**Interleukin-1 Induces Growth Arrest by Hypophosphorylation of the Retinoblastoma Susceptibility Gene Product RB***

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Interleukin-1 (IL-1) causes G0/G1 phase growth arrest in human melanoma cells, A375-C6. Because hypophosphorylation of the retinoblastoma susceptibility gene product, RB, is one of the key events responsible for G0/G1 phase growth arrest, we investigated whether IL-1 altered the phosphorylation status of RB protein in these cells. Exposure to IL-1 caused a time-dependent increase in hypophosphorylated RB that correlated with an accumulation of cells arrested in the G0/G1 phase. The ability of IL-1 to cause hypophosphorylation of RB and growth arrest was abrogated by the SV40 large T antigen, which binds preferentially to hypophosphorylated RB, but not by the K1 mutant of the T antigen, which is defective in binding to RB. Furthermore, the cells were protected from IL-1-inducible growth inhibition by ectopic expression of dominant-negative mutants of the RB gene, or the transcription factor E2F-1, which is a downstream target of RB. These results suggest that hypophosphorylated RB mediates the growth arrest induced by IL-1.

Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) are two important cytokines secreted primarily by activated macrophages and monocytes (1–5). These cytokines elicit diverse biological effects ranging from those that are beneficial to the host, such as recruitment of immunological and anti-tumor responses, to those that may have deleterious consequences, such as cell proliferation and tissue injury, and inflammatory responses that may lead to toxic shock and sepsis (1–5). In cell culture, these cytokines show pleiotropic effects on the growth of mammalian cells. The growth regulatory effects of IL-1 and TNF-α are cell type-dependent: the cytokines stimulate the growth of nontransformed cells such as fibroblasts (6, 7) and kidney epithelial cells (8) and transformed cells, such as glioblastoma (8–10) and renal carcinoma (11), but inhibit the growth of certain nontransformed fibroblast cells (12), and tumor cells, such as melanoma (8, 13), breast carcinoma (14), lung adenocarcinoma (15), ovarian carcinoma (16), and myeloid leukemia (17, 18). Although these cytokines show overlapping biological activities, their action is mediated by distinct cell surface receptors (19–21). Signal transduction and phenotypic alterations by IL-1 are mediated via IL-1-receptor type I (22–24), whereas two different TNF-α receptors mediate distinct phenotypic responses to TNF-α (25). The action of each of these cytokines is associated with diverse second messengers in the cytosol, which in turn activate DNA binding proteins, particularly AP-1 and NF-κB, that lead to induction of a number of immediate-early and delayed response genes (cited in Refs. 24, 26, and 27). The mechanism of action of these cytokines is, however, not known.

To understand the molecular basis for the pleiotropic effects of IL-1 on the growth of human cells, we are studying the gene programs associated with the actions of this cytokine (13). Human melanoma cells, A375-C6, show growth arrest in response to IL-1 or TNF-α and provide an excellent experimental model for exploring the molecular pathways that are initiated by the binding of these cytokines to the cognate cell surface receptors and that lead to a dose- and time-dependent growth arrest response (8, 13, 24). The action of IL-1 and TNF-α in the melanoma cells is dependent on induction of immediate-early genes (13), which are expected to initiate a cascade of events leading to growth arrest. Fluorescence-assisted cell sorting (FACS) analysis has revealed that IL-1 causes growth arrest in the G0/G1 phase of the cell cycle (24).

