Tumor Necrosis Factor α Inhibits Cyclin A Expression and Retinoblastoma Hyperphosphorylation Triggered by Insulin-like Growth Factor-I Induction of New E2F-1 Synthesis*

Received for publication, September 16, 2003, and in revised form, December 16, 2003
Published, JBC Papers in Press, December 16, 2003, DOI 10.1074/jbc.M310264200

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Cyclin A is required for cell cycle S phase entry, and its overexpression contributes to tumorigenesis. Release of pre-existing E2Fs from inactive complexes of E2F and hypophosphorylated retinoblastoma (RB) is the prevailing dogma for E2F transcriptional activation of target genes such as cyclin A. Here we explored the hypothesis that new synthesis of E2F-1 is required for insulin-like growth factor-I (IGF-I) to induce cyclin A accumulation and RB hyperphosphorylation, events that are targeted by tumor necrosis factor α (TNFα) to arrest cell cycle progression. We first established that IGF-I increases expression of cyclin A, causes hyperphosphorylation of RB, and augments the mass of E2F-1 in a time-dependent manner. As expected, E2F-1 small interfering RNA blocks the ability of IGF-I to increase synthesis of E2F-1. Most important, this E2F-1 small interfering RNA also blocks the ability of IGF-I to increase cyclin A accumulation and to hyperphosphorylate RB. We next established that TNFα dose-dependently inhibits IGF-I-induced phosphorylation of both RB and histone H1 by cyclin A-dependent cyclin-dependent kinases. Cyclin-dependent kinase 2 (Cdk2) mediates this suppression because co-immunoprecipitation experiments revealed that TNFα reduces the amount of IGF-I-induced cyclin A that binds Cdk2, leading to a reduction in Cdk2 enzymatic activity. TNFα antagonizes the ability of IGF-I to increase mass of both E2F-1 and cyclin A but not cyclin E or D1. The cytostatic property of TNFα is also shown by its ability to block IGF-I-stimulated luciferase activity of a cyclin A promoter reporter. Deletion of an E2F recognition site from this reporter eliminates the regulatory effects of both IGF-I and TNFα on cyclin A transcription, indicating the essential role of E2F-1 in mediating their cross-talk. Collectively, these results establish that TNFα targets IGF-I-induced E2F-1 synthesis, leading to inhibition of the subsequent accumulation in cyclin A, formation of cyclin A-Cdk2 complexes, hyperphosphorylation of RB, and cell cycle arrest.

* This work was supported by National Institutes of Health Grant AI50442 (to K. W. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Cell cycle aberrations occurring at the G1/S checkpoint often lead to uncontrolled cell proliferation, a hallmark of cancer. G1 phase progression is stimulated by extracellular growth factors (reviewed in Ref. 1), and this progression can be blocked by cytostatic cytokines (2, 3). Tumor necrosis factor α (TNFα) was first identified as a serum-derived substance that causes tumor cell death (4). Although the mechanisms of TNFα cytotoxicity are relatively well established (reviewed in Ref. 5), the cytostatic properties of TNFα that suppress cell cycle progression are largely unknown. We recently reported (6) that TNFα exerts its antiproliferative action on human breast cancer cells not by killing the cells but rather by preventing them from passing into S. Importantly, this cytostatic property of TNFα is manifested only when cells are released from a quiescent state into active cell cycle progression induced by the late G1, progression factor, insulin-like growth factor-I (IGF-I). Acting as the functional mediator of growth hormone and as a potent growth factor, IGF-I plays a role in promoting cell proliferation (7), survival (8), malignant transformation (9), and tumor progression (10). These biological actions of IGF-I are often related to cell cycle control through different signaling pathways (11).

Progression through the G1/S checkpoint is driven by coordinated activation of cyclin-dependent kinases (CDKs), which requires heterodimeric cyclin partners for enzymatic activity (12). Cyclin A-associated CDKs play a critical role in initiation of DNA replication and subsequent S phase entry by phosphorylating components of the DNA replication machinery (13). Activated CDKs can also hyperphosphorylate and inactivate the retinoblastoma (RB) tumor suppressor protein. It is believed that RB hyperphosphorylation causes release of E2F transcription factors from pre-existing RB-E2F complexes (14). These functional E2F transcription factors then bind to specific promoter regions to induce target genes whose products are necessary for S phase entry. Among the six members of the E2F family of transcription factors, E2F-1 possesses oncogenic properties both in vitro (15) and in vivo (16). Overexpression of E2F-1 is sufficient to induce quiescent cells to enter the S phase (17). The pivotal role of E2F transcription factors in driving the G1-S transition is largely mediated by their target genes such as G1 cyclins (18). We and others have shown that IGF-I acts on

