Identification of an estrogen receptor α non covalent ubiquitin-binding surface: role in 17β-estradiol-induced transcriptional activity

Valeria Pesiri, Piergiorgio La Rosa, Pasquale Stano and Filippo Acconcia*

Department of Science, Section Biomedical Science and Technologies, University Roma Tre, Viale Guglielmo Marconi, 446, 00146, Rome, Italy

*Author for correspondence (filippo.acconcia@uniroma3.it)

Summary

Ubiquitin (Ub)-binding domains (UBDs) located in Ub receptors decode the ubiquitination signal by non-covalently engaging the Ub modification on their binding partners and transduce the Ub signalling through Ub-based molecular interactions. In this way, inducible protein ubiquitination regulates diverse biological processes. The estrogen receptor alpha (ERα) is a ligand-activated transcription factor that mediates the pleiotropic effects of the sex hormone 17β-estradiol (E2). Fine regulation of E2 pleiotropic actions depends on E2-dependent ERα association with a plethora of binding partners and/or on the E2 modulation of receptor ubiquitination. Indeed, E2-induced ERα polyubiquitination triggers receptor degradation and transcriptional activity, and E2-dependent reduction in ERα monoubiquitination is crucial for E2 signalling. Monoubiquitinated proteins often contain UBDs, but whether non-covalent Ub–ERα binding could occur and play a role in E2–ERα signalling is unknown. Here, we report an Ub-binding surface within the ERα ligand binding domain that directs in vitro the receptor interaction with both ubiquitinated proteins and recombinant Ub chains. Functional analysis reveals that ERα residues leucine 429 and alanine 430 are involved in Ub binding. Moreover, impairment of ERα association to ubiquitinated species strongly affects E2-induced ERα transcriptional activity. Considering the importance of UBDs in the Ub-based signalling network and the central role of different ERα binding partners in the modulation of E2-dependent effects, our discoveries provide novel insights into ERα activity that could also be relevant for ERα-dependent diseases.

Key words: 17β-estradiol, Estrogen receptor, Ubiquitin, Ubiquitin binding domains, Signal transduction

Introduction

Ubiquitination influences the functions of the target proteins by either affecting their stability (i.e. Ub proteolytic functions) or endowing them with additionally signalling properties as well as by creating new surfaces for intra- and intermolecular interactions (i.e. Ub non-proteolytic functions). Thus, Ub is an intracellular messenger, whose nature as a signal resides in the Ub modification. Single Ub moieties (i.e. monoubiquitination) or polyubiquitin chains (e.g. K63-linked chains) attached to the substrate create structural determinants that can be further exploited for molecular interactions. Consequently, cells have evolved Ub receptors that bind to the ubiquitinated protein by non-covalently contacting the Ub modification through specific UBDs and in this way decode, transduce and amplify the ubiquitination signal diversity into regulation of physiological functions (e.g. cell proliferation and migration) (Acconcia et al., 2009; Husnjak and Dikic, 2012; Woelk et al., 2007).

The ERα is a ligand-activated transcription factor that mediates the cellular effects of the steroid hormone E2 through modifications in the pattern of gene expression. Regulation of ERα gene transcription requires the receptor recruitment to target gene promoters either directly through interaction with estrogen responsive elements (i.e. ERE sequences) or indirectly through ERα interaction with other transcriptional factors (e.g. AP-1; Sp-1) (Ascenzi et al., 2006; Smith and O’Malley, 2004). Mounting evidence indicates that ERα activities are modulated by Ub (La Rosa and Acconcia, 2011). Polyubiquitination controls the E2-dependent regulation of 26S-proteasome ERα degradation, which is interlaced with ERα transcriptional activity (La Rosa and Acconcia, 2011; La Rosa et al., 2012). Non-proteolytic Ub functions in E2:ERα signalling also occur and depend on ERα monoubiquitination, which is negatively modulated by E2 and controls E2-dependent cell proliferation (Eakin et al., 2007; La Rosa et al., 2011a; La Rosa et al., 2011b; Ma et al., 2010).

