Effects of TGF-β1 (transforming growth factor-β1) on the cell cycle regulation of human breast adenocarcinoma (MCF-7) cells

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Abstract The antiproliferative effects of TGF-β1 were investigated in a human breast adenocarcinoma cell line (MCF-7). We report that TGF-β1 inhibits proliferation through cell cycle arrest in G_1. A MCF-7 cell subline (MCF-7(−)), in which the type II TGF-β receptor is not detected, was shown to be resistant to TGF-β1 growth inhibitory effect. Cdk2 kinase activity was inhibited in the MCF-7 sensitive cell subline in parallel with the inhibition of cell cycle progression. In both sensitive and resistant cell lines, TGF-β1 treatment did not affect cdk2, cdk4, cyclin E and cyclin D1 mRNA and protein levels. However, in the MCF-7 sensitive cell subline, a time-dependent increase in cells positive for $p21^{WAF1/CIP1}$ nuclear localization was observed after TGF-β1 treatment. These findings suggest that TGF-β1 inhibition of MCF-7 cell proliferation is achieved through a type II receptor-dependent down-regulation of Cdk2 kinase activity without modification of Cdk and cyclin expression, but correlated with an increase in $p21^{WAF1/CIP1}$ nuclear accumulation.

Key words: TGF-β; Cell cycle regulation; Cyclin-dependent kinase (Cdk); $p21^{WAF1/CIP1}$; Adenocarcinoma

1. Introduction

Transforming growth factor-β1 (TGF-β1) inhibits epithelial cell proliferation by arresting the progression of the cell cycle at the G_1-to-S phase transition [1]. The molecular mechanisms of this growth inhibitory effect are still poorly understood and the signalling pathways used by TGF-β1 to arrest the cell cycle remain to be characterized. Three types of TGF-β receptors (type I (50 kDa), II (80–100 kDa), and III (200–400 kDa)) have been identified using cross-linking experiments with radiolabelled TGF-β1 [2]. Type I and type II receptors have recently been cloned and shown to encode serine-threonine kinases [3,4]. Type II TGF-β receptor is required for the antiproliferative activity of TGF-β1, whereas the type I mediates the induction of several genes implicated in the interactions between cell and extracellular matrix [5,6]. Type III receptor (or betaglycan) is not likely to mediate any of the TGF-β biological activities but is a direct regulator of its access to the signalling receptors [7]. TGF-β1 does not interfere with the binding of positive growth factors to their receptors or with their early signal transduction mechanisms. Previous work indicate that TGF-β affects late events in the mitogenic pathways, such as c-*myc* expression [8] and phosphorylation of the retinoblastoma gene product (Rb). TGF-β1 retains Rb in a hypophosphorylated state that may suppress progression into the S phase [1]. Rb binds to D-type cyclins and is phosphorylated by cyclin-dependent kinases [9]. These 'Cdk' kinases are related to $p34^{cdc2}$, an essential regulator of entry into mitosis, and are thought to be involved, associated with G_1 cyclins, in the progression in G_1 [10] and the regulation of the G_1-to-S phase transition [11]. It has therefore been hypothesized that TGF-β1 might regulate the expression or the activity of specific Cdk–cyclins complexes [12]. In mink lung epithelial cells, the growth inhibitory effect of TGF-β1 has been recently demonstrated to be linked to an inhibition of Cdk4 synthesis [13], which appears to prevent the activation of the cyclin E–Cdk2 complex [14]. Recently, the existence of negative regulators of cyclin–Cdk complexes, the CKIs (cyclin-dependent kinase inhibitors) $p16^{INK4}$, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, has been demonstrated [15]. It has been shown that in lung and mammary epithelial cells arrested in late G_1 by TGF-β1, the $p27^{KIP1}$ protein binds to cyclin E–Cdk2 complexes and prevents their activation [16,17]. A decrease in Cdk2 kinase activity induced by TGF-β1 was also accompanied by an induction of $p21^{WAF1/CIP1}$ in ovarian cancer cells [18]. Moreover, recent studies demonstrate that an additional CKI, called $p15^{INK4B}$, an inhibitor of Cdk4 and Cdk6 kinase activities, is strongly expressed after TGF-β1 treatment of human keratinocytes [19]. These observations suggest that different CKIs may be implicated in TGF-β1-mediated cell cycle arrest.

