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K252a Inhibits the Phosphorylation of pRb without Changing the Levels of G1 Cyclins and Cdk2 Protein in Human Hepatoma Cells

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A protein kinase inhibitor K252a suppressed the growth of HuH7 hepatoma cells and the hyperphosphorylation of retinoblastoma protein (pRb) at late G_1 phase of cell cycle. However, K252a treatment did not alter the levels of cyclin D1, cyclin E, cyclin A and Cdk2 protein bound to cyclin E or cyclin A. Therefore, the K252a inhibition of pRb phosphorylation is considered to be brought about probably by inhibiting the action of Cdk-cyclin complex rather than by changing its cellular level. These results also suggest that K252a is a useful tool for investigating the mechanism of phosphorylation of pRb mediated by Cdk-cyclin.

K252a is a protein kinase inhibitor isolated from the culture broth of Nocardiopsis species (1,2). As reported previously, K252a inhibited the proliferation of human hepatoma cells by inducing G_1 arrest (3). However, detailed mechanism of K252a action remained to be studied. The cell-cycle progression through G_1 is regulated by cyclins and cyclin-dependent protein kinases (Cdks) (4,5). Their actions are mediated probably by tumor suppresser proteins such as retinoblastoma protein (pRb) and p53 (6,7). Since the activity of pRb and p53 as cell cycle regulators depends on their phosphorylation state, it is supposed that K252a induces G_1 arrest in human hepatoma cells by inhibiting the phosphorylation of these proteins (8). The present study was carried out to analyze the effects of K252a on the phosphorylation of pRb.

MATERIALS AND METHODS

Materials. K252a was purchased from Kyowa Hakko, Co., Ltd. (Machida, Japan). Monoclonal antibodies to human Rb protein (G3-245), human cyclin D1 (G124-326), human cyclin E (HE67 and HE12) and human Cdk2 protein (G120-72) were purchased from Pharmingens Co. Ltd. (San Diego, USA). Monoclonal antibody to human cyclin A (CHLA-3,13) was purchased from Upstate Biotechnology Inc. (Lake Placid, USA). Peroxidase-labeled goat antibody to mouse IgM was purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, USA). Horseradish peroxidase-labeled horse antibody to mouse IgG was purchased from Vector Laboratories Inc. (Burlingame, USA).

Cell culture. The HuH7 and HepG2 human hepatoma cells were cultured in an RPMI1640 medium containing 1% fetal bovine serum (FBS) or in Dulbecco's modified MEM (DMEM) containing 5% FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were seeded in 60 mm dish (Falcon) at a density of 1×10^4 cells/cm^2. The cell growth was arrested by two days of serum-free culture. Then, HuH7 and HepG2 cells were stimulated with RPMI1640 + 1% FBS and DMEM + 5% FBS, respectively. K252a was added to the culture medium at the start of the serum-stimulation. The cells were incubated for indicated hours and counted with a hemocytometer.

Immuno-precipitations and Western blot analysis were carried out as described (5). Cells were lysed in ice cold lysis buffer (25 mM Tris-Cl, pH7.4, 2% NP40, 50 mM NaCl, 50 mM NaF, 0.5% Na deoxycholate, 1 mM Na orthovanadate, 1 mM PMSF, 0.2% SDS, 20 μM leupeptin, and 5 μg/ml aprotinin). The lysates were then cleared of insoluble debris by centrifugation for 15 min. Protein contents were determined by Bradford analysis (Bio-Rad protein assay) (9). Cell lysates were incubated with monoclonal antibody to cyclin E or A, and protein A-Sepharose beads (Pharmacia Biotech. AB, Uppsala, Sweden). Resulting precipitates (for $p33^{Cdk2}$) or whole cell lysates (for other proteins) were subjected for Western blot analysis using a 8% (for pRb) or 13% (for other proteins) SDS-PAGE. pRb, cyclins and $p33^{Cdk2}$ blotted on the membrane filters were visualized using monoclonal antibodies with peroxidase-labeled secondary antibodies and DuPont Western Blot chemiluminescence reagent (DuPont NEN Research Products, Boston).

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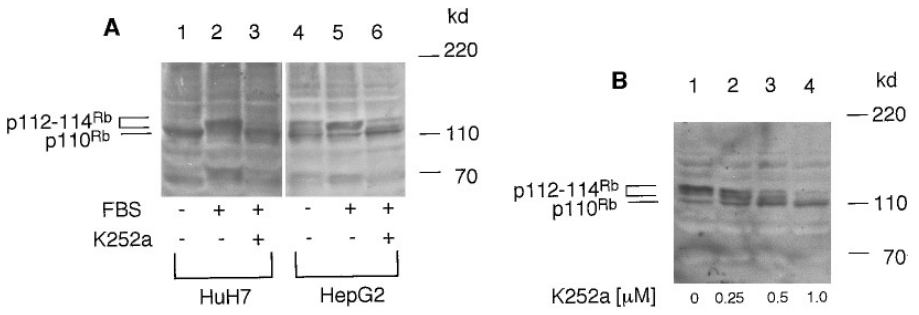

FIG. 1. K252a inhibits the serum-induced phosphorylation of retinoblastoma protein (pRb). (A) HuH7 and HepG2 cells were treated for 14 hours with 1% and 5% FBS, respectively. K252a (1 μM) was added into the growth media. pRb from these cells were subjected for immunoblot analysis. lane 1-3, HuH7; lane 4-6, HepG2; lane 1 and 4, serum free; lane 2 and 5, FBS; lane 3 and 6, FBS plus K252a. (B) HuH7 cells were incubated for 13 hours in the medium with 1% FBS and different concentrations of K252a. pRb from these cells were subjected for immunoblot analysis. lane 1, K252a 0 μM; lane 2, K252a 0.25 μM; lane 3, K252a 0.5 μM; lane 4, K252a 1 μM. $p110^{Rb}$, 110 kd dephosphorylated pRb; $p112\text{-}114^{Rb}$, 112-114 kd hyperphosphorylated pRb.

