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Tumor Necrosis Factor-α Induces Stress Fiber Formation through Ceramide Production: Role of Sphingosine Kinase

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Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that activates several signaling cascades. We determined the extent to which ceramide is a second messenger for TNF-α-induced signaling leading to cytoskeletal rearrangement in Rat2 fibroblasts. TNF-α, sphingomyelinase, or C_2-ceramide induced tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin, and stress fiber formation. Ly 294002, a phosphatidylinositol 3-kinase (PI 3-K) inhibitor, or expression of dominant/negative Ras (N17) completely blocked C_2-ceramide- and sphingomyelinase-induced tyrosine phosphorylation of FAK and paxillin and severely decreased stress fiber formation. The TNF-α effects were only partially inhibited. Dimethylsphingosine, a sphingosine kinase (SK) inhibitor, blocked stress fiber formation by TNF-α and C_2-ceramide. TNF-α, sphingomyelinase, and C_2-ceramide translocated Cdc42, Rac, and RhoA to membranes, and stimulated p21-activated protein kinase downstream of Ras-GTP, PI 3-K, and SK. Transfection with inactive RhoA inhibited the TNF-α- and C_2-ceramide-induced stress fiber formation. Our results demonstrate that stimulation by TNF-α, which increases sphingomyelinase activity and ceramide formation, activates sphingosine kinase, Rho family GTPases, focal adhesion kinase, and paxillin. This novel pathway of ceramide signaling can account for ~70% of TNF-α-induced stress fiber formation and cytoskeletal reorganization.

INTRODUCTION

Tumor necrosis factor-α (TNF-α) plays important roles in cancer, septic shock, cachexia, inflammation, autoimmunity, and wound healing (Heidecke *et al.*, 1999; Kakutani *et al.*, 1999; McCourt *et al.*, 1999; Williams *et al.*, 1999; Yazlovitskaya *et al.*, 1999). TNF-α is secreted by activated macrophages, B and T lymphocytes, and fibroblasts (Vilcek and Lee, 1991). TNF-α induces cytostatic and cytotoxic effects in tumor cell lines (Sugarman *et al.*, 1985; Obeid *et al.*, 1993). However, TNF-α also influences cell growth, differentiation and proliferation (Sugarman *et al.*, 1985; Kim *et al.*, 1991; Krasagakis *et al.*, 1995) and it stimulates liver regeneration (Rai *et al.*, 1998) and fibroblast division (Hanna *et al.*, 1999). This latter effect has implications for wound healing (Sugarman *et al.*, 1985; McCourt *et al.*, 1999), rheumatoid arthritis (Gerritsen *et al.*, 1998), neuroma formation after peripheral nerve damage (Lu *et al.*, 1997), pulmonary fibrosis (Miyazaki *et al.*, 1995), and chronic intestinal inflammatory disorders (Jobson *et al.*, 1998). The complex mechanisms by which TNF-α mediates diverse cell responses are not fully understood.

One pathway of TNF-α action is mediated through ceramide production (Hannun, 1994; Heller and Krönke, 1994; Kolesnick and Golde, 1994). Ceramides are lipid messengers that initiate apoptosis in tumor cell lines and in lymphocytes (Obeid *et al.*, 1993). Ceramides play important roles in the differentiation of HL-60 cells induced by vitamin D3 (Oka-

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Abbreviations used: BSA, bovine serum albumin; C_2-, acetyl; DMS, dimethylsphingosine; EGF, epidermal growth factor; FAK, focal adhesion kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; PAK, p21-activated protein kinase; PBS, phosphate-buffered saline; PI 3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SH, Src homology domain; SK, sphingosine kinase; sphingosine-1-phosphate, S1P; TNF-α, tumor necrosis factor-α.

zaki *et al.*, 1989), TNF-α and interferon-γ (Kim *et al.*, 1991). In contrast, ceramides stimulate cell division in quiescent Swiss 3T3 fibroblasts (Olivera *et al.*, 1992) and Rat2 fibroblasts (Hanna *et al.*, 1999). Ceramides mediate their effects by activating phosphoprotein phosphatases (Dobrowsky and Hannun, 1992), serine/threonine kinases (Liu *et al.*, 1994) that may increase Raf activity (Zhang *et al.*, 1997), and by inhibiting phospholipase D (Gómez-Muñoz *et al.*, 1994).

We showed that TNF-α and ceramides stimulate fibroblast division through activating tyrosine phosphorylation, Ras, and phosphatidylinositol 3-kinase (PI 3-K) (Hanna *et al.*, 1999). PI 3-K plays a central role in cell growth and proliferation (Roche *et al.*, 1994; Varticovski *et al.*, 1994). PI 3-K is also involved in cytoskeletal rearrangement (Wymann and Arcaro, 1994; Kotani *et al.*, 1994) and this could contribute to TNF-α-induced adhesion of leukocytes to endothelial cells and regulation of cell motility (Molony and Armstrong, 1991). Focal adhesion kinase (FAK) binds to PI 3-K (Guinebault *et al.*, 1995), which increases its activity (Chen *et al.*, 1996). The subsequent activation of small G proteins (Cdc42, Rac, and Rho) mediates actin cytoskeletal rearrangement (Wymann and Arcaro, 1994; Chant and Stowers, 1995; Nobes and Hall, 1995; Mackay and Hall, 1998). In fibroblasts, Rho, Rac, and Cdc42 regulate the formation of stress fibers, lamellipodia, and filopodia, respectively (Nobes and Hall, 1995). However, microinjection of mutants of Rho, Rac, and Cdc42 revealed that Rac and Cdc42 can also activate stress fiber formation in a Rho-dependent manner (Ridley *et al.*, 1992; Ridley and Hall, 1992; Chant and Stowers, 1995; Nobes and Hall, 1995) and that they are important for Ras transformation (Qiu *et al.*, 1997). These studies suggest the existence of a Ras-Cdc42-Rac-Rho GTPase cascade.

