Oncogenic Signals of HER-2/neu in Regulating the Stability of the Cyclin-dependent Kinase Inhibitor p27*

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Overexpression and activation of HER-2/neu, a proto-oncogene, play a pivotal role in cancer formation. Strong expression of HER-2/neu in cancers has been associated with poor prognosis. Reduced expression of p27kip1, a cyclin-dependent kinase inhibitor, correlates with poor clinical outcome in many types of carcinomas. Because many cancers with the overexpression of HER-2/neu overlap with those affected by reduced p27 expression, we studied the link between HER-2/neu oncogenic signals and p27 regulation. We found that down-regulation of p27 correlates with HER-2/neu overexpression. To address the molecular mechanism of this inverse correlation, we found that reduction of p27 is caused by enhanced ubiquitin-mediated degradation, and the HER-2/Grb2/MAPK pathway is involved in the decrease of p27 stability. Also, HER-2/neu activity causes mislocation of p27 and Jun activation domain-binding protein 1 (JAB1), an exporter of p27, into the cytoplasm, thereby facilitating p27 degradation. These results reveal that HER-2/neu signals reduce p27 stability and thus present potential points for therapeutic intervention in HER-2/neu-associated cancers.

The HER-2/neu oncogene (also named c-erbB-2) encodes a growth receptor tyrosine kinase, and amplification/overexpression of the human HER-2/neu gene are frequently found in human cancers, including breast, ovarian, lung, gastric, and oral cancers (1–3). Also, HER-2/neu overexpression correlates with a shorter survival rate in breast cancer patients. The molecular mechanism underlying how oncogenic signals of HER-2/neu affect the cell cycle machinery in affecting tumorigenicity is not completely determined. It is possible that HER-2/neu overexpression may stimulate cell proliferation through tyrosine kinase signaling to mediate mitogenic signals in promoting cell cycle progression. The cell cycle is regulated by both the positive and negative regulators. Cyclin and cyclin-dependent kinase (CDK)1 are positive regulators, whereas cyclin-dependent kinase inhibitors, including the Inhibitor of CDK4 family and the CIP/KIP family, are negative regulators (4).

p27, a CIP/KIP member, encodes a cyclin-dependent kinase inhibitor that causes G1 arrest by inhibiting the activities of G1/cyclin-CDKs. As a negative regulator of the cell cycle, p27 is a new class of tumor suppressor and is haplo-insufficient in tumor suppression (5, 6). In animal studies, the number of p27 gene copy can decide the rate of tumor formation, because p27 haplo-insufficient mice are hypersensitive to carcinogens (6).

Recently, reduced expression of p27 is frequently detected in human cancers, including breast (7, 8), prostate (9), gastric (10), lung (11), skin (12), colon (13), and ovarian cancers (14). Decreased expression of the p27 protein was shown to correlate with cancer development and poor survival, thus appearing as an important marker of cancer progression. Because p27 inhibits cyclin-CDK in a dosage-dependent manner to control cell cycle progression (15, 16), it is conceivable that decreased expression of p27 may result in abnormal cell proliferation in these cancers. However, the exact mechanism that underlies the decreased expression of p27 in cancer remains elusive. p27 is regulated post-transcriptionally through the ubiquitin-mediated proteasome degradation pathway (17); therefore, it is possible that reduced p27 in many types of cancer may be caused by the enhancement of ubiquitin-mediated p27 degradation.

Here, we assessed the roles of HER-2/neu signaling in regulating the stability of p27 and found that HER-2/neu activity specifically causes the decrease of p27 protein level by inducing the mislocation of p27 in the cytoplasm for ubiquitin-mediated degradation. Thus, this study provides an important mechanism link between two prognostic markers in cancers.

MATERIALS AND METHODS

Cell Culture—NIH3T3, 293T, B104–1–1 (18), B104–1–1/N-Grb2, B104–1–1/C-Grb2, SW3T3, SW3T3-X-1 (SW3T3 overexpressing HER2/neu), R1B/L17 cells (the mink lung epithelial cell line derivative) (15), human breast carcinoma cell lines MCF7 (does not overexpress HER-2/neu), and HER18 (MCF7 overexpressing HER2/neu) (19) were maintained in Dulbecco’s minimal essential media containing high glucose levels and 10% fetal calf serum. The use of B104–1–1, B104–1–1/N-Grb2, and B104–1–1/C-Grb2 has been described previously (20). A DEAE-dextran method was used to transiently transfect R1B/L17 cells as described previously (15). PD98059 (Calbiochem), LLL1 (Sigma), and MG132 (Sigma) were prepared in MeSO$_2$ for use.

