The Phosphodiesterase Inhibitor SQ 20006 Selectively Blocks Mitogen Activation of p70S6K and Transition to S Phase of the Cell Division Cycle without Affecting the Steady State Phosphorylation of eIF-4E*

(Received for publication, June 16, 1995, and in revised form, August 22, 1995)

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In quiescent cells high levels of protein synthesis are required in order to re-enter the cell cycle upon stimulation. Initiation of polypeptide synthesis is the step most often subject to regulation, controlled in part by phosphorylation of 40 S ribosomal protein S6 and a number of initiation factors. The kinase responsible for S6 phosphorylation is p70S6K. We now show that the p70S6K pathway can be selectively blocked by the aminopyrine analogue, SQ 20006. This agent is known to raise cAMP levels, resulting in activation of protein kinase A. We present evidence that the increase in cAMP is not responsible for the inhibitory effect observed. We also show that SQ 2006 can prevent the activation of p70S6K in a rapid and reversible manner. The compound does not exert its inhibitory activity on p70S6K but can inhibit in vitro two protein kinase C isozymes (α and γ). In a B lymphoblastoid cell line, treatment with SQ 20006 results in inhibition of protein synthesis at the initiation stage. In contrast, when tested directly upon the translational machinery in the reticulocyte lysate, inhibition is manifest at both the level of initiation and elongation. The role of protein kinase A in the modulation of p70S6K and the rate of translation is discussed.

Stimulation of cell growth and proliferation is initiated at the cell surface by specific ligand-receptor interactions. Such interactions lead to receptor dimerization, cross-phosphorylation (1) and recruitment of Src-homology 2-containing signal transducers, which dock at phosphorylated tyrosine residues (2). This in turn causes activation of signaling molecules by a variety of mechanisms, including tyrosine phosphorylation (3), conformational changes (4, 5), and translocation to the plasma membrane (6, 7). The signal is further propagated and amplified by cascades of cytosolic protein kinases (8, 9), which ultimately activate transcription factors in order to initiate metabolic processes necessary for growth.

One obligatory step required for progression through the cell cycle is the activation and maintenance of high rates of protein synthesis (10). Control of translation plays an important role in cell proliferation (reviewed in Ref. 11), with physiological regulation almost always exerted at the level of polypeptide chain initiation (12, 13). This phase is regulated, in part, by the phosphorylation of initiation factors involved in binding mRNA to the 40 S ribosomal subunit (11–16). The only protein in the ribosome that has been reported to undergo phosphorylation in vivo in response to a number of mitogens is the 40 S ribosomal protein S6 (17). This has been mapped to an area of the 40 S ribosomal subunit that is implicated in mRNA binding and is thought to reside near the tRNA acceptor site (17). S6 phosphorylation can be correlated with a selective translational up-regulation of a family of mRNAs encoding for proteins required for cell growth (18). The kinase believed to modulate the level of S6 phosphorylation is p70S6K, which is itself activated by phosphorylation (19–21, 23). However, the signaling pathway responsible for inducing p70S6K activation remains unidentified (24, 25). Contrary to other mitogen-regulated kinases, p70S6K activity remains high throughout G1, and microinjection of inhibitory antibodies at any time before S phase can block G1/S transition (26, 27).

The cap structure present on the 5′ end of mRNA facilitates its binding to the ribosome, a process mediated by three initiation factors (eIF-4A, -4B, and -4F) and ATP hydrolysis (11–13, 28, 29). eIF-4F is a cap binding protein complex composed of three subunits: eIF-4E, which specifically recognizes the cap structure (13); eIF-4A, an ATP-dependent, single strand RNA-binding protein with helicase activity (13, 28); and eIF-4y (p220, eIF-4G), whose function is unknown but whose integrity is required for eIF-4F complex activity (11–13). It is believed that eIF-4F functions to unwind secondary structure in the mRNA 5′-untranslated region to facilitate binding to the 40 S ribosomal subunit (11, 12, 28, 29). Consistent with its proposed regulatory role, eIF-4F exists in both phosphorylated and non-phosphorylated forms (11–13, 15) and is believed to be the least abundant of the initiation factors (15, 30). In response to the appropriate stimuli, increased levels of eIF-4E phosphorylation have been directly correlated with increased rates of translation in a variety of cell types (reviewed in Ref. 11). More recently, it has been proposed that in adipocytes the regulated phosphorylation of an eIF-4a-associated protein (PHAS-1, 4E-BP1) plays a role in modulating the availability of eIF-4E to enter the initiation pathway (31, 32). However, the role of phosphorylation of eIF-4E in this interaction is at present poorly defined, and moreover, it is not known whether such interactions occur in other cell types.

