Amplification, Expression, and Steroid Regulation of the Preprogalanin Gene in Human Breast Cancer

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Abstract

The GALN gene encodes the preprogalanin protein that is cleaved to liberate the galanin peptide, a neuropeptide and tumor cell mitogen, and the galanin message-associated peptide, which is of unknown function. GALN is located at chromosome 11q13, a frequently amplified locus in diverse tumor types including breast cancer. To determine whether GALN may contribute to the tumor phenotype resulting from 11q13 amplification, we examined GALN amplification and preprogalanin mRNA levels in breast tumors and cell lines. GALN was amplified in a subset of breast tumors and cell lines that carried 11q13 amplifications. Preprogalanin mRNA was expressed in the majority of breast cancer cell lines, but Northern analysis failed to demonstrate a relationship between GALN amplification and preprogalanin mRNA levels. Eight of eight estrogen receptor-positive cell lines expressed the preprogalanin message-associated peptide, which is of unknown function, but preprogalanin mRNA is expressed by breast cancer cells and is under steroid hormone control in estrogen receptor-positive cells, opening the wider question of the role of this steroid-regulated neuropeptide in the normal and cancerous breast.

Introduction

The amplification of band q13 of human chromosome 11 occurs in tumors of many human tissues and is of prognostic significance, especially in breast cancer, in which it may predict early relapse in patients with features of good prognosis (1–13). Amplifications at 11q13 in breast cancer comprise multiple amplicons that are highly variable in size (14–18). The genes within these amplicons have been the subject of intense investigation and are known to include CCND1 (encoding cyclin D1), INT-2 (encoding FGF-3), and EMS1 (encoding the cytoskeletal protein cortacin; Refs. 6 and 19). GALN (the gene encoding preprogalanin) is also located at 11q13 (20). Human galanin is a 30-amino acid nonamidated peptide (21) with potent mitogenic activity in small cell lung carcinoma cells (22–24). In normal physiology, galanin has reproductive, neuromodulatory, and endocrine roles (25, 26). The localization of the galanin gene to 11q13 and its mitogenic activity suggest that galanin could contribute to the phenotype conferred by 11q13 amplification, prompting this investigation of GALN amplification and preprogalanin mRNA expression and regulation in 20 human breast cancer cell lines and a panel of primary human breast carcinomas selected for 11q13 amplification.

Materials and Methods

cDNA Probes. Preprogalanin cDNA covered the entire galanin and galanin message-associated peptide coding region (22). Cyclin D1 cDNA covered the entire coding region and portions of the 5' and 3' untranslated regions and was provided by Drs. Yue Xiong and David Beach (Cold Spring Harbor, NY). FGF-3 cDNA was a 1-kb Scal fragment of human genomic DNA designated S66 (27), and the CD3 probe was a 0.8-kb EcoRI fragment from the human CD3 γ chain cDNA (28). Progesterone receptor cDNA covered the DNA binding domain and part of the A/B domain (29). The ribosomal 18S oligonucleotide was complementary to bases 151–180 of the rat sequence (30).

Cell Lines and Tissue Culture. Breast cancer cells were from the E. G. and G. Mason Research Institute (Worcester, MA; HBL-100, MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-330, and T-47D) and the American Type Culture Collection (Rockville, MD; all others) and were passaged as described previously (31) in RPMI 1640 supplemented with 5% FCS (RPMI 1640 s). Steroids were added in ethanol at 1000× final concentration.

Primary Breast Tumors. The breast tumor DNA samples analyzed in this study were prepared from patients who presented with infiltrating breast carcinoma at the Guy’s Hospital Clinical Oncology Unit between 1986 and 1988. The clinicopathological features have been described in an earlier study (32). Tumors were selected on the basis of INT-2 amplification (32).

Southern and Northern Analysis. For Southern and Northern analysis, 1 × 10^6 exponentially growing cells were plated in 50 ml of RPMI 1640 s and 5% FCS/150-cm² flask and grown to 1–2 × 10^7 cells/flask before harvest and analysis (33). Three separate preparations of DNA and RNA were analyzed. The gene copy number was calculated by correcting for the progesterone receptor signal (to control for multiple chromosome 11 copies) and normalizing against the signal generated by DNA extracted from peripheral blood lymphocytes. Northern blot signal intensities were corrected for loading by hybridization with the 18S oligonucleotide and normalized against the signal for T-47D cells. Both Southern and Northern analysis data are expressed as the mean normalized signal intensity of three determinations ± the SE.

