Transforming growth factor β targeted inactivation of cyclin E:cyclin-dependent kinase 2 (Cdk2) complexes by inhibition of Cdk2 activating kinase activity

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Transforming growth factor β (TGF-β)-mediated G_1 arrest previously has been shown to specifically target inactivation of cyclin D:cyclin-dependent kinase (Cdk) 4/6 complexes. We report here that TGF-β-treated human HepG2 hepatocellular carcinoma cells arrest in G_1, but retain continued cyclin D:Cdk4/6 activity and active, hypophosphorylated retinoblastoma tumor suppressor protein. Consistent with this observation, TGF-β-treated cells failed to induce $p15^{INK4b}$, down-regulate CDC25A, or increase levels of $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$. However, TGF-β treatment resulted in the specific inactivation of cyclin E:Cdk2 complexes caused by absence of the activating Thr^{160} phosphorylation on Cdk2. Whole-cell lysates from TGF-β-treated cells showed inhibition of Cdk2 Thr^{160} Cdk activating kinase (CAK) activity; however, cyclin H:Cdk7 activity, a previously assumed mammalian CAK, was not altered. *Saccharomyces cerevisiae* contains a genetically and biochemically proven CAK gene, *CAK1*, that encodes a monomeric 44-kDa Cak1p protein unrelated to Cdk7. Anti-Cak1p antibodies cross-reacted with a 45-kDa human protein with CAK activity that was specifically down-regulated in response to TGF-β treatment. Taken together, these observations demonstrate that TGF-β signaling mediates a G_1 arrest in HepG2 cells by targeting Cdk2 CAK and suggests the presence of at least two mammalian CAKs: one specific for Cdk2 and one for Cdk4/6.

The basic cell cycle control machinery is comprised of regulatory cyclin subunits complexed to catalytic serine/threonine cyclin-dependent kinase (Cdk) subunits that phosphorylate substrates in a cell cycle-specific fashion (1–4). Activation of Cdk2 by cyclin E in late G_1 at/near the G_1 restriction point and by cyclin A at the G_1-S phase transition, and cyclin B activation of CDC2 at the G_2/M phase transition suggests the involvement of these cyclin:Cdk complexes at specific cell cycle regulatory checkpoints (3, 4). In contrast, cyclin D-dependent activation of Cdk4 and Cdk6 (Cdk4/6) in cycling cells is constitutive throughout the cell cycle (5–10) and indicates a distinct role of cyclin D:Cdk4/6 complexes from that of cyclin E:Cdk2. Indeed, we previously have shown that cyclin D:Cdk4/6 complexes activate the retinoblastoma tumor suppressor protein (pRB) by hypo-phosphorylation in early G_1 (8–10), whereas cyclin E:Cdk2 complexes perform the initial inactivating, hyperphosphorylation of pRB at the late G_1 restriction point (8–13).

Cyclin:Cdk complexes are highly regulated at multiple levels. After cyclin synthesis, cyclin and Cdk proteins are assembled into inactive heterodimeric complexes that are further modified by phosphorylation of the Cdk subunit (4). Cdk2 contains two inhibitory phosphorylation sites at Thr^{14} and Tyr^{15} and one activating phosphorylation site at Thr^{160}. Wee1 phosphorylates Tyr^{15} on Cdk2 (21), whereas the Cdk2 Thr^{14} kinase remains unknown. Thr^{14}/Tyr^{15} are dephosphorylated by the dual-specificity CDC25 phosphatase family (A, B, C) (4). However, activation of cyclin E:Cdk2 complexes requires phosphorylation of Thr^{160} residue on Cdk2 by the Cdk activating kinase (CAK) (14).

The identity of the mammalian CAK has remained controversial in the literature. However, based primarily on *in vitro* biochemical assays, initial reports identified a putative mammalian CAK complex that is a member of the Cdk family, namely cyclin H:Cdk7:Mat1 (15, 16). Surprisingly, by both genetic and biochemical means, cyclin H:Cdk7 complexes subsequently were identified as a requisite component of TFIIH involved in phosphorylating the C terminal domain of the large subunit of RNA polymerase II (17–20). Consistent with these observations, inactivation of the *Saccharomyces cerevisiae* Cdk7 homologue, *KIN28*, results in loss of TFIIH activity with continued CAK activity (21). Significantly, Larochelle *et al.* (22) recently have demonstrated in *Drosophila* that Cdk7 inactivation leads only to a loss of CDC2 activity with continued cyclin E:Cdk2 activity. Moreover, several groups have identified the true *S. cerevisiae* CAK (*CAK1*/*CIV1*) gene that encodes a 44-kDa monomeric CAK protein (Cak1p) distinct from the proline-dependent Cdk family (23–25). Taken together, these observations challenge the perceived initial notion based on *in vitro* experiments that cyclin H:Cdk7 complexes function as a Cdk2 CAK in mammalian cells *in vivo*.

Cyclin D:Cdk4/6 complexes previously have been shown to be specifically targeted by transforming growth factor β (TGF-β) signaling in mediating a G_1 arrest by either induction of $p15^{INK4b}$, a negative regulator of Cdk4/6, down-regulation of CDC25A, or down-regulation of Cdk4 protein levels (26–28). In searching for a TGF-β-responsive cell line that growth arrests with continued cyclin D:Cdk4/6 activity, we uncovered the ability of HepG2 human hepatocellular carcinoma cells to undergo a TGF-β-mediated G_1 arrest independent of $p15^{INK4b}$ and CDC25A, which targets inactivation of cyclin E:Cdk2 complexes. We report here the biological regulation of Cdk2 CAK activity, independent of Cdk7 activity, by TGF-β signaling, while Cdk4/6 CAK activity remains unaffected.