Studies on cell cycle regulation have identified the product, RB, of the retinoblastoma susceptibility gene, Rb, as a key checkpoint control protein responsible for imposing a block in the cell cycle at the transition from the G1 to the S phase (28, 29). Mutations that functionally inactivate the Rb gene have been identified in a variety of human tumors (30). More recently, ectopic expression of the wild-type Rb gene can suppress the growth of tumor cells in culture or the formation of tumors in animal models (31, 32). The characterization of RB as a tumor suppressor has been further consolidated by the observations that three different transforming proteins encoded by DNA tumor viruses, adenovirus E1A, papovavirus large T antigen, and papillomavirus E7 protein, can bind to the RB protein at a domain called the A/B pocket and that the transforming functions of these proteins are linked to their ability to bind to RB protein (33–36). The RB protein is differentially phosphorylated during cell cycle progression (37–41). It is dephosphorylated (RB) during the early and mid G1 phase, then phosphorylated by cyclin/cyclin-dependent kinase complexes during late G1 and further in the S phase. Late in mitosis, it is enzymatically dephosphorylated, and cells entering the next G1 phase contain primarily RB. These observations coupled with the facts that the large T antigen preferentially binds to RB and induces G1-arrested cells to enter the S phase (42), suggest that RB causes G1 phase growth arrest and RB phosphorylation is linked to rescue from such an arrest.

The A/B pocket domain of RB can bind and inhibit the function of several cell cycle regulators such as the E2F family of transcription factors (28, 33, 35, 36, 43–46). The ability of RB...
protein to arrest the cell cycle depends on sequences necessary for its interaction with E2F (28, 33, 35, 36, 43–46). Ectopic overexpression of E2F-1, a member of the E2F-family, can rescue cells from RB-imposed G1 phase growth arrest, presumably by transactivating E2F-1 target genes that are necessary for entry and progression through the S phase (47–49). Moreover, a recent study has identified a carboxyl-terminal region within the A/B pocket domain, designated the C pocket, which binds to the tyrosine kinase, c-Abl (50, 51). RB can simultaneously bind to c-Abl and E2F; however, in the absence of RB protein, c-Abl does not bind to E2F (51). In this manner, RB acts a molecular “matchmaker” bringing together proteins that would not otherwise interact. Cotransfection studies suggest that coexpression of full-length RB along with an RB deletion mutant designated SE, which contains only the C pocket region (51), disrupts the ability of RB to suppress growth. The SE mutant binds to, and inhibits the function of, E2F, c-Abl, and perhaps other as yet unknown proteins. Despite inhibition of these protein activities, the SE mutant protects the cells from RB-imposed growth arrest (51). In other words, RB acts as a growth suppressor only when the A/B and the C pockets are in the cis configuration, suitable for molecular matchmaking, and RB function is inhibited by coexpression of the C pocket domain. Thus, the SE mutant acts as a dominant-negative inhibitor of RB function.

Because IL-1 and TNF-α often induce overlapping signal transduction and phenotypic effects, and because IL-1 causes cell cycle arrest in the G0/G1 phase, we sought to examine whether its action is mediated by RB. Data presented here suggest that growth arrest by IL-1 is dependent on RB function, but that by TNF-α is mediated by an RB-independent pathway.

MATERIALS AND METHODS

Cell Culture and Cytokines—Growth and maintenance of human melanoma cells, A375-C6, has been described previously (8). Human recombinant IL-1α (specific activity, 1.8 × 107 units/mg) was a gift from Craig Reynolds, Biological Response Modifiers Program, National Cancer Institute (Frederick, MD). Human recombinant TNF-α (specific activity, 4.8 × 107 units/mg) was purchased from R & D Systems (Minneapolis, MN).

Plasmid Constructs—The pSGS-SV40T antigen construct containing the cDNA encoding simian virus 40 (SV40) large T antigen subordinated in plasmid vector pSGS (Stratagene, La jolla, CA), and the pSGS-SV40 K1 construct encoding the K1 mutant of SV40 T antigen, which is deficient in transformation and RB-binding functions, were kindly provided by Mark Ewen (Harvard Medical School, Boston, MA). The deletion mutants of RB, designated ME, SE, and SE A, subordinated in eukaryotic expression vector pFLAG (IBI/Esamnt Kadak Co.) were kindly provided by Jiean Wang (University of California at San Diego, La jolla, CA). SE encodes the COOH-terminal amino acids 768–928 of RB; SE A encodes the COOH-terminal amino acids 785–806; and ME encodes the COOH-terminal amino acids 369–467 (46), were kindly provided by William Kaelin, Jr. (Harvard Medical School, Boston, MA).