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These observations have prompted us to investigate the role of p70S6k in G1 progression and in the phosphorylation of eIF-4E and its association with PHAS-I. Here we report on the properties of a p70S6k-specific inhibitor, SQ 20006; we show that SQ 20006 prevents the mitogen-induced activation of p70S6k and causes inhibition of protein synthesis initiation in Swiss 3T3 cells and Raji cells. SQ 20006 blocks entry of cells into S phase but does not affect the steady state phosphorylation of eIF-4E or its association with PHAS-I.

EXPERIMENTAL PROCEDURES

Materials—1-Ethyl-4-hydrazino-1H-pyrazolo-(3–4-b)-pyridine-5-carboxylic acid, ethyl ester, and hydrochloride (SQ 20006) as well as M1 and M5 polyclonal antisera to p70S6k were kindly provided by Dr. G. Thomas, Basel, Switzerland. Unless specified, chemicals were from Merck or Calbiochem. Media for cell culture and fetal bovine serum were from Bio-Whittaker (Dubboeco’s modified Eagle’s medium) or from Life Technologies, Inc. (RP1 M60). Recombinant baculovirus for protein kinase C isozymes were provided by Dr. S. Stabel (Cologne, Germany). Expression and partial purification of protein kinase Cs and determination of activities were carried out as described (33). The CAMP-dependent protein kinase was a gift of Dr. B. Herrmanns (Basel, Switzerland). p34cdc2/p53Cdk B from starfish oocytes was obtained from Dr. L. Meijer (Roscoff, France) and assayed as in Ref. 34.

Cell Culture and Cell Cycle Analysis—Swiss 3T3 fibroblasts were seeded and maintained as described previously (35). Cells at approximately 60–70% confluency were arrested in G1 by serum deprivation for 24 h in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum (serum starvation protocol) and resuspended to enter the cell cycle by adding Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (complete medium). Assessment of the resting state as well as determination of cell population in each phase of the cell cycle were carried out by labeling nuclei with propidium iodide (CytoTest kit, Becton-Dickinson). For this, 1 × 10^6 cells were analyzed for DNA content in a FACScan cell analyzer (Becton-Dickinson) employing the LYSIS II program, version 1.1. To monitor cell cycle progression into S phase, Swiss 3T3 cells were seeded at 2.5 × 10^5 cells/well in 200 μl in a 96-well plate in complete medium and allowed to grow for 48 h. Cells were then arrested following the serum starvation protocol and restimulated to enter the cell cycle by the addition of complete medium.

Finally, cells were labeled in a time course with 1 μCi of [3H]thymidine (DuPont NEN) well and after a 2-h pulse, cells were harvested with a PHD cell harvester (Cambridge Technology, Inc.). Raji cells (a B lymphoblastoid cell line, ATCC CCL 86) were maintained in mid-log phase in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamicin. Raji cells (1 × 10^6 cells in 200 μl) were seeded into a 96-well plate and pulse-labeled for 4 h with 2.5 μCi of [3H]thymidine (Amersham Corp.) as described (41). In the case of cyclin-dependent kinase 2, immunoprecipitation was carried out on 300 μg of total protein in Buffer D (25 mM Tris-HCl, pH 7.5, 60 mM β-glycerophosphate, 15 mM MgCl2, 15 mM EDTA, 0.1 mM NaF, 15 mM p-nitrophenylphosphate, 1 mM phenylmethylsulfonfluoride; 0.1% Nonidet P-40), and immunobilized on protein A-Sepharose beads. Pellets were washed with 3 × 1 ml of ice-cold Buffer D and 1 ml of ice-cold Buffer E (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100 and 0.5% sodium deoxycholate).

