The signal transducer and activator of transcription (Stat) gene family comprises seven members with similarities in their domain structure and a common mode of activation. Members of this gene family mediate interferon induction of gene transcription and the response to a large number of growth factors and hormones. Extracellular ligand binding to transmembrane receptors causes the intracellular activation of associated tyrosine kinases, phosphorylation of Stat molecules, dimerization, and translocation to the nucleus. Prolactin-induced phosphorylation of Stat5 is a key event in the development and differentiation of mammary epithelial cells. In addition to the crucial phosphorylation at tyrosine 694, we have identified an O-linked N-acetylglucosamine (O-GlcNAc) as another secondary modification essential for the transcriptional induction by Stat5. This modification was only found on nuclear Stat5 after cytokine activation. Similar observations were made with Stat1, Stat3, and Stat6. Glycosylation of Stat5, however, does not seem to be a prerequisite for nuclear translocation. Mass spectrometric analysis revealed a glycosylated peptide in the N-terminal region of Stat5. Replacement of threonine 92 by an alanine residue (Stat5a-T92A) strongly reduced the prolactin induction of Stat5a glycosylation and abolished transactivation of a target gene promoter. Only the glycosylated form of Stat5 was able to bind the coactivator of transcription CBP, an essential interaction for Stat5-mediated gene transactivation.

Signal transducer and activator of transcription (Stat) proteins are latent cytoplasmic transcription factors that mediate cellular responses to diverse cytokines, hormones, and growth factors (1). After binding of these ligands to their cell surface receptors Stat proteins are activated by tyrosine phosphorylation. Cytoplasmic tyrosine kinases, Janus kinases (Jaks), and members of the Src kinase family, mediate Stat activation (2). Tyrosine-phosphorylated Stat monomers can form dimers through reciprocal phosphotyrosine SH2 interactions; dimers translocate to the nucleus, bind to specific DNA-promoter elements, and induce target gene transcription (3). Seven Stat family members, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6 have been found in mammalian cells. They have diverse biological functions including roles in cell differentiation, development, proliferation, apoptosis, and inflammation (4–6). Constitutively activated Stat proteins have been observed in tumor cells, where they are able to contribute to the transformed phenotype (7).

The regulation of Stat activity is not restricted to tyrosine phosphorylation; serine phosphorylation has also been found to be important (8). The C-terminal regions of Stat1, Stat3, Stat4, and Stat5 contain phosphorylated serine residues required for full transcriptional activation (9). Members of the mitogen-activated protein kinases (MAPK) family, extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), and p38 MAPK have been implicated in the serine phosphorylation of Stat1, Stat3, and Stat5. Beuvink et al. (9) showed that serine 779 is a major phosphorylation site of Stat5a; the same is true for serine 725 (10).

N-Acetylglucosamine (O-GlcNAc) is a sugar residue that is used as a frequent post-translational modification of nuclear and cytoplasmic proteins. One molecule of GlcNAc is linked as a single monosaccharide to the hydroxyl group of serines or threonines and is not further elongated. Like phosphorylation, modification by O-GlcNAc is highly dynamic (11, 12) and can give rise to functionally distinct subsets of a protein. Proteins modified by O-GlcNAc can also be modified by phosphorylation (13). Over 50 proteins with O-GlcNAc modifications have been identified including RNA polymerase II, RNA polymerase II-associated transcription factors, nuclear pore proteins, the tumor suppressor protein p53, and c-Myc (14–17). Common sequence motifs, at which O-GlcNAc modifications occur in these diverse proteins, have not been identified (18), and O-GlcNAc modification of tyrosine residues has not been observed.

The attributes of O-GlcNAc modification are distinct from those of complex carbohydrates (19). O-GlcNAc modification of proteins is known to change in response to T cell activation, insulin signaling, glucose metabolism, and cell cycle progression (20–23); characteristics consistent with a function in signal transduction. O-GlcNAc modification is a dynamic process and inducible enzymes responsible for the attachment (O-GlcNAc transferase, OGTT) and for the removal (O-GlcNAcase) of this sugar moiety have been found in the nucleus and the cytoplasm of cells. The genes encoding these enzymatic activities have been cloned and characterized (24, 25). The N-termin-
nus of OGT contains multiple tetrastratopeptide repeats that mediate protein-protein interactions, which are critical for subcellular localization (24, 26, 27). Inactivation of the OGT gene in mouse cells has shown that OGT is required for embryonic stem cell viability and mouse ontogeny (27). Deglycosylation is also important, and the prevention of the removal of O-GlcNAc from proteins has been shown to be toxic to cells. This can be achieved by microinjection or overexpression of the galactosyltransferase enzyme causing the capping of the O-GlcNAc sugar residue or GSk3β was used instead of the previously described O-GlcNAcase activity (28). O-GlcNAcase activity can also be potently inhibited by O-(2-acetaminido-2-deoxy-β-D-glucopyranosylidene)amino-N-phenylcarbamamate (PUGNAc). PUGNAc is an O-GlcNAc analogue that prevents cycling of O-GlcNAc on proteins without significantly altering N-linked glycosylation or UDP-GlcNAc levels (29). PUGNAc inhibition rapidly increases O-GlcNAc levels in cells and testifies to the dynamic nature of this modification. Altered O-linked GlcNAc metabolism may play a role in human diseases including neurodegenerative disorders, diabetes mellitus, and cancer (12, 30).