DNA Transfections—Cells were transfected with various DNA constructs mixed in N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP) (from Boehringer Mannheim, as described previously (24). To obtain stable transfectants with the pSGS-based constructs, we performed cotransfections with vector pCB6 (24), which confers aminoglycosidic resistance, so as to allow selection of G418 sulfate-resistant cells. Stable transfectant cell lines were selected and maintained in growth medium supplemented with G418 sulfate (300 μg/ml).

Assay for [3H]Thymidine Incorporation—These studies were performed in 96-well plates and percent growth inhibition was calculated as described previously (8, 24).

FACS and Cell Cycle Distribution—Low density cultures (about 2 × 105 cells/T75 flask) were either left unexposed or exposed to IL-1 (100 units/ml) or TNF-α (100 units/ml) for 24, 48, or 72 h, then the DNA was stained with propidium iodide and subjected to FACS analysis at the Flow Cytometry Core Facility, University of Kentucky, as described (24). The number of cells in each compartment was obtained by using Modfit software (Verity Software, Topsham, ME).

Western Blot (Immunoblot) Analysis—Low density cultures (about 2 × 106 cells/T75 flask) were left unexposed or exposed to IL-1 (100 units/ml) or TNF-α (100 units/ml), for the indicated periods of time. Whole cell protein extracts were then prepared, and protein concentrations were determined as described previously (26). Unless otherwise indicated, 20 μg of each protein extract was electrophoresed on polyacrylamide-SDS gels. The proteins were transferred to nylon membranes, incubated with blocking solution (5% non-fat milk in 10 mm TrisCl and 150 mm NaCl) for 1 h, then probed with the indicated antibody (1 μg/ml in blocking solution), and finally incubated with 125I-protein A for 1 h. The anti-RB polyclonal antibody, C-15, which recognizes the 15 amino acids at the COOH-terminal end of RB, and the anti-E2F-1 monoclonal antibody, which recognizes aminoc acids 342–368 of E2F-1, were purchased from Sigma, and the rabbit anti-mouse IgG antibody was from Southern Biotechnology, Inc. (Birmingham, AL). Blots probed with the monoclonal antibodies were subsequently probed with the rabbit anti-mouse antibody, prior to incubation with 125I-protein A.

RESULTS

IL-1 and TNF-α Cause Hypophosphorylation of RB—Because the hypophosphorylated form of RB has been linked to growth arrest in the G0 phase of the cell cycle in certain cell types (52, 53), we sought to examine by Western analysis its phosphorylation status in response to IL-1 or TNF-α in A375-C6 cells. In addition, to determine whether the phosphorylation status of RB correlated with the extent of growth inhibition as judged by accumulation of cells in any particular phase of the cell cycle, we performed Western blot analysis and FACS analysis on cells from the same flask as follows. A375-C6 cells were exposed to IL-1 or TNF-α for various time intervals in T75 flasks and then appropriate portions of the cultures from each flask were used to prepare whole cell protein extracts for Western blot analysis or were simultaneously processed for FACS analysis. As seen in Fig. 1a, Western blot analysis of untreated A375-C6 cells primarily showed expression of a slow-migrating RB species, pRB (about 115 kDa). Cells treated with IL-1 or TNF-α showed a diminished expression of this slow-migrating species and a simultaneous increase in a relatively fast-migrating species of RB (Fig. 1a). The ratio of the fast-migrating form (RB) to the relatively slow-migrating form (pRB) increased over time. In untreated cells about 15% of the RB protein was in the RB form, whereas in cells treated with IL-1 about 25, 50, and 80% of the RB protein was in the RB form at 24, 48, and 72 h of exposure, respectively. Likewise, in cells treated with TNF-α, about 30, 50, and 85% of RB protein was in the RB form at 24, 48, and 72 h, respectively. Previous studies (Ref. 52 and references cited therein) have indicated that in cells whose growth is arrested, hypophosphorylation of RB may occur in parallel with a reduction in the amount of detectable RB protein. Because we loaded equal amounts (20 μg) of each extract protein, the data (Fig. 1a) suggest that compared with untreated cells, the total amount of RB was relatively lower in cells exposed to IL-1 or TNF-α (Fig. 1a; quantification of data not shown). FACS analysis indicated that IL-1 or TNF-α caused a time-dependent accumulation of cells in the G0/G1 phase of the cycle (Fig. 1b) and a simultaneous decrease in the number of cells in the S phase (Fig. 1c). Taken together, these data indicate that the time-dependent un(der)phosphorylation of RB by IL-1 or TNF-α correlates with increasing growth inhibition as indicated by the accumulation of cells in the G0/G1 phase and reduction of cells in the S phase.

