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**Calmodulin, a regulatory partner of the estrogen receptor alpha
in breast cancer cells**

Running title: Calmodulin regulates ER α

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Summary

Although calmodulin (CaM) interaction with estrogen receptor alpha (ER α) has been known for more than two decades, it is only recently that the molecular mechanism of CaM-mediated regulation of ER α is beginning to emerge. Others and we have identified a putative calmodulin binding site (P₂₉₅LMIKRSKKNLALSTADQMVS₃₁₇) in ER α , at the boundary between the hinge and the ligand binding domain. ER α mutations affecting its association with CaM have been reported to generate high basal, estrogen-independent transactivation activity, indicating that the P₂₉₅-T₃₁₇ sequence has an inhibitory function. Moreover, we found that a synthetic peptide (ER α 17p : P₂₉₅-T₃₁₁) containing residues crucial for CaM binding exerts estrogenic effects on breast carcinoma cells. Finally, computer-aided conformational studies revealed that the CaM binding site might associate with a region located downstream in ER α (the β turn/H4 region), this association likely resulting in an autoinhibitory folding of the receptor. Thus, we propose as a hypothesis that CaM acts as a positive regulator by relieving this ER α autoinhibition.

1. Historical background

First indications concerning a possible contribution of calmodulin (CaM) to the mechanism of action of estrogen receptor alpha (ER α) appear in studies of Auricchio's group published in the early '80s, where it was reported that a cytosolic CaM-dependent kinase could restore the estradiol (E₂) binding capacity of a crude ER α preparation inactivated beforehand by the action of a nuclear phosphatase (Auricchio et al., 1981a; Auricchio et al., 1981b; Migliaccio et al., 1984). Subsequent investigations performed by these authors suggested that this kinase, which was found to target the receptor on Tyr-537 (Castoria et al., 1993), might also confer E₂ binding ability to ER α synthesized *in vitro* (Castoria et al., 1993; Migliaccio et al., 1989; Migliaccio et al., 1991). Finally, they proposed that the loss of such a CaM-dependent phosphorylation of ER α in breast cancer may explain the decrease of hormone dependence that occurs during tumor progression (Castoria et al., 1996; Migliaccio et al., 1992). Because of its clinical implications, this hypothesis aroused in our team a considerable interest for Auricchio's studies. While we failed to reproduce in serially transplanted mouse mammary tumors these observations on estrogen binding (Piccart et al., 1998), we confirmed that CaM can associate with ER α in a Ca⁺⁺ dependent manner (Bouhoute, Leclercq, 1992; Bouhoute, Leclercq, 1995; Bouhoute, Leclercq, 1996), a property also described by these authors (Castoria et al., 1988). Nowadays, the involvement of a CaM-dependent kinase in ER α phosphorylation on Tyr-537, resulting in enhanced hormone binding capacity, appears at least questionable (Carlson et al., 1997; Yudt et al., 1999). In fact, some reported observations might result from the combined influence of various factors, such as a CaM-induced protection of ER α against proteolysis (Li et al., 2001), an

increase of Tyr-537 phosphorylation and a more stable association with chaperones (i.e. heat shock proteins, Hsp_s), these effects contributing altogether to increase ligand binding capacity.

While the implication of CaM in ER α phosphorylation is not at all substantiated, there is no doubt that CaM contributes to E₂-induced, receptor-mediated transcription. Early studies from our laboratory revealed that CaM increases the ability of the receptor to associate with estrogen response elements (EREs) (Bouhoute, Leclercq, 1995). Subsequent independent investigations (Biswas et al., 1998) indicated that it operates as a co-regulator of major importance for the formation of stable ER α -ERE complexes. The additional observations of these authors that a CaM antagonist (W7) abrogates the transactivation of an E₂-responsive promoter led them to conclude that CaM participates to ER α -mediated transcription. Extensive studies conducted by Sacks (Li et al., 2005; Li et al., 2001) and Ramos (Garcia Pedrero et al., 2002) definitely established this concept.