Here, we report a comparative study of TGF-β1 growth inhibitory effect on human breast adenocarcinoma sensitive (MCF-7) and resistant (MCF-7(−)) cell sublines. We have found that TGF-β1 arrests the cell cycle (i) in a type II TGF-β receptor-dependent manner, (ii) with a decrease in Cdk2 kinase activity but in the absence of Cdk4 protein regulation, and (iii) with a 3 fold increase in the percentage of cells displaying a $p21^{WAF1/CIP1}$ nuclear localization.

2. Materials and methods

2.1. Chemicals

Porcine TGF-β1 was purchased from R&D systems Inc. (Minneapolis, MN, USA). Propidium iodide and RNase A were obtained from Sigma. Bis(sulfosuccinimidyl) suberate (BS^3) was obtained from Pierce. [^{125}I]TGF-β1 (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from NEN.

2.2. Cell culture

MCF-7 (passage 150) and MCF-7 (−) (passage 300) were grown in a humidified atmosphere (5% CO_2/95% air) at 37°C in RPMI 1640 medium supplemented with sodium hydrogen carbonate (2 g/l), 2 mM glutamine, 1 μM insulin, gentamycin (25 mg/l) and 5% fetal calf serum (FCS). CCL-64 cells were grown in MEM medium supplemented with sodium hydrogen carbonate (2 g/l), 2 mM glutamine, gentamycin (25 mg/l) and 5% FCS.

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2.3. Cell growth experiments

Cells were plated in 35-mm Petri dishes at a density of 10,000 cells/cm^2. On the following day, the media were changed and TGF-β1 (40 pM) was added. The media were changed every 3 days and cell number was determined. Cell cycle phase distribution was determined by analytical DNA flow cytometry as previously described [20] using a Facscan flow cytometer (Becton Dickinson).

2.4. TGF-β1 receptor affinity labelling

Subconfluent cells were affinity labelled using 80 pM [^{125}I]TGF-β1 in presence or absence of 4 nM unlabelled TGF-β1. TGF-β1 binding sites on intact monolayers were cross-linked to [^{125}I]TGF-β1 using BS^3 according to [21]. Detergent extracts from affinity labelled cells were separated on 7% polyacrylamide gels under reducing conditions. For autoradiography, the dried gels were exposed to Hyperfilm MP (Amersham) at −70°C for 7 days.

2.5. Northern blot

Total cellular RNA was prepared using the one-step acid guanidinium isothiocyanate/phenol-chloroform extraction method [22], size fractionated (15 μg per lane) on 1.2% agarose-formaldehyde (2.2M) gels, blotted onto Hybond-N membrane (Amersham) by capillary transfer and UV cross-linked. mRNA expression was analysed using ^{32}P-labelled cDNA probes corresponding to the 1 kb *Bam*HI–*Bam*HI human cdk2 fragment, the 1.3 kb *Eco*RV–*Bgl*II human cyclin D1 fragment, the 1.4 kb *Bam*HI-*Hin*dIII human cyclin E fragment, and the 0.8 kb *Eco*RI–*Xho*I murine cdk4 fragment. In order to check for differences in the amounts of RNA transferred, the filters were probed with a 0.6 kb 36B4 cDNA fragment [23]. Signal intensities were evaluated by scanning densitometry of the autoradiograms.

2.6. Western blot, immunoprecipitation and kinase activity determination

Cell lysates were obtained as described in [10]. Total proteins (70 μg) were analysed by electrophoresis on polyacrylamide gels (12%) and transferred to nitrocellulose membrane by semi-dry blotting. Western Blot experiments were performed using rabbit antibodies raised against human Cdk2 specific carboxy-terminal peptide [24], against purified human recombinant cyclin A [25] and cyclin D1 [10]. For Cdk4 and cyclin E, rabbit antibodies raised against specific carboxy-terminal peptides were prepared and characterized as described in [10] for cyclin D1 antibodies. Immunoprecipitations and histone H1 kinase assays were performed as described in [24]. For p21$^{WAF1/CIP1}$ detection by Western blot after Cdk2 immunoprecipitation, a monoclonal antibody against p21$^{WAF1/CIP1}$ (Oncogene Science) was used at the dilution recommended by the manufacturer.

