A novel function of caspase-8 in the regulation of androgen-receptor-driven gene expression

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**Transcriptional regulation by the androgen receptor (AR)
is critical for male sexual development and prostate cancer.
In this study, we used an expression cloning strategy to
identify molecules that regulate AR-driven transcription.
Screening of a human cDNA library resulted in isolation of
caspase-8 (Casp8), an initiator caspase that mediates
death-receptor-induced apoptosis. Casp8 repressed AR-
dependent gene expression independently of its apoptotic
protease activity by disrupting AR amino-terminal
and carboxy-terminal (N/C) interaction and inhibiting
androgen-induced AR nuclear localization. Protein–
protein interaction analysis revealed that three motifs
in Casp8 specifically interacted with the motifs that
are known to be involved in AR N/C interaction.
Substitutions of the amino-acid residues critical for
AR–Casp8 interactions abolished the Casp8-mediated
inhibition of AR transactivation. In addition, knockdown
of Casp8 by RNA interference specifically affected the
androgen-dependent expression of AR-targeting genes in
LNCaP cells. These results indicate that Casp8 has a novel
function beyond its known role in the mediation of apoptosis.**

Introduction

The androgen receptor (AR) mediates androgen functions in
male sex differentiation, and mutations in AR cause androgen
insensitivity syndrome, which is characterized by incomplete
male sexual development (Quigley *et al*, 1995). AR signaling
plays a pivotal role in the development and maintenance of
the normal prostate and is linked to prostate tumorigenesis
and prostate cancer progression (Craft *et al*, 1999; Dehm and
Tindall, 2005; So *et al*, 2005).

AR is a ligand-activated transcription factor and contains
a carboxy-terminal ligand-binding domain (LBD), a central
DNA-binding domain, and an NH_2-terminal domain (Tsai and
O'Malley, 1994; Mangelsdorf *et al*, 1995). A unique property
of AR that distinguishes it from other steroid receptors is that
androgen binding stabilizes AR protein, whereas other ster-
oid receptors are downregulated by ligand binding (Langley
et al, 1995; Ikonen *et al*, 1997; Berrevoets *et al*, 1998; He and
Wilson, 2002). Androgen-induced AR stabilization results in
part from N-terminal and C-terminal (N/C) interaction
(Langley *et al*, 1995, 1998). AR N/C interactions are mediated
by two N-terminal motifs ([23]FQNLF[27] and [433]WHTLF[437]) and
the C-terminal AF2 (He *et al*, 2000, 2004). Several AR
mutations that are responsible for androgen insensitivity
syndrome disrupt the N/C interaction, suggesting the
physiological significance of this interaction (He and
Wilson, 2002; Wilson *et al*, 2003). Recent studies have
suggested that AR N/C interaction is modulated by AR
cofactors (Shenk *et al*, 2001; Bai *et al*, 2005; Hsu *et al*,
2005). Hence, the N/C interaction provides an additional
basis for AR regulation in response to various signals.

Studies have identified various cofactors and proposed that
they play important roles in the regulation of AR functions
(Brinkmann *et al*, 1999; Janne *et al*, 2000; Gelmann, 2002;
Heinlein and Chang, 2002). The detailed molecular mechan-
isms of AR cofactor action in modulation of AR-driven gene
expression are still elusive. Some AR cofactors possess his-
tone acetyltransferase activity or histone methyltransferase
activity; therefore, they may activate AR transactivation as
a result of their ability to remodel the chromatin structure
(Xu, 2005). On the other hand, negative AR cofactors such
as histone-deacetylase-containing complexes reduce AR
transcriptional activity by regulating the status of histone
acetylation (Baniahmad, 2005; Wang *et al*, 2005). Post-trans-
lational modifications of AR by cofactors such as CBP/p300
may modulate AR activity (Fu *et al*, 2003). Other AR cofactors
lacking such enzymatic activities may function through
multiple mechanisms, including control of ligand/AR binding
affinity (Dedhar *et al*, 1994; Sato *et al*, 1997), AR stability
(Lee and Chang, 2003a), AR subcellular localization (Poukka
et al, 2000), or recruitment of basal transcription machinery
(Lee and Chang, 2003b).

Caspases play critical roles in initiation and execution of
programmed cell death (Shi, 2002). They constitute a family
of cysteine proteases that cleave target proteins at sites next
to aspartic acid residues. Caspase-8 (Casp8) is the initiator
caspase for death-receptor-mediated apoptosis (Salvesen,
1999). Like other members of the caspase family, Casp8 is
initially synthesized as a zymogen with two death effector
domains at the N-terminus and a protease domain at the
C-terminus (Boatright and Salvesen, 2003). However, increas-
ing evidence suggests that caspases also have functions in
addition to their known apoptotic roles (De Maria *et al*, 1999;
Faouzi *et al*, 2001; Franchi *et al*, 2003; Kang *et al*, 2004). For
examples, Casp8 activates NF-κB signaling independently of
its activity as a proapoptotic protease (Chaudhary *et al*, 2000;
Hu *et al*, 2000). Further genetic and biochemical analyses
have shown that Casp8 functions both as a pivotal molecule
for death-receptor-induced apoptosis and as a selective signal
transducer for NF-κB activation by antigen receptor (Su *et al*,

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2005). During *Drosophila* oogenesis, loss of function of the *dcp-1* gene, which encodes a caspase (Drice), led to nurse cells defective in cytoskeletal reorganization and nuclear breakdown that normally accompany the apoptotic process (McCall and Steller, 1998). Expression of the active Drice (*tdcp-1*) led to widespread apoptosis and dying egg chambers similar to the normal nurse cell death that occurs in late oogenesis (Peterson *et al*, 2003). However, the cytoskeletal events induced by *tdcp-1* were significantly different from those normally seen in nurse cells during late oogenesis. These observations suggest that the cytoskeletal events may be Drice (caspase)-dependent but caspase-enzymatic activity-independent processes.

Using a human cDNA-expressing library, we isolated Casp8 and found that it inhibits AR transcriptional activity by disrupting AR N/C interaction and inhibiting androgen-induced AR nuclear localization.

Results

Casp8 is identified as a negative AR cofactor

We used a highly sensitive quantitative reporter assay (Pomerantz *et al*, 2002) to identify factors capable of regulating AR-dependent transcription. A pool of cDNAs in a mammalian expression vector was transiently transfected into PC3mm2 cells with the pGL3-4 × ARE-E4-luc reporter. From four pools (total 20 000 cDNAs), six positive cDNAs were obtained. One of these positive cDNAs encoded the full-length Casp8. To confirm the effect of Casp8 on AR-dependent transcription, the pGL3-4 × ARE-E4-luc reporter was cotransfected with expression plasmids for AR and/or Casp8 into PC3mm2 cells in the absence or presence of androgen (R1881). AR activated the reporter about 34-fold in the presence of androgen, and coexpressed Casp8 inhibited this activity in a dose-dependent manner (up to 13-fold) (Figure 1A). Casp8 also strongly inhibited AR-driven transcription from the natural prostate-specific androgen (PSA) enhancer (−4354 to −858) reporter construct (Figure 1B) (Schuur *et al*, 1996; Cleutjens *et al*, 1997) but did not inhibit Gal4-p53-driven transcription from the pGL3-4 × Gal4-E4-luc reporter (Figure 1C). Expression of Casp8 in transfected cells

was demonstrated by RT–PCR (Figure 1A, middle panel) and Western blot analysis (Figure 1A, bottom panel).

Similar assay was performed to determine whether expression of other caspases affects AR-driven transcription. AR activated the reporter about 36-fold in the presence of androgen, and coexpressed Casp8 inhibited this activity (12-fold) (Figure 2). In contrast, expression of Casp3, Casp6, Casp7, and Casp9 did not significantly inhibit AR-driven transcription from the pGL3-4 × ARE-E4-luc reporter.

