Stimulation of the breast cancer-derived MCF-7S cell line with insulin-like growth factor I (IGF-I; 20 ng/ml) leads to enhanced expression of cyclin D1, hyperphosphorylation of pRb, DNA synthesis, and cell division. 17β-Estradiol (E2; 10–10 M) is not able to stimulate proliferation of MCF-7S cells, although addition of E2 to serum-starved cells does result in induction of cyclin D1. However, in combination with submicromolar amounts of IGF-I (2 ng/ml), E2 induces cell proliferation. We have previously shown that the synergistic action of E2 and IGF-I emanates from the ability of both hormones to induce cyclin D1 expression and that IGF-I action is required to induce activity of the cyclin D1-CDK4 complex, which triggers cell cycle progression. Here, we show that IGF-I (but not E2) is able to induce nuclear accumulation of cyclin D1 by a phosphatidylinositol 3-kinase-dependent mechanism. Nuclear accumulation of cyclin D1 and cell cycle progression were also observed when LiCl, a known inhibitor of GSK3β, was added to E2-stimulated cells. Thus, inhibition of GSK3β activity appears to trigger nuclear accumulation of cyclin D1 and cell cycle progression. This notion was confirmed by overexpression of constitutively active GSK3β, which blocks IGF-I-induced nuclear accumulation of cyclin D1 as well as S phase transition.

The importance of estrogen action in the regulation of breast cancer cell proliferation has been demonstrated both in vivo and in vitro (1). Epidemiological studies have shown that estrogen exposure is a causative factor in the onset of breast cancer (2). Clinical studies in which the effects of pharmacological agents inhibiting the synthesis or action of estrogen have been studied have demonstrated the fundamental importance of estrogen-induced mitogenesis in breast cancer (3). From recent research, it has become clear that estrogens act in concert with other mitogens in the regulation of breast epithelial cell proliferation. In particular, synergistic effects on proliferation have been described with members of the insulin-like growth factor (IGF) family. The IGFs play important roles in the normal regulation of cell proliferation, differentiation, and apoptosis. Breast tumors have been shown to have defects in the regulation and function of the IGF ligands, binding proteins, and receptors (4, 5), and clinical trials targeting IGF signaling have been very successful in treating breast carcinomas (5, 6).

Originally, it was considered that IGF-I and 17β-estradiol (E2) manifest their mitogenic actions through separate pathways, but a growing body of evidence suggests that the IGF-I and E2-mediated signaling pathways are intertwined. We have reported previously that quiescent MCF-7S breast cancer epithelial cells resume the cell division cycle when IGF-I (20 ng/ml) is added to their phenol red- and serum-free medium (7). We have demonstrated that E2 by itself at concentrations ranging from 0.1 to 100 nM does not induce cell cycle progression in quiescent MCF-7S cells. However, a combination of submicromolar amounts of IGF-I and E2 synergistically induces cell cycle progression and proliferation (8).

It should be noted that, in contrast to other laboratory MCF-7 cell lines, MCF-7S cells are almost completely growth-arrested in G0/G1 by serum deprivation in estrogen-free medium, without the need for estrogen antagonists to reach quiescence. However, when the cells are cultured for >30 passages, they start to grow more rapidly and no longer show complete growth arrest upon serum withdrawal. The serum-starved MCF-7S cells become progressively less dependent on IGF-I with passage number. Eventually, proliferation of serum-starved MCF-7S cells can be induced by E2 without addition of IGF-I, as is observed in most laboratory strains of MCF-7. In vitro cultures of cancer cells are known to change their growth characteristics with time. The cultures become increasingly more independent of exogenously added growth factors because of the selective growth advantage of cells producing growth factors themselves. For this reason, no MCF-7S cells with a passage number over 25 were used in our experiments.

Using the MCF-7S cells as a model system, we investigated the pathways involved in the synergistic activation of cell cycle progression by IGF-I and E2. In contrast with several published reports, we could not detect any activation of cytoplasmic signaling cascades by E2 and any synergy in the activation of these cascades by IGF-I and E2. In terms of cell cycle-related molecules, we found that IGF-I dose-dependently raised cyclin D1 levels in serum-starved cells. Subsequent activation of the cyclin E-CDK2 complex, hyperphosphorylation of pRb, and DNA synthesis were detected only in cells treated with mitogenic concentrations of IGF-I. Treatment of cells with E2 also...

