O-Linked N-Acetylglucosamine Modification of Insulin Receptor Substrate-1 Occurs in Close Proximity to Multiple SH2 Domain Binding Motifs∗†

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Insulin receptor substrate-1 (IRS-1) is a highly phosphorylated adaptor protein critical to insulin and IGF-1 receptor signaling. Ser/Thr kinases impact the metabolic and mitogenic effects elicited by insulin and IGF-1 through feedback and feed forward regulation at the level of IRS-1. Ser/Thr residues of IRS-1 are also O-GlcNAc-modified, which may influence the phosphorylation status of the protein. To facilitate the understanding of the functional effects of O-GlcNAc modification on IRS-1-mediated signaling, we identified the sites of O-GlcNAc modification of rat and human IRS-1. Tandem mass spectrometric analysis of IRS-1, exogenously expressed in HEK293 cells, revealed that the C terminus, which is rich in docking sites for SH2 domain-containing proteins, was O-GlcNAc-modified at multiple residues. Rat IRS-1 was O-GlcNAc-modified at Ser914, Ser1009, Ser1036, and Ser1041. Human IRS-1 was O-GlcNAc-modified at Ser984 or Ser985, at Ser1011, and possibly at multiple sites within residues 1025–1045. O-GlcNAc modification at a conserved residue in rat (Ser1009) and human (Ser1011) IRS-1 is adjacent to a putative binding motif for the N-terminal SH2 domains of p85α and p85β regulatory subunits of phosphatidylinositol 3-kinase and the tyrosine phosphatase SHP2 (PTPN11). Immunoblot analysis using an antibody generated against human IRS-1 Ser1011 GlcNAc further confirmed the site of attachment and the identity of the +203.2-Da mass shift as β-N-acetylglucosamine. The accumulation of IRS-1 Ser1011 GlcNAc in HEPG2 liver cells and MC3T3-E1 preosteoblasts upon inhibition of O-GlcNAcase indicates that O-GlcNAcylation of endogenously expressed IRS-1 is a dynamic process that occurs at normal glucose concentrations (5 mM). O-GlcNAc modification did not occur at any known or newly identified Ser/Thr phosphorylation sites and in most cases occurred simultaneously with phosphorylation of nearby residues. These findings suggest that O-GlcNAc modification represents an additional layer of posttranslational regulation that may impact the specificity of effects elicited by insulin and IGF-1. Molecular & Cellular Proteomics 8:2733–2745, 2009.

Insulin receptor substrate-1 (IRS-1)† is a highly phosphorylated adaptor protein critical to insulin and IGF-1 receptor signaling. Many of the metabolic and mitogenic effects elicited by insulin and IGF-1 are mediated and modulated by posttranslational modifications of IRS-1, and tight regulation at the posttranslational level is crucial for maintaining insulin sensitivity and controlling growth factor-induced proliferation. Following hormonal stimulation, IRS-1 is phosphorylated by the receptor tyrosine kinases creating SH2 domain docking sites for downstream binding partners including the p85 regulatory subunits of phosphatidylinositol 3-kinase, Grb2, and the tyrosine phosphatase SHP2 (PTPN11) (1). Binding of p85 phosphatidylinositol 3-kinase and Grb2 activate the PI3K/Akt and Ras-MAPK pathways, respectively, whereas binding of SHP2 results in tyrosine dephosphorylation and signal attenuation (2). Positive and negative feedback regulation by Ser/Thr kinases, such as Akt (3), c-Jun N-terminal kinase (JNK) (4), S6K (5), and ERK (6), impact the interactions of IRS-1 with SH2 domain proteins and the receptor thereby affecting the duration and outcome of the signal. IRS-1 has been described as a central node for the integration of information regarding the nutrient and stress status of the cell (7). This information is encoded by site-specific phosphorylation by a number of kinases that regulate the specificity of effects that are elicited following receptor stimulation. Many sites of Ser/Thr phosphorylation have been identified on IRS-1, and cross-talk among Tyr and Ser/Thr phosphorylations at specific residues is evidence of dynamic and complex posttranslational regulation (8, 9). Inappropriate phosphorylation of IRS-1 resulting in the disruption of interactions of IRS-1 with binding partners

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Received, April 28, 2009, and in revised form, July 27, 2009
Published, MCP Papers in Press, August 11, 2009, DOI 10.1074/mcp.M900207-MCP200

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This paper is available on line at http://www.mcponline.org
is implicated in the development of insulin resistance (10) and altered IGF-1 signaling in breast cancer tissue (11, 12).

In addition to phosphorylation, Ser/Thr residues in IRS-1 are also dynamically modified by GlcNAc in a nutrient-responsive manner. As opposed to a negatively charged phosphate group, O-GlcNAcylation imparts a bulky, hydrophilic, electrostatically neutral moiety to Ser/Thr residues. The enzymes responsible for the incorporation and removal of the monosaccharide from proteins, O-GlcNAc-transferase and O-GlcNAcase, respectively, are localized in the cytoplasm and the nucleus of all eukaryotic cells (13, 14). Recent studies suggest that the activity of O-GlcNAc-transferase is regulated by insulin (15) and that localization of O-GlcNAc-transferase to the membrane is driven by direct association with phosphatidylinositol 3-phosphate (16). The abundance of O-GlcNAc modification on many proteins in the insulin signaling pathway increases with sustained high glucose and chronic insulin stimulation, and elevated O-GlcNAc modification of IRS-1 correlates with the development of insulin resistance in multiple cell types including 3T3-L1 adipocytes (17, 18), MIN6 pancreatic beta cells (19), Fao rat hepatoma cells (16), human aortic endothelial cells (20), and skeletal muscle (21). The impact of O-GlcNAcylation on insulin signaling and diabetic complications was reviewed recently (22, 23). The direct effect of O-GlcNAc modification on signaling via IRS-1 is not known because conditions that mimic those in the uncontrolled diabetic patient may also result in phosphorylation of IRS-1 at inhibitory sites (16, 24) and O-GlcNAc modification of other proteins in the insulin signaling pathway, such as the insulin receptor, Akt (18), FoxO (25), AMP-activated protein kinase (26), and β-catenin (17).