At present, it is not established whether TNF-α-induced cytoskeletal rearrangement is mediated by ceramide production. The present work shows that TNF-α, sphingomyelinase, and C_2-ceramide (a cell-permeable ceramide) activate Ras, PI 3-K, sphingosine kinase (SK), Cdc42, Rac, Rho, and p21-activated protein kinase (PAK) and cause the tyrosine phosphorylation of FAK and paxillin. This is the first comprehensive investigation to establish that ceramides can account for ~70% of the signaling cascade initiated by TNF-α that leads to stress fiber formation. We also demonstrated that ceramides stimulate SK activity downstream of PI 3-K activation rather than simply providing sphingosine for the reaction. SK activation is, therefore, part of the signaling pathway used by TNF-α and ceramides to increase stress fiber formation and it is compatible with the observed increase in fibroblast division.

MATERIALS AND METHODS

Materials

DMEM, penicillin, streptomycin, LipofectAMINE reagent and fetal bovine serum (FBS) were purchased from BRL Life Technologies (Burlington, ON, Canada). C_2-ceramide (*N*-acetyl-D-erythro-sphingosine) and dihydro-C_2-ceramide were obtained from BIOMOL (Plymouth Meeting, PA). Bovine serum albumin (BSA), perhexiline, desipramine, aprotinin, leupeptin, PI, sphingosine, dimethylsphingosine (DMS), and human TNF-α were purchased from Sigma Chemical (St. Louis, MO). Gö 6983 was obtained from Calbiochem (Hornby, ON, Canada). Rabbit polyclonal anti-p85α (sc-423), PAK, anti-FAK, and anti-pan Ras (sc-32) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-phosphotyrosine (05–321) was from Upstate Biotechnology (Lake Placid, NY). Thin layer chromatography plates of Silica Gel 60 were from BDH (Toronto, ON, Canada). Monoclonal anti-paxillin antibody was purchased from Transduction Laboratories (Lexington, KY) and Texas Red-X phalloidin was from Molecular Probes (Eugene, OR). [γ-^{32}P]ATP, anti-rabbit IgG linked to horseradish peroxidase and enhanced chemiluminescence kit were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, PQ, Canada). Sphingomyelinase was from ICN Biomedicals (Costa Mesa, CA). Rho cDNAs were generous gifts from Dr. Alan Hall (University College London, London, United Kingdom). The cDNAs for wild-type or inactive mutant RhoA (N19) were introduced into the *Bgl*II/*Xba*I sites of the green fluorescent protein (GFP) mammalian expression vector pEGFP-C1 (Clontech, Palo Alto, CA). Toxin B was a generous gift from Dr. G. Armstrong (University of Alberta, Alberta, AB, Canada).

Cell Culture and Preparation of Cell Membranes

The generation and characterization of Rat2 fibroblasts and fibroblasts stably expressing dominant-negative N17 H-Ras were described previously (Topp, 1981; Warner *et al.*, 1993). N17 H-Ras is preferentially GDP-bound and is thought to inhibit Ras guanine-nucleotide exchange factors, thereby preventing activation of endogenous Ras (Feig and Cooper, 1988). The levels of N17 Ras expression and the growth rates of the fibroblasts have been described (Hanna *et al.*, 1999). Fibroblasts were cultured until confluent in 10-cm dishes in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg of streptomycin/ml in a humidified atmosphere of 5% CO_2, 95% air at 37°C (Martin *et al.*, 1993). The medium for cells expressing cDNA for N17 Ras or empty vector was supplemented with 2.5 μg of puromycin/ml. Fibroblasts were then cultured overnight in DMEM containing 15 μM lipid-free BSA followed by the addition of C_2-ceramide, dihydro-C_2-ceramide, TNF-α, or sphingomyelinase, as indicated. Agonist concentrations used to produce cell responses were established from previous work (Hanna *et al.*, 1999). Ceramide and dihydro-C_2-ceramide were dissolved in dimethyl sulfoxide and the final concentration of dimethyl sulfoxide was 0.08%. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested by centrifugation, and resuspended in buffer A (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride). Cells were sonicated twice for 10 s and then centrifuged for 5 min at 800 × *g*. After discarding nuclei and unbroken cells, membrane and cytosolic fractions were prepared by centrifugation at 250,000 × *g* for 60 min. Membranes were washed and resuspended in buffer A.

Immunoprecipitation and Immunoblotting

To decrease nonspecific immunoprecipitation, cell lysates were preincubated with 40 μl of a 50% dilution of protein A-Sepharose in PBS for 2 h at 4°C. Samples were then centrifuged and supernatants were used for immunoprecipitation. FAK, paxillin, PI 3-K, and PAK were immunoprecipitated from cell lysates (300 μg of protein) by adding 5 μg of anti-phosphotyrosine antibodies for FAK and paxillin, or 2 μg of anti-p85α antibodies for PI 3-K or 3 μg of anti-PAK antibodies and incubating for 6 h at 4°C followed by adding 40 μl of a 50% dilution of protein A-Sepharose in PBS. The mixtures were incubated overnight at 4°C and immunoprecipitates were analyzed by SDS-PAGE (Hanna *et al.*, 1999).

