**GTPγS-induced actin polymerisation in vitro: ATP- and phosphoinositide-independent signalling via Rho-family proteins and a plasma membrane-associated guanine nucleotide exchange factor**

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**SUMMARY**

In a cell-free system from neutrophil cytosol GTPγS can induce an increase in the number of free filament barbed ends and massive actin polymerisation and cross-linking. GTPγS stimulation was susceptible to an excess of GDP, but not *Bordetella pertussis* toxin and could not be mimicked by aluminium fluoride, myristoylated GTPγS-G12 or Gβ12 subunits of trimeric G proteins. In contrast, RhoGDI and *Clostridium difficile* toxin B (inactivating Rho family proteins) completely abrogated the effect of GTPγS. When recombinant, constitutively activated and GTPγS-loaded Rac1, RhoA, or Cdc42 proteins alone or in combination were probed at concentrations >100 times the endogenous, however, they were ineffective. Purified Cdc42/Rac-interactive binding (CRIB) domain of WASP or C3 transferase did not prevent actin polymerisation by GTPγS. The action of GTPγS was blocked by mM [Mg2+] unless a heat- and trypsin-sensitive component present in neutrophil plasma membrane was added. Liberation of barbed ends seems therefore to be mediated by a toxin B-sensitive cytosolic Rho-family protein, requiring a membrane-associated guanine nucleotide exchange factor (GEF) for its activation by GTPγS under physiologic conditions.

The inefficiency of various protein kinase and phosphatase inhibitors (staurosporine, genistein, wortmannin, okadaic acid and vanadate) and removal of ATP by apyrase, suggests that phosphate transfer reactions are not required for the downstream propagation of the GTPγS signal. Moreover, exogenously added phosphoinositides failed to induce actin polymerisation and a PtdIns(4,5)P2-binding peptide did not interfere with the response to GTPγS. The speed and simplicity of the presented assay applicable to protein purification techniques will facilitate the further elucidation of the molecular partners involved in actin polymerisation.

Key words: Actin, Polymerisation, Cytoskeleton, Small G protein, Guanine nucleotide exchange factor, Signal transduction

**INTRODUCTION**

When stimulated with chemotactic agonists, neutrophils double their F actin content within seconds (Howard and Oresajo, 1985). This process was suggested to directly mediate lamellipod formation during locomotion and shape changes (Cooper, 1991; Theriot and Mitchison, 1991; Wymann et al., 1990; Mitchison and Cramer, 1996). Actin polymerisation is thought to be triggered by the removal of capping proteins from the filament’s barbed (+, fast growing) ends, allowing the addition of further monomeric G actin (Schafer and Cooper, 1995).

In neutrophils, more than half of the total actin is in its monomeric form and bound to thymosin β4 (Safer et al., 1991; Cassimeris et al., 1992) or profilin (Carlsson et al., 1977) in 1:1 complexes. While thymosin β4 serves solely as a buffer for G actin, profilin forms a productive complex that can directly add actin to barbed, but not pointed ends (see Pantaloni and Carlier, 1993, for references). It has been reported that the interactions between actin and various binding proteins like profilin (Lassing and Lindberg, 1985), CapZ (Heiss and Cooper, 1991) and gelsolin (Janmey and Stossel, 1989; Hartwig et al., 1996) can be disrupted by polyphosphoinositides (e.g. PtdIns 4-P and PtdIns(4,5)P2). How capping proteins are removed from the barbed ends to allow the growth of actin filaments due to surface receptor stimulation, however, is not clear. A major advancement in the understanding of these processes was the observation of Hall and coworkers, that constitutively activated small GTP-binding proteins of the Rho family could trigger cytoskeletal rearrangements (Ridley, 1994). While Rac induced membrane ruffles (Ridley et al., 1992), Rho mediated the formation of stress fibres (Ridley and Hall, 1992) and Cdc42Hs generated filopodia (Nobes and Hall, 1995). That these proteins indeed transduce signals from growth factor and seven transmembrane helix receptors was demonstrated by dominant negative forms
inhibiting agonist-induced actin rearrangements (Nobes and Hall, 1995; Kozma et al., 1995; Ridley, 1994). The state of Rho family proteins themselves is controlled by GTPase activating proteins (GAP) (Lamarche and Hall, 1994), guanine nucleotide dissociation inhibitors (GDI) and guanine nucleotide exchange factors (GEF) (Boguski and McCormick, 1993). GEFs of the DbI family contain as minimal elements a DbI homology (DH) and a pleckstrin homology (PH) domain (see Cerione and Zheng, 1996, for a recent review) of which the latter has been suggested to interact with polyphosphoinositides (Harlan et al., 1994). Some of the known GEFs act on several targets, e.g. DbI on Rho and Cdc42Hs (Hart et al., 1994; Cerione and Zheng, 1996), Tiam-1 on Rac1 and Cdc42Hs (Michiels et al., 1995), which might lead to a bifurcation of the incoming upstream signals. Downstream of Rho family proteins, various protein kinases are at work: Rho activates e.g. p164 Rho-associated kinase (p164Rho-K/ROKβ), p160 Rho-associated coiled-coil-containing protein kinase (p160ROCK) and protein kinase N (p128PKN/PRK12) (see Tapon and Hall, 1997, for a comprehensive list); Rac and Cdc42 interact with e.g. the protein Ser/Thr p65PA K and Tyr kinase p120 ACK (Manser et al., 1997). Some of the known GEFs act on several targets, e.g. the p164 Rho-associated coiled-coil-containing protein kinase (p160ROCK) and protein kinase N (p128PKN/PRK12) (see Tapon and Hall, 1997, for a comprehensive list); Rac and Cdc42 interact with e.g. the protein Ser/Thr p65PA K and Tyr kinase p120 ACK (Manser et al., 1994). While the latter two contain a CRIB (Cdc42/Rac-interactive binding; Burbelo et al., 1995) domain as is also present in W ASP (Wiskott-Aldrich syndrome protein; Aspenström et al., 1996; Symons et al., 1996), other Rac targets like p67phox (Prigmore et al., 1995) or POR-1 (partner of Rac-1; Van Aelst et al., 1996) interact differently. Of these molecules, Rho-K has been shown to mediate lysophosphatidic acid (LPA)/Rho-induced formation of stress fibers (Amano et al., 1997) and W ASP was reported to colocalize with Cdc42 in F-actin rich patches (Symons et al., 1996). Moreover, white blood cells of Wiskott-Aldrich syndrome patients show distorted shapes and a reduced number of microvilli compared to normal cells (Kirchhausen and Rosen, 1996).

