Mitogenic Signaling of Insulin-like Growth Factor I in MCF-7 Human Breast Cancer Cells Requires Phosphatidylinositol 3-Kinase and Is Independent of Mitogen-activated Protein Kinase*

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Addition of insulin-like growth factor I (IGF-I) to quiescent breast tumor-derived MCF-7 cells causes stimulation of cyclin D1 synthesis, hyperphosphorylation of the retinoblastoma protein pRb, DNA synthesis, and cell division. All of these effects are independent of the mitogen-activated protein kinase (MAPK) pathway since none of them is blocked by PD098059, the specific inhibitor of the MAPK activating kinase MEK1. This observation is consistent with the finding that the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), a strong inducer of MAPK activity in MCF-7 cells, effectively inhibits proliferation. The anti-proliferative effect of TPA in these cells may be accounted for, at least in part, by the MAPK-dependent stimulation of the synthesis of p21WAF1/CIP1, an inhibitor of cyclin/cyclin-dependent kinase complexes. In contrast, all of the observed stimulatory effects of IGF-I on cell cycle progression, cyclin D1 synthesis, and pRb hyperphosphorylation were blocked by the specific phosphatidylinositol 3-kinase inhibitor LY294002, suggesting that phosphatidylinositol 3-kinase activity but not MAPK activity is required for transduction of the mitogenic IGF-I signal in MCF-7 cells.

Insulin-like growth factor I (IGF-I)1 is a well established mitogen to many different cell types in vitro.

The components of the pathway transducing the mitogenic stimulus of IGF-I to the cell cycle machinery have been identified in part. The first step of IGF-I signaling involves its binding to the type 1 IGF surface receptor, consisting of two extracellular α-subunits and two membrane-spanning β-subunits (1). The activated receptor functions as a tyrosine kinase.

IRS-1, a 185-kDa protein, is an important substrate of this kinase and becomes extensively phosphorylated (2, 3). Activated IRS-1 then serves as a multi-isoform docking protein by binding through its phosphorylated residues to numerous SH2 domain-containing proteins. These include the p85 regulatory subunit of phosphatidylinositol 3-kinase 3-kinase (PI3-K) and the guanine-nucleotide exchange factor Grb2/SOS (4–8). Putative downstream effectors of PI3-K are the serine/threonine protein kinase PKB/Akt (11), the Rho family polyproteins Rac and Cdc42 (10), and the serine/threonine protein kinase PKB/Akt (11). Grb2/SOS binding subsequently results in activation of the proto-oncogenes p21ras and raf and the mitogen-activated protein kinase (MAPK)/extracellular response kinase (ERK) kinase MEK1, which activates the MAP kinases. Whether and how these pathways ultimately connect the surface receptor to the cell cycle components in the nucleus is still largely unknown.

The effectiveness of IGF-I as a mitogen of the human breast cancer-derived MCF-7 cell line is well documented (12–14). These cells arrest in the G0/G1 phase of the cell cycle upon withdrawal of serum, and the proliferative process may be restored by the addition of IGF-I at 10 ng/ml to the medium. In the present study, we have addressed the question whether the activation of the PI3-K and/or MAPK pathways is an essential step in the mitogenic action of IGF-I in MCF-7 cells.

The MAPK signal transduction cascade derives its name from findings that suggest a key role for MAPK (i.e. Erk-1 and -2) in the proliferative process. MAPK activity is elevated in response to many different growth factors. Furthermore, a large number of oncogenes share the activation of MAPK as a common pathway (15). The tumor promoting phorbol ester TPA is a strong inducer of MAPK activity and has been shown to be a mitogen of many different cell lines in vitro (16–18). However, addition of TPA to other cell types, including MCF-7 cells, leads to inhibition of their growth, although activation of MAPK is clearly observed (19–21). This observation raises the question whether MAPK activation plays a role in growth induction as well as in growth inhibition in MCF-7 cells. Alternatively, the PI3-K activation triggered by many growth factors and also seen after addition of IGF-I to MCF-7 cells may be involved in the transduction of the mitogenic signal. To establish their involvement in mitogenic signaling, we have used specific and, by numerous reports, well documented inhibitors of PI3-K (wortmannin (22) and LY294002 (23)) and of the MAPK-activating enzyme MEK1 (PD098059) (24).

