Structural basis for the nuclear import of the human androgen receptor

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Summary

Ligand-dependent nuclear import is crucial for the function of the androgen receptor (AR) in both health and disease. The unliganded AR is retained in the cytoplasm but, on binding 5α-dihydrotestosterone, it translocates into the nucleus and alters transcription of its target genes. Nuclear import of AR is mediated by the nuclear import factor importin-α, which functions as a receptor that recognises and binds to specific nuclear localisation signal (NLS) motifs on cargo proteins. We show here that the AR binds to importin-α directly, albeit more weakly than the NLS of SV40 or nucleoplasmin. We describe the 2.6-Å-resolution crystal structure of the importin-α–AR-NLS complex, and show that the AR binds to the major NLS-binding site on importin-α in a manner different from most other NLSs. Finally, we have shown that pathological mutations within the NLS of AR that are associated with prostate cancer and androgen-insensitivity syndrome reduce the binding affinity to importin-α and, subsequently, retard nuclear import; surprisingly, however, the transcriptional activity of these mutants varies widely. Thus, in addition to its function in the nuclear import of AR, the NLS in the hinge region of AR has a separate, quite distinct role on transactivation, which becomes apparent once nuclear import has been achieved.

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Introduction

The human androgen receptor (AR) plays a key role in development of male secondary sexual characteristics, and in the development and growth of the prostate (Quigley et al., 1995). Abnormalities of the AR gene or protein are associated with a wide range of clinical conditions, including androgen-insensitivity syndrome (AIS) (Brinkmann, 2001; Gottlieb et al., 1999; Quigley et al., 1995), prostate cancer (Heinlein and Chang, 2004; Linja and Visakorpi, 2004; Gottlieb et al., 1999; Stanford et al., 1997; Giovannucci et al., 1997), male breast cancer (Lobaccaro et al., 1993; Wooster et al., 1992), and spinal and bulbar muscular atrophy (Kennedy disease) (La Spada et al., 1991). Furthermore, modest increases in AR protein levels play a crucial role in the development of hormone refractory or hormone-independent prostate cancer (Chen et al., 2004).

In common with other members of the nuclear hormone-receptor family of transcription factors, the AR is constructed from a series of functional domains (Fig. 1). All receptors of this class have a common architecture based on a C-terminal ligand-binding domain (LBD), a N-terminal transactivation domain (TAD) and a central DNA-binding domain (DBD) that is linked to the LBD by a hinge region (Laudet and Gronemeyer, 2002; Brinkmann et al., 1989). In its unliganded state, the AR is located in the cytoplasm, complexed with Hsp90 and several other proteins (Fang et al., 1996; Georget et al., 2002; Kuil et al., 1995; Pratt et al., 2004). Ligand binding alters the conformation of the LBD (Wurtz et al., 1996; Kallenberger et al., 2003; Schaufele et al., 2005), and results in activation of the AR and its translocation to the nucleus (Simental et al., 1991; Jenster et al., 1993; Zhou et al., 1994; Georget et al., 1997; Georget et al., 1998; Tyagi et al., 2000), where it recognises and binds to androgen response elements (Shaffer et al., 2004) and activates the transcription of a range of target genes (Shang et al., 2002).

The nuclear import of the androgen receptor is crucial for its function. Although the import receptors for AR have not been investigated specifically, import is generally thought to be mediated through the classical pathway that employs importin-α and importin-β (Gorlich and Kutay, 1999; Macara, 2001; Stewart, 2006; Stewart, 2007), consistent with the finding that importin-α binds to the glucocroticoid receptor (Savory et al., 1999). In the classical nuclear-protein-import pathway, a positively charged nuclear localization signal (NLS) sequence motif (which is formed from either a single or bipartite cluster of Lys and Arg residues) is recognised in the cytoplasm by importin-α that serves as an adaptor to the nuclear transport factor importin-β. The importin-α–importin-β cargo import complex then moves through nuclear-pore complexes (NPCs) to the nucleus where it is dissociated by the Ras family GTPase Ran, thus releasing the AR; thereafter the importins are recycled to the cytoplasm to enable a further import cycle. Early immunostaining studies (Jenster et al., 1993; Simental et al., 1991; Zhou et al., 1994) demonstrated the importance of specific basic residues for nuclear localisation of the AR after androgen exposure, and these findings have been corroborated by more recent studies in which confocal microscopy was used to assess the nuclear import of the GFP-tagged AR (Poukka et al., 2000; Thomas et al., 2004) on a more dynamic timescale. These basic residues were proposed to constitute a bipartite NLS that contains two clusters of basic amino acids. In a bipartite NLS, the first (minor) cluster usually has two basic residues with an intervening stretch of ~ten residues

followed by a major cluster that commonly contains four lysine residues and contrasts with a monopartite NLS in which there is only a single cluster commonly containing five basic residues (Dingwall and Laskey, 1991). This putative bipartite NLS is highly conserved between many nuclear receptors, such as AR, glucocorticoid receptor, mineralocorticoid receptor and progesterone receptor (Fig. 1).

A number of mutations have been reported within the putative NLS sequence of the AR that are associated with AIS or prostate cancer (Marcelli et al., 1991; Gottlieb et al., 1999; Nazareth et al., 1999; Tilley et al., 1996; Wang and Uchida, 1997; Zoppi et al., 1992). However, it is not clear whether the nuclear import of these AR mutants is altered (so that the distribution of the activated receptor between the nucleus and cytoplasm is changed) and/or whether there are differences in their transcriptional activity. Moreover, several lysine residues within the AR NLS (629RKLKK633) are subject to acetylation by the transcriptional coactivators p300 (PCAF) and Tip60 (HTATIP), which in turn alters the transactivation functions of the AR (Fu et al., 2003; Fu et al., 2000; Fu et al., 2002; Gaughan et al., 2002). Given that these residues serve a role in both acetylation and binding to importin-α, it was important to explore how mutations within this region and other parts of the AR hinge region that have been shown to alter nuclear import can also influence subsequent transactivation.

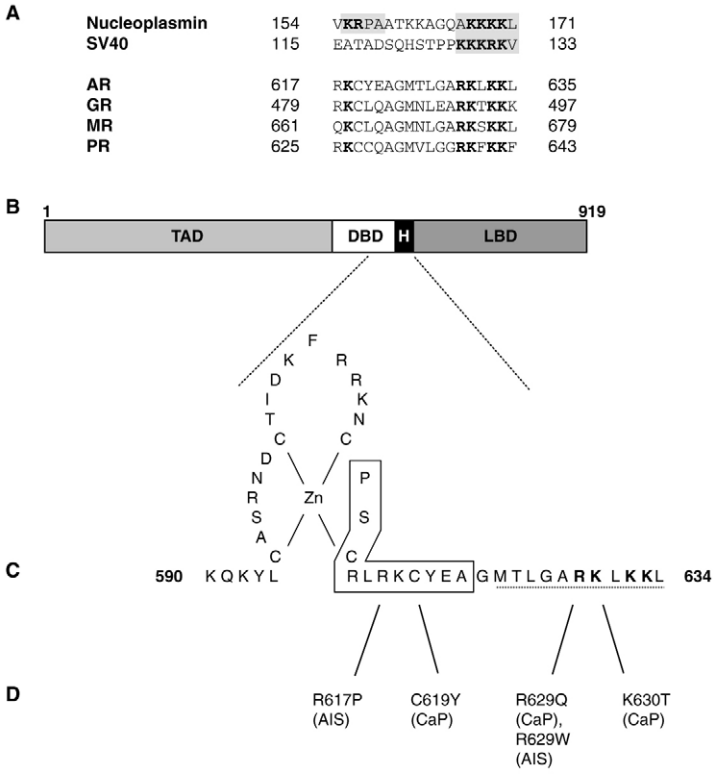

Fig. 1. The major NLS binding motif is conserved in the steroid receptor hinge region. (A) The amino acid sequences from the hinge regions of the androgen receptor (AR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), nucleoplasmin and the SV40 large T antigen were aligned using Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/). Conserved basic residues are in bold. The classical bipartite NLS is from nucleoplasmin, and binds to importin-α via its minor and major NLS motif, boxed in grey. SV40 is a classical monopartite NLS and binds to the major NLS-binding pockets of importin-α (area shaded grey). The sequence alignment of several nuclear hormone receptors reveals significant homology with these classical NLSs, with conserved basic residues in bold. (B) Scheme of the human AR, showing the N-terminal transactivation domain (TAD), DNA-binding domain (DBD), hinge domain (H) and ligand-binding domain (LBD). (C) The second zinc finger of the DBD and the hinge region. The ordered terminal α-helix of the DBD is boxed, and the unstructured hinge region is underlined. The conserved NLS motif is in bold. Only the stretch of positively charged residues (629RKLKK633; bold) lies within the unstructured hinge domain, whereas the charged residues of the C-terminal lie within the DBD α-helix. Adapted from Shaffer et al. (Shaffer et al., 2004). (D) Pathological substitutional mutations within this region that lead to AIS or prostate cancer (CaP).