The mechanism by which CaM enhances ER α -mediated transcription is still a matter of debate. The present paper, based on most recently reported data including ours, proposes a model aimed at explaining CaM involvement in this process. According to this model, CaM antagonizes the inhibitory activity of a receptor motif, the action of which is under the control of ligands as well as a panel of co-regulators. The potential implication of CaM in the regulation of ER α turnover rate, and thus in the control of ER α transactivation ability (Leclercq et al., 2006), is also evoked.

2. The regulatory function of CaM concerns the nuclear form of ER α

Intracellular visualization of ER α has revealed that its localization is mainly nuclear. This finding does not mean that ER α is sequestered within the nucleus since it has been shown to undergo a dynamic intracellular shuttling (Maruvada et al., 2003; Nonclercq et al., 2007; Stenoien et al., 2000) which seems to determine the type of response to estrogen signaling (Leclercq et al., 2006). Thus, estrogens elicit quasi-immediate responses *via* membrane associated form(s) of ER α that can interact with signaling cascades and thereby modulate *in fine* the transcription of target genes (Membrane-Initiated Steroid Signaling, MISS) (Acconcia, Kumar, 2006; Pedram et al., 2006; Song, 2007). In contrast, nuclear form(s) of ER α directly regulate gene expression (Nuclear Initiated Steroid Signaling, NISS), the receptor functioning in this case as an estrogen-dependent transcription factor (or as a co-regulator of other transcription factors).

Inasmuch as CaM regulates intracellular processes in both the cytoplasm and the nucleus, one may wonder whether it interacts with one or several forms of ER α . In order to explore this issue, Sacks and coll. (Li et al., 2003) have transfected cells with expression plasmids coding for peptides able to selectively inhibit the cytoplasmic or the nuclear form of CaM. Results of their study demonstrated that CaM exerts its action on nuclear ER α , since E₂-stimulated gene transactivation was abrogated when the CaM-inhibitory peptide accumulated within the nucleus, while it was only slightly attenuated when the peptide was targeted to the membrane and the cytoplasm. This study supported the concept that CaM influences the action of ER α acting as a transcription factor at promoter level.

3. CaM regulates ER α transactivation when complexed with Ca⁺⁺

Although Ca⁺⁺ binding appears essential for the regulatory function of CaM in many physiological processes, it is not always necessary. Indeed, Ca⁺⁺-free CaM (apo-calmodulin) modulates the activity of several proteins essential for cell function (Jurado et al., 1999). With regard to ER α , studies have demonstrated the requirement of Ca⁺⁺ not only for the formation of the CaM / ER α complex (Bouhoute, Leclercq, 1992) but also for its ability to regulate the transactivation ability of the receptor (Li et al., 2005). Thus, BAPTA-AM (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxymethyl)ester), a cell-permeable Ca⁺⁺ chelator, was shown to abrogate the enhancing effect of E₂ on ERE-driven gene transcription in ER α -positive breast cancer cells (MCF-7 cells). In these cells, ectopic expression (obtained by transfection) of a CaM mutant unable to bind Ca⁺⁺ produced a similar effect and, moreover, largely decreased the basal transcriptional activity of the receptor, unambiguously establishing the implication of the Ca⁺⁺~CaM complex in ER α -mediated transcription. Hence, Ca⁺⁺ oscillations that govern CaM activity may, as a metronome, regulate basal ER α -induced transactivation, contributing to its cyclic character (Métivier et al., 2003; Reid et al., 2003).

Because of what is said above, it should be noted that the abbreviation CaM used hereunder actually refers to Ca⁺⁺-complexed calmodulin.

