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A Novel Inhibitor of Cyclin-Cdk Activity Detected in Transforming Growth Factor β-Arrested Epithelial Cells

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Transforming growth factor β (TGF-β) is a potent inhibitor of epithelial cell growth. Cyclins E and A in association with Cdk2 have been shown to play a role in the G_1-to-S phase transition in mammalian cells. We have studied the effects of TGF-β-mediated growth arrest on G_1/S cyclins E and A. Inhibition of cyclin A-associated kinase by TGF-β is primarily due to a decrease in cyclin A mRNA and protein. By contrast, while TGF-β inhibits accumulation of cyclin E mRNA, the reduction in cyclin E protein is minimal. Instead, we find that the activation of cyclin E-associated kinase that normally accompanies the G_1-to-S phase transition is inhibited. A novel inhibitor of cyclin-Cdk complexes was detected in TGF-β-treated cell lysates. Inhibition is mediated by a heat-stable protein that targets both Cdk2 and Cdc2 kinases. In G_0-arrested cells, a similar inhibitor of Cdk2 kinase was detected. These data suggest the existence of an inhibitor of cyclin-dependent kinases induced under different conditions to mediate antiproliferative responses.

Control of the eukaryotic cell cycle occurs at key points in G_1 and at the G_2-to-M phase transition (41, 80, 85, 121, 122). These transitions are governed by multimeric protein complexes with serine/threonine kinase activity, whose catalytic subunits, proteins of 33 to 34 kDa, are regulated by phosphorylation and by periodic association with positive effector proteins, known as cyclins. Current understanding of cell cycle control derives largely from studies of yeast cells. The Cdc28 kinase in the budding yeast *Saccharomyces cerevisiae* and its homolog in the fission yeast *Schizosaccharomyces pombe*, Cdc2, appear to regulate both the G_1-to-S and G_2-to-M phase transitions (5, 81, 88, 93, 95). In *S. cerevisiae*, association of $p34^{Cdc28}$ with G1 cyclins, or Clns, is necessary for entry to S phase (12, 38, 77, 98, 115), while association with the mitotic or B-type cyclins, the Clbs, promotes entry into mitosis (30, 108). The first human homolog of $p34^{Cdc2/Cdc28}$ to be identified, Cdc2Hs, could complement p34 deficiency in both *S. cerevisiae* and *S. pombe* (16, 61). However, its action appears to be restricted to the G_2-to-M phase transition in humans (15, 89, 97, 110).

A number of Cdc2-related or cyclin-dependent kinases (Cdks) have subsequently been identified (23, 51, 61, 74, 78, 86, 102, 112). These kinases combine with different cyclins to govern different cell cycle transitions. One of these, Cdk2, appears to regulate G_1- and S-phase functions in human cells (62, 102, 113). The action of Cdk2 in complex with cyclin A is essential for progression through S phase and may play a role in the regulation of DNA synthesis (25, 32, 33, 83, 90, 123).

Several candidate G_1 cyclins, designated C, D, and E, were identified when human and *Drosophila* cDNA libraries were screened for sequences that could complement deletion of the *S. cerevisiae* Cln genes (51, 63, 65, 117). A cyclin D cDNA was also identified as corresponding to a gene whose G_1-specific expression is induced by colony-stimulating factor in murine macrophages (70). Overexpression of cyclin D1 has also been implicated in a number of human malignancies (46, 59, 75, 100). In addition to their ability to perform G_1 functions in yeast cells, a number of other recent observations support a role for cyclins D1 and E in the G_1-to-S phase transition. The activity of cyclin E-associated kinase, the level of cyclin E mRNA and protein, and the association of cyclin E with its catalytic partner, Cdk2, all rise in late G_1, peak at the G_1/S phase boundary, and decrease as cells progress into S phase (19, 52, 62). Overexpression of cyclins D1 and E in mammalian cells can accelerate progression through G_1 (82, 92, 96). Finally, microinjection of cyclin D1 antibodies blocks passage of fibroblasts from G_1 to S phase (3).

In *S. cerevisiae*, exposure to the mating pheromone, α-factor, induces G_1 arrest through the inactivation of the Cdc28 kinase at a G_1 restriction point called START (7, 40, 41). Inactivation of the kinase by mating pheromone appears to involve the binding of an inhibitory protein, Far1 (9, 87). In mammalian cells, commitment to a new round of DNA synthesis occurs at a similar point in G1 known as the restriction point (84, 85). Inhibition of protein synthesis or transfer to 1% serum in early G_1 prior to the restriction point prevents cell cycle progression and causes cells to enter into a quiescent state, G_0. After passing the restriction point, a cell is committed to a replication cycle and nutrient deprivation fails to prevent the transition to S phase (84, 121). Because of this apparent parallel between G_1 control in yeast and animal cells, it is of interest to determine whether mitogenic and growth-inhibitory signals in mammalian cells are mediated at the level of cyclins and cyclin-dependent kinases, as they are in yeast cells.

Transforming growth factor β (TGF-β) is a family of paracrine growth factors that mediate effects on cellular growth, cell-cell adhesion, and differentiation (66). In particular, TGF-β is a potent growth inhibitor of epithelial cells, lymphohemopoietic cells, and certain types of neuroectodermal cells. The mink lung epithelial cell line Mv1Lu is exquisitely sensitive to TGF-β; picomolar amounts can induce the G_1 arrest of asynchronously growing cells within a single cell cycle (57).

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This line can be rendered quiescent by contact inhibition when cells are grown to confluence. When cells are then replated at low density, they undergo parasynchronous reentry into the cell cycle. Exposure to TGF-β at any point up to 6 h from release of contact inhibition causes an arrest or delay of the cell cycle in late G_1.

Given the growing experimental evidence that cyclins E and A and their associated kinase Cdk2 play essential and positive roles in the G_1-to-S phase transition, the possibility that TGF-β might act through regulation of these kinases posed a compelling question. The present studies were undertaken to determine if TGF-β modulates the expression or activity of the G_1- and S-phase cyclins E and A to effect G_1 arrest. In the course of this work, we learned of another study that found that TGF-β inhibits the activation of cyclin E-Cdk2 kinase complexes (53). Our studies confirm this earlier result and expand on this observation. We have found that TGF-β regulates cyclin and Cdk activity at multiple levels, including the induction of a novel inhibitor of cyclin and Cdk activity. A similar study characterizing this Cdk inhibitor was published during revision of this report (91).

MATERIALS AND METHODS

Cell culture. Mv1Lu cells (CCL-64; American Type Culture Collection) were grown in alpha minimal essential medium supplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine, 100 mg of penicillin per ml, and 100 mg of streptomycin per ml. The cells were passaged every 2 to 3 days to maintain their exponential growth. Cultures were growth arrested by contact inhibition by allowing cultures to remain confluent for 72 h. Release from contact inhibition was achieved by trypsinizing cells and replating them at 2×10^6 to 2.5×10^6 cells per 150-mm-diameter dish. Cells were incubated in the presence of TGF-β1 (R&D) at 2 to 10 ng/ml for the times indicated in the figures.

Human mammary epithelial strains were grown in supplemented MCDB 170 medium (Clonetics Corporation) as described previously (106). Cultures were growth arrested by exposure to medium without epidermal growth factor (EGF), containing 5 μg of anti-EGF receptor monoclonal antibody (MAb) 225 per ml. Cells were restimulated to enter the cell cycle by being washed once with phosphate-buffered saline (PBS) and refed with medium containing 25 ng of EGF per ml as described elsewhere (107). TGF-β-exposed cells were fed with medium containing 5 ng of human recombinant TGF-β1 (gift of Genentech Incorporated) per ml.

Antisera. Preparation of rabbit polyclonal human cyclin E sera is described elsewhere (19). Anti-cyclin E MAbs E172 and E12 were provided by E. Lees and E. Harlow (Massachusetts General Hospital, Charlestown, Mass.). Anti-cyclin A MAb E67 was the gift of Julian Gannon and Tim Hunt. Other antibodies and their sources are as follows: rabbit polyclonal anti-human Cdk2, Upstate Biotechnology, Inc. (22); rabbit polyclonal anti-cyclin A serum (89), T. Hunter (Salk Institute, La Jolla, Calif.); rabbit antiserum to Cdc2 (COOH terminal), C. McGowan and P. Russell (Scripps Research Institute, La Jolla, Calif.); and mouse MAb to PSTAIRE, M. Yamashida and Y. Nagahama (Okazaki, Japan) (119). Preimmune sera were obtained from rabbits before immunization. MAb 225 to the EGF receptor was the gift of John Mendelsohn, Sloan Kettering Institute, New York, N.Y.

