IL-1β Suppresses Prolonged Akt Activation and Expression of E2F-1 and Cyclin A in Breast Cancer Cells


http://www.jimmunol.org/content/172/12/7272

References

This article cites 57 articles, 31 of which you can access for free at:

http://www.jimmunol.org/content/172/12/7272.full#ref-list-1

Subscription

Information about subscribing to _The Journal of Immunology_ is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
IL-1β Suppresses Prolonged Akt Activation and Expression of E2F-1 and Cyclin A in Breast Cancer Cells

Wen Hong Shen,* Steve T. Jackson,* Suzanne Broussard,* Robert H. McCusker,* Klemen Strle,* Gregory G. Freund,‡ Rodney W. Johnson,‡ Robert Danzter,§ and Keith W. Kelley²*

Cell cycle aberrations occurring at the G1/S checkpoint often lead to uncontrolled cell proliferation and tumor growth. We recently demonstrated that IL-1β inhibits insulin-like growth factor (IGF)-I-induced cell proliferation by preventing cells from entering the S phase of the cell cycle, leading to G0/G1 arrest. Notably, IL-1β suppresses the ability of the IGF-I receptor tyrosine kinase to phosphorylate its major docking protein, insulin receptor substrate-1, in MCF-7 breast carcinoma cells. In this study, we extend this juxtamembrane cross-talk between cytokine and growth factor receptors to downstream cell cycle machinery. IL-1β reduces the ability of IGF-I to activate Cdk2 and to induce E2F-1, cyclin A, and cyclin A-dependent phosphorylation of a retinoblastoma tumor suppressor substrate. Long-term activation of the phosphatidylinositol 3-kinase/Akt signaling pathway, but not the mammalian target of rapamycin or mitogen-activated protein kinase pathways, is required for IGF-I to hyperphosphorylate retinoblastoma and to cause accumulation of E2F-1 and cyclin A. In the absence of IGF-I to induce Akt activation and cell cycle progression, IL-1β has no effect. IL-1β induces p21Cip1/Waf1, which may contribute to its inhibition of IGF-I-activated Cdk2. Collectively, these data establish a novel mechanism by which prolonged Akt phosphorylation serves as a convergent target for both IGF-I and IL-1β; stimulation by growth factors such as IGF-I promotes G1-S phase progression, whereas IL-1β antagonizes IGF-I-induced Akt phosphorylation to induce cytostasis. In this manner, Akt serves as a critical bridge that links proximal receptor signaling events to more distal cell cycle machinery. The Journal of Immunology, 2004, 172: 7272–7281.

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.

Copyright © 2004 by The American Association of Immunologists, Inc.

0022-1767/04/$02.00

Downloaded from http://www.jimmunol.org/ by guest on September 25, 2017
of the E2F-1 transcription factor (17). More importantly, all these IGF-I-dependent mitotic effects are inhibited by TNF-α. In addition to cyclin A, IGF-I can also elevate other G1 cyclins, such as cyclin D1 (18) and cyclin E (19). Although important intracellular pathways, such as PI3K/Akt and mitogen-activated protein kinase, have been reported to mediate IGF-I-responsive expression of cyclin D1 (20), little direct evidence is yet available to demonstrate involvement of these two pathways in IGF-I-induced accumulation of cyclin A or cyclin E and the key G1 transcription factor, E2F-1. Moreover, it is unknown whether another important proinflammatory cytokine, IL-1β, causes cytostasis by suppressing these key G1 and S phase regulators.

We recently reported that the cytostatic property of IL-1β is associated with its ability to impair IGF-I-promoted juxtamembrane receptor signaling, specifically, tyrosine phosphorylation of insulin receptor substrate (IRS)-1 (7). In addition, IL-1β-induced G1 arrest reduces the number of IGF-I-induced cycling cells (S phase G2/M), suggesting that components at the G1/S transition point are targeted and regulated oppositely by IL-1β and IGF-I. In this study, we provide direct evidence to confirm this hypothesis by demonstrating that IGF-I increases abundance of cyclin A and E2F-1, the activity of Cdk2 and cyclin A, and both the expression and hyperphosphorylation of RB through a LY294002- and wortmannin-sensitive pathway. IL-1β suppresses all of these IGF-I-induced activities. Moreover, long-term Akt phosphorylation is identified not only as a critical protein that mediates this intracellular communication between IL-1β and IGF-I, but also as an intermediate link between receptor proximal signaling events and distal cell cycle machinery.

