Salicylate-induced Growth Arrest Is Associated with Inhibition of p70<sup>66k</sup> and Down-regulation of c-Myc, Cyclin D1, Cyclin A, and Proliferating Cell Nuclear Antigen*  

Received for publication, June 23, 2000, and in revised form, September 11, 2000  
Published, JBC Papers in Press, September 18, 2000, DOI 10.1074/jbc.M005545200

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Salicylate and its pro-drug form aspirin are widely used medicinally for their analgesic and anti-inflammatory properties, and more recently for their ability to protect against colon cancer and cardiovascular disease. Despite the wide use of salicylate, the mechanisms underlying its biological activities are largely unknown. Recent reports suggest that salicylate may produce some of its effects by modulating the activities of protein kinases. Since we have previously shown that the farnesyltransferase inhibitor L-744,832 inhibits cell proliferation and p70<sup>66k</sup> activity, and salicylate inhibits cell proliferation, we examined whether salicylate affects p70<sup>66k</sup> activity. We find that salicylate potently inhibits p70<sup>66k</sup> activation and phosphorylation in a p38 MAPK-independent manner. Interestingly, low salicylate concentrations (<250 μM) inhibit p70<sup>66k</sup> activation by phorbol myristate acetate, while higher salicylate concentrations (≥5 mM) are required to block p70<sup>66k</sup> activation by epidermal growth factor + insulin-like growth factor-1. These data suggest that salicylate may selectively inhibit p70<sup>66k</sup> activation in response to specific stimuli. Inhibition of p70<sup>66k</sup> by salicylate occurs within 5 min, is independent of the phosphatidylinositol 3-kinase pathway, and is associated with dephosphorylation of p70<sup>66k</sup> on its major rapamycin-sensitive site, Thr<sup>389</sup>. A rapamycin-resistant mutant of p70<sup>66k</sup> is resistant to salicylate-induced Thr<sup>389</sup> dephosphorylation.

Salicylate has been used since ancient times for its analgesic and anti-inflammatory properties. More recently, aspirin has received attention because of its protective effects against colon cancer (1, 2) and cardiovascular disease. Aspirin is a pro-drug form of salicylate that is rapidly hydrolyzed to salicylate in vivo (3). Aspirin is known to act by directly inhibiting cyclooxygenases 1 and 2 (COX1 and COX2),<sup>1</sup> thereby blocking the production of prostaglandins. Salicylate however, inhibits the synthesis of prostaglandins in vivo, but has little effect on COX1 and COX2 activities in vitro (3). Salicylate must therefore inhibit COX1 and COX2 activities in vivo through an alternate mechanism not involving direct effects on COX1 and COX2. It has recently been reported that salicylate potently inhibits transcription of the COX2 gene (4), although how salicylate abrogates COX2 transcription is unknown.

It is also becoming apparent that many of the biochemical effects of salicylate are independent of effects on cyclooxygenase activity. The increasing number of protein kinases reported to be modulated by salicylate might provide a potential explanation for the ability of salicylate to regulate COX2 transcription, as well as the cyclooxygenase-independent effects of salicylate. Previous studies in our laboratory demonstrating that the p38 MAPK inhibitor SB203580 potentiates PMA-induced p70<sup>66k</sup> activation (5), along with studies demonstrating that salicylate activates p38 MAPK (6) suggest that salicylate might regulate p70<sup>66k</sup> activity through a p38 MAPK-dependent mechanism.

Although there have been many recent discoveries involving the mechanisms of p70<sup>66k</sup> activation, the kinases that phosphorylate a number of the key regulatory sites of p70<sup>66k</sup> are unknown. Also unknown are the identities of the p70<sup>66k</sup> substrates responsible for mediating its biological effects. p70<sup>66k</sup> was among the first mitogen-activated protein kinases identified (7, 8), and has subsequently been shown to be important for G<sub>1</sub> cell cycle progression (9–11). Although the regulation of p70<sup>66k</sup> activity is complex, it is clear that the mammalian target of rapamycin, mTOR, plays a key role in p70<sup>66k</sup> activation. The immunosuppressant drug rapamycin acts by first binding FKBP12, and then forming a ternary complex with mTOR, resulting in mTOR inactivation. Through mechanisms not completely understood, mTOR regulates the phosphorylation of multiple sites on p70<sup>66k</sup>. Thr<sup>389</sup> was reported to be the major rapamycin-sensitive p70<sup>66k</sup> phosphorylation site because it is dephosphorylated with the most rapid kinetics, is required for p70<sup>66k</sup> activity, and a mutant in which Thr<sup>389</sup> is mutated to Glu displays partial rapamycin resistance (12).

