MOLECULAR MECHANISMS UNDERLYING IGF-I-INDUCED ATTENUATION OF THE GROWTH-INHIBITORY ACTIVITY OF TRASTUZUMAB (HERCEPTIN) ON SKBR3 BREAST CANCER CELLS

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The clinical usefulness of trastuzumab (Herceptin; Genentech, San Francisco, CA) in breast cancer treatment is limited by the rapid development of resistance. We previously reported that IGF-I signaling confers resistance to the growth-inhibitory actions of trastuzumab in a model system, but the underlying molecular mechanism remains unknown. We used SKBR3/neo cells (expressing few IGF-I receptors) and SKBR3/IGF-IR cells (overexpressing IGF-I receptor) as our experimental model. IGF-I antagonized the trastuzumab-induced increase in the level of the Cdk inhibitor $p27^{Kip1}$. This resulted in decreased association of $p27^{Kip1}$ with Cdk2, restoration of Cdk2 activity and attenuation of cell-cycle arrest in G_1 phase, all of which had been induced by trastuzumab treatment in SKBR3/IGF-IR cells. We also found that the decrease in $p27^{Kip1}$ induced by IGF-I was accompanied by an increase in expression of Skp2, which is a ubiquitin ligase for $p27^{Kip1}$, and by increased Skp2 association with $p27^{Kip1}$. A specific proteasome inhibitor (LLnL) completely blocked the ability of IGF-I to reduce the $p27^{Kip1}$ protein level, while IGF-I increased $p27^{Kip1}$ ubiquitination. This suggests that the action of IGF-I in conferring resistance to trastuzumab involves targeting of $p27^{Kip1}$ to the ubiquitin/proteasome degradation machinery. Finally, specific inhibitors of MAPK and PI3K suggest that the IGF-I-mediated reduction in $p27^{Kip1}$ protein level by increased degradation predominantly involves the PI3K pathway. Our results provide an example of resistance to an antineoplastic therapy that targets one tyrosine kinase receptor by increased signal transduction through an alternative pathway in a complex regulatory network.

Key words: *HER2/neu; IGF-I receptor; trastuzumab; $p27^{Kip1}$; Skp2; phosphatidylinositol-3-kinase*

HER2/neu, a member of the ErbB receptor family, is involved in development, cell proliferation, differentiation and oncogenesis.[1–3] Approximately one-third of breast cancers overexpress HER2/neu, and this overexpression contributes to epithelial cell transformation and predicts poor prognosis for breast cancer patients.[4–6] Trastuzumab (Herceptin; Genentech, San Francisco, CA) is a humanized blocking antibody against the HER2/neu receptor. While regarded as an example of a successful rational design drug based on identification of a novel molecular target,[7–9] its efficacy is limited because resistance develops rapidly in virtually all treated patients.[8,9]

We previously reported[10] that IGF-I signaling is associated with resistance to the growth-inhibitory actions of trastuzumab. Although the potential clinical relevance of our observation has been pointed out,[11] there are few data concerning the underlying mechanisms. IGF-I regulates both the expression and activity of many cell cycle–related proteins, including upregulation of cyclins and cyclin-associated Cdk activity and downregulation of $p27^{Kip1}$.[12–15] The growth-inhibitory activity of trastuzumab was also reported to involve $p27^{Kip1}$.[16–18] Together, these observations suggest interaction between IGF-I receptor signaling and trastuzumab at the level of the cell-cycle regulators.

Our current understanding of IGF-I signaling has been reviewed.[15,19,20] The IGF-I receptor initiates a strong proliferative and antiapoptotic signal.[20–23] Binding of IGF-I to the IGF-I receptor results in autophosphorylation of the receptor, leading to recruitment and phosphorylation of Shc and IRS-1 adaptor protein. This action of IGF-I results in activation of the Ras/Raf/MAPK pathway and/or the PI3K pathway, which influence cell proliferation and survival.[24,25]

Regulation of cell proliferation by cyclins and Cdks has also been reviewed.[14,26,27] The cell-cycle machinery is positively regulated by cyclins and Cdks and inhibited by Cdk inhibitors. $p27^{Kip1}$, a Cdk inhibitor, plays an important role in the control of cell proliferation, specifically in the G_1–S transition, where it inhibits cyclin E/Cdk2 and cyclin A/Cdk2 activities.[26–28] While $p27^{Kip1}$ mRNA levels appear to be constant throughout the cell cycle,[29] the $p27^{Kip1}$ protein level is decreased by mitogenic stimuli, allowing Cdk2 activation and cell-cycle progression into the S phase.[14,26,27,29,30] The protein level of $p27^{Kip1}$ is posttranscriptionally regulated through the ubiquitin/proteasome signaling pathway during progression from G_1 to S.[29–32] Reduced $p27^{Kip1}$ protein expression correlates with HER2/neu overexpression in breast cancer cells, and activation of HER2/neu leads to ubiquitin-mediated $p27^{Kip1}$ protein degradation.[33,34]

In the present study, we explored the molecular mechanisms by which IGF-IR signaling attenuates trastuzumab-induced growth inhibition of breast cancer cells. We found that the mechanisms involve IGF-I-mediated upregulation of ubiquitin-related $p27^{Kip1}$ degradation through increased expression of Skp2, the receptor component of an SCF ubiquitin ligase complex. This results in restoration of Cdk2 activity and attenuation of G_1 arrest. Furthermore, this action of IGF-IR signaling was due to activation of the PI3K signaling pathway.

MATERIAL AND METHODS

All cell culture material was from Invitrogen (Carlsbad, CA). Trastuzumab was purchased from the Oncology Pharmacy of the Jewish General Hospital (Montreal, Canada). PD98059, LY294002, propidium iodide, LLnL and transferrin were from Sigma (St. Louis, MO). Histone H1 was from Pharmingen (Mississauga, ON).

Abbreviations: Cdk, cyclin-dependent kinase; DTT, dithiothreitol; ECL, enhanced chemiluminescence; IGFBP-3, IGF binding protein-3; IGF-IR, IGF-I receptor; LLnL, *N*-acetyl-leucinyl-leucinylnorleucinal-H; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3-kinase; RIPA, radioimmunoprecipitation assay; SCF, Skp-cullin-F-box protein; SFM, serum-free medium.

