The Stat3/5 Locus Encodes Novel Endoplasmic Reticulum and Helicase-like Proteins That Are Preferentially Expressed in Normal and Neoplastic Mammary Tissue

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The signal transducers and activators of transcription (STAT) 5 and 3 are critical for mammary alveolar development during pregnancy and remodeling during involution. In the mouse, STAT3, STAT5a, and STAT5b are encoded by adjacent genes on chromosome 11 (60.5 cM). To identify additional genes in the Stat3/5 locus that may participate in normal and neoplastic development of the mammary gland, we have cloned and sequenced 500 kb and searched for genes preferentially expressed in mammary tissue. We identified six known genes and cloned two new genes, termed D11Lgp1 and D11Lgp2. Both genes are most highly expressed in normal mammary tissue and mammary tumors from several transgenic mouse models. LGP1 consists of 532 and 530 amino acids in mouse and human, respectively (88% similarity). A region in the carboxy-terminal half of LGP1 has limited homology with Arabidopsis thaliana GH3-like proteins. Immunofluorescence studies demonstrated that LGP1 is located in the nuclear envelope and the endoplasmic reticulum. LGP2 is a cytoplasmic protein of 678 amino acids.

**INTRODUCTION**

With each pregnancy, mammary tissue undergoes a cycle of proliferation, differentiation, and regression [1]. These events are controlled by cytokines and their downstream transcription factors. Stimulation of the prolactin receptor in mammary epithelium during pregnancy results in the activation of the signal transducer and activator of transcription-5 gene (Stat5), which in turn leads to the proliferation and differentiation of alveolar epithelium [2,3]. Upon weaning, mammary epithelial cells undergo apoptosis followed by tissue remodeling. Activated Stat3 seems to control mammary epithelial cell death [4], although the inducing signals remain elusive. In the mouse, Stat3, Stat5a, and Stat5b are next to one another on chromosome 11 at 60.5 cM [5]. Expression of Stat5a and Stat5b is highest in mammary tissue, which correlates with the defects seen in Stat5a-null mice [3].

Mice have been generated in which Stat5a [3] and Stat5b [6], alone or in combination [7], have been inactivated. Because Stat5a/b-null mice are infertile and have developmental problems that interfere with mammary studies, it will be necessary to inactivate this locus specifically in mammary epithelium using tissue-specific recombination. In addition, it is necessary to identify genes in this locus that may contribute to alveolar development and tumorigenesis, and whose expression may be altered through the deletion of the locus. To address these issues, we cloned and sequenced 500 kb from the Stat3/5 locus [5] and searched for genes expressed in mammary tissue and tumors. We identified six known genes. The gene Prf (polymerase I transcription releasing factor) is located upstream of Stat3. The genes Hcr1 (hypocretin/orexin), BEC2 (an ether-a-go-go K+ channel protein), Gcn5l2 (a histone acetyltransferase), Dnajc7 (mouse DnaJ homologue), and Cnp1 (29-, 3'-cyclic-nucleotide 3'-phosphodiesterase) are located downstream of Stat5b [5].

To identify new genes, we have used a combination of EST database searches, GENSCAN tools for exon prediction, and cDNA cloning. We cloned two novel genes, D11Lgp1 (Lgp1) and D11Lgp2 (Lgp2), that were expressed preferentially in mammary tissue and tumors.
RESULTS

Structure and Genes in the Mouse Stat5 Locus

To identify genes in the mouse Stat3/5 locus that are expressed preferentially in mammary tissue and tumors, we cloned and sequenced 500 kb (Fig. 1) and searched the NCBI database using the BLAST algorithm. In addition to Stat3 and Stat5a/b, we identified six known genes and two groups of expressed sequence tags (ESTs), which represented two or more exons of two new genes (Lgp1 and Lgp2). The gene Lgp1 is 4.6 kb, consists of nine exons, and is located 9 kb downstream of Stat5b. Lgp2 spans 9.2 kb and is 76.7 kb downstream of Stat5b. The gene sizes and intergenic sequences are drawn to scale.