The abbreviations used are: TNFα, tumor necrosis factor α; IGF-I, insulin-like growth factor-I; IRS-1, insulin receptor substrate-1; RB, retinoblastoma; CDK, cyclin-dependent kinase; Cdk2, cyclin-dependent kinase 2; MEM, minimum essential medium Eagle; FBS, fetal bovine serum; HRP, horseradish peroxidase; siRNA, small interfering RNA; GFP, green fluorescent protein.
key molecular events at the G1/S checkpoint, leading to inactivation of RB and elevation of G1 cyclins such as cyclin D1, E, and A (20), and A (21). However, there is no evidence that IGF-I augments G1 cyclin expression and RB hyperphosphorylation by up-regulating synthesis of E2F transcriptional factors.

In mammalian cells, three sets of G1 cyclin-Cdk complexes are sequentially assembled, thereby leading to the G1-S transition: D-type cyclins and Cdk4/6; cyclin E and Cdk2; and cyclin A and Cdk2 (12). Among these G1 cyclin proteins, cyclin A is synthesized at the onset of S phase and plays a critical role in initiation of DNA replication. Overexpression of cyclin A in cells from breast cancer patients is positively related to both early cancer relapse and death (22). Transgenic mice overexpressing cyclin A in the mammary glands exhibit significant preneoplastic changes, including nuclear abnormalities and hyperplasia (23). These effects can be mimicked in vitro by ectopic expression of cyclin A, which accelerates entry of G1 cells into the S phase (24). In contrast, microinjection of antisense directed at cyclin A or anti-cyclin A antibodies blocks DNA synthesis (25).

TNFa and IGF-I have been linked in our recent report (6) showing that TNFa blocks breast cancer cell growth by inhibiting IGF-I-induced tyrosine phosphorylation of the early adaptor docking protein, insulin receptor substrate-1 (IRS-1). Here we extend these early signaling events to the cell cycle machinery, focusing on an E2F-1 feed-forward loop acting at the G1/S checkpoint. We provide strong evidence that TNFa antagonizes IGF-I-induced molecular events during G1-S progression by reducing the expression of E2F-1, E2F-dependent cyclin A promoter activity, cyclin A expression, the activity of both cyclin A and Cdk2 in phosphorylating RB, and the formation of cyclin A-Cdk2 complexes. We establish that cyclin A expression and RB hyperphosphorylation directly result from IGF-I-induced synthesis of E2F-1, as shown by a failure of IGF-I to augment cyclin A expression and to hyperphosphorylate RB in cells containing E2F-1 small interfering RNA (siRNA). The requirement of E2F in mediating IGF-I induction of cyclin A expression is also demonstrated by IGF-I unresponsiveness in cells expressing a cyclin A promoter that lacks an E2F recognition site. These observations provide direct evidence for a new mechanism by which E2F-1 activates target genes by increasing its own synthesis in response to growth factors like IGF-I. Most important, these data establish that TNFa blocks cyclin A expression by inhibiting this E2F-1 feed-forward loop at the G1/S boundary.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—Human MCF-7 breast adenocarcinoma cells (ATCC) were cultured in maintenance medium consisting of minimum essential medium Eagle (MEM) supplemented with 10% fetal bovine serum (FBS, Sigma). Cells were seeded at 1 × 10^6 cells/ml in a volume of 2 ml of maintenance medium in 6-well Costar plates (Corning Glass). MCF-7 cells express estrogen receptors, and estrogen increases IGF-I-induced MCF-7 cell growth by enhancing IGF-I receptor signaling (26). Phenol red can bind to the estrogen receptor on MCF-7 cells and exhibit significant estrogenic activity (27). In order to avoid the influence of estrogen on cellular responses to IGF-I, all treatments were performed in phenol red-free medium. For each experiment, cells were washed three times with phosphate-buffered saline (PBS) followed by a 24-h incubation in culture medium consisting of phenol red-free MEM supplemented with 5 µg/ml human transferrin and 30 nM sodium selenite (Sigma). Human IGF-I (100 ng/ml) and varying concentrations of human TNFa (Intergen) were added to cells individually or in combination for 24 h prior to preparation of whole cell lysates.