Here we show that salicylate inhibits p70<sup>66k</sup> activity and phosphorylation in a p38 MAPK-independent manner. Salicy-
late inhibits PMA-induced \(p70^{65k}\) activation at low concentrations (\(\geq 250 \mu M\)), and both PMA and growth-factor-induced \(p70^{65k}\) activation at higher concentrations (\(\geq 5 \mu M\)). Salicylate-induced \(p70^{65k}\) dephosphorylation at Thr\(^{389}\) is not mediated through effects on the PI 3-kinase pathway. Finally, we demonstrate that salicylate and FTY induce the same effects on the levels of cell growth-associated proteins as the mTOR inhibitor rapamycin, specifically, down-regulation of c-Myc, cyclin D1, cyclin A, and PCNA protein levels.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, \(^{[3]}H\)Thymidine Incorporation Assays, and \(^{[35]}S\)Methionine/Cysteine Incorporation Assays—**Balb/MK cells were maintained as described previously (13). HEK 293 cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were treated as indicated in the figure legends with 1 \(\mu M\) PMA (Sigma), 20 \(\mu M\) SB203580 (Alexis), or 10 \(\mu M\) LY294002 (Sigma) dissolved in dimethyl sulfoxide. Sodium salicylate (Sigma) was prepared as a 3 \(M\) stock solution in water and diluted into medium immediately before use. One molar stock solutions of acetaminophen (Sigma) and indomethacin (Sigma) were prepared in ethanol and diluted into medium immediately before use.

For \(^{[3]}H\)thymidine incorporation assays, cells were plated at 20,000 cells/well in 24-well plates and incubated overnight. Cells were then pretreated as indicated in the figure and pulsed for 1 h with 20 \(\mu Ci/\text{well}\) of \(^{[3]}H\)thymidine (PerkinElmer Life Sciences), and the results were analyzed as described previously (13). \(^{[35]}S\)Methionine/cysteine incorporation was performed as with the \(^{[3]}H\)thymidine incorporation assays, except that the cells were pulsed with 20 \(\mu Ci/\text{well} Tran^{35}S\)-label (ICN) and the cell lysates were prepared in 0.1 \(\%\) NaOH containing 0.1% sodium dodecyl sulfate.

**Preparation of Cell Extracts—**Cell lysates were prepared as described previously (13) except in Fig. 6A. Since in Fig. 6A some of the proteins analyzed were nuclear, the extraction buffer was supplemented with 0.1% sodium dodecyl sulfate, and the lysates were sonicated prior to centrifugation. The protein concentrations of the extracts analyzed were nuclear, the extraction buffer was supplemented with 0.1% sodium dodecyl sulfate, and the lysates were sonicated prior to centrifugation. The protein concentrations of the extracts were quantitated using the Bradford assay (Bio-Rad), with bovine serum albumin as the standard.

**Immunoblotting and Kinase Assays—**Immunoblotting and kinase assays were performed as described previously (13) using the S6 peptide (Santa Cruz) as the substrate for \(p70^{65k}\). The results of kinase assays were visualized using a PhosphorImager and quantitated using ImageQuant software (Molecular Dynamics). Antibodies specific for \(p70^{65k}\) (catalog no. sc-230), c-Myc (catalog no. sc-764), cyclin A (catalog no. sc-586), cyclin D1 (catalog no. sc-450), or p27 (catalog no. sc-528) were obtained from Santa Cruz. Phosphorylation site-specific antibodies recognizing phospho-Thr\(^{389}\) \(p70^{65k}\) (catalog no. 9205), phospho-p38 MAPK (catalog no. 9211), and phospho-Thr\(^{202}\) Akt (catalog no. 9275) were obtained from New England Bioslabs. Antibodies specific for phospho-Erk1/2 (catalog no. V8031), the flag epitope tag (catalog no. F-3165), proliferating cell nuclear antigen (catalog no. NA03), and \(\beta\)-tubulin (catalog no. N357) were obtained from Promega, Sigma, Oncogene Science, and Amersham Pharmacia Biotech, respectively.