Materials and Methods

Cell culture and reagents

Human MCF-7 breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in MEM supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT). For experimentation, cells were plated at 1 × 10^4 cells/ml in a volume of 2 ml of maintenance medium in six-well Costar plates (Corning, Corning, NY). The next day, cells were washed with 0.15 M PBS followed by addition of 2 ml of maintenance medium in six-well Costar plates (Corning, Corning, NY). Following incubation for 24 h in culture medium consisting of phenol red-free MEM supplemented with 5 μg/ml human transferrin and 30 nM sodium selenite (Sigma-Aldrich, St. Louis, MO). Cells were treated with different concentrations of IL-1β with or without IGF-I (100 ng/ml, both from InterGen, Purchase, NY) for 24 h. Inhibitors LY294002 (50 μM), PD98059 (50 μM), rapamycin (50 nM), or wortmannin (100 nM) (all from Sigma-Aldrich) were added for 30 min before treatments with IGF-I (100 ng/ml) for either 15 min or 24 h. Abs used for immunoprecipitation and immunoblotting included rabbit anti-human cyclin A (H-432, sc-751; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human cyclin E (C-19, sc-198; Santa Cruz Biotechnology), mouse anti-human cyclin A (BF683, sc-239; Santa Cruz Biotechnology), mouse anti-human Cdk2 (D-12, sc-6248; Santa Cruz Biotechnology), mouse anti-human E2F-1 (KH95, sc-251; Santa Cruz Biotechnology), goat anti-human actin (I-19, sc-1616; Santa Cruz Biotechnology), mouse anti-human RB (554136, clone G3-245; BD PharMingen, San Diego, CA), rabbit anti-human phospho-RB (Ser780/81, sc-2450; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human phospho-RB (Ser780/81, sc-2450; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-rat ERK (K-23, sc-94; Santa Cruz Biotechnology), rabbit anti-rat p21 (C-19, sc-397; Santa Cruz Biotechnology, Santa Cruz, CA), and histone H1 (Roche Diagnostics, Indianapolis, IN) were used as CDK controls. Rabbit polyclonal rabbit anti-rat phospho-ERK (Thr202/Tyr204, E-4, sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-rat ERK (K-23, sc-94; Santa Cruz Biotechnology), rabbit anti-mouse IgG (Santa Cruz Biotechnology) and HRP-linked donkey anti-goat IgG (Santa Cruz Biotechnology) and HRP-linked donkey anti-rabbit and sheep anti-mouse IgG (Amersham, Arlington Heights, IL).

DNA synthesis

Following incubation for 24 h in serum-free MEM to synchronize cells in G0, MCF-7 cells were washed three times (400 × g), adjusted to 5 × 10^4 cells/ml in culture medium and plated into 96-well plates (Costar 3596, Corning) in a volume 200 μl per well. Cells were then treated in triplicate with wortmannin (100 nM; Sigma-Aldrich), LY294002 (50 μM), PD98059 (50 μM), or rapamycin (50 μM) for 30 min before IGF-I (100 ng/ml) treatment for an additional 24 h. Cells were pulsed with [3H]thymidine (1 μCi per well; 1 Ci = 37 GBq; ICN Pharmaceuticals, Costa Mesa, CA) and harvested onto fiberglass filters onto a 96-well PHD cell harvester (Cambridge Technology, Cambridge, MA), as we previously described (7). The filters were dried and then submerged in 3 ml of scintillation fluid, and [3H]-radioactivity was determined on a Beckman Coulter LS 6000 IC scintillation counter (Fullerton, CA).

Immunoblotting

Whole cell lysates were prepared from 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 PMSF, 1 mM NaF, 48 trypsin inhibitory units of aprotinin, 40 mM leupeptin, and 2 μg/ml pepstatin; Sigma-Aldrich). Following centrifugation at 16,000 × g at 4°C for 15 min, protein concentration in the supernatant was determined with a protein assay kit (BioRad, Hercules, CA). Equal amounts of protein in whole cell lysates (20–50 μg) were mixed with reducing sample buffer (0.92 M Tris-HCl, pH 8.8, 1.5% SDS, 4% glycerol, and 280 mM 2-ME) and separated in discontinuous SDS-PAGE gels. Proteins were transferred with a Bio-Rad Transblot electrophoretic transfer device to Immune-Blot polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature with 1% BSA or 5% nonfat milk dissolved in TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) supplemented with 0.1% Tween 20. The membranes were then incubated in the same blocking buffer with the following Abs for 1 h at room temperature. Blots were extensively rinsed and then incubated with an HRP-labeled species-matched secondary Ab for another 1 h. Immunoreactive bands were visualized using ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to B-PLUS X-ray film (Central Illinois X-ray, Bloomington, IN). Reprobing was performed following incubation of membranes in heated (>55°C) stripping buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris-Cl, pH 6.7) for 30 min. Intensity of immunoreactive protein bands on autoradiograms was quantified by scanning with an Agfa Duoscan T1200 scanner followed by analysis with GelAnalyzer 3.3 (San Magno, CA). Software (NucleoTech, San Mateo, CA) was used for minor variations in protein loading into the gels, data were calculated as a ratio of the densitometric intensity of the protein of interest relative to its loading control.

Immunoprecipitation, in vitro kinase assay, and in vivo association of Cdk2 and cyclin A

Following incubation with different concentrations of IL-1β and with or without IGF-I (100 ng/ml) for 24 h, MCF-7 cells were lysed with IGF-I lysis buffer (50 mM HEPEs, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10 mM 0.1% Nonidet P-40, 1 mM EDTA, 2.5 mM EGTA, 10 mM glycophosphate, with freshly added 50 mM NaF and 1 mM DTT, 1 mM PMSF, 1 mM NaF, 48 trypsin inhibitory units of aprotinin, 40 mM leupeptin, and 2 μg/ml pepstatin). Cdk2 or cyclin A was immunoprecipitated from 10 4 or 10 5 μg of protein (10–200 μg) from whole cell lysates. Immunoprecipitates were washed three times with CDK lysis buffer and twice with CDK buffer (50 mM HEPEs, pH 7.5, 10 mM MgCl2, 10 mM MnCl2, and 1 mM DTT). Kinase reactions were conducted by incubation of immune complexes in 50 μl reaction buffer (20 μM cold ATP, 5 μCi [γ-32P]ATP, and −0.5–1 μg CDK substrate) at 37°C for 30 min. Both a truncated RB protein (p56 RB, amino acids 379–928; QED, San Diego, CA) and histone H1 (Roche Diagnostics, Indianapolis, IN) were used as CDK substrates. Reactions were terminated by addition of 20 μl SDS sample buffer, and the samples were subsequently subjected to 10% SDS-PAGE. Following fixation in 20% methanol and 10% acetic acid for 30 min, gels were dried in a slab gel drier (SGD4050; Furina Scientific, Marietta, OH) and phoshorylation of p56 RB peptide or histone H1 was detected with either a PhosphorImager (Typhoon 8600; Molecular Dynamics, Piscataway, NJ) or with B-PLUS X-ray film (Central Illinois X-ray).