Flow cytometric analysis. Cells were trypsinized, washed with complete medium, and centrifuged at 1,000 rpm for 10 min. The medium was aspirated, and bare nuclei were prepared for DNA analysis by resuspension (10^6 cells per ml) in a solution of 0.1% Nonidet P-40, 0.1% sodium citrate, and 50 μg of propidium iodide per ml. Cells were incubated at 4°C in the dark for 4 to 36 h prior to DNA analysis by fluorescence-activated cell sorting (FACS), using a FACScan analyzer (Becton Dickinson).

Determination of labeling index. For autoradiographic analysis, human mammary epithelial cells (Fig. 2A) were exposed to 2-h pulses of 10 μCi of [^{3}H]thymidine per ml in MCDB 170 containing 3×10^{-7} M unlabeled thymidine. The cells were fixed in 100% methanol and processed for autoradiography by coating with NTB-2 nuclear track emulsion. The percentage of labeled nuclei was determined by counting over 400 cells per point from three different microscope fields.

Immunoblotting. Cells were lysed in modified radioimmunoprecipitation assay buffer (1% sodium deoxycholate–1% Triton X-100–0.01% sodium dodecyl sulfate [SDS]–150 mM NaCl–50 mM Tris [pH 7.5]–0.5 mM EDTA containing 50 mM NaF, 10 mM $NaPP_i$, 0.5 mM orthovanadate, and protease inhibitors [1 mM phenylmethylsulfonyl fluoride {PMSF} and 0.02 mg each of aprotinin, leupepsin, and pepstatin per ml]). Lysate protein was quantitated by Bradford analysis, and 50 to 100 μg of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% gels were used for cyclin E blots) (56). The transfer and blotting were done as described previously (19). Polyclonal rabbit anti-cyclin E serum was affinity purified and used at a 1:200 dilution on blots prepared from Mv1Lu cells. Anti-cyclin E MAb E12 was used at a concentration of approximately 5 μg/ml to detect human cyclin E.

Immunoprecipitation. Immunoprecipitations were performed on lysates containing 50 to 100 μg of protein, and complexes were adsorbed onto protein A-Sepharose beads. The depleted lysate was subjected to a second immunoprecipitation with polyclonal serum (anti-cyclin E, anti-cyclin A, or anti-Cdk2), and the precipitates were pooled. For both cyclins E and A, this was required to recover >90% of total cyclin-associated H1 kinase activity. The remaining steps of the histone kinase assays were as described previously (19). Whole rabbit serum and protein A beads were used as background controls. Background was less than 10% of the count on test samples and was subtracted before plotting the results.

To detect the presence of Cdk2 in anti-cyclin E immunoprecipitates, complexes were resolved by SDS-PAGE (11% gel) run at 60 mA (constant current) on 26-cm slab gels, proteins were transferred onto Immobilon, and the immunoblot was reacted with anti-PSTAIRE antibody at a dilution of 1:2,000. For detection of Cdk2 bound to human cyclin E in human cellular lysates, cyclin E was precipitated with anti-cyclin E MAb E172, and the immunoblotted protein was reacted with the Cdk2-specific anti-Cdk2 antibody (22).

Inhibitor assay. Lysates were prepared in buffer containing 0.1% Nonidet P-40, 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM PMSF, and 0.02 mg each of aprotinin, leupepsin, and pepstatin per ml. Following Bradford quantitation, lysates were either stored at −70°C or used immediately. Lysates of cells treated for various times with TGF-β, contact-inhibited Mv1Lu cells or EGF-starved human breast epithelial cells were mixed with lysates extracted from cells at the G_1-to-S phase transition, brought to 1 mM dithiothreitol, and incubated at 30°C for 15 min (unless otherwise indicated in the figure legends), and then cyclin or Cdk was immunoprecipitated. The histone H1 kinase activity of the immunoprecipitate was then assayed. Control immunoprecipitates from G_0/G_1-arrested cell lysate or active G_1/S lysate were assayed for H1 activity in parallel. All mixing assays were performed in antibody excess at least twice with different lysates to confirm results. Inhibition of H1 kinase

activity could also be detected when the cyclin or Cdk was immunoprecipitated from G_1/S-phase lysates, adsorbed onto protein A-Sepharose beads, washed once with lysis buffer, then mixed with TGF-β- or G_0-arrested lysate, and incubated for 15 min at 30°C.

Metabolic labeling and immunoprecipitation. For $^{32}P_i$ labeling, 3×10^6 contact-inhibited cells were plated on 150-mm-diameter dishes. At 14 h from release of contact inhibition, cells were washed twice with PBS, preincubated for 30 min with 10 ml of Dulbecco modified Eagle medium without phosphate, and then incubated for 2 h in 3 ml of phosphate-minus medium to which was added 1 mCi of $^{32}P_i$ (Amersham). Cells were washed twice with PBS and then lysed in 300 μl of the same lysis buffer as was used in inhibitor assays. Lysates were precleared with 5 μg of polyclonal immunoglobulin G2a and 50 μl of a 10% suspension of protein A-Sepharose. Immunoprecipitations were carried out with either 5 μg of polyclonal anti-Cdk2 antibody, 1 μg of anti-cyclin A MAb, or 1 μg of immunoglobulin G2a as negative control. Cellular lysate from one labeled p150 plate was used for each immunoprecipitation. Control plates of cells released from contact inhibition in the presence of TGF-β were also labeled, and lysates were reacted with anti-cyclin A antibody.

RNA extraction and Northern (RNA) blots. Total cellular RNA was extracted by the guanidine isothiocyanate-cesium chloride gradient method (10) and subjected to electrophoresis in 1.3% agarose–formaldehyde gels. RNA was blotted, and blots were hybridized with ^{32}P-labeled cDNA fragments and processed as described previously (120). Probes used were as follows: for cyclin E, a 1.2-kb *Hin*dIII fragment from human cDNA; and for cyclin A, a 322-bp fragment from human cDNA (65). Probes were labeled by using a random hexamer kit (Boehringer Mannheim) as instructed by the manufacturer.

RESULTS

TGF-β inhibits cyclin E- and cyclin A-associated kinases. To determine whether TGF-β-mediated G_1 arrest correlated with changes in cyclin E- and A-associated kinase activities, mink lung epithelial cells were growth arrested by contact inhibition and replated sparsely. Cells were either treated with TGF-β or not and were harvested at different times following release from quiescence. DNA content of recovered cells was assayed by propidium iodide staining and FACS analysis. Following release from contact inhibition, 60 to 70% of the cells exit G_0 and undergo parasynchronous reentry into the cell cycle. In the presence of TGF-β, however, cells arrest prior to S phase. Histone H1 kinase assays were performed on anti-cyclin E and anti-cyclin A immune complexes prepared from cell lysates recovered at successive times following release from contact inhibition. In the Mv1Lu cells, the activity of cyclin E-associated kinase rose in late G_1 phase, peaked at the G_1/S phase boundary, and declined prior to the peak in cyclin A-associated kinase in S phase (Fig. 1A). Both cyclin E- and cyclin A-associated kinases were strongly inhibited in the presence of TGF-β (Fig. 1A), consistent with a role for these kinases as targets of the antiproliferative signal.

