Cooperation between Fibroblast Growth Factor Receptor-4 and ErbB2 in Regulation of Cyclin D1 Translation*§

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Alterations in ErbB2 or fibroblast growth factor receptor-4 (FGFR-4) expression and activity occur in a significant fraction of breast cancers. Because signaling molecules and pathways cooperate to drive cancer progression, simultaneous targeting of multiple pathways is an appealing therapeutic strategy. With this in mind, we examined breast tumor cells for their sensitivity to the ErbB2 and FGFR inhibitors, PKI166 and PD173074, respectively. Simultaneous blocking of ErbB2 and FGFR-4 in MDA-MB-453 tumor cells had a stronger anti-proliferative effect than treatment with individual inhibitors. Examination of cell cycle regulators revealed a novel translation-mediated mechanism whereby ErbB2 and FGFR-4 cooperate to regulate cyclin D1 levels. Our results showed that FGFR-4 and ErbB2 via the MAPK and the phosphatidylinositol 3-kinase/protein kinase B pathways, respectively, both contribute to the maintenance of constitutive activity of the mammalian target of rapamycin translational pathway. Dual inhibition of these receptors strongly blocked S6 kinase 1 (S6K1) activity and cyclin D1 translation, as attested by a decrease in cyclin D1 mRNA association with polysomes. Ectopic expression of active protein kinase B or active S6K1 abrogated the dual inhibitor-mediated down-regulation of cyclin D1 expression, demonstrating the importance of these FGFR-4/ErbB2 signaling targets in regulating cyclin D1 translation. S6K1 has the central role in this process, since small interfering RNA-targeted S6K1 depletion led to a decrease in cellular S6K1 activity and, as a consequence, repression of cyclin D1 expression. Thus, we propose a novel mechanism for controlling cyclin D1 expression downstream of combined activity of ErbB2 and FGFR-4 that involves S6K1-mediated translation.

Deregulated activity of receptor tyrosine kinases (RTKs)† has been implicated in many human cancers. In particular, the overexpression of the ErbB2 receptor has been detected in ~30% of breast cancers and correlates with poor patient prognosis (1). Another 30% of breast tumors displayed overexpression of FGFR-4, a member of a different RTK family (2, 3). High expression of ErbB2 in cancer cells leads to ligand-independent constitutive activation of the receptor and its downstream signaling, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (P3K) pathways (4). FGFR also activates these major cellular signaling pathways (5). Although FGFR and ErbB2 activities are clearly important in physiological processes of development, aberrant expression of these receptors affects cell proliferation, migration, survival, and angiogenesis, all hallmarks of cancer progression (5–7). A number of studies have shown that inhibition of ErbB2 activity blocks proliferation of ErbB2-overexpressing cells (8–11). Recently, we have reported that down-regulation of FGFR-mediated signaling in breast tumor cells with high activity of these receptors also causes inhibition of cell proliferation (12). Although both classes of receptors are widely expressed in breast cancer and both have been implicated in cell cycle control, there have been no reports examining the functional cooperation between these receptor families, the topic of this paper.

The mTOR translational signaling pathway also participates in regulation of cell proliferation (13). Interestingly, the overexpression of the mTOR target, S6K1, is a negative prognostic indicator for breast cancer and amplification of both S6K1 and ErbB2 is associated particularly with poor survival (14–16). mTOR cooperates with MAPK and effectors of P13K to phosphorylate S6K1 (17). Activated S6K1 phosphorylates the 40S ribosomal subunit protein S6 (18), which promotes the translation of mRNAs containing 5′-terminal oligopyrimidine tracts. The translational repressor protein eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) is another downstream effector of mTOR. 4E-BP1 interacts with eIF4E and inhibits cap-dependent translation. Rapamycin, a bacterially derived drug that inhibits mTOR activity and phosphorylation of S6K1 and 4E-BP1, causes a proliferative block in many types of cells (19, 20). Some studies have identified cyclin D1 as a target of rapamycin-mediated inhibition of cell proliferation. However, depending on the cell type, this drug has been reported to regulate cyclin D1 expression by various means, including the control of mRNA and protein turnover, as well as translation (21, 22). Furthermore, rapamycin increases the expression of p27, an inhibitor of cyclin/cdk complexes, at both mRNA and protein levels (20, 23). Although these results indicate that mTOR activity is important for cell cycle progression, the mechanism of translational regulation in cell proliferation is still not fully understood.

