IUBMB *Life*, 63(1): 49–53, January 2011

Research Communication

17β-Estradiol Regulates Estrogen Receptor α Monoubiquitination

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Summary

Monoubiquitination is a nonproteolytic signal involved in a network of several different physiological processes. Recently, monoubiquitination has been discovered as a new post-transductional modification of the estrogen receptor α (ERα). However, at present no information is available about the role of the cognate ligand 17β-estradiol (E2) in modulating this receptor post-transductional modification. Thus, we studied the E2-dependent modulation of ERα monoubiquitination in different cell lines. Here, we report that ERα monoubiquitination is negatively modulated by E2. These results demonstrate that ERα monoubiquitination represents a new signalling modification that may modulate the E2:ERα-regulated cellular processes. © 2011 IUBMB

IUBMB ***Life*, 63(1): 49–53, 2011**

Keywords monoubiquitination; estrogen receptor; breast cancer; 17β-estradiol.

Abbreviations YY buffer, Yosef Yarden buffer; DCC, dextran-coated charcoal; EDTA, ethylenediaminetetra acetic acid; EGTA, ethylene glycol tetraacetic acid; GST, gluthation-S-transferase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

INTRODUCTION

The estrogen receptor α (ERα) mediates the pleiotropic effects of the sex hormone 17β-estradiol (E2). At present, there is a general agreement that the E2:ERα signaling is finely-tuned by receptor post-translational modifications, which impact on ERα functions and are modulated by E2 (*1–4*).

Among others (*e.g.,* phosphorylation, palmitoylation, methylation, and acetylation), ERα polyubiquitination (polyUbq) has long been recognized as a mean to regulate ERα turnover through the 26S proteasome (*5–7*). Indeed, ERα polyUbq increases on E2 binding and, as a consequence, ERα degradation occurs (*6*).

More recently, ERα monoubiquitination (monoUbq; *i.e.,* the attachment of one single Ub moiety to the receptor) has been found *in vitro* as a function of the activity of the BRCA1/BARD1 complex (*8, 9*). Interestingly, ERα monoUbq has been observed in cell lines also, but only under conditions in which either ERα or BRCA1 is exogenously overexpressed (*9*). Until now, these experimental settings have precluded the analysis of the endogenous E2-dependent modulation of ERα monoUbq. Therefore, to determine if E2 regulates ERα monoUbq, we used the double Ub binding domain of the ubiquitin (Ub) receptor Rabex5 (*10*) to capture ubiquitinated proteins from breast cancer cells. Here, we report that ERα monoubiquitination occurs endogenously in breast cancer cells and is negatively modulated by E2.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

All cell lines were grown as in ref. *11*. Specific antibodies against vinculin and flag epitope (Sigma, St. Louis, MO); ERα (D12) and Ub (P4D1; Santa Cruz Biotechnology, Santa Cruz, CA); and anti-ERβ (GeneTex) were used. The anti-Ub, Lys48-specific, and Lys63-specific antibodies were purchased by Millipore (Billerica, MA). The recombinant ERα and ERβ, the Alexa Fluor 488® and 456®, were purchased from Invitrogen (Madison, MA).

Cellular and Biochemical Assays

Before any cellular and biochemical assay, cells were grown in 1% dextran-coated charcoal medium for 24 h and then stimulated with E2 (10^{-8} M), or cycloheximide (CHX; 1 μg/mL) at the indicated time points. Cells were lysed in Yosef Yarden buffer (50 mM HEPES at pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM ethylenediaminetetra acetic acid, and 1 mM ethylene glycol tetraacetic acid) plus protease, and phosphatase inhibitors. Proteins were transferred on a nitrocellulose membrane (Whatman). Western blot, immunoprecipitation, and autoradiography were performed as in refs. *10* and *11*.

Additional Supporting Information may be found in the online version of this article.

Received 14 October 2010; accepted 3 December 2010

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ISSN 1521-6543 print/ISSN 1521-6551 online
DOI: 10.1002/iub.414

Gluthation-S-Transferase (GST) Pull-Down Assays

GST fusion proteins were expressed and purified as described and pull down experiments were performed as reported previously (*10*) by incubating 20 μg of GST-fusion proteins with 1 nM of human recombinant ERα for 1 h at 4 °C or 300 μg of proteins for 3 h at 4 °C. All experiments were normalized by running 1/20 of the pull down on an Sodium dodecyl sulphate - polyacrylamide gel electrophoresis gel. Proteins were detected by Comassie Brilliant Blue staining.

Confocal Microscopy Analysis

MCF-7 was stained with anti-Ub (1:30) and anti-ERα (1:30) antibodies following the procedure described in ref. *10*. For sequential costaining, cells were first stained for Ub, then the cells were blocked with normal goat serum (1%)-bovine serum albumin (2%) for 1 h and then further stained for ERα. Confocal analysis was performed using LCS (Leica Microsystems, Germany).