Materials and Methods

Cell Culture and Flow Cytometry. Human HepG2 hepatocellular carcinoma cells (American Type Culture Collection) were maintained in α-modified MEM, and HaCaT keratinocytes and HeLa cells in DMEM containing 5% heat-inactivated FBS as described

Abbreviations: Cdk, cyclin-dependent kinase; CAK, Cdk activating kinase; pRB, retinoblastoma tumor suppressor protein; TGF-β, transforming growth factor β; HGF, hepatocyte growth factor; HA, hemagglutinin; WCE, whole-cell extracts.

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(8, 29). Either 10 ng/ml TGF-β (R & D Systems) or 20 ng/ml hepatocyte growth factor (HGF) (Sigma) was added for 48 hr to 5×10^5 HepG2 cells per 10-cm dish (29). Flow cytometry was performed as described (8).

Immunoprecipitation and Immunoblot Analysis. Immunoprecipitations were performed as described (8) by addition of rabbit anti-Cdk2, anti-Cdk4, anti-Cdk6, anti-Cdk7, anti-cyclin E, anti-p15, anti-p16, anti-p21, or anti-p27 antibodies (Santa Cruz Biotechnology). For secondary immunoprecipitations, samples were diluted 1:33 with fresh E1A lysis buffer and reimmunoprecipitated as described above. Immunoblot analysis was performed as described (8) and probed with anti-pRB (PharMingen), anti-cyclin A, anti-cyclin E, anti-CDC25A, anti-p21, anti-p27, anti-Cdk2, anti-Cdk4, anti-Cdk6, anti-Cdk7 (Santa Cruz Biotechnology), and anti-pRB-phospho-Ser^{780} (kindly provided by Y. Taya, Tokyo Univ.). Rabbit anti-yeast Cak1p antibodies were generated against maltose binding protein (MBP)-Cak1p and purified over a MBP-Cak1p Sepharose column (P.K. and M.J.S., unpublished work). A total of 8×10^7 asynchronous HepG2 cells were incubated in 350 μl of hypotonic buffer [10 mM Hepes, pH 7.4/10 mM NaCl/1 mM EDTA/1 μg/ml aprotinin (Sigma)/1 μg/ml leupeptin (Sigma)/50 μg/ml PMSF (Sigma)/0.5 mM NaP_2O_7/0.1 mM $NaVO_4$/5.0 mM NaF] on ice for 30 min, then dounced on ice with an "A" pestle 20× and clarified, and 200 μl was injected into a Superose-12 gel filtration column attached to an FPLC (Amersham Pharmacia) equilibrated in kinase buffer. Column fractions (0.5 ml) were assayed for CAK activity by using baculovirus-produced cyclin B:CDC2-hemagglutinin (HA) complexes as a substrate (see below).

Kinase Assay. Kinase assays with anti-CDC2-HA (12CA5; Babco, Richmond, CA), anti-Cdk2, anti-Cdk4, anti-Cdk6, and anti-Cdk7 immune complexes were performed as described (8). TGF-β-treated whole-cell extracts (WCE) were mixed with 100 μg of control WCE, incubated for 1 hr followed by anti-cyclin E immunoprecipitation-kinase assay. Anti-cyclin E immune complexes were treated with 3.2 μg of glutathione *S*-transferase-CDC25A protein (kindly provided by H. Piwnica-Worms, Washington University) in 30 μl of kinase buffer (8) for 20 min at 30°C, stopped by addition of ice-cold 50 mM Na_3VO_4, and then incubated with 10 μCi of [γ-^{32}P] ATP and 2 μg of histone H1 as above. Reverse transcription–PCR was performed as described (9).

Activation of Baculovirus Cyclin B:CDC2-HA Complexes. High-5 insect cells (Invitrogen) were infected separately with HA-tagged CDC2 or 6× histidine-tagged cyclin B baculoviruses (kindly provided by D.O. Morgan, University of California, San Francisco) and purified as described (15). Cyclin B:CDC2-HA complexes (0.5 μg) were incubated with anti-Cdk7 antibody immunoprecipitates and WCE with 10 μCi of [γ-^{32}P] ATP in 25 μl of kinase buffer for 20 min at room temperature. Purified cyclin B:CDC2-HA complexes were incubated with WCE containing 100 μM ATP in kinase buffer for 20 min at room temperature followed by anti-HA immunoprecipitation-kinase assay. HepG2 WCE were incubated with rabbit anti-Cak1p or rabbit anti-mouse IgG antibodies plus protein A beads for 2 hr, clarified, and followed by readdition of antibodies for 24 hr. Depleted lysates were incubated with cyclin B:CDC2-HA complexes and ATP as above, followed by anti-CDC2-HA immunoprecipitation-kinase assay.

