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Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor β1

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ABSTRACT **One of the more intriguing aspects of transforming growth factor β1 (TGFβ1) is its ability to function as both a mitogenic factor for certain mesenchymal cells and a potent growth inhibitor of lymphoid, endothelial, and epithelial cells. Data are presented indicating that c-*myc* may play a pivotal role in both the mitogenic and antiproliferative actions of TGFβ1. In agreement with previous studies using C3H/10T½ fibroblasts constitutively expressing an exogenous c-*myc* cDNA, we show that AKR-2B fibroblasts expressing a chimeric estrogen-inducible form of c-*myc* (mycER) are able to form colonies in soft agar in the presence of TGFβ1 only when c-*myc* is activated by hormone. Whereas these findings support a synergistic role for c-*myc* in mitogenic responses to TGFβ1, we also find that c-*myc* can antagonize the growth-inhibitory response to TGFβ1. Mouse keratinocytes (BALB/MK), which are normally growth-arrested by TGFβ1, are rendered insensitive to the growth-inhibitory effects of TGFβ1 upon mycER activation. This ability of mycER activation to block TGFβ1-induced growth arrest was found to occur only when the fusion protein was induced with hormone in the early part of G_1. Addition of estradiol late in G_1 had no suppressive effect on TGFβ1-induced growth inhibition.**

Transforming growth factor β1 (TGFβ1), a multifunctional regulator of cell growth, differentiation, and morphogenesis, acts by interacting with a specific set of cell surface receptors, presumably followed by a series of molecular events leading to various effects on cells (1, 2). TGFβ1 has been shown to be stimulatory to the growth of certain mesenchymal cells in culture (3), while it is a potent inhibitor of growth of cells of lymphoid, endothelial, and epithelial origins both *in vitro* and *in vivo* (2, 4). Although the intracellular mechanisms leading to these pleiotropic effects of TGFβ1 remain poorly understood, evidence has suggested that the product of the c-*myc* protooncogene may function in both the mitogenic and antiproliferative effects of TGFβ1 (5–8).

TGFβ1 treatment of a mouse keratinocyte cell line (BALB/MK) results in the rapid downregulation of c-*myc* mRNA at the level of transcriptional initiation with a subsequent decrease of c-*myc*-encoded protein levels (7, 8). In a manner similar to that of TGFβ1, suppression of c-*myc* expression using antisense oligonucleotides has been shown to inhibit BALB/MK cells from entering S phase (7, 8). These observations taken together have led to the hypothesis that one mechanism by which TGFβ1 may act to inhibit BALB/MK cell growth is through suppression of c-*myc* expression.

Recent studies have implicated c-*myc* as a potential upstream positive regulator of cyclins A and E (9). The ability of these two cyclins to associate with and regulate the activity of cdk-2 (reviewed in ref. 10) is particularly interesting since this kinase has been implicated as a target of TGFβ1 growth-inhibitory signals in late G_1 (11). A possible link between c-*myc* and late G_1-phase targets of TGFβ1 growth-inhibitory signals led us to further investigate the importance of c-*myc* regulation by TGFβ1. To study c-*myc* effects, we have taken advantage of the system that utilizes the estrogen-inducible chimeric c-*myc*-encoded protein consisting of the coding region of human c-*myc* fused to the hormone-binding domain of the human estrogen receptor (mycER) (12). In support of a role for c-*myc* in growth suppression by TGFβ1, we find that mycER activation in BALB/MK keratinocytes is able to suppress the growth-inhibitory effect of TGFβ1. However, we also find that exogenous c-*myc* expression is required to allow TGFβ1 to induce anchorage-independent growth of AKR-2B fibroblasts in soft agar. This latter finding is in agreement with previous studies which showed that C3H/10T½ fibroblasts transfected with a c-*myc* cDNA are capable of anchorage-independent growth in soft agar when cultured in the presence of TGFβ1 (6). The results indicate that c-*myc* plays an important role in both the inhibitory and stimulatory responses to TGFβ1.

MATERIALS AND METHODS

Cell Culture. Noninfected BALB/MK cells were maintained in low calcium medium supplemented with 8% dialyzed fetal calf serum (JRH Biosciences) in 7% CO_2/93% air. The retroviral packaging cell lines ψ-2 and PA317 were grown in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum or fetal calf serum, respectively. Retroviral packaging cell lines producing infectious virus were maintained in phenol red-free DMEM containing the appropriate serum, which had been treated with charcoal to remove the estradiol (13). Retrovirally infected BALB/MK (MKmycER or MK-ΔmycER) cells were grown in 5% CO_2 in low calcium phenol red-free medium 154 (Cascade Biologics, Portland, OR) containing charcoal-treated, dialyzed fetal calf serum.