FGFR activity also contributes to cell proliferation via regulation of S6K1 phosphorylation (24, 25). However, how the FGFR-mediated S6K1 regulation is linked to the cell cycle machinery has not been described. In this work, we investi-
gated the mechanism by which FGFR-4 and ErbB2 receptor signaling contribute to S6K1-regulated cell proliferation in MDA-MB-453 breast cancer cells, a cell line in which both receptors are constitutively active. To block the receptors, we used the small molecule tyrosine kinase inhibitors PD173074 and PKI166 that selectively inhibit activation of FGFR and ErbB2, respectively. The combined use of the inhibitors causes significant down-regulation of mTOR pathway activity, inhibition of cyclin D1 translation, and, as a consequence, a strong block in cell proliferation. Our studies demonstrated that both MAPK and PI3K, acting downstream of FGFR and ErbB2, control S6K1 and 4E-BP1 phosphorylation, thereby cooperatively regulating the activity of the mTOR pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, Inhibitors, and Antibodies—MDA-MB-453 human breast cancer epithelial cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen) at 37 °C in a humidified CO2 (5%) incubator. The inhibitors were used as follows: the FGFR inhibitor, PD173074 (26), the ErbB2 inhibitor, PKI166 (27), and the mTOR inhibitor, rapamycin (Mitsubishi Chemical). The antibodies used for Western blotting and immunoprecipitation were as follows: polyclonal antibodies against FGFR-4 (C-16), cdk4 (H-22), obtained from Santa Cruz Biotechnology; monoclonal antibody against cyclin D1 from Novocastra Laboratories; polyclonal antibody against cyclin D1 from Chemicon International; polyclonal antibodies against p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), Akt, phospho-Akt (Ser473), mTOR, phospho-mTOR (Ser2448), S6K1, phospho-S6K1 (Thr421/Ser424), phospho-S6K1 (Thr389), S6, phospho-S6 (Ser240/Ser244), 4E-BP1, phospho-4E-BP1 (Ser65), and elf4E, all from Cell Signaling Technology; and a polyclonal antibody for ERβ2 for ErbB2 (21N) (28).

Fenomenology Assay—To determine cell proliferation, MDA-MB-453 cells were plated in 96-well plates and, after 24 h, treated with MeSO, PD173074 (2 μM), and/or PKI166 (2 μM). Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation kit I (Roche Applied Science) after 0, 4, and 8 days of treatment. To analyze the cell cycle, MDA-MB-453 cells were plated on 10-cm dishes. 24 h later, these cultures were treated with MeSO or inhibitors (2 μM) for 24 h and trypsinized and then the cells were processed for fluorescence-activated cell sorter analysis as previously described (29).

Western Blot Analysis—Cells were plated on 6- or 10-cm plates. 24 h later, the cultures were treated with MeSO or inhibitors (2 μM) for 8 h, cell lysates were prepared as above, and immunoprecipitation was done according to standard procedures as described previously (8). Signals were detected by ECL (Amersham Biosciences).

mRNA Isolation and Northern Blot Analysis—MDA-MB-453 cells were plated on 6-cm plates. After 24 h, cells were treated with MeSO or the indicated inhibitors (2 μM) and harvested at the indicated times. Total RNA (10 μg) was prepared with TRIzol reagent (Invitrogen). Northern blot analysis was performed as described previously (30). A CDNA clone for human cyclin D1 (obtained from B. Amati) was labeled and used as a probe. The mouse β-actin probe was obtained from Ambion. Levels of specific RNA were measured in a Molecular Dynamics PhosphorImager.

