsson, L. Poellinger, J.A. Gustafsson, Purification of the DNA binding form of dioxin receptor. Role of the Arnt cofactor in regulation of dioxin receptor function, J. Biol. Chem. 269 (1994) 4438–4449.
[14] J. McGuire, K. Okamoto, M.L. Whitelaw, H. Tanaka, L. Poellinger, Definition of a dioxin receptor mutant that is a constitutive activator of transcription, J. Biol. Chem. 276 (2001) 41841–41849.
[15] K. Numayama-Tsuruta, A. Kobayashi, K. Sogawa, Y. Fujii-Kuriyama, A point mutation responsible for defective function of the arylhydrocarbon-receptor nuclear translocator in mutant Hepa-1c1c7 cells, Eur. J. Biochem. 246 (1997) 486–495.

[16] I. Pongratz, C. Antonsson, M.L. Whitelaw, L. Poellinger, Role of the PAS domain in regulation of dimerization and DNA binding specificity of the dioxin receptor, Mol. Cell. Biol. 18 (1998) 4079–4088.
[17] P.L. Puri, M.L. Avantaggiati, C. Balsano, N. Sang, A. Graessmann, A. Giordano, M. Levrero, p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription, Genes Dev. 16 (1997) 369–383.
[18] S. Reisz-Porszasz, M.R. Probst, B.N. Fukunaga, O. Hankinson, Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT), Mol. Cell. Biol. 14 (1994) 6075–6086.
[19] M. Sawadogo, R.G. Roeder, Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region, Cell 43 (1985) 165–175.
[20] S.R. Sloan, C.-P. Shen, R. McCarrick-Walmsley, T. Kadesch, Phosphorylation of E47 as a potential determinant of B-cell-specific activity, Mol. Cell. Biol. 16 (1996) 6900–6908.
[21] K. Sogawa, R. Nakano, A. Kobayashi, Y. Kikuchi, N. Ohe, N. Matsushita, Y. Fujii-Kuriyama, Possible function of Ah receptor nuclear translocator (Arnt) homodimer in transcriptional regulation, Proc. Natl. Acad. Sci. USA 92 (1995) 1936–1940.
[22] H.I. Swanson, DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation, Chem. Biol. Interact. 141 (2002) 63–76.
[23] X.-H. Sun, D. Baltimore, An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers, Cell 64 (1991) 459–470.
[24] Y. Zhuang, P. Soriano, H. Weintrub, The helix–loop–helix gene E2A is required for B cell formation, Cell 79 (1994) 875–884.