Heregulin Induces Expression, ATPase Activity, and Nuclear Localization of G3BP, a Ras Signaling Component, in Human Breast Tumors

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Abstract

We have found using differential display of mRNA that the growth factor heregulin β1 (HRG), a combinatorial ligand for human epidermal growth factor receptors (HERs), induced expression of G3BP, the Ras GTPase-activating protein SH3 domain-binding protein, in breast cancer cells. G3BP is a downstream effector protein of Ras signaling with ATP-dependent RNase and helicase activities, which may link Ras signaling with RNA turnover and cell cycle progression. In human breast cancer cells, HRG induced G3BP mRNA and protein expression. Up-regulation of G3BP was found in MCF7 breast cancer cells overexpressing HER2. G3BP was also overexpressed in human breast tumors in parallel with HER2 overexpression and in an estrogen-independent manner, suggesting a role for G3BP in cancer progression. In addition, HRG stimulation of breast cancer cells promoted phosphorylation of G3BP and increased the association of G3BP with GTPase-activating protein, both of which are essential for G3BP activity. G3BP ATPase activity was also significantly increased by HRG treatment. Furthermore, HRG treatment resulted in G3BP translocation to the nucleus and colocalization with acetylated histone H3, a hallmark of active transcription sites. G3BP induction, phosphorylation, ATPase activity, and relocalization after HRG treatment could all be blocked by pretreatment with the anti-receptor HER2 monoclonal antibody Herceptin (trastuzumab), which may suggest additional applications for this therapeutic antibody. These findings demonstrate for the first time the receptor-dependent regulation of G3BP, a downstream effector of Ras signaling, by HRG, a growth factor with diverse functions in breast cancer cells.

Introduction

Abnormalities in the expression, structure, or activity of growth factors and/or their receptors contribute to the development and pathogenesis of cancers. For example, HER2 is overexpressed and/or amplified in a number of human malignancies, including breast cancer (1, 2). HRG, a combinatorial ligand for HER3 and HER4, is a secretory polypeptide that affects growth stimulation, differentiation, invasiveness, and motility of breast cancer cells (3). In mammary epithelial cells, HRG predominantly uses HER3/HER2 heterodimers to exert its biological effects (4). However, the underlying mechanisms of HRG cytoplasmic signaling leading to gene expression are not well established.

In many eukaryotic cells, the Ras protein represents one of the major signaling components of multiple receptor tyrosine kinase-mediated signal transduction pathways regulating cell proliferation, differentiation, and cytoskeleton changes (5). Signals from receptor tyrosine kinases such as HER2 activate Ras via guanine nucleotide exchange factors, which essentially convert the inactive GDP-bound form of Ras to the active GTP-bound Ras. Activation of Ras stimulates its downstream effectors, including the Raf/extracellular signal-regulated kinase kinase and phosphatidylinositols 3′-kinase that are involved in growth and survival functions (6). Naturally occurring mutations in Ras protein that are associated with the persistence of GTP-bound Ras lead to nonattenuation of growth signals in the absence of receptor-generated signals and eventually to oncogenic transformation (6). Ras GAP inactivates Ras by promoting the conversion of the active GTP-bound Ras to the inactive GDP-bound Ras (7). Therefore, GAP is thought to act as a physiological negative regulator of Ras signaling. In growth factor-stimulated cells, GAP is phosphorylated and interacts with cytoplasmic proteins and phosphorylated receptor tyrosine kinases. Furthermore, the SH3 domain of GAP has been shown to be essential for Ras signaling (8). GAP also promotes stress fiber formation by stimulating Rho activity, and blockage of the SH3 domain of GAP by a monoclonal antibody inhibits growth factor-triggered Rho-dependent cytoskeleton changes and cell survival (9, 10). Thus GAP is a critical effector of Ras.

Recent studies have suggested a role of G3BP, the GAP SH3 domain-binding protein, in the Ras signal transduction pathway. G3BP can interact efficiently with GAP in exponentially growing cells with activated Ras (11). G3BP is a ubiquitously expressed 68,000 protein with several unusual motifs. The COOH-terminal region of G3BP contains two ribonuclease protein motifs that are characteristic of RNA-binding proteins (12) and is thus implicated in RNA processing. Accordingly, G3BP also possesses phosphorylation-dependent RNase activity (11) and has been implicated in cleavage of the 3′ untranslated region of c-myc mRNA stimulating c-myc mRNA turnover. The integrity of the RNA-binding domain of G3BP has been shown to be essential for its function to promote the S phase of the cell cycle (13). In addition, G3BP has been shown to be identical to human DNA helicase VIII, an enzyme with a role in DNA replication, repair, and recombination (14), that was cloned in a different context. Recently, G3BP has been found to be up-regulated in proliferating retinal pigment epithelial cells (15) and overexpressed in human tumor cell lines and tumors (13). Together, current evidence suggests that G3BP may serve as an important downstream effector of Ras signaling and potentially influence RNA metabolism and DNA replication in a Ras-GTP-dependent manner. Very little is known, however, about potential growth factor regulation of G3BP in human malignancies. This report presents for the first time data that G3BP expression and phosphorylation, GAP interaction, and its nuclear functions are positively regulated by HRG, a growth factor with diverse functions in breast cancer cells.