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led to induction of cyclin D1, but, in the absence of IGF-I, did not lead to cell cycle progression. The absence of CDK4-specific pRb Ser780 phosphorylation after treatment of the cells with E2 suggested that the cyclin D1-CDK4 complex was not activated in these cells. We have shown that co-exposure of the cells to E2 and a submitogenic amount of IGF-I did activate the cyclin D1-CDK4 complex. From this, we concluded that IGF-I signaling is required for G1-to-S phase transition of E2 is present to induce cyclin D1 levels, a non-mitogenic amount of IGF-I suffices to activate the cyclin D1-CDK4 complex and to enter S phase. The nature of the role of IGF-I in the activation of the complex is not clear. The activity of the cyclin D1-CDK4 complex is dependent on multiple factors, including composition and subcellular localization of the complex and phosphorylation status of CDK4.

Here, we investigated the mechanism by which IGF-I induces the activation of the cyclin D1-CDK4 complex. We show that cyclin D1, although strongly induced, was not translocated to the nucleus in late G1 phase in E2-treated MCF-7S cells. Nuclear accumulation of cyclin D1 and activation of the cyclin D1-CDK4 complex were observed only in cells with elevated cyclin D1 levels subjected to the action of per se non-mitogenic amounts of IGF-I.

EXPERIMENTAL PROCEDURES

Reagents—The HA-GSK3β/S89A construct was a kind gift of Dr. B. Burgering. Anti-cyclin D1 (H-255 and M-20), anti-cyclin E (C-19), anti-CDK4 (C-22), anti-IAA probe (V-11), anti-p21Cip1/WAF1 (H-164), and anti-p27 Kip1 (C-25020) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-p21Cip1/WAF1 antibodies (G25220) and anti-GSK3β antibodies (G22320) antibodies were obtained from Transduction Laboratories (Lexington, KY). Anti-cyclin D1 monoclonal antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit antibodies were from Bio-Rad. Biotin-labeled goat anti-mouse antibody and Cy2-labeled streptavidin was from Amersham Biosciences (Arlington Heights, IL). Aprotinin, E2, leptomycin B, phenylmethylsulfonyl fluoride, and sodium 3-mercaptopropionic acid were from Sigma (St. Louis, MO). Aprotinin, E2, leptomycin B, and sodium 3-mercaptopropionic acid were from Sigma. E2 and sodium 3-mercaptopropionic acid were from Sigma.

Serum-starved cells were stimulated with IGF-I, E2, or combination of the two hormones. After 24 h of incubation, [3H]thymidine (2 Ci/ml) was added. 30 h after addition of the stimuli, cells were fixed with 10% formalin in PBS, washed with PBS, and lysed in sample buffer (50 mM Tris-HCl, pH 8.0, 5% SDS, 1% β-mercaptoethanol, and 0.1% glycerol). The samples were shocked, and a volume of lysate corresponding to 100–400 μg of protein was precleaved by incubation with 50 μl of 10% protein A-agarose beads for 1 h at 4°C. Lysates were incubated overnight at 4°C with 50 μl of 10% protein A beads coupled to the appropriate antibody. Beads were washed three times with immunoprecipitation buffer and resuspended in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% β-mercaptoethanol, and 10% glycerol). The samples were subjected to SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane.

For detection of proteins in total lysates, cells were seeded in six-well plates and 25- or 75-cm² cell culture flasks. After stimulation with hormones, cells were washed with ice-cold PBS and lysed in sample buffer. Lysates were boiled for 5 min, and protein concentrations were determined using the BCA protein assay (Pierce). Equal amounts of protein were loaded on an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane.

Specific proteins were detected by Western blotting. In brief, the polyvinylidene difluoride membrane was blocked with 2–3% nonfat milk or 0.5–2% BSA in 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.2% Tween 20 (Tris-buffered saline/Tween) for 1 h. Incubation with the primary antibody was performed overnight at 4°C in 0.1% blocking solution. The membrane was washed with Tris-buffered saline/Tween and exposed to horseradish peroxidase-conjugated secondary antibody for 1 h in Tris-buffered saline/Tween. For detection, the Western blot chemiluminescence reaction was added (PerkinElmer Life Sciences) was used.

Cdk2 Kinase Assay—The Cdk2 assay was performed as described previously (7). Briefly, cells were treated with the stimuli and then washed with PBS and lysed in immunoprecipitation buffer. After pre-clearing, the lysates were incubated overnight at 4°C with 50 μl of 10% protein A beads coupled to anti-Cdk2 antibody. Beads were washed several times and resuspended in kinase mixture. The mixture was incubated for 15 min at 30°C, after which the reaction was stopped with 4× sample buffer containing 400 μM dithiothreitol. The suspension was boiled and centrifuged, and the supernatant was run on SDS-polyacrylamide gel. The gel was dried and exposed to x-ray film. Levels of 3P incorporation in the substrate were quantified using a PhosphorImager (Amersham Biosciences).