Measurement of Protein Concentration, PAK, PI 3-K, and SK Activity

Protein concentrations were measured by the Bradford (Bradford, 1976) or bicinchoninic acid methods with the use of BSA as a standard. PI 3-K activity was measured after immunoprecipitation with anti-p85 antibody by determining the phosphorylation of PI

(Hanna et al., 1999). PAK activity was determined after immunoprecipitation with anti-PAK antibody by determining the phosphorylation of myelin basic protein (Yang *et al.*, 1998). SK activity was measured as described (Olivera *et al.*, 1999) with some modifications. Briefly, cell lysates (75 μg of protein) were incubated with sphingosine (50 μM) in the presence of 10 μCi [^{32}P]ATP for 30 min at 37°C. Sphingosine 1-[^{32}P]phosphate (S1P) was then extracted into water-saturated butan-1-ol. The butanol phase was washed three times with 2 M KCl and the remaining ^{32}P was determined by scintillation counting. The reaction depended absolutely on the addition of sphingosine and the labeled product was shown to be entirely S1P after chromatography on silica gel plates with the use of butanol/acetic acid/H_2O (3:1:1, v/v/v) for development.

Fluorescent Labeling of Filamentous Actin

Rat2 cells were cultured on glass coverslips in a 35-mm dish until confluent. Cells were then maintained in serum-free medium containing 0.1 mg/ml BSA for 24 h and then incubated with agonists. At the indicated times, cells were washed twice with PBS and fixed in 3.7% formaldehyde in PBS for 20 min at room temperature. Fixed cells were permeabilized by incubation with 0.2% Triton X-100 in PBS for 15 min and then blocked with 0.1% casein-PBS for 30 min. Filamentous actin was visualized with the use of Texas Red-conjugated phalloidin for 1 h. Coverslips were mounted on standard microscope slides in antifade medium containing *n*-propylgallate and glycerol to prevent photobleaching. Stress fibers were viewed on a Reichect Polyvar 2 microscope with the use of a 100× (numerical aperture 1.32) objective and were photographed on Kodak 400 plusX film. Stress fibers in Rat2 fibroblasts, transfected with inactive mutant (N19) or wild-type RhoA, were viewed with a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany). We used HeNe (543 nm) and Argon (488 nm) with an HFT 488/543 beam splitter.

Transient Transfection with Wild-Type and Mutant RhoA

Transient transfections with wild-type and mutant RhoA were performed with the use of LipofectAMINE reagent according to instructions from BRL Life Technologies. The GFP cDNA was fused to the N terminus of wild-type or the inactive mutant (N19) of RhoA. The chimeric cDNAs (1 μg) in pEGFP vectors were mixed with LipofectAMINE at room temperature for 60 min and then incubated overnight with Rat2 fibroblasts in serum-free DMEM. Fluorescence microscopy was used to visualize the green fluorescence of EGFP in fixed cells and to estimate the levels of transfection by RhoA wild-type or inactive mutant (N19). Expression and localization of EGFP-RhoA proteins were visualized by monitoring the green fluorescence either directly or with the use of anti-GFP antibodies and a secondary antibody conjugated to fluorescein isothiocyanate. There was no significant difference in the conclusions for the experiments between the two methods for visualizing GFP and for cells transfected with different levels of the wild-type or mutant RhoA. Stress fibers were visualized in the transfected cells and nontransfected cells on the same microscopic field.

RESULTS

TNF-α, Ceramide, and Sphingomyelinase Stimulate Tyrosine Phosphorylation of FAK and Paxillin and Formation of Stress Fibers

Treatment of Rat2 fibroblasts with 10 ng/ml TNF-α for 1 h increased stress fiber formation as indicated by increased phalloidin staining (Figure 1A). This effect was mimicked by treatment with 0.1 unit/ml sphingomyelinase or 40 μM C_2-ceramide. To define the signaling pathways involved, the tyrosine phosphorylations of FAK and paxillin were determined. Treatment of Rat2 fibroblasts with TNF-α, sphingomyelinase, or ceramide increased the tyrosine phosphorylation of paxillin and FAK by ~2.5–3.5-fold after 20–30 min (Figure 1, B and C).

Role of PI 3-K in TNF-α-, Sphingomyelinase-, and C_2-Ceramide-induced Stress Fiber Formation and Tyrosine Phosphorylation of FAK and Paxillin

PI 3-K is implicated in cytoskeletal rearrangement (Kotani *et al.*, 1994; Wymann and Arcaro, 1994; Nobes *et al.*, 1995), and we showed that TNF-α, sphingomyelinase, and C_2-ceramide stimulate PI 3-K activity in Rat2 fibroblasts (Hanna *et al.*, 1999). We, therefore, examined the role of PI 3-K in TNF-α-, sphingomyelinase-, and ceramide-induced stress fiber formation. Pretreatment of Rat2 fibroblasts with 20 μM Ly 294002 for 1 h partially blocked stress fiber formation by TNF-α (Figures 1A vs. 2A). However, Ly 294002 almost completely blocked the stimulation of stress fiber formation by C_2-ceramide and sphingomyelinase (Figure 1A vs. 2A). To investigate these differences further, we measured the tyrosine phosphorylations of FAK and paxillin. In agreement with the results in Figure 2A, pretreatment with Ly 294002 inhibited ~95% of the tyrosine phosphorylation of paxillin that was induced by C_2-ceramide and sphingomyelinase. Ly 294002 only blocked ~60% of the TNF-α-induced tyrosine phosphorylation (Figure 2B). Similar results were also obtained for the tyrosine phosphorylation of FAK (our unpublished results). The ceramide effect was specific because there was no significant increase in the levels of paxillin in anti-phosphotyrosine precipitates when cells were incubated with the relatively inactive ceramide analog, dihydro-C_2-ceramide (Figure 2B). Dihydro-C_2-ceramide (40 μM) was also ineffective at stimulating stress fiber formation (our unpublished results). Other studies showed that the tyrosine phosphorylation of FAK increases its association with PI 3-K leading to PI 3-K activation (Guinebault *et al.*, 1995; Chen *et al.*, 1996). Therefore, we tested whether TNF-α would have this effect and whether ceramide signaling could be involved. Treatment of cells with C_2-ceramide or TNF-α (Figure 3, A and B) increased the coimmunoprecipitation of FAK with PI 3-K in a time-dependent manner. As a control, we showed that the amount of PI 3-K in the immunoprecipitates was not affected significantly by the treatments with C_2-ceramide or TNF-α. Treatment of Rat2 fibroblasts with 10 ng/ml TNF-α, or 40 μM C_2-ceramide also increased the PI 3-K activity that coprecipitated with FAK by ~3-fold at 20 min (Figure 3C). Figure 3D shows that the TNF-α-induced stimulation of PI 3-K was accompanied by increased PI 3-K in anti-FAK immunoprecipitates. In control experiments, no PI 3-K activity was associated with beads in the absence of anti-FAK antibody (our unpublished results).

