

A novel inducible transactivation domain in the androgen receptor: implications for PRK in prostate cancer

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In addition to the classical activation by ligands, nuclear receptor activity is also regulated by ligand-independent signalling. Here, we unravel a novel signal transduction pathway that links the RhoA effector protein kinase C-related kinase PRK1 to the transcriptional activation of the androgen receptor (AR). Stimulation of the PRK signalling cascade results in a ligand-dependent superactivation of AR. We show that AR and PRK1 interact both *in vivo* and *in vitro*. The transactivation unit 5 (TAU-5) located in the N-terminus of AR suffices for activation by PRK1. Thus, TAU-5 defines a novel, signal-inducible transactivation domain. Furthermore, PRK1 promotes a functional complex of AR with the co-activator TIF-2. Importantly, PRK signalling also stimulates AR activity in the presence of adrenal androgens, which are still present in prostate tumour patients subjected to testicular androgen ablation therapy. Moreover, PRK1 activates AR even in the presence of the AR antagonist cyproterone acetate that is used in the clinical management of prostate cancer. Since prostate tumours strongly overexpress PRK1, our data support a model in which AR activity is controlled by PRK signalling.

Keywords: androgen receptor/nuclear hormone receptor/prostate cancer/protein kinase C-related kinase/transcriptional co-activator

Introduction

The androgen receptor (AR) is a member of the steroid hormone receptor family of ligand-activated transcription factors that regulate diverse biological functions including cell growth and differentiation, development, homeostasis and various organ functions in the adult (Mangelsdorf *et al.*, 1995; Cato and Peterzierl, 1998). The AR shares a common modular structure with other nuclear receptors and is composed of several domains that mediate DNA binding, dimerization, ligand binding and transcriptional activity (Mangelsdorf *et al.*, 1995). The ligand binding domain (LBD) performs a number of functions including

transcriptional repression or activation by generating the proper interaction surface for multiple partners, including co-repressors and co-activators (Renaud and Moras, 2000). Like other steroid receptors the AR contains a C-terminal ligand-dependent activation function 2 (AF-2) and an additional N-terminal ligand-independent activation function 1 (AF-1). The AR also harbours a less well-defined transcription activation unit termed TAU-5 (Jenster *et al.*, 1995; Jenster, 1999). The TAU-5 is located between amino acids 360 and 528 and is required for full activation of the AR (Jenster *et al.*, 1995; Bevan *et al.*, 1999). However, signals that control TAU-5, and thereby AR activity, are unknown. Recent data show agonist-induced interaction between the N-terminal domain (NTD) and the LBD of the AR, supporting the view that both the AF-1 and the AF-2 are required for transcriptional activation (Ikonen *et al.*, 1997; He *et al.*, 2000). It has been suggested that the ligand-induced conformational changes of the LBD in concert with physical interactions with the NTD render the AR fully active (Ikonen *et al.*, 1997; Bevan *et al.*, 1999).

Androgens and the AR are essential for the differentiation, development and maintenance of male reproductive functions and non-reproductive organs (Trapman and Brinkmann, 1996; Jenster, 1999). In the prostate, the AR is expressed in secretory epithelial cells that respond to androgens. According to the current model, the AR plays an important role in the development of prostate cancer that mainly originates from epithelial cells. Growth and survival of primary prostate cancer cells is critically dependent on androgens (Gregory *et al.*, 1998). Consequently, androgen ablation therapy is the standard clinical procedure used to inhibit prostate tumour growth. However, despite reduced circulating androgen levels and even in the presence of AR antagonists, most prostate cancers recur and progress to a terminal stage. The molecular mechanisms responsible for tumour recurrence are not well defined, but one possible scenario might be the stimulation of AR activity by cross-talk with other signalling pathways (Culig *et al.*, 1994).

In addition to the classical ligand-induced activation mechanisms, nuclear receptors use alternative strategies to transmit extracellular signals into altered gene expression programs. Growth factors or stimulation of protein kinases A (PKA) or C (PKC) activate the AR even in the absence of ligand, or show a synergistic effect with ligand (Trapman and Brinkmann, 1996). Growth factor-regulated signalling pathways also control the activity of nuclear receptor co-activators. Recent data show that co-activators of the p160 family such as SRC-1 or AIB1 are direct targets of mitogen activated protein (MAP) kinases, which modulate their transcriptional activity (Font de Mora and Brown, 2000; Rowan *et al.*, 2000). An alternative strategy to regulate co-factor function is employed by Rho

GTPases. RhoA induces translocation of the co-activator FHL2 from the cell membrane to the nucleus, leading to transcriptional activation of FHL2- and AR-dependent genes (Müller *et al.*, 2002).

By affecting multiple signalling pathways Rho family members regulate cell cycle progression, cellular transformation, metastasis and have been implicated in transcriptional regulation (Bar-Sagi and Hall, 2000). The Rho family GTPases work as molecular switches, being inactive when bound to GDP and active when bound to GTP. The exchange of GTP for GDP induces a conformational change that allows the GTPases to bind effector molecules such as the protein kinase C-related kinases (PRKs), which initiate downstream signalling events (Hall, 1998; Bishop and Hall, 2000). The PRKs constitute a subfamily of serine/threonine kinases that comprises PRK1 (also called PKN), PRK2 and PKN β (Mukai *et al.*, 1995; Palmer *et al.*, 1995; Oishi *et al.*, 1999). PRKs contain three highly-conserved regions: (i) an N-terminal regulatory domain spanning three repeats of charged amino acids with leucine zipper-like sequences (designated CZ region); (ii) a C-terminal catalytic domain; and (iii) a so-called D region located between the CZ region and the catalytic domain (Mukai *et al.*, 1995). RhoA binds to the CZ region through the RhoA effector motif class I in a GTP-dependent manner thereby enhancing PRK kinase activity. The physical interaction of PRK1 with transcription factors such as NeuroD2 or PCD17, and the nuclear localization suggest a potential role for PRKs in transcriptional regulation (Takanaga *et al.*, 1998; Shibata *et al.*, 1999).

Recently, we demonstrated that stimulation of the Rho signalling pathway induces translocation of the co-activator FHL2 to the nucleus leading to activation of the AR by FHL2 (Müller *et al.*, 2002). We now identify a novel pathway independent of FHL2 that links the RhoA effectors PRK1 and PRK2 to the transcriptional activation of AR. Here we show that stimulation of PRK1 signalling by extracellular stimuli or co-expression of constitutively active RhoA or PRK1 mutants results in ligand-dependent superactivation of AR-regulated genes. AR and PRK1 interact both *in vivo* and *in vitro*. Structure–function analyses demonstrate that the TAU-5 in the AR-NTD suffices for activation by PRK1, thus defining the TAU-5 as a novel, signal-inducible transactivation domain. In addition, PRK1 signalling also induces the transcriptional activity of mineralocorticoid receptor (MR), progesterone receptor (PR) and p160 co-activators, further supporting the importance of this signalling pathway for the transcriptional regulation of nuclear receptors. Our data show that prostate tumours highly overexpress PRK1. Importantly, PRK1 activates AR not only in the presence of agonists, but also when adrenal androgens or even the antagonist cyproterone acetate (CPA) are present. Taken together, our results demonstrate that the transcriptional activity of AR is controlled by PRK signalling in addition to ligands, providing an explanation for prostate cancer growth even during androgen ablation therapy.

