



Receptor for Activated C Kinase 1 Protein Binds to and Activates the Human Estrogen Receptor α

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The classical concept of estrogen receptor (ER) activation is that steroid passes the cell membrane, binds to its specific protein receptor in the cell's cytoplasm and the steroid-receptor complex travels to the nucleus where it activates responsive genes. This basic idea has been challenged by results of experiments demonstrating insulin-like growth factor 1 (IGF-1) activation of the ER in the complete absence of estrogen suggesting at least one other mechanism of ER activation not involving steroid. One explanation is that activation of the cell surface IGF-1 receptor leads to synthesis of an intracellular protein(s) able to bind to and stimulate the ER. Based on results using the two-hybrid system, coimmunoprecipitation and transfection-luciferase assays, we herein show that one of these proteins could well be receptor for activated C kinase 1 (RACK-1). Using the human ER type α (ER- α) as bait, a cloned complementary deoxyribonucleic acid (cDNA) library from IGF-1 treated human breast cancer MCF-7 cells was screened for ER- α – protein interactions. Many positive clones were obtained which contained the RACK-1 cDNA sequence. Coimmunoprecipitation of *in-vitro* translation products of the ER- α and RACK-1 confirmed the interaction between the two proteins. Transfection studies using the estrogen response element spliced to a luciferase reporter gene revealed that constitutive RACK-1 expression was able to powerfully stimulate ER- α activity under estrogen-free conditions. This effect could be enhanced by 17 β -estradiol (E2) and blocked by tamoxifen, an E2 antagonist. These results show that RACK-1 is able to activate the ER- α in the absence of E2, although together with the latter, enhanced effects occur. Since RACK-1 gene expression is stimulated by IGF-1, it is distinctly possible that RACK-1 is the mediator of the stimulatory effects of IGF-1 on ER- α .

Key words: Estrogen receptor, breast cancer, MCF-7, receptor for activated C kinase 1

INTRODUCTION

The classical concept of estrogen receptor (ER) activation is that 17 β -estradiol (E2) passes the cell membrane, binds to its specific protein receptor in the cell's cytoplasm and the steroid-receptor complex travels to the nucleus where it activates responsive genes.¹ In recent years, however, it has become increasingly clear that the ER can be activated by other cellular mechanisms. For example, there is evidence that the ER can be associated with the cell membrane and become activated in a similar manner as traditional cell-surface receptors.² Perhaps more significantly, it is clear from *in-vitro* culture studies that insulin-like-growth factor-1 (IGF-1), which acts upon cell-surface receptors, can activate the ER in the complete absence of E2.^{3,4} One possible explanation is that activation of the cell surface IGF-1 receptor leads to synthesis

of an intracellular protein(s) able to bind to and stimulate the ER. Using the two-hybrid system, coimmunoprecipitation and transfection studies on E2-dependent human breast cancer MCF-7 cells, it is herein reported that receptor for activated C kinase 1 (RACK-1), an intracellular G-protein-like molecule,⁵ is able to bind to and activate the human ER- α . As such, and because its gene expression is increased by some peptide growth factors,^{6,7} RACK-1 may well be the mediator of growth factor activation of ER- α .

MATERIALS AND METHODS

Two-hybrid system screen

The Matchmaker SMART Primer yeast two-hybrid system (Clontech, Oxford, UK) was used to identify intracellular protein(s) synthesized by human breast cancer MCF-7 cells, which are able to bind to the alpha form of ER (ER- α). The full-length human ER- α was cloned into pGBDKT7 (pGBDKT-ER) and expressed in yeast cells as shown by Western blot analysis (results not shown). When transcribed, this vector codes for a fusion protein containing the ER- α spliced to the deoxyribonucleic acid (DNA)-binding domain