Both the major and minor clusters of basic residues of the archetypal bipartite NLS from nucleoplasmin bind strongly to importin-α (Conti and Kuriyan, 2000; Fontes et al., 2000), therefore, it had been thought that both clusters of basic residues in the putative AR NLS bind to importin-α in a similar manner. However, recent structural studies have shown that the DBD is constructed from two zinc-finger motifs (Shaffer et al., 2004), and that the minor cluster of the putative AR bipartite NLS (residues 617-620) lies within the terminal α-helix of the second zinc finger and not within the flexible hinge region (Fig. 1C). NLSs are usually located in flexible regions of proteins such that they can adapt their conformation to complement the NLS-binding site of importin-α (reviewed by Stewart, 2007) and, therefore, it is possible that residues located within this α-helix might not be able to bind importin-α effectively. Indeed, mutations in the second zinc finger have been shown to disrupt AR conformation to a sufficient degree to interfere with its binding to DNA in the nucleus (Nazareth et al., 1999; Zoppi et al., 1992).

We therefore reinvestigated the precise mechanism by which the AR is imported into the nucleus. We describe here the structure of the complex between importin-α and the AR hinge region to define the interaction interface unequivocally, together with complementary biochemical and cellular transport studies to explore the effects of mutations in the AR hinge region on both its affinity for importin-α and its rate of nuclear accumulation. We show that although the AR binds only to the major NLS-binding site of importin-α (in a manner analogous to monopartite NLSs) the way in which it interacts with importin-α is different to that of other monopartite classical NLSs. Engineered mutations confirm that key residues in this region of the AR are crucial for binding. Moreover, mutations that attenuate the affinity of the AR NLS for importin-α are also important functionally in vivo and alter the nuclear import rate of GFP-AR fusion proteins in mammalian cell lines. We have explored the possibility that transcriptional activity of various AR mutants is related to nuclear import kinetics and whether this is consistent with various phenotypes of AIS and prostate cancer.

Results

Importin-α binds the AR in mammalian cell extracts and in vitro

Co-immunoprecipitation studies established that the AR binds to importin-α in mammalian cell lines. COS-7 cells were transfected with GFP-tagged wild-type AR (GFP-AR), which was then isolated from cell lysates by immunoprecipitation. The cell extracts were subsequently probed with importin-α antibodies, which confirmed the binding of importin-α to GFP-AR (Fig. 2C). Androgen increases expression of AR. Importin-α bound to AR in both

androgen-free and androgen-enriched conditions, but androgen exposure did not enhance AR binding to importin-α. These results were confirmed by densitometry of the gels.

To determine which region of the AR binds to importin-α, GST-fusion constructs of the different AR domains (TAD, DBD, H, HLBD, LBD; see Fig. 2A) were immobilised on glutathione resin; in vitro binding assays were performed using ΔIBB-importin-α – which lacks the importin-β-binding (IBB) domain, residues 70-529 – and samples probed for bound importin-α by western blot analysis. It is important to use ΔIBB-importin-α, because in the absence of importin-β the IBB domain has an autoinhibitory function, whereby it competes with the NLS for binding to importin-α and so results in the apparent affinity for NLSs being dramatically reduced (Goldfarb et al., 2004; Kobe, 1999). The affinity of ΔIBB-importin-α for NLS peptides is comparable with that of full-length importin-α in the presence of importin-β (Fontes et al., 2003b). As illustrated in Fig. 2B, importin-α clearly bound only to constructs containing the AR hinge domain, and not any of the other AR domains. These results indicate that importin-α binds exclusively and directly to the AR hinge region in vitro.

Structure of the importin-α–AR-NLS complex

The binding assays indicated that importin-α binds to full length AR in vivo and that this interaction requires the AR hinge region. We established the precise details of the interaction interface and identified the contribution made to the interaction by each residue in the hinge region by determining the crystal structure of the complex between importin-α and AR residues 617-634.

The complex was prepared by mixing crude lysates of bacterially expressed untagged importin-α and GST-AR (617-634). Thereafter, complex was purified using glutathione sepharose followed by gel filtration chromatography. Orthorhombic $P2_12_12_1$ symmetry crystals of the complex were grown using vapour diffusion in 100 mM HEPES pH 6.5, 0.7 M sodium citrate, 10 mM DTT, and diffracted to 2.6 Å on a laboratory X-ray source (Table 1). The importin-α–AR-NLS crystals were isomorphous with those formed between importin-α and a nucleoplasmin NLS peptide (Fontes et al., 2003a; Fontes et al., 2000) and were solved by molecular replacement using residues 70-496 of mouse importin-α (PDB accession code 1EJY) as a model. A clear horseshoe-shaped *Fo-Fc* difference density due to the AR-NLS peptide was seen over the major NLS binding site of importin-α (Fig. 3). No other significant difference density was present, especially in the vicinity of the secondary NLS binding site to which bipartite NLSs also bound (Conti et al., 1998; Conti and Kuriyan, 2000; Fontes et al., 2000; Fontes et al., 2003a). Residues 621-634 of the AR NLS were easily built into the difference density to give a model having an R-factor of 18.4% (R_{free}=22.9%) and excellent geometry after iterative cycles of refinement and rebuilding.

Importin-α is a banana-shaped molecule constructed from ten tandem Armadillo (ARM) repeats, each of which is based on three short α-helices. NLSs bind to the inner concave surface of importin-α at two sites. The major site, to which monopartite NLSs and the larger basic cluster of bipartite NLS binds, spans ARM repeats 1-4, whereas a secondary site, to which the smaller basic cluster of bipartite NLSs also binds, is located at ARM repeats 6-8 (reviewed by Stewart, 2007). At both sites, the interactions between NLS and importin-α is mediated by key Lys and Arg side chains of the NLS that are sandwiched between specific Trp residues of importin-α, together with positive charges of the NLS being neutralised by strategically-placed acidic residues. Residues 629-634 of the AR NLS bind to the major NLS binding site of importin-α in a similar way to that observed for monopartite NLSs, such as that of the SV40 large-T antigen, and IBB domain (Fig. 3D). The side chains of these residues interact with ARM repeats 2, 3 and 4 of importin-