Aliquots of Cdk2 immunoprecipitates that were used for the in vitro kinase activity assay and for measurement of in vivo association of cyclin A with Cdk2. Briefly, the Cdk2 immunoprecipitates were mixed with a nonreducing sample buffer (0.92 M Tris-HCl, pH 8.8, 1.5% SDS, 4% glycerol) and maintained at room temperature for 1 h before electrophoresis on 10% SDS-PAGE gels. Electrophoresis under nonreducing conditions prevented reduced H chain and L chain of the rabbit immunoprecipitating Ab from masking appearance of both cyclin A (~50 kDa) and Cdk2 (~30 kDa), respectively. As an additional control for these experiments, immunoblotting was conducted using a monoclonal mouse cyclin A Ab, which was subsequently detected with an HRP-conjugated sheep anti-mouse Ab.
Equal amounts of Cdk2 in each immunoprecipitate sample were confirmed by reprobing the same membrane with a monoclonal Cdk2 Ab.

Statistical analysis

Statistical analyses were performed using the Statistical Analysis System for Microsoft Windows (22). All data, including standardized densitometric intensities from replicate autoradiograms, were analyzed as a completely randomized design using standard ANOVA 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 a mean ± SEM. Two-sided values of *p* < 0.05 or **p** < 0.01 were considered statistically significant.

Results

IGF-I-induced elevation of both E2F-1 and cyclin A and hyperphosphorylation of RB occurs by a LY294002-sensitive, but PD98059- and rapamycin-insensitive pathway

IGF-I has long been known as a late G1 progression factor (23), which occurs by regulating expression of proteins at the G1/S checkpoint (24). Our recent experiments confirmed this idea by showing that IGF-I causes quiescent cells to exit G0, nearly doubling the proportion of cells in S phase from 8% to 15% (7). Cell cycle progression can be regulated by three major signaling pathways: PI3K (25), mammalian

A

**FIGURE 1.** DNA synthesis and expression of cell cycle proteins are induced by IGF-I via a PI3K/Akt-sensitive pathway. A, Inhibitors of PI3K (LY294002, 50 μM), mTOR (rapamycin, 50 nM), and ERK1/2 (PD98059, 50 μM) were added to human MCF-7 breast carcinoma cells 30 min before stimulation with IGF-I (100 ng/ml) for another 15 min. Activation of the three different pathways were analyzed by Western blot analysis with phospho-specific Abs, and total amount of Akt, p70S6K, and ERK were used as loading controls. B, Akt and p70S6K were activated by long-term exposure to IGF-I. The PI3K inhibitors, LY294002 and wortmannin (100 nM), both blocked Akt phosphorylation at Ser473. However, phosphorylation of p70S6K was blocked by LY294002, but not by wortmannin. C, MCF-7 cells were washed and arrested in G0 by culturing for 24 h in serum-free medium. The PI3K inhibitor LY294002 (50 μM), the ERK1/2 inhibitor PD98059 (50 μM) or the mTOR inhibitor rapamycin (50 nM) were added 30 min before IGF-I (100 ng/ml), and the treatments continued for an additional 18 h. After cell labeling with [3H]TdR for an additional 6 h, DNA synthesis was measured by [3H]TdR incorporation. Data represent the mean ± SEM of three independent experiments. Although all three inhibitors significantly reduced the ability of IGF-I to induce DNA synthesis, the inhibition caused by LY294002 was significantly greater than that caused by either PD98059 (*p* < 0.01) or by rapamycin (*p* < 0.05). D, MCF-7 cells were incubated with IGF-I (100 ng/ml) for 24 h following a 30 min preincubation with LY294002 (50 μM), PD98059 (50 μM), or rapamycin (50 nM). Western blot analysis was performed with specific Abs for E2F-1, cyclin A, and RB in whole cell lysates. β-actin was used as a loading control. A representative Western blot is shown, followed by graphs that represent the means ± SEM of densitometric ratios of each protein relative to the loading control in three independent experiments. ***, *p* < 0.01. E, Wortmannin (100 nM) was added to MCF-7 cells 30 min before IGF-I treatment for 24 h. Western blotting was performed as described in D.
target of rapamycin (mTOR) (26), and extracellular signal-regulated kinase (ERK)1 and ERK2 (27). Akt and p70S6K are downstream signaling effectors of PI3K and mTOR, respectively, and phosphorylation of Akt (Ser473) and p70S6K (Thr389) is required for full activation of Akt (28, 29) and p70S6K (28, 29). We first conducted experiments to determine whether IGF-I activates these proteins and whether blocking concentrations of LY294002 (30), rapamycin (31), and PD98059 (32) would specifically block them.