Monoubiquitinated proteins are often Ub receptors that possess at least one UBD (Woelk et al., 2006). Although many different UBDs exist, no specific conservation in terms of UBDs 3D-structure has been recognized. In fact, UbDs appear to share a common ‘shape’: structural folds that have a regular secondary structure (e.g. α-helix and/or Zn2+-finger) are thought to be the only UbDs common features (Dikic et al., 2009; Husnjak and Dikic, 2012; Woelk et al., 2007). Interestingly, ERα biochemical anatomy consists in six modular domains (A to F). The A/B, D and F domains do not display a folded structure, the C domain (i.e. the DNA-binding domain, DBD) consists in two Zn2+-finger motifs and the E domain (i.e. the ligand binding domain, LBD) is composed of 12 α-helices (Ascenzi et al., 2006). On this basis, we speculated that an UbD could be present in ERα. Here, we report the identification of an ERα Ub-binding surface (ERα-UBS) that is required for E2-dependent ERα transcriptional activity.
Results and Discussion
Identification of an Ub-binding surface on ERα

In vitro pull-down experiments were done to investigate the possibility that ERα binds Ub. We found that the A/B and the E domains but not the C domain were able to pull-down ubiquitinated species from total cellular lysates (Fig. 1A). Because the A/B domain is non-structured and displays weak association to ubiquitinated species when compared to the 12 α-helix-containing E domain, we focused on the E domain Ub-binding ability. Fig. 1B shows that this receptor portion is also able to pull-down recombinant K63-linked polyubiquitin chains, thus demonstrating that the ERα E domain non-covalently contacts Ub on the Ub-modified proteins. The E domain also bound recombinant K48-linked polyubiquitin chains but not purified mono-Ub (supplementary material Fig. S1A). Thus, as in the case of other UBDs (Woelk et al., 2007), the ERα Ub-binding surface (ERα-UBS) in the E domain displays preferential Ub-binding ability and could possess a low Ub-binding affinity.

We next investigated which portion of the ERα E domain is necessary for Ub-binding. The N-terminal part of the E domain (i.e. amino acids 301–439) but not the E domain C-terminus (i.e. amino acids 439–547) was able to pull-down ubiquitinated species from total cellular lysates (Fig. 1C) and recombinant K63-linked polyubiquitin chains (Fig. 1D) although with different amount of bound materials with respect to the intact E domain (Fig. 1A,B). This evidence indicates that the region of the ERα E domain that non-covalently associates in vitro with Ub is located within the protein region encompassing the amino acids 301–439. Interestingly, our data indicate that disruption of the ERα E domain reduces the ERα Ub binding abilities. Unfortunately, we could not narrow down a smaller section of the E domain that associated to ubiquitinated species by using rational deletions of the N-terminal protein portion (i.e. 301–439) (data not shown). Thus, it is most likely that the ERα-UBS requires an intact E domain 3D-folding. In turn, in silico molecular modelling experiments would help rationalize the biochemical structural requirements for ERα E domain:Ub interaction.

For these reasons, we decided to introduce specific point mutations within the minimal Ub-binding region (i.e. 301–439) in the context of the full-length E domain in order to find the critical residues required for Ub-binding. The residues I358 and the Q375 are important for in vitro ERα monoubiquitination (Eakin et al., 2007), thus they may be part of the ERα-UBS (Woelk et al., 2006). Because the E domain double-mutated in I358 and Q375 to A (IQAA) is native-like (Eakin et al., 2007) but did not display any significant differences in Ub-binding when compared with the wt