**Immunoprecipitation and Immunoblotting**—Whole cell lysates were prepared from 1 × 10^6 MCF-7 cells in whole cell lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, containing freshly added 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 48 trypsin inhibitory units of aprotinin, 40 µM leupeptin, and 2 µg/ml pepstatin; Sigma). Following centrifugation at 16,000 × g at 4 °C for 15 min, the amount of protein in the supernatant was determined with a protein assay kit (Bio-Rad). Antibodies used for immunoprecipitation and immunoblotting included rabbit anti-IRS-1 (Upstate Biotechnology, Inc. 66-248)), rabbit anti-cyclin A (Santa Cruz Biotechnology (H-432, sc-751)), rabbit anti-cyclin E (Santa Cruz Biotechnology (C-19, sc-198)), rabbit anti-Cdk2 (Santa Cruz Biotechnology (M2, sc-160)), mouse anti-cyclin A (Santa Cruz Biotechnology (BP683, sc-299)), mouse anti-Cdk2 (Santa Cruz Biotechnology (D-12, sc-6248)), mouse anti-E2F-1 (Santa Cruz Biotechnology (KH95, sc-251)), goat anti-actin (Santa Cruz Biotechnology (I-19, sc-1616)), mouse anti-phosphorysine P70 (Transduction Laboratories (P11120)), mouse anti-RB (Pharmingen (554136, clone G3-245)), horseradish peroxidase (HRP)-linked donkey anti-goat (Santa Cruz Biotechnology) and sheep anti-mouse IgG (Amersham Biosciences). Equal amounts of protein in whole cell lysates (~200 µg) were immunoprecipitated overnight at 4 °C. Antibodies used for immunoprecipitation were preincubated with protein G-Sepharose at 4 °C for 3 h before distribution into cell lysates. Immunoprecipitates or whole cell lysates were mixed with reducing sample buffer (0.92% Tris-HCl, pH 8.8, 1.5% SDS, 4% glycerol, and 280 mM β-mercaptoethanol) and separated on SDS-PAGE gels. Proteins were transferred with a Bio-Rad Trans-Blot electrophoretic transfer device to Immune-Blot polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% bovine serum albumin or 5% skim milk dissolved in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and 1% Tween 20. The membranes were then incubated in the same blocking buffer with the indicated antibodies for 1 h at room temperature. Blots were incubated with an HRP-labeled species-matched secondary antibody for another 1 h, and immunoreactive bands were visualized using enhanced chemiluminescence detection reagents (Amersham Biosciences). Blots were washed and quenched by incubation in membranes in heated (>55 °C) stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-CI, pH 6.7) for 30 min. Intensity of protein bands on autoradiograms was quantified by scanning with an Agfa Duoscan TI200 scanner followed by analysis using GelExpert 3.5 software (Bio-Rad).
Biomedicina de Valencia, Valencia, Spain). The CycA-79/100 construct is driven by the minimal promoter fragment displaying transcriptional activity of the 7.5-kb full promoter region of human cyclin A gene (28), whereas the CycA-54/100 construct contains a deletion in an E2F-1-binding site.

For transfection, cells were seeded at 5 × 10^5 cells/ml in a volume of 2 ml in 6-well Costar plates (Corning Glass) in maintenance medium. The following day, 60–80% confluent cells were transiently transfected in duplicate with 5 μg of the luciferase reporter constructs, CycA/79/100, CycA-54/100, or an empty pGL2 vector (Promega), using Lipofectamine 2000 (Invitrogen). Transfection efficiency was determined by co-transfection with a pEGFP-C1 expression vector (Clontech) that permits high level expression of green fluorescent protein (GFP). Analysis of 10,000 cells in an EPICS XL flow cytometer (Coulter) routinely showed that 30–40% of transfected MCF-7 cells were GFP-positive.

Medium containing transfection mixture was removed 2 h after transfection. Cells were washed with PBS three times, followed by addition of TNFα (10 ng/ml) or IGF-I (100 ng/ml) alone or in combination in serum-free and phenol red-free MEM for 60 h. Luciferase activity was measured in 20 μl of cell lysates using a luciferase assay system (Promega) on a Monolight 2010 luminometer (Analytical Luminescence Laboratory) for 12 s. All results were normalized to standard protein concentrations.

**E2F-1siRNA**—To create human E2F-1siRNA, two oligonucleotides were designed and synthesized based on the coding sequence of the human E2F-1 gene as follows: forward oligonucleotide, 5'-GGGCGTCTGTGGAGACAGCAGCTCCAGGAAGGCTTTT-3'; reverse oligonucleotide, 5'-AATTAAAAACCGCTCTGGAAGCTCTCTCTCTCTCTTGACACGAGCGCTCCAGGAAGGCGGGGACC-3'. The oligonucleotides were annealed and ligated into the pSilencer 1.0-U6 siRNA vector (Ambion), resulting in a siE2F-1 expression vector, U6/E2F1siRNA.

