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Identification of REPS2 as a putative modulator of NF-κB activity in prostate cancer cells

Fred Penninkhof[1], J Anton Grootegoed[1] and Leen J Blok*,[1]

[1]*Department of Reproduction and Development, Erasmus MC, Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands*

The protein REPS2 is implicated in growth factor receptor-mediated endocytosis and signalling, and its expression is downregulated in androgen-independent prostate cancer cells. Herein, the NF-κB subunit p65 is identified as a human REPS2 protein partner, interacting with the EH domain of REPS2. Using crystal structure data from literature and experimental data from yeast and mammalian two-hybrid analysis, the results indicate that the NPF-motif in p65 acts as binding site for the EH domain in REPS2. However, in cultured prostate cancer cells, the REPS2–p65 interaction is triggered upon stimulation with phorbol ester (PMA). This indicates that PMA-sensitive signalling pathways can affect the interaction between REPS2 and p65. During prostate cancer progression from androgen-dependent to androgen-independent growth, downregulation of REPS2 is accompanied by upregulation of NF-κB activity. This might involve loss of REPS2–p65 interaction, which would lead to increased NF-κB activity. Androgen-deprivation causes apoptosis of prostate cancer cells, and activated NF-κB is a known inhibitor of apoptosis. Hence, decreased expression of REPS2 might be a key factor, causing prostate cancer cells to become resistant to induction of apoptosis by androgen deprivation.
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Introduction

Growth of advanced prostate cancer initially is controlled by androgen ablation therapy. Reduction of the circulating androgen level to almost zero induces apoptosis of hormone-dependent normal prostate cells, and also induces apoptosis of prostate cancer cells. However, remaining prostate cancer cells progress from androgen-dependent towards androgen-independent growth, within a few years, and this means transition to uncontrollable cancer. To study molecular and cellular mechanisms involved in this transition, we have conducted a differential display PCR between androgen-dependent and androgen-independent prostate cancer cells (Chang *et al*., 1997). Clone JC19 was isolated as a transcript expressed at a five-fold higher level in an androgen-dependent prostate cancer cell line (LNCaP-FGC), as compared to three androgen-independent prostate cancer cell lines (LNCaP-LNO, PC3 and DU145). Furthermore, using a panel of androgen-dependent and androgen-independent human prostate cancer xenografts, a similar change in JC19 expression was observed (Chang *et al*., 1997). Clone JC19 was identified to encode REPS2 (RALBP1-associated Eps domain containing protein 2).

Although REPS2 is differentially expressed between androgen-dependent and androgen-independent prostate cancer cell lines, it is not androgen regulated (Chang *et al*., 1997). Furthermore, the REPS2 gene is located on the human X chromosome at Xp22, and transcribes into a single mRNA of approximately 7000 base pairs. This mRNA contains a maximum open reading frame (ORF), which encodes a protein of 659 amino acids, but on Western blot two REPS2 proteins were observed (Oosterhoff *et al*., 2003). The 78 kDa protein, REPS2a, is encoded by the maximal ORF that is present in the REPS2 mRNA. The other protein, REPS2b, is encoded by the same ORF but is shorter, with a molecular mass of 58 kDa and consisting of amino acid residues 140–659, because the second N-terminal methionine is used as a translation start (Oosterhoff *et al*., 2003). REPS2 was initially named POB1 (Partner Of RalBP1) (Ikeda *et al*., 1998), and identified as a 521-amino-acid protein that binds to RalBP1 (Rac/CDC42 GTPase-activating protein). The shorter form of REPS2 (REPS2b) is identical to the POB1 protein, except that POB1 has an additional glutamine residue inserted between amino acids 181 and 182.

REPS2 contains at least three different regions potentially involved in protein–protein interactions. First, at the C-terminus there is a coiled-coil protein–protein interaction domain, which is part of a larger region (amino acids 513–659) that is involved in RalBP1 binding (Ikeda *et al*., 1998). Second, there are two proline-rich motifs, PPTPPPRP (amino acids 476–483) and PPPPALPPRP (amino acids 512–521), which are putative binding sites for proteins containing an Src homology 3 (SH3) domain. The protein Grb2, containing an SH3 domain, has been reported to bind to REPS2

*Correspondence: LJ Blok; E-mail address: l.blok@erasmusmc.nl
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(Ikeda *et al*., 1998). Third, REPS2 contains one imperfect Eps15 homology (EH) domain (amino acids 16–69) and one consensus EH domain (amino acids 265–366). EH domain proteins are thought to play a role in receptor-mediated endocytosis (Santolini *et al*., 1999), and for REPS2 it has been demonstrated that the consensus EH domain binds Epsin and Eps15, two proteins that are implicated in endocytosis (Nakashima *et al*., 1999). In addition, it was shown that deletion mutants of REPS2 can inhibit internalization of EGF and insulin receptors (Nakashima *et al*., 1999). Based on these observations, REPS2 is thought to be involved in growth factor receptor signalling and internalization. Recently, it was shown that overexpression of REPS2 in prostate cancer cells leads to induction of apoptosis (Oosterhoff *et al*., 2003), which may point to an additional role for REPS2 in cellular mechanisms other than receptor internalization.

To study molecular and cellular aspects of the possible role of REPS2 in the transition of prostate cancer cells from androgen-dependent to androgen-independent growth in more detail, experiments were performed to identify novel protein partners of REPS2. Using a human prostate cDNA expression library, and yeast and mammalian two-hybrid strategies, REPS2 was found to interact with the NF-κB subunit RelA/p65.