To investigate whether expression of Casp8 affects levels of AR protein, we performed Western blot analysis of AR. Casp8
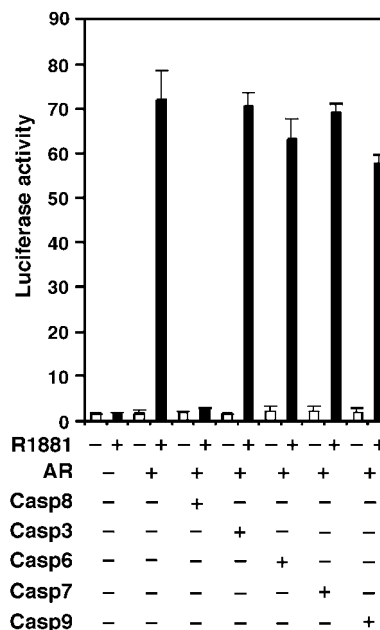
Figure 2 Caspases other than Casp8 do not inhibit AR-driven transcription. pGL3-4 × ARE-E4-luc reporter (100 ng), pcDNA-AR (30 ng), or indicated caspase-expressing constructs (30 ng) was cotransfected into PC3mm2 cells. Transfected cells were grown in the absence or presence of 10 nM R1881 for 48 h. Cells were then harvested and used for luciferase assay. Values represent mean±s.d. ($N = 3$).
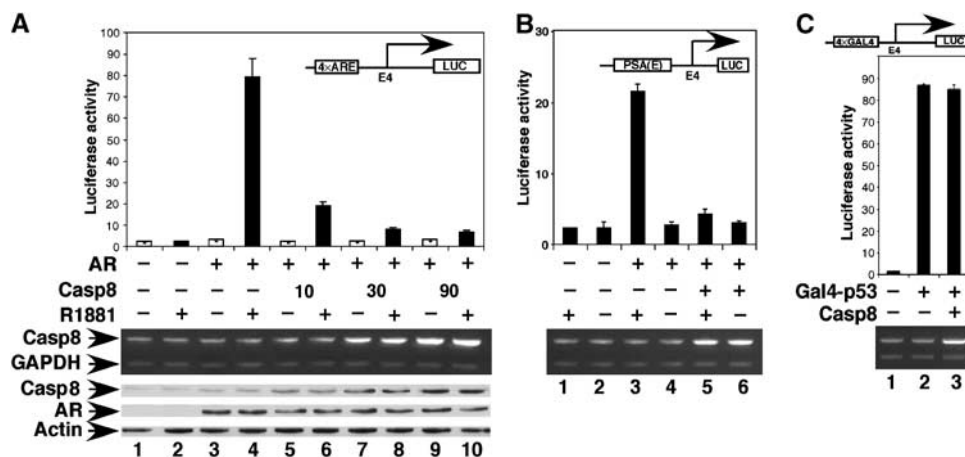

Figure 1 Casp8 downregulates AR transcriptional activity in a dosage-dependent manner. pGL3-4 × ARE-E4-Luc (**A**), pGL3-PSA(E)-E4-luc (**B**), or pGL3-4 × Gal4-E4-luc (**C**) reporter (100 ng), pcDNA-AR (30 ng) or pcDNA-Gal4-p53 (10 ng) and indicated amounts of pcDNA-Casp8 were cotransfected into PC3mm2 cells. Transfected cells were grown in the absence or presence of 10 nM R1881 for 48 h. Cells were then harvested for luciferase assay (top panel), RT–PCR (middle panel), and Western blot analysis (bottom panel). Values represent mean±s.d. ($N = 3$).

inhibited AR-driven promoter activity by 93% (14-fold; Figure 3A, top panel), and levels of AR protein decreased by 50% in the presence of Casp8 compared with those in the absence of Casp8 (middle panel, lane 6 versus lane 5). The decrease in AR protein by 50% only slightly lowered the AR-driven reporter activity (Figure 3A, lane 4 versus lane 5). These results indicated that the decrease in AR protein

expression induced by Casp8 expression did not account for loss of AR transcriptional activity mediated by Casp8.

As Casp8 is a cysteine protease, we next investigated whether Casp8 could degrade AR directly. The wild-type and enzymatic active site mutant (C345A) of Casp8 were expressed in and purified from PC3 cells (Figure 3B, lanes 2 and 3). Incubation of recombinant AR with wild-type (lane 5)
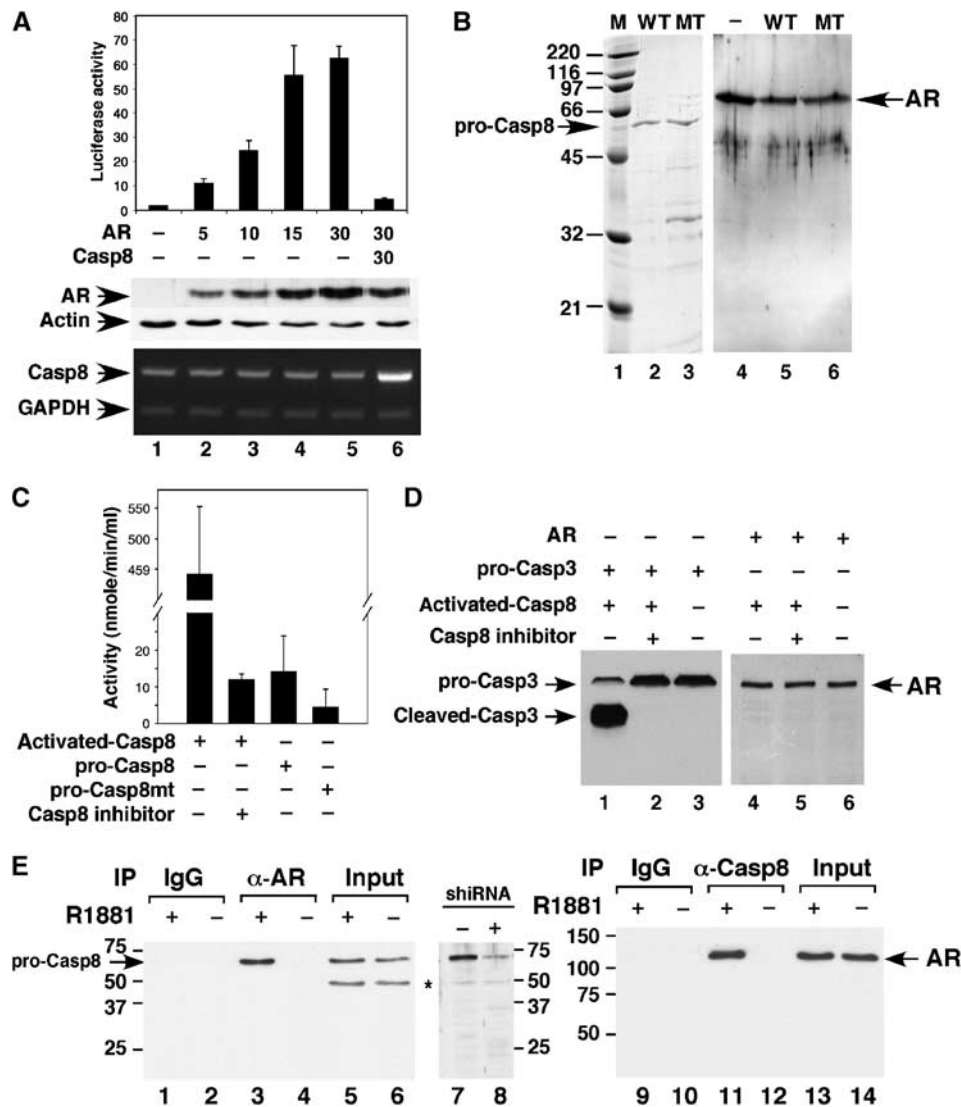
Figure 3 (**A**) Casp8-mediated inhibition of AR transcriptional activity did not result from AR degradation. pGL3-4 × ARE-E4-luc (100 ng), the indicated amounts of pcDNA-f:AR, and pcDNA-f:Casp8 were transfected into PC3mm2 cells. Transfected cells were grown in the presence of 10 nM R1881 for 48 h and then harvested for luciferase assay (upper panel), Western blot analysis (middle panel), and RT–PCR (bottom panel). Values represent mean ± s.d. ($N=3$). (**B**) Casp8 did not cleave AR *in vitro*. FLAG epitope-tagged Casp8 (wild-type) and mutant (C345A, which lacks enzymatic activity) were expressed in PC3 cells and immunopurified using M2-agarose beads. Wild-type (WT; lane 2) and mutant (MT; lane 3) Casp8 (100 ng) were analyzed using 12% SDS–PAGE and then stained with Coomassie blue R250. Lane 1, standard molecular weight markers (kDa) (Bio-Rad). Recombinant AR (40 µg) was incubated without (lane 4) or with wild-type (lane 5) or mutant (lane 6) Casp8 (50 µg) in a buffer containing 100 mM HEPES, pH 7.5, and 10 mM DTT in a total volume of 100 µl for 20 min at 37°C. Reaction mixtures were submitted for Western blot analysis. (**C**) Enzymatic activity assay of pro-Casp8 and activated casp8 by using the Caspase-8 Assay Kit (Sigma-Aldrich). In a total volume of 100 µl, 1.8 pmol of activated Casp8, pro-Casp8, or pro-Casp8mt were incubated with substrate (Ac-IETD-pNA) in the presence or absence of Casp8 inhibitor (Ac-IETD-CHO) at 37°C for 1 h and enzymatic activity was determined according to the manufacturer's instructions. (**D**) Activated Casp8 did not cleave AR. In a total volume of 100 µl, 3 pmol of pro-Casp3 (R&D Systems Inc.) or AR was incubated with 1.8 pmol of activated Casp8 at 37°C in the presence or absence of Casp8 inhibitor for 30 min. Reaction mixtures were submitted to Western blot with anti-Casp3 (lanes 1–3) (Cell Signaling) or anti-AR (lanes 4–6) antibodies. (**E**) Casp8 interacted with AR in LNCaP cells. LNCaP cells were grown in the presence (lanes 1, 3, 5, 9, 11, and 13) or absence (lanes 2, 4, 6, 10, 12, and 14) of 10 nM R1881 for 48 h. Whole-cell lysates were incubated with immobilized anti-AR (lanes 3 and 4) or anti-Casp8 (lanes 11 and 12) antibodies for 2 h at 4°C. After being washed, bound proteins were eluted with 0.1 M glycine, pH 2.5, and the eluates were neutralized with 1.5 M Tris–HCl, pH 8.8, and submitted to Western blot analysis with an anti-Casp8 or anti-AR antibody. Western blotting was also performed using whole-cell lysates derived from control (lane 7) and Casp8 shRNA-expressing LNCaP cells (lane 8) with the anti-Casp8 antibody to validate the specificity of anti-Casp8 antibody.