In the present study we show that the transcription factor Stat5a is modified by O-GlcNAc. The glycosylated form of Stat5a was mainly found in the nucleus of hormone-induced cells. Mass spectrometry analysis revealed an O-GlcNAc-modified peptide from the N-terminal region of Stat5a comprising threonine 92. Mutation of threonine 92 to an alanine (Stat5a-T92A) resulted in the loss of Stat5 glycosylation and failure in the transcriptional induction of a reporter gene construct. This loss of function is most likely due to the inability of Stat5a-T92A to interact with the coactivator of transcription CBP. Tyrosine and serine phosphorylation of Stat5a-T92A and the ability to specifically interact with its DNA response element were not impaired.

EXPERIMENTAL PROCEDURES

Cell Culture, Transient Transfections, and Luciferase Assays—SF9 cells were grown in ExCell400 medium without fetal calf serum. HC11 mammary epithelial cells were grown and treated with lactogenic hormones as described previously (31). COS7, HeLa, and 293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM l-glutamine, and phenol red. Transfections were performed using the calcium phosphate method. 2 μg of plasmid DNA of pMx-mStat5a, pcDNA3.1-mStat5a-T92A, pcDNA-PvrH, pCMV- CBP, or pCMV-βGal were used in the transfections. The transfected genes were expressed for 48 h before the cells were harvested. To monitor the p300-dependent transcriptional activation and transcription efficiency, the β-catenin luciferase reporter construct (2 μg) was cotransfected with an expression vector encoding the β-galactosidase gene (0.5 μg) driven by the CMV promoter. DNA was adjusted to 20 μg with sonicated salmon sperm DNA (Stratagene). COS7 cells were harvested 1 day after transfection; the cells were treated with 5 μg/ml ovine prolactin for 16 h before harvesting. Luciferase activity was determined in triplicate samples using the luciferase assay system as described previously (32). Total light emission was measured during the first 30 s in triplicate samples using the luciferase assay system as described previously (32). Eluted protein extracts were stored at −70 °C.

Galactosyltransferase Assay—In the galactosyltransferase assay, radioactively labeled UDP-galactose was transferred to a terminal GlcNAc sugar residues. The reaction was followed by PNGase F treatment which removed N-linked sugars. The remaining labeled proteins contained O-linked sugar residues. Stat5 protein was labeled with UDP-[6-3H]galactose (38.5 Ci/mmol) with autogalactosylated bovine milk galactosyltransferase (GalTase) as described previously (33). Ovalbumin (chicken egg white, fraction V, Sigma) served as a positive control in the labeling reaction. Labeled proteins were treated with PNGase F enzyme (Sigma). Avidin-Biotin Complex-DNA binding (ABC) Assay—Stat5 proteins were analyzed for their ability to specifically interact with the Stat5 DNA response element. DNA binding assays were carried out with the biotinylated Stat5 binding site present in the bovine β-casein promoter. Recombinant non-glycosylated, glycosylated Stat5a, or wild-type mouse mast/sta-T92A were immobilized to the 5′-end of double-stranded DNA. The immobilized Stat5 DNA oligonucleotide for 1 h at 4 °C, followed by incubation with streptavidin-coupled beads for 30 min at 4 °C and constant agitation. Beads were pelleted and washed three times with washing buffer containing 20 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT. Bound Stat5 was eluted under denaturing conditions, separated by SDS-PAGE (7.5%) and visualized by Western blotting with polyclonal Stat5a antibody (Zymed Laboratories Inc.).

Immunoprecipitation, Tryptic Digestion and Mass Spectrometry of Recombinant Stat5a—Recombinant Stat5a was modified with galactosyltransferase and radioactively-labeled UDP-galactose. Proteins generated by tryptic digestion of immunoprecipitated Stat5a were purified by RP-HPLC. RP-HPLC fractions were counted in a scintillation counter, and only radioactive fractions were used for mass spectrometry. MALDI-TOF mass spectra were acquired in the positive ionization mode using Hewlett-Packard G2025 mass spectrometer. Peptide samples were prepared for MALDI-TOF MS by diluting the sample solution with CHCA matrix solution (Hewlett-Packard, Palo Alto) to a final concentration of 0.1–1.0 pmol/μl. Mass spectra were calibrated using a mixture of standard peptides (34). PUGNAc and Glc/GlcN Treatment of HC11 Cells and Immune Fluorescence Microscopy—PUGNAc treatments were performed by replacing the media on confluent plates (80%) with fresh complete RPMI 1640 medium containing PUGNAc (40 μM) or glucose (5 mM) and galactosamine (7 mM). Stock solutions of PUGNAc were made in MilliQ water and sterilized prior to use. 16 h post-treatment cells were washed 2× with PBS and lysed or fixed for immunofluorescence studies.