Results

P65 and Clone4 were identified as binding partners of REPS2

To identify proteins that interact with the REPS2 protein, a human prostate cDNA expression library was screened with REPS2a (1–659) as a bait (Figure 1). Herein, we report on two proteins that showed binding to REPS2. One of the prey proteins that bound the REPS2 bait appeared to be a large fragment of the NF-κB subunit RelA/p65 protein (74–551). Since the relevant functional domains of p65 are located within this prey protein, the current clone was used as the basis for our further investigations. The second prey represented a large fragment of a hypothetical protein (GenBank accession number AF448860) and was named Clone4. In the present study, REPS2–Clone4 interactions serve as a control for the REPS2–p65 interaction.

The REPS2 EH domain binds to p65

To determine which part of REPS2 binds to p65 and Clone4, different REPS2 variants (Figure 1) were used in yeast two-hybrid analysis. Yeast strain AH109 was cotransformed with the REPS2-bait and p65- or Clone4-prey constructs as indicated in Table 1. Equal amounts of the cotransformation mix were plated out on medium that lacked tryptophan and leucine (only transformed cells survive), and on medium that lacked tryptophan, leucine, histidine and adenine (only transformants with interaction between bait and prey survive). When the REPS2 variants were cotransformed with empty prey vector, none of the cotransformants were able to survive the histidine and adenine selection (Table 1a; columns −T−L−H−A). This means that the REPS2 baits alone do not activate the histidine and the adenine marker in the yeast genome. However, when the REPS2 variants were cotransformed with the p65(74–551)-prey construct, six out of seven cotransformations resulted in yeast cotransformants that were able to grow on histidine- and adenine-minus plates (Table 1b; columns −T−L−H−A). All these six REPS2 variants have the EH domain in common, indicating that this domain of REPS2 binds to p65. When the REPS2 variants were cotransformed with Clone4 (Table 1c), only the cotransformants with a bait containing the C-terminal part of REPS2 were able to survive the histidine and adenine selection. These results show that the C-terminal part of REPS2 (amino acids 451–659) binds to Clone4, providing a control for specificity of binding of the REPS2 EH domain to p65.

The EH domain of REPS2 binds the NPF motif region of p65

The next step was to determine which region of p65 interacts with the EH domain of REPS2. An EH domain is a conserved protein–protein interaction domain, first identified in the tyrosine kinase substrate Eps15 (Wong *et al*., 1995). Different studies showed that EH domain-proteins preferentially bind to peptides or proteins containing an asparagine–proline–phenylalanine (NPF) motif (Fazioli *et al*., 1993; Salcini *et al*., 1997; Paoluzi *et al*., 1998), and such an NPF motif is present in the NF-κB p65 fragment (at amino-acid position 139–141). Alignment of human, mouse and chicken p65 shows that this NPF is conserved (Figure 2a). Furthermore, using structural NF-κB data from the literature, it was shown that the NPF sequence

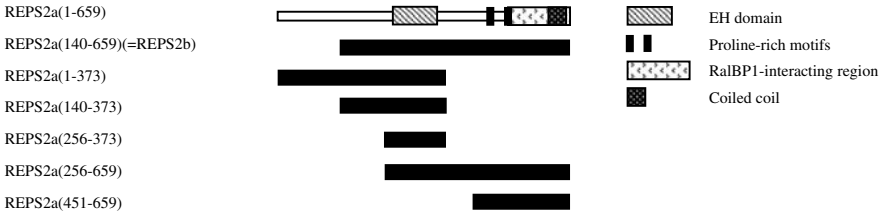

Figure 1 Schematic representation of seven REPS2 variants used in the present study

Table 1 The EH domain of REPS2 binds p65(74–263)

	GAL4-DBD	*GAL4-AD*	*Selection −T−L*	*Selection −T−L−H−A*
A	REPS2a(1–659)	Empty prey-vector	+	–
	REPS2a(140–659)	Empty prey-vector	+	–
	REPS2a(1–373)	Empty prey-vector	+	–
	REPS2a(140–373)	Empty prey-vector	+	–
	REPS2a(256–373)	Empty prey-vector	+	–
	REPS2a(256–659)	Empty prey-vector	+	–
	REPS2a(451–659)	Empty prey-vector	+	–
	—	Empty prey-vector	+	–
B	REPS2a(1–659)	p65(74–551)	+	+
	REPS2a(140–659)	p65(74–551)	+	+
	REPS2a(1–373)	p65(74–551)	+	+
	REPS2a(140–373)	p65(74–551)	+	+
	REPS2a(256–373)	p65(74–551)	+	+
	REPS2a(256–659)	p65(74–551)	+	+
	REPS2a(451–659)	p65(74–551)	+	–
	—	p65(74–551)	+	–
C	REPS2a(1–659)	Clone4	+	+
	REPS2a(140–659)	Clone4	+	+
	REPS2a(1–373)	Clone4	+	–
	REPS2a(140–373)	Clone4	+	–
	REPS2a(256–373)	Clone4	+	–
	REPS2a(256–659)	Clone4	+	+
	REPS2a(451–659)	Clone4	+	+
	—	Clone4	+	–
D	REPS2a(1–659)	p65(74–263)	+	+
	REPS2a(140–659)	p65(74–263)	+	+
	REPS2a(1–373)	p65(74–263)	+	+
	REPS2a(140–373)	p65(74–263)	+	+
	REPS2a(256–373)	p65(74–263)	+	+
	REPS2a(256–659)	p65(74–263)	+	+
	REPS2a(451–659)	p65(74–263)	+	–
	—	p65(74–263)	+	–