To analyze p70S6k and ERK2, equal amounts of total protein (5 μg) were resolved on SDS-PAGE as described (20, 40), and proteins were transferred to polyvinylidene difluoride (Millipore). The resulting membrane was decorated with either anti-ERK2 polyclonal antibody (40) or with the p70S6k M1 antibody (20) and revealed using the ECL system (Amersham Corp.). In order to assay protein kinase activity, equal amounts of total protein (50 μg) were immunoprecipitated with the p70S6k antibody M5 (20) or with an anti-ERK2 polyclonal antibody (40) and immobilized on protein A-Sepharose beads. Pellets were washed with 3 × 1 ml of ice-cold Buffer B followed by 1 ml of ice-cold Buffer C (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100 and 0.5% sodium deoxycholate) and resuspended in complete medium. For this, 1 ml of ice-cold Buffer D and 1 ml of ice-cold Buffer E (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol) were used.

RESULTS

SQ 20006 Specifically Blocks p70S6k Activation in Swiss 3T3 Cells—SQ 20006 is a methylxanthine analogue and a potent phosphodiesterase inhibitor (42). The drug was first employed in the characterization of S6 phosphorylation in order to mimic the effect of high concentration of CAMP (43). Although SQ 20006 caused an inhibition of S6 phosphorylation and protein synthesis initiation, it was shown that total changes in intracellular levels of cAMP were not involved in inhibiting either process. Evidence obtained in recent years suggests that p70S6k is the enzyme responsible for S6 phosphorylation in vivo (17). This prompted us to examine whether p70S6k might be the target of SQ 20006.

In order to test this hypothesis, we examined p70S6k activity in Swiss 3T3 fibroblasts. Cells were arrested in G0 by serum starvation for 24 h and restimulated with 10% serum in the presence or absence of SQ 20006. Fig. 1A shows that following re-entry into G1, p70S6k activity increases progressively, reaching a maximum at 1 h post stimulation. Contrary to epidermal growth factor stimulation (44), serum addition induces a sustained kinase activation that persists until transition to S phase. Addition of SQ 20006 at the time of serum stimulation completely prevents the activation of p70S6k (Fig. 1A).

The lack of kinase activity in the p70S6k immune complex is reflected by the absence of the character-
istic band shift on SDS-PAGE that accompanies p70S6k activation (Fig. 1C and Ref. 20). In order to examine whether this inhibition was specific for p70S6k, we analyzed the activation of ERK2, which lies on a parallel but distinct pathway from p70S6k (45). As shown in Fig. 1, B and D, ERK2 activity is totally unaffected by the presence of SQ 20006 in the medium. This suggests that SQ 20006 is specifically preventing the activation of p70S6k. Moreover, addition of SQ 20006 at any time in G1 fully reversed p70S6k activation (see below) without affecting the activation of ERK2 (data not shown).

SQ 20006 Does Not Directly Affect the Activity of p70S6k but Does Inhibit the Activity of Protein Kinase Cα and γ in Vitro—One possible explanation for the effects of SQ 20006 on the activation of p70S6k is that it is acting as a competitive inhibitor for ATP (46). In order to test whether SQ 20006 is a direct inhibitor of protein kinases, the compound was tested in vitro on numerous kinases, as summarized in Table I. The data presented show that p70S6k activity was unaffected by the presence of SQ 20006, even up to levels in excess of 1 mM. In agreement with the data presented in Fig. 1, ERK2 activity was similarly unaffected in these assays, as was the case with cAMP-dependent protein kinase (protein kinase A) and cyclin-dependent kinase 2/cyclin E, although p34cdc2 cyclin B was more sensitive to the inhibitor. We also tested the effect of SQ 20006 on the protein kinase C family of molecules; while little or no effect was observed on the majority of isoforms, both protein kinase Cα and protein kinase Cγ were inhibited by SQ 20006 in vitro, displaying an IC_{50} of 270 μM and 160 μM, respectively. These data suggest that protein kinase Cα and/or protein kinase Cγ may have a role in the activation of p70S6k. Indeed, it has been shown that p70S6k is activated in a biphasic manner following the addition of growth factors to cells, with part of the response regulated by a protein kinase C-dependent signaling pathway (44).