cDNAs and Transient Transfections—A plasmid encoding HA-tagged p85\(^{65k}\) was kindly provided by Joseph Avruch (Harvard Medical School, Cambridge, MA). The flag-\(\Delta\)N\(\Delta\)C\(\Delta\)T\(\Delta\)C construct was described previously (5). Flag-tagged wild type \(p70^{65k}\) was amplified from the plasmid encoding HA-p85\(^{65k}\) using PCR with the primer sets: 5'-TTTTGGATCCATACATGAGGACGGATGTAAGCAACAGGATATTGGTTTTCCCTAGATGTCACAGAGGGACGAATTCCCAATGTCG-3' and 5'-TTTTGATATCTCAGATCAGTACGATCACAAGGCGGTGGTGTTTTGGTTTTCCCTAGATGTCAGGAGGACGAATTCCCAATGTCG-3'. Underlined portions represent regions complementary to the plasmid template. PCR was performed using \(F\) \(F\) polymerase (Promega) according to the manufacturer’s instructions. The PCR product was digested with EcoRI and BamHI and subcloned into the pcDNA3.1 expression vector (Invitrogen). The insert was verified to be correct by DNA sequencing. For transfections, HEK 293 cells were seeded at 800,000 cells/100-mm dish and incubated overnight. The cells were transfected with \(6 \mu g\) of either the flag-p85\(^{65k}\) or flag-\(\Delta\)N\(\Delta\)C\(\Delta\)T\(\Delta\)C constructs using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Cell extracts were prepared as described above.

**RESULTS**

We previously observed that the p38 MAPK inhibitor SB203580 is able to potentiate the activation of \(p70^{65k}\) by PMA (5). These results are similar to those reported by others (14, 15) in which SB203580 activates MAPK by blocking a negative regulatory pathway mediated by p38 MAPK. Since sodium salicylate was shown to activate p38 MAPK (6), we hypothesized that salicylate might inhibit \(p70^{65k}\) by activating a negative regulatory pathway involving p38 MAPK. As expected, salicylate treatment of Balb/MK cells potently abrogated PMA-induced \(p70^{65k}\) activation (Fig. 1). Inhibition of \(p70^{65k}\) by salicylate was associated with a shift in electrophoretic mobility and decreased phospho-Thr\(^{389}\) staining, consistent with salicylate-inhibited \(p70^{65k}\) dephosphorylation.

The inhibition of \(p70^{65k}\) by salicylate, however, was independent of effects on p38 MAPK activity because SB203580 failed to block the inhibitory effect of salicylate on \(p70^{65k}\) activation even though SB203580 was still able to induce an increase in phospho-Erk1/2 staining in the presence of salicylate (Fig. 1). These results indicate that, under these experimental conditions, SB203580 blocked the effect of p38 MAPK on Erk1/2 phosphorylation. In addition, salicylate treatment did not induce a significant increase in the phosphorylation of p38 MAPK on sites required for activity. Together, these results demonstrate that salicylate inhibits \(p70^{65k}\) in a p38 MAPK-independent manner.

Because salicylate is an aspirin metabolite and its ability to inhibit \(p70^{65k}\) may play a role in the physiological responses elicited by aspirin and other salicylates, the ability of salicylate to inhibit \(p70^{65k}\) and cell proliferation was explored. To determine whether aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) and analgesic drugs affect \(p70^{65k}\) activity, Balb/MK cells were treated with various NSAIDs and cell extracts were prepared and assayed for \(p70^{65k}\) activity, \(p70^{65k}\) phosphorylation, and Erk1/2 phosphorylation (Fig. 2). Salicylate and aspirin both inhibited \(p70^{65k}\) activity while acetaminophen and indomethacin had little effect. As with salicylate, the inhibition of \(p70^{65k}\) by aspirin was associated with a shift in electrophoretic mobility and a decrease in the phospho-
Salicylate (Fig. 3). Both treatments stimulated p70s6k activity in a manner similar to rapamycin by inhibiting p70s6k activity by dephosphorylation of Thr389 suggests that salicylate inhibits kinases upstream of p70s6k, resulting in p70s6k inhibition. The phosphorylation of Thr389 is required for p70s6k catalytic activity (12). Decreased phosphorylation of Thr389 suggests that salicylate inhibits kinases upstream of p70s6k, resulting in p70s6k inhibition.