Yeast AH109 cells were cotransformed with REPS2-bait constructs combined with: (A) empty vector-prey, (B) p65(74–551)-prey, (C) Clone4-prey, (D) p65(74–263)-prey. Equal amounts of the cotransformation mix were plated out on medium that lacked tryptophan and leucine (−T−L; cotransformed cells survive) and on medium that lacked tryptophan, leucine, histidine and adenine (−T−L−H−A; only yeast cells with interaction between bait and prey proteins survive). After 3 days the plates were scored for yeast colonies: (+) = colonies present; (−) = no colonies present

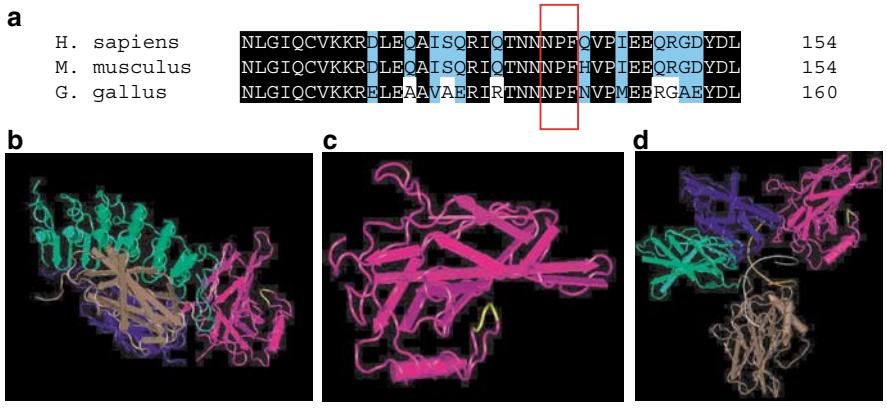

Figure 2 The NPF motif of p65 is present in the human, mouse and chicken proteins, and is located in a turn. (**a**) The p65 sequence from *Homo sapiens*, *Mus musculus* and *Gallus gallus* were aligned using the DNAMAN program. The red box indicates the NPF motif. (**b**) Structure of an I-κBα/NF-κB complex (Huxford *et al*., 1998). The complex shown consists of a part of mouse p65 (pink and blue), part of mouse p50 (brown) and part of human I-κBα (green). (**c**) Part of human p65 (pink) subunit from another I-κBα/NF-κB complex (Jacobs and Harrison, 1998). (**d**) Part of mouse p65 (pink and blue) and part of mouse p50 (brown and green) bound to the Ig/HIV-κB DNA site (grey and orange) (Chen *et al*., 1998). All three pictures were generated using the Cn3D 3D-structure viewer, which is available at the NCBI website. The NPF amino-acid motif is highlighted in yellow

is located in a turn at the surface of the p65 subunit (Figure 2b–d). Similarly, using solution structure data of two other EH–NPF complexes, the NPF was found to be located in a turn when bound into the pocket of the EH domain (De Beer *et al*., 2000). To collect experimental data to support the hypothesis further, yeast two-hybrid analysis was performed with p65(74–263), a truncated version of p65(74–551), which is missing 288 C-terminal amino-acid residues. It was observed that this truncated p65(74–263), which contains the NPF sequence, is still able to bind to REPS2 variants that contain the EH domain (Table 1d). This result is in agreement with the hypothesis that the p65-NPF motif is the site that binds to the EH domain of REPS2.

Tryptophan 323 in the core of the EH domain is important for REPS2–p65 binding in yeast

In Eps15, a tryptophan residue in the core of the second EH domain is critical for interaction with a peptide containing an NPF motif (De Beer *et al*., 1998). This interaction was lost when the critical tryptophan residue was replaced by an alanine residue, while substitution by a tyrosine residue affected the binding only slightly (De Beer *et al*., 1998). The respective tryptophan residue is highly conserved among EH domains, and in REPS2 this residue is located at position 323. To examine whether the conserved tryptophan at position 323 in the EH domain of REPS2 is involved in the binding between REPS2 and p65, this tryptophan was substituted for by either an alanine or a tyrosine residue in REPS2b, and the interaction between these REPS2b mutant proteins and p65 was measured in a quantitative yeast two-hybrid assay. An almost complete loss of reporter signal (beta-galactosidase activity) was observed in yeast cells expressing the REPS2b(W323A)-bait and p65(74–551)-prey, compared to yeast with the wild-type REPS2b-bait and p65(74–551)-prey combination (Figure 3a). This would be in agreement with the observations reported by De Beer *et al*. (1998). However, in the present experiments we found that the interaction was also lost for the REPS2b(W323Y)-bait (Figure 3a), which indicates that substitution of the tryptophan 323 residue in the EH domain of REPS2 by either an alanine or a tyrosine residue leads to loss of interaction with NF-κB/p65.

Clone4 binds to the C-terminal region of REPS2b, and it is unlikely that this interaction would be affected by the two point mutations (REPS2b(W323A) and REPS2b(W323Y), respectively). Indeed, wild-type and the two mutant REPS2 baits were found to bind the Clone4-prey construct with similar binding strength, as indicated by a comparable betagalactosidase reporter signal (Figure 3b).

Taken together, the results show that tryptophan 323 in the EH domain of REPS2 is critical for binding of REPS2 to p65 in yeast, lending further support that the EH domain of REPS2 binds the NPF motif(139–141) in p65.