Cloning and Characterization of Lgp1 and Lgp2

We used one EST from each group to search the NCBI dbEST database for additional ESTs. Four additional ESTs matched with the Lgp1 sequence and five ESTs matched with the Lgp2 sequence. The full-length cDNAs encoding Lgp1 and Lgp2 were cloned using computational analyses, RT-PCR, and 5’- and 3’-RACE techniques to obtain the full-length cDNA. Open arrows are RACE gene-specific primers. (D) Full-length cDNA obtained using 5’- and 3’-RACE.

FIG. 1. Structure of the locus containing Lgp1 and Lgp2 on mouse chromosome 11. The Stat5b part of the BAC clone spans approximately 171 kb and encompasses four known genes and two new genes. Lgp1 is 4.6 kb and 9.2 kb downstream of Stat5b. Lgp2 spans 9.2 kb and is 76.7 kb downstream of Stat5b. The gene sizes and intergenic sequences are drawn to scale.

FIG. 2. Strategy for isolating the full-length cDNAs for Lgp1 and Lgp2. (A) Exons were identified by EST search. The black solid line stands for genomic DNA, filled rectangles represent exons found by EST search, and the solid arrow 1 is the downstream RT-PCR primer. (B) GENSCAN software was used to predict additional exons in the genomic sequence. Open rectangles correspond to exons predicted by GENSCAN, and solid arrows 2-7 are the upstream RT-PCR primers. PCR was carried out using different combinations of the primers. (C) The PCR products were sequenced and aligned to the cDNA sequence. 5’- and 3’-RACE techniques were used to obtain the full-length cDNA. Open arrows are RACE gene-specific primers. (D) Full-length cDNA obtained using 5’- and 3’-RACE.

FIG. 3. Alignment of similar region among mouse and human LGP1, and an A. thaliana GH3-like protein (GenBank acc. no. AF279129) by CLUSTAL W (1.81) software. We found 29% of the amino acid residues in this region to be identical, and 47% to be conserved.

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human-LGP1
mouse-LGP1
arab-HGH3-like

LWPKQVYRTDGQGQAPRAALWGLCOCIGAAPYAAPGSGCGLMFLQLRPQ--HGLY 312
LWPKQYRTDGQGQAPRAALWGLCOCIGAAPYAAPGSGCGLMFLQLRPQ--HGLY 313
LWPKQYRTDGQGQAPRAALWGLCOCIGAAPYAAPGSGCGLMFLQLRPQ--HGLY 353

LLPDAFPEELPKEKQKEAEK REKQETELTUGMLRCLGDVVVGQ 372
LLEPVEELDPKEKQKEAEK REKQETELTUGMLRCLGDVVVGQ 373
AVPILEPELFELLQGEEQK PVQLTVQKEEYVRVQTVHYAGLYVRQGDDVVGQ 412

human-LGP1
mouse-LGP1
arab-HGH3-like

AVQCPFVRICRILQQVSRGLEDHLRALGRAVQWAGKLHGDVCVESSISDDSE 432
TYMQCFVRICRILQQVSRGLEDHLRALGRAVQWAGKLHGDVCVESSISDDSE 433
TYQAFQVRICRILQQVSRGLEDHLRALGRAVQWAGKLHGDVCVESSISDDSE 472

human-LGP1
mouse-LGP1
arab-HGH3-like

AGASPGRYVGLRHR--LSEENKMOLDCIGVARPSYKKEFFGSGVTVHVGQ 491
EGASPGRYVGLRHR--LSEENKMOLDCIGVARPSYKKEFFGSGVTVHVGQ 492
PG----HYLPMKQGETKNEVQLQCNCLDFRADIAG--TVVRRKWKTCIGALTEVKG 527

