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Regulatory function of the P295-T311 motif of the estrogen receptor α - does proteasomal degradation of the receptor induce emergence of peptides implicated in estrogenic responses?

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The way in which estrogen receptor α (ER α) mediates gene transcription and hormone-dependent cancer cell proliferation is now being largely reconsidered in view of several recent discoveries. ER α -mediated transcription appears to be a cyclic and transient process where the proteasome - and thus receptor degradation - plays a pivotal role. In view of our recent investigations, which demonstrate the estrogenic activity of a synthetic peptide corresponding to a regulatory motif of the receptor (ER α 17p), we propose that ER α proteasomal degradation could induce the emergence of regulatory peptide(s). The latter would function as a signal and contribute to the ER α activation process, amplifying the initial hormonal stimulation and giving rise to sustained estrogenic response.

Received December 21st, 2007; Accepted April 1st, 2008; Published April 18th, 2008 | **Abbreviations:** **AF:** activation function; **AR:** androgen receptor; **CaM:** calmodulin; **CHIP:** carboxyl terminus of Hsp70-interacting protein; **E2:** 17 β -estradiol; **E6-AP:** E6-associated protein; **EFP:** estrogen-responsive finger protein; **ERR:** estrogen related receptor; **ER α :** estrogen receptor α ; **LBD:** ligand binding domain; **MDM2:** murine double minute 2; **MHC:** major histocompatibility complex; **TAZ:** tamoxifen aziridine; **UPS:** ubiquitin proteasome system | Copyright © 2008, Gallo et al. This is an open-access article distributed under the terms of the Creative Commons Non-Commercial Attribution License, which permits unrestricted non-commercial use distribution and reproduction in any medium, provided the original work is properly cited.

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Introduction

Estrogen receptor α (ER α) is commonly depicted as a transcription factor which, once bound to the appropriate hormone (e.g., 17 β -estradiol; E₂), alters the expression of target genes. However, in view of discoveries made over the last decade, this simplistic concept must be reconsidered since the transcription of estrogen-regulated genes is actually a transient and cyclic mechanism involving successive recruitments and dissociations of a large number of corepressors and coactivators (see [Metivier et al., 2006] for review). These coregulators, which modulate ER α activity to the same extent as cognate ligands, should not be viewed as "accessory proteins", but rather as products of particular genes, historically called "master genes" [Britten and Davidson, 1969; O'Malley, 2006], that orchestrate coherent and synchronized events (see [Leclercq et al., 2006] for review). According to this view, the search for drugs able to specifically interfere with coregulator recruitment may open new therapeutic avenues for the treatment of ER α -related diseases [Galande et al., 2005; Geistlinger and Guy, 2003; Leduc et al., 2003; Norris et al., 1999; Rodriguez et al., 2004]. In order to design such compounds, one must have a clear understanding of the molecular mechanisms underlying the formation of active ER α oligomeric structures.

In this Perspective, we describe the regulatory function of the ER α P₂₉₅-T₃₁₁ amino-acid sequence, which harbors

a binding site for calmodulin (CaM) [Gallo et al., 2007a], a coregulator playing a role of major importance in the ER α mechanism of action (see [Li and Sacks, 2007] for review). In addition, we propose that ER α degradation product(s), including the P₂₉₅-T₃₁₁ sequence, may contribute to the activation process of the receptor.

Link between proliferation and ER α downregulation

Although involvement of ER α in the growth of hormone-dependent breast cancers has been clearly established, ER α expression in primary breast cancers is generally associated with a favorable prognosis, as compared to the ER α -negative phenotype. It is noteworthy that high amounts of ER α are correlated with a low expression of proliferation markers [Jensen et al., 2001], clearly indicating an inverse relationship between receptor level and cell proliferation.

An inverse correlation between ER α stability and ERE-dependent gene expression has also been reported, leading to the widely held - albeit subject to controversy [Alarid et al., 2003; Callige et al., 2005; Fan et al., 2003; Fan et al., 2004] - concept that the ubiquitin proteasome system (UPS) contributes to ER α -mediated gene transcription [Laios et al., 2005; Lonard et al., 2000; Reid et al., 2003].