To test whether E2F-1siRNA affects cyclin A expression and RB phosphorylation, U6/E2F1siRNA was introduced into MCF-7 cells by transfection as described above, resulting in MCF-7/E2F-1siRNA cells. Control cells were simultaneously transfected with the pSilencer 1.0-U6 siRNA control vector. Experiments were conducted during a period of 4–12 days after transfection. In control cells, no obvious disparity was found in the extent of inhibition of basal or IGF-I-induced E2F-1 protein expression. Expression of both E2F-1 and cyclin A, as well as phosphorylation of RB, was detected by Western blotting in MCF-7/E2F-1siRNA cells as well as control cells treated with IGF-I for 24 h.

**Statistical Analysis**—All statistical analyses were performed using the Statistical Analysis System for Windows (29). All data, including standardized densitometric intensities from replicate autoradiograms, were analyzed as a completely randomized design using standard analysis of variance procedures. Treatment differences were assessed by Duncan’s multiple range tests. All experiments were independently replicated at least three times, and data were summarized as means ± S.E. Two-sided p values of p < 0.05 or p < 0.01 were considered statistically significant.

**RESULTS**

**TNFα Blocks IGF-I-stimulated Cyclin A-dependent CDK Enzymatic Activity**—Extracellular growth factors stimulate G1 phase progression (reviewed in Ref. 1), which is often blocked by cytostatic cytokines (2, 3). We demonstrated recently (6) that IGF-I (100 ng/ml) increases the population of total cycling MCF-7 cells (S + G2/M) by 50%. Treatment with TNFα (5 ng/ml) prevents their entry into the S phase, leading to 90% inhibition of cells progressing through the G1 restriction point. Most important, TNFα does not inhibit G1 progression in the absence of IGF-I. A-type cyclin is synthesized at the onset of S phase and therefore plays a critical role in initiation of DNA replication (13). TNFα may act by impairing the ability of IGF-I to induce accumulation of cyclin A, which in turn reduces the enzymatic activity of cyclin A-associated CDK. To test this hypothesis, we used a cyclin A-specific antibody to precipitate its associated CDKs, and we measured the amount of cycline complexes to phosphorylate their substrates in vitro. Cyclin A-associated CDK activity, as assessed by phosphorylation of either truncated RB peptide or histone H1 substrates, was strongly induced by IGF-I (p < 0.01, Fig. 1). Simultaneous treatment of MCF-7 cells with TNFα dose-dependently impaired cyclin A-dependent CDK activity, as shown by elimination of IGF-I-promoted phosphorylation of both truncated RB and histone H1 (Fig. 1). TNFα, by itself, did not affect cyclin A-dependent CDK activity in the absence of IGF-I. These results show that TNFα blocks IGF-I-induced cyclin A-specific activity that leads to phosphorylation of RB.

**TNFα Suppresses IGF-I-Stimulated Cdk2 Activity and Formation of CyclinA-Cdk2 Complexes**—Because Cdk2 is a critical CDK that drives the G1-S transition (30), TNFα may specifically block the activity of Cdk2, which is the G1 CDK that associates with cyclin A. To test this possibility, Cdk2 was
immunoprecipitated from cells treated with TNFα, IGF-I, or their combination. Cdk2-specific enzymatic activity was measured with an in vitro CDK kinase assay. Cdk2 activity was significantly activated by IGF-I (p < 0.01), as shown by its ability to phosphorylate both RB (p56) and histone H1 substrates (Fig. 2A). Most important, TNFα dose-dependently inhibited, and at 1 ng/ml completely blocked, Cdk2 activity stimulated by IGF-I (p < 0.01). It is important to note that the inhibition caused by TNFα was detected only in the presence of IGF-I. These results establish that TNFα inhibits Cdk2-mediated RB phosphorylation induced by IGF-I.