human-LGP1
mouse-LGP1
arab-HGH3-like

AFRALIALARZPSPPP--PPMPVRVLR--HRHLAPIQVQVVS------ 530
SGVHALARZPSPPP--PPMPVRVLR--HRHLAPIQVQVVS------ 532

human-LGP1
mouse-LGP1
arab-HGH3-like

TFRQRFHFLGSSAGQ--QFMPVRVFSNAVQLIQCTGENVSSYFSTAF 578

Cloning and characterization of Lgp1 and Lgp2

We used one EST from each group to search the NCBI dbEST database for additional ESTs. Four additional ESTs matched with the Lgp1 sequence and five ESTs matched with the Lgp2 sequence. The full-length cDNAs encoding Lgp1 and Lgp2 were cloned using computational analyses, RT-PCR, and 5’- and 3’-RACE techniques to obtain the full-length cDNA. Open arrows are RACE gene-specific primers. (D) Full-length cDNA obtained using 5’- and 3’-RACE.

The open reading frames (ORFs) in the mouse and human LGP1 mRNAs were predicted to encode proteins of 532 and 530 amino acids, respectively. Mouse and human LGP1 exhibited a 73% identity and an 88% similarity. The amino terminus of LGP1 was rich in leucine residues, suggesting this protein might be a secretory or membrane-associated protein. Database analyses demonstrated a limited homology between LGP1 and an auxin-responsive GH3-like protein from Arabidopsis thaliana (29% identity and 47% similarity over a stretch of 253 amino acids; Fig. 3).
The ORFs in the mouse and human LGP2 mRNAs suggested a protein of 678 amino acid residues in both species. The identity and similarity of amino acid residues between mouse and human was 79% and 94%, respectively. We identified two conserved domains, DEAD/H and a helicase carboxy-terminal domain. The DEAD/H box domain (amino acids 2–170) in LGP2 showed a similarity of 49% with the DEAD-like helicases superfamily. A 70% similarity (amino acids 415–475) was seen with the helicase superfamily C-terminal domain. Further, there was a conserved ATP binding motif (PTGAGKT), an ATPase motif (DECH), and an unwinding motif (TAS) located within the DEAD/H domain. An RNA binding motif (QARGRARA) was found within the helicase C-terminal domain. A human RNA helicase gene, RAI1 (retinoic acid-induced gene; acc. no. AF038963), showed similarity to mouse and human LGP2.

Tissue Distribution of Mouse Lgp1 and Lgp2 mRNAs

We identified Lgp1 and Lgp2 ESTs in several cDNA libraries of mammary tumors from transgenic mice (Fig. 4C). The expression of Lgp1 and Lgp2 in mammary tissue during normal development and in several mouse mammary models was determined by northern blot analysis (Fig. 4A). High levels of Lgp1 mRNA were found in mammary tissue from mature virgins and at day 13 of pregnancy, and lower levels during lactation (Fig. 4A). The pattern of Lgp2 expression was different: mRNA levels were highest during pregnancy and low in the virgin and lactating mammary gland. Lgp1 and Lgp2 expression in liver was lower than in mammary tissue. We further analyzed Lgp1 and Lgp2 expression in mammary tumors from four different transgenic mouse models and in one ovarian tumor. The WAP-TAg [8,9] and WAP-int3 [10] mice express the SV40-T antigen and the int3/notch4 antigen, respectively, under control of the WAP gene promoter. The MMTV-neu [11] and MMTV-PyV-MT [12] mice express ErbB2 (avian erythroblastosis oncogene B-2) and the PyV-MT (polyoma virus middle T antigen) gene under control of the MMTV-LTR. Expression of Lgp1 and Lgp2 was detected in all tumors at levels similar to or higher than that in normal mammary tissue. However, their patterns were different (Fig. 4A). Notably, expression of Lgp2 was lower in the ovarian tumor.