[γ-^{32}P]ATP was from Amersham (Piscataway, NJ). IGFBP-3 and IGF-I were from Protigen (Mountain View, CA). Protein A-agarose, anti-IGF-IRβ, anti-c-myc, anti-p27^{Kip1} (C-19), anti- Cdk2 (M2), anti-Skp2, anti-α-tubulin and anti-α-ubiquitin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p27^{Kip1} (Ab-1) was from Neomarkers (Fremont, CA). Anti-phosphotyrosine-specific antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-Skp2 was from Zymed (South San Francisco, CA). Anti-phospho-MAPK, anti-total MAPK, anti-phospho-AKT and anti-AKT were from New England Biolabs (Mississauga, Ontario).

Cell lines

SKBR3/neo and SKBR3/IGF-IR are human breast cancer cell lines transfected with pcDNA3.1(+) and pcDNA3.1(+)/IGF-IR, respectively; and their characterization was described in our previous paper.[10]

Cell synchronization

To make SKBR3/neo and SKBR3/IGF-IR cells quiescent, they were seeded at a density of $8 \times 10^3/cm^2$ in the presence of serum. After 24 hr, cells were washed with PBS solution and incubated in SFM (McCoy's 5A supplemented with 40 μg/ml transferrin). Cells became quiescent after 72 hr in SFM, and medium was renewed every 24 hr.

Flow cytometry

SKBR3/neo and SKBR3/IGF-IR cells were plated in 100 mm dishes in the presence of 10% serum for 24 hr. When 30% confluent, cells were synchronized in serum-free conditions for 24 hr and either treated with 10 μg/ml trastuzumab or not for a further 24 hr. IGF-I was added at 40 ng/ml for a further 2, 4, 8, 16 or 24 hr incubation. Cells were then washed twice with ice-cold PBS solution and fixed in 70% ethanol at –20°C overnight. Cells were washed twice with ice-cold PBS solution again and resuspended in propidium iodide buffer [PBS (pH 7.4), 0.1%Triton-X 100, 0.1 mM EDTA (pH 7.4), 0.05 mg/ml RNase A, 50 μg/ml propidium iodide]. After 30 min of incubation at room temperature, the cell-cycle distribution was analyzed using a FACSCalibur flow cytometer (BD Biosciences, Burlington, MA). Triplicate experiments yielded similar results.

Trastuzumab and IGF-I treatment

SKBR3/neo and SKBR3/IGF-IR cells were passaged in McCoy's 5A medium supplemented with 10% FBS and 800 μg/ml G418 at 37°C and 5% CO_2. For studies assessing the effects of exposure to IGF-I and IGFBP-3 on IGF-IR activation in SKBR3/neo and SKBR3/IGF-IR cells, 70–80% confluent cultures were washed twice with ice-cold PBS solution and then cultured in SFM for 24 hr. During the last 15 min of culture, cells were treated with vehicle, 100 ng/ml IGF-I alone or 1 μg/ml IGFBP-3 at 37°C. Monolayers were quickly washed twice with ice-cold PBS solution and lysed with 0.4 ml of lysis buffer [10mM TRIS-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40 and 0.2 units/ml aprotinin]. For studies assessing the effect of IGF-I on regulating c-myc, quiescent SKBR3/neo and SKBR3/IGF-IR cells were stimulated by 40 ng/ml IGF-I for 1, 4, 8, 16 or 24 hr. After treatment, cells were lysed in RIPA buffer (0.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% NP-49, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.2 units/ml aprotinin). For studies assessing the effect of IGF-I on the trastuzumab-induced increase in p27^{Kip1}, SKBR3/neo and SKBR3/IGF-IR cells were plated in the presence of 10% serum for 24 hr. When 60% confluent, cells were treated with or without 10 μg/ml trastuzumab in SFM for 16 hr. Either 40 ng/ml IGF-I or IGF-I plus 1 μg/ml IGFBP-3 were added to cells for a further 12 hr. After treatment, cells were lysed in RIPA buffer. For studies assessing the effect of the proteasome inhibitor LLnL on IGF-I-induced p27^{Kip1} degradation, SKBR3/neo and SKBR3/IGF-IR cells were plated in the presence of 10% serum for 24 hr. When 60% confluent, cells were treated with or without 10 μg/ml trastuzumab in SFM for 16 hr. Either 40 ng/ml IGF-I or IGF-I plus 50 μg/ml LLnL were added to cells for a further 4, 8, 12 and 16 hr. After treatment, cells were lysed in RIPA buffer. For studies assessing the effects of 50 μm PD98059 and 15 μm LY294002 blockage of IGF-I-induced MAPK and AKT phosphorylation, respectively, SKBR3/neo and SKBR3/IGF-IR cells were plated in the presence of 10% serum for 24 hr. When 60% confluent, cells were treated with 10 μg/ml trastuzumab plus PD98059 or LY294002 in SFM for 16 hr. IGF-I (40 ng/ml) was added to cells for a further 8 hr. After treatment, cells were lysed in RIPA buffer.

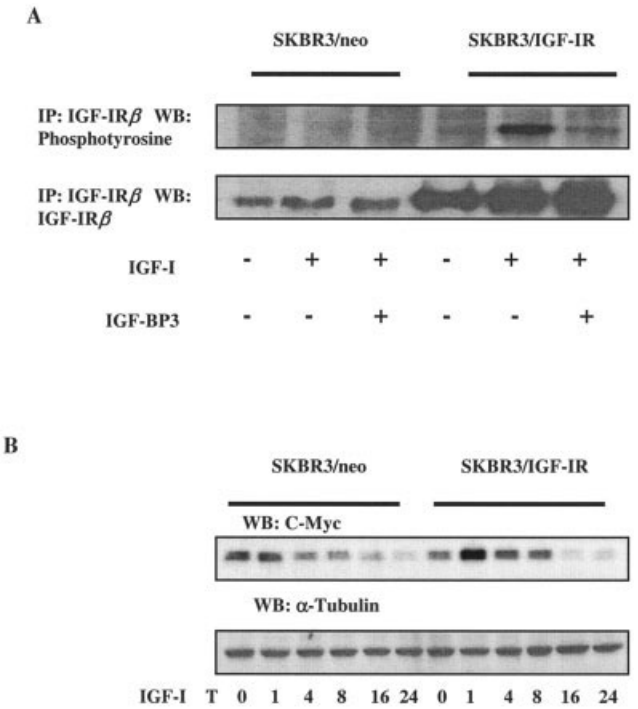

FIGURE 1 – Increasing IGF-IR expression in SKBR3 cells increases c-myc response. *(a)* IGF-IR phosphorylation of SKBR3/neo and SKBR3/IGF-IR cells in the presence or absence of IGF-I and IGFBP-3. Maximal receptor activation is seen in the SKBR3/IGF-IR cells in the presence of IGF-I; this is nearly abolished in the presence of IGFBP-3. *(b)* Effects of IGF-I on inducing c-myc in quiescent SKBR3/neo and SKBR3/IGF-IR cells. Time points (in hours) are shown.

