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Methylation, a key step for nongenomic estrogen signaling in breast tumors

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ABSTRACT

Estrogen receptor α (ERα) is a member of a large conserved superfamily of steroid hormone nuclear receptors which regulates many physiological pathways by acting as a ligand-dependent transcription factor. Evidence is emerging that estrogens also induce rapid signaling to the downstream kinase cascades; however the mechanisms underlying this nongenomic function remain poorly understood. We have recently shown that ERα is methylated specifically by the arginine methyltransferase PRMT1 at arginine 260 in the DNA-binding domain of the receptor. This methylation event is required for mediating the extra-nuclear function of the receptor which would thereby interact with Src/FAK and p85 and propagate the signal to downstream transduction cascades that orchestrate cell proliferation and survival. Of particular interest, a possible role of methylated ERα in mammary tumorigenesis is also evident by the fact that, as demonstrated by immunohistochemical studies on a cohort of breast cancer patients, ERα is methylated in normal epithelial breast cells and is hypermethylated in a subset of breast cancers. Hypermethylation of ERα in breast cancer might cause hyperactivation of cellular kinase signaling, notably of Akt, described as a selective survival advantage for primary tumor cells even in the presence of anti-estrogens. A detailed understanding of the molecular mechanisms that control estrogen signaling in breast cancer is a crucial step in identifying new effective therapies.

1. Introduction

Estrogens have multiple biological functions, notably a major role in breast tissue differentiation and in breast tumorogenesis through binding to two receptors, α and β. Here we will focus on ERα, which regulates many physiological pathways by acting as a ligand-dependent transcription factor. Besides its genomic function in the nucleus, estrogens can activate multiple signal transduction cascades in the extra-nuclear compartment (i.e., nongenomic mechanism) through interaction with and activation of signaling kinases such as IGF-1R, Src, PI3K, MAPK, EGFR and ErbB-2 [1,2]. Conversely, the cytoplasmic kinases can phosphorylate co-activators possibly modifying ERα activity [3].

Although estrogens have been associated with promotion and growth of breast cancer, many questions remain regarding the expression of its receptor during breast cancer evolution. Alterations in the response to estrogens are associated with a variety of hormone-dependent diseases, particularly breast cancer. About 15–25% of normal breast epithelial cells express ERα, and almost 70% of all breast cancers are ERα-positive and estrogen-dependent [4–6] indicating that estrogens promote breast cancer progression by stimulating malignant cell proliferation and that ERα is involved in early events in breast cancer. In addition, several lines of evidence suggest that most ERα-positive breast cancer cells stop expressing the receptor and become ER-negative due to the pressures exerted on ERα-positive cells by estrogen removal, hypoxia or overexpression of epidermal growth factor receptor (EGFR) or ErbB-2 in response to hyperactivation of MAPK signaling [7,8]. Therefore, gaining further insight into this complex network and into interactions with genomic and nongenomic ER pathways might help the development of targeted therapies.

2. Estrogen-induced nongenomic pathways

Even if significant progress has been made in understanding the molecular mechanisms underlying the nongenomic action of steroid receptors, several issues remain controversial, notably the nature of the receptors involved in nongenomic action and the molecular mechanisms that integrate hormonal action in the regulation of cell signaling pathways.

Accumulating evidence indicates that estrogens activate nongenomic pathways through a pool of conventional ERα located in the cytoplasm and/or at the plasma membrane. It has been described that ERα can be targeted to the cell membrane after palmitoyl acyl