Studies in other cell types have attributed differences in the
migration of RB protein in gel electrophoresis to the extent of underphosphorylation of the protein (52). Since the RB protein is completely dephosphorylated upon treatment with potato acid phosphatase, such a treatment has been used to identify unphosphorylated RB (52). To help determine whether the slow- and fast-migrating forms of RB represent phosphorylated and differentially underphosphorylated forms, respectively, of RB, we treated the protein extract from A375-C6 cells that had been exposed to IL-1 for 48 h, with vehicle or potato acid phosphatase, and then subjected these extracts to Western blot analysis. Data shown in Fig. 1 indicated that the vehicle-treated extract contained both the slow- and fast-migrating forms of RB (Fig. 1d), similar to those seen after 48-h exposure to IL-1 (compare with Fig. 1a). On the other hand, treatment of the extract with potato acid phosphatase resulted in disappearance of the slow-migrating species and simultaneous appearance of a fast-migrating species (Fig. 1d). This fast-migrating species of RB protein that formed after potato acid phosphatase treatment of the extract comigrated with the fast-migrating species of RB protein that was seen in the extract from cells treated with IL-1 for 72 h (Fig. 1d). These data indicate that the slow-migrating forms of RB represent differentially phosphorylated RB (pRB), whereas the fast-migrating form represents unphosphorylated RB (RB). Similar observations were made when extracts from TNF-α-treated cells were subjected to potato acid phosphatase (data not shown). These results indicate that IL-1 and TNF-α cause a time-dependent hypophosphorylation of RB.

Overexpression of SV40 Large T Antigen Protects Cells fromIL-1-or TNF-α-inducible Growth Inhibition—The SV40 large T antigen binds preferentially to the A/B pocket region of RB and other pocket-containing RB-related proteins such as p107, p130, or p300 (29) and thereby overcomes their growth suppressor effect. To determine whether SV40 large T antigen could overcome the growth-arresting actions of IL-1 or TNF-α, we transfected A375-C6 cells with a plasmid encoding the SV40 large T antigen. As a control, we transfected the cells with a plasmid expressing the K1 mutant of T antigen which does not bind to RB. Three different stable transfec tant cell lines for each plasmid, i.e. C6/T.L1, C6/T.L2, and C6/T.L3 for the large T antigen, or C6/K1.L1, C6/K1.L2, or C6/K1.L3 for the K1 mutant, were selected with G418 sulfate and examined for expression of T antigen or mutant T antigen by Western blot analysis using the anti-large T antigen antibody. Data shown in Fig. 2 for the cell lines C6/T.L1 or C6/K1.L1 are representative of the transfected cell lines. Parent A375-C6 cells did not express endogenous SV40 large T antigen, whereas C6/T.L1 cells expressed the 90–95-kDa SV40 large T antigen (Fig. 2a). C6/K1.L1 cells, on the other hand, expressed a relatively faster migrating form of T antigen (Fig. 2a), as expected based on the relatively smaller size of the K1 mutant cDNA compared with that of the large T antigen.