Western blots

Clarified protein lysates from each experimental condition (20–60 μg) were electrophoretically resolved on denaturing SDS-polyacrylamide gels (8–12%), transferred to nitrocellulose membranes and probed with the following primary antibodies: anti-c-myc, anti-p27^{Kip1}, anti-Skp2, anti-α-tubulin, anti-phospho-MAPK, anti-MAPK, anti-phospoAKT, anti-AKT. Proteins were revealed using horseradish peroxidase–conjugated antimouse or antirabbit antibodies.

Immunoprecipitation

Clarified protein lysates (200–300 μg/ml) were precleared with 25 μl of protein A-agarose and then precipitated with 2 μg of anti-IGF-IRβ, anti-Cdk2, anti-Skp2 or anti-p27^{Kip1} antibody and 25 μl of protein A-agarose overnight at 4°C. The next day, beads were collected by centrifugation and washed with lysis buffer. Samples were denatured with 25 μl of 2 $\times$ SDS-PAGE sample buffer and subjected to SDS-PAGE on a 12% gel. After separated proteins were transferred to membranes, membranes were probed with an

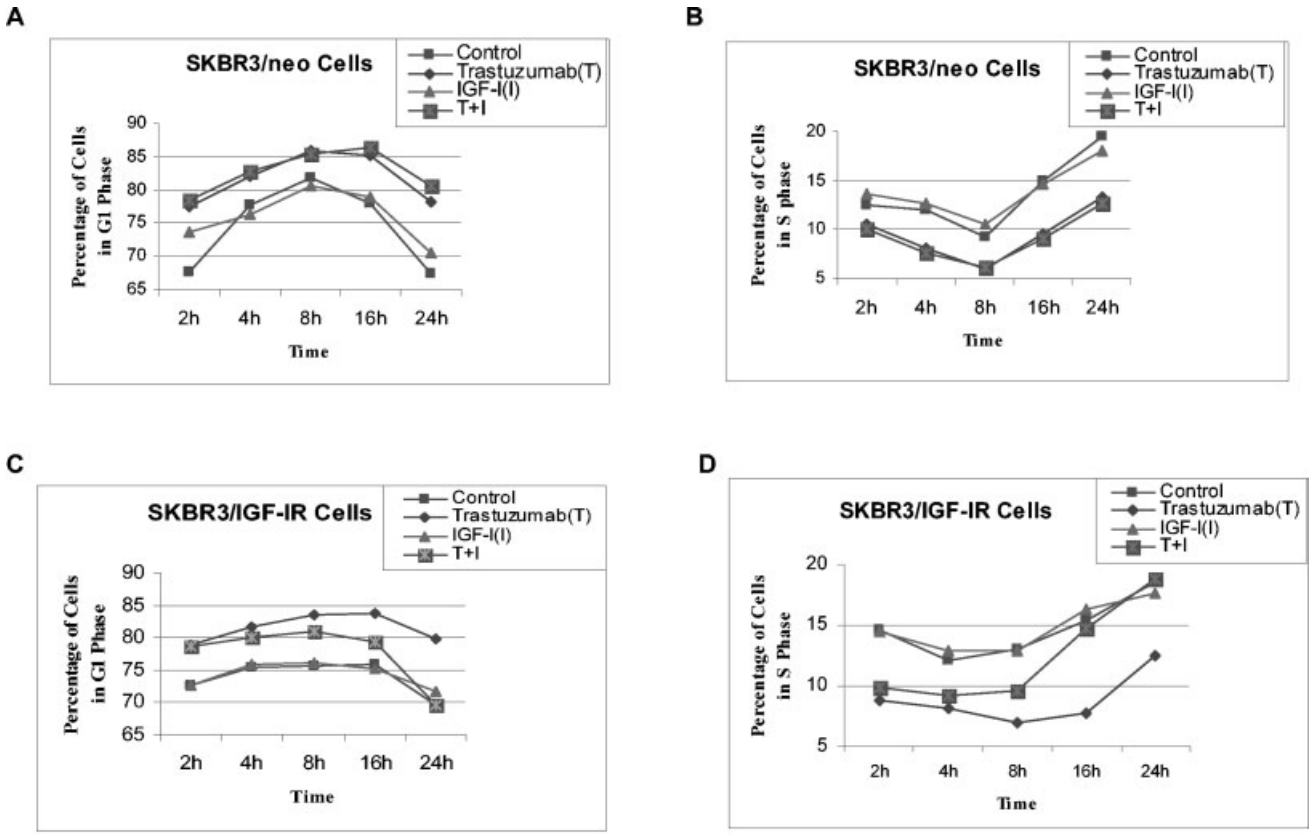

FIGURE 2 – IGF-I rescues SKBR3/IGF-IR cells from trastuzumab-induced G_1 arrest. After SKBR3/neo and SKBR3/IGF-IR cells were synchronized in SFM for 24 hr and treated or not with trastuzumab (10 μg/ml) for a further 24 hr, 40 ng/ml IGF-I were added and the cells collected at the times