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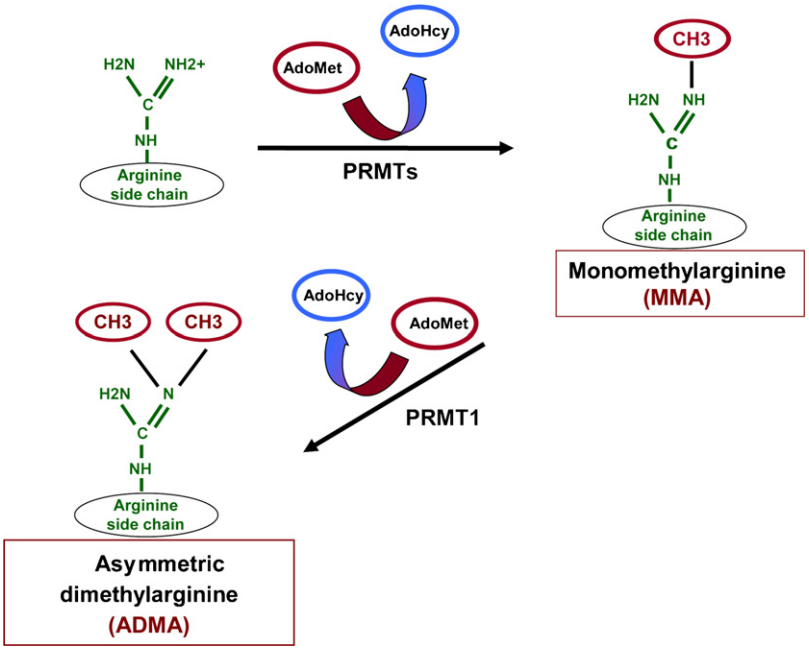

Fig. 1. Methylation on arginine residues catalyzed by PRMT1 (PRMT type I). Type I and type II enzymes all generate monomethylarginine (MMA) on one of the terminal guanidino nitrogen atoms catalyzing the transfer of a methyl group from AdoMet generating *S*-adenosylhomocysteine (AdoHcy) and monomethylarginine, then PRMT1 catalyzes specifically the generation of asymmetric dimethylarginine (ADMA).

transferase (PAT)-dependent S-palmitoylation of a cystein residue, promoting ERα interaction with caveolin-1 [9,10].

ERα membrane localization can also be mediated by its interactions with the membrane adaptor protein Shc [11] and with a variety of proximal signaling molecules such as G proteins [12]; Src kinase [13] and PI3K [14]. Other accessory proteins are known to modulate ERα interactions with Src and PI3K: for example, the scaffold protein MNAR promotes interactions with Src kinase and with the p85 subunit of PI3K, leading to increased Src kinase and PI3K activities, respectively [15–17]. Another modulator of the E2 signaling pathway is the adaptor protein p130 Cas which regulates the activation of Src kinase in human breast carcinoma T47D cells [18]. Differential complex formation activates two main pathways: the ras/MAPK and the PI3K/Akt pathways [1,12,14].

The activation of kinases triggers biological outcomes that are dependent or independent of transcription. For example, the activation of PI3K leads to increased cell proliferation [14] and abrogation of apoptosis through inactivation of BAD via phosphorylation [19]. More recently, E2 activation of PI3K/Akt signaling has been shown to inhibit the ATR pathway controlling cell cycle checkpoints and DNA repair [9] and the interaction of HDAC6 with membrane-localized ERα causing rapid deacetylation of tubulin which potentially contributes to cell migration and to aggressiveness of ERα-positive breast cancer cells [20]. Another role has been attributed to nongenomic estrogen signaling by Catalano and colleagues who have shown that E2-stimulated Src increases aromatase activity, revealing the existence of an autocrine loop between E2 and aromatase that could play a role in breast tumorogenesis [21].

3. Arginine methylation in cell signaling

Arginine methylation is becoming a known post-translational modification, as widely recognized as protein phosphorylation or acetylation. The methylation of arginine residues is catalyzed by the protein arginine N-methyltransferase (PRMT) family of enzymes. The complexity of the methylarginine mark is enhanced by the ability of this residue to be monomethylated, dimethylated symmetrically or asymmetrically. The PRMT family comprises 10 members classified as type I or type II, depending on whether they catalyze asymmetric or symmetric dimethylation, respectively. Fig. 1 illustrates the asymmetric dimethylation catalyzed by PRMT1. PRMTs are involved in a number of different cellular processes including transcriptional regulation, DNA repair, RNA metabolism and signal transduction [22]. Arginine methylation regulates these processes by blocking or promoting them mainly through modifications of protein–protein interactions. Even if signal transduction is not the major process regulated by arginine methylation, a growing body of data shows that several PRMTs are involved in several signaling pathways. For example PRMT1, has been involved in several receptor signaling pathways. It regulates interferon signaling by binding to the cytoplasmic region of the type 1 interferon receptor and also by turning off STAT1 activation [23]. Other data indicate that insulin treatment of cultured myotubes promotes PRMT1 accumulation and activation in the plasma membrane, suggesting that arginine methylation is involved in glucose metabolism [24]. Arginine methylation may also play an indirect role in cell signaling as it has been recently shown that PRMT1 methylation could interfere with Akt phosphorylation [25]. In addition, proteomic analysis has shown that many cell surface receptors and signaling molecules are methylated on arginine, suggesting that arginine methylation may play a more widespread role in signal transduction [26].

