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Regulation of Thrombin-Induced Stress Fibre Formation in Swiss 3T3 Cells by the 70-kDa S6 Kinase

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The signal transduction systems that mediate growth factor receptor-induced cellular shape change have not been fully elucidated, but are known to involve alterations in the state of actin filaments, termed stress fibres. It now appears from several studies that the GTP-binding protein, Rho, is involved. However, the mechanisms by which Rho is activated, and what effectors Rho in turn stimulates are largely matters of conjecture. The present work shows that thrombin is an effective stimulant of stress fibre formation in Swiss 3T3 cells. In addition, we show the 70 kDa form of S6 kinase ($p70^{s6k}$) to colocalise with stress fibres in both unstimulated and thrombin-activated cells. Coincident with the thrombin-induced formation of stress fibres is the elevated association $p70^{s6k}$ with the fibres. Pretreatment of cells with rapamycin, to inhibit $p70^{s6k}$ activation, inhibits thrombin-induced stress fibre formation and the associated presence of $p70^{s6k}$ on the fibres, supporting a role for $p70^{s6k}$ in thrombin-stimulated stress fibre formation. Thrombin is also shown to stimulate $p70^{s6k}$ activity and that this is inhibited by rapamycin. Thus, the data presented show that thrombin activates stress fibre formation through stimulation of $p70^{s6k}$ via a non-G_i pathway.

G-protein-coupled growth factor receptors of mouse 3T3 fibroblasts have been shown to induce a variety of cellular effects. These include shape change, DNA synthesis and cellular proliferation (1,2). Modulation of cellular shape change is a rapid response to growth factor activation, and underlying this effect is the remodelling of cellular actin filaments, termed stress fibres (3). Such alterations in stress fibres precede DNA synthesis by several hours, just as synthesis of DNA occurs several hours before mitosis. The temporal differences in activation of these varied cellular responses suggest that each may be ultimately controlled by separate initial signalling events at the receptors.

The thrombin receptor is known to be coupled to at least four GTP-binding proteins, G_i, G_q, G_{12} and G_{13} (4-7). Studies in this laboratory have shown that activation of DNA synthesis of our clone of Swiss 3T3 cells by thrombin is independent of G_i, but there is a dominant role of G_q in this response (8). A potential role of G_i in rapid cellular responses has yet to be uncovered for this agonist in our cells, although an involvement of G_i in mobilisation of Ca^{2+} in some 3T3 cells by thrombin and LPA is suggested (6,7,9). We have recently found that G_i plays an essential role in receptor-regulated mitosis, and so G_i can be seen as being important in the later events of the cell cycle (8). Johnson and coworkers (5) have shown that activated forms of G_{12} and G_{13} induce stress fibre formation. Similarly, the low molecular weight G-protein, Rho, has also been implicated in this response from several studies (9-11). However, how the signal is passed from one G-protein to the next and then on to actin polymerisation is unknown.

This study was initiated to examine potential downstream effector molecules capable of amplifying the initial receptor signal that could be involved downstream of a G-protein involvement in activation of stress fibre formation. A clear involvement of the 70 kDa form of S6 kinase ($p70^{s6k}$) has been found in the thrombin stimulation of stress fibre formation.

METHODS

Materials. Antibodies and blocking peptide to $p70^{s6k}$ were obtained from Santa Cruz (USA), texas red-X phalloidin from Molecular Probes (USA), Swiss 3T3 cells and fetal calf serum from P.A. Biologicals (Australia). DMEM and thrombin were from Sigma (USA), fluorescein-labelled anti-rabbit antibodies from Wellcome laboratories (Beckenham, UK), and HRP-labelled anti-rabbit antibodies were

