The Cyclin-dependent Kinase Inhibitor \( p21^{\text{CIP/WAF}} \) Is a Positive Regulator of Insulin-like Growth Factor I-induced Cell Proliferation in MCF-7 Human Breast Cancer Cells*

Joëlle Dupont‡, Michael Karas, and Derek LeRoith§

To study the role of IGF-I receptor signaling on cell cycle events we utilized MCF-7 breast cancer cells. IGF-I at physiological concentrations increased the level of \( p21^{\text{CIP/WAF}} \) mRNA after 4 h as well as protein after 8 h by 10- and 6-fold, respectively, in MCF-7 cells. This IGF-I effect was reduced by 50% in MCF-7-derived cells (SX13), which exhibit a 50% reduction in IGF-1R expression, demonstrating that IGF-1 receptor activation was involved in this process. Preincubation with the ERK1/2 inhibitor U0126 significantly reduced the IGF-1 effect on the amount of \( p21^{\text{CIP/WAF}} \) protein in MCF-7 cells. These results were confirmed by the expression of a dominant negative construct for MEK-1 suggesting that the increase of the abundance of \( p21^{\text{CIP/WAF}} \) in response to IGF-1 occurs via the ERK1/2 mitogen-activated protein kinase pathway. Using an antisense strategy, we demonstrated that abolition of \( p21^{\text{CIP/WAF}} \) expression decreased by 2-fold the IGF-1 effect on cell proliferation in MCF-7. This latter result is explained by a delay in G1 to S cell cycle progression due partly to a reduction in the activation of some components of cell cycle including the induction of cyclin D1 expression in response to IGF-1. MCF-7 cells transiently overexpressing \( p21 \) showed increased basal and IGF-I-induced thymidine incorporation. Taken together, these results define \( p21^{\text{CIP/WAF}} \) as a positive regulator in the cell proliferation induced by IGF-1 in MCF-7 cells.

Insulin-like growth factors are potent mitogens for the estrogen receptor-positive breast cancer cells including the MCF-7 human breast cancer cell line (1, 2). The proliferative effect is mediated mainly by the insulin-like growth factor-I receptor (IGF-1R), which can be inhibited by using a blocking antibody, \( \alpha \)IgR3 (3), or an antisense RNA (4) to this receptor. In vivo, some clinical and experimental data showed that the IGF-1R is involved in tumorigenesis of breast tissue (5). Furthermore, the tumorigenic potential of IGF-1R is usually dependent on the hyperactivation of IGF-1 signaling pathways.

The IGF-1R is a tyrosine kinase receptor, which is activated following ligand binding. Once autophosphorylated, IGF-1R binds and phosphorylates on tyrosine various substrates such as the insulin receptor substrates (IRS-1 to 4) (6–9) and Shc (10). These substrates serve as docking molecules for other proteins containing SH2 domains including the p85 regulatory subunit of PI3K and Grb2 that lead to the activation of the two main signaling pathways, the PI3K/Akt (11) and the MAPK pathways (12). In the ER-positive cell lines, IRS-1 is the main substrate activated by the IGF-1R in the growth stimulatory effects of IGF-1 (13, 14). The Shc pathway supports growth but also plays a role in the processes of cell motility and cell-cell aggregation (15). In MCF-7, the PI3K pathway but not the MAPK is crucial in the cell cycle progression induced by IGF-1 (1). The mechanisms by which IGF-1 induces cell proliferation involve a regulation of the cell cycle machinery. Progression through the cell cycle is regulated by sequential activation and subsequent inactivation of a series of cyclin-dependent kinases (CDKs) at different phases of the cell cycle (16, 17). In normal cells, the transition from G1 to S phase requires the activity of two classes of CDKs, CDK4/6, and CDK2. As cells emerge from quiescence in response to mitogenic stimuli, Dtype cyclins are synthesized and associate with CDK4/6. This cyclin D-CDK4/6 complex hyperphosphorylates the retinoblastoma protein (Rb), leading to its release from the transcription factor E2F (18, 19). The free transcription factor E2F then activates the genes responsible for cellular proliferation including cyclin E. Cyclin E binds then to CDK2 contributing to kinase activation and G1-to-S phase progression. CDK activity is regulated by multiple mechanisms, including phosphorylation (20–23) and association with both positive and negative regulatory proteins. CDKs activity can be inhibited by two different families of cyclin-dependent kinase inhibitors (CDKIs). Members of the INK4 family, including p15\(^{\text{INK4B}}\), p16\(^{\text{INK4A}}\), p18\(^{\text{INK4C}}\), and p19\(^{\text{INK4D}}\) bind specifically to monomeric CDK4/6 and prevent its association with a D-cyclin (24, 25). Members of the WAF1/Cip1 family, including p21\(^{\text{WAF1/Cip1}}\), p27\(^{\text{Kip1}}\), and p57\(^{\text{Kip2}}\) bind to G1 cyclin-Cdk complexes and not to monomeric cyclins or CDKs (18, 27).

