Nuclear Localization of STAT5A Modified with O-Linked N-Acetylglucosamine and Early Involution in the Mammary Gland of Hirosaki Hairless Rat*

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Hirosaki hairless rat (HHR) is a mutant strain spontaneously derived from Sprague-Dawley rats (SDR), and its inheritance is autosomal recessive. In addition to hair loss, female HHRs show involution of the mammary gland at an early stage of lactation. In the present study we investigated the mammary gland development in HHR. Morphological examinations revealed that HHR mammary glands are underdeveloped in virgins and exhibit distended alveoli on day 1 of lactation (L1), followed by involution. Milk secretion was observed on L1 in HHR. Whey acidic protein and other proteins were increased in milk of HHR and heterozygous rats on SDS-polyacrylamide gel electrophoresis. Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling assay revealed apoptosis in HHRs at an early stage of lactation. By Western blotting, signal transducer and activator of transcription (STAT)5A levels in cytoplasmic and nuclear fractions of the mammary glands were not different between HHR and SDR on L1 and L7. Nuclear localization of STAT5A in HHR and SDR was confirmed by immunohistochemistry. Tyr-phosphorylated STAT5A was not detected in HHR but was detected in SDR nuclear fractions. Several proteins modified with O-linked N-acetylgalosamine (O-GlcNAc) were detected in HHR nuclear extract on L1, although not in SDR or heterozygous rats by Western blotting. When HHR nuclear extract was applied to wheat germ agglutinin-agarose, a part of STAT5A was recovered in bound fractions. STAT5A of SDR or heterozygous rat nuclei were not bound to the lectin. Electrophoretic mobility shift assay revealed that STAT5A modified with O-GlcNAc is bound to the STAT5-responsive element. These results indicate that the mammary glands of HHR showed terminal differentiation for a short period, followed immediately by involution. In HHR, STAT5A is modified with O-GlcNAc but is not Tyr-phosphorylated. This type of glycosylation is suggested to be involved in the transient activation of STAT5A in HHR.

Signal transducer and activator of transcription (STAT) 2 proteins are latent cytoplasmic transcription factors that mediate cellular responses.

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2 The abbreviations used are: STAT, signal transducer and activator of transcription; EMSA, electrophoretic mobility shift assay; O-GlcNAc, O-linked N-acetylgalosaminylase; O-GlcNAcase, O-GlcNAc-selective N-acetyl-β-D-glucosaminidase; HHR, Hirosaki hairless rat; L1, day 1 of lactation; L7, day 7 of lactation; RT, reverse transcription; SDR, Sprague-Dawley rat; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling; WAP, whey acidic protein; WGA, wheat germ agglutinin.
loss, female HHR shows involution of the mammary gland at early stage of lactation, and most newborn rats die within a week because of less feeding.

In the present study we investigated the mammary gland development in HHR and found mature differentiation accompanying milk secretion at early stage of lactation followed by subsequent involution. In HHR, STAT5A is modified with O-GlcNAc but is not Tyr-phosphorylated on day 1 of lactation (L1). Furthermore, O-GlcNAcylated STAT5A is localized in nuclei of mammary epithelial cells and binds to the STAT5-responsive element.

EXPERIMENTAL PROCEDURES

Animals—Female HHRs and SDRs maintained in our department were housed in plastic cages in air-conditioned rooms with a 12-h light/dark cycle in the Institute for Animal Experiments of Hirosaki University School of Medicine and had free access to water and food. A minimum of four female rats of individual genotypes, wild (SDR), HHR, and heterozygous, were sacrificed at ages indicated for experiments. This study was carried out in accordance with the Guidelines for Animal Experimentation, Hirosaki University.