2.7. Immunofluorescence

Cells growing on glass coverslips were washed once with PBS and fixed in PBS/3.7% formaldehyde for 15 min at 4°C. Cell permeabilization was performed in PBS/0.25% Triton X-100 for 5 min at room temperature and methanol 100% for 10 min at −20°C. Cells were then washed twice in PBS and incubated in PBS/FCS 1% for 15 minutes at room temperature. Fixed and permeabilized cells were incubated overnight at 4°C with a monoclonal anti-p21$^{WAF1/CIP1}$ antibody (dilution 1:100; Oncogene Science) in PBS/FCS 1%. After three washes in the same buffer, cells were incubated with Texas red-conjugated sheep anti-mouse antibody (dilution 1:200; Amersham). Coverslips were washed twice with PBS/FCS 1%, three times with PBS and finally incubated for 10 min at room temperature with a 0.1 μg/ml solution of DAPI in PBS. After an additional rinse in PBS, coverslips were mounted on glass slides with Mowiol.

3. Results and discussion

3.1. TGF-β1 growth inhibitory effects

Growth and cell cycle parameters were examined in human

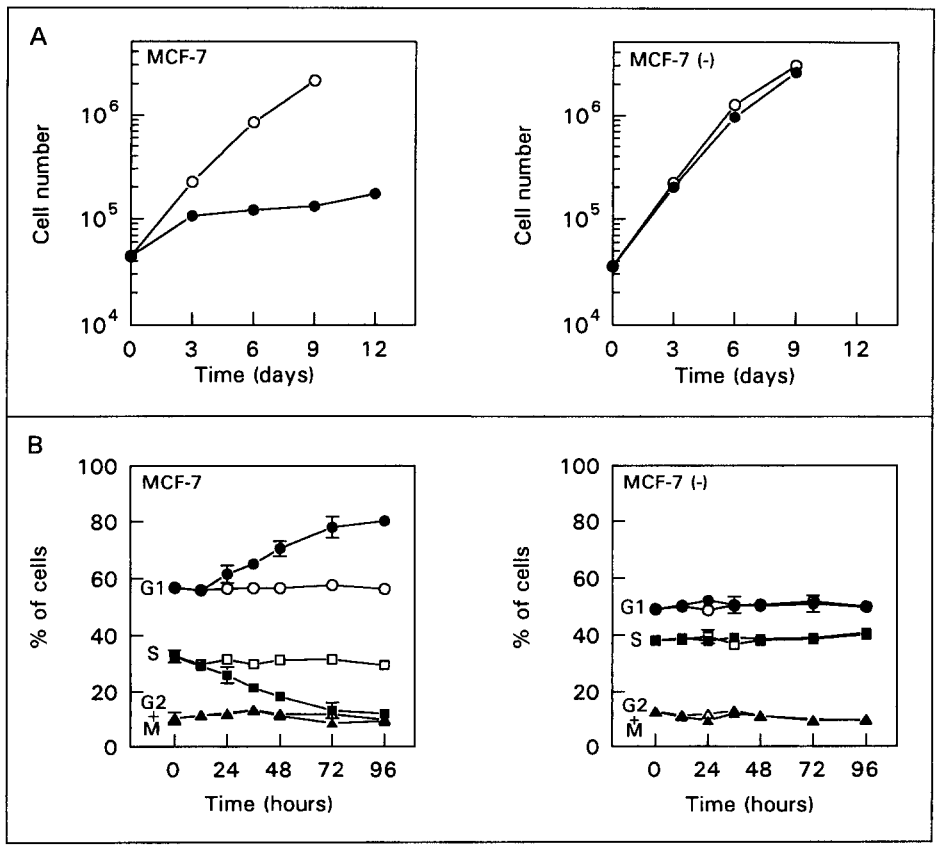

Fig. (A) Time–course of TGF-β1 growth inhibition effect on MCF-7 and MCF-7(−) cells untreated (open circles) or treated (filled circles) with TGF-β1 (40 pM). Each point is the mean of triplicate determinations. (B) Time–course effect of TGF-β1 (40 pM) on cell cycle distribution in MCF-7 and MCF-7(−) cells. Circles, squares and triangles are for G_1, S and G_2 + M phases, respectively. Open symbols correspond to control cells; filled symbols correspond to TGF-β1 (40 pM)-treated cells. Each point is the mean ± S.D. of three independent experiments.