To address whether ectopic overexpression of the T antigen or K1 mutant affected the RB phosphorylation status in response to IL-1 or TNF-α, the transfec tant cell lines, confirmed to express the large T antigen or the K1 mutant, were left unexposed or exposed to IL-1 or TNF-α for 48 or 72 h, and whole cell protein extracts were examined by Western blot analysis. Extracts from cells exposed to IL-1 for 48 h were treated in vitro with vehicle (v) or potato acid phosphatase (PAP) for 15 min and then subjected to Western blot analysis using the anti-RB antibody. These experiments used protein extracts from cells exposed to IL-1 for 72 h as a control for the fast-migrating RB species seen with IL-1 treatment. The slow-migrating differentially phosphorylated forms of RB (pRB) and the fast-migrating unphosphorylated form of RB (RB) are indicated (a and d).
TNF-α did not show increased hypophosphorylation of RB in response to IL-1 or K1 mutant are indicated. The bands corresponding to the large T antigen and a control were subjected to Western blot analysis using an anti-T antigen or the K1 mutant, respectively, or parent A375-C6 cells (29), it was possible that the rescue of A375-C6 cells by large T antigen from IL-1 or TNF-α action was owing to inhibition of another pocket-containing protein responsible for the G1/S checkpoint control and not RB itself. To directly determine whether RB was causally responsible for IL-1 or TNF-α-inducible growth arrest, we used the dominant-negative mutants of RB, designated SE and SEΔ. SE encodes the COOH-terminal amino acids, 768–928, of the RB protein, and SEΔ is a derivative of SE from which the amino acids required for E2F binding have been deleted. Because the SEΔ derivative does not bind to E2F, it does not disrupt the interaction of RB and E2F (51). Moreover, SEΔ is not homologous to other RB-related proteins and has been demonstrated to selectively bind proteins that do not bind to the carboxyl-terminal domains of the RB-related proteins p107 or p130 (51). SEΔ is not expected to disrupt p107- or p130-mediated complexes and is a specific inhibitor of RB function. For a control, we used another derivative of RB, designated ME, which lacks the NH₂-terminal amino acids of SEΔ, and therefore, does not provide the dominant-negative RB function (51). A375-C6 cells were stably transfected with plas-

**Fig. 2.** Overexpression of SV40 large T antigen rescues cells from IL-1- or TNF-α-inducible growth inhibition. a, transfectant cells express T antigen or K1 mutant. A375-C6 cells were transfected with pSG5-SV40 large T antigen plasmid encoding the SV40 large T antigen, or pSG5-K1 plasmid encoding the K1 mutant of T antigen, and stable transfectant cell lines were selected. Protein extracts from cell lines C6/T.L1 or C6/K1.L1 transfected with constructs encoding the T antigen or the K1 mutant, respectively, or parent A375-C6 cells (C6) for a control were subjected to Western blot analysis using an anti-T antigen antibody. The bands corresponding to the large T antigen and K1 mutant are indicated. b, transfectant cells expressing T antigen do not show increased hypophosphorylation of RB in response to IL-1 or TNF-α. The transfectant cells were left untreated (UT) or treated with IL-1 or TNF-α for 48 or 72 h as indicated, and whole cell protein extracts were examined by Western blot analysis for RB expression. As a control for the unphosphorylated form of RB, we used a protein extract (50 μg of protein) from A375-C6 cells (C6) which were exposed to IL-1 for 72 h (right panel). The differentially phosphorylated (pRB) and unphosphorylated (RB) forms of RB are shown. C6/T.L1 cells (right panel), which were left untreated or treated with IL-1 or TNF-α, expressed primarily pRB, whereas K1.L1 cells (left panel) showed hypophosphorylation of RB protein upon exposure to IL-1 or TNF-α. c, overexpression of T antigen abrogates the growth arrest response to IL-1 or TNF-α. The transfectant cell lines were left unexposed or exposed to IL-1 or TNF-α for 48 or 72 h, and the effect on growth was examined by [3H]thymidine incorporation studies. Percent growth inhibition is expressed as a function of cytokine exposure time.
mids encoding SE, SEΔ, or ME, then stable transfectant cell lines were selected with G418 sulfate-resistant transfectant cell lines were selected. Whole cell protein extracts were prepared from parent A375-C6 cells (C6) or the transfectant cell lines SE.L1 (C6/SE), SEΔ.L1 (C6/SEΔ), and ME.L1 (C6/ME) and subjected to Western blot analysis using the anti-RB antibody, C15. Arrows indicate position of SE or SEΔ (upper arrow; ~18 kDa) and ME (lower arrow; ~12 kDa) proteins. As expected, the parent cells did not show expression of RB mutants of such low molecular weight. [3H]thymidine uptake studies and viable counting of cells using the dye exclusion test revealed that the growth rate of the transfectants expressing SE, SEΔ, or ME was similar to that of the parent cells (data not shown).