In spite of the advancements in Rho family protein signalling, is it not clear how these events finally lead to the liberation of barbed ends, and requires the presence of a cytosolic, Rho family G protein and, in the presence of free Mg2+, a membrane-associated guanine nucleotide exchange factor (GEF) activity.

MATERIALS AND METHODS

Materials

ATPγS, ADP, GTP, GDP, apyrase grade V, cytochalasin B, heparin, sodium orthovanadate, pertussis toxin, and trypsin were from Sigma; Lymphoprep from Nycomed; Benzonase from Merck; diisopropyl fluorophosphatase (DFP) was purchased from Aldrich; leupeptin from Alexis Co.; pepstatin A, ATP, NAD+ from Fluka. Several batches of GTPγS (tetraethylammonium salt) were purchased from Sigma and Fluka. Phalloidin, rhodamine phalloidin, and N-(1-pyrene)iodocetamide were from Molecular Probes; GDPβS, DNase I and UDP-glucose from Boehringer Mannheim; genistein from ICN; G150 Sepharose (medium grade) from Pharmacia; okadaic acid from Calbiochem; and UDP-[14C]glucose was from Hartmann Analytic. PtdIns, PtdIns 4-P, and PtdIns(4,5)P2 from were from Fluka, dissolved in chloroform/methanol, dried, and sonicated in water before use.

Staurosporine was a kind gift from D. Fabbro, Ciba Geigy Ltd, Basel; wortmannin was kindly provided by T. G. Payne, Sandoz Pharma Ltd, Basel; rhodamine-labelled phosphoinositide-specific peptide (corresponding to gelsolin amino acids 160-169; Janmey et al., 1992) generously donated by P. Janmey, Brigham & Women’s Hospital, Boston; and buffy coats were kindly prepared by the Swiss Red Cross Transfusion Center, Fribourg.

GTPγS complexes were purified from baculovirus infected Sf9 cells as described (Kozasa and Gilman, 1995), myristoylated GαQ2 was purified from Escherichia coli cotransfected with a GαQ2 expression plasmid and N-myristoyl transferase (Lee et al., 1994; Mumby and Lider, 1994). Plasmids and baculovirus for the latter procedures were kindly obtained from A. G. Gilman, University of Texas, Dallas. RhoGDI, V12Rac1 and V14RhoA (Ridley et al., 1992; Ridley and Hall, 1992) and C3 transferase (kindly given by L. A. Feig, Tufts University, Boston; Dillon and Feig, 1995) were purified from E. coli as GST-fusion proteins. After thrombin digestion, the proteins were purified as described. E. coli expressed reference proteins (V12Rac1, V14RhoA, V12Cdc42 and N17Cdc42) successfully used in microinjections were also obtained from A. Ridley, LICR, London. W ASP expression plasmids to produce GST-fusions of W ASP (amino acids 48-321), W ASPACRIB (48-321 without the CRIB domain at 237-257) and W ASP-CRIB (235-268, with a deleted WIP-binding domain) were generously donated by U. Francke, Stanford University, Stanford (Symons et al., 1996). Clostridium difficile toxin B (Just et al., 1995) and Clostridium sordellii strain (strain 6018) lethal toxin (Just et al., 1996; Genth et al., 1996) were a generous gift from I. Just, University of Freiburg, Germany.

Neutrophil isolation and subcellular fractionation

Human neutrophils were isolated as described by Böyum (1968) and remaining erythrocytes were lysed by ammonium chloride treatment. Neutrophils were washed twice in 0.9% NaCl, 50 µM CaCl2 and resuspended in Hepes-potassium buffer (HKB, 135 mM KCl, 10 mM NaCl, 10 mM Hepes, 2 mM EDTA, pH 7.0, 108 cells/ ml) or Hepes-sodium buffer (HNaB, 138 mM NaCl, 4.6 mM KCl, 20 mM Hepes, pH 7.4). Membrane fractions were prepared from cells suspended in HKB without EDTA but supplemented with 3 mM MgCl2/2 mM EGTA.

Cells were disrupted in the above buffers supplemented with 1 mM PMSF, 0.5 mM diisopropyl fluorophosphatase (DFP), 20 µM leupeptin, 18 µM pepstatin, 12.5 units/ml benzene nuclease, and 5% glycerol by nitrogen cavitation (40 minutes, 35 bar) on ice. After centrifugation for 10 minutes at 1,300 g the supernatant was cleared for 45 minutes at 121,000 g.
To prepare plasma membranes and granules, the low speed supernatant from above was fractionated by a discontinuous sucrose gradient as described earlier (Abo and Segal, 1995). Plasma membranes and cytosol performed successfully in a cell-free NADPH oxidase assay (Abo and Segal, 1995). Samples were stored at −80°C without loss of activity.

Protein concentrations were determined with the Bradford (Bio-Rad) assay using BSA as a standard. ATP concentrations were determined using an ATP luciferase bioluminescence assay kit (CLSII, Boehringer Mannheim). Total ATP was measured by the release of bound nucleotides from proteins with 400 mM perchloric acid. Free ATP was determined by the direct addition of cytosolic samples to the luminescence assay.

In protease sensitivity experiments, plasma membranes (1.3 mg/ml of total protein) were incubated without or with 150 μg/ml trypsin for 2 hours at 37°C. DFP was added to 1 mM before membranes were applied to the actin polymerisation assay.

Analysis of actin aggregates

If not indicated otherwise, cytosol was diluted with HBK to 1.8 mg total protein/ml, and incubated at 37°C in the absence or presence of 50 μM GTP·S for 20 minutes. These samples were either: (i) fixed for microscopy with 10% p-formaldehyde for 10 minutes on ice, and supplied with 0.6 μM rhodamine phallolidin at least one hour before they were examined on a confocal microscope (Bio-Rad MRC 1024/ Nikon E800 optics), or (ii) centrifuged at 9,000 g, 5 minutes. The sediments were washed twice with HBK, denatured and applied to a 10% SDS-PAGE gel. Major actin-binding proteins were subjected to MALDI-TOF-MS on a PerSeptive Biosystem Voyager Elite mass spectrometer after in-gel trypsin digestion (for references see Cottrell and Sutton, 1996).