Results

The current dilemma in androgen ablation therapy is the aberrant regulation of AR activity. This clearly points out

the relevance of other yet undefined signalling pathways leading to AR-dependent tumour growth. Recently, we showed that Rho family members are overexpressed in human prostate tumours and that stimulation of the Rho signalling pathway leads to an agonist-dependent co-activation of AR-regulated target genes (Müller *et al.*, 2002). To further delineate the mechanisms of Rho signalling to AR, we tested the influence of Rho effectors on AR transcriptional activity. Therefore, we stimulated AR-dependent gene expression with physiological concentrations of the bioactive lipid sphingosine-1-phosphate (SPP) in the presence of AR and an MMTV luciferase reporter (MMTV-LUC). The serum component SPP activates small GTPases of the Rho family via G protein-coupled receptors (Pyne and Pyne, 2000). SPP stimulation of endogenous Rho GTPases increases the agonist-dependent activation of the MMTV-LUC reporter (Figure 1A; Müller *et al.*, 2002). Both the agonist and SPP-induced reporter activity is blocked by either the Rho-specific inhibitor *Clostridium botulinum* C3 exoenzyme (Busch and Aktories, 2000) or the dominant-negative RhoA mutant RhoA N19 (Figure 1A). In addition, the dominant-negative mutants of the Rho effectors PRK1 (dnPRK1) and PRK2 (dnPRK2) potently inhibit agonist and SPP-induced AR activity (Figure 1A). Wild-type PRK1 (PRK1wt) does not influence AR transcriptional activity (Figure 1A). In the absence of either AR or ligand the MMTV-LUC reporter is not influenced (our unpublished data). The dominant-negative PRK mutants also block ligand-dependent stimulation of AR by the constitutively active RhoA mutant RhoA V14 (Figure 1B), whereas the dominant-negative mutants Rac1 N17, Cdc42 N17 or Ras N17 fail to show any inhibitory effect (Figure 1B). All dominant-negative mutants are functional in our assay system and down-regulate the v-Src-induced activity of a SRE-LUC reporter (Chiariello *et al.*, 2001; our unpublished data). Furthermore, neither the various signal transduction factors nor the chemical inhibitors used in this study alter AR protein levels (see Supplementary figure 1, available at *The EMBO Journal* Online). Taken together, these data show for the first time an involvement of PRKs in RhoA-mediated activation of AR.

To examine the Rho→PRK signalling pathway leading to AR activation in more detail, we tested whether expression of GDP–GTP exchange factors (GEFs) is able to promote AR-dependent gene expression. GEFs bind small GTPases and are involved in promoting nucleotide exchange (Cerione and Zheng, 1996). The activated form of the Rho-specific GEF NET1 (NET1 Δ N) stimulates the AR- and agonist-dependent activity of MMTV-LUC in 293 cells to a similar extent as the constitutively active RhoA V14 (Figure 1C). We then used the N-terminus of PRK1 (PRK1.N), which acts as a dominant-negative mutant in RhoA- and NET1 signalling by binding to RhoA–GTP (Sahai *et al.*, 1998). When tested in our assay system PRK1.N blocks NET1 Δ N and RhoA V14 stimulated agonist-dependent AR activation (Figure 1C), further indicating the importance of PRK for AR activation. Accordingly, the PRK inhibitors Ro31-8220 or HA 1077 (Davies *et al.*, 2000) abolished agonist-dependent AR activation by RhoA V14 (Figure 1D). In contrast, blocking the MAP kinase