Sphingosine Kinase and Tyrosine Phosphorylation of FAK and Paxillin and Cytoskeleton Reorganization by TNF-α and Ceramide

SK activity can be increased by TNF-α (Xia *et al.*, 1998) and therefore we investigated whether ceramides could also mediate this action and thus increase stress fiber formation. Treatment of fibroblasts with TNF-α or C_2-ceramide increased SK activity by two- to threefold (Figure 4A). These effects were partially blocked by Ly 294002 and in fibroblasts

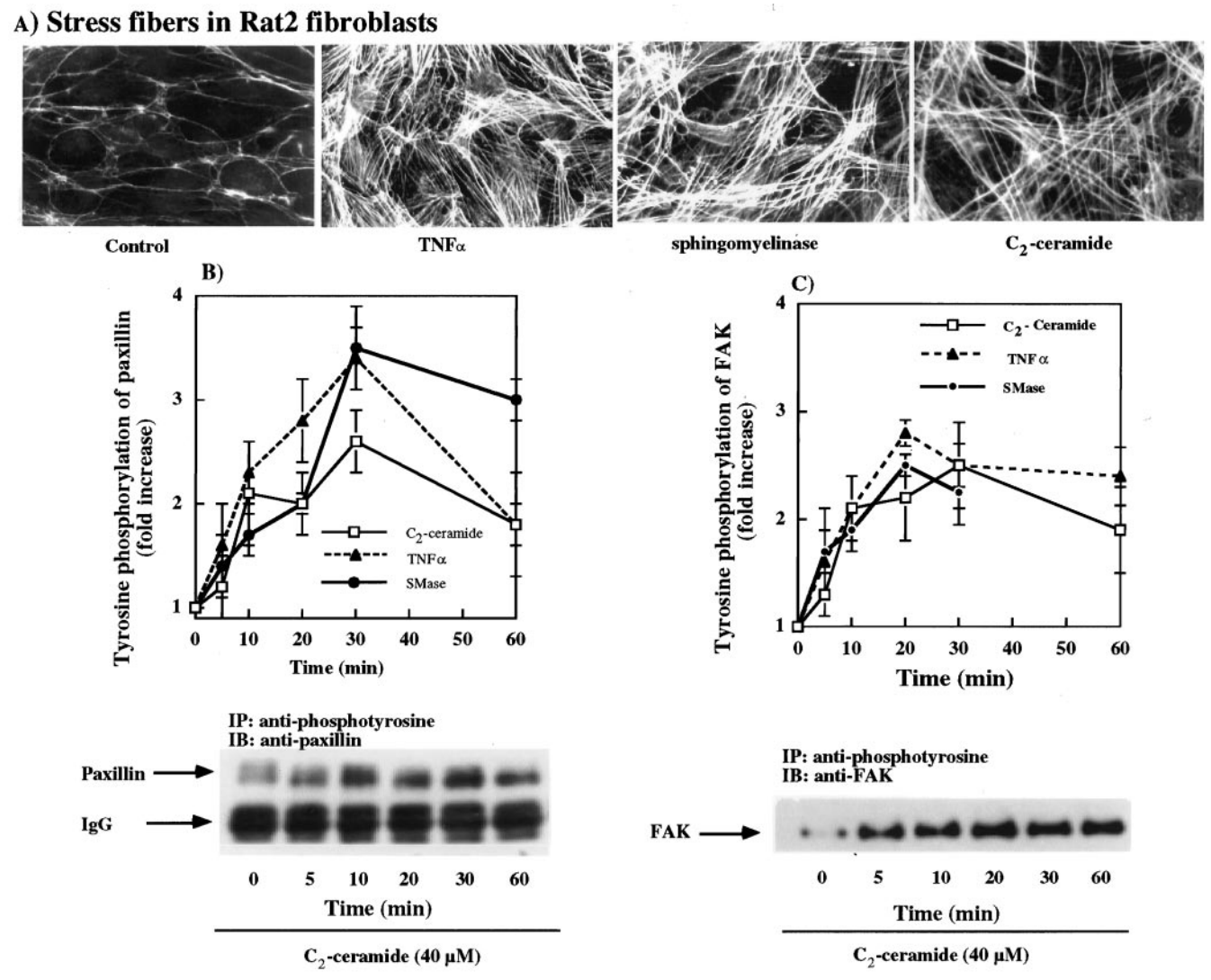

Figure 1. Stress fiber formation by TNF-α, sphingomyelinase, and C_2-ceramide. (A) Fibroblasts treated in the absence or presence of 10 ng/ml TNF-α, 0.1 unit/ml sphingomyelinase, or 40 μM C_2-ceramide for 1 h. Cells were then fixed, permeabilized, and stress fibers were visualized with the use of Texas Red-conjugated phalloidin. The results were reproduced in two further independent experiments. (B and C) Tyrosine phosphorylation of paxillin and FAK, respectively, is shown for Rat2 fibroblasts treated with 40 μM C_2-ceramide, 10 ng/ml TNF-α, or 0.1 unit/ml sphingomyelinase for various times. Results are means ± SEM of three independent experiments. The lower parts of B and C show representative Western blots for C_2-ceramide-induced tyrosine phosphorylation of paxillin and FAK.