To elucidate site-specific effects of O-GlcNAc modification on IRS-1-mediated signal transduction, we identified the sites of O-GlcNAc modification of rat and human IRS-1 by tandem mass spectrometry. To facilitate detection of the O-GlcNAc-modified peptides and assign the sites of modification, CID coupled with neutral loss-triggered MS3 and electron transfer dissociation (ETD) (27) tandem spectrometric approaches were used. Fragmentation of O-GlcNAc-modified peptides by ETD did not destroy the labile O-linkage (28) permitting direct detection of these peptides by the database searching algorithm ProteinProspector2 (29). O-GlcNAc modification occurred in close proximity to multiple SH2 domain binding motifs and within a region of IRS-1 shown previously to interact with the insulin and IGF-1 receptors (30).

**EXPERIMENTAL PROCEDURES**

**Generation of Wild Type and Mutant Rat and Human IRS-1 cDNAs—** His- and S-tagged rat IRS-1, amino acid residues 6–1235, in pTrEx vector (31) served as a template for site-directed substitution of O-GlcNAc-modified serine residues with alanine. Rat IRS-1 S1036A, S1041A, S1036A/S1041A, and S1009A were generated using oligonucleotide primers 5’-gccccccctccatctccacagctcgctgtgcttgtgcattcactc, 5’-gcccccccttcctccagctcgctgtgctgtgctgtgcattcactc, 5’-gccccccctccatctccacagctcgctgtgctgtgcattcactc, and 5’-ccatgaggccccctccatctccacagctcgctgtgctgtgcattcactc, respectively, using the QuickChange kit (Stratagene) according to the manufacturer’s recommendations. The cDNA encoding C-terminally hemagglutinin-tagged human IRS-1 was kindly provided by Adrian Lee (Baylor University) (27). Initial mass spectrometric analysis of the expressed protein did not reveal the sites of O-GlcNAc modification; therefore, the human IRS-1 cDNA was cloned into the NotI and PmlI sites of pTrEx4 vector (Novagen) to generate an N-terminally His- and S-tagged protein. A stop codon was inserted at the 3’ end of the coding sequence to remove the C-terminal hemagglutinin tag from the expressed protein. Mutagenesis reactions and manipulations of the cDNA were verified by sequencing.

**Cell Culture and Expression of IRS-1—** HEK293 cells were maintained in iscove’s modified Eagle’s medium (BioSource) containing 10% heat inactivated FCS with 1% antibiotic/antimycotic (Invitrogen). HEK293 cells were transiently transfected with rat or human IRS-1 cDNA using Polyfect transfection reagent according to the manufacturer’s recommendations (Qiagen). To facilitate mass spectrometric detection of O-GlcNAc-modified peptides, cells grown in 5 mM glucose were incubated with an O-GlcNAcase inhibitor (50 μM O-2-acetamido-2-deoxy-o-glucopyranosylidene) amino-N-phenylcarbamate (PUGNac); Toronto Chemicals) for 18 h prior to lysis. For co-immunoprecipitation experiments, cells were serum-starved for 16 h in the presence of absence of PUGNac and then stimulated with 100 mM insulin or 10 ng/ml IGF-1 (Sigma) for varying times. Cells were lysed in 300 mM NaCl, 50 mM sodium phosphate, pH 8, 10 mM imidazole, 10 mM β-mercaptoethanol, 1% Nonidet P-40, 50 μM PUGNac, EDTA-free protease inhibitor mixture (Roche Applied Science), 10 mM sodium fluoride, 500 μM sodium vanadate, 1 mM sodium pyrophosphate. His-tagged IRS-1 was enriched by incubation with nickel-nitritriacetic acid affinity resin (Novagen) for 1 h at room temperature followed by extensive washing with lysis buffer containing 20 mM imidazole. The protein was eluted in lysis buffer containing 250 mM imidazole and gel-purified by electrophoresis on 4–10% XT Criterion gels (Bio-Rad). For mass spectrometric analysis, the protein was stained with E-Zinc (Pierce), and the IRS-1 band was excised from the gel. Gel pieces were washed twice for 10 min with 100 mM ammonium bicarbonate, dehydrated with acetonitrile, and dried under vacuum. Cysteines were reduced with dithiothreitol and alkylated with iodoacetamide for 30 min at room temperature in the dark. Gel pieces were dehydrated with acetonitrile and dried completely prior to proteolytic digestion with trypsin (Promega) at 37 °C or with Glu-C (Roche Applied Science) at 25 °C for 18 h. Peptides were extracted with one wash of 25 mM ammonium bicarbonate for 20 min and three washes of 5% formic acid, 50% acetonitrile for 20 min each, dried under vacuum, reconstituted in 0.1% TFA, and cleaned up on C18 microspin columns (Nest Group). Prior to LC/MS analysis, the samples were reconstituted with 10 μL of 2% acetonitrile, 0.2% formic acid.

HEPG2 cells were grown in 10% FCS with low glucose (5 mM) Dulbecco’s modified Eagle’s medium. Murine MC3T3-E1 osteoblast precursor cells were grown in 10% FCS with low glucose (5 mM) minimum Eagle’s medium α (Invitrogen) with 1% penicillin/streptomycin (Mediatech). For overnight incubation in the absence or presence of 50 μM PUGNac, the medium was supplemented with 1% FCS.

**Immunoprecipitation and Western Blotting—** Wild type and mutant IRS-1 protein, isolated by nickel affinity chromatography or immunoprecipitation, was separated by SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with nonfat dry milk in Tris-buffered saline for detection by ECL or Odyssey blocking buffer (LicoR Biosciences, Inc.) prior to fluorescence detection. Antibodies used for immunoprecipitation and immunoblotting of endogenously expressed IRS-1 were purchased from Bethyl Laboratories (A301-158) and Santa Cruz Biotechnology (A-19), respectively. A pan-specific mono-
clonal anti-O-GlcNac (CTD 110.6) IgM generated against YSPTS(O-GlcNAc)PSK (32) was purchased for Western blot (Pierce) or immunoprecipitation (Covance). The presence of recombinant wild type or mutant IRS-1 was detected by probing for the S-tag with S-protein conjugated to HRP (Novagen) and visualized using West Pico enhanced chemiluminescent reagent (Pierce). Fluorescently labeled secondary antibodies, IRDye 800CW and 680, were purchased from Licor Biosciences, Inc.