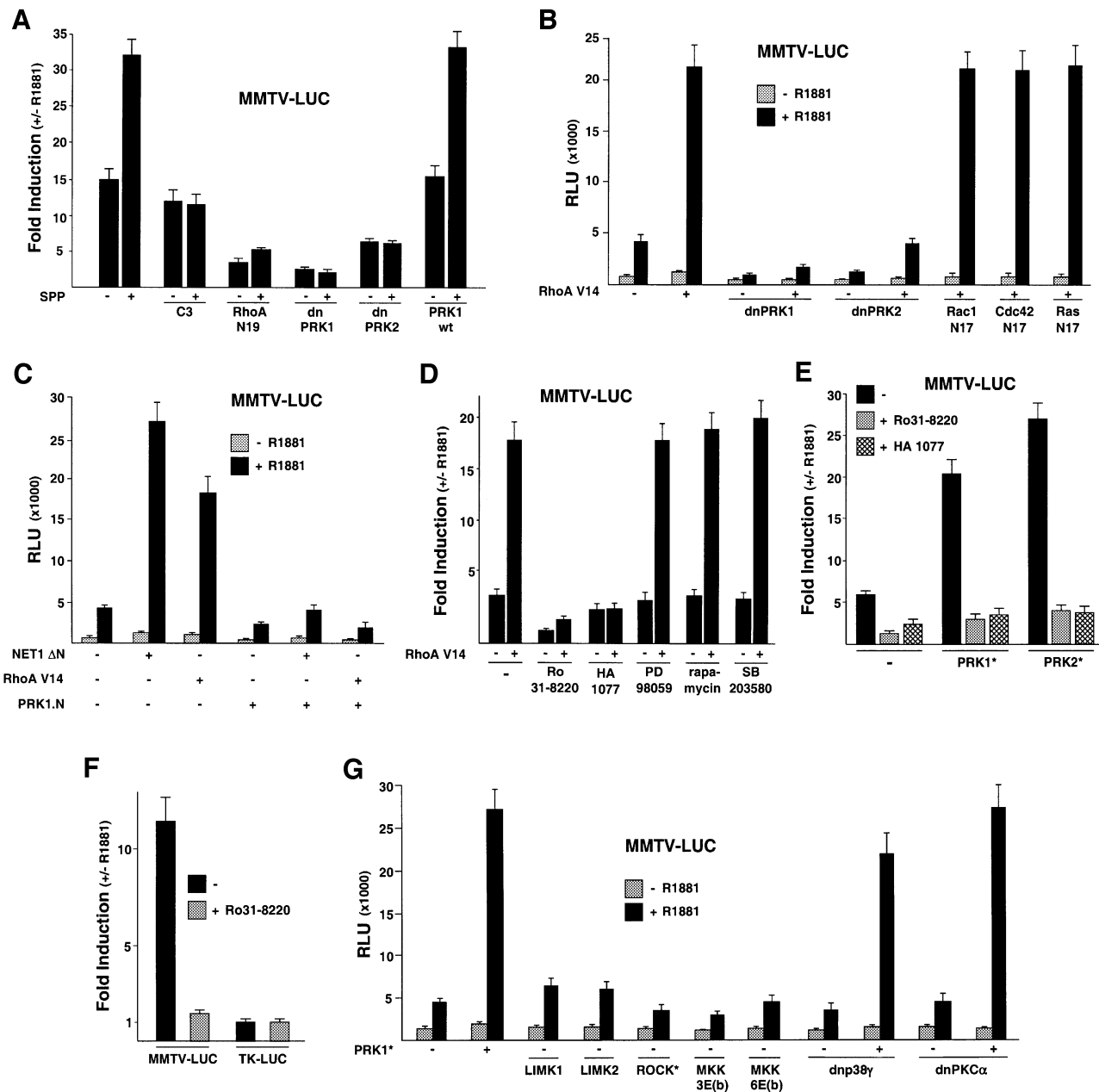


Fig. 1. The RhoA effectors PRK1 and PRK2 mediate transcriptional superactivation of the AR. 293 (A–E and G) and PC3-AR cells (F) were transfected with MMTV-LUC reporter and AR expression plasmids with or without 10^{-10} M R1881. (A) Stimulation of the endogenous Rho signalling pathway by SPP leads to the activation of AR. Activation is blocked either by C3 exoenzyme, or by dominant-negative forms of RhoA (RhoA N19), PRK1 (dnPRK1), PRK2 (dnPRK2), but not wild-type PRK1 (PRK1wt). Cells were treated with either vehicle or $1.2 \mu\text{M}$ SPP. (B) Dominant-negative PRK1 (dnPRK1) and PRK2 (dnPRK2), but not the dominant-negative forms of Rac1 (Rac1 N17), Cdc42 (Cdc42 N17) or Ras (Ras N17) block AR activation by constitutively active RhoA (RhoA V14). (C) The dominant-negative acting N-terminus of PRK1 (PRK1.N) blocks AR activation by RhoA V14 or by the RhoA-specific constitutively active GEF NET1 (NET1 Δ N). (D) Ligand-dependent activation of AR by RhoA V14 is blocked by the PRK inhibitors Ro31-8220 or HA 1077 but not by the MEK1 inhibitor PD 98059, the S6 kinase/TOR inhibitor rapamycin or the p38 MAP kinase inhibitor SB 203580. As indicated, cells were transfected with RhoA V14 expression plasmids and treated with either 5×10^{-7} M Ro31-8220, 3×10^{-5} M HA 1077, 50 ng/ml rapamycin, 10^{-5} M PD 98059 or 10^{-5} M SB 203580. (E) AR is blocked by the PRK inhibitors Ro31-8220 or HA 1077. Cells co-transfected with the constitutively active forms of PRK1 (PRK1*) or PRK2 (PRK2*) were cultured in the absence or presence of the PRK inhibitors Ro31-8220 (5×10^{-7} M) or HA 1077 (3×10^{-5} M). (F) The PRK inhibitor Ro31-8220 blocks endogenous AR activity in PC3-AR prostate tumour cells. Cells were treated with 7×10^{-7} M Ro31-8220. (G) Influence of Rho effector signalling molecules on AR activity. Wild-type LIMK1 and LIMK2, constitutively active Rho-kinase α (ROCK*), MKK3b [MKK3E(b)], or MKK6b [MKK6E(b)] and dominant-negative p38 γ (dnp38 γ) or protein kinase C α (dnPKC α) were analysed for their ability to modulate AR activity. Constitutively active PRK1 (PRK1*) was co-expressed as indicated.

pathway by the MEK1 inhibitor PD 98059, inhibiting S6 kinase/TOR with rapamycin, or using the p38 MAP kinase inhibitor SB 203580 does not interfere with the transcrip-

tional activation by RhoA V14 (Figure 1D). The effectiveness of the inhibitors (our unpublished data) was confirmed according to Müller *et al.* (2002). Next, we

analysed constitutively active mutants of PRK1 (PRK1*) and PRK2 (PRK2*). As demonstrated in Figure 1E, AR is strongly activated by both constitutively active mutants of PRK in an agonist-dependent manner and both PRK inhibitors Ro31-8220 or HA 1077 block this activation. Furthermore, Ro31-8220 also blocks endogenous AR transcriptional activity in PC3-AR prostate tumour cells (Figure 1F). As a control, we analysed thyroid hormone receptor α -dependent reporter activity, which is neither influenced by co-expression of PRKs nor by treatment with Ro31-8220 (our unpublished data). Since Ro31-8220 is known to also inhibit the kinases PKC α , RSK, MSK, S6K and GSK3 β (Davies *et al.*, 2000), we verified that these kinases do not influence AR activity (Figure 1G; our unpublished data). To further validate PRK as the downstream effector of Rho, we assayed several molecules involved in Rho signalling (Bishop and Hall, 2000). Neither wild-type LIM kinase1 (LIMK1) or LIM kinase2 (LIMK2), nor constitutively active Rho-kinase α (ROCK*), MKK3b [MKK3E(b)], or MKK6b [MKK6E(b)] are able to induce AR-dependent reporter expression (Figure 1G). In addition, neither dominant-negative mutants of p38 γ (dnp38 γ) or PKC α (dnPKC α) (Figure 1G), nor DMPK, PAK, CRK or MRCK α (our unpublished data), show any effect. Taken together, these results demonstrate that blocking the endogenous Rho \rightarrow PRK signalling pathway by PRK inhibitors or dominant-negative PRK mutants severely impairs agonist-dependent AR transactivation. In addition, our data show that constitutively active mutants of the Rho effector PRK stimulate agonist-dependent transcriptional activity of AR.

To examine the potential regulation of other steroid hormone receptors by PRK1 signalling the MMTV promoter was chosen because the receptors for glucocorticoids (GR), PR, MR and AR are known to regulate its expression in a ligand-dependent fashion (Schüle *et al.*, 1990). Co-expression of constitutively active PRK1 and MR or PR results in a ligand-dependent superactivation (Figure 2A), whereas the ligand-dependent transcriptional activity of GR is only marginally influenced. The same results were obtained when the activation assay was performed with different agonist concentrations for the various receptors (our unpublished data). When tested on the prostate-specific probasin promoter or on MMTV-LUC, PRK signalling stimulates agonist-dependent AR activity in 293 or DU145 human prostate tumour cells (Figure 2B). These results show that stimulation of AR by PRKs is functional in different cell types and not restricted to a special promoter context.

Using immunofluorescence analyses we investigated the subcellular distribution of AR and PRK proteins. AR and the constitutively active mutant PRK1* are present in the cytoplasm in the human 293 cell line in the absence of ligand, whereas addition of the AR agonist R1881 results in nuclear AR and triggers PRK1* nuclear localization (Table I). Equally, CPA promotes nuclear localization of both proteins. In contrast, the agonist-loaded mutant AR (AR Δ NLS) that is defective in nuclear translocation (Poukka *et al.*, 2000) does not support nuclear redistribution of PRK1* (Table I). In agreement with the transactivation data, agonist-loaded MR but not GR leads to enhanced nuclear translocation of PRK1* (Table I).

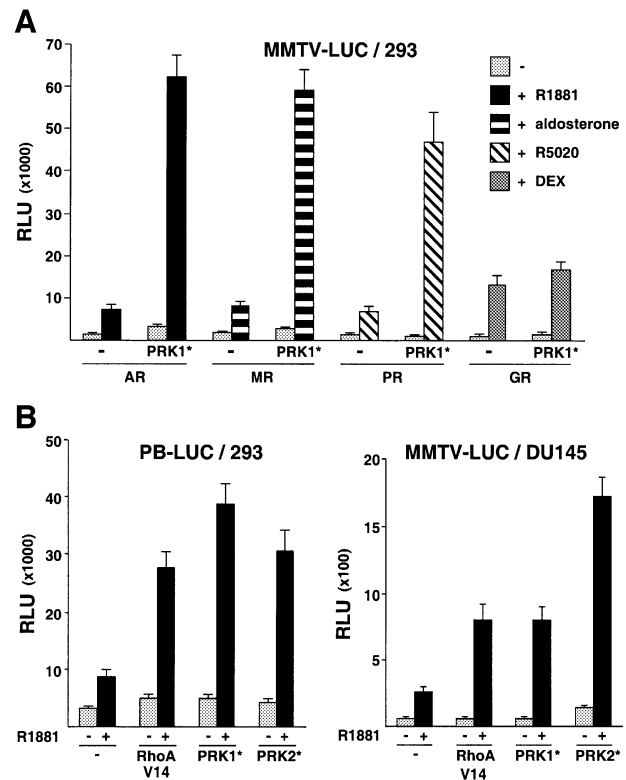


Fig. 2. Analysis of the PRK signalling. (A) PRK1 signalling mediates activation of AR, MR and PR, but not of GR. 293 cells were co-transfected with MMTV-LUC reporter and expression plasmids coding for either AR, MR, PR or GR in combination with or without PRK1*. Cells were treated with the corresponding agonists (10^{-10} M R1881, 10^{-9} M aldosterone, 10^{-9} M R5020 or 10^{-10} M DEX). (B) AR superactivation by PRK is independent of a particular promoter context or cell type. 293 and DU145 cells were stimulated with 10^{-10} M R1881 as indicated. Cells were co-transfected with AR, constitutively active RhoA V14, PRK1* or PRK2*, and either MMTV-LUC or PB-LUC.