or mutant (lane 6) Casp8 showed that caspase did not directly degrade AR. Enzymatic activity assay indicated that pro-Casp8 had very low (30-fold) proteinase activity than activated Casp8 (Figure 3C). Activated Casp8 cleaved recombinant pro-Casp3 (Figure 3D, lane 1), a well-known substrate of Casp8 but not AR (lane 4).

To further confirm the interaction of Casp8 with AR *in vivo*, we performed co-immunoprecipitation in LNCaP cells. Casp8 was co-immunoprecipitated with AR and AR was co-immunoprecipitated with Casp8 from the whole-cell extract made from LNCaP cells grown in the presence of androgen (Figure 3E, lanes 3 and 11). In the absence of ligand, no Casp8–AR interaction was detected (lanes 4 and 12). These results suggested that Casp8 interacted with AR in LNCaP cells in a ligand-dependent manner.

Additional analyses indicated that Casp8-mediated inhibition of AR transcriptional activity is not through a caspase-dependent apoptotic pathway, an Akt–Mdm2 pathway, or HDAC-containing corepressor complexes (see Supplementary data).
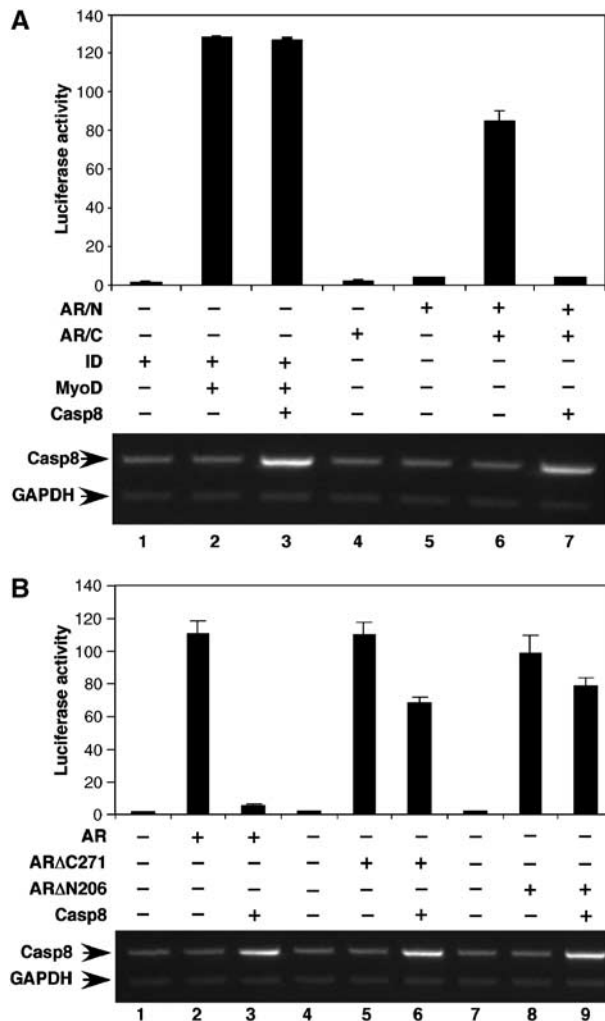
Casp8 disrupts AR N/C interaction

A unique property of AR is the androgen-induced intramolecular (N/C) interaction. The N/C interaction is required for AR function *in vivo* and androgen-induced AR protein stabilization. The mammalian two-hybrid system was used to evaluate the effect of Casp8 on AR N/C interaction. We observed a 21-fold activation of the reporter when cells were co-transferred with pACT-AR/N and pBIND-AR/C, indicating a strong AR N/C interaction (Figure 4A). Coexpression of Casp8 with pACT-AR/C and pBIND/N dramatically decreased the reporter activity, indicating that Casp8 inhibited AR N/C interaction. As a positive control, coexpression of pACT-ID with pBIND-MyoD resulted in activation (128-fold) of the reporter, which was not affected by co-transfection of pcDNA-Casp8. As negative controls, expression of neither pACT-AR/N nor pBIND-AR/C significantly activated the reporter activity. The androgen-induced AR N/C interaction stabilized AR (Langley *et al*, 1995, 1998); inhibition of this interaction by Casp8 might result in AR degradation (Figure 3A).