4. A CaM binding domain has been localized in ER α

Studies conducted in Sacks's laboratory as well as in ours localized the binding motif of CaM in a region of ER α extending from Pro-295 to Ser-317 (P₂₉₅LMIKRSKKNLALSTADQMVS₃₁₇), thus at the boundary between the hinge and the ligand binding domain (Gallo et al., 2007a; Li et al., 2005). Although the contribution of most of these aminoacid residues in terms of interaction with CaM is unknown, the importance of Ile-298, and Lys-299, 302 and 303 must, however, be stressed. Deletion or substitution of these residues not only abrogates the association of ER α with CaM (Garcia Pedrero et al., 2002; Li et al., 2005) but may also suppress the ability of E₂ to enhance the transcription of an ERE-driven reporter gene, as shown for I₂₉₈Q and K₂₉₉D mutated receptors (Li et al., 2005). Hence, the amino-terminal half of the P₂₉₅-S₃₁₇ sequence seems to contain the major determinants for ER α -CaM complexation.

To explore further ER α interaction with CaM, we synthesized a peptide containing these major determinants (ER α 17p : P₂₉₅-T₃₁₁), as well as two control peptides in which Lys-302 and 303 were substituted by alanines (ER α 17pAA) or glycines (ER α 17pGG) (Gallo et al., 2007a). As expected, while ER α 17p bound to CaM-Sepharose and inhibited the binding of ER α to immobilized CaM, the control peptides ER α 17pAA and ER α 17pGG both failed to show similar properties, confirming unambiguously the implication of the P₂₉₅-T₃₁₁ sequence - and in particular of residues Lys-302 and 303 - in CaM binding. Incidentally, ER α 17p behaved as a weak CaM antagonist as compared to a reference peptide derived from the CaM kinase II, suggesting that it may antagonize the ER α -CaM interaction without affecting main CaM-mediated processes. Toxicity tests confirmed this assumption. Indeed, MTT assays performed on MCF-7 cells exposed to ER α 17p at a dose

impeding the complexation of ER α with CaM (10 μ M) failed to disclose impairment of metabolic activity, while conventional CaM inhibitors displayed a detectable inhibitory effect at the same or even lower concentration. Hence, ER α 17p as well as ER α 17pAA and ER α 17pGG appeared as valuable tools to decrypt the function of ER α -CaM association (see # 8).

5. The CaM binding domain of ER α is located in a regulatory platform

Interestingly, Lys-299, 302 and 303 of ER α , the importance of which in CaM binding has been stressed above (# 4), are included in a nuclear localization signal (K₂₉₉RSKK₃₀₃), suggesting that binding of CaM to ER α may contribute to the intracellular shuttling of the latter (Sweitzer, Hanover, 1996). CaM may also protect the receptor against partial proteolysis, in view of the high sensitivity of the K₂₉₉-K₃₀₃ motif to trypsin-catalyzed cleavage (Seielstad et al., 1995). The fact that Lys-302 and 303 as well as Ser-305 are subjected to post-translational modifications that have been reported to play a critical role in ER α -mediated transactivation (K₃₀₂ and K₃₀₃ : acetylation (Wang et al., 2001) and mono-ubiquitination (Eakin et al., 2007; Heine, Parvin, 2007); S₃₀₅ : phosphorylation by PAK-1 and/or PKA (Wang et al., 2002; Zwart et al., 2007)) suggests a complementary regulatory control of CaM at this level. Finally, as described hereunder, CaM has also been shown to decrease the ability of ER α to bind E₂, a property most likely related to the proximity of the CaM binding motif to the ligand binding pocket. Hence, CaM appears as a crucial modulator of a regulatory platform playing an important role in the mechanism of action of ER α .

6. CaM modifies ER α binding properties

ER α exists under two forms that can be distinguished on the basis of their binding affinity for E₂ (K_d : ~0.06 nM and 0.8 nM respectively); a third form is totally unable to bind the hormone (Dayani et al., 1988). A phosphorylation/dephosphorylation process involving several enzymes favors the conversion of one form into another. Interestingly, the addition of CaM to an ER α preparation with high binding affinity promotes its conversion into the form with low binding affinity (increase of K_d value without decrease of binding capacity); in contrast, when the receptor preparation is characterized by a high K_d value, CaM only induces a weak loss of binding capacity that can be reversed by the CaM kinase inhibitor KN62 (Bouhoute, Leclercq, 1996).