Histological Analysis and Immunohistochemistry—For whole mount examination, mammary tissues from rats were fixed on glass slides with acetone overnight and stained with Harris’s hematoxylin according to the method described by Russo et al. (22). For histological examination, mammary tissues were fixed in 10% formaldehyde and embedded in the method described by Russo et al. (22). For histological examination, mammary tissues were fixed in 10% formaldehyde and embedded in paraffin. Tissue sections (4-μm thick) were routinely passed through xylene and a graded alcohol series and stained with hematoxylin and eosin. The area of the lobuloalveolar structures was measured with the NIH image software program in nine microscopic fields at 100-fold magnification. Immunohistochemical analysis for STAT5A was performed by the avidin-biotin-peroxidase complex (ABC) method (23) using anti-STAT5A antibody (L-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Affinity-purified, biotin-labeled goat anti-rabbit immunoglobulin G and the ABC complex (Vectastain ABC kit, PK4001) were obtained from Vector Laboratories Inc. (Burlingame, CA). The sites of peroxidase binding were determined by the diaminobenzidine method. Sections were then lightly counterstained with hematoxylin for microscopic examination.

Western Blotting—Nuclear or cytoplasmic extracts were prepared from rat mammary tissues, as described by Wheeler et al. (24). Proteins of these extracts were separated by SDS-PAGE (25) on 7.5% (w/v) polyacrylamide gels and electroblotted to Hybond nitrocellulose membranes (Amersham Biosciences) (26). Blots were probed with an anti-STAT5A antibody (1:1000, v/v) or an anti-Tyr-phosphorylated STAT5A antibody (catalog number 9351, Cell Signaling Technology, Inc., Beverly, MA) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; v/v; Amersham Biosciences). Signals were generated with an ECL kit (Amersham Biosciences) according to manufacturer’s protocol. Antibody against O-GlcNAc-modified proteins was purchased from Affinity BioReagents, Inc. (Golden, CO).

TUNEL Assay—Cell death was localized in tissue sections by TUNEL analysis (27). Paraffin tissue sections (4 μm) were dewaxed by 60 °C for 30 min and washed with toluene twice for 5 min each. Sections were hydrated through a graded series of ethanol and phosphate-buffered saline and then incubated with 20 μg/ml proteinase K in phosphate-buffered saline for 30 min at 37 °C. Thereafter, an ApopTag Plus peroxidase in situ apoptosis detection kit (Intergen Discovery Products, Purchase, NY) was used for nick-end labeling according to the manufacturer’s instructions. Fluorescein-linked nucleotides incorporated into DNA breaks were observed with a fluorescence microscope (Olympus BX60). Postweaning mammary tissue was included as a positive control.

Prolactin Determination—Rat serum prolactin concentrations were measured with a commercial EIA kit (Wako, Osaka, Japan), according to manufacturer’s protocol.

Lectin Affinity Chromatography—Lectin affinity chromatography of nuclear extract or cytoplasmic extract was carried out with wheat germ agglutinin (WGA)-agarose beads (Wako). Lectin beads, 500 μl of 50% (v/v) suspension in 20 mM Tris–HCl, pH 7.4, and 0.5 mM NaCl were incubated with 500 μl of nuclear or cytoplasmic extracts (500 μg of protein) for 1 h at room temperature. Protein concentrations were determined by Bradford’s method (28). Bound proteins were eluted off of the beads with 1 M N-acetylglucosamine in 20 mM Tris–HCl, pH 7.4, and 0.5 mM NaCl for 10 min at room temperature.

Electrophoretic Mobility Shift Assay (EMSA)—An EMA was carried out essentially as described by Schmitt-Ney et al. (29) and Li and Rosen (30). The double-stranded oligonucleotide of the β-casein promoter, containing the high affinity STAT5A binding site (−75 to −104, 5′-GGAGACTTCTGGGAATTAAGGGACTTTG-3′), was used as a probe. Because the whey acidic protein (WAP) promoter (−740 to −720) did not provide shifted bands, the β-casein promoter was used. Nuclear extracts were incubated with the probe labeled with 32P and then electrophoresed on a 4% nondenaturing polyacrylamide gel. The gel was dried and visualized by autoradiography. For the competition experiment a 10- to 50-fold excess of the unlabeled oligonucleotide was added. For the antibody supershift experiment, anti-STAT5A antibody or nonimmune serum was added to the reaction mixture containing labeled probe and proteins.

Amino Acid Sequencing—Proteins of rat milk were separated by SDS-PAGE on 12.5% (w/v) polyacrylamide gels and electroblotted to a polyvinylidene difluoride membrane. Blots were applied to a protein sequencer (model 492, Applied Biosystems). N-terminal amino acid sequencing was carried out as reported previously (31).