To demonstrate interaction between AR and PRK1, extracts from 293 cells transfected with expression plasmids for PRK1 and Flag epitope-tagged AR were immunoprecipitated using an α -Flag antibody (Figure 3A). Western blot analysis shows that AR and PRK1 interact weakly in the absence of ligand, whereas the Flag-AR-PRK1 complex is efficiently precipitated in the presence of the AR agonist R1881. No PRK1 was found in immunoprecipitated complexes using Flag transfected control extracts (Figure 3A), thus demonstrating specificity. Similar results were obtained with constitutively active PRK1 (our unpublished data). More importantly, AR and PRK1 endogenously expressed in primary human prostate tumours associate *in vivo* (Figure 3B). Extracts from primary human prostate tumours treated with R1881 were immunoprecipitated using an α -PRK1 antibody. As shown in Figure 3B, the endogenous AR is efficiently co-immunoprecipitated with the α -PRK1 antibody, thus demonstrating the relevance of this interaction *in vivo*. To further analyse the interaction, we performed *in vivo* GST pull-down experiments (Figure 3C). The fusion proteins GST-AR-NTD, GST-AR-DBD and GST-AR-LBD were co-expressed with either myc epitope-tagged constitutively active PRK1 (PRK1*) or wild-type PRK1

Table I. Subcellular localization of PRK1

Transfection	Ligand	PRK1 staining (%)	
		C	C + N and N
PRK1*	–	79	21
PRK1*	R1881	78	22
PRK1* + AR	–	77	23
PRK1* + AR	R1881	45	55
PRK1* + AR	CPA	52	48
PRK1wt + AR	–	100	0
PRK1wt + AR	R1881	97	3
PRK1* + AR ΔNLS	–	67	33
PRK1* + AR ΔNLS	R1881	72	28
PRK1* + GFP-MR	–	77	23
PRK1* + GFP-MR	aldosterone	56	44
PRK1* + GR-GFP	–	86	14
PRK1* + GR-GFP	DEX	79	21

293 cells were transfected with the indicated expression plasmids. Immunostained cells were analysed for exclusively cytoplasmic localization (C) or the appearance of cytoplasmic and nuclear (C + N) or nuclear (N) localization of PRK1* or PRK1wt in double-labelled cells ($n > 100$; $SD \leq 5\%$). Ligands (10^{-9} M R1881, 10^{-5} M CPA, 10^{-7} M aldosterone, 10^{-7} M DEX) were applied for 2 h before harvesting.

(PRK1wt) in 293 cells. Protein complexes were affinity purified from whole-cell extracts on glutathione–Sepharose and subsequently detected using either α -myc or α -PRK1 antibodies. As illustrated in Figure 3C, both PRK1wt and PRK1* specifically interact with the AR-NTD. In contrast, the AR-DBD or AR-LBD fails to do so. As expected, no interaction is observed with the control proteins GST or GST–GCNF. The AR-NTD also physically interacts with PRK1wt and PRK1* *in vitro* (Figure 3D). Pull-down experiments with bacterially expressed GST–AR-NTD fusion protein and [35 S]methionine-labelled *in vitro* translated PRK1wt and PRK1* show interaction with the AR-NTD or the AR-TAU-5 respectively, but not with the control proteins GST or GST–ROR β . In conclusion, this set of experiments demonstrates association of AR and PRK1 both *in vivo* and *in vitro*.

To delineate the domains in the AR that suffice for PRK1 signalling, we tested AR deletion mutants in transactivation assays. Deletion of the entire LBD creates the ligand-independent, constitutively active mutant AR_{1–627} (Jenster *et al.*, 1995). This mutant (MUT1) is strongly activated by PRK1 signalling (Figure 4A) thus demonstrating that a yet undescribed PRK-inducible transactivation function is located in the AR-NTD. Surprisingly, further analyses revealed that not the AF-1 located between residues 150–350, but the TAU-5 spanning residues 360–528 is responsible for the stimulation by PRK1* (Figure 4A). Conversely, when fused to a heterologous Gal4 DNA binding domain the resulting mutant Gal–TAU-5 suffices for potent transcriptional activation by constitutively active PRK1 (Figure 4B). Taken together, these results show that the AR-TAU-5 is the first PRK-inducible transactivation function described.

Recent data demonstrate that ligand-induced conformational changes of the AR-LBD in concert with physical interactions with the AR-NTD result in transcriptional

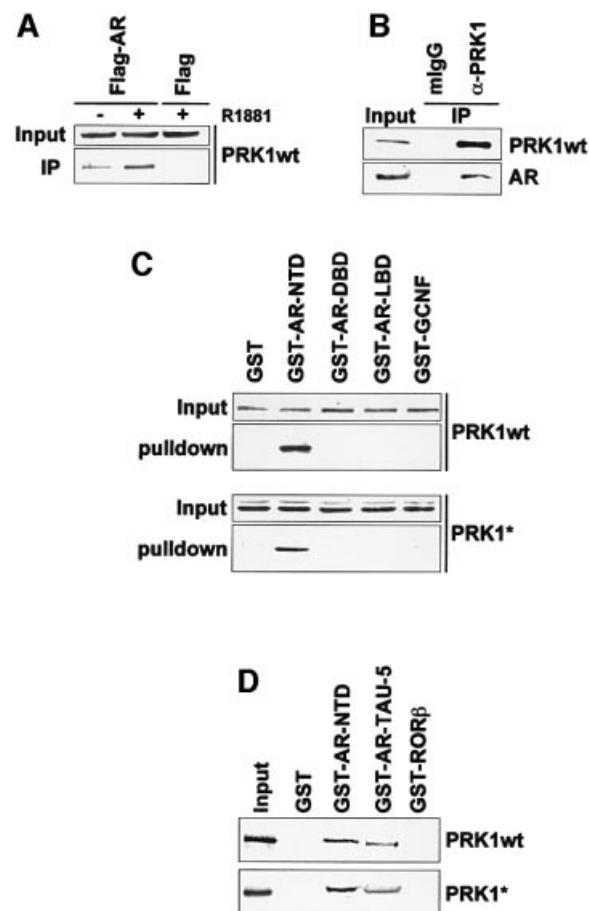


Fig. 3. PRK1 interacts with the AR *in vivo* and *in vitro*. (A) PRK1 co-immunoprecipitates with the AR. 293 cells transfected with either expression plasmids for wild-type PRK1 (PRK1wt), Flag-AR or Flag alone in the absence or presence of 10^{-10} M R1881. Extracts were immunoprecipitated with an α -Flag antibody. Five per cent of the extract used for immunoprecipitation was loaded as input. Western blots were decorated with an α -PRK1 antibody. (B) AR co-immunoprecipitates with PRK1. Extracts from primary human prostate tumours were immunoprecipitated with an α -PRK1 antibody or mouse IgG (control) in the presence of 10^{-10} M R1881. Five per cent of the extract used for immunoprecipitation was loaded as input. Western blots were decorated with α -AR and PRK1 antibodies. (C) PRK1 associates with the NTD of AR. *In vivo* GST pull-down assays were performed using lysates from 293 cells transfected with expression plasmids for either PRK1wt or myc-tagged PRK1* and GST fusion proteins of the indicated AR domains. Five per cent of the extract used for immunoprecipitation was loaded as input. Western blots were decorated with either α -PRK1 or α -myc antibodies. GST or GST–GCNF proteins were used as controls. (D) PRK1 interacts with the AR-NTD or the TAU-5 *in vitro*. GST pull-down assays were performed with [35 S]methionine-labelled PRK1wt or PRK1* with the corresponding bacterially expressed GST–AR fusion proteins. GST and GST–ROR β proteins were used as controls.