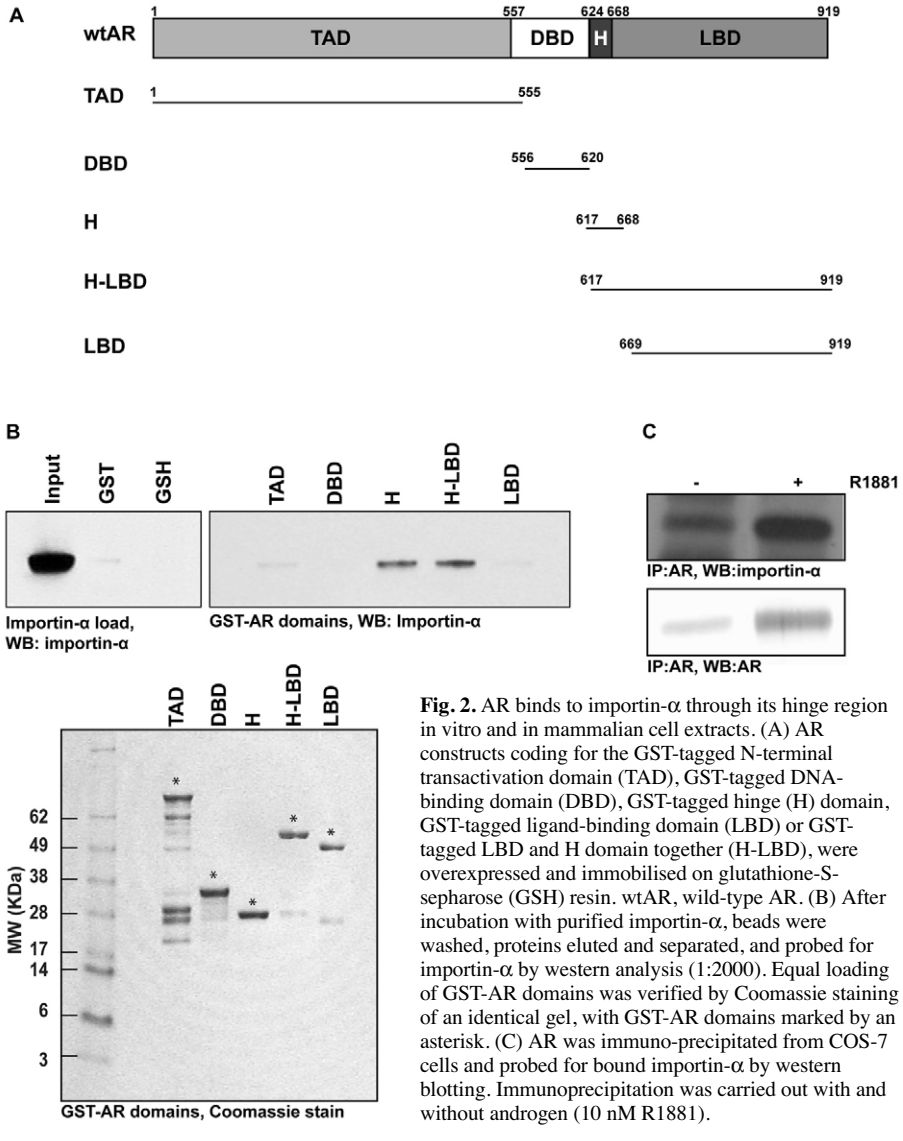

Fig. 2. AR binds to importin-α through its hinge region in vitro and in mammalian cell extracts. (A) AR constructs coding for the GST-tagged N-terminal transactivation domain (TAD), GST-tagged DNA-binding domain (DBD), GST-tagged hinge (H) domain, GST-tagged ligand-binding domain (LBD) or GST-tagged LBD and H domain together (H-LBD), were overexpressed and immobilised on glutathione-S-sepharose (GSH) resin. wtAR, wild-type AR. (B) After incubation with purified importin-α, beads were washed, proteins eluted and separated, and probed for importin-α by western analysis (1:2000). Equal loading of GST-AR domains was verified by Coomassie staining of an identical gel, with GST-AR domains marked by an asterisk. (C) AR was immuno-precipitated from COS-7 cells and probed for bound importin-α by western blotting. Immunoprecipitation was carried out with and without androgen (10 nM R1881).

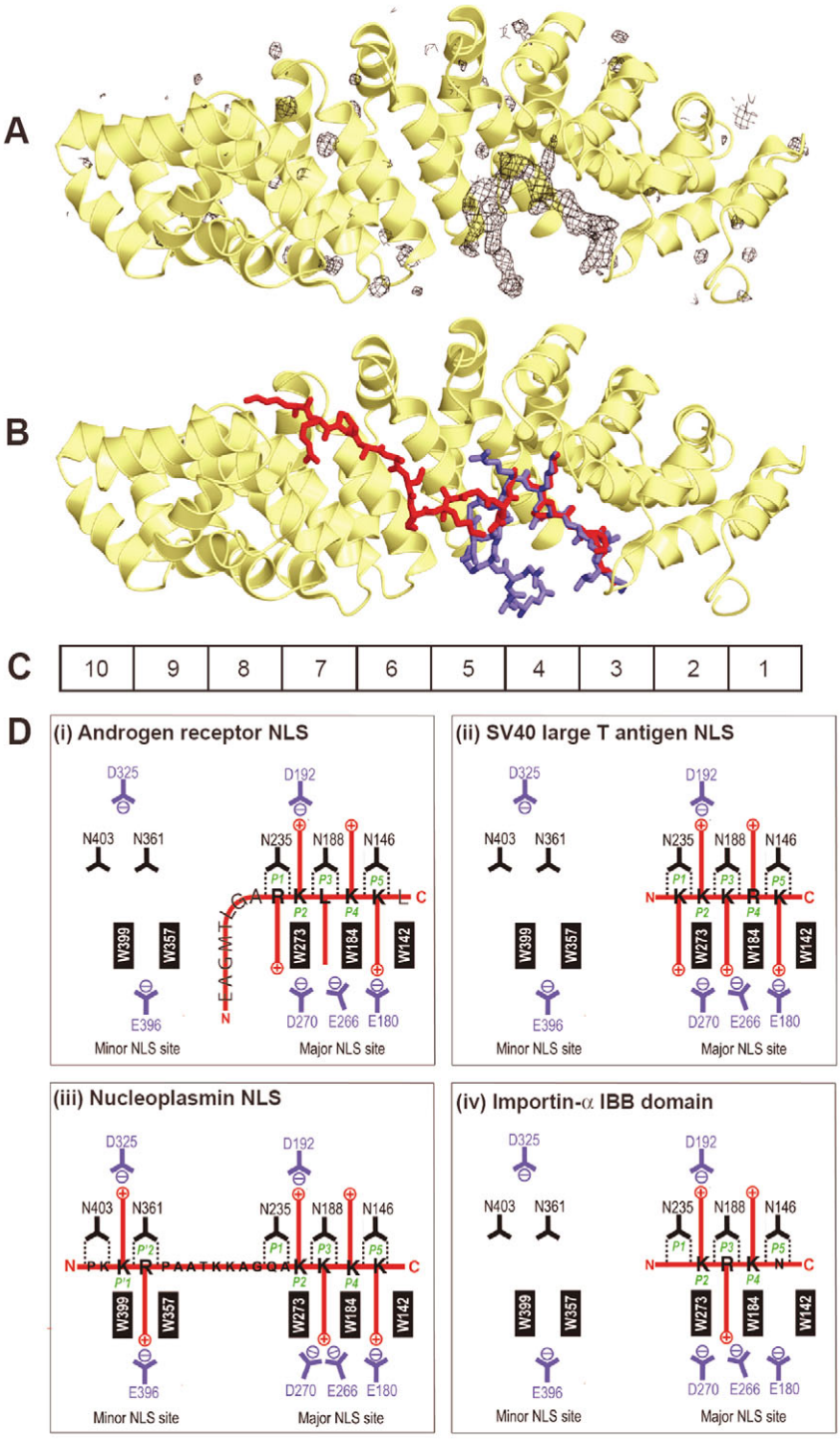

Fig. 3. The structure of the AR hinge region bound to importin-α. (A) 2.6 Å resolution *Fo-Fc* difference electron-density map (contoured at 3σ) showing the density resulting from the AR hinge region after subtraction of density due to importin-α in crystals of the complex. The structure of the underlying importin-α is shown in yellow. Electron density resulting from the AR hinge region was only seen over the primary NLS-binding site of importin-α. (B) Relationship between the binding sites on importin-α for the AR hinge region (blue) and the bipartite NLS from nucleoplasmin (red). Whereas the nucleoplasmin NLS binds to both the major and minor binding sites on importin-α, the AR hinge region binds to the major site together with an adjacent region of the importin-α surface that is not involved in the interaction with other NLSs. (C) Schematic illustration of the positions of the Armadillo (ARM) repeats of importin-α corresponding to the models shown in A and B. (D) Schematic representation of the interacting residues for different molecules (AR, SV40 large-T antigen, nucleoplasmin and the importin-α IBB domain) bound to importin-α. In all cases, binding at the major site involves insertion of side chains between a series of Trp residues (W142, W184, W273) on importin-α, complemented by H-bonds between key Asn (N146, N188, N235) and the NLS main-chain peptides, and also neutralization of negative charges by strategically placed acidic residues on importin-α. However, details of the interactions at the major site are different and, significantly, a series of residues (621EAGMTLGA628) immediately upstream of the negative cluster (629RKLKK634) in the AR hinge region bind to importin-α in a manner not seen in other NLSs. Only the bipartite nucleoplasmin NLS binds to the secondary site.

α to make contact with the P_1-P_5 binding pockets, through a combination of electrostatic and hydrogen bonds.

However, the way in which residues 621-628 of the AR NLS interact with importin-α is significantly different to that seen with other NLSs, either bipartite or monopartite, or the IBB domain of importin-α. Although the AR NLS peptide made contact with importin-α ARM repeat 5, at ARM repeat 6 the peptide curved away from importin-α and did not make contact with ARM repeats 7 and 8, as is seen for bipartite NLSs such as that from nucleoplasmin (Conti and Kuriyan, 2000; Fontes et al., 2000). There was no difference density over the minor NLS binding site of importin-α to indicate that the AR peptide might contact this site.

Crystals of importin-α in complex with amino acid residues 617-634 of AR were also generated by co-crystallising purified importin-α with a synthetic AR peptide that corresponded to residues 617-634. These crystals were generated under the same conditions as before and the resultant *Fo-Fc* difference density map showed the same horse-shoe shape observed with residues 621-628. Dissolution of the crystals followed by reverse-phase HPLC and mass

Table 1. Crystallographic-structure-determination statistics for importin-α:AR-NLS (617-634)

Data collection	
Space group	$P2_12_12_1$
Unit cell dimensions (Å)	*a*=78.26 *b*=89.79, *c*=98.13
Resolution range (Å)*	20-2.6 (2.74-2.60)
Total observations*	106744 (12729)
Unique reflections*	21477 (2765)
Completeness (%)*	98.0 (88.1)
R_{merge} (%)*	6.6 (26.2)
I/σ(I)*	14.8 (5.6)
Refinement	
Number of reflections (working, test)	19883 (1374)
R_{cry}:R_{free} (%)	18.4:22.9
Total number of non-H atoms	3401
Number of water molecules	33
r.m.s. deviation from ideal bond length (Å)	0.012
r.m.s. deviation from ideal bond angles (degree)	1.3
Ramachandran plot (%)	
Core region	95.4
Allowed region	4.1
Generously allowed region	0.3
Disallowed region	0.3

*Final resolution shell is given in parentheses.
r.m.s., root mean square.

spectrometry (MALDI) confirmed that the peptide was intact and had not been proteolysed.