As parameters for mitogenic IGF-I signaling and of growth inhibitory TPA signaling, we have studied the levels of the G1 cyclin D1, hyperphosphorylation of the retinoblastoma protein pRb, levels of DNA synthesis, and effects on cell numbers in cultures of MCF-7 cells. Induction of the D-type cyclins has
been shown to constitute an essential step in coupling of mitogenic signaling cascades to the cell cycle machinery. This has recently been confirmed for MCF-7 cells, in which mitogenic signals from different classes of receptors were shown to converge at and to strictly require enhanced cyclin D1/cdk4 activity to induce S-phase (25). Cyclins D2 and D3 are not expressed in MCF-7 cells, and thus cyclin D1 is the essential D-type cyclin (26). Moreover, moderate overexpression of cyclin D1 has been shown to induce cell cycle progression under otherwise growth inhibiting low serum conditions in MCF-7 cells (27). Subsequently, the rise in cyclin D1 levels and the enhanced activity of the cyclin D1/cdk4 complex, in cooperation with the cyclin E/cdk2 complex, lead to hyperphosphorylation of pRb and passage of the so-called restriction point in late G1. Progression through the cell cycle then becomes growth factor-independent, and DNA replication during S-phase followed by cell division during M-phase ensues. Growth inhibition agents on the other hand have been shown to often induce the synthesis of cyclin-dependent kinase inhibitors (CKIs). These relatively small proteins inhibit the activity of cyclin-cdk complexes, preventing pRb from being hyperphosphorylated and thus causing arrest in the G1 phase of the cell cycle. Addition of TPA to MCF-7 cells stimulates the expression of one of the CKIs, p21^{WAF1/CIP1}, which may account for the growth inhibition observed with this agent (28). Here we report on the effects of the specific PI3-K and MEK1 inhibitors on the growth-stimulating IGF-I signal and on the growth-inhibiting TPA signal to establish the signal transduction routes involved in these processes in MCF-7 cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies—** Anti-cyclin D1 (H-295), anti-cyclin E (C19), anti-cdk2 (M2), anti-cdk4 (C22), anti-p21^{WAF1/CIP1} (H-164), and anti-Erk2 (C14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-erbB2 (14001A) antibody was from PharMingen (San Diego, CA). Anti-pRb (14001A) antibody was from PharMingen (San Diego, CA). Anti-cyclin D1 antibody was obtained from Dr. D. Beach (Cold Spring Harbor, NY), is a 1325-base pair polymerase chain reaction fragment encompassing the entire published human cDNA sequence of cyclin D1, cloned in the pBluescript KS (29). The p21^{WAF1/CIP1} probe, kindly provided by Dr. R. H. Medema (Utrecht), is a 526-base pair human cDNA fragment. It was obtained by polymerase chain reaction amplification of a HeLa cell cDNA library using two oligonucleotide primers, a 5-primer (5'-GGAATTCATGCGAGACACGGCTG-3') and a 3-primer (5'-GGATCTCCTGTTGGGCGGAGAAGG-3'), based on the published sequence of the human p21^{WAF1/CIP1} gene (30). This fragment was cloned into the EcoRI site of pUCDNA3. As a probe for GAPDH the cDNA sequence of rat GAPDH cloned in pBR322 was used (31).

**Cell Culture—** MCF-7 cells were cultured in Dulbecco’s minimal essential medium/Ham’s F12 medium (1:1) supplemented with 5% fetal calf serum, glutamine (300 µg/ml), penicillin (100 IU/ml), and streptomycin (100 µg/ml) to a confluency of about 60%. Because of the known growth-stimulating effect of estrogens on MCF-7 cells (32), the cells were cultured for 24 h in phenol red-free medium containing 5% dextrose before starving the cells during 24 h in the same serum-free medium. LY294002 was purchased from Biomas Research Laboratories Inc., Plymouth Meeting, PA. PD98059 was from New England Biolabs, Beverly, MA, and wortmannin and rapamycin was from Sigma. Horseradish peroxidase-coupled antibodies (rabbit anti-mouse and goat anti-rabbit) were from Bio-Rad.

**MAP Kinase Assay—** Assays were performed in 24-well plates. Serum-starved cells were stimulated with IGF-I or TPA. After 20 h of incubation 3H-labeled thymidine (0.5 µCi/ml) was added. Twenty-six h after the addition of IGF-I or TPA, cells were fixed with 10% trichloroacetic acid, washed in water and incubated with 0.05% methylene blue for 30 min, again washed in 0.5% Tween 20, 0.1% BSA in PBS, and then resuspended in 1% dried milk. Cells were then fixed in 4% paraformaldehyde for 1 h, washed in 0.5% Tween 20, 0.1% BSA and incubated with 5 µg/ml 4,6-diamidino-2-phenylindole (DAPI) and 40 µg/ml propidium iodide (PI) for 30 min at room temperature. The cells were washed with PBS and treated with RNase A (12.5 µg/ml in sodium citrate (38 m M, pH 7.0)). DNA content was then determined by FACScan analysis (Becton Dickinson).

