Alternative O-Glycosylation/O-Phosphorylation of the Murine Estrogen Receptor β[†]

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ABSTRACT: Estrogen receptor β, a homologue to estrogen receptor α, is a new member of the steroid hormone receptor family. Recently, we documented that estrogen receptor α, like other transcription factors, is modified by O-linked *N*-acetylglucosamine (O-GlcNAc), a ubiquitous transitory posttranslational modification on nuclear and cytoplasmic proteins. Here, we report that estrogen receptor β is alternatively modified by either O-GlcNAc or O-phosphate. Lectin chromatography of in vitro translated protein first suggested that murine estrogen receptor β (mER-β) is O-GlcNAcylated. Structural characterization of the carbohydrate moieties on mER-β, overexpressed in insect Sf9 cells, confirmed the presence of O-GlcNAc. mER-β, overexpressed in mammalian cells, is also O-GlcNAcylated. The major site of O-GlcNAc on mER-β from Sf9 cells is Ser^{16} near the N-terminus. Concomitant analyses also documented the O-phosphorylation of mER-β at Ser^{16}. MALDI-TOF mass spectrometry showed alternative occupancy of this locus by these two abundant and dynamic posttranslational modifications. The localization of a major O-GlcNAc/O-phosphate site in proximity of the transactivation domain and as part of a PEST region (target sequences for rapid protein degradation) on mER-β suggests that these modifications may play a role in regulating estrogen receptor β transactivation and turnover.

Estrogen receptors (ERs)[1] belong to the ligand-activated steroid receptor superfamily (*1*). Signal transduction by the ER-α is initiated by the binding of its cognate ligand, estrogen, or by phosphorylation. Upon estrogen binding, the ER-α becomes hyperphosphorylated by several protein kinases at particular sites such as Ser^{104} and Ser^{106} by the cyclin A−CDK2 complex (*4*), Ser^{118} by mitogen-activated protein kinase (*5*), Ser^{128} by casein kinase II (*6*), Ser^{236} by protein kinase A (*7*), and Tyr^{537} by Src kinase (*8*). The phosphorylated ER-α dimerizes on its response element in the promoter regions of ER-responsive genes (*2*, *3*). In the absence of ligand, activation can also result from other kinase-mediated signal transduction cascades which modulate ER activation.

During recent efforts to elucidate estrogen regulation, ER-β, a homologue of ER-α, was cloned and characterized from several species (*9*−*11*). Functional studies on ER-β show that ER-β shares many features with mER-α but has a different tissue distribution (*12*), implying different roles of ER-β and ER-α. In addition, multiple isoforms of ER-β have also been reported (*13*). The knockout ER-β transgenic mouse has been generated which shows no significant detrimental effect (*14*), suggesting overlapping roles of the ERs in whole animals. Abnormalities in ER structure itself or ER signal transduction pathways, however, contribute to ER-related genetic disorders such as osteoporosis, cardiovascular disease, diabetes, and cancer (*15*).

O-GlcNAcylation, the attachment of *N*-acetylglucosamine to serine or threonine side chain hydroxyl groups, is an ubiquitous, but transitory modification analogous to phosphorylation that occurs on many cytosolic and nuclear proteins (*16*, *17*). The identity or close proximity of O-GlcNAcylation and O-phosphorylation sites on several proteins implies a reciprocal relationship between O-GlcNAcylation and phosphorylation on a growing list of proteins (*18*, *19*). Many of the characteristics and dynamics of O-GlcNAc, however, suggest its potential importance as a regulatory modification in its own right (*17*). Recent studies show that O-GlcNAcylation is required for life at the single cell level (*20*) and, thus, likely plays important roles in many aspects of protein function (*21*, *22*).

Our previous studies demonstrated that mouse ER-α is modified at several sites by O-GlcNAc, with a major site at Thr^{575} on the C-terminal end of the protein (*23*), a site at Ser^{10}, and a site within the transactivation domain at Thr^{50}

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[1] Abbreviations: mER-β, mouse estrogen receptor β; ERE, estrogen response element; O-GlcNAc, O-linked *N*-acetylglucosamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance chromatography; TFA, trifluoroacetic acid; galactosyltransferase, Galβ(1−4)galactosyltransferase; Gal, galactose; PNGase F, peptide *N*-glycosidease F; RCA I, *Ricinus communis* agglutinin I; His_6, six consecutive histidine residues; *E. coli*, *Escherichia coli*; ECL, enhanced chemiluminescence; HPAEC−PAD, high pH anion-exchange chromatography−pulsed amperometric detection; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; CHCA, α-cyano-4-hydroxycinnamic acid; LC/ESI-MS, liquid chromatography coupled electrospray ionization mass spectrometry.

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(manuscript submitted). We initiated the present study to determine whether the ER-β is also O-GlcNAcylated and to compare the glycosylation sites between the two ERs. Here we show that mER-β is modified by O-GlcNAc, that the major site is at Ser^{16} of mER-β, and that this O-GlcNAcylation site and/or adjacent hydroxy amino acids (Ser^{15} and Thr^{17}) are also alternatively O-phosphorylated, providing yet another important example of the reciprocal relationship often observed between these two ubiquitous and dynamic modifications. Major O-GlcNAc sites on both ER-α and ER-β are proximal to the transactivation domains and are within regions of high PEST scores (*24*), implying a role for O-GlcNAcylation/O-phosphorylation in regulation of transactivation and/or ER turnover.

EXPERIMENTAL PROCEDURES

Characterization of the Glycosylation State with Lectin RCA I Chromatography. mER-β cDNA was kindly provided by Dr. Gilles B. Kimblay in a pCMX vector. An in vitro coupled transcription/translation reaction was conducted using rabbit reticulocyte lysates, as described (Promega, Madison, WI). Galactosyltransferase labeling, β-galactosidase treatment, and subsequent RCA I chromatography followed the method described previously (*25*). Briefly, 30 μL of in vitro translated mER-β protein was labeled with 0.4 mM UDP-Gal, using 0.04 unit of galactosyltransferase at 4 °C overnight. Half of labeled product was treated with β-galactosidase in sodium phosphate buffer, pH 7.4, containing 0.1 unit of the enzyme (Sigma, St. Louis, MO), 0.01 unit of aprotinin, 1 μg of leupeptin, and 1 μg of α_2-macroglobulin at 37 °C overnight. Ten microliters or equivalent of the translation mixture was applied to 150 μL of RCA I Sepharose (EY Laboratories, San Mateo, CA) equilibrated with wash buffer containing 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, and 0.2% Nonidet P-40. The column was washed with wash buffer and challenged for nonspecific binding with 1 M GlcNAc in wash buffer. Bound material was then eluted with 1 M Gal in wash buffer. Aliquots of each fraction were monitored using liquid scintillation counting. The radioactive peak fractions were pooled, precipitated with trichloroacetic acid, and analyzed by 10% SDS−PAGE and fluorography.

Expression and Purification of mER-β. The mER-β cDNA was engineered with PCR (*26*). The coding sequence of His_6 tag was linked to the 3′ end of the cDNA, along with two restriction enzyme sites (*Bgl*II and *Nco*I) at 5′ and 3′ ends, respectively. The tagged cDNA fragment was subcloned into a baculoviral transfer vector pBlueBac3 (Invitrogen, San Diego, CA) via *Bgl*II and *Nco*I sites and verified by automated DNA sequencing with four internal sequencing primers covering the entire cDNA region. The recombinant virus of mER-β for expression in insect Sf9 cells was made with the transfection module by following the manufacturer's instruction (Invitrogen, San Diego, CA). To introduce appropriate restriction enzyme sites, mER-β cDNA in pBlueBac3 (Invitrogen, San Diego, CA) was subcloned into pBlueBacHis2 and excised using *Xho*I and *Eco*RI digestion. The resulting fragment was then subcloned into pcDNA3.1 (−) for expression in mammalian Cos-7 cells.

For site mapping, the His_6-tagged mER-β expressed in insect Sf9 cells was purified under denaturing conditions using Talon resin (Clontech, Palo Alto, CA). Briefly, infected cells were harvested, washed with phosphate-buffered saline buffer, and lysed in lysis buffer containing 6 M guanidinium chloride, 20 mM Tris, pH 7.9, and 0.1 M NaCl. The lysate was sonicated and centrifuged, and then the supernatant was mixed with Talon resin equilibrated with lysis buffer for 30 min. The resin was washed twice with washing buffer containing 8 M urea, 20 mM Tris, pH 7.9, and 0.1 M NaCl for 10 min and eluted with 0.3 M imidazole in washing buffer in a batchwise fashion. Protein eluted from the resin was subsequently purified by preparative SDS−PAGE with a prep-cell electrophoresis unit (Bio-Rad, Hercules, CA). The eluted protein was desalted and subjected to further characterization.