Reversed Phase HPLC and Mass Spectrometry—Digested peptides were separated by C18 reversed phase nano-LC using a 75-μm x 15-cm capillary column packed in house (YMC ODS-AQ 120A SS, Waters) with a gradient of 2–60% B in 110 min (B = 0.02% heptafluorobutyric acid or 0.2% formic acid in acetonitrile) at 180 nL/min using an Ultimate 3000 nanoflow system with Chromeleon 6.8 software (Dionex, Sunnyvale, CA). Eluted peptides were analyzed by CID or ETD MS/MS with an LTQ XL ion trap mass spectrometer equipped with ETD capability (ThermoFisher). Multiple mass spectrometric approaches were utilized to detect O-GlcNAc-modified peptides and confirm the assignments of the sites of O-GlcNAc modification. Data-dependent neutral loss-triggered MS3 acquisition facilitated the identification of candidate O-GlcNAc-modified peptides. The instrument was programmed to acquire MS/MS/MS (MS3) data on precursor ions that exhibited a neutral loss corresponding to N-acetylglucosamine (203.2, 101.6, 67.7, or 50 Da for the 1+, 2+, 3+, and 4+ charge states, respectively) upon collision-induced dissociation. A normalized collision energy of 35% was used for fragmentation by CID. The threshold for the selection of an ion for fragmentation was set to 500. In subsequent analyses, the instrument was programmed to acquire tandem mass spectra generated by alternating between fragmentation by CID and ETD or by ETD only on the five most abundant ions in the full mass spectrum survey scan (m/z range, 400–2000). The parameters used for ETD were as follows: 100-ms activation time; emission current, 130 μA; automatic gain control, 30,000; temperature, 170 °C; supplemental activation was turned off; isolation width, 4 Da. With the exception of LC-MS/MS analyses performed with a parent mass inclusion list, dynamic exclusion was enabled to exclude ions from MS/MS selection for 3 min after being selected three times in a 30-s window.

Tandem mass spectra were searched against a database of human or rat IRS-1 using Bioworks 3.3.1 for the following variable modifications: phosphorylation at serine, threonine, or tyrosine; O-GlcNAc modification of serine and threonine (+203.2 Da); and oxidation of methionine. Carbamidomethylcysteine was included as a static modification. The instrument was equipped with ETD capability (ThermoFisher). Multiple mass spectrometric approaches were utilized to detect O-GlcNAc-modified peptides and confirm the assignments of the sites of O-GlcNAc modification. Data-dependent neutral loss-triggered MS3 acquisition facilitated the identification of candidate O-GlcNAc-modified peptides. The instrument was programmed to acquire MS/MS/MS (MS3) data on precursor ions that exhibited a neutral loss corresponding to N-acetylglucosamine (203.2, 101.6, 67.7, or 50 Da for the 1+, 2+, 3+, and 4+ charge states, respectively) upon collision-induced dissociation. A normalized collision energy of 35% was used for fragmentation by CID. The threshold for the selection of an ion for fragmentation was set to 500. In subsequent analyses, the instrument was programmed to acquire tandem mass spectra generated by alternating between fragmentation by CID and ETD or by ETD only on the five most abundant ions in the full mass spectrum survey scan (m/z range, 400–2000). The parameters used for ETD were as follows: 100-ms activation time; emission current, 130 μA; automatic gain control, 30,000; temperature, 170 °C; supplemental activation was turned off; isolation width, 4 Da. With the exception of LC-MS/MS analyses performed with a parent mass inclusion list, dynamic exclusion was enabled to exclude ions from MS/MS selection for 3 min after being selected three times in a 30-s window.

Peptide Synthesis and Antibody Production—A peptide corresponding to human IRS-1 residues 1003–1019 with an O-linked-β-N-acetylglucosamine serine at 1011, Ac-CDTSPAAPVS(O-GlcNAc)YA-DMRTG1-amide, was synthesized and purified by Sussex Research (Ontario, Canada). An N-terminal Cys residue was included for conjugation of the peptide to keyhole limpet hemocyanin carrier protein, and a polyclonal antibody was generated in rabbit (Lampire Biological Laboratories, Pipersville, PA). To characterize the specificity of the antibody for the site of O-GlcNAc modification, wild type IRS-1 or a mutant of rat IRS-1 in which the site of O-GlcNAc modification was substituted with alanine (S1009A) was transiently expressed in HEK293 cells in the presence of the O-GlcNAcase inhibitor PUGNac. Nickel-purified proteins were separated by gel electrophoresis and transferred to nitrocellulose, and the membranes were probed with a 1:3000 dilution of rabbit sera followed by a fluorescently labeled secondary IgG. The protein was visualized using an Odyssey instrument (Licor Biosciences, Inc.). Removal of the site of O-GlcNAc modification at Ser1009 completely abolished antibody binding (supplemental Fig. 1). To confirm that the antibody recognized the monosaccharide and not the naked peptide backbone, a competition experiment was performed by preincubating the sera with either the unmodified or O-GlcNAc-modified peptide residues 1003–1019. Preincubation of the sera with the O-GlcNAc-modified peptide abolished antibody binding to rat IRS-1, whereas preincubation with the naked peptide did not alter antibody binding to IRS-1 (supplemental Fig. 1). To check the specificity of the antibody for N-acetylgalactosamine, a competition experiment was performed by preincubating the sera with 1 M N-acetylgalactosamine (Sigma) prior to probing IRS-1 by Western blot. Preincubation of the antibody with the monosaccharide greatly reduced the staining of the IRS-1 protein. To further confirm that the antibody recognized the monosaccharide and not the naked peptide backbone, human IRS-1 isolated from PUGNac-treated HEK293 cells was incubated in the presence or absence of β-N-acetyl-hexosaminidase (New England Biolabs). The reaction was performed at 37 °C for 18 h in reaction buffer supplied by the manufacturer. The protein was separated by gel electrophoresis and probed by Western blot. Treatment of IRS-1 with hexosaminidase abolished antibody recognition of the protein (supplemental Fig. 1).