expressing dominant/negative (N17) Ras. The involvement of SK activation in stress fiber formation was investigated by with the use of DMS to inhibit its activity. DMS blocked the effects of TNF-α and C_2-ceramide in stimulating the tyrosine phosphorylation of FAK and stress fiber formation (Figure 4, B and C), but the DMS-treated cells showed prominent cortical actin. To exclude the possibility that the effect of DMS resulted from an inhibition of protein kinase C (PKC) we also tested the effects of 20 μM sphingosine, which inhibits PKC (Hannun *et al.*, 1986) and 100 nM Gö 6983, which is a broad specificity protein kinase C inhibitor. Neither sphingosine nor 100 nM Gö 6983 inhibited the tyrosine phosphorylation of FAK and paxillin (our unpublished results). Also, Gö 6983 did not inhibit stress fiber formation in response to TNF-α and C_2-ceramide (our unpublished results). Sphingosine alone increased stress fiber formation through production of S1P because this effect was blocked by DMS.

Role of Rho Family G Proteins in Cytoskeleton Reorganization Produced by TNF-α, Sphingomyelinase, and Ceramide

Rho family G proteins play an important role in cytoskeletal organization (Ridley et al., 1992; Chant and Stowers, 1995; Nobes and Hall, 1995). Therefore, we investigated whether TNF-α and C_2-ceramide activate Cdc42, Rac, and RhoA to induce stress fiber formation. C_2-ceramide (Figure 5A) and

