p38 mitogen-activated protein kinase contributes to cell cycle regulation by cAMP in FRTL-5 thyroid cells

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Abstract
Objective: Thyrotropin activates the cAMP pathway in thyroid cells, and stimulates cell cycle progression in cooperation with insulin or insulin-like growth factor-I. Because p38 mitogen-activated protein kinases (p38 MAPKs) were stimulated by cAMP in the FRTL-5 rat thyroid cell line, we investigated (i) the effect of the specific inhibition of p38 MAPKs on FRTL-5 cell proliferation and (ii) the mechanism of action of p38 MAPKs on cell cycle control, by studying the expression and/or the activity of several cell cycle regulatory proteins in FRTL-5 cells.
Methods: DNA synthesis was monitored by incorporation of [3H]thymidine into DNA and the cell cycle distribution was assessed by fluorescence-activated cell sorter analysis. Expression of cell cycle regulatory proteins was determined by Western blot analysis. Cyclin-dependent kinase 2 (Cdk2) activity associated to cyclin E was immunoprecipitated and was measured by an in vitro kinase assay.
Results: SB203580, an inhibitor of α and β isoforms of p38 MAPKs, but not its inactive analog SB202474, inhibited DNA synthesis and the G1-S transition induced by forskolin plus insulin. SB203580 inhibited specifically p38 MAPK activity but not other kinase activities such as Akt and p70-S6 kinase. Treatment of FRTL-5 cells with SB203580 decreased total and cyclin E-associated Cdk2 kinase activity stimulated with forskolin and insulin. However, inhibition of p38 MAPKs by SB203580 was without effect on total cyclin E and Cdk2 levels. The decrease in Cdk2 kinase activity caused by SB203580 treatment was not due to an increased expression of p21Cip1 or p27Kip1 inhibitory proteins. In addition, SB203580 affected neither Cdc25A phosphatase expression nor Cdk2 Tyr-15 phosphorylation. Inhibition of p38 MAPKs decreased Cdk2-cyclin E activation by regulating the subcellular localization of Cdk2 and its phosphorylation on Thr-160.
Conclusions: These results indicate that p38 MAPK activity is involved in the regulation of cell cycle progression in FRTL-5 thyroid cells, at least in part by increasing nuclear Cdk2 activity.

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Introduction
Thyroid follicular cell proliferation is under the control of hormones and growth factors such as thyrotropin (TSH), insulin-like growth factor-I (IGF-I), and insulin which also activates the IGF-I receptor when used at a high concentration. Increased proliferation of thyroid cells is associated with several pathological states, including autoimmune diseases of the thyroid gland and thyroid carcinomas.