The changes in estrogen binding properties outlined here were found to be associated with an increase of ER α interaction with EREs (Bouhoute, Leclercq, 1995), enlightening the concept that CaM may confer to the ligand binding domain a conformation appropriate for co-activator recruitment, a step required for a stable association of the receptor with DNA. The potential dissociation of chaperones, which are known to maintain ER α in a high binding affinity, transcriptionally inactive form is an alternative interpretation. In fact, both possibilities are not mutually exclusive, co-regulator recruitment being often associated with the release of chaperones.

7. Tamoxifen modulates the complexation of CaM with ER α

Unexpectedly, the triphenylethylenic antiestrogen tamoxifen, which antagonizes ERE-dependent transcription, has been reported to increase the amount of ER α bound to CaM in MCF-7 cells (Li et al., 2001). The effect of the compound culminated at 0.25 to 0.5 μ M and was suppressed by E₂. Whether this observation is related to a stabilization of the ER α / CaM complex due to the interaction of the antiestrogen with the receptor ligand binding pocket or to a tamoxifen-induced protection of ER α against proteasomal degradation (Laïos et al., 2003; Leclercq et al., 1992; Wijayaratne, McDonnell, 2001) remains a matter of debate. Indeed, we have observed that the addition of 1 μ M tamoxifen (as well as of its steroidal analogues RU 39411 and RU 45144) to cytosolic ER α preparations perturbs the binding of the receptor to CaM-Sepharose (Bouhoute, Leclercq, 1992; Bouhoute, Leclercq, 1994). Exposure of MCF-7 cells to a triphenylethylenic antiestrogen like GW7604 (Wijayaratne, McDonnell, 2001), unable to induce receptor accumulation, may be an adequate approach to clarify this important issue.

Tamoxifen at a concentration of 1 μ M and higher displays a strong antitumor activity largely derived from its ability to interact with an array of targets implicated in the regulation of cell proliferation, including CaM (de Medina et al., 2004). Hence, the potential antagonism that tamoxifen exerts at this concentration against ER α -CaM association is not necessarily related to its capacity to fit within the ligand binding pocket of ER α . The study of a series of analogues and derivatives of tamoxifen with potent antitumor activity (Rowlands et al., 1997) confirmed this view. It revealed, indeed, that the inhibition of CaM / ER α complex formation is neither related to the affinity of these compounds for the receptor nor to their intrinsic anti-CaM activity (Fanidi et al., 1994; Hardcastle et al., 1995; Lopes et al., 1990). In fact,

the major determinant of the negative action of tamoxifen on CaM-ER α complex formation was found to be its phenylethoxyaminodialkyl side chain, which confers to this compound its pharmacological properties (de Medina et al., 2004).

Since the binding of CaM to ER α stabilizes the association of the latter with EREs, one may reasonably assume that tamoxifen at 1 μ M - which also interferes with CaM function at this concentration - would favor an ERE-independent anchorage of the receptor within the cell nucleus. This property of tamoxifen, along with the ability of this antiestrogen to induce the accumulation of a functionally inactive form of ER α in the nucleus (Laïos et al., 2003; Leclercq et al., 1992; Wijayarathne, McDonnell, 2001), may contribute to its antiproliferative activity.

8. The CaM binding domain inhibits ER α -mediated gene transactivation

Mutations (deletions/substitutions) of ER α affecting CaM binding have been reported to generate high basal, E₂-independent transcription of reporter genes (Gallo et al., 2007a; Li et al., 2005). From this finding one may surmise that the D/E border region to which CaM binds stabilizes the receptor in an inactive status. The fact that CaM contributes to E₂-induced gene transcription suggests further that its binding to this region may interfere with an auto inhibitory mechanism involving intramolecular interactions within ER α . Studies conducted with ER α 17p and its AA/GG derivatives unable to bind CaM (# 4) enabled us to confirm this hypothesis, as well as to decrypt the mechanism by which the CaM binding site operates.