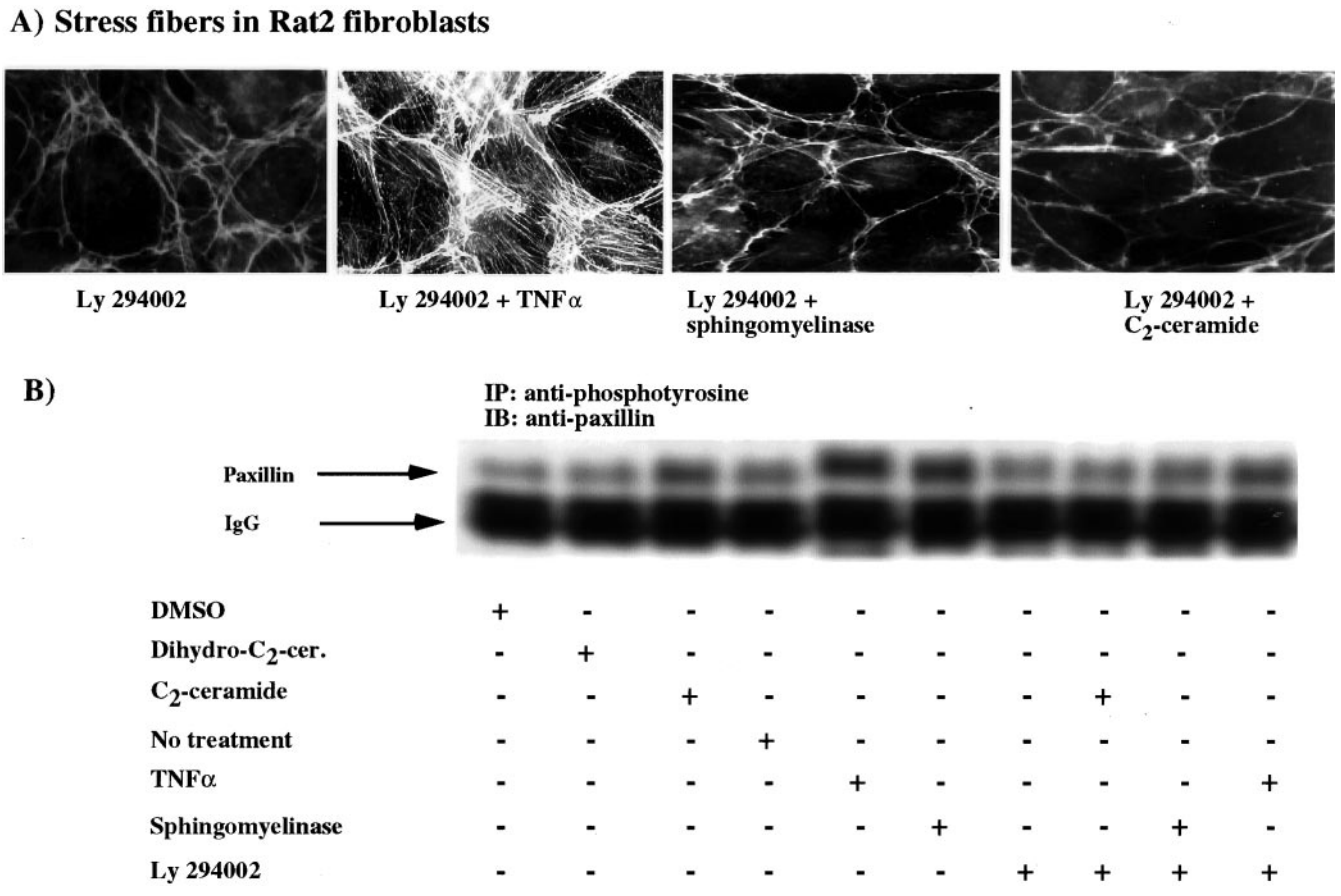

Figure 2. Involvement of PI 3-K in TNF-α-, sphingomyelinase-, and C_2-ceramide-induced stress fiber formation. (A) Rat2 fibroblasts were pretreated for 60 min with 20 μM Ly 294002. Cells were then treated with or without 10 ng/ml TNF-α, 0.1 unit/ml sphingomyelinase, or 40 μM C_2-ceramide for 60 min. Stress fibers were visualized with the use of Texas Red-conjugated phalloidin. These results can be compared directly to those shown in Figure 1A. (B) Tyrosine phosphorylation of paxillin after treatment of Rat2 fibroblasts with or without 20 μM Ly 294002 followed by adding 10 ng/ml TNF-α, 0.1 unit/ml sphingomyelinase, 40 μM C_2-ceramide, or 40 μM dihydro-C_2-ceramide for 30 min. Tyrosine phosphorylation of paxillin was assessed by with the use of anti-phosphotyrosine for immunoprecipitation and anti-paxillin for Western blotting. The results were reproduced in two further independent experiments.

TNF-α (our unpublished results) stimulated the translocation of Cdc42, Rac, and RhoA from cytosol to membranes in a time-dependent manner, and the effects on Rho were blocked by Ly 294002 (our unpublished results). Activated Cdc42 binds to PAK (Ottilie *et al.*, 1995) and coprecipitation of Cdc42 with PAK can be used as an indirect indication of Cdc42 activation. We established the effect of TNF-α and C_2-ceramide on Cdc42 activation by demonstrating that these agonists induced the physical association of Cdc42 with PAK (Figure 5B). Ly 294002 blocked these effects. C_2-ceramide and TNF-α increased PAK activity by ~2.3- and 2.1-fold, respectively, and this effect was inhibited by DMS, Ly 294002, or expression of N17 Ras (Figure 5C).

The role of Rho family proteins in TNF-α- and ceramide-induced stress fiber formation was also demonstrated with the use of toxin B from *Clostridium difficile*, which glucosylates Rho family proteins, thereby causing their inactivation (Just *et al.*, 1995). Toxin B strongly inhibited C_2-ceramide-induced tyrosine phosphorylation of FAK and paxillin (Figure 5, D and E). Pretreatment of fibroblasts with toxin B resulted in rounding of cells and blocked the C_2-ceramide-induced stress fiber formation (Figure 5F) and the association of FAK with PI 3-K (our unpublished results). Similar results to those seen with C_2-ceramide were obtained with the use of 10 ng/ml TNF-α (our unpublished results).

To establish further the role of RhoA in TNF-α- and C_2-ceramide-induced cytoskeletal rearrangement, we transiently transfected Rat2 fibroblasts with wild-type RhoA or inactive RhoA (N19), both with GFP attached at their N termini. Cells were then treated with TNF-α or C_2-ceramide for 15 min or 1 h. Cells transfected with GFP-tagged N19 RhoA or wild-type RhoA were identified by fluorescence microscopy. Nontransfected cells in the same microscopic field were used as internal controls. Treatment for 15 min with C_2-ceramide (Figure 6) or TNF-α (our unpublished results) increased cortical actin in cells that overexpressed wild-type, or N19 RhoA as assessed with Texas Red-conjugated phalloidin and its colocalization with the fluorescence of GFP. Incubation of Rat2 fibroblasts for 15 min with TNF-α or C_2-ceramide produced relatively little stress fiber formation.

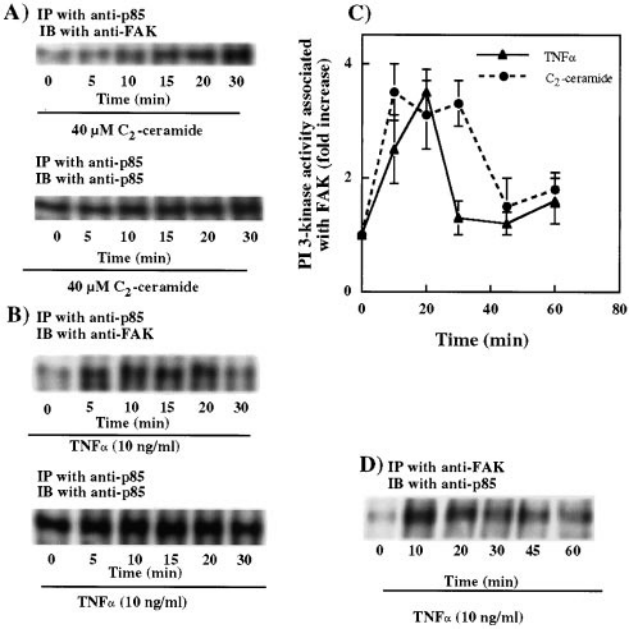

Figure 3. TNF-α and C_2-ceramide induce physical association between PI 3-kinase and FAK. (A and B) Physical association of FAK (A) with PI 3-K (B) after treatment of Rat2 fibroblasts with 40 μM C_2-ceramide or 10 ng/ml TNF-α for various times. The lower blots show that similar amounts of PI 3-K were used to measure the association of FAK to PI 3-K. The results were reproduced in two further independent experiments. (C) PI 3-K activity associated with the anti-FAK precipitate after treatment of Rat2 fibroblasts with 10 ng/ml TNF-α or 40 μM C_2-ceramide for various times. In a control experiment, no PI 3-K activity was detected in beads without anti-FAK antibody. Results are means ± SEM for three independent experiments. The blot in D is a representative Western blot to show the effect of TNF-α treatment on the detection of PI 3-K in anti-FAK immunoprecipitates.