Actin polymerisation assay in a cell-free system

The property of rhodamine phallolidin enhancing its fluorescence by approximately a factor of ten after binding to F actin (Huang et al., 1992) was exploited to measure increases in the amount of F actin in a cell-free system. The assay was set up to display a maximal sensitivity in cytosolic fractions (data not shown). If not indicated otherwise, reactions were carried out in a total volume of 35 μl HBK (with free Mg²⁺ at less than 10 μM, as calculated according to Martell and Smith, 1974) with 1.8 mg cytosolic protein/ml. After the indicated additions and preincubations (see below) either vehicle or GTP·S was added for 20 minutes at 37°C. Aliquots of 10 μl were then removed and added to 600 μl HBK with 16.5 nM rhodamine phallolidin. Fluorescence was measured 20 minutes later in a Perkin Elmer fluorescence spectrometer LS50B (Ex. 552 nm, slit 5 nm; Em. 580 nm, slit 20 nm). After subtraction of the background rhodamine phallolidin fluorescence, the fluorescence intensity is linearly related to the amount of F actin present (data not shown).

Free barbed ends assay

ATP·actin from rabbit skeletal muscles was isolated as described previously (Pardee and Spudich, 1982), labelled with N-(1-pyrene)iodoacetamide according to the method of DeNubile and Southwick (1988), and pyrenyl G actin was purified as in the method of MacLean-Fletcher and Pollard (1980), its concentration being determined according to the method of Carson et al. (1986). Cytosolic reaction mixtures were treated for 15 minutes as described above. Aliquots of 20 μl were subsequently mixed with 600 μl prewarmed HBK containing 0.5 μM pyrenyl G actin, and the kinetics of pyrenyl actin fluorescence was immediately followed at 27°C (Ex. 365 nm, slit 3 nm; Em. 387 nm, slit 10 nm). Under these conditions the initial rate of fluorescence increase is the measure of amount of free barbed ends added (Carson et al., 1986).

Bacterial toxins

Cytosolic fractions were preincubated with various toxins at 37°C before addition of GTP·S. B. pertussis toxin (pre-activated with 1 mM ATP and DTT for 15 minutes at 37°C) and C3 transferase were used in the presence of 20 μM NAD⁺ for ADP-ribosylations. C. difficile toxin B (Just et al., 1995) and C. sordellii (strain 6018) lethal toxin (Just et al., 1996; Popoff et al., 1996; Gentil et al., 1996) glucosylated small G proteins with 50 μM UDP-glucose as a co-substrate. The activities of toxin B and lethal toxin were tested at nM concentrations on NIH 3T3 fibroblasts and were found to induce the expected morphological changes (Just et al., 1996).

Toxin B-mediated glucosylation was assessed using 50 nCi UDP-[¹⁴C]glucose (50 μM final). After a 30 minute incubation at 37°C, samples were analysed for 10% trichloroacetic acid (TCA)-insoluble radioactivity or by SDS-PAGE followed by autoradiography.

Preloading of recombinant G proteins with guanine nucleotides

Gα2 (0.19 mg/ml) was preloaded with 0.1-1 mM GTP·S in the presence of 20 mM MgCl₂ for 1 hour at 37°C (Carty and Iyengar, 1994). Rac1, RhoA and Cdc42 were preloaded with a 5-10 mol excess of GTP or GTP·S in the presence of 10 mM EDTA for 20 minutes at RT, terminating the reaction by addition of 20 mM MgCl₂ (Self and Hall, 1995).

RESULTS

GTP·S triggers F actin assembly and cross-linking in vitro

When high speed cytosolic fractions from human neutrophils were incubated with GTP·S, the formation of actin filaments and aggregates was observed microscopically: F actin could be detected by rhodamine phallolidin staining both before (data not shown) and after fixation of GTP·S treated cytosol with p-formaldehyde, which was added to prevent further actin rearrangements (Fig. 1). While filament bundles and aggregates were numerous and of considerable size due to GTP·S addition (≤30 μm), untreated cytosols displayed only occasionally aggregates of much smaller size (∼10 μm). To determine changes in cross-linked F actin, GTP·S treated cytosolic fractions and controls were centrifuged at low speed and actin was quantified by Coomassie staining. Compared to controls, GTP·S caused typically a 6.8 (±2.5, s.d., n=4)-fold increase in sedimentable actin, resulting in the sedimentation of about 16% of the total actin present in the cytosol. Two major proteins co-sedimenting were identified as ABP-280 and α-actinin.

Taking advantage of the observation of Huang et al. (1992) that rhodamine phallolidin fluorescence increases about 10-fold upon its binding to F actin we have developed a functional reconstitution assay for actin polymerisation. Using the assay we found that ≥1 mg of total cytosolic protein/ml in the presence of ≥5 μM GTP·S was necessary to induce significant actin polymerisation. Higher concentrations of GTP·S up to 50 μM further increased the total amount of F actin detected after 20 minutes of incubation and reached a plateau thereafter (Fig. 2A). When the time course of actin polymerisation in response to different amounts of GTP·S was examined, we observed a strong effect of increasing GTP·S concentrations on the rate of actin polymerisation during the first 10 minutes of the reaction (Fig. 2B). The polymerisation process reached its maximum at about 10-20 minutes and F actin levels remained constant up to 40 minutes to decrease slightly to 80 minutes. The de novo polymerised actin seems therefore to remain stable, indicating
that the system reaches a new equilibrium at higher F actin levels due to GTP\textsubscript{S} stimulation. This can be explained by the observation that free barbed ends could not be detected beyond 30 minutes after GTP\textsubscript{S} addition (data not shown), indicating that the liberation of barbed ends is a transient process.