Fig. 1. Ub-binding ability of ERα. (A,C) Schematic of ERα (A, top) and ERα E (C, left) domain deletions are depicted. Amino acid positions and domains are indicated. In vitro pull-down assay using the indicated GST-tagged ERα constructs. GST-fusion proteins were incubated with total cellular lysates extracted from growing HeLa cells (A and C) or with synthetic polyUb2-7 linked by Lys63 (D) and analyzed by immunoblot as indicated. * indicates significant differences with respect to the relative wild-type sample. (B) Top: ClustalW (http://www.ebi.ac.uk/Tools/maa/clustalw2/) alignment of ERα E domain with RNF168 UMI domain (Pinato et al., 2011). Middle: Projected helices using the Helical Wheel Projections software tool (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Bottom: Three-dimensional structure of the human ERα LBD-E2 complex (1ERE) (Brzozowski et al., 1997); E2 has been removed. The structure is drawn in green; conserved amino acids between the ERα E domain and RNF168 UMI domain (Pinato et al., 2011). Middle: Projected helices using the Helical Wheel Projections software tool (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Bottom: Three-dimensional structure of the human ERα LBD-E2 complex (1ERE) (Brzozowski et al., 1997); E2 has been removed. The structure is drawn in green; conserved amino acids between the ERα E domain and RNF168 UMI domain (Pinato et al., 2011).
E domain (Fig. 2A), I358 and Q375 are not necessary for ERα-UBS. Interestingly, bioinformatic analysis identified L428, L429 and A430 in the E domain to have a spatial distribution reminiscent of the UBD called UMI (Pinato et al., 2011), with L428 buried in the E domain core protein matrix and L429 and A430 at least partially exposed to solvent (Fig. 2B). Consistently, while single substitution of L429 or A430 barely affected the Ub-binding abilities of the E domain (Fig. 2C), double mutation of L429 and A430 (L429A,A430G; LAAG) strongly prevented the E domain Ub-binding (Fig. 2C,D). Notably, introduction of the LAAG mutation in ERα did not significantly affect proper receptor folding, as indicated by circular dichroism (CD) on the E-domain GST-fusion proteins (supplementary material Fig. S1B).

Based on this evidence, we report that two Ub-binding surfaces could exist within ERα and that the residues L429 and A430 within the E domain are necessary for Ub-binding. Interestingly, as the A/B and the E domains (Ascenzi et al., 2006) are located at the N-terminus and at the C-terminus of the ERα monoubiquitination sites (K302 and K303) (Eakin et al., 2007) respectively, it is tempting to speculate that avidity-based Ub-binding interactions could direct intra-molecular communications between distant ERα domains. In this respect, structural cross-talk between distant ERα A/B and E domains has been reported to be required for the modulation of ERα activities (Metivier et al., 2002).

Role of ERα-UBS in E2-induced ERα gene transcription

Next, we sought to determine the role of ERα non-covalent Ub-binding on receptor transcriptional activity. Thus, the LAAG mutation was generated in the context of the full length receptor (wt) (Figs 3, 4) and transiently transfected in HeLa or HEK293 cells. Initially, we verified if the LAAG mutation in the context of the full length ERα reduces Ub-binding also in cells by evaluating if wt ERα was able to associate to ubiquitinated proteins in cells. The anti-Ub blot performed after wt ERα immunoprecipitation resulted in a clear smear only in HEK293 transfected with wt ERα but not in non-transfected HEK293 cells (Fig. 3A). Because ubiquitinated ERα interactors can in principle associate to the receptor both directly through the Ub-based modification and through a different protein surface (Woelk et al., 2006) and the anti-Ub antibody cannot distinguish between these two possibilities, we also performed wt ERα immunoprecipitation by treating the lysates with SDS in order to reduce the amount of receptor interactors (Woelk et al., 2006). Under these conditions, wt ERα was still able to associate with ubiquitinated proteins although less efficiently than in the non-treated lysates (Fig. 3A), thus demonstrating that ERα binds ubiquitinated proteins in cells. Prompted by these results, we next tested the ability of the LAAG mutant receptor to associate with ubiquitinated proteins in cells under the above-mentioned stringent conditions. Immunoprecipitation analysis revealed that introduction of the double mutation L429A,A430G in ERα strongly prevents the receptor ability to associate with ubiquitinated proteins (Fig. 3B). These data demonstrate that the ERα-UBS directs Ub-binding also in cells.

