Huntingtin interacting protein 1 modulates the transcriptional activity of nuclear hormone receptors

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Introduction

Endocytosis is important for receptor internalization, nutrient uptake, antigen presentation, pathogen internalization, and maintenance of plasma membrane surface area. Endocytosis occurs via several distinct pathways and requires coordinated interactions between a variety of molecules at the membrane and cell cortex. In yeast, a functional connection between the actin cytoskeleton and endocytosis has been firmly established (Geli and Riezman, 1998). Mutations in actin and in several actin-interacting partners of huntingtin (Kalchman et al., 1997; Wanker et al., 1997). Huntington’s disease is an inherited neuromuscular disorder caused by expansion of the codon CAG in the huntingtin gene, which leads to expression of a polyglutamine repeat–containing protein. In conclusion, we have discovered that HIP1 is a novel androgen receptor regulator, significantly repressing transcription when knocked down using a silencing RNA approach and activating transcription when overexpressed. We have also identified a functional nuclear localization signal at the COOH terminus of HIP1, which contributes to the nuclear translocation of the protein. In conclusion, we have discovered that HIP1 is a nucleocytoplasmic protein capable of associating with membranes and DNA response elements and regulating transcription.

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factor, the androgen receptor (AR; Chen et al., 2004). The AR is a member of the nuclear hormone receptor superfamily of transcription factors. The AR consists of an NH2-terminal domain containing polyglutamine and polyglycine repeats, which interacts with a series of transcriptional coregulators; a zinc finger DNA-binding domain; a hinge region encompassing nuclear localization signals; an acetylation site; and a COOH-terminal ligand-binding domain. The nuclear translocation of this transcription factor is dependent on the binding of androgen by the COOH-terminal ligand-binding domain. An actin-binding protein, filamin, was recently shown to interact with the receptor and to be required for translocation (Ozanne et al., 2000).

A subset of endocytic adaptor proteins including Eps15 and Epsin1 have been reported to undergo nucleocytoplasmic shuttling on the basis that their steady-state distribution becomes nuclear upon treating cells with an antifungal antibiotic, Leptomycin B, which inhibits nuclear export (Hyman et al., 2000; Vecchi et al., 2001). A strong argument against a nuclear distribution of these proteins is the absence of a nuclear subfraction under untreated conditions, although this can be explained by a high rate of nuclear export. A potential nuclear function for Eps15 and CALM was reported to be the regulation of transcription on the basis of their modulatory effects using a GAL4-based transactivation assay (Vecchi et al., 2001).

In this study, we have examined the effects of androgen treatment on the subcellular distribution of HIP1 and the effects of this protein on AR-mediated transcription. We have uncovered a functional association of HIP1 with androgen response elements (AREs), providing the first direct evidence for transcriptional modulation of hormone-responsive genes by an endocytic adaptor.

Results

HIP1 is both nuclear and cytosolic

A characteristic of prostatic tissue is a transcriptional response to androgen, which is mediated by the AR. We used an AR expressing prostate cancer cell line LNCaP to examine by subcellular fractionation changes in the distribution of the endogenous AR and HIP1 in response to treatment with a synthetic androgen, Mibolerone. Treating LNCaP cells with Mibolerone resulted in a 70% increase in nuclear AR but also resulted in a nuclear redistribution of HIP1 of up to 50% (Fig. 1 C). In contrast, clathrin, a binding partner of HIP1, remained entirely cytosolic (Fig. 1 A). This nuclear translocation was observed using confocal microscopy after Mibolerone treatment (Fig. 1 B). Translocation of endogenous AR and HIP1 was also induced by treating LNCaP cells with the physiological androgen dihydrotestosterone (unpublished data).

HIP1 associates with the AR

HIP1 was first identified as an interacting partner of a polyglutamine repeat–containing protein, huntingtin, in a yeast two-hybrid screen with a binding affinity inversely proportional to the size of the polyglutamine tract (Wanker et al., 1997). We tested whether HIP1 also interacts with the AR, also a polyglutamine repeat–containing protein, using immunoprecipitation.

