Rsk2 allosterically activates estrogen receptor α by docking to the hormone-binding domain

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We describe a novel mechanism for transcriptional regulation, in which docking of p90 ribosomal S6 kinase 2 (Rsk2) to the hormone-binding domain (HBD) of estrogen receptor α (ER α) induces a conformational change that enhances the transcriptional activation function contained in the HBD. A constitutively active mutant of Rsk2 specifically enhances ER α -mediated transcription by phosphorylation of Ser167 in ER α and by physically associating with residues 326–394 of the ER α HBD. The anti-estrogen 4-hydroxytamoxifen blocks Rsk2-mediated activation of ER α , by inducing a conformation of ER α in which the Rsk2 docking site is masked. Transcriptional activation and docking are specific for ER α and do not occur with the related isoform, ER β . ER α phosphorylation, docking and transcriptional activation are regulated by the Rsk2 N-terminal kinase domain. The allosteric regulation of a target protein, independent of phosphorylation, may be paradigmatic of a general function for protein kinase docking sites.

Keywords: allosteric/estrogen receptor α /p90 ribosomal S6 kinase 2/transcription

Introduction

The 90 kDa ribosomal S6 kinase (Rsk), a Ser/Thr protein kinase, is activated by mitogen-activated protein kinase (MAPK) (Zhao *et al.*, 1996). The four Rsk isoforms that have been identified each contain two non-identical kinase domains (Moller *et al.*, 1994; Fisher *et al.*, 1996; Yntema *et al.*, 1999). Upon MAPK phosphorylation, Rsk becomes activated and may be translocated into the nucleus (Chen *et al.*, 1992; Zhao *et al.*, 1995). Regulation of Rsk activity requires a cascade of phosphorylations that result from the action of MAPK, the C-terminal kinase domain (CTKD) of Rsk itself and 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Dalby *et al.*, 1998; Frodin *et al.*, 2000). The Rsks contain a docking site for MAPK at their extreme C-terminus and this docking site is a requirement for MAPK phosphorylation of Rsk (Figure 1A) (Smith *et al.*, 1999). MAPK phosphorylation causes displacement of an inhibitory α -helix from the substrate-binding region of the CTKD, which results in activation of the CTKD (Poteet-Smith *et al.*,

1999). The activated CTKD autophosphorylates Ser386, which results in the docking and activation of PDK1. Subsequently, PDK1 phosphorylates Ser227 in the N-terminal kinase domain (NTKD) of Rsk, which results in the activation of the NTKD (Frodin *et al.*, 2000). The NTKD is responsible for phosphorylation of exogenous substrates. The only known substrate for the CTKD is the autophosphorylation site.

A constitutively active Rsk2 can be generated by mutation of Tyr707 to alanine in the inhibitory α -helix [Rsk2(Y707A)] (Poteet-Smith *et al.*, 1999). This mutation results in Ser386 phosphorylation by the CTKD, which then initiates the other activation steps. Thus the requirement for active MAPK can be by-passed.

Estrogen receptor α (ER α) belongs to a superfamily of ligand-activated transcription factors the transcriptional activities of which can be stimulated further by second messenger pathways (Cho and Katzenellenbogen, 1993; Ignar-Trowbridge *et al.*, 1995; Enmark and Gustafsson, 1999). For example, epidermal growth factor (EGF) treatment enhances ER α -mediated transcription. Previously, we demonstrated that MAPK and Rsk1 can both directly phosphorylate ER α (Joel *et al.*, 1998a,b). However, because MAPK activates Rsk, it has not been possible to determine the importance of MAPK versus Rsk in ER α -mediated transcription.

Recent evidence suggests that the Rsk2 isoform may influence gene expression by affecting chromatin remodeling through phosphorylation of histone H3 (Sassone-Corsi *et al.*, 1999). Based on this evidence, it seems that Rsk2 plays a more important role in gene regulation than Rsk1 or Rsk3. Therefore, using the constitutively active Rsk2 mutant, Rsk2(Y707A), we determined the importance of Rsk2 for ER α -mediated transcription.

Rsk2, independently of MAPK, specifically activates ER α - and not ER β -mediated transcription. ER α and ER β are related isoforms that are similar in their DNA-binding domain (DBD) and hormone-binding domain (HBD). The ability of Rsk2 specifically to activate ER α depends not only on phosphorylation of ER α by Rsk2 but also on the ability of Rsk2 to physically associate with the HBD of ER α . We have identified residues in the HBD of ER α that are important for Rsk2 binding. Our data suggest a new mechanism for the activation of ER α function, in which the docking of a protein kinase (Rsk2) triggers a conformational switch and this activation is independent of, and additive to, the activation due to Rsk2-mediated phosphorylation of ER α . Importantly, the anti-estrogen 4-hydroxytamoxifen (4-OHT) blocks Rsk2-mediated activation of ER α , by inducing a conformation of ER α in which the Rsk2 docking site is masked.

Fig. 1. Schematic of Rsk2 and ER constructs. **(A)** Wild-type Rsk2 contains two kinase domains separated by a linker region. Phosphorylation by MAPK or mutation of Y707A leads to activation of the CTKD (1). Autophosphorylation by the CTKD (2) leads to activation of the NTKD by PDK1 (3). The locations of residues for mouse Rsk2 are shown. **(B)** The domains and locations of the residues for human wild-type ER α , rat wild-type ER β and the constructs used for this work are shown. A black bar indicates an inactivated region.

Results

Rsk2 specifically enhances ER α -mediated transcription

We first tested the ability of wild-type Rsk2 and constitutively active Rsk2 [Rsk2(Y707A)] to activate ER α -mediated transcription. Baby hamster kidney (BHK) cells were co-transfected with a construct encoding ER α , an estrogen-responsive element (ERE)-regulated luciferase reporter and a β -galactosidase expression vector to control for transfection efficiency. Additionally, the cells were transfected with a control vector or vectors encoding hemagglutinin (HA)-Rsk2, HA-Rsk2(Y707A) or

the N-terminal kinase-dead mutant, HA-Rsk2(K100A/Y707A). HA-Rsk2(K100A/Y707A) has the essential ATP-binding Lys100 of the N-terminal kinase domain mutated to alanine (Figure 1A). To decrease active MAPK levels, the cells were serum starved during the various treatments. Enhancement of ER α -mediated transcription by Rsk2 in BHK cells requires both ER α and the ERE, because no effect of Rsk2(Y707A) was observed in control experiments in which either the ER α construct or the EREs in the reporter was absent (data not shown).