Cell cycle progression in mammalian cells is driven by the sequential and periodic activation of a family of serine/threonine kinases named cyclin-dependent kinases (Cdks) (1, 2). Transition from G1 to S phase is regulated at several steps during cell cycle progression: cyclin D–Cdk4 and –Cdk6 complexes are active during the mid-G1 phase, whereas cyclin E–Cdk2 complexes are active at the G1-S transition. Activation of monomeric Cdk2 subunit requires as a first step the binding of its cyclin E partner. The resulting cyclin E–Cdk2 heterodimer remains inactive because of the presence of inhibitory phosphorylation of Tyr-15 and Thr-14 on Cdk2, close to its ATP binding site. Tyr-15 is phosphorylated by the mixed-lineage kinase Wee-1. Translocation of the cyclin E–Cdk2 complex in the nucleus contributes to the regulation of its activity. Activation of the cyclin E–Cdk2 complex is regulated by phosphorylation of Cdk2 on a conserved threonine in the activation loop (Thr-160) by Cdk activating kinase (CAK) and by subsequent dephosphorylation of Tyr-15 and Thr-14 amino acid residues by Cdc25A phosphatase. The Cip/Kip family of cyclin-dependent kinase inhibitor (CKI) proteins plays a crucial role in proliferation. It was initially assumed that the Cip/Kip proteins (p27kip1, p21Cip1, and p57Kip2) inhibit both Cdk4/6 and Cdk2 (1, 2). However, it is now recognized that cyclin D-dependent kinases sequester Cip/Kip proteins, thereby facilitating cyclin E–Cdk2 activation (3).
Proliferation, differentiation and function of the follicular thyroid cells are regulated by TSH. Most of these effects are mediated by a cAMP-mediated pathway that requires the activation of the cAMP-dependent protein kinase A (PKA). The TSH effects are mimicked by the diterpene forskolin which is a potent direct activator of adenyl cyclase. The FRTL-5 rat thyroid cell line has been extensively used to study the mechanisms that control G1-S progression. The effects of TSH and insulin/IGF-I on DNA synthesis in FRTL-5 cells are either synergistic or additive (4, 5), depending on the cell sublines cultured in different laboratories. In PC Cl3 and WRT rat thyroid cell lines, insulin/IGF-I is also a permissive factor for TSH stimulation of cell proliferation (4), although insulin itself acts as a mitogen in WRT cells (5). In our FRTL-5 cell line, forskolin alone does not stimulate DNA synthesis, in agreement with results showing that TSH alone has no effect on DNA synthesis in WRT thyroid cells (6). The cAMP/PKA-dependent pathway has been shown to activate p70-S6 kinase (S6K1) in thyroid cells (7), which is essential for cell cycle progression in many cell types. Although the activation of PKA was necessary for cell cycle progression, it has been suggested that PKA-independent mechanisms might be required in cAMP-mediated growth of thyroid cells (8, 9). Ras activity was required for DNA synthesis in TSH-stimulated WRT cells (10), but TSH did not activate directly the p42/p44 mitogen-activated protein kinase pathway in FRTL-5 cells and in dog thyrocytes (11, 12). In addition, TSH or cAMP did not activate directly the phosphatidylinositol-3 kinase (PI3K)/Akt pathway in dog thyrocytes (13) or FRTL-5 cells (14), but stimulated the formation of the PI3K/Akt/S6K pathway (5). In our FRTL-5 cell line, forskolin alone does not stimulate DNA synthesis, in agreement with results showing that TSH alone has no effect on DNA synthesis in WRT thyroid cells (6). The cAMP/PKA-dependent pathway has been shown to activate p70-S6 kinase (S6K1) in thyroid cells (7), which is essential for cell cycle progression in many cell types. Although the activation of PKA was necessary for cell cycle progression, it has been suggested that PKA-independent mechanisms might be required in cAMP-mediated growth of thyroid cells (8, 9). Ras activity was required for DNA synthesis in TSH-stimulated WRT cells (10), but TSH did not activate directly the p42/p44 mitogen-activated protein kinase pathway in FRTL-5 cells and in dog thyrocytes (11, 12). In addition, TSH or cAMP did not activate directly the phosphatidylinositol-3 kinase (PI3K)/Akt pathway in dog thyrocytes (13) or FRTL-5 cells (14), but stimulated the formation of the PI3K–Ras complex in the latter cell type (15).

Our previous studies established that p38 mitogen-activated protein kinases (p38 MAPKs) were expressed in FRTL-5 cells and were activated via a PKA-dependent signaling pathway (16). Although such a mechanism was not found in primary cultures of dog and human thyrocytes (17), we observed expression and phosphorylation of p38 MAPKs in pathologic human thyroid tissues (M Pomérance, paper in preparation). p38 MAPKs have been implicated in environmental stress responses, in the regulation of inflammation, and in apoptosis (18). Recently, it was shown that p38 MAPKs are also involved in proliferation and differentiation of several cell types (19, 20). However, the exact mechanisms of p38 MAPK-mediated regulation of cell proliferation remain largely unknown.

p38 MAPK activity is inhibited by the pharmacologic inhibitor SB203580, a pyridine-imidazole compound that specifically targets the α and β isoforms (21), and by expression of dominant-negative mutants of p38 MAPK isoforms. In this study, we examined the role of p38 MAPKs in thyroid cell cycle regulation, mostly using the inhibitory properties of SB203580. To exclude possible non-specific effects of SB203580, we checked that this compound did not interfere with other signaling pathways that may be involved in the regulation of FRTL-5 cell proliferation, such as the PI3K/Akt/S6K pathway. We show that inhibition of p38 MAPKs decreased the G1-S phase progression and cyclin E–Cdk2 kinase activation in FRTL-5 cells stimulated with forskolin plus insulin. The decrease in Cdk2 activation could not be explained by the inhibitory phosphorylation of Cdk2 on Thr-160 or by changes in the amounts of several key proteins involved in cell cycle control. The mechanism by which p38 MAPKs interfere with G1-S phase progression may involve, at least in part, the nucleocytoplasmic localization of Cdk2.