Transfections, Retroviral Infections, and Selection Procedure. The calcium phosphate coprecipitation method was used to transfect PA317 cells with 20 μg of plasmid pMV-7mycER (12) and ψ-2 cells with 20 μg of plasmid pMV-7ΔmycER (12). Medium containing recombinant mycER retrovirus produced by the PA317 cell line was transferred to ψ-2 cells and incubated at 37°C overnight in the presence of Polybrene (8 μg/ml). After infection with the mycER retrovirus or transfection with pMV-7ΔmycER, retrovirus-producing ψ-2 cells were selected for 10 days in phenol red-free medium containing G418 (400 μg/ml) (Geneticin, GIBCO). Medium from the ψ-2 cells containing ecotropic retroviruses was transferred to BALB/MK cells and incubated at 37°C in the presence of Polybrene (6 μg/ml) for 12 hr, after which time the infected BALB/MK cells were placed into phenol red-free medium

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Abbreviations: TGFβ1, transforming growth factor β1; EGF, epidermal growth factor.
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154. Twenty-four hours later G418 was added to the BALB/MK culture medium at a final concentration of 100 μg/ml and G418-resistant cells were selected for 10 days. G418-resistant colonies were pooled and, after 2 months of culture, MKmycER cells were discarded and replaced with liquid nitrogen stocks of MKmycER cells obtained from the initial selection procedure.

Soft Agar Assays. Retrovirally infected or noninfected AKR-2B cells were plated at 7500 cells per plate in soft agar made of DMEM minus phenol red containing 10% charcoal-stripped fetal calf serum. Soft agar plates contained TGFβ1 (R & D Systems) at the concentrations indicated and were untreated or treated with 2 μM estradiol throughout the experiment. Ten to 14 days after plating, colonies >750 μm were counted by an Omnicon colony counter (Bausch & Lomb).

Immunoprecipitations. Logarithmically growing MK and MKmycER cells (4×10^6 cells) were washed twice with phosphate-buffered saline (PBS; pH 7.4) and starved of methionine for 30 min in methionine-free DMEM (warmed to 37°C) and labeled with 250 μCi of [^{35}S]methionine per ml (1 Ci = 37 GBq) in fresh methionine-free DMEM for 10 min prior to lysis. Cells were washed twice with PBS and lysed in antibody lysis buffer [50 mM Tris·HCl, pH 8.0/50 mM NaCl/0.5% Triton X-100/0.5% deoxycholate/0.5% SDS/50 mM NaF/100 μM $NaVO_4$/75 μg of phenylmethylsulfonyl fluoride (PMSF) per ml/0.1 TIU (trypsin inhibitor units) of aprotinin per ml/1 μg of leupeptin per ml/8 mM iodoacetamide]. Anti-c-Myc polyclonal antibody 50-4 (gift of S. Hann, Vanderbilt University) was incubated on ice for 3 hr in the presence or absence of an approximately equal molar ratio of the immunogenic bacterially produced c-Myc protein (gift of S. Hann) as a block to show specificity of the antisera. The cell lysates were divided in half and, after the blocking step, each antibody preparation was added to the cell lysates and rocked overnight at 4°C. After immunoprecipitation with staphylococcal enterotoxin A, pellets were washed four times with RIPA buffer (50 mM Tris·HCl, pH 8.0/150 mM NaCl/1% Triton X-100/0.5% deoxycholate/0.1% SDS/50 mM NaF/100 μM $NaVO_4$/75 μg of PMSF per ml/0.1 TIU of aprotinin per ml/1 μg of leupeptin per ml/8 mM iodoacetamide) and separated by SDS/10% PAGE. Gels were enhanced, dried, and exposed to x-ray film for 5 days.

