Alternative O-Glycosylation/O-Phosphorylation of Serine-16 in Murine Estrogen Receptor β

POST-TRANSLATIONAL REGULATION OF TURNOVER AND TRANSACTIVATION ACTIVITY*

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O-Linked N-acetylgalactosamine (O-GlcNAc) is a dynamic post-translational modification abundant on nuclear and cytoplasmic proteins. Recently, we demonstrated that the murine estrogen receptor-β (mER-β) is alternatively O-GlcNAcylated or O-phosphorylated at Ser16. Analyses of mER-β containing mutations in the three adjacent hydroxyl amino acids at this locus confirmed that Ser16 is the major site of O-GlcNAc modification on mER-β and that mutants lacking hydroxyl amino acids at this locus are glycosylation-deficient. Pulse-chase studies in transfected Cos-1 cells demonstrate that the turnover rate of the mutant containing a glutamic acid moiety at Ser16, which mimics constitutive phosphorylation at this locus, is faster than that of the wild type receptor. Whereas, the mutant without hydroxyl amino acids at this locus is degraded at a slower rate, indicating that O-GlcNAc/O-phosphate at Ser16 modulates mER-β protein stability. Luciferase reporter assays also show that the Ser16 locus mutants have abnormal transactivation activities, suggesting that the two alternative modifications at Ser16 on mER-β may also be involved in transcriptional regulation. DNA mobility shift assays show that the mutants do not have altered DNA binding. Green fluorescence protein constructs of both wild type and mutant forms of mER-β show that the receptor is nearly exclusively localized within the nucleus. It appears that reciprocal occupancy of Ser16 by either O-phosphate or O-GlcNAc modulates the degradation and activity of mER-β.

A large number of proteins, ranging from cytokeskeletal components to nuclear proteins, are dynamically modified by O-linked N-acetylgalactosamine (O-GlcNAc)† in virtually all higher eukaryotes, including plants and fungi (for reviews see Refs. 1 and 2). Many O-GlcNAcylated proteins are regulatory proteins, such as transcription factors (3, 4), nucleoporins (5), and cytokeskeletal proteins (6, 7). The attachment and removal of O-GlcNAc by specific enzymes (8–13) is rapid and analogous to the dynamics of O-phosphate controlled by kinases and phosphatases (14, 15). O-GlcNAc sites resemble phosphorylation sites, and in some cases the two modifications are mutually exclusive (16). For example, in the case of estrogen receptor β (ER-β) (17), SV-40 large T antigen (18), and the c-Myc oncogene (19, 20), O-GlcNAc and O-phosphate compete for the same hydroxyl moiety. Like phosphorylation, O-GlcNAcylation is responsive to the cell cycle (21), extracellular signals (22), glucose metabolism (23), and to the growth state of the cell (24). Other studies suggest that O-GlcNAcylates plays important roles in the regulation of transcription and translation (16, 25–27).

The estrogen receptor (ER) is a central component of estrogen regulation (28, 29). Recently ER-β, a homologue of ER-α, was discovered in various species (30–33). ER-β not only shares many structural and functional features with ER-α but also has distinctive characteristics, such as different tissue distribution (31) and differential ligand responsiveness (34). High expression of ER-β in human brain, cardiovascular system, thymus, bone, kidney, lung, urogenital tract, and gastrointestinal tract suggests that ER-β has significant roles in cellular functions not previously thought to involve estrogens. Such ER-β-mediated functions might include effects on memory and reproduction, enhancement of T-cell immunity, vascular lesion protection, and colon cancer protection. In addition, ER-β appears to be the only estrogen receptor type expressed in the embryonic central nervous system, implying that ER-β is important to early embryonic development. Transgenic mice with only mER-β deleted (35) and double mER-α/mER-β knockouts (36) have been generated. Female ER-β knockout mice develop follicular arrest and anovulation, indicating that both ER-β and ER-α are required for the maintenance of germ and somatic cells in the postnatal ovary. Data from these mice further suggest that several specialized functions are mediated distinctly between ER-α and ER-β. However, because ER-β was only recently discovered (30–33), much work remains toward elucidating its functions and relationships to ER-α.