Endogenous AR and HIP1 were coimmunoprecipitated from the LNCaP cell line (Fig. 2 A). This has been confirmed independently by band identification using mass spectroscopy (unpublished data). We have also confirmed this interaction using transfected COS7 cells in a mammalian two-hybrid screen with the AR coactivator Tip60 as a positive control (Brady et al., 1999; unpublished data).

We used a GAL4-based transactivation assay in an attempt to identify domains of HIP1 with potential nuclear functions (Fig. 2 B). Expression constructs encoding the GAL4 DNA-binding domain fused to full-length HIP1, a construct bearing the FxDxF/coiled coil and I/LWEQ domains (ΔANTH), and a construct consisting of the COOH-terminal I/LWEQ domain alone were cotransfected along with a reporter plasmid encoding the luciferase gene under the transcriptional control of a GAL4-responsive promoter into AR-null COS7 cells. GAL4–HIP1 and GAL4–ΔANTH produced a three- to fourfold transactivation over the basal value produced by GAL4 alone (Fig. 2 B). Although the degree of transactivation was lower than that produced by E2F1, an established trans-
activator, it was statistically significant and at a similar level to that observed for endocytic proteins previously reported to undergo nuclear-cytoplasmic shuttling (Vecchi et al., 2001).

We attempted to narrow down the binding site for the AR in HIP1 by expressing and purifying GST-tagged domain constructs of HIP1 encompassing the coiled coil domain, the coiled coil/DxF region, and the COOH-terminal I/LWEQ domain from *Escherichia coli*. We then incubated these recombinant domains with lysate extracted from COS7 cells transfected with the AR and, after immunoprecipitation with a polyclonal AR antibody, blotted for the AR, GST, and HIP1. Equal quantities of the AR were immunoprecipitated in the conditions used. A HIP1 blot detected an association with the FxDxF/coiled coil domain and this was confirmed using an antibody raised against GST (Fig. 2 C). From these data we conclude that HIP1 and the AR associate, and that this association requires the central FxDxF/coiled coil domain of HIP1.

**HIP1 is a transcriptional regulator of hormone receptors**

We investigated the effects of ectopic HIP1 expression on the transcriptional activity of the AR using a luciferase reporter construct driven by a prostate-specific antigen (PSA) promoter, pPSALuc. COS7 cells were transfected with 0, 50, 100, 200, and 400 ng of pPSALuc. Cells were treated with 10 nM Mibolerone (bicalutamide) for 48 h and luciferase activity was normalized for transfection efficiency as determined by β-galactosidase assays and expressed relative to vehicle-treated singly transfected AR-positive cells. Data shown represent the results of three independent experiments ± SD. ( Insets ) Lysates were prepared and run at 50 μg per lane before blotting for HIP1 and AR. (B) COS7 cells were transfected with 0, 50, 100, 200, and 400 ng of a minimal ARE luciferase reporter construct (pARE-Luc). After 48 h, cells were lysed and luciferase assays were performed as described. Data shown represent the means of three independent experiments ± SD.

FxDxF/coiled coil domain and this was confirmed using an antibody raised against GST (Fig. 2 C). From these data we conclude that HIP1 and the AR associate, and that this association requires the central FxDxF/coiled coil domain of HIP1.
signaling. Coactivation was also observed when a minimal ARE reporter construct was used, further arguing against surrogate effects on the activities of other transcription factors (Fig. 3 B).

To determine whether the effects on AR-mediated transcription reflected an association between HIP1 and AREs, or were less direct, chromatin immunoprecipitations (ChIP) were undertaken using antibodies against HIP1 and the AR. Sequences corresponding to the proximal (AREI) and distal (AREIII) AREs were amplified by PCR (Fig. 4 A). ChIP assays were performed over 4 h after treatment of LNCaP cells with Mibolerone. A temporal recruitment of HIP1 and the AR to both AREI and AREIII was observed, though the temporal recruitment of HIP1 differed somewhat from that of the AR (Fig. 4 B). HIP1 was recruited within 1 h of Mibolerone treatment to AREI at levels that were sustained over the 4-h treatment period in contrast to the biphasic recruitment of the AR, which peaked at 1 and 4 h maxima. Recruitment of the AR to AREIII was also biphasic, resembling the pattern of recruitment to AREI (Fig. 4 B). In contrast, the recruitment of HIP1 to AREIII was monophasic with maximal recruitment detectable 1 h after stimulation with Mibolerone. In a reChIP assay, immunoprecipitated chromatin extracts were blotted for HIP1 and the AR after 2 h of treatment with Mibolerone. HIP1 and the AR were reciprocally immunoprecipitated and associated with AREI but not with a non-ARE–containing region of the PSA promoter (AREX, Fig. 4 C).