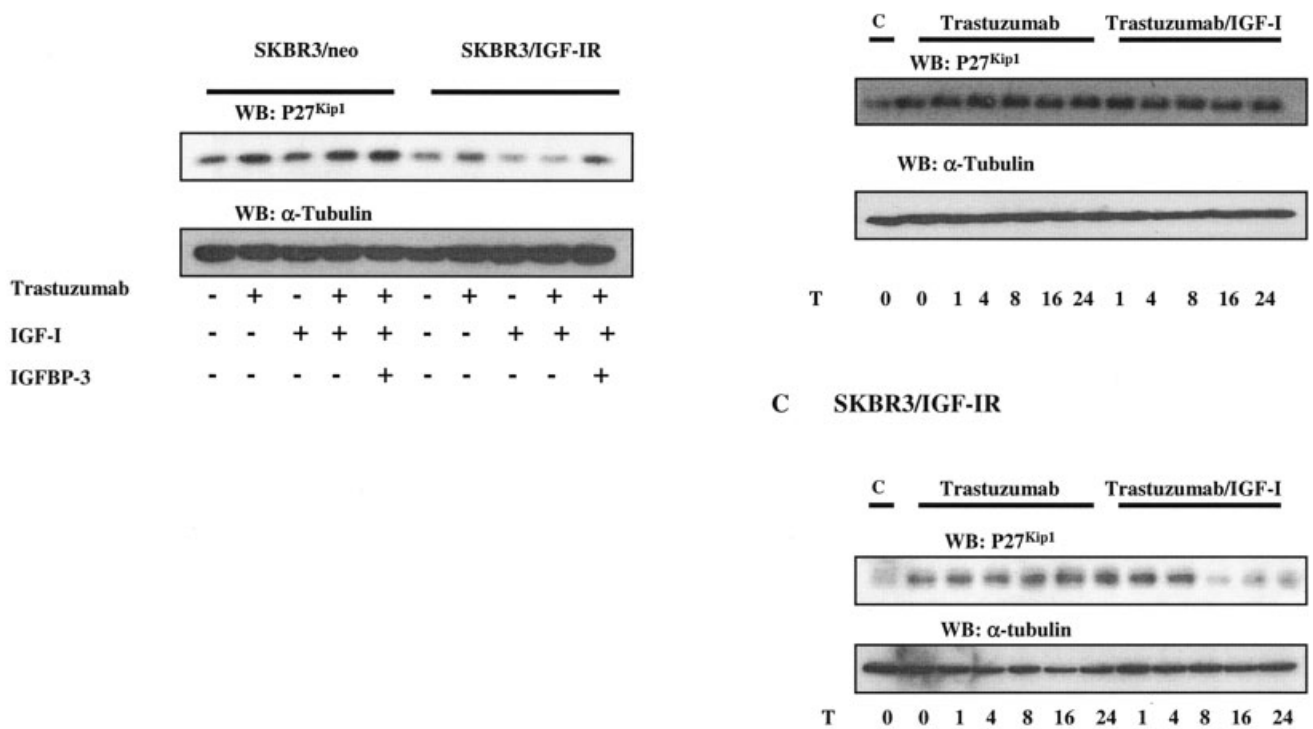

FIGURE 3 – IGF-I antagonizes trastuzumab-induced increase in $p27^{Kip1}$ in SKBR3/IGF-IR cells. *(a)* After SKBR3/neo and SKBR3/IGF-IR cells were treated or not with trastuzumab (10 μg/ml) in SFM for 16 hr, IGF-I (40 ng/ml) alone or with IGFBP-3 (1 μg/ml) were added for a further 12 hr in SFM. $p27^{Kip1}$ and α-tubulin expression were determined by Western blotting analysis. *(b,c)* Time course study of IGF-I on regulation of $p27^{Kip1}$ expression in SKBR3/neo *(b)* and SKBR3/IGF-IR *(c)* cells. SKBR3/neo or SKBR3/IGF-IR cells were treated with trastuzumab as in *(a),* and 40 ng/ml IGF-I were added; cell lysate was collected at the times indicated thereafter (in hours) for Western blotting.

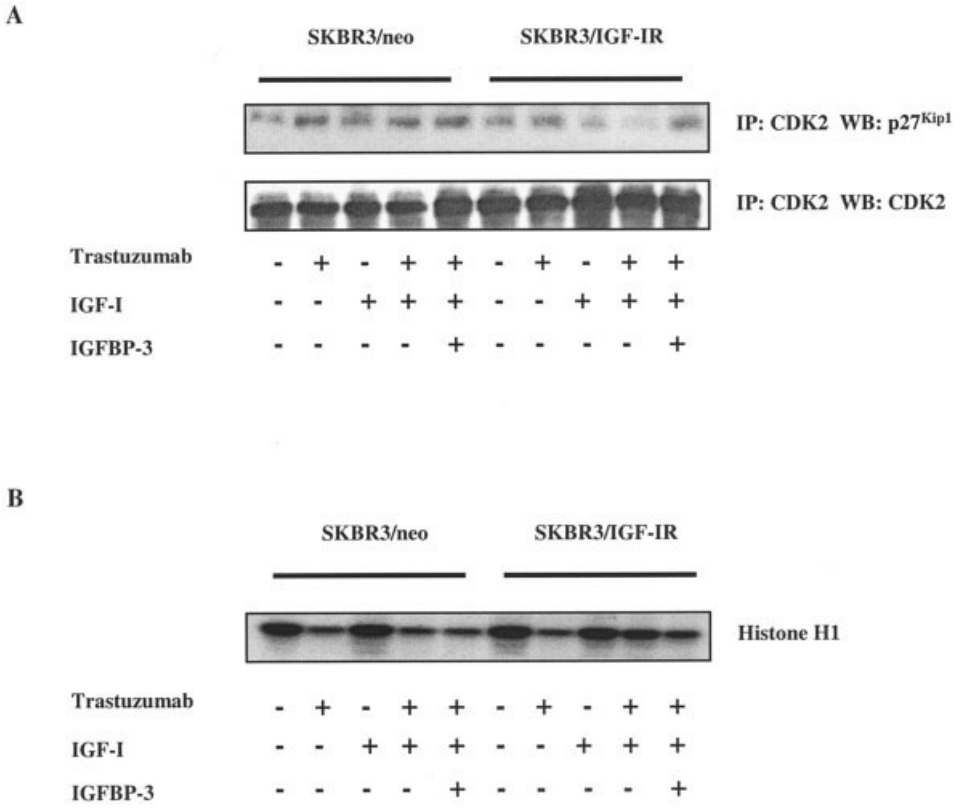

FIGURE 4 – IGF-I releases $p27^{Kip1}$ from Cdk2 and restores Cdk2 activity inhibited by trastuzumab in SKBR3/IGF-IR cells. After SKBR3/neo and SKBR3/IGF-IR cells were treated or not with trastuzumab (10 μg/ml) in SFM for 16 hr, IGF-I (40 ng/ml) was added alone or with IGFBP-3 (1 μg/ml) for a further 12 hr in SFM. *(a)* $p27^{Kip1}$ association with Cdk2 complexes was assessed through immunoprecipitation experiments in serum-free conditions. *(b)* Precipitates recovered with antibody to Cdk2 were assayed for histone H1 kinase activity in serum-free conditions.

antiphosphotyrosine-specific antibody, anti-IGF-IRβ, anti-Cdk2 antibody, anti-$p27^{Kip1}$ antibody or anti-α-ubiquitin followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL.