To determine whether ectopic expression of SE, SEΔ, or ME could rescue the cells from growth arrest by IL-1 or TNF-α, the transfectant cell lines, SE.L1, SEΔ.L1, or ME.L1, were left unexposed or exposed to IL-1 or TNF-α for various intervals of time, and the effect on growth was examined by [3H]thymidine incorporation studies. ME.L1 cells showed growth inhibition in response to IL-1 and TNF-α with kinetics similar to those seen in parent A375-C6 cells (Fig. 3, b and c). Similarly, the growth of SE.L1 and SEΔ.L1 transfectant cells was inhibited by TNF-α (Fig. 3b). By contrast, SE.L1 and SEΔ.L1 transfectant cells were relatively resistant to the growth arresting action of IL-1: a maximum of about 25–35% growth arrest was seen in 48 or 72 h (Fig. 3c). Three different transfectant cell lines for SE, SEΔ, and ME were examined for expression of the RB derivatives and susceptibility to IL-1 or TNF-α, and the observations (data not shown) were similar to those described for Figs. 3, a–c. These results suggest that the dominant-negative mutants of RB, SE, and SEΔ, protect cells from IL-1 but not from TNF-α action. Thus, RB plays a role in the growth-arresting action of IL-1, but may be a dispensable component of the TNF-α-inducible growth arrest pathway. The accumulation of hypophosphorylated RB upon exposure to TNF-α, therefore, seems to be a consequence of G0/G1 phase growth arrest mediated by another checkpoint control protein. Further studies
will examine whether the RB-related proteins, p107 or p130, mediate the action of TNF-α in these cells.

To determine whether ectopic expression of SEα, which protected the cells from IL-1- but not TNF-α-inducible growth inhibition, affected the phosphorylation status of RB in response to the cytokines, SEΔL1 cells were left unexposed or exposed to IL-1 for 48 or 72 h, and whole cell protein extracts were examined by Western blot analysis for RB expression. As seen in Fig. 3d, IL-1 caused a time-dependent hypophosphorylation of RB in the SEΔL1 cells, in a manner similar to that seen in the parent cells (compare with Fig. 1). Since IL-1-inducible growth arrest was abrogated in SEΔL1 cells, these results suggest that SEΔ provides protection from IL-1 action without interfering with the ability of the cytokine to cause hypophosphorylation of RB.