**Northern Blotting—** Cells from 25-cm² flasks were washed with PBS and lysed in RNAzol B (Biotex Laboratories Inc., Houston, TX). Total RNA was extracted and precipitated according to the manufacturer’s instructions.
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Fig. 1. G1 phase progression of serum-starved MCF-7 cells after stimulation with IGF-I. Serum-starved cells were stimulated with 10 ng/ml IGF-I and harvested after different periods. Cell lysates were used for Western blotting and for measurement of cdk2 activity. Western blots were analyzed for the amount of cyclin D1 and cyclin E protein and for the phosphorylation state of pRb (top band is hyperphosphorylated pRb, bottom band is hypophosphorylated pRb). Cdk2 activity was measured as described under "Experimental Procedures." Instructions and dissolved in sterile water. Equal amounts of RNA (10 μg) were glyoxylated in 50% Me2SO and size-separated by electrophoresis in 0.8% agarose gel. RNA was transferred to nylon membrane (GeneScreen, NEN Life Science Products) and cross-linked to the membrane by UV irradiation. The blots were prehybridized for 4 h at 42 °C in 50% deionized formamide, 1 M NaCl, 50 mM Tris-HCl, pH 7.5, 0.2% BSA, 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, and 200 μg/ml denatured herring sperm DNA. Hybridization was performed overnight in the same solution to which the specific 32P-labeled cDNA probes (106 cpm/ml) were added. The cDNA probes were labeled by random priming using Rediprime (Amersham Life Sciences Inc., Little Chalfont, UK). Membranes were exposed to Fuji x-ray films at −80 °C.

PKB Activity Assay—Cells were washed in PBS and lysed in IP buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 mM Na3VO4). Lysates were pre-cleared by incubation with 25 μl of 10% protein A-agarose beads pre-coupled to anti-PKB antibody for 2 h at 4 °C. Beads were washed twice in IP buffer, twice in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.5 mM LiCl, 200 μM Na3VO4, and once in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 200 μM Na3VO4. Beads were then resuspended in 25 μl of kinase mix (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM ATP, 50 μM γ-[32P]ATP per sample). The mixture was incubated for 20 min at 30 °C, after which the reaction was stopped with 8 μl of 4 × sample buffer (200 mM Tris-HCl, pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromphenol blue, 40% glycerol). The suspension was boiled for 5 min and centrifuged, and the supernatant was run on a 15% SDS-polyacrylamide gel. The gel was dried and exposed to Fuji x-ray film.

RESULTS

IGF-I Stimulates G1 Progression, DNA Synthesis, and Proliferation of MCF-7 Cells—We studied, in a time course experiment, the progression of serum-starved MCF-7 cells through the G1 phase of the cell cycle after addition of IGF-I to the culture medium. Serum-starved MCF-7 cells were given IGF-I (10 ng/ml) and were harvested at different time points thereafter. Protein levels of cyclin D1 and cyclin E and phosphorylation of pRb were determined by Western blotting. Furthermore, the activity of cdk2 and [3H]thymidine incorporation into DNA was measured. Fig. 1 shows that the level of cyclin D1 protein is clearly increased 4 h after the addition of IGF-I and that it remains at a high level until about 24 h after stimulation. While the level of cdk4 protein does not change upon IGF-I addition (data not shown), Cyclin E levels become elevated at about 20 h after IGF-I addition, are maximal at about 24 h, and then rapidly decrease. Phosphorylation of pRb can first be seen at 16 h after IGF-I addition and increases strongly until at least 32 h after stimulation. Cdk2 activity, due to complex formation of cyclin E and of the more abundantly expressed S-phase cyclin A with cdk2, is observed from 24 h post-stimulation onward. These data are in agreement with the results on [3H]thymidine incorporation. DNA synthesis starts to increase strongly 24 h after the addition of IGF-I to the medium and reaches a maximum around 27 h post-stimulation (Fig. 2A). After 48 h a clear increase in cell number is observed, and after 72 h the initial number of cells has tripled (Fig. 2B). The proliferation rate of the cells is dependent on the IGF-I concentration. A concentration of 10 ng/ml results in a rate comparable to that observed with 10% fetal calf serum (FCS) (Fig. 2C). Taken together, these data show that addition of IGF-I to the culture medium of quiescent MCF-7 cells results in progression through the G1 phase of the cell cycle indicated by increased levels of cyclins D1 and E and by phosphorylation of pRb. S-phase entry indicated by the increase in cdk2 activity and DNA synthesis, followed by proliferation.