Ionization Efficiency and Estimated Stoichiometry of O-GlcNAc-modified Peptides—The peptide Ac-Cys-1003–1019-amide was synthesized with and without the O-GlcNAc modification at Ser1011. The concentrations of the purified peptides were determined by amino acid analysis (Anaspec, San Jose, CA). To compare the ionization efficiencies and the possible loss of the labile monosaccharide in the ionization source of the mass spectrometer, equal molar ratios of O-GlcNAc-modified and unmodified peptides were combined and analyzed using the LC-MS/MS parameters described above. The peak areas of the unsmoothed extracted ion chromatograms corresponding to the doubly charged ions of the unmodified and O-GlcNAc-modified synthetic peptides were determined using the Qual Browser feature in Xcalibur 2.0. The loading equimolar amounts of the synthetic peptides resulted in equal areas under the base peak ion chromatograms suggesting that the O-GlcNAc modification was not lost upon ionization in the source and that the O-GlcNAc-modified peptide was ionized as efficiently as the unmodified peptide (supplemental Fig. 3).

Phosphopeptide Interaction with SH2 Domain Protein Array—To determine candidate SH2 domain-containing proteins that may interact with a putative SH2 domain binding motif at Y1012ADM, an SH2 domain protein array was probed with a synthetic peptide corresponding to human IRS-1 phosphorylated at Tyr1012. The peptide Ac-Cys-1003–1019-amide phospho-Tyr1012, synthesized and purified by Anaspec, was biotinylated using maleimide-polyethylene glycol-
RESULTS

To identify the sites of O-GlcNAc modification of human and rat IRS-1, the proteins were transiently expressed in HEK293 cells grown in the presence of the O-GlcNAcase inhibitor PUGNAc. His-tagged IRS-1 was enriched by nickel affinity chromatography and gel-purification. The IRS-1 protein was excised from the gel and digested with trypsin and/or Glu-C. Extracted peptides were separated and analyzed by LC-MS/MS using collision-induced dissociation with neutral loss-triggered MS3 and electron transfer dissociation. Trypsin digestion yielded 84 and 75% sequence coverage of the 131-kDa rat and human IRS-1 proteins, respectively.

O-GlcNAc Modification of Rat IRS-1 at Ser\textsuperscript{1036} and Ser\textsuperscript{1041} and Human IRS-1 Residues 1025–1045—O-GlcNAc modification of Ser\textsuperscript{1036} was identified previously by a chemical derivatization approach utilizing β-elimination and Michael addition with dithiothreitol (31). However, the presence of O-GlcNAc modification elsewhere in the protein, which has 181 Ser and 63 Thr residues, was evident following immunoblot analysis of a mutant of IRS-1 in which the site of O-GlcNAc modification had been substituted with alanine (S1036A). Removal of this site of O-GlcNAc modification reduced but did not completely abolish immunoreactivity of the pan-specific anti-O-GlcNAc antibody CTD 110.6 against IRS-1 as determined by Western blot (31). To facilitate the detection of other sites of O-GlcNAc modification, rat IRS-1 was analyzed by ETD MS/MS. This methodology is amenable to the detection of labile amino acid modifications and leaves O-GlcNAc Ser intact during fragmentation of the peptide backbone for MS/MS analysis (28). The fragmentation pattern of the O-GlcNAcylated tryptic peptide 1027–1074 of rat IRS-1 by ETD is consistent with the co-elution of a mixture of mono-O-GlcNAc-modified peptides in which only the site of modification at Ser\textsuperscript{1036} could be resolved (Fig. 1A). LC-MS/MS analysis of the S1036A mutant of IRS-1 revealed a second site of O-GlcNAc modification within residues 1032–1041 (Fig. 1B). The observed molecular mass of the O-GlcNAc-modified peptide 1021–1051 S1036A following Glu-C digestion was 3083.1 Da. The increase in mass of 203.9 Da above the calculated mass of the unmodified peptide, 2879.2 Da, is consistent with the calculated average mass of N-acetylglucosamine (203.2 Da). The major fragment ions in the CID tandem mass spectrum (m/z 1440.3 and 960.4) represent the characteristic neutral loss of GlcNAc from the doubly and triply charged precursor ions. Because the retention of the monosaccharide on a series of b ions starting with residue 1041 is consistent with O-GlcNAc modification at this residue, this amino acid was replaced by site-directed mutagenesis. LC-MS/MS analysis of rat IRS-1 following site-directed substitution of Ser\textsuperscript{1036} and Ser\textsuperscript{1041} with alanine did not reveal O-GlcNAc modification at any other sites within residues 1027–1073 consistent with the assignment of O-GlcNAc modification at Ser\textsuperscript{1036} and Ser\textsuperscript{1041}. Furthermore, O-GlcNAc modification at other sites within the protein (Ser\textsuperscript{1009} and Ser\textsuperscript{914}; described below) was observed suggesting that modification at residues 1036 and 1041 is not required for subsequent O-GlcNAcylation within other regions of the protein.

The homologous tryptic peptide in human IRS-1, residues 1029–1074, was observed in multiple posttranslationally modified forms: O-GlcNAc-modified, phosphorylated at Ser\textsuperscript{1043}, O-GlcNAc-modified and phosphorylated at Ser\textsuperscript{1043}, and doubly phosphorylated at Ser\textsuperscript{1041} and Ser\textsuperscript{1043} (Fig. 2 and Table I). The CID tandem mass spectra of the tryptic peptide 1029–1074 confirmed the newly identified sites of phosphorylation at Ser\textsuperscript{1041} and Ser\textsuperscript{1043}. However, complete neutral loss of the monosaccharide obscured determination of the site(s) of O-GlcNAc modification (Fig. 2A). CID MS/MS of the Glu-C-digested peptide 1024–1052 (m/z 1418.5) yielded partial retention of the N-acetylglucosamine (supplemental Fig. 3).

The presence of fragment ions corresponding to the calculated m/z of b\textsubscript{17} GlcNAc and y\textsubscript{11} GlcNAc is consistent with co-elution and simultaneous analysis of two mono-O-GlcNAc-modified peptides with sites of glycosylation in the N and C termini. Similar to the homologous peptide in rat IRS-1, fragmentation by ETD was not sufficient to resolve the site(s) of O-GlcNAc modification within this peptide. Thus, additional approaches are required to resolve the site(s) of O-GlcNAc modification within residues 1025–1045 of human IRS-1.