Cdk2 assay

Cdk2 activity was determined as described by Zi *et al.*[35] Cells were lysed in RIPA buffer as described above. Clarified protein lysates (200 μg) were subjected to immunoprecipitation in lysis buffer at 4°C overnight in the presence of anti-Cdk2 antibody and protein A-agarose beads. Phosphorylation of histone H1 was measured by incubating the beads with 40 μl of "hot" kinase solution {0.25 μl (2.5 μg) of histone H1, 0.5 μl of [γ-^{32}P]ATP, 0.5 μl of 0.1 mM ATP and 38.75 μl of kinase buffer (50 mM TRIS-HCl, pH 7.4, 10 mM $MgCl_2$ and 1 mM DTT)} for 30 min at 30°C. The reaction was stopped by boiling the samples in SDS buffer for 5 min. Samples were analyzed by 12% SDS-PAGE and the gels dried and subjected to autoradiography.

RESULTS

Increasing IGF-IR expression in SKBR3 cells increases c-myc response

We have previously shown that overexpression and activation of IGF-IR attenuates trastuzumab-induced growth inhibition in breast cancer cells.[10] We used the SKBR3/neo and SKBR3/IGF-IR cell lines to study the tyrosine phosphorylation level of IGF-IR by immunoprecipitation with an anti-IGF-IRβ antibody followed by antiphosphotyrosine immunoblotting. As expected, the highest level of phosphorylated IGF-IR was seen in the SKBR3/IGF-IR cell line in the presence of IGF-I. In the presence of both IGF-I and IGFBP-3, IGF-IR phosphorylation was reduced (Fig. 1*a*). These results provide evidence that IGF-IR is functional in SKBR3/IGF-IR cells but not in SKBR3/neo cells.

Subsequently, we examined the effect of IGF-I in regulating its downstream target in SKBR3/neo and SKBR3/IGF-IR cells. IGF-I substantially increased c-myc protein level following 1 hr incubation in quiescent SKBR3/IGF-IR cells but had only a small effect in SKBR3/neo cells (Fig. 1*b*).

Activation of IGF-IR releases SKBR3/IGF-IR cells from trastuzumab-induced G_1 arrest

Since the reduction in cell proliferation induced by trastuzumab is associated with an increased proportion of cells in G_1 phase,[16,17] we examined the role of IGF-IR signaling in rescuing trastuzumab-induced G_1 arrest. After both SKBR3/neo and SKBR3/IGF-IR cells were synchronized in SFM for 24 hr, they were treated or not with 10 μg/ml trastuzumab for a further 24 hr. IGF-I (40 ng/ml) was added, and cell-cycle profiles were analyzed by flow cytometry. Control SKBR3/neo cells were induced to arrest in G_1 phase by trastuzumab treatment. Trastuzumab also induced G_1 arrest in SKBR3/IGF-IR cells in serum-free conditions. However, SKBR3/IGF-IR cells were rescued from trastuzumab-induced G_1 arrest by IGF-I (Fig. 2). Indeed, SKBR3/IGF-IR cells were pushed to S phase by IGF-I in the presence of trastuzumab. In contrast, IGF-I did not show any effect on cell-cycle regulation in the SKBR3/neo cell line in the presence of trastuzumab (Fig. 2).

IGF-IR activation antagonizes trastuzumab-induced increase in $p27^{Kip1}$

To identify the molecular basis of the cell-cycle regulation by IGF-IR signaling, both SKBR3/neo and SKBR3/IGF-IR cells were treated with 10 μg/ml trastuzumab in SFM for 16 hr. After incubation with IGF-I alone or IGF-I plus IGFBP-3 for a further 12 hr, cell lysates were obtained for Western blots. The $p27^{Kip1}$ level was increased by trastuzumab treatment in both SKBR3/IGF-IR and SKBR3/neo cells; however, IGF-I attenuated the trastuzumab-induced increase in $p27^{Kip1}$ only in SKBR3/IGF-IR cells, and IGFBP-3 restored trastuzumab actions (Fig. 3*a*). The time course of downregulation of $p27^{Kip1}$ by IGF-I in SKBR3/IGF-IR cells is shown in Figure 3*c*. However, IGF-I did not show the same effect in SKBR3/neo cells (Fig. 3*b*).

IGF-IR-mediated attenuation of trastuzumab-induced inhibition of Cdk2 activity is associated with decreased association of $p27^{Kip1}$ with Cdk2

$p27^{Kip1}$ directly inhibits Cdk2 activity, which is crucial for cells to progress through the G_1–S transition. Trastuzumab interference with HER2/neu receptor signaling causes Cdk2 inactivation at least in part by increasing $p27^{Kip1}$ association with Cdk2.[17] We therefore examined whether IGF-I-induced attenuation of trastuzumab action involved modulation of $p27^{Kip1}$/Cdk2 stoichiometry. Immunoprecipitation experiments showed that trastuzumab increased $p27^{Kip1}$ association with Cdk2 in both SKBR3/neo and SKBR3/IGF-IR cells in serum-free conditions; however, in SKBR3/IGF-IR cells, IGF-I attenuated this association. IGFBP-3 restored the Cdk2–$p27^{Kip1}$ association induced by trastuzumab (Fig. 4*a*). Consistent with Western blot and immunoprecipitation results, Cdk2 activity was markedly decreased by trastuzumab treatment in both SKBR3/neo and SKBR3/IGF-IR cells, as expected; this decrease was blocked by IGF-I in SKBR3/IGF-IR cells only. IGFBP-3 could block this IGF-I action (Fig. 4*b*).

IGF-I regulates $p27^{Kip1}$ protein through enhancement of ubiquitin-mediated degradation

While $p27^{Kip1}$ is downregulated as a consequence of HER2/neu signaling through enhanced ubiquitin-mediated degradation,[33,34] $p27^{Kip1}$ mRNA appears to be stable during the progression from G_1 to S phase induced by growth factors.[29] We investigated the possibility that the downregulation of $p27^{Kip1}$ by IGF-IR activation involves the ubiquitin/proteasome pathway. We observed that trastuzumab decreased levels of the F-box protein Skp2, which is a ubiquitin ligase for $p27^{Kip1}$,[36–38] but that this decrease was attenuated by IGF-I in SKBR3/IGF-IR cells (Fig. 5*a*). Immunoprecipitation further showed that IGF-I increased the Skp2 and $p27^{Kip1}$ association (Fig. 5*b*). IGF-I did not show the same effects in SKBR3/neo cells (data not shown).