Results

TGF-β-Mediated G_1 Arrest Targets Cdk2. To further understand the regulation of pRB in early G_1, we searched for a cell line that elicited a G_1 cell cycle arrest in response to TGF-β signaling, but failed to inactivate cyclin D:Cdk4/6 complexes. To this end, we previously have found (8) that TGF-β treatment of human

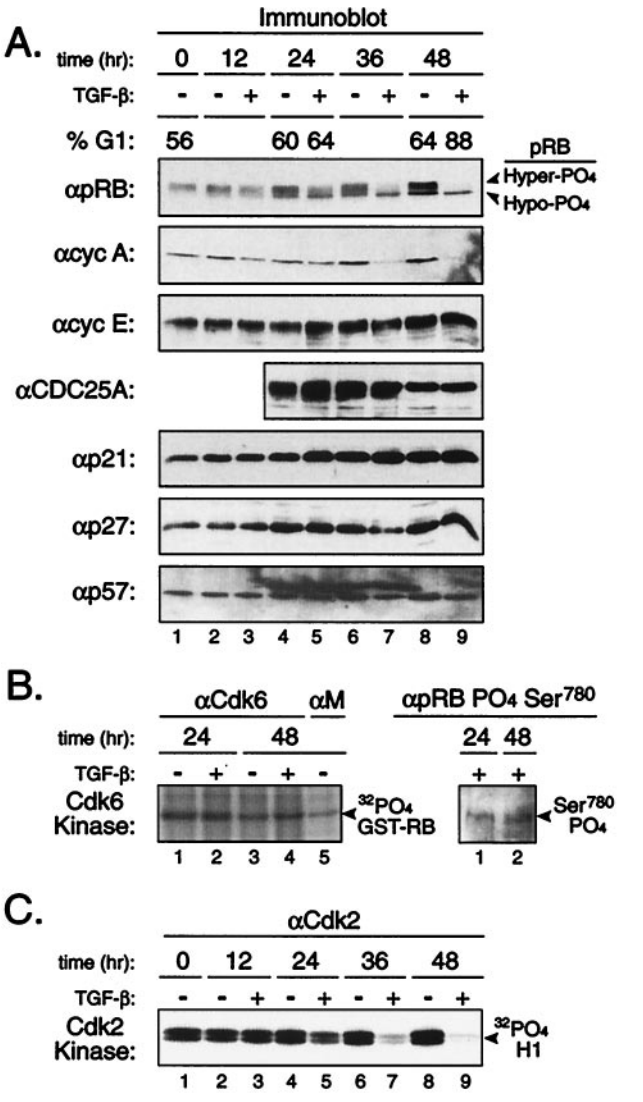

Fig. 1. TGF-β treatment induces a G_1 arrest of human HepG2 hepatocellular carcinoma cells. (*A*) Time course immunoblot analysis with indicated antibodies of cell lysates from control and TGF-β-treated cells. Note loss of pRB hyper-phosphorylation (slower migrating species) and appearance of hypo-phosphorylated pRB (fastest migrating species) beginning at 24 hr. (*B*) Cdk6 kinase activity (*Left*) and immunoblot detection of phospho-Ser^{780} on hypo-phosphorylated pRB at 48 hr after TGF-β treatment (*Right*). (*C*) Cdk2 kinase activity during TGF-β-treated time course.

HepG2 hepatocellular carcinoma cells resulted in a G_1-specific cell cycle arrest (88% G_1) compared with untreated cells (64% G_1) (Fig. 1*A*). Given a ≈30-hr cell cycle, this observation suggested that the cells were arresting in the first early G_1 phase encountered after addition of TGF-β. Anti-pRB immunoblot analysis of TGF-β-treated HepG2 cells over a 48-hr time course showed loss of the slower migrating, inactive hyper-phosphorylated forms of pRB associated with late G_1, S, and G_2/M phases of the cell cycle and the appearance of the faster migrating, active hypo-phosphorylated pRB species present in early G_1 as early as 24 hr after treatment (Fig. 1*A*). Immunoblot analysis also showed loss of cyclin A expression during the time course, but continued cyclin E expression in TGF-β treated HepG2 cells (Fig. 1*A*).

Down-regulation of CDC25A phosphatase levels and induction of p21^{Cip1} and p27^{Kip1} Cdk inhibitors previously have been

identified as mechanisms of TGF-β-mediated arrest (3, 28). However, immunoblot analysis over the 48-hr time course detected no change of CDC25A, p21, p27, or p57 protein levels in TGF-β-treated HepG2 cells compared with untreated cells (Fig. 1*A*). In addition, we observed no loss of Cdk2, Cdk4, or Cdk6 protein levels in TGF-β-treated cells (data not shown). Anti-Cdk6 and anti-Cdk2 immunoprecipitation-kinase assays performed on cellular lysates from control and TGF-β-treated cells showed no alteration of Cdk6 activity at 24 and 48 hr post-TGF-β treatment (Fig. 1*B Left*). *In vitro*, Cdk4/6 previously have been shown to preferentially phosphorylate Ser^{780} on pRB (30). Anti-pRB-phospho-Ser^{780} immunoblot analysis showed the continued presence of phospho-Ser^{780} at 24 and 48 hr post-TGF-β treatment (Fig. 1*B Right*). Consistent with these observations, no induction of $p15^{INK4b}$ mRNA or protein was detected in control or TGF-β-treated cells (data not shown). In contrast and in close agreement with loss of pRB hyper-phosphorylation, Cdk2 activity was noticeably diminished after TGF-β treatment as early as 24 hr and markedly absent at 36 and 48 hr (Fig. 1*C*). These observations demonstrated that Cdk4/6 complexes are not the target of TGF-β signaling in HepG2 cells, but rather cyclin E:Cdk2 complexes are targeted for inactivation.