activation of the AR whereby p160 co-activators promote these functional interactions (Ikonen *et al.*, 1997; Bevan *et al.*, 1999). To further analyse the functional consequences of PRK1 signalling, we performed mammalian two-hybrid assays with Gal–AR-LBD and VP16–AR-NTD fusion proteins. In agreement with previous data (Ikonen *et al.*, 1997) the LBD and the NTD interact in a ligand-dependent manner, which is reflected in agonist-induced reporter activity (Figure 4C). In the presence of constitutively active PRK1 the functional interaction of

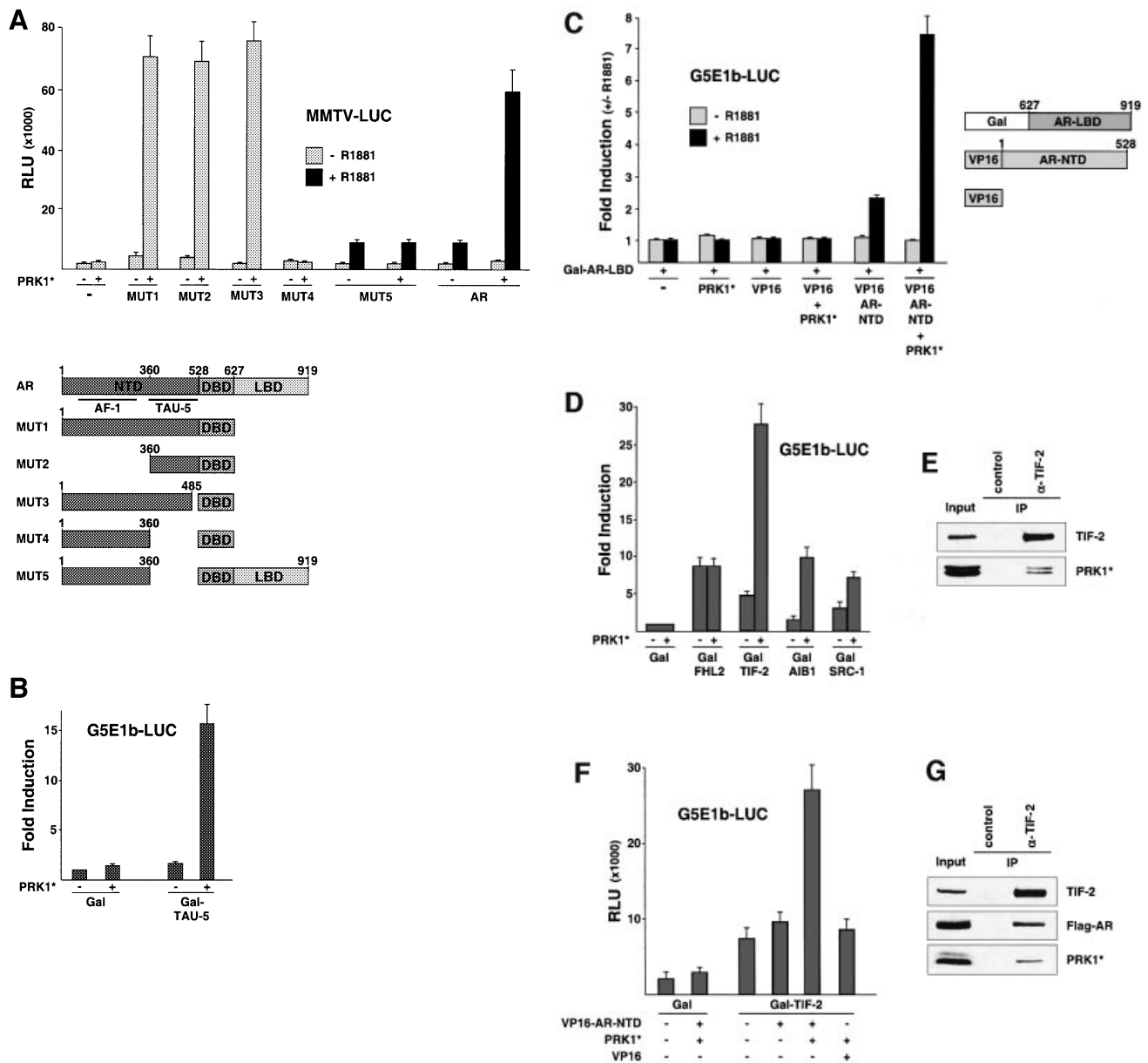


Fig. 4. Activation mode of PRK1. **(A)** The AR N-terminal TAU-5 domain suffices for transcriptional activation by PRK1. The schematically depicted AR deletion mutants were analysed on the MMTV-LUC reporter in 293 cells with or without co-expression of PRK1*. Deletion mutants containing the LBD of AR were analysed with or without 10^{-10} M R1881. **(B)** The activity of Gal-TAU-5 was analysed on the Gal-dependent reporter G5E1b-LUC in the absence or presence of PRK1*. Relative Gal activity was set to 1. **(C)** PRK1 signalling enhances the N- and C-terminal interaction of AR in an agonist-dependent manner. 293 cells co-transfected with the G5E1b-LUC reporter and Gal-AR-LBD expression plasmids were stimulated with 10^{-10} M R1881. As indicated, PRK1*, VP16 and VP16-AR-NTD were co-expressed. **(D)** Co-factor activation by PRK1. Gal fusion proteins of the co-activators FHL2, TIF-2, AIB1 and SRC-1 were tested on G5E1b-LUC in 293 cells with PRK1* as indicated. Relative Gal activity was set to 1. **(E)** PRK1 associates with the transcriptional co-activator TIF-2 *in vivo*. 293 cells were transfected with expression plasmids for myc-tagged constitutively active PRK1 (PRK1*). Extracts were immunoprecipitated with α -TIF-2 or control antibodies. Five per cent of the extract was loaded as input. Western blots were decorated with α -TIF-2 or α -myc antibodies. **(F)** PRK1 signalling enhances the interaction of TIF-2 and AR-NTD. 293 cells were transfected with the G5E1b-LUC reporter. As indicated, Gal, Gal-TIF-2, PRK1*, VP16 and VP16-AR-NTD were co-expressed. **(G)** AR and PRK1 co-immunoprecipitate with TIF-2. 293 cells were transfected with expression plasmids for myc-tagged constitutively active PRK1 (PRK1*) and AR (Flag-AR). Extracts were treated with α -TIF-2 or control antibodies to immunoprecipitate endogenous TIF-2. Five per cent of the extract was loaded as input. Western blots were decorated with α -TIF-2, α -Flag or α -myc antibodies.

the AR-LBD with the NTD is greatly enhanced, thus providing a mechanistic explanation for the stimulation of AR activity (Figure 4C). Since PRK1 neither contains an autonomous transactivation domain nor phosphorylates the AR, nor enhances the physical interaction between the AR-LBD and NTD in GST pull-down assays (our unpublished data), we tested whether PRK1 signalling

might stimulate AR by modulating the transcriptional activity of AR-associated co-factors (Figure 4D). The co-activator FHL2 does not respond to PRK1*, clearly demonstrating that PRK1 signalling is independent of FHL2 (Figure 4D). In contrast, the transcriptional activity of the p160 co-activators TIF-2, AIB1 and SRC-1 is significantly stimulated by PRK1* (Figure 4D).