**GTP\textsubscript{S} is the only nucleotide to stimulate actin polymerisation**

Addition of 50 \mu M GTP\textsubscript{S} to cytosolic reaction mixtures increased the amount of filamentous actin by a factor of 5.17 ± 0.59 (mean ± s.e.m., \(n > 30\)). All tested batches of GTP\textsubscript{S} exhibited the same effect, which was not mediated by lithium ions associated with GTP\textsubscript{S}: LiCl up to 2 mM did not affect F actin levels (data not shown). Other nucleotides tested (ATP, ATP\textsubscript{S}, ADP, GDP, GDP\textsubscript{S}, all at 100 \mu M) failed to display any stimulatory action on actin polymerisation (\(n \geq 3\)). GTP at 100 \mu M induced a slight increase in F actin, and was at 1 mM about 50% as effective as GTP\textsubscript{S} (data not shown). The absence of a stimulatory action of 100 \mu M ATP or ATP\textsubscript{S} on the polymerisation process demonstrates that the effect of GTP\textsubscript{S} does not involve the transfer of its \(\gamma\)-thiophosphate group to ADP by a nucleoside diphosphokinase (Parks and Agarwal, 1973).

When an excess of GDP (1 mM) was added together with GTP\textsubscript{S}, the effect of the latter was completely inhibited, which is a further indication of the specific action of GTP\textsubscript{S} on a signalling molecule (Fig. 3). ADP or UDP (1 mM) added shortly before GTP\textsubscript{S} had no effect, but prolonged preincubations increasingly lowered basal and GTP\textsubscript{S}-controlled F actin levels (data not shown).

**GTP\textsubscript{S} increases the number of free barbed ends**

An excess (30 \mu M) of DNase I, sequestrating monomeric actin

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**Fig. 1.** GTP\textsubscript{S}-mediated actin polymerisation and cross-linking.

Cytosols from human neutrophils at a concentration of 1.8 mg/ml of total protein in HKB were incubated for 20 minutes in the absence (−, −GTP\textsubscript{S}) or presence of 50 \mu M GTP\textsubscript{S} (+, +GTP\textsubscript{S}). (A,B) Samples for confocal microscopy were subsequently fixed with \(p\)-formaldehyde and stained with rhodamine phalloidin for F actin. (A) Largest aggregate found in sample; (B) representative field of view. Bar, 50 \mu m. (C) Alternatively, the treated samples were centrifuged at low speed, and washed sediments were subjected to SDS-PAGE and Coomassie blue staining. Total protein applied before sedimentation (T) corresponds to \(1/10\) of the material yielding the sediments in first two lanes (Sedimented). Positions of molecular mass standards are indicated to the right (in kDa). Data are representative for \(\geq 3\) experiments. Asterisks indicate the positions of ABP-280 and \(\alpha\)-actinin (apparent molecular mass 105 kDa), as identified by peptide mass fingerprinting analysis.

**Fig. 2.** Effect of increasing concentrations of GTP\textsubscript{S} on end point and kinetics of actin polymerisation. (A) Reaction mixtures with 1.8 mg cytosolic protein/ml were incubated with the indicated concentrations of GTP\textsubscript{S} for 20 minutes before F actin was quantified (mean ± s.e.m., \(n \geq 3\)). (B) Samples were incubated with the indicated concentrations of GTP\textsubscript{S} for the indicated times before aliquots were removed for F actin determination (data representative for 6 experiments).
Inhibition of GTP\textsuperscript{s}\textsubscript{S}-mediated actin polymerisation (Fig. 3). Cytochalasin B, on the other hand, did not affect the basal signal, but potently blocked the GTP\textsuperscript{s}\textsubscript{S}-induced increase in F actin. This suggests that the barbed ends (fast growing, + ends) of actin filaments have to be accessible for GTP\textsuperscript{s}\textsubscript{S} to be able to trigger polymerisation. To test if the number of barbed ends indeed increased, cytosolic reaction mixtures were stimulated with 50 \textmu M GTP\textsuperscript{s}\textsubscript{S} for 15 minutes at 37°C and aliquots were subsequently tested for barbed end nucleation activity (Carson et al., 1986). While cytosol incubated in the absence of GTP\textsuperscript{s}\textsubscript{S} did not trigger an increase in pyrenyl actin fluorescence, GTP\textsuperscript{s}\textsubscript{S} treated samples led to a rapid incorporation of pyrenyl actin into filaments, and thus to an increase in fluorescence (Fig. 4). Cytochalasin B (5 \textmu M) in the measurement cuvette blocked this increase, while variations of the actin concentration did not influence the initial rate of the process. Altogether, this illustrates that GTP\textsuperscript{s}\textsubscript{S} promoted an increase of available barbed ends. Interestingly, no free barbed ends could be detected 30 minutes after addition of GTP\textsuperscript{s}\textsubscript{S} at 37°C (data not shown). However, when samples were collected 15 minutes after GTP\textsuperscript{s}\textsubscript{S} addition and then kept on ice, cytosol retained its nucleating activity for prolonged times. This might indicate that at 37°C, but not 0°C, GTP\textsuperscript{s}\textsubscript{S} triggers some feedback reactions leading to recapping of barbed ends.

The role of protein-\textsubscript{g}, lipid kinases and phosphatases

The effect of two broad band protein kinase inhibitors, genistein targeting protein tyrosine kinases (Akiyama and Ogawara, 1991) and staurosporine affecting Ser/Thr kinases (Tamaoki, 1991), was examined. Both inhibitors (genistein at 50 \textmu M and staurosporine at 500 nM) did not interfere with GTP\textsuperscript{s}\textsubscript{S}-mediated in vitro actin polymerisation. Wortmannin, at nM concentrations a specific inhibitor of PI 3-kinases (Arcaro and Wymann, 1993; Yano et al., 1993), affects at \mu M concentrations some other lipid and protein kinases (see Wymann et al., 1996, for references). Even at 1 \mu M, however, the substance remained without effect (Table 1).

When cytosol was exposed to 0.5 units/ml of apyrase (converting ATP and ADP to AMP and P\textsubscript{i}; Kettlun et al., 1982) for 70 minutes on ice, >99% of the free ATP remaining after cell fractionation (initially 2.5 \mu M) was hydrolysed resulting in an [ATP] of about 22 nM. The amount of protein-bound ATP, however, was only reduced from 2.1 \mu M to approx. 220 nM. At the same time, GTP\textsuperscript{s}\textsubscript{S} was still able to produce a >2-fold increase in F actin, the partial inhibitory effect of apyrase being best explained by the loss of actin-bound ATP. Similarly, prolonged incubations with 1 mM ADP before GTP\textsuperscript{s}\textsubscript{S} addition decreased subsequent actin polymerisation, most likely also interfering by the elimination of ATP-actin from the system. The above, the inhibitor data and the fact that addition of 1 mM ATP to cytosolic reaction mixtures did not enhance the GTP\textsuperscript{s}\textsubscript{S}-mediated increase in F actin (data not shown), suggest that lipid- and protein kinase activities are not involved in the GTP\textsuperscript{s}\textsubscript{S}-mediated process.