REPS2 variants that have the EH domain and lack the C-terminal region bind to p65(74–551) in COS-1 cells, but none of the variants bind to p65 in LNCaP cells

From the literature it is known that NF-κB transcription factors are absent in yeast, and bait–prey binding between (putative) NF-κB pathway proteins in yeast, therefore, does not involve endogenous NF-κB pathway regulators. To study the interaction between REPS2 and p65 in a mammalian environment, a two-hybrid assay

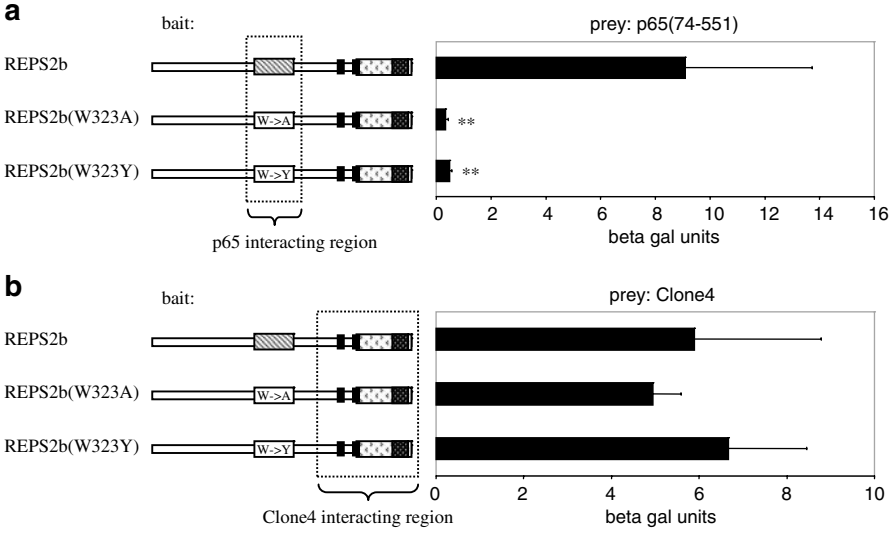

Figure 3 Quantitative two-hybrid assay demonstrating that tryptophan 323 in REPS2 is important for binding to p65. Yeast strain Y190 harboring the REPS2b-, the REPS2b(W323A)- or the REPS2b(W323Y)-bait in combination with the p65(74-551)-prey (**a**) or the Clone4-prey (**b**) were cultured. From the cultures, extracts were prepared and β-galactosidase activity was measured. The figure shows the average of three independent experiments for each combination. Differences between wild-type and mutant REPS2b were considered significant (**) at $P<0.01$, as determined by paired Student's *t*-tests

was performed in COS-1 cells. Based on the results obtained with the yeast two-hybrid assays (Table 1b), six out of the seven REPS2 variants were expected to bind to p65(74–551). However, using the mammalian two-hybrid assay in COS-1 cells, only three REPS2-bait variants bound to the p65(74–551)-prey, as indicated by the binding signal (Figure 4a; prey: p65(74–551)).

Different factors might be responsible for the failure of several REPS2-baits to generate a binding signal when cotransfected with p65(74–551)-prey: the constructs may not be expressed; the bait-hybrid may not be able to translocate to the nucleus; or REPS2, which is known to induce apoptosis when overexpressed, may also induce apoptosis under these particular circumstances. To exclude these possibilities, REPS2 variants were also cotransfected with Clone4. It was observed that Clone4 bound to all the REPS2 baits that contained the C-terminal region (Figure 4a; prey: Clone4). These results are in agreement with the data obtained from the yeast two-hybrid assays (Table 1c) and show that all REPS2 hybrid proteins are suitable as bait in the mammalian two-hybrid assays in COS-1 cells.

To study the interaction between REPS2 and p65 in human prostate cells, the mammalian two-hybrid assays were also performed using the androgen-dependent human prostate cancer cell line LNCaP. In contrast to what was observed for COS-1 cells, none of the REPS2-bait variants bound to the p65(74–551)-prey, as indicated by lack of a binding signal (Figure 4b). However, the control binding of REPS2-bait variants to the Clone4-prey also gave negative results (Figure 4b), so that we cannot exclude any possible confounding factors, which may exert an effect in LNCaP cells.

REPS2–p65 and REPS2–Clone4 binding occurs in LNCaP cells after stimulation with PMA

In COS-1 cells the luciferase signals obtained with REPS2 and p65 were higher than the signals produced by positive control binding between p53 and Large T antigen (Figure 4a). In contrast, in LNCaP cells no binding signal for REPS2–p65 or REPS2–Clone4 could be detected, whereas the positive control showed a high binding signal (Figure 4b). A possible explanation for the lack of binding signal in the experimental bait–prey assays in LNCaP cells is that LNCaP cells, compared to COS-1 cells, may contain additional pathways that suppress REPS2–p65 and REPS2–Clone4 interactions. As p65 is a subunit of the NF-κB complex, it was speculated that regulators of the NF-κB pathway could play a role. To test this, mammalian two-hybrid assays were conducted in the presence of the phorbol ester PMA, an activator of the NF-κB pathway. It was observed that for some variants of REPS2, binding to p65 and Clone4 was highly induced by PMA (Figure 5). Expression of REPS2b and p65, and control-binding between p53 and Large T antigen was only marginally affected by PMA, which indicates that the observed effect was not due to increased protein expression, but rather supports the idea that PMA modulates pathways