For immunofluorescence experiments cells were fixed in 5% methanol for 10–15 min. Fixed cells were washed twice with 10 min with PBS, then for 20 min with methanol-PBS mixture, followed by a wash step for 5 min with PBS. Coverslips were blocked for 30 min at room temperature with cold jellyfish gelatin. Cells were incubated with a 1:400 dilution of polyclonal anti-Stat5a antibody (primary antibody) overnight at 4 °C, followed by three wash steps for 10 min at room temperature with PBS. Secondary antibody incubation with anti-rabbit Alexa Fluor 555-conjugated (1:1000) was performed for 1 h at room temperature followed by three wash steps for 10 min at room temperature with PBS. Cell nuclei were visualized using DAPI staining.

Immunoprecipitation, Lectin Affinity Chromatography, and Western Blot Analysis—Protein concentrations were determined by the Brad-
ford method. Stat5a was immunoprecipitated from 0.2 to 0.5 mg protein of whole cell extract with a specific antiserum (rabbit anti-mouse Stat5a, Zymed Laboratories Inc.) and captured by incubation with protein A-Sepharose beads. CBP protein or glucocorticoid receptor was immunoprecipitated from 0.5 to 1.0 mg of protein of whole cell extracts with anti-CBP antibody or anti-GR antibody (Santa Cruz Biotechnology).

Immunoprecipitation of O-GlcNAc-modified proteins was performed with a specific monoclonal O-GlcNAc antibody using Protein G-Sepharose (Cnb): Lectin affinity chromatography was carried out with wheat germ agglutinin-agarose beads (Amersham Biosciences) using 2.0–4.0 mg of whole cell protein extract. Lectin beads were incubated with protein extracts for 1 h at room temperature. Glycosylated proteins were eluted off the bead with 1 M GlcNAc for 10 min at room temperature. Immunoprecipitates were subjected to SDS-PAGE (7.5%), and Western blot analyses were performed as described previously using polyacrylamide membranes (Schleicher and Schuell). Membranes were incubated with a monoclonal phosphotyrosine-specific Stat5 antibody (1:5000, Zymed Laboratories Inc.), Stat5a antiserum (1:5000, Zymed Laboratories Inc.), or O-GlcNAc antiserum (1:1000, NanoTools).

Coimmunoprecipitation experiments—CoS7 or 293T cells were transfected with expression plasmids encoding Stat5a-T92A and the PRL receptor, CBP, or GR. Two days after transfection, the cells were treated with 5 µg/ml ovine PRL for 30 min before harvesting. Whole cell extracts were prepared as described above. Protein extracts from cells expressing transfect CBP or GR (293T cells) were incubated together with protein fractions containing glycosylated or nonglycosylated Stat5 from Sf9 cells, or Stat5a-T92A with a 2 µg of CBP specific antibody (Santa Cruz Biotechnology) for 1 h at 4°C and constant agitation. Protein A-Sepharose-coupled beads (Pierce) were added for 1 h. The beads were pelleted and washed three times with incubation buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 10 mM KCl, 0.1 mM NaVO₄, 1 mM EDTA, 1 mM DTT). The immunoprecipitates were separated by SDS-PAGE (7.5%), and the Western blot analyses were performed as described previously using polyacrylamide membranes (Schleicher und Schuell). Membranes were incubated with a monoclonal phosphotyrosine-specific Stat5 antibody (1:5000, Zymed Laboratories Inc.), Stat5a antiserum (1:5000, Zymed Laboratories Inc.), or O-GlcNAc antiserum (1:1000, NanoTools).

RESULTS

Stat5a Is Modified with O-Linked N-Acetylgalactosamine Residues—Post-translational modifications serve as on-off switches or modulators of protein activity. Many cytoplasmic and nuclear proteins such as transcription factors, RNA polymerase II, oncoproteins, nuclear pore proteins, viral proteins, and tumor repressor proteins have been found to be modified by O-GlcNAc at serine and threonine residues (11). We have investigated the modification of Stat5a by O-GlcNAc in HC11 mouse mammary epithelial cells. These cells are responsive to lactogenic hormone induction and express Stat proteins endogenously (35). Treatment of HC11 cells with prolactin, insulin, and dexamethasone (DIP) causes the activation of Stat5 and milk protein expression.