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Abbreviations: G_i, G_q, G_{12}, and G_{13}, are different members of the GTP-binding protein family; $G_{i\alpha}$, the α-subunit of G_i; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate buffered saline; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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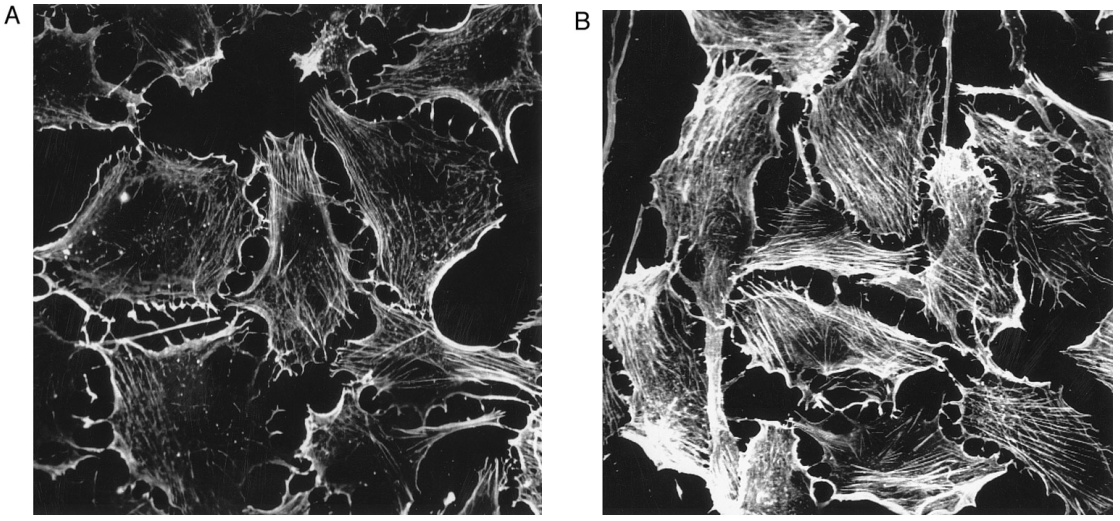

FIG. 1. Thrombin stimulates stress fibre formation in Swiss 3T3 cells. (a) Unstimulated cells had relatively few actin filaments as visualised with texas red-X phalloidin, and those present were short and disorganised. In contrast (b) thrombin stimulation (3 U/ml) for 2 hours induced the formation of dense networks of actin filaments. This effect was also seen at later time points of stimulation.

from Amersham (UK). The S6 kinase assay kit was from Upstate Biotechnology Inc. (USA).

Cell preparation for immunoprecipitation. Swiss 3T3 cells were seeded in 250 ml tissue culture flasks (Nunc) at a concentration of 15,000 per ml in Dulbecco's Modified Eagles Medium (DMEM) with bicarbonate containing penicillin and streptomycin and 10% fetal calf serum (FCS), and grown over 4 days to confluency and quiescence at 37°C in 95% O_2/5% CO_2. This depleted medium was then removed and replaced with DMEM with bicarbonate without FCS, in some cases in the presence of pertussis toxin (200 ng/ml), and cells were left overnight. Thrombin was subsequently added for specified times with or without a 1 hour preincubation with rapamycin. Flasks were then placed on ice and the incubation medium removed and replaced firstly with 5 ml of DMEM, and then with a hypotonic buffer (Tris/HCl, 10 mM, pH 7.9; KCl, 10 mM; $MgCl_2$, 1 mM). These and all further steps were carried out at 0-4°C. After 2 min, this medium was removed and replaced with Triton X-100 lysis buffer (Tris/HCl, 10 mM pH 7.9; Triton X-100, 0.3%; sucrose, 0.3 M; $MgCl_2$, 1.5 mM; PMSF, 40 μg/ml; Na pyrophosphate, 10 mM; NaF, 20 mM; Na_3VO_4, 2.7 μM and EDTA, 1 mM). Cells were scraped from the flask, and cell lysates were homogenised with 3 strokes with a glass-teflon homogeniser. Cell lysates were placed in tubes and centrifuged for 20 min at 17,400 $\times$ g. The resulting supernatant was used for immunoprecipitation.

Immunoprecipitation. The fraction described above was incubated gently overnight with $p70^{s6k}$ antibodies as indicated in the figures in the presence or absence of blocking peptide, and the following day antibodies were precipitated with protein A-sepharose (4 mg dry weight per sample). The precipitates were then washed twice with 1 ml of TBS (Tris/HCl, 50 mM pH 7.4; NaCl, 0.2 M) containing Na pyrophosphate, 5 mM, NaF, 10 mM, Na_3VO_4, 1.35 mM, and once with TBS without phosphatase inhibitors.

$p70^{s6k}$ activity measurement. $p70^{s6k}$ was immunoprecipitated from cellular lysates, as described above. The beads were then incubated with an S6 kinase assay cocktail as described by the manufacturer. Briefly, the assay determined the phosphorylation of a peptide substrate (AKRRRLSSLRA) of S6 kinase in the presence of [γ^{32}P]-ATP and inhibitors of protein kinases A and C and calmodulin dependent protein kinases. Controls for inter-protein phosphorylation and peptide block of antibodies were carried out, and the results shown are representative of S6 kinase activity to its recognised peptide substrate alone. No inter-protein phosphorylation was observed in the kinase reactions, and peptide-blocked antibodies precipitated no kinase activity (not presented).