TPA Inhibits DNA Synthesis and Proliferation—The phorbol ester TPA strongly activates MAPK and is a known inhibitor of growth in MCF-7 cells. We confirmed this in a growth assay in which serum-starved cells were given 0.5% FCS, 5% FCS, or 5% FCS in combination with 10 ng/ml TPA. Addition of 5% FCS clearly stimulates proliferation. When TPA is added simultaneously, this stimulation is inhibited (Fig. 2D). Addition of IGF-I to quiescent MCF-7 cells results in an 18-fold increase in [3H]thymidine incorporation into DNA between 20 and 26 h after stimulation in comparison to unstimulated control cells. Addition of TPA completely abolishes this increase in DNA synthesis (Fig. 2E).

Effects of Inhibitors on Cyclin D1 and p21WAF1/CIP1 Expression—To investigate the signal transduction routes by which IGF-I stimulates and TPA inhibits cell cycle progression in MCF-7 cells, we studied the effects of both of these agents on cell cycle progression in MCF-7 cells in the presence of specific inhibitors of key enzymes in the PI3-K and MAPK signal transduction pathways. As parameters for cell cycle progression, we studied the levels of cyclin D1 and, because TPA is known to induce synthesis of this protein (28), also of the CKI p21WAF1/CIP1. In addition, we measured hyperphosphorylation of pRb and DNA synthesis. MCF-7 cells, synchronized by serum starvation, were preincubated for 10 min with the specific PI3-K inhibitor LY294002 (50 μM) or the p70S6K inhibitor rapamycin (20 ng/ml). PI3-K activation by IGF-I and the effectiveness of LY294002 to block PI3-K activation in these cells were confirmed using an assay for PKB, an established downstream target of PI3-K (11)(see "Activation of PKB by IGF-I" below). After these preincubations, IGF-I (10 ng/ml) or TPA was added. In some of the experiments described below, 100 ng/ml TPA was added to the cells, and in others 10 ng/ml was used. No significant differences in the effects of TPA at these two concentrations have been observed. The concentration of 10 ng/ml is sufficient for the maximal growth inhibitory effect. Cells were harvested 4 h later and cell lysates were analyzed by Northern and Western blotting.

The results are shown in Fig. 3. Fig. 3A is a graphic representation of the PhosphoImager analysis of multiple Northern blots (as indicated in the figure legend) and Fig. 3B is a representative Western blot. The IGF-I-induced increase in cyclin D1 mRNA and protein is completely abolished by addition of LY294002. Identical results were obtained using the unrelated PI3-K inhibitor wortmannin (data not shown). Distinct inhibition, although not complete, is also observed when the cells are preincubated with rapamycin. Surprisingly, TPA was found to induce cyclin D1 expression to an extent comparable to that...
observed with IGF-I. This TPA-induced increase in cyclin D1 is completely inhibited by LY294002 and partly by rapamycin, both at the mRNA and protein levels (Fig. 3, A and B). This suggests that both IGF-I and TPA induce the synthesis of cyclin D1 mRNA and protein in quiescent MCF-7 cells via a PI3-K-dependent signal transduction route.

Stimulation with IGF-I results in a slight increase in mRNA levels of the CKI p21WAF1/CIP1, when compared with untreated quiescent cells. This increase is not influenced by preincubation with LY294002 or rapamycin (Fig. 4A). The IGF-I-induced increase in p21WAF1/CIP1 protein is less pronounced (Fig. 4B).

As expected, TPA causes a strong induction in both mRNA and protein levels of p21WAF1/CIP1 (Fig. 4, A and B). This induction is not inhibited by LY294002 or rapamycin. Addition of TPA to randomly growing cells also results in p21WAF1/CIP1 induction, ng/ml, open circles), or TPA (100 ng/ml, closed triangles) and were harvested after the indicated periods. Labeled thymidine (2 μCi/ml) was added to the culture medium 1 h before harvesting. Incorporation into DNA was measured as described under “Experimental Procedures.” Indicated values are means of triplicate wells.