IGF-1 has been shown to regulate both the expression and the activity of various molecules involved in the cell cycle progression. In Rat L6E9 myoblasts, IGF-1 increases cyclin D1,
and CDK4 gene expression and Rb phosphorylation (28). In MCF-7 cells, IGF-1 induces cyclin D1 expression and hyperphosphorylation of Rb through the PI3K and not the MAPK pathway (1). IGF-1 has also been shown to regulate the CDKIs. In melanoma cells, IGF-1 is involved in the redistribution of p27, which is a mechanism for growth arrest (29). Surprisingly, in a previous study from our laboratory and in a good agreement with another study, we showed that p21CIP/WAF expression is increased by IGF-1 in MCF-7 cells (1, 2). Here, we investigated the regulation of p21CIP/WAF by IGF-1 and the role of p21CIP/WAF in the mitogenic effects of IGF-1 in MCF-7 cells. Based on the signal transduction pathways activated in MCF-7 cells and the effects of inhibitory agents as well as those of the expression of dominant negative constructs, we showed that the ERK1/2 MAP kinase is important in the up-regulation of both mRNA and protein of p21CIP/WAF after IGF-1 stimulation. Using an antisense of p21CIP/WAF to inhibit p21CIP/WAF expression, we show that cell proliferation induced by IGF-1 is reduced by at least 2-fold following p21CIP/WAF down-regulation.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Antibodies—* The radionucleide $[^{32}P]dCTP$ (6000 Ci/mmol) and $[^{3}H]thymidine$ was purchased from PerkinElmer Life Science Products (Boston, MA). Recombinant human IGF-1 was obtained from Genentech (San Francisco, CA). ICI 182,780 was kindly supplied by Dr. Alan Wakeling at Zeneca Pharmaceuticals (Macclesfield, England). $[17\beta$-Estradiol and PI3K-specific inhibitors LY294002 were from Sigma (Lyon, France). Both MEK1/2-specific inhibitor U0126 and p38 MAPK-specific inhibitor SB202190 were from Calbiochem (La Jolla, CA). The JNK1/2-specific inhibitor SP600125 was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, MA). The stock solutions of pharmacological inhibitors were all prepared in Me2SO at a concentration of 1000-fold, so that when they were added to the culture medium, the concentration of Me2SO was below 0.1%. Antibodies to p38, ERK2, JNK1, p21CIP/WAF, CDK4, CDK6, cyclin D1, and cyclin E were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to $\beta$-actin was from Sigma. Polyclonal antibodies to phospho-Akt (Ser-473), phospho-ERK1/2, phospho-p38, phospho-JNK1/2, phospho-Rb, Rb, and Akt protein were purchased from New England Biolabs Inc. (Beverly, MA). All these antibodies were used with a 1:1000 dilution in Western blotting. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins (1:5000) were purchased from Amersham Biosciences. Enhanced chemiluminescence reagents were obtained from PerkinElmer Life Science Products.