O-GlcNAc Modification of Rat IRS-1 Ser\textsuperscript{914}—Rat IRS-1, residues 891–915, was observed either phosphorylated at 891 or O-GlcNAc-modified at Ser\textsuperscript{914} and phosphorylated at 891 (Fig. 3 and Table I). These modifications flank the SH2 domain binding motif at Y\textsuperscript{906}V, which when phosphorylated by the insulin receptor creates a docking site for Grb2 and signal transduction to the Ras-MAPK pathway (33). The CID tandem mass spectrum of the doubly charged precursor at m/z 1419.2 confirmed the previously reported phosphorylation at Ser\textsuperscript{891} (34) and revealed O-GlcNAc modification at Ser\textsuperscript{914} (Fig. 3A). Following collision-induced dissociation, the major fragment ions in the spectrum at m/z 1317.7 and 1268.8 correspond to neutral loss of the GlcNAc and the subsequent loss of phosphoric acid, respectively. Partial retention of GlcNAc on the y series of ions generated a complex tandem
mass spectrum that gave poor peptide probability scores by automated database searching algorithms, such as SEQUEST. This behavior contributes to the difficulty in identifying O-GlcNAc-modified peptides by conventional MS/MS sequencing approaches. This peptide was detected as a result of triggering the acquisition of an MS3 spectrum following neutral loss of the monosaccharide. The chromatographic separation and an estimate of the relative stoichiometry of the differentially modified forms of this peptide of IRS-1 isolated from cells treated with the O-GlcNAcase inhibitor are shown in Fig. 3B. O-GlcNAc modification of the homologous human IRS-1 peptide, 892–922, was not observed. However, this peptide was phosphorylated at Ser892 (34), and a novel site of phosphorylation was identified at Ser918 in human IRS-1 (supplemental Fig. 4).

Fig. 1. A, ETD MS/MS of rat IRS-1 revealed a mixture of mono-O-GlcNAcylated forms of the tryptic peptide 1027–1073. The fragmentation pattern of the precursor at m/z 1133.3 confirms the identity of the peptide and the previously identified site of O-GlcNAc modification at Ser1036. The observation of the c10 ion with and without GlcNAc is consistent with the co-elution of peptides O-GlcNAc-modified at Ser1036 and C-terminal to this residue. The c and z ions are labeled according to O-GlcNAc modification at Ser1036; c10 indicates the unglycosylated c10 ion. Fragment ions with GlcNAc are indicated by an asterisk. The calculated and observed molecular masses of peptide 1027–1073 GlcNAc were 4527.5 and 4528.8 Da, respectively. B, O-GlcNAc modification of rat IRS-1 at Ser1041 was revealed following substitution of Ser1036 with alanine. CID MS/MS of the precursor at m/z 1028.7 confirms the identity of the Glu-C-digested peptide 1021–1051 (S1036A) with GlcNAc. The calculated and observed molecular masses of the O-GlcNAc-modified peptide were 3082.4 and 3083.1 Da, respectively. Based on the series of b ions and the b22 ion that retained the monosaccharide, the fragmentation pattern is consistent with the assignment of the O-GlcNAc modification at residue 1041, which was further confirmed by site-directed mutagenesis. b and y ions are labeled according to the unmodified peptide. Fragment ions that retained the GlcNAc moiety are indicated with an asterisk. Within the peptide sequence shown, the amino acid that was substituted by site-directed mutagenesis, S1036A, is underlined, and the newly identified site of O-GlcNAc modification is indicated with a "g."
IGF-1 receptors (30). The presence of the b8 GlcNAc ion in the CID tandem mass spectrum of the doubly charged precursor ion at m/z 1459.3 is consistent with O-GlcNAc modification of the peptide 1029−1074. The calculated and observed molecular masses of the O-GlcNAc-modified peptide were 4376.8 and 4374.9 Da, respectively. Neutral loss of N-acetylglucosamine (68.3) from the doubly charged precursor at m/z 1459.3 yielded the most abundant ion at m/z 1391.5. Complete neutral loss of the GlcNAc from fragment ions precluded determination of the site of modification. The b and y ions are labeled according to the unmodified peptide. Ions retaining the GlcNAc modification are indicated with an asterisk. B, CID MS/MS of the doubly charged precursor ion at m/z 1419.3 is consistent with phosphorylation of peptide 1029−1074 at Ser1043. The calculated and observed molecular masses of the phosphorylated and O-GlcNAc-modified peptide 1029−1074 further confirms phosphorylation at Ser1043. The calculated and observed molecular masses of the phosphorylated and O-GlcNAc-modified peptide were 4457.8 and 4454.7 Da, respectively. Ions retaining the GlcNAc modification are indicated with an asterisk. C, MS/MS of the triply charged precursor at m/z 1445.4 is consistent with phosphorylation at Ser1041 and Ser1043. The calculated and observed molecular masses of the phosphorylated peptide were 4335.6 and 4333.2 Da, respectively. Within the peptide sequence, the sites of phosphorylation are indicated with a “p.”


O-GlcNAc Modification of IRS-1

Phosphorylated forms of these peptides are also shown, p, phosphorylation, g, O-GlcNAc modification. References previously reporting sites of posttranslational modification are indicated in parentheses. The following chemical modifications occurred during sample preparation (data not shown). Peptides with N-terminal glutamine were partially cyclized to pyroglutamic acid. Peptides were observed with and without sites of posttranslational modification are indicated in parentheses. The following chemical modifications occurred during sample preparation.

| Residues | Peptide | Site | Observed m/z | Observed mass | Calculated mass | Site assignment |
|----------|---------|------|--------------|---------------|----------------|----------------|
| 981–998  | AVPSGGQYMTMQMSCPR | 984 or 985g | 1139.95 | 2278.6 | 2278.6 | CID |
| 999–1016 | QSYDTPAPVSYADMR  | 1009g | 1095.3 | 2184.9 | 2184.9 | CID/ETD |
| 1029–1074 | ATMAASSSSAApSPTGPQGAELAAHSSLll | 1021–1051 (S1036A) | 1043p | 1419.3 | 4254.8 | 4255.6 | CID |

* Expected mass of peptide 1021–1051 following site-directed mutagenesis of S1036A.