Results

To examine whether GALN is located within the amplified regions at 11q13, Southern blots of DNA from tumors selected for 11q13 amplification were hybridized with preprogalanin cDNA. A representative blot is shown in Fig. 1. Of the 25 tumors showing amplification at 11q13 using FGF-3 cDNA, 8 also showed amplification of GALN using preprogalanin cDNA. This analysis was extended to 20 breast cancer cell lines, where Southern analysis of 3 separate DNA preparations identified 3 cell lines (MDA-MB-134, MDA-MB-175, and ZR-75-1) with GALN copy numbers greater than 2 (Fig. 2, bottom panel). The Southern blots were rehybridized with cyclin D1 cDNA.
and four cell lines were identified (MDA-MB-134, MDA-MB-361, MDA-MB-175, and ZR-75–1) with CCND1 gene copy numbers greater than 2 (Fig. 2). Comparison of GALN and CCND1 amplification showed that three of these cell lines harbored amplifications of both genes (Fig. 2). These three cell lines also carried EMS1 amplification (Ref. 13; Fig. 2). None of the cell lines that showed EMS1 amplification in the absence of CCND1 amplification (13) also showed GALN amplification.

Northern blot analysis was used to determine whether GALN amplification resulted in altered preprogalanin mRNA levels (Fig. 2, top panel). Preprogalanin mRNA was detected in the human breast cancer cells as a major transcript of 0.9 kb, estimated relative to the ribosomal subunits, and two minor transcripts of 5.8 and 3.6 kb, which were seen on either side of the 28S ribosomal subunit (Fig. 2, top panel). Preprogalanin mRNA was detected in the human breast tumors carrying GALN amplification by Southern analysis. A representative Southern blot is shown. Blood DNA is loaded as a control, and the CD3 loading control is also indicated. Top panel, Southern blot hybridized with FGF-3 cDNA, which produces three BamHI bands at 8.4, 5.6, and 2.8 kb. Tumors carrying INT-2 amplifications are indicated with an A. Bottom panel, rehybridization of the same Southern blot with preprogalanin cDNA. Tumors carrying GALN amplification are indicated with an A.

The effect of estrogen addition was studied in serum-free medium (Fig. 3, top panel). Preprogalanin mRNA levels fell by 50% in response to serum withdrawal for 24 h, indicating that serum factors were responsible for the maintenance of basal levels of expression. The addition of estrogen at time 0 prevented this fall in the preprogalanin mRNA level and resulted in an increase in preprogalanin mRNA of 60% over basal levels and 200% over the time-matched control after 24 h.

The contribution of estrogen to the ability of serum to maintain basal preprogalanin mRNA levels was examined in more detail by the addition of antiestrogens hydroxytamoxifen and ICI 164384 to cultures of T-47D cells in media containing 5% normal serum. (Fig. 3, bottom panel). In the presence of normal serum, estradiol increased the preprogalanin mRNA levels by 30%. Both antiestrogens reduced the preprogalanin gene expression in a concentration-dependent manner to ~50% of control levels at antiestrogen concentrations in excess of 10 nM. The addition of a 10-fold excess of estradiol to cells simultaneously treated with 100 nM of either antiestrogen resulted in a complete reversal of the antiestrogen effect.

The ability of other steroid hormones to regulate preprogalanin mRNA expression was also examined in the presence of 5% normal serum. In MCF-7 cells, the progestin ORG2058 (10 nM) increased preprogalanin mRNA levels 2-fold after a 24-h exposure, whereas the androgen dihydrotestosterone (10 nM) and the glucocorticoid Dex (100 nM) were without effect (Fig. 4, inset). The progestin effect was studied further in the highly progestin-sensitive T-47D cell line (Fig. 4). ORG2058 provoked a time-dependent increase in preprogalanin mRNA.

These results show that GALN is amplified in a subset of breast cancers that carry 11q13 amplifications. Breast cancer cells expressed the GALN gene, but GALN amplification did not lead to increased preprogalanin mRNA levels; rather, preprogalanin mRNA levels are under steroid hormone control.
Fig. 2. Comparison of GALN amplification and preprogalanin mRNA levels in human breast cancer cell lines. Top panel, Northern analysis of 20 breast cancer cell lines using preprogalanin cDNA. Results from three separate total RNA preparations were corrected for loading using hybridization with the 18S oligonucleotide and normalized against the T-47D signal. Error bars, SE. ER status is indicated (+, positive; −, negative), and cell lines are arranged in descending ER level from left to right. Inset, Northern blot showing preprogalanin mRNA. Arrows indicate the position of the 18S and 28S ribosomal subunits. Bottom panel, Southern analysis of 20 breast cancer cell lines using preprogalanin cDNA. The GALN gene copy number was calculated by correcting for progesterone receptor signal and normalizing against the signal from peripheral blood lymphocytes. Error bars, SE. The gene copy number for EMS1 (13) and CCND1 is given where amplified.