Furthermore, we performed transient transfection assays using different AR truncations to determine if intact AR is necessary for Casp8-mediated transcriptional inhibition. ARΔC271 (with the C-terminal 271 amino-acid residues deleted) and ARΔN206 (with the N-terminal 206 amino-acid residues deleted) still had transactivation activities but no N/C interaction. ARΔC271 and ARΔN206 activated luciferase reporter activity (58- and 56-fold, respectively) to an extent similar to that of the full-length AR (71-fold) (Figure 4B). In contrast to the full-length AR, transactivations by the ARΔC271 and ARΔN206 truncations were only slightly inhibited by Casp8.
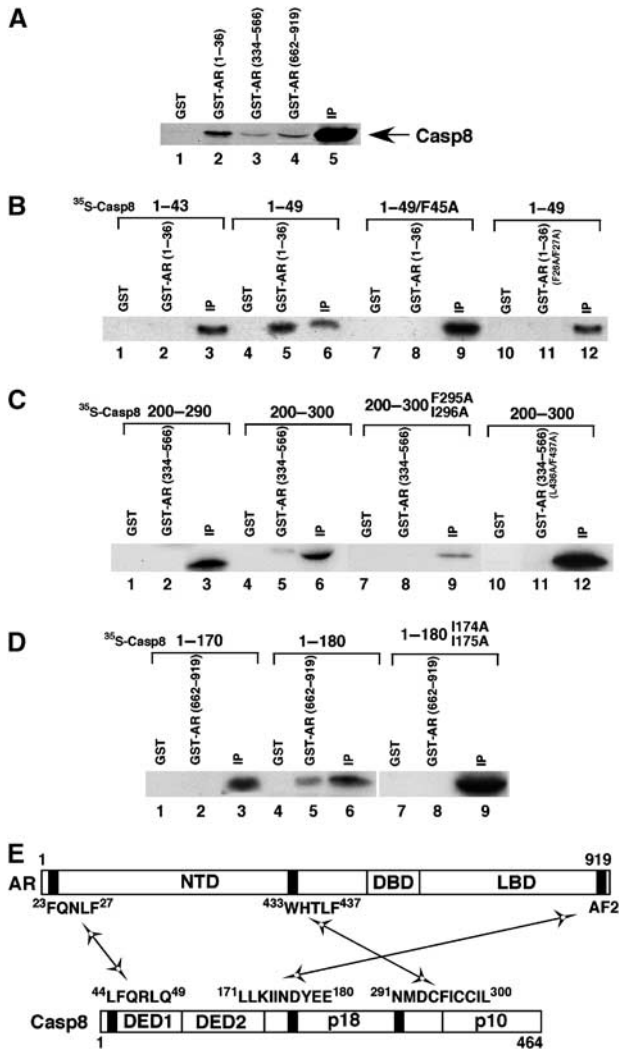
Casp8 physically interacts with AR

Three motifs ([23]FQNLF[27], [433]WHTLF[437], and the C-terminal AF2) have been found to mediate AR N/C interaction (He *et al*, 2000; He and Wilson, 2002). Our hypothesis was that Casp8 physically interacts with these motifs, thereby disrupting AR N/C interaction. To investigate this possibility, we performed *in vitro* protein–protein pull-down assays. Figure 5A shows that Casp8 binds to AR (1–36) (lane 2),

Figure 4 Casp8 disrupts the AR N/C interaction. (**A**) The mammalian two-hybrid assay was performed in PC3mm2 cells. Cells were transfected with pACT-AR/N, pBIND-AR/C, or both. Cells were transfected with pACT-ID or pBIND-MyoD as control. Transfected cells were grown in the presence of 10 nM R1881 for 48 h and then harvested for luciferase assay. Casp8 Expression was detected by RT–PCR (bottom panel). (**B**) An intact AR is required for Casp8-mediated inhibition of AR transactivation. pGL3-4 × ARE-E4-luc, pcDNA-AR, pcDNA-ARΔC271, or pcDNA-ARΔN206 and pcDNA-Casp8 were cotransfected into PC3mm2 cells. Cells were grown in the presence of 10 nM R1881 for 48 h and then harvested for luciferase assay. Values represent mean ± s.d. (*N* = 3).

AR (334–566) (lane 3), and AR (662–919) (lane 4) but not glutathione *S*-transferase (GST) (lane 1). To identify domains in Casp8 that interact with AR, the various truncations of Casp8 were expressed as S[35]-labeled proteins and incubated with GST (Figure 5B, lanes 1, 4, 7, and 10; Figure 5C, lanes 1, 4, 7, and 10; Figure 5D, lanes 1, 4, and 7), GST-AR (1–36) (Figure 5B, lanes 2, 5, 8, and 11), GST-AR (334–566) (Figure 5C, lanes 2, 5, 8, and 11), or GST-AR (662–919) (Figure 5D, lanes 2, 5, and 8) fusion proteins immobilized on glutathione-agarose beads. As shown in Figure 5B, the N-terminal part (amino-acid residues 1–49) of Casp8 strongly bound to GST-AR (1–36) (lane 5) but not GST alone (lane 4), whereas further deletion of six residues ([44]LFQRLQ[49]) resulted in the complete loss of this interaction (lane 2). Mutation of F45A on Casp8 (1–49) completely abolished

Figure 5 Casp8 physically interacts with AR. (**A**) Casp8 interacts with multiple regions in AR. GST, GST-AR (1–36), GST-AR (334–566), or GST-AR (662–919) was immobilized on glutathione-Sepharose beads and incubated with ^{35}S-labeled Casp8. Proteins retained on the beads were separated by SDS–PAGE and the gel was analyzed by autoradiography. Five percent of input (IP) for the interaction assay was loaded on the same gel. (**B–D**) Identification of motifs that mediate Casp8–AR interactions. GST, GST-AR (1–36), GST-AR (1–36/F26A/F27A), GST-AR (334–566), GST-AR (334–566/L436A/F437A), or GST-AR (662–919) was immobilized on glutathione-Sepharose beads and incubated with ^{35}S-labeled Casp8 (1–43), Casp8 (1–49), Casp8 (1–49/F45A), Casp8 (200–290), Casp8 (200–300), Casp8 (200–300/F295A/I296A), Casp8 (1–170), Casp8 (1–180), or Casp8 (1–180/I174A/I175A) as indicated. After being washed, beads were submitted to SDS–PAGE and the gel was analyzed by autoradiography. The input (IP) (5%) for the interaction assay was loaded onto the same gel. (**E**) Diagram of motifs in AR and Casp8 that are involved in Casp8–AR interactions. Black boxes represent motifs that mediate Casp8–AR interactions.

this interaction (Figure 5B, lane 8). Furthermore, mutation of F26A/F27A in AR (1–36) also lost this interaction (Figure 5B, lane 11). These results indicate that the motif [44]LFQRLQ[49] in Casp8 interacts with the AR motif [23]FQNLF[27], which has been shown to be involved in AR N/C interaction. Similar analyses (Figure 5C and D) revealed that amino-acid residues 291–300 ([291]NMDCFICCIL[300]) of Casp8 weakly interacted with the second motif ([433]WHTLF[437]) in AR that is involved in AR

N/C interaction, and amino-acid residues 171–180 ([171]LLKIINDYEE[180]) of Casp8 interacted with the LBD of AR (probably the AF2). Motifs involved in Casp8–AR interactions are diagrammed in Figure 5E.

### *Interacting motifs are required for Casp8-mediated inhibition of AR-driven transcription*

To further confirm the function of the motifs in Casp8, we generated point mutations in Casp8, which selectively abolished the interactions with the motifs in AR (Figure 5B–D, lane 8). Consistent with protein–protein pull-down results, the mutations F45A and I174A/I175A in Casp8 dramatically abolished Casp8-mediated inhibition of AR transcriptional activity, and the double mutation F295A/I296A partially affected this activity (Figure 6A). These results suggest that three AR-binding motifs in Casp8 are essential for the observed strong inhibition of AR-driven transcription by Casp8.

Furthermore, three AR mutants (L26A/L27A, L436A/F437A, and L26A/L27A/L436A/F437A) that have been shown to be defective in the N/C interaction (He *et al*, 2000) were used in the luciferase assays. As shown in Figure 6B, AR mutants L26A/L27A and L26A/L27A/L436A/F437A lost inhibitory effects of Casp8 to a large extent, whereas AR mutant L436A/F437A had a mild effect. This might reflect the weak interaction between motif [433]WHTLF[437] in AR and motif [291]NMDCFICCIL[300] in Casp8 (Figure 5C, lane 5). Thus, these results further confirmed that physical interactions between the motifs in Casp8 and AR are essential for Casp8-mediated inhibition of AR-dependent transcription.