We found that Rsk2(Y707A) enhanced ER α -mediated transcription by ~100% in the absence of estradiol, compared with the vector control (Figure 2A). Addition of estradiol plus Rsk2(Y707A) increased transcription by >200% compared with the vector control with estradiol. Ectopic addition of wild-type Rsk2 also enhanced ER α -mediated transcription in both the absence and presence of estradiol. The increases in transcription were greater for Rsk2(Y707A) than Rsk2 in both the absence and presence of estradiol. However, upon EGF activation, there was no statistical difference in the ability of Rsk2 and Rsk2(Y707A) to stimulate transcription. These observations are in agreement with results from *in vitro* kinase assays in which Rsk2(Y707A) has a higher basal activity than Rsk2 but has a similar activity upon activation by EGF (Poteet-Smith *et al.*, 1999).

The ability of Rsk2(Y707A) to enhance transcription was not due to changes in the relative expression levels of ER α that result from co-expression of ER α with Rsk2(Y707A) (Figure 2A, inset). The NTKD activity of Rsk2 was responsible for the increase in transcription because the inactive mutant Rsk2(K100A/Y707A) produced the same transcriptional response as the vector control (Figure 2A). EGF treatment further stimulated the ability of Rsk2(Y707A) to activate transcription (Figure 2A). This effect may be due to activation of endogenous MAPK and Rsk, or to the ability of active MAPK to stimulate the kinase activity of Rsk2(Y707A) further, or a combination of both (Poteet-Smith *et al.*, 1999). Overexpression of either Rsk2 or Rsk2(Y707A) enhanced EGF stimulation of ER α -mediated transcription, which suggests that the amount of endogenous Rsk2 in BHK cells limits the transcriptional response to EGF. The transcriptional response with either Rsk2 or Rsk2(Y707A) in the presence of estradiol was greater than the sum of the transcriptional responses obtained with either Rsk2 construct or estradiol alone. Thus both Rsk2 and Rsk2(Y707A) synergistically enhance transcription in the presence of estradiol.

To decrease MAPK activity below that present in serum-starved BHK cells, we added the MAPK kinase inhibitor, PD98059. Controls indicated that PD98059 was effective at inhibiting endogenous MAPK and Rsk2 activities (data not shown). Although PD98059 increased basal transcription, the percentage increases in transcription induced by Rsk2(Y707A) were the same with or without PD98059 (Figure 2B). Although the mechanism by which PD98059 enhances basal transcription is not clear, these data further suggest that Rsk2 and not MAPK is important in ER α -mediated transcription.

To determine if the effect of Rsk2 is specific for ER α -mediated transcription, we also tested the ability of Rsk2 to activate ER β -mediated transcription, using the same

Fig. 2. Constitutively active Rsk2, in the absence of active MAPK, specifically enhances ER α -mediated transcription. (A) BHK cells were co-transfected with ER α , an ERE-regulated luciferase reporter and β -galactosidase expression vectors. Additionally, the cells were transfected with a control vector or vectors encoding HA-Rsk2, HA-Rsk2(Y707A) or HA-Rsk2(K100A/Y707A), serum-starved and treated with vehicle (–), 10 nM estradiol (E₂), 100 ng/ml EGF or both E₂ and EGF. Luciferase and β -galactosidase activity were determined 19 h after hormone addition. The luciferase data were divided by the β -galactosidase activity to control for differences in transfection efficiency. To facilitate comparison between the different experiments, the data were normalized so that, in the absence of activated Rsk2, the response to vehicle addition by ER α was zero and the response of ER α to E₂ was 100. The inset shows immunoblots of lysates of BHK cells that were transfected with ER α , ER α and HA-Rsk2, or ER α and HA-Rsk2(Y707A). (B) BHK cells were transfected and treated as for (A) except that during hormone treatment either 2 μ M of PD98059 or vehicle was also added. Data were normalized as in (A). (C) BHK cells were transfected and treated with vehicle or E₂ as for (A) except that an expression vector encoding ER β was used. Data were normalized to those obtained for ER α as in (A), then expressed as the percentage increase relative to the vehicle control with ER β . (D) MCF-7 cells were co-transfected with ERE-regulated luciferase reporter and β -galactosidase expression vectors. Additionally, the cells were transfected with a control vector or the vector encoding HA-Rsk2(Y707A). Transfected cells were serum-starved and treated with the indicated agent. After 5.5 h, the cells were lysed, and luciferase and β -galactosidase activity determined. Data were normalized as in (A). In each panel, values are means $\pm$ 1 SEM. *** P < 0.0005, ** P < 0.01 and * P < 0.05 (Student's t -test) obtained by comparing the response obtained with vector control with that obtained with the vector encoding HA-Rsk2(Y707A).

reporter system described above and co-transfecting with an ER β construct. Constitutively active Rsk2 did not significantly influence ER β -mediated transcription in the absence of estradiol and had only a small effect in the presence of estradiol (Figure 2C). These results are in marked contrast to those seen for ER α . These data, together with those presented in Figures 5B and D, and 6A, suggest that Rsk2 is specific for the ER α isoform.

The ability of constitutively active Rsk2 to stimulate ER α -mediated transcription was also observed in MCF-7 cells, a human breast cancer cell line containing endogenous ER α (Figure 2D). These experiments were similar to those described above except that it was not necessary to co-transfect with the ER α construct. In MCF-7 cells, Rsk2(Y707A) enhanced transcription over that observed with the vector control in both the absence and presence of estradiol. Furthermore, Rsk2(Y707A) synergistically increased transcription in the presence of estradiol. The effects of Rsk2(Y707A) are less than those

observed in BHK cells. We did not detect any differences in the specific activity towards S6 peptide of immunoprecipitated HA-Rsk2(Y707A) isolated from either BHK or MCF-7 cells in *in vitro* kinase assays (data not shown). Thus the diminished response may reflect cell-specific differences in co-activator expression.