The ER α P₂₉₅-T₃₁₁ sequence is a regulatory platform

The P₂₉₅-T₃₁₁ sequence, located between the D- (hinge) and E- (Ligand Binding Domain; LBD) domains, appears to be involved in both the stability and the transcriptional activity of the receptor. This short sequence, actually situated in the AF-2a (autonomous activation function) domain [Norris et al., 1997; Pierrat et al., 1994], can be considered as a platform for various posttranslational modifications such as phosphorylation [Lee and Bai, 2002; Wang et al., 2002], acetylation [Wang et al., 2001], SUMOylation [Sentis et al., 2005] and monoubiquitination [Eakin et al., 2007; Heine and Parvin, 2007]. This motif also contains the third nuclear localization signal of the receptor [Picard et al., 1990; Ylikomi et al., 1992], as well as a proteolysis site [Seielstad et al., 1995]. Finally, as stated above, the P₂₉₅-T₃₁₁ sequence includes a binding site for CaM [Bouhoute and Leclercq, 1995; Castoria et al., 1988; Gallo et al., 2007a; Garcia Pedrero et al., 2002; Li et al., 2005], a coregulator which enhances both the transactivation [Biswas et al., 1998; Garcia Pedrero et al., 2002; Li et al., 2003; Li et al., 2005] and the stabilization of the receptor [Castoria et al., 1988; Li et al., 2001] by impeding its E6-AP- (E6-Associated Protein) mediated polyubiquitination [Li et al., 2006]. It is obvious that these dual effects do not fit with the concept of a relationship between ER α -induced transcription and receptor proteolysis.

In order to further elucidate the role of the P₂₉₅-T₃₁₁ segment, we have synthesized a peptide with the same sequence: ER α 17p (P₂₉₅LMIKRSKKNLSLALSLT₃₁₁). Surprisingly, this peptide elicits estrogenic responses in ER α -expressing breast carcinoma cells [Gallo et al., 2008; Gallo et al., 2007a; Gallo et al., 2007b]. Thus, ER α 17p stimulates both cell proliferation and ERE-dependent transcription. This is associated with receptor downregulation, occurring through an increase of ER α degradation rate and a decrease of ER α mRNA level (Figure 1). Interestingly, it should be stressed that the latter response is typical of that elicited by agonist ligands. In spite of the fact that ER α 17p binds to CaM and inhibits its association with ER α , its estrogenic action cannot be totally ascribed to a CaM-dependent mechanism since two ER α 17p analogs, unable to associate with CaM, displayed estrogenic properties with a slightly higher efficiency [Gallo et al., 2007a].

The mechanism by which ER α 17p and its analogs operate is not established as yet, although their intracellular penetration seems to be required (see below). In this regard, it should be stressed that ER α , like other nuclear hormone receptors, is subject to a constant trafficking between various intracellular compartments and targets, especially in the absence of (anti)estrogenic stimulation [Kumar et al., 2006; Leclercq et al., 2006; Maruvada et al., 2003; Pick et al., 2007; Stenoien et al., 2000]. Hence, we may logically propose that our peptides may interfere with this process, as described for conventional ligands. Note, however, in this context we do not know if a particular or all forms of ER α (nuclear,

cytoplasmic, membrane-associated, posttranslationally modified, etc.) are implicated in the mode of action of ER α 17p.

Molecular mechanisms leading to ER α 17p-induced estrogenic effects

Recent investigations from our laboratory have revealed that ER α 17p binds to purified recombinant human ER α [Gallo et al., 2007b], most likely disrupting intramolecular interactions suspected to confer upon the receptor an inactive conformation (i.e., association between the S₃₀₅-T₃₁₁ sequence and the β -turn/H4 composite motif [Jacquot et al., 2007]). The additional finding that, in MCF-7 cells, ER α 17p dissociates by competition with ER α -Hsp70 complexes [Gallo et al., 2008], suggests a complementary mechanism that may contribute to the agonistic property of the peptide. According to these observations, we assume that the inactive, chaperone-stabilized, unliganded ER α adopts a structure where the hinge region is "closed" by an Hsp70-stabilized intramolecular interaction between the S₃₀₅-T₃₁₁ sequence and the β -turn/H4 motif. Agonist-induced Hsp dissociation destabilizes this inactive folding, provoking the "opening" of the hinge with a concomitant exposure of the P₂₉₅-T₃₁₁ motif. CaM, by associating with this motif, would stabilize the activated ER α oligomeric structure. This proposal, which of course requires further experimental validation, could be extended to other coregulators which modulate molecular interactions involving the P₂₉₅-T₃₁₁ sequence.

