Cooperative Regulation of p70S6 Kinase by Receptor Tyrosine Kinases and G Protein-Coupled Receptors Augments Airway Smooth Muscle Growth †

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Cooperative Regulation of p70S6 Kinase by Receptor Tyrosine Kinases and G Protein-Coupled Receptors Augments Airway Smooth Muscle Growth†

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ABSTRACT: We have previously demonstrated that concomitant activation of receptor tyrosine kinases and certain G protein-coupled receptors (GPCRs) can promote a synergistic increase in the rate of airway smooth muscle cell (ASM) proliferation. Here we clarify the role of p70S6 kinase (p70S6K) as an integrator of receptor tyrosine kinase and GPCR signaling that augments ASM DNA synthesis by demonstrating that specific p70S6K phosphorylation sites receive distinct regulatory input from GPCRs that promotes sustained kinase activity critical to mitogenesis. Prolonged stimulation of ASM cells with EGF and thrombin induced a greater than additive effect in levels of p70S6K phosphorylated at residue T389, whereas a significant but more modest increase in the level of T229 and T421/S424 phosphorylation was also observed. The augmenting effects of thrombin could be dissociated from p42/p44 MAPK activation, as selective inhibition of thrombin-stimulated p42/p44 failed to alter the profile of cooperative p70S6K T389 phosphorylation, p70S6K kinase activity, or ASM [^{3}H]thymidine incorporation. Thrombin stimulated a sustained increase in the level of Akt phosphorylation and also augmented EGF-stimulated Akt phosphorylation. The cooperative effects of thrombin on Akt/p70S6K phosphorylation and [^{3}H]thymidine incorporation were all attenuated by heterologous expression of G$\beta\gamma$ sequestrants. These data suggest that PI3K-dependent T389/T229 phosphorylation is limiting in late-phase p70S6K activation by EGF and contributes to the cooperative effect of GPCRs on p70S6K activity and cell growth.

p70S6K is a highly regulated kinase whose activation promotes both cell hyperplasia and hypertrophy. Activation of p70S6K is a complex process involving the sequential phosphorylation of at least 13 residues by multiple kinases, some as yet unidentified. Phosphorylation occurs initially on a subset of "proline-directed sites" (S411, S418, T421, and S424, for which proline is located at the +1 position) in the autoinhibitory domain of the C-terminus (*1*−*3*). These rapamycin- and wortmannin-resistant sites are rapidly phosphorylated in response to mitogen, and although several proline-directed kinases, including p42/p44 mitogen-activated protein kinase (MAPK),[1] have been shown to phosphorylate these residues in vitro, (*4*) the kinase(s) responsible in vivo remains undetermined. Analyses using various truncation and substitution mutants suggest that phosphorylation of these residues serves to induce a conformational change releasing the catalytic domain from the C-terminal autoinhibitory domain, thus providing access to additional phosphorylation sites (*2*).

To achieve full p70S6K activity, the wortmannin- and rapamycin-sensitive residues T229 in the catalytic domain and S371 and T389 in the linker domain must be phosphorylated (*5*−*7*). Although the downstream kinase of phosphoinositide 3′-kinase (PI3K), 3′-phosphoinositide-dependent protein kinase 1 (PDK1), appears to be the specific kinase for T229 (*8*), the kinase(s) responsible for phosphorylating T389 is unestablished with recent studies suggesting roles for mTOR (*9*), NEK6/7 (*10*), PDK1, protein kinase C (PKC) ζ, and autophosphorylation (*11*).

Questions of p70S6K regulation are frequently examined using transformed cell lines as convenient expression systems, but such an approach can be problematic in that such cells typically have aberrant signaling elements that contribute to transformation or immortalization, and such signals (e.g., constitutive Ras activity or lack of PTEN activity) can be modifiers of p70S6K activity. Moreover, such dysregulated (oncogenic) signaling precludes analyses linking p70S6K signaling and physiologic cell growth.

In an attempt to examine regulation of p70S6K activation under physiologically relevant conditions, we have utilized

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[1] Abbreviations: ASM, airway smooth muscle; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; MAPK, mitogen-activated protein kinase; p70S6K, p70S6 kinase; PTX, pertussis toxin; PKC, protein kinase C; PI3K, phosphoinositide 3′-kinase; PDK1, 3′-phosphoinositide-dependent protein kinase 1; RTK, receptor tyrosine kinase.

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primary cultures of human airway smooth muscle (ASM) which retain many of the properties of differentiated ASM cells in vivo and have been used as a model system for analysis of regulation of cell growth, contraction, migration, and cytokine secretion [reviewed by Billington and Penn (*12*)]. Human ASM cells respond to numerous mitogenic agents, many of which are found to be upregulated in the airways of asthmatics, and an increased rate of ASM proliferation is an important feature of the airway remodeling that contributes to the pathogenesis of chronic asthma (*12*−*14*). We have demonstrated previously that combined activation of receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) induces synergistic proliferation of human ASM cells that is associated with an increase in late-phase activity of p70S6K (*13*). Moreover, the cooperative effect on cell growth extends to various pairings of RTK (PDGF and EGF) and GPCR agonists (thrombin, serotonin, carbachol, angiotensin, histamine, and lysophosphatidic acid) (*13*−*16*) and is consistently observed in contractile, but not noncontractile, cell types (*15*).

To explore the mechanisms and mitogenic effect of cooperative regulation of p70S6K by combined RTK and GPCR stimulation, we chose to initially investigate EGF and thrombin-induced regulation of p70S6K, given that both agents alone are mitogenic but when combined are synergistic in promoting both ASM growth and late-phase p70S6K activity. Here we detail the relative contributions and pathways by which EGF and thrombin effect time-dependent p70S6K phosphorylation to augment ASM proliferation, and identify an important role for G protein $\beta\gamma$ subnits in mediating cooperative signaling by thrombin and EGF.

EXPERIMENTAL PROCEDURES

Materials. All phospho-p70S6K and phospho-Akt antibodies were obtained from Cell Signaling Technology (Beverly, MA) except for phospho-T229p70S6K (R&D Antibodies, Benicia, CA). p70S6K antibody (C-18) was from Santa Cruz Biotechnology (Santa Cruz, CA). IRDye 700 or 800 secondary antibodies were from Rockland (Gilbertsville, PA). Pertussis toxin (PTX), thrombin, and bisindolylmaleimide I (Bis I) were from Calbiochem (San Diego, CA). All other materials were obtained from Sigma (St. Louis, MO). pQE6GαiG203A was provided by A. Gilman (University of Texas Southwestern Medical Center, Dallas, TX).

Cell Culture. Human ASM cultures were established as described previously (*17*) from human tracheae obtained from lung transplant donors. Characterization of these cells with regard to immunofluorescence of smooth muscle actin and agonist-induced changes in cytosolic calcium has been reported previously (*17*, *18*). Third to sixth passage cells, or fifth to eighth passage cells stably selected after retroviral infection as described below, were plated at a density of 10^4 cells/cm^2 in 6- or 24-well plates and maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum. Cells were growth-arrested after being washed twice in PBS and after addition of Ham's F-12 medium supplemented with 0.1% bovine serum albumin (BSA) to cells for 24 h prior to assay. One hour before the initial stimulation, cells were washed twice and refed Ham's F-12 medium with 0.1% BSA.