IGF-I is a late G1 progression factor (31), so we focused on the G1-S transition where the association of Cdk2 with cyclin A is critical for S phase entry. In order to investigate the physical association between cyclin A and Cdk2, mass of cyclin A was measured in Cdk2 immunoprecipitates derived from cells treated with IGF-I and TNFα. These co-immunoprecipitation experiments showed that IGF-I caused an 18-fold increase in the amount of cyclin A that associates with Cdk2 (Fig. 2B). This induction was not due to an increase in Cdk2 protein, as shown by equal amounts of Cdk2 in each immunoprecipitate. Co-treatment with TNFα dose-dependently decreased the amount of IGF-I-induced cyclin A that associated with Cdk2 (Fig. 2B). TNFα alone did not alter the association of Cdk2 with cyclin A, consistent with the findings of TNFα alone on Cdk2 activity (Fig. 2A). These results establish that TNFα impairs the ability of IGF-I to induce the formation of cyclin A-Cdk2 complexes.

**Fig. 2.** TNFα blocks IGF-I-induced Cdk2 activity by preventing the formation of Cdk2-cyclin A complexes. A, Cdk2 was immunoprecipitated (IP) from MCF-7 cells treated as in Fig. 1. Cdk2 activity was measured with truncated RB (p56) and histone H1 substrates. E, cyclin A association with Cdk2 was detected by Western blotting of the Cdk2 immunoprecipitates. Densitometric analyses (mean ± S.E.) were performed on three independent experiments, as shown in the graphs. ***, p < 0.01 compared with the cells treated with IGF-I (100 ng/ml) alone.

**IGF-I up-regulates Cyclin A Expression, Accompanied by a**
Progressive Elevation of E2F-1 and Hyperphosphorylation of RB—Cyclin A is synthesized at the onset of S phase (13), and cyclin A induction is mediated by the E2F-1 transcription factor (32). Although IGF-I is known to promote cyclin A accumulation (33), it is unknown whether IGF-I can increase synthesis of E2F-1. To test this possibility, cells were exposed to IGF-I for different times within a single population doubling time (~24 h (34)) following a G₁ arrest by serum deprivation (Fig. 3). As expected, IGF-I time-dependently increased cyclin A accumulation, with a significant rise occurring 6 h after the MCF-7 breast cancer cells were released from the G₁ arrest by IGF-I. Activated cyclin A-CDK complexes phosphorylate RB and maintain it in an inactive state. In accord with these concepts, IGF-I increased the ratio of hyperphosphorylated versus hypophosphorylated RB, an effect that was most prominent at 24 h (Fig. 3). RB acts as a transcriptional repressor by sequestering E2F-1 that is needed to activate genes whose products are necessary for passage of cells through the G₁/S checkpoint. Although E2F-1 activity is considered to be regulated mainly through its temporal association with pocket family proteins like RB (14), the data in Fig. 3 show a direct induction of E2F-1 expression by IGF-I. At the same time that cyclin A expression was increased (6 h), there was a significant and progressive increase in expression of E2F-1 and hyperphosphorylation of RB (Fig. 3). These results establish that E2F-1-steady-state protein expression is induced by IGF-I in a time-dependent fashion.

**TNFα Dose-dependently Inhibits IGF-I-stimulated Hyperphosphorylation of RB and Up-regulation of E2F-1 and Cyclin A but Not Cyclin E.** Western blotting was performed on MCF-7 cells treated with different doses of TNFα in the absence and presence of IGF-I (100 ng/ml) for 24 h. The results are representative of at least three independent experiments. The loading control, β-actin, varied less than 5% in all experiments.

FIG. 4. TNFα dose-dependently inhibits IGF-I-stimulated hyperphosphorylation of RB and expression of E2F-1 and cyclin A but not cyclin E. Western blotting was performed on MCF-7 cells treated with different doses of TNFα in the absence and presence of IGF-I (100 ng/ml) for 24 h. The results are representative of at least three independent experiments. The loading control, β-actin, varied less than 5% in all experiments.
residue than that caused by TNFα. The combination of IGF-I and TNFα led to a response similar to IGF-I alone (data not shown). It is therefore unlikely that phosphorylation of Ser307 alone accounts for the significant impairment caused by TNFα in the ability of IGF-I to induce tyrosyl phosphorylation of IRS-1.

Degradation of IRS-1 is another possible mechanism for down-regulation of its activity and may play an important role in attenuating insulin/IGF-I signaling. Consistent with our findings (39) and those of others (40), we found that IGF-I caused a 36% reduction in the mass of IRS-1 within 10 min \((p < 0.05, n = 3)\), and this time-dependent reduction was associated with both serine and tyrosine phosphorylation of IRS-1 (Fig. 5). Because IRS-1 phosphorylation at Ser307 (equivalent to human Ser312) plays an essential role in mediating its proteasomal degradation (41), this process may well serve as a mechanism by which IGF-I attenuates its own signaling and desensitizes MCF-7 cells to IGF-I. However, the loss of IRS-1 mass was only found when cells were stimulated with IGF-I but not with TNFα. Similar results were obtained in the presence of both IGF-I and TNFα (data not shown). Collectively, these data indicate that neither IRS-1 degradation nor phosphorylation of IRS-1 at Ser307 is likely to be responsible for TNFα antagonism of IGF-I-induced tyrosine phosphorylation of IRS-1.

