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Gene regulation in an MCF-7 cell line that naturally expresses an estrogen receptor unable to directly bind DNA

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Abstract

In the described studies, we have developed a variant of the MCF-7 cell line, M-ERd3/g8, for analysis of 17-β-estradiol (E_2)-action without direct DNA interaction by E_2-receptors. M-ERd3/g8 cells principally express the estrogen receptor α (ER) form ERΔ3 which, due to skipping of exon 3, lacks the second zinc finger of ER that is required for direct DNA interaction. This was achieved by introduction of siRNA targeting exon 3 to a Tamoxifen-selected MCF-7 variant, TMX 2-11, expressing approximately equal amount of full-length ER and ERΔ3 proteins. M-ERd3/g8 cells exhibited a normal nuclear ER localization, and ERΔ3 expression levels were similar to those for full-length ER protein in MCF-7 cells. Ser 118 phosphorylation of the ERΔ3 was triggered by E_2 treatment. The expression of several well characterized E_2-responsive markers was strongly modified in the M-ERd3/g8 cells. The E_2-induction of progesterone receptor (PR) and HEM45 mRNAs was abolished. The effect on pS2 mRNA expression was complex: the pS2 mRNA levels fell ∼50-fold in control M-ERd3/g8 cells. There was E_2-induction of pS2-expression but with an altered temporal pattern. This was blocked by inhibitors of the p42/44 mitogen activated protein (MAP) kinase and inositol triphosphate (PI3) kinase pathways suggesting a role for cytoplasmic signaling pathways.

Gene array analysis and real-time polymerase chain reaction (PCR) studies identified several genes whose expressions were induced in E_2-treated M-ERd3/g8 cells. These included A-Myb, a homolog to the avian myoblastosis virus oncogene, carbonic anhydrase XII (CAXII), chemokine ligand 12 (CXCL-12), early growth response 3 (EGR 3), fibrinogen B β (FibBβ), along with serine protease 23 (SPUVE). The responses fell into several temporal patterns. A-Myb, CAXII, CXCL-12 and EGR 3 were E_2-induced within 2 h. The expression of CXCL-12 and EGR 3 was persistent to 24 h, while that of A-Myb and CAXII was not persistent in M-ERd3/g8 cells. FibBβ and SPUVE expression was not induced until times later than 6 h. Expression of none of the genes was elevated prior to 2 h, but the utilization of a 24 h time point for the gene array analysis may have eliminated the most transiently responsive genes. Immediate early 3 (IE3) was down-regulated by E_2 in the M-ERd3/g8 cells but was transiently up-regulated during the 2–6 h period in MCF-7 cells. Basal levels of several of the genes were strongly reduced in M-ERd3/g8, compared to MCF-7. The studies suggest that M-ERd3/g8 cells provide a new model for studies of E_2-action without direct ER binding to DNA and where E_2-action must be via alternate pathways.

Keywords: Estrogen receptor; Alternate splicing; Gene expression/regulation; siRNA; Real-time PCR analysis; Gene array analysis

1. Introduction

Estrogen action is generally regarded as mediated via the ligand activated nuclear receptors estrogen receptor α (ER) and estrogen receptor β (ER β) (Nilsson et al., 2001). Steroid receptors act as ligand activated signal transducers with a domain for direct binding to specific DNA sites. Interaction of ER with 17-β-estradiol (E_2) affects conformation, and interaction with DNA plus co-regulators, and leads to

Abbreviations: CAXII, carbonic anhydrase XII; CXCL-12, chemokine ligand 12; DC10, 10% DCS in DMEM; DCS, donor calf serum; DMEM, Dulbecco's modified Eagle's medium; E_2, 17-β-estradiol; EGR 3, early growth response 3; ER, estrogen receptor α; FibBβ, fibrinogen B β; IE3, immediate early 3; MAPK, mitogen activated protein kinase; PCR, polymerase chain reaction; PI3, inositol triphosphate; PR, progesterone receptor; RT, reverse transcriptase; SPUVE, serine protease 23

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enhanced transcriptional activation of target genes. In breast cancer, there is often an initial acquisition of increased ER expression, which leads to hormone-dependent growth and the possibility of intervention to block growth signal with antiestrogens and aromatase inhibitors (Osborne, 1998).

There is evidence that E_2 can activate gene expression without direct DNA binding by ER. Non-genomic actions of E_2 include effects on calcium flux that occur too rapidly to be mediated by transcriptional effects of nuclear ER (Nadal et al., 2001). Non-genomic effects are best documented for the central nervous and vascular systems (Falkenstein et al., 2000). Multiple studies have found immunoreactive ER α antigen on the surface of both naturally ER-positive cells (Watson et al., 2002) and in cells transfected with ER expression constructs (Razandi et al., 1999). There are data indicating clustering of membrane ER to caveolae, a finding which supports the association of ER with other specific membrane signaling molecules (Chambliss et al., 2000). ER transfection to CHO cells resulted in detectable membrane ER, which mediated mitogen activated protein kinase (MAPK) action by E_2 and activation of the cAMP and inositol triphosphate (PI3) pathways (Razandi et al., 1999).