To extend our observations to a different cell type and to ensure that the regulation of cyclin-associated kinase activities seen in the Mv1Lu cell line was not an artifact of immortalization, we have examined a nonimmortal human breast epithelial cell strain. The human mammary epithelial cell strain 184SK was derived from normal breast tissue as described previously (106). These cells can be G_0 arrested by incubation for 48 h with an antibody specific for the EGF receptor and restimulated to enter the cell cycle by subsequent removal of

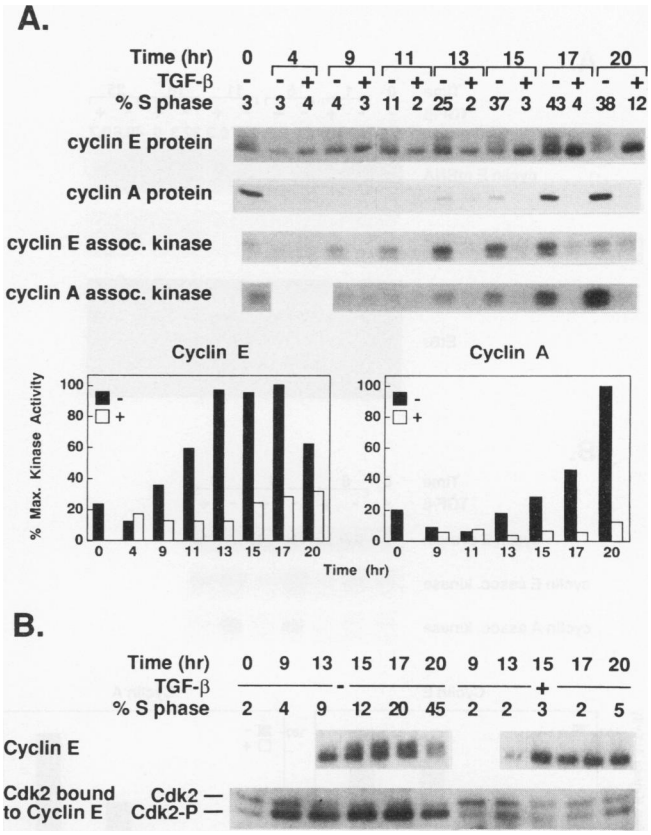

FIG. 1. Effects of TGF-β on expression and associated kinase activities of cyclins E and A following release from quiescence. (A) Mv1Lu cells were released from contact inhibition and grown for the intervals indicated in the presence (+) or absence (−) of TGF-β. The percentage of cells in S phase was determined by FACS analysis as described in Materials and Methods. The profile of cyclin E and cyclin A protein expression during the G_0-to-S phase transition and in TGF-β-blocked cells was assayed by Western analysis. The histone H1 kinase activity in anti-cyclin E and anti-cyclin A immunoprecipitates was assayed in aliquots of lysate containing 200 μg of protein. Quantitation of kinase activity was obtained by scintillation counting of excised histone H1 bands. (B) In a second experiment, Mv1Lu cells were released from contact inhibition and replated in the presence (+) or absence (−) of TGF-β. The percentage of S phase cells at different time intervals was determined as for panel A. Whole cell lysates were immunoblotted and reacted with anti-cyclin E polyclonal serum. To detect Cdk2 bound to cyclin E, anti-cyclin E immune complexes were recovered from 300 μg of protein lysate, resolved by SDS-PAGE (11% gel), and immunoblotted, and the blot was probed with anti-PSTAIRE antibody. The mobilities of hypophosphorylated Cdk2 (Cdk2) and the faster-migrating phosphorylated Cdk2 (Cdk2-P) are indicated.

antibodies and incubation with EGF (107). As with Mv1Lu cells, when these cells were restimulated with EGF in the presence of TGF-β, entrance to S phase was inhibited. Human breast epithelial cells were assayed for cyclin E-associated protein kinase activity at different intervals after release from G_0. At 14 h following restimulation, the population was predominantly at the G_1/S phase boundary (Fig. 2A) (107). Cyclin E- and cyclin A-associated kinases in the breast cell strain showed the same pattern of activation and TGF-β inhibition as in the Mv1Lu cell line (Fig. 2B).

Cyclin mRNA and protein in TGF-β-treated cells. Having ascertained that TGF-β inhibits the activities of cyclin E- and cyclin A-associated kinases, we wished to determine whether

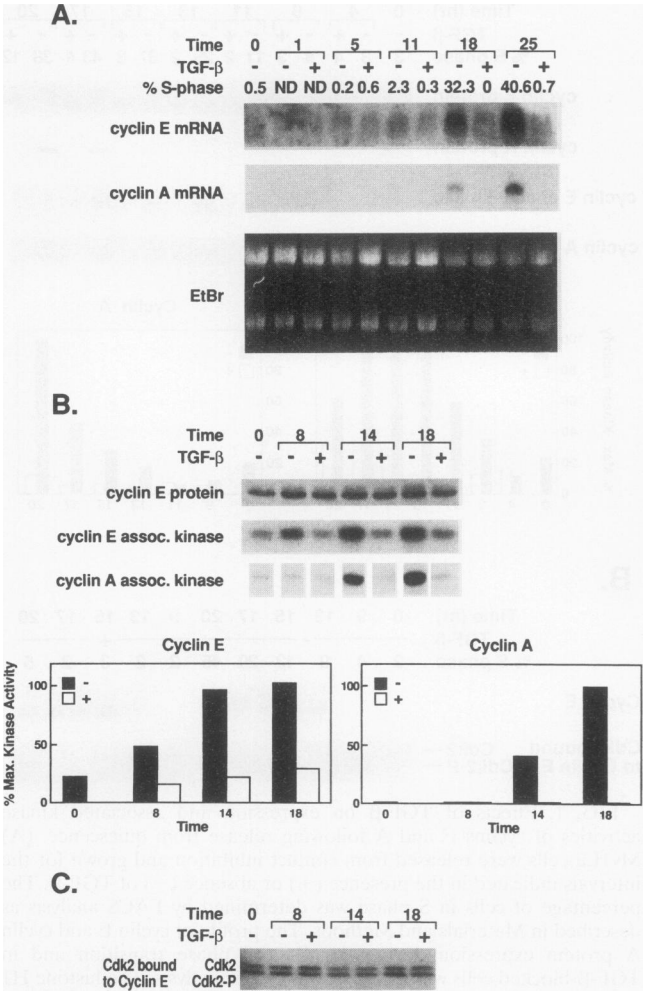

FIG. 2. Effects of TGF-β on cyclin expression and associated kinase activity in human breast epithelial cells restimulated after EGF starvation. (A) The human breast epithelial strain 184SK was grown to midconfluence and then exposed to anti-EGF receptor MAb 225 (5 μg/ml) for 48 h. Cells were then washed and refed with complete medium containing 25 ng of EGF per ml with (+) or without (−) TGF-β and grown for the indicated times. Cells were harvested for RNA extraction, and 10 μg of total cellular RNA from each time point was fractionated on a 1.3% agarose–formaldehyde gel and transferred to nylon filter. The filter was sequentially probed with cDNAs to human cyclin E and cyclin A. Total RNA in the original gel was stained with ethidium bromide (EtBr). The percentage of cells in S phase at each time point was determined by labeling index (see Materials and Methods). (B) Cells were grown to midconfluence and EGF starved as described for panel A. Cells were restimulated with EGF, with (+) or without (−) TGF-β, and lysates were prepared at the indicated intervals. Cells were lysed, and 100 μg of protein was resolved by SDS-PAGE (7.5% gel). Immunoblotting was performed with anti-cyclin E MAb E12 as described in Materials and Methods. Anti-cyclin E MAb E172 was used to precipitate cyclin E from 200 μg of protein lysate, and the complexes were assayed for histone H1 kinase activity. Cyclin A immune complexes were recovered from 50 μg of protein lysate at each time point, using anti-cyclin A MAb E67, and H1 kinase activity was assayed. Radioactivity in the histone bands was quantitated by scintillation counting and plotted as a function of time. (C) Cyclin E immune complexes prepared as described for panel B were separated by SDS-PAGE, and Cdk2 isoforms (designated as for Fig. 1) were detected by immunoblotting using anti-Cdk2 antibody.

the inhibition was due to loss of cyclin expression or rather due to posttranslational regulation of these proteins. Northern blot analysis was performed in order to examine levels of cyclin E and A mRNAs in the presence or absence of TGF-β (Fig. 2A). In the 184SK cells, cyclin E mRNA was expressed at a low but detectable level in G_0 cells and in cells early in G_1. As cells approached the G_1/S boundary, cyclin E message increased dramatically. Cyclin A mRNA was undetectable in arrested cells, and its rise late in G_1 occurred subsequent to that of cyclin E mRNA. The accumulation of both cyclin E and cyclin A mRNAs was inhibited in TGF-β-treated cells. In the Mv1Lu cell line, the pattern of cyclin E and cyclin A mRNA expression during the G_0-to-S phase transition and following TGF-β treatment was identical to that seen in the 184SK strain (data not shown). Similar data have recently been reported for a human keratinocyte cell line (28).