Previously, we mapped the major O-GlcNAc site to Ser16 near the amino terminus of mER-β and showed that this same hydroxyl moiety is alternatively modified by O-phosphate (17). Here, we report that these two alternative modifications play distinct roles in modulating mER-β degradation and function.

MATERIALS AND METHODS

mER-β O-GlcNAc Site Mutants and Related Plasmid Constructs—The mER-β cDNA was engineered using an established method, as described in our previous studies (17). To introduce the appropriate restriction enzyme sites, mER-β cDNA in pBlueBac 3 (Invitrogen, San Diego, CA) was subcloned into pBlueBacHi2 and excised using XhoI and EcoRI digestion. The resulting fragments were then subcloned into...
pcDNA3.1(−) for functional studies. All constructs were verified by automated DNA sequencing.

Altered Sites I (Promega, Madison, WI) was used to mutate mER-β Ser29, mER-β cDNA in pcDNA3.1(−) was subcloned into pAlter I via XbaI and EcoRI sites. The mutated expression primers (shown in parenthesis) were used, S16A, CTTCGCCGAGTGTAGCAGCGGGGAGGACTGATGTC, S16E, CCTTCACTTCACTCGGCCGGGAACTCAGTATGTC containing changes (with underline) of the Ser16 to Ala or Glu along with the changes of two adjacent Ser32 and Thr37 to Ala and Val were synthesized and used, respectively, in the mutagenesis process. The mutants were named as S16A and S16E accordingly, as illustrated below in Fig. 1. The mutated DNAs were verified by automated DNA sequencing with serial internal primers covering the entire coding region and subcloned back into pcDNA3.1(−) and other plasmids for functional studies.

To study the subcellular localization of mER-β, mER-β cDNA in pAlter I was digested with BglII and EcoRI and subcloned into GV vector pEGFP-C1 (CLONTECH, Palo Alto, CA) in-frame. The final engineered mER-β cDNAs in pEGFP-C1 were verified by automated DNA sequencing. To immunoprecipitate mER-β expressed in mammalian cell lines, a FLAG tag encoding the peptide epitope, DYKDDDDK, was incorporated into the carboxyl-terminal end of mER-β cDNA using polymerase chain reaction (37). All constructs were verified by automated DNA sequencing.

Characterization of the Glycosylation of the mER-β O-Glcnac Mutant—Expression and purification of mER-β proteins from Sf9 cells were described previously (17). The O-Glcnac moieties on purified mER proteins were labeled with galactosyltransferease by transferring [3H]galactose from UDP-[3H]galactose, as described previously (38). The tritium-labeled protein was separated from unincorporated UDP-[3H]galactose using a 1.5 × 30-cm Sephadex G-50 column in 50 mM ammonium formate, 0.1% (w/v) SDS. For tritium images, the protein was resolved on 10% SDS-PAGE gel, and the gel was stained with Coomassie Blue R-250, impregnated with 1M salicylic acid for 30 min, dried under vacuum, and exposed to x-ray film. The tritium-labeled protein was separated from unincorporated UDP-[3H]galactose using a 1.5 × 30-cm Sephadex G-50 column in 50 mM ammonium formate, 0.1% (w/v) SDS. For tritium images, the protein was resolved on 10% SDS-PAGE gel, and the gel was stained with Coomassie Blue R-250, impregnated with 1M salicylic acid for 30 min, dried under vacuum, and exposed to x-ray film at −70 °C for 2 days. For trityl map comparison, the labeled proteins were digested with 2% (w/v) trypsin (sequencing grade, Roche Molecular Biochemicals, Indianapolis, IN) in 0.1 M ammonium bicarbonate (pH 7.4) at 37 °C overnight. Separation of [3H]galactose-labeled tryptic mER glycopeptides was achieved on a C8 column, reversed phase column (3.2 × 100 mm, Amersham Pharmacia Biotech, Piscataway, NJ) using a 0–60%(v/v) gradient of acetonitrile in 0.1%(w/v) trifluoroacetic acid over 90 min at a flow rate 0.1 ml/min on a SMART system (Amersham Pharmacia Biotech, Piscataway, NJ) in 0.1M ammonium bicarbonate (pH 7.4) over-night. Trypsin digestion of mER proteins from Sf9 cells was achieved on a C8 column, reversed phase column (3.2 × 100 mm, Amersham Pharmacia Biotech, Piscataway, NJ) using a 0–60%(v/v) gradient of acetonitrile in 0.1%(w/v) trifluoroacetic acid over 90 min at a flow rate 0.1 ml/min on a SMART system (Amersham Pharmacia Biotech, Piscataway, NJ). Fractions were collected (0.2 ml/fraction) and counted.