*Cell Culture and Stable Transfection—* MCF-7 and T47D cells from ATCC were cultured in IMEM (MCF-7) or DMEM (T47D) supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 $\mu$g/ml). MCF-7 cells stably transfected with an
IGF-1 (10 nM) cells were trypsinized, pelleted and washed twice in PBS. Cell pellets were resuspended in the citrate buffer (250 mM sucrose, 40 mM trisodium citrate, pH 7.6, 5% Me_SO) and stored at -70°C. Nuclei were obtained by incubation of the cell s in 300 µl of solution A (3.4 mM trisodium citrate, pH 7.6, 1 mM Tris, 3 mM spermine tetrahydrochloride, 0.2% Nonident P40, 100 µg/ml ribonuclease A, and 500 µg/ml trypsin inhibitor) for 5 min at room temperature and in 300 µl of solution B (3.4 mM trisodium citrate, pH 7.6, 1 mM Tris, 3 mM spermine tetrahydrochloride, 0.2% Nonidet P40, 100 µg/ml ribonuclease A, and 500 µg/ml trypsin inhibitor) for 30 min at room temperature. Finally, the nuclei were stained with 30 µl of propidium iodide solution (1 mg/ml) and DNA staining was analyzed by flow cytometry on a FACScan using CellQuest software. Cell cycle analysis was performed using ModFitLT software Version 2 (Verify Software House, Inc).

Immunoprecipitation and Immunoblotting—Cells were prepared in lysis buffer A (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P40) containing various protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml apropin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate). Lysates were centrifuged at 12,000 × g for 20 min at 4°C and the supernatants were aliquoted and protein concentrations determined using the BCA protein assay. The immunocomplexes were precipitated with 40 µl of protein A-agarose for 1 h at 4°C and the supernatants were aliquoted and protein concentrations determined using the BCA protein assay. After quantification by densitometry, membranes were stripped and probed with the actin antibody as a loading control. The error bars represent the mean ± S.D. from three separate experiments.

IGF-1 (10 nM) cells were trypsinized, pelleted and washed twice in PBS. Cell pellets were resuspended in the citrate buffer (250 mM sucrose, 40 mM trisodium citrate, pH 7.6, 5% MeSO) and stored at -70°C. Nuclei were obtained by incubation of the cell s in 300 µl of solution A (3.4 mM trisodium citrate, pH 7.6, 1 mM Tris, 3 mM spermine tetrahydrochloride, 0.2% Nonident P40, 100 µg/ml ribonuclease A, and 500 µg/ml trypsin inhibitor) for 5 min at room temperature and in 300 µl of solution B (3.4 mM trisodium citrate, pH 7.6, 1 mM Tris, 3 mM spermine tetrahydrochloride, 0.2% Nonidet P40, 100 µg/ml ribonuclease A, and 500 µg/ml trypsin inhibitor) for 30 min at room temperature. Finally, the nuclei were stained with 30 µl of propidium iodide solution (1 mg/ml) and DNA staining was analyzed by flow cytometry on a FACScan using CellQuest software. Cell cycle analysis was performed using ModFitLT software Version 2 (Verify Software House, Inc).

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Role of p21<sup>CIP/WAF</sup> in Proliferative Effects of IGF-1R

RESULTS

IGF-1 Induced p21<sup>CIP/WAF</sup> Expression in MCF-7 Cells—MCF-7 cells were partially synchronized in the G<sub>0</sub> phase of the cell cycle by serum deprivation and using the anti-estrogen ICI 182, 780 (10 nM) for 48 h. Under these conditions, more than 75% of cells were synchronized in G<sub>0</sub> (data not shown). Next, cells were treated with IGF-1 for various times. IGF-1 (10 nM) increased p21<sup>CIP/WAF</sup> mRNA level maximally after 4 h of stimulation. This effect was maintained for 12 h and abolished after 24 h of stimulation (Fig. 1A). p21<sup>CIP/WAF</sup> protein increased 7-fold after 12 h of IGF-1 stimulation (Fig. 1B). This increase was observed for up to 24 h of IGF-1 treatment. However, after 48 h of stimulation, the effect of IGF-1 was abolished. Thus, the time course for p21<sup>CIP/WAF</sup> protein expression correlates well with the expression of p21<sup>CIP/WAF</sup> mRNA induced by IGF-1. In order to confirm that IGF-1 increases p21<sup>CIP/WAF</sup> expression through the IGF-1R, MCF-7-derived cells (SX13) which exhibit a 50% reduction in IGF-1R expression (2, 4) were stimulated with IGF-1 (10 nM) for 4 h. As shown in Fig. 2, the increase in p21<sup>CIP/WAF</sup> mRNA level in response to IGF-1 was significantly reduced in SX13 cells as compared with the MCF-7/neo cells. Taken together, these data demonstrate that IGF-1-R activation is necessary to increase p21<sup>CIP/WAF</sup> expression in MCF-7 cells.