Two protein phosphatase inhibitors, okadaic acid (Hardie et al., 1991) and vanadate (Gordon, 1991), were tested on GTP\textsuperscript{s}\textsubscript{S}-stimulated actin polymerisation (Table 1). Okadaic acid at 0.5 \mu M did not display a significant effect, while vanadate at 3 mM slightly reduced both, the basal and GTP\textsuperscript{s}\textsubscript{S}-induced F actin levels. The fold increase in polymerised actin triggered by GTP\textsuperscript{s}\textsubscript{S}, however, remained unchanged. Vanadate seems therefore to mediate its weak action not via inhibition of protein phosphatases, but rather by its direct interaction with...
actin filaments, as it has been reported before (Combeau and Carlier, 1988).

Involvement of Rho-family but not trimeric GTP-binding proteins

The results presented above point to GTP-binding proteins being the mediators of the observed rise in F actin. Candidates might thus be either trimeric or small G proteins. To test the importance of trimeric G proteins, cytosols were pretreated with \(B. pertussis\) toxin, or aluminium fluoride, recombinant \(G\beta\gamma\) or GTP\(\gamma\)S-loaded \(G\alpha\) were added. None of these treatments, however, showed any inhibitory or stimulatory effect (see Table 1).

\(C. difficile\) toxin B (inactivating Rho proteins by glucosylation using UDP-glucose as a co-substrate; Just et al., 1995), on the other hand, completely inhibited GTP\(\gamma\)S-mediated actin polymerisation in a concentration dependent way. This inhibition correlated with the incorporation of \([14\text{C}]\)glucose into total TCA-precipitatable protein and into a \(G_{\alpha2}\) subunit of trimeric G proteins (Dillon and Feig, 1995, for references), virtually eliminating all Rho proteins due to their dissociation from bacterial toxins to Rho proteins.

<table>
<thead>
<tr>
<th>Permutations of Rho proteins§</th>
<th>Action of treatment</th>
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<tbody>
<tr>
<td>See (V12\text{Rac1}, V14\text{RhoA}, V12\text{Cdc}42)</td>
<td>Activated (G\alpha2) subunits</td>
</tr>
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\(\text{GST-WASP-CRIB domain} 30 \mu M\)

\(\text{GST-WASP-GRB2 domain} 30 \mu M\)

\(\text{GST-WASP-CRIB domain} 30 \mu M\)

\(\text{Additions} 2 \mu M ADP-ribosylation of RhoA, B, C\)

\(\text{Additions} 5 \mu M ADP-ribosylation of RhoA, B, C\)

\(\text{Additions} 10 \mu M ADP-ribosylation of RhoA, B, C\)

Membrane-associated protein(s) reverse the inhibitory action of \(Mg^{2+}\)

As shown in Fig. 7A, free \(Mg^{2+}\) inhibits GTP\(\gamma\)S-induced actin polymerisation in a concentration dependent way. While the basal level of F actin was not affected significantly, GTP\(\gamma\)S-mediated increases were totally abolished at 1 mM free \(Mg^{2+}\). This behaviour of the cytosolic system suggests, that it does not contain a guanine nucleotide exchange factor (GEF) activity that would allow the nucleotide exchange on the aspersed G protein at physiological \(Mg^{2+}\) concentrations.

Table 1. Treatments not affecting GTP\(\gamma\)S-mediated actin polymerisation

<table>
<thead>
<tr>
<th>Additions</th>
<th>Max. concentrations used</th>
<th>Action of treatment</th>
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<tbody>
<tr>
<td>Genistein</td>
<td>50 (\mu M)</td>
<td>Broad protein tyrosine kinase inhibitor</td>
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<tr>
<td>Stauroporine</td>
<td>500 (nM)</td>
<td>Broad protein Ser/Thr kinase inhibitor</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>1 (\mu M)</td>
<td>PI 3-kinase inhibitor</td>
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<tr>
<td>PPI-binding peptide</td>
<td>10 (\mu M)</td>
<td>Binds PtdIns(4,5)(P_2)</td>
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<tr>
<td>Na-orthovanadate</td>
<td>3 (mM)</td>
<td>Broad protein phosphatase inhibitor</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>250 (nM)</td>
<td>Broad protein phosphatase inhibitor</td>
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<tr>
<td>Aluminium fluoride*</td>
<td>20 (mM) Na(\text{II})</td>
<td>Activator of trimeric G proteins</td>
</tr>
</tbody>
</table>

\(B. pertussis\) toxin** 10 \(\mu M\) AlCl\(3\)

\(G\alpha2\) GTP\(\gamma\)S* 2 \(\mu g/ml\) ADP-ribosylation of \(G\alpha2\) subunits

\(G\beta\gamma\) 2 \(\mu g/ml\) \(G\beta\gamma\) subunit of trimeric G proteins

\(C. sordellii\) lethal toxin strain 6018*** 10 \(\mu g/ml\) Glucosylation of Rac1, Ras, Rap, Rat

\(C. difficile\) toxin B (inactivating Rho proteins by glucosylation using UDP-glucose as a co-substrate; Just et al., 1996), could not prevent the action of GTP\(\gamma\)S signalling. C3 transferase, ADP ribosylating RhoA-C (Dillon and Feig, 1995), did not produce any effect on the system at 5 \(\mu g/ml\). When concentrations where elevated to 10 \(\mu M\) as 12, 6/6 or 4/4/4 \(\mu M\) sets were equally ineffective. GST-WASP fusion proteins were added to the cytosol to bind GTP-loaded Cdc42: GST-WASP (amino acids 48-321) and WASPACRIB (48-321, a.a. 237-257 deleted, used as a negative control) at 3 \(\mu M\), and the GST fusion of the WASP CRIB domain (235-268) up to 30 \(\mu M\) all failed to interfere with GTP\(\gamma\)S signalling. C3 transferase, ADP ribosylating RhoA-C (Dillon and Feig, 1995), did not produce any effect on the system at 5 \(\mu g/ml\). When concentrations where elevated to 100 \(\mu g/ml\), an approx. 40% drop in GTP\(\gamma\)S-mediated actin polymerisation could be achieved. This reduction, however, was independent of the presence of NAD* and heat inactivation of C3 (Table 1). In the presence of 25 \(\mu M\) PtdIns, reported to stimulate the access of bacterial toxins to Rho proteins due to their dissociation from RhoGDI (see Dillon and Feig, 1995, for references), virtually no effect of C3 transferase was observed (data not shown). Protein and lipid kinase and phosphatase inhibitors were preincubated with cytosol for 10 minutes at 37°C before GTP\(\gamma\)S addition.