Western Blot and Autoradiography. Western blotting was conducted by the following standard procedures. Proteins were resolved on 10% SDS−PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Membranes were probed with either monoclonal antibody RL-2, specific for the O-GlcNAc moieties on proteins, or a monoclonal antibody against His_6 tag (Clontech, Palo Alto, CA). His_6-tagged mER-α expressed and purified from *Escherichia coli* (Cheng and Hart, unpublished data) was used as a negative control for RL-2 antibody. The immunoreactive protein was detected with a horseradish peroxidase conjugated secondary antibody followed by ECL (Amersham, Piscataway, NJ). For tritiated samples, the gel was stained with Coomassie Blue R250, impregnated with 1 M salicylic acid for 30 min, dried under vacuum, and exposed to X-ray film at −70 °C overnight.

Electrophoresis Mobility Shift Assay. To determine DNA binding activity of mER-β expressed from insect Sf9 cells, the whole cell lysate was assayed according to published methods (*27*). Typically, 2 μL of the lysate was used in each binding reaction. DNA binding reactions were 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 BSA, and 50 ng/μL poly[d(I−C)/(I−C)]. One nanogram of ^{32}P-labeled double-stranded oligonucleotide probe containing a consensus ERE sequence from chicken vitellogenin (5′-CTAGAAAGTCAGGTCACAGTGACCTGATCATT-3′) was used in 20 μL reactions. Preincubations with all the reaction components except labeled probe were conducted on ice for 15 min. After addition of the labeled probe, the binding reactions were allowed to incubate on ice for 15 min. The ER−ERE complex was then resolved on native 6% polyacrylamide gel. The gel was fixed, dried, and exposed to X-ray film. The antibody against ER-β (Calbiochem, La Jolla, CA) and excess unlabeled ERE probe were added as indicated.

Galactosyltransferase Labeling. The carbohydrate moieties on purified protein were probed with bovine milk galactosyltransferase (Sigma, St. Louis, MO) using UDP-[6-^{3}H]Gal (38 Ci/mmol; Amersham, Piscataway, NJ) according to a published method (*28*). To achieve maximum labeling efficiency, the protein was first denatured by boiling in 0.1% SDS for 5 min. SDS binding was neutralized with 1% Triton X-100, and the mixture was labeled at 4 °C overnight and chased with 0.4 mM cold UDP-Gal for 4 h to saturate potential sugar acceptor sites. The labeled protein was desalted over a 1.5 × 30 cm Sephadex G-50 column equilibrated with 50 mM ammonium formate and 0.1% SDS

to remove unincorporated UDP-Gal, dried down, and precipitated by acetone at a ratio of 8:1 (v/v).

Characterization of the Sugar Moieties. For PNGase F digestion, 5 μg of [^{3}H]Gal-labeled protein was treated with 0.5 unit of PNGase F using the buffer that the manufacture supplied (New England BioLabs, Beverly, MA). An equal amount of [^{3}H]Gal-labeled ovalbumin was used as a positive control for the reaction. Half of the volume of each sample was applied to an 8% SDS−PAGE gel. The gel was stained with Coomassie Blue, impregnated with 1 M salicylic acid, dried, and exposed to X-film at −80 °C for 2 days. The remaining half of each sample was subjected to Sephadex G-50 chromatography.

For the characterization of the sugar moieties on the protein, the labeled sugar moieties were removed from the protein by alkali-induced β-elimination in the presence of 0.1 M NaOH and 1 M $NaBH_4$ at 37 °C overnight. After neutralization with acetic acid, β-eliminated products were fractionated on a 1.5 × 30 cm Sephadex G-50 column in 50 mM ammonium formate and 0.1% SDS. V_i fractions were pooled, lyophilized, and resuspended in 1 mL of water and then mixed with 0.25 mL of 25% KCl. The precipitated SDS was removed by centrifugation, and the supernatant was passed through a Superdex peptide column (Pharmacia, Piscataway, NJ) in 0.2 M ammonium acetate at a flow rate of 0.1 mL/min. The tritiated portion of the eluate was pooled, desalted by passage over a 1 mL column of Dowex AG50W-X8 (200−400 mesh, H^+ form, Bio-Rad), and further analyzed by HPAEC-PAD on a CarboPak MA-1 column using a 30 min isocratic gradient of 0.2 M NaOH at a flow rate of 0.4 mL/min (Dionex Corp., Sunnyvale, CA) (*29*).

Mapping the O-GlcNAc Modification Sites. The tritium Gal-labeled protein was digested with trypsin at a 1:20 (w/w) ratio at 37 °C overnight. Tryptic peptides were first resolved on a reverse-phase C_{18} column (Pharmacia, Piscataway, NJ) with a 0−90% B (0.1% TFA/60% acetonitrile) gradient over 90 min and a flow rate 0.1 mL/min. Subsequently, a second dimension of the major Gal-labeled tryptic peptides was separated on the same column with a shallow 15−45% B (0.1% TFA/60% acetonitrile) gradient over 90 min at the same flow rate. Digestion of selected fractions with proline endopeptidase (Seikagaku Corp., Tokyo, Japan) was done according to instructions given by the manufacturer. Proline endopeptidase digested tryptic peptides were separated using the same conditions as the second dimension. The glycopeptides were analyzed using MS and manual Edman degradation.

Mass Spectral Analysis and Sequence Determination of Tryptic Peptides. The identity of glycopeptides was determined by automated Edman gas-phase sequencing or by using MS. MALDI-TOF mass spectra were acquired in the positive ionization mode using a Hewlett-Packard G2025A mass spectrometer. Peptide samples were prepared for MALDI-TOF MS by diluting the sample solution with CHCA matrix solution (Hewlett-Packard, Palo Alto, CA) to a final concentration of 0.1−1 pmol/μL. After an aliquot (0.8 μL) of this solution was applied onto a metal target, salts were washed away by dipping the target briefly into a solution of 0.1% TFA. The sample target was dried in vacuo and then placed into the mass spectrometer. Mass spectra were calibrated externally using a mixture of standard peptides. Three spectra were collected for each sample, and the average *m*/*z* values with error ranges are reported. Protein ladder sequencing was achieved by limited digestion of samples with aminopeptidase M (slurry form; Boehringer Mannheim, Indianapolis, IN). Briefly, a 0.5 μL volume of sample was mixed with 2.0 μL of aminopeptidase M (1:50 dilution in 0.1 M Tris-HCl, pH 7.5). At desired time points, 0.8 μL of digest solution was removed and mixed with 0.4 μL of 10% (v/v) TFA + 0.8 μL of CHCA matrix solution. A 0.8 μL volume of this solution was dried on the MALDI target and washed with 0.1% TFA as above. Mass spectra were then acquired. LC/ESI-MS/MS was performed as described (*30*). The exact position of O-GlcNAc modification was also determined by manual Edman degradation, as described previously (*18*).

MALDI-TOF and LC/ECI-MS/MS Analysis of the Phosphorylated Peptide of mER-β. An aliquot of mER-β peptides carrying the major O-GlcNAc site was either treated with 0.5 unit of alkaline phosphatase (Promega, Madison, WI) or mock treated using the buffer supplied by the manufacturer at 37 °C for 30 min in 5 μL volume. The digestion mixture (1 μL) was mixed with matrix α-cyano-4-hydroxycinnamic acid (1 μL) in 50% acetonitrile/0.3% TFA. The identities of the peptides were examined using MALDI-TOF MS (Voyager, PerSeptive Biosystems, Framingham, MA). A LC/ESI-MS/MS approach using a capillary C_{18} column linked to an ion-trap mass spectrometer (LCQ, ThermoQuest, Needham Heights, MA) was used to determine the phosphorylation site on the peptide. LC/ESI-MS/MS was performed as described (*31*).