Transfections—Cells were seeded in 6- or 12-well plates (106 cells/plate) 1 day before transfection. Transfections were done using the Dac30 transfection reagent (Europgentech, Seraing, Belgium). 2 μg Dac30 and 1–2 μg/well plasmid DNA were used, each diluted in 500 μl of HBS (150 mM NaCl and 20 mM HEPES, pH 7.4). The DNA and Dac30 dilutions were then added, gently mixed, and left at room temperature for 30 min. Immediately before transfection, the cell medium was refreshed using 1 ml/well. The DNA/Dac30 mixture was then added dropwise to the cells under continuous swirling, after which the cells were incubated for 3–4 h at 37°C. Subsequently, the transfection mixture was replaced by phenol red- and serum-free medium to serum-starve the cells.

Immunofluorescence Staining and Microscopy—For detection of specific immunofluorescence, cells were seeded in 12-well plates (106 cells/plate) on 12-mm-diameter glass coverslips. After stimulation with hormones, cells were washed twice with ice-cold PBS, and the coverslips were transferred into humid incubation chambers, where they were kept until the immunofluorescence staining procedure of the cells was completed. To fix the cells, they were incubated with paraformaldehyde for 15 min at room temperature. Next, the coverslips were washed three times with PBS, and then added dropwise to the cells under continuous swirling, after which they were incubated for 3–4 h at 37°C. Subsequently, the coverslips were replaced by phenol red- and serum-free medium to serum-starve the cells.

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cells were grown in 75-cm² culture flasks and serum-starved for 26 h. 20 ng/ml IGF-I (I(20)), 2 ng/ml IGF-I (I(2)), 1 nM E₂ or a combination of 2 ng/ml IGF-I and 1 nM E₂ (I(2)E₂) was added for 16 h. Cell lysates were subjected to immunoprecipitation (IP) with anti-cyclin D1/2 monoclonal antibody. The precipitated proteins were size-separated on an SDS-polyacrylamide gel and analyzed by Western blotting. Co-immunoprecipitation of CDK4, p27, and p21 was visualized with the appropriate antibodies. Presence of the proteins in the total lysate is shown on the same blot. In the immunoprecipitated samples of the p27 blot, an additional band was detected, migrating slightly faster than p27. This is the IgG light chain for the immunoprecipitation antibody that also reacted with the anti-mouse secondary antibody.

**RESULTS**

**IGF-I and E₂ Do Not Affect the Composition of the Cyclin D1-CDK4 Complex**—We have previously shown that submitogenic amounts of IGF-I in combination with E₂ synergistically induce proliferation of the MCF-7S human breast tumor epithelial cell line, whereas E₂ on its own does not induce proliferation in this particular subline of MCF-7 (8). Although both hormones induce cyclin D1 protein expression, we demonstrated that in these cells, IGF-I action is required to induce cyclin D1-CDK4 complex-specific phosphorylation of serine 780 in pRb (8). To establish this particular role of IGF-I, the composition of the cyclin D1-CDK4 complex was studied after addition of IGF-I and/or E₂ to the cells. Serum-starved MCF-7S cells were given no mitogens, mitogenic amounts of IGF-I (20 ng/ml), mitogenic amounts of IGF-I (2 ng/ml), a combination of submitogenic amounts of IGF-I (2 ng/ml) and E₂ (1 nM), or E₂ (1 nM). After 16 h of stimulation, the cells were lysed. In the lysate, elevated cyclin D1 levels were detected in cells treated with IGF-I (20 ng/ml), with E₂, and with a combination of IGF-I (2 ng/ml) and E₂. The hormones did not significantly alter CDK4 and p27 levels, but p21 was up-regulated when the cells were treated with mitogenic amounts of IGF-I (Fig. 1, Total lysate). For detection of proteins associated with cyclin D1, cyclin D1 was immunoprecipitated. Co-immunoprecipitation of CDK4, p21, and p27 was detected by Western blotting. Although the absolute amount of immunoprecipitated cyclin D1 and of co-immunoprecipitated CDK4, p27, and p21 varied in the five immunoprecipitated samples, we found no obvious differences when comparing the relative content of these proteins in the cyclin D1 complexes from cells treated with a mitogenic stimulus (20 ng/ml IGF-I or 2 ng/ml IGF-I and 1 nM E₂) or with a non-mitogenic stimulus (2 ng/ml IGF-I or 1 nM E₂) (Fig. 1, IP). Conceivably, other proteins associated with the complex may regulate the activity of CDK4. A number of reports have shown CDC37, HSP90, and calmodulin to be involved in the regulation of CDK4 activity (9–11). However, we could not detect any of these proteins in our co-immunoprecipitation studies, although they were readily detected in the total lysate samples. Moreover, treatment of the cells with the different hormones had no effect on the levels of any of the three proteins in the total lysates (data not shown).