We, therefore, treated the cells with C_2-ceramide for 1 h to induced stress fiber formation as in Figure 1. Treatment of cells transfected with wild-type RhoA-GFP with C_2-ceramide produced stress fibers as visualized with Texas Red-conjugated phalloidin and its colocalization with the green fluorescence of GFP (Figure 6). However, C_2-ceramide did not stimulate stress fiber formation in cells transfected with inactive N19 RhoA-GFP. As a control, C_2-ceramide did induced stress fiber formation in neighboring fibroblasts that were not transfected with the inactive RhoA mutant (Figure 6). Similar results (our unpublished results) were obtained by with the use of TNF-α. Treatment with C_2-ceramide for 1 h caused rounding and apparent retraction of fibroblasts transfected with N19 RhoA (Figure 6). Prolonging the incubation to 3 h lead to the detachment of the cells containing N19 RhoA from the monolayer (our unpublished results).

Role of Ras in Cytoskeleton Reorganization by TNF-α, Sphingomyelinase, and Ceramide

There is indirect evidence implicating Ras in cytoskeletal rearrangement (Rodriguez-Viciana *et al.*, 1997). We showed previously that TNF-α, sphingomyelinase, and C_2-ceramide increase Ras-GTP concentrations in Rat2 fibroblasts (Hanna *et al.*, 1999). Therefore, we tested whether Ras is involved in cytoskeletal rearrangement caused by TNF-α, sphingomyelinase, and ceramide. Expression of dominant/negative Ras (N17) in fibroblasts almost completely blocked stress fiber formation by sphingomyelinase and C_2-ceramide (Figures 1A vs. 7A). In contrast, N17 Ras expression appeared to inhibit stress fiber formation by TNF-α only partially. To assess this effect further, we measured the tyrosine phosphorylation of FAK and paxillin. Expression of N17 Ras caused ~90–100% inhibition of tyrosine phosphorylation of FAK and paxillin by C_2-ceramide and sphingomyelinase. In contrast, the TNF-α effect was inhibited by an average of 66% for paxillin and 89% for FAK (Figure 7, B and C).

DISCUSSION

The present study established that ceramide signaling mediates ~70% of the cytoskeletal rearrangement produced by TNF-α. We also provided the novel observation that ceramides activate SK and this is involved in stress fiber formation (Figure 8).

Inhibition of PI 3-K with Ly 294002 completely blocked stress fiber formation by sphingomyelinase and C_2-ceramide. In contrast, Ly 294002 only partially inhibited the TNF-α-induced stress fiber formation and tyrosine phosphorylation of FAK and paxillin, implying that TNF-α also uses PI 3-K-independent pathway(s) for inducing stress fiber formation. This conclusion is supported because Ly 294002 had no significant inhibitory effect on TNF-α-induced activation of PAK or FAK and paxillin phosphorylation in fibroblast expressing N17 Ras (our unpublished results). The ceramide effects were specific because dihydro-C_2-ceramide did not stimulate PI 3-K (Hanna *et al.*, 1999), tyrosine phosphorylation of paxillin (Figure 2), and stress fiber formation (our unpublished results). Phosphorylation of FAK on Tyr397 induces binding to PI 3-K through Src homology 2 domains of p85 (Chen *et al.*, 1996) and increases PI 3-K activity (Sonoda *et al.*, 1999). This implies that FAK is upstream of PI 3-K. However, activation of PI 3-K causes tyrosine phosphorylation of FAK and cytoskeletal rearrangement (Kotani *et al.*, 1994; Nobes *et al.*, 1995), implying that FAK is also downstream of PI 3-K.

TNF-α also increased SK activation and this was partially blocked by Ly 294002 or expression of N17 Ras. In contrast, the effect of ceramide was almost completely blocked (Figure 4A). SK is, therefore, downstream of Ras and PI 3-kinase (Figure 8). SK is upstream of PAK activation, the tyrosine phosphorylations of FAK and paxillin, and stress fiber formation because DMS, an SK inhibitor, partially blocked the effects of TNF-α and C_2-ceramide on these responses. TNF-α through activation of sphingomyelinase could also increase sphingosine production and thus also provide the substrate for SK. However, C_2-ceramide is not metabolized to sphingosine by Rat2 fibroblasts to a significant extent compared with long-chain ceramides (Hanna *et al.*, 1999). Stimulation of SK by TNF-α was also reported in endothelial cells and this increased the expression of adhesion molecules (Xia *et al.*, 1998). Generation of internal S1P prevents ceramide-induced apoptosis and provides survival and proliferative signals that are also used by platelet derived- and nerve growth factors (Wang *et al.*, 1997). Activation of SK in Rat2