The Inhibition of p70S6k Activity Induced by SQ 20006 Is Reversible—To further characterize the kinetics of p70S6k inhibition by SQ 20006, quiescent Swiss 3T3 fibroblasts were stimulated with serum for 1 h and then treated with SQ 20006; extracts were then prepared at different times following incubation, and the activation state of p70S6k was monitored by SDS-PAGE and immunoblotting. The data presented in Fig. 2A shows that p70S6k is partially inactivated within 10 min of exposure of the cells to SQ 20006 and fully inactivated within 30 min. Next, we examined whether SQ 20006 addition is toxic to cells, leading to general cell death. Swiss 3T3 fibroblasts rendered quiescent by serum deprivation were restimulated to grow in the presence of SQ 20006 for 1 h. Cells were then washed free of SQ 20006, fresh medium was added, and p70S6k activation was examined by Western blot analysis. The data presented in Fig. 2B indicate that within 60 min, full activation of p70S6k was observed, as judged by its retarded mobility on SDS-PAGE, comparable with that obtained following 1 h of incubation with growth factors (see Ref. 16).
inhibition is then bypassed, allowing cells to progress to S phase. This could account for this observation. Whether such early inhibition of phosphodiesterase and a subsequent rise in the levels of cAMP causes acute effects on the inhibition of polysome assembly and protein synthesis initiation when added to cells re-entering the cell cycle from a quiescent state. An essential step in this process is the phosphorylation of 40 S ribosomal protein S6 (17). Since SQ 20006 blocks p70S6k activation (Fig. 1, A and C), this could account for this observation. Whether such early inhibition is then bypassed, allowing cells to progress to S phase, is not known. To address this possibility, we have examined the effect of SQ 20006 addition on G1 progression in Swiss 3T3 fibroblasts. Quiescent cells were stimulated with serum in the absence or presence of SQ 20006 or aphidicolin (to arrest cells in G1), and progression through the cell cycle, with DNA synthesis clearly observed at 24 h (panel E). This is prevented by the inclusion of either SQ 20006 or aphidicolin (Fig. 3, I and G, respectively) to the restimulated cells or the addition of SQ 20006 to an asynchronous cell population (panel H). These data suggest that SQ 20006 induces a G1 block that is not bypassed by the cell.

In order to investigate in more detail the inhibition of S phase entry following SQ 20006 addition, we first considered the response of cells to increasing doses of the drug. Fig. 4 shows that the concentration of SQ 20006 required to inhibit S phase entry by 50% (IC50) is 0.2 mM, with full block at concentrations of 0.5 mM and above (Table I). We have also compared the effect of SQ 20006 addition with that of a potent inhibitor of the p70S6k activation pathway, the immunosuppressant, rapamycin. As shown in Table I, rapamycin decreases the extent of S phase transition to 48% in Swiss 3T3 cells, whereas SQ 20006 fully blocks DNA synthesis. One possible explanation for the inhibitory effect of SQ 20006 could be that it is due to inhibition of phosphodiesterase and a subsequent rise in the levels of cAMP. To test this, cells were incubated in the presence of 8-Br-cAMP. As shown in Table II, 1 mM 8-Br-cAMP did not block entry of cells into S phase whether added at the start of the incubation or at any time during the G1 period (data not shown).

To complement these studies, aliquots of cells treated as above for 1 h were also analyzed for the activation state of p70S6k. While the addition of SQ 20006 (Fig. 2) or rapamycin (Fig. 5) to serum-stimulated cells prevented the activation of p70S6k, neither 8-Br-cAMP nor the phosphodiesterase inhibitor, IBMX was effective at altering the activity of p70S6k (Fig. 5).