MAP Kinase ERK1/2 Is Involved in p21<sup>CIP/WAF</sup> Expression Induced by IGF-1—In order to study the signaling pathways involved in the increase of p21<sup>CIP/WAF</sup> protein level by IGF-1 in MCF-7 cells, inhibitors specific for PI3K, p38, JNK1, and Akt kinases, revealed that protein levels were similar among the samples. Fig. 3 indicates ERK1/2 were used. First, we determined the active concentration of each inhibitor. MCF-7 cells were treated for 1 h with the inhibitors prior to the IGF-1 stimulation (10 nM, 10 min). Western-blot analysis revealed the dose-dependent inhibition by LY294002 (PI3K pathway), SB202190 (p38 pathway), SP600125 (JNK1/2 pathway), and U0126 (ERK1/2 pathway), respectively. 2 µM U0126 was capable of inhibiting ERK1/2 phosphorylation induced by IGF-1 (data not shown). In the same manner, 50 µM SB202190, 50 µM SP600125, and 50 µM LY294002 inhibited p38, JNK1/2, and Akt phosphorylation (data not shown). Western blot analyses performed to detect all forms of ERK1/2, p38, JNK1, and Akt kinases, revealed that protein levels were similar among the samples.

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that the JNK1/2 pathway inhibitor SP600125, the p38 pathway inhibitor SB202190 and the PI3K pathway inhibitor LY294002 did not affect the increase in the p21CIP1/WAF protein level induced by IGF-1. However, the addition of U0126 reduced by 80% the increase in the p21CIP1/WAF expression induced by IGF-1 stimulation. Thus, in MCF-7 cells, the increase in the p21CIP1/WAF protein level by IGF-1R activation is apparently mediated through the MAPK ERK1/2 pathway.

In order to confirm these data, MCF-7 cells were transiently transfected with a dominant negative MEK-1 construct (MEK1/H11002/H11002). Upon IGF-1 stimulation, there was a 70% reduction in ERK1/2 phosphorylation after transfection of the MEK-1 dominant negative construct (Fig. 4A). Western-blot analysis was performed on MCF-7 transfected with MEK1/H11002/H11002 and with the empty vector as a control (Fig. 4B). Upon IGF-1 stimulation, in cells transfected with the empty vector, p21CIP1/WAF protein level was increased by 7-fold as compared with unstimulated cells. However, when MCF-7 cells were transfected with a dominant negative MEK-1 the increase in expression of p21CIP1/WAF by IGF-1 was reduced by about 80%. Thus, the inhibition of the ERK1/2 pathway, either by specific inhibitors or by the expression of a dominant negative MEK-1 in MCF-7 results in inhibition of the increase of p21CIP1/WAF protein expression by IGF-1.

Growth Inhibition of MCF-7 Cells in Response to IGF-1 by Stable Expression of p21CIP1/WAF Antisense Ribonucleic Acid—

In order to understand the role of p21CIP1/WAF in the mitogenic effect of IGF-1, we generated MCF-7/p21CIP1/WAF AS (antisense) clones. The MCF-7/p21CIP1/WAF clones were developed by stable transfection of MCF-7 cells with the pBK-CMV-AS-p21CIP1/WAF expression vector as described under “Experimental Procedures.” Thirty G418 resistant clones were analyzed by immunoblotting, using an anti-p21CIP1/WAF antibody. Control cells are designated as Neo 1 and Neo 2, and five individual clones stably transfected with p21CIP1/WAF antisense construct are designated as p21AS C1 to p21AS C5. Panel B, MCF-7 cells expressing either the neomycin resistance gene alone (Neo 1) or the p21CIP1/WAF antisense construct (p21AS C1) were plated at the same density (3000 to 5000 cells per well). Both cell lines were maintained in SFM in the absence or presence of either IGF-1 (10 nM) or estradiol (1 nM) for 48 and 72 h. Cell number was then determined each day indirectly using the colorimetric MTT method. Results are expressed as the mean ± S.D. of percentage of cell number increase as compared with cells maintained in SFM. The results are obtained from two independent experiments using quintuplet measurements per experiment for each condition. *, p < 0.05 indicates a significant IGF-1 effect.