RESULTS

Characterization of the O-Glycosylation State of mER-β. Galactosyltransferase will transfer a Gal residue from UDP-Gal to any terminal GlcNAc moiety to form Galβ1,4GlcNAc-R, where R can be almost any substrate, including a protein. Galactosyltransferase is used widely as an in vitro probe for O-GlcNAc (*28*). After reaction with galactosyltransferase, a RCA I lectin column will specifically bind terminal Gal residues and indicate whether Gal was transferred to GlcNAcs on mER-β. In vitro translated mER-β protein from rabbit reticulocyte lysate that had been treated with galactosyltransferase was specifically retained by RCA I lectin resin and eluted with 1 M Gal **(**Figure 1A,B). The mER-β protein, therefore, contains terminal GlcNAc moieties. The combined use of galactosyltransferase and RCA I adds an additional level of specificity to these experiments above that obtained by simply using GlcNAc-specific lectins, such as wheat germ agglutinin (WGA).

mER-β was overexpressed in Cos-7 cells and insect Sf9 cells to investigate the respective abilities of mammalian or insect cells to modify mER-β with O-GlcNAc (Figure 1C). mER-β expressed in both mammalian and insect cells contains O-GlcNAc moieties, as detected using an O-GlcNAc specific antibody, RL2. Insect Sf9 cells efficiently attach posttranslational modifications at sites similar to those of mammalian cells, such as phosphorylation on human ER-α (*32*), O-GlcNAcylation on human cytomegalovirus basic base protein-1 (*33*), and O-GlcNAcylation on keratin (*8*, *18*, *34*). Because the insect Sf9 cell expression system is a eukaryotic expression system which is able to produce a large quantity of biologically active protein (*27*), mER-β was overexpressed

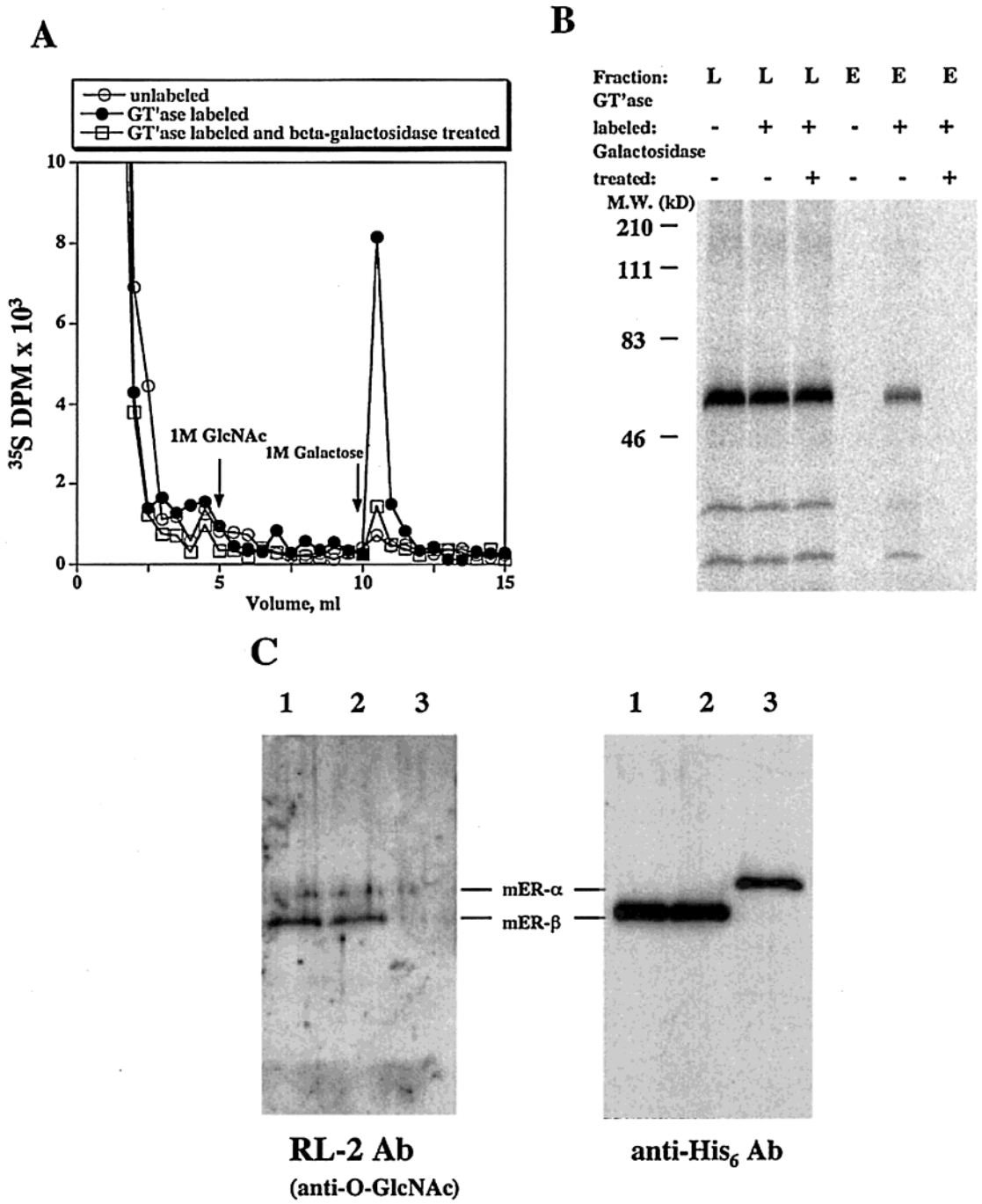


FIGURE 1: In vitro translated mER-β or mER-β expressed in mammalian cells is modified by O-GlcNAc. (A) The ^{35}S-mER-β (open circle) generated by in vitro translation in reticulocyte lysates was labeled by galactosyltransferase (GT'ase) and UDP-Gal and either mock (no enzyme) treated (closed circle) or treated galactosidase (open square). The same amount of each reaction mixture was applied to *R. communis* agglutinin I (RCA I) columns. The columns were washed, challenged with 1 M GlcNAc, and eluted with 1 M Gal. Each fraction was monitored using liquid scintillation counting. (B) Aliquots of each fraction were pooled, desalted, and analyzed by 10% SDS−PAGE and fluorography. Abbreviations: L, load; E, eluted with Gal. (C) Mammalian Cos-7 cells were transfected with His_6-tagged mER-β cDNA using the liposome method. The expressed protein was purified under the same scheme as described for insect Sf9 cells. Purified proteins were resolved on 10% SDS−PAGE gel in duplicate, and the gels were transferred to nitrocellulose membranes. The membranes were immunoblotted with either RL-2 (left panel), specific for the O-GlcNAc moieties on proteins, or anti-His_6 monoclonal antibody (right panel) and visualized with ECL. His_6-tagged mER-α protein expressed and purified from *E. coli* was used as a negative control for RL-2 antibody. Lanes: 1, mER-β from mammalian Cos-7 cells; 2, mER-β from insect Sf9 cells; 3, mER-α from *E. coli*.

in insect SF9 cells to isolate enough mER-β protein for carbohydrate characterization and site mapping. The expression of mER-β in Sf9 cells produced a high yield (Figure 2A) and biologically active mER-β as determined in a DNA mobility shift assay (Figure 2B).

mER-β was purified from Sf9 under denaturing conditions, using 6 M guanidine or 8 M urea, to inactivate glycosidases and phosphatases so as to preserve the carbohydrate or phosphate moieties on mER-β during purification. Because we linked a His_6 tag to the C-terminal end of the protein, we achieved a purification to over 90% homogeneity in one step of metal affinity chromatography (Figure 2C, lane E). To remove other minor contaminants, which could interfere with later sugar or phosphate analyses, we further isolated