To determine the salicylate concentration range required to inhibit p70s6k, Balb/MK cells were stimulated with PMA or EGF + IGF-1 in the presence of increasing concentrations of salicylate (Fig. 3). Both treatments stimulated p70s6k activity to similar levels in the absence of salicylate. p70s6k was inhibited by almost 40% in the presence of 250 μM salicylate when PMA was used as the stimulus (Fig. 3A). For comparison, administration of a single analgesic antiinflammatory dose of salicylate to patients yields a serum salicylate concentration of about 400 μM (3). Near complete inhibition of PMA-stimulated p70s6k activity, however, required 5 mM salicylate. ImmunobLOTS performed using antibodies to p70s6k (second panel) or phospho-Thr389 p70s6k (third panel) demonstrated that inhibition is associated with p70s6k dephosphorylation. Dephosphorylation was apparent for both the p70 upper bands and p85 lower bands alternative translational products of the S6 kinase gene.

Interestingly, activation of p70s6k by EGF and IGF-1 was only inhibited by higher (≥5 mM) concentrations of salicylate (Fig. 3B). The results of the kinase assays in Fig. 3 (A and B) were normalized to control and plotted in Fig. 3C. These data suggest that salicylate may act on two distinct targets during p70s6k activation. One putative target would be sensitive only to high salicylate concentrations and would be required for activation of p70s6k by both PMA and EGF + IGF-1. The other putative target would be sensitive to lower concentrations of salicylate and would be required only for activation of p70s6k by PMA. Thus, at low concentrations (≥1 mM), salicylate mimics the down-regulation of PKCs by chronic PMA treatment, which specifically blocks p70s6k activation in response to PMA treatment (5). At high concentrations (≥5 mM), salicylate acts in a manner similar to rapamycin by inhibiting p70s6k activity irrespective of the stimulus. The millimolar concentrations of salicylate required to inhibit p70s6k activation regardless of the stimulus are similar to those reported by others to induce effects on p38 MAPK activity (6), IxB phosphorylation (6, 16), and cellular growth (17).

To determine whether salicylate acts rapidly to inhibit p70s6k, consistent with a relatively direct effect on the p70s6k signaling pathway, time course experiments were performed. Balb/MK cells were treated with 20 mM salicylate in normal growth medium (Fig. 4A). At 5 min salicylate induced a 53% inhibition of p70s6k activity, indicating a rapid effect of salicylate on p70s6k activity. p70s6k inhibition reached a plateau at 20 min and was associated with a shift in p70s6k electrophoretic mobility and a loss of phospho-Thr389 immunostaining. The observation that salicylate rapidly inhibited p70s6k activity suggests p70s6k inhibition was independent of effects on COX2, because COX2 regulation at the transcriptional level (4) could not ex-
The presence of salicylate and the results analyzed as described under "Experimental Procedures." The data presented represent the average of triplicate determinations ± standard deviation.

In order to determine whether the rapid inhibition of p70s6k by salicylate correlates with a rapid inhibition of protein synthesis, the rate of protein synthesis was determined after various salicylate pretreatment intervals by monitoring the incorporation of [35S]labeled methionine and cysteine into cellular protein (Fig. 4C). Salicylate induced a greater than 50% inhibition of protein synthesis after a 5-min pretreatment, followed by a 1 h pulse with [35S]Met/Cys in the continued presence of salicylate. This inhibition was maximal because no further inhibition occurred after a 2-h pretreatment. Since protein synthesis is required throughout the cell cycle in order for cells to proliferate (17), the inhibition of protein synthesis by salicylate may play a role in salicylate-induced growth arrest.

In order to gain some insight into how salicylate might inhibit p70s6k by salicylate, we made use of a rapamycin-resistant deletion mutant of p70s6k (ΔNT/ΔCT). We have previously shown that wild-type p85s6k becomes dephosphorylated on Thr389 in response to treatment with FTI, rapamycin, and the PI 3-kinase inhibitor LY294002. The Thr389 site of ΔNT/ΔCT, however, is unaffected by FTI or rapamycin treatment, and is only dephosphorylated in response to LY294002 treatment (5). To determine the effect of salicylate on Thr389 phosphorylation of both p70s6k and ΔNT/ΔCT, HEK 293 cells were transfected with either construct and left untreated or treated with 20 mM salicylate, 5 mM rapamycin, or 10 mM LY294002 (Fig. 5). Analysis of the resulting cell extracts by immunoblotting with antibodies specific for phospho-Thr389 p70s6k (P-T389 IB), the flag epitope (Flag IB), or Akt phosphorylated on Thr389 (P-308 Akt IB) had a rapid effect on DNA synthesis, the magnitude of the effect was small at early time points, and gradually increased to approximately 50% inhibition of [3H]thymidine incorporation after a 2-h salicylate pretreatment. One of the earliest observations that may relate to the ability of salicylate to inhibit cell proliferation involves the ability of salicylate to inhibit protein synthesis without inhibiting the uptake of amino acids (16).