Mutations in the AR hinge region influence binding affinity for importin-α

To test our model for the structure of the importin-α–AR-NLS interface in vitro, we used isothermal titration calorimetry (ITC) to quantify the effects of mutating key residues within the AR hinge region on its affinity for importin-α (Table 2). Importin-α binding affinity was reduced for mutations of single Lys residues (Lys630, Lys632, Lys633), with the greatest binding contribution from Lys630 – consistent with its contact with the P2-binding pocket of importin-α (Conti and Kuriyan, 2000; Fontes et al., 2000; Hodel et al., 2001). However, the most striking effect was evident with the triple Lys-to-Ala substitution KKK630/632/633AAA, which completely abolished importin-α binding. Although it appeared from our structural model that AR Met624 might make a hydrophobic contact with importin-α, mutation of this residue did not generate a significant change in importin-α binding affinity, suggesting that this interaction is relatively weak.

There have been several reports of point mutations within the AR sequence (617-634) that are associated with the clinical phenoptypes of AIS and prostate cancer (Marcelli et al., 1991; Gottlieb et al., 1999; Nazareth et al., 1999; Tilley et al., 1996; Wang and Uchida, 1997; Zoppi et al., 1992). ITC was used to determine the binding affinities of these known human mutations for importin-α (Table 2). These show that mutations within the AR that bind to the major NLS-binding pockets of importin-α (i.e. R629W, R629Q and K630T) had significantly reduced affinity for importin-α, whereas other AR mutations in the N-terminus of the peptidethat do not form part of the NLS (R617P, C619Y) did not alter binding affinity.

These results indicated that the AR has an essentially monopartite NLS (comprised of residues 629-634) that binds to the major NLS-binding pockets of importin-α and that mutation of AR residues within this region significantly weakened its affinity for importin-α. Consistent with this hypothesis, the Arg-to-Ala substitutions R617A and K618A only reduced the ITC K_d of the AR peptide for ΔIBB-importin-α by twofold (to 10±2 μM), whereas the corresponding mutation in the nucleoplasmin peptide (nucleoplasmin KR155,156AA) reduced its K_d 12-fold to 2.5 μM, comparable with that of the wild-type AR peptide. We further

Table 2. Binding measurements of mutant AR NLS peptides to importin-α, using ITC at 10°C

NLS peptide	Sequence	K_d (μM)	*n*	ΔH (kcal/mol)
Control peptides				
Nucleoplasmin	^{155}**KR**PAATKKAGQA**KKKK**^{170}	0.19±0.02	0.98	–8.8±0.05
SV40	^{126}P**KKKRK**V^{132}			
		0.31±0.15*	1*	–4.0±1.02*
		0.98±0.08**	1.44**	–7.8±0.76**
wtAR	^{617}RKCYEAGMTLGA**RKLKKL**^{634}	5±0.1	1.13	–8.0±0.03
AR NLS mutants to test structure model				
AR K630A	RKCYEAGMTLGAR**A**LKKL	600±10	1.13	–4.5±0.10
AR K632A	RKCYEAGMTLGARKL**A**KL	15±0.2	1.11	–6.8±0.02
AR K633A	RKCYEAGMTLGARKLK**A**L	72±2	0.90	–4.7±0.09
AR KKK630,2,3AAA	RKCYEAGMTLGAR**AL AA**L	No binding		No binding
AR M624D	RKCYEAG**D**TLGARKLKKL	7±0.1	0.94	–7.1±0.02
AR mutants associated with pathological phenotypes				
AR R617P (AIS)	**P**KCYEAGMTLGARKLKKL	7±0.3	0.82	–4.8±0.04
AR C619Y (CaP)	RK**Y**YEAGMTLGARKLKKL	5±1	0.96	–7.8±0.02
AR R629W (AIS)	RKCYEAGMTLGA**W**KLKKL	60±2	1.20	–1.4±0.01
AR R629Q (CaP)	RKCYEAGMTLGA**Q**KLKKL	48±2	1.19	–3.6±0.06
AR K630T (CaP)	RKCYEAGMTLGAR**T**LKKL	140±10	0.94	–2.6±0.30

Control peptide were nucleoplasmin, SV40 and wtAR NLSs. Mutant peptides were NLS regions containing amino acid substitutions within the interface that binds to importin-α, and exhibited reduced binding affinity. AR mutants associated with pathological phenotypes contain substitution mutations that occur naturally in AIS or prostate cancer (CaP), and also exhibited reduced affinity for importin-α.

For SV40, the two sets of values represent the binding to the major and minor importin-α NLS-binding sites: *, binding to major importin-α binding site; **, binding to minor importin-α binding site.

No binding, no binding could be demonstrated.

Mutated amino acid residues are in bold.

validated this model physiologically for the binding interface of importin-α–AR (i.e. importin-α in complex with the whole of the AR) by carrying out nuclear import assays in living cultured mammalian cells.

Affinity of the AR for importin-α influences nuclear import

Nuclear import of wild-type AR (wtAR) and a series of AR mutants was investigated by transfecting COS-7 cells with the AR fused to green fluorescent protein (GFP-AR) and using fluorescence microscopy to determine its distribution between nucleus and cytoplasm following androgen exposure. wtAR was predominately cytoplasmic in the absence of androgen, but was rapidly imported into the nucleus within 30 minutes of androgen exposure. All mutations within the region that our crystal structure indicated were in intimate contact with importin-α (K630A, K632A, K633A, KKK630,632,633AAA, M624D) showed delayed nuclear import (see Fig. 4 and Table 3). Only partial nuclear localisation was evident for the K630A, K632A, K633A mutants at 30 minutes after androgen exposure, although by 48 hours nuclear localisation had reached the same level as seen with wtAR. The M624D mutant showed delayed import at 30 minutes and, although most cells demonstrated nuclear localisation at 48 hours, some cells in the field exhibited a localised pattern of cytoplasmic fluorescence. The NLS-null triple mutant (KKK630/632/633AAA) showed no nuclear localisation even after 48 hours, indicating that nuclear import was completely abrogated. Our results confirm previous the results of studies that reported altered localisation of the AR with similar Lys-Ala mutations (Jenster et al., 1993; Thomas et al., 2004), and extend them by showing that altered subcellular localisation is associated with reduced binding affinity for importin-α and that the effect is primarily at the level of the rate of import rather than influencing the final equilibrium concentrations present in the cytoplasm and nucleus.

Several pathological AR mutants associated with AIS (e.g. point mutation R629W) or prostate cancer (e.g. point mutation R629Q) have reduced binding affinity for importin-α (Table 2). We therefore examined whether these mutations can alter the subcellular localisation of the AR in the presence of androgens. Only partial nuclear localisation of the R629W, R629Q, K630T mutants was evident at 30 minutes (Fig. 4), although subcellular localisation was