4. Methylation of ERα, a prerequisite for kinase recruitment

The molecular mechanisms that integrate hormonal action in the regulation of cell signaling pathways remain poorly understood. It is not clear how ERα triggers the formation of a multiprotein complex, what the kinetics of this formation might be, and how the signals are initiated and terminated. Recent studies of our group have established a novel mechanism of ERα regulation through arginine methylation by PRMT1 [27]. We have discovered that PRMT1 transiently methylates arginine 260 (R260) within the ERα DNA-binding domain rapidly after E2 treatment. Mechanistically

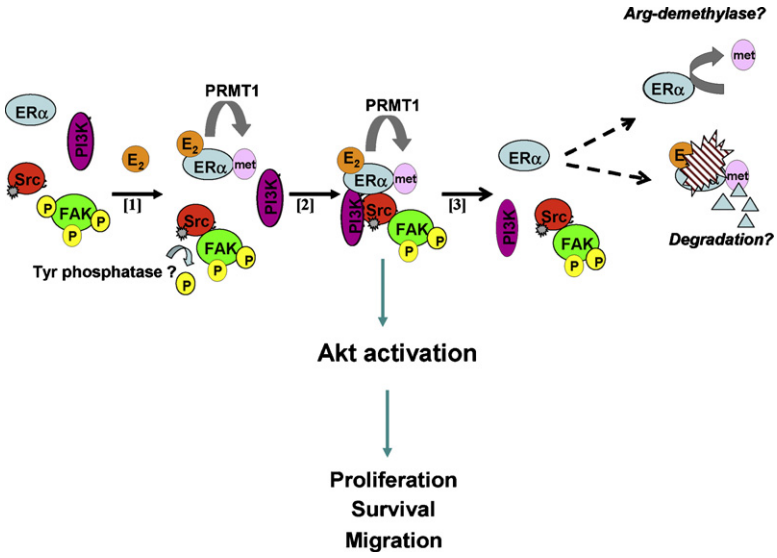

Fig. 2. Model of the assembly and regulation of the ERα macromolecular complex involved in nongenomic signaling. In the absence of hormone, ERα, p85, Src and FAK are not associated (1). E2 induces ERα methylation *via* PRMT1 and partial FAK dephosphorylation triggering Src/FAK interaction (2). These two events are necessary for the association of ERα, Src, p85 and FAK in a macromolecular complex (3). Complex formation elicits Akt activation which regulates cellular processes. Then, the main complex dissociates concomitantly to the disappearance of methylated ERα. Rapid down-regulation of ERα methylation and/or Src activity induces the dissociation of the complex and ultimately the extinction of downstream kinase activation. Two non-exclusive mechanisms can lead to the rapid loss of methylated ERα and the dissociation of the complex: (a) proteasome-mediated protein degradation may selectively degrade the modified form of the receptor; (b) methylation of ERα could be reversed by an unknown arginine-demethylase.

this methylation promotes the interaction of ERα with the p85 subunit of PI3K and Src and thereby the activation of cytoplasmic Akt signaling cascades. Our data strongly implicate the methylation of ERα by PRMT1 as a critical upstream signal for the formation of this complex. In fact, the silencing of endogenous PRMT1 is sufficient to inhibit the estrogen-dependent formation of this complex, the activation of Akt and cyclin D1 and inducing cell cycle arrest [27]. In the future, it will be interesting to explore the factors/events that trigger PRMT1 activation and allow E2-induced arginine methylation of ERα, and to elucidate whether this methylation cross-talks with other modifications on the receptor or its associated proteins. Our preliminary results suggest that upon E2 stimulation PRMT1 activity is increased concomitantly to the methylation of ERα (data not shown).

