We investigated the role of 14-3-3 protein in insulin-like growth factor-I (IGF-I) receptor signaling. It has been previously shown that 14-3-3 protein negatively regulates cell cycle especially in response to p53-sensitive DNA damage. In this study we demonstrated that 14-3-3 protein is a positive mediator of IGF-I receptor-induced cell proliferation. Treatment with IGF-I increased 14-3-3 protein and protein levels about 4-fold, in a time-dependent manner in MCF-7 breast cancer cells. Preincubation with the phosphoinositide 3'-kinase inhibitor LY294002 significantly reduced the effects of IGF-I on 14-3-3 gene expression in these cells, suggesting that this effect of IGF-I occurs via the phosphoinositide 3'-kinase pathway. 14-3-3 protein is induced by IGF-I in MCF-7 cells, which express wild-type p53, as well as in MCF-7 cells transfected with a small interfering RNA targeting duplex that reduced p53 expression levels. These results suggest that IGF-I induces 14-3-3 protein in a manner that is independent of p53. Using the small interference RNA strategy, we demonstrated that a 70–75% reduction of 14-3-3 protein levels resulted in a similar decrease in the effects of IGF-I on cell cycle progression and proliferation in MCF-7 cells. This effect was also associated with a reduction in IGF-I-induced cyclin D1 expression. Taken together, these results suggest that 14-3-3 protein positively mediates IGF-I-induced cell cycle progression.

The insulin-like growth factor I receptor (IGF-IR) is a receptor tyrosine kinase that plays a critical role in cell survival and proliferation. Cells that lack the IGF-IR cannot be transformed by most oncogenes, with the exception of v-Src (1) and G12V (2). Following ligand-induced autophosphorylation of the IGF-IR, the receptor forms a complex with the Shc protein (3) and the various insulin receptor substrate molecules (4–7). The IGF-IR then phosphorylates these docking proteins on specific tyrosine residues. This leads to activation of two main signaling events: the mitogen-activated protein kinase (MAPK) pathway, which is implicated in receptor-mediated mitogenesis and transformation, and the phosphoinositide 3'-kinase (PI3K) pathway. Although the PI3K pathway has been implicated in the transmission of cell survival signals (8, 9), it may also be important for IGF-I-induced progression of the cell cycle (10).

The progression of a cell through the G1 phase of the cell cycle is regulated by the sequential expression and degradation of G1 cyclins and the resulting activation and inhibition of cyclin-dependent kinases (CDKs). The activity of CDKs is also influenced by the concentration of specific protein molecules known as cyclin-dependent kinase inhibitors (e.g. p27). Cyclin D1 is the regulatory subunit of CDK4/6, which catalyzes the phosphorylation of retinoblastoma protein (pRb). Phosphorylated pRb fails to bind and inactivate E2F, an S phase transcription factor (11). Cyclin D1 overexpression plays an essential, albeit subsidiary, role in the transition between the G1 and S phase in proliferating cells. It has been reported that the accumulation of cyclin D1 in cyclin D1-overexpressing tumors (12) accelerates the cell cycle by decreasing the duration of the G1 phase (13).

IGF-I has been shown to regulate both the expression and the activity of various molecules involved in progression of the cell cycle. Several studies have reported that IGF-I induces cyclin D1 expression in a number of tumor cell lines, including breast cancer cells (14–18). The idea that regulation of cyclin D1 plays an essential role in the mitogenic actions of IGF-I is consistent with studies showing that expression of antisense cyclin D1 in human pancreatic cancer cells abolished the ability of IGF-I to stimulate proliferation of these cells (16). IGF-I also induces the expression of cyclin E, which is involved in late G1 phase, in MCF-7 human breast cancer cells (19). In skeletal muscle satellite cells, IGF-I induces cyclin A2 (20). It has been also reported that IGF-I-stimulated proliferation of primary satellite cells was associated with the activation of PI3K/Akt and the down-regulation of cell cycle inhibitor p27kip1 (21). In MCF-7 cells, IGF-I induces the expression of p21 via the PI3K pathway to inhibit UV-induced cell cycle arrest (21).

The 14-3-3 proteins are a family of highly conserved acidic proteins that includes at least seven different mammalian isoforms (α, β, δ, ε, γ, η, and σ) (22). These proteins are a part of an emerging family of proteins and protein domains that bind to serine and/or threonine-phosphorylated residues in a context-specific manner, analogous to the Src homology 2 and phosphotyrosine-binding domains (23). 14-3-3 proteins bind and regulate key proteins involved in various physiological processes such as intracellular signaling (e.g. MEK kinase, Raf, and mixed lineage kinase) (24–26), cell cycling (e.g. Weel, CDK2, and Cdc25) (27–29), apoptosis (e.g. BAD and ASK-1) (30, 31), and transcriptional regulation (e.g. DAF-16, TAZ, and p53) (32–34).

