The estrogen receptor α:insulin receptor substrate 1 complex in breast cancer: structure–function relationships

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Background: Insulin receptor substrate 1 (IRS-1) is a signaling molecule that exerts a key role in mediating cross talk between estrogen receptor α (ERα) and insulin-like growth factor 1 (IGF-1) in breast cancer cells. Previously, we demonstrated that a fraction of IRS-1 binds ERα, translocates to the nucleus, and modulates ERα-dependent transcription at estrogen response elements (ERE). Here, we studied structure–function relationships of the ERα:IRS-1 complex under IGF-1 and/or estradiol (E2) stimulation.

Materials and methods: ERα and IRS-1 deletion mutants were used to analyze structural and functional ERα/IRS-1 interactions. IRS-1 binding to ERE and IRS-1 role in ERα-dependent ERE transcription was examined by chromatin immunoprecipitation and gene reporter analysis, respectively. The requirement for IRS-1 in ERα function was tested with RNAi technology.

Results: Nuclear translocation of IRS-1 was induced by E2, IGF-1, and a combination of both stimuli. ERα:IRS-1 binding was direct and involved the activation function-1 (AF-1)/DNA binding domain (DBD) region of ERα and two discrete regions of IRS-1 (the N-terminal pleckstrin homology domain and a region within the C-terminus). IRS-1 knockdown abrogated IGF-1-dependent transcriptional activity of unliganded ERα, but induced the activity of liganded ERα.

Conclusions: ERα/IRS-1 interactions are direct and involve the ERα AF-1/DBD domain and IRS-1 domains mapping within N- and C-terminus. IRS-1 may act as a repressor of liganded ERα and coactivator of unliganded ERα.

Key words: estrogen receptor alpha (ERα), insulin receptor substrate 1 (IRS-1), breast cancer

introduction

Insulin-like growth factor-1 (IGF-1) and 17β-estradiol (E2) have been shown to act in synergy, stimulating breast cancer cell growth and survival [1, 2]. The functional interactions between E2 and IGF-1 signaling systems involve several transcriptional and posttranscriptional mechanisms. For example, IGF-1 can affect estrogen receptor α (ERα) action by enhancing its expression and potentiating its transcriptional activity in a ligand-independent manner [3–7]. On the other hand, E2 can enhance IGF-1 signaling by upregulating the expression of IGF-1 [8], IGF-1 receptor [9], and some IGF-1 binding proteins [10]; ERα also stimulates transcription and enhances stability of insulin receptor substrate 1 (IRS-1), a major IGF-1 signaling molecule [11–13].

IRS-1 is a 130–180 kDa docking protein containing two conserved domains within the N-terminal portion. The PH (pleckstrin homology) domain mediates interactions with phospholipids and proteins containing acidic motifs. The phosphotyrosine-binding (PTB) domain couples IRS-1 with the phosphorylated IGF-1 receptor. The IRS-1 C-terminus contains several serine and tyrosine residues that can modulate its activity. The major intracellular pathways stemming from IRS-1 are activated upon its tyrosine phosphorylation and subsequent recruitment of downstream signaling molecules through Src homology domain-type interactions [14, 15].

Numerous studies have shown that in breast cancer cells, IRS-1 signaling regulates cell proliferation, survival, and drug resistance. IRS-1 is also a key molecule sustaining efficient E2/IGF-1 cross talk [11, 16]. Recently, we described that in addition to its function as a signaling molecule, IRS-1 might affect nuclear processes. Specifically, IRS-1 can be found in the nucleus in breast cancer cells where it can interact with ERα. In breast tumors, nuclear colocalization of IRS-1 and ERα negatively correlated with tumor grade, size, mitotic index, and lymph node involvement in ductal breast cancer tissues [17]. The function of nuclear IRS-1 in the regulation of steroid receptor function is not well defined; our data

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indicated that nuclear IRS-1 can act as transcriptional regulator of liganded ERα at estrogen response elements (ERE) in DNA [18].

In this study, we examined how E2, IGF-1, and the combination of both factors regulate IRS-1 nuclear translocation, its binding with ERα, and its effects on ERα-mediated transcription. Furthermore, using different deletion mutants of IRS-1 and ERα, we characterized structure–function relationships in the ERα:IRS-1 complex.