Discussion

The amplifications at 11q13 are complex. In a single breast tumor sample, up to four different independently amplified regions at 11q13 have been found (15). Amplicons at 11q13 can span all four regions, just a single region, or two or more discrete regions. In addition, the number of times that a region is amplified within a large spanning amplicon can vary (15). Of the genes now known to reside within these amplicons, CCND1 and EMS1 have emerged as likely candidates driving the emergence of cell populations with 11q13 amplifications. Given the complexity of 11q13 amplification, it is probable that other genes remain to be identified; however, the absence of an increase in preprogalanin mRNA in cell lines with GALN amplification makes GALN an unlikely 11q13 candidate oncogene in breast cancer.

An answer to the question of why GALN amplification does not increase preprogalanin mRNA levels is provided by the observation of estrogen and progestin regulation of preprogalanin gene expression. Similar observations have been made in rats, in which estrogen treatment results in a rapid time- and dose-dependent increase in pituitary preprogalanin mRNA (35, 36). Progestin in this system is thought to blunt the estrogen effect (34), but the direct induction of preprogalanin mRNA by ORG2058 in human breast cancer cells

Fig. 3. Estrogen and antieslrogen regulation of preprogalanin mRNA levels. Top panel, T-47D cells were grown in medium containing 5% dextran-charcoal-treated FCS, harvested, and plated in medium containing 1% dextran-charcoal-treated FCS, which was changed to serum-free medium 24 h later (0 h) in the presence (•) or absence (□) of 10 nM estradiol. The preprogalanin mRNA level at the indicated times was measured by Northern analysis of 20 µg of total mRNA. Bottom panel, MCF-7 cells were treated with the indicated concentrations of antiestrogens ICI 164384 (■) and hydroxytamoxifen (□) or with 100 nM antiestrogens combined with 1000 nM estradiol (▲, ○) or with 10 nM estradiol alone (▲), all for 24 h in normal serum-containing media before Northern analysis using preprogalanin cDNA and quantification by densitometry. The results are expressed as a percentage of the preprogalanin mRNA level found in time-matched vehicle-treated control cells.
indicates that progesterins exert a novel modulatory role in breast cancer cells. Although DEX has been shown to enhance galanin levels in the rat vas deferens, epididymis, and anterior pituitary (37, 38), no effect was observed in breast cancer cells. Because amplification at 11q13 is associated with ER positivity (5, 9, 10), it is probable that steroidal regulation abrogates the effects of GALN amplification in most steroid-insensitive tumor types, especially small cell lung carcinoma, in which galanin acts as a mitogen (22-24) and 11q amplifications are frequent (39).

The observation that galanin is expressed by many breast cancer cell lines opens the question of what role this neuropeptide may play in the normal physiology of the breast and in breast cancer. In normal physiology, galanin controls the pituitary secretion of prolactin (26, 27, 32), a hormone necessary for mammary development and lactation (40, 41), and one that is mitogenic for human breast cancer cells (42). Galanin has been postulated to influence food consumption, gastrointestinal smooth muscle function, and insulin release (26, 27, 32), crucial parameters involved in the maternal adaptation to lactation (43). Galanin immunoreactivity has been observed in mammalian gland nerve endings located in the stroma surrounding lactating lobules and adjacent to smooth muscle cells of the nipple (44). Thus, secretion of galanin by mammary epithelial cells could allow mammary epithelia to influence a number of factors required for successful lactation.

References


Fig. 4. Regulation of the preprogalanin mRNA level by steroid hormones. T-47D cells were treated with 10 nM ORG2058 (B) or vehicle (C) in medium containing normal 5% FCS for the indicated times before Northern analysis of preprogalanin mRNA and quantification by densitometry. /«.ve/, MCF-7 cells were treated with vehicle (CON), the progestin ORG2058 (10 nM), the androgen dihydrotestosterone (10 nM), or the glucocorticoid DEX (100 nM) before Northern analysis using preprogalanin cDNA.


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