Analysis of Endogenous p70S6K/Akt/p42/p44 Phosphorylation. Cells were grown to near confluence in 6-well plates and growth-arrested for 24 h in serum-free medium as described previously (*13*) and then stimulated with indicated agents for 0−4 h. Cells were then washed once with cold TBS and then solubilized in a 50 mM Tris buffer (pH 8.0) containing 150 mM NaCl, 20 mM NaF, 5 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, 0.1 M PMSF, and 1% (v/v) NP-40 (lysis buffer) for 30 min at 4 °C. Following scraping, cell lysates were centrifuged at 13200*g* for 10 min. Protein quantification was performed on supernatants using a Nanodrop (Rockland, DE) spectrophometer and BSA as a standard. Samples were then boiled for 5 min in 1% sodium dodecyl sulfate (SDS) sample buffer and 30 μg of protein electrophoresed on either a 7.5% (to observe the phosphorylation-induced shift) or 10% SDS−polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently probed with antibodies that recognize total p70S6K or forms phosphorylated at specific residues, and either IRDye 700 or 800 secondary antibodies conjugated with infrared fluorophores. Bands were visualized and directly quantified using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE), and typically normalized to the value obtained for the 30 min EGF-stimulated condition. Because a homogeneous population of cells was plated prior to stimulation, minimal variability of total p70S6K levels among conditions was observed, and for analyses of endogenous p70S6K, normalization of phospho-p70S6K values to values assessed for total p70S6K did not affect results.

Retroviral Infection. Stable expression of GFP, GRK2CT−GFP [a chimera of the C-terminus of G protein-coupled receptor kinase 2 (GRK2) and green fluorescent protein (GFP)], and GαiG203A was achieved by retroviral infection. GRK2-(495−689) (GRK2-CT) (*20*) was subcloned into pEGFPN1 by PCR cloning. HindIII−NotI fragments, encoding GFP or GRK2-CT−GFP, were cloned into the retrovirus expression vector pcLNCX2. The sequence encoding the open reading frame of pQE6GαiG203A (*21*) was PCR cloned into HindIII- and SalI-digested pLNCX2. Retrovirus for expression of GFP, GRK2CT−GFP, and GαiG203A was produced by transfecting GP2-293 cells with pLNCX2-GFP, pLNCX2-GRK2CT-GFP, or pLNCX2-GαiG203A, each with the pVSV-G vector which encodes the pantropic (VSV-G) envelope protein. Supernatants were harvested 48 h after transfection and used to infect human ASM cultures, with effective virus concentrations established by immunoblot analysis. Infected cells typically exhibited 50−90% GFP expression within 48 h (direct visualization by fluorescence microscopy), and selection to homogeneity with 250 μg/mL G418 was rapid (7 days). Expression of GαiG203A was confirmed by immunoblotting as described above, and by immunocytochemistry using an anti-Gαi1 antibody (Santa Cruz) and FITC-conjugated secondary antibodies as described previously (*19*).

Assays of p70S6K activity, [^{3}H]thymidine incorporation, and phosphoinositide hydrolysis were performed as described previously (*13*). For experiments examining p70S6K activity, naïve primary ASM cultures were plated in 10 cm plates, grown to near confluence, growth-arrested, and stimulated as described previously (*13*). For experiments examining cultures infected with retrovirus, the assay was miniaturized by plating cells in 60 mm plates.