Fig. 2. DNA synthesis and proliferation of MCF-7 cells in response to IGF-I and TPA. A, [3H]thymidine incorporation in MCF-7 cells. Serum-starved cells were given no mitogens (crosses), IGF-I (20 ng/ml, open circles), or TPA (100 ng/ml, closed triangles) and were harvested after the indicated periods. Labeled thymidine (2 μCi/ml) was added to the culture medium 1 h before harvesting. Incorporation into DNA was measured as described under “Experimental Procedures.” Indicated values are means of triplicate wells. B, cells were serum-starved and subsequently maintained for up to 3 days in serum-free medium (open triangles) or in medium containing 10 ng/ml IGF-I (closed triangles). Every 24 h the cell number was determined using the methylene blue staining assay as described under “Experimental Procedures.” Indicated values are means of triplicate wells. C, serum-starved cells were cultured for 4 days in medium containing 5 or 10% FCS (hatched bars) or IGF-I (open triangles) in increasing concentrations (0.2, 1, 5, and 10 ng/ml, crossed bars). After this incubation methylene blue staining was performed. D, after starvation, cells were cultured for up to 3 days in serum-free medium containing 0.5% FCS (closed circles), 5% FCS (closed squares), or 5% FCS plus 10 ng/ml TPA (open squares). Cell numbers were determined every 24 h. E, serum-starved cells were given IGF-I (10 ng/ml), TPA (10 ng/ml), or a combination of IGF-I and TPA (both at 10 ng/ml). Labeled thymidine (2 μCi/ml) was added after 20 h of incubation, and incorporation was measured 6 h later as described under “Experimental Procedures.” Indicated values are means of triplicate wells.
Effects of Inhibitors on p21WAF1/CIP1 induction—Northern (A) and Western (B) blot analyses of p21WAF1/CIP1 mRNA and protein levels in control cells and in IGF-I- or TPA-treated cells. Serum-starved cells were cultured for 4 h with 10 ng/ml IGF-I or with 100 ng/ml TPA present in the medium, with or without preincubation with 50 μM LY294002 (LY) or 20 ng/ml rapamycin (RA) for 10 min. Equal RNA loading was checked by hybridization of the Northern blot to a GAPDH-specific probe.

which is not inhibited by LY294002 (data not shown). This suggests that induction of p21WAF1/CIP1 by TPA in quiescent and growing MCF-7 cells is independent of PI3-K. Subsequently, we studied the involvement of the MEK1/MAPK pathway in the induction of cyclin D1 and p21WAF1/CIP1. Serum-starved cells were incubated for 5 h with IGF-I (10 ng/ml) or TPA (10 ng/ml) with or without a preincubation of 1 h with 40 μM PD098059, a specific inhibitor of MAPK/ERK kinase (MEK1). Cell lysates were used for Northern and Western blotting to determine the effects on cyclin D1 and p21WAF1/CIP1 mRNA and protein levels. The results are shown in Fig. 5. Fig. 5A shows a representative Northern blot (top part) and a graphic representation of the PhosphorImager analysis of five independent Northern blot experiments (lower part). A representative Western blot is shown in Fig. 5B. Neither the IGF-I nor the TPA-induced increase in cyclin D1 mRNA and protein levels is inhibited by preincubation with PD098059, although activation of MAPK is completely inhibited by the concentration of PD098059 used in these experiments (Fig. 5C and Ref. 59). In contrast, PD098059 does inhibit the strong induction of p21WAF1/CIP1 mRNA and protein upon addition of TPA (100 ng/ml). The same results were obtained when TPA was added to randomly growing MCF-7 cells in 5% FCS-containing medium (data not shown). This suggests that in both quiescent and cycling MCF-7 cells p21WAF1/CIP1 induction by TPA is dependent on the MAPK route of signal transduction.

Effects of Inhibitors on pRB Phosphorylation—The inhibitors LY294002 and rapamycin affect the increase in cyclin D1 levels induced by IGF-I and TPA. The increase in p21WAF1/CIP1 caused by TPA is blocked by PD098059. Therefore, these inhibitors are also expected to influence pRB hyperphosphorylation. To test this hypothesis, serum-starved cells were incubated with IGF-I (10 ng/ml) or TPA (100 ng/ml), with or without preincubation with 50 μM LY294002 (10 min), 20 ng/ml rapamycin (10 min), or 20 μM PD098059 (1 h). Cell lysates were used for Western blotting. Fig. 6 shows that 16 h after addition of IGF-I to the starved cells, pRB has become markedly hyperphosphorylated. This phosphorylation is completely inhibited by preincubation with LY294002 and partly by rapamycin. Preincubation with PD098059 for 1 h does not have an inhibitory effect. Addition of TPA to the cells does not cause hyperphosphorylation of pRB, and preincubation with LY294002 and rapamycin does not influence this finding. Preincubation with PD098059, however, leads to a slight recurrence of pRB hyperphosphorylation (Fig. 6, rightmost lane). This recurrence may be explained by the inhibition of p21WAF1/CIP1 induction caused by PD098059. By inhibiting p21WAF1/CIP1 induction, PD098059 prevents blocking of the activity of the cyclin D1-cdk4 complex. The reduction in the amount of p21WAF1/CIP1 together with the unaffected induction of cyclin D1 observed with TPA may result in the reappearance of a small amount of the hyperphosphorylated form of pRB.