TGF-β Inactivation of Cdk2 Is Independent of Cdk Inhibitors. Cdk2 initially becomes activated at/near the late G_1 restriction point by association with cyclin E (1). We assayed for the assembly of cyclin E:Cdk2 complexes by anti-cyclin E immunoprecipitation followed by reimmunoprecipitation with either anti-cyclin E or anti-Cdk2 antibodies and observed similar levels of assembled cyclin E:Cdk2 complexes (Fig. 2*A*). Although we did not detect increased levels of p21 or p27 in TGF-β-treated cells (Fig. 1*A*), p27 can be displaced from other cyclin:Cdk complexes, resulting in an increased association of p27 with cyclin E:Cdk2 complexes (31). Therefore, we analyzed for association of p21 and p27 with Cdk2, Cdk6, and/or cyclin E from control and TGF-β-treated HepG2 cells (Fig. 2 *B* and *C*). We detected a consistent minor decrease of p21 or p27 associated with either cyclin E:Cdk2 and/or cyclin D:Cdk6 complexes in TGF-β-treated cells compared with controls. Recently, we have shown that HGF induces p27 in HepG2 cells (29), and as an internal control, we readily detected the association of p27 with cyclin E:Cdk2 complexes in HGF-treated cells (Fig. 2*C*).

p27 Cdk inhibitory activity originally was identified by performing WCE mixing experiments between TGF-β-treated and untreated cells (11). Therefore, to exclude/include the involvement of an unknown Cdk inhibitor induced by TGF-β, we performed a series of extract mixing experiments between treated and untreated HepG2 cells. Increasing amounts of WCE from 48 hr TGF-β-treated HepG2 cells were mixed with untreated HepG2 WCE followed by anti-cyclin E immunoprecipitation-kinase assays (Fig. 2*D*). We detected no alteration of cyclin E:Cdk2 activity from control lysates when increasing amounts of TGF-β-treated WCE were titered in (Fig. 2*D*, lanes 2–4). These observations suggest that an unknown Cdk inhibitor (other than p21, p27, or p57) was likely not involved in TGF-β-mediated inactivation of Cdk2 in HepG2 cells.

Activation of cyclin E:Cdk2 complexes requires dephosphorylation of phospho-Thr^{14}/Tyr^{15} residues on Cdk2 by CDC25A (4). Although we detected no decline in CDC25A protein levels (Fig. 1*A*), CDC25A could be inactivated or mislocalized in TGF-β-treated HepG2 cells. Treatment of anti-cyclin E:Cdk2 immune complexes from both control cells with glutathione *S*-transferase (GST)-CDC25A resulted in a 2.5-fold increase in activity (Fig. 2*E*, lane 3 vs. 4). Although cyclin E:Cdk2 complexes from TGF-β-treated cells showed a dramatic loss of activity (Fig. 2*E*), treatment with GST-CDC25A also increased the kinase activity 2.5-fold (Fig. 2*E*, lane 1 vs. 2). However, this level of activity was still below the activity of untreated cyclin E:Cdk2 complexes from control cells (Fig. 2*E*, lane 2 vs. 3). Therefore, inactivation of cyclin E:Cdk2 complexes in TGF-β-treated cells was not caused by increased levels of phospho-Thr^{14}/Tyr^{15} on Cdk2.

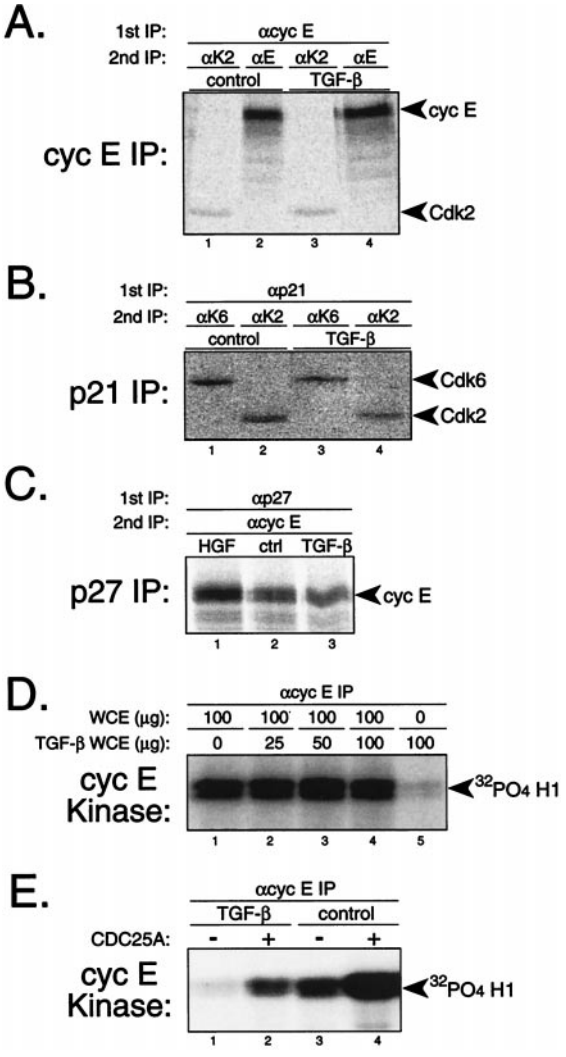

Fig. 2. TGF-β inactivation of Cdk2 is independent of Cdk inhibitors. (*A*) Primary anti-cyclin E immunoprecipitation (IP) followed by reimmunoprecipitation with anti-Cdk2 or anti-cyclin E antibodies from ^{35}S-methionine-labeled control and TGF-β-treated HepG2 cells. (*B* and *C*) TGF-β treatment of HepG2 cells does not alter p21 or p27 levels associated with cyclin:Cdk complexes. Note increased association of cyclin E with p27 in HGF-treated cells. (*D*) *In vitro* mixing experiment of increasing amounts of TGF-β-treated WCE into control WCE followed by anti-cyclin E immunoprecipitation-kinase assay. (*E*) TGF-β treatment of HepG2 cells does not result in increased Thr^{14}/Tyr^{15} phosphorylation. Anti-cyclin E immunoprecipitates from control and TGF-β-treated cells were incubated with glutathione *S*-transferase-CDC25A protein followed by Cdk2 kinase assay.