Materials and Methods

Cell Cultures and Reagents. SKBR3 human breast cancer cells (16) were maintained in RPMI 1640 supplemented with 10% FCS. MCF7 and MCF7/HER2 (transformed MCF7 cells that express high levels of the HER2 receptor;
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Ref. 17) breast cancer cells were maintained in DMEM with 10% FCS. The following antibodies were used: (a) anti-G3BP and anti-GAP (Transduction Laboratories, Franklin Lakes, NJ); (b) anti-T7 (Novagen, Milwaukee, WI); (c) anti-HER2 (Neomarkers, Fremont, CA); (d) anti-vinclin (Sigma Chemical Co., St. Louis, MO); (e) anti-acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY); and (f) antimonospecific horseradish peroxidase-conjugate (Amersham, Piscataway, NJ). Alexa 546-labeled goat antirabbit and Alexa 488-labeled goat antimonospecific secondary antibodies and the DNA-intercalating fluorescent dye ToPro3 were purchased from Molecular Probes (Eugene, OR). In all experiments, HRG (Neomarkers) and HCT (Genentech, San Francisco, CA) were used at concentrations of 1 and 10 nM, respectively. Stock chemicals were from Sigma Chemical Co.

Differential Display Analysis. SKBR3 cells were serum-starved for 24 h and then treated with 1 nM HRG for 8 h. Total RNA was isolated using Trizol reagent (Life Technologies, Inc., Rockville, MD). Differential display was performed as described previously (18) using the Delta Differential Display kit. Amplified DNA was separated on an agarose gel, and the bands were purified and cloned into a PCR2.1 vector using the TOPO cloning kit (Invitrogen, Carlsbad, CA). Five to 10 independent clones were isolated, miniprepared, and sequenced at The University of Texas M. D. Anderson Cancer Center Core facility. Sequences were compared with GenBank sequences using a BLAST search program.

Northern Blot Hybridization. Total cytoplasmic RNA was isolated using the Trizol reagent, and 20 μg of RNA were analyzed via Northern hybridization using a 1752-nucleotide cDNA of human G3BP cDNA sequence corresponding to nucleotides 23–1774. rRNA (28 S and 18 S) was used to assess the integrity of the RNA, and the blots were routinely reprobed with human glyceraldehyde-3-phosphate dehydrogenase cDNA for RNA loading and transfer control as described previously (18). In some experiments, cells were treated with 50 μg/ml cycloheximide (a translational inhibitor; Sigma Chemical Co.) or 10 μg/ml actinomycin D (a transcriptional inhibitor; Sigma Chemical Co.) in the presence of HRG treatment (1 nm).

Cell Labeling, Cell Extracts, and Immunoprecipitation. An equal number of cells was metabolically labeled for 4–8 h with 100 μCi/ml [32P]ATP in methionine-free medium containing 2% dialyzed fetal bovine serum in the presence or absence of the indicated treatments. Cells were lysed in radioimmunoprecipitation assay buffer, and the DNA-intercalating fluorescent dye ToPro3 was purchased from Molecular Probes (Eugene, OR). In all experiments, HRG (Neomarkers) and HCT (Genentech, San Francisco, CA) were used at concentrations of 1 and 10 nM, respectively. Stock chemicals were from Sigma Chemical Co.

Results and Discussion

Identification of G3BP as a Growth Factor-regulated Gene. To identify genes whose expression in human breast cancer cells may be modulated by HRG, we prepared total RNA from SKBR3 breast cancer cells with or without HRG treatment. cDNAs were first synthesized using a reverse transcriptase in the presence of [γ-33P]ATP and subjected to PCR. A total of 90 reactions were performed using nine 3’ degenerate oligodeoxynucleotidic acid primers and ten 5’ random primers for each treatment. Analysis of PAGE gels showed amplification of a number of bands ranging from 100 nucleotides to 1800 nucleotides, and a majority of the bands were of equal intensity. Using these bands as internal controls, we analyzed for bands with differences in intensity in the HRG-treated lane. This analysis resulted in the identification of a 1752-bp differential band induced by HRG (Fig. 1A). The differentially expressed gene product was then amplified, cloned, and sequenced. Five clones were sequenced, and all five sequences were identical to G3BP from nucleotides 23–1774 (G3BP, GenBank accession number XM_032733).