Because of our past observations that FTI rapidly inhibited both p70s6k activity and cell proliferation, we examined whether salicylate could rapidly inhibit DNA synthesis by performing [3H]thymidine assays (Fig. 4B). Although salicylate...
inhibited ΔNT/ΔCT phosphorylation on Thr<sup>389</sup>. As expected, rapamycin had no effect on ΔNT/ΔCT phosphorylation at Thr<sup>389</sup>. Immunoblotting with antibodies specific for the flag epitope tag present on both proteins indicated that the expression levels of the constructs were similar in each of the four treatments. These results suggest that salicylate did not primarily inhibit Thr<sup>389</sup> phosphorylation of p70<sup>66k</sup> by a mechanism involving the PI 3-kinase pathway. Similar results were also obtained in COS 7 cell transfections, and the splice variant p85<sup>66k</sup> behaved identically to p70<sup>66k</sup> with respect to salicylate-induced inhibition and dephosphorylation (data not shown). To further demonstrate that salicylate does not act through the PI 3-kinase pathway, we examined the effect of salicylate on the phosphorylation of Akt on Thr<sup>389</sup> using a phosphospecific antibody. Phosphorylation of Akt on Thr<sup>389</sup> is catalyzed by the kinase PDK1 through a PI 3-kinase-dependent mechanism (18) and thus serves as a marker for in vivo PDK1 activity. Salicylate treatment had no effect on Thr<sup>389</sup> phosphorylation, while LY294002 caused a marked reduction in Thr<sup>389</sup> phosphorylation. Moreover, salicylate abrogated PMA-induced activation of p70<sup>66k</sup> (Figs. 1 and 3), which occurs through a PI 3-kinase-independent pathway (5, 19). Together these results suggest that salicylate acts like rapamycin and FTI rather than PI 3-kinase inhibitors to block p70<sup>66k</sup> activity.

There is an increasing body of evidence implicating the mTOR signaling pathway (20, 21), and more specifically p70<sup>66k</sup> (9–11), in regulating cell proliferation. Little is currently known regarding the identity of the downstream targets of p70<sup>66k</sup> involved in cell cycle regulation or how these targets regulate cell cycle events. In order to more fully characterize the effects of salicylate, rapamycin, and FTI on the cell cycle, rapidly growing Balb/MK cells were treated for 24 or 48 h with each agent and cell extracts were prepared. Analysis of the extracts was performed with antibodies specific for c-Myc, cyclin D1, PCNA, and cyclin A (Fig. 6A). The levels of these proteins were previously reported to be regulated by either rapamycin (22–28) or salicylate (29), and are known to play a role in cell proliferation. The results revealed that the most rapid and dramatic effect of the three agents was c-Myc down-regulation. c-Myc down-regulation at 24 h correlated closely with Thr<sup>389</sup> dephosphorylation of p70<sup>66k</sup>, suggesting that the mTOR/p70<sup>66k</sup> pathway may play a role in regulating c-Myc levels in Balb/MK cells as was reported in Epstein-Barr virus immortalized B-cell lines (22). At 48 h, rapamycin, salicylate, and FTI treatment continued to suppress c-Myc levels and also induced a down-regulation in the levels of cyclin D1, PCNA, and cyclin A. The two c-Myc bands likely represent the 67- and 64-kDa isoforms, termed c-Myc1 and c-Myc2, respectively (30). The three cyclin D1 bands observed may result from alternative splicing (31) and/or phosphorylation. Although rapamycin up-regulates p27 in T cells (32), neither rapamycin, salicylate, nor FTI up-regulates p27 in Balb/MK cells. The decrease in the levels of c-Myc, cyclin D1, PCNA, and cyclin A was highly reproducible and not due to unequal protein loading because equal amounts of total protein were loaded into each lane based on protein assays, and based on immunoblotting with a β-tubulin antibody as a loading control. The ability of salicylate and FTI to induce the same decreases in the levels of c-Myc, cyclin D1, PCNA, and cyclin A as rapamycin may indicate a possible role for the mTOR/p70<sup>66k</sup> signaling pathway in the growth inhibitory actions of these drugs. Importantly, the changes observed in the levels of proliferation-associated proteins correlated well with the inhibition of DNA synthesis (Fig. 6B). Rapamycin inhibited DNA synthesis by approximately 50% and 75% at 24 and 48 h, respectively, while high concentrations of salicylate and FTI were able to inhibit DNA synthesis to a greater extent. The increased inhibition induced by high salicylate and FTI concentrations may result from the inhibition of pathways in addition to the mTOR/p70<sup>66k</sup> pathway. FTI inhibits the farnesylation of a number of proteins, so its effects on cell proliferation represent an integration of the effects of FTI on all farnesylated proteins. Similarly, salicylate inhibits NF-κB activity (33) as well as affecting the activity of several different protein kinases (6, 34, 35). Indeed, we have observed inhibition of NF-κB DNA binding activity in Balb/MK cells at salicylate concentrations similar to those required to inhibit p70<sup>66k</sup> (data not shown). Thus, salicylate, like FTI, probably inhibits cell proliferation through actions on multiple signaling pathways. Since high concentrations of salicylate (≥5 mM) are required to