Cell Synchronization and Nuclear Labeling. MK, MKmycER, and MK-ΔmycER cells were grown to ≈70% confluency in 24-well plates in the presence of epidermal growth factor (EGF; 4 ng/ml) at which time the EGF was removed for 3 days to arrest cells at the G_0/G_1 boundary. Cells were then restimulated to enter the cell cycle by addition of EGF to the culture medium and parallel wells were treated with TGFβ1 (10 ng/ml), estradiol (2 μM), 4-hydroxytamoxifen (2 μM; gift of M. Seyfred, Vanderbilt University), TGFβ1 and estradiol, or TGFβ1 and 4-hydroxytamoxifen at the times indicated. At the time of restimulation with EGF, [^{3}H]thymidine (5 μCi/ml) was added to the wells and cells were cultured in the above conditions for 18 hr, after which time they were fixed with trichloroacetic acid and exposed to emulsion, and the percentage of nuclear labeling was determined. In each case, at least 1000 nuclei were counted in triplicate and averaged to generate the columns in the figures.

Kinetic Analyses. Cells were synchronized as described above and were either untreated or treated with TGFβ1, estradiol, or TGFβ1 and estradiol as described. At the times indicated, wells were pulsed with [^{3}H]thymidine (5 μCi/ml) for 1 hr and subsequently fixed with ascorbic acid followed by scintillation analysis of trichloroacetic acid-precipitable material.

RESULTS

***c-myc* Is Required for the Mitogenic Effects of TGFβ1.** A role for *c-myc* in the mitogenic activities of TGFβ1 derives from previous studies showing that overexpression of *c-myc* from a transfected cDNA can allow C3H/10T½ fibroblasts to form colonies in soft agar in the presence of TGFβ1 (6). In agreement with these findings, we found that AKR-2B fibroblasts infected with a recombinant retrovirus constitutively expressing an estrogen-inducible form of the c-Myc protein, mycER (12), were also able to form colonies in soft agar in the presence of TGFβ1 in a hormone-dependent manner (Fig. 1*A*). AKR-2B cells expressing mycER that were plated in soft agar in the presence of estradiol and in the absence of TGFβ1 did not form any colonies (data not shown), and uninfected AKR-2B cells did not form any visible colonies in soft agar with or without estrogen and/or TGFβ1 (Fig. 1*B*). These results show that neither *c-myc* nor TGFβ1 alone is sufficient to elicit a mitogenic response on these cells, but the synergistic effect of both suggests that *c-myc* overexpression may enhance the mitogenic effects of TGFβ1 or provide a cellular signaling pathway that TGFβ1 cannot activate. In addition, because we were able to reproduce effects seen in a system that utilized a nonchimeric *c-myc* construct (6), our data indicate that the mycER fusion protein can be used as a reliable tool for studying the effects of inducible *c-myc* overexpression in cultured cells.

mycER Induction Blocks TGFβ1 Inhibition. To circumvent the potential problems of toxicity and/or apoptosis produced by overexpressing an oncogene such as *c-myc* (14, 15), we infected BALB/MK cells with the recombinant retrovirus that expresses the estrogen-inducible mycER protein. Immunoprecipitation analysis verified that expression of the mycER protein was achieved (Fig. 2, compare lanes 1 and 3). The mycER fusion protein ran consistently at the predicted size of 105 kDa and could be immunoprecipitated with anti-c-Myc antibodies (Fig. 2) and anti-ER antibodies (data not shown). G418-resistant MKmycER cells were pooled and tested for their sensitivity to TGFβ1. After synchronization at the G_0/G_1 boundary by EGF deprivation, MKmycER cells were stimulated to enter G_1 by the addition of EGF to the cultures followed by the addition of TGFβ1, estrogen, a combination of TGFβ1 and estrogen, or EGF alone as a control. At the time of EGF addition, [^{3}H]thymidine was added to the medium and cells were cultured for 18 hr in the conditions described above, after which time the cells were fixed and the percentage of nuclear labeling was determined for each condition (Fig. 3*A*).

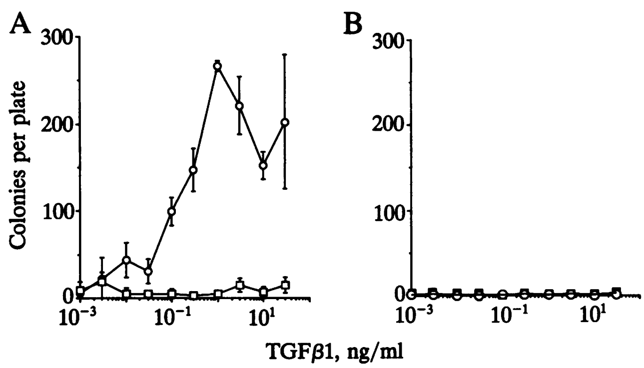

FIG. 1. Activation of mycER in AKR-2BmycER cells allows TGFβ1-induced growth of colonies in soft agar. Retrovirally infected (*A*) or noninfected (*B*) AKR-2B cells were plated at 7500 cells per ml in soft agar in the presence of increasing concentrations of TGFβ1 as indicated above. Plates were also treated with estradiol (○) or untreated (□) as described. Ten to 14 days after plating, the number of colonies >750 μm was determined. Shown are the means of colony counts done in triplicate ± 1 SD. Data are representative of two independent experiments.