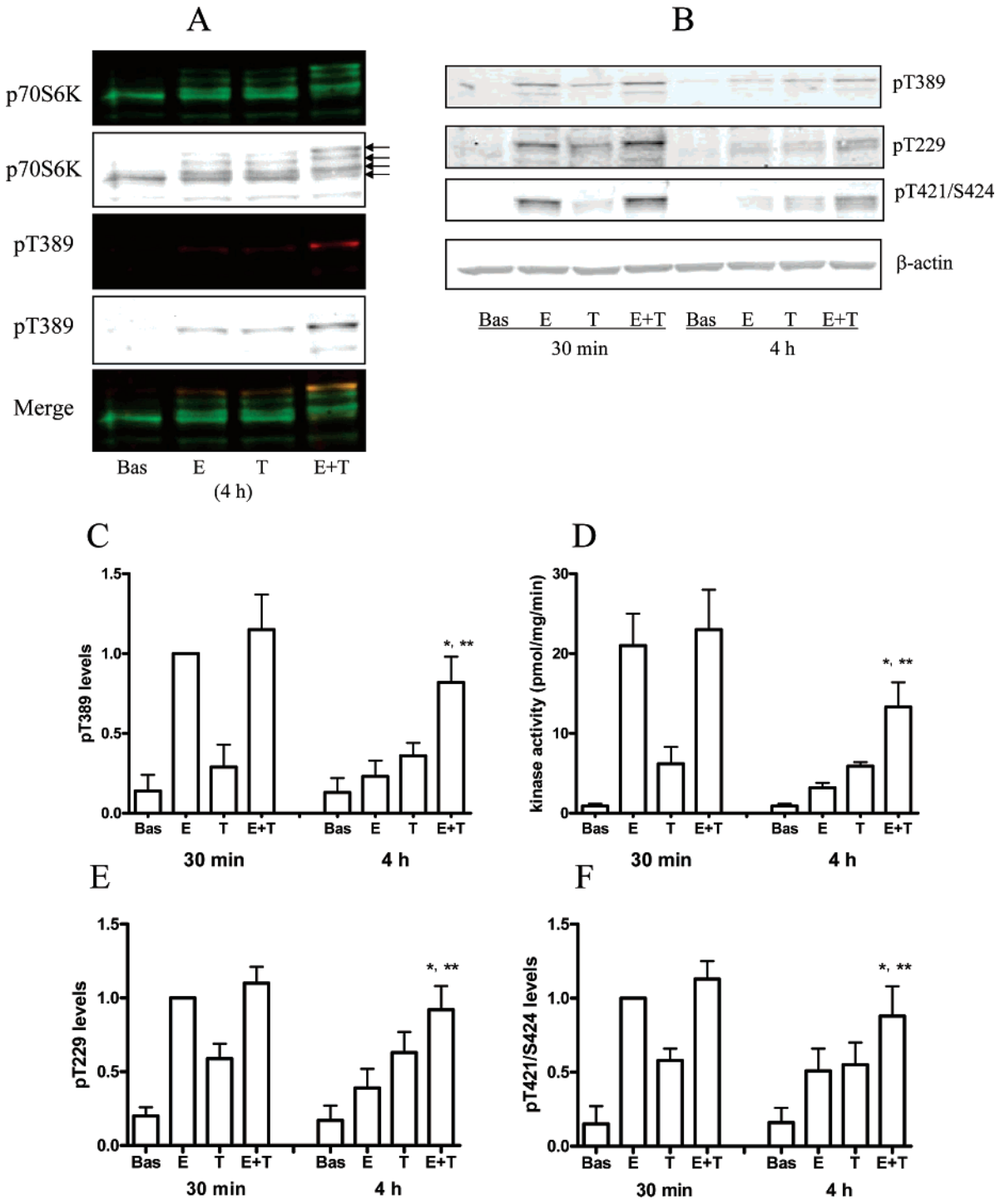

FIGURE 1: Regulation of p70S6K phosphorylation activity by EGF and thrombin. Human ASM cells were treated for 30 min or 4 h in the presence of vehicle (Bas), 10 nM EGF (E), 1 unit/mL thrombin (T), or 10 nM EGF with 1 unit/mL thrombin (E+T). Reactions were terminated, and cell lysates were subjected to immunoblot analysis of total p70S6K, phospho-T389 (pT389), phospho-T229 (pT229), phospho-T421/S424 (pT421/S424), or β-actin (A, B, and D−F), or total p70S6K was immunoprecipitated for subsequent analysis of p70S6K activity (C) as described in Experimental Procedures. In panel A, total p70S6K and pT389 were simultaneously assessed by dual labeling with a polyclonal anti-p70S6K and monoclonal pT389 antibody, and distinct fluorescently labeled secondary antibodies (total p70S6K, IRDye 700 secondary Ab; pT389, IRDye 800 secondary Ab). The blot that is shown is representative of six experiments. Arrows point to four distinct specific bands resolved in the total p70S6K blot. A feint, apparently nonspecific band is observed above the (slowest-migrating, most intense) pT389 band. A parallel blot probed with nonimmune IgG and IRDye 800 secondary Ab was essentially blank (not shown). Panel B represents a single blot probed for pT229 and then stripped and reprobed for the indicated proteins. Panels C−F depict bar graphs of mean ± standard error values for kinase activity (C, $n = 5$) and quantified phosphoprotein band (D−F, $n = 9-10$) values obtained from direct quantification of the secondary antibody binding as described in Experimental Procedures. Raw values for the 30 min EGF-stimulated condition for each experiment were set to a value of 1.0 and all other values normalized accordingly.

Statistically significant differences among groups were assessed by analysis of variance (ANOVA), and Tukey post-hoc tests for specific pairwise differences, with p values of <0.05 sufficient to reject the null hypothesis.

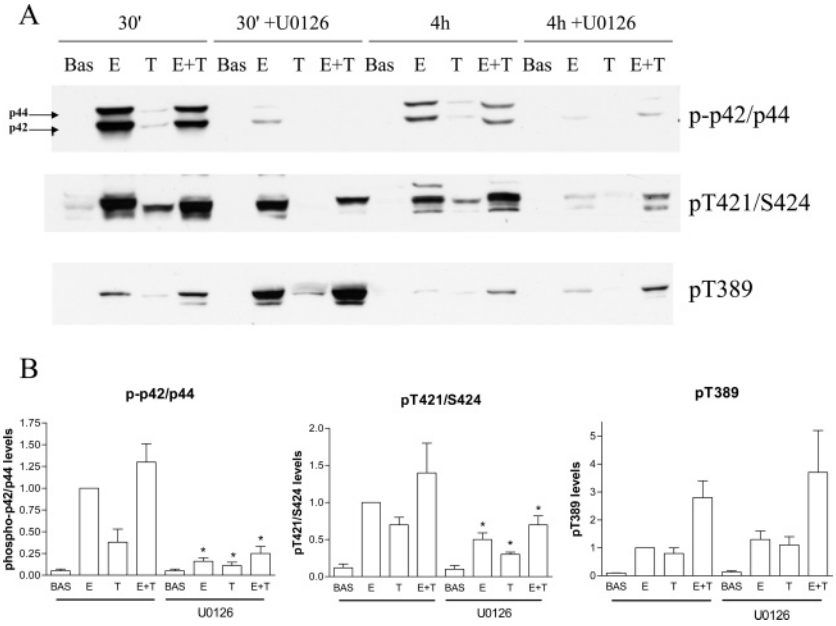

FIGURE 2: Effect of p42/p44 inhibition on p70S6K phosphorylation. Human ASM cells were treated for 30 min or 4 h in the presence of EGF (E), thrombin (T), or both. Where indicated, cells were pretreated for 30 min with the p42/p44 inhibitor U0126 (10 μM). Phosphorylation of p42/p44 at T202/Tyr204 and T185/Tyr187 (panel A) and p70S6K at T421/S424 (B) and T389 (C) were assessed by immunoblotting with phospho-specific antibodies as described in Experimental Procedures. Representative blots (panel A) and graphs depicting mean $\pm$ standard error values of quantified phosphoprotein bands from 4 h experiments (panel B, $n = 8-9$) are presented. Raw values for the 4 h EGF-stimulated condition (vehicle-pretreated) for each experiment were set to a value of 1.0 and all other values normalized accordingly. Asterisks indicate a p of <0.05 for the effect of U0126 pretreatment on the corresponding stimulated value in vehicle-pretreated cells.

RESULTS

Prolonged Stimulation with EGF and Thrombin Induces an Enhanced Mobility Shift of p70S6K and Synergistic Increase in the Level of p70S6K Phosphorylated at T389. Having previously shown combined stimulation with EGF and thrombin synergistically increases late-phase p70S6K activity in ASM cultures (*13*), we sought to determine the phosphorylation profile of p70S6K under these conditions. Dual probing of total p70S6K and that phosphorylated at T389 (pT389) demonstrated that stimulation for 4 h with EGF or thrombin caused a mobility shift in p70S6K, and that the pT389 species migrated primarily with the slowest-migrating band (Figure 1A). Combined EGF and thrombin stimulation resulted in a more pronounced shift in bands to the slower-migrating forms and a greater increase in pT389 p70S6K levels, with a feint, faster-migrating band also observed. Interestingly, EGF- and thrombin-stimulated pT389 levels exhibited no cooperativity with stimulation for 30 min, whereas greater than additive effects were observed at 4 h (Figure 1B,C). Changes in p70S6K kinase activity exhibited a profile similar to that of changes in pT389 levels (Figure 1D). Collectively, these data suggest that p70S6K phosphorylated at T389 is among the most hyperphosphorylated isoforms and is a reliable marker of full p70S6K activation as suggested previously (*22*, *23*).

Thrombin Also Increases the Level of EGF-Stimulated Phosphorylation of T229 and T421/S424. To determine whether the increase in the level of p70S6K phosphorylated at T389 represented a selective increase in the level of T389 phosphorylation or whether other phosphorylation events were similarly affected, we examined the phosphorylation state of T421/S424 as well as T229 using phospho-specific antibodies. T229 is situated in the catalytic domain of p70S6K, and in addition to T389, its phosphorylation is necessary for full activation of the protein. Residues T421 and S424 are situated in the autoinhibitory/pseudosubstrate domain of the C-terminus, and phosphorylation of these residues is proposed to be one of the early steps of p70S6K activation (*2*). Stimulation with EGF and thrombin for 4 h, but not 30 min, significantly increased the level of phosphorylation of residues T229, and T421 and S424, relative that observed with stimulation with EGF or thrombin alone (Figure 1B,E,F). However, the increases were not as dramatic as those observed for pT389 levels, being roughly additive (for pT229) or less than additive (for pT421/S424).