TGF-β Inhibition of the Activating Thr^{160} Phosphorylation on Cdk2. Activation of cyclin E:Cdk2 complexes requires phosphorylation of Thr^{160} on Cdk2 by CAK (4, 14). Cdk2 protein previously has been shown to migrate as two distinct bands on SDS/PAGE (32). Phosphorylation of Thr^{160} on Cdk2 results in a shift to the faster migrating, active form; however, Cdk2 migration is unaffected by the phosphorylation status of Thr^{14} and/or Tyr^{15} residues (4, 32). TGF-β-treated cells contained a marked reduction of the faster migrating, phospho-Thr^{160} active form of Cdk2 when assayed directly (Fig. 3*A*). Importantly, to exclude the possibility that loss

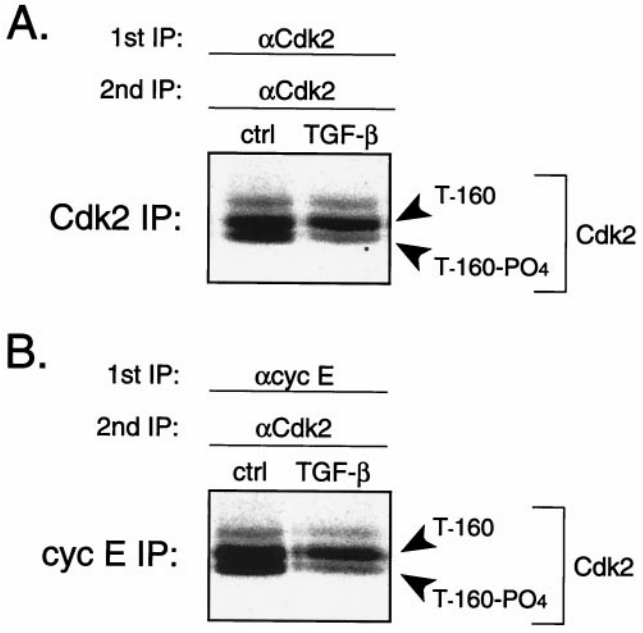

Fig. 3. Loss of Cdk2 Thr^{160} phosphorylation in TGF-β-treated HepG2 cells. Primary immunoprecipitation (IP) with anti-Cdk2 (*A*) and anti-cyclin E (*B*) antibodies followed by reimmunoprecipitation with anti-Cdk2 antibodies from ^{35}S-methionine-labeled control (ctrl) and TGF-β-treated cells. Cdk2 migrates as two species: the faster migrating, active form contains phospho-Thr^{160} whereas the slower migrating, inactive forms contain nonphosphorylated Thr^{160}. The presence of phospho-Thr^{14} and phospho-Tyr^{15} on Cdk2 do not affect migration (32).

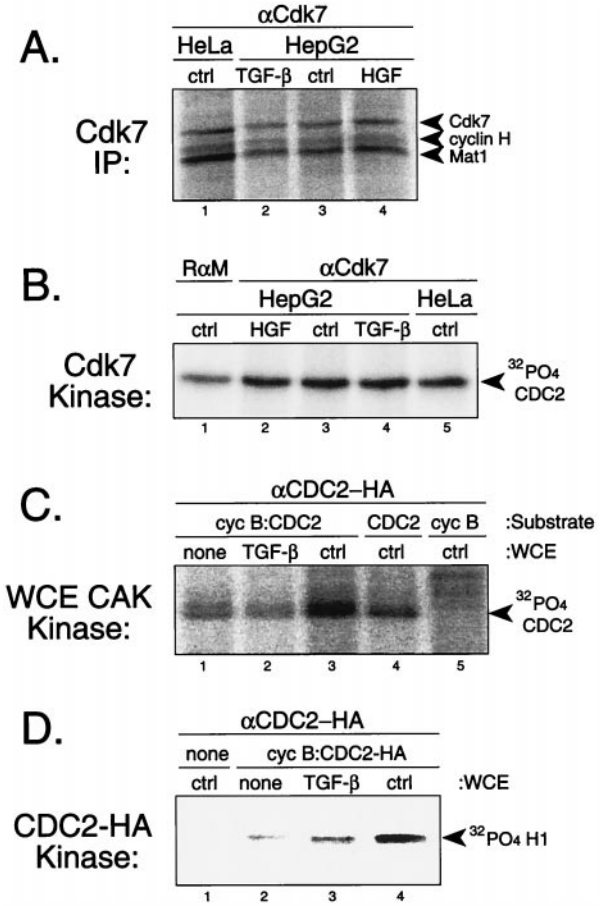

Fig. 4. Whole-cell lysates from TGF-β-treated HepG2 cells contain decreased CAK activity, but retain cyclin H:Cdk7 activity. (*A*) Immunoprecipitation (IP) with anti-Cdk7 antibodies from ^{35}S-methionine-labeled control (ctrl), TGF-β-, and HGF-treated lysates and control HeLa cell lysate. Positions of cyclin H, Cdk7, and Mat1 as indicated. (*B*) Anti-Cdk7 immunoprecipitation-kinase assay from control (ctrl), TGF-β-, and HGF-treated HepG2 cells and control HeLa cell lysates using purified cyclin B:CDC2-HA complexes as a substrate. (*C*) Whole-cell lysates from control (ctrl) and TGF-β-treated cells were incubated with γ^{32}P-ATP and purified cyclin B:CDC2-HA complexes, CDC2-HA alone, or cyclin B alone as substrates. (*D*) Whole-cell lysates from control (ctrl) and TGF-β-treated cells were incubated with ATP and purified cyclin B:CDC2-HA complexes followed by anti-HA immunoprecipitation and CDC2-HA activation by incubation with γ^{32}P-ATP and histone H1 as a substrate. None indicates no lysate or no cyclin B:CDC2-HA added; RαM, rabbit anti-mouse IgG negative control.