Next, we addressed the question of whether p70S6k and protein synthesis inhibition by SQ 20006 after the restriction point (47) are critical to S phase transition. For these studies, quiescent cells were induced to grow by the addition of serum, SQ 20006 was added at various times, and DNA synthesis was monitored at 14 h following stimulation. Fig. 6A shows that induction of DNA synthesis was very sensitive to the presence of SQ 20006 during the first 10 h following serum stimulation, with sensitivity decreased by 12 h. The addition of SQ 20006 to cells during S phase transition (i.e. 12–18 h) did not significantly affect the extent of [3H]thymidine incorporation into DNA (data not shown). Accordingly, withdrawal of the drug early in G1 allowed cells to regularly proceed to S phase, whereas later removal caused a delay in the transition to S (Fig. 6B and Table III).

Since SQ 20006 yields an apparent G1/S block, we decided to test whether the drug might also function in a manner similar to known inhibitors of S phase entry. This is the case for hydroxyurea, an inhibitor of the DNA precursor pool synthesis. Cells were restimulated with serum for 15 h, in the absence or presence of either SQ 20006 or hydroxyurea, the drugs were removed by washing the cells, and DNA synthesis was measured at 2-h intervals during the following 20 h by pulse labeling with [3H]thymidine, as described. The data presented in Table III show that release of the cells from the hydroxyurea block was rapid, with cells progressing into S phase within 4 h. On the contrary, cells released from SQ 20006 block required 15 h to reach mid-S phase, independent of whether SQ 20006 was added at the start of the incubation (Table III) or during G1 (data not shown). Taken together, the data above suggest that SQ 20006 is not directly involved in the inhibition of DNA replication but rather it blocks early in G1 and in a reversible manner.

SQ 20006 Inhibits DNA and Protein Synthesis in the B Lymphoblastoid Cell Line, Raji—To complement the above studies with Swiss 3T3 cells, we have also examined the effect of SQ 20006 upon DNA and protein synthesis in the B lymphoblastoid cell line, Raji, maintained in the mid-log phase of cell growth. Raji cells were incubated in the absence or presence of different concentrations of SQ 20006 (Fig. 7A, left panel) or rapamycin (Fig. 7A, right panel) for 20 h, prior to measuring the rates of DNA or protein synthesis, as described under “Experimental Procedures.” Panel A shows that, as with Swiss 3T3 cells, SQ 20006 inhibited DNA synthesis, with an IC50 of 0.1–0.2 mM; inhibition of protein synthesis was also evident over this concentration range. However, the two structurally unrelated p70S6k inhibitors, SQ 20006 and rapamycin, display a different potency with regard to inhibition of DNA synthesis. The former fully blocks it (Fig. 7A, left panel), whereas the latter displays only a weak effect (Fig. 7A, right panel). In Swiss 3T3 cells, rapamycin has been reported to slightly decrease the rate of initiation and block the preferential translation of a class of mRNAs (18, 48). To analyze whether SQ 20006 was affecting the initiation phase of translation, extracts were prepared from control or SQ 20006-treated cells, and their ribosomes were analyzed by sucrose density centrifugation. Relative to the control cells, polysome profiles obtained from SQ 20006-treated Raji cells (Fig. 7B) show a clear decrease in...
Selective Inhibition of p70\(^{S6k}\) and G\(_1\) Progression

Fig. 3. SQ 20006 treatment prevents entry into S phase in Swiss 3T3 cells after serum starvation/stimulation. Quiescent Swiss 3T3 fibroblasts were stimulated with serum, and progression into the cell cycle was scored by flow cytometric measurement of the DNA content at 8, 12, 16, 20, 24, and 32 h (panels A–F); cells shown in panel G were incubated for 20 h in the presence of 5 \(\mu\)g/ml aphidicolin, and those in panel I were incubated for the same time in the presence of 1 \(\text{mM}\) SQ 20006. Asynchronous cultures of Swiss 3T3 cells treated for 24 h with 1 \(\text{mM}\) SQ 20006 are shown in panel H.

heavy polysomes, which is suggestive of a decrease in the initiation rate.