Co-immunoprecipitation experiments performed with an α -TIF-2 antibody and cell extracts from 293 cells transfected with PRK1* show that endogenously expressed TIF-2 interacts efficiently with PRK1* *in vivo* (Figure 4E). Since PRK1* interacts with both AR and TIF-2, we then asked whether PRK1* would be able to promote a functional interaction between the two proteins. Mammalian two-hybrid assays with Gal-TIF-2 and VP16-AR-NTD fusion proteins show low reporter activity reflecting weak interaction (Figure 4F). In the presence of limited amounts of PRK1* Gal-TIF-2 activity is only weakly stimulated, whereas the functional interaction between Gal-TIF-2 and VP16-AR-NTD is significantly enhanced (Figure 4F). In addition, immunoprecipitation of endogenously expressed TIF-2 with an α -TIF-2 antibody results in the co-precipitation of AR and PRK1* (Figure 4G). This data further suggest the formation of a functional complex consisting of TIF-2, AR and PRK1*.

Since p160 co-activators and Rho family members are overexpressed in human prostate tumours (Gregory *et al.*, 2001; Müller *et al.*, 2002), we analysed the expression of PRK1 in human prostate in normal and pathologic situations. Immunohistochemical analyses of PRK1 expression were performed in tissue sections obtained from radical prostatectomies, which contained areas of benign prostate as well as prostate carcinoma. Neither in the secretory epithelium of normal prostate tissue nor in the cells of the normal basal compartment was significant PRK1 immunoreactivity detected (Figure 5A, arrowheads). All cancer specimens obtained from ten different radical prostatectomies revealed a robust increase in PRK1 immunoreactivity (Figure 5B, arrowheads). We also compared AR expression in normal prostate versus prostate tumours in the same cancer specimens used above. As indicated for the majority of prostate tumours (Leav *et al.*, 2001), AR expression is not significantly altered (Figure 5C and D, arrowheads). In addition, western blot analysis from resected human prostate tissue shows that PRK1 is overexpressed in prostate tumours compared with normal prostate (Figure 5E). Taken together, our data demonstrate strong overexpression of PRK1 in prostate carcinoma and co-expression of AR and PRK1 *in vivo*.

Finally, we analysed whether stimulation of AR transcriptional activity by Rho \rightarrow PRK signalling is regulated by clinically relevant AR ligands. During testicular androgen ablation therapy, adrenal androgens present in the peripheral blood play an important role in AR-dependent prostate cancer progression, since adrenal glands continue to supply the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione. These androgen metabolites exhibit weak agonist activity, but are metabolized into potent agonists such as dihydrotestosterone in the prostate (Straub *et al.*, 1998; Gregory *et al.*, 2001). Thus, we tested whether PRK1 signalling would be able to stimulate AR activity at physiological concentrations of the adrenal androgens DHEA and androstenedione in 293 cells that do not metabolize androgens. At concentrations of 10^{-10} M DHEA or 10^{-8} M androstenedione, respectively, AR is barely activated by these weak androgens (Figure 6A). Importantly, however, PRK1 signalling strongly induces AR activity in the presence of the adrenal androgens

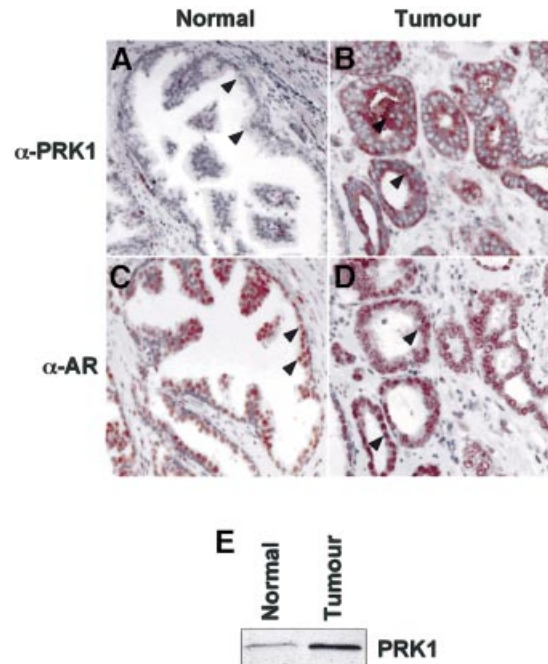


Fig. 5. Overexpression of PRK1 in human prostate cancer. Little PRK1 immunoreactivity is detected in the secretory epithelium of normal prostate (A, arrowheads), but strong PRK1 immunoreactivity is detected in prostate tumours (B, arrowheads). AR immunostaining reveals the presence of AR in benign secretory cells of the gland (C, arrowheads), as well as in carcinoma cells (D, arrowheads). All sections were taken from the same radical prostatectomy specimen. Magnification: $\times 250$. (E) Normal human prostate and prostate tumour extracts were analysed in western blots using an α -PRK1 antibody.

(Figure 6A). We then tested the anti-androgen CPA, which is commonly used during androgen ablation therapy to block the effects of androgens (Leewansangtong and Crawford, 1998). As expected, CPA at 10^{-5} M only marginally activates the AR (Figure 6B). However, PRK signalling potently stimulates AR activity in the presence of the antagonist CPA. Combined with the observed overexpression of PRK in prostate tumours (Figure 5) these results might, at least in part, provide an explanation for the clinically well-documented observation (Jenster, 1999) that during prostate cancer progression AR-dependent gene expression is upregulated in the absence of testicular androgens and even in the presence of antagonists.

Discussion

Here we have identified a novel signalling pathway that links the RhoA effectors PRK1 and PRK2 to the transcriptional activation of the AR (Figure 7). The combined use of either the PRK inhibitors Ro31-8220 or HA 1077, or dominant-negative and constitutively active mutants of PRK1 show that PRK is the Rho effector leading to AR-dependent activation. Since the PRK inhibitor Ro31-8220 also blocks GSK3 β , S6K, RSK, MSK and PKC α activity (Davies *et al.*, 2000), we excluded that these kinases mediate the regulation of AR by PRK signalling. Furthermore, by ruling out other major signalling pathways we demonstrate specificity for PRKs (Figure 1D, E and G).