The Increased Level of T389 Phosphorylation Is Unaffected by Inhibition of p42/p44. Experiments were undertaken to identify upstream signals through which thrombin augments EGF-stimulated p70S6K activation. p42/p44 MAPK is a critical mitogenic signaling element in ASM cells (*13*), and is able to phosphorylate p70S6K in a cell-free assay (*4*). We therefore considered whether the cooperative effect of combined EGF and thrombin stimulation on phosphorylation and ASM [^{3}H]thymidine incorporation extended to p42/p44 phosphorylation, and whether p42/p44 inhibition differentially affected phosphorylation at residues T421 and S424 or residue T389. As shown in Figure 2, levels of phospho-p42/p44 after stimulation for 4 h with EGF and thrombin were similar to those stimulated by EGF alone. Inhibition of p42/p44 by pretreatment of human ASM cells with the MEK inhibitor U0126 (10 μM) partially inhibited phosphorylation of T421/S424, yet did not inhibit phosphorylation of T389, or affect EGF-, thrombin-, or EGF- and thrombin-stimulated p70S6K kinase activity (data not shown).

We further extended our analysis to demonstrate that thrombin-mediating signaling to p42/p44 is dissociated from the cooperative effect of thrombin on EGF-stimulated p70S6K activity and DNA synthesis in ASM. Pretreatment

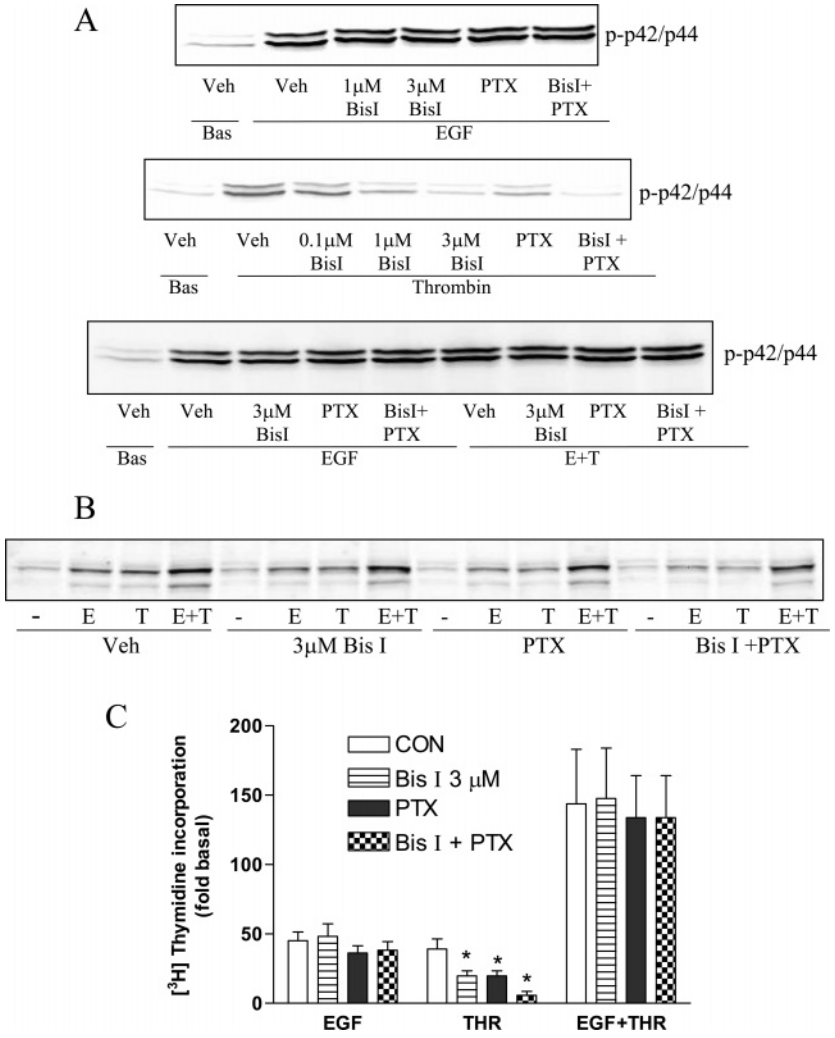

FIGURE 3: Effect of specific inhibition of thrombin-stimulated p42/p44 phosphorylation on cooperative signaling by EGF and thrombin. (A and B) Where indicated, human ASM cells were pretreated with vehicle, Bis I (0.1−3 μM, 30 min), pertussis toxin (PTX, 100 ng/mL, 8 h), or 3 μM Bis I and 100 ng/mL PTX. Cells were treated with vehicle (Bas), EGF, thrombin, or EGF and thrombin for 4 h, and cell lysates were generated for analysis of phospho-p42/p44 (A, representative of four experiments) and pT389 p70S6K levels (B). Pretreatment with Bis, PTX, or Bis and PTX had no significant effect on EGF-, thrombin-, and EGF- and thrombin-stimulated pT389 levels, with mean values from eight experiments differing by less than 10% (data not shown). In panel C, cells in 24-well plates were pretreated as in panels A and B, stimulated as indicated for 24 h, and then loaded with [^{3}H]thymidine (1 μCi/mL) for an additional 24 h. Following cell lysis, [^{3}H]thymidine incorporation (mean ± standard error values, $n = 5$) was assessed as described in Experimental Procedures.

of ASM cultures with the PKC inhibitor Bis I caused a dose-dependent inhibition of thrombin-stimulated p42/p44 phosphorylation. Bis I (3 μM) significantly inhibited (4 h) thrombin-stimulated, but not EGF-stimulated, p42/p44 phosphorylation (Figure 3A). Pretreatment with pertussis toxin (PTX), which ADP ribosylates Gαi, also inhibited thrombin-stimulated p42/p44 phosphorylation, and combined 3 μM Bis I and PTX pretreatment further reduced the level of thrombin-stimulated p42/p44 phosphorylation without affecting p42/p44 phosphorylation stimulated by EGF. Thus, selective inhibition of p42/p44 phosphorylation by thrombin can be achieved by pretreatment with Bis I and PTX. However, pretreatment of ASM with Bis I and PTX was shown to have no effect of EGF- and thrombin-stimulated p42/p44 phosphorylation. Induction of (4 h) pT389 levels (Figure 3B) and p70S6K kinase activity (not shown) stimulated by EGF, thrombin, or EGF and thrombin was not significantly affected by pretreatment with Bis I, PTX, or Bis I and PTX (Figure 3B). Pretreatment with Bis I, PTX, or Bis I and PTX was able to significantly inhibit [^{3}H]-thymidine incorporation stimulated by thrombin, but not that stimulated by EGF or EGF and thrombin (Figure 3C). Thus, thrombin-stimulated signaling via PKC, Gαi, and p42/p44 is dispensable in the potentiating effect of thrombin on p70S6K activity and [^{3}H]thymidine incorporation in ASM.