Synchronous AR binding is not a requisite for the association of HIP1 with AREs, and HIP1 may therefore potentially associate with other promoters/response elements and regulate the transcriptional activity of other nuclear hormone receptor proteins. Indeed, cotransfection of HIP1 with α and β isoforms of the estrogen receptor enhanced their transcriptional response to estradiol treatment. The coactivation effect of HIP1 was appreciably greater than that of one of the best-characterized estrogen receptor coactivators, p300 (Fig. S1 A and B, available at http://www.jcb.org/cgi/content/full/jcb.200503106/DC1; Hanstein et al., 1996). Furthermore, increasing quantities of HIP1 cotransfected into COS-7 cells along with the glucocorticoid receptor produced progressive transcriptional coactivation of this receptor (Fig. S1 C). Interestingly, when HIP1 is cotransfected into COS7 cells along with a fusion of GAL4 with the ligand-binding domain (GAL4-LBD) of the glucocorticoid receptor, no additional transcriptional enhancement is observed (Fig. S1 D). This implies that the effects of HIP1 on other nuclear hormone receptors may depend on the NH2-terminal domains of these proteins. HIP1 therefore affects the transcriptional activity of other members of the nuclear hormone receptor family and this is characteristic of many AR coregulators.

HIP1 levels affect the rate of AR degradation

We examined whether endogenous HIP1 was also required to sustain AR transcriptional activity by taking a silencing RNA (siRNA) approach to knockdown HIP1 in LNCaP cells (Fig. 5 A). HIP1 levels were reduced by 70–80% using this approach, as were levels of PSA, an androgen-responsive gene product for which AR activity is required. Protein levels of the AR were also reduced and quantitative reverse transcriptase PCR for HIP1, HIP1R, AR, and PSA was used to determine whether the reduction in the protein levels was reflected at the mRNA level. HIP1 mRNA was significantly reduced as predicted from the siRNA targeting of this protein, as were the mRNA levels of PSA, which reflects both the decreased level of AR in the treated cells and perhaps reduced transcriptional activity although it was not possible to differentiate between these two factors (Fig. 5 B). Strikingly, the mRNA levels of the AR itself were unaffected, and this implied that the reduction in the protein levels of the AR reflected an effect on protein rather than mRNA turnover. We explored this further by repeating the siRNA experiment and at 36 h after treatment inhibiting new protein synthesis by treating the cells with cycloheximide. Lysates were then prepared at two hourly time points after the application of the cycloheximide block and blotted for the AR, HIP1, and β-tubulin. The half-life of the AR was found to be reduced threefold in cells treated with siRNA-targeting HIP1 versus control siRNA (Fig. 5 C). HIP1 therefore reduces the rate of AR degradation. It is not currently known what the mechanism for AR degradation may be. There is evidence that the AR is ubiquitinated and that this enhances its transcriptional activity, whereas treatment with proteasomal inhibitors reduces the rate of AR dissociation from the AREs and AR-mediated transcription (Beitel et al., 2002; Burgdorf et al., 2004). However, treatment of cells with MG132, a proteasomal inhibitor, has not been shown to increase the protein levels of
the AR appreciably and so a direct link between AR ubiquitination and AR degradation is yet to be made (Tanner et al., 2004).