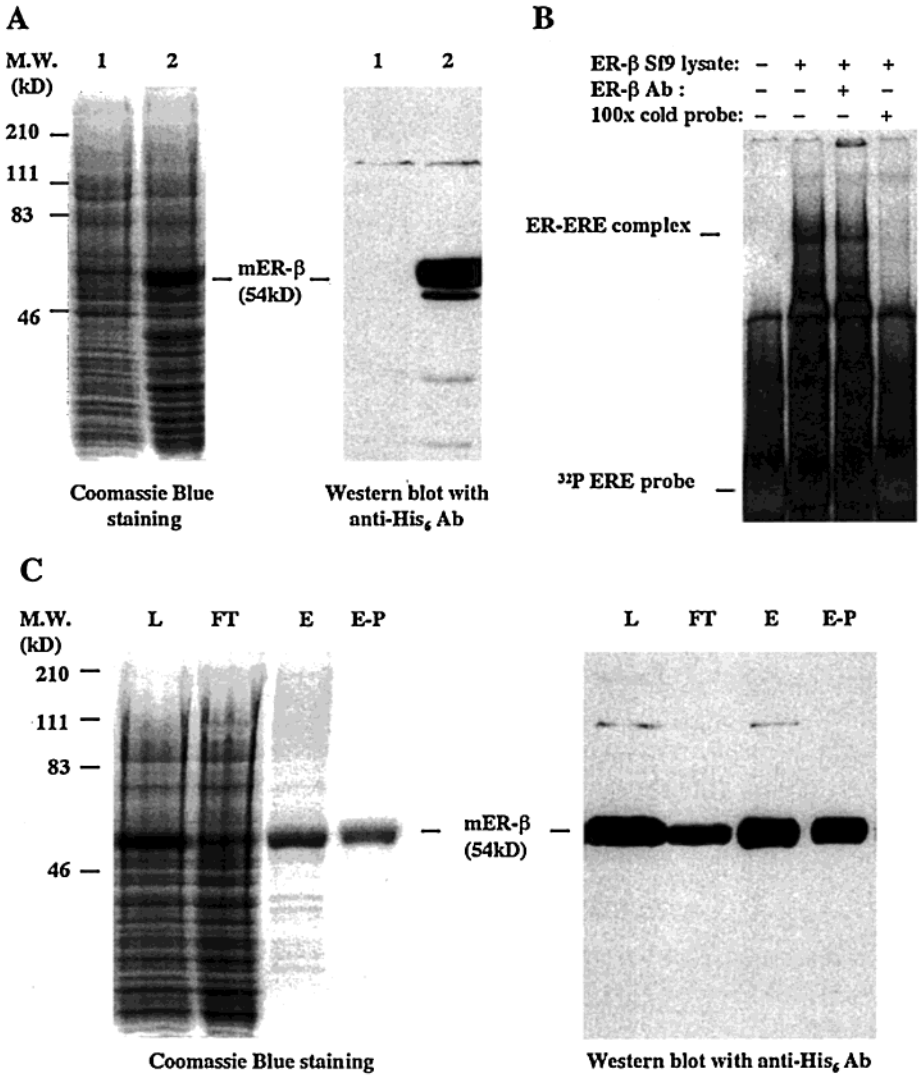


FIGURE 2: Expression and purification of His$_6$-tagged mER-β in insect Sf9 cells. (A) The cell lysate from either mock-infected Sf9 cells (lane 1) or mER-β-infected Sf9 cells (lane 2) was resolved on 10% SDS−PAGE. The gel was either stained with Coomassie Blue (left panel) or immunoblotted with anti-His$_6$ antibody (right panel). (B) The electrophoresis mobility shift assay was used to examine the DNA binding ability of the recombinant mER-β. A ^{32}P-labeled consensus vitellogenin ERE probe and the mER-β-infected Sf9 cell lysate were used in the assay. Excess unlabeled ERE probe (100×) and the antibody against ER-β were used to control for specificity. (C) The mER-β expressed in Sf9 cells was purified with metal affinity resin (lanes L, FT, and E are load, flow through, and elute) and subsequently purified by preparative SDS−PAGE (lane E-P is prep-cell elute). Aliquots of each fraction were resolved on 10% SDS−PAGE. The gel was either stained with Coomassie Blue (left panel) or immunoblotted with anti-His$_6$ antibody (right panel).

mER-β using preparative SDS−PAGE. After these two purification steps, recombinant mER-β was purified to a single band on a Coomassie Blue stained SDS−polyacrylamide gel (Figure 2C, lane E-P).

Linkage Analysis of the Sugar Moieties. RCA I chromatography and RL-2 immunoblots indirectly indicate that mER-β is modified by O-GlcNAcylation. Therefore, to directly determine the presence of O-GlcNAc on mER-β and to establish its carbohydrate linkage, the GlcNAcs on purified mER-β were enzymatically labeled with [^{3}H]Gal, and treated with PNGase F, to remove any putative asparagine-linked glycans. Serine (threonine) O-glycans were released by dilute alkali-induced β-elimination (*28*). The labeled sugar moieties were resistant to PNGase F treatment, indicating that GlcNAc-terminating N-glycans are not present on mER-β (Figure 3A). As the control for PNGase F activtiy, ovalbumin was susceptible to this treatment. The labeled carbohydrates on mER-β were susceptible to β-elimination (Figure 3B). The β-elimination reaction products behave as disaccharides when fractionated using gel filtration chromatography (data not shown) and migrate at the same position as standard Galβ1−4GlcNAcitol on high pH anion-exchange chromatography (Figure 3C). mER-β, therefore, is modified by O-GlcNAc.

Identification of Attachment Sites for O-GlcNAc on mER-β. The sites of O-GlcNAc modifications were mapped by first digesting [^{3}H]Gal-labeled mER-β with trypsin and fractionating the tryptic fragments by reverse-phase C_{18} chromatography. There was only one major tritium peak in the tryptic map of [^{3}H]Gal-labeled mER-β, and it comigrated with a significant UV peak (Figure 4A). Endoproteinase Asp-N also generated a similar single tritium peak in the RP-HPLC chromatogram (data not shown). These data suggest that there is only one major site of O-GlcNAc addition on mER-β detected by exogenous galactosyltransferase labeling.

Use of a shallower gradient resolved the UV peak into a small UV peak containing most of the tritium counts,

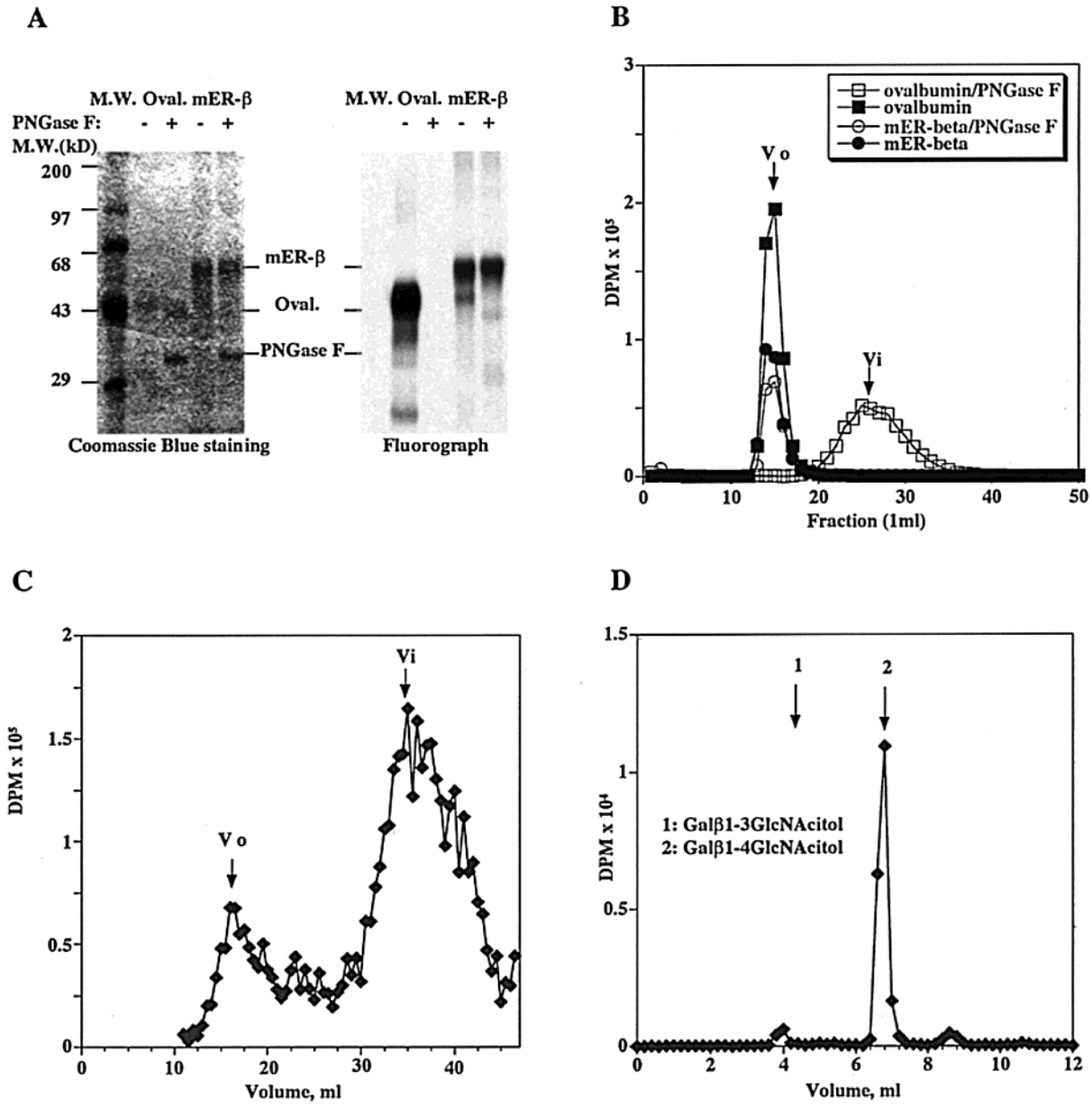


FIGURE 3: Characterization of the sugar moieties attached to mER-β. (A) PNGase F treatment of ^{3}H-Gal-labeled mER-β purified from Sf9 cells. Purified proteins were enzymatically labeled with ^{3}H-Gal, treated in the absence (−) or presence (+) of PNGase F, and resolved by 10% SDS−PAGE. ^{3}H-Gal-labeled ovalbumin was used as a positive control for PNGase F. The gel was stained with Coomassie Blue and exposed to X-ray film for 2 days. (B) PNGase F treatment of ^{3}H-Gal-labeled mER-β analyzed by G-50 chromatography. ^{3}H-Gal-labeled ovalbumin and mER-β treated either with (open circle and square) or without (closed circle and square) PNGase F were subjected to Sephadex G-50 gel filtration. Each fraction was monitored using liquid scintillation counting. (C) β-Eliminated glycans of ^{3}H-Gal-labeled mER-β analyzed by G-50 chromatography. The products of β-elimination were resolved on a Sephadex G-50 gel filtration column. Abbreviations: V_0, void volume; V_i, included volume. (D) The V_i peak from (C) was analyzed on a Dionex CarboPAc-MA1 column. Arrows 1 and 2 indicate the elution position of Galβ1−3GlcNAcitol and Galβ1−4GlcNAcitol standards, respectively.