RESULTS

The effects of serum and K252a on the phosphorylation state of pRb were demonstrated in Fig.1. In serum-free culture, HuH7 and HepG2 cells did not proliferate. Their pRb remained to be dephosphorylated and formed a major band at 110 kd. On addition of serum into the serum-free culture, cells began to proliferate. pRb isolated from these serum-stimulated cells migrated at 112-114 kd, indicating that this pRb was heavily phosphorylated (10). K252a added simultaneously with serum inhibited this serum-induced phosphorylation of pRb (Fig.1A). The inhibitory effect of K252a on pRb phosphorylation was dose-dependent as demonstrated in Fig.1B.

The effects of serum and K252a on the cellular levels of cyclins were examined next. Fig.2A demonstrates that the serum treatment increased cellular levels of cyclin D1. K252a added simultaneously with serum did not suppress this serum-induced increase in cellular levels of cylcin D1 (Fig.2A). Furthermore, the levels of cyclin E and A were not altered by K252a treatment (Fig.2B). In addition, K252a did not alter the levels of Cdk2 protein bound to cyclin E or cyclin A (Fig.3).

DISCUSSION

K252a is a protein kinase inhibitor that induces G_1 arrest in human hepatoma cells as reported previously (3). Present study disclosed that K252a inhibited the phosphorylation of

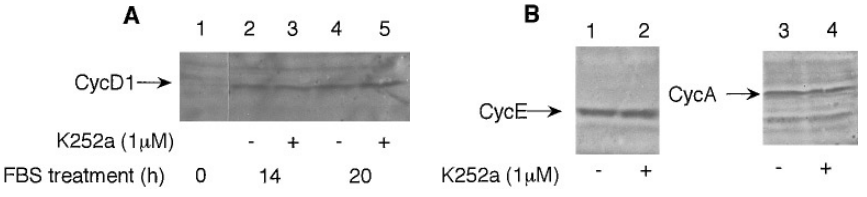

FIG. 2. Effects of K252a on the cyclin levels of HuH7 hepatoma cells. (A) The levels of cyclin D1 proteins. HuH7 cells were treated for different times (lane 1, 0 hr; lane 2 and 3, 14 hr; lane 4 and 5, 20 hr) with 1% FBS only (lane 2 and 4) or 1% FBS plus 1 μM K252a (lane 3 and 5). (B) The levels of cyclin E and cyclin A proteins. HuH7 cells were preincubated without (lane 1,3) or with 1 μM K252a (lane 2,4) and stimulated with 1% FBS for thirteen hours (cyclin E, lane 1,2) or twenty hours (cyclin A, lane 3,4).

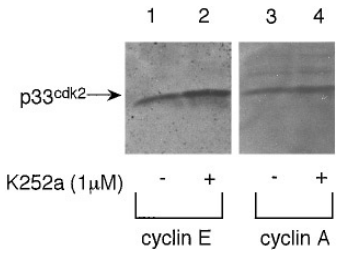

FIG. 3. The levels of Cdk2 protein of HuH7 cells. HuH7 cells were treated for fourteen hours with 1% FBS (lane 1,3) or 1% FBS plus 1 μM K252a (lane 2,4). Cdk2 proteins bound to cyclin E (lane 1,2) or cyclin A (lane 3,4) were isolated from them and immunoblotted.

pRb of HuH7 hepatoma cells without changing the levels of cyclins and Cdk2 protein. It is known that hyperphosphorylation of pRb at G_1 phase of cell cycle is necessary for G_1/S transition (8). Therefore, it is conceivable that the inhibition of pRb phosphorylation by K252a is responsible, at least partly, for the G_1 arrest induced by this protein kinase inhibitor.

The pRb is phosphorylated by Cdk-cyclin complex (6). Cyclins are supposed to regulate Cdk activity by changing their nuclear levels during the cell cycle progression (4,11). In the present study, serum treatment of human hepatoma cells was disclosed to increase pRb phosphorylation and nuclear cyclin D1. This provides further data for this supposition. On the other hand, K252a did not change the levels of cyclin D1, E and A. This suggests that K252a inhibits serum-induced pRb phosphorylation by interfering the Cdk2 activity directly. Recently, it has been reported that Cdk2 activity is regulated by threonine phosphorylation mediated by the Cdk-activating kinase (12,13,14). Therefore, it is conceivable that K252a inhibits pRb phosphorylation by changing the phosphorylation state of Cdk2-cyclin complexes. However, electrophoretic mobility of cyclin E and A and Cdk2 was not altered by K252a treatment, suggesting that K252a did not change the phosphorylation state of these proteins remarkably (15). Other possible mechanisms such that K252a inhibits pRb phosphorylation by altering the function of Cdk inhibitory subunits cannot be ruled out completely (16,17). The cell cycle regulation by Cdk-cyclin complexes and pRb is considered to be mediated by protein phosphorylation. However, detailed mechanisms of phosphorylation of cell cycle regulatory proteins remain to be studied. A novel protein kinase inhibitor K252a may be a useful tool for analyzing the mechanisms of phosphorylation of these proteins and cell cycle regulation.

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