Transcriptional Up-Regulation of Paxillin Expression by Heregulin in Human Breast Cancer Cells¹

Ratna Vadlamudi, Liana Adam, Ben Tseng, Louis Costa, and Rakesh Kumar²

Cell Growth Regulation Laboratory, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030 [R. V., L. A., B. T., R. K.]; and Unidade de Oncologia, Hospital de Santa Maria, Lisbon 1500, Portugal [L. C.]

Abstract

Activation of heregulin (HRG) signaling has been implicated in the development of aggressive phenotype in breast cancer cells. The mechanisms through which HRG regulates the progression of breast cancer cells to a more invasive or motile phenotype are currently unknown. Because the process of cell migration must involve dynamic changes in the formation of new focal adhesions at the leading edge and dissolution of preexisting focal points, we explored the potential HRG regulation of paxillin, a major component of focal adhesion. Here, we report that HRG stimulation of noninvasive breast cancer MCF-7 cells resulted in the up-regulation of paxillin mRNA and protein. The observed HRG stimulation of paxillin mRNA expression was completely blocked by actinomycin D (a transcriptional inhibitor) as well as by cycloheximide (a protein synthesis inhibitor), suggesting the involvement of an inducible protein factor(s) and transcriptional regulation of paxillin mRNA by HRG. Extension of these observations to other HRG-responsive human cell lines also demonstrated that HRG has a significant capacity to up-regulate the paxillin expression. Furthermore, the levels of paxillin expression were closely linked with the coexpression of human epidermal growth factor receptor 2 (HER2/HER3 receptors in breast cancer cell lines and in grade III human breast tumors. This study is the first demonstration of regulation of paxillin expression by a polypeptide growth factor, and it suggests a potential role for paxillin in the HER2 pathway in breast cancer.

Introduction

Overexpression of the HER2 (also known as c-erbB2 or c-neu) receptor is associated with increased progression and metastasis, an aggressive clinical course, and decreased disease-free survival in human breast cancer (1). Recently, two additional members, HER3 and HER4, have been added to the HER family. All of these receptors share sequence homology with the tyrosine kinase domain of HER1 (2, 3). The regulation of HER family members is complex: they can be transactivated by heterodimeric interaction between two HER members and, thus, can use multiple pathways to execute their biological functions (4). For example, HER3 and HER4 receptors bind to more than a dozen isoforms of the HRGs (5) and can activate the HER2 receptor as a result of heterodimeric interactions between receptors. It is believed that, among the HER family, the HER2/HER3 dimer complex elicits the most potent mitogenic signal (4). In addition to HER2 overexpression, accumulating evidence suggests that the HRG pathway may be involved in the progression of breast cancer cells to a more invasive phenotype (6, 7). Despite the widely acknowledged role of HER2 in breast cancer, the mechanism through which HRG participates in breast cancer progression remains elusive.

The exposure of cells to growth factors has been shown to cause cytoskeleton reorganization, formation of lamellipodia, and altered cell morphology and, accordingly, has been implicated in stimulating cell migration and invasion (8). The leading edge of a motile cell is composed of thin protrusions of membrane that continuously extend and retract, mediating the initial stage of cell movement and determining the direction of advance. The cell migration also involves a change in cytoskeleton actin stress fibers that end in focal adhesions, which are points of attachment of the plasma membrane to the substratum. The focal adhesion points play an important role in the regulation of cell motility because they involve cycles of formation of cell adhesion and cell spreading by disassembling the components of cell adhesion (9). Increased cell spreading contributes to a increased cell migration and invasiveness.

Growth factor stimulation of cells is accompanied by a rapid increase in tyrosine phosphorylation of focal adhesion proteins, notably paxillin and FAK. The activation of focal adhesion complexes, in turn, initiates a cascade of interactions with other proteins containing SH2/SH3 domains such as pS6, v-Crk, and vinculin (10, 11). Because paxillin has been shown to be phosphorylated in vitro by FAK (12), it is believed to be a major substrate for FAK. Recently, both paxillin and FAK have been shown to be phosphorylated on tyrosine residues by a number of growth factors, including platelet-derived growth factor (13), vascular endothelial growth factor (14), insulin growth factor I (15), hepatocyte growth factor (16), and stem cell factor (17).