Fluorescence histochemistry of Swiss 3T3 cells. Immunohistochemical analyses were carried out essentially as described previously (12). Cells were seeded onto glass coverslips in DMEM with 10% FCS at 30,000 per ml and allowed to attach overnight. The medium was then changed to DMEM without FCS, in the presence or absence of pertussis toxin (200 ng/ml), and cells were incubated for a further 24 hrs. Cells were then activated with thrombin for specified times with or without pretreatment with rapamycin for 1 hour, and fixed with Zamboni's fixative for 30 min. Cells were washed 5 times with PBS and then left overnight in PBS at 4°C. Samples were then treated with PBS/1% BSA/0.1% SDS for 30 min and then the primary antibody was added in PBS/1% BSA. This was left on the cells overnight at 4°C, and cells were then washed 5 times with PBS. Samples were then incubated with anti-rabbit FITC-labelled secondary antibody for 1 hr at room temperature in PBS/1%BSA, and washed 5 times with PBS. Cover slips were then placed upside down on a microscope slide on a glycerol mount. In some cases, cells were double-labelled with Texas Red-X phalloidin, which was added at a 1:40 dilution for 20 min on top of the secondary antibody incubation. Cellular fluorescence was visualised with a Leica (Germany) confocal microscope. There was no cross-over of fluorescence between FITC and Texas Red wavelengths.

Measurement of DNA synthesis. Cells were seeded at 15,000 per ml in 96 well plates and grown to confluency over 4 days. Following subsequent overnight starvation, cells were incubated with or without rapamycin for 1 hour and then stimulated with thrombin at various concentrations. After 24 hrs, [^{3}H]-thymidine (5μCi/ml) was added to each well, and after a further 24 hrs the samples were frozen with dry ice and thawed three times to lyse the cells. Radioactivity was assessed by harvesting insoluble material onto filter paper and counting in a scintillation counter.

RESULTS

Unstimulated Swiss 3T3 cells contained stress fibres, as visualised with fluorescently-labelled phalloidin, but these were relatively few in number, non-parallel in