Next, we determined whether the reduction in ERα Ub-binding could affect basal ERα transcriptional activity. As shown in Fig. 3C, equal expression of either wt ERα or of ERα LAAG...
mutant triggered the activation of the artificial ERE-containing reporter constructs (i.e. 3xERE-TATA, pERE) (La Rosa et al., 2012) to comparable levels. ERα binds DNA to ERE sequences as a homodimer (Ascenzi et al., 2006) and co-immunoprecipitation analysis revealed that both wt and mutant receptors were equally able to dimerize (Fig. 3D), thus further suggesting that receptor dimerization is not affected by the LAAG mutation. Moreover, ERα-based signalling is a function of receptor intracellular localization (i.e. nuclear and extra-nuclear) (Ascenzi et al., 2006; La Rosa et al., 2012). Thus, we also tested the effect of LAAG mutation on ERα sub-cellular distribution. Immunofluorescence analysis showed that both GFP-ERα and GFP-ERα LAAG mutant have the same nuclear, cytoplasmic and membrane-tethered localization (Fig. 3E). Thus, we conclude that mutation of L429 and A430 residues does not influence basal ERα transcriptional activity (e.g. ERE binding), receptor dimerization and sub-cellular localization.

Analysis of the ability of E2 to trigger ERα transcriptional activity revealed that the introduction of the LAAG mutation in ERα strongly reduced, with respect to the wt ERα, the receptor ability to activate the 3xERE-TATA reporter constructs as a dose-dependent function of E2 (Fig. 4A) without affecting E2 binding affinity (Table 1). This effect was still evident when the E2-induced ERα LAAG mutant transcriptional activity was tested in the presence of a reporter construct containing the ERE sequence in the context of the protein complement 3 (pC3) natural promoter (La Rosa et al., 2011b) (Fig. 4B). Besides the direct association of ERα to ERE located in the target gene promoters (Reid et al., 2003), the E2-activated receptor can regulate the transcription of non-ERE containing genes (e.g. cyclin D1) through indirect association with other transcription factors (e.g. AP-1, Sp-1) (Ascenzi et al., 2006; Marino et al., 2002; Marino et al., 2003). Even in this case, mutation of the L429 and A430 residues within ERα prevented the E2-induced wt ERα mediated cyclin D1 promoter activation (pD1) (Fig. 4C). Therefore, mutation of the ERα-UBS prevents the E2-dependent activation of direct and indirect ERα transcriptional activity. Finally, we investigated the physiological expression of the endogenous E2-responsive Bcl-2 gene (Accorcia et al., 2005b). Dose-response analysis revealed that the LAAG mutation reduced the ability of the E2:ERα complex to increase the Bcl-2 cellular levels as compared with the wt ERα (Fig. 4D). Taken together, these data demonstrate that the reduction in ERα non-covalent Ub-binding results in an overall lower ERα transcriptional activity.

In conclusion, we identify here the structural determinants required for ERα to non-covalently associate to Ub and further demonstrate the involvement of the ERα-UBS in the E2-induced ERα transcriptional activity. E2-dependent productive gene transcription involves the sequential formation of macromolecular complexes centred on ERα (Manavathi et al., 2012; Tarallo et al., 2011). Many of the ERα binding partners are either ubiquitinated proteins or enzymes of the ubiquitination cascades (i.e. Ub-ligases) (La Rosa and Accorcia, 2011; Métivier et al., 2012).