The methylated form of ERα is only detectable in the cytoplasm; this indicates that the modification of the receptor occurs in this compartment, possibly implicating that PRMT1 is located in the same compartment. This is in accordance with observations showing that the protein is localized in the nucleus and the cytoplasm. Recently Coté group described seven PRMT1 isoforms with the V2 isoform localized exclusively in the cytoplasm [28], making it a good candidate as the enzyme responsible for ERα methylation.

Additionally, E2-induced methylation of ERα was inhibited by the Src family tyrosine kinase inhibitors PP1 and SU6656, indicating that ERα methylation is dependent on the activation of the c-Src family of tyrosine kinases [27]. These results confirm the essential role of Src kinase in the rapid action of estrogens on steroid receptors, as clearly demonstrated in experiments with embryonic fibroblasts derived from src−/−mice [29]. Contrarily to wild-type cells, these Src-deficient cells did not show a rapid response to ER activation. In line with this, we found that the Src substrate FAK participates in this signaling complex and that Src activity is required both for FAK interaction with Src and for complex stability. Given the key role of the dual kinases Src/FAK in cell migration [30], we can speculate that the complex containing methylated ERα/Src/PI3K/FAK could be involved in cell adhesion and migration. These data support a model (Fig. 2) in which PRMT1 activation could initiate the rapid estrogens action responsible for methylating ERα, which would thereby interact with Src/FAK and p85, and propagate the signal to downstream transduction cascades. Rapid down-regulation of ERα methylation and/or Src activity may serve to control rapid physiological responses of the estrogens, inducing the dissociation of the complex and ultimately the extinction of downstream kinase activation.

As we mentioned previously, the appearance of methylated ERα is transient after treatment with estrogens, suggesting the existence of mechanisms that rapidly demethylate R260 or remove the modified form of ERα [27]. We can imagine two non-exclusive mechanisms: (a) proteasome-mediated protein degradation may selectively degrade the modified form of the receptor. Although our results show that MG132 treatment did not significantly affect the down-regulation of ER methylation [27], we cannot completely rule out that arginine methylation affects ERα stability. Future studies, using distinct experimental conditions (different inhibitors, concentration and time of treatment) will be aimed to state this point. (b) ERα methylation can be reversed by an unknown arginine-demethylase leading to the translocation of the receptor to the

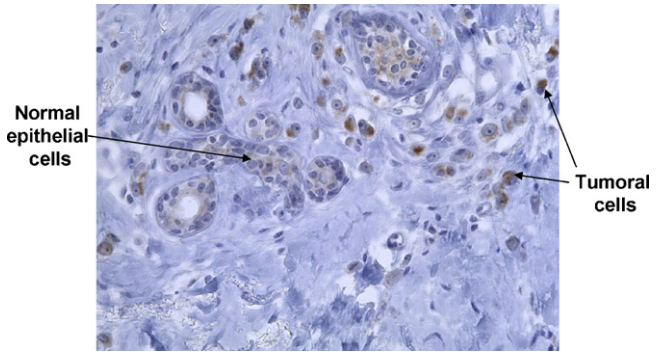

Fig. 3. Expression of ERα in breast cancer tissues. Immunohistochemical analysis of human breast cancer sample with an antibody directed against ERα methylated on R260 (anti-ERα(CH3)R260). Methylated ERα localizes in the cytoplasm of tumor cells (high level of expression) and in their normal counterparts (low level of expression) (obj 40×).

