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 (ERα) mediates gene transcription and hormone-dependent cancer cell proliferation is now being largely reconsidered in view of several recent discoveries. ERα-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 (ERα17p), we propose that ERα proteasomal degradation could induce the emergence of regulatory peptide(s). The latter would function as a signal and contribute to the ERα 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: 17β-estradiol; E6-AP: E6-associated protein; CHIP: estrogen-responsive finger protein; ERR: estrogen related receptor; ERα: 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.

Cite this article: Nuclear Receptor Signaling (2008) 6, e007

Introduction

Estrogen receptor α (ERα) is commonly depicted as a transcription factor which, once bound to the appropriate hormone (e.g., 17β-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 ERα 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 ERα-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 ERα oligomeric structures.

In this Perspective, we describe the regulatory function of the ERα 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 ERα mechanism of action (see [Li and Sacks, 2007] for review). In addition, we propose that ERα degradation product(s), including the P295-T311 sequence, may contribute to the activation process of the receptor.

Link between proliferation and ERα downregulation

Although involvement of ERα in the growth of hormone-dependent breast cancers has been clearly established, ERα expression in primary breast cancers is generally associated with a favorable prognosis, as compared to the ERα-negative phenotype. It is noteworthy that high amounts of ERα 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 ERα 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 ERα-mediated gene transcription [Laios et al., 2005; Lonard et al., 2000; Reid et al., 2003].
The ERα P_{295}-T_{311} sequence is a regulatory platform

The P_{295}-T_{311} 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 P_{295}-T_{311} sequence includes a binding site for CaM [Bouhoute and Leclercq, 1995; Castoria et al., 1988; Gallo et al., 2007a; Garcia Pedroso et al., 2002; Li et al., 2005], a coregulator which enhances both the transactivation [Biswas et al., 1998; Garcia Pedroso 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 ERα-induced transcription and receptor proteolysis.

In order to further elucidate the role of the P_{295}-T_{311} segment, we have synthesized a peptide with the same sequence: ERα17p (P_{295}LMIKRSKKNSLALSLT_{311}). Surprisingly, this peptide elicits estrogenic responses in ERα-expressing breast carcinoma cells [Gallo et al., 2008; Gallo et al., 2007a; Gallo et al., 2007b]. Thus, ERα17p stimulates both cell proliferation and ERE-dependent transcription. This is associated with receptor downregulation, occurring through an increase of ERα degradation rate and a decrease of ERα 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 ERα17p binds to CaM and inhibits its association with ERα, its estrogenic action cannot be totally ascribed to a CaM-dependent mechanism since two ERα17p analogs, unable to associate with CaM, displayed estrogenic properties with a slightly higher efficiency [Gallo et al., 2007a].

The mechanism by which ERα17p 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 ERα, 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 ERα (nuclear, cytoplasmic, membrane-associated, posttranslationally modified, etc.) are implicated in the mode of action of ERα17p.

Molecular mechanisms leading to ERα17p-induced estrogenic effects

Recent investigations from our laboratory have revealed that ERα17p binds to purified recombinant human ERα [Gallo et al., 2007b], most likely disrupting intramolecular interactions suspected to confer upon the receptor an inactive conformation (i.e., association between the S_{295}T_{311} sequence and the β-turn/H4 composite motif [Jacquot et al., 2007]). The additional finding that, in MCF-7 cells, ERα17p dissociates by competition with ERα-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 ERα adopts a structure where the hinge region is "closed" by an Hsp70-stabilized intramolecular interaction between the S_{295}T_{311} 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 P_{295}-T_{311} motif. CaM, by associating with this motif, would stabilize the activated ERα oligomeric structure. This proposal, which of course requires further experimental validation, could be extended to other coregulators which modulate molecular interactions involving the P_{295}-T_{311} sequence.

Hence, ERα17p and its analogs, regardless of their ability to interact with CaM, would activate ERα 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 ERα P_{295}-T_{311} motif has a repressive function

The model outlined above postulates the existence of an autoinhibitory function associated with the ERα P_{295}-T_{311} sequence. In support to this view, we and others have observed that partial or complete deletion of the P_{295}-T_{311} 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 E_2, 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., R_{529}KLKKLGN_{536} sequence; Figure 2A) [Haelens et al., 2007], suggesting that other nuclear receptors may be subject to such autoregulation.

Genetic arguments support this concept of ERα autoinhibition. Indeed, a missense point mutation resulting
Figure 1. Agonistic properties of ERα.