Considering the differences in the magnitude of inhibition observed between SQ 20006 and rapamycin, we asked whether SQ 20006 might act on translational targets other than p70\(^{S6k}\). One potential site of regulation is initiation factor 4E (eIF-4E), which is a limiting factor in the process of initiation and has been demonstrated to undergo phosphorylation in response to numerous growth factors and hormones in a variety of cells (11–13). Recently it has been suggested that, in addition to phosphorylation, interaction of eIF-4E with other proteins, such as PHAS-I (4E-BP1 (31, 32, 49)) may play a role in translational control. Therefore, we have looked at the association between eIF-4E and PHAS-I following treatment of cells with SQ 20006, by isolation of the former on m\(^7\)GTP-Sepharose, as described under “Experimental Procedures.” As shown in Fig. 7C, prolonged treatment of Raji cells with SQ 20006 did not affect the interaction of eIF-4E with its inhibitory partner PHAS-I. We have also examined the phosphorylation status of eIF-4E by vertical slab isoelectric focusing and immunoblotting. Fig. 7D shows that SQ 20006 has no effect on the steady state phosphorylation of eIF-4E. In order to test whether SQ 20006 has a direct inhibitory effect on the translational machinery, we have also employed the reticulocyte lysate translation system. This in vitro system is unique, as it maintains high levels of protein synthesis, which is largely independent of any requirement for S6 phosphorylation; indeed, p70\(^{S6k}\) activity in the reticulocyte lysate is low relative to that found in mitogen-stimulated cells.\(^3\) As shown in Fig. 8A, time course and dose-response experiments indicate that SQ 20006 displayed a significant inhibition of translation at concentrations comparable with those used above. Analysis of polysomes by sucrose density gradient centrifugation (Fig. 8B) shows that SQ 20006 induces a weak and incomplete disaggregation of ribosomes from polysomes and a rise in the content of free 80S ribosome couples. This was also seen in the presence of the elongation inhibitor, emetine, suggesting that part of the inhibitory effect of SQ 20006 is due to activation of low levels of nuclease. However, this level of nuclease is insufficient to account for the large inhibition of translation shown in panel A (data not shown). An alternative conclusion is that SQ 20006 induces a weak inhibition at the level of initiation, with a dominant effect at the level of elongation, the latter possibly through activation of eEF-2 kinase via protein kinase A (50). The inhibition of translation in the reticulocyte lysate appears to be an in vitro effect; treatment of intact reticulocytes (37) with 1 \(\text{mM}\) SQ 20006 for 90 min did not affect the rate of translation in derived lysates, polysome disaggregation, the

\(^3\) V. Frost and S. J. Morley, unpublished data.
association between eIF-4E and PHAS-I, or the steady state phosphorylation of eIF-4E (data not shown). This possibly reflects the lack of requirement of this translation system for S6 phosphorylation and activation of p70S6k.

**DISCUSSION**

In many cell systems examined, stimulation of quiescent cells to re-enter the cell cycle with serum or growth factors causes an immediate drop in the intracellular concentration of cAMP (51). Concomitant with this event is the activation of kinase cascades, which mediate the mitogenic effect of growth factors (11, 17), leading to evidence of a negative correlation between higher levels of cAMP and mitogenesis. This has been shown in studies that have addressed the role of the cAMP-dependent protein kinase (protein kinase A) during cell cycle progression in yeast (52), during meiotic maturation of Xenopus oocytes (53) and directly on intracellular signaling pathways (54). A number of hypotheses have been proposed to explain the mechanism by which raised levels of cAMP yield a G1 block; these include inhibition of activation of the ras kinase (54) and increased levels of p27Kip, which in turn inhibits cyclin-dependent kinase 4 activity (55).