Ser167 is important in Rsk2 activation of ER α -mediated transcription

Previously, using [³²P]orthophosphate labeling, we determined that Ser167 of ER α is phosphorylated *in vivo* in response to activation of the MAPK pathway (Joel *et al.*, 1998a). To determine whether Ser167 is phosphorylated in response to estradiol, we immunoblotted lysates of ER α -expressing BHK cells with an anti-pSer167 antibody. The lysates were obtained from cells that had been treated with estradiol, EGF or vehicle before lysis. The specificity of the anti-pS167 antibody is shown in Figure 4B, in which the anti-pS167 antibody recognizes wild-type ER α

but not the mutant ER α (S167A), in which Ser167 is replaced by alanine. Ser167 is phosphorylated in response to activation of the MAPK pathway by EGF, but not by hormone addition (Figure 3A). As we have demonstrated previously, phosphorylation of Ser118 causes a shift in the mobility of ER α under particular electrophoretic conditions, whereas phosphorylation of Ser167 does not cause an upshift (Joel *et al.*, 1998b). Thus, in contrast to Ser167, Ser118 is phosphorylated both in response to activation of the MAPK pathway and by hormone binding. Thus Ser118 and Ser167 are differentially phosphorylated in response to known activators of ER α -mediated transcription.

To determine the importance of Ser167 phosphorylation in the activation of transcription, we tested whether Rsk2(Y707A) could enhance transcription of the mutant ER α (S167A) (Figure 1B). ER α and ER α (S167A) produced similar transcriptional responses in the absence of Rsk2(Y707A) (Figure 3B). However, the response of ER α (S167A) to Rsk2(Y707A) was substantially diminished compared with that of wild-type ER α (Figure 3B). Thus, Rsk2 is less efficient in stimulating ER α -mediated transcription when Ser167 is mutated to alanine. However, Rsk2(Y707A) is able to enhance transcription by ER α (S167A) above that of the wild-type receptor without Rsk2(Y707A) (Figure 3B). These results are not due to differences in protein expression levels, as ER α and ER α (S167A) are expressed to the same extent (Figure 3B, inset).

Further support for the importance of Ser167 phosphorylation in transcription is provided by the ability of Rsk2(Y707A) to stimulate transcription by the deletion mutant ER α (1–281) (Figure 3C), which lacks the HBD (Figure 1B). The stimulation is not due to differences in protein expression levels, as ER α and ER α (1–281) are expressed to the same extent (Figure 3C, inset).

Taken together, these data suggest that phosphorylation of Ser167 by Rsk2 is important for ER α -mediated transcription. However, Rsk2 can still activate the mutant ER α (S167A), although at a reduced level compared with wild-type ER α . We conclude, therefore, that Rsk2 activates ER α -mediated transcription both through phosphorylation of Ser167 and by an additional, unidentified, mechanism.

Rsk2 phosphorylation and docking sites in ER α are distinct entities

Some kinases associate physically with their substrates at a site distinct from the phosphorylation site (Pawson and Nash, 2000). Based on these observations, it seemed reasonable to test whether or not Rsk2 could co-immunoprecipitate with ER α . To this end, we transfected BHK cells with either a construct encoding ER α or a control vector. Additionally, the cells were transfected with constructs encoding HA-tagged wild-type or mutant Rsk2s. The cells were serum starved and ER α was immunoprecipitated. As shown in Figure 4A, both HA-Rsk2 and HA-Rsk2(Y707A) co-precipitated with ER α . Thus, either wild-type or constitutively active Rsk2 can associate with ER α . However, the N-terminal kinase-dead mutant Rsk2(K100A/Y707A) did not co-immunoprecipitate with ER α (Figure 4A). The subcellular distributions of HA-Rsk2, HA-Rsk2(Y707A) and HA-Rsk2(K100A/Y707A) as determined by immunofluorescence were very

Fig. 3. Ser167 is important in Rsk2 activation of ER α -mediated transcription. (A) BHK cells were transfected with a vector encoding ER α , treated with E₂, EGF or vehicle, then lysed and aliquots immunoblotted. (B) BHK cells were transfected and treated with vehicle or E₂ as in Figure 2A except that an expression vector encoding the mutant ER α (S167A) was also used. Data were normalized as in Figure 2A. Means $\pm$ SE are shown. ***P* < 0.005 and **P* < 0.05, obtained by comparing the response obtained with ER α and HA-Rsk2(Y707A) with that obtained with ER α (S167A) and HA-Rsk2(Y707A). The inset shows immunoblots of lysates of BHK cells transfected with ER α and HA-Rsk2(Y707A) or ER α (S167A) and HA-Rsk2(Y707A). (C) BHK cells were transfected and treated with vehicle or EGF as in Figure 2A except that an expression vector encoding ER α (1–281) was also used. Data were normalized initially as in Figure 2A, then expressed as the percentage increase relative to the vehicle control with ER α (1–281). Values are means $\pm$ SE. ****P* < 0.005, obtained by comparing the response obtained with ER α and HA-Rsk2(Y707A) with that obtained with ER α (1–281) and HA-Rsk2(Y707A). The inset shows immunoblots of lysates of BHK cells transfected with ER α and HA-Rsk2(Y707A) or ER α (1–281) and HA-Rsk2(Y707A).

similar (data not shown). These results suggest that the NTKD of Rsk2 influences the ability of Rsk2 to physically associate with its exogenous substrates.

To determine whether Rsk2 phosphorylation and docking to ER α are correlated, we assessed the ability of HA-Rsk2(Y707A) to co-immunoprecipitate with ER α (S167A). Similar amounts of ER α and ER α (S167A) were immunoprecipitated and, suprisingly, similar amounts of HA-Rsk2(Y707A) were present in both the

Fig. 4. Rsk2 physically associates with ER α . (A) BHK cells were co-transfected with control vector or a vector encoding ER α , plus expression vectors encoding HA-tagged wild-type Rsk2, HA-Rsk2(Y707A) or HA-Rsk2(K100A/Y707A), then serum-starved, lysed, and ER α and associating proteins immunoprecipitated with anti-ER α antibody. Aliquots of the immunoprecipitates and lysates were immunoblotted. (B) BHK cells were co-transfected with a vector encoding HA-tagged HA-Rsk2(Y707A) plus control vector or a vector encoding wild-type ER α , or ER α (S167A). Immunoprecipitates and immunoblots were performed as in (A). (C) BHK cells were co-transfected as for (B) except that a vector encoding ER α (1–281) was used instead of ER α (S167A). Immunoprecipitates and immunoblots as in (A).