**E₂ in Combination with IGF-I Induces Nuclear Accumulation of Cyclin D1 during Late G₁ Phase**—To establish whether IGF-I affects the subcellular localization of cyclin D1 during the G₁ phase of the cell cycle, we stained serum-starved and hormone-treated cells for cyclin D1 expression in a time course experiment for up to 20 h after stimulation. We found that mitogenic stimuli were able to change the subcellular localization of cyclin D1. In serum-starved cells, a low level of cyclin D1 was present, as shown in Fig. 1. The localization of cyclin D1 in these cells was predominantly perinuclear (Fig. 2A). In cells treated with non-mitogenic concentrations of either IGF-I or E₂, the localization of the cyclin D1 protein was not changed. Only in a small percentage of cells was nuclear fluorescence detected, 4 and 2% for IGF-I (2 ng/ml)- and E₂-treated cells, respectively (Fig. 2A and B). However, treatment of the cells with mitogenic concentrations of IGF-I or with non-mitogenic concentrations in combination with E₂ did result in a change in localization. We first observed this change around 16 h after addition of the stimulus (data not shown). Therefore, the cells
in the experiments shown were all fixed and stained after 17 h of stimulation. In 94 and 87% of cells treated with 20 ng/ml IGF-I or with 2 ng/ml IGF-I and 1 nM E2, respectively, the cyclin D1 staining was nuclear with a uniform distribution (Fig. 2, A and B), except for the nucleoli that appeared unstained. This accumulation in the nucleus seems essential for cell cycle progression, which may be explained by the fact that the pRb protein, one of the major CDK4 targets, is also localized in the nucleus during G0/G1 phase (12, 13).

Our data show that mitogenic concentrations of IGF-I (20 ng/ml) and submitogenic amounts of IGF-I (2 ng/ml) in combination with E2 induced nuclear accumulation of cyclin D1, whereas IGF-I (2 ng/ml) and E2 (1 nM) separately did not. This suggests that an IGF-I-activated signal is essential for nuclear accumulation. However, the elevation of cyclin D1 levels seems to be essential as well because IGF-I (2 ng/ml) did not induce nuclear accumulation. This suggests that a threshold in cyclin D1 levels must be reached before an IGF-I-activated signal facilitates the accumulation of the protein in the nucleus.

**Nuclear Accumulation of Cyclin D1 Is Regulated via the PI3K Pathway**—Previously, we demonstrated that neither the ERK2 kinase pathway nor the PI3K pathway is significantly activated after treatment of MCF-7S cells with E2, whereas stimulation with IGF-I leads to the activation of both pathways in a dose-dependent manner (8). Using specific inhibitors, we have shown activation of the PI3K pathway by IGF-I to be essential for cell cycle progression. In particular, this pathway was shown to be involved in the regulation of cyclin D1 mRNA and protein levels (7, 14). Here, we investigated whether the PI3K or ERK pathway is involved in the transduction of the IGF-I signal leading to nuclear accumulation of cyclin D1. To this end, inhibitors of these pathways were added to serum-starved MCF-7S cells 1 h prior to stimulation with hormones.

The PI3K inhibitor LY294002 (25 μM) completely abolished nuclear accumulation of the cyclin D1 protein in late G1 phase. In cells treated with the LY294002 inhibitor and mitogenic amounts of IGF-I or a combination of submitogenic amounts of IGF-I and E2, the percentage of cells showing nuclear accumulation of cyclin D1 was reduced to background levels (Fig. 3). The ERK pathway is not critically involved in induction of nuclear accumulation because the MEK inhibitor PD098059 (40 μM) did not significantly block translocation of cyclin D1 to the nucleus. Some residual cytoplasmic staining was visible in cells treated with PD098059 in combination with either 20 or 2 ng/ml IGF-I together with 1 nM E2 (Fig. 3A). However, this effect may well be due to an aspecific stress response to the presence of the inhibitor in the medium for 18 h.