**TABLE II**

Effect of SQ 2006 addition on S phase entry

| Addition          | [3H]Thymidine % inc |
|-------------------|---------------------|
| None              | 100                 |
| SQ 2006           | 0.5                 |
| Rapamycin         | 48                  |
| 8-Br-cAMP         | 105                 |

**FIG. 4.** Dose-response effect on S phase entry by SQ 2006. Increasing amounts of SQ 2006 were added to quiescent Swiss 3T3 fibroblasts at the time of stimulation with serum. To estimate DNA synthesis, at 14 h (mid S phase) cells were pulse-labeled with 1 μCi of [3H]thymidine for 2 h and harvested as described under “Experimental Procedures.” The data are representative of those obtained in three separate experiments.

**FIG. 5.** Rapamycin, but not 8-Br-cAMP or IBMX inhibit the activation of p70S6K. Quiescent cells were stimulated with phosphate-buffered saline (lane 1) or serum (lanes 2-5) for 1 h, in the absence (lanes 1 and 2) or presence of 1 mM IBMX (lane 3), 20 ng/ml rapamycin (lane 4), or 1 μM 8-Br-cAMP (lane 5). Cell extracts were prepared and p70S6K activation was then monitored by SDS-PAGE, as described.

**FIG. 6.** The effect of time course of addition and withdrawal of SQ 2006 on S phase entry. Panel A, 0.5 mM SQ 2006 was added to quiescent cells at 0, 2, 4, 6, 8, 10, and 12 h (lanes 2 to 8) following stimulation with serum. [3H]thymidine was added at the beginning of the incubation, and cells were harvested at 14 h. Values are expressed as percentage of [3H]thymidine incorporation relative to untreated cells (lane 1). Panel B, 0.5 mM SQ 2006 was added to quiescent cells at the time of stimulation with serum. The drug was then removed by washing aliquots of cells and replacing the medium at 0, 2, 4, 6, 8, 10, and 12 h (lanes 2-8). Cells were labeled and harvested as described in panel A. Lane 1, untreated cells.

**TABLE III**

Effect of SQ 2006 withdrawal on S phase entry

| Addition          | Hours |
|-------------------|-------|
| SQ 2006           | 15    |
| Hydroxyurea       | 4     |
Recent data obtained using a lymphoid cell line has indicated another target of protein kinase A to be the inactivation of p70S6k (56). On the other hand, previous data on S6 phosphorylation in Swiss 3T3 fibroblasts have shown that changes in total intracellular levels of cAMP were not involved in the inhibition of either S6 phosphorylation or protein synthesis initiation (43). The data presented in Fig. 1 show that in Swiss 3T3 cells, SQ 20006 is a potent inhibitor of the pathway leading to p70S6k activation, although not a direct inhibitor of the activated kinase itself in vitro. Panel C, Raji cell extracts were prepared as in panel B, and eIF-4E was isolated as described under “Experimental Procedures.” Proteins recovered from the affinity resin were subjected to SDS-PAGE, resolved proteins were transferred to polyvinylidene difluoride, and eIF-4E and PHAS-I were identified using specific antisera. Panel D, samples prepared as in panel C to enrich for eIF-4E were subjected to one-dimensional vertical slab isoelectric focussing and immunoblot analysis with antisera specific for eIF-4E, as described. The migration of the phosphorylated form of eIF-4E is indicated.