Akt Phosphorylation Is Similarly Enhanced by Thrombin. EGF and thrombin are each able to induce ASM cell proliferation via both p42/p44- and PI3K-mediated pathways (*24*−*26*). Inhibition of PI3K markedly inhibits EGF- and thrombin-stimulated p70S6K activation and ASM cell proliferation (*25*), and as in other systems, pT389 phosphorylation is wortmannin-sensitive (data not shown). Having established no role for p42/p44 in thrombin's effect on EGF-stimulated p70S6K activity, we examined the potential role of PI3K in this phenomenon. Direct assessment of chronic stimulation of PI3K activity is problematic, in that PI3K activity assessed by in vitro kinase activity from anti-pTyr precipitates is not readily detectable after stimulation of cells for 30 min (*13*). To gain some insight into the activity of PI3K after prolonged stimulation, we therefore examined Akt

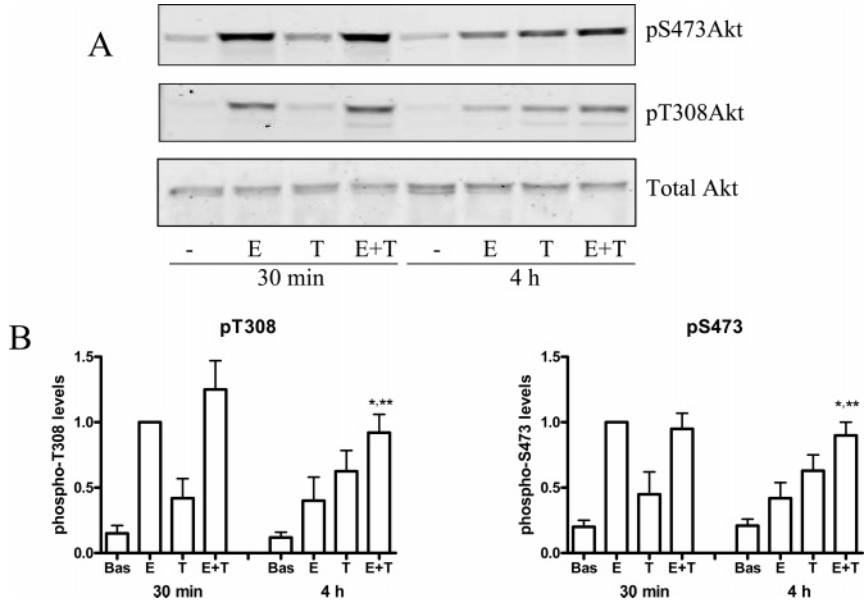

FIGURE 4: Regulation of Akt phosphorylation in human ASM cells. Cells were treated for 30 min or 4 h in the presence of EGF (E), thrombin (T), or both. Phosphorylation of Akt at T308 (pT308) and S473 (pS473) was assessed by immunoblotting with phospho-specific antibodies. Representative blots (A) and graphs depicting mean ± standard error values of quantified phosphoprotein bands (B, $n = 6$) are presented. Raw values for the 30 min EGF-stimulated condition for each experiment were set to a value of 1.0 and all other values normalized accordingly.

phosphorylation. Akt is regulated by PI3K through activation of PDK1; thus, Akt phosphorylation serves as an effective readout for PI3K activity (*27*−*29*).

To assess the regulation of Akt by EGF and thrombin, immunoblots were probed for Akt phosphorylated at T308 (pT308) and S473 (pS473) (Figure 4). Phosphorylation of these two residues confers full activity upon Akt (*29*). Treatment with EGF or thrombin for 30 min stimulated both T308 and S473 phosphorylation, while the effect of combined EGF and thrombin treatment was similar to that stimulated by EGF alone. At 4 h, pT308 and pS473 levels were significantly greater in cells stimulated with EGF and thrombin compared to levels in cells stimulated by EGF or thrombin alone.

Interestingly, we observed that whereas the level of EGF-stimulated Akt phosphorylation decreased dramatically from the 30 min to 4 h time point, the level of Akt phosphorylation by thrombin did not. A more detailed examination of the kinetics of Akt phosphorylation suggested that thrombin phosphorylation of Akt at both T308 and S473 remains relatively sustained throughout 4 h of stimulation (data not shown). Whereas Akt phosphorylation by EGF is strong for 30−60 min and then wanes, stimulation by EGF and thrombin exhibits a significantly weaker diminution of Akt phosphorylation over the course of 10 min to 4 h, suggesting the influence of sustained Akt phosphorylation by thrombin.

Expression of Gβγ Sequestrants Attenuates Activation of Akt and p70S6K by Thrombin. Recent studies using the Chinese hamster embryonic fibroblast cell line IIC9 have revealed sustained PI3K/Akt activation by thrombin that is mediated through a Gβγ-dependent mechanism (*27*, *28*). We therefore considered whether a similar Gβγ-mediated PI3K/Akt phosphorylation in ASM serves as a mechanism for the late-phase increase in p70S6K activity that occurs with combined EGF and thrombin treatment. The C-terminus of G protein-coupled receptor kinase 2 (GRK2CT) has been employed as an effective sequestrant of Gβγ subunits when expressed in intact cells (*30*). To overcome limitations associated with poor transient transfection efficiency and the inability to generate stable lines with sufficient expression through conventional approaches (not shown), we generated retrovirus for GFP and GRK2CT−GFP and infected ASM cultures (see Experimental Procedures). Cultures expressing each construct were rapidly established following selection for 7 days (Figure 5), and EGF- and thrombin-stimulated Akt and p70S6K activation were subsequently analyzed. The profile of time-dependent Akt phosphorylation by EGF was similar in cells expressing GRK2CT−GFP and GFP (Figures 6 and 7). However, thrombin-stimulated Akt phosphorylation observed in GFP-expressing cells was attenuated in cells expressing GRK2CT−GFP. In addition, the relatively sustained EGF- and thrombin-stimulated Akt phosphorylation observed in GFP-expressing cells was significantly suppressed in GRK2CT−GFP-expressing cells, in which the profile of phosphorylation was more similar to that induced by stimulation with EGF alone. The cooperative effect of EGF and thrombin reflected in the 4 h time point was significantly inhibited.

Analysis of the effects of GRK2CT−GFP expression on thrombin-stimulated p70S6K phosphorylation revealed a similar attenuation of time-dependent T389 phosphorylation stimulated by either thrombin or EGF and thrombin (Figures 6 and 7). To account for possible nonspecific actions of GRK2CT−GFP and further establish the role of Gβγ in thrombin-mediated regulation of Akt and p70S6K, we generated an additional ASM culture stably expressing a point mutant of Gαi1 (GαiG203A) specifically engineered as a powerful Gβγ sequestrant (*21*). GαiG203A expression attenuated the late-phase phosphorylation of Akt and p70S6K phosphorylation by thrombin and by EGF and thrombin (Figure 7). Effects of GRK2CT−GFP and GαiG203A expression on p70S6K phosphorylation were reflected in assays of kinase activity, which demonstrated a reduction in p70S6K activity stimulated by treatment with thrombin