* Indicates peptides in which the site(s) of O-GlcNAc modification could not be assigned.

O-GlcNAc modification occurred at either 984 or 985, residues underlined.

tive abundance of differentially modified forms of rat IRS-1 peptide 997–1014 isolated from cells treated with the O-GlcNAcase inhibitor are shown in Fig. 5E.

Human IRS-1 was phosphorylated and O-GlcNAc-modified at the homologous residues Ser1005 and Ser1011, respectively. Interestingly, phosphorylation at Ser1005 decreases in euglycemic hyperinsulinemic clamp studies (36) indicating that modifications within this region of IRS-1 are involved in acute responses to insulin in vivo. The CID tandem mass spectrum of human IRS-1 residues 999–1016 at m/z 1081.7 is consistent with the assignment of O-GlcNAc modification at Ser1011 (Fig. 5D).

The presence of this site-specific glycosylation in endogenously expressed IRS-1 was confirmed by Western blotting and tandem mass spectrometry. IRS-1 was immunoprecipitated from HEPG2 human hepatoma cells or murine MC3T3-E1 osteoblast precursor cells grown in 5 mM glucose in the absence or presence of PUGNAC. Tandem mass spectrometric analysis of IRS-1 isolated from MC3T3-E1 cells confirmed O-GlcNAc modification of mouse IRS-1 at Ser1005 (supplemental Fig. 6) Using an antibody generated against a synthetic peptide corresponding to human IRS-1 residues 1003–1019 Ser1011 GlcNAc (supplemental Fig. 1), the immunoprecipitated IRS-1 was probed for O-GlcNAc modification at Ser1011 (human) or Ser1005 (mouse) by Western blot analysis (Fig. 6). IRS-1 was O-GlcNAc-modified under basal conditions, and the extent of O-GlcNAc modification increased following inhibition of O-GlcNAcase suggesting dynamic cycling of the modification under basal conditions. These data further confirm the identification and assignment of the +203.2-Da mass shift as an O-GlcNAc modification of mouse, rat, and human IRS-1 at residues Ser1005, Ser1009, and Ser1011, respectively.

Ser1011 GlcNAc Is Adjacent to a Putative Binding Site for the N-terminal SH2 Domains of p85α,β Subunits of PI3K and the Tyrosine Phosphatase SHP2 (PTPN11)—O-GlcNAc modification of human IRS-1 at Ser1011 occurs immediately adjacent to a putative SH2 domain binding motif, Y1012ADM. Phosphorylation of this tyrosine has been reported (37), although this YYXXM motif, which is preceded by hydrophobic amino acids and does not possess the Tyr + 1 methionine residue, has not been observed by in vitro phosphorylation by the insulin receptor nor has phosphorylation at this residue been detected in recent studies characterizing the temporal dynamics of insulin-stimulated tyrosine phosphorylation (9, 35, 38). This motif is one of the nine YYXXM motifs in IRS-1, six of which are
To ascertain candidate binding partners for this particular motif, we probed an SH2 domain protein array with an HRP-labeled synthetic peptide corresponding to human IRS-1 residues 1003–1019, phospho-Tyr1012 (Fig. 7). The peptide interacted with the N-terminal SH2 domains of the p85α/H9251 and p85β/H9252 regulatory subunits of PI3K and, consistent with phosphotyrosine peptide pulldown experiments in C2C12 cells (39), with the N-terminal SH2 domain of the tyrosine phosphatase SHP2 (PTPN11).

Because O-GlcNAc modification and tyrosine phosphorylation of IRS-1 occur at substoichiometric levels and the protein has been shown to be localized to different subcellular pools depending on the cell type, we questioned whether these modifications were occurring on the same pool of IRS-1. To probe the O-GlcNAc-modified fraction of IRS-1 for insulin-stimulated tyrosine phosphorylation, rat IRS-1 was isolated from HEK293 cells that had been incubated overnight with the O-GlcNacase inhibitor in the absence or presence of 100 nM insulin. Total O-GlcNAc modification of IRS-1

**Fig. 3.** Phosphorylation and O-GlcNAc modification of rat IRS-1 at Ser891 and Ser914. A, CID MS/MS of the doubly charged precursor at m/z 1419.2 corresponds to residues 891–915 phosphorylated at Ser891 and O-GlcNAc-modified at Ser914. The b and y ions are labeled according to the peptide phosphorylated at 891. Ions retaining the GlcNAc modification are indicated with an asterisk. The calculated and observed molecular masses of the phosphorylated and O-GlcNAc-modified peptide were 2837.9 and 2836.4 Da, respectively. B, chromatographic separation of differentially modified forms of residues 891–915. Base peak chromatograms of the doubly charged ions representing the differentially modified peptides of rat IRS-1 residues 891–915 isolated from HEK293 cells treated with the O-GlcNAcase inhibitor are shown. Based on the sum of the peak areas of the 2+ and 3+ charge state ions, the estimated stoichiometry of modification was as follows: 19% unmodified, 75% phosphorylated, and 6% phosphorylated and O-GlcNAcylated. Phosphorylation is indicated by p in A and P in B. NL, normalized intensity.

**Fig. 4.** O-GlcNAc modification of human IRS-1 residues 981–998. CID MS/MS of the doubly charged precursor at m/z 1139.4 corresponds to residues 981–998 O-GlcNAc-modified at Ser984 or Ser985 (residues underlined in sequence). The b and y ions are labeled according to the unmodified peptide. Ions retaining the GlcNAc modification are indicated with an asterisk. The retention of the monosaccharide on the b8 ion indicates that the O-GlcNAc modification occurs in the N terminus of the peptide. The calculated and observed molecular masses of the O-GlcNAc-modified peptide were 2278.6 and 2277.8 Da, respectively.
recombinant IRS-1 was enriched by nickel-nitrilotriacetic acid affinity chromatography. The O-GlcNAc-modified fraction of IRS-1 was immunoprecipitated using a pan-specific anti-O-
GlcNAc antibody (CTD 110.6) or the anti-human Ser1011 GlcNAc IRS-1 antibody. Immunoprecipitates were probed for global phospho-Tyr and phosphorylation of IRS-1 at Tyr989 (human), FIG. 5.