**RESULTS**

**FGFR-4 and ErbB2 Cooperate to Maintain Constitutive Activation of MAPK and PI3K Signaling and to Drive Proliferation of MDA-MB-453 Breast Tumor Cells**—The MDA-MB-453 breast tumor cells display active FGFR-4 that promotes constitutive MAPK signaling. We have previously shown that proliferation of these cells depends mainly on FGFR-MAPK-mediated signaling (12). Interestingly, MDA-MB-453 cells also upregulate ErbB2, leading to its constitutive activation. In this study, we asked what signaling pathways were driven by ErbB2 in these cells and whether there was any cooperation between the two receptors in terms of the proliferative response. To address this question, we used small molecule tyrosine kinase inhibitors selective for FGFRs (PD173074) (26) and for ErbB receptors (PKI166) (27). The treatment of the cells with PD173074 or PKI166 led to decreased activity of FGFR-4 or ErbB2, as shown by the lower phosphotyrosine content of the respective immunoprecipitated receptor (Fig. 1A). The activity of the MAPK and PI3K pathways was examined by determining phospho-ERK1/2 and phospho-PKB levels as a readout for the activation status of the respective pathway (Fig. 1B). As previously shown, the inhibition of FGFR-4 blocks activity of the MAPK pathway in MDA-MB-453 cells (12). The loss of ErbB2 activity results in the down-regulation of PI3K signaling, whereas simultaneous targeting of the receptors with PD173074 and PKI166 suppressed the activity of both pathways (Fig. 1B). Interestingly, we consistently observed that simultaneous treatment with both inhibitors led to a stronger decrease in phospho-PKB levels in comparison with treatment with PKI166 alone, suggesting a possible cooperation between FGFR-4 and ErbB2 in the regulation of this pathway.

The proliferation of MDA-MB-453 cells in response to individual or combined inhibitor treatment was examined next. Cells treated with either PKI166 or PD173078 were blocked by 26 and 61% in proliferation, respectively. Proliferation of the cells treated simultaneously with both inhibitors was completely blocked (Fig. 1C). To explore the anti-proliferative effects of the inhibitors at the level of the cell cycle, we performed flow cytometry analyses. The inhibition of ErbB2 signaling did not lead to a dramatic change in G1-accumulated cells (+2%), nor did cells accumulate in any other phase of the cell cycle (Fig. 1D and data not shown); however, as shown above, this increase was sufficient to slow down proliferation over the time course. PD173078 treatment led to a strong increase in G1-accumulated cells (+24%, Fig. 1D) as previously reported (12), and treatment with both inhibitors led to a further increase in G1 accumulation (+28%) (Fig. 1D). Taken together, these results suggest that FGFR-4 and ErbB2 cooperate to control cell proliferation via their major downstream effector pathways,
MAPK and PI3K. Based on cell counts and the cell cycle analysis, it appears that the combined activity of these pathways has a major impact on G1 regulators.

**FGFR-4 and ErbB2 Cooperate to Regulate Cyclin D1 Expression**

Cyclin D1 is a major cell cycle regulator in breast tumor cells with active FGFR signaling (12). As a result, we examined the effect of dual inhibitor treatment on cyclin D1 expression. The treatment of MDA-MB-453 cells with PD173074 caused a decrease in cyclin D1 mRNA (Fig. 2A), which we have previously shown was due to transcriptional regulation (12), and protein expression (Fig. 2B). Neither cyclin D1 mRNA nor its protein levels were altered in PKI166-treated cultures (Fig. 2, A and B). Intriguingly, cells treated with both inhibitors had