FIG. 1. mER-β mutants used in this study. Wild type mER-β cDNA was mutated at the Ser16 by changing either Ser → Ala or Ser → Glu along with changing Ser32 and Thr37 to Ala and Val. The two mutants were named S16A and S16E, respectively. All constructs were engineered to incorporate a FLAG tag at their carboxyl terminus.

Electrophoretic Mobility Shift Assay—To determine the DNA binding activity of the ER-β mutants, extracts of mER-transfected Cos-1 cells were made according to published methods (39). The assay was carried out in binding buffer containing 20 mM HEPES, pH 7.4, 50 mM KCl, 1 mM dithiothreitol, 10% glycerol, 50 nM estradiol, 0.5 mg/ml bovine serum albumin, 50 ng/μl poly(I/C-dI/C-I). The antibody against ER-β (CalBiochem, La Jolla, CA) and excess unlabeled ER-β probe were added as indicated.

Characterization of Subcellular Localization—To examine subcellular localization of mER-β, the GFP fusion constructs of mER-β cDNA were transfected into mammalian cell lines, as described above. After 1 day, fresh media and 17β-estradiol at the final concentration of 20 nM were added. Fluorescence images were recorded using a digital camera (Hamamatsu, Tokyo, Japan).

RESULTS

Mutation of the O-GlcNAc/O-Phosphorylation Locus on mER-β—Our previous studies demonstrated that mER-β is modified alternatively by O-GlcNAc or O-phosphorylation at Ser16 (17). To reveal biological roles of the O-GlcNAcylation/O-phosphorylation on mER-β, we mutated Ser16 into either Ala or Glu along with Ser15 and Thr27 into Ala and Val to generate two mutants, designated mER-β S16A and S16E, respectively, as illustrated in Fig. 1. The S16E mutant is designed to mimic the constitutive phosphorylation of Ser16 (40, 41). Ser15 and Thr27 were concomitantly mutated to eliminate the possibility for promiscuous O-glycosylation/O-phosphorylation at this locus, which might occur even when the observed major acceptor site (Ser16) is eliminated.

mER-β O-GlcNAc Site Mutant Is Glycosylation-deficient—To

Luciferase Activity Assays—All transfections were done with Cos-1
assess the glycosylation state of mER-β O-GlcNAc site mutants, the protein of the S16A mutant expressed and purified from insect Sf9 cells was probed for O-GlcNAc using galactosyltransferase and UDP-[3H]galactose (38). As shown in Fig. 2A, the protein of the S16A mutant was labeled approximately 5-fold less than the wild type, suggesting that the mutant S16A protein is deficient in O-GlcNAc. To further compare tryptic maps between the wild type and the S16A mutant, labeled proteins were digested with trypsin and the tryptic glycopeptides were resolved on a reverse phase column. As shown in Fig. 2B, tryptic glycopeptide maps from the S16A mutant (top panel) showed only baseline levels of radioactivity, whereas the wild type (bottom panel) showed the same major tryptic glycopeptide (retention time = 42 min) and smaller labeled peaks at retention times of 20, 36, and 54 min, respectively, previously seen for mER-β (17). Previous analyses (17) of the smaller labeled peaks in (Fig. 2B, bottom panel) indicate that they likely result from incomplete proteolysis. This conclusion is supported by their disappearance in the mutant (Fig. 2B, top panel). Thus, we conclude that the mER-β mutants at the Ser16 locus are glycosylation-deficient.