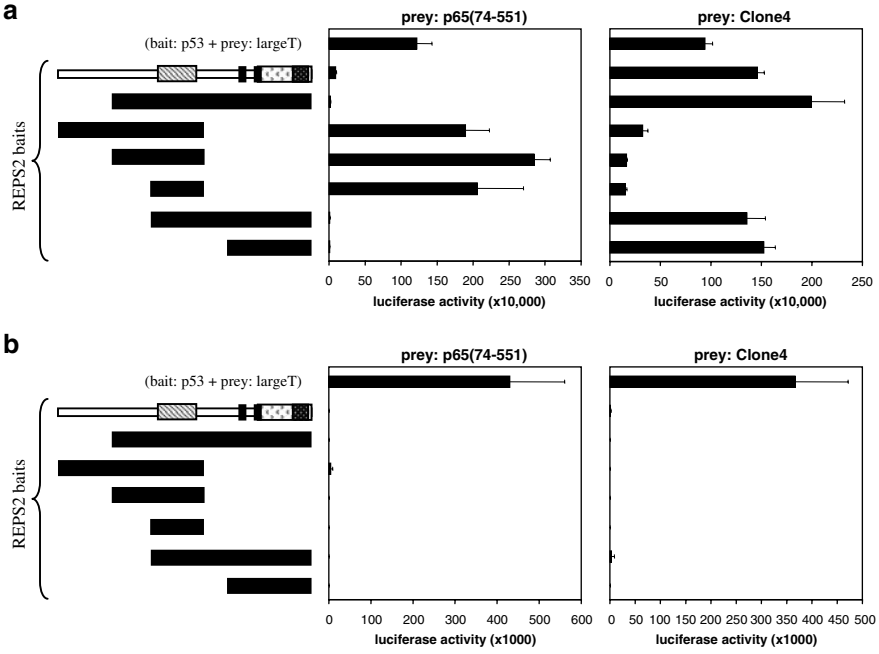

Figure 4 REPS2–p65 and REPS2–Clone4 binding in COS-1 and LNCaP cells. REPS2 bait constructs (left side of the figure) and p65- or Clone4-prey constructs (top of the figures) were cotransfected with the GAL4-luciferase reporter in COS-1 cells (**a**) or in LNCaP cells (**b**). Besides these bait and prey constructs, the positive control constructs p53-bait and Large T antigen-prey were also used in this assay. At 48 h after cotransfection, luciferase activity was measured in the cells. For each mammalian two-hybrid assay, two control assays were conducted: empty bait vector + prey and bait + empty prey vector. The control assays generated, compared to the experimental assays, only a low luciferase signal. This experiment has been repeated three times, and the figure shows the data from one representative experiment

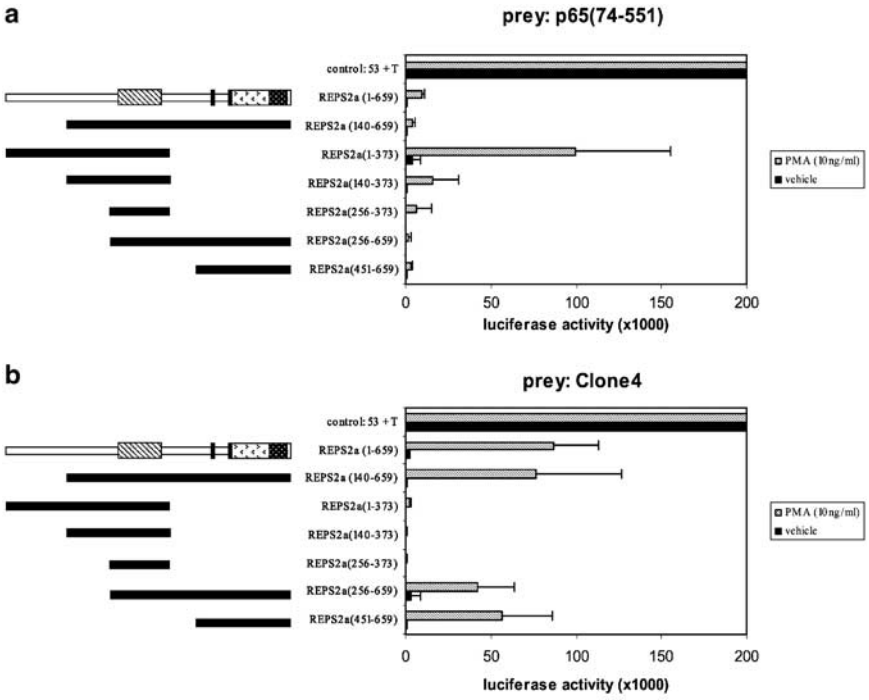

Figure 5 REPS2–p65 and REPS2–Clone4 binding occurs in LNCaP cells after stimulation with PMA. The assays in this figure were performed in the same way as described for Figure 4, except that 24 h after transfection the cells were treated with 10 ng/ml PMA or vehicle

that stimulate binding of REPS2 to p65 and Clone4 in LNCaP cells.