ER α and ER α (S167A) immunoprecipitates (Figure 4B). We conclude that the ability of Rsk2 to dock is not dependent on the presence of a phosphorylation site motif.

To assess whether the regions of ER α that flank the Rsk2 phosphorylation site are important for Rsk2 docking, we next tested the ability of HA-Rsk2(Y707A) to co-precipitate with the deletion mutant ER α (1–281). These experiments were similar to the co-immunoprecipitation experiments described above. The relative amounts of ER α and ER α (1–281) that immunoprecipitated and the amounts of HA-Rsk2(Y707A) present in the lysates with ER α and ER α (1–281) were similar (Figure 4C). However, barely detectable amounts of HA-Rsk2(Y707A) co-immunoprecipitated with ER α (1–281) compared with wild-type ER α . These data strongly suggest that the Rsk2 docking site is distinct from the phosphorylation site motif.

Rsk2 docks to the HBD of ER α

We next wanted to identify the Rsk2 docking site in ER α . From the results shown in Figure 4C, we knew that the docking site is not contained within the N-terminal region of ER α . Therefore, we tested the ability of the isolated HBD of ER α to co-precipitate with HA-Rsk2(Y707A). These experiments were similar to the co-immunoprecipitation experiments described above, except that HA-Rsk2(Y707A) was immunoprecipitated. The deletion mutants ER α (1–181) and ER α (282–595) were expressed

as fusion proteins with green fluorescent protein (GFP). A nuclear localization signal (NLS) was also fused to the mutant ER α (1–181). The mutant ER α (1–181) contains only the AF1 region, and ER α (282–595) contains just the HBD (Figure 1B). As shown in Figure 5A, HA-Rsk2(Y707A) associated specifically with ER α (282–595) in both the presence and absence of estradiol. No binding of ER α (1–181) to HA-Rsk2(Y707A) was detected (Figure 5A). We note that the expression level of the ER α (1–181) construct was lower than that for ER α (282–595) and the amount of ER α (1–181) bound could be less than our detection limit. However, these data are in agreement with Figure 4C. We conclude, therefore, that the docking of Rsk2 to ER α is hormone independent and the docking site is contained within the HBD, from residues 282 to 595.

To assess the selectivity of this interaction, we next determined whether or not the HBD of ER β could co-immunoprecipitate with HA-Rsk2(Y707A). A chimera consisting of the N-terminus of ER α with the C-terminus of ER β was constructed (ER $\alpha\beta$) (Figure 1B). We constructed this chimera because it allowed us to immunoprecipitate with an anti-ER α antibody and then determine the relative levels of ER α and ER $\alpha\beta$ that were expressed. Similar amounts of ER α and ER $\alpha\beta$ were immunoprecipitated and similar amounts of HA-Rsk2(Y707A) were present in the lysates (Figure 5B). Importantly, however, the anti-HA immunoblot of the immunoprecipitates shows that HA-Rsk2(Y707A) associates with ER α to a much greater extent than with ER $\alpha\beta$ (Figure 5B). These results demonstrate that HA-Rsk2(Y707A) binds selectively to the HBD of ER α .

To delineate the docking site further, we tested the ability of the fragments myc-ER α (301–527), myc-ER α (282–394) and myc-ER α (326–394) to co-immunoprecipitate with HA-Rsk2(Y707A) (Figure 1B). The fragments contain an NLS in addition to the myc tag. ER α (301–527) lacks helix 12 (H12), which is known to bind a number of co-activators and is critical for estradiol-dependent transcriptional activation (Mak *et al.*, 1999). ER α (282–394) contains helix 2–helix 6 and ER α (326–394) contains helix 3–helix 6. Remarkably, these fragments associated with HA-Rsk2(Y707A), as seen from the anti-myc immunoblots of the immunoprecipitates (Figure 5C and D). The anti-myc immunoblot of the lysates in Figure 5D shows that myc-ER α (282–394) electrophoreses as a doublet. Presumably, the lower band is a breakdown product with deletions from the C-terminus end. However, we observed that only the upper molecular weight band co-immunoprecipitated with HA-Rsk2(Y707A), a result that testifies to the high specificity of the interaction.

Taken together, our results demonstrate that the Rsk2 docking site is contained within residues 326–394 in ER α . To confirm that binding of this fragment is specific, we tested the ability of an ER β fragment, myc-ER β (211–302), to bind HA-Rsk2(Y707A). This region of ER β is ~57% homologous to the region of ER α from 301 to 394. An extremely faint band that corresponded to myc-ER β (211–302) was observed in the anti-myc immunoblot of the immunoprecipitated HA-Rsk2(Y707A) (Figure 5D). These results are in contrast to those obtained with myc-ER α (326–394), myc-ER α (282–394) and myc-ER α (301–

Fig. 5. Rsk2 specifically binds to the region from 326 to 394 of the HBD of ER α . (A) BHK cells were co-transfected with a vector encoding a deletion mutant of ER α fused to GFP and either HA-tagged RSK2(Y707A) or control vector, then treated with E₂ or vehicle. Immunoprecipitates were obtained as in Figure 4 except that HA-Rsk2(Y707A) and associated proteins were immunoprecipitated using anti-HA antibody. Aliquots of the immunoprecipitates and lysates were immunoblotted. (B) BHK cells were transfected with a vector encoding HA-Rsk2(Y707A) and either control vector or a vector encoding wild-type ER α or the chimera ER $\alpha\beta$. Immunoprecipitates and immunoblots as in Figure 4. (C) BHK cells were transfected with a vector encoding a myc-tagged fragment of ER α and either HA-Rsk2(Y707A) or control vector. Immunoprecipitates and immunoblots as in (A). (D) BHK cells were transfected with a vector encoding a myc-tagged deletion fragment of ER α or ER β . Additionally, the cells were co-transfected with either a vector encoding HA-Rsk2(Y707A) or control vector. Immunoprecipitates and immunoblots as in (A). (E) Schematic showing homology between the region in ER α that binds to Rsk2 and the analogous region of ER β . The secondary structure of these regions is also shown, in which the rectangles represent helices (Shiau *et al.*, 1998).

527). The relative amount of myc-ER β (211–302) present in the lysates was comparable with that obtained with the myc-tagged ER α fragments that bind HA-Rsk2(Y707A). Therefore, taken together, these data provide strong evidence that the binding of HA-Rsk2(Y707A) to ER α (326–394) is highly specific and selective (Figure 5E).