The O-Glycosylation/O-Phosphorylation Site Mutants of mER-β Have Altered Turnover Rates—PEST regions in proteins, enriched with Pro, Glu, Ser, and Thr, have been proposed to be responsible for the rapid degradation of certain proteins (42, 43). Our earlier studies documented that O-GlcNAcylation sites on mER-α (44) and mER-β (17) are in regions of the proteins that have high PEST scores. This observation suggests that one likely role of O-GlcNAc on ER is to modulate ER protein stability. Therefore, we directly examined the relative turnover rates in vivo of the wt and mutant mER-β proteins using pulse-chase analyses. Transfected Cos-1 cells were pulse-labeled with [35S]-labeled amino acids in vivo for 3 h and chased for up to 6 h. Quantitative results averaged from three independent experiments are shown in Fig. 3. Compared with wt, the degradation of the S16A mutant appears to be slower, whereas the degradation of the S16E mutant appears to be much faster. Assuming rough linearity, the wt mER-β turned over rapidly in the presence of estrogen, with an average half-life of 7–8 h, similar to the range of ER-α reported previously (45). In contrast, the S16A mutant has a prolonged average half-life of about 15–16 h, and the S16E mutant has a shortened average half-life of about 4–5 h. These findings suggest that O-phosphorylation on the Ser16 of mER-β results in accelerated degradation of mER-β as mimicked by the S16E mutant, whereas O-glycosylation, which blocks phosphorylation, would be predicted to result in stabilization of the protein.

The O-Glycosylation/O-Phosphorylation Site Mutants of mER-β Have Altered Transactivation Activities—The fact that the major O-GlcNAc site on mER-β is located within the transactivation domain of the protein led us to examine the role of O-GlcNAc/O-phosphate at this site in ER-mediated transcriptional activation. The transactivation activities of the mutants were measured by cotransfection of mER-β cDNAs with an ERE-linked luciferase reporter gene in Cos-1 cells. As summarized in Fig. 4, the S16E mutant has elevated transactivation activities compared with wt-mER-β. However, the S16E mutant is not further stimulated by estrogen, suggesting that the S16E is constitutively active under these conditions. In contrast, the S16A has only basal activity with minor stimulation by estrogen. The relatively modest level of stimulation seen in this system may reflect the lack of appropriate coactivators in the Cos-1 cells used. Nonetheless, it appears that the alternative post-translational modification of Ser16 of mER-β is not only crucial to achieve normal levels of ER-mediated transac-

FIG. 2. The Ser16 to Ala mutant is poorly glycosylated in vivo. A, the wt and the S16A mutant mER-β were expressed and purified from insect Sf9 cells. Purified proteins were enzymatically labeled with UDP-[3H]galactose using galactosyltransferase. [3H]Galactose-labeled proteins were resolved by 10% SDS-PAGE gel electrophoresis. Gels were impregnated with 1 μl salicylic acid, dried, and exposed to x-ray film at −80 °C for 2 days. Scintillation counting of the bands indicated that the band from wild-type contained ~1.77 × 106 dpm and that from the mutant contained ~3.61 × 106 dpm. B, [3H]Galactose-labeled proteins were digested with trypsin. Tryptic peptides were resolved on a C18 reversed phase column. The column was developed with a 90-min linear gradient of 0–60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid from 6 to 96 min at a flow rate of 0.1 ml/min. Eluates were collected and counted. Upper panel, the S16A mutant tryptic profile; lower panel, the wt tryptic profile. Note: Smaller labeled peaks likely result from incomplete proteolysis of the major glycosylation site.
activation but also is important to estrogen responsiveness of the receptor.