**Materials and methods**

**Materials**

SB203580, SB202274, SKF86002, PD169316, LY294002, calf thymus histone H1 and forskolin were purchased from Calbiochem EMD Biosciences Inc. (Darmstadt, Germany), propidium iodide was from Sigma-Aldrich Co. (St Louis, MO, USA), [methyl-3H]thymidine and [γ-32P]ATP (specific activity: 3000 Ci/mmoll) were from PerkinElmer Inc. (Boston, MA, USA) and protein G-Sepharose was from Amersham Biosciences (Little Chalfont, Bucks, UK). Retinoblastoma (Rb) protein (sc4112) and antibodies directed against Cdk2 (sc-163), Cdk4 (sc-260), cyclin E (sc-481), p21[Sup] (sc-397), p38 MAPK (sc-535) and Akt1 (sc-1619) were purchased from Santa Cruz Bio-technology Inc. (Santa Cruz, CA, USA). Monoclonal antibodies against p27[Sup]2 (13231A) and β-tubulin (60181A) were obtained from BD Biosciences Pharmingen (San Diego, CA, USA). Antibodies against Cdc25A phosphatase, phospho-Akt1 (Ser-473), and phosphotyrosine (PY20) were obtained from Upstate Inc. (Charlottesville, VA, USA). Antibodies against phospho-S6K1 (Thr-389), phospho-Cdk2 (Thr-160), and phospho-p38 MAPK (Thr180/Tyr182) were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibodies against mouse and rabbit IgG were obtained from Promega Corp. (Madison, WI, USA); those against sheep and goat IgG were from DakoCytomation (Glostrup, Denmark).

**Cell culture and transfections**

Rat thyroid follicular FRTL-5 cells were kindly provided by Dr M Eggo (Birmingham, UK). Cells were routinely cultured in Coon’s F-12 medium (Biochrom AG, Berlin, Germany) supplemented with GlutaMAX (2 mmol/l), and with 5% heat-inactivated newborn calf serum (Invitrogen), and three hormones: bovine TSH (0.5 mU/ml), insulin (10 μg/ml), and transferrin.
(5 μg/ml) (all Sigma Chemical Co.). For starvation, subconfluent cells were cultured in basal medium without serum and hormones for at least 72 h. Attempts to obtain stable transfectants expressing dominant negative p38α-AGF and p38β-AGF were performed as follows: FRTL-5 cells grown in 60 cm² plates under negative p38 MAP kinase (AGF and p38) were selected with 0.2 mg/ml G418 for 2 weeks.

**Cell extracts**

To prepare whole cell lysates, cells were collected and resuspended in cold lysis buffer A (10 mmol/l Tris/HCl, pH 7.5, 250 mmol/l NaCl, 10 mmol/l EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.05% SDS, 1 mmol/l Na₃VO₄, and a protease inhibitor mixture (1 mmol/l 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) 1 mmol/l benzamidine, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin A)). The lysates were centrifuged at 10 000 g for 10 min and assayed for protein using bicinchoninic acid-protein assay reagents (Biocult Inc, Rockford, IL, USA). For Western blot analysis with phospho-antibodies, cell extracts were prepared as described previously (16). To study the subcellular localization of Cdk2, nuclear and cytoplasmic extracts were prepared as follows. Cells were resuspended in buffer (20 mmol/l Hepes pH 7.9, 1.5 mmol/l MgCl₂, 200 mmol/l sucrose, 10 mmol/l sodium glycerophosphate, 10 mmol/l KCl, 1 mmol/l dithiothreitol, 1 mmol/l Na₃VO₄, a protease inhibitor cocktail, 0.5% (w/v) Triton X-100), lysed for 15 min on ice, and then spun at 10 000 g for 10 min at 4°C. The nuclear pellets were washed with the same buffer and the purity of the nuclei was checked by optical microscopy. The nuclear pellets were resuspended in buffer containing 20 mmol/l Hepes pH 7.9, 400 mmol/l NaCl, 10 mmol/l sodium glycerophosphate, 1.5 mmol/l MgCl₂, 1 mmol/l dithiothreitol, 1 mmol/l Na₃VO₄ and the protease inhibitor mixture, and then incubated for 30 min at 4°C. The samples were centrifuged at 10 000 g for 15 min at 4°C, and the supernatants were removed and stored in aliquots at −80°C.