designated as fraction A, and an adjacent large UV peak, designated as fraction A′ (Figure 4B). Since these two fractions were very close to one another on the elution profile, they may originate from the same peptide backbone but differ by their posttranslational modification states. MALDI-TOF MS was used to measure the mass of peptides in each fraction. Fraction A produces major ions at *m*/*z* 1925.0 ± 0.8, 2128.4 ± 0.2, and 2291.2 ± 0.6 (Figure 5A), corresponding to unmodified peptide, GlcNAcylated peptide (203.4 unit observed shift, 203.20 unit calculated), and Gal-labeled GlcNAcylated peptides (366.2 unit observed shift, 365.34 unit calculated), respectively. The ion at *m*/*z* 2004.8 is shifted 79.8 units relative to the unmodified peptide ion and may correspond to the phosphorylated peptide (calculated mass shift 79.98 units) with no glycosylation (see below). Previous studies on pure synthetic glycopeptides by both MALDI-TOF and electrospray (ES) MS have shown considerable loss of O-GlcNAc from the glycopeptide within the mass spectrometer or very poor relative detection of the glycopeptide versus its unmodified counterpart, thus resulting in an artificially large signal for the unmodified peptide (*33*). Thus, it is not possible to draw conclusions regarding the relative quantities of these peptides based on the abundance of these ions in MALDI-TOF mass spectra. Fraction A′, however, contains only a single ion at *m*/*z* 1924.3 ± 0.1 (Figure 5B), corresponding to the *m*/*z* value detected for the

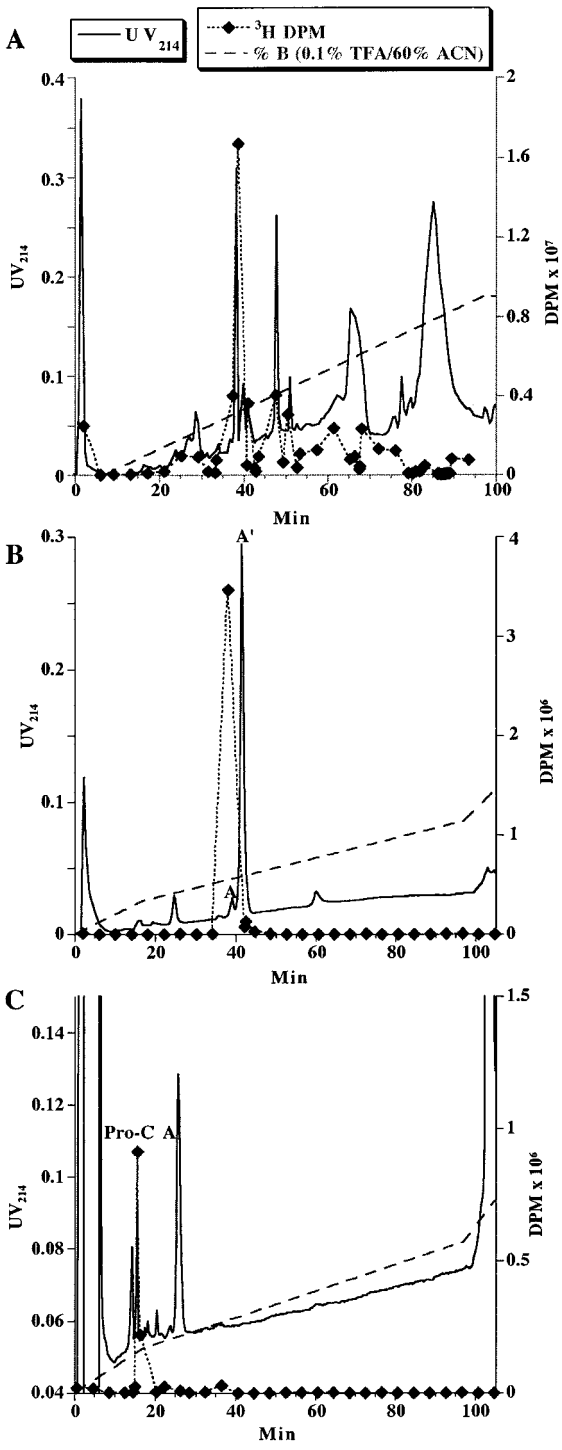


FIGURE 4: RP-HPLC analysis of tryptic ^{3}H-Gal-labeled mER-β peptides. (A) The first dimension of RP-HPLC analysis of tryptic ^{3}H-Gal-labeled mER-β peptides. ^{3}H-Gal-labeled mER-β tryptic peptides were applied to a C_{18} column. The column was developed with a 90 min linear gradient (wide broken line) of 0−60% acetonitrile (ACN) in 0.1% TFA at a flow rate of 0.1 mL/min. (B) The major ^{3}H-Gal-labeled mER-β tryptic fragment eluting from an RP C_{18} column at 38 min as shown in panel A was further purified by a shallow gradient of 15−45% acetonitrile in 0.1% TFA over 90 min. The fraction carrying most of the tritium counts and the adjacent fraction containing the major UV peak are denoted as A and A′, respectively. (C) The major ^{3}H-Gal-labeled mER-β tryptic fragment A, eluting from the second dimension as shown in panel B, was digested with Pro-C at 37 °C overnight and purified by a shallow gradient of 15−45% acetonitrile in 0.1% TFA over 90 min. The fraction carrying most of the tritium counts was designated as Pro-C A and further subjected to MALDI-TOF MS analysis.

unmodified peptide in fraction A, within the error range of the instrument. Therefore, the unmodified peptide in fraction A′ was used to determine the amino acid sequence of the peptide.

The fact that the *m*/*z* value of the unmodified peptide (1924.3 ± 0.1, Figure 5B) does not match calculated values for tryptic peptides of mER-β required de novo sequencing of the peptide. Several attempts to obtain gas-phase Edman sequence data were unsuccessful. This peptide was also resistant to digestion by aminopeptidase M, producing no change in the MALDI-TOF mass spectrum (data not shown). A modified scheme was developed to determine the internal sequence of the peptide. Fraction A was digested with proline-specific endopeptidase, and the resulting peptides were separated using RP-HPLC. The tritium peak was shifted from the original 40 min elution to an earlier point at 18 min (Figure 4C), indicating that the peptide is susceptible to proline-specific endopeptidase digestion. The MALDI-TOF spectrum of the peptide in fraction A′, after brief digestion with Pro-C, contained a single ion at *m*/*z* 1174.1 ± 0.5 (Figure 5C). This peptide was further digested with aminopeptidase M, generating a sequence ladder (Figure 5D). The masses of the ladder ions, 1175.7 ± 0.3, 1088.8 ± 0.4, 1001.4 ± 0.4, 900.2 ± 0.3, and 842.8 ± 0.3, correspond to sequential loss of Ser, Ser, Thr, and Gly from the N-terminus of the peptide. This sequence corresponds to peptide (^{14}P)-SSTGNLEGGPVR^{26} from mER-β with calculated *m*/*z* 1174.25. MALDI-TOF analysis of the Gal-labeled GlcNAcylated Pro-C A fragment in Figure 4C yielded two ions with *m*/*z* values of 919.7 ± 0.1 and 1283.6 ± 0.4 (Figure 5E). These masses correspond to ^{15}SSTGNLEGGP^{24} (calculated *m*/*z* 918.94) and Gal-GlcNAc-^{15}SSTGNLEGGP^{24} (calculated *m*/*z* 1284.28), with a difference of 363.9 representing Gal-GlcNAc (calculated *m*/*z* 365.3).