**Fig. 1.** Effect of FGFR and ErbB2 receptor-targeted inhibitors on signaling molecules and proliferation of MDA-MB-453 breast cancer cells. A, MDA-MB-453 cells were treated with Me2SO (DMSO), the FGFR inhibitor PD173074 (PD), and/or the ErbB2 inhibitor PKI166 (PKI) at concentrations of 2 μM for 30 min. Receptors were immunoprecipitated (IP) from 1 mg of protein lysates. After immunoblotting with a phosphotyrosine-specific antibody (PTyr), the membranes were reprobed to control for FGFR-4 and ErbB2 protein levels. B, cells were treated with the indicated inhibitors harvested after 8 h, and protein lysates were analyzed by immunoblotting with phosphospecific antibodies against ERK1/2 (ERK1/2_P) and PKB (PKB_P). The membrane was reprobed to control for ERK1/2 and PKB protein levels. C, MDA-MB-453 were treated with Me2SO (DMSO) (●), PD173074 (●), PKI166 (●), or PD173074 and PKI166 (×) at a concentration of 2 μM each. Cell proliferation was measured by MTT assays at days 0, 4, and 8. Assays were performed in triplicates, and mean values ± S.D. are shown with error bars. D, cells were treated with the indicated inhibitors (2 μM) for 24 h. Cells were collected, and the cell cycle distribution was analyzed by flow cytometry. The increase in percentage of cells in the G1 phase of the cell cycle in inhibitor-treated compared with Me2SO-treated cells is indicated. The table shows data from two independent experiments.

**Fig. 2.** Effect of FGFR and ErbB2 inhibitors on cyclin D1 expression and protein synthesis. A, MDA-MB-453 cells were treated with the indicated inhibitors (2 μM). At the indicated times, cells were harvested and cyclin D1 mRNA was examined by Northern blot analysis using a specific probe. Equivalent loading of RNA was confirmed by methylene blue staining of 18 and 28 S rRNA. The graph in the lower panel shows normalized cyclin D1 mRNA quantification measured by PhosphorImager. B, cells were treated with the indicated inhibitors for 8 h, and protein lysates were analyzed by immunoblotting with cyclin D1 and cdk4 antibodies. Equivalent loading was confirmed by reprobing with anti-tubulin antibody. DMSO, Me2SO. C, cells were treated with inhibitors for 8 h and pulse-labeled for the last 1 h with [35S]methionine, and protein lysates were prepared. Cyclin D1 and cdk4 were immunoprecipitated (IP) from the lysates with specific antibodies, proteins were separated on a 10% SDS-PAGE, and the level of [35S]methionine incorporation was determined by PhosphorImager scanning. Graphs represent data from four independent experiments, and the mean values ± S.D. are shown with error bars. PD, the FGFR inhibitor PD173074; PKI, the ErbB2 inhibitor PKI166; rap, rapamycin.

**S6K1-mediated Cyclin D1 Translation**

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**FGFR-4 and ErbB2 Cooperate to Regulate Cyclin D1 Expression**—Cyclin D1 is a major cell cycle regulator in breast tumor cells with active FGFR signaling (12). As a result, we examined the effect of dual inhibitor treatment on cyclin D1 expression. The treatment of MDA-MB-453 cells with PD173074 caused a decrease in cyclin D1 mRNA (Fig. 2A), which we have previously shown was due to transcriptional regulation (12), and protein expression (Fig. 2B). Neither cyclin D1 mRNA nor its protein levels were altered in PKI166-treated cultures (Fig. 2, A and B). Intriguingly, cells treated with both inhibitors had
the same level of cyclin D1 mRNA as PD173074-treated cells (Fig. 2A), whereas the level of cyclin D1 protein was reduced (Fig. 2B). The expression of cdk4, the partner of cyclin D1, was not affected by any treatment (Fig. 2B). The PD173074-mediated block and the dual inhibitor-mediated block in cell proliferation correlated well with changes in cyclin D1 expression (Fig. 1C). These data suggested that the FGF and ErbB2 receptors cooperate via the downstream MAPK and PI3K pathways to control cyclin D1 expression and implied that simultaneously blocking the receptors affects cyclin D1 at a post-transcriptional level.