**Western blot analysis and immunoprecipitation**

For Western blot analysis, proteins were denaturated by boiling in Laemmli sample buffer, resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in Tris–buffered saline containing 0.1% Tween-20 (TBS/T) and 5% non-fat dry milk or 3% bovine serum albumin and then incubated overnight with the appropriate primary antibody. The membranes were washed with TBS/T and then incubated for 1 h at room temperature with a 1:25 000 dilution of appropriate horseradish peroxidase-conjugated secondary antibody. Antibody binding was revealed by the ECL system (Amersham Biosciences). For immunoprecipitation, cell lysates (200–300 μg protein) were incubated for 2 h at 4°C with 2 μg Cdk2, Cdk4 or cyclin E antibody. The immunocomplexes were immunoprecipitated by protein G-Sepharose. Immune complexes were pelleted by centrifugation at 10 000 g and washed twice with buffer A and a further three times with kinase assay buffer (20 mmol/l Hepes pH 7.4, 5 mmol/l MgCl₂, 2 mmol/l EGTA, 1 mmol/l Na₃VO₄, 2 mmol/l dithiothreitol) prior to kinase assays.

**Measurement of DNA synthesis**

Triplicate cultures of quiescent FRTL-5 cells (0.5–1 × 10⁴ cells/ml) were pretreated with inhibitors or vehicle (Me₂SO) for 1 h and then stimulated with forskolin (10 μmol/l), insulin (10 μg/ml) or both, and pulsed with 10⁻⁶ mol/l thymidine and 1 μCi/ml [methyl-³H]thymidine 3 h prior to the end of the treatment protocol. The cells were washed twice with ice-cold phosphate buffered saline (PBS), precipitated with ice-cold 5% trichloroacetic acid, and lysed with 0.02 mol/l NaOH in 1% SDS. The radioactivity was counted by liquid scintillation spectrometry (Packard-Cambera, Meriden, CT, USA).

**Flow cytometry**

Following treatment of quiescent cells as above, cells were harvested by trypsinization, washed twice in PBS, and then fixed with ice-cold 70% ethanol. Cells were washed twice with PBS and stained with 50 μg/ml propidium iodide containing 100 μg/ml RNase for 30 min. Cell cycle analysis was performed using a fluorescence-activated cell sorter (FACS) and Cell Quest software version 1.2 (BD Biosciences). At least 10 000 cells were analyzed per sample. The cell cycle distribution was quantified using the ModFit LT software (Verity Software House Inc., Topsham, ME, USA).

**In vitro kinase assays**

Cdk4 activity was assayed by incubating immunoprecipitates with 2 μg Rb protein, used as a substrate, for 20 min at 30°C in kinase assay buffer containing 40 μmol/l ATP and 5 μCi [γ⁻³²P]ATP. Cdk2 activity was assayed by incubating immunoprecipitates with 10 μg histone H1, used as a substrate, for 10 min at 30°C in kinase assay buffer containing 40 μmol/l ATP and 5 μCi [γ⁻³²P]ATP. The reactions were stopped by the addition of Laemmli sample buffer. Samples were subjected to SDS-PAGE. The gels were dried and phos-
phorylated Rb protein or H1 histone was quantified by InstantImager analysis (Packard-Canberra).

**Statistical analysis**

Statistical significance was assessed using Student’s t-tests. P values less than 0.05 were considered to be significant.

**Results**

*Inhibition of p38 MAPKs correlates with inhibition of G1–S transition in FRTL-5 thyroid cells*

FRTL-5 cells, forced to quiescence by hormonal and serum withdrawal, were pretreated for 1 h with SB203580, a specific p38 MAPK inhibitor, or with the vehicle alone (Me2SO), and then treated with 10 µg/ml insulin plus 10 µmol/l forskolin for different periods of time. Fig. 1A shows that 5 µmol/l SB203580 inhibited by 45–50% the increase in DNA synthesis induced by forskolin plus insulin, as followed by incorporation of [methyl-3H]thymidine into DNA. The inhibitory effect of SB203580 was maximal at 24–30 h after the stimulation, i.e. at the stage where the cells progressively reached G1–S transition. The results of five independent experiments (Fig. 1B) indicated that the effect of SB203580 was highly reproducible (46 and 41% inhibition at 24 and 30 h of treatment respectively). Fig. 1C shows that forskolin alone had no effect on cell cycle progression, while the effect of insulin alone was at least 10-fold lower than that of forskolin plus insulin. SB203580 had no effect on insulin-induced DNA synthesis. As a control, we observed that LY294002, a specific PI3K inhibitor, inhibited in a concentration-dependent manner the insulin-induced DNA synthesis (not shown). This suggests that p38 MAPK acts only when the cAMP pathway and the insulin/IGF-I pathway are activated simultaneously. To rule out a potential nonspecific effect of SB203580, we used SB202474, which has no effect on p38 MAPK activity, as a negative control; similar results were obtained whether SB202474 was present or not. As shown in Fig. 2, SB203580 inhibited DNA synthesis induced by a 24 h treatment with forskolin plus insulin in a dose-dependent manner. The half-maximum effect was obtained at 1 µmol/l, whereas the inactive molecule had no significant effect. In addition, other pyridine-imidazole p38 MAPK inhibitors, such as SKF86002 and PD169316, also inhibited growth-stimulated DNA synthesis (data not shown). Then, we examined the cell cycle distribution by FACS analysis after 24 h and 30 h of stimulation of cells with forskolin plus insulin in the presence or absence of SB203580. Figure 3 shows the results of two independent experiments. SB203580 treatment reduced the number of cells in S phase by 34–54% at 24 h. This reduction