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(ER-BD) of a transcription factor for a number of yeast reporter genes, including MEL-1 expression. In parallel, messenger ribonucleic acid was extracted from cultured MCF-7 cells grown in the presence and absence of IGF-1, reversed transcribed and subjected to amplification by PCR using SMART primers. The PCR products and linearized plasmid containing the yeast reporter gene(s) activation domain (pGADT7) were cotransfected into yeast cells containing pGBDKT-ER. This system allows generation of a complementary deoxyribonucleic acid (cDNA) library (pGADT7-cDNA) by intracellular recombination (Clontech). Protein interaction with the ER was heralded by growth on Trp-minus agar plates and MEL-1 reporter gene activity, as indicated by development of a blue color when these colonies were incubated with X- α -gal. A selection of positive clones was re-grown in selective nutrient medium and the plasmids extracted according to the Matchmaker manufacturer's instructions. Inserts in the purified plasmids were sequenced by Functional Genomics (University of Birmingham, West Midlands, UK). The screen was performed 4 times, consisting of 100 15 cm petri-dishes for each.

Coimmunoprecipitation

As a confirmatory test for positive interaction, a coimmunoprecipitation and denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure was used as previously described in detail⁸ and compatible with the Clontech plasmids. pGBDKT-ER and a cloned SMART-derived plasmid coding for full-length RACK1 (obtained from the two-hybrid screen) were translated separately using a reticulocyte lysate system (Promega) in the presence of S³⁵-methionine (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions. In this system, c-myc protein is tagged onto the ER-fusion protein to facilitate coimmunoprecipitation studies. The translation products were mixed together, allowed to incubate at RT, and then subjected to coimmunoprecipitation using an antibody against c-myc of the ER fusion protein. Prior to denaturation with SDS, the products were coimmunoprecipitated using an antibody against the c-myc moiety of the ER fusion protein. Denatured sample was then subjected to PAGE together with Rainbow Markers (mw, molecular weight, arrowed) (GE Healthcare) and the gel autoradiographed.⁸

Transfection-luciferase assay

MCF-7 cells (1×10^5) were seeded into 6-well plates. After 24 h, plasmid estrogen response element (ERE)-LUC (1 μ g), a plasmid containing the ERE fused to the luciferase reporter gene,³ or ERE-LUC together with the RACK-1 expressing pCMV-SPORT6 (MRC Geneserve, Mill Hill, London, UK)

(1 μ g each) were transfected into the cells using Fugene Reagent (Roche, Burgess Hill, UK) in a ratio of 6 μ L/ μ g DNA. After 24 h, cells were treated with either basal medium or medium containing E2 (10^{-8} mol/L) and/or tamoxifen (10^{-7} mol/L), as indicated. A modified estrogen-free medium was used, deficient in phenol-red and using charcoal-stripped fetal calf serum.^{3,4} Following 4 h incubation, cells were lysed, mixed with luciferase substrate (Brite-Glo, Promega) and emitted signal assessed using a luminometer.

RESULTS

Two-hybrid screen

Some interacting sequences contained a number of relatively early stop codons in all reading frames and did not match known polypeptides, and were thus probably "false positives." However, the vast majority of clones examined contained large sequences identical to the human RACK-1 gene without intervening intronic regions. An example of partial typical sequences is shown in Figure 1. Moreover, in the four screens examined, there tended to be a higher number (about 3:1) of positive clones when cDNA libraries from IGF-1 treated MCF-7 cells were used. This latter observation could be significant because IGF-1 has been shown to stimulate RACK-1 gene expression and to interact with the IGF-1 receptor.^{6,7}

Coimmunoprecipitation

Denaturing polyacrylamide electrophoresis of the precipitated proteins yielded two bands that migrated approximately to the