Effects of Inhibitors on DNA Synthesis—Subsequently, we studied the effects of LY294002 and PD098059 on DNA synthesis. Addition of IGF-I to serum-starved MCF-7 cells results in a distinct increase in [3H]thymidine incorporation into DNA (Fig. 7). Pretreatment of the cells with LY294002 completely abolishes this increase. When the cells are pretreated with PD098059 the DNA synthesis level reaches about 60% of that observed without inhibitors. The fact that PD098059 does to some extent affect the level of DNA synthesis without influencing cyclin D1 levels and pRB hyperphosphorylation may result from disturbances caused in numerous intracellular processes by blocking the MAPK signaling pathway for over 20 h.

In addition, the effects of LY294002 and PD098059 on cell cycle phase distribution were studied by FACS scan analysis. Cells were serum-starved and then maintained in serum-free medium or incubated with 10 ng/ml IGF-I for 30 h with or without preincubation with LY294002 (50 μM) or PD098059 (20 μM) for 10 and 60 min, respectively. After fixation of the cells, FACS scan analysis was performed. Of the cells maintained in serum-free medium for 30 h the majority is in the G0/G1 phase of the cell cycle (Fig. 8, top left panel). The same results are obtained for cells serum-starved for the usual 24 h (data not shown). Incubation with IGF-I results in a decrease of G0/G1 cells and a clear increase of the number of cells in G2/M- and S-phase (top right panel). Pretreatment with LY294002 completely abolishes this IGF-I-induced effect. In contrast, PD098059 only slightly decreases the effect of IGF-I (lower panels), confirming the data of the thymidine incorporation studies.

Activation of PKB by IGF-I—Activation of PI3-K by IGF-I in MCF-7 cells and the effectiveness of LY294002 in inhibiting this activation was monitored using an assay for the downstream PI3-K target PKB (11). Serum-starved cells were treated with IGF-I (10 ng/ml) with or without preincubation with LY294002 (50 μM) for 10 min or PD098059 (20 μM) for 1 h. Cells were lysed, PKB was immunoprecipitated from the lysates, and its kinase activity was determined using histone 2B as a substrate and [γ-32P]ATP as phosphate source. The labeled product was run on a SDS-polyacrylamide gel and detected by autoradiography. Treatment with IGF-I results in a 9-fold activation of PKB after 15 min, when compared with quiescent cells. This activation is sustained for at least 3 h (Fig. 9). Pretreatment with LY294002 completely abolishes the IGF-I-induced activation of PKB. PD098059 does not affect PKB activation by IGF-I. These data confirm that IGF-I indeed activates the PI3-K/PKB pathway in these cells and that this pathway is specifically inhibited by LY294002.
DISCUSSION

In recent years, many reports have been published on the physiological effects of insulin and IGF-I and the signal transduction pathways leading to these effects. The general conclusion from these studies is that the PI3-K pathway is mainly associated with the metabolic properties of insulin and IGF-I, such as stimulation of glucose uptake by cells (34, 35), regulation of glucose/glycogen metabolism (36, 37), and stimulation of protein synthesis (38). From studies using antibodies against p21ras or dominant negative mutants of p21 ras, it was concluded that the signaling pathway involving p21 ras, raF, MEK1, and MAPK is essential in the transmission of the growth-promoting signals of insulin and IGF-I (39–41). Our results obtained with MCF-7 cells seem to contradict this general notion.