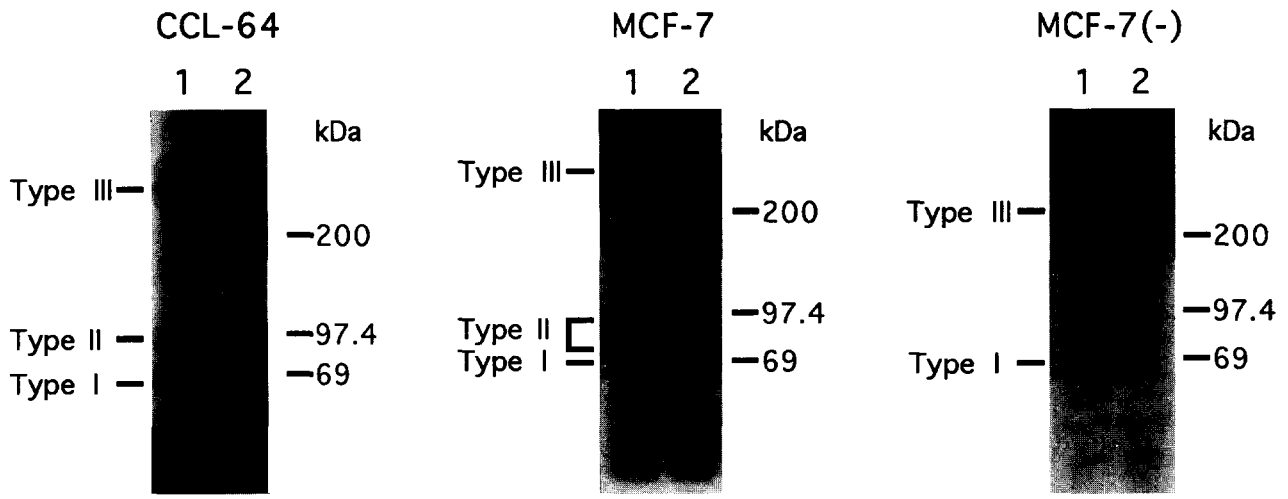

Fig. 2. Chemical cross-linking of ^{125}I-labeled TGF-β1 performed on CCL-64, MCF-7 and MCF-7(−) cells in the absence (lanes 1) or presence (lanes 2) of a 50 fold excess of unlabeled TGF-β1. The position of molecular size markers are indicated.

breast adenocarcinoma cell sublines (MCF-7 and MCF-7(−)) treated with TGF-β1. As shown in Fig. 1A, the addition of TGF-β1 (40 pM) to MCF-7 cells resulted in a time-dependent inhibition of cell proliferation. The half-maximal effect was observed with 5 pM TGF-β1 (data not shown). MCF-7(−) cell proliferation was not affected by TGF-β1 (Fig. 1A) even at concentrations as high as 100 pM (data not shown). A time–course study of the cell cycle distribution in MCF-7 cell sublines treated with TGF-β1 (40 pM) was performed using flow cytometry. MCF-7 cells showed little change until 24 hours of TGF-β1 treatment, then the percentage of cells in S phase began to fall while the percentage of cells in G_1 phase increased (Fig. 1B). Under the same conditions, TGF-β1 did not affect the distribution in the cell cycle phases of MCF-7(−) cells. In the MCF-7 cell line, TGF-β1 inhibition of proliferation can be correlated with a specific arrest in the G_1 phase of the cell cycle. In the literature, there are conflicting reports concerning the sensitivity of MCF-7 cells to TGF-β1 [26,27]. These discrepancies might be due to the use of MCF-7 cell sublines cultivated at different passages, the cells diverging with time in culture. In our study, MCF-7(−) cells were at passage 300 while MCF-7 cells were at passage 150.