An intramolecular interaction exists in Casp8

When studying Casp8–AR interactions, we observed that GST-AR (1–36) strongly interacted with ^{35}S-labeled Casp8 (1–100) and Casp8 (1–150) (Figure 7A, lanes 2 and 5) but not with Casp8 (1–200) (lane 8). These observations led us to hypothesize that there is an intramolecular interaction in Casp8 molecule that inhibits intermolecular interaction between AR (1–36) and Casp8 (1–200). The *in vitro*-translated, ^{35}S-labeled Casp8 (150–200) (Figure 7A, lane 12) bound to GST-Casp8 (1–49) (lane 11) and not to GST (lane 10) beads. This result confirms that there is an intramolecular interaction in the Casp8 molecule. The mutant (F45A) of Casp8 (1–49) completely abolished this intermolecular interaction (Figure 7A, lane 14), indicating that the intramolecular interacting motif overlaps with the motif ([44]LFQRLQ[49]) involved in intermolecular interaction with AR.

A model of the interactions between Casp8 and AR is shown in Figure 7B. The intramolecular interactions in Casp8 involve the motif (amino-acid residues 44–49) and a motif located within amino-acid residues 150–200 (not mapped in detail). Our studies suggested that three motifs in Casp8 are involved in the intermolecular interactions with AR. These are as follows: motif [44]LFQRLQ[49], which mediated a strong interaction (Figure 5B, lane 5; 17% input was pulled down) with AR motif [23]FQNLF[27]; motif [171]LLKIINDYEE[180], which mediated an intermediate interaction (Figure 5D, lane 5; 4% input was pulled down) with AR LBD; and motif [291]NMDCFICCIL[300], which mediated a weak interaction (Figure 5C, lane 5; 2% input was pulled down) with AR motif [433]WHTLF[437].
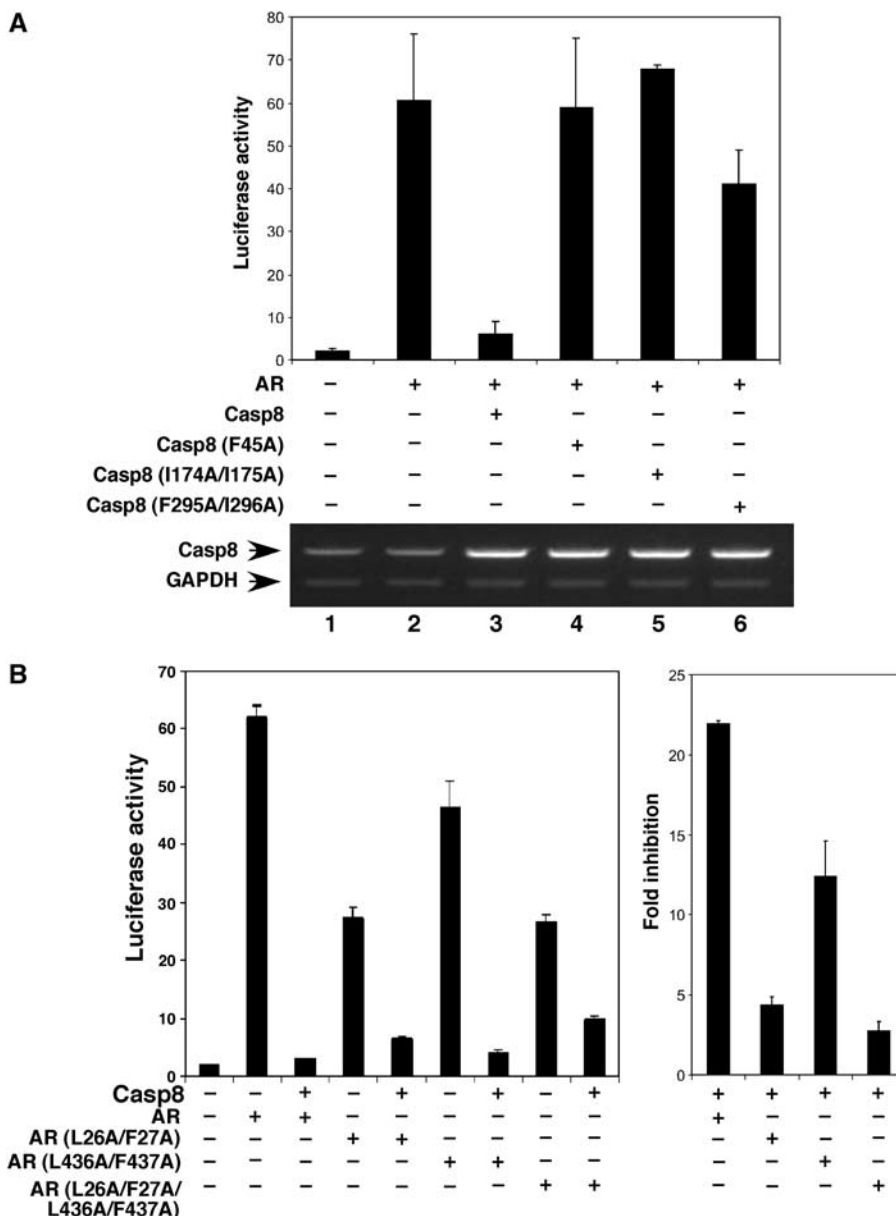
Figure 6 Mutations in Casp8–AR interaction motifs abolish Casp8-mediated inhibition of AR transactivation. (**A**) Casp8 with mutations in interaction motifs lost its ability to inhibit AR-driven transcription. PC3mm2 cells were transfected with pGL3-4 × ARE-E4-luc, pcDNA-AR, pcDNA-Casp8 (wild-type), or pcDNA-Casp8 (F45A, I174A/I175A, or F295A/I296A) mutants. Transfected cells were grown in the presence of 10 nM R1881 for 48 h and then harvested for luciferase assay (top panel) and RT–PCR. (**B**) Mutations in interaction motifs in AR abolish Casp8-mediated inhibition of AR transcriptional activity. PC3mm2 cells were transfected with pGL3-4 × ARE-E4-luc, pcDNA-Casp8, pcDNA-AR (wild-type), or pcDNA-AR (L26A/F27A, L436A/F437A, or L26A/F27A/L436A/F437A) mutants. The transfected cells were grown in the presence of 10 nM R1881 for 48 h and then harvested for luciferase assay (left panel). Fold inhibition of wild-type and mutant AR by Casp8 is represented on the right. Values represent mean ± s.d. (*N* = 3).

RNA interference of Casp8 leads to altered expression of AR target genes

To further confirm a role for Casp8 in the regulation of AR target genes *in vivo*, we performed RNA interference assay. Infection of LNCaP with a retrovirus expressing shCasp8D-1 (lane 2) and shCasp8D-3 (lane 4) reduced the level of Casp8 protein by 50 and 70%, respectively (Figure 8A). However, infection of LNCaP cells with a retrovirus expressing shCasp8D-2 (Figure 8A, lane 3) only slightly decreased the level of Casp8 protein. The levels of AR protein or β-actin were not affected by these shRNAs (Figure 8A, middle and

bottom panels). Northern blot analysis demonstrated that the decrease in the levels of Casp8 induced by shRNAs resulted in a dramatic increase (up to 5.6-fold) in the levels of PSA gene expression in the presence of androgen (Figure 8B, lanes 2 and 4 versus lane 1). These shRNAs did not significantly affect the androgen-independent expression of PSA (Figure 8B, lanes 6–8 versus lane 5) or β-actin (bottom panel) gene.

We also investigated, using real-time PCR, whether decreased Casp8 expression alters expression of other known AR target genes. Casp8 downregulation enhanced expression

of *Nkx3.1* and *KLK2* genes and inhibited expression of *Maspin* (Table I), consistent with previous observations that AR pathway positively regulated expression of *Nkx3.1* (Prescott *et al*, 1998; Bhatia-Gaur *et al*, 1999) and *KLK2* (Wang *et al*, 2006), but repressed *Maspin* expression genes (Zhang *et al*, 1997). However, the Casp8 downregulation did not affect expression of *TFF1* and *DIO1* genes, which are regulated by the estrogen and thyroid hormone receptors, respectively (see Supplementary data). These results indicated that Casp8 specifically modulates expression of AR target genes. Consistent with this conclusion, Casp8 overexpression in LNCaP cells inhibited expression of the PSA gene (Figure 8C, lane 2 versus lane 1).