*Actin polymerisation was studied in the absence of further added GTP\(\gamma\)S.

**Used with 20 \(\mu M\) NAD*.

***Used with 50 \(\mu M\) UDP-glucose and 0.1-0.4 mM free Mn\(\text{II}\) for up to 1 h, before complexation of divalent cations with EDTA and addition of GTP\(\gamma\)S.

†At the highest concentrations used, the 3\(C\) transferase preparations reduced polymerised actin by ca. 40%. This reduction, however, also occurred with heat inactivated preparations.

§Added to a total concentration of 12 \(\mu M\). Combinations of 12, 6/6, 4/4/4 \(\mu M\) \(V12\text{Rac1}, V14\text{RhoA}, V12\text{Cdc}42\) were tested.

¶Where tested in the system containing plasma membrane and Mg\(\text{II}\) (see Fig. 7B for conditions). Identical results were obtained in the cytosolic system without membranes and Mg\(\text{II}\).
As many GEFs are membrane associated, we reconstituted our system by the addition of neutrophil plasma membrane. As demonstrated in Fig. 7B, addition of plasma membrane protein to 100 to 300 µg/ml, but not neutrophil granule membranes (data not shown), rescued the stimulatory effect of GTPgS in the presence of 1 mM free Mg2+. While the response to GTPgS reached a plateau at about 180 µg membrane protein/ml, the basal levels of F actin slightly increased in a linear fashion. In the absence of free Mg2+ we could not detect any influence of plasma membrane preparations on actin polymerisation (data not shown). Dominant negative N17Cdc42 (5 µM) added to the system (+membranes and Mg2+) in order to test whether the putative GEF would interact with Cdc42, could not prevent actin polymerisation.

As it has been reported before that PtdIns(4,5)P2 exhibits GDP-dissociation activity on Cdc42 (Zheng et al., 1996), we tested the effects of pure polyphosphoinositides in cytosolic reaction mixtures. Unexpectedly, PtdIns, PtdIns4P and PtdIns(4,5)P2 all inhibited GTPgS-mediated actin polymerisation completely in the absence of free Mg2+ at 25, 80 and 25 µM, respectively, and none of the tested lipids induced actin polymerisation by itself. Moreover, 10 µM of a rhodamine-labelled PtdIns(4,5)P2-binding peptide derived from gelsolin (Janmey et al., 1992) did not block the action of GTPgS (higher concentrations of the peptide interfered with the assay and caused actin polymerisation). If free Mg2+ was set to 1 mM in cytosolic reaction mixtures, on the other hand, lipids compensated the inhibitory action of free Mg2+ on GTPgS-mediated action polymerisation (EC50 s for PtdIns, PtdIns4P and PtdIns(4,5)P2 were 10, 100, and 120 µM, respectively), but did not induce an increase in F actin (≤ 240 µM). The decrease in potency of PtdIns > PtdIns4P > PtdIns(4,5)P2 is unlikely to be related to a previously observed GDP dissociation activity on Cdc42 and fits better with the lipid-induced dissociation of Rho proteins from RhoGDI (Chuang et al., 1993).

When plasma membrane preparations were exposed to trypsin or heat and then added to cytosol containing 1 mM free Mg2+, the membrane-mediated rescue of the GTPgS-triggered response was eliminated (Fig. 7B,C). Most of the membrane associated activity could be preserved, if the serine protease inhibitor DFP was added before trypsin. These results further demonstrate that the active plasma membrane constituent which permits the action of GTPgS in the presence of high free Mg2+ is a protein and not a lipid.

In summary, our results reveal that GTPgS triggers actin polymerisation by the action of a toxin B-sensitive, RhoGDI-binding Rho family-related protein, which requires under physiologic conditions the presence of a membrane associated, putative GEF activity.
DISCUSSION

GTPγS-mediated actin polymerisation: an in vitro approach

Although numerous actin-interacting proteins have been isolated, it is presently not clear how signals emerging from cell surface receptors trigger actin polymerisation. It has been recognised, however, that Rho family proteins relay these signals and that constitutively activated Rac, Rho and Cdc42 cause cytoskeletal rearrangements by themselves (Ridley, 1994; Ridley and Hall, 1992; Nobes and Hall, 1995; Nobes et al., 1995). Nevertheless, it is not understood how these small G proteins might cause e.g. the liberation of barbed ends to allow the elongation of actin filaments. This motivated us to develop in vitro assays to elucidate the mechanisms of signal-induced actin polymerisation.

We observed that GTPγS addition to neutrophil cytosolic fractions caused the appearance of F actin bundles and networks that grew so large that they could be easily viewed by light microscopy and sedimented by low speed centrifugation. Size, stability and appearance of the networks can be explained by the presence of ABP-280 (branching F actin at high angles; Gorlin et al., 1990), α-actinin (an actin bundling and network-forming crosslinker; Otto, 1994) and further unidentified actin-binding proteins. As cytosol after high speed centrifugation contains only filaments of less than 20 actin monomers (DiNobili and Southwick, 1988), these observations illustrate the dramatic effect of GTPγS on the elongation of actin filaments.

Based on the previously described increase in rhodamine phalloidin fluorescence upon binding to F actin (Huang et al., 1992) we have composed an optimized assay to determine F actin changes in cytosolic samples. In comparison to other methods, we have drastically reduced both the amount of rhodamine phalloidin used and lowered the amount of total cytosolic protein required to about 40 μg/sample. The major advantage of the assay is, however, that the F actin content can be determined directly after sample treatment and that no centrifugation, washing steps or release of labelled phalloidin are required. The in vitro assay presented here is therefore the first fast and simple enough to be used along with protein purification schemes designed to identify molecules involved in the actin polymerisation process.