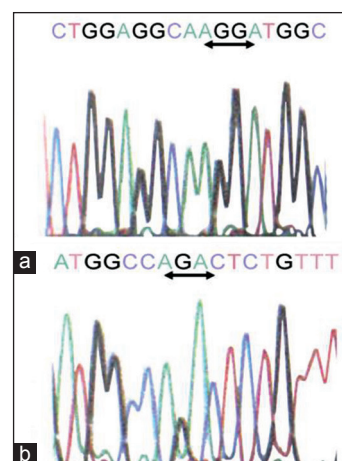


Figure 1. Partial sequences of complementary deoxyribonucleic acid (cDNA) obtained from a clone showing the interaction with the human ER- α in the yeast two-hybrid system. The sequences are identical to the human receptor for activated C kinase 1 cDNA in exons 5 and 6 (5'-CTGGAGGCAAGGATGGC-3' [a]), and 7 and 8 (5'-ATGGCCAGACTCTGTTT-3' [b]). The arrows and highlighted bases show the exon boundaries and intronic regions are clearly absent

predicted molecular weight positions for ER and RACK-1 fusion proteins [70 and 40 kDa, respectively, Figure 2].

Transfection-luciferase assays

As expected, E2 powerfully stimulated luciferase, and thus ER, activity and this effect was considerably reduced by coincubation with tamoxifen [Figure 3]. Of considerable interest, transfection of the cells with RACK-1 also considerably stimulated luciferase activity, strongly suggesting that a RACK-1/ER complex is able to activate the ER. Further support for this latter concept is provided by the observation that tamoxifen abolished this stimulatory effect of RACK-1 [Figure 3]. In further transfection-luciferase studies, the effect of constitutive RACK-1 expression in combination with a maximal dose E2 (10^{-7} mol/L) was investigated [Figure 4]. A powerful stimulatory effect of both RACK-1 and E2 on luciferase activity was observed and, in combination, the effects of both were enhanced to a greater effect when compared to RACK-1 or E2 alone [Figure 4].

DISCUSSION

These results provide strong evidence that RACK-1 is able to bind to and activate the human ER and that there might also be an interaction with E2. In addition, the E2 antagonist, tamoxifen, appears to be able to reduce this effect, even in the complete absence of E2-like activity.

Since its discovery as an intracellular protein with similarities to β -subunits of G-proteins, a whole wealth of potential functions for RACK-1 have been described, including its participation as a scaffold protein,⁹ activator of protein kinase C¹⁰ and binder and activator of inositol trisphosphate receptors.¹¹ Of relevance to the results of the present study, similar binding of RACK-1 to the

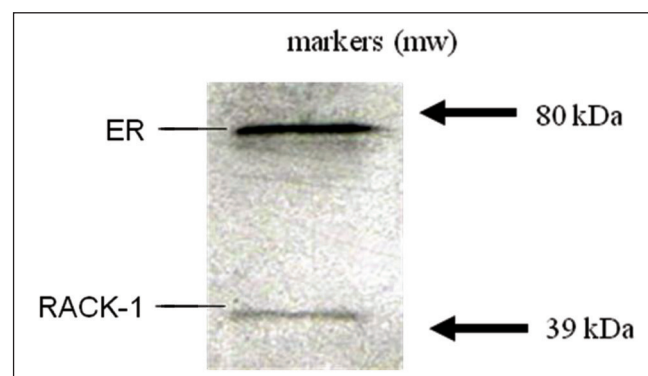


Figure 2. Denaturing polyacrylamide gel electrophoresis of coimmunoprecipitated proteins yielded after incubation together of the *in-vitro* translation products of estrogen receptor (ER) and receptor for activated C kinase 1 (RACK-1). The visible bands migrated approximately to the predicted molecular weight positions for ER and RACK-1 fusion proteins (70 and 40 kDa, respectively)

androgen receptor has also been described,⁹ although in this case, the binding appears to inhibit the activity of the steroid receptor. Nevertheless, the present results confirm a potentially very important interaction between RACK-1 and steroid receptors. As such, they describe a novel intracellular interaction and, since RACK-1 functions and abnormalities have been described in a number of tumorous tissues and, indeed, human breast cancer MCF-7 cells,^{12,13} there may be significance in these findings to the etiology of breast cancer. Moreover, these findings may partially explain the somewhat paradoxical observation that the majority of breast cancers in postmenopausal women are E2-dependent and respond to tamoxifen therapy, despite the fact that available biologically active estrogens are limited in such subjects. It could well be that the ER in breast cancer cells of postmenopausal women responds not only to steroids but also other cellular factors of which RACK-1 is a prime candidate. In addition, as demonstrated by the transfection studies, this effect of RACK-1 is abolished by tamoxifen, perhaps indicating that the antimetastatic effects of this drug are not only through blockade of the effects of E2.