To further characterize the mechanism of stimulation of $p27^{Kip1}$ degradation by IGF-I, we used the highly specific proteasome inhibitor LLnL. As shown in Figure 6*a,* the previously observed IGF-I-induced decrease of $p27^{Kip1}$ was completely blocked in the presence of LLnL. Furthermore, IGF-I increased $p27^{Kip1}$ ubiquitination and the ubiquitinated $p27^{Kip1}$ was markedly increased by blocking the proteasome pathway by LLnL (Fig. 6*b*). These results provide evidence that the IGF-I-induced reduction in $p27^{Kip1}$ level involves the ubiquitination/proteasome pathway.

IGF-I-induced decrease in $p27^{Kip1}$ involves the PI3K pathway

Upon activation by IGF-I binding, the IGF-IR tyrosine kinase leads to downstream activation of major signaling pathways, including the Ras/Raf/MAPK pathway and the PI3K pathway. To investigate the role of these pathways in linking IGF-IR signaling to antagonism of trastuzumab action in our system, we used PD98059 and LY294002, specific inhibitors of MAPK and PI3K, respectively. PD98059 at 50 μM reduced IGF-I-induced phosphorylation of MAPK, and LY294002 at 15 μM reduced IGF-I-induced phosphorylation of AKT in SKBR3/IGF-IR cells (Fig. 7*a*). Total levels of MAPK and AKT were not altered by either inhibitor.

Growth factor–induced degradation of $p27^{Kip1}$ proceeds through both the Ras/MAPK and PI3K pathways by multiple posttranslational regulation.[14,27,39] Therefore, we examined the role of IGF-I stimulation of the Ras/MAPK and PI3K pathways in the attenuation of the increase in $p27^{Kip1}$ induced by trastuzumab. Figure 7*b* shows that MAPK inhibition by PD98059 has a small effect on the basal level of $p27^{Kip1}$ and the trastuzumab-induced increase in $p27^{Kip1}$ and that IGF-I can still diminish the trastuzumab-induced increase in $p27^{Kip1}$ in SKBR3/IGF-IR cells. However, blockade of the PI3K pathway by LY294002 increased the basal level of $p27^{Kip1}$. IGF-I did not attenuate the trastuzumab-induced increase in the $p27^{Kip1}$ level in the presence of LY294002. This provides evidence for an important role of the PI3K pathway in the action of IGF-I as an attenuator of trastuzumab-induced accumulation of $p27^{Kip1}$.

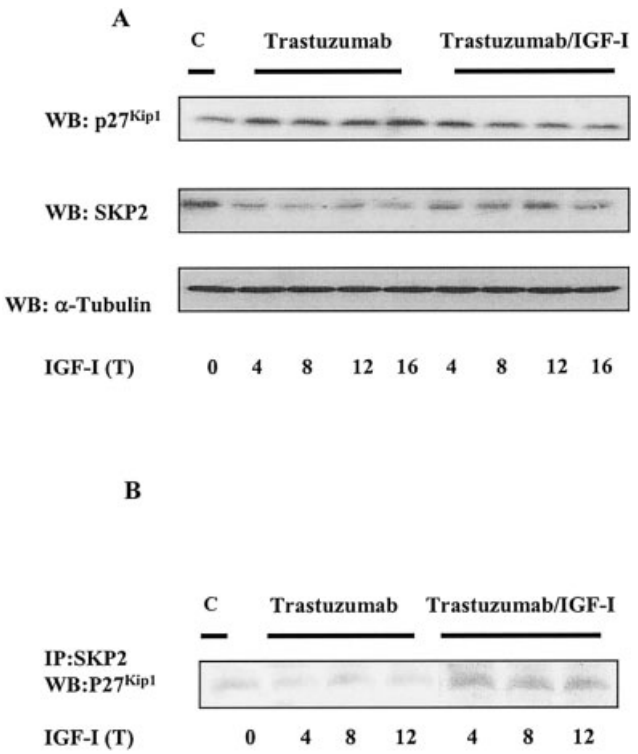

FIGURE 5 – IGF-I-induced decrease in $p27^{Kip1}$ is accompanied by increasing Skp2 expression and $p27^{Kip1}$ association with Skp2 in SKBR3/IGF-IR cells. After SKBR3/IGF-IR cells were treated or not with trastuzumab (10 μg/ml) in SFM for 16 hr, IGF-I (40 ng/ml) was added for a further 4, 8, 12 or 16 hr in serum-free conditions. *(a)* Expression of $p27^{Kip1}$, Skp2 and α-tubulin was determined by Western blotting. *(b)* $p27^{Kip1}$ association with Skp2 complexes was assessed through immunoprecipitation.

Finally, we examined the roles of the MAPK and PI3K pathways in the effects of IGF-I on Skp2 modulation. Trastuzumab reduced Skp2 levels. Consistent with the above results, PD98059 failed to influence the IGF-I-induced increase in Skp2, while LY294002 eliminated this increase (Fig. 7*b*). This provides evidence that the IGF-I-induced decrease in $p27^{Kip1}$ is correlated with a PI3K pathway–dependent increase in IGF-I-induced Skp2.

DISCUSSION

Although trastuzumab has important activity against HER2/neu-positive metastatic breast cancer,[7–9] development of resistance to this agent is common clinically.[8,9] Previous studies have shown that overexpression of HER2/neu is necessary but not sufficient to predict responsivity to trastuzumab.[17] Our data support the view that the Cdk inhibitor $p27^{Kip1}$ is a common downstream target of the HER2/neu and IGF-IR signaling pathways in breast cancer cells. We propose that each of these pathways acts in part by increasing the degradation of $p27^{Kip1}$ through activation of the PI3K signaling pathway and that increased activation of IGF-IR can compensate for the loss of HER2/neu function induced by trastuzumab treatment. This could account for the observed attenuation of trastuzumab action under conditions where IGF-I signaling is increased.[10]

In view of the modern concepts of networks of interacting intracellular signaling pathways, as distinct from multiple noninteracting signal-transduction systems,[27,39,40] we speculate that in-