![Fig. 6. Salicylate, rapamycin, and FTI induce similar changes in the levels of proliferation-associated proteins. A, Balb/MK cells were plated at 700,000 cells/100-mm dish and incubated 24 h. Cells were treated with inhibitors at the indicated concentrations in normal growth medium for either 24 or 48 h, and cell extracts were prepared. Cell extracts were analyzed as described in Fig. 1 by immunoblotting with antibodies specific for phospho-Thr<sup>389</sup> p70<sup>66k</sup> (p-T389 IB), c-Myc, cyclin D1, PCNA, cyclin A, p27, or β-tubulin. B, Balb/MK cells were plated at 20,000 cells/well in 24-well plates and incubated 24 h. Cells were incubated with the same treatments used in A and incubated for either 24 or 48 h and pulsed for 1 h with [³H]thymidine. Results were analyzed and presented as described in Fig. 4B.](http://www.jbc.org/)
inhibit p70^{66k} activation in response to serum (Fig. 2) or EGF + IGF-1 (Fig. 3), it is unclear whether salicylate-induced inhibition of p70^{66k} activity and cell proliferation is physiological because serum salicylate concentrations of \(-2.5\) mM are the highest achieved clinically (3). Chronic salicylate treatment may allow inhibition of p70^{66k} at lower salicylate concentrations (Fig. 6A). Further studies will be necessary to determine whether salicylate-induced p70^{66k} inhibition and growth arrest occur at physiologically relevant concentrations of salicylate.

**DISCUSSION**

We report the first evidence that the mTOR/p70^{66k} pathway may play a role in mediating some of the biological effects of salicylate. The effect of salicylate on p70^{66k} is apparent within 5 min of treatment. p70^{66k} inhibition is not general to all NSAIDs and analgesic antipyretic drugs because acetaminophen and indomethacin have no effect on p70^{66k} activity.

Mechanistically, low concentrations of salicylate (\(\leq 1\) mM) act similarly to the down-regulation of PKCs by inhibiting p70^{66k} activation by PMA, but not by EGF + IGF-1. High concentrations of salicylate (\(\geq 5\) mM) may appear to act similarly to rapamycin and FTI to inhibit p70^{66k} irrespective of the stimulus used. The ability of salicylate to preferentially inhibit PMA-induced p70^{66k} activation at low concentrations, but to inhibit p70^{66k} activation regardless of the stimulus at higher concentrations, suggests that salicylate may prove to be a useful tool to help identify and distinguish different upstream activators of p70^{66k}.