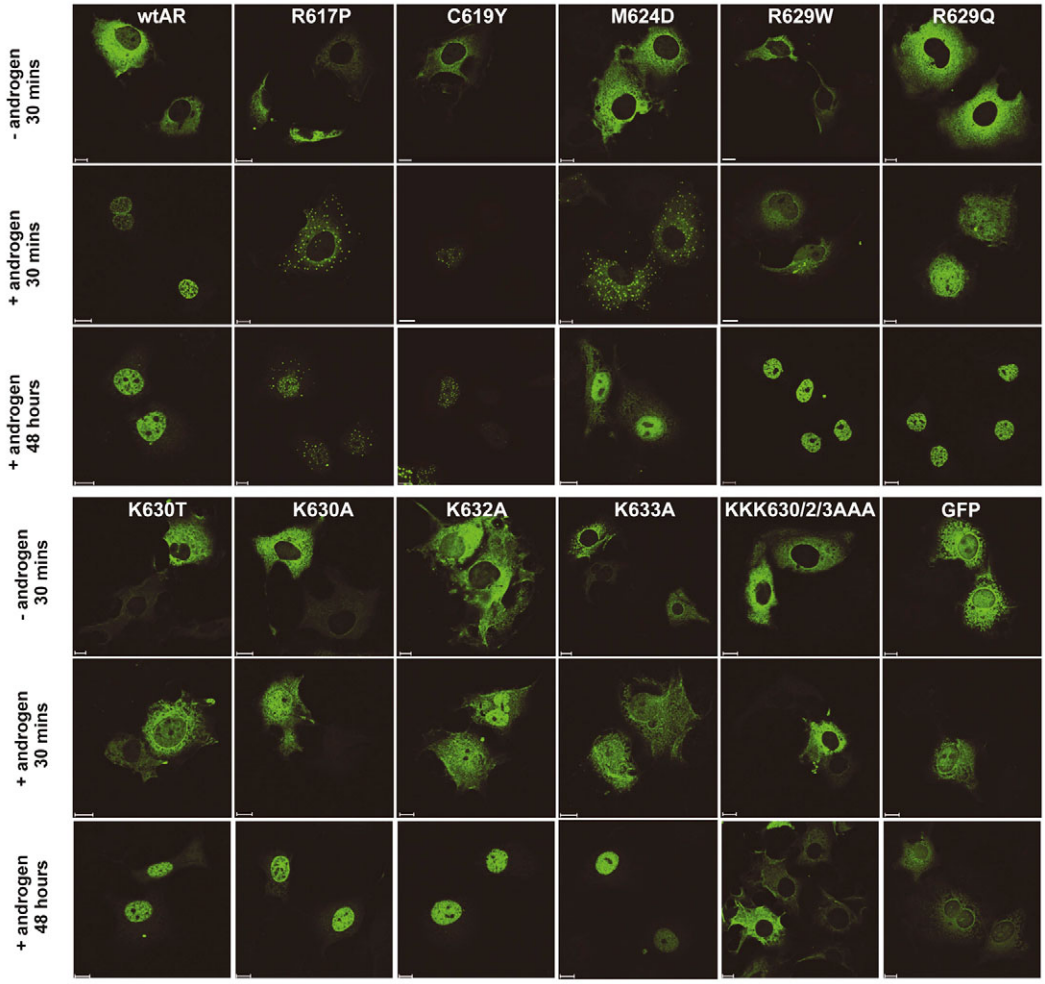

Fig. 4. The AR hinge region has an intrinsic nuclear import function in live cells. COS-7 cells were transfected with GFP-AR constructs corresponding to wtAR, or disease-related mutations (R617P, C619Y, R629W, R629Q, K630T) or mutations predicted to alter nuclear import from the crystal structure (M624D, K630A, K632A, K633A, KKK630/2/3AAA). Cells were treated with 10 nM non-hyrolysible androgen (R1881, + androgen) or vehicle (ethanol,–androgen) for 30 minutes or 48 hours, fixed with methanol and mounted in Vectorshield. Cells were imaged by confocal microscopy. GFP is shown in green. Images of the two top rows show the cytoplasmic location of wtAR and the different AR mutants in the absence of androgen. The middle and bottom rows show the nuclear translocation of the different AR constructs in the presence of androgen after 30 minutes and 48 hours, respectively. Scale bars, 10 μM.

Table 3. Nuclear import of GFP-wtAR and AR mutants in COS-7 cells. determined using confocal microscopy at 30 minute and 48 hour timepoints

AR protein	Region of mutation	Localisation after androgen exposure T_0	T_{30min}	T_{48hrs}
Control proteins				
GFP only		C=N	C=N	C=N
GFP-wtAR	^{617}RKCYEAGMTLGARKLKKL^{634}	C>N	N	N
AR NLS mutants to test structure model				
GFP-AR K630A	RKCYEAGMTLGAR**A**LKKL	C>N	C=N	N
GFP-AR K632A	RKCYEAGMTLGARKL**A**KL	C>N	C=N	N
GFP-AR K633A	RKCYEAGMTLGARKLK**A**L	C>N	C=N	N
GFP-AR KKK630,2,3AAA	RKCYEAGMTLGAR**A**L**AA**L	C	C	C
GFP-AR M624D	RKCYEAG**D**TLGARKLKKL	C>N	C>N	N
AR mutants associated with pathological phenotypes				
GFP-AR R617P (AIS)	**P**KCYEAGMTLGARKLKKL	C>N	C>N	N
GFP-AR C619Y (CaP)	RK**Y**YEAGMTLGARKLKKL	C>N	N	N
GFP-AR R629W (AIS)	RKCYEAGMTLGA**W**KLKKL	C>N	C=N	N
GFP-AR R629Q (CaP)	RKCYEAGMTLGA**Q**KLKKL	C>N	C=N	N
GFP-AR K630T (CaP)	RKCYEAGMTLGAR**T**LKKL	C>N	C=N	N

Nuclear import was determined using confocal microscopy at 30-minute and 48-hour time points.

A construct expressing GFP only was transfected as a control because is sufficiently small to passively diffuse through the nuclear pore complex and equilibrate between the cytoplasm and nucleus. GFP-wtAR localised to the cell nucleus within 30 minutes of androgen exposure. Mutant AR proteins contained NLS regions with amino acid substitutions within the binding interface that binds to importin-α, and reduced nuclear import at 30 minutes. AR mutants associated with pathological phenotypes contain substitution mutations that occur naturally in AIS or CaP, and also reduced nuclear import at 30 minutes. The AR-NLS-null mutant (KKK630,2,3AAA) abrogated nuclear import, even at 48 hours. C, cytoplasm; N, nucleus.

Mutated amino acid residues are in bold.

predominately nuclear for the three mutants after 48 hours of exposure. Our results indicate that affinity of AR mutants for importin-α influences their nuclear import rate in vivo. Moreover, several pathological AR mutations also exhibit delayed nuclear import.

Influence of hinge residues on transactivation

The AR is acetylated in vivo through Lys residues of its 630KLKK633 motif by the transcriptional co-activators p300, P/CAF and Tip60, and both p300 and Tip60 augment the androgen-induced transactivation of the AR (Fu et al., 2003; Fu et al., 2000; Fu et al., 2002; Gaughan et al., 2002). It is possible that these residues can serve a role in both acetylation and binding to importin-α; therefore it was important to explore how mutations within this region and other parts of the NLS that have been shown to alter nuclear import also influence subsequent transactivation. This was investigated using the GFP-wtAR and GFP-AR mutations described earlier.

AR transactivation following androgen exposure was measured using ARE_2-TATA-EIB-luc transcriptional reporter constructs transfected into COS-7 cells. The results are illustrated in Fig. 5. GFP alone did not have an effect on transactivation. AR transactivation was induced by androgen, with greater than tenfold increases in reporter activity for wtAR. The NLS-null triple mutant (KKK630/632/633AAA) markedly attenuated androgen-induced transactivation compared with wtAR. Some single-residue mutants (R629W, K630T, K630A, K632A, K633A) seemed to augment transactivation 1.5- to fourfold greater than observed with wtAR. Other mutants (R617P, C619Y, M624D) attenuated transactivation with little androgen-induced response. The R617P and C619Y mutants served as negative controls because these mutations are within the DBD and have been reported to reduce binding of the AR to DNA-response elements, and attenuate AR transactivation (Zoppi et al., 1992; Nazareth et al., 1999). The relative expression of the different GFP-AR mutants was determined by western blotting (for GFP) and densitometry to be broadly similar, and any minor differences were shown not to influence transactivation (data not shown).

These results indicate that reduced binding affinity for importin-α influences nuclear import at the 30-minute and 48-hour time points, but does not correlate with subsequent transactivation, which appears to be largely independent.

Discussion

The AR is a nuclear transcription factor whose subcellular nuclear localisation is determined by ligand binding (Simental et al., 1991; Jenster et al., 1993; Zhou et al., 1994; Georget et al., 1997; Georget et al., 1998; Tyagi et al., 2000). Previous studies of AR nuclear

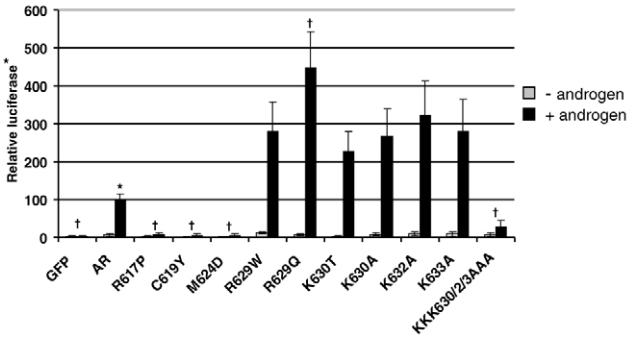

Fig. 5. The effect of hinge-region mutations on AR transactivation. COS-7 cells were transfected with GFP-tagged AR constructs, BOS β-galactosidase expression construct, and the androgen-responsive ARE_2-TATA-EIB-luc reporter gene. After treatment with 10 nM R1881 (black bars) or vehicle (grey bars) for 48 hours, cells were harvested and assayed for luciferase activity. Transfection efficiency was normalised to β-galactosidase activity and is shown relative to wtAR (*). Mutations of the 630KLKK633 motif alter transactivation. $^{\dagger}P<0.001$ compared with wtAR. *P*-values for all other samples were between 0.002 and 0.018.

import have highlighted the importance of key residues involved in nuclear import (Jenster et al., 1993; Poukka et al., 2000; Thomas et al., 2004; Zhou et al., 1994) but did not establish the precise nuclear import pathway that was employed. In this study, we have shown a direct interaction between the nuclear import receptor importin-α and the AR, determined the crystal structure of the binding interface of importin-α–AR-NLS, and defined its relationship to nuclear import and subsequent transactivation.