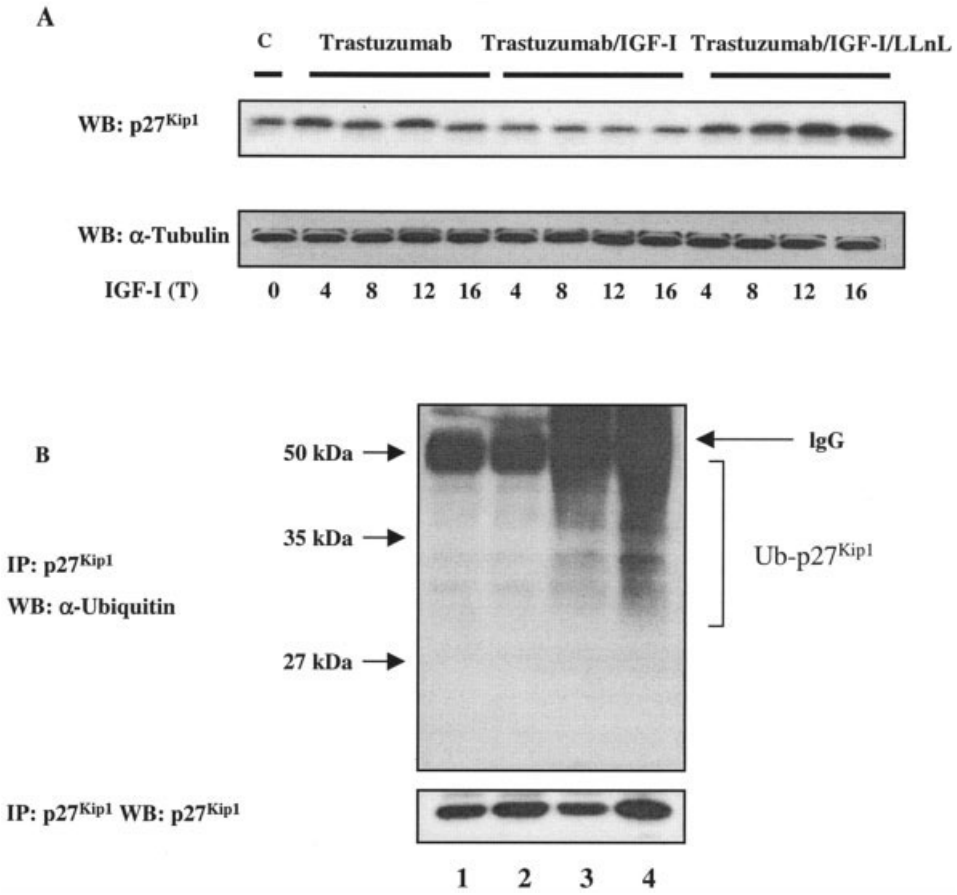

FIGURE 6 – IGF-I enhances ubiquitin-mediated degradation of $p27^{Kip1}$ in SKBR3/IGF-IR cells. *(a)* After SKBR3/IGF-IR cells were treated in the presence or absence of trastuzumab (10 μg/ml) in SFM for 16 hr, IGF-I (40 ng/ml) alone or with LLnL (50 μg/ml) was added at the times indicated (in hours) and $p27^{Kip1}$ and α-tubulin levels were determined by Western blot analysis. *(b)* After SKBR3/IGF-IR cells were treated or not with trastuzumab (10 μg/ml) in SFM for 16 hr, IGF-I (40 ng/ml) alone or with LLnL (50 μg/ml) was added for a further 12 hr. IGF-I-induced $p27^{Kip1}$ ubiquitination was examined by immunoprecipitation of $p27^{Kip1}$ followed by immunoblotting with anti-α-ubiquitin MAb. The same blots were reprobed with anti-$p27^{Kip1}$ MAb. Lane 1, control; lane 2, trastuzumab treatment; lane 3, trastuzumab treatment followed by IGF-I; lane 4, trastuzumab treatment followed by IGF-I and LLnL.

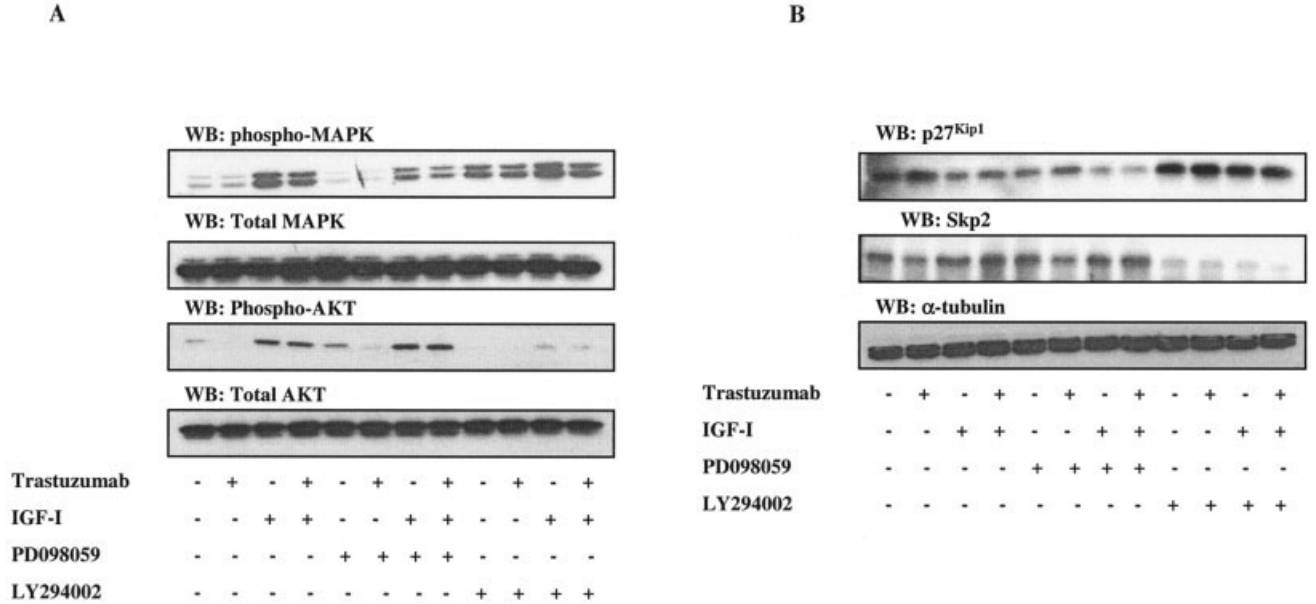

FIGURE 7 – IGF-I regulates $p27^{Kip1}$ mainly through the PI3K pathway. After SKBR3/IGF-IR cells were preincubated with trastuzumab (10 μg/ml), PD98059 (50 μm) or LY294002 (15 μm) was added for 16 hr under serum-free conditions. IGF-I (40 ng/ml) was added for a further 8 hr. *(a)* Phospho-MAPK, total MAPK, phospho-AKT and total AKT were determined by Western blot analysis. *(b)* $p27^{Kip1}$, Skp2 and α-tubulin levels were determined by Western blot analysis.