MCF-7 cells were treated for 30 min with each inhibitor before IGF-I stimulation. As shown in Fig. 1A, IGF-I (100 ng/ml for 15 min) induced site-specific phosphorylation of Akt, p70S6K, and ERK1/2. Moreover, the inhibitors potently and specifically blocked IGF-I-stimulated activation of each pathway. For example, PD98059 and rapamycin independently prevented IGF-I from phosphorylating ERK1/2 and p70S6K, respectively, whereas LY294002 blocked the ability of IGF-I to phosphorylate both Akt and p70S6K. LY294002 inhibits PI3K by competing with ATP for binding to the p110 catalytic subunit (30), whereas another commonly used selective PI3K inhibitor, wortmannin, covalently binds and inhibits PI3K (33). This mechanistic difference did not distinguish their specificity in blocking IGF-I early signaling, as shown by similar inhibition of short-term IGF-I-induced activation of both Akt and p70S6K by wortmannin (data not shown). These data are consistent with our earlier findings with murine myeloid progenitor cells (34). Interestingly, we found that both Akt and p70S6K, but not ERK1/2 (data not shown), remained phosphorylated as long as 24 h following addition of IGF-I (Fig. 1B). As expected, we observed a similar specificity of inhibition in prolonged IGF-I-induced activation of Akt and p70S6K by LY294002 and rapamycin as was observed with the shorter, 15 min exposure to IGF-I. In contrast, wortmannin specifically blocked IGF-I-induced prolonged Akt phosphorylation at Ser473 without affecting sustained activation of p70S6K.

We then tested the ability of these pharmacologic compounds at the same concentrations to inhibit IGF-I-induced DNA synthesis. As we previously reported (7), IGF-I significantly (p < 0.01) increased DNA synthesis in MCF-7 cells (Fig. 1C). In the presence of the PI3K inhibitor, LY294002, the ability of IGF-I to promote DNA synthesis was completely blocked. Similar experiments with wortmannin (100 nM) showed that it inhibited IGF-I-induced DNA synthesis by 76 ± 2% (p < 0.01, data not shown). Rapamycin was not as potent as LY294002 in reducing IGF-I-induced DNA synthesis. The ERK1/2 inhibitor, PD98059, was the least efficacious in reducing the ability of IGF-I to promote DNA synthesis. Indeed, the inhibition caused by LY294002 was significantly greater than that caused by either PD98059 (p < 0.01) or rapamycin (p < 0.05). This inhibition was not due to cytotoxicity because in the absence of IGF-I, none of the inhibitors reduced basal [3H]TdR incorporation into DNA (Fig. 1C) or cell viability (data not shown). These results confirm those of others (35, 36) and point to the importance of the PI3K-related pathway in mediating IGF-I-induced DNA synthesis in MCF-7 cells.

As an important G1 cyclin, cyclin A is synthesized at onset of the S phase (10). Expression of cyclin A is mediated by the E2F-1 transcription factor (37) and activation of E2F-1 is directly related to hyperphosphorylation of the RB protein (38). To investigate the role of IGF-I in regulating these critical G1/S checkpoint proteins, we analyzed expression of G1 cyclins and E2F-1, as well as RB phosphorylation in cells treated with IGF-I for 24 h. IGF-I caused a dramatic accumulation in cyclin A (Fig. 1, D and E), although it did not affect expression of either cyclin E or cyclin D1 in these cells (17). IGF-I also increased expression of E2F-1 and caused RB phosphorylation at both Ser107/11 and Thr356 (Fig. 1E). IGF-I increased (p < 0.01) the amount of RB (as determined by relative densities of both the hypo- and hyperphosphorylated forms) compared with that of MCF-7 cells in medium only. LY294002 completely blocked IGF-I-induced E2F-1 and expression of both cyclin A and RB (Fig. 1D). Similar inhibition was observed with wortmannin treatment for 24 h (Fig. 1E).
with G₁ cyclins (41). In this study we focused upon cyclin A activity in the absence of IGF-I.

cause the amount of this protein (Fig. 1C), but not that of cyclin E or cyclin D1 (data not shown), was increased by 24 h treatment with IGF-I in MCF-7 cells. To investigate the physical association between cyclin A and Cdk2, amount of cyclin A was measured in Cdk2 immunoprecipitates derived from cells treated with IGF-I, IL-1β, or both. These communoprecipitation experiments showed that IGF-I greatly increased 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. Cotreatment with IL-1β dose dependently reduced IGF-I-induced formation of the cyclin A-Cdk2 complexes. IL-1β alone did not alter the association of Cdk2 with cyclin A. These data establish that IL-1β prevents formation of cyclin A-Cdk2 complexes only in the presence of IGF-I.

**IL-1β targets IGF-I-stimulated, cyclin A-dependent Cdk activity to inhibit RB phosphorylation**

Because IL-1β impairs formation of IGF-I-induced cyclin A-Cdk2 complexes, cyclin A itself may serve as a target for the antagonism between IL-1β and IGF-I. To test this idea, we precipitated cyclin A instead of Cdk2 with a specific Ab from cells treated with different concentrations of IL-1β with or without IGF-I (100 ng/ml) and measured the ability of cyclin A-associate Cdk to phosphorylate their substrates in vitro. As expected, IGF-I increased enzymatic activity of cyclin A-associated Cdkks, leading to phosphorylation of both histone H1 and a truncated RB peptide (Fig. 3). IL-1β inhibited this IGF-I-stimulated cyclin A-dependent Cdk activity in a dose-dependent manner. In contrast, IL-1β itself did not affect cyclin A-dependent phosphorylation of either truncated RB or histone H1. These results show that IL-1β suppresses IGF-I-triggered cyclin A expression and enzymatic activity that leads to RB phosphorylation and inactivation.