To obtain some insight into whether the RTK inhibitors act at the level of cyclin D1 translation, we compared their effects with those of rapamycin, an inhibitor of mTOR (35). MDA-MB-453 cultures were treated with the inhibitors and then labeled with [35S]Met, and the incorporation of label into cyclin D1 was quantified. In PD173074-treated cultures, there was a 36% decrease in [35S]Met-labeled cyclin D1 (Fig. 2C), which is likely to reflect the reduced cyclin D1 mRNA expression in these cells (Fig. 2A). As expected, in PKI166-treated cells [35S]Met-labeled cyclin D1 levels were the same as in Me2SO-treated control cells (Fig. 2C), reflecting the fact that this inhibitor had no effect on cyclin D1 expression (Fig. 2, A and B). In cultures treated with the combination of receptor inhibitors, the level of [35S]Met-labeled cyclin D1 was ~20% decreased in comparison with its level in cells treated with PD173074 alone. Inhibition of mTOR with rapamycin resulted in a 15% decrease in [35S]Met-labeled cyclin D1 levels. The incorporation of [35S]Met into cdk4 was unaffected by the inhibitors (Fig. 2C). Because rapamycin had no effect upon cyclin D1 mRNA expression (data not shown), the results suggested that translational control accounts for ~15–20% cyclin D1 levels. Interestingly, the enhanced reduction (~20%) of [35S]Met incorporation into cyclin D1 in the presence of both RTK inhibitors compared with treatment with PD173074 alone was similar to the effect of rapamycin. These results suggested that the FGF and ErbB2 receptors and their downstream signaling pathways cooperate in the regulation of cyclin D1 translation. Moreover, they suggested that the cooperation is targeted to mTOR-dependent translation.

**FGF and ErbB2 Receptors Cooperate to Regulate Activity of the mTOR Pathway**—To gain insight into the molecular mechanism regulating cyclin D1 translation downstream of FGFR-4 and ErbB2, we examined the phosphorylation status of mTOR and its two effectors, S6K1 and 4E-BP1. One of the major phosphorylation sites thought to be important in controlling mTOR activity is Ser2448 (36), which was shown to be a target of the PKB pathway (37, 38). S6K1 activation is a multistep process that begins with the phosphorylation of amino acid residues Ser411, Ser418, Thr421, and Ser424 within the autoinhibitory domain by proline-directed kinases such as MAPK (17, 39). This process is followed by mTOR-mediated phosphorylation of Thr389, an indicator of S6K1 activity (18). mTOR signaling also controls the phosphorylation of 4E-BP1 on multiple residues (40). Its final phosphorylation on Ser65 results in 4E-BP1 dissociation from eIF4E and activation of cap-dependent translation (41). Only simultaneous inhibitor treatment, resulting in complete down-regulation of PKB activity (Fig. 1B), resulted in a strong decrease in mTOR phosphorylation at the PKB target site Ser2448 (Fig. 3A). Inhibition of FGFR-4 activity caused a decrease in the phosphorylation of S6K1 on MAPK-regulated sites (Thr421/Ser424). As a consequence, S6K1 activity measured by phospho-Thr389 levels was diminished, as were levels of phosphorylated S6 (Fig. 3A). PD173074 treatment also decreased 4E-BP1 phosphorylation, as confirmed by an accumulation of the hypophosphorylated faster-migrating form of the protein and by a decrease in phospho-Ser65 levels (Fig. 3A). Inhibition of ErbB2 signaling had no effect on S6K1 activity, although a decrease in phospho-4E-BP1 levels was observed (Fig. 3A). Finally, dual inhibitor treatment completely blocked S6K1 activity, as confirmed by the absence of phosphorylation on Thr389 and on its substrate, S6. Phospho-4E-BP1 levels were also strongly decreased in these cells (Fig. 3A). To confirm the importance of FGFR-4 and ErbB2 activity in the regulation of 4E-BP1, we performed m’GTP-Sepharose pull-down assays. m’GTP mimics the cap structure of mRNA and can be used to affinity-purify eIF4E and its associated proteins. The treatment of cells with PD173074 or PKI166 caused an increase in eIF4E/4E-BP1 association compared with control Me2SO treatment (Fig. 3B), reflecting the 4E-BP1 phosphorylation status (Fig. 3A). Importantly, the association of eIF4E and 4E-BP1 was the highest in lysates from cells simultaneously treated with both inhibitors (Fig. 3B). Taken together, these results showed that, in MDA-MB-453 cells, combined FGFR-4 and ErbB2 signaling was required to maintain full activity of mTOR effector molecules.