Tryptophane 323 in the core of the EH domain is important for REPS2–p65 binding in mammalian cells

As shown in Figure 3, in yeast, tryptophan 323 in the EH domain of REPS2(140–659) is important for binding between REPS2(140–659) and p65(74–551). The outcome of similar experiments using mammalian COS-1 and LNCaP cells is shown in Figure 6. As binding between the REPS2(1–373)-bait and the p65-prey produced the highest luciferase signal in LNCaP cells upon treatment with PMA (Figure 5a), this bait was used in the mammalian two-hybrid assays. Figure 6a and b show that substitution of tryptophan 323 to alanine has a dramatic effect on REPS2(1–373)–p65(74–551) binding in COS-1 and LNCaP cells. When tryptophan 323 was substituted for a tyrosine, however, the effect on binding between REPS2(1–373) and p65(74–551) was less severe in both cell lines. These results are in agreement with the results obtained by De Beer *et al*. (1998) for the binding between a mutated EH domain and an NPF containing peptide (De Beer *et al*., 1998). It should be noted that the background signals (Figure 6a; panel on the right), which are produced by wild-type REPS2a(1–373) and the two substitution mutated baits, although less high, resembles the signals that are obtained when p65 prey is cotransfected (Figure 6a; panel on the left). Possibly, there is some binding of an endogenous protein with transactivating activity to the REPS2 baits. In fact, this endogenous protein with transactivating capacity could very well be p65. LNCaP cells probably have less endogenous p65 available for binding to the REPS2 baits (Figure 6b; panel on the right).

Discussion

Using the yeast two-hybrid system, it was shown that REPS2 was identified as a binding partner of the NF-κB subunit RelA/p65. Furthermore, it was demonstrated that the Eps15 homology domain within REPS2 (EH domain, located at amino acids 265–366) is responsible for this interaction. It has been described that EH domain-proteins preferentially bind to peptides or proteins containing an asparagine–proline–phenylalanine (NPF) motif (Fazioli *et al*., 1993; Salcini *et al*., 1997; Paoluzi *et al*., 1998), and the p65 sequence contains such an NPF sequence at amino acids 139–141. Using crystal structure data from the literature (Chen *et al*., 1998; Huxford *et al*., 1998; Jacobs and Harrison, 1998), it was determined that the NPF in p65 is located at the surface of the protein in a turn of a loop. The position of the NPF in p65 is in agreement with protein-structure data from De Beer *et al*. (2000), and suggests that the NPF domain can act as a docking site for proteins like REPS2. Experimental data provided further back-up for this hypothesis: when a large portion of p65 was deleted, the yeast two-hybrid assay still showed normal interaction between the EH domain of REPS2 and p65(74–263). Additional support

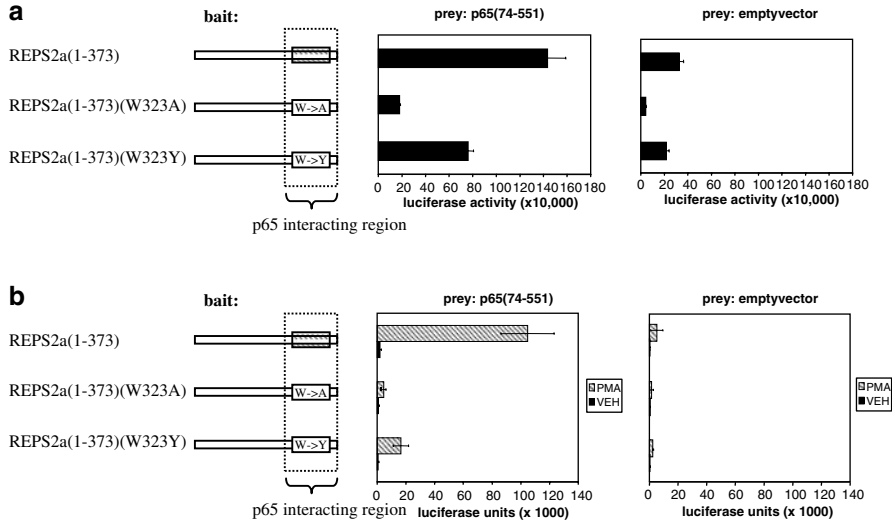

Figure 6 The conserved tryptophan in the EH domain of REPS2 is important for REPS2–p65 binding in mammalian cells. REPS2a(1–373), REPS2a(1–373)(W323A) and REPS2a(1–373)(W323Y) were used as baits in mammalian two-hybrid assays. COS-1 (top) and LNCaP cells (bottom) were cotransfected with reporter, bait and p65(74–551)-prey construct (left side), or the empty-prey vector (right side), as indicated in the figure. (**a**) COS-1 cells were left untreated for 48 h, and then luciferase activity was measured. (**b**) LNCaP cells were treated with PMA or vehicle starting at 24 h after transfection, and assayed for luciferase activity 48 h after transfection

came when the role of a highly conserved tryptophan in the core of the REPS2 EH domain was studied in quantitative yeast two-hybrid assays. In agreement with De Beer *et al*. (1998), it was shown that this tryptophan in the EH domain of REPS2 is critical for binding to the NPF-containing protein sequence of p65.

Although the data obtained with the yeast two-hybrid system demonstrated that REPS2 is able to bind to p65, it cannot be concluded that this interaction has a physiological role in the cell. Therefore, the interaction was studied also in mammalian cells, and it was observed that there are significant differences between REPS2–p65 binding in yeast as compared to mammalian cells. First, it was observed that not all REPS2 variants, which – on the basis of the yeast data were thought to interact with p65 – did interact in COS-1 cells. In fact in mammalian cells, the C-terminal part of REPS2 was found to inhibit the interaction between REPS2 and p65. Second, in the human prostate cancer cell line LNCaP, all interactions between EH domain-containing variants of REPS2 and p65 are below detection level. Because p65 is part of the NF-κB complex, it was speculated that NF-κB pathway regulators may suppress REPS2–p65 interaction in LNCaP cells. To modify the activity of NF-κB pathway regulators the assays were conducted in the presence of PMA, which is an activator of the NF-κB pathway. In line with the observations for COS-1 cells, it was observed that in LNCaP cells, in the presence of PMA, significant binding occurred between REPS2 and p65.