### Casp8 inhibits the androgen-driven AR nuclear localization

We next investigated whether Casp8 affects the interaction of AR with DNA in LNCaP cells. Nuclear extracts (NE) were prepared from control, Casp8-expressing, and Casp8

shRNA-expressing LNCaP cells and submitted to electro-phoretic mobility shift assays with an ARE-containing probe. While a strong signal of AR–DNA complex was observed in LNCaP NE (Figure 9A, lanes 3 and 5), this signal was decreased six-fold by Casp8 overexpression (lane 4) and increased 1.5-fold by Casp8 downregulation via shRNA expression (lane 6). Mutations of the conserved nucleotides in ARE (lanes 7–12) or addition of the cold probe (lanes 2 and 8) specifically abolished AR–DNA complex formation, indicating specificity of the AR–DNA complex. These results suggested that inhibition of AR-driven transcription by Casp8 might be through decreased AR–DNA interaction in the presence of Casp8.

To investigate whether Casp8 affects androgen-driven AR nuclear localization, the EGFP-Casp8 fusion protein was expressed in LNCaP cells by transient transfection. The EGFP-Casp8 protein inhibited AR-driven transcription and interacted with AR similarly to the non-fused Casp8 (data
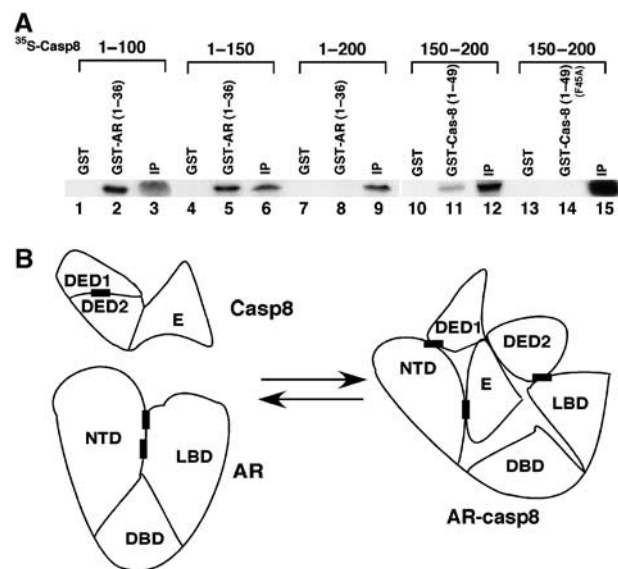
Figure 7 (**A**) An intramolecular interaction exists in Casp8 molecule. GST, GST-AR (1–36), GST-Casp8 (1–49), or GST-Casp8 (1–49/F45A) mutant was immobilized on glutathione-Sepharose beads and incubated with ^{35}S-labeled Casp8 (1–100), Casp8 (1–150), Casp8 (1–200), or Casp8 (150–200) as indicated. After being washed, the beads were submitted to SDS–PAGE and the gel was analyzed by autoradiography. The input (IP) (5%) for the interaction assay was loaded onto the same gel. (**B**) Diagram of Casp8–AR interactions. Black boxes represent motifs that mediate Casp8–AR interactions.
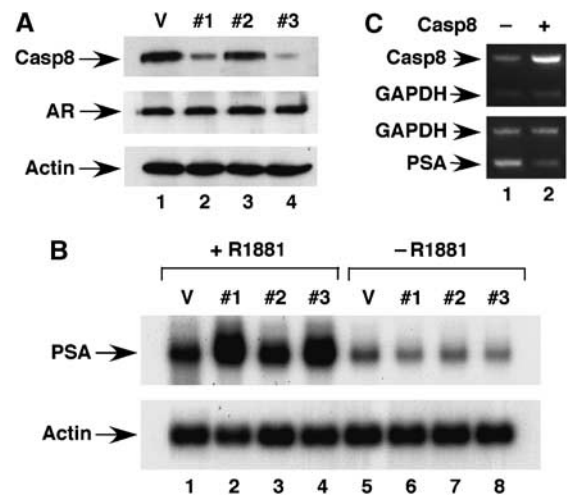
Figure 8 Casp8 regulates AR transactivation *in vivo*. (**A**) Knockdown of Casp8 expression by shRNAs in LNCaP cells. LNCaP cells were infected with vector control retrovirus (lane 1) or Casp8 shRNA-expressing virus (lanes 2–4). Infected cells were analyzed by Western blot. (**B**) Downregulation of Casp8 expression affected expression of AR target gene. Retrovirus-infected LNCaP cells were grown in the presence (lanes 1–4) or absence (lanes 5–8) of 10 nM R1881 for 24 h. Gene expression was detected by Northern blot. (**C**) Casp8 overexpression inhibited PSA gene expression. LNCaP cells were seeded in 6-cm dishes and transfected with 2 μg of control vector pCNDA3.1 (lane 1) or pcDNA-Casp8 (lane 2). Cells were grown in the presence of 10 nM R1881 for 24 h and RNAs were prepared from transfected cells for RT–PCR analysis to detect Casp8 (top panel) and PSA (bottom panel).

Table I Data of real-time PCR assay

| | $2^{-\Delta\Delta C_t}$ | | | | | |
| | LNCaP + vector | | LNCaP + shRNA | | Fold change[a] | |
	Average	s.d.	Average	s.d.	Average	s.d.
NKX3.1	10.96	0.51	19.77	3.17	1.80	0.20
KLK2	10.89	0.76	16.81	0.76	1.69	0.55
Maspin	5.15	0.54	1.46	0.01	−3.53	0.36

[a]For *NKX3.1* and *KLK2*, fold change = relative quantification of the Casp8 knockdown group/relative quantification of the control group; for *Maspin*, fold change = −relative quantification of the control group/relative quantification of the Casp8 knockdown group.

A

B
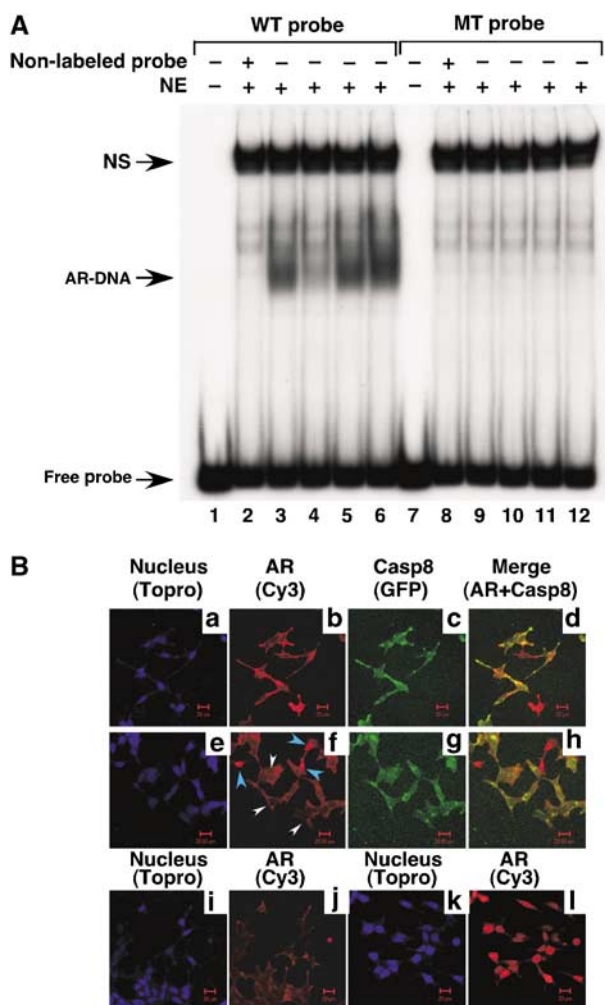
Figure 9 (**A**) Casp8 inhibits AR interaction with DNA in LNCaP cells. NE were prepared from LNCaP (lanes 2, 3, 8 and 9) or LNCaP trasfected with pcDNA-Casp8 (lanes 4 and 10), retrovirus vector (lanes 5 and 11), or Casp8 shRNA (#3) (lanes 6 and 12). (**B**) Casp8 expression inhibited the androgen-driven AR nuclear localization. LNCaP cells or LNCaP cells transfected with pcDNA-EGFP-Casp8 were grown in the absence (panels a–d, i, j) or presence (panels e–h, k, l) of 10 nM R1881. Signals were detected for nucleus (blue) (panels a, e, i, k), AR (red) (panels b, f, j, l), and EGFP-Casp8 (Green) (panels c, g) under a confocal microscope. In panel f, EGFP-Casp8-positive and -negative cells are indicated by white and blue arrows, respectively. Panels d and h are merged images of panels b and c and f and g, respectively.