To address whether the steady-state protein levels were reduced in parallel with mRNA, we performed Western blot (immunoblot) analysis of cyclin E and cyclin A proteins. In Mv1Lu cells released from contact inhibition, the cyclin E protein level rose modestly as cells progressed through G_1 (Fig. 1A). However, the change in cyclin E protein levels was minimal compared with the dramatic changes observed in mRNA levels. Furthermore, the electrophoretic mobility of a portion of the cyclin E protein decreased as cells approached the G_1/S phase boundary. The decreased mobility of cyclin E is a result of phosphorylation, as demonstrated by treatment of immunoprecipitated cyclin E with potato acid phosphatase (18). TGF-β prevented the phosphorylation of cyclin E that gave rise to the mobility shift on gel electrophoresis that normally accompanies the transition from late G_1 into S phase. Surprisingly, TGF-β had little impact on cyclin E protein accumulation even though the accumulation of cyclin E mRNA was strongly inhibited. By contrast, the rise in cyclin A protein that normally follows that of cyclin E was inhibited in TGF-β-treated cells in parallel with inhibition of cyclin A mRNA accumulation (Fig. 1A).

As 184SK human breast cells progressed from G_0 into S phase, the cyclin E protein underwent a similar modest increase in level and a mobility shift on gel electrophoresis as was noted in the mink cells (Fig. 2B). Again, treatment with TGF-β inhibited cyclin E phosphorylation but not the accumulation of cyclin E protein. The appearance of cyclin A protein followed the onset of cyclin E phosphorylation in the 184SK cells and was prevented in cells treated with TGF-β (data not shown).

While the inhibition of cyclin A-dependent kinase activity seen in TGF-β-treated cells reflects the reduction in mRNA and protein levels, the inhibition of cyclin E by TGF-β is more complex. Although cyclin E mRNA is reduced in TGF-β-treated cells, the lack of a parallel reduction of cyclin E protein suggests that regulation of cyclin E mRNA in itself is not responsible for TGF-β-mediated inhibition of cyclin E-associated kinase activity. On the other hand, TGF-β treatment clearly mediates a reduction in cyclin E phosphorylation, and although it has not been shown that this modification is of regulatory importance, in all cases examined to date, phosphorylation of cyclin E accompanies kinase activation.

TGF-β inhibits activation of cyclin E-Cdk2 complexes. Cyclin E associates with Cdk2 as its kinase partner (19, 52, 62). We wished to determine whether TGF-β inhibited cyclin E-dependent kinase activity by modulating either the extent of Cdk2 binding to cyclin E or the phosphorylation state of bound Cdk2 or both. Cell lysates were prepared from Mv1Lu cells as in Fig. 1A, and anti-cyclin E immune complexes were recovered. These were resolved by SDS-PAGE and blotted. The blot was reacted with anti-PSTAIRE antibody (reactive against

several cyclin-dependent kinases, including Cdk2), revealing the immunoprecipitated Cdk2. To verify that cyclin E was binding Cdk2 specifically in the mink cells, cyclin E immunoprecipitates were boiled in 2% SDS, then diluted 10-fold, and reimmunoprecipitated with anti-Cdk2 antibody specific for the unique carboxy-terminal portion of Cdk2 (22). Complexes were resolved, immunoblotted, and reacted with anti-PSTAIRE antibody. The mobility of Cdk2 was identical to that of the PSTAIRE reactive species bound to cyclin E (data not shown). In the Mv1Lu cells, Cdk2 was observed complexed to cyclin E in contact-inhibited cells but with approximately equal amounts present in phosphorylated and hypophosphorylated forms, as determined by electrophoretic mobility (35). As cells exited G_0 and progressed through G_1, the amount of Cdk2 in anti-cyclin E immunoprecipitates increased. The predominance of a higher-mobility species of Cdk2 bound to cyclin E (Fig. 1B) indicates that the associated Cdk2 became progressively phosphorylated on Thr-160 (19, 35). Following entrance to S phase, the amount of Cdk2 in anti-cyclin E immunoprecipitates and the phosphorylation of the complexed Cdk2 decreased. In the presence of TGF-β, the level of Cdk2 complexed to cyclin E and its phosphorylation state remained similar to that seen in G_0 cells. Thus, treatment of Mv1Lu cells with TGF-β inhibited phosphorylation of cyclin E and of cyclin E-bound Cdk2. In addition, the total amount of Cdk2 bound to cyclin E was reduced, but this may be a consequence of the effect of TGF-β on the phosphorylation of Cdk2 (see Discussion). Similar results have been reported by Koff et al. (53).

In the human mammary epithelial cell line, there was very little increase in the total amount of Cdk2 bound to cyclin E as cells progressed from G_0 to S phase. Cyclin E was immunoprecipitated from these cells with anti-cyclin E MAb E172, rather than with the polyclonal anti-E antiserum, and resolved Cdk2 was immunoblotted with the anti-Cdk2-specific antibody. TGF-β treatment did not reduce either the total amount of Cdk2 bound to cyclin E or the phosphorylation (Thr-160) of bound Cdk2 (Fig. 2C). Thus, in these human breast epithelial cells, one must invoke a mechanism independent of cyclin-Cdk binding and Cdk activation by phosphorylation to account for the observed lack of kinase activity.

An inhibitor of cyclin and Cdk kinase activity in TGF-β-treated cells. While cyclin E-Cdk2 complexes were readily detected in TGF-β-treated cells, in both the Mv1Lu cell line and the 184 mammary epithelial strain, the activity of these kinase complexes was inhibited. We wished to determine whether TGF-β-induced arrest was accompanied by the presence of an inhibitor of cyclin-associated kinase activity. Lysates were prepared from Mv1Lu cells 13 to 15 h from release of contact inhibition, at which time DNA analysis revealed 16 to 26% S-phase cells (G_1/S lysates). Lysates were also recovered from cells that had been released from contact inhibition at the same time but were treated with TGF-β and were therefore blocked in G_1 (TGF-β lysates). Active cyclin E kinase complexes were immunoprecipitated from G_1/S lysates and collected on protein A-Sepharose beads. The beads were mixed with an equal amount of protein lysate from TGF-β-treated cells and incubated at 30°C for 15 min. TGF-β-treated cells contained an activity capable of inhibiting cyclin E-dependent kinase immunoprecipitated from G_1/S phase cells (Fig. 3A, first panel). When TGF-β lysates were mixed with G_1/S lysates prior to immunoprecipitation of cyclin E, the same or greater degrees of inhibition of cyclin E-associated kinase activity were seen. Cyclin A-associated kinase as well as Cdk2 activities were inhibited to similar degrees when TGF-β lysates and G_1/S lysates were mixed prior to immunoprecipitation or when TGF-β lysates were mixed with immunoprecipitated cyclin A or Cdk2 from G_1/S lysates (Fig. 3A). At 24 h after release from contact inhibition, the Mv1Lu cell population was enriched for G_2/M-phase cells. Cdc2 activity in lysates prepared from such cell populations was inhibited by an activity in TGF-β lysates (Fig. 3A). Although the Cdc2 inhibition was less dramatic, the result was consistent on repeated assays.