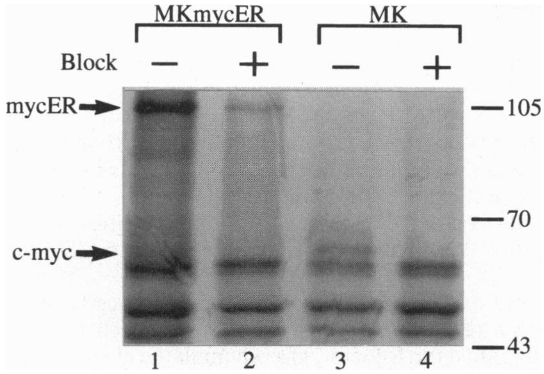

FIG. 2. Expression of the mycER fusion protein in mouse keratinocytes. Proliferating MKmycER cells (lanes 1 and 2) and MK cells (lanes 3 and 4) labeled with [^{35}S]methionine as described. Cell lysates were divided in half and incubated overnight with rabbit polyclonal anti-c-Myc antibody. Before the antiserum was added to the samples, it was incubated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of bacterially produced c-Myc protein as a block to show antibody specificity. Immunoprecipitates were separated by SDS/PAGE and the gel was enhanced, dried, and exposed to film. Numbers on the right indicate protein size standards (kDa) (GIBCO).

Cells that were unstimulated with EGF showed essentially no labeling of their nuclei (data not shown), indicating that they were unable to enter the cell cycle. TGFβ1 treatment of MKmycER cells in the absence of estrogen resulted in >70% inhibition of nuclear labeling, while estrogen treatment alone, which activates mycER, did not significantly affect the amount of nuclear labeling as compared to that of the control cells (EGF alone). However, in the presence of estrogen added early in G_1 the ability of TGFβ1 to inhibit nuclear labeling was diminished. Unexpectedly, estrogen addition late in G_1 showed no affect on the ability of TGFβ1 added either early or late in G_1 to inhibit the entry of MKmycER cells into S phase (Fig. 3B). Control MK cells lacking mycER did not respond to treatment with estrogen or 0.1% ethanol (final concentration of estrogen solvent), nor did estrogen affect the ability of TGFβ1 to inhibit the uninfected MK cells from entering S phase (data not shown).

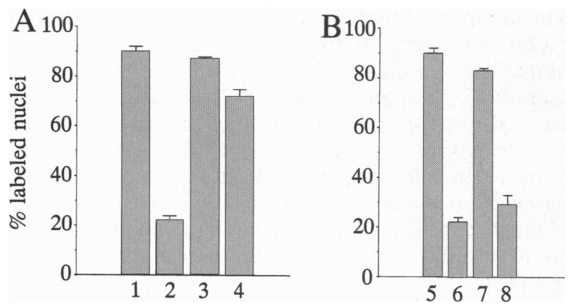

FIG. 3. Estrogen-dependent loss of TGFβ1 sensitivity in MKmycER cells. MKmycER cells were arrested for 3 days in medium lacking EGF as described. The medium was changed and EGF was added to restimulate the cells to enter the cell cycle. (A) At the time of EGF restimulation, TGFβ1, estradiol, or TGFβ1 and estradiol were added to the cultures. Cells were cultured for 18 hr in the above conditions in the presence of [^{3}H]thymidine, after which time they were fixed in ascorbic acid and exposed to emulsion, and the percentage of nuclear labeling was determined. Columns: 1, EGF; 2, EGF + TGFβ1; 3, EGF + estradiol; 4, EGF + estradiol + TGFβ1. (B) Cells were arrested and restimulated with EGF in the presence of [^{3}H]thymidine as above. Ten hours after EGF addition, estrogen, TGFβ1, or estrogen and TGFβ1 were added to the culture medium. Cells were cultured for 8 hr more, after which time the cells were fixed and nuclear labeling was determined as described. Columns: 5, EGF; 6, EGF + TGFβ1; 7, EGF + estrogen; 8, EGF + estrogen + TGFβ1. Each column represents the mean of three independent counts of at least 1000 nuclei ± 1 SD.