HRG has been shown to be involved in the morphogenesis and ductal migration of mammary epithelium cells (18, 19). Ectopic delivery of HRG via implanted pellets has been shown to induce proliferation and differentiation of mammary epithelium (19). Furthermore, targeted expression of a HRG transgene resulted in persistence of terminal end buds and late development of mammary adenocarcinomas, suggesting that HRG may inhibit signals that normally lead to the terminal differentiation (20). Recently, HRG has been shown to promote formation of actin-containing motile structures, motility and invasiveness of breast cancer cells (21), and growth and differentiation of breast cancer cells (22). While studying the early regulation of paxillin phosphorylation by HRG, we noticed that a longer HRG treatment of MCF-7 cells was accompanied by an increase in the steady-state levels of paxillin protein. Because modulation of paxillin expression by growth factors has not been described before, this study was initiated to characterize the HRG regulation of paxillin expression in breast cancer cells. Here, we demonstrate that HRG-stimulated increased cell motility was associated with transcriptional up-regulation of paxillin expression in MCF-7 cells. Furthermore, coexpression of HER2 and HER3 receptors in breast cancer cell lines as well as in grade III breast tumors correlated well with the levels of paxillin. These results suggest that increased paxillin expression may constitute an integral part of HER2 pathway and may be associated with the development and/or maintenance of the motility or invasiveness of breast cancer.

¹ The abbreviations used are: HER, human epidermal growth factor receptor; HRG, heregulin; FAK, focal adhesion kinase; ECL, enhanced chemiluminescence; mAb, monoclonal antibody; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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To whom requests for reprints should be addressed, at Cell Growth Regulation Section (Box 36), The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-3558; Fax: (713) 745-3792; E-mail: rkumar@notes.mdacc.tmc.edu.

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HRG Up-Regulates Paxillin Protein in Breast Cancer Cells. Recently, we have demonstrated that HRG-β1 is a very potent cell motility factor for MCF-7 breast cancer cells (Ref. 21; Fig. 1, A and B). Treatment of MCF-7 cells with HRG for 16 h results in cell spreading, bipolar cell shape, and cell scattering, which are typical characteristics of a motile cell (Fig. 1A, compare bottom and top). While studying the role of paxillin modification in the action of HRG, we observed that HRG treatment of MCF-7 cells led to an enhancement of paxillin expression. Because induction of paxillin expression by a polypeptide factor(s) has not been shown before, we pursued this unexpected finding by characterizing HRG regulation of paxillin expression. MCF-7 cells were stimulated with HRG for various times, and total lysates were analyzed by Western blotting using well-characterized antipaxillin mAbs. As shown in Fig. 1C, HRG had a dual effect on paxillin expression: an early (<1 h treatment) increased phosphorylation, as seen by mobility shift (compare Lane 4 with Lane 2).
to increased synthesis of newly transcribed mRNA and/or enhanced stability of paxillin mRNA. To delineate this possibility, we examined the effect of actinomycin D, an inhibitor of transcription on paxillin mRNA levels. Pretreatment of cells with actinomycin D completely abolished the HRG-mediated induction of paxillin, suggesting that HRG regulates paxillin at transcriptional level. To address the issue of translational regulation, we have used cycloheximide, a translational inhibitor. It was interesting to note that cycloheximide abolished the ability of HRG to induce paxillin mRNA. These findings suggest that a newly synthesized protein(s) is involved in the observed HRG up-regulation of paxillin mRNA. HRG-mediated induction of paxillin mRNA was also blocked by pretreating cells with tyrophostin (a tyrosine kinase inhibitor), suggesting the involvement of HRG-initiated receptor dependent phosphorylation/signaling in the induction of paxillin mRNA (Fig. 2C, Lane 8). In brief, these results implied that HRG regulates paxillin expression at the transcriptional level.