![Fig. 3. Effect of PI3K and ERK inhibition on the subcellular localization of cyclin D1 in IGF-I- and E2-treated MCF-7S cells.](http://www.jbc.org/Downloaded from)

![Fig. 4. Phosphorylation of GSK3β in IGF-I- and E2-treated MCF-7S cells.](http://www.jbc.org/Downloaded from)

**Submitogenic Concentrations of IGF-I Inactivate GSK3β Kinase Activity by Phosphorylation of Serine 9**—GSK3β is a well documented downstream target of PI3K. Activation of PI3K leads to activation of protein kinase B (AKT-1), which has been...
shown to inactivate GSK3β by specific phosphorylation. Recently, GSK3β has been implicated in the regulation of the relocalization of cyclin D1 from the nucleus to the cytoplasm during S phase (15). To investigate whether nuclear accumulation of cyclin D1 in G1 phase is regulated by GSK3β, we first determined whether the GSK3β kinase was inhibited upon...
Phosphorylation of Ser 9, which may be detected by Western concentrations of IGF-I led to a phosphorylated state of GSK3.

47650

The subcellular localization of cyclin D1 in IGF-I- and E2-treated E2 in the presence of higher concentrations of LiCl, no significant difference was observed compared with their counterparts without LiCl. The higher level of cyclin D1 in IGF-I (2 ng/ml)-treated cells in the presence of LiCl may explain the observed increase in numbers of cells showing nuclear localization of cyclin D1.

To investigate whether the nuclear accumulation of cyclin D1 observed in cells stimulated with IGF-I (2 ng/ml) and E2 in the presence of LiCl leads to cell cycle progression, we studied the levels and activities of proteins involved in G1-to-S phase transition. The activity of the cyclin E-CDK2 complex was monitored in cells stimulated for 22 h with hormones in the presence or absence of 10 mM LiCl. The cells were lysed, and CDK2 was immunoprecipitated. Kinase activity was measured using histone H1 as substrate. Fig. 5D shows that addition of IGF-I (20 ng/ml) as well as of a combination of IGF-I (2 ng/ml) and E2 (1 nM) in the absence of LiCl led to a clear increase in active cyclin E-CDK2 complex activity in the cells, whereas treatment of the cells with either IGF-I (2 ng/ml) or E2 increased the activity of the kinase only slightly. In serum-starved and IGF-I (2 ng/ml)-treated cells, addition of 10 mM LiCl raised background CDK2 activity slightly, whereas it had little additional effect on the already active CDK2 in IGF-I (20 ng/ml)- or IGF-I (2 ng/ml/E2)-treated cells. However, a large effect was seen in cells treated with E2. In the presence of LiCl, the CDK2 kinase activity became almost equal to the activity in samples of cells treated with mitogenic concentrations of IGF-I or with a combination of IGF-I and E2. This again suggests that LiCl can mimic the action of IGF-I when used in combination with E2. Surprisingly, in cells treated with only low amounts of IGF-I in the presence of LiCl, nuclear accumulation of the weakly induced cyclin D1 was observed, but no activation of CDK2 ensued.

Phosphorylation of the pocket protein pRb is a subsequent important event in G1-to-S phase progression. We studied the phosphorylation status of pRb in cells treated with E2, IGF-I, or with mitogenic amounts of IGF-I or with a combination of IGF-I and E2. This again suggests that LiCl can mimic the action of IGF-I when used in combination with E2. Surprisingly, in cells treated with only low amounts of IGF-I in the presence of LiCl, nuclear accumulation of the weakly induced cyclin D1 was observed, but no activation of CDK2 ensued.

A number of studies have shown that the cyclin D1 protein is stabilized by inhibition of GSK3β (15, 17). Therefore, we investigated whether LiCl by itself has an effect on cyclin D1 levels in MCF-7S cells. Fig. 5C shows that when LiCl was present in the medium of serum-starved or hormone-treated cells for 16 h, a rise in cyclin D1 levels was observed in all of the samples compared with their counterparts without LiCl. The higher level of cyclin D1 in IGF-I (2 ng/ml)-treated cells in the presence of LiCl may explain the observed increase in numbers of cells showing nuclear localization of cyclin D1.

In vivo addition of non-mitogenic concentrations of IGF-I to serum-starved MCF-7S cells. In vivo, GSK3β is rendered inactive by phosphorylation of Ser9, which may be detected by Western blotting using an antibody specifically recognizing phospho-GSK3β Ser9. As shown in Fig. 4, Ser9 of GSK3β remained phosphorylated for at least 4 h after addition of IGF-I (2 ng/ml) to the cells, whereas addition of E2 did not lead to increased phosphorylation of GSK3β at any time. Addition of mitogenic concentrations of IGF-I led to a phosphorylated state of GSK3β for at least 16 h.