Finally, since the predominant number of clones were obtained from IGF-1 treated cells, and since IGF-1 can stimulate RACK-1 production,⁶ the results suggest that RACK-1 might be able to mediate the effects of IGF-1 on ER activity. Further quantitative studies are required to confirm this latter hypothesis.

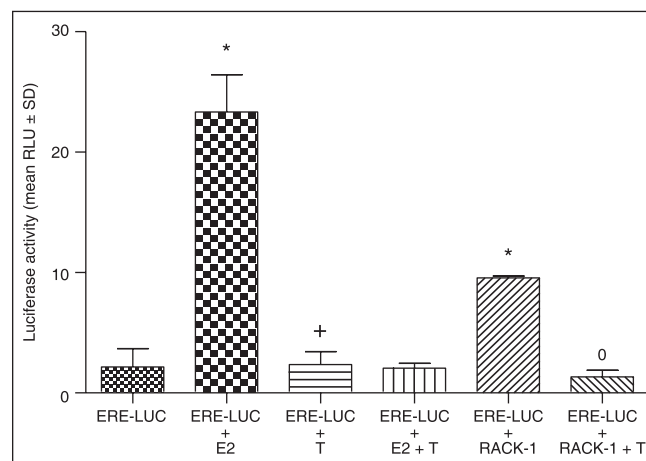


Figure 3. Stimulation of luciferase activity by E2 (10^{-8} mol/L) and cotransfected receptor for activated C kinase 1 (RACK-1) in MCF-7 cells transfected with estrogen receptor (ER)-LUC. Both effects were abolished by tamoxifen (T, 10^{-7} mol/L). Each treatment was tested in triplicate and the results are expressed as mean relative light units $\pm$ SD. Both E2 and RACK-1 had no effect on luciferase activity in cells transfected with an ERE-minus luciferase plasmid (data not shown). Statistical analysis, performed using Prism GraphPad, was by *t*-tests using pooled estimates of error and Bonferroni's correction for multiple group analysis; * $P < 0.001$ versus control (estrogen response element [ERE]-LUC); + $P < 0.001$ versus ERE-LUC + E2; $P < 0.001$ versus RACK-1 + ERE-LUC

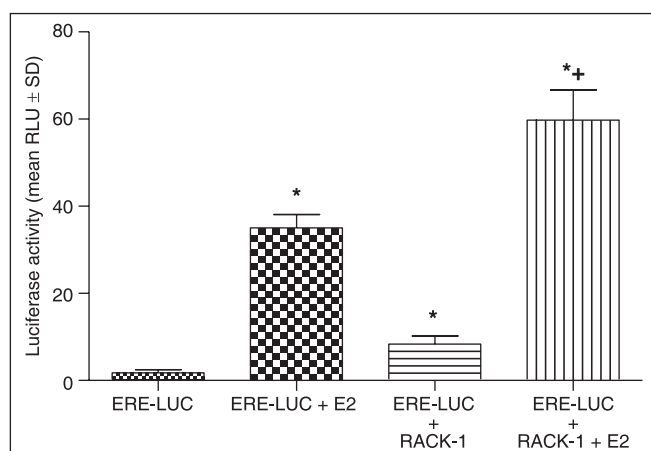


Figure 4. Effect of E2 (10^{-7} mol/L) on luciferase activity in MCF-7 cells transfected with estrogen response element (ERE)-LUC alone and ERE-LUC together with receptor for activated C kinase 1 expressing pCMV-SPORT6. Each treatment was tested in triplicate and the results are expressed as mean relative light units $\pm$ SD * $P < 0.001$ versus ERE-LUC; + $P < 0.01$ versus E2 (10^{-7} mol/L)

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DISCLOSURE

No conflict of interest.

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