**IL-1β dose- and time-dependent inhibition of IGF-I up-regulated expression of cyclin A and E2F-1 and hyperphosphorylation of RB**

The ability of IL-1β to inhibit IGF-I-stimulated cyclin A activity and RB phosphorylation (Fig. 3) may directly result from a reduction in IGF-I-induced accumulation of cyclin A (Fig. 1D). Because IGF-I induces synthesis of new E2F-1 protein, which is required for cyclin A accumulation (17), IL-1β may also suppress IGF-I-induced E2F-1 expression. To test this hypothesis, we analyzed expression of both cyclin A and E2F-1, as well as different phosphorylation forms of RB, in MCF-7 cells treated with different concentrations of IL-1β in the absence and presence of IGF-I (100 ng/ml). A concentration-dependent reduction in IGF-I activity by IL-1β was detected in the amount of cyclin A and E2F-1 (Fig. 4A).

Several experiments were then conducted to more fully characterize the role of IL-1β in inhibiting IGF-I-induced expression and phosphorylation of RB. Intensity of both the hypo- and hyperphosphorylated forms of RB was expressed as both a ratio (ppRB:pRB) and sum (ppRB and pRB) of RB. This summary revealed that IL-1β significantly impaired IGF-I-induced hyperphosphorylation of RB as well as the total amount of RB, although the former occurred at a five-fold lower concentration. We then used site-specific RB Abs to more specifically begin to determine the phosphorylation sites on RB that are regulated by both IGF-I and IL-1β (Fig. 4C). These experiments demonstrated that as little as 1 ng/ml IL-1β potently impaired both IGF-I induced phosphorylation of residues pSer807/811 and pThr536 of RB. To determine the time course in which IL-1β acts to inhibit these G₁-S regulators that are induced by IGF-I releasing the cells from G₀, we treated MCF-7 cells simultaneously with IL-1β and IGF-I for different periods of time and analyzed the expression of E2F-1, cyclin A and RB, as well as site-specific...
phosphorylation of RB at either Ser\textsuperscript{807/811} or Thr\textsuperscript{356}. Consistent with our recent report (17), we found IGF-I induces all three G\textsubscript{1} regulators in a time-dependent manner (Fig. 4D). A dramatic inhibition by IL-1\textbeta/H9252 occurred at 12 h, and some inhibition was observed at earlier time points. Because MCF-7 cells require at least 12 h to exit G\textsubscript{1} to begin synthesizing DNA, this finding is consistent with the idea that IL-1\textbeta-induced G\textsubscript{1} arrest (7) results from the inhibitory effects of IL-1\textbeta on IGF-I-induced G\textsubscript{1}-S regulators. Importantly, these reductions caused by IL-1\textbeta were manifested only in the presence of IGF-I. To determine whether cyclin A is the only G\textsubscript{1} cyclin that is regulated by the combination of IL-1\textbeta and IGF-I, we also analyzed the amount of cyclin E and cyclin D1. We found no effect of IL-1\textbeta on the amount of these two proteins, either in the absence or presence of IGF-I (data not shown). These data indicate that IL-1\textbeta inhibits both the expression and phosphorylation status of RB protein, but the phosphorylation state of RB is reduced at lower concentrations of IL-1\textbeta than concentrations required to reduce the amount of RB. Collectively, these results establish that IL-1\textbeta inhibits the ability of IGF-I to up-regulate key proteins during the G\textsubscript{1}-S transition.

FIGURE 4. IL-1\textbeta dose dependently inhibits IGF-I-stimulated accumulation of E2F-1 and cyclin A as well as hyperphosphorylation and amount of RB. A, Western blot analysis was performed with specific Abs to cyclin A or E2F-1 on whole cell lysates of MCF-7 cells treated with different concentrations of IL-1\textbeta in the absence and presence of IGF-I (100 ng/ml) for 24 h. B, The same whole cell lysates from all treatments were blotted with the G3-245 mouse anti-human RB Ab. Density of the hypo- and hyperphosphorylated forms of RB was determined, and the results presented as both a ratio of hyperphosphorylated to hypophosphorylated RB (ppRB/\text{pRB}) and total amount of RB (ppRB + \text{pRB}). Graphs (A and B, bottom) represent the mean \pm SEM of densitometric ratios of the appropriate protein relative to the loading control from three independent experiments (n = 3). C, Independent experiments, including the same treatments, were then conducted with site-specific RB Abs in 8% polyacrylamide gels. IGF-I increased phosphorylation of residues pSer\textsuperscript{807/811} (pS\textsuperscript{807/811}) and pThr\textsuperscript{356} (pT\textsuperscript{356}) of RB, and these events were inhibited by 1 ng/ml IL-1\textbeta. These changes in RB site-specific phosphorylation were much more prominent than the changes of total RB amount, as shown by analyzing the same samples in 10% polyacrylamide gels and blotting with Ab G3-245. The loading control, \textbeta-actin, varied \textless10% in all experiments. *, p < 0.05; **, p < 0.01. D, The kinetics of IL-1\textbeta inhibition on IGF-I-induced cyclin A, E2F-1, and RB are represented. MCF-7 cells were treated with IL-1\textbeta (20 ng/ml), IGF-I (100 ng/ml), or both for the indicated times. Expression of cyclin A, E2F-1, and RB protein was detected by Western blotting.
**IL-1β induces expression of p21<sup>Cip1/Waf1</sup>**