Importin-α binds to the AR in mammalian cell extracts and in vitro

Studies focussing on the nuclear import of other hormone receptors have reported the binding of importins to the glucocorticoid receptor (GR). Importin-α has been shown to bind to a classical NLS site in the GR (amino acid residues 479-497, termed GR NLS1 site) (Savory et al., 1999) and, more recently, importins 7 and 8 have been reported to interact with the GR, either through an unspecified site within the GR LBD (termed the GR NLS2 site) or indirectly through its NLS1 site by binding with importin-α (Freedman and Yamamoto, 2004). Here, we show that importin-α binds to the AR in mammalian cell extracts, although this binding is not enhanced by androgen (Fig. 2C). One would intuitively expect an enhancement of binding in the presence of ligand, given that the AR translocates to the cell nucleus upon androgen exposure (Georget et al., 1997; Georget et al., 1998; Jenster et al., 1993; Poukka et al., 2000; Simental et al., 1991; Thomas et al., 2004; Tyagi et al., 2000; Zhou et al., 1994). Such ligand-enhanced binding has been reported for GR binding to importin-α, but we did not observe this with the AR, even when co-immunoprecipitating with anti-AR or anti-importin antibodies, which suggests that the nuclear import of liganded AR results more from its release from cytoplasmic components rather than from enhanced exposure of its NLS.

Importin-α binds to the AR hinge region (Fig. 2B), confirming previous studies which indicated that residues within this region determine AR nuclear localisation (Jenster et al., 1993; Poukka et al., 2000; Thomas et al., 2004; Zhou et al., 1994). The direct interaction between the AR NLS and importin-α is consistent with the classical nuclear-protein-import machinery mediating AR nuclear import. Furthermore, heat shock proteins (such as Hsp90) bind to the AR in proximity to this region and dissociate following conformational changes in the LBD induced by ligand binding (Georget et al., 2002; Kuil et al., 1995; Marivoet et al., 1992; Pratt et al., 2004; Kallenberger et al., 2003; Schaufele et al., 2005; Wurtz et al., 1996; Savory et al., 1999). It has been proposed that the dissociation of Hsp90 facilitates greater exposure of the hinge regions of steroid receptors (Kuil et al., 1995; Savory et al., 1999), although our results suggest that such processes do not enhance the binding of the AR to importin-α, at least in our cell-line assays. However, the AR was overexpressed in our cultured cell assays and it is possible that binding of importin-α to the AR in the absence of androgen was due to relatively low levels of Hsp90, because Hsp90 normally masks the NLS site.

Structure of the binding interface in the importin-α–AR complex

The molecular details of the binding of NLSs to importin-α have been established from the crystal structures of nucleoplasmin, SV40 and several other NLS-containing proteins bound to importin-α (Conti and Kuriyan, 2000; Conti et al., 1998; Fontes et al., 2003a; Fontes et al., 2000). Nucleoplasmin contains a classical bipartite NLS (Fig. 1A), which consists of a minor and major component, and binds to importin-α as illustrated in Fig. 3C. The SV40 large T antigen contains a monopartite NLS and binds to importin-α most strongly at the major site. Although residues 617-634 of the AR show sequence homology with the putative bipartite NLS shared by many hormone receptors, our crystal structure of the importin-α–AR complex shows that the AR binds to importin-α primarily through residues 629-634 (629RKLKKL634) and that the more N-terminal residues (617RK618) do not bind. Thus, binding of the AR NLS to importin-α is different to that described for nucleoplasmin, and more in keeping with that of the SV40 monopartite NLS (Fig. 3D). The failure of residues 617-618 to bind is consistent with their being an integral component of the second zinc finger. In addition to the crystal structure, the ITC data confirm that AR residues 629-634 (629RKLKKL634) provide the predominant binding to importin-α and indicate that the strongest interaction occurs through Lys630. This residue occupied the P2-binding pocket of importin-α that has been shown with other NLSs to be the most important contact for importin-α binding (Hodel et al., 2001; Makkerh et al., 1996; Robbins et al., 1991). Mutation of the N-terminal residues in the AR peptide (R617P, C619Y, analogous to the minor component of the nucleoplasmin NLS) have been reported to reduce AR binding to DNA (Nazareth et al., 1999; Zoppi et al., 1992) but did not reduce binding affinity for importin-α. Furthermore, the structure of the AR-DBD has been recently reported (Shaffer et al., 2004) and the location of AR residues 617 and 618 lie within the terminal α-helix of the second zinc finger, whereas residues 629-634 (629RKLKKL634) lie within the unstructured and flexible hinge region (Fig. 1C). That importin-α only binds to residues 629-634 (629RKLKKL634) is therefore completely consistent with the structure of the AR. We also show that pathological mutations associated with AIS (i.e. point mutation R629W) or prostate cancer (i.e. point mutations R629Q, K630T) show a reduced binding affinity for importin-α that has not been reported previously.

Binding affinity for importin-α and nuclear import are related, but the hinge region has a distinct function on transactivation

The functional significance of altered NLS-binding affinity for importin-α and its effect on localisation of NLS peptides has been reported in yeast cells (Hodel et al., 2006). Here, we show that the binding affinity for importin-α also influences nuclear import of whole proteins in mammalian cells. The reduced binding affinity for a range of AR mutants to importin-α (AR K630A, K632A, K633A, KKK630,632,633AAA) was determined by ITC, and their subsequent delay in nuclear import was illustrated by confocal microscopy. This observation was most striking for the NLS triple mutant KKK630,632,633AAA, which completely abrogated nuclear import at 48 hours, and suggests that the physiological significance of the AR NLS2 domain is different to that proposed previously (Poukka et al., 2000). The altered import of several of these mutants has been previously reported (Jenster et al., 1993; Thomas et al., 2004) but here we show that this is associated with reduced binding affinity for importin-α. We show this influence of binding affinity on import kinetics is also evident in naturally occurring pathological mutations, such as mutations R629W or R629Q and K630T, that are associated with AIS or prostate cancer, respectively.

It is apparent that there are additional factors that influence nuclear import of the AR. We observed that the nuclear import of the R617P mutant was delayed at 30 minutes – perhaps owing to disruptions in AR DBD structure and sequestration in

cytoplasmic aggregates. Several mutations within the AR zinc finger domains that do not involve the putative NLS (C579F, F582Y) have been reported to reduce nuclear localisation of the AR (Kawate et al., 2005). From inspection of the structure of the zinc fingers of the DBD domain, it is likely that such mutations interfere with the conformation of the protein in the zinc-finger DBD region and thereby generate proteins in which the AR structure was at least locally disrupted. Such a structural disruption could potentially mask the NLS and thus reduce nuclear import of the AR. Indeed, mutations of key Cys residues within the AR DBD zinc fingers have been reported to cause cytoplasmic aggregations after androgen exposure (Jenster et al., 1993; Kawate et al., 2005), perhaps as a direct result of such structure disruption. It is therefore possible that mutations of the minor basic cluster located in the second zinc-finger (R617, K618) can also disrupt its structure, and so reduce nuclear accumulation – either through indirectly preventing binding to importin-α in the cytoplasm or interfering with the binding of the AR to DNA in the nucleus (Nazareth et al., 1999; Zoppi et al., 1992). The R617P mutation is likely to disrupt the AR DBD structure as it reduces the binding affinity of the AR to DNA response elements (Zoppi et al., 1992) and forms cytoplasmic aggregates after androgen exposure that resemble those of other DBD mutants that abolish the DBD zinc finger structure (Jenster et al., 1993). Although speculative, the R617P mutant may form these aggregates as a result of disruption in binding to Hsp90. Nuclear import of the AR is facilitated by Hsp90 (Georget et al., 2002), perhaps owing to stabilisation of the AR in an active confirmation (Kuil et al., 1995; Georget et al., 2002). However, Hsp90 fails to colocalise with cytoplasmic aggregates of various AR hinge mutants (Thomas et al., 2004), and specific inhibition of Hsp90 with geldanamycin can precipitate formation of cytoplasmic aggregates and reduce nuclear import (Marcelli et al., 2006). It is therefore feasible that the R617P mutant forms cytoplasmic aggregates and has delayed nuclear import as a result of altered protein conformation and reduced binding to Hsp90.