In the present study we show that IGF-I induces cyclin D1 mRNA and protein synthesis in MCF-7 cells. The levels of cdk4, the catalytic partner of cyclin D1, are not changed by IGF-I, and the expression of the CKI p21WAF1/CIP1 is stimulated only very moderately. Furthermore, IGF-I stimulation results in hyperphosphorylation of pRb, DNA replication, and an increase in cell numbers. Since cdk4 and p21WAF1/CIP1 levels do not change significantly, the increase in cyclin D1 protein

FIG. 5. Effect of MAPK inhibition on cyclin D1 and p21WAF1/CIP1 induction. Northern (A) and Western (B) analysis of cyclin D1 and p21WAF1/CIP1 mRNA and protein levels in control cells and IGF-I- or TPA-treated cells. Serum-starved cells were cultured for 5 h in the presence of 10 ng/ml IGF-I or 10 ng/ml TPA with or without preincubation with 40 μM PD098059 (PD) for 1 h. Equal RNA loading was checked by hybridization of the Northern blot to a GAPDH-specific probe. The lower part of A shows a graphic representation of the PhosphorImager analysis of multiple Northern blots. The levels in control cells were set at 1, and the inductions in other lanes were calculated accordingly. For cyclin D1, the indicated values are means of five (IGF lanes) and four (TPA lanes) experiments, respectively. For p21WAF1/CIP1 five (IGF lanes) and six (TPA lanes) experiments were performed. C, MAPK activation by IGF-I. Serum-starved cells were stimulated for 5 min with IGF-I (20 ng/ml) with or without preincubation for 1 h with PD098059 (20 μM). Positions of the unphosphorylated (p42) and phosphorylated (pp42) forms of MAPK are indicated.

FIG. 6. Effect of PI3-K and MAPK inhibition on the phosphorylation state of pRb. Serum-starved cells were stimulated with 10 ng/ml IGF-I or 100 ng/ml TPA. Preincubations were for 10 min with 50 μM LY294002 (LY) or 20 ng/ml rapamycin (RA) or for 60 min with 20 μM PD098059 (PD). Cells were harvested 16 h after stimulation. Positions of the hypophosphorylated (pRB) and hyperphosphorylated (ppRB) forms are indicated.
seems to be the trigger of the growth-promoting process induced by IGF-I. Preincubation of the cells with LY294002, a specific inhibitor of PI3-K (23), blocks IGF-I-induced cyclin D1 expression. Furthermore, LY294002 completely inhibits pRb hyperphosphorylation and stimulation of DNA synthesis by IGF-I. Inhibition of IGF-I-induced cyclin D1 expression was also observed when wortmannin, a PI3-K inhibitor unrelated to LY294002 (22), was used (data not shown), again indicating that this enzyme is involved in transducing the IGF-I stimulus to cyclin D1 expression. No complete, but still considerable, inhibition of cyclin D1 induction was seen after preincubation with rapamycin, a specific inhibitor of p70S6K (42). The effect of rapamycin on pRb hyperphosphorylation was identical to the effect of LY294002, i.e. complete inhibition of IGF-I-induced hyperphosphorylation was observed. From these data we conclude that activation of the PI3-K pathway is an essential step in proliferative signaling of IGF-I in MCF-7 cells. This conclusion is supported by the finding that the inhibitor PD98059 does not influence the enhanced cyclin D1 expression nor the hyperphosphorylation of pRb induced by IGF-I, indicating that the MEK1-MAPK route is not involved in this process. The effectiveness of the inhibitor is indicated by the fact that the moderate and transient MAPK activation observed in IGF-I-stimulated MCF-7 cells is completely abolished by PD98059 at the concentration used in our experiments (Fig. 5C). Even the much stronger and longer lasting MAPK activation by TPA in these cells is fully blocked by the inhibitor at this concentration (59).