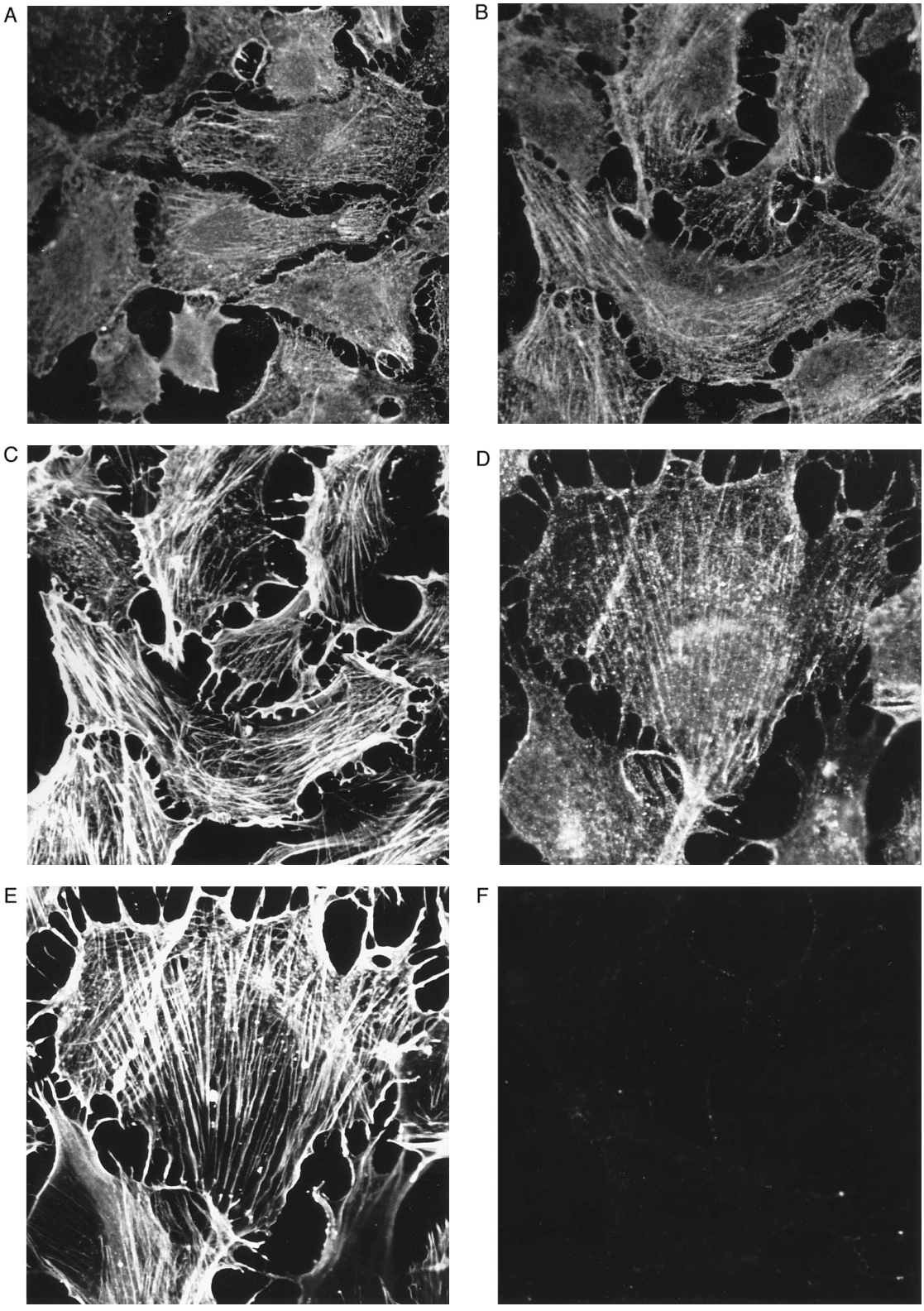

FIG. 2. p70 S6 kinase colocalises with stress fibres in Swiss 3T3 cells. Swiss 3T3 cells were double labelled with $p70^{s6k}$ antibodies and phalloidin. (a) $p70^{s6k}$ antibodies were used to localise this protein in unstimulated cells by fluorescence immunohistochemistry. Following incubation for 2 hours with thrombin (3 U/ml), cells were processed either for $p70^{s6k}$ immunohistochemistry (b), or phalloidin staining of stress fibres (c). The same cells were double labelled in (b) and (c). Higher power view of cells double labelled for $p70^{s6k}$ (d) and actin stress fibres (e) are also shown. It can be seen that there was a precise co-labelling of actin and $p70^{s6k}$. The specificity of the $p70^{s6k}$ antibody for the antigen is indicated by the ability of the antigenic peptide to block antibody staining (f).