RESULTS

The ERα Colocalizes With Ub

To unravel the topology of the ERα modification with Ub, confocal microscopy experiments were performed in ductal carcinoma cells (MCF-7), which express endogenous Ub and ERα. Figure 1A (upper row) shows that Ub and ERα partially colocalized in exponentially growing MCF-7 cells. Moreover, the internal control of the sequential costaining demonstrates the specificity of the merged signals (Fig. 1, lower row). However, the anti-Ub antibody used for the staining procedure does not distinguish between the different Ub-based modifications (*i.e.,* monoUbq and polyUbq) (*12*). Therefore, two different anti-Ub antibodies, which selectively recognize either Lys48-based or Lys63-based Ub chains (*13*), were used to discriminate between monoUbq and polyUbq. Notably, neither the Lys48-based nor the Lys63-based Ub chain-dependent signal superimpose to the ERα-dependent signal (Fig. 1B). Therefore, these observations indicate that under basal conditions monoUbq could be the Ub-based ERα modification.

Endogenous ERα is Monoubiquitinated

To study the endogenous ubiquitination of the ERα in breast cancer cells, the region (*i.e.,* 75 amino acids) encompassing the double Ub binding domain (*i.e.,* RUZ:MIU) of the Ub receptor Rabex5 fused in frame with GST (S75) (*10*) was employed as a powerful Ub-binding reagent. Pull down experiments were performed using S75 as the bait to capture ubiquitinated proteins extracted from ERα-positive (*i.e.,* MCF-7) breast cancer cells. This reagent was able to pull down ERα from untreated MCF-7 cells but not from the ERα-negative HeLa cells (Fig. 2A). To confirm the specificity of the ERα signal, MCF-7 cells were further treated for 24 H with the protein synthesis inhibitor CHX, which is known to reduce the ERα cellular content

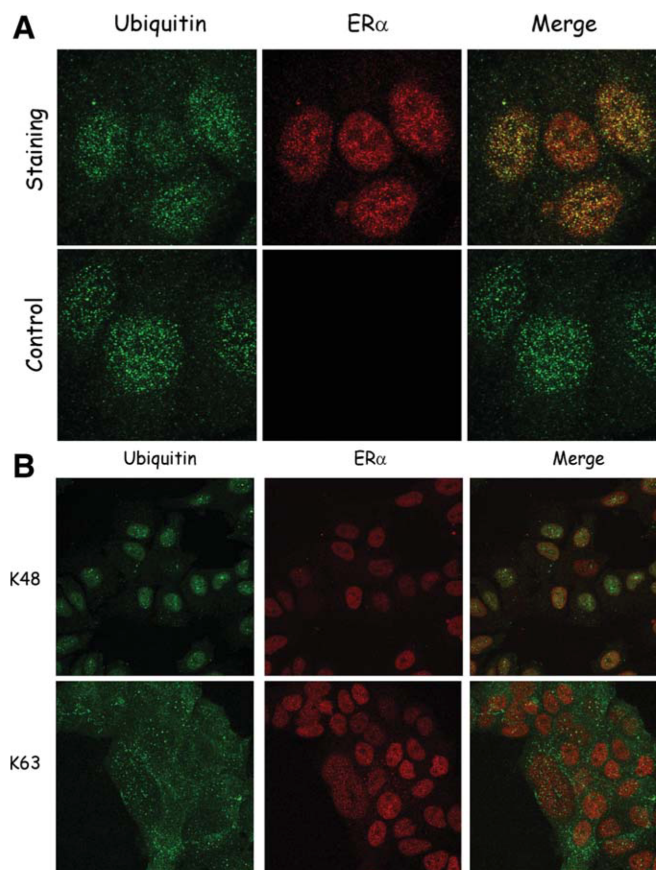

Figure 1. ERα and Ub colocalization. Confocal microcopy analysis of MCF-7 cells kept in growing conditions and stained for Ub (green) or ERα (red) detected by the corresponding secondary antibodies (upper row) and without using the ERα primary antibody and using both secondary antibodies (lower row) (A). Confocal microcopy analysis of MCF-7 cells kept in growing conditions and stained for both K48-based or K63-based Ub chains (green) or ERα (red) (B).

(*5*) (Fig. 2A, inputs). CHX treatment prevents the ability of S75 to pull down ERα from MCF-7 cells (Fig. 2A). In addition, S75 was able to pull down ERα also from the ERα-devoid human embryonic kidney 293 cells transiently endowed with the flag-tagged ERα (Supporting Information Fig. 1A). Thus, these data strongly confirm the specificity of the ERα:S75 interaction.