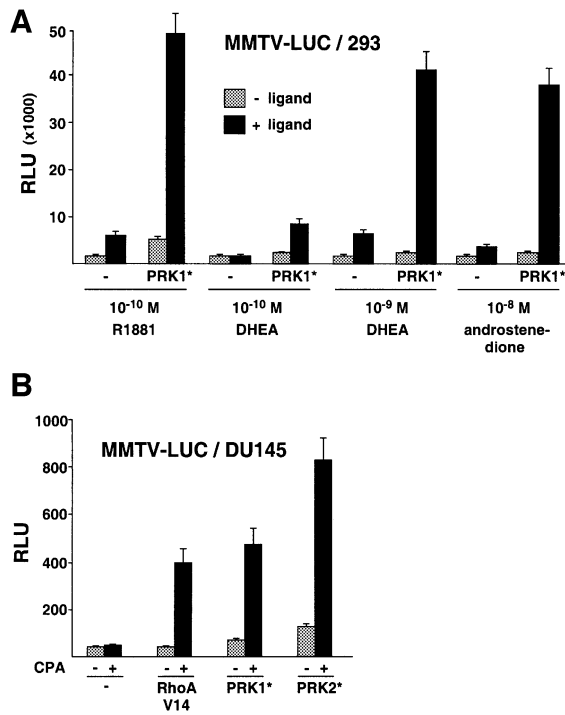


Fig. 6. Ligand-selective stimulation of AR transcriptional activity by PRK. (A) Super-activation of the AR by PRK1 signalling in the presence of adrenal androgens. 293 cells were co-transfected with AR and MMTV-LUC in the presence or absence of the indicated androgens. (B) PRK signalling activates the AR in the presence of the anti-androgen CPA. DU145 cells were stimulated with or without 10⁻⁵ M CPA and co-transfected with the indicated expression plasmids.

So far, only the transcription factors ATF-2, MEF-2A and GATA-4 are known to be regulated by Rho→PRK signalling (Charron *et al.*, 2001; Marinissen *et al.*, 2001). Our results clearly demonstrate that the mechanism of AR activation is distinct from the p38γ-dependent Rho→PRK signalling that activates these transcription factors, since we excluded the upstream activators MKK3/6 of p38 kinases as regulators of AR (Figure 1G). Furthermore, neither dominant-negative mutants of p38 nor the p38 kinase inhibitor SB 203580 blocked stimulation of AR activity by PRK, demonstrating that p38α/β are also not involved (Figure 1D and G).

In this manuscript we demonstrate that constitutively active PRK1 stimulates the p160 co-activators TIF-2, AIB1 and SRC-1 (Figure 4D). In contrast, PRKs do not stimulate FHL2 transcriptional activity unravelling an alternative, FHL2-independent pathway for Rho→PRK signalling to the AR (Figures 4D and 7; Müller *et al.*, 2002). In addition, PRK robustly modulates AR activity in FHL2-deficient cells further supporting a FHL2-independent pathway (our unpublished data). At this point, the relative importance of FHL2-dependent versus FHL2-independent RhoA signalling is not defined. The relative abundance of FHL2 and PRK1 might determine how Rho signalling acts on AR. In cells that express little or no functional FHL2, PRK1 signalling might be dominant.

Structure–function analyses identified that the yet ill-defined TAU-5 region suffices for PRK1-mediated acti-

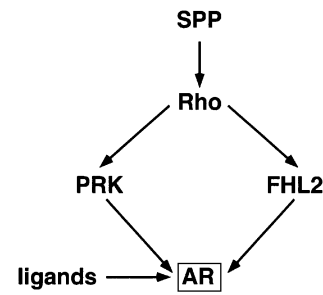


Fig. 7. Model of the Rho signalling pathway controlling AR transcriptional activity.

vation of AR. So far TAU-5 was considered as a constitutively active transactivation domain necessary for maximal AR activity only (Jenster *et al.*, 1995; Bevan *et al.*, 1999). In contrast, our results establish that the TAU-5 is a novel, PRK-inducible transactivation domain. Therefore, AR activity is not only mediated by the N-terminal AF-1 but in addition is also controlled by the signal-regulated TAU-5. As shown by immunoprecipitation analyses and pull-down experiments, PRK1 and AR associate *in vivo* and *in vitro*. Indeed, the TAU-5 represents not only a novel PRK-inducible transactivation domain but also suffices for physical association with PRK1.

PRK1 signalling enhances the transcriptional activity of SRC-1, AIB1 and TIF-2 (Figure 4D). TIF-2 and PRK1* interact *in vivo* (Figure 4E and G), but phosphorylation of TIF-2 by PRK1* is not detected (our unpublished data). As shown in mammalian two-hybrid assays PRK1* supports the formation of both transcriptionally active NTD–LBD and NTD–TIF-2 complexes (Figure 4). This would suggest that binding of PRK1* to TIF-2 and the TAU-5 of AR results in a complex that contains all three proteins and is mediating PRK1 signalling. Since TIF-2 is able to activate MR and PR (Voegel *et al.*, 1996; Fuse *et al.*, 2000) the association of TIF-2 and PRK1* might explain our observation that these receptors are also stimulated by PRK1*. The reason for the unresponsiveness of GR are unknown.

We analysed the expression of the RhoA effector PRK1 in normal human prostate and prostate cancer and revealed strong overexpression in tumours. Our data corroborate earlier results that the PRK isoform PKNβ is barely detected in adult tissue but strongly upregulated in tumours (Oishi *et al.*, 1999). In addition, we recently showed a dramatic increase in Rho expression in poorly differentiated human prostate tumours (Müller *et al.*, 2002). Therefore, prostate tumours not only overexpress Rho GTPases, but also Rho effectors such as PRK1, thus indicating that overexpression of Rho signalling molecules might be a common feature in prostate tumourigenesis.

Of special importance is our observation that PRK signalling stimulates AR not only in the presence of agonists but also in the presence of CPA. The anti-androgen CPA is frequently used in prostate cancer therapy to block AR-dependent tumour growth, though the undesired, partial agonistic function of CPA is well known (Wong *et al.*, 1993). Importantly, even at ligand

concentrations where the antagonist CPA exhibits no partial agonistic function PRK signalling potently stimulates AR transcriptional activity (Figure 6B). During testicular androgen ablation therapy the concentrations of adrenal androgens such as DHEA and androstenedione in the peripheral blood of human males is of importance to control the growth of prostate tumours. Our data now show that AR is strongly stimulated by PRK1 signalling in the presence of physiological concentrations of DHEA and androstenedione. Prostate tumours not only overexpress co-activators such as TIF-2 and FHL2, and Rho GTPases, but also show a dramatic increase in PRK expression (Figure 5; Gregory *et al.*, 2001; Müller *et al.*, 2002). Therefore, our results provide, at least in part, an explanation for the clinically well documented observation that during prostate cancer progression AR-dependent gene expression is upregulated in the absence of testicular androgens or even in the presence of antagonists such as CPA. Consequently, PRK might be a promising therapeutic target and inhibitors of PRK signalling may turn out to be beneficial in the treatment of prostate cancer.