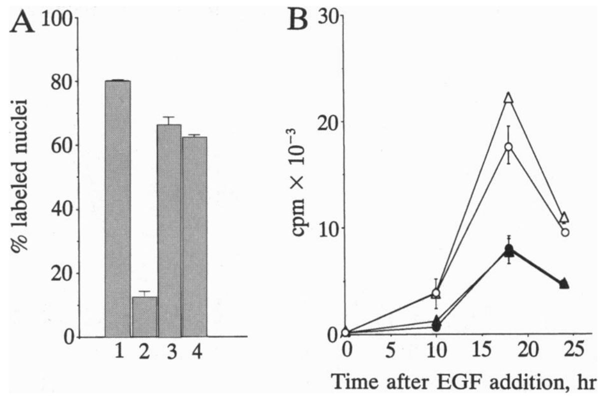

FIG. 4. Evidence that the ER domain of mycER does not contribute to the effects of the fusion protein in MK cells. (A) MKmycER cells were synchronized as described. EGF-restimulated cells were treated with TGFβ1 (column 2) or 4-hydroxytamoxifen (column 4) or estradiol (column 3) in the presence of TGFβ1 or with EGF alone (column 1). [^{3}H]Thymidine was added at the time of EGF restimulation, and, after 18 hr of culture under the above conditions, cells were subjected to autoradiography and percentage of nuclear labeling was determined. Columns show averages of three independent counts of at least 1000 nuclei ± 1 SD. (B) MKΔmycER cells were cultured, synchronized, and treated with TGFβ1 and estradiol at the time of EGF addition as described. Cell cultures were pulsed for 1 hr with [^{3}H]thymidine at the times indicated, after which time the cells were fixed and trichloroacetic acid-precipitable material was analyzed by scintillation counting. Means of triplicate cultures ± 1 SD are shown. ○, EGF control; ●, TGFβ1; △, estradiol; ▲, TGFβ1 + estradiol.

As a control for contributions of the estrogen-binding domain (ER) to the above observations, the estrogen antagonist 4-hydroxytamoxifen was used in place of estrogen. 4-Hydroxytamoxifen had an effect on TGFβ1-induced inhibition of MKmycER growth similar to that observed with estrogen treatment (Fig. 4A). Based on studies of both chimeric and native estrogen receptor proteins (16–18), these results indicate that the suppressive effects of mycER activation on TGFβ1-induced growth arrest are due to functions arising from the Myc domain and that the ER domain does not contribute to these effects. In further support of this, kinetic data and nuclear labeling experiments (data not shown) showed that MK cells expressing ΔmycER (12, 14) were not responsive to estrogen treatment and remained sensitive to TGFβ1 (Fig. 4B). The ΔmycER protein has a deletion of amino acids 106–143 in the putative transactivating domain of c-Myc, rendering it inactive in a variety of c-Myc-related activities (14, 19–21). Since this mutant of c-Myc is unable to block TGFβ1-induced inhibition, we hypothesize that the transactivation function of c-Myc contributes to the mechanism by which c-Myc blocks the antiproliferative actions of TGFβ1.

Effects of mycER Activation on Cell Cycle Kinetics. Studies using Rat1a cells expressing the mycER protein have shown that activation of the fusion protein is sufficient to allow these cells to form soft agar colonies or to allow monolayer cultures to enter the cell cycle under low serum conditions (9, 12, 22). In contrast to these observations, we found that mycER activation alone was not sufficient to allow the AKR-2B fibroblast cell line to form colonies in soft agar (Fig. 1). In addition, we found that mycER activation was not able to stimulate EGF-deprived BALB/MK cells to enter S phase (Fig. 5A). From these results, it is evident that induction of mycER is not sufficient for all cell types to escape from a G_0 arrest or elicit a transformed phenotype. BALB/MK cells, unlike Rat1a cells, require more than c-*myc* overexpression alone to proceed through G_1 into S phase. In addition, mycER induction did not shorten the duration of G_1 phase in MKmycER cells (Fig. 5B), an effect previously observed with