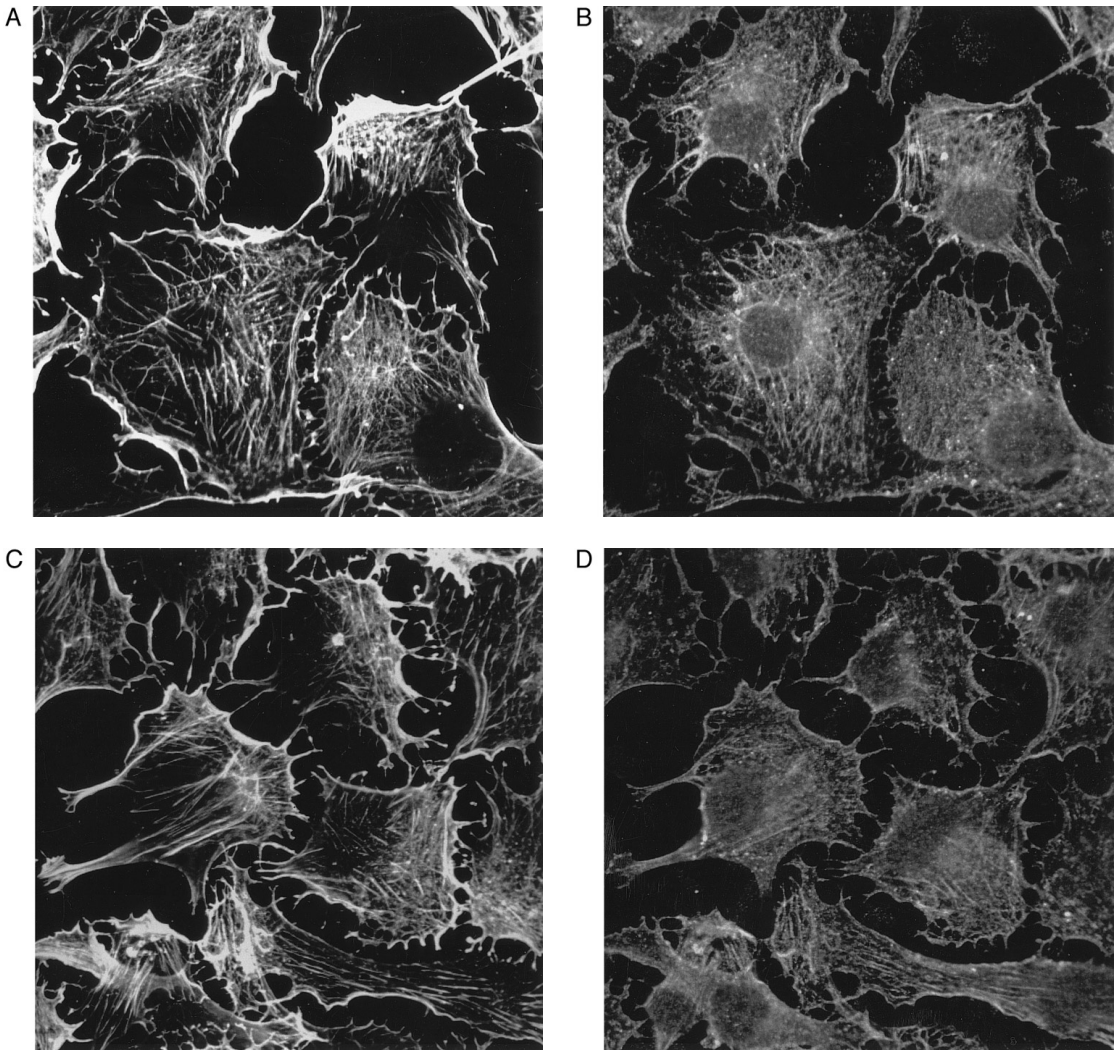

FIG. 3. Rapamycin, but not pertussis toxin, inhibits thrombin-stimulated actin polymerisation and $p70^{s6k}$ co-localisation. Swiss 3T3 cells were pre-incubated for 1 hour with rapamycin, stimulated with thrombin (3 U/ml) for two hours, and then fixed and double labelled for $p70^{s6k}$ immunohistochemistry and texas red-X phalloidin staining of stress fibres. Rapamycin (100 nM) inhibited the ability of thrombin to stimulate stress fibre formation, as indicated in two representative fields (a,c), and the appearance of the actin filaments is similar to that observed in control cells seen in previous figures. Cells double labelled with $p70^{s6k}$ antibodies showed that the same structures were labelled, and that correlating with a drop in actin polymerisation was a similar decrease in associated $p70^{s6k}$ (b,d). To examine the effect of pertussis toxin, Swiss 3T3 cells were preincubated for 24 hours with 200 ng/ml pertussis toxin and then stimulated for 2 hours with thrombin. Pertussis toxin had no obvious inhibitory effect on the formation of actin filaments (e).

arrangement, short and usually did not extend to the plasma membrane (fig 1a). Thrombin stimulation induced the formation of more actin filaments, and these were of consistently greater length than in control cells. Thrombin activated cells also often extended long cellular processes (fig 1b). In all cases, these processes contained long stress fibres that radiated to the end of the processes. Thrombin-stimulated stress fibre formation was observed following 2 hours of stimulation, the earliest time point examined, and fibre density was further increased following 4 hours of activation. Overnight stimulation with thrombin also resulted in elevated stress fibres, which were greater in number but somewhat less organised than after the shorter times of thrombin incubation.

Double labelling of these cells with a $p70^{s6k}$ antibody showed that much of this protein co-localised with actin filaments (fig 2). In unstimulated cells, $p70^{s6k}$ immunohistochemistry showed this protein to be present both attached to disorganised actin filaments, as well as being present as diffuse staining in the cytosol (fig 2a). The latter probably represents $p70^{s6k}$ that is linked to monomeric actin or as free cytoplasmic $p70^{s6k}$. Double labelling of $p70^{s6k}$ and actin showed that, following stimulation with thrombin, the association of $p70^{s6k}$ (fig 2b) with the elevated number of actin filaments (fig