O-GlcNAc Modification of IRS-1

Fig. 5. O-GlcNAc modification of rat IRS-1 at Ser<sup>1009</sup> and human IRS-1 at Ser<sup>1011</sup>. A, CID MS/MS of rat IRS-1 peptide 997–1014 GlcNAc is consistent with O-GlcNAc modification at Ser<sup>1009</sup>. Neutral loss of 101.7 from the doubly charged precursor at m/z 1095.3 to m/z 993.6 triggered acquisition of an MS3 spectrum aiding the detection of this peptide. The calculated and observed molecular masses of the O-GlcNAc-modified peptide were 2189.4 and 2188.6 Da, respectively. The b and y ions are labeled according to the unmodified peptide. Ions that retained the GlcNAc are indicated with an asterisk. B, ETD MS/MS of this peptide at m/z 1094.8 further confirms the site of O-GlcNAc modification. The c and z ions are labeled according to O-GlcNAc modification at Ser<sup>1009</sup>. Ions with the GlcNAc modification are indicated with an asterisk. C, CID MS/MS of the doubly charged precursor ion at m/z 1135.0 is consistent with O-GlcNAc modification of rat IRS-1 at Ser<sup>1009</sup> and phosphorylation at Ser1003. The calculated and observed molecular masses of the O-GlcNAc-modified and phosphorylated peptide were 2269.4 and 2268.0 Da, respectively. The b and y ions are labeled according to phosphorylation at Ser<sup>1003</sup>. Ions retaining the GlcNAc modification are indicated with an asterisk. In the peptide sequence, phosphorylation and O-GlcNAc modification are indicated by p and g, respectively. D, CID MS/MS of human IRS-1 peptide 999–1016 O-GlcNAc-modified at Ser<sup>1011</sup> and phosphorylation at Ser1003. The calculated and observed molecular masses of the O-GlcNAc-modified and phosphorylated peptide were 2269.4 and 2268.0 Da, respectively. The b and y ions are labeled according to phosphorylation at Ser<sup>1003</sup>. Ions that retained GlcNAc are indicated with an asterisk. E, CID MS/MS of human IRS-1 peptide 999–1016 O-GlcNAc-modified at Ser<sup>1011</sup>, MS/MS of the precursor at m/z 1081.6 is consistent with O-GlcNAc modification at residue 1011. The b and y ions are labeled according to the unmodified peptide. The calculated and observed molecular masses of the O-GlcNAc-modified peptide were 2161.3 and 2161.4 Da, respectively. Ions retaining the GlcNAc modification are indicated with an asterisk. In the peptide sequence, phosphorylation and O-GlcNAc modification are indicated by p and g, respectively. Base peak chromatograms of the doubly charged ions representing the differentially modified peptides of rat IRS-1 residues 997–1014 isolated from HEK293 cells treated with the O-GlcNAcase inhibitor are shown. Based on the sum of the peak areas of the 2+ and 3+ charge state ions, the estimated stoichiometry of modifications were as follows: 62% unmodified, 22% phosphorylated, 11% O-GlcNAcylated, and 5% phosphorylated and O-GlcNAcylated. Phosphorylation is indicated by P. NL, normalized intensity.

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a known site of receptor-mediated tyrosine phosphorylation that creates a docking site for the p85 subunits of PI3K (7). The detection of tyrosine phosphorylated IRS-1 in the O-GlcNAcylation fraction of IRS-1 suggests that O-GlcNAc-modified IRS-1 remains available for insulin-stimulated tyrosine phosphorylation (supplemental Fig. 7).

**DISCUSSION**

To elucidate the mechanism by which O-GlcNAc modification by O-GlcNAc-transferase may impact insulin and IGF-1 signaling at the level of IRS-1, we identified the sites of O-GlcNAc modification on the rat and human homologs of IRS-1 expressed in HEK293 cells. These studies revealed that the C terminus of IRS-1, which is rich in protein interaction docking sites, is O-GlcNAc-modified at multiple residues within a region of IRS-1 spanning ~130 residues.

Fragmentation of peptides by CID and ETD revealed four sites of O-GlcNAc modification at residues Ser914, Ser1009, Ser1036, and Ser1041 in rat IRS-1. Because not all of these residues are conserved in human IRS-1, the human homolog was also characterized. Analysis of human IRS-1 revealed novel sites of phosphorylation at Ser918, Ser1041, and Ser1043 and O-GlcNAc modification at Ser984 or Ser985, at Ser1011, and possibly at multiple sites between residues 1025–1045 (Table I). A sequence alignment of rat and human IRS-1 (residues 892–1059) with the observed sites of posttranslational modification and the proximity of the O-GlcNAc-modified residues to SH2 domain binding motifs, interaction surfaces for the insulin and IGF-1 receptors, and two human polymorphisms (40, 41) is shown in Fig. 8. Interestingly, the sites of O-GlcNAc modification observed occur within a region of the protein that is unique to IRS-1 and is not conserved in IRS-2.

O-GlcNAc modification of human IRS-1 at 984/985 occurs in close proximity to the DY989MTM docking site for the p85 subunit of PI3K. The effect of O-GlcNAc modification at the Tyr5 position on receptor-mediated phosphorylation at Tyr989 and on SH2 domain interaction will be examined in future studies. This site of O-GlcNAc modification also occurs within residues 950–986 to which the insulin and IGF-1 receptors have recently been shown to bind (30). A single amino acid polymorphism, G972R, within this region has been shown to impact the kinetics of the receptor-substrate interaction and autophosphorylation of the insulin receptor and attenuate signaling to both the PI3K/Akt and Grb2/ERK axes (30, 36). Thus, it is conceivable that O-GlcNAc modification at this site could impinge on insulin signaling by multiple mechanisms.