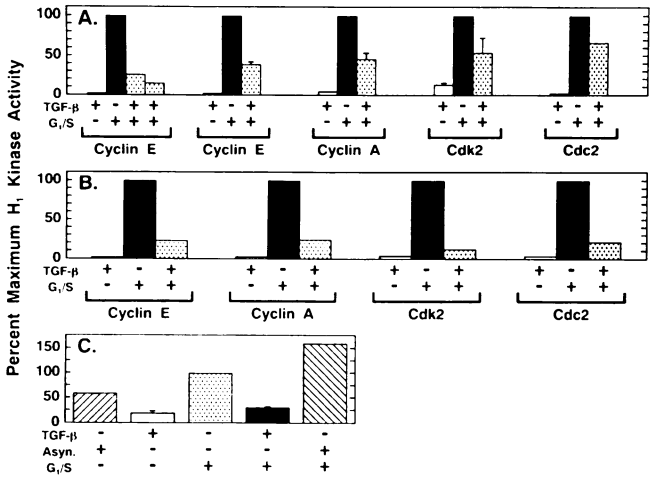

FIG. 3. An inhibitor of cyclin and Cdk activity in TGF-β-treated cells. (A) Mv1Lu cells were released from contact inhibition and grown for 13 to 15 h with TGF-β (TGF-β lysate) or without TGF-β (G_1/S lysate). TGF-β lysate containing 100 μg of protein was mixed with 50 μg of protein of G_1/S lysate, incubated for 15 min at 30°C, and then assayed for cyclin- or Cdk-associated kinase activity following immunoprecipitation. The radioactivity incorporated in histone H1 bands was quantitated by scintillation counting and plotted as a percentage of maximum activity in the histograms after subtraction of background. Open bars represent the kinase activity in cyclin or Cdk precipitated from TGF-β-blocked cell lysates (TGF-β); closed bars represents the activity of the cyclin or Cdk in the lysate recovered from cells at the G_1-to-S phase transition (G_1/S); shaded bars represent the kinase activity of samples in which G_1/S lysate was mixed with TGF-β lysate. In the first group of bars in panel A, cyclin E was immunoprecipitated from G_1/S lysate containing 50 μg of protein and then mixed with TGF-β-treated lysate containing either 100 (first shaded bar) or 150 (second shaded bar) μg of protein. In all of the other experiments, lysates were mixed prior to the immunoprecipitation of the indicated cyclin or Cdk. Where Cdc2 kinase was assayed, lysates from cell populations enriched for G_2/M-phase cells were used rather than G_1/S-phase lysates. (B) Mixing experiments were carried out as described above; however, the G_1/S lysate was recovered from the human breast epithelial strain 48R 10 h after EGF-starved cells were restimulated with EGF. In the Cdc2 mixing assay, a G_2/M-enriched population was obtained by lysing cells 18 h after restimulation with EGF. Open bars represent the kinase activity in TGF-β-treated Mv1Lu cells, recovered 15 h after release from contact inhibition; closed bars represent maximal kinase activity in human breast cells not treated with TGF-β (at the G1-to-S or G_2-to-M phase transition); shaded bars represent kinase activity in samples in which the TGF-β-treated Mv1Lu cell lysate was mixed with the 48R breast cell lysate prior to immunoprecipitation of the cyclin-associated or Cdk kinase. (C) Lysate was recovered from an Mv1Lu cell population enriched for cells at the G_1-to-S phase transition (G_1/S) as described for panel A. Asynchronously growing Mv1Lu cells were growth arrested by treatment with TGF-β for 12 h and then assayed for inhibitory activity as described for panel A. As a control, lysate was also prepared from asynchronously growing cells. No excess of inhibitor activity was demonstrable in asynchronous (Asyn.) cell lysates. The standard error of repeated assays is indicated by error bars.

Mink inhibitor acts on human cyclin and Cdk activities. The human breast epithelial strain 48R was G_0 arrested by block-

age of EGF receptor signal transduction. In this cell line, reentry to cell cycle occurs more rapidly and with slightly greater synchrony than in the 184SK cells described above (44, 107). G_1/S- and G_2/M-phase lysates were recovered at 10 and 18 h, respectively, following restimulation with EGF. Mixing of TGF-β lysates from Mv1Lu cells with human breast epithelial cell lysates caused inhibition of cyclin E-associated, cyclin A-associated, and Cdk2 kinase activities in the G_1/S-phase human breast cell lysates and inhibition of Cdc2 kinase activity in the G_2/M-phase lysate (Fig. 3B). This result indicates that the TGF-β-induced inhibitory activity is capable of functioning across mammalian species.

Inhibitor is present in G_1 cells. Mv1Lu cells released from contact inhibition in the presence of TGF-β are thought to progress to a point late in G_1 (45, 57, 76). From the data presented above, it was not clear if TGF-β acts to maintain an inhibitor normally present in quiescent cells, if inhibitor activity reflects the G1-arrested state of TGF-β-treated cells, or, alternatively, if TGF-β specifically induces an inhibitor normally not present in cycling cells.

We wished to determine whether the inhibitory activity could be detected in cells whose asynchronous growth was arrested in G_1 by TGF-β independent of prior arrest in G_0. When asynchronously growing Mv1Lu cells were treated with TGF-β, they underwent an appreciable reduction in S-phase cells by 8 h, and by 12 h, G_1 arrest was observed with a DNA profile of 90% G_1-phase, 5% S-phase, and 5% G_2/M-phase cells. TGF-β-treated Mv1Lu cells have been shown to reenter S phase within 2 to 3 h of TGF-β removal, consistent with a late G_1 arrest (45). Lysates prepared from asynchronous cells that were treated with TGF-β for 12 h exhibited inhibitory activity toward cyclin E-associated H1 kinase (Fig. 3C). Thus, TGF-β-induced G_1 arrest correlates with the presence of inhibitor activity independent of emergence from quiescence. As a control, lysate prepared from asynchronously growing cells was mixed with G_1/S lysate. No excess of inhibitor was present in the asynchronous cell lysate. These experiments do not address the question of whether TGF-β actually induces an inhibitor not normally present in cycling cells or promotes maintenance of inhibitory activity extant in G_1 cells independent of TGF-β treatment.

Kinetics of inhibitor appearance during the G_0-to-S phase transition. To determine if inhibitor activity was present in G_1, cells were lysed at intervals from release of contact inhibition without TGF-β and lysates were assayed for the ability to inhibit cyclin E-associated kinase activity in G_1/S lysates. Inhibitor activity was present and maximal in G_0 and early G_1 and decreased as cells progressed toward the G_1-to-S phase transition. The decrease in inhibitor activity corresponded to the onset of cyclin E-associated kinase activity (Fig. 1A) and to the progressive loss of sensitivity to TGF-β as cells progress through G_1 toward S phase (Fig. 4). Thus, inhibitor activity is present in G_0 and in early G_1 independent of TGF-β treatment. That loss of inhibitor activity corresponds to the point at which cells are no longer sensitive to TGF-β-induced G_1 arrest suggests that stabilization or maintenance of Cdk inhibition is one of the major mechanisms of TGF-β-mediated arrest.

Inhibitor activity present in G_0 cells. The inhibitory activity detected in G_0 cells was similar to that detected in TGF-β-arrested cells. Mv1Lu cells that have been contact inhibited for 3 to 5 days accumulate in a quiescent state. Lysates from contact-inhibited cells exhibit inhibitory activity toward cyclin E-associated, cyclin A-associated, and Cdk2 kinase activities (Fig. 5A). Likewise, lysates from human breast epithelial strain 48R, G_0 arrested by EGF starvation, contained inhibitor activity (Fig. 5B).

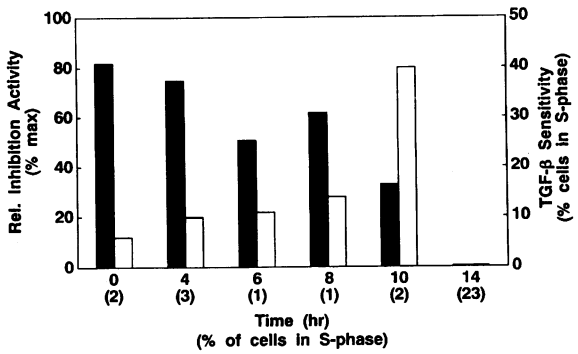

FIG. 4. Kinetics of inhibitor activity from G_0 to S phase. Lysates were prepared from contact-inhibited cells and at intervals following release from contact inhibition during progression from G_0 to S phase. No TGF-β was added in this series of assays. Lysates were assayed for inhibitor activity against cyclin E-associated kinase in G_1/S lysates recovered 14 h following release from quiescence. The DNA profile of cells was assayed at each time point by FACS, and the percentage of cells in S phase at each time point is indicated. Inhibitor activities in lysates are plotted as a function of time. To correlate the presence of inhibitor in early G_1 with the window of sensitivity to TGF-β, parallel plates of cells were transferred to medium containing TGF-β at intervals during the G_0-to-S phase transition and allowed to grow for 16 h. Cells were then recovered for DNA analysis. Entrance to S phase following TGF-β treatment is plotted as a function of the time at which cells were transferred into TGF-β-containing medium.