To detect O-GlcNAc modification of Stat5 enzymatic reactions were used. Whole cell extracts of HC11 cells were immunoprecipitated with an antibody directed against the C terminus of Stat5a. Immunopurified Stat5a was treated with bovine galactosyltransferase in the presence of UDP-[6-3H]galactose. In this reaction the radioactive sugar moiety is transferred to terminal N-acetylgalactosamines (GlcNAc), irrespective of their O- or N-linkage. Subsequent treatment with the endoglycosidase PNGase F removes the N-linked but not the O-linked moieties. The radiolabeled proteins were visualized by autoradiography upon gel electrophoresis. Stat5a was strongly labeled with [6-3H]galactose in the galactosyltransferase reaction (Fig. 1A, lane 5), and only a part of the label could be removed by PNGase F treatment (Fig. 1A, lane 6). The protein band with the molecular weight of about 50 kDa in Fig. 1, lane 5 corresponds to the heavy chain of the IgG antibody used in the Stat5a immunoprecipitation reaction. Because N-acetylgalactosamine sugar residues are not charged and Stat5 is most likely modified with only one or two sugar residues, no change in the electrophoretic mobility of Stat5 is observed after PNGaseF treatment.

We conclude that Stat5a contains O-linked GlcNAc. Ovalbumin served as a positive control for the labeling reaction and showed a complete removal of N-linked sugars in the PNGase F reaction (Fig. 1A, lanes 1 and 2). The protein band with the molecular weight of about 50 kDa in Fig. 1, lane 5 corresponds to the heavy chain of the IgG antibody used in the Stat5a immunoprecipitation reaction.

Activation of Stat5a Induces Its Modification with GlcNAc Residues—O-GlcNAc transferase is expressed ubiquitously and has been found in the cytoplasm and in the nucleus of mammalian cells. Its TPR domain is thought to mediate protein-protein interactions (36) and the activity of the enzyme can be directly regulated by the concentration of UDP-GlcNAc (24). We employed wheat germ agglutinin (WGA) lectin affinity chromatography assays to study the glycosylation status of endogenous Stat5 in the cytoplasm and in the nucleus of HC11 mammary epithelial cells grown in the absence and presence of lactogenic hormones (Fig. 1B). WGA lectin has a specific affinity for terminal GlcNAc residues and allows the separation of Stat5a into glycosylated and non-glycosylated fractions (37).

Stat5a was visualized in the individual fractions by gel electrophoresis and immunoblotting. WGA-bound, glycosylated Stat5a was preferentially found in the nuclear fraction of HC11 cells (Fig. 1B, lanes 3 and 4) and increased after lactogenic hormone stimulation of the cells (lane 4). The lower molecular weight proteins observed may represent proteolytic fragments. O-GlcNAc competition in the WGA binding reaction confirmed the specificity of the interaction. No Stat5 was retained when 200 µM GlcNAc was added (data not shown). The translocation of total Stat5 to the nucleus upon hormone treatment of the cells is apparent in Fig. 1B, lanes 5–8. Only 10% of the protein introduced in the WGA affinity chromatography assays (lanes 1–4) have been used in these Western blot experiments. Stat5a was found glycosylated mainly in the nuclear fraction after hormone stimulation indicating that glycosylation of Stat5a might be a hormonally regulated process or occur as a function of its nuclear translocation.

Stat1, Stat3, and Stat6 Are Also Modified by O-GlcNAc—Seven Stat family members have been identified in mammals, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6 (38). We performed lectin affinity chromatography experiments to investigate if O-GlcNAc glycosylation is a feature restricted to Stat5 or if other members of the Stat family also exhibit this modification. Stat1, Stat3, Stat5b, and Stat6 were activated in HC11 cells by treatment with IFN-γ, EGF, prolactin or IL-4, respectively. Whole cell protein extracts were prepared and subjected to WGA affinity chromatography. WGA-bound and immunoprecipitable Stat molecules were detected by Western blotting (Fig. 1C). O-GlcNAc modification of Stat1, Stat3, Stat5, and Stat6 was found after cytokine treatment.

Glycosylated Stat5 Fractions Are Tyrosine- and Serine-phosphorylated and Are Able to Bind DNA—To explore possible effects of Stat5 glycosylation on DNA binding and phosphorylation, we used purified Stat5a obtained from insect cells infected with recombinant baculoviruses. We have previously shown that tyrosine phosphorylated, activated Stat5a can be obtained from extracts of SF9 cells simultaneously infected with baculoviruses encoding Stat5 and Jak2 (39). SF9 cells properly carry out post-translational modifications of recombinant proteins such as the tyrosine phosphorylation of Stat5 or the O-GlcNAcylation of human cytomegalovirus basic protein 1 and keratins 8, 13, and 18 (40).

We investigated whether the glycosylation of Stat5 is compatible with phosphorylation of the molecule. For this purpose
we prepared glycosylated and non-glycosylated fractions of Stat5a by WGA affinity chromatography. The WGA-bound fraction was eluted under native conditions with 1M GlcNAc and considered as the glycosylated fraction. The flow-through contained non-glycosylated Stat5. Stat5 was immunoprecipitated from these fractions. Upon electrophoresis and blotting the filters were probed with antisera specific for tyrosine-phosphorylated Stat5, antisera specific for Stat5 phosphorylated at serine 779 or antisera specific for O-GlcNAc (Fig. 2). We observed that glycosylated and non-glycosylated Stat5a isoforms were recognized by phosphotyrosine and phosphoserine 779-specific antibodies (Fig. 2, A and B). The specificity of the antibody directed against phosphoserine 779 was established by Beuvink et al. (9). In addition to the lectin affinity chromatography experiments, immunoprecipitations were performed with an antibody specific for O-GlcNAc (41). Immunoprecipitation of glycosylated Stat5a with the O-GlcNAc specific antibody corroborated the results obtained by lectin purification (compare Fig. 2B, lanes 1 and 3). The signal for serine phosphorylation seems to be stronger in the glycosylated fractions compared with the non-glycosylated Stat5a fraction. The basic activation mechanism is not affected by glycosylation of Stat5a. Reciprocal effects of serine phosphorylation or threonine phosphorylation and glycosylation of Stat5a have not been investigated (11).