Exposure of MCF-7 cells to these three synthetic peptides was found to induce responses normally observed under estrogenic stimulation (i.e. ER α down regulation, induction of pS2 and progesterone receptor, growth stimulation...) (Gallo et al., 2007a; Gallo et al., 2007b; Gallo et al., 2008a). Assessment of a panel of breast cancer cell lines revealed that the trophic effect of these peptides was restricted to ER α -positive cell lines, establishing the implication of the receptor in their biological effect. With regard to the underlying molecular mechanism, we opted for a peptide-induced disruption of putative inhibitory interactions between the P₂₉₅-T₃₁₁ sequence and a cognate motif localized elsewhere in the receptor (or in another protein). Disruption of these inhibitory interactions would unblock the transactivation capacity of the receptor. A computer-based modeling approach described hereunder, and the finding that ER α 17p associates with human recombinant ER α (Gallo et al., 2007b) as well as with the chaperone Hsp-70 (Gallo et al., 2008a) provided theoretical and experimental arguments in favor of this concept.

Our modeling approach identified indeed within the ligand binding domain of ER α a motif theoretically able to establish intramolecular interactions with the P₂₉₅-T₃₁₁ sequence (Jacquot et al., 2007). This putative motif includes the β turn/H4 subregion of the D₃₅₁-Q₄₁₄ sequence, which contains the H3-H5/ β hairpin domain involved in the recruitment of co-regulators harboring an LxxLL consensus motif (Fig. 1). Thus, we postulate that the estrogenic response induced by ER α 17p stems from the fact that this peptide competitively suppresses the inhibitory interaction between the P₂₉₅-T₃₁₁ sequence and the β turn/H4 region. Conformational changes associated with this relaxation process, along with the release of Hsp-70 (Gallo et al., 2008a), would favor the recruitment of LxxLL co-regulators. In this context, it should

be stressed that an ELISA-type assay of ER α binding to an LxxLL co-regulator motif revealed that ER α 17p modulates the association of a highly purified recombinant receptor to this motif, in a same manner as a control LxxLL peptide (Gallo et al., 2007b).

Pursuing this line of reasoning, one may logically postulate that co-regulators recruited at the D/E border region of ER α may, like ER α 17p, abrogate the auto-inhibition resulting from the mutual interactions between the P₂₉₅-T₃₁₁ sequence and the β turn/H4 region. One may anticipate that CaM would be a privileged actor of this regulatory mechanism, which modulates *in fine* ER α -mediated gene transcription, even if ER α 17p and its AA/GG derivatives peptides exert their stimulating effect in a CaM-independent fashion.

9. CaM contributes to the to regulation of ER α turnover rate

The rate at which ER α is synthesized and degraded (turnover) is a major factor regulating the cell responsiveness to various ligands. Studies conducted in various laboratories have demonstrated the implication of the ubiquitin-proteasome system (UPS) in the elimination of the mature receptor which has achieved transactivation and has become functionally obsolete (Alarid et al., 1999; El Khissiin, Leclercq, 1999; Laïos et al., 2005; Nawaz et al., 1999a; Wijayaratne, McDonnell, 2001). Natural and pharmacological ligands either accelerate (estrogens, pure antiestrogens) or reduce (partial antiestrogens) the proteasomal degradation of ER α (Laïos et al., 2005; Read et al., 1989; Wijayaratne, McDonnell, 2001) by selecting UPS with high or low degradation rate.