**Knock-down of E2F-1 Expression by siRNA Abrogates IGF-I-induced Cyclin A Synthesis and RB Hyperphosphorylation**—Release of E2F from pre-existing RB-E2F complexes following RB hyperphosphorylation is the best described mechanism for E2F activation (14). However, an untested hypothesis is that new E2F synthesis also contributes to E2F-dependent gene transcription by enlarging the E2F pool. In order to investigate this possibility, we designed siRNA specific for E2F-1, constructed an expression plasmid U6/E2F-1siRNA, and introduced it into MCF-7 cells. Expression of cyclin A as well as E2F-1 in MCF-7/E2F-1siRNA cells was compared with control cells simultaneously transfected with empty pSilencer 1.0-U6 siRNA vector. As expected, IGF-I augmented expression of E2F-1, cyclin A, and hyperphosphorylated RB in MCF-7 cells transfected with the control plasmid \((p < 0.01, n = 4; \text{Fig. 6})\). In contrast, synthesis of E2F-1 protein induced by IGF-I was abrogated by the U6/E2F-1siRNA \((p < 0.01), \text{indicating that E2F-1siRNA successfully blocks the ability of IGF-I to induce new E2F-1 synthesis. Most important, the U6/E2F-1siRNA plasmid also blocked the ability of IGF-I to increase expression of cyclin A and hyperphosphorylation of RB (Fig. 6). When summarized over four independent experiments, the MCF-7/E2F-1siRNA caused a modest but non-significant reduction in basal E2F-1 accumulation, with no reduction in basal expression of either cyclin A or hyperphosphorylation of RB. It is therefore possible but unlikely that basal levels of E2F-1 are sufficient to drive cyclin A synthesis in the absence of growth factors. These data clearly establish that IGF-I-induced E2F-1 synthesis is required for cyclin A synthesis. Therefore, enlargement of the pool with newly synthesized E2F-1 in response to growth factors such as IGF-I offers a novel explanation for E2F regulation of its targeted genes. Most important, instead of being only released and regulated by RB, E2F-1 is also required for IGF-I-responsive RB phosphorylation. These results are consistent with our hypothesis that IGF-I stimulation of E2F-1 synthesis triggers a G1-S feed-forward cycle, leading to cyclin A accumulation and RB hyperphosphorylation.

**E2F Mediates IGF-I-stimulated Cyclin A Promoter Activity**, an Event Blocked by TNFα—Structural analysis of the cyclin A promoter region has revealed that residues 79/+100 constitute the smallest functional fragment (28). An important advantage of this minimal functional region is that it contains two E2F recognition sites (32), which minimizes nonspecific binding of other possible transcription factors on the full-length cyclin A promoter. In order to determine whether E2F mediates IGF-I-induced cyclin A transcription, and whether this induc-
**Fig. 7. TNFα inhibits the ability of IGF-I to promote E2F-1-dependent cyclin A expression.** MCF-7 cells were transiently transfected with a luciferase reporter plasmid (CycA-79/+100) that is driven by the minimal promoter fragments required for transcription of cyclin A. A single E2F-1-binding site in this construct was deleted, resulting in plasmid CycA-54/+100. After 2 h of incubation in transfection reagents, cells were treated with IGF-I (100 ng/ml) or TNFα (10 ng/ml) alone or their combination for 60 h. Luciferase activity was analyzed in whole cell lysates and normalized to total protein concentration. Results are expressed as the mean ± S.E. of three independent experiments. ***, p < 0.01.

**DISCUSSION**

Elevated plasma concentrations of IGF-I (10, 42) and increased activity of components of the IGF-I signaling pathway (43–45) have been implicated in tumor progression. IGF-I is well known to act on cell cycle machinery as a late G1 progress factor (31), primarily by regulating expression of checkpoint proteins at the G1–S restriction point (20, 46). IGF-I hyperphosphorylates RB (20), leading to the release of free E2F-1. Here we establish that IGF-I significantly increases cyclin A promoter activity, and this induction was completely blocked by TNFα (Fig. 7). TNFα alone did not affect cyclin A promoter activity. To investigate further the role of E2F in mediating IGF-I-dependent cyclin A transcription, a shorter construct, CycA-54/+100, was also used, with an E2F recognition site deleted. Luciferase activity of this shorter form of the cyclin A promoter was not induced by IGF-I (Fig. 7). This is the first report to demonstrate a requirement for E2F in mediating IGF-I-stimulated cyclin A transcription, an event that is blocked by TNFα.