Hence, ER α 17p and its analogs, regardless of their ability to interact with CaM, would activate ER α by relieving an auto-inhibitory folding and provoking concomitantly the dissociation of Hsp70. Likewise, we assume that CaM would produce a similar conformational change, accounting for the antiestrogenic activity of CaM antagonists.

The ER α P₂₉₅-T₃₁₁ motif has a repressive function

The model outlined above postulates the existence of an autoinhibitory function associated with the ER α P₂₉₅-T₃₁₁ sequence. In support to this view, we and others have observed that partial or complete deletion of the P₂₉₅-T₃₁₁ sequence leads to receptor mutants characterized by a constitutively high level of transcriptional activity [Gallo et al., 2007a; Li et al., 2005]. Even in the absence of E₂, these mutated receptors appear unstable, most likely because of rapid degradation. This could be explained by their potential inability to establish association with Hsp70. Interestingly, a motif with a repressive activity has also been recently localized in the hinge region of the human androgen receptor (AR) (i.e., R₆₂₉KLKLLGN₆₃₆ sequence; Figure 2A) [Haelens et al., 2007], suggesting that other nuclear receptors may be subject to such autoregulation.

Genetic arguments support this concept of ER α autoinhibition. Indeed, a missense point mutation resulting

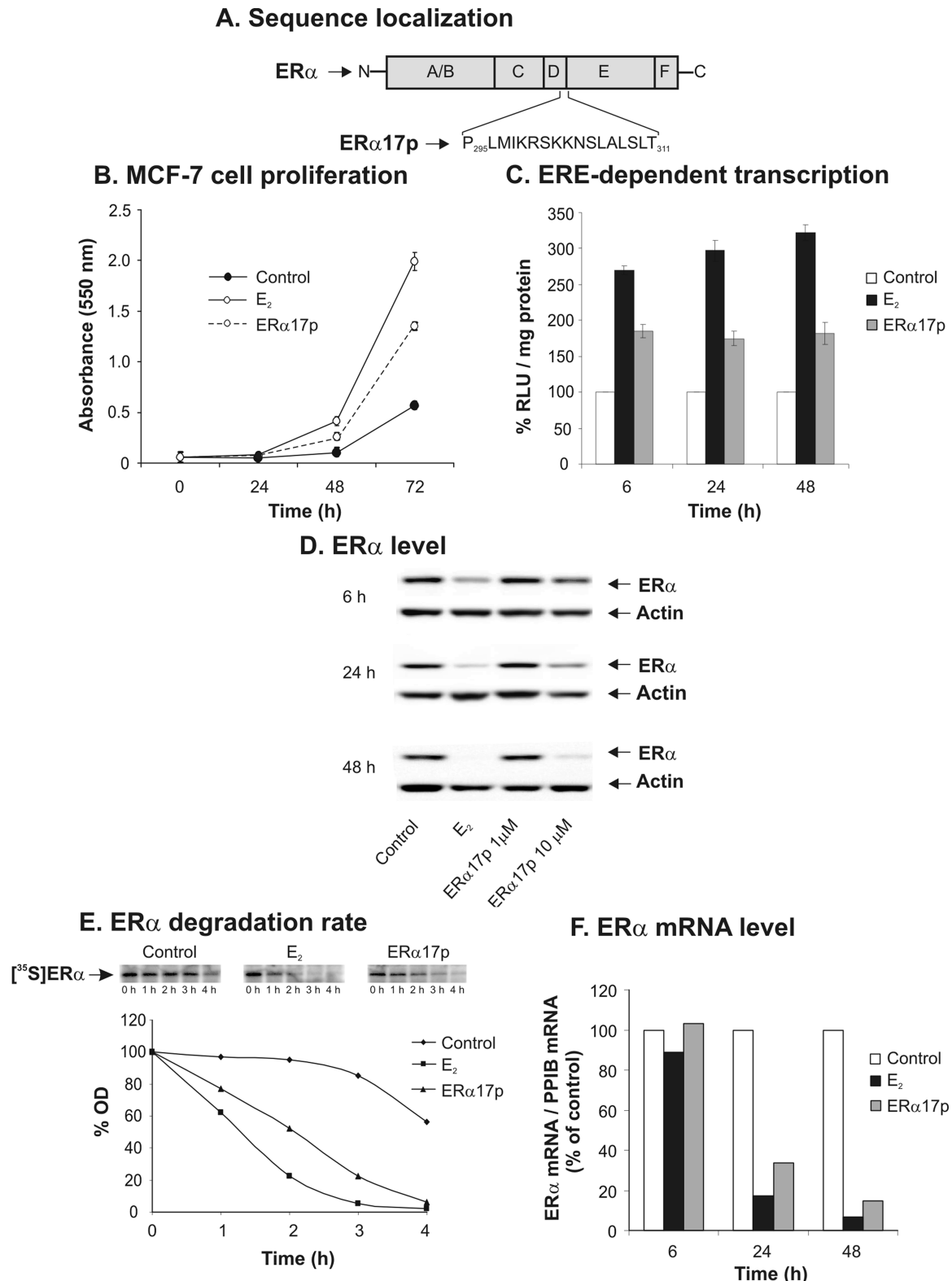