Since the AR is a nuclear transcription factor whose subcellular localisation is determined by its NLS, we sought to determine whether the NLS also has a role on transactivation that was distinct from its role in nuclear import. The NLS triple mutant (KKK630,632,633AAA), which abrogated AR import, also markedly attenuated androgen-induced transactivation compared with wtAR. Some single-residue mutations (R629W, K630T, K630A, K632A, K633A) seemed to augment transactivation, whereas others (M624D) attenuated transactivation. Overall these results imply that nuclear localisation of the AR is required for a full androgen-induced response, but the hinge has a separate, distinct role in transactivation that is apparent once nuclear localisation has been attained. Whereas the effect of the triple Lys mutation is to completely abrogate androgen-induced transactivation, the results obtained with the single-site mutants show either augmentation or attenuation of transactivation. Unravelling the cause of this is complicated by the large number of coregulators which have been shown to interact with the AR at sites overlapping with the hinge, including coactivators such as p300, P/CAF (Fu et al., 2000; Fu et al., 2002), Tip60 (Brady et al., 1999; Gaughan et al., 2002), Ubc9 (Poukka et al., 1999), SNURF (Moilanen et al., 1998; Poukka et al., 2000), ARIP3 (Moilanen et al., 1999), and co-repressors, such as HDAC1 (Gaughan et al., 2002; Fu et al., 2003), NCoR (Cheng et al., 2002), and cyclinD1 (Reutens et al., 2001). Lys mutations within the AR 630KLKK633 motif have been reported to alter the binding of co-regulators p300, HDAC1 and NCoR (Fu et al., 2003; Fu et al., 2002), and also attenuate coactivator-enhanced transactivation (Fu et al., 2002). It is quite plausible that mutations within the NLS and DBD interfere with binding of some of the other co-regulators listed above, and this may therefore modify transactivation by altering the composition of the AR transcription complex (Louie et al., 2003; Shang et al., 2002). A recent study on the glucocorticoid receptor (GR) demonstrated that the selective substitutions of its NLS with the SV40 NLS did not alter the nuclear localisation of mutant GR or recruitment to the MMTV promoter. These substitutions did, however, attenuate both the recruitment of the GR-transcription complex and of mutant GR transactivation, implying a direct function of its hinge region on co-regulator assembly (Carrigan et al., 2007). Haelens et al., have also recently shown that AR hinge residues have a function on transactivation that is independent on other functions, such as DNA binding (Haelens et al., 2007).

As a consequence, single-residue mutations in the NLS are predicted to have effects on AR transactivation that are highly dependent on cellular context, co-regulator expression patterns and the promoter used in reporter assays. This highlights the hazards of using reporter assays in an attempt to understand the mechanistic effects of single-site mutations within this region in AIS and related conditions. The triple-Lys mutant, however, provides a potent tool to distinguish between genomic and non-genomic AR functions through the clear-cut inhibition of both nuclear entry and AR transactivation.

In summary, we have demonstrated that importin-α binds to the AR hinge region, and have established the structure of this binding interface. The way in which AR-NLS binds to importin-α differs from the binding observed with classical monopartite and bipartite NLSs, and biochemical analysis demonstrates that, in vivo, altered binding affinity influenced the nuclear import of a range of AR mutants mediated by importin-α, including several mutants associated with different clinical phenotypes. The wide range of transcriptional activities of hinge and DBD mutants indicates the hinge region has a distinct role on transactivation that is separate from its role on nuclear import. Further studies are needed to clearly define the role of the AR hinge region in this process.

Materials and Methods

Unless otherwise stated all chemicals were purchased from Sigma Aldrich.

Vectors and reagents

The following plasmids have been described previously: pSV40-AR_o containing the full-length AR gene (kindly donated by Albert Brinkmann, Erasmus University, Rotterdam, The Netherlands) (Brinkmann et al., 1989) and pET30a (Novagen) containing the untagged importin-α construct [mouse importin-α 2 (Accession: NP_034785 XP_992959), residues 70-529, kindly donated by Yoshiyuki Matsuura, MRC LMB, Cambridge, UK] (Matsuura and Stewart, 2005).

The following AR domains were generated by PCR using pSV40-AR_o as a template and cloned into the pGEX 4T1 vector (Amersham): transactivation domain (TAD, residues 1-555), DNA-binding domain (DBD, residues 556-620), hinge domain (H, residues 617-668); hinge- and ligand-binding domain (HLBD, residues 617-918); LBD (residues 669-919) and AR NLS region (residues 617-635).

To generate the GFP-tagged AR, the full-length AR gene was amplified by PCR using pSV40-AR_o as a template and cloned into the pQBI 25/50 fc3 vector (Qbiogene). Site-directed mutagenesis was performed to generate the following GFP-AR mutants: R617P; C619Y; R629W; R629Q; K630T; M624D; K630A; K632A; K633A; KKK630,632,633AAA. To generate GST-tagged importin-α, the pET30a importin-α ΔIBB construct (ΔIBB importin-α, residues 70-529) was used as a template, amplified by PCR and cloned into pGEX-4T1. Primers and restriction sites used to generate these constructs are illustrated in supplementary material Tables S1 and S2. All constructs were fully verified for integrity by sequencing.

The following antibodies were used: AR441 (Dako Cytomation) for western blotting of the AR, N-20 (Santa Cruz Biotechnology) for immunoprecipitation of the AR, and ab22534 (Abcam) for western blotting of importin-α.

Mammalian cell culture and transfection

COS-7 cells were purchased from the Cancer Research UK cell bank and were routinely cultured in DMEM supplemented with 10% foetal bovine serum (FBS; Labtech). For transfection cells were grown to 40% confluence and transfected using FuGENE6 reagent (Roche) according to the manufacturer's instructions. Prior to androgen treatment cells were washed in PBS and grown in Phenol-Red-free RPMI supplemented with charcoal-stripped FBS (Perbio) for 24 hours. Cells were androgen treated with either the DHT analogue R1881 (10 nM) or an equal volume of vehicle (ethanol).

Bacterial cell culture and transformation

BL21-DE3 RIL (Stratagene) were transformed with the various pGEX-4T1 constructs under manufacturer's instructions and cultured in 2×TY medium at 37°C to an optical density of 0.3 (A 600 nm), before reducing the growth temperature to 20°C and inducting cells with 1 mM isopropyl thio β-D-galactoside (IPTG). Cells were then grown for a further 16 hours at 20°C prior to pelleting and resuspension on ice in buffer A [50 mM Tris, 150 mM NaCl, 4 mM DTT, 1 mM phenyl-methyl-sulfonyl fluoride (PMSF), enzyme inhibitors (Complete, Roche)]. All subsequent steps were carried out a 4°C.

Western blotting

COS-7 cells were washed in PBS, pelleted and protein lysates obtained by resuspending pellets in modified RIPA buffer [50 mM Tris pH 7.8, 150 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 0.5% sodium deoxycholate, 1 mM DTT, mammalian protease inhibitor cocktail (Roche), 20 mM N-ethylmaleimide, 0.1% NP40] and passed five times through a 26G needle. Lysates were centrifuged and the supernatants retained as the soluble protein lysates. Equivalent concentrations of total protein, determined by Bradford assay, were compared using SDS-PAGE and western analysis. Proteins were detected using rat anti-importin-α antibody (1:2000) (Abcam) or mouse anti-AR antibody (1:2000) (AR441, Dako Cytomation). Mouse anti-actin (1:5000, Abcam) was used as a loading control. Proteins were visualised using anti-mouse or anti-sheep HRP-conjugated secondary antibodies (1:1000, Dako Cytomation) and ECL-Plus (Amersham Biosciences). If the detected signal was beyond the dynamic range of film, diaminobenzidine (Vector Laboratories) was used for detection.

Immunoprecipitation

Prior to immunoprecipitation, COS-7 cells were plated into 10-cm^2 dishes and grown to 60% confluence. Medium was changed to Phenol-Red-free DMEM supplemented with 10% charcoal-stripped serum and cells were transfected with 2 μg of each GFP-AR construct and Fugene6 reagent before growing for a further 24 hours. Cells were starved in this androgen-free medium for a further 24 hours and then treated with either R1881 or vehicle for 30 minutes. Cells were lysed and immunoprecipitated in the presence of R1881 or vehicle at 4°C. The AR was immunoprecipitated from 750 μg of whole-cell lysate using 3 μl rabbit anti-AR antibody (N-20, Santa Cruz Biotechnology) and protein G sepharose (Amersham). Control immunoprecipitations had an equivalent concentration of sheep IgG added (Vector Laboratories). Sepharose was washed five times in high-stringency RIPA buffer supplemented with 2% NP40, sample buffer was then added and protein analysed by SDS-PAGE and western blotting.