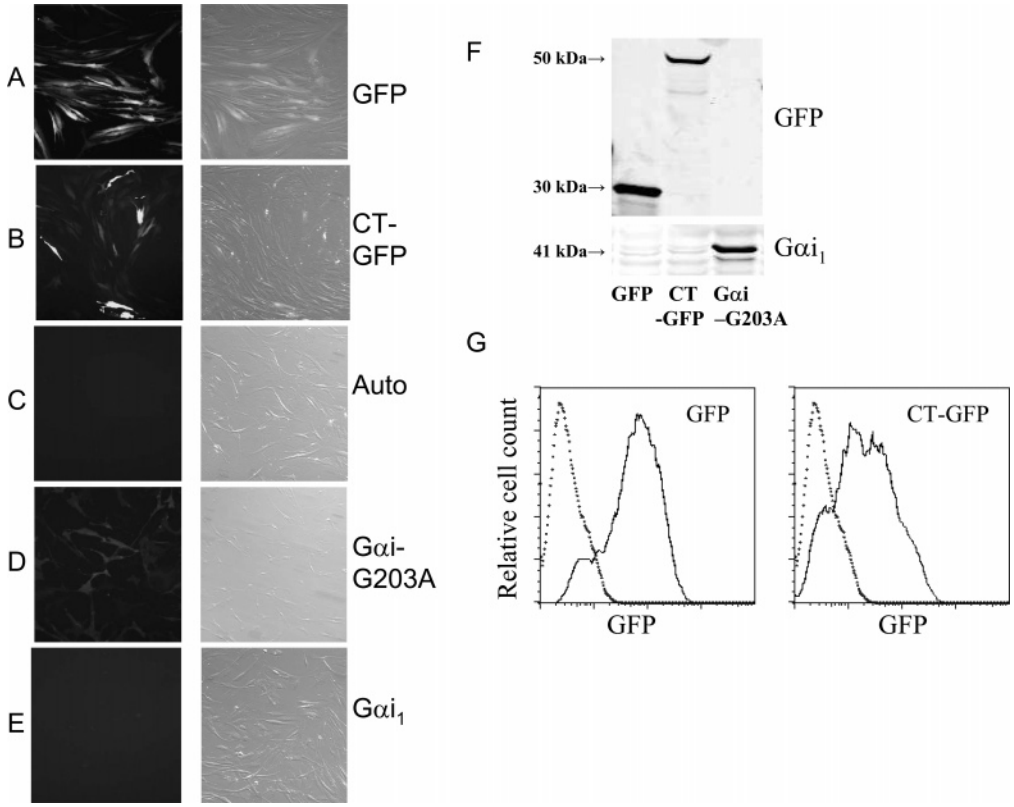

FIGURE 5: Establishment of ASM cultures stably expressing GFP, GR2CT−GFP, or GαiG203A. Third-passage ASM cells were infected with retrovirus to induce expression of GFP (A), GRK2CT−GFP (B), or GαiG203A (D) as described in Experimental Procedures. Forty-eight hours after infection, cells were selected with 250 μg/mL G418. One week later, GFP fluorescence or immunostaining for $G\alpha i_1$ was examined (left panels). Panels at the right represent Hoffman modulation light field images. Autofluorescence of noninfected ASM cells is depicted in panel C, and immunostaining of endogenous Gαi1 is depicted in panel E. Panel F depicts immunoblots probed with anti-GFP (top) and anti-Gαi1 (bottom). Panel G depicts flow cytometry analysis of GFP fluorescence in a GFP and GRK2CT−GFP line. The dotted line represents the cellular autofluorescence in a non-GFP-expressing ASM line.

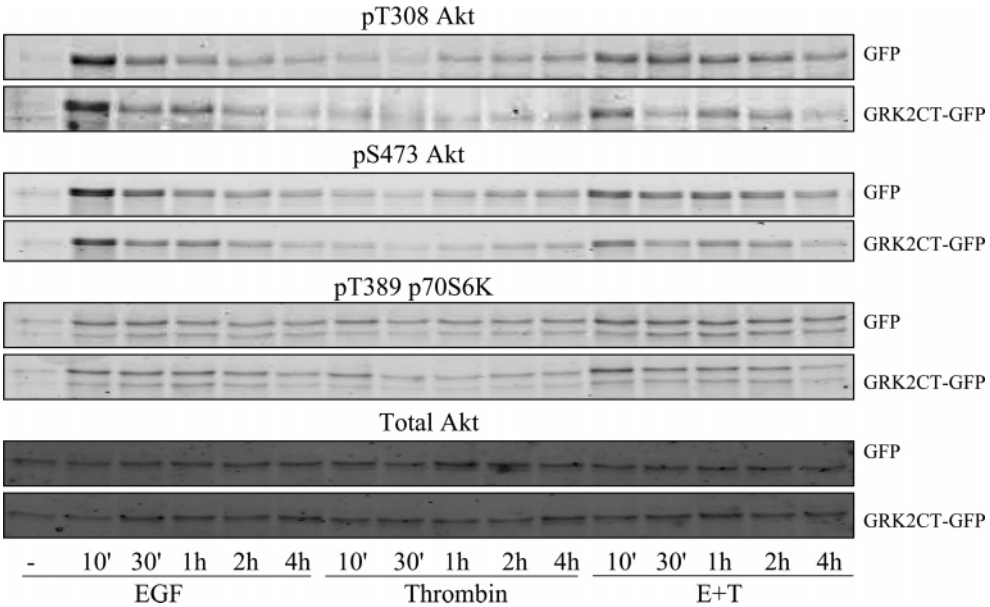

FIGURE 6: Effects of GRK2CT−GFP expression on Akt, p70S6K phosphorylation in human ASM cells. ASM cells were infected with retrovirus to achieve stable expression of GFP or GRK2CT−GFP as depicted in Figure 5. Cells grown to near confluence in six-well plates were growth arrested and stimulated with EGF, thrombin, or both (E+T), and levels of phospho-Akt (pT308), phospho-Akt (pS473), and phospho-p70S6K (pT389) were assessed by immunoblotting. Blots are representative of results obtained in three separate sets of lines derived from distinct donors.

or EGF and thrombin for 4 h in the GRK2CT−GFP and GαiG203A groups relative to that of the GFP group. Differences among the GFP, GRK2CT−GFP, and GαiG203A groups were not observed for 30 min values of pS473Akt, pT389p70S6K levels, or p70S6K kinase activity, for any of the stimulatory conditions (data not shown).

Interestingly, the inhibitory effects of GRK2CT−GFP on Akt/p70S6K phosphorylation and p70S6K activity occurred