STAT5A cDNA Sequencing—Total RNAs were extracted from the mammary gland on L1 using an RNasey mini kit (Qiagen, Hilden, Germany). Seven primer pairs were designed for reverse transcription (RT) polymerase chain reaction, and RT-PCR was performed with an Access RT-PCR System (Promega). The RT-PCR products were loaded on agarose gel (1%) electrophoresis, and resolved bands were recovered from the gel using the QIAquick gel extraction kit (Qiagen). These were sequenced with a Big Dye terminator kit (PE Biosystem, Foster City, CA) and an ABI DNA sequencer Model 310 (PE Biosystems).

RESULTS

Early Involvement of HHR Mammary Glands in Lactation—Whole mount examination of HHR mammary glands was performed at the virgin stage (up to 12 weeks old) and on the 1st and 7th days of lactation, L1 and L7, as described under “Experimental Procedures.” During the virgin stage, HHR showed underdeveloped mammary glands with smaller alveoli and fewer branching, as compared with the SDR (Fig. 1, A and B). No differences in lobuloalveolar structure were, however, observed between HHR and SDR on L1 (Fig. 1, C and D). HHR glands showed involution and decreased lobuloalveolar structures on L7, whereas SDR maintained well developed structures (Fig. 1, E and F). Heterozygous rats showed normal mammary gland development patterns like those of SDRs (data not shown). Histological examination of HHR mammary glands was also performed on these time points. No differences were observed between HHR and SDR in virgins (Fig. 2, A and B). HHR glands on L1 showed mostly distended alveoli, and glandular acini were more expanded and more
developed as compared with those of SDRs (Fig. 2, C and D). HHR glands on L7 showed involution, whereas SDRs maintained well-developed structures of lactating mammary glands (Fig. 2, A and B). The area of lobuloalveolar structures in HHR on L1 occupied 48% of the mammary gland area, comparable with the value for the mammary gland of SDR (41%). In HHR the value decreased to 18% on L7, although it did not in SDR (Fig. 2G).

Apoptosis Induction in HHR Mammary Glands in Lactation—To examine the early involution of HHR mammary glands, apoptotic cell death was evaluated by the TUNEL assay as described under “Experimental Procedures.” On L1, TUNEL-positive cells occupied 2.5% of alveolar epithelial cells in HHR, although only 0.2% in SDR. Similar results were also obtained on L7 (Fig. 3). Heterozygous rats as well as SDR showed very low TUNEL-positive cells (data not shown). These results indicated that the accelerated involution of HHR mammary gland in lactation was due to apoptosis induction.

WAP Levels in HHR Milk—Although the mammary glands of HHRs were involuted early in lactation and these rats could not raise their pups on L7, the glands showed expanded acini and milk secretion on L1. To examine whether milk protein profiles were different between HHRs and SDRs, milk collected from these rats, including heterozygous rats at L1, were analyzed by SDS-PAGE (Fig. 4). Protein staining revealed three major bands (67, 37, and 28 kDa) in SDR milk, whereas two additional bands (77 and 21 kDa, marked with asterisks in Fig. 4) along with two minor proteins (34 and 32 kDa) in HHR and heterozygous rats. By N-terminal amino acid sequencing, the 67-, 37-, and 28-kDa bands were identified as serum albumin, β-casein, and α-casein, respectively. The 21-kDa protein observed in HHR and heterozygous rats was identified as WAP. The 77-kDa protein could not be identified by this procedure. These results indicate that levels of four proteins including WAP are increased in HHR milk, raising the possibility of enhanced activities of some transcription factors.