3.2. *MCF-7(−) cells lack type II TGF-β receptor*

By performing cross-linking experiments, we investigated whether the lack of TGF-β1 growth inhibitory effect on MCF-7(−) cells can be correlated with a modification of TGF-β receptor expression (Fig. 2). CCL-64 cells were used as a positive control since these cells are known to express three detectable TGF-β1 binding proteins. The overall expression of TGF-β receptors was lower in MCF-7 cell sublines compared to CCL-64 cells. In MCF-7 cells, a specific labelling with [^{125}I]TGF-β1 was detectable above 200 kDa (type III receptor) and between 70 and 90 kDa, suggesting an overlap of type I and II TGF-β receptors. In MCF-7(−) cells, no specific labelling could be observed at a position corresponding to type II TGF-β receptor, whereas type I TGF-β was detected as a single labelled protein (70 kDa). Thus, the inability of TGF-β1 to block the cell cycle progression in G_1 and to inhibit proliferation of MCF-7(−) cells correlates with the fact that the type II TGF-β receptor is undetectable in these cells. Furthermore, it has been previously reported that MCF-7 cells resistant to the TGF-β growth inhibitory effect do not express mRNA for the type II TGF-β receptor [4]. Both type II and type I receptors are required for TGF-β's biological effects [28]. However, the inactivation of type II receptor induces a specific abrogation of the TGF-β1 antiproliferative effect without affecting the TGF-β1 effects on extracellular matrix [6]. We have shown that TGF-β1 is able to induce its own transcription in MCF-7(−) cells that are insensitive to its growth inhibitory effect [29], and to increase the expression of the *jun* gene family [30]. Hence, in MCF-7 cells, the modification of TGF-β1 sensitivity in long-term tissue culture specifically affects the growth inhibitory response in correlation with the loss of the type II TGF-β receptor.

3.3. *Inhibition of Cdk2 kinase activity*

It has recently been reported that, in normal mink lung epithelial cells, TGF-β1 inhibition of the G_1-to-S phase transition was related to a lack of Cdk2–cyclin E complex activity [13,14]. We therefore investigated the regulation of this kinase activity in MCF-7 cell sublines during TGF-β1 treatment. In MCF-7

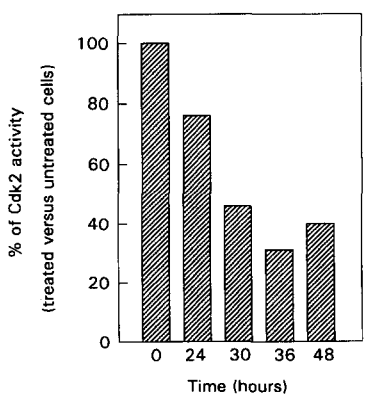

Fig. 3. Kinetic study of the TGF-β1 effect on Cdk2 activity in MCF-7 cells. Kinase activities were assayed on histone H1 after immunoprecipitation with anti-Cdk2 antibodies on 750 μg total proteins. Results are expressed as a percentage of kinase activity in treated vs. untreated cells.