To exclude that ERα was pulled down through receptor association with other ubiquitinated proteins, a pull down assay was done by using S75 as the bait and the commercially available human recombinant purified ERα protein. As shown in Fig. 2B (middle panel), the S75 pulled down the recombinant ERα also. Remarkably, the ERα pulled down by S75 immunoreacted with an anti-Ub antibody (Fig. 2B, upper panel) also, thus demonstrating S75 binds ERα because of its Ub binding ability (Supporting Information Fig. 1B). Interestingly, Ub immunopre-

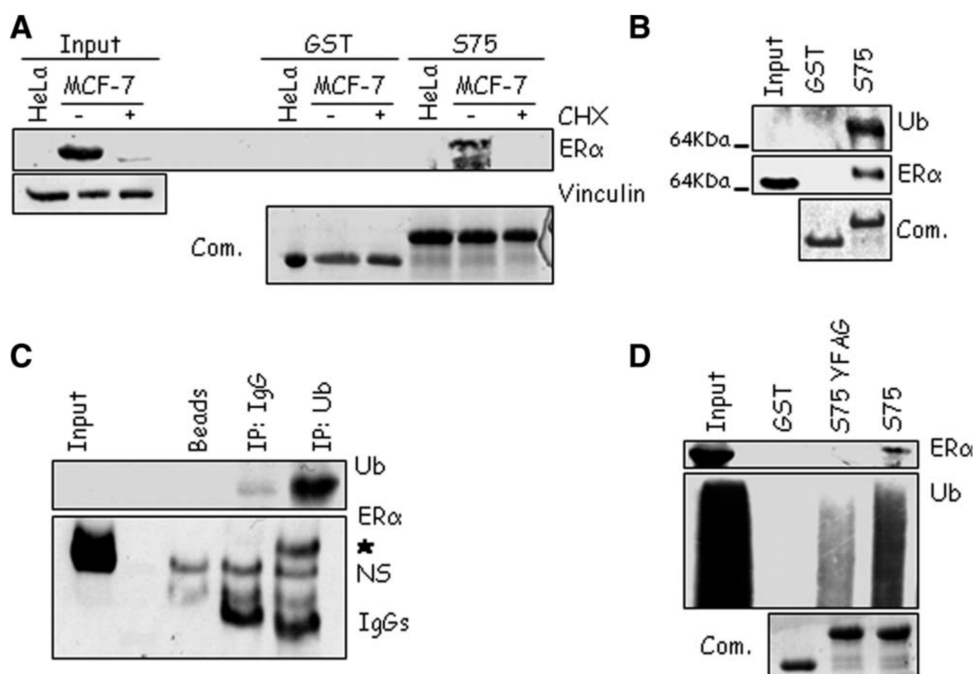

Figure 2. MonoUbq of the ERα. Western blot analysis of pull down assays were done using GST as negative control and the GSTRUZ:MIU fragment of the Rabex5 (S75) (A, B, and D) or the GST-RUZ:MIU fragment of the Rabex5 (S75) mutated in Y25F, A58G (D) immobilized on GSH beads as baits. Fusion proteins were incubated either with protiens extracted from HeLa cells or MCF-7 cells treated 24 H with CHX (A) and then analyzed by immunoblot. D: GST, GST-S75 YFAG, and GST-S75 were incubated with proteins extracted from exponentially growing MCF-7 cells and then analyzed by immunoblot. GST and GST-RUZ:MIU fragment of the Rabex5 (S75) were incubated with human recombinant ERα (B). Bound proteins were analyzed by Western blot using either an anti-ERα and an anti-Ub (B) antibody. (C) Immunoprecipitation of human recombinant ERα was performed either with the anti-Ub antibody, with unrelated IgGs or control protein A beads-only as negative controls. Immunoprecipitated proteins were analyzed by Western blot using either an anti-ERα and an anti-Ub antibody. The star (*) indicates the ERα immunoreactive band; NS: nonspecific. Figures show representative blots of two independent experiments.

cipitation of the human recombinant ERα further revealed that a fraction of this purified receptor is monoubiquitinated (Fig. 2C).

To definitely confirm this notion, proteins extracted from MCF-7 cells were further subjected to a pull down assay by using as baits both S75 and mutant S75 (Y25F, A58G), which displays a reduced ability to bind to ubiquitinated species (*11*). Figure 2D shows that the binding of S75 to ERα was prevented by the S75 Y25F, A58G mutation. As expected the S75 Y25F, A58G pulled down a reduced amount of ubiquitinated species with respect to the wt S75 (Fig. 2D). Notably, no ERα association to GST was detected and all the above described differences do not depend on a different quantity of both GST and GST-S75 (Figs. 2A, 2B, and 2D). These data strongly demonstrate that a fraction of the endogenous ERα is monoubiquitinated in MCF-7 cells under basal conditions.