Rsk2 docking to the HBD influences phosphorylation and transcriptional activity of ER α

To determine whether or not the docking of Rsk2 to ER α has functional significance, we first examined the ability of Rsk2(Y707A) to phosphorylate ER α and ER α (1–281). Rsk2 enhanced the phosphorylation of Ser167 in wild-type ER α and the ER α (1–281) fragment to the same extent (Figure 4C). ER α (1–281) does not contain the Rsk2 docking site and these data, therefore, suggest that Rsk2 does not have to physically associate with its substrate in order for phosphorylation of the substrate to occur. Interestingly, the level of Ser167 phosphorylation for the chimera ER $\alpha\beta$ was reduced compared with wild-type ER α (Figure 5B). Extended exposure of the anti-pS167 immunoblot, however, shows that the chimera is only poorly phosphorylated (data not shown). Thus the chimera

ER $\alpha\beta$ is a worse substrate for Rsk2 than either the deletion mutant or wild-type ER α . We have also determined that HA-Rsk2(Y707A) has a greater affinity for HBD of ER α than the HBD of ER β (Figure 5B). Therefore, taken together, these results suggest a model in which the HBD sterically hinders Ser167 phosphorylation and, in the context of the holoreceptor, efficient phosphorylation of Ser167 can only occur when Rsk2 docks and induces a conformational change that exposes Ser167.

We next asked whether Rsk2 docking influences ER α -mediated transcription by testing the ability of Rsk2(Y707A) to enhance the transcriptional response of the chimeras ER $\beta\alpha$ and ER $\alpha\beta$. The chimera ER $\beta\alpha$ contains the N-terminal region of ER β and the HBD of ER α (Figure 1B). The kinase-dead mutant Rsk2(K100A/Y707A) was used as a negative control in these experiments because it controls for Rsk2(Y707A) effects that are not regulated by the NTKD. Our results show that both chimeras had a weaker transcriptional response to Rsk2(Y707A) compared with that observed with ER α (Figure 6A). Additionally, the effects of estradiol and Rsk2(Y707A) were additive for the chimeras, as opposed to the synergism that was observed for ER α . The

Fig. 6. Rsk2 activation requires AF2 activity. (A) BHK cells were co-transfected with an expression vector encoding ERα, ERβ, ERαβ or ERβα plus vectors encoding HA-Rsk2(Y707A) or the N-terminal-kinase-dead mutant, Rsk2(K100A/Y707A), as well as reporter and β-galactosidase expression vectors. The transfected cells were treated with vehicle or E₂ and the data analyzed as in Figure 2A. Data were normalized initially as in Figure 2A, then expressed as the percentage increase relative to the vehicle control with either ERβ, ERαβ or ERβα. Values are means ± SEM. ****P* < 0.0005, ***P* < 0.01 and **P* < 0.05, obtained by comparing the response obtained with vector encoding Rsk2(K100A/Y707A) with that obtained with the vector encoding HA-Rsk2(Y707A). The inset shows immunoblots of lysates of BHK cells transfected with ERα and HA-Rsk2(Y707A) or ERαβ and HA-Rsk2(Y707A). (B) BHK cells were transfected, treated and the data analyzed as in (A) except that the cells were transfected with a vector encoding ERα or ERα-AF1. Values are means ± SEM. ***P* < 0.01 and **P* < 0.05, obtained by comparing the response obtained with ERα with that obtained with ERα-AF1. The inset shows immunoblots of lysates of BHK cells transfected with ERα and HA-Rsk2(Y707A) or ERα-AF1 and HA-Rsk2(Y707A). (C) BHK cells were transfected and serum-starved as in (A), then treated with either 1 μM ICI 182,780 or 0.5 μM 4-OHT. Control transfections were treated with vehicle or E₂. Data were normalized so that in the absence of activated Rsk2 the response to vehicle addition by ERα was 100. Data shown are from one experiment that is representative of two experiments each performed in quadruplicate. (D) BHK cells were co-transfected with a vector encoding HA-Rsk2(Y707A) plus a control vector or a vector encoding ERα, then serum-starved and treated with vehicle or 0.5 μM 4-OHT. Immunoprecipitates and immunoblots were performed as in Figure 4A. (E) BHK cells were co-transfected with a vector encoding ERα and either a control vector or a vector encoding HA-Rsk2(Y707A), then treated with E₂, 4-OHT or vehicle, lysed and aliquots immunoblotted.

differences in response to Rsk2 for the chimera ERαβ and wild-type ERα were not the result of protein expression effects because the relative levels of ERα and ERαβ were similar, as were the levels of HA-Rsk2(Y707A) (Figure 6A, inset).

In Figure 5B, low levels of Ser167 phosphorylation were observed for the chimera ERαβ. However, the ratio of Rsk2(Y707A) to ER constructs used for transfection was much higher in the transcription experiments than for the co-immunoprecipitation experiments. Thus, under the

conditions of the transcription experiments, it is likely that ERαβ was phosphorylated on Ser167 to a greater extent than for the co-immunoprecipitation experiments. Therefore, it seems reasonable to suggest that the Rsk2 activation of ERαβ is the result of phosphorylation of Ser167, which affects AF1 function. We have not observed enhanced phosphorylation of residues in the HBD of ERα in response to EGF treatment (Joel *et al.*, 1998a). Thus, the ability of Rsk2 to enhance transcription of ERβα suggests that Rsk2 docking influences the

activity of AF2. Moreover, the data suggest that for full activation by Rsk2, both the AF1 and AF2 domains of ER α are required.

To confirm that Rsk2 influences AF2 activity, we tested the ability of Rsk2(Y707A) to enhance the transcriptional activity of a mutant ER α that has a defective AF2 domain (ER α -AF1). We found that the mutant ER α -AF1 can still respond to estradiol but has a weaker transcriptional response than wild-type ER α (Figure 6B). The responses of ER α -AF1 to Rsk2(Y707A) were also diminished compared with those observed with wild-type ER α . These results are not due to differences in protein expression levels, as ER α and ER α -AF1 are expressed to the same extent (Figure 6B, inset). These data support a novel model in which Rsk2 docking influences the activity of AF2.