**methods and results**

**E2 and IGF-1 modulate nuclear translocation of IRS-1 and its recruitment to ERE sites**

Previously, we reported that IRS-1 colocalizes and coprecipitates with ERα in ER-positive MCF-7 cells and that a fraction of IRS-1 can be translocated to the nucleus together with liganded ERα [18]. Here, we asked whether IRS-1 could be transported to the nucleus in response to IGF-1 or IGF-1 plus E2 treatments. Under serum-free medium conditions, IRS-1 was present mainly in the cytoplasm. The addition of E2 for 1 or 4 h significantly increased nuclear abundance of IRS-1 and reduced its cytoplasmic content (Figure 1A). Similar effects were seen with the combination of E2 and IGF-1. IGF-1 alone minimally increased IRS-1 nuclear translocation at 4 h (Figure 1A).

![Figure 1.](image-url)  
**Figure 1.** Insulin receptor substrate 1 (IRS-1) associates with the pS2 ERE motif in insulin-like growth factor 1 (IGF-1) and estradiol (E2)-treated MCF-7 cells. (A) MCF-7 cells synchronized in serum-free medium were left untreated or were treated with 10 nM E2 and/or 20 ng/ml IGF-1 for 24 h. The abundance and localization of IRS-1 was analyzed by western blotting using 50 μg of cytoplasmic and nuclear proteins. The purity of cytoplasmic and nuclear protein markers, respectively. The antibodies (Abs) used were described previously [18]. (B) Chromatin immunoprecipitation assays were carried out as described previously [18]. Briefly, MCF-7 cells were treated with 10 nM E2 and/or 20 ng/ml IGF-1, for 1, 4, 8, 12, and 24 h. Next, the cells were cross-linked with paraformaldehyde and chromatin–protein complexes were immunoprecipitated with a specific IRS-1 Ab. The presence of pS2 ERE in the resulting immunoprecipitates was analyzed by PCR [18].

Next, we analyzed the association of IRS-1 with ERE sequences within the pS2 gene promoter (Figure 1B). We found that E2 stimulated IRS-1 loading on pS2 ERE from 1 h to 12 h, reaching the maximum at 4 h, which was concomitant with the increased nuclear translocation of IRS-1 (Figure 1A and B). On the other hand, IGF-1 stimulation produced two peaks in the IRS-1 binding on pS2 ERE promoter, at 4 h and 12 h. The addition of E2 significantly improved IGF-1-induced recruitment of IRS-1 on pS2 ERE at 4 h. Interestingly, at 8 h, IGF-1 was loaded on pS2 in response to E2 but not under IGF-1 or E2 plus IGF-1, indicating involvement of IRS-1 in IGF-1 signaling at these time points.

**characteristics of the ERα:IRS-1 complex**

To characterize the region of IRS-1 responsible for ERα binding under different stimuli, we employed IRS-1 truncation mutants (depicted in Figure 2A) [15]. The glutathione S-transferase fusion protein incorporating IRS-1 (GST-IRS-1) mutants were incubated with 100 μg of either cytoplasmic or nuclear proteins obtained from MCF-7 cells stimulated with E2 and/or IGF-1, or left untreated. In unstimulated cells, the strongest ERS binding mapped within the first 300 amino acids of IRS-1 (M1); a less efficient binding was also detected with the last 500 amino acids corresponding to the mutants M4 and M5 (Figure 2B). The IRS-1 M1 region contains the PH domain and a portion of the PTB domain [14, 19]. The absence of binding with the M2 mutant, containing 97 amino acids of the PTB domain, indicates that this domain is not involved in ERα:IRS-1 interactions (Figure 2B). ERα binding to IRS-1 M1, M4, and M5 domains occurred under all stimulation conditions (Figure 2C), indicating that these interactions are not affected by conformational changes or phosphorylation induced by stimulation with IGF-1 and/or E2. The question whether ERα:IRS-1 binding is direct or requires other proteins was addressed with the GST-IRS-1 mutants with a synthetic ERα protein. The results demonstrated efficient ERα binding to M1, and to a lesser extent to M4 and M5 in vitro (Figure 2D), indicating that ERα directly interacts with IRS-1.