Mutation of the O-Glycosylation/O-Phosphorylation Site on mER-β Does Not Affect DNA Binding—To compare the binding of the wild type and mutant forms of mER-β to the DNA response elements, we tested cell extracts from mER-β-expressing Cos-1 cells with both wild type and mutant mER-β cDNAs. The same distribution of the wt receptor was observed for other cell lines, including HeLa and MCF-7 cells, independent of the presence or the absence of its cognate ligand estrogen. The same distribution of the wt receptor was also observed for other cell lines, including HeLa and MCF-7 cells (data not shown).

Earlier studies on nuclear pore proteins suggested that O-GlcNAcylation is likely involved in nuclear transport (5, 46–48). Because the functionality of the estrogen receptor relies on its proper subcellular localization, we also examined the subcellular localization of the two glycosylation/phosphorylation site mutants as GFP fusion proteins. Both mutants are also exclusively localized to the nucleus (data not shown), suggesting that modifications at Ser16 are not involved in mediating mER-β's nuclear transport (Fig. 3).

Fig. 3. The modification state of Ser16 of mER-β affects the protein's turnover rate. Cos-1 cells transfected with either the wild type or mutated mER-β were metabolically labeled with 35S-protein Express label for 3 h and then chased with unlabeled rich medium for various time points as indicated in the figure. Cell lysates were prepared as described under “Materials and Methods.” Samples, containing 5 × 107 dpm per lysate, were immunoprecipitated with anti-FLAG antibody M2, and isolated proteins were resolved by electrophoresis on 10% SDS-PAGE gels. Gels were fixed, dried, and exposed to x-ray film for 30 min. 35S images from three separate experiments were processed with an IS-1000 Digital imaging system. The density values of mER-β bands from every time point are converted into percentages relative to the zero time point of each sample. The average percentages along with standard deviations are plotted.

Fig. 4. Modification of Ser16 of mER-β modulates the protein's transactivation activity. Cos-1 cells were cotransfected with the reporter plasmid ERE-TATA-TK-Luc, internal control β-galactosidase expression plasmid, and the expression vector (V) containing wt or mutant mER-β cDNA. At 24 h post-transfection, 20 nM 17β-estradiol (E2) in fresh medium was added, and the cells were incubated overnight. Cells were then harvested, and luciferase assays were performed. All activities were normalized with activities obtained from equal numbers of cells cotransfected with wt-β-galactosidase. The relative luciferase activity was set as 1 for the activity in cells transfected with the vector alone.

Fig. 5. Modification of Ser16 of mER-β does not affect the protein's DNA binding. The mER-β DNAs were transfected into Cos-1 cells by the liposome method. Cell extracts were prepared for the electrophoretic mobility shift assay. The vitellogenin estrogen response element (ERE) DNA probe was labeled with 32P using the Klenow fragment of DNA polymerase. The specificity of the assay was demonstrated by cold probe competition (100-fold excess) and mER-β antibody blocking of the interaction. Note: mER-β levels are less in the S16E mutant due to its rapid degradation (see text and Fig. 3).

Since the discovery of the O-GlcNAc modification, several functional roles have been postulated, such as modulation or mediation of protein-protein interactions, regulation of nuclear transport, transient regulation of phosphorylation site availability, and modulation of protein turnover (1, 2). In this study, we provide evidence that O-GlcNAc on mER-β has a reciprocal relationship with phosphorylation by capping a phosphorylation site that modulates mER-β degradation and is also important for the receptor's transactivational activity.