Glycosylated and non-glycosylated Stat5a fractions were tested for their ability to specifically interact with the Stat5 response element. DNA binding assays were carried out with the binding site present in the bovine β-casein gene promoter. The biotinylated response element and the interacting proteins were purified with streptavidin beads. Bound Stat5 was visualized by Western blotting (Fig. 2C). Both, glycosylated and non-glycosylated Stat5a bound to the DNA response element (lanes 3 and 4), and no difference between the two isoforms of the molecule could be detected with respect to the DNA binding ability. We assume that the slight difference between signal intensities observed in Fig. 2C, lanes 3 and 4 might be due to non-equal loading in the input lanes (Fig. 2C, lanes 1 and 2).

**Stat5a Glycosylation Can Be Enhanced by Treatment of Cells with PUGN Ac and Glucose/Glucosamine**—We investigated whether Stat5 glycosylation can be affected by treatment of cells with the inhibitor of O-GlcNAcase, PUGN Ac. PUGNAc is a nontoxic compound that causes the accumulation of O-GlcNAc-modified proteins. This increase can be as much as 2-fold.
in certain cell types (29). UDP-GlcNAc and UDP-GalNAc are mainly synthesized from glucose by the hexosamine biosynthetic pathway. Glucose and glucosamine entering the cell can be processed to UDP-GlcNAc. Elevated UDP-GlcNAc levels enhance the glycosylation of proteins by O-GlcNAc transferase (OGT) (42, 26). Treatment with the O-GlcNAcase inhibitor PUGNAc and addition of glucose (Glc) and glucosamine (GlcN) to growth medium has been shown to increase protein O-GlcNAcylation in many cell types.

We treated HC11 cells for 16 h with growth medium supplemented with 5 mM glucose/7 mM glucosamine or 40 mM PUGNAc. Cells were stimulated with the lactogenic hormones DIP for 30 min before harvesting. Nuclear extracts were prepared, and protein glycosylation was examined after gel electrophoresis and Western blotting. The RL-2 antibody recognizes O-GlcNAc-modified proteins with the amino acid sequence PV(S/T) (43). The O-GlcNAc modification status of Stat5a was investigated by wheat germ agglutinin affinity chromatography. The Stat5a protein bound to the lectin beads was visualized after elution and Western blotting with a specific Stat5a antibody. After PUGNAc or Glc/GlcN treatment of HC11 cells a 2–3-fold increase in nuclear protein glycosylation was detected in comparison to not treated cells (Fig. 3A). Treatment of the cells with the O-GlcNAcase inhibitor PUGNAc or supplementation of the growth medium with glucose and glucosamine resulted in a more than 10-fold increase in Stat5 O-GlcNAc modification (Fig. 3A, right panel). Comparing PUGNAc and Glc/GlcN treatment, a similar quantitative increase in general protein glycosylation was detected. Growth medium supplemented with Glc/GlcN was used in subsequent experiments.

Stat5 translocates from the cytoplasm to the nucleus upon stimulation with the lactogenic hormones prolactin, dexamethasone, and insulin. A biochemical analysis of the cytoplasmic and nuclear localization of Stat5a upon lactogenic hormone treatment, shown in Fig. 1C, confirms these findings and allows a semiquantitative determination of this subcellular distribution. The cell line and hormonal treatment are equal in the experiments shown in Fig. 1C and in Fig. 3.

O-GlcNAcylation of Stat5 Does Not Influence Its Nuclear Transport—We tested if O-GlcNAc modification of Stat5a might influence its nuclear translocation. For this purpose HC11 cells were treated with 5 mM glucose and 7 mM glucosamine for 16 h and induced with lactogenic hormones. The increase in protein glycosylation was tested in an aliquot of the
cells with the O-GlcNAc-specific antibody RL-2 as described above. The cells were fixed, and Stat5a was detected with a specific antibody and visualized with rhodamine by immune fluorescence microscopy. The nuclei were stained with DAPI (Fig. 3B). Lactogenic hormone treatment causes the translocation of cytosolic Stat5a to the nucleus in cells grown in regular medium (Fig. 3B, left panel). The hormones had the same effect on Stat5 translocation in cells grown in medium containing glucose and glucosamine (Fig. 3B, right panel). Treatment with glucose and glucosamine increased Stat5a glycosylation, but did not influence hormone-dependent nuclear translocation.