In the UPS, target proteins committed to degradation are first tagged by ubiquitination (Deshaies, 1999). The first step of this process is the formation of a thioester bond between ubiquitin and a Cys residue of an ubiquitin-activating enzyme (E1). The second step consists in the transfer of activated ubiquitin from E1 to a Cys residue of a member of the ubiquitin-conjugating enzymes (E2). This is followed by a third step where ubiquitin is transferred from the E2 enzyme to a Lys residue of the target protein, thanks to the activity of a specific ubiquitin ligase (E3). This mechanism proceeds cyclically, grafting additional ubiquitin moieties to those already present in the target protein. E6AP has been identified as an E3 ubiquitin ligase implicated in ligand-induced elimination of steroid hormone receptors, (Khan et al., 2006; Nawaz et al., 1999b). Remarkably, CaM has been shown to attenuate the E6AP-mediated ubiquitination of ER α , as well as to decrease the capacity of E6AP to associate with the receptor, suggesting that it may protect the latter against proteasomal degradation (Li et al., 2006). The finding that the proteasome inhibitors MG-132 and lactacystin prevent a permeable CaM antagonist from reducing ER α level in MCF-7 cells is consistent with this assumption. This protective effect of CaM may be a key factor by which it may modulate ER α turnover rate and influence thereby events depending on ER α level.

Based on these considerations, we propose that the proteasomal degradation of ER α resulting from the release of bound CaM generates ER α 17p-like peptides that, like CaM, might relieve ER α auto-inhibition (Gallo et al., 2008b). ER α proteasomal degradation occurring during the successive steps of this process would facilitate the access to EREs of newly synthesized receptors, after release of bound

Hsp-70 and association with CaM, to initiate further transactivation cycles. Successive occurrences of these events would guarantee high gene expression. Of course, other actors may be implicated in this scheme, in view of the fact that the CaM binding site of ER α is located in a platform for various posttranslational changes.

10. Inappropriate CaM - ER α complexation may be involved in breast pathology

Events affecting interaction between ER α and CaM may be expected to have pathological consequences. According to the concept outlined in section 8 and 9, one may anticipate that high amounts of CaM would favor extensive relaxation of ER α , with a concomitant loss of the inhibitory effect exerted by the CaM binding site on the recruitment of LxxLL motif-containing co-regulators. Remarkably, high CaM levels have been reported to frequently occur in breast cancers (Singer et al., 1976), especially those classified as ER α -positive (Krishnaraju et al., 1991), suggesting an implication of CaM in breast cancer development and / or progression. This would explain the synergistic effect of anti-CaM drugs on the antagonistic activity of antiestrogens (Frankfurt et al., 1995; Strobl, Peterson, 1992).

The BRCA-1 protein plays a critical role in the control of ER α function. When bound to BARD-1, BRCA-1 indeed provokes the mono-ubiquitination of Lys-302 and 303 in ER α (Eakin et al., 2007). Inasmuch as these residues are implicated in the association of ER α with CaM (# 4), this posttranslational modification of the receptor may decrease its association with EREs with a concomitant arrest of transcription

initiation (Heine, Parvin, 2007). Hence, loss of function mutations of BRCA-1, known to enhance the risk of breast cancer, could result in a disruption of this negative regulation of ER α -mediated transactivation. Alternatively, alterations within the P₂₉₅-T₃₁₁ sequence may also contribute to breast cancer emergence. Proteolytic cleavage of the K₂₉₉RSKK₃₀₃ motif (Seielstad et al., 1995), frequently recorded in breast cancer samples (Maaroufi et al., 2000), is likely to withdraw ER α from the action of repressors like BRCA-1, the oligomeric nature of ER α maintaining such cleaved receptors in a compact structure still able to mediate transcription (Kraus et al., 1995). Finally, missense mutation causing substitution of Lys-303 by an arginine, which is known to confer a high cell sensitivity to E₂ (Fuqua et al., 2000), most probably facilitates receptor activation, with a concomitant enhancement of cell proliferation. Interestingly, this K₃₀₃R mutation has been frequently reported in pre-neoplastic and neoplastic mammary tissues (Fuqua et al., 2000) as well as in high-grade breast tumors (Conway et al., 2005).