LiCl, an Inhibitor of GSK3β, Induces Nuclear Accumulation of Cyclin D1 and, in Combination with E2, Leads to G1-to-S Phase Transition—Subsequently, we used LiCl, a chemical inhibitor of GSK3β, to establish the role of the kinase in the regulation of the subcellular localization of cyclin D1 during G1 phase. LiCl inhibits GSK3β by competition for Mg2+ binding, but not for ATP or substrate binding, and has been shown to be highly specific for GSK3β (16). We determined whether inhibition of GSK3β by LiCl leads to nuclear accumulation of cyclin D1 and to cell cycle progression. Immunofluorescence staining of cyclin D1 showed that treatment of quiescent cells with 10 mM LiCl for 17 h led to an increase in nuclear cyclin D1. The percentage of cells showing nuclear cyclin D1 in samples treated with mitogenic amounts of IGF-I or with submitogenic amounts of IGF-I in combination with E2 was not significantly changed by LiCl addition (Fig. 5, A and B). In untreated and IGF-I (2 ng/ml)-treated cells, however, nuclear staining was observed in 25 and 61% of the cells, respectively, compared with 1 and 4% without LiCl. The largest difference was observed with E2, showing 83% of the cells with nuclear localization of cyclin D1 in the presence of LiCl versus only 2% in the absence of LiCl. Because of considerable variations in LiCl concentrations used in published reports, we initially tested concentrations ranging from 2.5 to 50 mM. In cells treated with E2 in the presence of higher concentrations of LiCl, no significant increase in the percentage of cells with nuclear cyclin D1 was observed compared with the percentage of cells treated with E2 in the presence of 10 mM LiCl. In cells treated with E2 in the presence of lower concentrations of LiCl, the percentage of cells with nuclear localization of cyclin D1 was not or only slightly raised compared with background levels (data not shown). In subsequent experiments, 10 mM LiCl was used to inhibit endogenous GSK3β activity.

Progression through S phase was further monitored by a DNA synthesis assay. Serum-starved MCF-7S cells were treated with IGF-I and E2 in the presence or absence of LiCl.
After 24 h, [3H]thymidine was added; the cells were harvested after further incubation for 6 h; and [3H]thymidine incorporation was measured by liquid scintillation counting. Fig. 5F (left panel) shows that E₂ by itself induced only a 13-fold increase in [3H]thymidine incorporation compared with background incorporation in unstimulated cells during the same time period. Mitogenic amounts of IGF-I induced a 142-fold increase in [3H]thymidine incorporation, whereas IGF-I at a non-mitogenic concentration induced only a small increase, comparable with the amount of incorporation observed with E₂. A combination of IGF-I (2 ng/ml) and E₂ induced a 134-fold increase.

Addition of LiCl to serum-starved cells induced an 8-fold increase in [3H]thymidine incorporation, whereas IGF-I at a non-mitogenic concentration induced only a small increase, comparable with levels with IGF-I or E₂ combined with submitogenic concentrations of IGF-I. This correlates well with the observed activation of CDK2 and hyperphosphorylation of pRB in E₂-treated cells in the presence of LiCl. DNA synthesis in E₂-stimulated cells in the presence of higher concentrations of LiCl up to 25 mM was not significantly different from that observed in E₂-treated cells in the presence of 10 mM LiCl (Fig. 5F, right panel). Higher concentrations of LiCl (50 mM) resulted in low levels of [3H]thymidine incorporation, although nuclear accumulation of cyclin D1 was observed. This may be due to cytotoxicity or aspecific inhibition of enzymes essential for progression through the cell cycle.

We conclude that inhibition of GSK3β by LiCl leads to nuclear accumulation of cyclin D1 and raises steady-state cyclin D1 levels. Under these conditions, addition of E₂ to the cells leads to CDK2 activation, pRB phosphorylation, and DNA synthesis. LiCl treatment thus substitutes for IGF-I, which is able to suppress GSK3β activity via PI3K. Inhibition of GSK3β thus seems a key event in the regulation of cell cycle progression in MCF-7S cells.