Anti-estrogens inhibit Rsk2 docking with ER α

To determine the effectiveness of anti-estrogens in preventing constitutively active Rsk2 from enhancing ER α -mediated transcription, we compared the ability of ICI 182,780 and 4-OHT to inhibit the stimulation by Rsk2(Y707A). Both anti-estrogens decreased transcription below that observed for vehicle addition with the vector control in either the presence or absence of Rsk2(Y707A). Therefore, in the experiment shown in Figure 6C, the data were normalized so that with the vector control the response to vehicle addition by ER α was 100. In this manner, it can be seen that both ICI 182,780 and 4-OHT prevent Rsk2(Y707A) from stimulating ER α -mediated transcription. However, the antagonist ICI 182,780 enhances degradation of the receptor, which is probably the basis for its ability to inhibit ER α -mediated transcription (Joel *et al.*, 1998b). Therefore, the data obtained with ICI 182,780 further demonstrate that ER α is required for Rsk2(Y707A) stimulation of transcription. The antagonist 4-OHT inhibits ER α -mediated transcription by regulating the ability of the AF2 region to interact with co-activators (Shiau *et al.*, 1998). Therefore, the fact that 4-OHT can block Rsk2-mediated activation of ER α strongly supports the conclusion from Figure 6B that the AF2 region influences the ability of Rsk2 to activate ER α -mediated transcription.

Since 4-OHT inhibited the ability of constitutively active Rsk2 to stimulate ER α -mediated transcription, we tested whether this anti-estrogen could inhibit the association between HA-Rsk2(Y707A) and ER α . Approximately equal amounts of ER α with and without 4-OHT were immunoprecipitated, and comparable amounts of HA-Rsk2(Y707A) were present in the lysates. Interestingly, however, HA-Rsk2(Y707A) did not co-immunoprecipitate with ER α complexed to 4-OHT (Figure 6D). These results suggest that the affinity of HA-Rsk2(Y707A) for ER α is much lower when the receptor is bound to 4-OHT than when the receptor is unliganded. These data further support a role for Rsk2 docking in activation of ER α -mediated transcription.

We observed that Rsk2 phosphorylated Ser167 to the same extent in either wild-type ER α or ER α (1–281) (Figure 4C), which suggested that the docking of Rsk2 to ER α is not required for phosphorylation of Ser167. If this hypothesis is correct, then constitutively active Rsk2 should phosphorylate ER α in the presence of 4-OHT. We

observed that Ser167 phosphorylation by constitutively active Rsk2 was unaffected by the ligand complexed to ER α (Figure 6E). These results demonstrate that the abilities of Rsk2 to dock and phosphorylate ER α are independent of one another.

Discussion

We propose the following novel mechanism for transcriptional activation, in which the docking of a protein kinase to its effector can, even in the absence of phosphorylation, regulate effector function. In the case we have studied, docking of the Rsk2 protein kinase to the ER α transcription factor induces a conformational switch in the HBD of ER α (Figure 7A). This switch enhances the activity of the transcriptional activation function (AF2), which is present in the HBD of ER α . In addition to this novel mode of activation, Rsk2 is also able to influence the transcriptional activation function present in the N-terminal domain (AF1) of ER α .

Our model proposes that Rsk2 has two independent modes of action in influencing ER α -mediated transcription. The first mode of Rsk2 action involves the ability of the NTKD to enhance AF1 activity by phosphorylation of Ser167. The evidence for this conclusion is supported by

Fig. 7. Model of Rsk2 activation. (A) Schematic of Rsk2 activation of ER α -mediated transcription. See text for details. (B) Ribbon representation of the ER α HBD complexed to either estradiol or 4-OHT. These models were generated by RasMol and are based on the coordinates under Protein Data Bank entry codes 1ERE and 3ERT (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). Ligands are represented in green. Residues have been colored pink (301–341), orange (H3), blue (363–371), purple (H5 and H6) and red (H12).

the following observations: (i) the N-terminal kinase-dead Rsk2, that is unable to phosphorylate Ser167, does not enhance ER α -mediated transcription; and (ii) the mutant S167A-ER α has diminished transcriptional activity compared with the wild-type receptor in the presence of constitutively active Rsk2. The ability of Rsk2 to phosphorylate its effector is not regulated by a docking site because the fragment ER α (1–281) and ER α complexed to 4-OHT were phosphorylated as efficiently as the unliganded wild-type ER α . However, Rsk2 phosphorylates the chimera ER $\alpha\beta$ poorly as compared with ER α . We propose that in the context of the holoreceptor, the phosphorylation of Ser167 is sterically hindered by the HBD. Thus Rsk2 docking induces a conformational change in the HBD, which exposes Ser167. Presumably, the conformational change induced by 4-OHT binding also permits Rsk2 access to Ser167. ER α (1–281) is phosphorylated efficiently because the HBD is not present. In the case of the chimera ER $\alpha\beta$, Ser167 is phosphorylated poorly because Rsk2 cannot dock with the HBD of ER β and induce a conformational change.

It is of interest to note that Ser167 is phosphorylated in response to activation of the MAPK pathway but not by hormone addition. Co-activators may interact with ER α differently, depending on whether or not Ser167 is phosphorylated. Thus, ER α -regulated genes may be differentially activated depending on the status of Ser167 phosphorylation.

In the second mode of Rsk2 action, the docking of Rsk2 to the HBD of ER α influences the activity of AF2. This conclusion is based on the following observations: (i) the N-terminal kinase-dead Rsk2 does not dock with ER α , nor activate transcription; (ii) docking of Rsk2 is specific for ER α and not for the related isoform, ER β , thus replacement of the HBD of ER β with that for ER α allows the chimera ER $\beta\alpha$ to be activated to a greater extent by Rsk2 than ER β ; (iii) the ability of Rsk2 to enhance ER α -mediated transcription is influenced by mutations that are known to affect AF2 activity; and (iv) the anti-estrogen 4-OHT, which is known to inhibit AF2 activity, prevents Rsk2 stimulation of ER α -mediated transcription and docking with ER α .