11. Perspectives and conclusions

The concepts outlined in the previous sections primarily concern ER α -mediated gene transactivation in breast cancer cells. These concepts are most probably valid for ER α -positive cells where the receptor acts as a transcription factor in the nucleus. Whether they are also relevant to cells with a different intracellular ER α distribution remains a matter of speculation. Potential implication of CaM in the nuclear translocation of the receptor (Sweitzer, Hanover, 1996) as well as in its intracellular trafficking (Maruvada et al., 2003; Stenoien et al., 2000) suggests that it might not be the case. However, the polyproline II / α -helix mixed secondary

structure of the CaM binding site (P₂₉₅-T₃₁₁ sequence), extensively discussed in previous publications of our group (Gallo et al., 2007b; Jacquot et al., 2007), suggests that this motif might interact with a number of regulatory proteins containing polyproline-binding modules implicated in both non genomic and genomic estrogenic responses. Co-activators PELP1/MNAR (Nair, Vadlamudi, 2007) and PNRs (Zhou et al., 2006) involved in gene transactivation, and the adaptor protein Grb2 belonging to the MAPK pathway are candidates for such potential regulatory processes (Yang et al., 2004). Therefore, interference of CaM with ER α functions other than these directly associated with gene transactivation might be of importance.

On the other hand, the inhibition exerted by the P₂₉₅-T₃₁₁ sequence on ER α -mediated transcription could be exploited for the design of drugs directed against AF-2-dependent transactivation. The recent identification in the androgen receptor (AR) of a surface cleft adjacent to this activation function and designated as BF3 has led to the development of drugs that modulate the association of this receptor with its co-regulators (Estebanez-Perpina et al., 2007). Interestingly, superimposition of AR on the ER α backbone revealed that the ER α -corresponding BF3 domain is composed of residues located in the β turn/H4 subregion, implicated in the inhibitory interaction exerted by the P₂₉₅-T₃₁₁ sequence on the recruitment of co-activators at AF-2 (Y. Jacquot, unpublished data, see #8, Fig. 1). Structure activity relationships established for AR may therefore orient the design of drugs for the treatment of breast cancer, especially those which fail to respond to antiestrogens.

Our review being devoted to ER α in the context of estrogen dependence of breast cancer, we did not discuss the function of the association of CaM with AR

(Cifuentes et al., 2004), nor its interaction with the orphan estrogen-related receptor γ (ERR γ) (Hentschke et al., 2003). The latter receptor, unable to bind E₂, is nevertheless of peculiar interest because it has been proposed as a new target for the endocrine therapy of breast cancer (Ariazi, Jordan, 2006). Of note, sequence alignment of ER α with ERR γ disclosed in the latter a homology with the N-terminal part of the P₂₉₅-T₃₁₁ motif, crucial for CaM binding (# 4). Consequently, one may anticipate that the mechanisms discussed here for ER α may to some extent apply to ERR γ .

Further studies are warranted to lend experimental support to the concepts outlined in the current review. Indeed, some of our proposals still remain quite speculative. Nevertheless, we consider that they may open new perspectives toward the understanding of clinical disorders associated with nuclear receptor dysfunctions and pave the way to the development of new therapeutical approaches. This should be an incentive for further research on other aspects of CaM-nuclear receptor interaction.