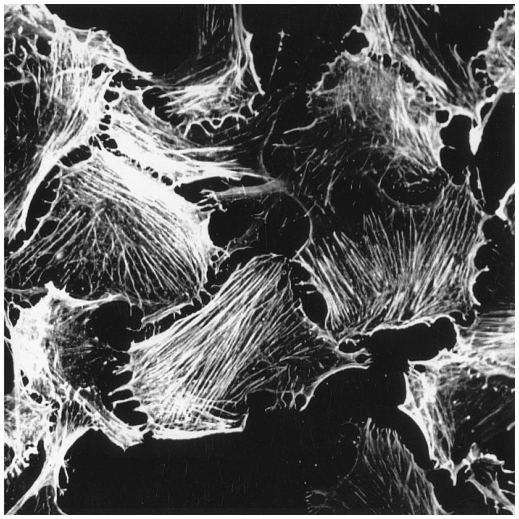

FIG. 3—*Continued*

2c) was greatly enhanced. Such activated cell cultures showed less non-filamentous $p70^{s6k}$, suggesting either the recruitment of this molecule from a free cytoplasmic store or the polymerisation of monomeric actin which had $p70^{s6k}$ already associated. Co-localisation of $p70^{s6k}$ with actin filaments was preserved in all cell preparations, and was particularly obvious at higher magnification (fig 2d,e). Preincubation of the $p70^{s6k}$ antibody with the immunising peptide blocked all staining of stress fibres, showing the antibody specificity (fig 2f). There was no cross-over of fluorescence between the FITC label of the $p70^{s6k}$ labelling and the Texas Red-X label of the phalloidin.

Pretreatment of cells with rapamycin inhibited both the thrombin-stimulated formation and parallel organisation of stress fibres (fig 3a,c), and also the association of $p70^{s6k}$ to actin filaments (fig 3b,d) as again shown by double labelling. This effect was seen at a concentration of 20 nM rapamycin, and was increased with elevated rapamycin concentrations. Typically, rapamycin reversed the effect of thrombin to induce actin polymerisation. In parallel, the cells appeared partially impaired in their substrate contact, as evidenced by a generally smaller population of cells with fewer extending processes.

This study also examined the effect of pertussis toxin on stress fibre formation. While pertussis toxin induced some morphological changes in the Swiss 3T3 cells, it did not appear that this toxin inhibited the ability of thrombin to induce stress fibre formation or the association of $p70^{s6k}$ with the stress fibres (fig 3e).

The effect of thrombin on $p70^{s6k}$ activity was also examined directly. $p70^{s6k}$ was immunoprecipitated from cells that were quiescent or were activated with thrombin for 30, 60 or 120 mins. Thrombin induced a 4-fold stimulation of $p70^{s6k}$ activity which was maximal after 30 min of stimulation, and this level of activation was maintained at the 2 hour time point (60 min time point shown, fig 4). The activation of $p70^{s6k}$ activity by thrombin was totally abolished by preincubation of cells with 50 nM rapamycin (fig 4), but pertussis toxin (200 ng/ml) did not reduce this response (fig 4).

Such $p70^{s6k}$ immunoprecipitates were also assessed for potential modification of the enzyme due to thrombin stimulation. As can be seen by Western analysis, the $p70^{s6k}$ antibody was highly specific for the desired protein (fig 5). Thrombin induced a mobility shift of $p70^{s6k}$ on SDS-PAGE to a slightly higher molecular weight following either 30, 60 (not shown) or 120 min of activation, and the protein became a broader band, probably indicating a phosphorylation event. This mobility shift was inhibited by rapamycin (fig 5), in parallel with the inhibition of $p70^{s6k}$ enzymatic activity.

Pertussis toxin (100 ng/ml) had little effect on thrombin stimulation of the mobility shift of $p70^{s6k}$ on SDS-PAGE (not presented).

The effect of rapamycin on DNA synthesis stimulated by thrombin was also examined. Rapamycin had only a minor inhibitory effect on thrombin stimulation of DNA synthesis (fig 6).

DISCUSSION

The present work has shown that the 70 kDa form of S6 kinase ($p70^{s6k}$) is involved in the thrombin receptor regulation of stress fibre formation in Swiss 3T3 fibroblasts. The colocalisation of $p70^{s6k}$ with actin stress fibres suggested that this enzyme may be involved in the polymerisation of actin filaments. To test this, cells