As indicated in the Introduction, REPS2 expression is significantly reduced in androgen-independent prostate cancer (Chang *et al*., 1997; Oosterhoff *et al*., 2003). Based on these findings, and in view of indications that REPS2 is involved in growth factor signaling (Nakashima *et al*., 1999), it was hypothesized that REPS2 might play a role in the transition of prostate cancer from androgen-dependent towards androgen-independent growth. Therefore, the newly identified interaction between REPS2 and the NF-κB subunit p65 could also be of importance for our understanding of prostate cancer progression. During prostate cancer progression, and parallel to loss of REPS2 expression, the NF-κB pathway becomes much more active (Palayoor *et al*., 1999; Chen and Sawyers, 2002). In a developing cancer, activation of the NF-κB pathway would provide the cells with additional cell survival proteins (Van Antwerp *et al*., 1996; Wang *et al*., 1996; Sumitomo *et al*., 1999). For example, Mayo *et al*. (1997) showed that cells stably transfected with an oncogenic form of Ras die when the NF-κB pathway is blocked. In case of prostate cancer, activation of the NF-κB pathway would imply that the developing cancer becomes much more resistant to removal of androgens (androgen-ablation therapy). Figure 7 shows a sequence of events that may illustrate different and subsequent stages in the development of prostate cancer. Three stages of prostate cancer are depicted. In Figure 7a, an oncogenic signal induces cell growth and cell death pathways in prostate cells. Because, for unknown reasons, the cell death signal is to weak, the prostate cells develop into cancer cells. In Figure 7b, cell death is stimulated because the cancer cells are treated with androgen ablation therapy. The NF-κB pathway cannot prevent this therapy-induced apoptosis, because the pathway is inhibited by REPS2. In Figure 7c, some prostate cancer cells manage to escape cell death with the help of additional cell survival proteins. The observed downregulation of REPS2 during this stage of the disease could be responsible

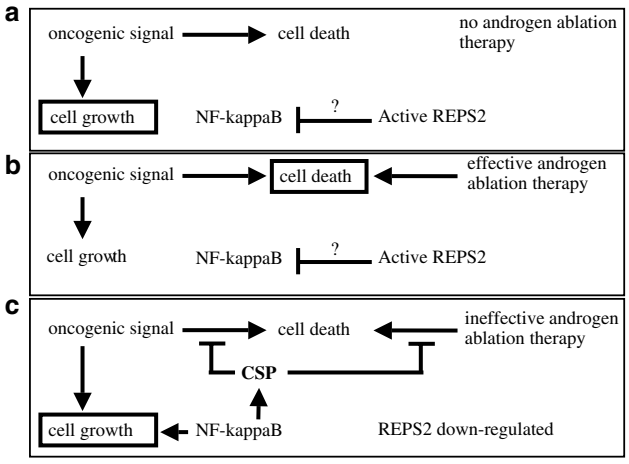

Figure 7 Lack of the REPS2–p65 interaction in relation to prostate cancer. (**a, b**) Androgen-dependent prostate cancer. The growth signal overrules the endogenous cell death signal (**a**). However, upon ablation of androgens an additional cell death signal is activated and the cells die of apoptosis (**b**). In androgen-independent prostate cancer (**c**), REPS2 expression is relatively low. Lack of REPS2–p65 (NF-κB subunit) interaction is hypothesized to contribute to constitutive activity of the NF-κB pathway. This constitutive activity could then be responsible for expression of extra cell survival proteins (CSP), which would result in survival of prostate cancer cells during androgen ablation

for, or contribute to, upregulation of cell survival proteins through loss of inhibition of the NF-κB pathway.

Many reports in literature describe an antiapoptotic role for the NF-κB pathway; however, there are also some reports that show a proapoptotic role for the NF-κB pathway. In case of prostate cancer for example, Ling *et al*. (2003) showed that increased NF-κB pathway activity in LNCaP cells promotes cell survival, whereas Kimura and Gelmann (2002) showed a proapoptotic effect of NF-κB signalling in the same cell line. As the NF-κB pathway is highly active in androgen-independent prostate cancer (Palayoor *et al*., 1999; Chen and Sawyers, 2002), and this activity does not result in cell death, the antiapoptotic effect of NF-κB is depicted in the model (Figure 7). Besides a role in regulating apoptosis, active NF-κB is also able to stimulate proliferation through upregulation of genes that are involved in cell growth (Figure 7c). For example, recently it has been shown that the activated NF-κB pathway, in androgen-independent prostate cancer cells, is important for upregulation of interleukin-6 (IL-6) (Park *et al*., 2003; Zerbini *et al*., 2003). Since IL-6 has also been reported to stimulate growth of prostate cancer cells (Steiner *et al*., 2003), these cells will become more and more independent of growth factors and androgens.

The molecular mechanism through which down-regulation of the REPS2 protein contributes to upregulation of NF-κB activity is not clear at the moment. Because proteins with which REPS2 interacts are all cytoplasmic in localization (Grb2, RalBP1, Epsin, Eps15) and because REPS2 itself was also reported to be located predominantly in the cytoplasm (Oosterhoff *et al*., 2003), the interaction between REPS2 and p65 probably takes place in the cytoplasm. It can be speculated that REPS2 may inhibit NF-κB activity by retaining p65 in the cytoplasm, but the exact mechanism needs to be studied further.