The inhibitor is a heat-stable protein. Lysates from TGF-β-treated cells prepared in the absence of protease inhibitors or in the presence of 1% Triton X-100 and 1% sodium deoxycholate did not exhibit inhibitor activity. Inhibitor activity was also lost when lysates were repeatedly freeze-thawed. Sensitiv-

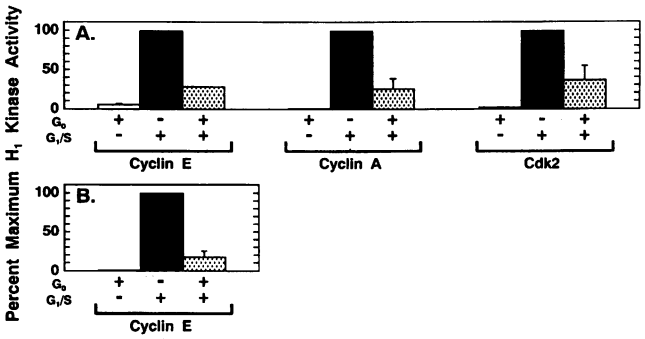

FIG. 5. Quiescent cells contain an inhibitor of cyclin E- and cyclin A-associated kinase and Cdk2 kinase. (A) Mv1Lu cells were contact inhibited for 72 h and lysed as described in Materials and Methods (G_0 lysate). The DNA profile of contact-inhibited cells was 95% G_0/G_1 phase, 2% S phase, and 3% G_2/M phase. G_0 lysate (100 μg) was mixed with 50 μg of G_1/S lysate (prepared as in Fig. 1A), and H1 kinase activity was determined. The histone H1 kinase activities in anti-cyclin or anti-Cdk2 immunoprecipitates prepared from the G_0 cells, the G_1/S-phase cells, and the mixed lysates are plotted in open, closed, and shaded bars, respectively. (B) Quiescence was induced in the human breast epithelial strain by treatment of midconfluent cultures with anti-EGF receptor MAb 225 (5 μg/ml) for 48 h prior to cell lysis (G_0). Lysates recovered 10 h after restimulation with fresh medium and EGF were enriched for G_1/S-phase cells (G_1/S). Anti-cyclin E immunoprecipitates were prepared from the G_0, the G_1/S, and the mixed lysates and assayed for histone H1 activity. As in Fig. 3, the standard error of repeated assays is indicated.

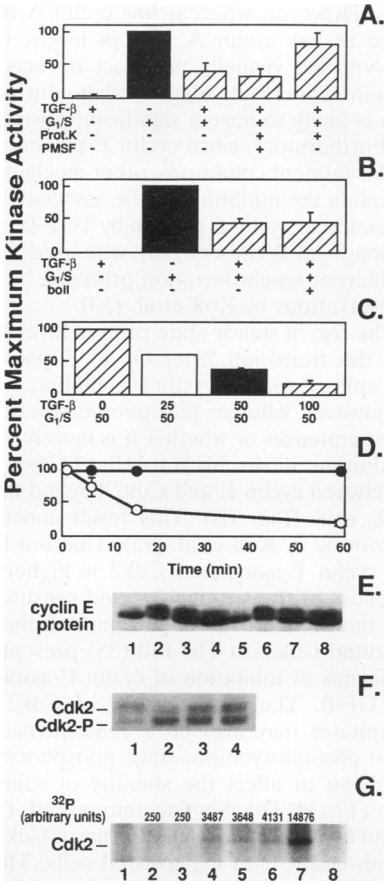

FIG. 6. Biochemical nature of the inhibitor. G_1/S lysate and TGF-β lysate were prepared as for Fig. 3. (A) H1 kinase activity was determined in 100 μg of protein of TGF-β lysate and in 50 μg of protein of G_1/S lysate following immunoprecipitation with anti-cyclin E antibody. Aliquots of 100 μg of TGF-β lysate protein were either kept on ice, treated with proteinase K that had been inactivated by PMSF (PMSF), or treated with active proteinase K (Prot.K) for 1 h, followed by the addition of PMSF. These TGF-β lysates were then incubated with 50-μg samples of G_1/S lysate, cyclin E was immunoprecipitated, and histone H1 kinase activity was assayed (striped bars). (B) Mixing experiments were carried out as for panel A. Anti-cyclin E-precipitable H1 kinase activities in TGF-β, G_1/S, and mixed lysates are displayed by open, closed, and striped bars, respectively. However, in the assay quantitated in the second striped bar, prior to mixing of the TGF-β lysates with G_1/S lysates, the inhibitor-containing lysate was boiled for 5 min, precipitated proteins were cleared by centrifugation, and the remaining supernatant was mixed with the G_1/S lysate and incubated as usual. (C) Increasing amounts of inhibitor lysate (25, 50, and 100 μg) were mixed with 50 μg of G_1/S lysate and incubated for 15 min at 30°C prior to cyclin E immunoprecipitation and histone H1 assay. Percent kinase activity is plotted as a function of the amount of inhibitor added. (D) G_1/S lysate (closed circles) and G_1/S lysate mixed with TGF-β-blocked lysate (open circles) were incubated at 30°C for the times indicated prior to anti-cyclin E immunoprecipitation. Precipitates were then assayed for H1 kinase activity, and scintillation counting of the histone bands was quantitated and graphed. Error bars indicate standard error where shown. (E) Whole-cell lysates were resolved by SDS-PAGE (7.5% gel), immunoblotted, and probed with anti-cyclin E antibody. Fifty micrograms of protein of TGF-β lysate was resolved in lanes 1 and 5, and 50 mg of G_1/S lysate was run on lanes 2 and 6. In lanes 3 and 7, 50 μg of G_1/S and 50 μg of TGF-β lysate proteins were mixed before loading on the gel. In lanes 4 and 8, aliquots of 50 μg of G_1/S and 100 μg of TGF-β lysate proteins were mixed before loading. Samples loaded on lanes 1 to 4 were incubated at 4°C prior to loading. In lanes 6 to 8, the samples were mixed, brought to 1 mM dithiothreitol, and incubated at 30°C for 15 min before they were loaded on the gel. (F) Anti-cyclin E immune complexes were resolved by electrophoresis at 60 mA on a 26-cm SDS–11% polyacrylamide gel and blotted. The mobilities of hypophosphorylated and phosphorylated forms of Cdk2, designated Cdk2 and Cdk2-P, are indicated. Cyclin E-bound Cdk2 was detected by reacting the blot with anti-PSTAIRE antibody. Lane 1 shows the Cdk2 bound to cyclin E in 250 μg of TGF-β lysate. The Cdk2 bound to cyclin E in the same amount of G_1/S lysate is seen in lane 2. In lane 3, 250 μg of G_1/S lysate was incubated with 250 μg of TGF-β lysate; in lane 4, 250 μg of G_1/S lysate was incubated with 500 μg of TGF-β lysate prior to anti-cyclin E immunoprecipitation. (G) Cells were released from contact inhibition with or without the addition of TGF-β. Between 14 and 16 h from release from G_0, cells were labeled with $^{32}P_i$. Radioactivity incorporated into Cdk2 present in cyclin A or Cdk2 immunoprecipitates is shown. Cdk2 was not demonstrable in cyclin A immunoprecipitated from increasing amounts of TGF-β-treated cells (lanes 2 and 3). Lane 4 shows the Cdk2 bound to cyclin A in cells labeled during the G_1-to-S phase transition (the percentage of cells in S phase rose from 21% at 14 h to 38% at 16 h). Lysates from cells labeled during the G_1-to-S phase transition were mixed with equal amounts of unlabeled TGF-β-treated cellular lysate (lane 5) or unlabeled contact-inhibited cell lysate (lane 6) for 30 min prior to immunoprecipitation of cyclin A. Lane 7 shows Cdk2 precipitated with anti-Cdk2 antibody from G_1/S-phase cells. Lanes 1 and 8 are negative control immunoprecipitations with polyclonal immunoglobulin G2a from TGF-β-treated and G_1/S-phase lysates, respectively. On quantitation by PhosphorImager, the radioactivity incorporated in Cdk2 in lanes 2 and 3 (TGF-β-arrested cells) was negligible compared with the control level (lanes 1 and 8). Quantitation of radioactivity incorporated in cyclin A-bound Cdk2 in G_1/S-phase cells (lane 4) showed no diminution following incubation with unlabeled inhibitor lysates (lanes 5 and 6).

ity to treatment with proteinase K demonstrated that the inhibitor is a protein (Fig. 6A).