A numbers of studies examined interaction of ER with other transcription factors in the absence of ER binding to DNA. Activation of a collagenase promoter lacking an identified ERE involved the AP1 site in the promoter (Webb et al., 1995). There could be direct tethering of ER to other transcription factors via protein–protein interaction. The AP1 component Jun interacted with ER in a mammalian cell two-hybrid screen (Jakacka et al., 2001), and interaction of ER and SP1 in co-immunoprecipitation assays has been reported (Porter et al., 1997). Alternatively, cytoplasmic ER may activate AP1, or other transcription factors, via non-genomic pathways (Bjornstrom and Sjoberg, 2004).

Principal evidence for E_2-action being via the known ER and ER β is correlation between their expression and E_2 effects. This correlation breaks down in some known cases such as in a study of hippocampal neurons from ER knockout mice (Gu et al., 1999) and studies on breast tumor cells such as SKBR cells that lack classical ER and ER β (Filardo et al., 2000).

There are reports that a G-coupled receptor, GPR30, can mediate plasma membrane effects of E_2, transfection of GPR30-negative MDA-MB-231 can confer E_2-activation of membrane based receptors and linked cytoplasmic signaling pathways such as the ERK1&2 MAPK pathway (Filardo et al., 2000). GPR30 transfection to HEK293 lead to acquisition of E_2 binding with the characteristic of a high affinity receptor, and siRNA against GPR30, in [ER, ER β]-negative SKBR3 cells, lead to a reduction in E_2 binding (Thomas et al., 2005). This is fairly good evidence of its action as an alternative estrogen receptors, though there is not yet evidence for direct interaction with the isolated protein.

Our experimental approach to the investigation of E_2-actions that do not require DNA binding is to utilize MCF-7-derived cells expressing a form of ER that cannot directly bind DNA. Transfection of ER constructs, together with suitable reporter constructs, has had good success in defining regions of the ER and specific pathways of ER action. However, there are several limitations on the use of ER expression constructs in cell biology applications. A first problem is that transiently transfected and adenovirus delivered ER has the risk of overloading the cells to which the construct is delivered. However, if delivery is to only a fraction of the cells, then any changes to the phenotype may be masked by the signal from the unmodified cells. A second problem is that ER expressed from expression constructs in ER-negative cells may not fully reflect natural ER function. Studies have generally found reduced growth responses in cells re-transfected with full-length ER expression constructs (Jiang and Jordan, 1992; Zajchowski et al., 1993). In addition, cells expressing transfected ER have generally failed to reactivate classic markers of ER action such as progesterone receptor (PR) (Oesterreich et al., 2001), although we and others have shown that some genes are estrogen-regulated in ER transfected cells (Pentecost, 1998; Oesterreich et al., 2001).

We previously cloned an MCF-7 variant, TMX 2-11, from a Tamoxifen-selected population that expresses approximately equal amounts of full-length 66 kDa ER and the 61 kDa ERΔ3 form (Fasco et al., 2003). Human ER transcripts are prone to aberrant splicing, leading to generation of ER Δ variant transcripts lacking one or more exons (Fasco, 1997; Murphy et al., 1997). ERΔ3 lacks the sequences of exon 3, which encodes one of the ER zinc fingers. This ER has intact protein sequence beyond the skipped exon region as the open reading frame is maintained. ERΔ3 has been shown to not bind DNA when transfected constructs are used (Wang and Miksicek, 1991), but it should still be able to dimerize (Tamrazi et al., 2002). There were several reports that ERΔ3 is a dominant negative inhibitor of ER action (Wang and Miksicek, 1991; Erenburg et al., 1997). However, in our prior study, there was no evidence that the presence of ERΔ3 affected the action of the full-length ER protein. We have co-transfected HeLa cells with ER and ERΔ3 expression constructs and find that an excess of the ERΔ3 construct is required for it to block ER-mediated expression from a reporter construct (see supplementary data).

In the current study, we modified the TMX 2-11 cells in order to probe the action of an ER form that cannot bind DNA. This was achieved by the use of expression constructs that generated siRNA targeting the exon missing from the ERΔ3 variant, resulting in selective removal of the full-length ER. The expression and regulation of ER in these ERΔ3 cells was then studied, and the ability of E_2 to regulate classical and novel estrogen modulated genes was determined.

2. Methods

2.1. siRNA expression constructs

The siRNAs were expressed from pSilencer 1.0 (Ambion, Austin, TX) under the control of a U6 Pol III promoter.