To confirm the sequence of the unmodified peptide, the peptide in fraction A′ was microsequenced by LC-MS/MS. The resulting CID spectrum reveals a sequence for the mER-β peptide, ^{7}MNYSVPSSTGNLEGGPVR^{26}, with 59.0 amu added to the first methionine residue of the peptide (Figure 6). Interestingly, this is not a tryptic peptide of mER-β. Yet all of the y′ ions have the correct predicted mass, and the *m*/*z* values of all of the b′ ions are 59.0 amu higher than expected. This suggests a new second internal methionine start site that is carbamoylated at the N-terminus (mass shift of 43.08) and oxidized at the methionine side chain (mass shift of 16.00), for a total mass shift of 59.08. These modifications are consistent with the peptide's being resistant to aminopeptidase M and gas-phase sequencing. Carbamoylation is known to result from exposure to urea, and methionine oxidation is a common artifact in peptide purification, especially in oxidative buffers. This mER-β peptide also has a proline in the correct position to produce the Pro-C digestion products of the unmodified and Gal-GlcNAc peptides in fractions A and A′.

After the sequence of the glycopeptide was determined, the position of the modification site was determined by manual Edman degradation of the Gal-labeled, GlcNAcylated Pro-C A peptide. All of the tritium counts were released at the second degradation cycle (Figure 7), indicating that the major O-GlcNAcylation site on mER-β is Ser^{16}.

The mER-β O-GlcNAcylation Site at Ser^{16} Is Also Phosphorylated. The studies above (Figure 5) indicated that there

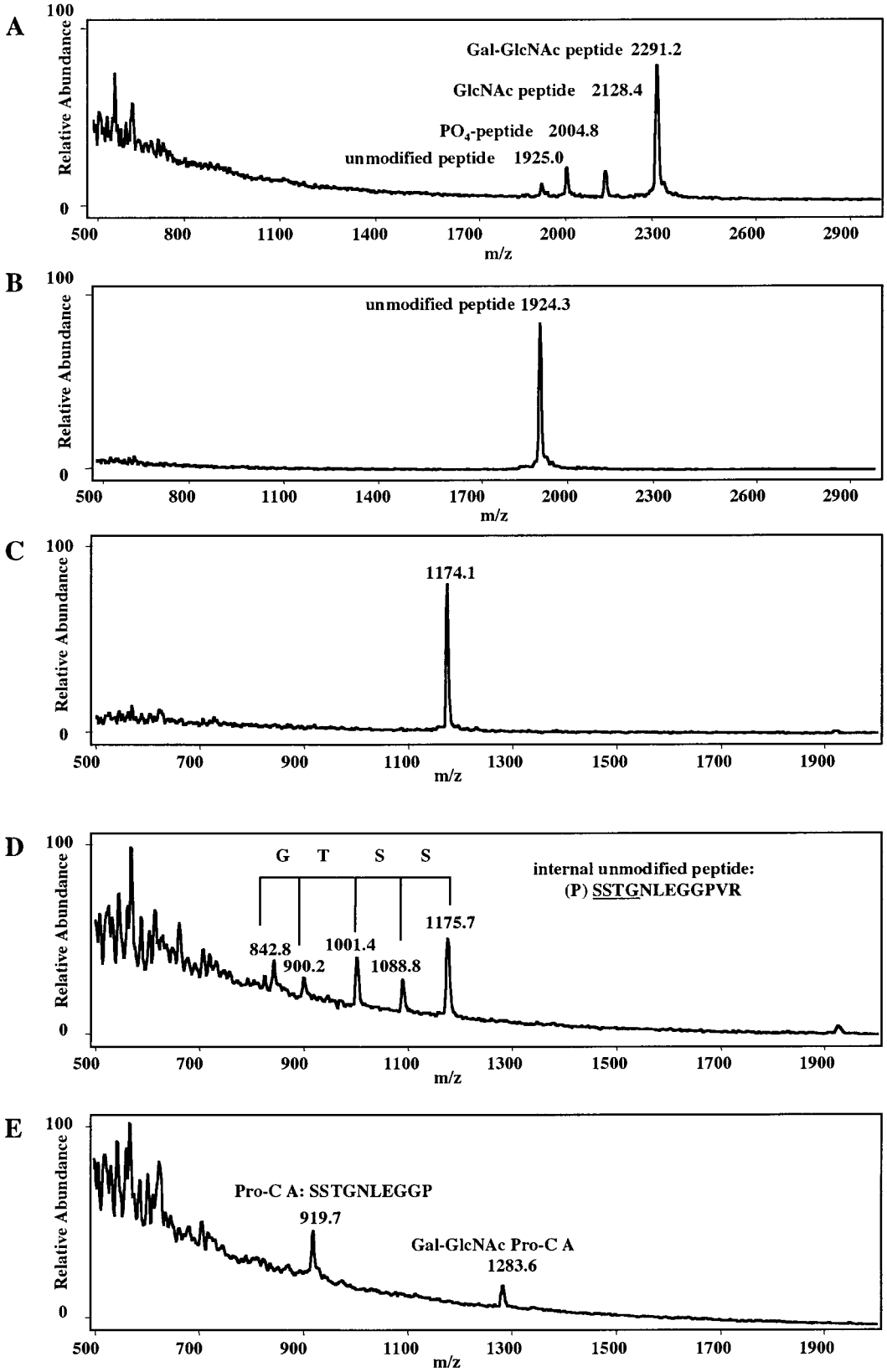
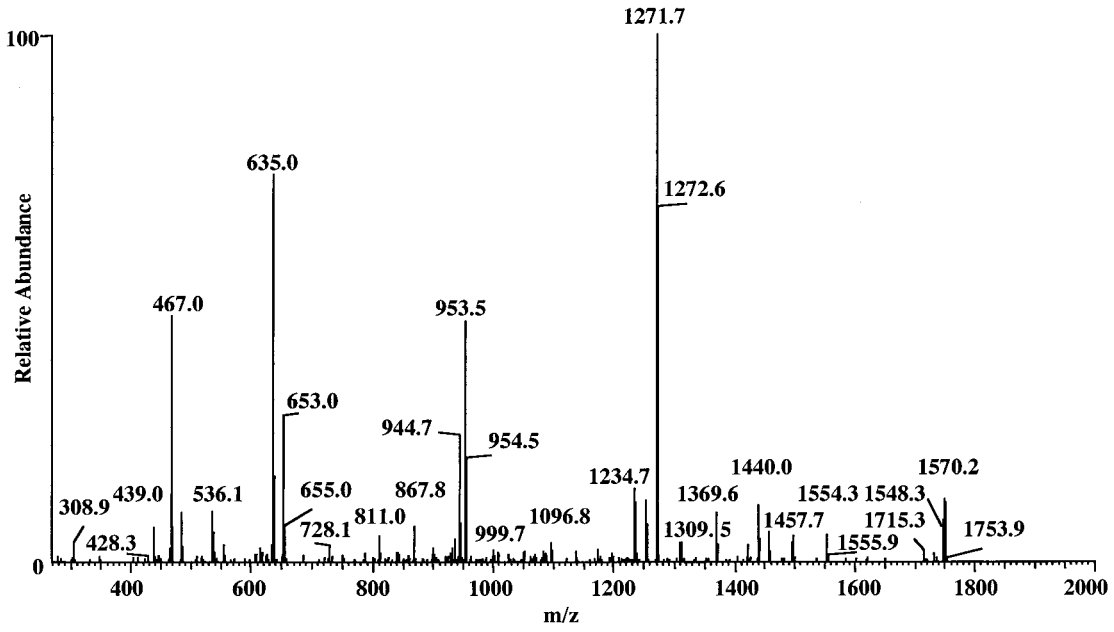


FIGURE 5: MALDI-TOF MS and sequence analysis of the major ^{3}H-Gal-labeled mER-β tryptic fragments. (A) An aliquot of fraction A from the second dimension as shown in Figure 4B was mixed with the matrix α-cyano-4-hydroxycinnamic acid (CHCA) and detected by MALDI-TOF MS. Unmodified, GlcNAc, Gal-GlcNAc, and phosphorylated forms of the peptide A are indicated. (B) An aliquot of fraction A′ from the second dimension as shown in Figure 4B was mixed with the CHCA matrix and detected by MALDI-TOF MS. Only one mass peak corresponding to unmodified peptide A as shown in panel A was detected. (C) An aliquot of the fraction A′ was digested with proline endopeptidase at 37 °C for 30 min. The digestion mixture was detected by MALDI-TOF MS. (D) An aliquot of peptide A was first digested with proline endopeptidase as shown in panel C. The digestion mixture was then digested with aminopeptidase M for 3 min. The final digestion mixture was characterized by MALDI-TOF MS. The deduced internal sequence of the peptide A after proline endopeptidase cleavage is shown. (E) An aliquot of Pro-C-digested fraction A purified by RP C_{18} chromatography as shown in Figure 4C was examined by MALDI-TOF MS. The unmodified and Gal-GlcNAc forms are indicated.