The GSK3β(S9A) Mutant Inhibits Nuclear Translocation of Cyclin D1—To further investigate the involvement of GSK3β in induction of cyclin D1 accumulation in the nucleus during G₁ phase, MCF-7S cells were transfected with the HA-tagged GSK3β(S9A) mutant. This mutant lacks the Ser9 phosphorylation site, making it inactive by protein kinase B. However, GSK3β(S9A) can still be inactivated by LiCl (20). After transfection, the cells were steroid hormone-deprived and serum-starved for 24 h. Subsequently, no mitogens, IGF-I (20 ng/ml), or IGF-I (2 ng/ml) in combination with E₂, was added in the presence or absence of 25 mM LiCl. 25 mM LiCl was used to ensure optimal inhibition of the overexpressed GSK3β without aspecific inhibitory effects on cell cycle progression in MCF-7S cells (Fig. 5F, right panel). 17 h after addition of the hormones, cells were fixed and stained for HA and cyclin D1 expression. We found that transfection of the cells with GSK3β(S9A) had no effect on the localization of cyclin D1 in serum-starved cells. Stimulation of GSK3β(S9A)-transfected cells with mitogenic amounts of IGF-I or with a combination of non-mitogenic amounts of IGF-I and E₂ did not result in nuclear accumulation of cyclin D1, whereas cyclin D1 was nuclear in the non-transfected cells in the same experiment. In the presence of LiCl, the nuclear accumulation in the stimulated transfected cells was restored (Fig. 6). GSK3β inactivation is thus a necessary step in the accumulation of cyclin D1 in the nucleus in late G₁ phase.

**DISCUSSION**

Estrogen and IGF-I are hormones involved in a wide variety of processes regulating proliferation, apoptosis, and differentiation in mammalian cells (21, 22). In MCF-7S cells, a combination of IGF-I (2 ng/ml) and E₂ induced a marked increase, comparable with the amount of incorporation observed with E₂. A combination of IGF-I (2 ng/ml) and E₂ induced a 134-fold increase.

Addition of LiCl to serum-starved cells induced an 8-fold increase in [3H]thymidine incorporation, whereas IGF-I at a non-mitogenic concentration induced only a small increase, comparable with levels with IGF-I or E₂ combined with submitogenic concentrations of IGF-I. This correlates well with the observed activation of CDK2 and hyperphosphorylation of pRB in E₂-treated cells in the presence of LiCl. DNA synthesis in E₂-stimulated cells in the presence of higher concentrations of LiCl up to 25 mM was not significantly different from that observed in E₂-treated cells in the presence of 10 mM LiCl (Fig. 5F, right panel). Higher concentrations of LiCl (50 mM) resulted in low levels of [3H]thymidine incorporation, although nuclear accumulation of cyclin D1 was observed. This may be due to cytotoxicity or aspecific inhibition of enzymes essential for progression through the cell cycle.

We conclude that inhibition of GSK3β by LiCl leads to nuclear accumulation of cyclin D1 and raises steady-state cyclin D1 levels. Under these conditions, addition of E₂ to the cells leads to CDK2 activation, pRB phosphorylation, and DNA synthesis. LiCl treatment thus substitutes for IGF-I, which is able to suppress GSK3β activity via PI3K. Inhibition of GSK3β thus seems a key event in the regulation of cell cycle progression in MCF-7S cells.

The GSK3β(S9A) Mutant Inhibits Nuclear Translocation of Cyclin D1—To further investigate the involvement of GSK3β in induction of cyclin D1 accumulation in the nucleus during G₁ phase, MCF-7S cells were transfected with the HA-tagged GSK3β(S9A) mutant. This mutant lacks the Ser9 phosphorylation site, making it inactive by protein kinase B. However, GSK3β(S9A) can still be inactivated by LiCl (20). After transfection, the cells were steroid hormone-deprived and serum-starved for 24 h. Subsequently, no mitogens, IGF-I (20 ng/ml), or IGF-I (2 ng/ml) in combination with E₂, was added in the presence or absence of 25 mM LiCl. 25 mM LiCl was used to ensure optimal inhibition of the overexpressed GSK3β without aspecific inhibitory effects on cell cycle progression in MCF-7S cells (Fig. 5F, right panel). 17 h after addition of the hormones, cells were fixed and stained for HA and cyclin D1 expression. We found that transfection of the cells with GSK3β(S9A) had no effect on the localization of cyclin D1 in serum-starved cells. Stimulation of GSK3β(S9A)-transfected cells with mitogenic amounts of IGF-I or with a combination of non-mitogenic amounts of IGF-I and E₂ did not result in nuclear accumulation of cyclin D1, whereas cyclin D1 was nuclear in the non-transfected cells in the same experiment. In the presence of LiCl, the nuclear accumulation in the stimulated transfected cells was restored (Fig. 6). GSK3β inactivation is thus a necessary step in the accumulation of cyclin D1 in the nucleus in late G₁ phase.