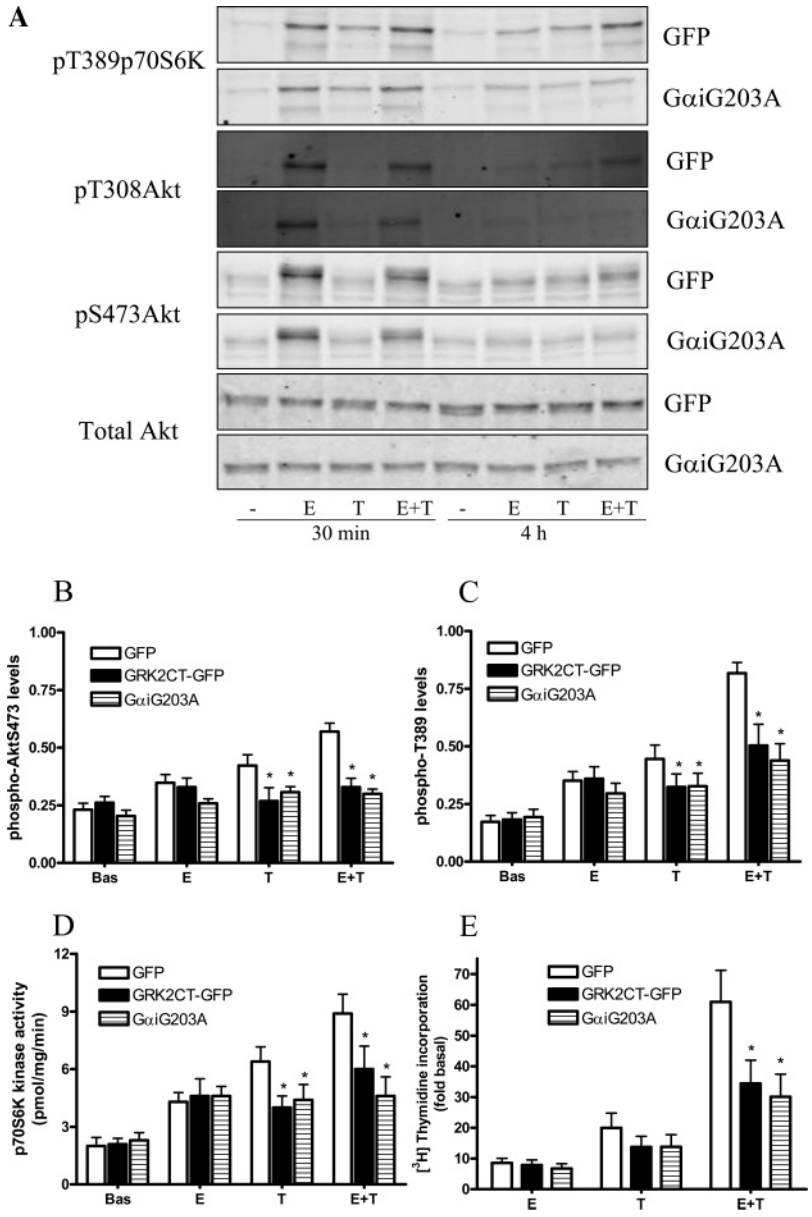

FIGURE 7: Effects of expression of G$\beta\gamma$ sequestrants on regulation of kinase phosphorylation, activity and ASM [^{3}H]thymidine incorporation. Human ASM cells stably expressing GFP, GRK2CT−GFP, or GαiG203A grown to near confluence in six-well plates were growth arrested and stimulated with EGF (E), thrombin (T), or both (E+T) for 0 min, 30 min, and 4 h, and pT308Akt, pS473Akt, total Akt, and pT389p70S6K levels were assessed by immunoblotting. Blots of pS473Akt and pT389p70S6K regulation in matched GFP, GRK2CT−GFP, and GαiG203A lines were quantified and band intensities for the 4 h time points plotted in panels B and C ($n = 6$). Raw values for the 30 min GFP EGF-stimulated condition for each experiment were set to a value of 1.0 and all other values normalized accordingly. p70S6K activity in cell lysates obtained from 60 mm dishes plated and treated in parallel was assessed in vitro as described in Experimental Procedures and is presented in panel D ($n = 6$). Panel E depicts [^{3}H]thymidine incorporation stimulated by EGF, thrombin, or both in GFP-, GRK2CT−GFP-, or GαiG203A-expressing lines ($n = 6$ paired observations for GFP vs GRK2CT−GFP, $n = 5$ paired observations for GFP vs GαiG203A). Asterisks indicate a p of <0.05, GFP vs GRK2CT−GFP or GαiG203A group, for thrombin- and EGF- and thrombin-stimulated conditions.

despite a small but statistically significant increase in the extent of thrombin receptor−G protein coupling as assessed by phosphoinositide assays [thrombin-stimulated phosphoinositide hydrolysis for 30 min in GFP-expressing cells is (1.9 ± 0.3)-fold greater than basal values, compared to (2.4 ± 0.3)-fold greater than basal values in GRK2CT−GFP expressing cells ($n = 5$ paired observations; $p < 0.05$)]. These findings suggest that although GRK3 appears to be a more effective regulator of PAR1 phosphorylation and signaling than GRK2 (*31*), GRK2CT has the capacity to mitigate thrombin receptor desensitization and regulate thrombin signaling in ASM cells. This result further supports the notion that PI3K/Akt/p70S6K regulation by thrombin or EGF and thrombin is dissociated from phospholipase C as well as PKC activity.

Last, the reductions in late-phase p70S6K activity observed in GRK2CT−GFP- and GαiG203A-expressing cells were associated with reductions in the cooperative effect of EGF and thrombin on ASM [^{3}H]thymidine incorporation (Figure 7E). The group effects on Akt phosphorylation, p70S6K phosphorylation and activity, and [^{3}H]thymidine incorporation were independent of genetic or clonal variability, being observed in several different sets of GFP-, GRK2CT−GFP-, and GαiG203A-expressing cells, each set of cell lines derived

from a different donor ASM culture. Collectively, these findings suggest an important role of thrombin-mediated G$\beta\gamma$-dependent p70S6K activation in the cooperativity of EGF and thrombin promoting enhanced ASM proliferation.

DISCUSSION

Increased airway smooth muscle mass is now recognized as a feature of airway remodeling that contributes to the pathogenesis of asthma. Causal factors of excessive growth of ASM are not known, but an attractive hypothesis is that exaggerated presentation of mitogenic agents in the airway promotes such growth. Several studies have reported levels of both polypeptide growth factors (*32*) and numerous contractile agents capable of mitogenic signaling to be elevated in the airways of asthmatic patients (reviewed in Billington and Penn) (*12*).

We and others recently demonstrated significant positive cooperativity between growth factors and procontractile GPCR agonists in stimulating ASM cell proliferation and DNA synthesis. However, mechanistic insight into this phenomenon has been elusive, despite a growing body of literature elucidating the crosstalk between RTK and GPCR signaling pathways in more artificial cell systems.

We previously identified increased late-phase p70S6K activity to be associated with the cooperative action of RTK and GPCR agonists on ASM growth (*13*). This finding suggested a valuable model system for exploring the relative contributions of RTK- and GPCR-derived signals to p70S6K phosphorylation and activation, and possibly clarifying some fundamental mechanisms of p70S6K activation (*22*, *23*, *33*). The goal of this study was to identify the specific contribution of the GPCR signaling pathway that influences RTK-mediated regulation of p70S6K. Because both receptors for EGF and thrombin utilize common downstream effectors to activate p70S6K, and these effectors (PI3K, PDK1, and mTOR) are critical for p70S6K activation, direct inhibition of these molecules provides no insight into the mechanism or locus of cooperative signaling. Thus, we focused on targeting putative GPCR signaling intermediates upstream of these effectors in an attempt to identify specific GPCR-activated elements of p70S6K activation.

We had previously excluded transactivation of the EGF receptor as a mechanism by which thrombin augments p70S6K activity and ASM cell growth (*13*).

Diacylglycerol-activated PKC has been shown to activate p70S6K in cardiomyocytes (*34*), and late-phase activation of p70S6K by EGF was reported to be sensitive to PKC inhibition by a staurosporine analogue in 3T3 cells (*35*). In contrast to these studies' findings, we found pharmacological inhibition of conventional PKCs with Bis I to have no effect on EGF-stimulated or EGF- and thrombin-stimulated p70S6K phosphorylation or ASM DNA synthesis. Additional studies using various inhibitors capable of inhibiting novel and atypical PKC also had no effect on p70S6K activation in ASM cells (data not shown).