GTPγS liberates barbed ends
GTPγS-mediated actin polymerisation could be demonstrated before in permeabilized neutrophils (Therrien and Naccache, 1989; Bengtsson et al., 1990; Downey et al., 1990; Redmond et al., 1994) and was shown to be driven by the availability of free barbed ends (Tardif et al., 1995). In our cell-free system, both the end point levels and the rate of GTPγS-induced actin polymerisation increased with the amount of stimulator added to cytosol. Together with the observations that GTPγS increases the availability of free barbed ends and that cytochalasin B blocks GTPγS-mediated actin polymerisation, this suggests that GTPγS transiently removes capping proteins from free barbed ends or forms them de novo, similarly to what probably happens in vivo upon stimulation.

The role of protein-, lipid kinases and phosphatases, and ATP
To elucidate the role of protein and lipid kinases, we attempted

Fig. 7. Inhibition of GTPγS-triggered actin polymerisation by free Mg2+ and reversal by membrane proteins. (A) Increasing amounts of MgCl2 were added to cytosolic reaction mixtures yielding the indicated free [Mg2+]. Subsequently, samples were supplemented with 50 μM GTPγS (closed circles) or vehicle only (open circles) and incubated as above. (B) Cytosolic samples containing 1 mM free Mg2+ were combined with plasma membranes with the indicated quantity of membrane protein, before GTPγS or vehicle was added. Alternatively, membranes denatured by boiling were used in the presence of GTPγS (filled square). (C) Cytosolic samples as in B were supplemented with plasma membranes pretreated in the absence (-) or presence of trypsin (+) or trypsin and diisopropyl fluorophosphate (+/DFP). In all membrane samples DFP was brought to 1 mM before being added to cytosolic reaction mixtures. After all described incubations, F actin was quantified as above (mean ± s.e.m., n≥3; deviations omitted where smaller than symbols).
to block the GTP$\gamma$S-mediated actin polymerisation with broad band inhibitors for protein tyrosine kinases (genistein; Akiyama and Ogawara, 1991), Ser/Thr protein kinases (staurosporine; Tamaoki, 1991) and PI 3-kinases (wortmannin; see Wymann et al., 1996). Even the use of elevated inhibitor concentrations did not interfere with the polymerisation response. This finding clearly excludes a variety of src- and EGF receptor-like tyrosine kinases, members of the protein kinase C family, and PI 3-kinase and related proteins from being downstream elements for the primary target of GTP$\gamma$S in cytosolic fractions. This contrasts with the proposed involvement of a protein tyrosine kinase downstream of Rho in bombesin and lysosphosphatidic acid-induced stress fibre formation in fibroblasts (Ridley and Hall, 1994).

As a variety of kinases might still escape the treatments above, apyrase was used to remove ATP from cytosol to eliminate phosphorylation reactions completely. Free ATP in cytosol was already lowered to about 2.5 $\mu$M after cell fractionation and decreased by a factor of $>100$ due the addition of apyrase. The GTP$\gamma$S-induced actin polymerisation response was lowered in parallel to about $1/3$. As protein-bound ATP decreased to approximately $1/10$ of the initial concentration within the same time, it must be assumed that ATP was also lost from actin. It is most likely that this loss of the nucleotide interfered with the ability of actin to polymerise, but that ATP was not required for downstream kinase reactions. This is in agreement with the observations that addition of mM ATP did not enhance GTP$\gamma$S-induced actin polymerisation and that mM ADP only showed inhibitory activity when incubated for prolonged periods allowing ATP/ADP exchange on actin. Redmond et al. (1994) have shown previously in streptolysin-O permeabilised neutrophils that degradation of extracellular ATP does not abrogate GTP$\gamma$S-stimulated increases in F actin.

A further argument against the involvement of phosphorylation reactions is that the process takes place in an excess of EDTA, leaving most kinases inactive due to their dependence on divalent cations. These findings exclude the participation of a number of protein and lipid kinases that were proposed to act downstream of Rho family proteins (see Introduction). Additionally, these results exclude an involvement of Rac-induced PtdIns(4,5)P$_2$ synthesis as described by Hartwig and coworkers (1995) in permeabilised platelets.

In neutrophils and platelets, okadaic acid was reported to alter cell shape and to inhibit agonist-mediated changes in F actin (Kreienbuhl et al., 1992; Downey et al., 1993; Yano et al., 1995). While it was suggested that this occurs without the involvement of myosin light chain phosphorylations, it was shown in fibroblasts that RhoA regulates myosin phosphatase through Rho-kinase (Kimura et al., 1996). In vitro actin polymerisation by GTP$\gamma$S as shown here, on the other hand, was not affected by okadaic acid, nor o-vanadate, suggesting that the effects of protein phosphatases observed in neutrophils would be localised up-, but not downstream of the target of GTP$\gamma$S.

The cytosolic target of GTP$\gamma$S

As GTP$\gamma$S can act as an activator for trimeric and small G proteins, we modulated in vitro actin polymerisation by stimulators and inhibitors for both protein families. B. pertussis toxin, which inhibits G$\alpha$2 subunits by ADP-ribosylation, was shown previously to inhibit agonist mediated F actin increases, but did not interrupt the stimulation conferred by GTP$\gamma$S in permeabilized neutrophils (Redmond et al., 1994). Likewise B. pertussis toxin left the GTP$\gamma$S-mediated actin polymerisation in the cell free system intact. Activators of trimeric G protein controlled pathways, aluminium fluoride, GTP$\gamma$S-loaded myristoylated G$\alpha$2 subunits, and G$_{\beta\gamma}$2 complexes also failed to stimulate actin polymerisation in cytosolic reaction mixtures. Although trimeric G proteins certainly mediate agonist-induced actin polymerisation in intact cells (Shefcky et al., 1985), they are not necessary to carry on the GTP$\gamma$S signal in the cytosol. All this is in agreement with the fact that trimeric G proteins require an excess of free magnesium for their activation both in vivo and in vitro (Gilman, 1987), and could thus not exchange GTP for GTP$\gamma$S under the standard conditions of the assay.