Mitogen-dependent progression through G<sub>1</sub>-S transition is regulated by an integrated interplay of G<sub>1</sub> CDKs, cyclins, and CDK inhibitors. As the first recognized CDK inhibitors, p21<sup>Cip1/Waf1</sup> can induce G<sub>1</sub> arrest and block S phase entry by inactivating Cdkks (42). A defect in p21<sup>Cip1/Waf1</sup> increases susceptibility to chemically induced skin carcinoma formation (43), and over-expression of p21<sup>Cip1/Waf1</sup> effectively suppresses tumor growth (44). IL-1β has been shown to induce p21<sup>Cip1/Waf1</sup> in human melanoma cells, leading to G<sub>1</sub> arrest (45). IL-1β may also stimulate expression of p21<sup>Cip1/Waf1</sup> in our system, which may contribute to the impairment of IGF-I-induced Cdk2 (Fig. 2A) activity. To test this idea, p21<sup>Cip1/Waf1</sup> was analyzed in MCF-7 cells treated with increasing concentrations of IL-1β. As shown in Fig. 5, IL-1β dose-dependently induces p21<sup>Cip1/Waf1</sup>. Significant induction occurred when cells were treated with as little as 0.1 ng/ml IL-1β, suggesting that the induction of p21<sup>Cip1/Waf1</sup> may contribute to IL-1β inhibition of IGF-I-stimulated Cdk2 activation.

**IL-1β inhibits IGF-I-induced sustained phosphorylation of Akt, but not p70<sup>S6K</sup> or ERK**

IGF-I up-regulates both cyclin A and E2F-1 through a LY294002-sensitive pathway (Fig. 1C), indicating that PI3K/Akt-related pathway may be responsible for this regulation. A logical question is whether the IGF-I-induced activation of Akt is also a target for IL-1β. To test this possibility, we determined whether IL-1β inhibits not only the PI3K/Akt pathway but also the mTOR/p70<sup>S6K</sup> and ERK1/2 pathways, which are activated by short-term treatment (15 min) with IGF-I in MCF-7 cells (Fig. 1B). As shown in Fig. 6, activation of both PI3K/Akt and mTOR/p70<sup>S6K</sup> pathways remained detectable even following a 24 h continuous exposure to IGF-I. Cotreatment with IL-1β reduced IGF-I-induced Akt phosphorylation by 75%. IL-1β pretreatment for up to 24 h did not inhibit the immediate (15 min) IGF-I-induced activation of PI3K/Akt, mTOR/p70<sup>S6K</sup>, or ERK1/2 pathways (data not shown).

![Figure 5](link)  **FIGURE 5.** Induction of p21<sup>Cip1/Waf1</sup> by IL-1β. Following 24 h serum deprivation, human MCF-7 cells were treated with different doses of IL-1β for 24 h. Expression of p21<sup>Cip1/Waf1</sup> was analyzed by immunoblotting, and β-actin was used as a loading control. A densitometric analysis (mean ± SEM) of three independent experiments is shown in the graph. *, p < 0.05; **, p < 0.01 as compared with the medium control.

![Figure 6](link)  **FIGURE 6.** IL-1β targets prolonged Akt activation, but not activation of mTOR, to suppress IGF-I receptor signaling. Site-specific Ser/Thr phosphorylation of Akt and p70<sup>S6K</sup> was analyzed by Western blot lysates from MCF-7 cells treated with IGF-I (100 ng/ml), IL-1β (10 ng/ml), or their combination for 24 h. Amount of Akt and p70<sup>S6K</sup> was verified by stripping and reprobing the same membrane with Abs directed to these proteins. The two graphs represent the mean ± SEM of the densitometric ratio of phospho-proteins relative to total amount of the proteins from three independent experiments; **, p < 0.01.

The ability of IGF-I to induce sustained p70<sup>S6K</sup> phosphorylation was unaffected by IL-1β, suggesting that IL-1β targets activation of Akt, but not mTOR/p70<sup>S6K</sup>, to inhibit IGF-I receptor signaling. The IGF-I-activated ERK1/2 pathway was transient, remaining detectable when cells were exposed to IGF-I for 15 min (Fig. 1B) but not for 24 h (data not shown). We did not detect any reduction in ERK phosphorylation when MCF-7 cells were coincubated with both IL-1β and compared with IGF-I alone for 10, 20, or 40 min, which were all times when IGF-I was able to induce ERK1/2 phosphorylation (data not shown). Similar results were observed at 24 h, indicating that the ERK1/2 pathway is unlikely to be targeted by IL-1β. These experiments point to PI3K/Akt signaling as the critical pathway that is responsible for IL-1β inhibition of the ability of IGF-I to promote cell proliferation and increase expression of critical G<sub>1</sub>/S checkpoint proteins.