Both EGF and thrombin are strong activators of p42/p44 MAPK in ASM cells (*26*). A role for p42/p44 in p70S6K phosphorylation and activation in various cells has been suggested but remains subject to debate. Following the observation by Mukhopadhyay et al. that p42/p44 can phosphorylate p70S6K in vitro (*4*), some studies have suggested similar regulation occurs in intact cells (*36*−*38*), whereas others found p42/p44 and p70S6K to lie on distinct and entirely separate pathways (*39*−*42*). In the study presented here, the p42/p44 inhibitor U0126 significantly reduced the level of T421/S424 phosphorylation induced by all agents, but despite this effect, phosphorylation of T389 and p70S6K kinase activity was not affected. To further clarify the relationship between thrombin-stimulated p42/p44 activation and its potential role in augmenting EGF-stimulated p70S6K activity and ASM [^{3}H]thymidine incorporation, we established the means of selectively inhibiting thrombin-stimulated p42/p44 activation (Figure 4). Although combined inhibition of PKC and $G_{\alpha i}$ significantly inhibited thrombin-stimulated p42/p44 phosphorylation and DNA synthesis, the effect of thrombin on EGF-stimulated T389 phosphorylation and DNA synthesis was minimally affected. Collectively, these data suggest no role for p42/p44 in the cooperative activation of p70S6K by EGF and thrombin. Moreover, because the level of T412/S424 phosphorylation can be significantly reduced without reducing the level of T389 phosphorylation or kinase activity, the role of p42/p44 in activating p70S6K in ASM appears to be innocuous, and the failure of p42/p44 inhibition to inhibit T389 phosphorylation or p70S6K activity under conditions of inhibited T412/S424 phosphorylation suggests that phosphorylation of proline-directed residues is not limiting under the many stimulatory conditions that were examined.

Two recent studies by Goel et al. (*27*, *28*) have described a sustained activation of PI3K, and consequently Akt phosphorylation, by thrombin that was determined to be essential for G1 phase progression in IIC9 cells. Expression of G$\beta\gamma$ sequestrants inhibited this sustained PI3K/Akt activation (*27*). Because we observed a similar sustained phosphorylation of Akt by thrombin in ASM cells, we considered whether G$\beta\gamma$-mediated PI3K activation represented a GPCR-specific signal contributing to the synergistic activation of p70S6K. Stable expression of either GRK2CT−GFP or GαiG203A caused an attenuation of the sustained phosphorylation of Akt and p70S6K by thrombin while significantly reducing the cooperativity of thrombin and EGF in increasing the level of p70S6K phosphorylation and activity (Figures 6 and 7). These effects on p70S6K activation were associated with a significant reduction in the ability of thrombin to accelerate EGF-mediated proliferation of ASM cells, implicating an important role for G$\beta\gamma$-mediated PI3K activation in mitogenic signaling by GPCRs, particularly in the context of concomitant receptor tyrosine kinase activation.

Interestingly, the G$\beta\gamma$-mediated activation of Akt and p70S6K occurs despite the inability to detect p110PI3Kγ in ASM cultures (*13*), suggesting that either undetectable levels of PI3Kγ mediate this effect or, alternatively, p110PI3Kβ serves as the G$\beta\gamma$ effector (*44*−*46*). Stephens et al. (*44*) originally identified G$\beta\gamma$-responsive PI3K activity in cells lacking detectable p110PI3Kγ. Subsequent studies by Kurosa et al. (*46*) and Maier et al. (*45*) determined that p110PI3Kβ could be activated by G$\beta\gamma$ in vitro and in intact cells. Moreover, G$\beta\gamma$-stimulated p110PI3Kβ activity in vitro was greatly increased by addition of phosphotyrosyl peptide (*46*), consistent with the cooperative effects of receptor tyrosine kinase and GPCR activation on Akt phosphorylation in IIC9 cells subsequently observed in Goel et al. (*27*) and on Akt,

p70S6K phosphorylation, and cell proliferation in ASM observed in this study.

The failure of GRK2CT−GFP or GαiG203A to fully inhibit the cooperative effect of thrombin on EGF-stimulated p70S6K activity and proliferation in ASM cells has several possible explanations. One reason might be that G$\beta\gamma$ signaling is an important but not the sole mechanism by which thrombin or other GPCRs enhance the effect of growth factors on p70S6K activity and cell growth. This is suggested by our data that demonstrate a greater effect of GRK2CT−GFP or GαiG203A expression on S473Akt phosphorylation than on pT389 phosphorylation, and further suggested by data demonstrating that thrombin enhances EGF-stimulated Akt phosphorylation more modestly than it increases p70S6K activity. Another possible explanation could be the inability to express (or limited efficacy of) GRK2CT−GFP or GαiG203A to levels sufficient to fully inhibit G$\beta\gamma$-mediated signaling. Indeed, if some level of G$\beta\gamma$ signaling is required for cell growth or survival, expression of G$\beta\gamma$-mediated sequestrants would be self-limiting. Direct evidence characterizing the association of GRK2CT−GFP and GαiG203A with G$\beta\gamma$ during chronic thrombin treatment would help clarify this issue. However, GPCR-mediated transmembrane and second-messenger signaling under chronic stimulatory conditions are typically highly compartmentalized events that are difficult to assess without direct visualization approaches. Daaka et al. (*47*) have reported G$\beta\gamma$ association with overexpressed GRKs is observed only briefly after GPCR activation. Yet effects of ectopic GRKCT expression on numerous integrative cell/tissue functions dependent upon chronic GPCR signaling clearly occur (*30*, *48*, *49*). Moreover, effects of chronic thrombin treatment on Akt and p70S6K phosphorylation and/or activity evidenced in Goel et al. (*27*) and our study are robust, are observed in two different cell types, and are attenuated by four different constructs capable of G$\beta\gamma$ sequestration.

G$\beta\gamma$ sequestration had essentially no effect on EGF-stimulated [^{3}H]thymidine incorporation, and caused a greater reduction in the level of [^{3}H]thymidine incorporation stimulated by combined EGF and thrombin than by thrombin alone. These results suggest that G$\beta\gamma$-mediated effects on p70S6K are less critical in cells stimulated with thrombin alone, and that other regulatory input may be limiting. Conversely, with EGF stimulation, late-phase PI3K/PDK1 phosphorylation of T389/T229 may be the critical limiting factor such that when provided by thrombin-dependent G$\beta\gamma$ release, an increased level of activation of p70S6K occurs.

Thrombin, via activation of proteinase-activated receptors, has the capacity to promote release of G$\beta\gamma$ from the $G_{q/11}$, G_i, and $G_{12/13}$ families of heterotrimeric G proteins. Given its relative abundance in most cell types, Gi would be predicted to a likely source of G$\beta\gamma$ subunits in mediating the observed effects of thrombin in ASM cells. However, pretreatment of cells with PTX did not significantly affect the synergy induced by thrombin with respect to p70S6K activity and DNA synthesis, implicating another G protein as the critical source of G$\beta\gamma$. Future studies requiring the application of either genetic or molecular strategies will serve to further dissect the signaling events immediately upstream and downstream of G$\beta\gamma$ promoting the activation of PI3K.

In summary, our findings identify a novel mechanism whereby GPCRs promote cooperative activation of p70S6K resulting in enhanced ASM cell proliferation. Identifying mitogenic signals specifically generated by G protein-coupled receptors should aid in the development of therapies or prophylactic strategies that reverse or deter aberrant smooth muscle growth that contributes to various diseases.

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