Identification of an O-GlcNAc-modified Peptide in the N-terminal Region of Stat5a by Mass Spectrometry—The comparison of sites of O-GlcNAcylation in different proteins has not yielded a consensus sequence at which the addition of O-GlcNAc occurs (44). To determine the site within the Stat5a molecule we used MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) analysis (34). Recombinant Stat5a was expressed in Sf9 insect cells and purified on an antibody affinity column. The eluate was further fractionated by lectin binding to obtain glycosylated Stat5a. Glycosylated Stat5a was digested with trypsin and analyzed by mass spectrometry. We detected a peptide, representing amino acid positions 87–109, which was modified by an O-GlcNAc residue. The peptide comprises two threonines at positions 92 and 97, which could be O-GlcNAcylation sites (Fig. 4A). We used site-directed mutagenesis to investigate the glycosylation site with regard to the function of Stat5a as a transcriptional inducer. For this purpose, we substituted threonine 92 with an alanine residue. The mutant Stat5a (Stat5a-T92A) was tested for glycosylation, DNA binding ability upon cytokine induction and transactivation potential. Cells were transfected with Stat5a-T92A, induced with prolactin and Stat5 was obtained by immunoaffinity chromatography with a Stat5-specific antibody. Glycosylation of Stat5a was detected with an antibody specific for O-GlcNAc (Fig. 4B). Immunodetection re-
revealed a reduction in the glycosylation of Stat5a-T92A in comparison with wild-type Stat5a (compare Fig. 4B, lane 2 and lane 3). The remaining glycosylation signal observed stems from endogenously expressed Stat5a in this cell line (compare Fig. 4B, upper panel, lanes 1 and 2). Replacement of threonine 92 by alanine is accompanied by a strong reduction in Stat5 glycosylation. The phenotypic consequences of other mutations in the identified peptide, e.g. in threonine 97, are worthwhile pursuing and will be addressed in future experiments.

We also tested if the specific DNA binding ability of Stat5 is affected by the mutation of threonine 92 to alanine. Stat5a or Stat5a-T92A and the prolactin receptor were expressed in COS7 cells, and the cells were induced with prolactin. Equal amounts of whole cell extracts were used in the DNA binding assay. The biotinylated response element with the bound proteins were purified with streptavidin beads. Bound Stat5 was visualized by Western blotting (Fig. 4B). Wild-type Stat5a and Stat5a-T92A did not differ in their binding ability to bind the DNA response element (Fig. 4B, lanes 6 and 7).

We also compared the transactivation potential of wild-type Stat5a and Stat5a-T92A. The Stat5 variants, the prolactin receptor, and a Stat5 responsive luciferase construct were expressed in 293T cells (Fig. 4C). Upon prolactin stimulation, cells transfected with wild-type Stat5a showed an 8-fold induction of luciferase activity (Fig. 4C, lanes 1 and 2). No luciferase activity was observed in cells transfected with Stat5a-T92A upon prolactin stimulation (Fig. 4C, lanes 3 and 4). We also derived Stat5 variants in which small deletions were introduced. Deletion mutants of Stat5a, which lack a short sequence comprising threonine 92 were also not able to induce gene transcription (not shown).

**O-GlcNAc Modification of Stat5 Is Required for the Interaction with the Coactivator of Transcription CBP**—We tested whether O-GlcNAc modification of Stat5 affects protein interactions and examined the interaction of the glucocorticoid receptor, N-Myc-interacting protein (Nmi), the corepressor of transcription N-CoR, and the coactivator of transcription CBP (45, 46).

The GR functions as a ligand-dependent coactivator of Stat5 and can be detected in immunoprecipitates of Stat5. Complex formation between Stat5 and the GR diminishes the glucocorticoid response of a GRE-containing promoter, and enhances Stat5-dependent transcription in a synergistic manner (32). We tested whether the glycosylation status of Stat5a influences the
interaction with GR by coimmunoprecipitation experiments. Whole cell extracts were prepared from 293T cells transfected with a GR expression vector and incubated with recombinant expressed glycosylated or non-glycosylated Stat5a. GR was immunoprecipitated and complexed Stat5a was visualized by immunoblots with a specific antibody against Stat5a (bottom panel). The blots were reprobed with an antibody raised against GR to show equal protein loading (top panel). The negative control (Co) consists of protein extracts with Sepharose beads without antibody to detect unspecific binding of Stat5a to Sepharose beads. Both glycosylated and non-glycosylated Stat5a interacted equally well with the glucocorticoid receptor. B, recombinant glycosylated or non-glycosylated Stat5a, and Stat5a-T92A overexpressed in HeLa cells were incubated with endogenous CBP protein from 293T cells. Specific interactions of CBP with Stat5a were examined in immunoafinity chromatography experiments using a specific antibody raised against CBP. In immunoblots, interaction of individual Stat5a fractions was visualized with an specific antibody against Stat5a (bottom panel). The blots were reprobed with antibody raised against CBP to show equal protein loading (top panel). The negative control (Co) consists of protein extracts with Sepharose beads without antibody to examine unspecific binding of Stat5a. Glycosylated Stat5a protein interacted preferentially with CBP protein. Mutation of the glycosylation site at threonine 92 abolishes Stat5a interaction with CBP and is possibly the reason for the failure of Stat5a-T92A to achieve transactivation upon prolactin induction.