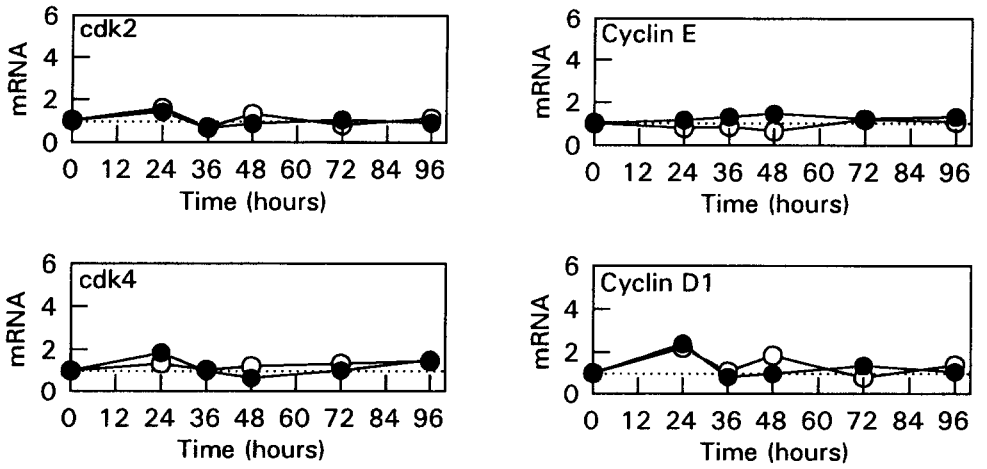

Fig. 4. Time-course effects of TGF-β1 (40 pM) on cdk2, cdk4, cyclin D1 and cyclin E mRNA expression in MCF-7 and MCF-7(−) cells. mRNA levels (normalized to 36B4 mRNA by densitometric scanning) are expressed as a ratio between TGF-β1-treated and untreated cells. Filled circles correspond to MCF-7 cells; open circles correspond to MCF-7(−) cells.

sensitive cells, the overall histone H1 kinase activity associated with immunoprecipitates of Cdk2 was found to decrease in parallel with the occurrence of cell cycle parameter modifications (Fig. 3). In this cell line, cyclin A associated kinase activity was also decreased by 25% after 30 h of treatment. Since Cdk2 is known to be predominantly in complex with cyclin A and to be involved in S phase progression [24], this result might in part reflect the inhibition of entry into S phase. In MCF-7(−) cells, neither the Cdk2 nor the cyclin A-associated kinase activities displayed any significant changes upon TGF-β1 treatment (data not shown).

3.4. Expression of cyclins and Cdks

We investigated in the two MCF-7 cell sublines the TGF-β1 effects on the expression of Cdks and cyclins implicated in G_1-to-S phase progression. The relative levels of cdk4, cdk2, cyclin D1 and cyclin E mRNA were analysed by Northern blotting of total RNA from TGF-β1 (40 pM) treated and un-

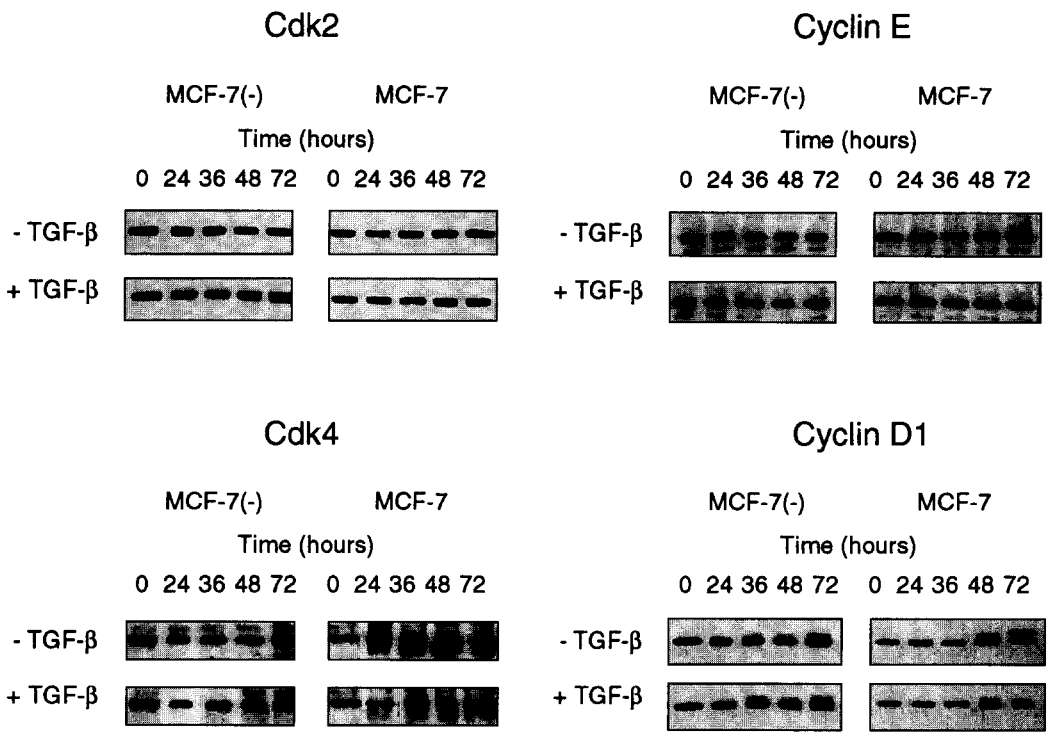

Fig. 5. Time-course effects of TGF-β1 (40 pM) on Cdk2, Cdk4, cyclin D1 and cyclin E protein expression in MCF-7 and MCF-7(−) cells. The identity and the significance of the upper band labelled with the anti-cyclin D1 antibody, present in several samples, is under investigation.