Table 1. E2 binding to wild-type and mutant ERα

<table>
<thead>
<tr>
<th>IC50 (M)</th>
<th>Kit</th>
<th>ERα Rec</th>
<th>ERα wt</th>
<th>ERα LAAG</th>
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<tr>
<td>E2</td>
<td>1.0×10^-8</td>
<td>3.0±0.3×10^-8</td>
<td>7.0±0.9×10^-10</td>
<td>2.6±0.4×10^-10</td>
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</table>

In vitro ERα affinity (as indicated by the IC50 value) was determined using the HitHunter EFC assay (Discovery) according to manufacturer instructions for E2 towards either recombinant ERα protein (ERα Rec), wild-type or LAAG mutant receptors (ERα wt or ERα LAAG, respectively) extracted from transfected HEK293 cells. Kit indicates the value of E2 affinity to ERα as reported in the manufacturer’s specification, and has been introduced as a positive control reference; the value is similar to that measured with ERα Rec (Panvera). Results show that the E2 affinity to ERα is the same as that for the mutant ERα LAAG. Overall affinity of ERα wt and ERα LAAG with respect to ERα Rec can be ascribed to the fact that ERα wt and ERα LAAG E2 binding abilities were measured in equivalent amounts of total cellular lysates.
et al., 2003; Reid et al., 2003). Thus, our discoveries open the possibility that the assembly of E2-dependent ERα-based macromolecular complexes could be due to the presence of ERα UBS (present results), ERα monoubiquitination (Eakin et al., 2007; La Rosa et al., 2011a) or both. Remarkably, the ERα extranuclear signalling also integrates and controls receptor transcriptional activity in the modulation of the E2-dependent cellular processes (e.g. proliferation) (Ascenzi et al., 2006; La Rosa et al., 2012). Thus, ERα Ub-binding could play critical roles in the modulation of ERα nuclear and extranuclear activities and of E2-regulated cellular processes.

Materials and Methods

Cell culture and reagents

HeLa and Hek293 cells were grown as previously described (La Rosa et al., 2011b). Specific antibodies against FLAG epitope (M2) and vinculin (Sigma, St. Louis, MO, USA); anti-ERα (HC-20)-anti-Bel-2 (C-2) and anti-ubiquitin (P4D1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. All other antibodies were purchased from Cell Signalling Technology Inc. (Beverly, MA, USA). All the products were from Sigma-Aldrich (St. Louis, MO, USA). Analytical or reagent grade products, without further purification, were used.

Plasmids and constructs

The reporter plasmid 3xERE TATA, protein complement 3 (pC3) and the pXP2-D1-2966 plasmid (pD1) and the pDNA FLAG-ERα have been described (La Rosa et al., 2011b). The pEGFP-ERα was obtained by subcloning the ERα ORF from the pCGS-HED (Acconcia et al., 2005a) into the pEGFP-C2 obtained by Dr Simona Polo, IFOM, Milan.

Site-directed mutagenesis of the ERα L429 and A430 residues were performed by using the QuikChange kit (Stratagene, La Jolla, CA) and the following oligonucleotides: 5′-GAGATCTCTCACATGCCTGGGCTACATCATCGTGGTTC-3′ (for single L429A mutation) on the wt ERα ORF used as template; 5′-ATCTTCGACATGCTGCTGG-3′ (9 for amino acid sequence data). Plasmids were then sequenced to verify the introduction of the desired mutations. pGEX-6P-3 ERα E domain I358A, Q375A was subcloned from pET151-ERα LBD I358A, Q375A generously provided by Dr Rachel Klevit (Eakin et al., 2007). To engineer constructs harboring ERα deletions domains, PCR amplification was performed with the oligonucleotides in supplementary material Table S1.

PCR products were then cloned into pGEX-6P-3 (obtained by Dr Simona Polo, IFOM, Milan) using BamHI/XhoI sites. All constructs were sequence verified.