Immunofluorescence and Confocal Imaging. SKBR3 cells were plated on glass coverslips in 6-well culture plates. When the cells were approximately 50% confluent, they were serum-starved for 48 h. Separate wells were treated with 1 mM HRG for 0, 30, or 60 min, and then the cells were rinsed in PBS and fixed in cold 100% methanol for 10 min. After fixation, cells were processed for immunofluorescence staining of T7-G3BP and either GAP or acetylated histone H3, and then they were counterstained with ToPro3. Fluorescence labeling was visualized using a Zeiss LSM 510 microscope and a ×40 objective.

Fig. 1. HRG induces G3BP mRNA and protein expression. A, a single 32P-labeled band of approximately 1.8 kb was identified by differential display as up-regulated by treatment of SKBR3 cells with 1 nM HRG for 8 h. B, SKBR3 cells were treated with 1 nM HRG for 8 h, and isolated RNA was analyzed by Northern hybridization using the G3BP cDNA. C, SKBR3 cells were treated with 1 nM HRG for 8 h, and protein lysates were analyzed by SDS-PAGE with a monoclonal antibody against G3BP. D, SKBR3 cells were treated with 1 nM HRG in the presence or absence of actinomycin D or cycloheximide, and the isolated RNAs were analyzed by Northern hybridization.
Working from this initial finding, we performed Northern blot hybridization using the 1.8-kb G3BP cDNA as a probe to determine whether G3BP RNA can be modulated by HRG in tumor cells. HRG treatment of SKBR3 breast cancer cells was accompanied by a significant increase in the G3BP mRNA level (Fig. 1B). G3BP protein levels were also induced after HRG treatment (Fig. 1C). Pretreatment of cells with actinomycin D, an inhibitor of transcription, abolished the HRG-mediated induction of G3BP mRNA expression, suggesting the need for continuous RNA synthesis in the observed increased expression of G3BP mRNA in HRG-treated cells (Fig. 1D). Pretreatment with the translational inhibitor cycloheximide only modestly affected mRNA levels (Fig. 1D). This is the first report of HRG induction of the Ras signaling component G3BP. Previous studies failed to show increased G3BP expression in fibroblast cell lines with stimulation by serum (11, 12) or epidermal growth factor (12). This differential response could be due to differences in the tissues of origin of the cell lines examined or to the differential responsiveness of breast cancer cells to growth factors. However, G3BP has been shown to promote S-phase entry (13) and to be up-regulated in proliferating retinal pigment epithelial cells (15). Thus, our finding of increased levels of G3BP after HRG growth factor stimulation may indicate a role for G3BP in HRG-induced promotion of breast cancer cell growth.

G3BP Expression in Murine Tissues and Human Breast Cancer. To explore the significance of G3BP in human breast cancer progression, we examined G3BP protein expression levels in normal mouse tissues and in human breast tumors. Previous reports have indicated that G3BP is ubiquitously expressed (12). However, when a multiorgan murine tissue protein blot was probed for G3BP expression, high levels of G3BP protein were expressed in the liver, heart, lung, uterus, and testes, but extremely low levels of G3BP protein were expressed in several other organs (Fig. 2A). The significance of this tissue-specific expression remains to be investigated. Because G3BP expression was increased in HER2-overexpressing cells (Fig. 2B) and was highly inducible in breast cancer cells (Fig. 1C), we investigated whether G3BP protein expression was altered in paired normal human breast epithelium and breast carcinoma biopsy samples.
As shown in Fig. 2C, G3BP expression was dramatically increased in eight of eight tumors as compared with the adjacent normal tissue, which showed little or no G3BP expression. These same tumor samples also had elevated levels of the HER2 receptor (Fig. 2C), opening the possibility of growth factor-mediated up-regulation of G3BP in these tumors. Blots were reprobed for vinculin as a loading control. Finally, we examined a possible relationship between tumor ER status and G3BP up-regulation (Fig. 2D). There was no apparent correlation in this sample set. Additional studies using a large number of clinical samples are needed to confirm these findings.