Transcriptional regulation by HIP1 is distinct from lipid binding and requires a COOH-terminal NLS

HIP1 has in the past been reported to play a role as an adaptor in clathrin-dependent membrane trafficking of growth factor receptors through the binding of phosphoinositides and clathrin (Hyun et al., 2004). It was therefore important to dissect the transcriptional effects of HIP1 from its other established functions. To do this, mutations were made in the phosphoinositide-binding ANTH domain of HIP1 based on the strong homology with other ANTH domain–containing proteins (Fig. 6A). The crystal structure of the ANTH domain of clathrin assembly lymphoid myeloid leukemia protein (CALM) in complex with a soluble short-chain (diC8) L-<sub>H</sub>9251,D-<sub>H</sub>9251-myophosphatidylinositol-4,5-bisphosphate has been resolved and the strong homology with

HIP1 therefore enabled us to identify conserved basic residues predicted to lie in the lipid binding pocket (Ford et al., 2001). Note key lipid binding residues (red); sequence identity (red); mutated residues (red arrowheads); and predicted NLS using http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl (blue amino acids). Sequence identifications: HsHIP1 (NP_005329), HsHIP1R (NP_003950), Sp putative clathrin coat assembly protein (NP_596345), ScSla2p (NP_014156), Xl Hip1-prov protein (AAH77182), and RnAP180 (CAA48748). An I/LWEQ module sequence alignment prepared using CLUSTALW incorporating the predicted fourth α helix of the module is shown (McCann and Craig, 1999). Additional sequence identifiers: HsTalin1 (AAF27330) and SpSla2p (NP_594069). (B) Coomassie-stained gel of a sedimentation assay with phosphatidylinositol (PI) and brain (Folch) liposomes. P, pellet; S, supernatant. Liposomes were incubated with 5 μM of the indicated proteins: GST, GST-HIP1N [aa 1–310], GST-HIP1N K/R [aa 1–310 K56E/K58E]. (C) Isolation of an enriched clathrin-coated vesicle fraction from LNCaP cells. LNCaP cells were transfected with pCDNA3 HIP1 R1005E or HIP1 K56E/K58E or vector alone. 48 h posttransfection they were disrupted by homogenization and CCV fractions were isolated using a protocol adapted from Hirst et al. (2004). Coomassie blue–stained gel (left) and Western blots of equal protein loadings of homogenate (H), high speed supernatant (HSS), and CCV fractions from the isolation procedure.

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liposomes but the wild-type HIP1 ANTH domain bound effectively to liposomes produced from bovine brain lipid extract (Folch lipids; Fig. 6 B). However, the double lysine mutation knocked out lipid binding as predicted.

To determine whether the K56E/K58E mutation affected the subcellular distribution of HIP1, LNCaP cells were transfected with Myc-His HIP1 or the HIP1 K56E/K58E double mutant. Enriched clathrin-coated vesicle fractions were prepared and blotted for clathrin, HIP1, and adaptors. The double mutant of HIP1 was significantly de-enriched from the CCV fraction relative to the wild-type protein (Fig. 6 C). The lipid binding mutation may therefore increase the size of the “free” or cytosolic pool of HIP1 available to associate with the AR and/or alternative scaffolds such as DNA response elements and transcription complexes.