Figure 1 Effect of SB203580 on [3H]thymidine incorporation into DNA. Quiescent FRTL-5 cells were pretreated without or with 5 µmol/l SB203580 for 1 h before stimulation with 10 µmol/l forskolin or 10 µg/ml insulin or both. Uptake of [3H]thymidine was measured as described in the Materials and methods section. (A) [3H]Thymidine incorporation was measured as a function of stimulation time by forskolin plus insulin. Data obtained without (○) or with (●) SB203580 are the means±s.e.m. of triplicate wells. (B) [3H]Thymidine incorporation was measured at 24 and 30 h of stimulation by forskolin plus insulin. Data obtained without (solid bars) or with (open bars) SB203580 are the means±s.e.m. of five independent experiments. (C) Cells were stimulated with forskolin (▲, △) or insulin (■, □) without (solid symbols) or with (open symbols) SB203580. S.E.M. values, lower than 10% of the mean (triplicate wells), were omitted for clarity. Similar results were obtained in three independent experiments. *P < 0.05, **P < 0.005, ***P < 0.001 relative to control cells.
was 26–28% after 30 h of stimulation. Concurrently, cells in G0/G1 phase increased by 10–15%. Because FRTL-5 cells usually start synthesizing DNA 12 to 16 h after stimulation, only a few cells had reached G2/M at the time of harvest.

To exclude the possible nonspecific effects of SB203580 on other important signaling pathways involved in FRTL-5 cell proliferation, we examined the effect of the drug on the PI3K/Akt pathway and the S6K1 pathway. As shown in Fig. 4A, using an antiphospho-Akt antibody we found that phosphorylated Akt was detected in insulin-treated, but not in forskolin-treated FRTL-5 cells. However, SB203580 had no effect on Akt phosphorylation. SB203580 also had no effect on forskolin-induced phosphorylation of S6K1 when referred to the amount of S6K1 protein. We used rapamycin, a specific inhibitor of the mTOR protein kinase involved in S6K1 phosphorylation, as a positive control of inhibition of S6K1 phosphorylation. As expected, rapamycin blocked forskolin-induced S6K1 phosphorylation (Fig. 4B). We attempted to express dominant negative forms of p38α and p38δ MAPK (in which the TGY motif in the activation loop is mutated to AGF) in FRTL-5 cells. All our attempts to obtain viable FRTL-5 cells expressing the p38αAGF mutant were unsuccessful, whereas we did obtain FRTL-5 cells that stably expressed p38δAGF. These latter cells showed a normal proliferative response to forskolin plus insulin when studied by incorporation of [methyl-3H]thymidine into DNA (data not shown), suggesting that p38δ MAPK is not involved in regulation of cell proliferation, in contrast to p38α MAPK.

Involvement of p38 MAPKs in Cdk2 activation by forskolin and insulin

Because the inhibitory effect of SB203580 occurred at the stage where cells reached G1–S transition, we studied the immunoprecipitated activity of Cdk4 and Cdk2 kinases in the presence or absence of SB203580 in FRTL-5 cells stimulated by forskolin plus insulin. No effect of SB203580 was observed on endogenous Rb phosphorylation, using an antibody directed against Ser-780, which is targeted by Cdk4. On the other hand, immunoprecipitation with antibodies directed against Cdk2 or cyclin E followed by kinase assays on histone H1 showed an increase in endogenous total Cdk2 and cyclin E-associated kinase activity, beginning 16 h after stimulation (Fig. 5A and B). The activity was maximal between 24 and 30 h, at which time cells initiated de novo DNA synthesis (Figs 1A and 3). Treatment of FRTL-5 cells with SB203580 (5 μmol/l) reduced total and cyclin E-associated Cdk2 activity by 40–50%. The quantification of histone H1 phosphorylation obtained in 3 to 5 independent experiments (Fig. 5C) indicated that this inhibitory effect
was highly reproducible. We checked that SB203580 had no direct inhibitory effect on Cdk2 kinase activity measured in vitro (data not shown).