14-3-3 protein (also known as human mammary epithelial marker 1 or HME1) is expressed in epithelial cells. It was originally identified as a p53-inducible gene that is responsive to DNA-
damaging agents (35). 14-3-3σ apparently sequesters the mitotic initiation complex, cdc2-cyclinB1, within the cytoplasm after DNA damage. This prevents cdc2-cyclin B1 from entering the nucleus, where it would normally initiate mitosis. In this manner, 14-3-3σ induces G2 arrest and allows the repair of damaged DNA (35). In this study, we have investigated the effects of IGF-I on regulation of 14-3-3σ and the role of this protein in IGF-I-induced mitogenesis in MCF-7 cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The radionuclide \([^{32}P]dCTP\) (6000 Ci/ mmol) was purchased from PerkinElmer Life Sciences. Recombinant human IGF-I was obtained from Genentech (San Francisco, CA). The PI3K-specific inhibitor LY294002 was purchased from Sigma. The MEK1/2-specific inhibitor UO126 was from Calbiochem (La Jolla, CA), and the JNK1/2-specific inhibitor SP600125 was from Biomol Research Laboratories Inc. (Plymouth Meeting, MA). The siRNA duplexes and pS3 siGENE SMART pool were purchased from Dharmacon Research. The MEK1/2-specific inhibitor UO126 was from Calbiochem (La Jolla, CA), and the PI3K-specific inhibitor LY294002 was purchased from Sigma. The human IGF-I was obtained from Genentech (San Francisco, CA). The radionuclide \([^{32}P]dCTP\) (6000 Ci/ mmol) was purchased from PerkinElmer Life Sciences. Recombinant phospho-Akt (Ser 473), Akt, phospho-MAPK (ERK1/2) (Thr 202/Tyr204), MAPK, phospho-JNK1/2Thr183/Tyr185, JNK1, and p53 polyclonal antibodies, Cell Signaling (Beverly, MA); and anti-rabbit IgG and anti-mouse IgG secondary horseradish peroxidase-conjugated antibodies, Amersham Biosciences. Enhanced chemiluminescence reagents were obtained from PerkinElmer Life Sciences.

Cell Culture—MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells were cultured in MEM supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 

Western blotting—Total cell lysates were prepared on ice with lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 5% glycerol, 10 mM NaF, 0.3 mM NaMo, 1 mM NaVO3, and 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) containing various protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml apro tin). The lysates were centrifuged for 20 min at 4 °C, and the insoluble fraction was discarded. The total protein concentration in the soluble lysate was measured using a BCA protein assay kit (Pierce). Samples containing 50 or 100 

The siRNA duplexes were 21 base pairs. They were generated with the following three siRNA duplexes: 117, 5'-GAGCAGAAAACGCCCGCCUCG-3'; 371, 5'-GGGUGACUAUACUCCGCUAC-3'; and 641, 5'-AGAGACCAACCCCAGUAUG-3'. The control siRNA was a GFP RNA oligonucleotide that was co-transfected with the siRNA oligonucleo tides for 24 h, according to the manufacturer’s recommended protocol. Northern blot analysis was used to determine transfection efficiency. From this experiment, we chose the most effective 14-3-3σ siRNA 117 (see Fig. 4). We checked the GenBank TM data base, and found that siRNA 117 is 14-3-3σ-specific.

Cell Cycle Assays—The cells were transiently transfected with 100 nM of either GFP siRNA or 14-3-3σ siRNA. Twenty-four hours later, the cells were transfected to 24-well tissue culture plates (4 \(\times 10^4\) cells/well) in MEM with 10% FBS. After 15 h of incubation, the medium was switched to MEM containing 1% FBS for 16 h. For cell cycle analysis, the CycleTest PLUS DNA reagent (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used. After 16 h of serum starvation, the cells were cultured in FBS-free and phenol red-free MEM in the presence or absence of 50 nM IGF-I. At the indicated times, the cells were harvested by trypsinization and counted with a hemacytometer.