The COOH-terminal I/LWEQ domain has regularly spaced, conserved amino acids believed to comprise four α-helices, and in Sla2p and HIP1R this domain binds to F-actin (Engqvist-Goldstein et al., 1999; Legendre-Guillemin et al., 2002). Mutation of a conserved residue, arginine-958, in Sla2p ablates actin binding (McCann and Craig, 1999). Although by sequence alignment this arginine residue (R1005) is also present in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain fragment and actin (Senetar et al., 2004). Indeed binding is absent if a larger expression construct incorporating an upstream fragment and actin (Senetar et al., 2004). Mutations of a conserved residue, arginine-958, in Sla2p ablates actin binding (McCann and Craig, 1999). Although by sequence alignment this arginine residue (R1005) is also present in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain fragment and actin (Senetar et al., 2004). Indeed binding is absent if a larger expression construct incorporating an upstream fragment and actin (Senetar et al., 2004). Mutations of a conserved residue, arginine-958, in Sla2p ablates actin binding (McCann and Craig, 1999). Although by sequence alignment this arginine residue (R1005) is also present in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain fragment and actin (Senetar et al., 2004). Indeed binding is absent if a larger expression construct incorporating an upstream fragment and actin (Senetar et al., 2004). Mutations of a conserved residue, arginine-958, in Sla2p ablates actin binding (McCann and Craig, 1999). Although by sequence alignment this arginine residue (R1005) is also present in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain fragment and actin (Senetar et al., 2004). Indeed binding is absent if a larger expression construct incorporating an upstream fragment and actin (Senetar et al., 2004). Mutations of a conserved residue, arginine-958, in Sla2p ablates actin binding (McCann and Craig, 1999). Although by sequence alignment this arginine residue (R1005) is also present in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain fragment and actin (Senetar et al., 2004). Indeed binding is absent if a larger expression construct incorporating an upstream fragment and actin (Senetar et al., 2004). Mutations of a conserved residue, arginine-958, in Sla2p ablates actin binding (McCann and Craig, 1999). Although by sequence alignment this arginine residue (R1005) is also present in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain fragment and actin (Senetar et al., 2004). Indeed binding is absent if a larger expression construct incorporating an upstream fragment and actin (Senetar et al., 2004). Mutations of a conserved residue, arginine-958, in Sla2p ablates actin binding (McCann and Craig, 1999). Although by sequence alignment this arginine residue (R1005) is also present in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain fragment and actin (Senetar et al., 2004). Indeed binding is absent if a larger expression construct incorporating an upstream fragment and actin (Senetar et al., 2004). Mutations of a conserved residue, arginine-958, in Sla2p ablates actin binding (McCann and Craig, 1999). Although by sequence alignment this arginine residue (R1005) is also present in HIP1, there is only limited biochemical evidence for an association between a recombinantly expressed I/LWEQ domain fragment and actin (Senetar et al., 2004). Indeed binding is absent if a larger expression construct incorporating an upstream fragment and actin (Senetar et al., 2004).

Figure 7. Characterization of an NLS at the COOH terminus of HIP1. COS7 cells were transfected with GFP-HIP1R (aa 992–1009; A) or GFP- HIP1NLS (aa 992–1009; B) 48 h before imaging and fixation. Nuclei were stained with DAPI. GFP-HIP1R is predominantly cytosolic, whereas GFP-HIP1 has a nucleocytoplasmic distribution. Bars, 80 μm. (C) COS7 cells were transfected with GFP-HIP1, GFPHIP1_R1005E, GFPHIP1R, and GFP alone and were fractionated to give nuclear (N) and cytoplasmic (C) fractions, which were resolved by SDS-PAGE (50 μg per lane) and blotted for GFP, lamin B, and clathrin. (D) The degree of translocation of GFP, GFPHIP1, GFPHIP1_R1005E, GFPHIP1R, and clathrin was quantitated by densitometric analysis. Data shown represent the means of five independent experiments ± SD.

Given this ambiguity and in light of the nuclear role that we have uncovered for HIP1, we undertook algorithmic searches for other motifs within this COOH-terminal domain. We identified a putative NLS at the COOH terminus between amino acids 996 and 1009 resembling the consensus RK|x[RK]x[SRK]|x[4–6]RRK, which is strikingly absent in other proteins with talin-like domains (Fig. 6 A; Cokol et al., 2000). This implied an alternative role for R1005 in nuclear transport. We therefore mutated this residue to a glutamate and tagged GFP expression vectors with the HIP1 NLS, the mutated NLS, and the equivalent sequence region in HIP1R. Confocal imaging revealed that the GFP-HIP1 construct has an incomplete but clear nuclear colocalization in comparison to GFP-HIP1R (Fig 7, A and B). Subcellular fractionation revealed that this amounted to an approximate doubling in the amount of nuclear GFP when compared with GFP-HIP1R, the R1005E mutant, or GFP alone (Fig. 7, C and D). This indicates that an NLS within HIP1 itself can contribute to nuclear import and predicts an interaction between HIP1 and importins. A large number of imported proteins contain multiple or bipartite NLS motifs, which cumulatively result in high efficiency of import. Given the fact that the AR contains NLS motifs in its hinge region, we cannot rule out the possibility that other NLS-containing proteins may translocate into the nucleus in a complex with HIP1. This would explain why, in COS7 cells cotransfected with HIP1 and the AR, the nuclear translocation of both is androgen-responsive and more efficient than that of the GFP-tagged minimal NLS (Georget et al., 2002; Saporita et al., 2003).