FIG. 5. p21CIP1/WAF antisense overexpression reduces IGF-1 induced cell proliferation in MCF-7 cells. Panel A, p21CIP1/WAF protein expression in MCF-7 cells stably transfected with a p21CIP1/WAF antisense construct. MCF-7 cells were stably transfected with pBK-CMV (Neo) or pBK-CMV-AS-p21CIP1/WAF, as described under “Experimental Procedures.” 50 μg of protein from whole cell lysates was subjected to SDS-PAGE followed by immunoblotting with a monoclonal p21CIP1/WAF antibody. Control cells are designated as Neo 1 and Neo 2, and five individual clones stably transfected with p21CIP1/WAF antisense construct are designated as p21AS C1 to p21AS C5. Panel B, MCF-7 cells expressing either the neomycin resistance gene alone (Neo 1) or the p21CIP1/WAF antisense construct (p21AS C1) were plated at the same density (3000 to 5000 cells per well). Both cell lines were maintained in SFM in the absence or presence of either IGF-1 (10 nM) or estradiol (1 nM) for 48 and 72 h. Cell number was then determined each day indirectly using the colorimetric MTT method. Results are expressed as the mean ± S.D. of percentage of cell number increase as compared with cells maintained in SFM. The results are obtained from two independent experiments using quintuplet measurements per experiment for each condition. *, p < 0.05 indicates a significant IGF-1 effect.
IGF-1 in MCF-7/p21CIP/WAF antisense cells correlates with an increase in the proportion of cells in G1, suggesting a delay in G1 phase of the cell cycle in the MCF-7/p21CIP/WAF antisense cells. Thus, the decrease in proliferation rate in response to IGF-1 stimulation, 3% of C1 cells and 22% of Neo 1 cells progressed through S phase. IGF-1 treatment further improved thymidine incorporation in T47D p21CIP/WAF cells compared with Neo and T47D cells.

G1 Growth Arrest in Response to IGF-1 in MCF-7 p21CIP/WAF Antisense Cells—FACS scan analysis was used to determine the effects of IGF-1 on the distribution of cells through the phases of the cell cycle in the MCF-7/p21CIP/WAF antisense clones, C1, and C2 and their controls, Neo 1 or Neo2. Neo and p21CIP/WAF antisense cells were synchronized for 48 h using the anti-estrogen ICI 182,780 (time 0) and stimulated with IGF-1. As shown in Fig. 6B similar results are observed in T47D as with MCF-7 cells. Indeed, thymidine incorporation is higher in T47D p21CIP/WAF cells than T47D control cells in the basal state (p < 0.05) and once again IGF-1 increased cell proliferation (p < 0.05). G1 Growth Arrest in Response to IGF-1 in MCF-7 p21CIP/WAF Antisense Cells—FACS scan analysis was used to determine the effects of IGF-1 on the distribution of cells through the phases of the cell cycle in the MCF-7/p21CIP/WAF antisense clones, C1, and C2 and their controls, Neo 1 or Neo2. Neo and p21CIP/WAF antisense cells were synchronized for 48 h using the anti-estrogen ICI 182,780 (time 0) and stimulated with IGF-1. Thymidine incorporation is higher in T47D p21CIP/WAF cells than T47D control cells in the basal state (p < 0.05) and once again IGF-1 increased cell proliferation (p < 0.05).