We interpret these data as evidence for an E2F-1-centered feed-forward loop that is activated by IGF-I to promote G1-S progression. This IGF-I-induced cascade of events is significantly impaired by very low concentrations of TNFα.

These data offer evidence for a new mechanism on how E2F-1 abundance induces target gene expression and subsequent cell cycle progression through G1-S. E2F was originally described as a HeLa cell factor required for transcriptional activation of the viral E2 promoter (47). Although RB is the best known regulator of E2F, activation of E2F-dependent transcription is likely to involve other regulatory processes. Overexpression of E2F-1 in quiescent cells leads to activation of genes required for DNA synthesis, including the E2F-1 gene itself (48). Activity of E2F-1 in DNA binding and transcription is enhanced by forming a heterodimeric complex with DP-1 (48). Phosphorylation of E2F-1 on Ser323 and Ser337 prevents its interaction with RB, regardless of the phosphorylation status of RB (49), whereas phosphorylation of E2F-1 on Ser376, a specific CDK site, promotes RB binding (50). Acetylation of E2F-1 can augment its own DNA binding activity, increase its transcriptional activity, and prolong its half-life (51). E2F activity is also affected by subcellular localization (52, 53) and ubiquitin-directed degradation (54, 55). However, the possibility that E2F-1 synthesis is regulated by growth factors such as IGF-I has not been adequately explored.

Here we demonstrate that new synthesis of E2F-1 in response to growth factors is required for it to induce downstream genes such as cyclin A (Fig. 6), which leads to inactivation of RB and cell transition through the G1-S checkpoint. We used the relatively new approach of RNA interference, which employs the induction of sequence-specific gene silencing by small double-stranded RNA, targeting a corresponding mRNA for degradation (56). The absence of mRNA as a template causes mRNA translation and protein synthesis to stop. Therefore, this approach is useful to distinguish between protein synthesis and protein degradation when an increased steady-state expression of a protein is observed, such as E2F-1 in response to IGF-I (Figs. 3 and 4). By using E2F-1-specific siRNA, we found that the ability of IGF-I to increase expression of E2F-1 protein was completely blocked (Fig. 6). Complete inhibition indicates that IGF-I primarily induces E2F-1 protein synthesis rather than inhibiting its degradation. As a result of this siRNA knockdown, IGF-I is no longer able to induce cyclin A expression and RB hyperphosphorylation (Fig. 6). E2F-1 promotes transcription of its own gene (48, 57). This self-regulation may contribute to elevated abundance of E2F-1 in response to IGF-I, which leads to cyclin A induction and RB hyperphosphorylation, eventually promoting G1-S progression. On the other hand, it is possible that a small primary (initiating) increase in RB phosphorylation could secondarily be amplified through a positive feedback cycle to result in a much higher degree of subsequent RB phosphorylation. In this scenario, blocking the feedback cycle with E2F-1siRNA could then greatly reduce the extent of RB phosphorylation following IGF-I stimulation even if RB phosphorylation was the primary (initiating) event. Regardless of the interpretation, these collective results provide direct evidence for an indispensable role for new E2F-1 synthesis in up-regulating cyclin A expression, which suggests a novel mechanism for E2F-1 regulation of its downstream genes through altering its own synthesis. Most significant, recognition of the requirement of new E2F-1 synthesis for IGF-I-responsive cyclin A accumulation and RB hyperphosphorylation provides a novel mechanism to supplement the conventional regulatory relationship between RB and E2F-1.