**DISCUSSION**

Estrogen and IGF-I are hormones involved in a wide variety of processes regulating proliferation, apoptosis, and differentiation in mammalian cells (21, 22). In MCF-7S cells, a combination of IGF-I (2 ng/ml) and E₂ induced a 134-fold increase.

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thus directly induced nuclear accumulation of cyclin D1 once GSK\(3\beta\) was inactivated. However, we found that non-mitogenic concentrations of IGF-I (in the presence or absence of E\(_2\)) inhibited the GSK\(3\beta\) kinase for only 4–6 h and that nuclear accumulation of cyclin D1 was first observed after 16 h. If inactivation of GSK\(3\beta\) induces nuclear accumulation of cyclin D1 directly by regulating cyclin D1 phosphorylation, we should be able to observe the accumulation much before 16 h after addition of IGF-I to the cells. Second, if nuclear accumulation is exclusively caused by decreased nuclear export by impeding the association of CRM1 with cyclin D1, this would suggest that there is constitutive import of the complex into the nucleus. Addition of leptomycin B, a known inhibitor of CRM1, to MCF-7S cells should thus lead to nuclear accumulation of cyclin D1. However, we found that addition of leptomycin B to the cells for 3 h in mid-late G1 phase did not lead to nuclear accumulation of cyclin D1 (data not shown). Taken together, these data suggest that nuclear accumulation of cyclin D1 is indirectly regulated by GSK\(3\beta\) via a process of activated import rather than inhibited export by CRM1.

Treatment of MCF-7S cells with E\(_2\) resulted in induction of cyclin D1, but not in nuclear accumulation of cyclin D1, as long as GSK\(3\beta\) was not inactivated. Treatment of serum-starved MCF-7 cells with submitogenic amounts of IGF-I (2 ng/ml) inactivated GSK\(3\beta\), but still did not induce nuclear accumulation of cyclin D1. Only a combination of the two hormones induced nuclear accumulation of cyclin D1, suggesting that inactivation of GSK\(3\beta\), as well as a certain threshold level of cyclin D1, is required for the change in cyclin D1 localization to occur. In fibroblasts, a protein named SSceCKS has been shown to bind cyclin D1 in a phosphorylation-dependent manner and to act as a cytoplasmic anchor (26–28). Stimulation of the fibroblasts with mitogens was shown to cause down-regulation of the protein and, in addition, to induce phosphorylation of the protein by protein kinase C. Both effects together result in nuclear accumulation of cyclin D1 (26–28). Conceivably, this mechanism also operates in MCF-7S cells. This would suggest that cyclin D1 is bound to SSceCKS, retaining it in the cytoplasm, as long as GSK\(3\beta\) is active. When GSK\(3\beta\) is inactivated by IGF-I stimulation of the cells, the scaffolding protein is down-regulated and phosphorylated by protein kinase C, making it unable to retain the cyclin D1 protein in the cytoplasm as soon as the threshold level is reached. However, in MCF-7S cells, inhibition of protein kinase C did not result in retention of cyclin D1 in the cytoplasm (data not shown). This finding suggests that SSceCKS is not involved in the regulation of the subcellular localization of cyclin D1 in MCF-7S cells. Nevertheless, other scaffolding proteins may bind cyclin D1 and retain it in the cytoplasm until both GSK\(3\beta\) is inactivated and the cyclin D1 threshold level is reached.

We found that low concentrations of IGF-I in the presence of LiCl to some extent induced cyclin D1 protein levels as well as nuclear accumulation of cyclin D1, but that no cell cycle progression ensued. This would imply that not only a threshold level of cyclin D1 must be reached before the protein can translocate to the nucleus, but that a threshold level of cyclin D1 or of cyclin D1-CDK4 complex activity must be reached as well before the cells progress through the cell cycle.

Based on our findings, we propose a model (Fig. 7) in which the synergy of IGF-I and E\(_2\) signaling is explained by regulation of cyclin D1 levels and by GSK\(3\beta\) inactivation. High levels of IGF-I are capable of inducing cyclin D1 and of inactivating GSK\(3\beta\). E\(_2\) may substitute for IGF-I in its capacity to enhance cyclin D1 levels. Because much lower concentration of IGF-I is needed to suppress GSK\(3\beta\) activity, per se non-mitogenic amounts of IGF-I in synergy with E\(_2\) may now trigger proliferation of MCF-7S cells.

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