of phospho-Thr^{160} on total Cdk2 from TGF-β-treated cells was caused merely by the reduction of cyclin A expression (Fig. 1*A*), we directly examined cyclin E-associated Cdk2 and also found a significant 4.5-fold reduction of phospho-Thr^{160} Cdk2 from TGF-β-treated cells (Fig. 3*B*). In contrast, control cells contained both the faster migrating phospho-Thr^{160} form and the slower migrating, inactive forms of Cdk2. Two-dimensional tryptic phosphopeptide mapping of Cdk2 revealed no alteration of phospho-Thr^{14}/Tyr^{15} status between control and TGF-β-treated cells (data not shown); however, consistent with the observations of others (32), the tryptic Cdk2 peptide containing phospho-Thr^{160} was unresolvable. Thus, TGF-β treatment resulted in the specific absence of the CAK phosphorylated Thr^{160} site on Cdk2 *in vivo*.

TGF-β Inhibits Cdk2 CAK Activity Independent of Cyclin H:Cdk7. Several *in vitro* studies previously have indicated that cyclin H:Cdk7:Mat1 complexes may be a mammalian CAK (15, 16). We detected no alteration of cyclin H:Cdk7:Mat1 complexes from control, HGF-, and TGF-β-treated HepG2 cells and control HeLa cellular lysates (Fig. 4*A*). Anti-Cdk7 immunoprecipitation-kinase assays from all cellular lysates using purified baculovirus produced cyclin B:CDC2-HA (HA tagged) complexes as a substrate also showed no alterations in cyclin H:Cdk7 activity (Fig. 4*B*). Cyclin B:CDC2-HA complexes were chosen as a substrate because of high background activities of baculovirus produced cyclin A:Cdk2 complexes in our hands (data not shown) and as previously noted elsewhere (15). These observations demonstrate that loss of Cdk2 CAK activity in TGF-β-treated cells occurs in the presence of assembled and active cyclin H:Cdk7 complexes.

We next examined CAK activity in lysates from TGF-β-treated HepG2 cells. WCE from control and TGF-β-treated cells were incubated with γ^{32}P-ATP and purified cyclin B:CDC2-HA complexes as a substrate (Fig. 4*C*). Phosphorylation of CDC2-HA was readily detectable when incubated with control lysates (Fig. 4*C*, lane 3); however, CDC2-HA phosphorylation was substantially reduced when incubated with TGF-β-treated cell lysates (Fig. 4*C*, lane 2). Negative controls showed similar levels of CDC2-HA phosphorylation. To exclude the possibility of Thr^{14}/Tyr^{15} phosphorylation on the exogenous CDC2-HA substrate, cyclin B:CDC2-HA complexes were incubated with control or TGF-β-treated cellular lysates then immunoprecipitated with anti-HA antibodies and analyzed for activation of cyclin B:CDC2-HA kinase activity (Fig. 4*D*). Cyclin B:CDC2-HA complexes incubated with untreated, control lysates showed a marked activation of CDC2-HA kinase activity (Fig. 4*D*, lane 4), whereas incubation with TGF-β-treated lysates showed only a minor increase (Fig. 4*D*, lane 3) compared with the negative control (Fig. 4*D*, lane 2). Taken together, these observations are consistent with TGF-β signaling specifically inhibiting Cdk2 CAK activity in HepG2 cells that is exclusive of Cdk7 activity.