**Discussion**

Acting as a prototypical proinflammatory cytokine, IL-1β is recognized as one of the most pleiotropic cytokines involved in a variety of different biological events. IL-1β inhibits IGF-I-induced tyrosine phosphorylation of IRS-1 and prevents IGF-I from causing cells to enter the S phase of the cell cycle, leading to G<sub>1</sub> arrest (7). In this report, we dissected the cell cycle machinery at the G<sub>1</sub>-S boundary to reveal Akt as a key convergent protein that is targeted by both IGF-I and IL-1β to regulate cell cycle progression. We recently established that IGF-I induces synthesis of E2F-1 transcription factor (Fig. 1D), which is responsible for promoting expression of downstream genes like cyclin A (17). IGF-I promotes...
transition through the G1 restriction point by inducing cyclin A accumulation and RB hyperphosphorylation (Fig. 1D). A LY294002-and wortmannin-sensitive, but rapamycin- and PD98059-insensitive, pathway mediates IGF-I-induced DNA synthesis (Fig. 1C), hyperphosphorylation of RB, and accumulation of both cyclin A and E2F-1 (Fig. 1D). IL-1β suppresses IGF-I-induced activation of Akt (Fig. 6). As a consequence, IL-1β antagonizes the ability of IGF-I to trigger activation of Cdk2 (Fig. 2), accumulation of both cyclin A and E2F-1 (Fig. 4A), expression and hyperphosphorylation of RB (Fig. 4, B and C), and ultimately, G1-S progression. In the absence of IGF-I, IL-1β does not affect any of these molecular events, which is consistent with our previously established model that the major inhibitory properties of proinflammatory cytokines on growth of breast cancer cells are manifested prominently in the presence of growth factors (7).

IGF-I receptor signaling occurs via binding to its transmembrane receptor and activation of the type I IGF receptor’s intrinsic tyrosine kinase activity. This tyrosine kinase activity leads to recruitment and activation of the IRS docking proteins and cytoplasmic signaling enzymes. These early membrane signal transduction events have been widely investigated and elucidated. Similarly, the major downstream consequences of these early signaling events, in this case cell cycle progression, have also been studied and clarified to a large extent. However, understanding the links between upstream signaling events and downstream cell cycle progression is rather limited. How do cells select from a variety of signaling cascades that are initiated by growth factors to cause them to pass through the restriction point of the cell cycle? Data in this report provide evidence that IGF-I causes changes directed toward S phase entry by increasing the amount of cyclin A and E2F-1 and the subsequent hyperphosphorylation and inactivation of RB. These events and passage of cells through the cell cycle are completely blocked by PI3K inhibitors, but ERK1/2 or mTOR inhibitors do not affect them.

The ERK1/2 (46, 47) and mTOR (48–50) pathways can be involved in the G1-S transition by regulating cyclin D1 expression. However, our results demonstrate that IGF-I induces G1-S key regulators by activating the PI3K/Akt signaling cascade or related kinases in a manner independent of ERK1/2 and mTOR. The PI3K-specific inhibitor, wortmannin, blocks IGF-I and mTOR. The PI3K-specific inhibitor, wortmannin, blocks IGF-I– and mTOR–responsive prolonged activation of Akt, but not that of p70S6K (Fig. 1B). Blockage of prolonged PI3K/Akt activation with wortmannin also suppresses the ability of IGF-I to elevate both E2F-1 and cyclin A and to hyperphosphorylate RB (Fig. 1E). Interestingly, IL-1β acts just like wortmannin by inhibiting IGF-I-induced Akt phosphorylation at Ser473 (Fig. 6), up-regulation of both E2F-1 and cyclin A (Fig. 4A) and phosphorylation of RB (Fig. 4, B and C). LY294002 blocks phosphorylation of both Akt and p70S6K (Fig. 1B). However, blockage of the mTOR pathway with rapamycin does not affect the ability of IGF-I to stimulate accumulation of cyclin A, activation of E2F-1 or phosphorylation of RB (Fig. 1D), the key molecular indicators for passage into the S phase in response to IGF-I. Wortmannin blocks the activation of both Akt and p70S6K evoked by short pulse of IGF-I (data not shown). However, the inhibition remains at 24 h for only IGF-I–stimulated phosphorylation of Akt, but not that of p70S6K (Fig. 1B). These data suggest that the PI3K/Akt pathway may not be responsible for IGF-I–induced prolonged activation of p70S6K. Although it is well accepted that mTOR/p70S6K resides downstream of PI3K/Akt (51), the mTOR pathway can also be regulated independently of PI3K (52, 53). Other signaling molecules, such as newly synthesized amino acids (52, 53), may mediate prolonged IGF-I–induced signals associated with activation of mTOR/p70S6K. These data not only provide direct evidence that identifies an important role for PI3K/Akt in mediating the engagement of IGF-I–stimulated early signaling events to subsequent cell cycle progression, but also suggest that IL-1β specifically targets the PI3K/Akt pathway to suppress IGF-I–induced key G1-S regulators. Our results suggest that other signaling events downstream of PI3K/Akt mediate IGF-I–induced key G1-S regulators in an mTOR/p70S6K–independent manner.