GST-pull-down assays

BL21 DE3-RIL cell pellets expressing pGEX-4T1 protein constructs were resuspended in buffer A on ice and lysed by sonication (Misonix Inc.). All subsequent steps were carried out a 4°C. The supernatants were obtained by centrifuging cell lysates (40,000 ***g*** for 45 minutes), filtered (0.4 μm) and applied to glutathione sepharose resin (Amersham) for 1 hour. The resins were then washed in buffer A to remove non-specific protein binding.

In vivo binding assays

COS-7 cells were transfected with the GFP-wtAR vector (pQBI25/50fc3 wtAR) and grown in androgen enriched or vehicle treated media. Cells were lysed and supernatants analysed for AR and importin-α by immunoprecipitation and western blotting.

In vitro binding assays

The AR domains (TAD, DBD, H, HLBD, LBD) were expressed as GST-fusion proteins in BL21-DE3 RIL cells (Fig. 2A) and pulled down on glutathione resin as described above. The resins were washed in buffer A, proteins separated on SDS-PAGE and visualised by Coomassie staining. Resin volumes were then adjusted for equalised protein levels and incubated with 2 mg untagged importin-α in buffer A for 2 hours. Samples were washed with buffer A, and proteins separated by SDS-PAGE and visualised by Coomassie staining to detect the presence of bound importin-α. Because of similarities in molecular weight of importin-α and co-existing proteins in these assays, subsequent western blotting for importin-α was carried out on the samples. The samples were diluted 1:1000 and proteins separated on SDS-PAGE and transferred onto nitrocellulose membrane. The samples were probed for importin-α using Ab22534 (1:2000; Abcam). Blots were incubated using the anti-rat HRP-conjugated secondary antibody (Ab6734, 1:5000; Abcam) and visualised using ECL-Plus (Amersham Biosciences).

Crystallisation, data collection and structure determination

The AR NLS and untagged importin-α were transformed and expressed in BL21-DE3 RIL cells. Cell pellets were resuspended in buffer A and all subsequent steps were carried out at 4°C. Cells were lysed by passing through an Emulsiflex C5 high-pressure cell homogeniser (Glen Creston) in the presence of DNAse (Sigma). The supernatants were obtained by centrifugation (40,000 ***g*** for 45 minutes) and filtered (0.4 μm). Supernatant from 4L of AR NLS culture was incubated with glutathione sepharose resin (Amersham) for 1 hour and then washed in buffer B (50 mM Tris-HCl, 300 mM NaCl, 4 mM DTT), prior to incubation with supernatant from 6 L importin-α culture for 4 hours. The complex was washed with buffer A, resuspended in buffer C (20 mM Tris-HCl, 50 mM NaCl, 4 mM DTT) and incubated with thrombin (Sigma) for a further 16 hours. The importin-α–AR-NLS complex was eluted, incubated with p-aminobenzamidine (Sigma) for 1 hour and purified over an S-75 gel filtration column. 10 litres of culture yielded 15 mg of pure protein complex visualised as two bands on a Coomassie-stained SDS-PAGE gel. The protein complex was concentrated to 20 mg/ml, snap frozen in liquid nitrogen and stored at –80°C.

To determine conditions favourable for crystallisation of the importin-α–AR-NLS complex, parameters were screened around commercial conditions (Molecular Dimensions MD1-01) and those described for the crystallisation of the importin-α–nucleoplasmin complex (Fontes et al., 2000). 2 μl of protein solution and 2 μl of reservoir solution were suspended from a coverslip by hanging-drop method over a reservoir of 500 μl. The largest crystals were obtained using reservoir conditions of 100 mM HEPES pH 6.5, 0.7 M sodium citrate, 10 mM DTT.

Crystals were picked up in a cryoloop and transiently exposed to cryoprecipitate [reservoir solution containing 23% glycerol (v/v)], after which the crystal was vitrified in a stream of anhydrous nitrogen at 100 K. A 2.6 Å resolution data set (Table 1) was collected at 100 K in house using a Rigaku X-ray generator equipped with Osmic mirrors and a Mar345dtb image plate detector. Data were processed using MOSFLM (CCP4, 1994) and reduced using SCALA and TRUNCATE (CCP4, 1994). Free-R flags were set to be identical to those used for the structure of the importin-α–nucleoplasmin-NLS complex (Fontes et al., 2000). A structural model was produced using residues 70-496 of importin-α in the structure of the importin-α–nucleoplasmin-NLS complex (PDB accession number 1EJY) and, after rigid body refinement using REFMAC5 (CCP4, 1994), a clear difference density was visible into which residues 621-634 of the AR NLS were built. After iterative cycles of refinement with REFMAC5 and model building using O (Jones et al., 1991) and the addition of 33 water molecules, a final structure with an R-factor of 18.8% (FreeR=22.9%) and excellent geometry was produced (Table 1). Asn239 of importin-α is a Ramachandran outlier as in other mouse importin-α structures (Fontes et al., 2000; Kobe, 1999; Matsuura and Stewart, 2005). Coordinates and structure factors have been deposited with the protein data bank (PDB) with accession code 3BTR.

To explore the possibility of peptide proteolysis within the crystals of protein complex, crystals were dissolved in 0.1% trifluoroacetic acid (TFA) and analysed by reverse-phase chromatography (HPLC) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. This confirmed the peptide was intact.

ITC-binding studies

Importin-α (residues 70-529) inserted into the GST-fusion expression vector pGEX-4T1, was transformed and expressed in BL21 DE3-RIL cells. The supernatant was incubated with glutathione sepharose resin for 4 hours and the resin then washed in buffer B, prior to resuspension in buffer D (100 mM HEPES, pH 7.4, 50 mM NaCl, 4 mM DTT) and incubated with thrombin (Sigma) for a further 16 hours. The importin-α protein was eluted, incubated with p-aminobenzamidine (Sigma) for 1 hour and purified over an S-75 gel filatration column; 12 litres of culture yielded 100 mg of pure protein visualised as a single band on a Coomassie-stained SDS-PAGE gel.

The peptides used in the isothermal titration calorimetry (ITC) experiments were synthesised to greater than 95% purity and analysed by reverse phase chromatography and laser desorption mass spectrometry (ADL Advanced Biomedical). ITC was used to investigate the binding of AR NLS peptides to importin-α using a VP-ITC calorimeter (MicroCal Inc., CA). Experiments were carried out in 100 mM HEPES pH 7.4, 50 mM NaCl, 4 mM DTT at 10°C. The cell contained 1.36 ml importin-α solution (typically 100 μM), and peptide (typically 2 mM) was injected in 5 μl aliquots every 4 minutes, typically to a four- to fivefold molar excess. The concentrations of the peptides were typically 20 times greater than that of importin-α. If possible, protein concentration was selected to be at least five times greater than that of the estimated dissociation constant. The manufacturer's ORIGIN software fitted titration curves and yielded the stoichiometry (N), the binary equilibrium constant K_a (K_d^{-1}) and the enthalpy of binding (ΔH) from the data. The dissociation constant (K_d) was subsequently calculated ($K_d=1/K_a$).

Confocal nuclear-import assays

Subcellular localisation assays were performed as described (Whitaker et al., 2004). Briefly, COS cells were grown on glass coverslips and transfected with GFP-tagged AR constructs using Fugene6 (Roche). Cells were starved in Phenol-Red-free medium, supplemented with 10% charcoal-stripped serum for 24 hours. This medium was replaced with pre-warmed medium containing either vehicle (ethanol) or R1881 for either 30 minutes or 48 hours. Cells were fixed in 100% methanol for 10 minutes at –20°C before mounting with Vectashield containing DAPI (Vector Laboratories). GFP-AR and GFP-AR mutants were visualised using a Zeiss Meta 510 confocal microscope using a 63× objective.

Transcriptional activity

COS cells were grown in 24-well plates to 40% confluence. Prior to transfection cells were washed and medium was changed to Phenol-Red-free DMEM with charcoal-stripped serum as described. Using Fugene6, cells were transfected with 75 ng per well GFP-tagged AR constructs, 50 ng per well ARE_2-TATA-EIB-luc construct, and co-transfected with 25 ng/well BOS-β-galactosidase (Mizushima and Nagata, 1990) as a control for transfection efficiency. GFP alone was used as a negative control and had no effect on AR transactivation. Twelve hours after transfection, medium was replaced with medium containing either 10 nM R1881 or vehicle (ethanol) and cells were harvested 48 hours later. Luciferase activity was measured using the Luclite luciferase assay kit (Packard Biosciences). Results were normalised using β-galactosidase activity with a Galacton Galactolite assay kit (Tropix). All assays were completed on at least three separate occasions. Error bars indicate standard deviation from the mean. *P*-values were calculated using a two-tailed *t*-test.

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