In *S. cerevisiae*, a 40-kDa heat-stable protein (p40) that binds to $p34^{Cdc28}$ and inhibits its mitotic kinase activity has been identified (72, 73, 94). To determine whether the inhibitors present in TGF-β lysate and in lysates from quiescent cells were heat stable, lysates were boiled for 5 min and precipitated proteins were cleared by centrifugation. The remaining supernatants contained full inhibitory activity (Fig. 6B), indicating that the inhibitor is a heat-stable protein.

When increasing amounts of inhibitor lysate were mixed with fixed amounts of G_1/S lysate, the degree of inhibition increased, indicating that it was dosage dependent (Fig. 6C). When constant amounts of lysates were mixed and incubated at 30°C for increasing lengths of time, the degree of inhibition increased with apparent first-order kinetics consistent with a saturable, inhibitor-limited phenomenon (Fig. 6D). Inhibitor activity itself was stable following incubation for 60 min at 30°C, so that saturation is not the result of inhibitor inactivation (data not shown). The first-order kinetics are consistent with a simple binding event.

The following experiment demonstrated that inhibitor was present in and could be recovered from cyclin-Cdk complexes. Cyclin A-Cdk2 complexes were immunoprecipitated from G_1/S-phase cells with MAb E67 and collected on protein A-Sepharose. TGF-β lysate was added to these kinase complexes, and the mixtures were incubated at 30°C as described in Materials and Methods. Incubation of cyclin A immunoprecipitates with TGF-β lysate inhibited the cyclin A-associated kinase to 38% of the control value (standard error = ±21%). Inhibited cyclin A immune complexes were washed extensively and then boiled for 5 min in lysis buffer. Inhibitor released from boiled complexes inhibited cyclin A-associated histone H1 kinase activity to 49% of the control value (standard error = ±13%, based on three independent experiments). A control

immunoprecipitation of G_1/S lysate with an unrelated MAb (MAb 225) failed to bind the inhibitor present in TGF-β-treated cell lysates. When these nonspecific complexes were boiled after incubation with TGF-β lysate, the supernatant did not inhibit cyclin A-associated kinase when added to cyclin A immunoprecipitates in G_1/S lysates (kinase activity, 106% of the control value). Thus, the inhibitor binds to and can be released from cyclin A-Cdk2 complexes.

Inhibition is not likely to be mediated by cyclin E or Cdk2 dephosphorylation. To address whether the heat-stable inhibitory proteins have cyclin E- or Cdk2-directed phosphatase activities, a series of experiments were performed. First, lysates of G_1/S and TGF-β-treated cells were prepared, mixed, and incubated as described above. A portion of the mixed lysate was used to immunoblot cyclin E protein (Fig. 6E). The rest of the lysate was used to assess the Cdk2 bound to cyclin E, using the strategy described for Fig. 1B. There was no shift in the mobility of cyclin E on Western blots, nor was there any loss of the more rapidly migrating species (Thr-160 phosphorylated) of Cdk2 in cyclin E immunoprecipitates with the addition of increasing amounts of TGF-β lysate (Fig. 6F). To address the possibility that dephosphorylation events that do not affect electrophoretic mobility occurred, Mv1Lu cells were metabolically labeled with $^{32}P_i$ as they approached the G_1-to-S phase transition. Phosphate-labeled G_1/S-phase lysates were mixed with equal amounts of unlabeled TGF-β lysate or lysate from contact-inhibited cells, and cyclin A was immunoprecipitated with a MAb. A control anti-Cdk2 immunoprecipitate was run alongside the cyclin A immunoprecipitates. Radioactivity in Cdk2 bands was quantitated with a PhosphorImager using Molecular Dynamics ImageQuant software and is shown in Fig. 6G. While mixing of lysates showed the predicted inhibition of cyclin A kinase in G_1/S lysates (not shown), there was no reduction in Cdk2-bound radioactive phosphate in cyclin A-Cdk2 complexes following treatment with inhibitor lysate.

Finally, inhibitor reactions could be carried out with equal efficacy in the presence or absence of either 1 μM okadaic acid, an inhibitor of most protein serine/threonine phosphatases (11), or 0.5 mM sodium orthovanadate, an inhibitor of protein tyrosine phosphatases (8, 31, 50, 111). When immunoprecipitated cyclin E from G_1/S lysate was mixed with TGF-β lysate in the presence of these phosphatase inhibitors, no loss of inhibition was seen (data not shown). Thus, it is unlikely that inhibition is mediated by a cyclin E- or a Cdk2-directed phosphatase.

DISCUSSION

TGF-β modulates cyclin expression and activity. TGF-β is a polypeptide growth factor with pleiotropic effects on growth and differentiation (reviewed by Massague [66]). Although first identified as a growth stimulator of mesenchymal cells (99), it has growth-inhibitory effects on all normal epithelial and endothelial cells examined to date (44, 68, 71, 103, 109). TGF-β mediates a reversible growth arrest in late G_1 phase of the cell cycle (45, 57, 76, 103). The present studies were undertaken to study the effects of TGF-β on cell cycle regulators of the G1-to-S phase transition.

In both human breast epithelial cells of finite life span and the immortal Mv1Lu cell line, TGF-β modulates G_1/S cyclins and associated kinase activities at multiple levels. TGF-β inhibits accumulation of cyclin E and cyclin A mRNAs. This observation was also reported recently by Geng and Weinberg (28). Abundance of mRNA is likely the major level at which cyclin A is regulated by TGF-β, since the levels of both cyclin A protein and its associated kinase activity closely parallel mRNA levels. However, whereas low cyclin A mRNA levels were reflected in low cyclin A protein levels, restriction of cyclin E mRNA had virtually no effect on accumulation of cyclin E protein. This result suggests that control of cyclin E accumulation is likely to have a significant posttranscriptional component. Furthermore, since cyclin E protein accumulates under TGF-β treatment conditions, other mechanisms must be invoked to explain the inhibition of the associated kinase. The lack of suppression of cyclin E protein by TGF-β contrasts with the data of Geng and Weinberg (28), who used a different cell type and a different synchronization protocol, but is in agreement with observations by Koff et al. (53).

Although the rise in steady-state protein level that normally accompanies the transition from G_0 to S phase occurs on schedule, phosphorylation of cyclin E is inhibited by TGF-β. It is not clear, however, whether phosphorylation of cyclin E has regulatory consequences or whether it is merely a reflection of activity. In addition, in the Mv1Lu cells, TGF-β prevents the association between cyclin E and Cdk2 beyond the basal level existing in G_0 cells (Fig. 1B). This result concurs with that reported previously by Koff et al. (53). That our basal level of detection of cyclin E-associated Cdk2 is higher may simply reflect differences in the specificities and sensitivities of antisera used. In the Mv1Lu cells, impairment of phosphorylation of cyclin E-bound Cdk2 on Thr-160 (35) presents one of the major mechanisms of inhibition of cyclin E-associated kinase activity by TGF-β. The low recovery of Cdk2 in cyclin E immunoprecipitates may also be a consequence of reduced Cdk2 Thr-160 phosphorylation, since phosphorylation at this position is known to affect the stability of some cyclin-Cdk combinations (17, 34, 79). Similar amounts of Thr-160-phosphorylated and hypophosphorylated forms of Cdk2 were found associated with cyclin E in G_0-arrested cells. These data are consistent with the possibility that similar inhibitory mechanisms are responsible for density-mediated and TGF-β-mediated arrest.