IGF-I increases DNA synthesis (Fig. 1C), an indicator of cells entering the S phase, by 40-fold in MCF-7 cells. Consistent with elimination of IGF-I–induced increase in cyclin A, E2F-1 and RB phosphorylation (Fig. 1D), LY294002 completely abrogates the ability of IGF-I to induce DNA synthesis (Fig. 1C). Another PI3K–specific inhibitor, wortmannin, also significantly suppresses IGF-I–induced DNA synthesis. The failure of IGF-I to promote DNA synthesis in the presence of these inhibitors confirms an essential role of PI3K, or other PI3K-related kinases (54), in mediating IGF-I–induced cell proliferation. Structural and mechanistic differences between wortmannin and LY294002 may be responsible for their different levels of inhibition. Wortmannin inhibits PI3K by covalently binding to the p110 catalytic subunit (33), whereas LY294002 competes with ATP for binding to p110 (30). In addition, the shorter half-life (~90 min) of wortmannin (55) may also explain its reduced potency in inhibiting the relative long process of induction of DNA synthesis.

Growth factors often trigger a rapid burst of signaling, such as phosphorylation of the growth factor receptor and receptor adaptor proteins, recruitment and activation of PI3K and phosphorylation of Akt, all of which occur within seconds and subside within an hour, even in the continuous presence of growth factors (55). In comparison, over 12 h are required for a G0 fibroblast to progress to the S phase, of which 8–10 h are required for cells to reach the G1-S restriction point (56). It has been difficult to explain why a transient signal, lasting <60 min, is sufficient to drive cells through the G1 restriction point, which begins 8 h after exposure to growth factors. Recent experiments have revealed that there is an additional wave of signaling in response to growth factor stimulation. Although the first burst of signaling is dispensable, the second wave, which occurs 3–7 h poststimulation, is required for DNA synthesis and S phase entry (55, 57). In our experimental setting, activation of the PI3K/Akt pathway, shown by phosphorylation of Akt at Ser473, remains detectable following constant IGF-I stimulation for 24 h (Figs. 1B and 6) at a level that is comparable to 15 min of IGF-I stimulation (Fig. 1A). In agreement with the two-wave concept, inhibition of Akt activation with LY294002 (Fig. 1D) and wortmannin (Fig. 1E) eliminates the increase in E2F-1/cyclin A and RB hyperphosphorylation following a 24-h continuous exposure to IGF-I. Interestingly, prolonged activation of both Akt and p70S6K is blocked by LY294002, whereas wortmannin only specifically blocks Akt phosphorylation (Fig. 1B). More importantly, the sustained, but not the immediate, IGF-I–responsive activation of Akt is largely inhibited when cells are simultaneously exposed to IL-1β (Fig. 6), in a manner similar to wortmannin. In contrast, although activation of p70S6K is also detectable after a 24-h exposure to IGF-I, IL-1β does not affect the ability of IGF-I to phosphorylate p70S6K at Thr389 (Fig. 6). Because a PI3K–related pathway and Akt activation mediate IGF-I–promoted G1-S transition and cell proliferation in an mTOR/p70S6K–independent manner (Fig. 1D), IL-1β inhibits IGF-I–induced signaling (Figs. 2, 3, and 4) by targeting the pathway that is essential for IGF-I–to promote cell cycle transition (Fig. 6). As for ERK1/2 pathway, IGF-I–induced ERK1/2 phosphorylation can be detected only with a 15-min treatment (Fig. 1A), but the signal is not detectable at 24 h (data not shown). These results exclude a possible role for the ERK1/2 pathway in mediating either IGF-I stimulation or IL-1β inhibition of cyclin A, phosphorylation of RB or E2F-1.
Cells are responsive to extracellular growth factors only during an interval between the early to mid-G1 phase and the restriction point of the cell cycle (58). Phosphorylation of RB marks the end of this interval and enables cells to transit through the restriction point, leading to completion of the cell cycle even in the absence of growth factors. RB phosphorylation is directly related to the activity of G1 CDKs, accumulation of G1 cyclins, and activation of E2F transcription factors. In this report, we demonstrate in MCF-7 breast cancer cells that IGF-I activates Cdk2, the vital G1 CDK that is necessary for cells to pass through the G1/S restriction checkpoint. This event occurs concurrently with accumulation of cyclin A and its association with Cdk2, hyperphosphorylation of RB and elevation and activation of E2F-1. Significantly, all these cell cycle-accelerating properties of IGF-I are eliminated when MCF-7 cells are simultaneously exposed to the proinflammatory cytokine, IL-1β. These data also establish an essential role for PI3K/Akt pathway, through mTOR/p70S6K and ERK-independent mechanisms, as a convergent target in mediating IGF-I-induced G1/S transition and IL-1β-induced antagonism. IL-1β impairs IGF-I-induced Akt activation. In the absence of Akt activation by IGF-I, IL-1β does not affect any of the G1-S regulators.

As a surrogate risk factor for development of various cancers, elevated concentrations of IGF-I are associated with uncontrolled cell proliferation by activating key signaling enzymes, such as PI3K/Akt, and promoting cell cycle progression by triggering critical G1-S regulators, such as RB, E2F, and G1 cyclin. The present results establish an intracellular mechanism of cross-talk between the immune (IL-1β) and neuroendocrine (IGF-I) systems, both of which are expressed in the microenvironment of tumors, in regulating the growth of breast cancer cells. In this scenario, leukocyte-derived IL-1β manifests its cytostatic G1 arresting property only when cells are driven into a cycling state by IGF-I. Identification of prolonged PI3K/Akt activation as an intracellular target for this IL-1β-IGF-I interaction in regulating breast cancer cell cycle progression not only confirms the requirement of sustained IGF-I signaling for the transition through the restriction point, but also identify cytoplasmic proteins that provide a link between juxtamembrane early signaling events and the nuclear cell cycle machinery.

References