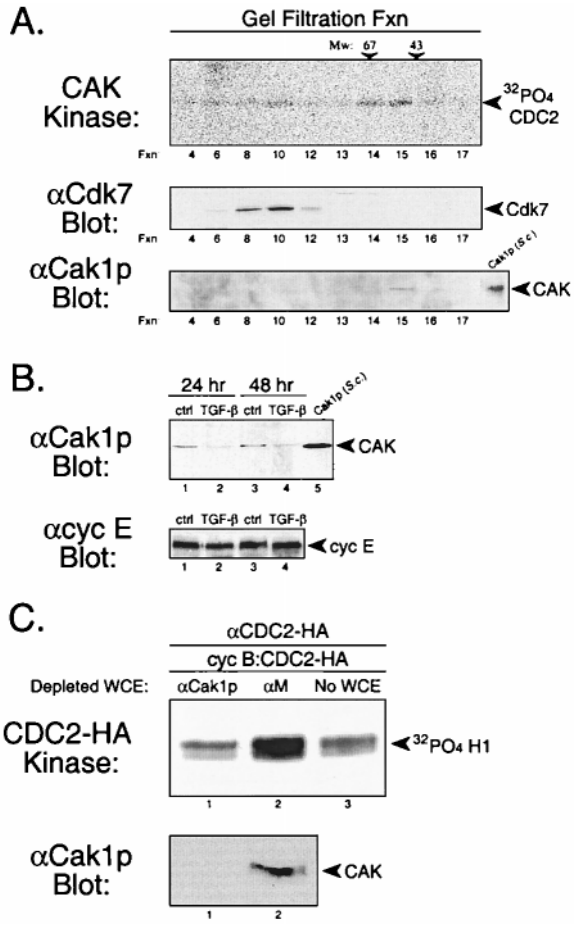

Fig. 5. TGF-β down-regulates a 45-kDa putative human CAK protein. (*A*) Superose-12 gel filtration fractionated of HepG2 lysates was analyzed for CAK activity by incubation with baculovirus-produced cyclin B:CDC2-HA complexes as a substrate (*Top*). Low-level CAK activity peak present in fraction 10 (>158 kDa) and a high-level CAK activity peak present in fraction 15 (≈48 kDa). Immunoblot analysis of gel filtration fractions with anti-Cdk7 (*Middle*) shows Cdk7 protein and activity (data not shown) present in fraction 10. Immunoblot analysis using anti-yeast Cak1p antibodies (*Bottom*) revealed a cross-reacting putative human CAK protein present in fraction 15 (and to a lesser extent fraction 14) at ≈45 kDa. Gel filtration molecular mass markers as indicated. (*B*) Immunoblot analysis of control (ctrl) and TGF-β-treated HepG2 whole-cell lysates probed with anti-yeast Cak1p antibodies (*Upper*). Anti-cyclin E immunoblot of the same filter was used to normalize for protein loading (*Lower*). (*C*) HepG2 whole-cell lysates were sequentially depleted with rabbit anti-Cak1p (αCak1p) or rabbit anti-mouse IgG (αM) antibodies then incubated with purified cyclin B:CDC2-HA complexes followed by anti-HA immunoprecipitation and assayed for CDC2-HA activation (*Upper*). No WCE, background levels of cyclin B:CDC2 activity in the absence of incubation with WCE. Anti-Cak1p immunoblot analysis showed specific loss of the 45-kDa CAK band from the anti-Cak1p depleted lysates (*Lower*).

TGF-β Signaling Down-Regulates a 45-kDa Protein with CAK Activity. To further dissect regulation of CAK activity by TGF-β, we chromatographically separated lysates from untreated HepG2 cells on a Superose-12 gel filtration column. CAK activity of column fractions was observed in two peaks: a low level activity peak was present in fraction 10 (>158 kDa) and a higher level activity peak was present in fraction 15 (and 14) (≈48 kDa) (Fig. 5*A*, *Top*). Cdk7 protein and kinase activity (data not shown) were confined to the low-level CAK activity peak present in fraction 10 and absent from the higher level CAK activity peak present in fraction 15 (Fig. 5*A*, *Middle*).

The *S. cerevisiae* CAK1 gene encodes a 44-kDa protein

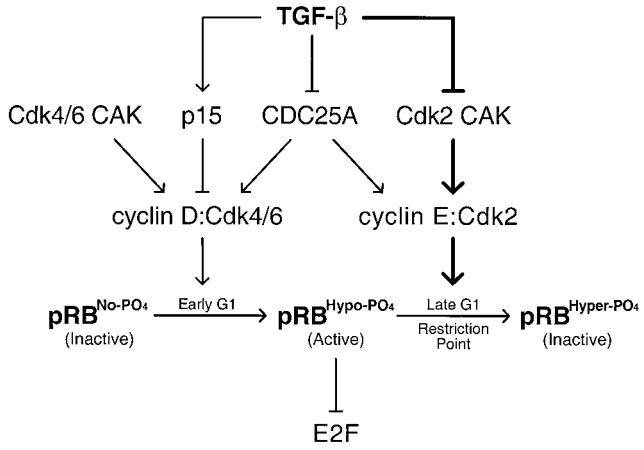

Fig. 6. Pathways of TGF-β mediated G_1 arrest. In cells containing an inducible $p15^{INK4b}$ or down-regulated CDC25A gene, cyclin D:Cdk4/6 complexes are targeted for inactivation by TGF-β signaling. In HepG2 cells, the inability of TGF-β to induce $p15^{INK4b}$ or down-regulate CDC25A levels results in continued cyclin D:Cdk4/6 activity because of presumed continued Cdk4/6 CAK activity. However, cyclin E:Cdk2 complexes are inactivated because of down-regulation of Cdk2 CAK activity. Cyclin D:Cdk4/6 complexes activate the pRB in early G_1 by hypophosphorylation (8–10). Cyclin E:Cdk2 complexes perform the initial inactivating hyper-phosphorylation of pRb at the late G_1 restriction point transition (8–10).

(Cak1p; refs. 23–25), which is similar in molecular mass to the CAK activity we observed in fraction 15 from HepG2 cells (≈48 kDa). Unexpectedly, rabbit antibodies directed against the yeast Cak1p protein cross-reacted with a purified 45-kDa related human protein with CAK activity (P.K. and M.J.S, unpublished observation). Immunoblot analysis using rabbit anti-yeast Cak1p antibodies detected a cross-reacting 45-kDa human protein present in column fraction 15 (and lightly in fraction 14) that was absent from fraction 10 (Fig. 5*A*, *Bottom*). Consistent with loss of Cdk2 activity, the 45-kDa putative CAK protein was down-regulated at 24 and 48 hr after TGF-β-treated cells (Fig. 5*B*, *Upper*). To control for protein loading, the same filter was reprobed with anti-cyclin E antibodies (Fig. 5*B*, *Lower*). In addition, we detected the presence of the 45-kDa putative CAK protein in HaCaT keratinocytes and Hep3B hepatocellular carcinoma cells; however, it was markedly reduced in both HeLa cervical carcinoma or HT1080 fibrosarcoma cells (data not shown).