Western Blot Analysis—Total cell lysates were solubilized in lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, 1 μg each of aprotinin, leupeptin, and pepstatin per ml) and were processed as described previously (15). Analyses were performed on 12% polyacrylamide gels with a 5% polyacrylamide stacking gel. After electrophoretic transfer (Amer sham Pharmacia Biotech) of protein from SDS polyacrylamide gels to DAPI, 4,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; CIP, CDK interaction protein; KIP, kinase inhibitor protein; JAB, Jun activation domain-binding protein; CMV, cytomegalovirus.

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The abbreviations used are: CDK, cyclin-dependent kinase; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; p27kip1, a cyclin-dependent kinase inhibitor; JAB1, an exporter of p27, into the cytoplasm, thereby facilitating p27 degradation.
polynylidine difluoride membranes (Millipore), the membranes were blocked with the buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 50 μg/ml Blotto (Bio-Rad) for 1 h at room temperature and incubated for 1 h at room temperature with the following primary antibodies: polyclonal anti-p21 antibody (Santa Cruz), polyclonal anti-actin antibody (sigma), polyclonal anti-p27 antibody, monoclonal anti-p27 antibody (Transduction Laboratories), and monoclonal anti-FLAG antibody (M2) (Sigma). Subsequently membranes were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. Following several washes, membranes were incubated with a chemiluminescence (ECL) system (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

Northern Blot Analysis—Total RNAs were isolated using Qiagen RNeasy kits. Each sample containing 20 μg of total RNAs was used for Northern blot analysis. RNAs were transferred to GeneScreen Plus membranes (NEN Life Science Products) using a Turboblotter system (Schleicher & Schuell). The p27 and glyceraldehyde-3-phosphate dehydrogenase cDNA probes were labeled by a random-primer DNA labeling kit (Roche Molecular Biochemicals). The glyceraldehyde-3-phosphate dehydrogenase probe was used to indicate the integrity and equal amounts of loading for each RNA sample.

The Cdk-associated Histone H1 Kinase Assay—The cells were lysed in lysis buffer as described above, and the protein concentration was quantified. 1 μg of cell lysate was immunoprecipitated with anti-Cdk2 antibody (PhosphoGen). The immunoprecipitates were assayed for histone H1 kinase activity as described previously (15).

Metabolic Labeling, Immunoprecipitations, and Half-life Determination—Cells were maintained and grew to 70% confluency. The cellular proteins were pulse-labeled with 3H-methionine (100 μCi/ml) for 3 h in methionine-free media and chased with cold Met for 0, 2, or 5 h; p27 was then immunoprecipitated from each lysate with 10 μg/ml of p27 antibody, separated by SDS-PAGE, and analyzed by phosphorimaging (Molecular Dynamics) or autoradiography. The intensity of radioabeled p27 was quantitated by the Imagequant program of the PhosphorImager. The half-life of the protein was determined graphically according to procedures described previously (21).

In Vitro Degradation Assays—A polymerase chain reaction-generated fragment of the p27 cDNA containing the full-length coding region was subcloned into pET21a (Novagen) to yield a construct that encodes p27 with a FLAG-tag sequence. The protein was expressed in BL21 (DE3), and FLAG-tagged proteins were prepared as described previously (15). 100 μg of cell lysate prepared from NIH3T3 or B104–1-1 cells was incubated with 100 ng of affinity-purified FLAG-tagged p27 protein at 37 °C for 0, 0.5, 3, and 19 h in a buffer containing 10 μM Tris-HCl, 75.5 mM MgCl2, 2 μg of ubiquitin, 2 μM ATP, 1 μM dithiothreitol, 15 mM phosphocreatine, 1 μM of creatine phosphokinase according to the procedure described previously (13).

Immunofluorescence—Endogenous p27 or JAB1 subcellular localization was detected in NIH3T3 or B104–1-1 cells. Cells were prepared and seeded onto chamber slides with 2 × 105 cells per well 1 day prior to staining. Cells were then fixed with methanol/acetone (1:1, v/v) at room temperature for 2 min and stained for 1 h with rabbit anti-p27 (Santa Cruz) or goat anti-JAB1 (Santa Cruz) antibody followed by a 1-h incubation with Cy3-conjugated anti-rabbit antibody (Zymed Laboratories Inc.) or Texas red-conjugated anti-goat antibody (Jackson Research Laboratories). For studying subcellular localization of exogenous p27 or JAB1, Rb-L17 cells were transiently co-transfected with pcMV-Her-2/neu (a point mutation at the transmembrane domain results in constitutive activation of Her-2/neu) (18, 22) and pcMV-FLAG-p27, or pcMV-FLAG-JAB1. 24 h after transfection, 2 × 105 cells were seeded onto tissue culture chamber slides (Nunc). 2 days later, cells were fixed and stained as mentioned above. Monoclonal anti-FLAG antibody (M2) (Sigma) was used in detecting p27 or JAB1 expression. In this case, the fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (Jackson Research Laboratories) was used. For all staining, cells were incubated with 0.1 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) (Sigma) to stain the nuclei. Immunofluorescence was detected using a BX50 fluorescent microscope (Olympus).