It has been reported that C. difficile toxin B glucosylates a broad range of Rho family proteins (Rho, Rac, Cdc42, but not Ras) at the residue corresponding to threonine 37 in RhoA (Just et al., 1995). Moreover, it was demonstrated that microinjected glucosylated RhoA produced a dominant negative effect and disassembled actin filaments in fibroblasts (Just et al., 1995). When toxin B was tested in the cell free actin polymerisation assay for its potential to interfere with GTP$\gamma$S stimulation, it was found that the dose dependent inhibition correlated with the incorporation of $[14C]$glucose into protein in particular with the labelling of a 20 kDa protein family. C. sordellii lethal toxin with Rac1, Ras, Rap andRal as targets (Just et al., 1996; Genth et al., 1996), on the other hand, was not effective.

As a further strategy to block Rho family protein signalling we used recombinant RhoGDI to complex the putative small G protein, and RhoGDI was indeed capable to block the action of GTP$\gamma$S at 50 $\mu$M. The effect of RhoGDI on exocytosis in mast cells (Mariot et al., 1996) or on receptor-mediated endocytosis in HeLa cells (Lamaze et al., 1996) was previously taken as proof for the involvement of Rho-related proteins in these cell responses. Together with the inhibitory action of toxin B, our data clearly demonstrate the involvement of a Rho family member in the GTP$\gamma$S-mediated actin polymerisation response.

The membrane associated GEF activity

We found that the actin polymerising action of GTP$\gamma$S was very sensitive to free magnesium ions. This fits well with the fact that small G proteins can exchange their loaded guanine nucleotide in vitro as long as Mg$^{2+}$ is complexed, but that the GDP/GTP exchange is hindered if this requirement is not met (Self and Hall, 1995). In vivo, at mM free Mg$^{2+}$ concentrations, the nucleotide exchange is accelerated by guanine nucleotide exchange factors (GEFs; Boguski and McCormick, 1993). Our data illustrate that the cytosolic fractions are devoid of a GEF activity that could mediate nucleotide exchange on the target G protein mediating actin polymerisation.

GEFs are often localised to membrane fractions (Bokoch et al., 1994; Whitehead et al., 1995; Chardin et al., 1996; Paris et al., 1997) and re-addition of plasma membranes to the system at 1 mM Mg$^{2+}$ indeed rescued the effect of GTP$\gamma$S, while neutrophil granule membranes remained ineffective. On the other hand, it has been shown that PtdIns(4,5)P$_2$ can accelerate the dissociation of GDP from human Cdc42 (Zheng et al., 1996) and stimulate GEFs by binding to pleckstrin homology domains (Paris et al., 1997). Working with Xenopus egg
extracts, Ma and coworkers (1998) have proposed that 4,5-phosphorylated phosphoinositides trigger actin polymerisation either by direct action on small G proteins or serving as docking site for GEFs.

The sensitivity of the plasma membrane activity to heat and trypsin demonstrated here, however, illustrates clearly that either the GEF itself or an essential proteinous GEF activator is associated with the plasma membrane and that carried over lipids do not act by themselves.

In the absence of free Mg$^{2+}$ phosphoinositides inhibited GTP$^\gamma$S-induced actin polymerisation. This reason for this is not clear, but it supports the notion that eventually present, but undetectable traces of cytosolic phosphoinositides are not essential to promote actin polymerisation. This is agreement with the negative results obtained with a gelsolin-derived PtdIns(4,5)$P_2$-binding peptide and excludes the involvement of processes like PtdIns(4,5)$P_2$ synthesis as they were proposed to be of importance in permeabilized platelets downstream of Rac (Hartwig et al., 1995).

When free Mg$^{2+}$ was present, the above situation was reversed, and phosphoinositides now restored the ability of GTP$^\gamma$S to polymerise actin even in the absence of plasma membrane. The order of potency for PtdIns, PtdIns 4-P and PtdIns(4,5)$P_2$ best correlated with the previously reported capacity of the lipids to dissociate Rac/RhoGDI complexes (Chuang et al., 1993). That dissociation indeed occurred was confirmed by the increased availability of targets for toxin B-[14 C]-glucosylation (data not shown). Although hypothesetical, it is tempting to speculate that PtdIns releases a further small G protein from RhoGDI, which would, in contrast to the primary GTP$^\gamma$S target, possess a suitable GEF activity in the cytosol. As the cytosol reflects a very disorganised neutrophil which was once capable of performing specialised tasks like chemotaxis and phagocytosis, it is likely that the randomised system represents the multitude of signalling pathways necessary to concert the cell’s actions.

While this work was being prepared for submission, Zigmond and coworkers (1977) published data supporting a role for a small G protein in a cell free actin polymerisation system. Using cytosol from D. discoideum mutants lacking trimeric G protein subunits they showed in an elegant way that these are not the targets for GTP$^\gamma$S. Furthermore, they successfully induced actin polymerisation in vitro with 100 nM Cdc42 purified from Sf9 insect cells, but not from E. coli. In our study, 10 μM concentrations, exceeding 100 times the ones in cytosolic fractions for Rac and Rho and 1,000 times for Cdc42 were still found to be unsuccessful in inducing actin polymerisation.

While isoprenylation seems to be important for membrane translocation and GEF-mediated GDP/GTP exchange (Bockoch et al., 1994), non-isoprenylated Rho family proteins usually interact with and activate their downstream partners normally. When produced in E. coli, Rho bound to and stimulated the activity of protein kinase N (Watanabe et al., 1996); Rac reconstituted cell-free NADPH oxidase (Abo and Segal, 1995); and Cdc42 induced N-WASP-mediated F actin depolymerisation (Miki et al., 1998) and IQGAP1-mediated F actin crosslinking (Fukata et al., 1997). The failure of massive amounts of recombinant Rac, Rho and Cdc42 to induce actin polymerisation in a pure cytosolic system, the fact that C3 transferase, N17Cdc42, the WASP-CRIB domain (this work) and a Cdc42-binding PAK-derived peptide did not block GTP$^\gamma$S action (Zigmond et al., 1997), indicate that the endogenous small G protein might be different from the ones above.

The availability of a simple assay system for signal-induced F actin using fully fractionated material will certainly help to further elucidate the molecular mechanisms leading to actin polymerisation and allow a better definition of the cell compartments involved in the process.

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