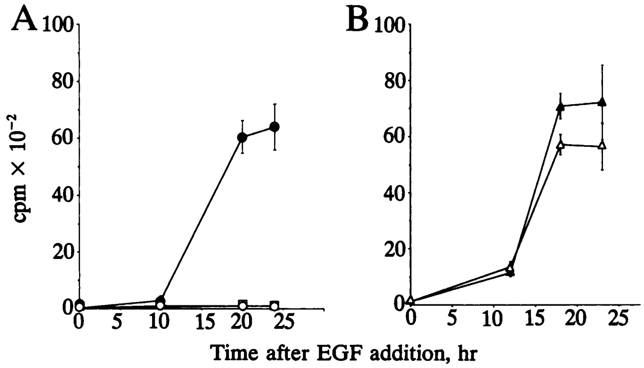

FIG. 5. Effects of mycER induction on EGF deprivation and length of G_1 in BALB/MK cells. MKmycER cells were synchronized and subjected to kinetic analyses as described in the legend to Fig. 4*B*. (*A*) MKmycER cells were restimulated with medium containing EGF (●) or given medium without EGF in the presence (○) or absence (□) of estradiol. (*B*) MKmycER cells were restimulated with EGF in the presence (▲) or absence (△) of estradiol. Points on the curves are means of scintillation counts of triplicate wells ± 1 SD. Data shown are representative of at least three experiments, each done in triplicate.

overexpression of c-*myc* (23) and certain G_1 cyclins in other cell types (24–26).

DISCUSSION

At least three reports have claimed that c-*myc* overexpression is unable to block the growth-inhibitory effects of TGFβ1 (27–29). In all three of these studies, however, overexpression of c-*myc*-encoded protein was not shown and only two presented data showing that exogenous c-*myc* mRNA was being produced (27, 28). The clonal selection procedures used these investigators may have actually selected for populations of cells that were deficient in overexpression of c-*myc*-encoded protein or had acquired unwanted genetic alterations that circumvented the effects of c-*myc* overexpression. Such a hypothesis is supported by data indicating that under certain circumstances cells overexpressing c-*myc* will go through apoptotic cell death unless they acquire other genetic alterations, such as *bcl-2* overexpression, that allow them to survive in culture (14, 15, 30, 31). Our experimental approach differed from those described above in that we used a form of c-*myc* that was expressed constitutively in our cells in a suppressed state. This unique feature of the mycER fusion protein prevented potential cytotoxicity of functional c-*myc* during the selection procedures and subsequent culturing, while it allowed temporal induction of c-*myc* function at the posttranslational, rather than the transcriptional, level when desired.

Coincident with a G_1 growth arrest, TGFβ1 treatment of certain cell types has been shown to rapidly decrease the levels of c-*myc* RNA and protein (7, 8). Because c-*myc* is required for progression of cells through G_1 (7, 8, 32), it has been suggested that the c-*myc* gene product may be an important target of the antiproliferative signals induced by TGFβ1 (7, 8). In further support of this hypothesis, the experiments described in this report indicate that induction of c-Myc protein, at least in the early part of G_1, in BALB/MK cells can prevent TGFβ1 from inducing a state of growth arrest. Unexpectedly, induction of c-*myc* in late G_1 does not suppress the ability of TGFβ1 to inhibit the keratinocytes. Since we have not yet determined the mechanism by which c-*myc* acts to block TGFβ1 at certain times in G_1 and not at other times; we can only speculate as to why there is a temporal dependency on the ability of c-*myc* to affect the antiproliferative actions of TGFβ1.

Considering the recent evidence suggesting the possible involvement of downregulation or inactivation of cyclins A and E (33), cdk-2 (11), and cdk-4 (34) in the TGFβ1 signaling pathway, one possibility is that these cyclins and kinases could be downstream targets of c-*myc*. In support of this hypothesis are data showing that c-*myc* may upregulate the expression of cyclins A and E (9). These findings would suggest that mycER activation early in G_1 may be blocking TGFβ1-induced growth inhibition by increasing the activity of TGFβ1-sensitive cdks that are active in the latter half of G_1. In a similar manner, c-*myc* could affect the activities of the cdks by directly or indirectly downregulating the levels or activities of negative regulators of the cdks such as p21 (35–37), p16 (38), or Kip-1 (39).