The NF-κB pathway is constitutively active in many advanced tumours, and is considered a potential target for cancer drug development (Garg and Aggarwal, 2002). Since the mechanisms that are responsible for high NF-κB activity in tumours are not known, studies on the REPS2–p65 interaction may provide additional information with regard to control of activity of the NF-κB pathway during progression of prostate and potentially other cancers.

Materials and methods

Constructs

The complete REPS2a ORF (Oosterhoff *et al*., 2003) was used as a template to amplify the seven REPS2 variants (Figure 1). In order to facilitate subcloning, an *Eco*RI site was added to the N-terminal primers and a *Sal*I site to the C-terminal primers. The Zero Blunt TOPO PCR Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to clone the PCR products into the pCR-Blunt II-TOPO vector. The REPS2 variants were subsequently subcloned, using the *Eco*RI and *Sal*I sites, into the pGBKT7 (the bait vector from the BD Matchmaker Two-Hybrid System 3, BD Biosciences Clontech, Palo Alto, CA, USA) and into the pM vector (the bait vector from BD Matchmaker Mammalian Two-Hybrid Assay Kit, BD Biosciences Clontech). All seven REPS2 variants have been sequenced. The QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to construct the REPS2 fragments containing the W323A and W323Y pointmutations. The isolated yeast two-hybrid prey plasmids pACT2clone4 (unknown hypothetical protein) and the pACT2clone8 (encodes for p65(74–551)) were used as template to PCR the Clone4 fragment and the p65 fragment. Again, to facilitate subcloning an *Eco*RI site was added to the N-terminal primer for the Clone4 fragment and a *Sal*I site to the C-terminal primer. For the p65 fragment N-terminal a *Bam*HI site was introduced and C-terminal a *Hin*dIII site. The PCR fragments were cloned into the pCR-Blunt II-TOPO vector and subsequently subcloned into the pVP16 vector (the prey vector from the Mammalian two-hybrid system, BD Biosciences Clontech). DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany) and the DNA Gel Extraction Kit from Millipore (Millipore Corporation, Bedford, MA, USA) was used to isolate fragments from gel. The Rapid DNA Ligation Kit (Roche, Mannheim, Germany) was used for the ligation reactions.

Yeast two-hybrid screen

The BD Matchmaker Two-Hybrid System 3 (BD Biosciences Clontech) was used for the yeast two-hybrid screen. The pGBKT7-REPS2a bait construct was introduced into the *S. cerevisiae* AH109 strain and protein expression was verified on Western blot (not shown). The AH109 strain with the REPS2a bait was used to screen the Human Prostate Matchmaker cDNA Library (BD Biosciences Clontech). The screening was performed as described in the BD Matchmaker Two-Hybrid System 3 manual, with the exception that carrier DNA from

Salmon Testes was used (Sigma-Aldrich Corporation, St Louis, MI, USA). From yeast cells that survived binding selection the prey plasmid was rescued according to the protocol described by Hoffman and Winston (1987).

Yeast two-hybrid assays

The *S. cerevisiae* AH109 strain was used in the qualitative yeast two-hybrid assays as shown in Table 1. The AH109 yeast strain was cotransformed, with the appropriate bait and prey vectors, according the Quick and Easy TRAFO Protocol (Gietz and Woods, 2002). Since the AH109 strain has low expression of the *lacZ* reporter gene the *S. cerevidiae* Y190 strain, which has high expression of the *lacZ* gene, was used in the quantitative yeast two-hybrid assays as shown in Figure 3. A liquid culture assay, which used ONPG as substrate (Yeast Protocols Handbook; BD Biosciences Clontech manual PT3024-1), was used to measure the activity of the *lacZ* reporter. Because the Y190 is relatively difficult to cotransform the Y190 strain was first transformed with the appropriate prey vector and then the appropriate bait vector was introduced.

Mammalian two-hybrid assays

The BD Matchmaker Mammalian Two-Hybrid Assay Kit (BD Biosciences Clontech) was used for the mammalian two-hybrid assays. Instead of the pSG5CAT reporter vector, which is the reporter from the Mammalian Two-Hybrid Assay Kit, a 5 × GAL4 binding site-luciferase reporter was used. COS-1 and LNCaP cells were cotransfected using Fugene 6 Transfection Reagent (Roche, Indianapolis, IN, USA) with 200 ng DNA mix (80 ng bait, 80 ng prey and 40 ng reporter) per well of a 24 wells plate. PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich Corporation) was dissolved in 100% ethanol and used in a 10 ng/ml concentration. COS-1 cells were maintained in DMEM/F12 containing 5% dextran-coated charcoal treated fetal calf serum (DCC-FCS). LNCaP were maintained in RPMI 1640 containing 10% fetal calf serum (FCS) and were used between passages 27 and 37. Both cell lines were cultured at 37°C in a 5% CO_2 atmosphere and standard antibiotics were added to the media. Luciferase activity was determined 48 h after the start of transfection with the Topcount NXT™ microplate luminescence counter (Packard Bioscience BV; Meriden, CT, USA). Cells were lysed with a standard luciferase lysis buffer and subsequently a part of the lysate was transferred to the Topcount 96 wells microplate. Just prior to the measurement, Steady Glo substrate (Promega, Madison, WI, USA) dissolved in Steady Glo buffer was added to the wells.

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