In the human mammary epithelial strain, however, TGF-β-induced inhibition of cyclin E-associated kinase was not accompanied by a reduction in the association of cyclin E with Cdk2 or in the phosphorylation of the kinase. Thus, in this strain, the action of a Cdk2 inhibitor may represent the major mechanism of cyclin E-associated kinase inhibition. The difference in the effect of TGF-β on cyclin E-Cdk2 association in the mink cell line and the human mammary epithelial strain may be cell type specific or may reflect differences in cell cycle regulation in immortal cells versus cells of finite life span. Alternatively, the mode of presynchronization, contact inhibition versus mitogen removal, may contribute to different physiologies of Cdk2 regulation.

A recent report by Ewen et al. suggests that regulation of Cdk4 is important for the TGF-β response in mink lung epithelial cells (24). Constitutive overexpression of Cdk4, shown to be a partner for D-type cyclins (47, 69), was found to render cells insensitive to TGF-β-mediated G_1 arrest (24). Furthermore, TGF-β was found to repress Cdk4 protein levels, consistent with the idea that Cdk4 is central to the response to TGF-β. It is not yet clear whether the activity of Cdk4 is directly antagonistic to the effects of TGF-β or whether Cdk4 might function by titrating the Cdk2 inhibitor described in this report as has been suggested by Polyak et al. (91). These authors showed that Cdk4-cyclin D complexes could remove the inhibitor from Cdk2-cyclin E complexes, reactivating them in vitro (91).

Novel inhibitor(s) of cyclin-Cdk complexes. We have identified a novel heat-stable activity in TGF-β-treated Mv1Lu cells that can inhibit active cyclin E- and cyclin A-associated kinases

and Cdk2 and Cdc2 kinases. A similar activity is detected in TGF-β-arrested and G_0-arrested human mammary epithelial cells (2a) (Fig. 5B). The inhibitor does not modify the mobility of cyclin E protein on SDS-PAGE, nor does it remove the activating phosphate on Thr-160 in Cdk2 (Fig. 6). The presence of orthovanadate and okadaic acid did not prevent inhibitor action; thus, it seems unlikely that inhibition is due to the action of a phosphatase on cyclin E or on Cdk2. This is of interest in light of the recent report of cyclin-dependent interactor 1 (Cdi1), a protein with sequence similarity to tyrosine and dual-specificity phosphatases that was shown to interact with Cdc2, Cdk2, and Cdk3 and whose induction of cell cycle delay is dependent on phosphatase activity (37). Our results suggest that the inhibitor induced during TGF-β-mediated arrest is not Cdi1. The definitive demonstration that inhibitor activity does not involve the action of a phosphatase awaits the large-scale purification of inhibitor and authentic phosphatase assays.

Inhibitor activity is detectable when TGF-β mediates the G_1 arrest of asynchronously growing cells. It is present in cells released from contact inhibition in the presence of TGF-β, and its activity decreases as cells approach the G_1-to-S phase transition. A similar activity was also present in contact-inhibited G_0-arrested Mv1Lu cells and in EGF-starved G_0 human breast epithelial cells. Inhibition of cyclin E-associated kinase was not seen when active G_1/S lysate was mixed with G_1/S lysate or with lysate from G_2/M cells or asynchronously growing cells. Thus, the cyclin E-inhibitory activities detected in these assays were confined to G_0 and early G_1. At this point, we cannot say whether TGF-β directly induces an inhibitory activity or stabilizes activities normally present in G_0 and G_1.

Earlier work demonstrated the presence of a heat-stable Cdc2 inhibitor protein, called inhibitor of maturation factor (IMF), present in G_1-phase HeLa cell lysates (1). When lysates from G_1 cells were mixed with lysates from G_2- and M-phase cells, G_2/M-phase lysates could no longer induce germinal vesicle breakdown and chromosome condensation when injected into *Xenopus* oocytes. IMF was identified as the protein that caused the inhibition of maturation-promoting factor in G_2/M-phase lysates. As it has now been shown that maturation-promoting factor is Cdc2 kinase (4, 14, 20, 27, 55, 60, 61, 114), the IMF activity detected in these studies may correspond to the Cdc2-inhibitory component that we detected in TGF-β-arrested G_1 cells.

A model of inhibitor action. The inhibitor detected in TGF-β-treated cells acts on already active cyclin E-Cdk2 and cyclin A-Cdk2 kinase complexes and immunoprecipitable Cdk2. Increasing the amount of inhibitor lysate added to G_1/S lysate increased the kinase inhibition. The inhibitory reaction appeared to follow first-order kinetics, implying that inhibition may be due to a simple stoichiometric binding event. This view is supported by our observation that inhibitory activity could be released from inhibited cyclin A-Cdk2 complexes by boiling. The released inhibitor was shown to act on cyclin A-associated kinase immunoprecipitated from fresh G_1/S lysates. This model is also supported by the data of Polyak et al., which indicate that TGF-β-induced Cdk2 inhibition in mink lung epithelial cells acts via a stoichiometric mechanism (91). Furthermore, that report suggests that the TGF-β inhibitor is distinct from another recently described Cdk inhibitor, $p21^{CIP1/WAF1}$ (21, 36, 39, 118). However, the TGF-β inhibitor, as judged from the molecular weight, is likely to be identical to a 28-kDa inhibitor of Cdks found in G_1 cycling cells and in cells arrested in G_1 by the drug Lovostatin, as well as in quiescent fibroblasts (42).

An attractive functional model is that the inhibitor binds to the Cdk protein, thereby blocking or altering the conformation of the catalytic domain, rendering it inactive. Our results and those of Koff et al. (53) suggest, in addition, that in Mv1Lu cells, TGF-β inhibits the action of the Cdk-activating kinase (CAK) (104, 105) on Cdk2. It is conceivable that the same inhibitor molecule that could bind and inactivate phosphorylated Cdk2 could also bind unphosphorylated Cdk2 and prevent its activation by CAK, simply by occupying the catalytic cleft (13, 101). It will be of interest to determine whether the inhibitor identified in the present studies can also prevent CAK activation of Cdk2. The same inhibitor may prevent premature activation of cyclin-Cdk complexes by inhibiting phosphorylation of new complexes and by inhibiting the action of any residual phosphorylated complexes remaining in early G_1 following cytokinesis. This model, however, is at odds with our results with human mammary epithelial cells; in the latter case, inhibited cyclin E-Cdk2 complexes in vivo were not limited for Cdk2 phosphorylation. Further investigation will be required to resolve this issue.

TGF-β resistance and loss of cell cycle control in cancer. Resistance to the growth-inhibitory effects of TGF-β is seen in many malignant epithelial cell lines (reviewed by Massague et al. [67] and by Filmus and Kerbel [26]). Several mechanisms have been proposed to explain these observations. In certain cases, cells have lost intact TGF-β receptors (2, 49, 54, 58). Mutational inactivation of tumor suppressor genes RB1 and p53 has been implicated in the development of TGF-β resistance (29, 116). TGF-β prevents the phosphorylation of pRb in G_1, thereby maintaining it in its growth-suppressive state (57). Mutations of RB1 and p53 that abrogate the growth-inhibitory properties of these genes are common in human malignancy (for reviews, see references 6, 43, and 64) and may promote TGF-β-resistant cell growth.

The results of the present study and other recent inhibitor reports suggest an additional level at which the growth-inhibitory signals of TGF-β may be interrupted in human malignancy. Altered expression or activity of cyclins E and A and of Cdk2 may promote unregulated growth by overcoming the inhibitory responses that occur in normal cells. Overexpression of cyclins E and D has been shown to accelerate G_1-to-S phase transit in mammalian cells (82, 92, 96). Indeed, aberrant expression of cyclin E has been demonstrated in human breast cancer cell lines and in primary breast carcinoma tumors (48). Finally, functional inactivation of inhibitors of Cdks described in this and other reports may provide yet another means of escape from negative growth-regulatory signals.

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