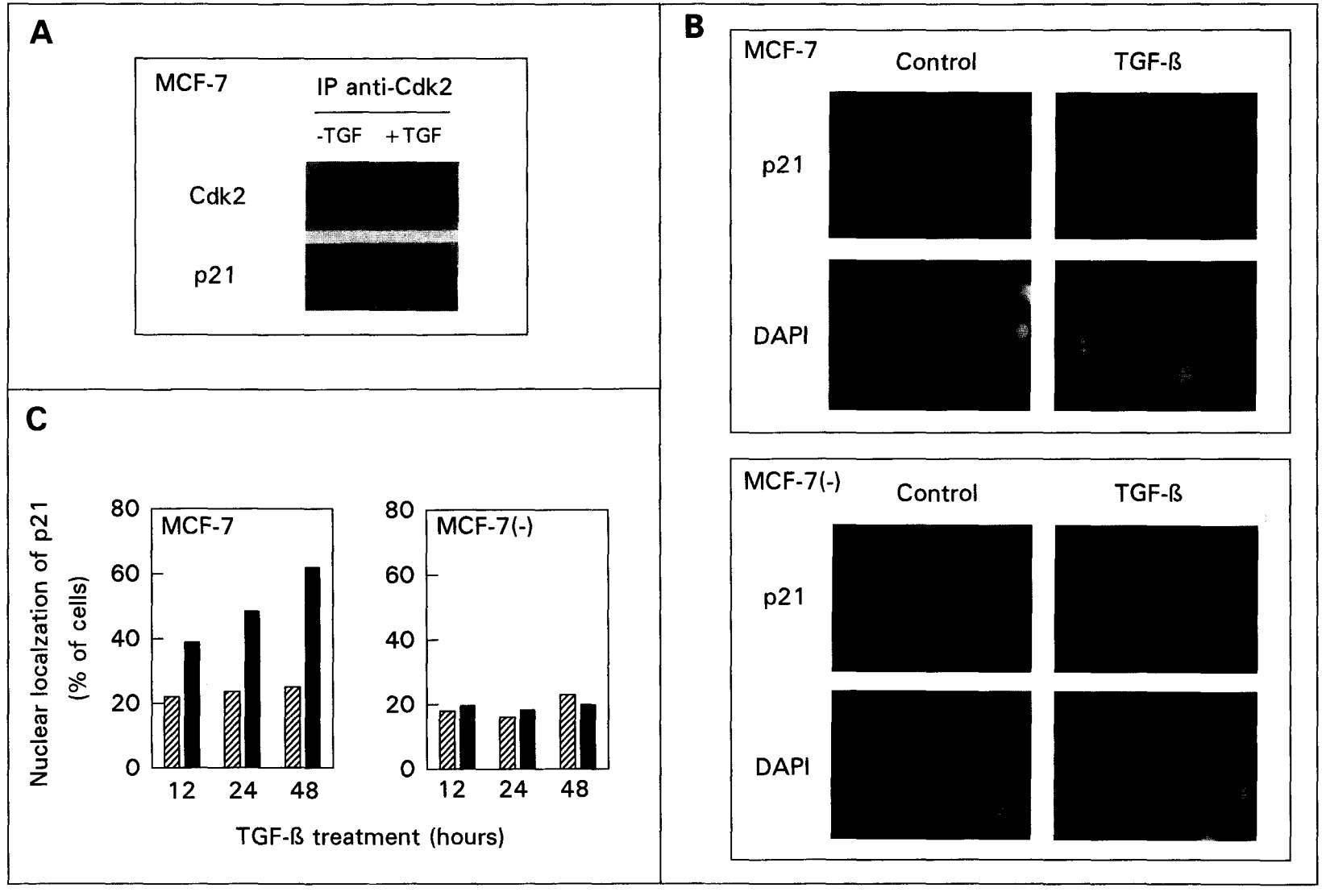

Fig. 6. Regulation of $p21^{WAF1/CIP1}$ in TGF-β1-treated MCF-7 cells. (A) Western blot showing expression of Cdk2 and $p21^{WAF1/CIP1}$ after Cdk2 immunoprecipitation in 48 h TGF-β1 (40 pM)-treated or untreated MCF-7 sensitive cells. (B) Immunofluorescence analysis of $p21^{WAF1/CIP1}$ localization in MCF-7 and MCF-7(−) cells untreated or treated with TGF-β1 (40 pM) for 48 h. Nuclei are visualized by DAPI staining. (C) Percentage of cells exhibiting nuclear localization of $p21^{WAF1/CIP1}$ at various times after TGF-β1 treatment (40 pM).

treated MCF-7 and MCF-7(−) cells. As shown in Fig. 4, there was no detectable variation of these transcripts after TGF-β1 treatment either in MCF-7 or in MCF-7(−) cells. In addition, Western blot analysis of cellular extracts did not reveal any modification of Cdk4, Cdk2, cyclin D1 and cyclin E protein levels induced by TGF-β1 treatment in any of the two cell lines (Fig. 5).

Several controversial observations have recently been reported about TGF-β1 effects on the expression of cell cycle regulatory proteins. In human keratinocytes, TGF-β1 decreases Cdk4, Cdk2 and cyclin E at mRNA and protein levels [31]. In normal mink lung epithelial cells (CCL-64), a decrease in Cdk4 protein level in the absence of mRNA regulation was reported [13]. Moreover, in these cells, constitutive over-expression of Cdk4 suppresses the agreement with those previously reported in CCL-64 cells by Koff et al. [14]. However, in MCF-7 cells sensitive to the TGF-β1 antiproliferative effect, we did not detect any decrease in Cdk4 protein levels (Fig. 5), whereas TGF-β1 reduced Cdk4 protein levels in normal endometrial cells (data not shown). The discrepancies in TGF-β1 effects between normal cells (CCL-64, keratinocytes and endometrial cells) and human mammary adenocarcinoma cells (MCF-7) may be cell specific and/or may reflect differences in cell cycle regulation between normal and tumoral cells.