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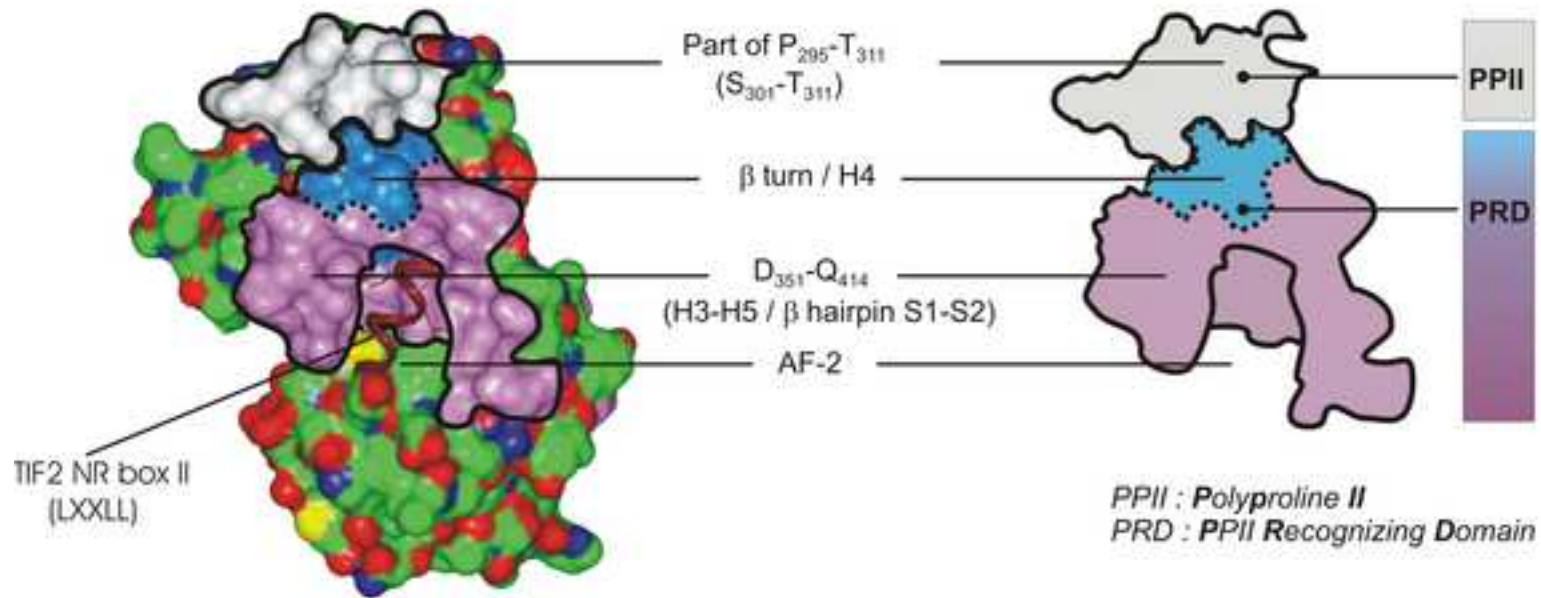
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Legend to the figure**Putative mechanism whereby CaM and ER α 17p induce estrogenic responses.**

The three-dimensional model represented here is based on X-ray crystallography analysis of the ligand binding domain of ER α complexed with the estrogenic core of raloxifene (Wärnmark et al., 2002). It also incorporates our own structural studies of intramolecular interactions between, on the one hand, a part of the CaM binding motif (P₂₉₅-T₃₁₁) adopting a helical polyproline II (PPII) conformation (the S₃₀₅-T₃₁₁ sequence), and, on the other hand, the β turn/H4 subregion of a huge polyproline recognizing domain (PRD) delineated by residues D₃₅₁-Q₄₁₄ (Jacquot et al., 2007). The PRD, which contains the H3-H5 subregion as well as the β hairpin structure (S1, S2 anti-parallel β strands) regulates the conformation of AF-2 (Kong et al., 2005; Wärnmark et al., 2002). Interactions between the CaM binding motif and the PRD are thought to confer to AF-2 a conformation inappropriate for the recruitment of LxxLL-containing co-regulators. CaM, by binding to the P₂₉₅-T₃₁₁ sequence, relieves this autoinhibition. ER α 17p, by binding to the β turn/H4 subregion of the PRD, produces a similar effect.

Theoretical background (from X-ray data)



Antagonism of CaM and ERα17p on the P₂₉₅-T₃₁₁ -mediated inhibition