The Endogenous ERα MonoUbq is Modulated by E2

Next, we asked whether E2 may modulate endogenous ERα monoUbq. To this purpose, both MCF-7 cells and another ductal carcinoma cell line (T47D-1) were treated with E2 and then subjected to the S75-based pull down assay. Remarkably, in unstimulated MCF-7 and also in T47D-1 cells monoubiquitinated ERα was pulled down by S75, thus indicating that ERα monoUbq occurs in different ERα-containing cells. Interestingly, in the presence of E2, ERα monoUbq was reduced (Fig. 3). Notably, no ERα association to GST was detected,

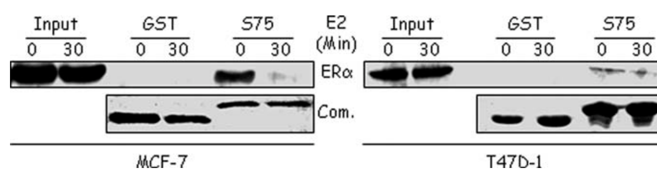

Figure 3. E2-dependent modulation of ERα monoUbq. Western blot analysis of pull down assays were done incubating GST and the GSTRUZ:MIU fragment of the Rabex5 (S75) immobilized on GSH beads with proteins extracted from MCF-7 or T47D-1 cells treated with E2 and then analyzed by immunoblot. Figures show representative blots.

S75 bound to ubiquitinated proteins (data not shown) and all of the above described differences do not depend on a different quantity of both GST and GST-S75 (Fig. 3). These data demonstrate that ERα is endogenously monoubiquitinated in cells and that E2 rapidly reduces ERα monoUbq in breast cancer cells.

DISCUSSION

The main goal of this work was to define if ERα monoUbq is influenced by E2. The ERα ubiquitination has been extensively studied by exploiting experimental settings in which either the ubiquitination machinery (*i.e.,* Ub and ligases) or the substrate (*i.e.,* ERα) are over-represented with respect to the physiological conditions or the 26S proteasome is chemically inhibited (*e.g.,* MG-132) [see for example, refs. *14* and *15*)]. Although these approaches are well established and informative, those tools can strongly affect the overall balance of the Ub-based network (*13*) in turn producing conclusions with incomplete physiological relevance.

For these reasons, we decided to study the ERα ubiquitination by minimizing the exogenous manipulation of the Ub system endogenous components, and thus we employed the double Ub binding domain (RUZ:MIU; *i.e.,* S75) of the Rabex5 (*10*) as a powerful Ub-binding reagent for both monoubiquitinated and polyubiquitinated proteins. By using this alternative approach, we were able to not only confirm that ERα is monoubiquitinated (*9*) but also demonstrate that endogenous ERα monoubiquitination occurs in breast cancer cells (present data).

Moreover, this method allowed us to demonstrate for the first time the E2-dependent modulation of this new ERα post-traslational modification. In particular, we found that E2 diminishes ERα monoUbq in two breast cancer cell lines. Remarkably, as this E2 effect occurs in a time frame (30 min) that is not consistent with the induction of the E2-triggered ERα degradation (2 h) (*5*), monoUbq does not function as a proteolitic mark in ERα signaling. On the contrary, it is well known that E2 increases ERα polyUbq (*6*). This apparent contradiction can be reconciled by considering that monoubiquitination of proteins represent an intracellular signal, which does not lead to 26S proteasome-mediated degradation (*16*) but is rather necessary to regulate several cellular processes (*e.g.,* endocytosis, intracellular trafficking).

The monoubiquitinated receptor appears to localize in the nucleus of breast cancer cells in distinct nuclear spots (Fig. 1). Particulate ERα nuclear localization corresponds to sites where the receptor is transcriptionally active (*17*). Therefore, it is tempting to speculate that basal monoUbq may serve to address ERα to those nuclear sites where transcription needs to efficiently take place after E2 stimulation (*5, 17*). Thus, it is anticipated that ERα monoUbq will play critical roles in the modulation of both the ERα activities and the E2-regulated cellular processes.

ACKNOWLEDGEMENTS

This study was supported by grants from Ateneo Roma Tre (to F. A.) and Ministero della Salute (Strategico 2008; to M. M.). The authors wish to thank Dr. Isabella Ponzanelli, IFOM, Milan, Italy, for the generous gift of MCF-7, T47D-1, and HeLa cells; Dr. Lorenza Penengo, University of Piemonte Orientale, Novara, Italy, for the generous gift of pGEX-6P2-S75, pGEX-6P2-S75 Y25F, and A58G plasmids; and Dr. Chiara Cenciarelli, University Roma Tre, Roma, Italy, for helpful discussion on confocal microscopy data.

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