not shown). Control and EGFP-Casp8-transfected LNCaP cells were grown in the absence or presence of androgen for 48 h and submitted to immunostaining for AR. The androgen-driven nuclear translocation of AR was clearly demonstrated in control LNCaP cells (Figure 9B, panel l versus panel j). However, this translocation was inhibited in EGFP-Casp8-expressing LNCaP cells (panel f, indicated by white arrows) but not in cells lacking EGFP-Casp8 expression (indicated by blue arrows). These observations indicate that Casp8 inhibited the androgen-dependent AR nuclear translocation.

Discussion

In an effort to better understand the disparate mechanisms of AR activation and repression, we used an expression cloning

approach to find new factors, new mechanistic insights, and new pathways. In the present study, we found that Casp8 physically and functionally interacted with AR and inhibited AR-driven transcription by disrupting AR N/C interaction and inhibiting the androgen-driven AR nuclear translocation. Our results suggest the existence of a functional link between Casp8 and AR signalings and indicate that Casp8 has a novel role beyond its activity as a cell death protease.

Identification of Casp8 as a negative AR cofactor

Transcriptional corepressors mediate repression by various nuclear receptors (Baniahmad, 2005; Wang et al, 2005). Some nuclear receptors (including retinoid receptor, thyroid hormone receptor, vitamin D receptor, and certain orphan receptors) that are not associated with heat-shock proteins in their unliganded state repress transcription by recruitment of corepressor complexes (Tsai and O'Malley, 1994; Heinlein and Chang, 2002). In contrast, unliganded steroid receptors (including AR) generally associate with heat-shock proteins and, upon ligand binding, dissociate from the heat-shock proteins, translocate to the nucleus, and associate with coactivators or corepressors to activate or repress target genes (Brinkmann et al, 1999; Janne et al, 2000; Gelmann, 2002; Haendler, 2002; Heinlein and Chang, 2002). The molecular mechanisms that underlie the selectivity of coactivators versus corepressors by liganded AR have remained elusive. It has been shown that some proteins repress AR-dependent transcription by physically interacting with AR to affect subcellular localization of the latter (Poukka et al, 2000) or AR transactivation (Baniahmad, 2005; Wang et al, 2005). Similarly, Casp8 negatively regulates AR-driven transcription. First, physical Casp8–AR interactions are supported by co-immunoprecipitation and GST-fusion protein pull-down data. Second, Casp8 strongly inhibited androgen-dependent reporters in the transient transfection assay, independently of its protease activity. Third, two shRNAs that specifically targeted Casp8 mRNA decreased the levels of Casp8 protein and hence affected androgen-driven gene expression in LNCaP cells. Together, these results clearly show that Casp8 has a pathway-specific and factor-specific role in the inhibition of AR signaling.

Casp8 regulates AR N/C interaction

The existence of AR N/C interaction provides an additional regulation point for AR-driven transcription. Indeed, MAGE-11 was identified as a positive AR cofactor that specifically bound the AR N-terminal [23]FQNLF[27] motif (He and Wilson, 2002). This interaction increased exposure of AF2 for the recruitment of SRC/p160 coactivators. It was reported that various cofactors differentially influenced AR N/C interaction, although the relevance of the effects of their cofactor functions on AR-driven transcription was not firmly established by detailed deletions and site-specific point mutations (Bai et al, 2005). Similarly, p53 was shown to repress androgen-induced transcription of PSA gene (Shenk et al, 2001). This inhibitory effect coincided with disruption of AR N/C interaction and inhibition of AR interaction with DNA.

The results presented here clearly link Casp8-mediated inhibition of AR-driven transcription to its ability to disrupt AR N/C interaction. We found that three motifs in Casp8 mediated interactions with AR and that these motifs were similar to the LXXLL motif, known to mediate nuclear

receptor cofactor interaction. More importantly, we found that these motifs specifically interacted with AR motifs that mediate AR N/C interaction. The interactions between the motifs in Casp8 and the motifs in AR disrupted AR N/C interaction and consequently inhibited AR-driven transcription. These studies were well controlled by using site-specific point mutations on both Casp8 and AR.

Casp8 overexpression inhibited the androgen-driven AR nuclear translocation. Our preliminary observation indicated that recombinant Casp8 directly inhibited the AR–ARE interaction (data not shown). These results suggest that Casp8 inhibited AR-driven transcription by dual mechanisms, inhibiting the androgen-driven AR nuclear translocation and AR–ARE interaction.

The AR signaling pathway has multiple functions in male sexual development and maintenance through regulation of many genes whose functions are required for cell proliferation and differentiation. Identification and characterization of cofactors that are able to regulate AR-dependent gene expression are critical to understanding the molecular mechanisms of AR action in these processes. Our findings reveal a novel function of Casp8 in the regulation of AR function and suggest a possible linkage between the AR signaling pathway and the Casp8-mediated apoptotic process.

Materials and methods

Cell culture, chemicals, and antibodies
LNCaP and PC3mm2 cells were cultured in RPMI 1640 medium (Cellgro) with 5% (v/v) fetal bovine serum (HyClone). Androgen-free medium was composed of phenol red-free RPMI 1640 medium (Cellgro) and 5% (v/v) dextran/charcoal stripped fetal bovine serum. The synthetic androgen R1881 and transfection reagents (Lipofectamine and Lipofectamine 2000) were obtained from New England Nuclear and Invitrogen, respectively. Mouse anti-human Casp8, Casp3, and monoclonal anti-actin antibodies were purchased from Cell Signaling and Sigma, respectively. The rabbit anti-AR antibody was described previously (Yu *et al*, 2001). The cDNA fragment of human Casp8 encoding amino-acid residues 185–447 was subcloned into the vector pET15d (Novagene), expressed as a 6His-tagged protein in *Escherichia coli* BL21, and purified using NTA Ni^{2+} agarose. The SDS–PAGE-purified recombinant protein (10 mg) was sent to Sigma Genosys for polyclonal antibody production. The antibody was purified through a 6His-Casp8 (185–447) affinity column.

Library screening
E. coli (DH10B) was transfected with DNA from four wells of an arrayed human colon cDNA library (Origene) and plated to obtain about 100 colonies per plate (100 mm). Colonies were scraped onto two plates, and the plates were grown at 37°C overnight. One plate was stored as a stock at 4°C and the other plate was used to make plasmid DNA. PC3mm2 cells (5×10^4) were plated in each well of a 24-well plate 24 h before transfection. A total of 700 ng of DNA was transfected using Lipofectamine, including pRL-CMV (5 ng), pGL3-4 × ARE-E4-luc (100 ng), pcDNA-AR (30 ng), and pooled DNA (565 ng). Control transfection contained pcDNA3.1 (565 ng) instead of pooled cDNA. Transfected cells were grown in the presence of 10 nM R1881 for 48 h and then harvested for luciferase assay. Fold stimulation was calculated for each sample by dividing the value for luciferase activity by the value of control sample. The pool was considered positive if it stimulated or inhibited the luciferase reporter more than two-fold. Colonies on the stock plate of a positive pool were scraped onto 10 duplicated plates (10 colonies per plate) and plates were grown at 37°C overnight. Ten plates were stored as stocks at 4°C and another 10 plates were used to make plasmid DNA. DNA from each plate was assayed. Individual colonies on the positive stock plate (10 colonies per plate) were grown, and plasmid DNA was isolated for the assay. Positive clones were identified by DNA sequencing analysis.