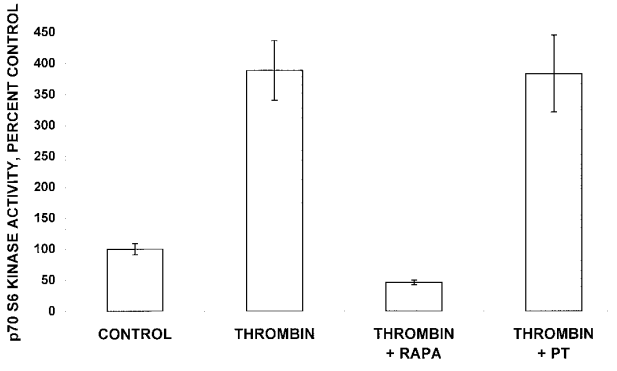

FIG. 4. Thrombin stimulates $p70^{s6k}$ activity in Swiss 3T3 cells: Inhibition by rapamycin but not pertussis toxin. Serum starved Swiss 3T3 cells were preincubated with or without pertussis toxin (PT, 200 ng/ml, overnight) or rapamycin (rapa, 50 nM, 1 hour) and then stimulated for 60 min with thrombin (1 U/ml). Cells were extracted with a triton X-100 containing buffer, and samples immunoprecipitated with a $p70^{s6k}$ antibody. Washed immunoprecipitates were then assayed for $p70^{s6k}$ activity. Results are expressed as a percentage of peptide substrate phosphorylation by $p70^{s6k}$ from unstimulated cells as the mean+/−SEm of 4-7 samples from 2–3 separate experiments.

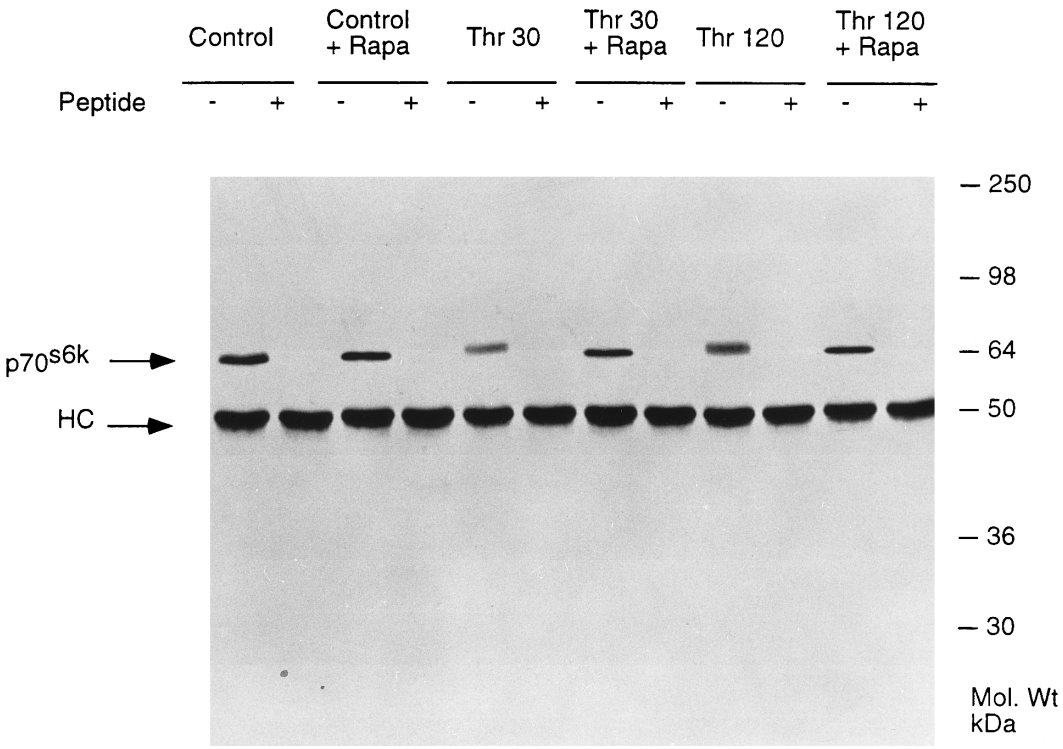

FIG. 5. Thrombin induces a molecular weight shift of $p70^{s6k}$ on SDS-PAGE: Inhibition by rapamycin. Serum-starved Swiss 3T3 cells were preincubated with or without rapamycin (rapa, 50 nM) for 60 min and then cells were further incubated with or without thrombin (1 U/ml). Cells were dissolved with a triton X-100 containing buffer and $p70^{s6k}$ antibodies added to samples in the presence (+) or absence (−) of blocking antigenic peptide. Immunoprecipitates were washed and proteins resolved on SDS-PAGE and western blotted and probed for $p70^{s6k}$. Positions of the heavy chain of the precipitating $p70^{s6k}$ antibodies, $p70^{s6k}$ itself and molecular weight standards are shown.

were pretreated with rapamycin to inhibit the enzyme FRAP that is responsible for activation of $p70^{s6k}$ (13). Consistent with a role for $p70^{s6k}$ in cytoskeletal organisation, rapamycin inhibited thrombin induced stress fibre formation. Thrombin was also an effective stimulus of $p70^{s6k}$ activity, and this was totally inhibited by rapamycin, showing that the effects of rapamycin could be accounted for by an action on this enzyme. The activation by thrombin and inhibition by rapamycin of $p70^{s6k}$ activity were mirrored by changes in electrophoretic mobility of $p70^{s6k}$, such that thrombin reduced mobility relative to $p70^{s6k}$ from control cells, and this was blocked by rapamycin. These results are consistent with thrombin activation of $p70^{s6k}$ occurring via phosphorylation of the enzyme, and this is due to FRAP activation, which is sensitive to rapamycin.