Found:	1735.4	1621.7	1457.7	1370.5	1271.7	1174.0	1087.8	1000.8	898.6	841.5	728.1	614.5	485.3	428.3	ND	ND	ND
Calculated:	1734.9	1620.8	1457.6	1370.5	1271.4	1174.3	1087.2	1000.1	899.0	841.9	727.8	614.7	485.6	428.5	371.5	274.3	175.2

	y17	y16	y15	y14	y13	y12	y11	y10	y9	y8	y7	y6	y5	y4	y3	y2	y1
	M	N	Y	V	S	P	S	S	T	G	N	L	E	G	G	P	V	R
	b1	b2	b3	b4	b5	b6	b7	b8	b9	b10	b11	b12	b13	b14	b15	b 16	b17

Found:	ND	303.9	468.0	555.4	654.0	751.1	838.8	925.1	1026.2	1084.6	1198.7	1311.6	1440.0	1496.8	1554.3	1651.4	1750.2
Calculated: (+59.0)	191.2	305.3	468.5	555.6	654.7	751.8	838.9	926.0	1027.1	1084.1	1198.2	1311.4	1440.5	1497.6	1554.6	1651.7	1750.8

FIGURE 6: Mass spectrum of LC/ESI-MS/MS analysis of the unmodified peptide containing the O-GlcNAc site. An aliquot of the unmodified peptide from the second dimension as shown in Figure 4B was subjected to LC/ESI-MS/MS analysis. For clarity, the observed and calculated b and y ions of the peptide are listed below the spectrum.

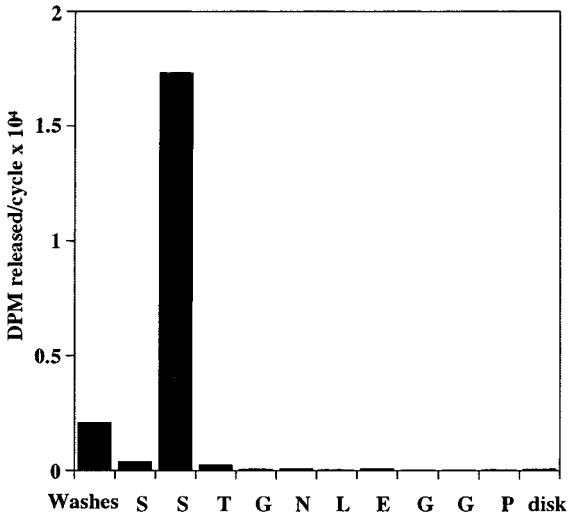


FIGURE 7: The major site of O-GlcNAcylation on mER-β is Ser[16]. The fraction of the peptide Pro-C A from Figure 4C was coupled to a Sequelon-AA disk (Millipore), and manual Edman degradation reactions were done. The released tritium counts from each cycle were monitored using liquid scintillation counting.

is a phosphorylated species in the MALDI-TOF spectra from the peptide carrying the major O-GlcNAc site of mER-β. The presence of phosphate on this peptide was confirmed by alkaline phosphatase digestion (Figure 8A). In addition, on the basis of the spectrum in Figure 8B, it is clear that either the phosphorylated or the glycosylated species exist alternatively without further modification, implying that these two types of posttranslational modification are mutually exclusive on this peptide.

The attachment site of the phosphate on the peptide was examined using LC/ESI-MS/MS. There are five potential phosphorylation residues on the peptide of MNYVSPSST-GNLEGGPVR. Figure 8C shows the MS/MS spectrum of the phosphorylated peptide ion at *m/z* 2005.1. The identification of the ions including *m/z* at 467 (b_3), 653.1 (b_5), 1253.7 ($y_{12} + 80$), and 1351.4 ($y_{13} + 80$) indicates that the tyrosine at the third position and the serine at the fifth position do not have phosphate addition. Loss of phosphate during fragmentation dehydrates the amino acid for a combined loss of 98 amu. The presence of a 908.1 ion (b_8 minus 98) suggests that the serine (Ser[16]) in the eighth position of this peptide is phosphorylated. Additional evidence for phosphorylation at this position comes from further fragmentation of the LC-MS/MS ion 1351.5 ($y_{13} + 80$) which yields the LC-MS/MS/MS ion at *m/z* 1254 ($y_{12} + 80$), which in turn was further fragmented, yielding a 1069.6 ion ($y_{11} - 98$) and a 999.9 ion (y_{10}) (data not shown). These CID spectra are most consistent with a phosphate at the serine (Ser[16]) in the eighth position of this peptide. The poor fragmentation within this region of the peptide and the presence of additional fragment ions suggest a possible mixture of modified peptides phosphorylated at any one of the potential residues in the serine/threonine patch from the seventh to

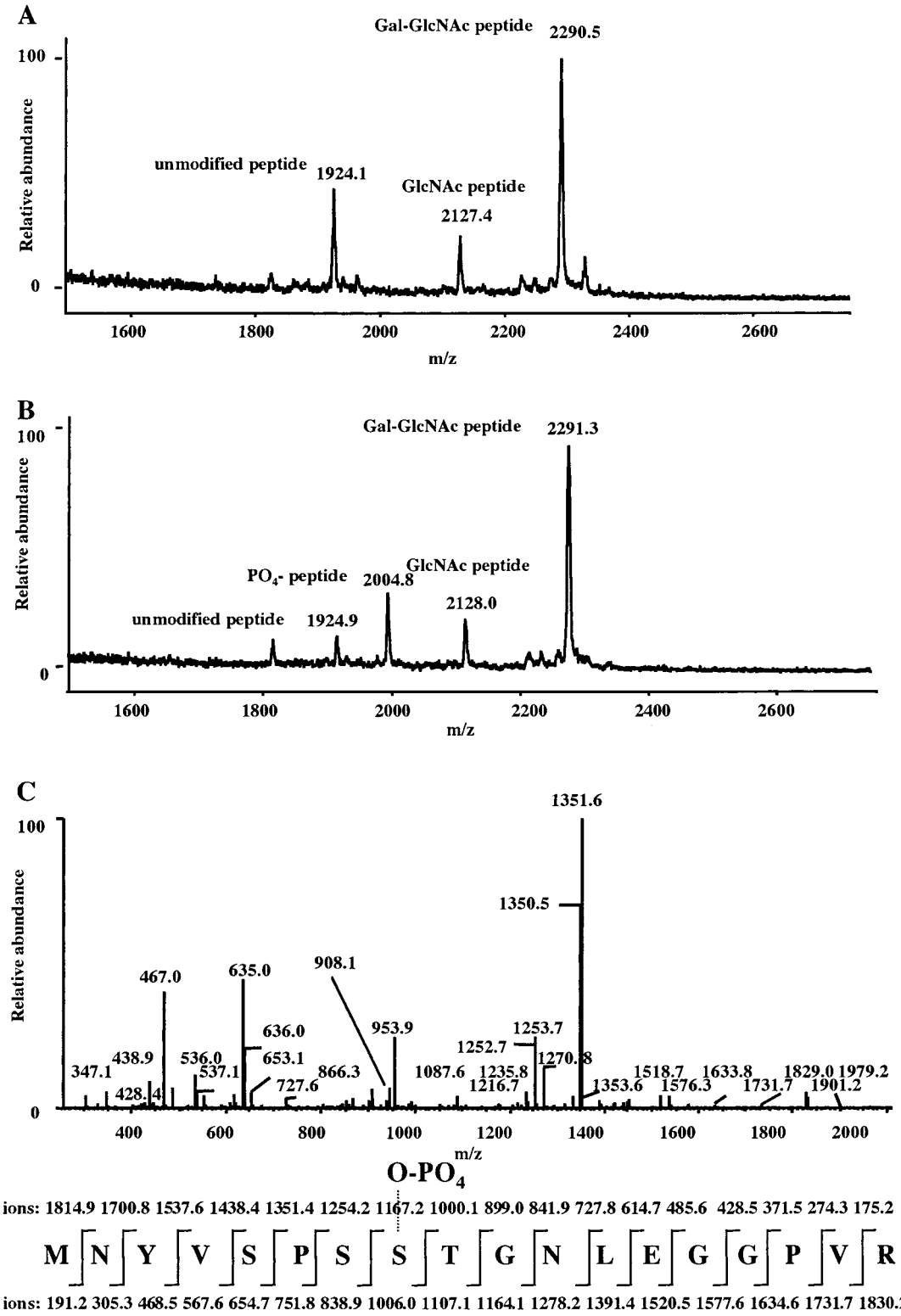


FIGURE 8: MALDI-TOF and LC/ESI-MS/MS analysis of the mER-β peptides carrying the major O-GlcNAc/O-phosphate site. An aliquot of mER-β peptides carrying the major O-GlcNAc site was treated with 0.5 unit of alkaline phosphatase in a 5 μL reaction at 37 °C for 30 min. The reaction mixture (1 μL) was mixed with matrix α-cyano-4-hydroxycinnamic acid (1 μL) in 50% acetonitrile/0.3% TFA and analyzed using MALDI-TOF MS. Panels: A, treated with the enzyme; B, treated without the enzyme. Panel C: An aliquot of Gal-labeled peptide carrying the major O-GlcNAc site on mER-β as shown in panel B was subjected to LC/ESI-MS/MS analysis. The MS/MS spectrum from the phosphorylated species with 2004.8 Da (calculated 1005.1 Da) is shown. The sequence and predicted b and y ions of the peptide with the addition of phosphate (80 Da) at the eighth position are listed below the spectrum. The mass of the unmodified peptide has a 59 Da addition due to carbamoylation and oxidation on the first methionine residue.

the ninth position. Nonetheless, the results strongly suggest that the phosphate is most evident at the eighth position. Combined with our MALDI data, we conclude that the major O-GlcNAc site at Ser[16] is also alternatively phosphorylated.