Ectopic E2F-1 Expression Rescues Cells from IL-1- and TNF-α-inducible Growth Arrest—RB is involved in direct binding and inhibition of various key proteins, such as those of the E2F family, that are required for S phase entry (29, 36). Members of the E2F family of transcription factors have been shown to bind to the A/B pocket region of RB and RB-related proteins, p107 or p130. Because the experiments using the dominant-negative mutants of RB, SE, and SEΔ indicated that RB was required for the growth inhibitory effects of IL-1, but not TNF-α, we sought to determine whether E2F could rescue the cells from the action of these cytokines. To address this question we took advantage of the fact that E2F-1, a member of the E2F family, can overcome the growth suppressor effects of RB and that this effect is dependent on the ability of E2F-1 to cause transactivation via its target sequence (46). A375-C6 cells were transfected with an expression plasmid for E2F-1, and, for a control, a transactivation-deficient deletion construct, E2F-1(1–368), and stable transfectant cell lines were selected with G418 sulfate. We then prepared protein extracts from the transfectant cells and, for another control, from parent A375-C6 cells and performed Western blot analysis to ascertain expression of the transfected plasmids. The parent A375-C6 cells did not show detectable expression of endogenous E2F-1 protein (Fig. 4a, right panel); whereas cells transfected with the E2F-1 expression plasmid showed a 55-kDa band for E2F-1, and those transfected with the E2F-1(1–368) expression plasmid showed a 50-kDa band for the E2F-1 mutant (Fig. 4a, both panels), as expected.

We then exposed the transfectant cells to IL-1 or TNF-α for 24, 48 or 72 h and performed [3H]thymidine incorporation experiments, to determine their susceptibility to the cytokines. The E2F-1 transfectant cell line, E2F-1.L1, showed 5, 10, or 30% growth inhibition in response to IL-1 in 24, 48, or 72 h, respectively (Fig. 4b), and <5, 15, or 35% growth inhibition in response to TNF-α in 24, 48, or 72 h, respectively (Fig. 4c). On the other hand, the E2F-1(1–368) transfectant cell line E2F-1(1–368).L1 showed about 20, 50, or 80% growth inhibition in response to IL-1, in 24, 48, or 72 h, respectively (Figs. 4b and 4c). Three different transfectant cell lines for E2F-1 or E2F-1(1–368) were examined in this manner and the results (not shown) were similar to those described above in Fig. 4, a–c. These data suggest that ectopic expression of E2F-1 can rescue the melanoma cells from growth arrest by IL-1 and are consistent with the view that RB plays a role in IL-1 action. However, the rescue of cells expressing E2F-1 from the growth arresting action of TNF-α is particularly interesting, because the data in Fig. 3 suggest that the action of this cytokine is not mediated via RB hypophosphorylation. It is possible, therefore, that the action of TNF-α is mediated by an RB-related protein, perhaps p107, p130, or another checkpoint control protein whose expression or function may be modulated or circumvented by expression of E2F-1. As the transactivation-deficient mutant of
E2F-1 did not rescue the cells from IL-1 or TNF-α action, the data imply that the transactivation function of ectopically expressed E2F-1 is required for protection from IL-1-evoked hypophosphorylation of RB or TNF-α-mediated growth arrest via an RB-related protein.