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Figure 4 SB203580 has no effect on the PI3K and S6K1 pathways. (A) Cells were pretreated for 1 h without or with 5 μmol/l SB203580 before stimulation (1 or 3 h) with 10 μmol/l forskolin or 10 μg/ml insulin or both. Cell lysates were analyzed by SDS-PAGE and immunoblotting with antibodies specific for phospho (P)-Akt and total Akt. (B) Cells were pretreated without or with 5 μmol/l SB203580 or 20 nmol/l rapamycin, and unstimulated (0) or stimulated for 30 min by 10 μmol/l forskolin. Cell lysates were analyzed by SDS-PAGE and immunoblotting with antibodies specific for phospho (P)-S6K1 and total S6K1. Similar results were obtained in two separate experiments. Wb, Western blot.

Expression of several cell cycle regulatory proteins upon SB203580 treatment

We studied whether the decrease in Cdk2 activity induced by SB203580 could be due to its effects on expression of Cdk2, cyclin E, and the CKIs p27Kip1 and p21Cip1. Quiescent cells were stimulated with forskolin plus insulin for 24 and 30 h in the presence or absence of SB203580, and Western blot analyses for Cdk2, cyclin E, p27Kip1 and p21Cip1 were performed. Figure 6 shows that anti-Cdk2 antibodies recognized two main forms of Cdk2 proteins (33 and 34 kDa). The level of the 33 kDa activated form was very low in quiescent cells and increased 24 h and 30 h after the treatment of cells with forskolin plus insulin. The presence of SB203580 during the cell stimulation did not affect significantly the expression of the 33 and 34 kDa forms. Cyclin E was barely detectable in quiescent cells and increased 24 and 30 h after stimulation. However, the presence of SB203580 did not affect the levels of cyclin E (Fig. 6). To determine whether SB203580 affected the expression of CKIs, their amounts were determined by Western blot analysis, p27Kip1 expression was high in quiescent cells and decreased at 24 and 30 h after stimulation (Fig. 6). p27Kip1 expression could not be detected at 48 h (data not shown). This downregulation coincides with the increase in Cdk2 kinase activity (see Fig. 5) and in Cdk2 and cyclin E expression. However, the level of p27Kip1 protein was not significantly affected by SB203580 treatment (Fig. 6). In contrast, p21Cip1 expression was very low in quiescent cells and transiently increased 24 h after the stimulation of cells with forskolin plus insulin. Nevertheless, SB203580 did not influence p21Cip1 protein levels (Fig. 6). Therefore, the p38 MAPK inhibitor did not modify the expression of Cdk2, cyclin E, and the CKIs under study.

p38 MAPK inhibition did not affect Cdk2 Tyr-15 phosphorylation or Cdc25A expression

We studied whether SB203580 affected the inhibitory phosphorylation of Cdk2 on Tyr-15 which maintains Cdk2 in an inactive form. Extracts from FRTL-5 cells stimulated for 24 and 30 h with forskolin plus insulin in the presence or absence of SB203580 were immunoprecipitated with an anti-Cdk2 antibody and probed with anti-phosphotyrosine antibody. Tyrosine phosphorylation increased in correlation with Cdk2 expression, but no significant change in phosphotyrosine content was observed following SB203580 treatment (Fig. 7A). As Cdc25A phosphatase regulates the activity of the Cdk2–cyclin E complex, we examined the effect of SB203580 on its expression. Cdc25A, which appeared as 3 bands ranging from 65 to 72 kDa on Western blots, was constitutively expressed throughout the cell cycle and its expression did not undergo major changes in the presence of SB203580 (Fig. 7B). Therefore, Cdk2 phosphorylation on Tyr-15 and Cdc25A expression were unlikely to be involved in the p38 MAPK effect on Cdk2 activity.