Previous studies on eukaryotic initiation factor 2-associated protein p67 and on the transcription factor Sp1 showed that O-GlcNAc removal from both p67 and Sp1 targets them for rapid degradation by the proteasome (25, 49). Studies on the mutated forms of mER-β not only provide additional evidence...
for O-GlcNAc regulating protein degradation but also suggest that the saccharide may act by blocking the addition of phosphate, which itself targets the protein for rapid degradation. To further understand the relative roles of O-GlcNAc versus O-phosphate at Ser\textsuperscript{16}, we compared the wt mER-\textbeta to both the S16A and the S16E mutants, the latter of which mimics constitutive phosphorylation (40, 41, 50, 51). The simplest interpretation of the data is that the S16E mutant behaved as the phosphorylated form of the protein resulting in accelerated degradation, whereas the S16A mutant behaved analogous to the “capped” glycosylated form of the protein that slowed degradation. Because many of the known O-GlcNAc sites are located near proline residues, glycosylation sites adjacent to acidic amino acids will have high intrinsic PEST scores, whereas others may be dependent upon phosphorylation to target PEST-mediated degradation (42, 43). It has been suggested that phosphorylation can change Ser or Thr residues into negatively charged residues so as to convert some imperfect PST sequences into PEST degradation signals. In contrast, O-GlcNAcylation could prevent these phosphorylation effects by either competing at the same hydroxyl directly or by changing the protein conformation indirectly to mask the charged regions, as has been suggested for p53 (52).

The N terminus of mER-\textbeta, which harbors the major O-GlcNAc site, mediates the receptor’s transactivation functions, which in turn activates target genes (53, 54). Our luciferase reporter data show that the extent of transactivation is dependent upon the modification of the hydroxyl group of Ser\textsuperscript{16}. These data suggest that O-GlcNAc/O-phosphate at this site directly plays a role in modulating mER-\textbeta-mediated transactivation. Earlier studies suggested that O-GlcNAcylation modulates transactivation by mediating the appropriate protein-protein interactions of many transcription factors such as Sp1 (3, 26, 27). Recent in vivo studies showed that the concentration of these transcriptional activator proteins is regulated by the proteasome-mediated degradation pathway, and the rate of degradation of activators by the proteasome correlates with activation domain potency in vivo (55). Consistent with these earlier reports on other transcription factors, the alternate O-GlcNAc/O-phosphorylation of mER-\textbeta appears to be involved in both degradation and transactivation functions of the molecule.

Based on our in vivo \textsuperscript{32}P orthophosphate labeling studies (data not shown), Ser\textsuperscript{16} is one of several phosphorylation sites on mER-\textbeta. It is likely that Ser\textsuperscript{16} is a regulatory site with typically low occupancy and rapid cycling. Earlier studies on transcription factor Sp1 have suggested that, upon glucose starvation, Sp1 undergoes rapid deglycosylation and becomes more susceptible to proteasome degradation (49). Recently, it has been reported that ER-\alpha is rapidly degraded by the 26 S proteasome upon estrogen stimulation (56). O-GlcNAc trans-erase activity is exquisitely sensitive to concentrations of UDP-GlcNAc/UDP, which are in turn highly sensitive to energy metabolism (11, 23). Thus, in energy-rich conditions, O-GlcNAc levels would be expected to increase, in turn preventing degradation of certain proteins, such as ER-\beta.

Our green fluorescence protein fusion results revealed that there is no significant redistribution of mER-\beta in mammalian cells induced by the mutations, excluding the possibility that nuclear localization requires either phosphorylation or O-GlcNAcylation at the Ser\textsuperscript{16} of mER-\bet. However, we did observe some minor nuclear pattern changes, such as a clustering of ER-\beta within the nucleus upon estrogen treatment (Fig. 6). This clustering phenomenon is similar to that seen for ER-\alpha using a similar approach (57).

The occurrence of O-GlcNAcylation sites in the key regulatory regions of some oncogenes and tumor suppressors, such as c-Myc, p53, and SV-40 large T-antigen (18–20, 52), reinforces the potential regulatory significance of this modification. If a reciprocal relationship between O-GlcNAcylation and O-phosphorylation is found to be common, then it will be important to carefully evaluate the respective roles of these distinct modifications. Generally, this will not be possible by direct site-directed mutagenesis approaches but rather will require novel methods, such as the chemi-enzymatic synthesis of site-specifically modified glyco- and phospho-forms of these regulatory proteins (58–60). The interplay between O-GlcNAc and O-phosphate on mER-\beta Ser\textsuperscript{16} demonstrated in this study provides another excellent example where both of these modifications work in a coordinated manner to regulate the activity of a key regulatory protein.

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