Figure 1. Agonistic properties of ER α . A. Localization of the P₂₉₅-T₃₁₁ sequence within ER α . B. MCF-7 cell proliferation. Cells were grown in the absence (control) or presence of ER α 17p at 10 μ M or E₂ at 0.1 nM taken as a reference. Cell growth was measured by crystal violet staining. C. ERE-dependent transcription. MVLN cells (MCF-7 cells stably transfected with a pVit-tk-Luc reporter plasmid (Pons et al., 1990) were incubated in the absence (control) or presence of ER α 17p at 10 μ M or E₂ at 0.1 nM. Luciferase activity, assayed by luminometry, was normalized according to protein concentration in cell extracts. D. ER α level. MCF-7 cells were cultured in the absence (control) or presence of ER α 17p at 1 and 10 μ M or E₂ at 0.1 nM. ER α and actin levels in cell extracts were determined by Western blot analysis. E. ER α degradation rate. [³⁵S]methionine labeled MCF-7 cells were maintained in culture without (control) or with ER α 17p at 10 μ M or E₂ at 1 nM. Immunoprecipitated [³⁵S]ER α was submitted to SDS-PAGE, revealed by autoradiography and quantified by measuring band intensities. F. ER α mRNA level. MCF-7 cells were incubated in the absence (control) or presence of ER α 17p at 10 μ M or E₂ at 0.1 nM. ER α mRNA levels were measured by NASBA (Nucleic Acid Sequence Based Amplification; (Compton, 1991; Verjat et al., 2004) and normalized according to PPIB mRNA levels. Figures A, B, C and D were adapted from (Gallo et al., 2007a) (Mol Cell Endocrinol 268, 37-49), figures E and F from (Gallo et al., 2008) (J Steroid Biochem Mol Biol, doi:10.1016/j.jsbmb.2007.12.012).