Flow Cytometric Analysis—The cells were seeded at a density of 60 to 70% in 100-mm-diameter plates in MEM containing 10% FBS for 24 h. After 16 h of serum starvation, the cells were cultured in FBS-free and phenol red-free MEM in the presence or absence of 50 nM IGF-I. At the indicated times, the cells were washed with ice-cold PBS and collected. According to the manufacturer’s protocol, 1 \(\times 10^7\) cells were washed three times with buffer solution from the kit. The cell pellets were then resuspended in 1 ml of buffer solution and were frozen at −80 °C until further analysis. According to the manufacturer’s protocol, isolate, and stain cell nuclei from frozen cell suspensions, the samples were filtered using a 3-μm cell strainer (Fisher), and the DNA content was stained and determined by flow cytometry. All analyses were carried out on a FACSCalibur using CellQuest Software (Becton Dickinson, Mountain View, CA).

RESULTS

IGF-I Increases 14-3-3σ Expression—It is well known that the IGF-IR plays an important role in cell proliferation and survival. We therefore examined whether the biological function of 14-3-3σ, which is known to negatively regulate cell cycle progression, is linked to IGF-I function. To determine the 14-3-3σ mRNA levels after IGF-I stimulation, serum-starved MCF-7 cells were treated with 50 nM IGF-I for 2–8 h. 14-3-3σ mRNA levels were then determined by Northern blot. As shown in Fig. 1A, IGF-I treatment increased 14-3-3σ mRNA levels in a time-dependent manner. 14-3-3σ mRNA levels were elevated 4-fold after 8 h of exposure to IGF-I. To examine 14-3-3σ protein levels, MCF-7 cells were starved in MEM containing 0.2% FBS, and then the cells were treated with 50 nM IGF-I for 6–18 h. 14-3-3σ protein levels were determined by Western blot. As shown in Fig. 1B, IGF-I treatment increased 14-3-3σ protein levels in a time-dependent manner. 14-3-3σ protein levels were elevated 4-fold after 12 h of exposure to IGF-I.

Inhibition of PI3K Blocks IGF-I-induced 14-3-3σ Expression—It is well established that IGF-I receptor activation results in the initiation of the MAPK, PI3K/AKT, and JNK1/2 signaling pathways. To identify which of the above the pathway(s) is involved in the regulation of 14-3-3σ expression, we used specific pharmacological inhibitors corresponding to each of these three pathways (U0126, LY294002, and SP600125 for MAPK, PI3K/AKT, and JNK1/2 pathways, respectively). Serum-starved cells were treated with the various inhibitors for 1 h prior to stimulation with IGF-I (50 nM). The use of phospho specific antibodies in Western blot analysis revealed that U0126, LY294002, and SP600125 inhibited IGF-I-induced activation of ERK1/2, Akt, and JNK1/2, respectively, as shown in Fig. 1C. The total levels of ERK1/2, Akt, and JNK1 immunoreactivity were similar among the samples, indicating that these effects were not due to differences in the total amounts of these proteins. Fig. 2B indicates that neither U0126 (a MAPK pathway inhibitor) nor SP600125 (a JNK1/2 pathway inhibitor) affected IGF-I-induced expression of 14-3-3σ. However, we found that the PI3K inhibitor LY294002 (50 μM) reduced IGF-I-induced 14-3-3σ mRNA levels under conditions that blocked Akt phosphorylation. Thus, the IGF-I-induced expression of 14-3-3σ in MCF-7 cells is mediated specifically through the PI3K pathway.
The cellular proliferation rates of si-α cells with that of si-GFP cells. The cells were plated at a density of 4 × 10^4 cells/well in 24-well plates and incubated in the presence or absence of IGF-I (50 nM) for 42, 60, or 90 h. IGF-I-induced cell growth was reduced by 40% in si-α cells, as compared with si-GFP cells (Fig. 5B). Thus, a reduction in the expression of 14-3-3-α decreases IGF-I-induced cell proliferation in MCF-7 cells.

14-3-3-α Is Important for IGF-I-induced Cell Cycle Progression—We next examined whether 14-3-3-α is important in IGF-I-induced cell cycle progression. Fluorescence-activated cell sorting analysis was used to determine the effects of IGF-I on the distribution of si-GFP and si-α MCF-7 cells in the various phases of the cell cycle. The cells were serum-starved for 16 h after transfection and stimulated with IGF-I (50 nM) for the indicated times. Representative results are shown in Table I. After 18 h of IGF-I stimulation, 30% of si-GFP cells and 19% of si-α cells progressed to the S phase. After 24 h of IGF-I stimulation, 55% of si-GFP cells and 45% of si-α cells progressed to the S phase. Thus, the decrease in proliferation rate in response to IGF-I in si-α cells correlates with an increase in the proportion of cells in G2, suggesting that these cells exhibit a delay in progression from G2 to S. These data show that 14-3-3-α expression is specifically required in IGF-I-induced cell cycle progression. We used Northern blot analysis to examine the expression of 14-3-3-α in si-α and si-GFP cells. These experiments showed that 14-3-3-α mRNA levels were reduced by 75% in si-α cells, as compared with si-GFP cells (Fig. 6).