On the basis of this preliminary characterization of the HIP1 mutants, we predicted that the lipid binding mutant (K56E/K58E) might enhance the ability of HIP1 to coactivate the AR by increasing the available cytosolic pool of HIP1 for nuclear import. We also hypothesized that reducing nuclear import with the R1005E mutation might conversely reduce the transcriptional activity of the AR by having a dominant negative effect on the nuclear import of both HIP1 and, owing to their association, the AR. To test this, we transfected mutant and wild-type HIP1 into COS7 cells and examined the Mibolerone response of the pPSALac reporter construct. The K56E/K58E HIP1 mutant produced a twofold greater enhancement of transcription than wild-type HIP1 under conditions of androgen stimulation (Fig. 8 A). In contrast, the R1005E mutation repressed transcriptional activ-
ity by fourfold. Examining androgen-treated cells expressing the R1005E mutant we noted that there was a reduction in the apparent nuclear translocation of the AR in cells coexpressing the AR and the mutant (Fig. 8 B and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200503106/DC1).

Discussion

Nuclear translocation of membrane trafficking proteins

It has previously been proposed that certain endocytic proteins undergoing nucleocyttoplasmic shuttling might function as protein scaffolds in the nucleus and exert regulatory effects on transcription (Vecchi et al., 2001). Endocytic proteins first reported to undergo nucleocyttoplasmic shuttling were not found to be stimulated to translocate to the nucleus upon treatment with epidermal growth factor or phorbol ester (Vecchi et al., 2001). In contrast, we report here that HIP1 translocates to the nucleus and that this translocation is androgen inducible (Fig. 1). The theme of a direct integration between signal-dependent membrane trafficking events and transcription is a new and emerging one. The only other example of a stimulus-induced nuclear translocation of an endocytic protein is the EGF-dependent translocation of APPL (adaptor protein containing PH domain, PTB domain, and leucine zipper motif) to the nucleus, which complexes with histone deacetylase multiprotein complex to regulate chromatin structure and transcription (Miaczynska et al., 2004). In contrast, HIP1 associates with a transcription factor, the AR, and perhaps other members of the nuclear hormone receptor family to enhance their transcriptional activity.

The overexpression of HIP1 in prostate cancer, as an endocytic protein capable of translocating to the nucleus and co-activating androgen-dependent transcription, is of potential importance in the study of hormonal responses in prostate cancer. A major focus of the prostate cancer field thus far has been on androgen hypersensitivity and kinase-dependent cross talk between growth factor pathways and nuclear hormone receptors rather than adaptor-based cross talk acting on both promoters and membranes (Chen et al., 2004; Culig, 2004). Our work now suggests that HIP1 may be capable of such integration given the association with AREs and the coactivation of AR-mediated transcription (Figs. 3 and 4).

HIP1 as a transcriptional regulator of the AR

It remains to be shown whether the association between HIP1 and the AR and between HIP1 and DNA is indirect or direct. Immunoprecipitation has demonstrated an association between the AR and the FxDxF/coiled coil domain of HIP1 (Fig. 2, A and C). Mammalian two-hybrid assays suggest that the NH2-terminal domain of the AR is a potential binding site for HIP1 (unpublished data). However, this region also binds other transcriptional coregulators, which could act as a molecular bridge to HIP1, and so more detailed mapping of the interaction site in the AR is required (Metzler et al., 2001; Sampson et al., 2001; Waelter et al., 2001).