Effect of SB203580 on subcellular localization and Thr-160 phosphorylation of Cdk2

Cytosolic and nuclear extracts were prepared from FRTL-5 cells to study the effect of SB203580 on subcellular localization of Cdk2 and cyclin E. SB20474 was
used as a negative control to validate the specificity of SB203580. Nuclear and cytosolic extracts from FRTL-5 cells stimulated with forskolin plus insulin in the presence of SB203580 or SB202474 were analyzed by Western blot (Fig. 8). Beta-tubulin was used as a cytoplasmic marker. Cyclin E was barely expressed in nuclear and cytosolic fractions from quiescent cells, consonant with the results obtained with whole cell extracts (see Fig. 6). After 16 h of stimulation with forskolin plus insulin, cyclin E was found in the cytosolic and nuclear fractions. However, its amount was not significantly affected in either compartment by SB203580 or SB202474. Significant amounts of Cdk2 were detected in the nuclear fraction of stimulated cells, whereas Cdk2 was essentially cytosolic in quiescent cells. Furthermore, the level of Cdk2 within nuclei of SB203580-treated cells was repeatedly decreased compared with that present in nuclei of SB202474-treated cells. Using an antibody specific for Thr-160-phosphorylated Cdk2, we observed that forskolin plus insulin induced the phosphorylation of Thr-160 within 16 to 30 h in the nuclei whereas negligible phosphorylation occurred in the cytosolic fraction. A marked reduction of the activating Thr-160 phosphorylation of Cdk2 occurred in nuclei from SB203580-treated cells compared with nuclei of SB202474-treated cells. The same extracts were monitored for their Cdk2 kinase activity. The treatment of the cells by SB203580 decreased Cdk2 activity in the nuclei, compared with that of SB202474-treated cells, with a time course similar to that of Thr-160 phosphorylation. These results suggest that the nuclear translocation of Cdk2, but not its activating phosphorylation, is specifically targeted by p38 MAPK inhibition.

Discussion

Depending on the cell type and stimulus, p38 MAPKs can have either a positive or a negative role in the regulation of cell cycle progression. p38 MAPKs have an inhibitory role in cell cycle progression in various cell types (19, 20). Conversely, there is accumulating evidence that activated p38 MAPKs are involved in positive control of cell proliferation. Thus, p38 MAPKs appear to be required for the proliferation of Swiss 3T3 cells or
airway smooth muscle cells stimulated by fibroblast growth factor-2 (22, 23), of hematopoietic cells stimulated by cytokines (24, 25), and of vascular smooth muscle and pancreatic stellate cells stimulated by platelet-derived growth factor (26, 27). p38 MAPKs are also required for CD40-induced proliferation of B-lymphocytes (28), and for cell cycle progression of breast cancer, melanoma and chondrosarcoma cell lines (29–31). However, the signaling pathways and identities of signaling key intermediates, which mediate p38 MAPK involvement in cell cycle progression, are poorly characterized.

In previous studies, we have reported that the p38 MAPK pathway was stimulated by forskolin in FRTL-5 cells (16) and that forskolin-stimulated p38 MAPK phosphorylation was inhibited by SB203580 (32). Here, data obtained using thymidine incorporation and FACS analysis show that inhibition of p38 MAPKs decreased significantly DNA synthesis by FRTL-5 cells stimulated by forskolin plus insulin. We performed several control experiments that argue in favor of a specific p38 MAPK-mediated effect of SB203580 on FRTL-5 cell proliferation. The effect of SB203580 on DNA synthesis was not obtained with SB202474, a structural pyridine-imidazole analog which does not inhibit p38 MAPK activity. It has been reported that SB203580 reduced the phosphorylation and activation of Akt in T cells independently of p38 MAPK activity (33). Such a mechanism is unlikely to occur in FRTL-5 cells because: (i) SB203580 did not affect the slow proliferative effect of insulin, (ii) SB203580 did not affect the phosphorylation of Akt by insulin, and (iii) we observed no effect of forskolin on the phosphorylation of Akt as previously reported (13, 14). Previous studies reported that SB203580 does not affect the PI3K or the S6K1 pathways in non-thyroid cells (34). We also exclude an effect of SB203580 on forskolin-stimulated S6K1 phosphorylation in FRTL-5 cells. A similar result was also reported in feline cardiomyocytes, where forskolin activates both p38 MAPKs and S6K1 (35). It has also been reported that a high concentration of SB203580 reduced the activity of c-Jun N-terminal kinases (JNKs) in rat ventricular myocytes and in transfected COS-7 cells (36, 37), but JNKs were not activated either by cAMP in FRTL-5 cells (11) or by IGF-I in human thyrocytes (38). These results indicate that SB203580 inhibited G1-S transition in FRTL-5 cells by specifically targeting p38 MAPK isoform(s).