DISCUSSION

O-GlcNAc modification of serine and threonine residues of nuclear and cytoplasmic proteins is a post-translational modification thought to modulate their functional activity (11). Perturbations in the regulation of O-GlcNAc addition have been implicated in the etiology of diabetes type II, cancer, and neurodegenerative diseases (51, 52, 12). Many transcription factors have been shown to be O-GlcNAcylated such as p53, c-Myc, and the SV40 T antigen (53, 54, 16). We could assign a function to the O-linked glycosylation of Stat5 and show that it regulates interaction with the transcriptional coactivator CBP.

We identified the O-GlcNAc modification of the transcription factor Stat5a in its N terminus. This domain of the protein is known to be important for protein interactions and tetramer formation of Stat5 dimers on target gene promoters (55). Glycosylated Stat5a was found to be localized mainly in the nucleus after cytokine stimulation of mammary epithelial cells (56, 57). This implies that Stat5a is glycosylated by the O-GlcNAcase either in the nucleus or after induction on the way to the nucleus. It will be interesting to investigate whether O-GlcNAc transferase is itself hormonally regulated or if Stat5 access to the enzyme is only possible in the nucleus. Whether glycosylation of Stat5a occurs in response to other transcription factors is an open question. Whether glycosylation of Stat5a occurs in response to other Stat5-activating ligands will be answered in future studies.

Modification by O-GlcNAc was also observed with other members of the Stat gene family (Stat1, Stat3, Stat5b, and Stat6). O-Linked glycosylation might be a general feature of Stat activation by cytokines. The activity of other transcription factors such as p53, c-Myc, and Sp1 (17, 58, 16) are also regulated by glycosylation and coactivator interaction.

Glycosylation of Stat5a can be increased by treating HC11 mammary epithelial cells with the O-GlcNAcase inhibitor PUGNAc or by supplementing the growth medium with glucose and glucosamine. High glucose and glucosamine causes increased sugar levels inside the cell and an increase in protein glycosylation. Haltiwanger et al. (29) showed that the O-GlcNAcase inhibitor PUGNAc caused approximately a 2-fold increase in general O-GlcNAc modification in HT29 cells which...
also can be reached by treating cells with high glucose and glucosamine. We have shown that Stat5a glycosylation levels can be increased more than 10-fold. Kreppel and Hart (26) showed dependence of OGT on UDP-GlcNAc levels resulting in increased glycosylation of control peptides.

It has been proposed that O-GlcNAc may play a role in nuclear transport. Early reports showed that the lectin wheat germ agglutinin blocked energy-dependent nuclear transport, suggesting that O-GlcNAc played a role in the transport process. Many nuclear proteins are modified with O-GlcNAc, and O-GlcNAcylated proteins shuttle between cytoplasm and nucleus. This observation led to the hypothesis that O-GlcNAc serves as alternative nuclear localization signal. Monsigny and co-workers (59) showed that the addition of carbohydrates, including O-GlcNAc, to proteins can induce the nuclear transport of heterologous proteins without NLS sequence. This carbohydrate-dependent transport is energy-dependent but does not demand cytosolic factors required by typical nuclear transport mechanisms. Stat proteins, however, also interact with conventional nuclear transport proteins. An interaction of Stat1 with importin-α, one of the subunits of the nucleocytoplasmic transport machinery, has been described. The crucial residues in Stat1 are located within the DNA binding domain (60, 61). Our experiments show that simply increasing Stat5 glycosylation by treatment of cells with high glucose/glucosamine was not sufficient to influence the translocation from the cytoplasm to the nucleus.

O-GlcNAc is also able to influence DNA binding ability of transcription factors like p53. It has been reported that O-GlcNAc modification of the p53 tumor suppressor protein increases its DNA binding activity, presumably by disrupting an intramolecular interaction (16). This observation is different from the situation encountered with Stat5a. Binding to DNA does not seem to be dependent on its glycosylation status.

Prolactin treatment of cells causes activation of Stat5 within a few minutes. This activation does not require ongoing protein synthesis, but tyrosine kinase inhibitors prevent Stat5 activation. Also treatment of activated Stat5 with a tyrosine-specific protein phosphatase in vitro abolished its DNA binding activity (62, 63). The phosphorylation of tyrosine 694 seems to be the crucial switch that causes dimerization of Stat5 monomers, translocation to the nucleus and the ability to bind specific response elements (64, 65). Phosphorylation of Stats on serine residues 725 and 779 seems constitutive in many organs, and hormonal states and mutagenesis of these residues had no effects on prolactin-induced transcription (9, 10, 1). Several groups have documented an apparent reciprocity between O-GlcNAc and O-phosphorylation (13, 66). O-GlcNAcylation occurs at sites on the protein backbone that are similar to those modified by many kinases, and O-GlcNAc is reciprocal with phosphorylation. Thus a given serine/threonine residue may exist in three states: glycosylated, phosphorylated, or unmodified (19). We analyzed the reciprocity of O-GlcNAc modification and phosphorylation of Stat5. Lecitin affinity chromatography and immunoprecipitation experiments showed that tyrosine and serine phosphorylation of Stat5a residues amenable to our investigation are not affected by O-GlcNAc modification.