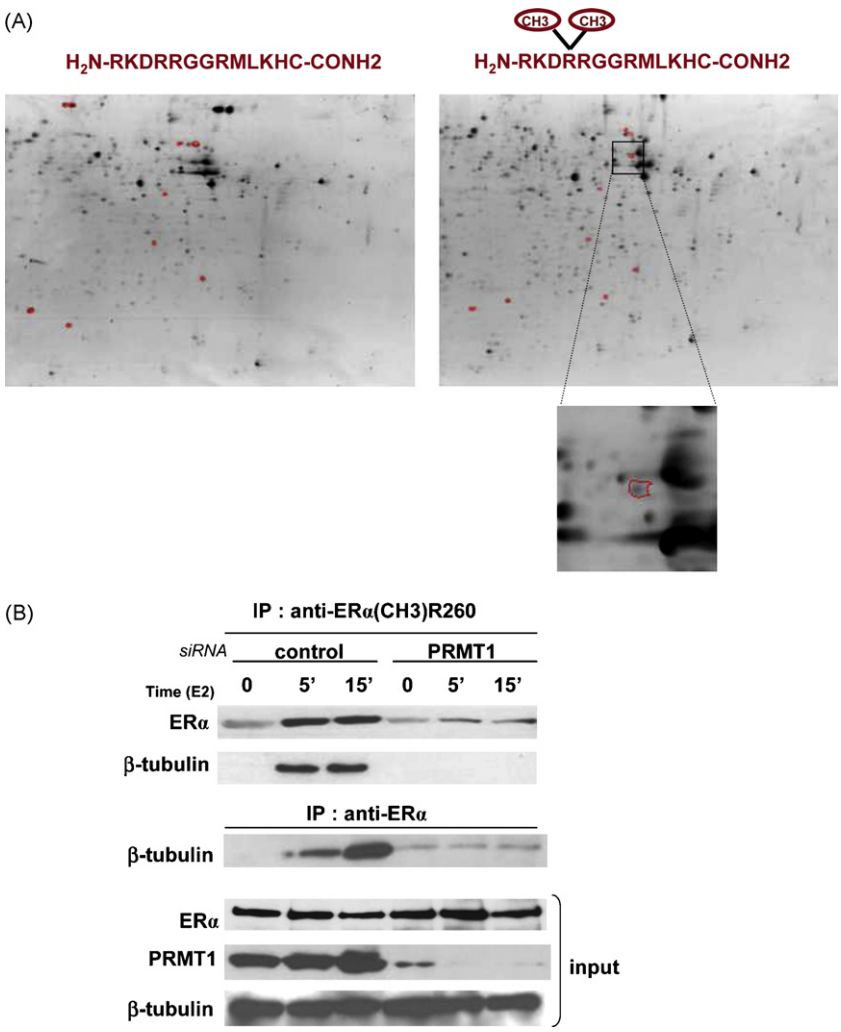

Fig. 4. β-Tubulin interacts with methylated ERα after rapid E2 induction. (A) Silver-stained gel obtained by *in vitro* pull-down assays and 2D-PAGE-based proteome analyses (see text). Represented spots (circled by red lines), representing proteins differentially associated with methylated or nonmethylated peptides, were analyzed by mass spectrometry-based microsequencing and identified using database search algorithms. The position of β-tubulin spot in the gel is shown in the enlarged region. (B) MCF-7 cells were transfected with control siRNAs or with specific PRMT1 siRNAs. Cell extracts from estrogen-deprived cells ($t = 0$) or stimulated with 10^{-8} M estradiol for the indicated times were immunoprecipitated with the anti-ERα(CH3)R260 and anti-ERα antibodies, followed by western blotting with antibodies against ERα and β-tubulin. The amount of ERα, β-tubulin, and PRMT1 in the different samples was determined by western blot. (For interpretation of the references to color in the citation of this figure, the reader is referred to the web version of the article.)

nucleus. Indeed, R260 lies within one of the nuclear localization signals of ERα, so methylation at this site might interfere with ERα nuclear import. The first enzyme described as capable to reverse the methylation of arginine residues is JMJD6 [31], although it remains unclear whether JMJD6 activity is limited to histones or it is also able to demethylate nonhistone proteins. So far, although we are analyzing the functional relationships between ERα and JMJD6, the possibility also exists that other types of arginine-demethylases have yet to be discovered.