Materials and methods

Plasmids

The following plasmids were described previously: AR, PR, MR, GR, Gal-TIF-2, Gal-SRC-1, Gal-FHL2, CMX-Flag, CMX-GST, MMTV-LUC, PB-LUC, TK-LUC and G5E1b-LUC (Müller *et al.*, 2000); RhoA V14, Rac1 N17, C3, Cdc42 N17, Ras N17, LIMK1, LIMK2, RhoA N19, PRK2* (tr.PRK2wt), NET1 ΔN and Gal-AIB1 (Müller *et al.*, 2002); PRK1.N (PKN.N) (Sahai *et al.*, 1998); PRK1* (mycΔNPRK1) and PRK1wt (Flynn *et al.*, 1998); dnPRK1(PRK1 K644E) (Takahashi *et al.*, 1998); dnPKCα (PKCαK368R) (Überall *et al.*, 1997); dnp38γ [p38γ(AF)], MKK3E(b) and MKK6E(b) (Wang *et al.*, 2000); ROCK* (ROKα1-543) (Leung *et al.*, 1996); GST-AR-NTD (Alen *et al.*, 1999); MUT1 to MUT5 (pARS, 106, 126, 99 and 113), Gal-TAU-5 (G106) (Jenster *et al.*, 1995); VP16-AR-NTD, Gal-AR-LBD and pFlag-AR (Ikonen *et al.*, 1997). dnPRK2 (PRK2 KD1) was kindly provided by B.L.Quilliam, Indianapolis; GST-TAU-5 (GST-AR 360-546), GFP-MR, GR-GFP and ARANLS (pSG5-ARA612-633) by A.C.Cato, Karlsruhe; GST-RORβ by E.Greiner, Heidelberg; GST-GCNF by H.Greschik, Strasbourg. To construct CMX-GST-AR-DBD, CMX-GST-AR-LBD, and pGEX4T1-AR-LBD the corresponding fragments (AR-DBD: amino acids 539-623; AR-LBD: amino acids 624-919) were PCR amplified and inserted at the *Bam*HI site of CMX-GST and pGEX4T1 (Pharmacia). All plasmids were verified by double-stranded sequencing.

Transfections

DU145 and 293 cells were cultured in DMEM and PC3-AR in Ham's F-12 supplemented with 10% double-stripped fetal calf serum (dsFCS). Transient transfection assays were carried out in 12-well plates (1×10^5 cells per well) as described previously (Müller *et al.*, 2002). After transfection, cells were cultured in DMEM or Ham's F-12 supplemented with 0.5% dsFCS. The total amount of transfected DNA was kept constant (4 μg) by adding the corresponding amounts of empty expression plasmids and pUC18. The following amounts per well were used: 500 ng reporter plasmids MMTV-LUC, PB-LUC or G5E1b-LUC; 25 ng expression plasmids for AR, PR, ARANLS, GFP-MR, GR-GFP, GR, MR, dnPKCα, PRK1*, PRK2*, PRK1wt, VP16-AR-NTD or Gal-AR-LBD; 10 ng expression plasmids for Gal-AIB1, Gal-SRC-1, Gal-TIF-2 or Gal-FHL2; 12.5 ng expression plasmids for MKK3E(b), MKK6E(b), or ROCK*. One hundred nanogrammes of all other expression plasmids were transfected per well. Chemicals were obtained as indicated: PD 98059, SB 203580, SPP (BioMol); Ro31-8220 (Alexis Biochemicals); HA 1077 (Calbiochem); R5020, aldosterone, dexamethasone, DHEA and androstenedione (Sigma); R1881 and CPA (Schering AG, Berlin); rapamycin (ICN Biomedicals Inc.). All chemicals were applied for a total of 20 h except PC3-AR cells, which were treated for 8 h. Luciferase activity was assayed as described previously (Müller *et al.*, 2002). All experiments were repeated at least five times.

Immunofluorescence

Transfected cells were analysed essentially as described previously (Müller *et al.*, 2002). Cells were seeded on coverslips coated with fibronectin and gelatin. Primary antibody staining was performed with the indicated dilutions: α-AR (N-20) (1:4000; Santa Cruz), α-myc (1:10 000; Santa Cruz) and α-PRK1 (1:2000; Transduction Lab). Subcellular localization was visualized using secondary Alexa Fluor 488- and 546-labelled antibodies (1:4000; Molecular Probes). Nuclei were stained with 1 μg/ml DAPI (Roche).

Co-immunoprecipitation assays and western blot analyses

293 cells were transfected in 15 cm dishes with 10 μg of PRK1wt, PRK1*, Flag-AR or CMX-Flag and cultured in the presence of 10^{-10} M R1881 as indicated. Total cell extract was prepared in the presence or absence of 10^{-10} M R1881 in IP buffer (50 mM Tris-HCl pH 8.0, 170 mM NaCl, 0.1% NP-40, 20% glycerol, 50 mM NaF, 2 mM NaV, 0.2 mM DTT, 1 μg/ml BSA, 0.1 mM Pefabloc). Extracts from normal human prostate and prostate tumour tissue were prepared in the presence of 10^{-10} M R1881 in IP buffer. Following centrifugation, pre-cleared supernatants were incubated for 2 h with M2 α-Flag antibody (Sigma), α-TIF-2 (Transduction Lab), or α-PRK1 antibody (Transduction Lab) and GammaBind™-Sephacryl 4B (Pharmacia) in IP buffer. Precipitated protein complexes were subsequently washed four times either in the presence or absence of 10^{-10} M R1881 and subsequently analysed on 10% SDS polyacrylamide gels. Western blots were decorated with M2, α-AR 441 (Santa Cruz), α-TIF-2, α-PRK1 or α-cyclinA (control) antibodies. Secondary antibody and chemoluminescence procedure was performed according to the manufacturer's instructions (Amersham).

In vivo GST pull-down assays

For *in vivo* GST pull-down experiments, 293 cells grown in 15 cm dishes were co-transfected with 10 μg of expression vectors for GST, GST-AR-NTD, GST-AR-DBD, GST-AR-LBD, GST-GCNF, myc-tagged PRK1* or PRK1wt. Cells were grown for 18 h in DMEM containing 0.5% dsFCS with or without 10^{-10} M R1881. Whole-cell extracts were prepared by repeated freeze and thaw cycles either in NENT75-Mo buffer (20 mM Tris pH 6.8, 75 mM NaCl, 1 mM EDTA, 0.1% NP-40, 25% glycerol, 20 mM Na-molybdate) for cells transfected with myc-ΔNPRK1 or in buffer B1 (20 mM HEPES-KOH pH 7.7, 500 mM KCl, 25 mM MgCl₂, 100 μM EDTA pH 8.0, 10 mM DTT, 0.15% NP-40) for cells transfected with PRK1wt. GST fusion proteins were immobilized on glutathione-Sephacryl (Pharmacia) for 1 h at 4°C. After washing, bound proteins were analysed by SDS-PAGE, followed by immunodetection with α-myc or α-PRK1 antibodies. Equal expression of GST fusion proteins was controlled in western blots using an α-GST antibody. All antibodies were obtained from Santa Cruz.

In vitro GST pull-down assays

Expression of GST fusion proteins (Pharmacia) and the coupled *in vitro* transcription-translation reaction (Promega) were performed according to the manufacturer's instructions. GST pull-down assays were performed as previously described (Müller *et al.*, 2000) using buffer containing 250 mM KCl. Ten per cent of the *in vitro* translated proteins were loaded as input.

Immunohistochemistry

Stainings were performed using a protocol for antigen retrieval and indirect immunoperoxidase as described previously (Müller *et al.*, 2000). α-PRK1 antibody (Transduction Lab) was used at a dilution of 1:50, anti-mouse IgG (1:500; Dako) was used as secondary antibody and immunoreactions were visualized with the ABC-complex diluted 1:50 in PBS (Vectastain, Vector).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 388) and the Schering AG, Berlin to RS. We thank G.Baier, A.O.Brinkmann, M.Brown, A.C.Cato, F.Claessens, J.Han, K.S.Erdmann, O.A.Janne, L.Lim, Y.Ono, J.Palvimo, P.J.Parker, B.L.Quilliam, A.Soler, R.Treisman and U.Wetterauer for generously providing reagents and support, the members of the Schüle lab especially Philip Hublitz and Thomas Günther for fruitful discussion. Special thanks to Ellen Paggen for excellent technical assistance.

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*Received June 19, 2002; revised November 6, 2002;
accepted November 14, 2002*