The function of the I/LWEQ domain of HIP1 has previously proven difficult to confirm despite strong sequence homology with well-characterized actin-binding proteins (Legendre-Guillemin et al., 2002; Senetar et al., 2004). We have identified an NLS within the COOH-terminal I/LWEQ domain, which promotes the nuclear localization of GFP, and we believe that this distinguishes HIP1 from its actin-binding homologues (Figs. 6 A and 7). This motif may also explain a nuclear pool of variable size observed in transfected and untransfected COS7 cells by others (unpublished data). The NLS, although not strong enough to localize GFP constitutively to the nucleus, suggests that HIP1 may therefore have additional nuclear functions and transcriptional effects that are independent of hormonal stimulation and AR expression.

HIP1 is believed to be recruited from the cytosol to membranes through the binding of phosphoinositides by the ANTH domain. We have demonstrated that the nuclear translocation of HIP1 is an alternative dynamic event using cytosolic HIP1. Ablating lipid binding and therefore membrane recruitment with a double lysine mutation in the ANTH domain increases the transcriptional coactivation of HIP1 (Figs. 6 and 8). The
NLS in HIP1 is also clearly equally important for the coactivator function of HIP1 because the R1005E mutation within this motif converts HIP1 from a coactivator to a potent corepressor (Fig. 8 A). Although this mutant can still bind to the AR (not depicted), the steady-state distribution of AR in R1005E-transfected cells is altered such that the AR appears largely cytoplasmic in certain cells (Fig. 8 B).

Other groups have in the past reported a requirement for F-actin binding proteins in the nuclear translocation of the AR although HIP1 itself has not been found so far to bind to F-actin other than in vitro in biochemical experiments (Ozanne et al., 2000; Schrantly et al., 2004; Senetar et al., 2004). Our findings imply that the R1005E mutation exerts its influence on AR signaling as a nuclear trafficking mutant by in part interfering with nuclear entry after androgen treatment (Fig. 8 B and Fig. S2).

Previously, it has been reported that the AR shuttles in and out of the nucleus several times after androgen treatment (Tyagi et al., 2000). Given that the NLS in HIP1 is weak and that the AR contains its own NLS motifs in the hinge domain, it is unlikely that the R1005E HIP1 mutant could block the nuclear translocation of the AR. The more plausible explanation must therefore be that the association between HIP1 and the AR occurs to some degree in the cytoplasm and affects the cycling and turnover of the receptor. A role for HIP1 in regulating the degradation or turnover of the AR is implied by the reduction in steady-state AR levels induced by HIP1 siRNA and the increased rate of AR degradation after the imposition of a cycloheximide block (Fig. 5). A link between nuclear translocation of the AR and its degradation was made when lysine mutations in the NLS of AR were shown to delay nuclear entry of the protein in response to ligand and inhibit proteasomal degradation (Thomas et al., 2004). Degradation of native AR by a cytosolic complex incorporating the E3 ubiquitin ligase, Hsc70, interacting protein (CHIP), was recently reported (Thomas et al., 2004). We therefore hypothesize that the R1005E mutant delays AR nuclear translocation in response to ligand, thus making the receptor available to such a complex for degradation and so repressing transcription. However, other factors that may contribute to the striking repressive effect of the R1005E mutant on transcription by the AR include an alteration in the steady-state nucleocytoplasmic distribution of the AR or disruption to the assembly of an active AR transcription complex on promoters.