Rsk2 docks at a site between residues 326 and 394 of the HBD of ER α (Figure 5E). Interestingly, estradiol does not influence the ability of Rsk2 to dock with ER α . However, the anti-estrogen, 4-OHT efficiently prevents Rsk2 from associating with ER α . To understand the mechanism by which 4-OHT prevents Rsk2 association with ER α , we examined the crystal structure of the HBD of ER α complexed with either estradiol or 4-OHT (Figure 7B). The striking difference between these two structures is in the location of helix 12 (H12) (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). This helix is known to bind various co-activators and is important for AF2 activity (Mak *et al.*, 1999). In the complex with 4-OHT, H12 occludes part of the co-activator groove, which consists of residues between H3 and H5 (Shiau *et al.*, 1998). In the complex with estradiol, H12 does not occupy the co-activator groove. Thus we suggest that Rsk2 does not bind to ER α complexed with 4-OHT because H12 is blocking the Rsk2 docking site. This hypothesis would predict that residues 342–394 which include H3–H6 are extremely important for Rsk2 binding. This prediction is supported by our

findings that the Rsk2 docking site is between residues 326 and 394.

Sequence alignment between ER α s from different species indicates that the residues comprising the region from 342 to 394 are fairly highly conserved. Thus the allosteric activation of ER α -mediated transcription by Rsk2 may have been conserved through evolution. Sequence alignment between human ER α , glucocorticoid receptor, androgen receptor, progesterone receptor and mineralocorticoid receptors shows low homology in the region from 342 to 394. Thus we would predict that Rsk2 docks with ER α but not with other members of the steroid receptor superfamily. It may be that ER α is a unique member of the steroid receptor superfamily in its ability to be activated allosterically by Rsk2.

Although allosteric regulation of ER α by Rsk2 may be unique among the steroid receptor superfamily, it is unlikely that ER α is the only Rsk2 effector regulated in this fashion. It is possible that this allosteric activation is a general mechanism for Rsk2 action. It is also possible that docking of Rsk2 to its effectors influences Rsk2 activity. This mechanism of allosteric activation by an effector has been reported for the protein kinase PDK1 (Frodin *et al.*, 2000).

In summary, we suggest that in the absence of estradiol, activated Rsk2 interacts with the HBD of ER α , and induces a conformational change, which permits Rsk2 phosphorylation of Ser167. Estradiol is known to induce a conformational change in ER α (Fritsch *et al.*, 1992). We suggest, however, that the conformational switch induced by hormone binding is probably different from that induced by Rsk2 binding. We argue that if Rsk2 and estradiol induced the same conformation, then one would expect to observe an increased affinity of ER α for Rsk2 in the presence of estradiol. The increased Rsk2-binding affinity would result from the reduction in free energy cost for Rsk2 docking in the presence of estradiol, which arises because in this situation the Rsk2 does not have to perform work to induce the conformational change. Therefore, since we did not observe an increase in Rsk2 binding upon estradiol addition, we suggest that the conformational switches produced by Rsk2 and estradiol are different from one another but are not mutually exclusive.

The mechanisms by which Rsk2 and estradiol influence ER α -mediated transcription also appear to be different and independent of one another. This conclusion is based on the observation that constitutively active Rsk2 and estradiol synergistically enhance transcription. Both modes of Rsk2 action appear to be required for this synergism because Rsk2 and estradiol produced only additive responses with the chimeras ER $\alpha\beta$ and ER $\beta\alpha$. One possible model for the synergistic activation is that estradiol and Rsk2 recruit different co-activators, each of which influences RNA polymerase II activity. Another possibility is that Rsk2 phosphorylates a co-activator and that the affinity of this phosphorylated co-activator for ER α is enhanced by the conformational change induced by hormone binding.

Rsk2 transcriptional activation and docking are specific for ER α and do not occur with the related isoform ER β . However, EGF enhances both ER α - and ER β -mediated transcription (Joel *et al.*, 1998b; Tremblay *et al.*, 1999). These results suggest that MAPK is more important for

ER β than ER α activation and suggest a possible mechanism by which EGF could differentially regulate ER α and ER β within the same cell. The temporal expression of MAPK can vary within the same cell type depending on the stimulus (Grewal *et al.*, 1999). Furthermore, these temporal differences result in different cellular responses. Thus, in cells containing both ER α and ER β , a rapid burst of MAPK activity may be just sufficient to activate Rsk2, which could result in ER α stimulation, whereas a sustained elevation of MAPK activity could result in activation of both ER α and ER β .

Finally, we note the exciting possibility that constitutively active Rsk2 may play a role in breast cancer, especially since we observed that constitutively active Rsk2 synergistically enhanced ER α -mediated transcription in the presence of estradiol. We speculate that a constitutively active Rsk2 mutant could influence several pathways that lead to the transformed phenotype, one of which would include the enhancement of ER α -mediated transcription.

Materials and methods

Materials

The monoclonals α -78 and EVG-F9 were provided by Dr Traish (Joel *et al.*, 1998b). The polyclonals anti-pS167-ER α and anti-GFP were provided by Upstate Biotechnology and Dr Silver (Seedorf *et al.*, 1999), respectively. The vectors pMN9, pKR7-GFP and pKR7-myc were donated by Dr Macara (University of Virginia).

Expression vectors and receptor mutants

A schematic of the constructs used in this study is shown in Figure 1. Drs Chambon, McDonnell and Gustafsson provided HEGO (Green *et al.*, 1986), ER α -AF1 (Tzukerman *et al.*, 1994) and pCMV5-ER β (Kuiper *et al.*, 1996), respectively. The HA-tagged Rsk2 and Rsk2(Y707A) and ER α (S167A) constructs were described previously (Joel *et al.* 1998a; Poteet-Smith *et al.*, 1999). All mutant constructs used in this work were produced by PCR and the sequences verified.

Transcriptional analysis

BHK and MCF-7 cells were transfected, treated and assayed for luciferase and β -galactosidase as described previously (Joel *et al.*, 1998a,b).