Kinetic experiments have suggested that cells must pass through a restriction point in late G_1, at which time a labile protein, called the R factor, has accumulated above a threshold necessary for progression of the cells into S phase (40–43). Cyclins A and E have been shown to satisfy the criteria for the R protein and have led to the hypothesis that cyclin production and inactivation may form the molecular basis of the R factor (41). It appears from preliminary experiments that a block to TGFβ1-induced growth arrest can be exerted by c-*myc* induction as late as 9 hr into G_1 (M.G.A. and H.L.M., unpublished data), which correlates approximately with the putative restriction point (42). This result makes it interesting to speculate that induction of c-*myc* prior to the R point may upregulate the expression or associated activity of cyclins A and E and create an environment in the cell that is unable to be negatively regulated by TGFβ1. Thus, the window of time during which c-*myc* can elicit an effect on these cell cycle-regulated proteins may be limited to the part of G_1 when these proteins normally must accumulate above a necessary threshold for cell cycle progression. After the cells have passed the restriction point, c-*myc* may no longer have the ability to regulate the expression or activity of the cyclins and associated kinases. Alternatively, if c-*myc* retains the ability to regulate these factors after the restriction point, then perhaps TGFβ1 may be causing irreversible changes to the cell that cannot be overcome simply by induction of c-*myc*.

Although the actual function of c-*myc* remains elusive, it is believed that c-*myc* function is required in the early half of G_1 because levels of c-*myc* RNA and protein peak at this point in the cell cycle (44, 45). However, there is evidence which suggests that c-*myc* may also function in the latter half of G_1 and perhaps during S phase (7, 46, 47). Although controversial, it has been reported that the c-Myc protein has been detected in several cell types in high molecular weight complexes containing DNA polymerase α and several other enzymes necessary for DNA replication (47). These data might suggest a functional role for the c-Myc protein in late-G_1 complexing prior to the onset of S phase as well as during the replicative phase itself. In addition, other studies have found that in *Xenopus laevis* oocytes c-*myc* exists as a massive maternal store of stabilized, cytoplasmic protein which is triggered by fertilization to rapidly migrate into the nuclei of the cleaving embryo (46). The authors suggested from these observations that the maternal store of c-*myc* may be involved in controlling the rate of DNA replication during early development of *X. laevis*. What is more interesting about this phenomenon is that there is no significant transcription during these early cleavages until the midblastula transition (48, 49). In this respect, the *Xenopus* studies also suggested that c-*myc* could potentially have a nontranscriptional function in the cell cycle. Whether this nontranscriptional function is required in the latter part of G_1 or in S phase remains to be determined.

If c-*myc* were to have a late G_1 function that is targeted by TGFβ1-induced negative signals, then one would predict that induction of mycER late in G_1 should supply the cell with enough c-Myc protein to counter the effects of TGFβ1. To reconcile this prediction with the observation that c-*myc* induction late in G_1 does not block TGFβ1, we propose that a period of G_1 may exist during which c-*myc* must be present or must accumulate to allow for proper timing of its interactions with proteins required for progression into S phase. In this respect, c-*myc* could be a candidate for the R factor. The labile nature of c-*myc* (50) and evidence that c-*myc* is constitutively expressed in two chemically transformed cell types (51), features that were originally proposed to be characteristic of the putative R factor (40, 42, 43), also support such a hypothesis. The presence of excess exogenous c-*myc* up to the restriction point may prevent TGFβ1 from blocking cell cycle progression by increasing the abundance of active replication complexes. However, after the restriction point, TGFβ1 may irreversibly block complexing involving endogenous or exogenous c-*myc* and induction of c-*myc* would no longer have an effect on the ability of TGFβ1 to induce growth arrest.

In certain cell types, TGFβ1 has the potential to stimulate growth given that the environment of the cell is appropriate for such an event (6). It appears that c-*myc* creates an environment in AKR-2B and C3H/10T½ cells (6) that allows TGFβ1 to be mitogenic, perhaps by providing a signal that is absent, but nonetheless required, for anchorage-independent proliferation of the cells. Recently it has been shown that overexpression of cyclin A in NRK and NIH 3T3 fibroblast cell lines can cause proliferation in the absence of cell adhesion (52). Because c-*myc* has been implicated in controlling the expression of cyclin A (9), it is possible that c-*myc* induction in the AKR-2B cells could result in cyclin A expression that, combined with stimulation by TGFβ1, allows these fibroblasts to form colonies in soft agar. Although the mechanisms through which c-*myc* affects TGFβ1 function remain unknown, the data presented in this report provide compelling evidence that the product of the c-*myc* protooncogene plays a mechanistic role in both the stimulatory and antiproliferative actions of TGFβ1.

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