The finding that activation of MAPK is not required for mitogenic signaling of IGF-I in these cells is surprising, since the involvement of the p21ras signaling pathway in mitogenic stimulation by IGF-I is well documented. Since p21ras is the activator of the p21ras-raf-MEK1-MAPK route, these studies (39–41) have suggested that this pathway is important in IGF-I-induced proliferation. However, in addition to being activated directly by the IGF-I receptor, PI3-K has also been shown to be a possible downstream target of p21ras (43). From our data, we cannot exclude the existence of an IGF-I signaling route involving the type 1 receptor, activation of p21ras, and subsequent activation of PI3-K, and thus these data are not necessarily contradicting our findings. However, other investigators (44, 45) have found that induction of cyclin D1 expression is inhibited by overexpression of dominant negative MAPK mutants and is stimulated by constitutively active MAPK. These data clearly indicate the importance of the MEK1-MAPK route in stimulation of cyclin D1 expression and cell division, and our present results are in obvious conflict with these findings. The experiments using dominant negative and constitutively active constructs of MAPK have been performed in fibroblasts, and their results may be indicative of essential differences in IGF-I signaling in fibroblasts on one hand and MCF-7 breast cancer cells on the other. Growth factors like epidermal growth factor stimulate proliferation of many different cell types but are not mitogenic in MCF-7 cell cultures (14). However, these factors do cause transient activation of MAPK upon addition to serum-starved MCF-7 cells, comparable to the level of activation observed with IGF-I (46). The physiological substrate of the insulin and type I IGF receptor, IRS-1, has been shown to activate PI3-K directly by binding to its p85 subunit (47). IRS-1 is not phosphorylated by the epidermal growth factor and platelet-derived growth factor receptors. This observation again hints to the important role of PI3-K in the induction of mitogenesis by IGF-I in MCF-7 cells.
Further study on the signal transduction pathways of IGF-I in other cell types will be necessary to resolve this matter. In support of our findings in MCF-7 cells, IGF-I has recently been reported to activate PI3-K in C2C12 myoblasts. IGF-I elicits a mitogenic response in these cells that exhibit only minimal MAPK activity after IGF-I stimulation. Surprisingly, PI3-K activation was not blocked by wortmannin, and the authors suggest a wortmannin-insensitive signaling event involving association of PI3-K with IRS-1 (49). Finally, several reports have recently been published (50–52) in which the anti-apoptotic effect of IGF-I in several cell types is shown to be mediated by the PI3-K signaling pathway.

In addition to the effects of the mitogen IGF-I, we have determined the effects of the growth inhibitor TPA on the levels of cyclin D1, cdk4, and p21WAF1/CIP1 and on hyperphosphorylation of pRb. The growth inhibitory properties of TPA in MCF-7 cells have been described several years ago (19, 53). Growth inhibition and induction of differentiation by TPA have also been documented in other cell types, e.g. keratinocytes (21, 54) and melanoma-derived cells (20, 55). In contrast, TPA has been shown to stimulate the growth of several other cell types, e.g. T-lymphocytes (16), adrenocortical cells (17), and fibroblasts (18). We show here that TPA induces cyclin D1 mRNA and protein expression in MCF-7 cells to an extent comparable to the induction observed with IGF-I. Like IGF-I, TPA does not affect cdk4 levels. Furthermore, TPA very strongly induces expression of p21WAF1/CIP1 mRNA and protein, and no hyperphosphorylation of pRb is observed after TPA addition to serum-starved MCF-7 cells. TPA also induces p21WAF1/CIP1 when it is added to randomly growing MCF-7 cells. From this we conclude that, despite the clear induction of cyclin D1, TPA has growth inhibitory properties in these cells because of its strong stimulatory effect on p21WAF1/CIP1 production. This probably results in inactivation of the cyclin D1-cdk4 complex and consequently pRb remains hypophosphorylated. Cyclin D1 induction by TPA is inhibited by LY294002 and, to a lesser extent, by rapamycin. PD098059 does not influence stimulation of cyclin D1 transcription. From these data we conclude that active PI3-K is a prerequisite for the induction of cyclin D1 by TPA, whereas the MEK1-MAPK route is not involved. So far no data have been published on TPA-induced signaling via PI3-K. However, the reproducible and clear effect of the PI3-K inhibitor on cyclin D1 induction by TPA suggests that TPA is able to activate PI3-K in the MCF-7 cell line.

Conversely, TPA induction of p21WAF1/CIP1 is insensitive to LY294002 and rapamycin, whereas PD098059 does inhibit TPA-induced p21WAF1/CIP1 expression. This suggests that p21WAF1/CIP1 induction by TPA is mediated via the MEK1-MAPK pathway and that the PI3-K route is not involved. Induction of p21WAF1/CIP1 by TPA has been reported previously in human fibroblasts, in several leukemic cell lines, and in MCF-7 cells (28, 56, 57). Furthermore, for PC12 neuronal cells it has been shown that the MEK1-MAPK pathway is involved in the activation of p21WAF1/CIP1 expression (58). Our data now show that the same is true for MCF-7 cells. In conclusion, our data lead to the following model. In human MCF-7 breast carcinoma cells, active PI3-K is necessary for the transmission of the growth stimulatory IGF-I signal, whereas the MEK1-MAPK route is not involved in mitogenic signaling. Conversely, transmission of the growth inhibitory signal of TPA, resulting in high levels of p21WAF1/CIP1 does not require active PI3-K but involves the MEK1-MAPK pathway.

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