Salicylate does not act on the PI 3-kinase pathway to inhibit p70^{66k} because: (a) salicylate inhibits p70^{66k} activation by PMA, which occurs independently of the PI 3-kinase pathway (19); (b) salicylate does not inhibit the phosphorylation of Akt on Thr^{308}, an event catalyzed by PKD1 in a PI 3-kinase-dependent mechanism (18); (c) salicylate induces only a weak dephosphorylation of \(\Delta\)NT/\(\Delta\)CT at Thr^{308}, while the PI 3-kinase inhibitor LY294002 induces potent dephosphorylation of \(\Delta\)NT/\(\Delta\)CT at Thr^{308}. We (5) and others (36, 37) have previously argued that rapamycin acts through a protein phosphatase to induce p70^{66k} dephosphorylation, based on the observation that rapamycin-resistant deletion mutants of p70^{66k} remain phosphorylated on Thr^{308} in the presence of rapamycin. In addition, the phosphatase inhibitor calyculin A can partially reverse the effects of rapamycin on p70^{66k} (38, 39). One interpretation of these results is that the kinase that normally phosphorylates Thr^{308} is still active in the presence of rapamycin, but the phosphatase that normally dephosphorylates Thr^{308} does not dephosphorylate the truncation mutants. Recent reports demonstrating that both mTOR (40) and PKD1 (41) are able to phosphorylate Thr^{308}, however, raise an alternate possibility. mTOR might normally phosphorylate Thr^{308}, rendering the site rapamycin-sensitive. Thr^{308} of the rapamycin-resistant deletion mutants, however, may be a better substrate for PKD1 than for mTOR, rendering the mutants rapamycin-insensitive, but still sensitive to PI 3-kinase inhibitors. This model is consistent with our results involving FTI (5, 13) and salicylate-induced inhibition of p70^{66k}. The idea that salicylate (1, 2), FTI (42, 43), and rapamycin analogs (23, 44) may be useful anti-cancer drugs, and the observation that these agents all inhibit p70^{66k}, might indicate a common role for p70^{66k} in some of the actions of these drugs.

The observation that salicylate and FTI mimic rapamycin in their ability to induce growth arrest and c-Myc, cyclin D1, cyclin A, and PCNA down-regulation suggest the possibility that inhibition of the mTOR/p70^{66k} pathway may play an important role in the cytostatic effects of salicylate and FTI. These results are consistent with those of others showing that rapamycin can induce down-regulation of c-Myc (22), cyclin D1 (23, 24), cyclin A (27, 28), and PCNA (25, 26), and that induction of growth arrest by salicylate is associated with cyclin D1 down-regulation (29). Although salicylate can inhibit p70^{66k} phosphorylation, inhibit cell proliferation, and induce down-regulation of c-Myc, cyclin D1, PCNA, and cyclin A at relatively high concentrations (\(>2\) mM), it is unclear whether clinically relevant concentrations of salicylate (\(\leq 2.5\) mM) are able to produce these effects in vivo. It may be possible, however, to design salicylate analogs that would more potently and specifically inhibit p70^{66k} activity in vivo.

Interestingly, the maximal growth inhibition induced by salicylate exceeds that observed in response to rapamycin, as we observed previously with FTI (13). This increased inhibition probably results from the ability of salicylate to inhibit other targets besides p70^{66k}. Salicylate, for example, inhibits NF-\(\kappa\)B (33), which is involved in regulating cell proliferation and is implicated in tumorigenesis (45). Salicylate may inactivate NF-\(\kappa\)B by inhibiting the protein kinases IkB kinase-\(\beta\) (34) and RSK2 (35). Salicylate inhibition of IkB kinase-\(\beta\) was shown to result from competition for ATP binding (34). Competitive inhibition of ATP binding to IkB kinase-\(\beta\) by salicylate provides a potential explanation for the ability of salicylate to inhibit the activity of multiple protein kinases. Although the ability of FTI and salicylate to inhibit multiple signaling pathways makes it more difficult to decipher which pathways are mediating the effects observed, the ability of signal transduction inhibitors to simultaneously inhibit multiple signaling pathways may be important for their ability to inhibit tumorigenic cell growth.

The mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor U0126 for example, which inhibits both the MAPK and p70^{66k} pathways, blocks the anchorage-independent growth of Ki-Ras-transformed fibroblasts (46). The mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor PD98059, however, which also inhibits the MAPK pathway, but only weakly inhibits the p70^{66k} pathway, must be combined with rapamycin to block the anchorage-independent growth of Ki-Ras-transformed fibroblasts.

Together, these observations represent the first report that p70^{66k} is a potential target of salicylates. Further studies will be necessary to determine the role of salicylate inhibition of p70^{66k} in the many biological effects of salicylate, and whether clinically relevant concentrations of salicylate are able to inhibit p70^{66k} activity in vivo.

**Acknowledgments**—We thank Merck Pharmaceuticals for supplying FTI and Joseph Avruch for supplying p85^{66k} cDNA.

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J. Biol. Chem. 2000, 275:38261-38267.
doi: 10.1074/jbc.M005545200 originally published online September 18, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005545200

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