**HRG Regulates Paxillin Expression in Cancer Cell Lines.** To determine whether the observed induction of paxillin is a restricted effect of HRG in MCF-7 cells or whether it could be demonstrated in other HRG-responsive cells, we examined the effect of HRG on paxillin expression in human colorectal CaCO-2 and FET cells and ovarian cancer SKOV-3 cells. As illustrated in Fig. 3, HRG treatment was associated with variable (2–4-fold) but significant up-regulation of paxillin mRNA (Fig. 3A) and protein (Fig. 3B). These results suggested that up-regulation of paxillin expression may be a general HRG response in cancer cell lines and was not restricted to MCF-7 breast cancer cells.

**Paxillin Expression and Breast Cancer.** Because HER family members are overexpressed in a number of tumors and cancer cell lines, we explored the potential relationship between the levels of HER family members and paxillin expression. As shown in Fig. 4 (left), HER2-overexpressing cell lines and cells with coexpression of HER2 and HER3 receptors exhibited elevated levels of paxillin, as compared to low HER2-expressing cell lines or HER2 expression with undetectable HER3 level. Paxillin expression does not correlate well with HER1 expression. Expression of another cytoskeletal protein vinculin was not affected by the status of HER family members.

We next examined the expression of paxillin in a small number of tumor biopsies. We analyzed three samples each from grade II, grade III node-negative, and grade III node-positive breast tumor biopsies. Various characteristics tumor biopsies including proliferative index, estrogen receptor/progesterone receptor, and node status are presented in Table 1. Grade III node-positive specimens (metastatic), in general, had higher levels of HER2 and HER3 than did grade III node-negative or grade II tumors. Curiously, the level of paxillin expression was also elevated in HER2/HER3-overexpressing tumors (Fig. 4, right). Expression of another cytoskeletal protein vinculin was not effected by the expression
level of HER2/HER3 and remained same between various stages of tumors. Additional studies using large number of clinical samples are needed to validate these preliminary findings.

An important finding emerging from this study is increased expression of paxillin by HRG via transcriptional regulation. To the best of our knowledge, this is the first report to demonstrate the up-regulation of paxillin expression by a polypeptide growth factor. The finding that a translation inhibitor cycloheximide inhibits paxillin mRNA expression is interesting because it indicates the potential requirement of synthesis of a new factor during HRG up-regulation of paxillin expression. In the past, src-induced morphological transformation has been also shown to be dependent on the synthesis of new protein because src-induced morphological changes can also be blocked by cycloheximide (24). It will be interesting to determine whether paxillin is one of the proteins whose synthesis may be required for src-induced morphogenesis because its expression is affected by cycloheximide. In addition, it is also possible that the factor required to induce paxillin expression by HRG pathway may be same as that required by src transformation because of the known cross-talks between these pathways (24).

Paxillin expression has been detected in many tissues: at high levels in circulating lymphocytes, not at all in platelets, and at very low levels in brain tissue (25). An increase in the paxillin expression has been correlated with the transition of prostate carcinomas to metastatic carcinomas (26). Preferential increase in paxillin but not FAK was also reported in experimental nephrotic syndrome, in which paxillin overexpression was speculated to have a role in adherence and tissue repair (27). Here, we demonstrated an increase in the paxillin expression with HER2/HER3 pathway and grade III breast cancer tumors. Because cell motility is dependent on the dynamic disassembly and subsequent reassembly of focal adhesions, its induction by HRG pathway may provide an advantage and contribute to increased metastatic potential of cells with activated HER2 signaling.

Recently, HER2 transformed NIH3T3 cells have been shown to exhibit abnormalities in cytoskeleton structure. In this study, the paxillin was not tyrosine-phosphorylated but accompanied by an increased association with FAK (28). In this context, we have also observed that HRG does not regulate tyrosine phosphorylation of paxillin. Failure of activated HER2 or HRG to induce tyrosine phosphorylation of paxillin raises the possibility that increased expression of paxillin without tyrosine modification may function as a dominant negative molecule and may confer cancer cells an advantage in migration by interfering with the attachment with extracellular matrix, and this may involve blocking or sequestering signaling molecules or interfering with signal transduction from focal adhesions.

In our findings, we have established transcriptional induction of paxillin by a potent cell migratory factor, the HRG, in breast cancer cells.

References


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