In conclusion, the field of endocytosis is now developed enough for network theory to be applied to the large inventory of adaptors and their protein–protein and protein–lipid interactions (Praefcke et al., 2004). In contrast, mapping adaptor interactions at a nuclear and promoter level, be it by ChIP-on-ChIP or ChIP display, is only just beginning (Barski and Frenkel, 2004; Praefcke et al., 2004; Wang, 2005). HIP1 is an example of an emerging subset of adaptor proteins capable of nuclear translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery. It and other adaptors have been linked with cancer translocation and associating with promoters and transcriptional machinery.
NIPT-40 followed by 10× vigorous vortexing. Nuclei were pelleted by cen-
trifugation at 14,000 rpm for 30 s. The cytoplasmic fraction was removed,
and nuclei were washed twice in homogenization buffer with NP-40 and
resuspended in 200 μl of the same buffer. Nuclei were solubilized by soni-
cation, and protein concentrations of nuclear and cytoplasmic fractions
were determined using the BCA protein assay (Pierce Chemical Co.).
Equal amounts of protein from all fractions were boiled in 2× Laemmli
sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose.
Membranes were blotted with antibodies to AR (Santa Cruz Biotechnol-
yogy, Inc.) and HIP1 (NOVUS Biologicals), as well as anti-lamin B (Santa
Cruz Biotechnology, Inc.) and clathrin heavy chain (Transduction Labora-
tories) that were used as nuclear and loading control probes. Primary an-
tibodies were followed up with appropriate HRP-conjugated secondary
antibodies (Dako). Immunoreactive bands were visualized with ECL using
SuperSignal substrate (Pierce Chemical Co.). Blots were scanned using a
densitometer (model FL-5000; Fuji) and band densities were quantitated
using ImageQuant software. Gel and blot images were prepared for the
illustrations with the use of Adobe Photoshop software.

CCV isolation
CCVs were isolated from LNCaP cells growing on six to eight 75-cm² tissue
culture flasks using an adaptation of an existing protocol (Hirst et al.,
2004). Protein levels were assayed, and equal protein loadings of the
fractions were blotted after SDS-PAGE.

Cell culture and microscopy
COS7 and LNCaP lines were grown in DMEM and RPMI-1640 media,
respectively, supplemented with 10% FBS or charcoal-stripped FBS
(Invitrogen). Cells were grown in the presence of charcoal-stripped medium for at
least 24 h before transfection with a PSA luciferase reporter construct (pPSALuc) and
and grown in 6-well dishes (Myoclon). Cells were lysed after 48 h and analyzed
using lipofectamine (Invitrogen). Cells were lysed after 48 h and analyzed
at least three independent experiments performed in triplicate
and grown in steroid-depleted media for 48 h pretrans-
fection with a PSA luciferase reporter construct (pPSALuc) and
and grown in DME and RPMI-1640 media,
respectively, supplemented with 10% FBS or charcoal-stripped FBS
(Invitrogen) according to the manufacturer's instructions. Real-time PCRs
were amplified using the following primers: ARE I, TCTGCCCTTGTCCCTAGAT, and
ARE II, AACCTTCATCCCCAGGACT, to amplify 235 bp of the proximal
PSA promoter, encompassing the ARE I (Fig. 4 A); ARE III, CCTCCCGAG-
GTCAGCTTCTAGAT, and ARE III, GCCCTGTAATCCCGACCTTT, to am-
plify the distal ARE III; ARE X, GCCACTGCTGCTGCTGCGTCTGGA,
and ARE XII, GAGAAGGTGCTGAGCC, to amplify a noneARE-containing por-
tion of the PSA promoter. PCR products were resolved, dried, and then ex-
posed to X-ray film for 2–12 h. ChIP data are representative of triplicate
experiments performed using similar passage number LNCaP cells.

Online supplemental material
Fig. S1 shows coactivation of estrogen and glucocorticoid receptors by
HIP1. HIP1 was cotransfected into COS7 cells with the estrogen or gluco-
corticoid receptors along with appropriate luciferase reporter constructs.
Lysates were assayed for luciferase activity. Experiments were performed
in triplicate and SDs are shown. Fig. S2 shows the effect of the HIP1
R1005E mutant on the nucleocytoplasmic distribution of the AR. LNCaP
cells were transfected with Myc-tagged wtHIP1 or HIP1 R1005E and frac-
tionated after androgen treatment. Nuclear and cytosolic fractions were
resolved by SDS-PAGE and blotted for the AR and Myc illustrated with rep-
resentative blots (Fig. S2 A). Fractions were also blotted for lamin B and
clathrin as nuclear and cytosolic control proteins (Fig. S2 C). The degree
of translocation of the AR and HIP1 was quantitated by densitometric anal-
ysis of the blots (Fig. S2 B). Experiments were performed five times and
SDs are shown. Online supplemental material is available at http://
www.jcb.org/cgi/content/full/jcb.200503106/DC1.

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