To analyze whether Lgp1 was expressed in tissues other than the mammary gland, we probed additional northern blots and searched all available EST databases. Membranes containing poly(A)+ RNA from 10 mouse tissues were hybridized with an Lgp1 cDNA probe, which detected the expected RNA species of 2.2 kb (Fig. 4B). Lgp1 mRNA levels were highest in liver. Greatly reduced levels were detected in kidney, heart, and brain. The Lgp2 cDNA hybridized with an RNA of 2.4 kb, and expression was detected in testis, heart, spleen, and lung (Fig. 4B). The results from mouse EST database searches confirmed this tissue distribution. Out of 1,955,169 mouse EST sequences, Lgp1 sequences were detected in liver, kidney, embryo, skin, and mammary gland tumors (Fig. 4C). Lgp2 sequences were detected in liver, spleen, testis, embryo, mammary gland tumors, and normal mammary gland tissue (Fig. 4C).
Localization of Mouse LGP1 and LGP2 within Cells
To identify whether the predicted open reading frames in Lgp1 and Lgp2 mRNA encoded proteins and to determine their localization in the cell, we cloned the respective cDNAs into mammalian expression vectors and analyzed the recombinant proteins. A myc tag was fused to the C termini of LGP1 and LGP2, and antibodies against the myc tag were used to identify the two proteins. HeLa cells were transfected with the LGP1 and LGP2 expression vectors and the presence of the respective proteins was analyzed by immunohistochemistry and western blots. Based on confocal microscopy, LGP1 was located in the endoplasmic reticulum (ER) and LGP2 was evenly distributed in the cytoplasm (Figs. 5A and 5B). We also generated antibodies against mouse LGP1, which recognized LGP1 in transfected HeLa cells (Fig. 5E). We detected the endogenous protein in the mammary epithelial cell line HC11 using immunohistochemistry. Although the signal was rather weak, LGP1 was located in the ER (data not shown). Medium and cell lysates were collected 48 hours after transfection and analyzed by immunoblot. The concentrated medium was immunoprecipitated with anti-myc antibodies, followed by immunoblot analysis to identify whether the recombinant proteins were secreted into the medium (Fig. 5C). Recombinant LGP1 and LGP2 of the predicted sizes were detected in cell lysates, but not in the medium. LGP1 appeared as two bands on Tris-glycine gels; the upper band was more pronounced after 48 hours, suggesting that LGP1 may undergo posttranslational modifications. After 48 hours, LGP1 levels had decreased sharply compared with LGP2 at the same time point (Fig. 5D). This suggested that overexpression of LGP1 might be toxic. Alternatively, LGP1 might be less stable than LGP2.

Immunohistochemical analyses demonstrated that LGP1 was located in the nuclear envelope, the ER, and to some extent in circular structures adjacent to the nuclear envelope. To verify that these structures were part of the ER or nuclear envelope, we co-stained with antibodies against the myc tag and calreticulin (a protein of the ER; Fig. 6). The bright circular structures were seen in anti-myc and anti-calreticulin staining at 12 hours and 24 hours after transfection, but very few were seen at 6 hours or later than 48 hours (data not shown). In merged images, the circular structures appeared yellow, suggesting that LGP1 is located in the ER. These experiments also demonstrated that the ER had undergone proliferation, a phenomenon frequently observed in cells where large amounts of protein are expressed, such as HMG-CoA reductase [13]. In our experiment, the proliferation of the ER may have accommodated the overexpression of LGP1.

**DISCUSSION**

Here we report the identification and characterization of a novel nuclear envelope/endoplasmic reticulum-resident protein (LGP1) and a cytoplasmic protein (LGP2) that are preferentially expressed in normal and neoplastic mammary tissue. The respective genes are located in the Stat5 locus, which is involved in mammary development.

There are two lines of evidence that suggest LGP1 is an ER resident protein: it possesses a hydrophobic N terminus and immunofluorescence studies demonstrated subcellular localization of myc-tagged LGP1 in the ER. This observation is supported by co-staining for the ER protein calreticulin. We hypothesize that LGP1 is a new type of ER resident protein that lacks homology to any of the known proteins resident in the ER. Based on the hydrophobic N terminus, it is possible that LGP1 is a secreted protein. However, we were unable to detect it in the supernatant of cells. A region in the
C-terminal part of LGP1 resembles GH3-like proteins. The GH3 protein was first identified in soybeans as an early auxin-responsive gene [14]. Auxins are a class of plant hormones that affect a wide range of growth and developmental processes [15]. GH3 is one of the most specifically auxin-regulated genes [16]. In A. thaliana, the family of GH3-like proteins shares 25–29% identity with LGP1 over a stretch 280 amino acids. The functions of GH3 proteins are not known, and no mammalian homologues have been described.