**FGF-4 and ErbB2 Signaling Control the Polysomal Distribution of Cyclin D1 mRNA**—To further examine the involvement of FGFR-4 and ErbB2 in cyclin D1 translation, we ana-
lyzed the distribution of cyclin D1 mRNA on polysome profiles. Following the treatment of MDA-MB-453 cells with inhibitors, total cell extracts were separated on sucrose gradients and fractions were analyzed for cyclin D1 mRNA. The distribution of β-actin mRNA was followed as a control. PKI166 treatment did not affect the polysomal distribution of cyclin D1 mRNA. In contrast, PD173074 treatment caused a 9% decrease in cyclin D1 mRNA polysome association (Fig. 4), reflecting a moderate decrease in its translation. The major change in translation was observed after dual inhibitor or rapamycin treatment reflected by a decrease in polysome-associated cyclin D1 mRNA of 16 and 17%, respectively (Fig. 4). These results showed that FGFR-4 activity alone can influence cyclin D1 translation to a minor extent, but importantly, combined signaling from FGFR-4 and ErbB2 has a significant effect on this regulation. Additionally, the level of S6K1 activity following inhibitor treatments (Fig. 3A) paralleled the loss of polysomal-associated cyclin D1 mRNA (Fig. 4), suggesting that S6K1 has an important role in this process.

**Ectopic Expression of Active PKB and S6K1 Rescues Cells from Inhibitor-mediated Cyclin D1 Loss**—Because dual inhibitor treatment strongly down-regulated PKB phosphorylation (Fig. 1B) and resulted in the inhibition of S6K1 phosphorylation (Fig. 3A), we asked whether active PKB could rescue the activity of S6K1 and thus restore cyclin D1 expression. To examine this possibility, we transiently expressed a membrane-targeted constitutively active PKB (Myr-PKB) in MDA-MB-453 cells by retroviral infection. EGFP-expressing virus-infected cells served as control and displayed a complete loss of cyclin D1 in response to simultaneous treatment with both inhibitors (Fig. 5) as did the parental cells (Fig. 2B). In contrast, cells expressing active PKB were resistant to dual inhibitor treatment, i.e. the cyclin D1 level was not further down-regulated in comparison with cells treated with PD173074 alone. As mentioned above, this level probably reflects the consequences of decreased cyclin D1 transcription resulting from FGFR inhibition. Importantly, active PKB also rescued S6K1 activity as measured by S6 phosphorylation, (Fig. 5, PD+PKI lane), strengthening our hypothesis that S6K1-mediated translational activity is involved in cyclin D1 regulation. Finally, we directly tested the role of S6K1 in the regulation of cyclin D1 expression by infecting cells with a retrovirus expressing an active form of the kinase (S6K1-E389D) (34). Similar to the results observed with active Myr-PKB, active S6K1 had no effect upon the basal level of cyclin D1 (DMSO lanes). This finding suggested that cyclin D1 expression might already be at a maximum in these tumor cells. Importantly, in dual inhibitor-treated cells, active S6K1 restored cyclin D1 levels to a similar extent as the active PKB did (Fig. 5). These results confirmed the involvement of PKB-S6K1 signaling in regulation of cyclin D1 expression downstream of FGF and ErbB2 receptors.