Contrary to other mitogen-induced protein kinases (such as ERK2; Fig. 1), p70S6k activity remains high throughout G1, and microinjection of inhibitory antibodies at any time in G1 can block the G1/S transition (26, 27). Considering the low turnover rate of SQ 20006 and that it was not toxic to cells, we set out to examine whether prolonged inhibition of p70S6k was involved in blocking transition to S phase. As shown by FACScan analysis, SQ 20006 addition to cells yields an apparent G1/S block, similar to that seen with aphidicolin (Fig. 3 and Table II), which could be reversed by removing the drug (Table III). We conclude that the inhibition set by SQ 20006 cannot be bypassed and does not allow cells to progress to S phase. In
similar experiments, the immunosuppressant rapamycin is known to cause only a delay in the transition to S phase despite full inhibition of p70S6k activity (Table II and Refs. 20, 22, and 57). Furthermore, we observed that release from SQ20006 did not allow a rapid initiation of DNA replication. Cells appeared to require at least 10 h to enter S phase (Table III), indicating that SQ20006 is an early G1 blocker. Treatment of cells with SQ20006 at different times following restimulation with serum (Fig. 6) showed that the compound could set an effective block only before the restriction point (47). In agreement with published data, this implies that p70S6k function is necessary throughout the G1 phase of the cell cycle (26). Accordingly, withdrawal of the drug from the medium within the first 2 h of exposure allowed cells to proceed to S phase, whereas removal at times after 4 h did not prevent the inhibitory effect of SQ20006 on the transition to S phase (Fig. 6B and Table IIII). These data suggest that as a result of, or as a consequence of, the inactivation of p70S6k, cells are returned to an early point in G1, possibly to enable the cells to resynthesize labile proteins necessary for G1 progression. Indeed, immunoprecipitation of cyclin-dependent kinase 2 with anti-cyclin E antiserum indicated a dramatic drop in cyclin-dependent kinase 2 activity 10 h following serum stimulation in the presence of SQ20006 (data not shown) without affecting the activity of this kinase in vitro (Table I).

Early studies with SQ20006 in Swiss 3T3 cells showed that the addition of the drug decreased the mobilization of 80 S ribosomes into polysomes (43). This is indicative of a lesion at the level of polypeptide chain initiation. We now show that SQ20006 can inhibit translation initiation in the B lymphoblastoid cell line, Raji, when maintained in the mid-log phase of growth (Fig. 7A, left panel). The effect of SQ20006 on translation was more pronounced than that seen with rapamycin (Fig. 7A, right panel). Because both SQ20006 and rapamycin inhibit the activation of p70S6k, we have studied other aspects of protein synthesis initiation in an attempt to explain the different effects of these compounds on protein synthesis. In mammalian cells, there is evidence for the regulation of translation by phosphorylation of initiation factors and their associated proteins (PHAS-I, 4E-BP1) involved in binding mRNA to the 40 S ribosome (11–13, 16, 29, 31, 32, 49). Although S6 phosphorylation may play a role in the selective binding of mRNA species to ribosomes (17, 18, 48), the activity of initiation factors, such as the eIF-4F complex, may also influence the selection of mRNA from the cellular pool for translation (11–13). It is believed that the eIF-4F complex functions to unwind secondary structure in the mRNA 5'-untranslated region to facilitate binding of the 40 S ribosome. As with S6, in response to the appropriate stimuli, increased levels of eIF-4E phosphorylation have been directly correlated with increased rates of translation in a variety of cell types (11–13). The data presented in Figs. 7 and 8 show that SQ20006 affected neither the association between eIF-4E and PHAS-I nor the phosphorylation status of eIF-4E in Raji cells or reticulocyte lysate, respectively. Together these data confirm the finding that eIF-4E lies on a
signaling pathway distinct from that of p70S6k (39) and suggest that protein kinase Cε and/or protein kinase Cγ is not involved in the serum-stimulated phosphorylation of ElF-4E in vivo. At this time, the exact function of the coordinate phosphorylation of S6 and ElF-4E in recruiting mRNA to the ribosome is not understood, although each appears to be mediated by separate signaling pathways.

Acknowledgments—We are grateful to Dr. G. Thomas (Basel, Switzerland) for providing SQ 20053 and antibodies to p70S6k. In addition we are indebted to Drs. B. Hemmings (Basel, Switzerland), S. Stabel (Cologne, Germany), and L. Meijer (Roscoff, France) for providing protein kinase A, protein kinase C isoforms, and p34\(^{cdk2}\)/cyclin B, respectively. We thank Dr. M. Hümblin for critical review of the manuscript. We are also grateful to I. Fernandez for secretarial assistance and F. Wüttig for help with photography.

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