3.5. Regulation of $p21^{WAF1/CIP1}$ in TGF-β1-treated MCF-7 cells

An increase in $p21^{WAF1/CIP1}$ has been recently reported in TGF-β1-treated ovarian adenocarcinoma cells [18]. We therefore checked for the presence of $p21^{WAF1/CIP1}$ in Cdk2 immunocomplexes from MCF-7 cells (Fig. 6A). We found that $p21^{WAF1/CIP1}$ was associated with Cdk2 both in control and TGF-β1-treated cells. However, due to the low level of $p21^{WAF1/CIP1}$ detected in Cdk2 immunoprecipitates, it was difficult to conclude that TGF-β1 induces a variation of $p21^{WAF1/CIP1}$ in these complexes. We therefore decided to investigate by immunofluorescence whether $p21^{WAF1/CIP1}$ subcellular localization was regulated (Fig. 6B and C). In 25% of control MCF-7 cells, $p21^{WAF1/CIP1}$ was detected in the nucleus. After TGF-β1 treatment, we observed a time-dependent increase in the number of $p21^{WAF1/CIP1}$ nuclear positive cells. 48 h after TGF-β1 treatment, 62% of cells were positive. No such variations were observed in TGF-β1-resistant MCF-7(−) cells under the same conditions. These results indicate that TGF-β1 induces a nuclear accumulation of $p21^{WAF1/CIP1}$ in MCF-7 sensitive cells. $p21^{WAF1/CIP1}$ exists in association with active as well as inactive kinase complexes, and a very low variation in the level of inhibitor present in the complex could lead to an abrupt transition from a fully active to an essentially inactive kinase [19]. Thus, it is conceivable that an undetectable variation in the association of $p21^{WAF1/CIP1}$ with Cdk2 complexes could lead to inhibition of this kinase activity in MCF-7 cells. How the fine-tuning of this interaction is regulated by TGF-β1 and what is the significance of nuclear localization of $p21^{WAF1/CIP1}$ are therefore important questions that remain to be investigated.

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