A. Localization of the P295-T311 sequence within ERα. B. MCF-7 cell proliferation. Cells were grown in the absence (control) or presence of ERα17p at 10 μM 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 ERα17p at 10 μM or E2 at 0.1 nM. Luciferase activity, assayed by luminometry, was normalized according to protein concentration in cell extracts. D. ERα level. MCF-7 cells were cultured in the absence (control) or presence of ERα17p at 1 and 10 μM or E2 at 0.1 nM. ERα and actin levels in cell extracts were determined by Western blot analysis. E. ERα degradation rate. [35S]methionine labeled MCF-7 cells were maintained in culture without (control) or with ERα17p at 10 μM or E2 at 1 nM. Immunoprecipitated [35S]ERα was submitted to SDS-PAGE, revealed by autofluorography and quantified by measuring band intensities. F. ERα mRNA level. MCF-7 cells were incubated in the absence (control) or presence of ERα17p at 10 μM or E2 at 0.1 nM. ERα 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).
Similarly, a small percentage of ligand-bound receptor induces synthesized receptors generates a signal appropriate for property explained by the fact that ligand binding to newly vanishes upon treatment with conventional ligands, a cells, accumulated ER\textsubscript{1996; Katzenellenbogen et al., 1983}. Thus, in MCF-7 access of other ligands [Borras et al., 1994; Borras et al., 530] [Harlow et al., 1989; Robertson et al., 1981], blocking covalently with the hormone binding pocket of ER\textsubscript{α} demonstrated with cells labeled with \textsuperscript{14}C tamoxifen aziridine ([\textsuperscript{14}C]TAZ), a partial antiestrogen which reacts covalently with the hormone binding pocket of ER\textsubscript{α} (Cys 530) [Harlow et al., 1989; Robertson et al., 1981], blocking access of other ligands [Borras et al., 1994; Borras et al., 1996; Katzenellnogen et al., 1983]. Thus, in MCF-7 cells, accumulated ER\textsubscript{α} covalently liganded with \textsuperscript{14}C 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 \textsuperscript{14}C TAZ-ER\textsubscript{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 E\textsubscript{2} (sub-K\textsubscript{d} doses) or short E\textsubscript{2} pulses are sufficient to produce optimal transactivation, as well as the degradation of the whole receptor pool [El Khissiin et al., 2000; Gylling 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\textsubscript{α} constitute such (a) signal(s), the “missing link” connecting receptor degradation and transactivation?

In view of the estrogenic activity of ER\textsubscript{α}17p, one may reasonably wonder whether there is a physiological, proteasome-mediated production of peptide(s) with similar estrogenic properties. Insofar as ER\textsubscript{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\textsubscript{α} breakdown which accompanies transactivation generates peptide(s) that, like ER\textsubscript{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

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**Figure 2. Sequence alignment of AR, ERR\textbeta, and ERR\textγ with ER\textalpha.** A. AR E621-G643 versus ER\textalpha P295-T311. The AR inhibitory motif (R\textsubscript{295}K\textsubscript{L}K\textsubscript{L}G\textsubscript{N}3\textsubscript{38}) delineated by Haelens et al. (Haelens et al., 2007) is boxed. B. Orphan receptors ERR\textbeta (C\textsubscript{158}-A\textsubscript{315}) and ERR\textgamma (C\textsubscript{240}-E\textsubscript{419}), gaps within the orphan receptor regions corresponding to the C-terminus part of the ER\textalpha P295-T311 sequence are boxed. Sequences were downloaded from the ExPASy Proteomics Server (http://au.expasy.org) (accession numbers: ER\textalpha: P03372, AR: P10275, ERR\textbeta: O95718 and ERR\textgamma: P62508). Alignments were carried out using T-Coffee server (http://tcoffee.vital-it.ch) (Notredame et al., 2000). Hyphens (-) indicate sequence, a characteristic which might signify (a) signal(s), the "missing link" connecting receptor degradation and transactivation.

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**Is an endogenous production of ER\textalpha17p-like peptides conceivable?**

Direct interaction of a ligand with ER\textalpha is not an absolute prerequisite for its proteasomal degradation, as demonstrated with cells labeled with \textsuperscript{3}H tamoxifen aziridine ([\textsuperscript{3}H]TAZ), a partial antiestrogen which reacts covalently with the hormone binding pocket of ER\textalpha (Cys 530) [Harlow et al., 1989; Robertson et al., 1981], blocking access of other ligands [Borras et al., 1994; Borras et al., 1996; Katzenellnogen et al., 1983]. Thus, in MCF-7 cells, accumulated ER\textalpha covalently liganded with \textsuperscript{3}H 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 \textsuperscript{3}H TAZ-ER\textalpha degradation [Borras et al., 1996]. Similarly, a small percentage of ligand-bound receptor genera...
Figure 3. Induction of ERα activation/degradation cycles by putative peptides generated by the proteasomal degradation of ERα. In the absence of hormone, ERα-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 ERα 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 ERα proteolytic profiles. In this regard, it should be stressed that several ways have been described by which ERα is polyubiquitinated and thus tagged for proteasomal degradation. While CHIP (Carboxyl Terminus of Hsp70-Interacting Protein) exclusively polyubiquitates unliganded ERα [Fan et al., 2005; Tateishi et al., 2004], MDM2 (Murine Double Minute 2) seems to promote both basal and E2-associated ERα 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 ERα 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 ERα 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 ERα, subjected to proteasomal degradation under agonist stimulation [Chaturvedi et al., 2001]. Hence, our hypothesis could apply not only to ERα 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 ERα17p-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 y-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 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.

Acknowledgements
This work has been supported by grants from the Belgian Fund for Medical Scientific Research (Grant n° 3.4512.03), the CGRI/CNRS/FRNS (Grant n° 18217), the Fonds Jean-Claude Heuson, the Fondation MEDIK, ‘Les Amis de l’Institut Bordet’ and the Lambeau-Marteaux Foundation. Dominique Gallo is a recipient of a grant from the Fonds Jean-Claude Heuson. Guy Laurent is Senior Research Associate of the National Fund for Scientific Research (Belgium). The authors are also grateful to Jeanne Richard for her kind secretarial assistance.

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Perspective

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