The p38 family of MAPKs is composed of four members, p38α, β, γ, and δ (18). In the human thyroid gland, the mRNAs of p38α and p38δ MAPK isoforms are predominantly expressed, compared with those of p38β and γ isoforms (39). Only p38α and β MAPKs are inhibited by SB203580, thus our data suggest that the α isoform is involved in the control of G1–S transition in FRTL-5 cells. Accordingly, we could not
obtain stable transfectants expressing the dominant negative form of the p38α isoform, whereas we obtained transfectants expressing the dominant negative form of the p38δ isoform in FRTL-5 cells. These results suggest that the dominant negative α isoform interfered with FRTL-5 cell proliferation.

Cotreatment of FRTL-5 cells with TSH and insulin/IGF-I increases the expression of cyclins D and E, and thereby increases the corresponding associated kinase activities (4). Our data indicate that SB203580 reduced the activity of Cdk2, but not that of Cdk4. The levels of cyclin E or Cdk2 proteins were not affected by SB203580 treatment. One mechanism of regulation of Cdk2–cyclin E activity is through its inhibition by p27Kip1 and p21Cip1 (1, 2), but these proteins are also shown to be essential activators of cyclin D complex formation (2). When we examined the effect of forskolin plus insulin stimulation on the expression of these proteins, only p27Kip1 showed a decrease 24 h after stimulation as previously observed (4), whereas p21Cip1 was increased. To our knowledge, there was no previous report on the regulation of the expression of p21Cip1 in thyroid cells. We observed no effect of SB203580 on the regulation of p27Kip1 and p21Cip1 expression by forskolin plus insulin. An effect of SB203580 on the stabilization of the Cdk2–cyclin E–CKI(s) complex is unlikely because p27Kip1 and p21Cip1 expression is downregulated at 30 h of stimulation by forskolin plus insulin.

Cdk2 activity has been shown to be negatively regulated by phosphorylation on Thr-14 and Tyr-15 during S phase and to be positively regulated by Cdc25A phosphatase (1, 2). In this work, we did not detect any significant effect of p38 MAPK inhibition on Tyr-15 phosphorylation of Cdk2. We found that Cdc25A was constitutively expressed throughout the cell cycle in FRTL-5 cells. Furthermore, SB203580 treatment had no effect on Cdc25A expression. We conclude that the low kinase activity of cyclin E–Cdk2 complexes in SB203580-treated cells is not due to inhibitory phosphorylation of Thr-14 and Tyr-15 on Cdk2. Anyhow, the role of Cdc25A in the control of Cdk2 activity is now subject to controversy.

Several studies have highlighted the importance of the nucleocytoplasmic distribution of Cdk/cyclins complexes in cell cycle control (40, 41), including dog thyroid cells (42). Recently, it has been shown that the nucleocytoplasmic translocation of Cdk2 is under the control of p42/p44 MAPKs in fibroblasts (43, 44). Cytoplasmic mislocalization of Cdk2 has also been reported to be a key event in vitamin D-mediated growth inhibition of prostate cancer cells (45) and in irradiation-induced apoptosis in mouse mesangial cells (46). In this study, we show that the subcellular localization of Cdk2 provides a novel mechanism whereby p38 MAPKs could regulate cell cycle progression. Our results show a decrease in the amount of Cdk2 within nuclei following p38 MAPK inhibition. Furthermore, cyclin E shuttled between the nucleus and the cytoplasm as reported in other cell types (40, 41), but SB203580 had no effect on its translocation in FRTL-5 cells. This finding suggests that the p38 MAPK pathway is not involved in the regu-
lution of the subcellular localization of this cyclin in FRTL-5 cells.

In fibroblasts, it has been shown that p42/p44 MAPKs are necessary for Cdk2 Thr-160 phosphorylation (47, 48), although it may be independent of their impact on Cdk2 subcellular localization (48). p42/p44 MAPKs could be involved in regulation of CAK or phosphatase activities. Here, we show that in FRTL-5 cells, p38 MAPKs have an impact on Thr-160 phosphorylation of Cdk2 and on its activity. In nuclei from SB203580-treated cells, we observe a decrease in the amount of Cdk2, which likely accounts for the decrease in its phosphorylation on Thr-160, and therefore for the decrease in its activity. Taken together, our results suggest that inhibition of p38 MAPK activity reduces Cdk2 expression by decreasing nuclear Cdk2 available for cyclin E binding and for phosphorylation by CAK of the resulting complex in rat thyroid cells. Although the mechanisms underlying impaired Cdk2 translocation described in this study require further investigation, these data indicate a role for p38 MAPKs in the regulation of cycle progression in rat FRTL-5 thyroid cells.

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