Localization of Unphosphorylated STAT5A in Nuclei of HHR Mammary Glands—Because STAT5A is a key transcription factor involved in the development of the mammary gland and WAP gene expression (30), we examined its expression in HHR mammary glands by Western blotting and immunohistochemistry. Protein levels of STAT5A (subunit M, of 94 kDa) in cytoplasmic and nuclear fractions were not different between HHR and SDR on L1 and L7 (Fig. 5, A and B). Because phosphorylation of STAT5A at Tyr-694 is reported to be essential for its nuclear migration and transcriptional activity (2), nuclear fractions were also examined with an antibody against Tyr-phosphorylated STAT5A. The phosphorylated form was not detected in HHR but was detected in SDR (Fig. 5C). Phosphorylated protein was also observed in heterozygous rats on L1 but not on L7. STAT5B was only faintly stained in cytoplasmic fractions of both HHRs and SDRs on L1 and L7 (data not shown). Immunohistochemistry was performed to demonstrate nuclear localization of STAT5A. In virgin rats, STAT5A was demonstrated in nuclei of mammary gland cells of SDRs, although only weakly in HHRs (Fig. 6, A and B). On L1 most nuclei of lobuloalveolar cells were heavily stained for STAT5A in HHRs, whereas some nuclei were weakly stained in SDRs (Fig. 6, C and D). On L7 HHRs lacked lobuloalveolar structures and showed ductal structures. Both nuclei and cytoplasm of ductal cells were heavily stained for STAT5A (Fig. 6E). SDR exhibited distended lobuloalveolar structures, and some nuclei were strongly stained (Fig. 6F). These results indicate that STAT5A is localized in nuclei of mammary gland cells in HHRs as well as SDRs.

Sequence of HHR STAT5A cDNA—The sequencing of whole HHR STAT5A cDNA was carried out as described under “Experimental Procedures.” The deduced amino acid sequence did not differ from the rat STAT5A sequence reported to date (7).
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Serum Concentrations of Prolactin—Because prolactin is known to induce activation of STAT5A in the mammary gland (2), serum levels were assayed in HHRs and SDRs. Prolactin concentrations were the highest at pregnancy (20 days postcoitus), and decreased on L1 in both HHRs and SDRs. The values were not different between HHRs and SDRs at individual time points except on L7 (Fig. 7). These results suggested that the activation of STAT5A in HHRs on L1 was not due to altered prolactin levels.

Modification of STAT5A with O-Linked N-Acetylgalosamine in HHR Mammary Glands—STAT5A was localized in nuclei of HHR mammary gland cells but was not phosphorylated. Recently, it was reported that STAT5A is modified with O-linked GlcNAc for transcriptional activation (32). To examine whether STAT5A in the HHR mammary gland was modified by glycosylation, cytoplasmic and nuclear fractions of HHR and SDR were subjected to WGA affinity chromatography. The flow-through fractions and bound fractions eluted with 1 M GlcNAc were resolved by SDS-PAGE. Protein staining patterns of bound fractions were not significantly different between SDRs and HHRs on L1 and L7 (data not shown). In Western blotting with an antibody recognizing O-GlcNAc-modified proteins, however, several proteins ranging from 97- to 30-kDa were detected in the nuclei of the HHR mammary gland on L1, although not in SDR or heterozygous rats. Such bands were not observed in HHRs on L7 (Fig. 8A). With Western blotting we examined whether bound fractions include STAT5A. The protein was detected in HHR nuclei on L1 (Fig. 8B) but hardly in SDR or heterozygous rat nuclei. Bound fractions of HHR or SDR cytoplasmic fractions did not contain STAT5A (Fig. 8D), whereas unbound fractions did (Fig. 8, C and E).

Binding of O-GlcNAcylated STAT5A to STAT5-responsive Element—Nuclear extracts from HHR and SDR mammary glands on L1 were subjected to EMSA with a probe for STAT5. Although STAT5A levels in nuclear extracts were not different between HHRs and SDRs (Fig. 5B), HHRs showed an increased amount of shifted band as compared with SDRs and heterozygous rats (Fig. 9A). The band was specific for the STAT5-responsive element, because it was lost with increasing amounts of unlabeled probe. To determine whether the band includes STAT5A, EMSA was performed in the presence of the anti-STAT5A antibody. Although supershifted band was not evident, the band was decreased by increasing amounts of the antibody (Fig. 9B). Further-
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**FIGURE 7.** Quantitation of serum prolactin concentrations in HHRs (open circles) and SDRs (closed circles). Sera were collected from HHR and SDR at virgin stage (12 weeks old), day 20 of pregnancy (P20, L1, and L7) and assayed for prolactin as described under "Experimental Procedures."

more, to examine whether O-GlcNAcylated STAT5A binds to the STAT5-responsive element, HHR nuclear extracts bound to WGA-agarose were also subjected to EMSA. Band shift occurred in bound fraction, indicating that O-GlcNAcylated STAT5A binds to the element (Fig. 9C).