Expression of Cyclin D1 in Response to IGF-I Is Reduced in si-α MCF-7 Cells—To determine why G2 to S progression is delayed in MCF-7 14-3-3-α siRNA cells in response to IGF-I, we transiently transfected either si-GFP (as a control) or si-α into MCF-7 cells (Fig. 7A). We then examined the levels of cyclin D1, which is essential for progression through G3 phase (11, 38,
FIG. 2. IGF-I activates 14-3-3σ through the PI-3 kinase pathway. A, inhibition of the Akt, ERK, and PI3K pathways diminishes the effects of IGF-I on 14-3-3σ levels. MCF-7 cells were cultured in 60-mm dishes (5 × 10⁵ cell/dish). Serum-starved cells were pretreated in either the presence (+) or absence (−) of the PI3K inhibitor LY294002 (50 μM), the ERK1/2 inhibitor UO126 (5 μM), or JNK1/2 inhibitor SP600125 (50 μM) for 1 h. The cells were then incubated with or without IGF-I (50 nM) for 10 min. The phosphorylation states of Akt, ERK1/2, and JNK1/2 were determined by immunoblotting with phospho-specific antibodies, as described under "Experimental Procedures." The membranes were then stripped and reblotted with antibodies that equally recognize the phospho- and non-phospho-state of the proteins, to measure the total Akt, ERK1/2, and JNK1 immunoreactivity levels.

B, serum-starved MCF-7 cells were pretreated for 1 h in the presence or absence of the PI3K inhibitor LY294002 (50 μM), the MAPK inhibitor UO126 (5 μM), or the JNK1/2 inhibitor SP600125 (50 μM). The cells were then treated with or without IGF-I (50 nM) in the serum-free and phenol red-free IMEM for 6 h. Total RNA was extracted from the cells and subjected to 14-3-3σ Northern blot analysis. The membranes were stripped and reprobed with a cDNA probe specific for GAPDH, as a loading control. These results are representative of three separate experiments.
Thirty-nine). Twenty-four hours after transfection, the si-GFP and si-α cells were serum-starved for 16 h and then stimulated with IGF-I (50 nM) for 8 h. As shown in Fig. 7B, cyclin D1 immuno-reactivity in response to IGF-I is reduced by 65% in si-α cells, as compared with the si-GFP cells. Thus, the delay in G1 to S progression in response to IGF-I in si-α MCF-7 cells is associated with a reduction in the expression of cyclin D1. We also examined the expression of cyclin D1 in the si-GFP and si-α cells without IGF-I stimulation at 8 h. They have similar cyclin D1 expression levels (data not shown).

**DISCUSSION**

The 14-3-3 protein is induced under various biological conditions, including cell cycle arrest, differentiation, and apoptosis (23, 40–42). 14-3-3α has been described as a negative cell cycle regulator in HCT116 cells, because it is induced in response to DNA damage and leads to G2/M arrest (43). Cells that lack 14-3-3α are defective in the maintenance of G2 arrest (43). 14-3-3α has also been described as a potent inhibitor of cell proliferation in many breast cancer cell lines. For example, in MDA-MB-361, MCF-7, and HER-18 breast cancer cells, overexpression of 14-3-3α blocks cell growth and entry into the S phase of the cell cycle (28). 14-3-3α also can bind to and inhibit various cyclin-dependent kinases, CDK2, CDC2, and CDK4, which regulate cell cycle progression and cell proliferation, and represent a novel class of CDK inhibitors (28).

We have now evaluated the effects of IGF-I on 14-3-3α mRNA and protein levels and found that the PI3K signaling pathway plays a critical role in stimulating 14-3-3α gene expression in response to IGF-I. Binding of IGF-I to its receptor is followed by a rapid phosphorylation of several protein substrates that leads to the activation of the PI3K and MAPK signaling pathways. The MAPK pathway is closely associated with gene expression and cell proliferation. IGF-I has also been shown to strongly activate MAPK in MCF-7 and T47D breast cancer cells (44). Activation of the PI3K/Akt pathway has been shown to be important for cell survival, because Akt can di-
directly phosphorylate and modulate a set of protein substrates that are critical for apoptosis, including BAD (45, 46), caspase-9 (47), forkhead proteins (48, 49), and the NF-κB activating kinase, IKK (50, 51). These are a number of proteins that are regulated downstream of IGF-I activated PI3 kinase.