Transcription complexes comprise a large number of proteins and secondary modifications are thought to regulate the composition of such complexes in different cell types (67). Many proteins modified with O-GlcNAc play key roles in the organization and assembly of the cytoskeleton, including cytokeratins, neurofilaments, talin, vinculin, and synapsin (68–71). Site-specific glycosylation of the transcription factor Sp1 for example blocks its oligomerization in vitro (72). It has also been suggested that O-GlcNAc might play a role in protein degradation, protein expression, and transactivation by Sp1 and estrogen receptor (58, 73, 74). Signal-induced modifications might affect associations with other proteins or regulate intrinsic activities. O-GlcNAc seems to be able to do both. The ubiquitous transcription factor Sp1 is extensively modified by O-GlcNAc. Sp1 becomes hyperglycosylated in response to hyperglycemia or elevated glucosamine levels (75). Increased O-GlcNAc modification of Sp1 prevents its degradation via the proteasome (58). O-GlcNAc modification can also inhibit the interaction of an Sp1 peptide with TATA-binding protein-associated factor (TAF110) or holo-Sp1 (76).

The coactivator of transcription CBP interacts with the transactivation domain of Stat5 at the C terminus. The glucocorticoid receptor interacts with the N terminus of the molecule, as it is also known for the corepressor of transcription N-CoR and Nmi (46, 50). Interestingly, Stat5a glycosylation is required for molecular interaction with the coactivator of transcription CBP in vitro, but not with the glucocorticoid receptor, N-CoR or Nmi. Mutation of threonine 92 results in a reduced Stat5a glycosylation and a transactivation-deficient Stat5a variant. Stat5a-T92A cannot be glycosylated and is unable to induce transcription and to interact with CBP. If the mutation of threonine 92 to alanine influences the structure of the Stat5a N terminus is under investigation.

There is a seeming discrepancy between the mapped interaction site of CBP with Stat5 at the C terminus and the effect observed for the O-GlcNAc modification at the N terminus. An explanation might lie in the occurrence of multiple interaction sites. For the CBP interaction with Stat1 e.g. the transactivation domain at the C terminus is required in addition to a second interaction domain at the N terminus (77). The mapping of Stat5a interaction with CBP has been carried out with bacterially expressed GST fusion proteins, which were probably not modified (50). It is also possible that the interaction of CBP with the transactivation domain of Stat5a might be stabilized by the glycosylated N-terminal domain. Zhu et al. (46) described an association of Nmi with the coiled-coil region of Stat5b in a yeast two-hybrid screen, and demonstrates that Nmi augments Stat-mediated transcription in response to cytokine stimulation by enhancing the association of CBP with Stat5. In our assay system we were not able to detect any difference in the interaction of Nmi with Stat5a dependent on its glycosylation state.

Yang et al. (78) reported that the O-GlcNAc transferase interacts with a histone deacetylase complex by binding to the corepressor mSin3A, demonstrating that OGT and mSin3A cooperatively repress transcription. The authors examined proteins that are modified in their transactivation domain, like the Sp1 activation domain, that is subjected exclusively to O-GlcNAcylation. Here, coexpression of mSin3A and OGT can inhibit both basal and Sp1-driven transcription suggesting a general repression of transcription in a gene-specific manner. A different observation was made by Du et al. (75), the authors showed an induction of plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation in response to hyperglycemia-induced mitochondrial superoxide overproduction. Hyperglycemia increased Sp1 glycosylation 1.7-fold, and in luciferase reporter assays a 3-fold increase in plasminogen activator inhibitor-1 promoter luciferase reporter gene could be observed. It seems that OGT is interacting with mSin3A and is repressing a certain set of genes. This might not be generally true, but restricted to a subset of promoters.

Reversible secondary modifications offer an attractive possibility to regulate complex protein-protein interactions (79). Tyrosine phosphorylation on tyrosine residue 694 of Stat5a is an example for such a crucial modification event. Recently, argi-
nine methylation of Stat1 by PRMT1 has been described to be required for transcriptional activation, for IFN-mediated gene induction and anti-proliferation (80). O-GlcNAc modification raises the possibility of additional control of signaling beyond the binary model (phosphorylation on/off) and complicates the interpretation of functional analyses of phosphorylation (79). Determining how O-GlcNAc fits into existing models of transcription, translation, and signaling, as well as understanding the role of this modification in disease states such as cancer, diabetes, and Alzheimer’s disease, will be of future importance (19).

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