HRG-dependent Phosphorylation of G3BP. G3BP RNase activity is phosphorylation dependent both in vitro and in vivo (11). To determine whether G3BP phosphorylation was altered by HRG, serum-starved SKBR3 cells were metabolically labeled with [32P]orthophosphoric acid for 24 h and then stimulated with HRG for 1 h. Results indicate a large induction of G3BP phosphorylation after HRG treatment (Fig. 3A). This HRG effect was HER2 receptor dependent because pretreatment of cells with the HER2 blocking antibody HCT blocked the HRG-dependent increase in G3BP phosphorylation. Thus, G3BP expression and phosphorylation are increased by HRG. We next sought to determine whether HRG altered G3BP activity.

HRG Stimulated G3BP ATPase Activity. The RNase and helicase activities of G3BP have been shown to be ATP dependent (11, 12). To determine whether HRG regulates the putative functions of G3BP by influencing its ATPase activity, we measured the ATPase activity of immunoprecipitated G3BP at different time points after HRG treatment using total breast mRNA as a substrate for G3BP. Results indicate that G3BP ATPase activity is increased >2-fold after HRG treatment.
6 h of HRG treatment but is not increased after 2 h HRG treatment. (Fig. 3B). New G3BP protein synthesis (Fig. 1C) and subsequent activation may be necessary for a detectable change in G3BP ATPase activity by this assay. Increased ATPase activity was dependent on HER2 signaling and G3BP phosphorylation because pretreatment of cells with HCT was able to suppress the HRG-induced increase. This effect was selective for G3BP because total cellular ATPase activity was not significantly altered by HRG (Fig. 3C). To our knowledge, this is the first demonstration of growth factor-induced activation of ATPase activity associated with a specific cellular protein. Our findings raise the possibility that other functions of G3BP, such as nuclear and cytoplasmic helicase activity (14) and inhibition of the ubiquitin protease USP10 (20), may also be increased after HRG treatment. However, these data clearly demonstrate that HER2 is an integral part of HRG regulation of G3BP, presumably due to the formation of HER3/HER2 heterodimers (4). HRG signaling leads to phosphorylation of G3BP and increased ATPase activity, which may regulate the downstream functions of G3BP, including control of DNA transcriptional processing (14), RNA turnover (11), and protein stability (19).

**HRG Rapidly Redistributes G3BP in Breast Cancer Cells.** The known functions of G3BP, including GAP binding, catalysis of RNA degradation, and helicase activity, are dependent on specific localization of G3BP within subcellular compartments. We next sought to determine the influence of HRG on the subcellular distribution of transfected T7-G3BP. SKBR3 cells were treated with HRG for various lengths of time, and T7-tagged G3BP was localized using immuno-fluorescence labeling and confocal microscopy. In serum-starved control cells, T7-G3BP protein was spread diffusely in the cytoplasm (Fig. 4A). This result is in agreement with previous reports on the normal subcellular distribution of G3BP (12). After 30 min of HRG treatment, G3BP moved to distinct vesicle-like structures in the cell cytoplasm and to distinct bands at the periphery of the cell (Fig. 4B). At this time point, T7-G3BP was colocalized with GAP within these distinct cytoplasmic locations (Fig. 4C), consistent with the previously reported cell fractionation studies (11). However, by 60 min, T7-G3BP had again localized within the cell, becoming once more diffusely spread in the cytoplasm with some perinuclear and cell membrane accumulation. Importantly, T7-G3BP was also localized to distinct nuclear regions, where it colocalized with acetylated histone H3, a marker of active transcription (Fig. 4D). This nuclear localization was selective because G3BP did not colocalize with acetylated histone H4 (data not shown). A nuclear localization and function for G3BP has been implicated previously (14), but the current report is the first to localize G3BP to specific functional regions of the nucleus after growth factor treatment. Pretreatment with HCT effectively blocked both the colocalization of G3BP with GAP (Fig. 4E) and the nuclear movement of G3BP (Fig. 4F), demonstrating that these changes were HER2 dependent. This distinct temporal movement of T7-G3BP within the cell after HRG treatment could imply a rapid activation and/or initiation of cytoplasmic activity of G3BP, followed closely by translocation and function in specific nuclear domains. Both changes could facilitate G3BP promotion of S-phase entry and cell cycle progression after growth factor stimulation (13).

Together, the results presented here have shown that G3BP is a HRG-inducible gene product that is up-regulated in breast tumors. HRG and HER2 signaling modulated G3BP phosphorylation, which is necessary for its RNase (11) and ATPase (the present study) activities.

In addition, HRG also promoted subcellular redistribution and nuclear colocalization of G3BP, a downstream effector of Ras signaling, with acetylated histone H3. In summary, this report presents several unique findings regarding growth factor regulation of the expression, phosphorylation, activity, and nuclear translocation of G3BP.

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**References**


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