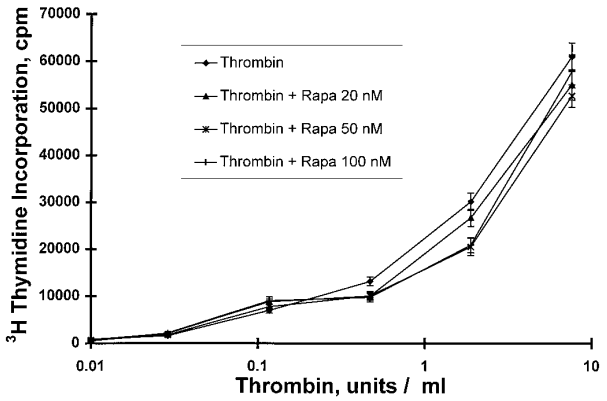

FIG. 6. Rapamycin does not inhibit thrombin stimulated DNA synthesis of Swiss 3T3 cells. Serum-starved Swiss 3T3 cells were pretreated with or without rapamycin (rapa) at the concentrations shown for 60 min and then further incubated with or without thrombin for 24 hours. [^{3}H]-Thymidine was then added and cells left for a further 24 hours. DNA synthesis was assessed by incorporation of label into insoluble cellular material, and results are expressed as the mean+/−SEm from 8 separate samples for each point.

Previous studies have implicated the GTP-binding protein, Rho, in the stimulation of stress fibres by hormone receptors. Some of the evidence for this correlation comes from the effects of C3 botulinum toxin on the response (9-11), as this toxin inhibits the activity of Rho. The effect of this toxin on the thrombin stimulation of $p70^{s6k}$ has not yet been examined in the cells used in this study.

It was of interest to try to determine the potential role of certain trimeric GTP-binding proteins in the coupling of the thrombin receptor to $p70^{s6k}$ and stress fibre formation. Pertussis toxin had little or no effect on either response to thrombin, suggesting that G_i is not involved. In these cells the DNA synthesis induced

by thrombin is also insensitive to pertussis toxin but there appears to be a large component stimulated by a G_q pathway (8). It appears likely that the coupling of the thrombin receptor to $p70^{s6k}$ and stress fibre formation is via a non-G_i and non-G_q pathway. Johnson and coworkers (5) have recently shown that activated forms of G_{12} and G_{13}, but not G_q or G_i, will induce stress fibre formation. Experiments are now being conducted to examine the G-proteins and subsequent coupling systems involved in the stimulation of $p70^{s6k}$ by thrombin. It has been shown that phosphatidylinositol 3-kinase (PI3K) can activate $p70^{s6k}$ in certain cells (14). Experiments are now examining the role of PI3K in thrombin-stimulated stress fibre formation and $p70^{s6k}$ activation by thrombin in Swiss 3T3 cells. Chou and Blenis (16) have presented evidence that $p70^{s6k}$ is regulated in NIH 3T3 cells by the G-proteins Cdc42 and Rac1. The present study would suggest that in Swiss 3T3 cells this is not the case, as neither of these G-proteins is implicated in stress fibre formation (9-11).

The dissociation between the mitogenic stimulus, which appears to be largely via G_q, and the $p70^{s6k}$ activating pathway is highlighted further by the relative insensitivity of thrombin stimulation of DNA synthesis to rapamycin. Circumstantial evidence has previously been used to suggest a role of $p70^{s6k}$ in growth factor-stimulated mitogenesis in several cells (reviewed in 15). However, our and other (17) data show in fibroblasts that the pathways are largely dissociable, and in the present study $p70^{s6k}$ plays little role in receptor-mediated DNA synthesis.

Thus, there appear to be distinct and separable cellular events stimulated by the thrombin receptor. These appear to diverge very early in the signalling pathway, and the combination of our data and that of others suggests that it may occur at the level of the coupling G-protein. This may imply that the thrombin receptor can couple, probably in a competitive manner, to at least three distinct G-proteins, each of which controls a separate cellular signalling system for a defined cellular response.

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