O-GlcNAc modification of human IRS-1 at Ser1011 is adjacent to a YXXM motif that resides within a sequence

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**FIG. 6.** O-GlcNAc modification of endogenously expressed IRS-1 at Ser1011. IRS-1 was immunoprecipitated from HEPG2 human liver cells (left panel) or MC3T3-E1 mouse osteoblasts (right panel) grown in 5 mM glucose and in the presence or absence of 100 μM O-GlcNAcase inhibitor PUGNAc for 18 h. The membrane was probed with an anti-IRS-1 Ser1011 GlcNAc antibody generated against a peptide corresponding to human IRS-1 residues 1003–1019 O-GlcNAc-modified at Ser1011. Lanes 1 and 2 represent 5% of IRS-1 immunoprecipitated from 6 mg of MC3T3-E1 cell lysate. Lanes 3 and 4 represent 2 and 5%, respectively, of IRS-1 immunoprecipitated from 22 mg of cell lysate. IP, immunoprecipitate; WB, Western blot; Ab, antibody; h, human; r, rat; m, mouse.

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**FIG. 7.** Interaction of phospho-Tyr1012 with N-terminal SH2 domains of SHP2 (PTPN11) and p85α and p85β subunits of PI3K. An IRS-1 peptide, Ac-CDTSPAAPVSpYADMRTGI-NH2, was biotinylated and incubated with streptavidin-HRP prior to incubation with the SH2 domain protein array. The nitrocellulose was spotted twice with 100 ng of the recombinant SH2 domain from each protein indicated. Proteins with two SH2 domains are indicated (D1/D2). BTK, Bruton agammaglobulinemia tyrosine kinase; CRKL, v-crk sarcoma virus homolog like; GRAP, Grb2 related adaptor protein; TNS, tensin1; HCK, hemopoietic cell kinase; CSK, c-src tyrosine kinase; FES, feline sarcoma oncogene; GFR, Rap guanine nucleotide exchange factor 5; MATK, megakaryocyte-associated tyrosine kinase; LCK, lymphocyte-specific protein tyrosine kinase.
conserved in mammalian IRS-1 homologs, PVSYYADM. The presence of O-GlcNAc modification at this site in endogenously expressed human and mouse IRS-1 was confirmed by Western blot analysis using a site-specific antibody. Based on the interaction of the corresponding phospho-Tyr peptide with the SH2 domains of p85 PI3K and SHP2, phosphorylation of Tyr1012 may contribute to PI3K/Akt signaling, or conversely, it may facilitate Tyr dephosphorylation thereby attenuating signaling through IRS-1. Based on previous studies illustrating the critical role of other SH2 binding motifs in mediating the interactions of IRS-1 with PI3K (42) and SHP2 (2), the extent to which phosphorylation at this Tyr impacts insulin signaling is not clear. The effect of O-GlcNAc modification at Ser1011, which may alter phosphorylation at Tyr1012 or the binding of SH2 domains to this motif, will be addressed in future studies.

As a highly posttranslationally modified adaptor protein that interacts with many binding partners, dynamic cross-talk among modifications of IRS-1 may contribute to specificity of effects elicited following receptor stimulation. Reversible posttranslational modifications acting either independently or in combination may induce or antagonize protein interactions by multiple mechanisms (for a review, see Ref. 43). O-GlcNAc modification mutually excludes phosphorylation of identical or adjacent residues in several proteins; therefore, we analyzed the phosphorylation state of the O-GlcNAc-modified peptides observed. In our mass spectrometric characterization of recombinant IRS-1 isolated from cells treated with PUGNAC or stimulated with insulin for varying times, we did not observe evidence for reciprocal or mutually exclusive O-GlcNAc modification and Ser/Thr phosphorylation within the proteolytic peptides examined. In fact, most of the O-GlcNAc-modified peptides observed were also phosphorylated at nearby serines or threonines within consensus sequences for proline-directed kinases. Because of the difficulty in resolving the site(s) of O-GlcNAc modification of human IRS-1 to specific residue(s) within peptide 1035–1045, SSSSSASAPPT, which is phosphorylated at Ser1041 and Ser1043, we cannot rule out the occurrence of reciprocity of modifications within this peptide. The bottom up approach of proteolytically digesting the protein prior to MS analysis limits the observable connectivity of modifications to short peptide sequences. We probed for potential reciprocity of O-GlcNAc modification and insulin-stimulated tyrosine phosphorylation of the intact protein by immunoblot analysis and found that the O-GlcNAc-modified fraction of IRS-1 remained an available substrate for insulin-stimulated tyrosine phosphorylation. These observations suggest that rather than mutually exclusive site-specific competition with phosphorylation the O-GlcNAc modification may provide an additional layer to the complex posttranslational regulation of IRS-1.

The investigation of the sites and functional effects of O-GlcNAc modification on many proteins has been neglected because of the difficulty in detecting the modification by conventional tandem mass spectrometric approaches. However, electron transfer dissociation and robust search algorithms providing automated identification of O-GlcNAc-modified peptides will greatly facilitate the advancement of this field. Furthermore, middle down approaches utilizing ETD will permit analysis of the temporal dynamics of O-GlcNAc modification and connectivity of this modification to other modifications within larger peptide sequences.

The function of O-GlcNAc modification of IRS-1 is unclear. It may affect protein interactions either by blocking hydrophobic interactions, by recruiting a different subset of binding partners, or by altering subcellular localization. Furthermore, the effects may vary by the cellular milieu; e.g., they may be cell-specific. Given the critical role of the posttranslational modifications of IRS-1 in mediating and modulating insulin and IGF-1 receptor signaling, in vivo studies concerning the effects of the detected sites of O-GlcNAc modification on insulin and IGF-1 receptor signaling seem warranted.

**Acknowledgments**—The data were obtained on instrumentation housed in the Mass Spectrometry Facility at the Medical University of South Carolina. We thank Jennifer R. Bethard for maintaining the MS instruments, Katy A. Robinson for assistance with tissue culture, and Drs. Morris White and Adrian Lee for generously providing the rat and human IRS-1 cDNA, respectively.

*This work was supported, in whole or in part, by National Institutes of Health Grants U24 DE016508 (to L. E. B.), P20RR017696 from the National Center for Research Resource (to L. E. B.) through the South Carolina Center of Biomedical Research Excellence for Oral Health, and DK002001 (to M. G. B.). This work was also supported by American Cancer Society Institutional Research Grant IRG 97-219-08 (to L. E. B.).
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