RESULTS

HER-2/neu Overexpression/Activation Results in Reduced p27 Expression—To investigate whether HER-2/neu affects the expression of p27, we used NIH3T3 cells, B104–1-1 cells (derived from NIH3T3 overexpressing constitutively active HER-2/neu) (18), MCF7, and HER18 cells (derived from MCF7 overexpressing HER-2/neu) (19) to examine the role of HER-2/neu signaling in regulating the protein level of p27 by immunoblotting. As demonstrated in Fig. 1A, the constitutive activation (B104–1-1) or overexpression (HER-18) of HER-2/neu results in the decreased expression of p27. Interestingly, ΔN-Grb2, which is an amino-terminal deletion mutant of Grb2 and acts as a dominant mutant of Grb2 to block HER-2/neu signaling through the Grb2 pathway (20), can alleviate the decreased protein expression of p27 in B104–1-1 cells. On the other hand, ΔC-Grb2 (20), which has minimum effect in blocking HER-2/neu signaling, does not block the down-regulation of p27 in B104–1-1 cells. In addition, when cells were co-transfected with p27 and increasing amounts of Her2/neu, the level of p27 was diminished as detected by immunoblotting (Fig. 1A). To determine whether the HER-2/Grb2/PAK pathway is involved in regulating p27 protein level, we further examined the effect of MAPK inhibitor on p27 protein level in B104–1-1 and HER18 cells. The level of p27 protein is rescued when these cells are treated with MAPK inhibitor PD98059 (Fig. 1B), suggesting that the MAPK pathway of HER-2/neu signals is responsible for down-regulation of p27 protein. The decreased level of p27 protein is not due to down-regulation of mRNA, because each cell line contains the same amount of p27 transcripts (Fig. 1C). To study the consequence of decreased p27 in regulating Cdk activity, we measured the Cdk2-associated histone H1 kinase activity in cells. We found that Cdk2 kinase activity was markedly increased in B104–1-1 compared with NIH3T3, which reflects the reduction of both Cdk2-associated p27 (Fig. 1C) and total p27 level (Fig. 1D) in B104–1-1 cells. Interestingly, the level of p21, another CIP/KIP member, is up-regulated in B104–1-1 cells (Fig. 1D), indicating that
HER-2/neu signals affect the expression of p27 or p21 (23) in a different manner. In conclusion, our results indicate that HER-2/neu signals cause the down-regulation of p27 specifically.

HER-2/neu Overexpression/Activation Accelerates the Turnover Rate of p27—To study whether HER2-mediated down-regulation of p27 results from increased turnover rate of p27, we determined the half-life of p27 by pulse-chase experiment in cell lines with different HER-2/neu statuses, including HER-2/neu overexpression/activation cell lines (B104–1-1, B104–1-1/ΔC-Grb2, and SW3T3-X-1 cells) and non HER-2/neu overexpression/activation cells (NIH3T3, B104–1-1/ΔN-Grb2, and SW3T3 cells). A pulse-chase analysis of p27 protein clearly indicated that radiolabeled p27 protein decreased faster in HER-2/neu overexpression/activation cells than that in non HER-2/neu overexpression/activation cells (Fig. 2A). Images of the radiolabeled p27 protein were analyzed and quantitated by a PhosphorImager (Molecular Dynamics) to calculate the half-life of the p27 protein according to procedures described previously (21). C, half-life of p27 in indicated cell lines with different HER-2/neu status. The half-life of p27 was calculated using the methods described in B. D, in vitro ubiquitin-mediated degradation of p27. 100 μg of cell lysate prepared from NIH3T3 or B104–1-1 cells was incubated with 100 ng of affinity-purified FLAG-tagged p27 protein at 37°C for 0 min, 30 min, 3 h, or 19 h. The level of p27 in each reaction was immunoblotted with anti-M2. E, p27 is increased in the presence of 26 S proteasome inhibitors. B104–1-1 cells were treated with Me2SO, LLLnL (500 μM), or MG132 (50 μM) for 16 h. Me2SO treatment was used as a control. 100 μg of each cell lysates were immunoblotted with anti-p27. Levels of actin were shown as equal loading control.