LGP2 is a cytoplasmic protein that shares similarities with members of the DEAD/H box family of proteins. Two conserved regions, the DEAD and helicase C-terminal domain, were identified in LGP2. RNA helicases exhibit several functions including translational initiation, ribosome biogenesis, nuclear mRNA export, RNA degradation, and nuclear as well as mitochondrial RNA processing. Other proteins of this family have been implicated in cell growth, division, and differentiation [17]. Therefore, RNA helicases represent key elements in the regulation of different cellular processes. Except for the conserved functional domains, sequences vary greatly between the RNA helicases. The biological function of LGP2 is unknown.

Expression of Lgp1 and Lgp2 was highest in normal mammary tissue and mammary tumors from several transgenic mouse models. Lgp1 mRNA levels were similar in mammary tissue from virgin and pregnant mice, suggesting that the gene is not under the control of lactogenic hormones. Together with the reduced expression during lactation, this suggests that Lgp1 is expressed in the stromal and epithelial compartments. High levels of Lgp1 mRNA were detected in mammary tumors from four different transgenic mouse models, suggesting that it serves as a marker for transformed mammary epithelium. The pattern of Lgp2 expression was different from that of Lgp1 with maximal mRNA levels during pregnancy and low in the virgin and lactating state. Such expression profiles are different from those observed with genes under lactogenic control and therefore point to potentially new control mechanisms. Although Lgp2 mRNA was found in every mammary tumor tested, its pattern of expression was distinct from that of Lgp1. This suggests that Lgp1 and Lgp2 are characteristic of specific cell types or stages of tumor progression. Like Stat5, Lgp1 and Lgp2 are preferentially expressed in mammary tissue. This raises the possibility that the promoters of these genes in the Stat5 cluster are under coordinate control. Furthermore, it is possible that Lgp1 has a role in normal [3,18] and neoplastic [19] mammary development.

**MATERIALS AND METHODS**

Cloning of Lgp1 and Lgp2 cDNA. We cloned and sequenced two BACs from the mouse Stat3/5 locus that spanned 500 kb in genomic sequence, and searched the NCBI database with this sequence using the BLAST algorithm. Six additional known genes and two groups of expressed sequence tags (ESTs), which represented two or more exons of two new genes (D11LGPI, GenBank acc. nos. AF316996 and AF316998; D11LGPI, GenBank acc. nos. AF316999 and AF317000), were identified. One EST from each group (Lgp1, AW763596 from mammary gland tumor NCI_CGAP_Mam3; Lgp2, AW414261 from mammary gland tumor NCI_CGAP_Mam3) were used to search the NCBI dbEST database for additional ESTs. Using the GENSCAN program, the putative coding exons of Lgp1 and Lgp2 were predicted. To clone the full-length cDNA, oligonucleotides from the predicted exons were designed, RT-PCR was carried out using total RNA from mouse liver and virgin mammary tissue, followed by
sequence analyses. The RT-PCR fragments were aligned in using the Sequencher 4.0.5 program. The 5’ and 3’ ends of the full-length cDNAs were isolated using the GeneRacer kit (Advanced RACE Method for Amplification of Full-Length cDNA Ends, Invitrogen, L1500-01).