5. ERα methylation in breast tumors

Given the fact that ERα was methylated in human breast cells, we checked its expression in human breast tissue by immunohistochemical analysis using an anti-methyl-ERα antibody [27]. In confirmation of biochemical data, methylated ERα was faintly detectable in the cytoplasm of normal epithelial cells and showed only a diffuse expression (Fig. 3). On the contrary, its expression in tumor tissue varied in intensity from faint to strong polarized and granular staining. The analysis of a cohort of 164 invasive breast cancer tumors showed that around 50% of the tumors expressed high levels of methylated ERα [27]. Interestingly, the presence or absence of methylated ERα did not correlate with the clinical classification of ER positive or negative tumors [27]. In fact, this seemingly paradoxical result is not surprising because only tumors showing ERα nuclear staining were included in the population of ERα-positive cases. Further studies will be necessary to determine if methylated ERα could constitute a new marker of breast tumorigenesis. The present results, together with the observations in breast cell lines, indicate that ERα is methylated in breast under physiological conditions and that this process is deregulated in breast tumors by an unknown mechanism. Moreover, deregulation of the demethylation mechanism (loss of expression or loss of enzyme activity) as well as hyperactivation of the methylase activity of PRMT1 can be responsible for the high levels of methylated ERα found in a subset of breast cancers. Whether this modification of ERα is a cause or a consequence of cancer needs to be addressed.

6. Methylated ERα also recruits β-tubulin

To better understand the role of methylated ERα and identify proteins specifically interacting with the methylated form of

ERα, we established a proteomic approach using *in vitro* pull-down assays and 2D-PAGE-based proteome analyses. Asymmetrically methylated or nonmethylated peptides encompassing R260 and adsorbed to Sepharose beads were incubated with HeLa cell extracts. After extensive washing, the proteins associated with each peptide were eluted, fractionated on SDS acrylamide gels, and silver-stained (Fig. 4A). Individual differentially represented spots were analyzed by mass spectrometry and identified using database search algorithms. Among the identified proteins that associated preferentially to the methylated peptide, we identified β-tubulin (Fig. 4A) as a partner of methylated ERα and we focused on this protein because previous reports have shown that endogenous ERα can associate with microtubules [32] and that estrogen rapid actions induce the interaction of ERα with tubulin and HDAC6 at the plasma membrane and tubulin deacetylation [20]. We then addressed the question of whether the ability of ERα to bind β-tubulin requires the methylation of R260. Cell extracts from MCF-7 cells stimulated with E2 were immunoprecipitated with anti-ERα(CH3)R260 or anti-ERα. The precipitates were analyzed by immunoblotting with anti-ERα, and anti-β-tubulin (Fig. 4B). By correlating with the ERα methylation peak, β-tubulin associated with ERα with a similar kinetic in both immunopreciptations. Most importantly, the silencing of endogenous PRMT1 was sufficient to inhibit the estrogen-dependent association of ERα and β-tubulin (Fig. 4B). These findings collectively established that methylation is required for the interaction of ERα with β-tubulin.

7. Conclusions

As nongenomic estrogen signaling seems to play a pivotal role in breast tumorigenesis, deciphering the initial mechanisms that start the process appears crucial. New insights in estrogen signaling have emerged since the discovery of arginine methylation as a new ERα post-translational modification essential for nongenomic estrogen signaling regulating the complex formation containing Src/PI3K and FAK. These results raise several questions about the physiological role of ERα methylation, how it is regulated and turned up in breast tumors, and whether a relationship exists between ERα hypermethylation and breast cancer development and/or progression. To address the physiological relevance of ERα methylation *in vivo*, we are currently producing knock-in mice carrying mutations in R260 of the estrogen receptor. These knock-in mice will provide a key resource for the in vivo validation of many of these *in vitro* findings. Finally, because ERα is hypermethylated in breast tumors, it is tempting to speculate that this deregulation may be involved in breast cancer development and may contribute to resistance to hormonal therapy. This hypothesis is supported by previous reports demonstrating that hyperactivation of cellular kinase signaling, notably of Akt, is probably the most frequent lesion occurring in human cancers. The overall idea would then be that ERα hypermethylation leads to Akt hyperactivation, described as a selective survival advantage for primary tumor cells, even in the presence of anti-estrogens.

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