**S6K1 Is an Important Regulator of Cyclin D1 Expression**—To directly establish the role of S6K1 in the regulation of cyclin D1 expression, we used a specific siRNA to knock down its expression in MDA-MB-453 cells. The transfection of cells with two different S6K1 siRNAs, S6K1A and S6K1B, covering either the coding or non-coding sequences, respectively, resulted in a significant decrease in S6K1 levels when compared with control

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**Fig. 4. Cyclin D1 mRNA distribution on polysomes from inhibitor-treated cells.** MDA-MB-453 cells were treated with the indicated inhibitors or Me2SO (DMSO) for 8 h, and polysomes from cellular lysates were fractionated on sucrose gradients. Northern blots were performed on the indicated gradient fractions for cyclin D1 and β-actin mRNA. The percent of indicated mRNAs present in the polysomal fractions (6–12) is shown to the right of the autoradiographs. The densitometric analyses of the signals obtained from Northern blot PhosphorImager scanning are shown as a graphic representation on the right. PD, the FGFR inhibitor PD173074; PKI, the ErbB2 inhibitor PKI166; Rap or rap, rapamycin.
with ErbB2, regulates mTOR-dependent cyclin D1 translation. Simultaneous inhibition of FGFRs depends largely on FGFR/MAPK-mediated transcriptional regulation of the D-type cyclins (12). In this study, we describe a novel interaction between FGFR-4 and ErbB2 in the regulation of cyclin D1 expression. Inhibitor-mediated inactivation of FGFR and MAPK signaling caused down-regulation of cyclin D1, whereas the combination of inhibitors and, to a smaller extent, FGFR-4 and ErbB2 activity in MDA-MB-453 breast cancer cells infected with a retrovirus expressing active S6K1 (S6K1-E389D,E) were transfected with control LacZ siRNA and two S6K1 siRNAs (S6KA and S6KB). Two days after transfection, cells were cultured. Cell lysates were analyzed by immunoblotting with antibodies against cyclin D1, phospho-S6 (S6_P), and S6K1. Equivalent loading was confirmed by reprobing with anti-MAPK antibody. The numbers represent the percentage of measured decrease in S6 phosphorylation and cyclin D1 expression compared with LacZ-transfected cells normalized to MAPK expression.

**DISCUSSION**

The process of cell proliferation is controlled by an integrated array of signaling pathways. We have previously shown that the proliferation of breast cancer cells displaying high activity of FGFRs depends largely on FGFR/MAPK-mediated transcriptional regulation of the D-type cyclins (12). In this study, we describe a novel interaction between FGFR-4 and ErbB2 in regulating cyclin D1 translation. Inhibition of FGFR-4 and ErbB2 activity in MDA-MB-453 breast cancer cells had a cooperative effect on down-regulation of cyclin D1 levels and resulted in a strong block in cell proliferation. Based on the results presented here, we propose a model of RTK cooperation in regulating cyclin D1 expression. FGFR signaling stimulates cyclin D1 transcription, and FGFR, in cooperation with ErbB2, regulates mTOR-dependent cyclin D1 translation (Fig. 7).

Previous reports have shown that signaling from FGFR or ErbB2 is involved in S6K1 activation (24, 25, 42). Here we demonstrate that each receptor class can contribute to S6K1 activity by regulating distinct pathways. Inhibitor-mediated inactivation of FGFR and MAPK signaling caused down-regulation of S6K1 phosphorylation at MAPK-directed sites in the autoinhibitory domain, resulting in a partial block of S6K1 activity. Inhibition of the MAPK and PKB pathways, as a consequence of combined PD173074 and PKI166 treatment, caused a complete block of S6K1 activity. Furthermore, the inhibition of MAPK activity alone or simultaneous down-regulation of MAPK and PI3K activity by the selective inhibitors, UO126 and wortmannin, had the same effect on S6K1 phosphorylation as receptor inhibitors, proving the importance of these pathways in S6K1 regulation in MDA-MB-453 cells (data not shown). Although several earlier studies demonstrate that the MAPK pathway is not necessary for S6K1 activation (43, 44), recent reports demonstrate the importance of this pathway in S6K1 phosphorylation (45, 46). These studies together with our results suggest that, depending upon the cell type and nature of the stimulant, different pathways control S6K1 activation.