Unfortunately, as has been observed with other antibodies, the rabbit anti-yeast Cak1p antibodies failed to immunoprecipitate an active kinase (unpublished observation). Therefore, to test whether the 45-kDa protein was indeed a human CAK, we sequentially depleted HepG2 whole-cell lysates with rabbit anti-yeast Cak1p or control rabbit anti-mouse IgG antibodies, then incubated the depleted lysates with cyclin B:CDC2-HA complexes and assayed for activation of CDC2-HA kinase activity (Fig. 5*C*, *Upper*). Anti-Cak1p depleted HepG2 lysates (lane 1) showed a marked reduction in the ability to activate cyclin B:CDC2-HA complexes compared with control anti-mouse IgG depleted lysates (lane 2) or negative control (lane 3). In addition, anti-Cak1p immunoblot analysis demonstrated the specific loss of 45-kDa CAK band from the anti-Cak1p-depleted lysates (Fig. 5*C*, *Lower*). These observations directly linked the 45-kDa anti-Cak1p cross-reacting protein to CAK activity.

Discussion

TGF-β-mediated G_1 growth arrest previously has been shown to specifically target inactivation of cyclin D:Cdk4/6 complexes by either induction of $p15^{INK4b}$ or down-regulation of CDC25A and Cdk4 protein levels (26–28). We report here the ability of TGF-β

to arrest human HepG2 hepatocellular carcinoma cells by targeting inactivation of cyclin E:Cdk2 complexes and thus, leaving pRB in its active, hypo-phosphorylated form (see Fig. 6). These observations are consistent with our previously demonstrated physiological role for cyclin D:Cdk4/6 complexes in activating pRB by hypo-phosphorylation and for cyclin E:Cdk2 complexes in inactivating pRB by hyper-phosphorylation at the late G_1 restriction point (8–10).

We observed a specific loss of the activating Thr^{160} phosphorylation on Cdk2. Biochemical analyses revealed inhibition of CAK activity and down-regulation of a 45-kDa putative human CAK protein levels in TGF-β-treated cells. Therefore, we conclude that TGF-β signaling induces a G_1 cell cycle arrest in HepG2 cells by a mechanism of inhibiting Cdk2 CAK activity. Moreover, the presence of active cyclin D:Cdk4/6 complexes in TGF-β-treated cells argues for at least two distinct CAKs: one specific for Cdk2/(CDC2) that is negatively regulated by TGF-β and one specific for Cdk4/6 that remains unaffected.

Cyclin H:Cdk7 complexes initially were classified as a mammalian CAK based primarily on *in vitro* biochemical experiments (15, 16). However, recently Larochelle *et al.* (22), using *Drosophila* genetics, demonstrated that embryos harboring a temperature-sensitive Cdk7 allele that was incubated at the nonpermissive temperature contained active cyclin E:Cdk2 complexes, but inactive CDC2 complexes. Significantly, this observation excludes Cdk7 as a Cdk2 CAK *in vivo* in higher eukaryotes and suggests that it could be only a CDC2 CAK, if at all. Importantly, both genetic and biochemical studies have demonstrated that cyclin H:Cdk7 complexes are a requisite component of TFIIH (17–20). Indeed, the presumed role of mammalian Cdk7 as both CAK and the TFIIH kinase are in direct contradiction with the generally observed expansion of a single yeast gene into a gene family in higher eukaryotes as opposed to compressing two independent yeast genes, *CAK1* and *KIN28*, into a single mammalian gene. In addition, *in vivo*, cyclin:Cdk complexes are proline-dependent Ser/Thr kinases (4); however, the activating Thr residues of Cdk2 (T-Y-T^{160}*-H-E-V), CDC2 (V-Y-T^{161}*-H-E-V), and Cdk6 (A-L-T^{177}*-S-V-V) are not followed by proline residues. Moreover, we did not detect any alteration of cyclin H:Cdk7 kinase activity in response to TGF-β treatment of HepG2 cells. Furthermore, the apparent low level of the 45-kDa human CAK protein in HeLa cells would serve to explain the absence of this CAK activity peak in the original studies using HeLa cell lysates (15).

Although our results demonstrate a TGF-β-mediated inhibition of Cdk2 CAK activity, we cannot formally exclude the possibility of TGF-β-dependent induction of an unknown Thr^{160} specific phosphatase. Currently, the only assumed Thr^{160} phosphatase is Cdk-associated phosphatase (KAP) (33, 34); however, recent work of Cheng *et al.* (35) has shown that the major Thr^{160} phosphatase acting on Cdk2 belongs to the type 2C family of protein phosphatases. Our experiments specifically focused on cyclin E:Cdk2 heterodimeric complexes and therefore potentially exclude any involvement of KAP. In addition, in an attempt to rule out any *in vitro* artifacts associated with the preparation of lysates, we detected the absence of the activating CDC2-HA phosphorylation from TGF-β-treated lysates containing broad phosphatase inhibitors (Na_3VO_4, NaF, and PP_i).

In conclusion, we present here a mechanism for TGF-β-mediated G_1 cell cycle arrest that targets inactivation of cyclin E:Cdk2 complexes by inhibition of Cdk2 CAK activity, while cyclin D:Cdk4/6 complexes remain active. Our observations argue for the existence of at least two members of a larger mammalian CAK family: one specific for Cdk2/(CDC2) and one specific for Cdk4/6.

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