HER-2/neu Signaling Is Involved in the Regulation of p27 Nuclear Localization—p27 was shown to be transported to the cytoplasm for degradation (24). To determine whether HER-2/neu signals are involved in the degradation of p27 by affecting its localization, we analyzed the subcellular localization of p27 in the presence of activating HER-2/neu signals as detected by immunofluorescence (green, bottom panels). Empty vector transfection (CMV) was used as a control. Cells that received indicated plasmids were seeded at 2 × 10⁵ in chamber slide. Cells were fixed, and p27 were immunodetected with monoclonal anti-FLAG antibody followed by FITC-conjugated anti-mouse immunoglobulin. DAPI staining was used to show the localization of nuclei.
HER-2/neu Signaling and p27 Expression

**DISCUSSION**

HER-2/neu overexpression plays an important role in promoting tumorigenicity in colon, gastric, breast, lung, and ovarian cancers. Interestingly, these cancers overlap with those affected by reduced p27 expression. Now both are significant prognostic markers for breast cancer. Accordingly, we screened the protein expression of HER-2/neu and p27 in primary breast tumor samples by immunohistochemistry. Among the 60 cases we have studied, we have found that there was a trend for a decreasing degree of p27 staining with increasing HER-2/neu expression, suggesting a link between HER-2/neu expression and p27 regulation. These results are consistent with the biochemical studies described here in HER2/neu overexpressing cell lines. This study showed that HER-2/neu oncogenic signals can cause reduced expression of the haplo-insufficient tumor suppressor p27 (6). We have investigated the molecular mechanism of HER-2/neu signals in down-regulating p27. First, we used isogenic cell lines that only differ in HER-2/neu expression of an activated HER-2/neu (HER-2/neu) and empty vector transfection (CMV) was used as a control. Cells were fixed and analyzed for immunofluorescence using monoclonal anti-FLAG antibody followed by FITC-conjugated anti-mouse immunoglobulin (green, bottom panels). Nuclei were visualized by DAPI staining of DNA.

**FIG. 4.** Constitutively active HER-2/neu can stimulate the accumulation of JAB1 in the cytoplasm. B104–1-1 and NIH3T3 cells were fixed and analyzed for the localization of JAB1. The localization of JAB1 was analyzed by indirect immunofluorescence staining using polyclonal anti-JAB1 antibodies followed by Texas red-conjugated anti-goat immunoglobulin (red, top panels). R1B/L17 cells were transfected with plasmid-encoding FLAG-tagged-JAB1 (CMV-FLAG-JAB1) in the presence or absence of plasmid-encoding activating HER-2/neu (CMV-HER-2/neu). Empty vector transfection (CMV) was used as a control. Cells were fixed and analyzed for immunofluorescence using monoclonal anti-FLAG antibody followed by FITC-conjugated anti-mouse immunoglobulin (green, bottom panels). Nuclei were visualized by DAPI staining of DNA.

[Image: 63x439 to 283x729]

FIG. 5. A model for p27 down-regulation induced by HER-2/neu oncogenic signals.

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cytoplasm, including tuberous sclerosis complex (30) and Barrett’s adenocarcinoma (31). Moreover, several anchorage-transformed cells have misplacement of p27 into cytoplasm (32). The mechanism of mislocation of p27 in these observations remains elusive. It is possible that mislocation of p27 in the cytoplasm could trigger degradation and loss of function. Because p27 is degraded faster in HER-2/neu-overexpressing cells, we investigated its subcellular localization and found that p27 is excluded from the nucleus in the presence of constitutively active HER-2/neu. It could be argued that cytoplasmic mislocation per se may not be sufficient to account for p27 degradation. We then further examined whether JAB1, a p27 exporter, can be affected by HER-2/neu activity to facilitate p27 degradation. It was shown that overexpression of JAB1 resulted in reduced expression of p27 (24). In addition, JAB1 was shown to mediate p27 degradation in a proteasome-dependent manner, because proteasome inhibitor LLnL can interfere with JAB1 to export p27 from the nucleus to the cytoplasm (24). We found that HER-2/neu signals enhance p27 degradation through ubiquitination. Also, we found that JAB1 is co-localized with p27 when HER-2/neu activity is high. Thus, HER-2/neu signals are directed to regulate the JAB1 activity, thereby affecting the localization and stability of p27. Investigating the link between HER-2/neu signals and JAB1 regulation will shed light on how HER-2/neu signals influence the turnover rate of p27.

Recent studies have shown that anti-HER-2/neu monoclonal antibody has an effect in growth inhibition and up-regulating p27 in HER-2/neu-overexpressing cancer cell lines (33), which supports our studies that HER-2/neu signals can cause reduction of p27. The inhibitory effect of HER-2/neu antibody was successfully used in the treatment of human breast cancers (29, 34). It is conceivable that regulating the level of p27, at least in part, accounts for the treatment outcome of these diseases. In conclusion, our studies suggest that p27 and JAB1 are the downstream targets of HER-2/neu oncopgenic signals, and they may be useful targets for therapeutic intervention in HER-2/neu-associated tumors.

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