Construction of plasmids
Plasmids pGL3-4 × ARE-E4-Luc, pGL3-PSA(E)-E4-luc, pcDNA-AR, pcDNA-ARΔN206 (with 206 amino-acid residues from the N-terminus of AR deleted), and pcDNA-ARΔC271 (with 271 amino-acid residues from the C-terminus of AR deleted) were described previously (Liu *et al*, 2003; Gao *et al*, 2004). Plasmids pCMVhAR L26A/F27A, L436A/F437A, and L26A/F27A/L436A/F437A were kindly provided by Dr Elizabeth M Wilson (The University of North Carolina) (He *et al*, 2000). All Casp8 cDNA fragments were amplified using PCR with specific oligonucleotides and cloned into the vectors pET15d, pGEX-2TL (Amersham Biosciences), and pcDNA3.1 (Invitrogen). Fragments were expressed as 6His-tagged (via pET15d) or GST-fusion (via pGEX-2TL) proteins in *E. coli* BL21 and purified through NTA Ni^{2+} agarose or glutathione-Sepharose columns, respectively. DNA fragments encoding AR N-terminus (amino-acid residues 1–661) and C-terminus (amino-acid residues 662–919) were amplified by PCR and subcloned into the pACT and pBIND vectors (Promega) to generate pACT-AR/N and pBIND-AR/C, respectively. The Casp8 site-specific point mutations were prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing.

Cell transfection and luciferase assay
All transfection assays were performed according to the manufacturer's protocol for Lipofectamine or Lipofectamine 2000 (Invitrogen). Briefly, cells were seeded in 6-well plates (4×10^5 LNCaP, 3×10^5 PC3mm2) or 24-well plates (5×10^4 PC3mm2 cells) 24 h before transfection. The total amounts of DNA for each transfection reaction were adjusted to 1500 ng (six-well plate) and 300 ng (24-well plate) by the addition of pcDNA3.1 plasmid. The transfected cells were incubated in the absence or presence of 10 nM R1881 for 36–48 h and assayed for luciferase activity.

RT–PCR analysis
RNA was isolated from cells using the TRIzol Reagent (Invitrogen), and cDNA synthesis was performed using SuperScript III First-Strand Synthesis System for RT–PCR (Invitrogen). Amplification of Casp8 and GAPDH cDNA was performed in one reaction (see Supplementary data for primer sequences). PCR conditions were as follows: 94°C for 2 min followed by 23 cycles at 94°C for 30 s, 53°C (for Casp8) or 57°C (for PSA) for 30 s, and 72°C for 30 s. Plasmids with Casp8 or PSA cDNA inserts were used as positive controls, and water was used as a negative template control. PCR products were separated by 1.5% agarose gel electrophoresis and quantified using a Gel Doc 1000 apparatus (Bio-Rad).

Real-time PCR
Total RNAs were isolated from cultured cells using the TRIzol Reagent and reverse transcribed using the Reaction Ready First-Strand cDNA Synthesis Kit (SupperArray Bioscience Corp.). The cDNA products were PCR-amplified (40 cycles of 30 s at 94°C; 20 s at 55°C; 30 s at 72°C) with the RT^2 real-time SYBR green PCR master mix and the gene-specific primer sets for human *PSA* (PPH01002A), *NKX3.1* (PPH02267A), *KLK2* (PPH01003A), Maspin (PPH00695A), and β-*actin* (PPH00073A) genes (SupperArray Bioscience Corp.) by SmartCycler II (Cepheid). Raw data processing and quantification were performed with the SmartCycler software (version 2.0C). The $2^{-\Delta\Delta C_t}$ method was used to determine the relative quantification (in the presence of ligand versus in the absence of ligand) of target gene expression (Livak and Schmittgen, 2001). For *NKX3.1* and *KLK2*, the equation fold change = relative quantification of the Casp8 knockdown group/relative quantification of the control group. For *Maspin*, the equation fold change = −relative quantification of the control group/relative quantification of the Casp8 knockdown group.

Mammalian two-hybrid analysis
Mammalian two-hybrid analysis was performed using the Check-Mate Mammalian Two-Hybrid System (Promega). PC3mm2 cells were transfected with the pG5-luc reporter and pACT-AR/N, pBIND-AR/C, individually or together. The pRL-CMV plasmid was included as an internal control. Transfected cells were incubated for 36–48 h with medium containing 10 nM R1881 and then harvested for luciferase assay. The plasmids pACT-ID and pBIND-MyoD provided with the kit were used as controls.

GST affinity matrix-binding assay

The GST and GST-fusion proteins were expressed using the vector pGEX-2TL (+) in bacteria and immobilized on glutathione-Sepharose-4B beads (Amersham Biosciences). Then, 10 μl of beads (with 1 μg of immobilized protein) was incubated with 5 μl of rabbit reticulocyte lysate containing ^{35}S-labeled proteins in a final volume of 200 μl in a buffer containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 20% glycerol, 2 mM DTT, 150 mM KCl, 0.1% NP-40, and 0.5 mg/ml bovine serum albumin for 2 h at 4°C. The beads were washed five times (1 ml each) with the incubation buffer, boiled in 10 μl of 2× SDS gel sample buffer, and submitted to SDS–PAGE. The gel was analyzed using autoradiography.

Western blot analysis

Proteins (20 μg) were separated by 10% (for AR) or 12% (for Casp8) SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with the primary antibody (anti-FLAG M2 monoclonal, 1:2000; anti-Casp8, 1:1000; anti-Casp3, 1:1000; or anti-AR polyclonal, 1:5000). After washing, the membrane was incubated with an HRP-conjugated secondary antibody (anti-rabbit or anti-mouse IgG) for 2 h. Immunoreactive bands were visualized with the ECL Advance Western Blotting Detection system (Amersham Biosciences).

RNA interference

Three sets of oligos (Casp8D-1, Casp8D-2, and Casp8D-3) (see Supplementary data for the sequences) were synthesized, annealed, and subcloned into *Bgl*II/*Hin*dIII sites of pSuperior.retro.puro (OligoEngine) to generate pCasp8D-1, pCasp8D-2, and pCasp8D-3, respectively. Similarly, a set of nonspecific control oligos was synthesized and subcloned into pSuperior.retro.puro. Retroviral production and infection of LNCaP cells were performed as reported previously (Gao *et al*, 2005). Whole-cell lysates (20 μg of protein) made from infected cells were submitted to Western blot analysis with anti-Casp8, anti-AR, and anti-actin antibodies. The mRNAs isolated from the infected cells were submitted to Northern blot and RT–PCR as described previously (Gao *et al*, 2005).

Electrophoretic mobility shift assay

The ARE-containing wild-type and mutant probes derived from the PSA gene (−152 to −174) (Liu *et al*, 2003) were labeled with [α-^{32}P]dCTP using a fill-in reaction with the Klenow enzyme. The NE were made from cultured cells, and electrophoretic mobility shift assays were performed as described previously (Liu *et al*, 2003).

Immunohistochemistry staining

LNCaP cells were fixed and permeabilized with cold acetone, blocked with 4% fish gel, and incubated with the rabbit anti-AR antibody (1:1000 dilution) at 4°C for 12 h. Cells were incubated with Cy3-conjugated goat anti-rabbit IgG (1:500 dilution; Jackson ImmunoResearch Laboratories Inc.) at 25°C for 1 h. The nuclei were counterstained with Topro (1:10 000 dilution; Jackson ImmunoResearch Laboratories Inc.). The slides were viewed under a confocal microscope.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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