**DISCUSSION**

The present study revealed that in melanoma cells, A375-C6, IL-1, or TNF-α, invoke hypophosphorylation of RB, and the extent of hypophosphorylation correlates with inhibition of growth and accumulation of cells in the G1/G0 phase of the cell cycle. Since underphosphorylated RB has been implicated in the G1/G0 phase growth arrest caused by another cytokine, transforming growth factor-β (52, 53), we sought to determine whether RB was functionally involved in the action of IL-1 or TNF-α. We began by asking whether the SV40 large T antigen, which has been shown to preferentially overcome the growth suppressor action of hypophosphorylated RB, could abrogate the growth arresting action of the cytokines. Previous studies (52, 53) evaluating the role of hypophosphorylated RB in TGFB-β-inducible G1/G0 growth arrest in other cell types have shown that the SV40 large T antigen can rescue the cells from the G1/G0 block. The rescue action of the large T antigen is attributed to its ability to displace the key proteins, particularly those of the E2F family, that are sequestered in the A/B pocket domain by hypophosphorylated RB, to sequester hypophosphorylated RB, and to trigger the phosphorylation of RB (35, 52). Our studies in the melanoma cells indicated that the large T antigen, but not the K1 mutant, which cannot bind to RB, could rescue the cells from the action of IL-1 or TNF-α. This observation suggested that hypophosphorylated RB, a target of the large T antigen, was likely to be involved in the action of these cytokines. However, because the large T antigen is known to bind to other key G1/G0 checkpoint control proteins, such as the RB-related proteins p107 and p130, the experiments with the large T antigen did not conclusively identify RB as a functional mediator of either IL-1 or TNF-α action. To definitively identify RB as a mediator of IL-1- or TNF-α-inducible growth arrest, we used dominant-negative mutants of RB. The SEA mutant selectively inhibits the function of RB, but not of the other key G1/G0 checkpoint control proteins, by interfering with the ability of RB to act as a molecular matchmaker and assemble protein complexes (51). Ectopic expression of SEA abrogated the ability of IL-1, but not of TNF-α, to cause growth arrest in the melanoma cells, implying that RB is an important mediator of IL-1-inducible growth arrest. However, because growth arrest by IL-1 was not completely blocked by SEA, we cannot exclude the possibility that other checkpoint control proteins are also involved in IL-1 action. By contrast, IL-1 causes growth stimulation in human fibroblast cells, WI-38, or human glioblastoma cells, U373-MG, and does not invoke hypophosphorylation of RB.2 The cell surface receptor, IL-1-receptor type I, is involved in both the growth inhibitory and growth stimulatory actions of IL-1 in different cell lines (22–24). The induction of RB hypophosphorylation in the melanoma cells, but not in the fibroblasts or glioblastoma cells, suggests that cell type-specific differences characterized by the signaling components that are located downstream of the receptor and that lead to the differential regulation of RB phosphorylation are responsible for the pleiotropic growth effects of IL-1. Further studies directed toward identifying the individual components of the signaling pathway responsible for hypophosphorylation of RB will help delineate the molecular basis of the pleiotropic growth effects of IL-1.

The mechanism(s) by which RB mediates growth arrest is an area of intense investigation. The dominant-negative mutants of RB disrupt the capacity of RB to form protein complexes, but they do not alter the hypophosphorylation status of RB (Ref. 51; this study), nor do they restore E2F transactivation function in transfectant cells (51), suggesting that rescue from hypophosphorylated RB-mediated growth inhibition can occur in the absence of functional E2F. Nevertheless, ectopic overexpression of one of the RB-target proteins, E2F-1, provides protection from the growth inhibitory action of hypophosphorylated RB (Ref. 46; this study). It is obvious, therefore, that the dominant-negative mutants of RB and ectopically overexpressed E2F-1 use different mechanisms to overcome the effect of hypophosphorylated RB. This hypothesis is being tested so as to elucidate the mechanism(s) by which IL-1-inducible accumulation of hypophosphorylated RB causes growth inhibition.

Although the biological actions of IL-1 and TNF-α are initiated by the binding of the cytokines to distinct cell surface receptors (22–25), these cytokines generally show overlapping biological activities and the induction of similar immediately early gene programs (13). However, by using the dominant-negative mutants of RB, the present study has revealed that the G1/G0 growth arresting action of IL-1 is dependent on the hypophosphorylation of the RB protein, but the action of TNF-α is independent of RB hypophosphorylation. Because E2F-1 overexpression rescues the cells from the action of TNF-α, an RB-related protein like p107 or p130 may possibly mediate the action of this cytokine. The ability of the dominant-negative mutants of RB to selectively overcome the action of IL-1, and not that of TNF-α, indicates the usefulness of these mutants in dissecting the growth inhibitory pathways regulated by RB and other related checkpoint control proteins.

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