To map ERα regions involved in IRS-1 binding, we first used ERα deletion mutants lacking the activation function-1 (AF-1)/DNA binding domain (DBD) or activation function-2 (AF-2) domain [20] (Figure 3). Using GST pull-down assays, we demonstrated that IRS-1 binds to AF-1/DBD, but not to AF-2 (Figure 3A and B). Interestingly, stimulation with E2, IGF-1, or both increased AF-1/DBD/IRS-1 binding in the nucleus, decreasing their cytoplasmic interactions (Figure 3A). A more detailed mapping of ERα:IRS-1 interfaces was done using additional GST-ERα truncation mutants (Figure 3C). Specifically, we tested A1 and A2 mutants that include the AF-1 domain, A3 that includes a part of the AF-1 domain and the entire DBD, A4 that covers the AF-2 domain and a part of DBD, and A5 that includes a major portion of the AF-2 domain [21] (Figure 3C). The results confirmed that IRS-1 binds to the AF-1/DBD domain of ERα (Figure 3D).

**effects of IRS-1 knock down on ERα-mediated transcription at pS2 ERE**

To investigate functional interactions between IRS-1 and the ERα AF-1 domain, we employed a luciferase transcription reporter assays (Figure 4). HEK293 cells (ERα negative, IRS-1 positive) were transiently cotransfected with the ERE-responsive luciferase reporter plasmid and a plasmid encoding ERα (pSG5-HeG6, Figure 4B), ERα with C-terminal truncation (encoding ERα AF-1/DBD domain pSG5-HE15, Figure 4C), or AFα with N-terminal truncation (encoding ERα AF-2/DBD domain, pSG5-HE19, Figure 4D), or an empty vector (pSG5, Figure 4A) [22]. To test the role of IRS-1 in ERα-mediated transcription, IRS-1 levels were downregulated by 70% using anti-IRS-1 siRNA, as described before [23]. We observed a significant increase of E2-induced ERE transcription in the absence of IRS-1 (Figure 4B). In contrast, downregulation of IRS-1 reduced ERE
transcription in IGF-1 and IGF-1 plus E₂-treated cells (Figure 4B). IRS-I knock down did not significantly influence ERE-mediated transcription in HeLa cells expressing the AF-2/DBD region of ERα (Figure 4D), while a significant decrease of ERα transactivation was observed in cells expressing the AF-1/DBD region in response to IGF-1 stimulation.

**discussion**

ERα/IGF-1 cross talk is known to influence breast cancer cell proliferation, survival, transformation, migration, and invasion [2, 24, 25]. IRS-1 is a major substrate of the IGF-1 receptor and a crucial molecule mediating ERα/IGF-1 interactions [1, 2, 14]. In breast cancer, IRS-1 overexpression has been associated with the development of the transformed phenotype, hormone independence, and drug resistance [2]. These effects have been attributed to increased IRS-1 tyrosine phosphorylation and potentiation of its signaling through the antiapoptotic Akt pathway [2, 25]. In addition to its conventional role as signal transducing molecule, IRS-1 has been found in the nuclear compartment in several cell types [15, 17, 18, 26, 27]. Recently, we demonstrated that nuclear IRS-1 is present in ERα-positive breast tumors and cell lines.