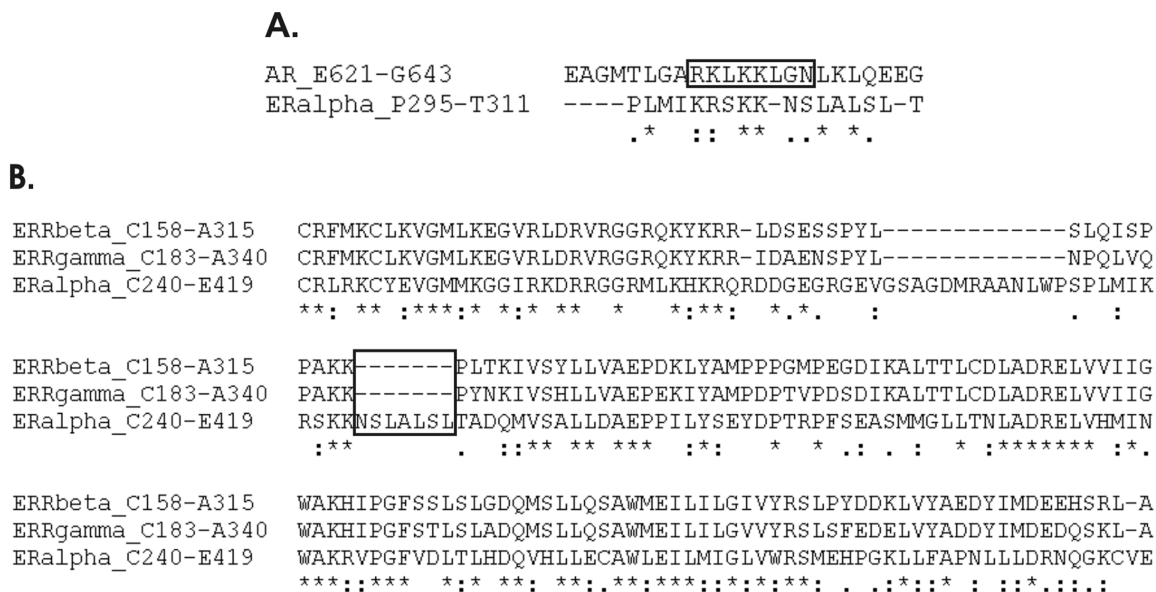


Figure 2. Sequence alignment of AR, ERR β and ERR γ with ER α . A. AR E₆₂₁-G₆₄₃ versus ER α P₂₉₅-T₃₁₁. The AR inhibitory motif (R₆₂₉KLKGLN₆₃₆) delineated by Haelens et al. (Haelens et al., 2007) is boxed. B. Orphan receptors ERR β (C₁₅₈-A₃₁₅) and ERR γ (C₁₈₃-A₃₄₀) versus ER α (C₂₄₀-E₄₁₉), gaps within the orphan receptor regions corresponding to the C-terminus part of the ER α P₂₉₅-T₃₁₁ sequence are boxed. Sequences were downloaded from the ExPASy Proteomics Server (<http://au.expasy.org>) (accession numbers: ER α : P03372, AR: P10275, ERR β : O95718 and ERR γ : P62508). Alignments were carried out using T-Coffee server (<http://tcoffee.vital-it.ch>) (Notredame et al., 2000). Hyphens (-) indicate a one-residue gap, stars (*) notify identical amino acids, colons (:), and full points (.) highlight amino acids that belong to a 'strong' and a 'weak' group, respectively.

in a K303R substitution was shown by Fuqua et al. to increase the sensitivity of the receptor to E₂ [Fuqua et al., 2000; Herynk and Fuqua, 2004]. This functional ER α alteration, which could be explained by a decrease of the autoinhibition exerted by the P₂₉₅-T₃₁₁ sequence, has been found associated with high tumor grade [Conway et al., 2005]. On the other hand, according to sequence alignments (Figure 2B), orphan estrogen receptors (Estrogen receptor-Related Receptors; especially ERR β and γ) display a gap in the region corresponding to the ER α P₂₉₅-T₃₁₁ sequence, a characteristic which might explain the constitutive activity of these receptors.

Hence, one could speculate that drugs which enhance the repressive action of the P₂₉₅-T₃₁₁ sequence may be of therapeutic interest for the treatment of ER α -related diseases, especially in cases of antiestrogen resistance.

Is an endogenous production of ER α 17p-like peptides conceivable?

Direct interaction of a ligand with ER α is not an absolute prerequisite for its proteasomal degradation, as demonstrated with cells labeled with [³H] tamoxifen aziridine ([³H]TAZ), a partial antiestrogen which reacts covalently with the hormone binding pocket of ER α (Cys 530) [Harlow et al., 1989; Robertson et al., 1981], blocking access of other ligands [Borras et al., 1994; Borras et al., 1996; Katzenellenbogen et al., 1983]. Thus, in MCF-7 cells, accumulated ER α covalently liganded with [³H]TAZ vanishes upon treatment with conventional ligands, a property explained by the fact that ligand binding to newly synthesized receptors generates a signal appropriate for inducing [³H]TAZ-ER α degradation [Borras et al., 1996]. Similarly, a small percentage of ligand-bound receptor

generates a response which also affects unliganded receptors, since very low amounts of E₂ (sub-K_d doses) or short E₂ pulses are sufficient to produce optimal transactivation, as well as the degradation of the whole receptor pool [El Khissiin et al., 2000; Gyling and Leclercq, 1988; Otto, 1995]. In fact, such behavior suggests the existence of (a) molecular signal(s) able to amplify the cellular response to hormonal stimulation. Could (a) peptide(s) derived from the hormone-induced proteasomal processing of ER α constitute such (a) signal(s), the "missing link" connecting receptor degradation and transactivation?