Although both mTOR targets, S6K1 and 4E-BP1, were affected by inhibitor treatment, our results suggest that S6K1 plays a more important role in translational control of cyclin D1. The combination of inhibitors and, to a smaller extent, PD173074 alone caused a shift of cyclin D1 mRNA from the
polysomal fraction to the monosomal fraction, indicating a decrease in translational efficiency. This pattern of polysomal distribution correlated well with the activation status of S6K1, but not that of 4E-BP1, following inhibitor treatment. Although 4E-BP1 was also affected by PKI166 alone, this did not change the translational state of cyclin D1 mRNA. Moreover, the depletion of S6K1 by siRNA as well as rescue experiments with ectopically expressed active-PKB and active-S6K1 verified the important role of the S6K1 pathway in the regulation of cyclin D1 translation. Interestingly, the human cyclin D1 mRNA has a putative 5′-terminal oligopyrimidine motive (47), consistent with our hypothesis that it is regulated by S6K1.

Control of cyclin D1 expression occurs by various mechanisms. For example, proteasome-mediated degradation has been reported to influence cyclin D1 protein levels (48). This possibility was excluded in MDA-MB-453 cells, because the ~15-min half-life of cyclin D1 protein remained constant in the inhibitor-treated cells (data not shown). With respect to mTOR-mediated control of cyclin D1 expression, rapamycin treatment of NIH3T3 fibroblasts enhances cyclin D1 protein degradation and lowers the level of cyclin D1 by increasing mRNA turnover (21). In our studies, rapamycin treatment resulted in decreased cyclin D1 mRNA polysomal association and, as a consequence, a lower protein level without affecting cyclin D1 mRNA (data not shown). In serum-starved NIH3T3 fibroblasts, overexpression of eIF4E, which titrates out 4E-BP1, caused redistribution of cyclin D1 mRNA from the nucleus to the cytoplasm and thus increased its translation (49, 50). However, in our experiments, the inhibitor-mediated increase in 4E-BP1/eIF4E association did not lead to decreased cytoplasmic cyclin D1 mRNA as assessed by polysomal fractionation and by Northern blotting of cytoplasmic and nuclear fractions (data not shown). The mechanism of cyclin D1 regulation downstream of mTOR described here differs from those discussed above, clearly showing that cyclin D1 is a target of S6K1-mediated translational control. Importantly, considering that down-regulation of S6K1 activity correlated with the inhibition of MDA-MB-453 cell proliferation, our data are in agreement with studies showing that microinjection of antibodies against S6K1 leads to a G1 block in the cell cycle (13).

Translation is becoming an increasingly attractive target for cancer control. For example, it was recently shown that, in oncogenic glioma cells, the primary effect of Ras and PKB signaling on gene expression occurred at the translational rather than the transcriptional level (51). The data presented here indicate that, in breast cancer cells with constitutive MAPK and PI3K signaling, translation is an important component of cyclin D1 regulation. We considered it probable that cyclin D1 is not the only mRNA affected in these cells. Future work will be aimed at identifying additional targets. Moreover, blocking both pathways with the RTK inhibitors had the strongest impact on cell proliferation, which paralleled the effects on mTOR and S6K activity, showing the importance of mTOR as a cancer target.

Both ErbB2 and FGFRs have been implicated in breast cancer development. As such, there are a number of ErbB2- or FGFR-directed therapeutics in various stages of preclinical or clinical development for cancer treatment (52, 53). Our studies show that targeting both FGFR and ErbB2 has a cooperative effect on blocking cell proliferation and down-regulating cyclin D1 expression. Thus, our results confirmed that the FGFR and ErbB2 tyrosine kinase families represent exciting targets for anti-cancer therapeutic agents. Moreover, they demonstrated the potential of simultaneously blocking multiple RTKs and, more generally, multiple signaling pathways for future treatment modalities.

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