For example, in 32D cells, IGF-I up-regulates Id2 gene expression via the PI3K pathway (52), and Zhang et al. (53) reported that IGF-I up-regulates MT1-MMP (the membrane type 1 matrix metalloproteinase) via the PI3K/Akt pathway. It was also reported that IGF-I induces vascular endothelial growth factor expression through the PI3K pathway in human osteoblast-like MG63 cells (54). Activation of the PI3K pathway is important not only for cell survival but also for cell proliferation (10, 20).

It has previously been reported that 14-3-3 is induced by p53 in response to γ irradiation and other DNA-damaging agents (35). On the other hand, Akt has been shown to directly phosphorylate and regulate several components in the p53 pathway, including p21 and MDM2 (21). However, in this study, we showed that IGF-I-induced expression of 14-3-3 persisted even when p53 expression was reduced by siRNA in
MCF-7 cells. Although 14-3-3σ is induced in response to IGF-I in MCF-7 cells, which express wild-type p53, the up-regulation of 14-3-3σ in response to IGF-I appears to be p53-independent. In a previous study, we showed that IGF-I reduces p53 expression by inducing MDM2-dependent degradation of p53 via the p38 MAPK pathway in response to 4-nitroquinoline 1-oxide-induced DNA damage in NIH3T3 cells (55). However, in this study, we found that IGF-I increases p33 expression in MCF-7 cells. It has also been reported that IGF-I increases p33 expression in response to UV-induced DNA damage in MCF-7 cells (21). In different cells, with different signaling systems, the IGF-IR may trigger different cellular tumor surveillance machinery. In MCF-7 cells, it is possible that IGF-I may activate p14ARF, which in turn activates p53 (21). Because p33 functions as a gatekeeper against abnormal cell growth and genomic instability, it is conceivable that abnormal proliferation signals, such as high levels of IGF-I, may activate the p53 pathway, which restrains the abnormal cell proliferation.

In MCF-7 cells, Dufourny et al. (10) showed that certain stimulatory effects of IGF-I on cell cycle progression, such as the accumulation of cyclin D1 and hyperphosphorylation of pRb, were blocked by the specific PI3K inhibitor LY294002. From these results, they suggested that PI3K activity, but not MAPK activity, was required for transduction of the mitogenic IGF-I signal in these cells. In a previous study, we showed that IGF-I induced p21 expression through the MAPK pathway is involved in the mitogenic effects of IGF-I (56). Thus, both PI3K and MAPK are important signaling pathways involved in the effects of IGF-I on cell cycle progression in MCF-7 cells.

In HCT116 cells, 14-3-3σ might block entry into mitosis by anchoring the cdc2/cyclin B1 complex in the cytoplasm after treatment with adriamycin, which causes DNA damage (43). In MCF-7 cells, in contrast, we show for the first time that IGF-I induces 14-3-3σ through the PI3K/Akt pathway and that this is involved in the mitogenic effects of IGF-I, because abrogation of 14-3-3σ expression by transient transfection of siRNA targeting 14-3-3σ in MCF-7 decreased the mitogenic effects of IGF-I by 40%. Moreover, we show that this reduction in mitogenic activity is correlated with a delay in G1/S progression. This indicates that, at least in MCF-7 cells, 14-3-3σ plays a positive role in growth factor-induced mitogenesis.

Here, we have shown for the first time that a reduction in 14-3-3σ gene expression in MCF-7 cells is associated with a decrease in IGF-I-induced cyclin D1 expression. These data probably explain the reduction in the cell proliferation and the delay in cell cycle progression. Cyclin D1 plays an important role in controlling the cell cycle in mammary tissue, and clinical studies of human breast cancer have confirmed its importance. For example, overexpression of cyclin D1 was observed in about 50% of human mammary carcinomas that were evaluated (57, 58). Recently, it was reported that deregulated cyclin D1 expression may play an important role in canine mammary carcinogenesis and that canine malignant mammary tumors exhibiting high levels of cyclin D1 expression show higher rates of proliferation than cyclin D1-negative tumors (59).

In summary, we have delineated the molecular mechanisms by which 14-3-3σ expression is increased in response to IGF-I in MCF-7 cells. We showed that IGF-I up-regulation to 14-3-3σ is independent of p53. Using siRNA to specifically reduce 14-3-3σ expression, we demonstrated that 14-3-3σ is a key positive regulator in IGF-I-induced cell proliferation. Moreover, we showed for the first time that a reduction in 14-3-3σ gene expression is associated with a decrease in the cyclin D1 expression in MCF-7 cells. Future studies will focus on further characterizing the interaction between the IGF-IR, 14-3-3σ, and cyclin D1.

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