**DISCUSSION**

The present study has revealed that HHR mammary glands are underdeveloped in virgin rats and exhibited distended alveoli on L1 but involution on L7 due to apoptosis (Figs. 1–3). In milk obtained from HHR and heterozygous rats some proteins were increased, and one of them was identified as WAP. STAT5A is a transcription factor that regulates mammary gland maturation and expression of milk proteins including WAP (3, 7, 30, 33). Western blotting and immunohistochemical examination have revealed that STAT5A is localized in nuclei of HHR mammary gland cells but is not Tyr-phosphorylated (Fig. 5 and 6). We had considered a possibility that the STAT5A gene of HHR was mutated and that the mutant protein could not be phosphorylated at the Tyr-694 residue. Sequencing of whole HHR STAT5A cDNA obtained by RT-PCR revealed an identity with the STAT5A amino acid sequence reported to date (2), ruling out such a possibility.

O-GlcNAc modification of Ser and Thr residues of nuclear and cytoplasmic proteins is proposed to modulate their functional activity, and O-GlcNAcylated proteins shuttle between cytoplasm and nucleus (34), suggesting that O-GlcNAc serves as a nuclear localization signal. Transcription factors such as STAT5, p53, c-Myc, and SV40 T antigen have been shown to be O-GlcNAcylated (12, 35, 36). Gewinner et al. (32) have reported that O-GlcNAc glycosylation is involved in activation of STAT5A. In the present study, STAT5A was demonstrated to be O-GlcNAcylated, and such modified STAT5A was only detected in nuclei of HHR on L1 (Fig. 8B). Furthermore, O-GlcNAcylated STAT5A was shown to bind to the STAT5-responsive element with an enhanced affinity (Fig. 9). Although we had examined whether the addition of an antibody against O-GlcNAc-modified proteins in the EMSA resulted in alterations in a shifted band, the results were negative (data not shown). Therefore, instead of the antibody, WGA-agarose was used to purify O-GlcNAcylated proteins including STAT5A, and bound fractions were subjected to the assay. This exhibited a shifted band with a similar mobility as the band bound by STAT5A (Fig. 9, B and C). Furthermore, in Western blotting of bound fractions to WGA-agarose with an anti-O-GlcNAc-modified protein antibody, several proteins including a 94-kDa protein were detected in HHR nuclear extract, and the STAT5A band of 94-kDa was also demonstrated (Fig. 8, A and B). These results strongly support the hypothesis that STAT5A is modified with O-Glc-
receptor coactivators SRC-1, and SRC-3, peroxisome proliferator-activated receptor (PPAR)-binding protein (PPB), and PPAR-interacting protein (PRIP) exhibit defective development of the mammary gland (44–47). SRC-1-deficient mice and PRIP-deficient mice show a normal elongation of ducts but decreased number of ductal branches during puberty and decreased numbers and small size of alveoli during pregnancy (44, 47). The PRIP-deficient mice also show decreased numbers of lobuloalveolar during lactation and failed to nurture pups. Like these mutant mice, HHRs show underdeveloped mammary gland during the virgin stage but well developed structure on L1. Expression of these nuclear receptor coactivators was retained in the mammary glands of HHRs (data not shown). Thus, these coactivators are unlikely to be responsible for disorders in mammary gland development of HHRs.

The development of epithelial organs such as the mammary gland and hair depends on a series of sequential interactions between epithelial cells and adjacent mesenchymal cells (48). Overexpression of parathyroid hormone-related protein interferes with hair follicle development (49) and impairs branching morphogenesis during mammary gland development and lobuloalveolar development during pregnancy (50). Although underdevelopment of the mammary gland and hair loss in HHR seem to reflect disorders in epithelial-mesenchymal interactions, enhanced expression of the protein was not observed in these organs by immunohistochemistry (data not shown).

In conclusion, the present study indicated that the mammary glands of HHR show terminal differentiation for a short period at early stage of lactation, immediately followed by involution. In HHR, STAT5A is modified with O-GlcNAc but is not Tyr-phosphorylated on L1. This type of glycosylation is suggested to be involved in the transient activation of STAT5A in HHR.

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