Expression of Cyclin D1 and Its Association with CDK4 and CDK2 and the Level of Rb Phosphorylation in Response to IGF-1 Is Reduced in MCF-7 p21CIP/WAF Antisense Cells—In order to explain further the G1 growth arrest in MCF-7 p21CIP/WAF antisense cells in response to IGF-1, we studied some cell cycle components. The elements of the cell cycle we examined included cyclin D1 expression and its association with CDK4 or CDK6 (Fig. 7, A and B) and the level of Rb phosphorylation and cyclin E expression (Fig. 8). Neo and p21CIP/WAF antisense cells were synchronized for 48 h by serum deprivation and then stimulated with IGF-1 for the indicated times. As shown in the time course experiment of Fig. 7A, cyclin D1 immunoreactivity in response to IGF-1 is reduced by about 2-fold in MCF-7 p21CIP/WAF antisense compared with the Neo cells. Consequently, we showed a marked reduction in the association of cyclin D1 with CDK4 or CDK6 in MCF-7 p21CIP/WAF antisense compared with the Neo cells (Fig. 7B). However, these results were observed with a decrease in cyclin D1 protein levels but without any changes in CDK4/CDK6 protein levels (data not shown) in p21CIP/WAF antisense compared with the Neo cells. Finally, we determined the phosphorylation state of Rb and the expression level of cyclin E. As shown in the time course experiment of Fig. 8, similar to the cyclin D1 expression, phosphorylation of Rb (Fig. 8A) and protein level of cyclin E (Fig. 8B) were significantly reduced in MCF-7/p21CIP/WAF antisense cells as compared with Neo cells. Thus, the G1 growth arrest in response to IGF-1 in MCF-7/p21CIP/WAF antisense cells is due at least to some extent to the reduction of the activation of several components of the cell cycle including the cyclin D1 expression.

DISCUSSION

The cyclin-dependent kinase inhibitor, p21CIP/WAF is induced in various biological situations including cell cycle arrest (32), differentiation (33, 34), and apoptosis (35, 36). Several studies described p21CIP/WAF as a potent inhibitor of cell proliferation in various cell culture models (37). For instance, in A431 cells, EGF inhibited cell growth by increasing p21CIP/WAF protein levels and, more specifically, the half-life of p21CIP/WAF mRNA (38). Similarly, treatment with fibroblast growth factor (FGF) induces an increase in p21CIP/WAF mRNA and protein levels and results in inhibition of cellular proliferation in
MCF-7 (39). p21 inhibits CDKs that function in the G1 and S phases. A recent study shows that p21CIP/WAF is an important physiological target of antiestrogen action that inhibits cdk4 in addition to cdk2 in human breast cancer cells (40). Interestingly, in a previous study, we have observed that IGF-1 increases the protein levels of p21CIP/WAF (2). The protein levels of p21CIP/WAF are known to be regulated by post-translational degradation and also by transcriptional activation, although the exact mechanism of protein degradation is unclear (41). Here, we studied the regulation of p21CIP/WAF mRNA and protein levels by IGF-1 and found that the MAPK ERK1/2 signaling pathway is involved in the activation of p21CIP/WAF gene expression by IGF-1 stimulation. In PC12 and Hela cells, some studies have suggested that the p21CIP/WAF gene expression is regulated at the level of transcription, primarily through the MAP kinase pathway in response to growth factors (42). In the differentiating chondrogenic cell line MCT as well as in primary mouse chondrocytes, the Raf-1/MEK/ERK pathway is the major regulator of p21CIP/WAF gene transcription (43). The MAPK pathway is also involved in p21CIP/WAF induction by TGFβ in the HaCaT cells (44). However, p21CIP/WAF was also reported to be regulated by a MAP kinase independent pathway (45). Two studies demonstrated that the PI3 kinase pathway, which is regulated by growth factors and which up-regulates AKT kinase activity, was involved with the modulation of p21CIP/WAF levels (46, 47). Moreover, a recent study showed that the stability of p21CIP/WAF is regulated by AKT-mediated phosphorylation at residue Ser-146 (48). In MCF-7 cells, Dufourny et al. (1) showed that some stimulatory effects of IGF-1 on cell cycle progression such as cyclin D1 synthesis and pRb hyperphosphorylation were blocked by the specific PI3K inhibitory LY294002. From these results, they suggested that the PI3K activity but not MAPK activity was required for transduction of the mitogenic IGF-1 signal in these cells (1). In the present study, we showed that the IGF-1 induced p21CIP/WAF expression through the MAPK ERK1/2 is involved in the mitogenic IGF-1 effect. Thus, both PI3K and MAPK are important signaling pathways involved in the IGF-1 effect on the cell cycle progression in MCF-7 cells.