In view of the estrogenic activity of ER α 17p, one may reasonably wonder whether there is a physiological, proteasome-mediated production of peptide(s) with similar estrogenic properties. Insofar as ER α -dependent transcription is impeded by inhibitors of proteasome [Laios et al., 2005; Lonard et al., 2000; Reid et al., 2003], it seems logical to surmise that ER α breakdown which accompanies transactivation generates peptide(s) that, like ER α 17p, could be involved in the receptor activation process (Figure 3). From a general point of view, one may propose that the UPS not only eliminates misfolded, damaged or obsolete proteins, but also generates signaling peptides.

If our hypothesis is correct, pure antiestrogens such as fulvestrant would not induce the emergence of such peptide(s) with stimulatory properties. The observation that the binding of pure antiestrogen causes the receptor to be processed by other UPS components - as compared to agonist binding - supports this view. Estrogen- and pure antiestrogen-induced proteasomal degradation indeed occur in different cellular compartments: while the

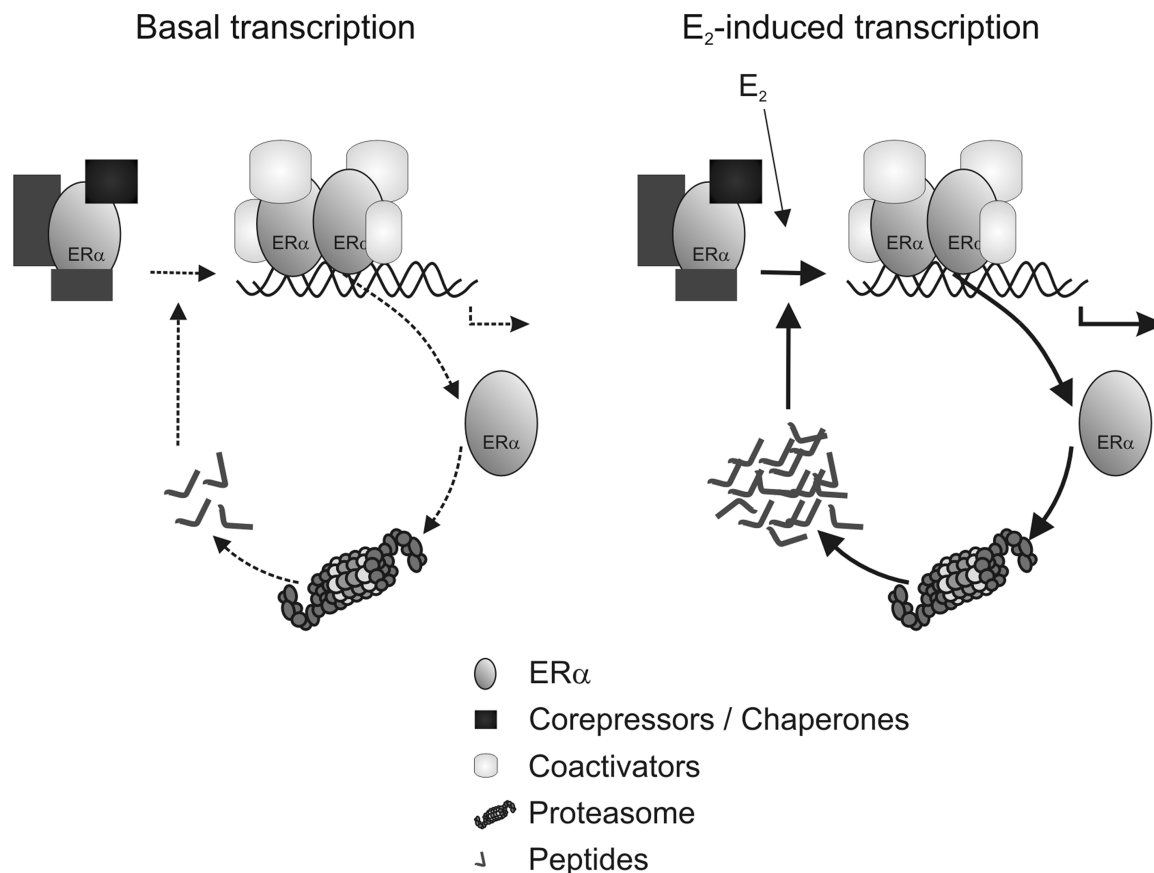


Figure 3. Induction of ER α activation/degradation cycles by putative peptides generated by the proteasomal degradation of ER α . In the absence of hormone, ER α -induced gene transcription is maintained at basal level by a low production of peptides originating from receptor proteasomal degradation. Agonists enhance the production of such peptides, giving rise to sustained ER α activation/degradation cycles. This enhancing signal would persist until receptor mRNA depletion.

first takes place in the cytosol, the latter implies a nuclear proteasome system [Callige et al., 2005; Nonclercq et al., 2007], suggesting that agonists and antagonists may generate dissimilar ER α proteolytic profiles. In this regard, it should be stressed that several ways have been described by which ER α is polyubiquitinated and thus tagged for proteasomal degradation. While CHIP (Carboxyl Terminus of Hsp70-Interacting Protein) exclusively polyubiquitinates unliganded ER α [Fan et al., 2005; Tateishi et al., 2004], MDM2 (Murine Double Minute 2) seems to promote both basal and E $_2$ -associated ER α degradation [Duong et al., 2007; Saji et al., 2001]. By contrast, EFP (Estrogen-responsive Finger Protein) and E6-AP ubiquitin ligases seem to be good candidates for the specific processing of agonist-bound receptor [Khan et al., 2006; Li et al., 2006; Nakajima et al., 2007; Nawaz et al., 1999].