GST pull-down assays and circular dichroism

GST fusion proteins were expressed, purified and as described in (Belenghi et al., 2003) except that all E2-ERα domain encoding constructs were prepared in the presence of 20 μM E2. Pull-down experiments were performed as previously reported (La Rosa et al., 2011b; Penengo et al., 2006) by incubating 30 μg of GST-fusion proteins with either 300 ng of growing HeLa cells total lysate for 3 hours at 4°C or with 0.5 μg of polyUb-2 linked by Lys63 (Boston Biochem) for 1 hour at 4°C in the presence of 20 μM E2. All experiments were normalized by running 1/10 of the pull-down on an SDS-PAGE gel. Proteins were detected by Comassie Brilliant Blue staining (Com.). For circular dichroism analysis GST-fusion proteins were eluted and recovered from the beads by treatment with 20 mM free glutathione in 50 mM Tris HCl (pH 9) buffer. The elution buffer, which is unsuitable for spectral measurement in the far UV region, was exchanged by dialyzing the proteins (two times) against 100 volumes of 25 mM sodium phosphate (pH 7.5), 150 mM NaF at 4°C, for 10 hours.

UV absorption spectroscopy

The molar extinction coefficients at 280 nm (ε280) of GST-WT and GST-LAAG fusion proteins (476 residues, 54.8 kDa), calculated by the method of Gill and von Hippel (Gill and von Hippel, 1989), resulted to be 65,110 cm⁻¹ M⁻¹. The proteins were centrifuged (16,100 g, 10 minutes, 15,000 rpm) to remove traces of scattering particles, and their absorption spectra were recorded on an Agilent HP8453 diode array spectrophotometer, using 1 cm quartz cell. Samples were perfectly transparent at all wavelengths greater than 300 nm. The protein concentrations were measured by reading the absorption at 280 nm.

Circular dichroism measurements

CD spectra in the far-UV region were recorded at 16°C using a Jasco J-600 spectropolarimeter and 0.05 cm quartz cells. GST-WT and GST-LAAG fusion proteins were measured at a concentration of 0.075 mg/ml. For each sample, nine sequential spectra were recorded and averaged. Data are shown, without smoothing, in terms of mean molar ellipticity per residue.

Cellular and biochemical assays

Protein extraction, immunoprecipitation assays and western blots as well as HeLa and Hek293 cells transfection, luciferase assays and confocal microscopy analysis were performed as previously described (La Rosa et al., 2011b).

Statistical analysis

Statistical analysis was performed using the ANOVA test with the InStat3 software system (GraphPad Software Inc., San Diego, CA). In all analyses, P<0.01 was considered significant, but for densitometric analysis P<0.05 was considered significant. Data are means±s.d. of three independent experiments.

Author contributions

V.P. performed pull-down, transfection, western blot analysis, confocal microscopy, binding and circular dichroism experiments; P.L.R. performed the dimerization assay; P.S. performed circular dichroism and analyzed the relative data; F.A. conceived the idea and planned the research, prepared the plasmids, performed the transfection and binding experiments, analyzed the data and wrote the paper.

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Supplementary material available online at http://jcs.biologists.orglookup/suppli doi/10.1242/jcs.123307/-/DC1

References


**Fig. S1.** (A) In vitro pull-down assay using the indicated GST-tagged ERα constructs. GST-fusion proteins were incubated with synthetic polyUb2-7 linked by Lys48 or mono ubiquitin and analyzed in immunoblot as indicated. (B) Circular dichroism spectra of GST-WT and GST-LAAG fusion proteins in 25 mM sodium phosphate (pH 7.5), 150 mM NaF, 16 °C.
Table S1. List of oligonucleotides used.

<table>
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<th>Construct (amino acids)</th>
<th>ABC (3-256)</th>
<th>C (181-256)</th>
<th>E (301-547)</th>
<th>ERα 301-439</th>
<th>ERα 439-547</th>
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<tr>
<td>Primer Reverse</td>
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