The induction of p21CIP/WAF gene expression by IGF-1 appears to be contradictory to the previously described role of p21CIP/WAF as a negative regulator of cell growth via its ability to inhibit the activity of CDKs. However, the observed effects of IGF-1 on p21CIP/WAF expression suggest that the regulation of this gene is complex and may be influenced by multiple signaling pathways in different cell types and conditions.
Role of p21<sup>CIP/WAF</sup> in Proliferative Effects of IGF-1R

In the levels of p21CIP/WAF in the rapidly proliferating tumors, progression. In addition, several studies show a marked increase in cyclin E expression by overexpression of p21CIP/WAF cDNA antisense in normal myeloid progenitor cells (57). Another study indicate that tumors arising in MMTV-ras/p21<sup>CIP/WAF</sup>−/− mice displayed higher S-phase fractions and correspondingly increased cyclin D1 and E/CDK activity than MMTV-ras tumors. In contrast, MMTV-myc/p21<sup>CIP/WAF</sup>−/− tumors had lower S-phase fractions and levels of cyclin D1 and E/CDK activity than MMTV-myc tumors (58). Thus, p21<sup>CIP/WAF</sup> might play a role in promoting either growth arrest or proliferation, depending on the specific cellular context.

In our study, we showed for the first time that a reduction of p21<sup>CIP/WAF</sup> expression in MCF-7 cells is associated with a reduction in the IGF-1-induced cyclin D1 expression. Consequently, the levels of CDK4/cyclin D1 and CDK6/cyclin D1 complexes as well as the Rb phosphorylation and the level of cyclin E are reduced in response to IGF-1. These data probably explain the delay in cell cycle progression and the 2-fold reduction in the IGF-1 induced cell proliferation. Cyclin D1 plays an important role in the cell cycle control of the mammary gland and clinical studies of human breast cancer confirm its importance. For example, cyclin D1 is overexpressed in the majority of human breast cancers (59). Moreover, p21<sup>CIP/WAF</sup> does correlate with cyclin D1 overexpression; 93% of breast tumors that overexpressed D-type cyclins also overexpressed p21<sup>CIP/WAF</sup>, suggesting that a relationship exists between p21<sup>CIP/WAF</sup> and cyclin D1 levels in some human malignancies (60). Our results suggest that in addition to regulating the activity of cyclin/CDK complexes through direct interaction, p21<sup>CIP/WAF</sup> can also either directly or indirectly lead to changes in cyclin D1 protein levels. A study analyzing the effects of loss of both p21<sup>CIP/WAF</sup> and p27 in mouse embryo fibroblasts demonstrated not only a loss of cyclin D/CDK activity but also markedly reduced cyclin D protein levels (61). Cyclin D1 has been also reported to be less stable in cells lacking p21<sup>CIP/WAF</sup> and p27<sup>kip1</sup>, suggesting that the CIP/KIP protein levels were required to maintain cyclin D1 protein levels (53). Although it is unclear whether the effect of p21<sup>CIP/WAF</sup> on cyclin D1 is direct or indirect, there is some evidence for p21<sup>CIP/WAF</sup>-induced transcription of cyclin D1 (59) possibly through pRb activation. A recent study has also demonstrated that in Ras-transformed cells, cyclin D1 is required to protect p21<sup>CIP/WAF</sup> from proteasomal degradation (26).

In summary, we have elucidated the molecular mechanisms involved in the increase of p21<sup>CIP/WAF</sup> expression in response to IGF-1 in MCF-7 cells. Using antisense strategy, we also demonstrated that p21<sup>CIP/WAF</sup> is a positive key regulator in the IGF-1 induced cell proliferation. Moreover, we showed for the first time that a reduction of p21<sup>CIP/WAF</sup> protein is associated to a decrease in the cyclin D1 expression in MCF-7 cells. Further studies will enable us to understand the interaction between IGF-1R, p21<sup>CIP/WAF</sup>, and cyclin D1.

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