**RNA analyses.** Mouse multiple tissue northern blots containing 2 μg of poly (A)+ RNA from ten tissues were purchased (Clontech). Mammary tissue northern blots were prepared with 40 μg total RNA from six mouse tumors (MMTV-neu, WAP-Int3, MMTV-Py-MT, and WAP-Tag), normal mouse mammary tissue (virgin 10 weeks, pregnant day 13, lactation day 3), and mouse liver. PCR fragments were used as hybridization probes. The Lgp1 probe was generated with primers 5’-ATCCTCACCAGCACAACACC-3’ and 5’-CAGATG-GACTTTGAGGACAG-3’ and spanned 1.2 kb. The LGP2 probe was generated with primers 5’-AGGGTGTATAGTGACCGAG-3’ and ‘GATGCCGTCATTGAGCACA-3’ and spanned 1 kb. The probes for northern blot analyses were labeled with [α-32P] using the Prime-it II random priming kit (Stratagene), and diluted in Express Hybridization Solution (Clontech). Blots were hybridized at 65°C overnight and washed according to the manufacturer’s instructions.

**Construction of expression vectors.** The full-length coding regions of Lgp1 with EcoRI/XhoI overhangs and Lgp2 with NotI/Apal overhangs were generated using PCR catalyzed by Platinum Taq HIFI (Gibco BRL). The Lgp1 cDNA was constructed with primers 5’-GGAACTCATGCTCTGGGCTGCTG-3’ and 5’-GCTCTAGAGGATCCCTCTCTGGAG-3’. The Lgp2 cDNA was constructed with primers 5’-AGGGTGTATAGTGACCGAG-3’ and 5’-ACCGGGCGGGGCGGAGGACGAGAGACGAC-3’. The Lgp1 and Lgp2 cDNAs were cloned into pcDNA3/Myc/His mammalian expression vector (Invitrogen) using EcoRI/XhoI restriction sites for Lgp1 and NotI/Apal for Lgp2. Myc and His epitope tags were fused in-frame at the C terminus of Lgp1 and Lgp2. The integrity of the constructs was confirmed by DNA sequencing.

**Transient expression of Lgp1 and Lgp2 in mammalian cells.** HEK cells were grown and maintained in DMEM medium, supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), 100 units/ml penicillin, and 100 μg/ml streptomycin. We seeded 2 to 3 × 10^6 cells into individual wells of a six-well plate 24 h before the transfection. Plasmid DNA for transfection was prepared using the Qiagen maxi-prep protocol. FuGENE6 transfection Reagent (3 μl; Roche Molecular Biochemicals) and 1 μg plasmid DNA were incubated for 30 min at room temperature in 100 μl serum-free medium. Cells at 60-80% confluency were transfected with the FuGENE6-DNA complexes in 1 ml serum-free medium for 5-6 h at 37°C, and then 1 ml medium with 10% (v/v) fetal bovine serum was added. Transfected cells were cultured at 37°C (5% CO2). Cells and medium were harvested 6, 12, 24, and 48 h after the start of transfection. Antibodies. A peptide specific for mouse LGP1 (ELGLRLINSEENRDLKD) was conjugated to keyhole limpet hemocyanin and used for immunization of rabbits. The specificity of the antiserum was analyzed by western blots using protein extract of HEK cells transfected with the LGP1 expression vector. Antibody against the myc tag was purchased (Invitrogen).

**Immunoprecipitation and immunoblotting.** Medium (3 ml) was collected by Centrifuge-10 columns (Amicon) to 500 μl, and 2 μg anti-myc antibody was added and incubated with rotation for 1 h at 4°C. Immune complexes were sedimented with 60 μl immobilized Protein A beads (Sigma) overnight at 4°C. The beads were washed three times in 1× lysis buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP-40, 2 mM EDTA, 10 mM NaF, 50 μg/ml PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM sodium orthovanadate), resuspended in 100 μl 2× loading buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.06% bromophenol blue), boiled for 5 min, and centrifuged briefly. Transfected HeLa cells were washed in ice-cold PBS and scraped into 1 ml PBS, centrifuged briefly, and resuspended in 100 μl 2× lysis buffer, rotated for 1 h at 4°C, and centrifuged at 13,000g for 10 min at 4°C to remove insoluble debris. Protein (25 μg) from cell lysates and protein (40 μl) from medium were loaded to precasted 8% Tris-Glycine gels, and proteins were transferred to PVDF membranes as instructed by the manufacturer (Novex).

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**REFERENCES**


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