In summary, we assume that agonist-induced proteasomal degradation of ER α may generate a positive feedback loop involving receptor degradation product(s). In this context, the UPS would not only contribute to promoter clearance, a step required for initiation and progression of new transcription cycles [Lonard et al., 2000; Reid et al., 2003], but also enhance ER α activation. Even if our hypothesis is only based on indirect evidence, such a mechanism of response amplification is not unheard of. Indeed, a peptide (receptorphin)

corresponding to a transmembrane segment of the opioid receptors has been reported to activate the latter [Kampa et al., 2001]. Interestingly, these receptors are, like ER α , subjected to proteasomal degradation under agonist stimulation [Chaturvedi et al., 2001]. Hence, our hypothesis could apply not only to ER α and/or other nuclear receptors, but also to a number of UPS-targeted proteins. Assessment of function and fate of proteasome-processed peptides would provide important information in this regard.

Perspectives

In order to test our hypothesis, we are currently trying to decipher the peptidome of MCF-7 cells under various experimental conditions (e.g., treatment with agonists or pure antiestrogens). To date, studies carried out by using a classical LC-MS/MS approach have failed to reveal the presence of ER α 17p-like peptides. Such a methodology, commonly used for detection of easily ionizable peptides in relatively high amounts, seems to be inappropriate in our case (hypothetical non-tryptic peptides in low concentrations). To alleviate this drawback, we have considered using N-terminal sulfonation because it facilitates the sequencing of newly formed peptides by predominantly inducing the formation of γ -type ion series during MS/MS fragmentation. Derivatized peptides will be separated before analysis by liquid chromatography, and different ionization/detection methods such as

MALDI-TOF/TOF or ESI-Orbitrap will be used. Alternatively, affinity chromatography on CaM- or Hsp70-immobilized matrices could be used to concentrate peptides of interest from cell extracts and conditioned culture media. Finally, peptide detection and/or extraction will also be performed using immunological approaches.

Our hypothesis, if confirmed, brings forth new perspectives in the ever-evolving field of cell signaling. In addition, clinical consequences would be considerable with regard to both diagnosis and therapy. From a diagnostic point of view, one may indeed speculate that in estrogen-related diseases such as breast cancers, these putative peptides would be overexpressed and perhaps secreted or displayed in association with the MHC (Major Histocompatibility Complex). In a therapeutic context, one may conceive the design of specific inhibitors or anticipate immunotherapeutic strategies.

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