Figure 3.  Estrogen receptor α (ERα) domains involved in insulin receptor substrate 1 (IRS-1) binding. (A) We expressed and purified the GST-ERα fusion proteins with activation function-1/DNA binding domain or activation function-2 deletions, as described previously [15]. MCF-7 cells were left untreated or treated with 10 nM estradiol, or 20 ng/ml of IGF-1 or both (B) for 24 h and then lysed. Hundred microgram of cytoplasmic or nuclear proteins were precipitated with 10 μg of GST (V) or different GST-IRS-1 truncation mutants coupled to glutathione-Sepharose. IRS-1 and GST content in precipitates were determined by western blotting (WB). Twenty microgram of total lysates were loaded as control (Lysate [Lys]). (C) A more detailed mapping was carried out with shorter GST-ERα fragments. (D) Cell lysates were precipitated with 10 μg of GST (V) or different GST-ERα truncation mutants coupled to glutathione-Sepharose. IRS-1 and GST content in precipitates were determined by WB.
Our results indicated that the interaction between IRS-1 and ERα does not require intermediating proteins as it can occur in vitro between GST-IRS-1 mutants and synthetic ERα. Two binding sites for ERα were mapped on IRS-1. One site mapped within the N-terminal portion of IRS-1 containing the PH domain, while the second localized within the C-terminus of IRS-1. These results are consistent with previously published observations that nuclear IRS-1 can interact with other proteins (e.g. the T antigen of JCV virus) via the PH domain. The binding site for IRS-1 on ERα was mapped in the AF-1/DBD domain that contains several serine residues responsible of ligand-independent transactivation of ERα. However, because the ERα:IRS-1 complex can bind to ERE under E2, which must engage an unoccupied DBD domain, we speculate that ERα binding to IRS-1 is mediated mostly by AF-1.

Nuclear translocation of IRS-1 and its interaction with ERE could be induced by both E2 and IGF-1, but with different dynamics and efficiency. E2 activates continuous presence of IRS-1 on ERE, while IGF-1 stimulates intermittent IRS-1 interaction with these sites. Notably, IRS-1 recruitment to ERE in response to E2 and IGF-1 resembles that of liganded or unliganded ERα, respectively. The differential recruitment of the ERα:IRS-1 complex could be explained by the nature of ERα activation in response to E2 or IGF-1. In particular, E2 directly activates ERα by binding to the AF-2 domain. Instead, activation of ERα by IGF-1 is indirect and mediated by Erk1/2 and Akt kinases that phosphorylate ERα AF-1 domain on serine residues 118 and 167, respectively. Notably, the recruitment of

Figure 4. Insulin receptor substrate 1 is a transcriptional coregulator of estrogen receptor α. The experiments were carried out using HeLa cells that are ERα negative and IRS-1 positive. All transfection mixtures contained the reporter plasmid, ERE-Luc, encoding the firefly luciferase complementary DNA under the control of the TK promoter and three estrogen response element sequences and, as internal control, the plasmid pRL-Tk (Promega) encoding Renilla reniformis luciferase. The cocktail was cotransfected with either the empty vector pSG5 (A), pSG5-HeG0 encoding ERα (B), pSG5-HE15, and pSG5-HE19, code for a C-terminal truncated receptor (activation function-1/DNA binding domain (DBD), amino acids 1–281) (C) and for the N-terminal truncated receptor (activation function-2/DBD, amino acids 179–575) (D), respectively. The luciferase activity was measured using Dual luciferase assay System (Promega Madison, WI). IRS-1 knock down was obtained by transfecting cells with pSilencer IRS-1 plasmid (shIRS1) [23] or with a control scrambled shRNA (Scrambled). Transfections and luciferase assays were carried out as described previously [18]. The results represent mean ± standard deviation of five independent experiments.
IRS-1 on ERE site in response to a combination of IGF-1 and E2 was greater than that seen with either IGF-1 or E2 alone, confirming synergistic effects of both mitogens on ERα.

Finally, we investigated the relevance of IRS-1/ERα interaction in ERα-dependent transcription in response to E2 and/or IGF-1 stimulation. Using IRS-1 RNAi technology, we confirmed that IRS-1 might act as a repressor of liganded ERα on ERE [18]. It is worth noting that the effects of IRS-1 knock down were not noticeable in cells expressing the AF-1 or the AF-2 truncated mutants of ERα. This is in agreement with IRS-1 function since the absence of IRS-1 reduces the recruitment of protein kinases that phosphorylate serine residues within the AF-1 domain inducing ligand-independent activation of ERα [16, 33]. On the other hand, our results indicated that IRS-1 might be a coactivator of unliganded (IGF-1 transactivated) ERα. The negative effects of IRS-1 towards liganded ERα were abrogated under combined E2 plus IGF-1 treatment, indicating that cooperation of both stimuli might be optimal for ERα transcriptional response. In conclusion, our data indicate that IRS-1 interacts directly with ERα in the nucleus of breast cancer cells and plays a key role in the regulation of balanced transcription of liganded and unliganded ERα.

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