Immunoprecipitations and immunoblots

For Figures 4, 5B and 6D, BHK cells were transfected in 150 mm dishes with calcium phosphate and 10 μ g of control vector or an expression vector encoding an ER α construct. Additionally, the cells were co-transfected with 15 μ g of control vector or an expression vector encoding the wild-type or a mutant Rsk2. At 19 h post-transfection, the cells were washed with phosphate-buffered saline (PBS) and placed in Dulbecco's modified Eagle's medium (DMEM). After 24 h of serum starvation, the cells were washed with PBS, lysed and ER α was immunoprecipitated with α -78 (Joel *et al.*, 1998a). For Figure 5A, C and D, BHK cells were transfected, lysed and the immunoprecipitates treated as described above except that 17.5 μ g of either control vector or ER construct and 7.5 μ g of either control vector or mutant RSK construct were used in the transfection. Additionally, 25 μ g of the purified monoclonal antibody 12CA5 were used for the immunoprecipitation. Immunoblots were detected using either anti-mouse or anti-rabbit secondary IgGs conjugated to horseradish peroxidase (HRP). To detect ER α (1–281), the anti-ER α monoclonal, EVG-F9 and anti-pS167 were labeled directly with HRP. To detect the myc-tagged constructs, the anti-myc monoclonal, 9e10 was labeled directly with HRP. Antigen-antibody interactions were detected with chemiluminescence reagent.

Acknowledgements

We thank Ian G. Macara for helpful discussions. This work was supported by Research Project Grant #TBE-97523 from the American Cancer Society.

References

- Brzozowski, A.M. *et al.* (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, **389**, 753–758.
- Chen, R.H., Sarnecki, C. and Blenis, J. (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.*, **12**, 915–927.
- Cho, H. and Katzenellenbogen, B.S. (1993) Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators. *Mol. Endocrinol.*, **7**, 441–452.
- Dalby, K.N., Morrice, N., Caudwell, F.B., Avruch, J. and Cohen, P. (1998) Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. *J. Biol. Chem.*, **273**, 1496–1505.
- Enmark, E. and Gustafsson, J.A. (1999) Oestrogen receptors—an overview. *J. Intern. Med.*, **246**, 133–138.
- Fisher, T.L. and Blenis, J. (1996) Evidence for two catalytically active kinase domains in pp90rsk. *Mol. Cell. Biol.*, **16**, 1212–1219.
- Fritsch, M., Leary, C.M., Furlow, J.D., Ahrens, H., Schuh, T.J., Mueller, G.C. and Gorski, J. (1992) A ligand-induced conformational change in the estrogen receptor is localized in the steroid binding domain. *Biochemistry*, **31**, 5303–5311.
- Frodin, M., Jensen, C.J., Merienne, K. and Gammeltoft, S. (2000) A phosphoserine-regulated docking site in the protein kinase Rsk2 that recruits and activates PDK1. *EMBO J.*, **19**, 2924–2934.
- Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P. and Chambon, P. (1986) Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature*, **320**, 134–139.
- Grewal, S.S., York, R.D. and Stork, P.J. (1999) Extracellular-signal-regulated kinase signalling in neurons. *Curr. Opin. Neurobiol.*, **9**, 544–553.
- Ignar-Trowbridge, D.M., Pimentel, M., Teng, C.T., Korach, K.S. and McLachlan, J.A. (1995) Cross talk between peptide growth factor and estrogen receptor signaling systems. *Environ. Health Perspect.*, **103**, Suppl. 7, 35–38.
- Joel, P.B., Smith, J., Sturgill, T.W., Fisher, T.L., Blenis, J. and Lannigan, D.A. (1998a) pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser167. *Mol. Cell. Biol.*, **18**, 1978–1984.
- Joel, P.B., Traish, A.M. and Lannigan, D.A. (1998b) Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. *J. Biol. Chem.*, **273**, 13317–13323.
- Kuiper, G.G., Enmark, E., Peltö-Huikko, M., Nilsson, S. and Gustafsson, J.A. (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl Acad. Sci. USA*, **93**, 5925–5930.
- Mak, H.Y., Hoare, S., Henttu, P.M. and Parkerm, M.G. (1999) Molecular determinants of the estrogen receptor-coactivator interface. *Mol. Cell. Biol.*, **19**, 3895–3903.
- Moller, D.E., Xia, C.H., Tang, W., Zhu, A.X. and Jakubowski, M. (1994) Human rsk isoforms: cloning and characterization of tissue-specific expression. *Am. J. Physiol.*, **266**, C351–C359.
- Pawson, T. and Nash, P. (2000) Protein-protein interactions define specificity in signal transduction. *Genes Dev.*, **14**, 1027–1047.
- Poteet-Smith, C.E., Smith, J.A., Lannigan, D.A., Freed, T.A. and Sturgill, T.W. (1999) Generation of constitutively active p90 ribosomal S6 kinase *in vivo*. Implications for the mitogen-activated protein kinase-activated protein kinase family. *J. Biol. Chem.*, **274**, 22135–22138.
- Sassone-Corsi, P., Mizzen, C.A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A. and Allis, C.D. (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. *Science*, **285**, 886–891.
- Seedorf, M., Damelin, M., Kahana, J., Taura, T. and Silver, P.A. (1999) Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase. *Mol. Cell. Biol.*, **19**, 1547–1557.
- Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A. and Greene, G.L. (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*, **95**, 927–937.
- Smith, J.A., Poteet-Smith, C.E., Malarkey, K. and Sturgill, T.W. (1999) Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK *in vivo*. *J. Biol. Chem.*, **274**, 2893–2898.
- Tremblay, A., Tremblay, G.B., Labrie, F. and Giguere, V. (1999) Ligand-

- independent recruitment of SRC-1 to estrogen receptor β through phosphorylation of activation function AF-1. *Mol. Cell*, **3**, 513–519.
- Tzukerman,M.T., Esty,A., Santiso-Mere,D., Danielian,P., Parker,M.G., Stein,R.B., Pike,J.W. and McDonnell,D.P. (1994) Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol. Endocrinol.*, **8**, 21–30.
- Yntema,H.G. *et al.* (1999) A novel ribosomal S6-kinase (RSK4; RPS6KA6) is commonly deleted in patients with complex X-linked mental retardation. *Genomics*, **62**, 332–343.
- Zhao,Y., Bjorbaek,C., Weremowicz,S., Morton,C.C. and Moller,D.E. (1995) *RSK3* encodes a novel pp90rsk isoform with a unique N-terminal sequence: growth factor-stimulated kinase function and nuclear translocation. *Mol. Cell. Biol.*, **15**, 4353–4363.
- Zhao,Y., Bjorbaek,C. and Moller,D.E. (1996) Regulation and interaction of pp90(rsk) isoforms with mitogen-activated protein kinases. *J. Biol. Chem.*, **271**, 29773–29779.

*Received November 8, 2000; revised April 4, 2001;
accepted May 16, 2001*