creased signaling at and downstream of the IGF-IR represents only one of several possible molecular mechanisms of trastuzumab resistance. Studies of paired clinical tumor specimens obtained prior to and after development of resistance to trastuzumab will be required to determine if a single mechanism predominates in the development of resistance in the clinic. This information will be

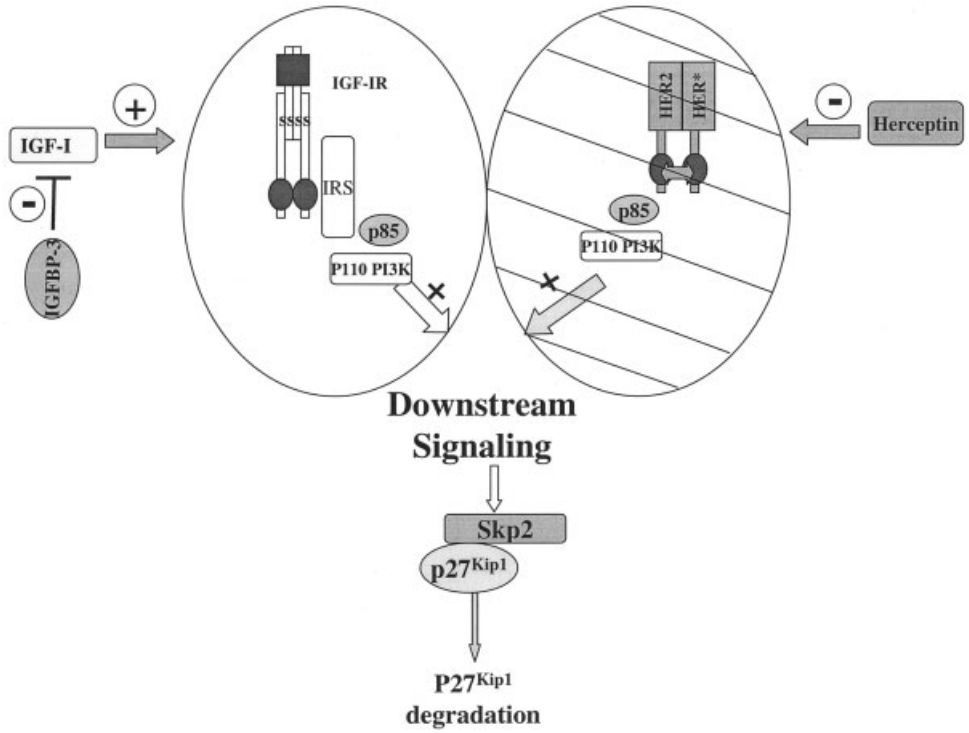

FIGURE 8 – Model of a mechanism by which IGF-I signaling attenuates trastuzumab-induced inhibition of breast cancer cell growth. Shaded area denotes signaling inhibited by trastuzumab. IGF-I signaling compensates for loss of HER2/neu-dependent PI3K activation. HER* indicates another member of the epidermal growth factor receptor family.

helpful in the design of novel approaches to delay or prevent trastuzumab resistance.

It has previously been shown that HER2/neu signaling reduces $p27^{Kip1}$ levels by enhancing $p27^{Kip1}$ degradation through ubiquitination.[33,34] Furthermore, it is known that by interfering with HER2/neu receptor signaling, trastuzumab increases $p27^{Kip1}$, promotes $p27^{Kip1}$ and Cdk2 association and inhibits Cdk2 activity.[16–18] In our model system, IGF-I attenuated the increase in $p27^{Kip1}$ induced by trastuzumab in SKBR3 cells overexpressing IGF-IR. Furthermore, the decrease in $p27^{Kip1}$ by IGF-I is accompanied by an increase in Skp2 expression and an increase in Skp2–$p27^{Kip1}$ association. Skp2 has been identified as a ubiquitin ligase for $p27^{Kip1}$ and is required for its ubiquitination and consequent degradation.[36–38] LLnL, a highly specific proteasome inhibitor, completely blocked the ability of IGF-I to reduce the $p27^{Kip1}$ protein level, which had been increased by trastuzumab pretreatment. Our data also show that IGF-I increases $p27^{Kip1}$ ubiquitination, and the ubiquitinated $p27^{Kip1}$ was markedly increased after blockage of the proteasome pathway by LLnL. These data suggest that IGF-I-induced antagonism of trastuzumab-induced G_1 arrest involves targeting of $p27^{Kip1}$ to the ubiquitin/proteasome degradation machinery. Consequently, IGF-I decreased the association of $p27^{Kip1}$ with Cdk2, markedly increased Cdk2 activity and released cells from G_1 arrest, which had been suppressed by trastuzumab in this cell line.

The proteasome plays a central role in the degradation of key regulatory proteins of the cell cycle and has therefore become an important therapeutic target for diseases involving cell proliferation, notably cancer.[41] Proteasome inhibition induces apoptosis, sensitizes cancer cells to traditional tumoricidal agents and overcomes drug resistance of cancer cells.[42,43] Our data combined with these results provide a rationale for further examination of proteasome inhibitors as potential therapeutic agents, even for trastuzumab-resistant tumors.

The PI3K pathway is downstream of both HER2/neu and IGF-R signaling. Activation of this pathway promotes cell growth, survival and resistance to treatment.[41,42] Deregulation of this pathway, by either gene amplification of PI3K or mutational loss of PTEN, is common in human cancers, including breast cancer.[25,44,45] Although the PI3K pathway can phosphorylate forkhead transcription factors (AFX) and inhibit AFX-mediated transcription of $p27^{Kip1}$, downregulation of $p27^{Kip1}$ mRNA is not associated with growth factor–mediated cell-cycle progression[29] and not often observed in human cancer.[32] Instead, intracellular levels of $p27^{Kip1}$ are highly regulated by 2 posttranslational mechanisms, ubiquitin proteasome-mediated degradation[29–32] and subcellular localization.[46–48] The IGF-IR-mediated resistance to trastuzumab treatment predominantly involved the PI3K pathway, leading to enhanced degradation of $p27^{Kip1}$.

Finally, our data are consistent with the model shown in Figure 8. The PI3K pathway leads to increased $p27^{Kip1}$ degradation. Both IGF-IR and HER2/neu can activate PI3K. Trastuzumab blockade of HER2/neu-induced PI3K signaling leads to growth inhibition in cells where HER2/neu is overexpressed, but this can be compensated for by increased IGF-IR signaling. It remains to be seen if, in general, the effectiveness of blockade of a particular tyrosine kinase receptor will clinically be limited by the activity of other receptors.

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