Only a paucity of evidence is available to explain how TNFα...
acts on the cell cycle machinery as a cytostatic cytokine. For example, TNF induces p21Cip1/Waf1 and the binding of p21Cip1/Waf1 to Cdk2/4 to arrest cell growth (58). TNFα synergizes with the prototypical cytostatic cytokine, interferon, to induce p16INK4a, p21Cip1/Waf1, and p27kip1 and to suppress expression of cyclin D1 and Cdk2 (59) and activity of cyclin A and Cdk2 (60). Progression through the cell cycle is arrested when TNFα induces cyclin D degradation (61). In all these reports, the presence of 10% FBS may provide an alternative interpretation of these data. In this scenario, TNFα would act by suppressing cell cycle progression induced by numerous growth factors contained in FBS, including up to 200 ng/ml IGF-I (62). This interpretation is consistent with our hypothesis that TNFα arrests cell cycle progression only when the cycling machinery is initiated by signals from extracellular growth factors. Indeed, although the critical S phase marker cyclin A is elevated by IGF-I (Fig. 3), this increase in cyclin A is dose-dependently eliminated by TNFα, with a 100% blockage occurring at a concentration of only 1 ng/ml (Fig. 4). This antagonism between TNFα and IGF-I-induced cyclin A is accompanied by a similar negative association between TNFα and IGF-I on the phosphorylation status of the RB tumor suppressor as well as mass of the E2F-1 transcription factor (Fig. 4). Consistent with these findings, the ability of IGF-I to activate the cyclin A promoter is blocked by TNFα (Fig. 7). IGF-I-induced cyclin A at both the transcriptional and steady-state protein levels directly results in an increase in cyclin A-associated CDK kinase activity, as demonstrated by phosphorylation of both a truncated RB protein and histone H1 (Fig. 1). Whereas TNFα alone does not affect cyclin A activity, it dose-dependently reduces IGF-I-stimulated phosphorylation of RB and histone H1 (Fig. 1). A similar result is found when Cdk2 activity is determined by immunoprecipitating Cdk2 instead of cyclin A. Once again, IGF-I-induced Cdk2 enzymatic activity is inhibited by TNFα (Fig. 2A). Moreover, our results in Fig. 2B establish that IGF-I leads to a significant increase in the amount of cyclin A-Cdk2 complexes. Most important, these same experiments provide direct evidence that TNFα inhibits Cdk2 activity not directly but by reducing the amount of cyclin A in the IGF-I-induced cyclin A-Cdk2 complexes (Fig. 2B). The reduction of cyclin A association with Cdk2 may be a direct result of TNFα-induced inhibition of cyclin A expression (Fig. 4).

Consistent with the idea that the cytostatic properties of TNFα are detected only in the presence of IGF-I, we have identified IRS proteins as a direct target of TNFα in inhibiting IGF-I-induced neuroprotection (63), skeletal muscle protein synthesis (64), and breast cancer cell growth (65). In all these reports, TNFα consistently inhibits IGF-I-induced tyrosine phosphorylation of IRS proteins. Potential mechanisms responsible for the negative regulation of IRS-1 involve site-specific serine phosphorylation of IRS-1 (35, 38) and its subsequent degradation (36, 39, 40). However, our results (Fig. 5) question whether either of these mechanisms explain the ability of TNFα to inhibit IGF-I-promoted tyrosine phosphorylation of IRS-1. Although we confirm that the well accepted inhibitory residue of Ser307 is phosphorylated by TNFα, IGF-I itself causes more prominent phosphorylation of the same residue. Furthermore, phosphorylation of Ser307 and tyrosine phosphorylation occurs in a nearly identical time course (Fig. 5). Although these findings confirm that both events are associated, they do not prove that one event is responsible for the other. Furthermore, the IRS-1 blots in Fig. 5 show a time-dependent decline in IRS-1 mass induced by IGF-I but not by TNFα. Phosphorylation of IRS-1 at Ser307 may be responsible for the IGF-I-induced reduction in IRS-1 mass and downstream signaling, which may provide a self-limiting feedback mechanism.
regulate endogenous E2F-1 synthesis, which is necessary for transcriptional induction of genes important for S phase entry. This novel idea complements well the RB regulatory mechanism for E2F-dependent gene transcription by emphasizing the importance of a variant pool of total E2F-1 instead of only “free E2F” in controlling its downstream genes and driving cell cycle progression. Most important, these data clearly begin to reveal the molecular mechanisms by which proinflammatory cytokines, e.g., TNFα, halts IGF-I-promoted cell cycle progression by depressing the activity of key cell cycle regulators at the G1/S checkpoint. In the absence of these proteins, as occurs in quiescent cells, TNFα has little effect. These results support a new model in which an E2F-1 feed-forward loop serves as a target for TNFα to impair the ability of growth factors such as IGF-I to promote passage of breast cancer cells through the G1/S checkpoint. These data should contribute to a better understanding of how progression through the cell cycle is regulated by proteins that are invariably expressed in the microenvironment of tumors, proinflammatory cytokines, and growth factors.

Acknowledgment—We thank Dr. Vicente Andrés (Instituto de Medicina de Valencia, Valencia, Spain) for kindly providing cyclin A luciferase plasmid.

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