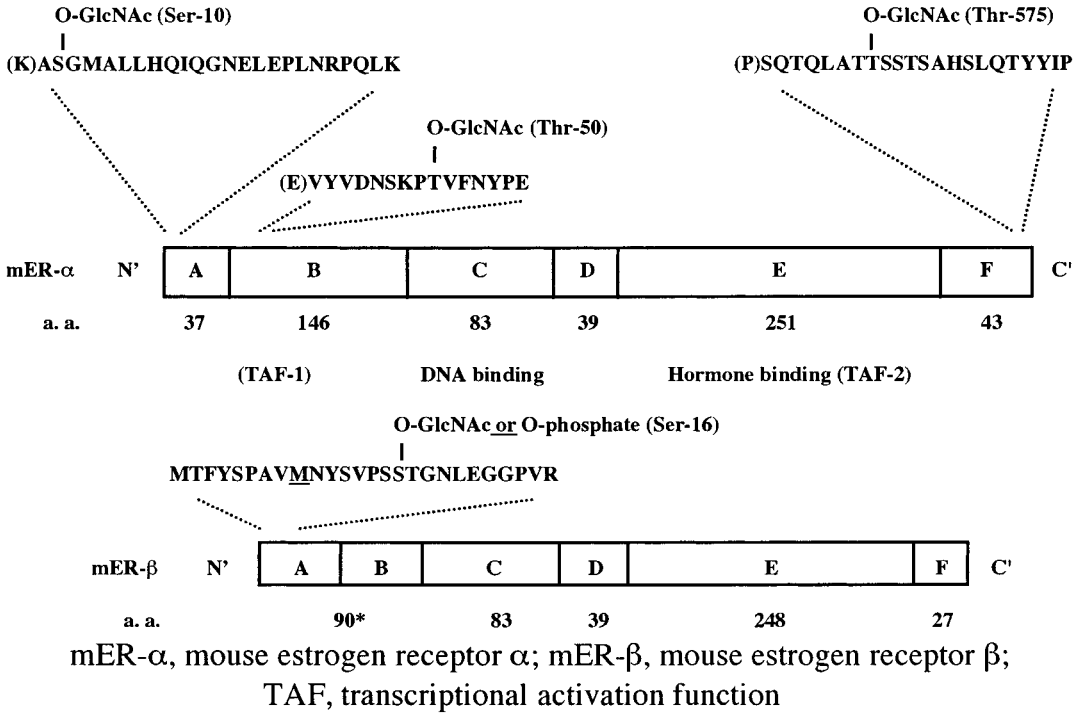


FIGURE 9: Known O-GlcNAcylation sites on mouse ERs. Estrogen receptors are illustrated as several distinctive domains. N′ and C′ are N-terminal and C-terminal ends. A major O-GlcNAc/O-phosphate site on mER-β is located at Ser[16] near the N-terminus. The underlined methionine is likely additional translation start codon. The asterisk (*) indicates the length of the short form of domains A and B. Two additional O-GlcNAc sites on mER-α were recently mapped at Ser[10] and Thr[50] (manuscript submitted).

DISCUSSION

In this study, we have demonstrated that mER-β is alternatively modified by O-GlcNAc or O-phosphate in the proximity of the transactivation domain and within a region with a high PEST score. The major O-GlcNAc site accessible to the galactosyltransferase probe is located at Ser[16], which is also phosphorylated in a subset of molecules from the same source. Although there are several consensus N-linked glycosylation sites (N-X-S, X except proline) existing in the mER-β amino acid sequence, our data indicate that mER-β is not modified by complex or hybrid N-linked glycosylation, consistent with its nuclear/cytoplasmic localization.

We have estimated the minimal stoichiometry of the O-GlcNAcylation on Ser[16] on the basis of the specific activity of the [^{3}H]Gal label in galactosyltransferase-treated mER-β. It is approximately 6.7%, which is similar to the stoichiometry of O-GlcNAc on mER-α. Although we have attempted to preserve the sugar moieties on the protein throughout the purification processes, this estimate of stoichiometry is likely an underestimate. First, the high expression of mER-β in Sf9 cells may result in underglycosylation. Second, the enzymatic and chemical labile nature of O-GlcNAc moieties would result in additional loss during the protein purification. Third, the labeling efficiency of the galactosyltransferase based on previous studies (*28*) is poor. Therefore, the real stoichiometry of the O-GlcNAcylated mER-β is likely much higher. Figure 9 summarizes the O-GlcNAc sites mapped on ERs. To date, there is no apparent consensus sequence motif for the attachment of O-GlcNAc on the proteins studied. However, many documented O-GlcNAc sites have high PEST scores. The major O-GlcNAc sites on ERs fall into this category. The peptide carrying the major O-GlcNAc site on mER-β has the highest PEST motif score (-7.63) in the entire protein sequence. Likewise, the region containing the major O-GlcNAc site Thr[575] on mER-α also has the highest PEST score (9.28) in that protein. The PEST motif has been proposed to be the signal responsible for rapid protein degradation (*24*). The similarity between these two closely related ER members suggests that the role of the glycosylation on ER is to modulate protein degradation. Many PEST motifs serve as constitutive degradation signals. However, some serve as conditional degradation signals that are activated by phosphorylation. However, our site-mapping data suggest that the site bearing the major O-GlcNAc site on mER-β also is phosphorylated (Figure 8). It is conceivable that the phosphorylation of this peptide converts this locus from a latent PEST signal into a highly active one. In contrast, O-GlcNAc addition at this same site might be expected to have the opposite effect, thus blocking rapid degradation. Using site-directed mutagensis, we have obtained direct evidence that indeed the Ser[16] site of mER-β has this role in regulating the receptor's turnover (manuscript in preparation). These site-specific constructs also suggest a role for the respective forms of posttranslational modifications in the ability of the receptor to transactivate its target genes (manuscript in preparation).

One surprising finding of this study is that the recombinant mER-β expressed in Sf9 cells uses the second internal methionine (Met) as the start site. The expression vector we chose was a transcription vector. The N-terminal part of the mER-β cDNA coding sequence subcloned in the expression vector was with the full length of the short form of mER-β ([1]MTFYSPAVMNYSVPSSTGNLEGGPVR[26]). Under normal circumstances, Sf9 cells should express the protein with the full length starting from the first starting codon of the

cDNA. However, we actually isolated the tryptic N-terminal peptide from the second Met. On the basis of the facts that the carbamylation commonly occurs at the N-terminus of the protein and there is not a trypsin site in front of the second Met, we speculate that the real starting codon of mER-β expressed in insect Sf9 cells is likely its second internal Met. Recently, several reports described additional amino acids at the N-terminal end of ER-β (*35*, *36*). One study has showed that the short form of ER-β transactivates but not as actively as the long form of ER-β (*37*). Our observation that mER-β may translate from its second internal codon provides support for the notion that the short form of ER-β exists as a functional expression variant. To validate our observation, we conducted a nucleotide subsequence motif search and found that there is, indeed, a translation initiation site existing upstream of the second internal Met of the short form of the mER-β cDNA.

ER regulation remains at the center stage of human steroid hormone regulation and related breast cancer studies. Our studies provide an additional dimension for the understanding of ER-mediated signal transduction regulation by alternative posttranslation modifications. Although the experimental evidence for the functional roles of O-GlcNAc and its complex interplay with phosphorylation is becoming evident, the role(s) of the O-GlcNAcylation of the ERs remain(s) to be determined. This study provides a solid biochemical foundation for functional studies using site-directed mutagenesis of O-GlcNAc/O-phosphate sites on mER-β to elucidate a role for the reciprocal O-glycosylation/O-phosphorylation on ERs.

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