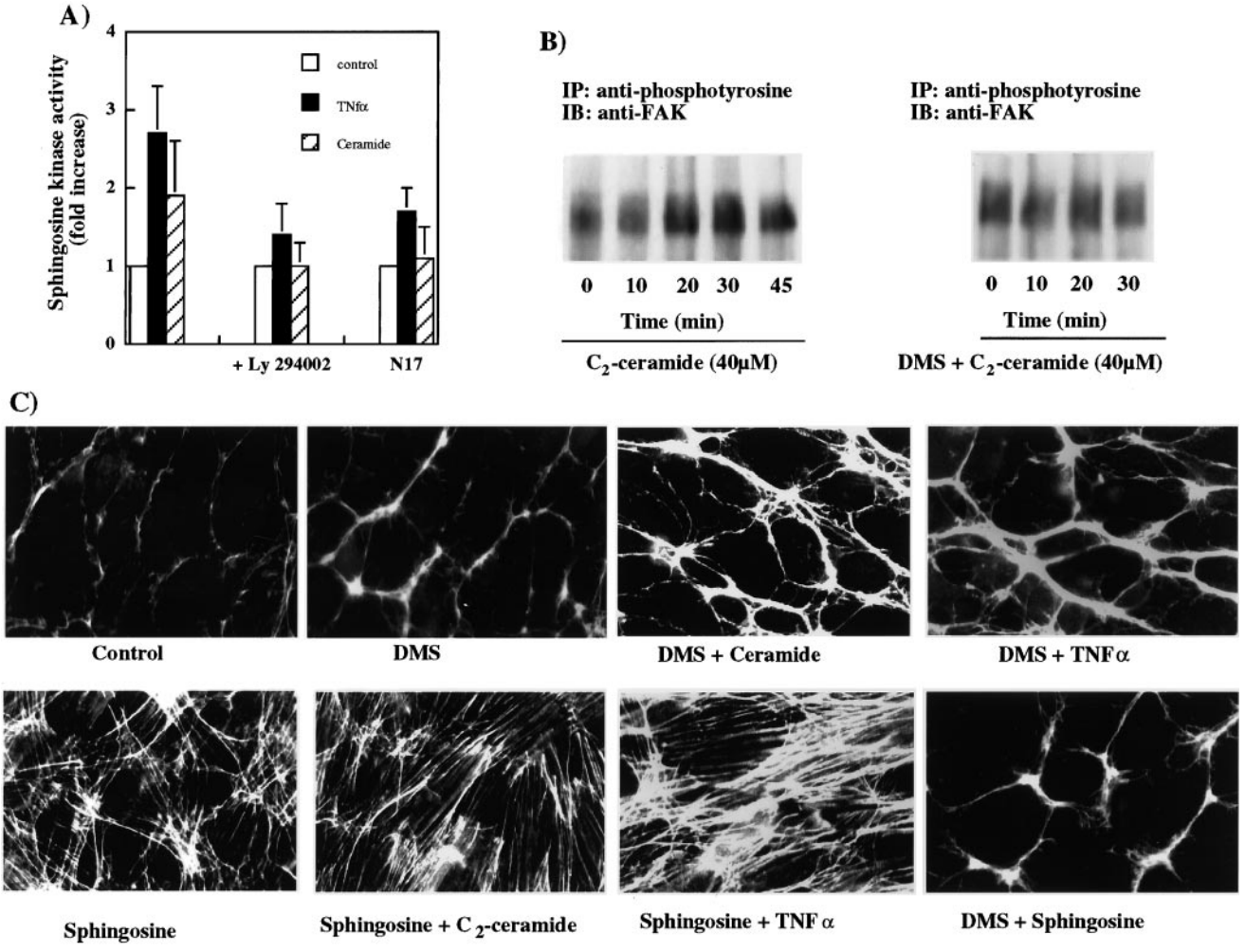

Figure 4. TNF-α and C_2-ceramide treatment increases sphingosine kinase activity that is upstream of the tyrosine phosphorylation of FAK and stress fiber formation. (A) Activation of sphingosine kinase by 40 μM C_2-ceramide or 10 ng/ml TNF-α is attenuated by inhibiting PI 3-kinase with Ly 294002, or in fibroblasts expressing N17 Ras. Results are means ± SEM for three independent experiments. (B) Effects of treating Rat2 fibroblasts with DMS in inhibiting the effects of TNF-α and C_2-ceramide in stimulating the tyrosine phosphorylation of FAK. (C) Distribution of stress fibers in Rat2 fibroblasts that were pretreated with 10 μM DMS and then treated with 10 ng/ml TNF-α, 40 μM C_2-ceramide, or 20 μM sphingosine.

fibroblasts is compatible with the TNF-α and ceramide effects in stimulating cell division rather than apoptosis (Hanna *et al.*, 1999). Exogenous S1P can also stimulate cell division and induce stress fiber formation (Wang *et al.*, 1997). Most extracellular effects of S1P are mediated through activation of cell surface endothelial differentiation gene receptors. It was recently proposed that internally generated S1P might be secreted and thereby provide an autocrine/paracrine signal through stimulation of endothelial differentiation gene receptors (Hobson *et al.*, 2001).

We investigated whether C_2-ceramide might increase diacylglycerol production by acting as a substrate for phosphatidylcholine:ceramide phosphocholine transferase thereby stimulating stress fiber formation through protein kinase C. However, 100 nM Gö 6983, a broad specificity PKC inhibitor, had no significant effect on PI 3-K activation and stress fiber formation (our unpublished results). Sphingosine, which also inhibits PKC activity (Hannun *et al.*, 1986), in fact, increased stress fiber formation through increased S1P production.

Activation of Rac, Cdc42 and Rho induces cytoskeletal rearrangement in fibroblasts, leading to the formation of lamellipodia, filopodia, and stress fibers, respectively (Nobes and Hall, 1995). These Rho family proteins are involved in cytoskeletal rearrangement induced by TNF-α and C_2-ceramide because toxin B blocked their effects on paxillin phosphorylation and stress fiber formation. Second, expression of dominant/negative RhoA blocked TNF-α- and C_2-ceramide-induced stress fiber formation. Third, TNF-α and C_2-ceramide induced the translocation of Rho, Cdc42, and Rac-1 to membranes, association of Cdc42 with PAK, and increased PAK activity. TNF-α and C_2-ceramide induce the formation of cortical actin after 15 min in fibroblasts transfected with wild or mutant RhoA. Stress fiber formation at

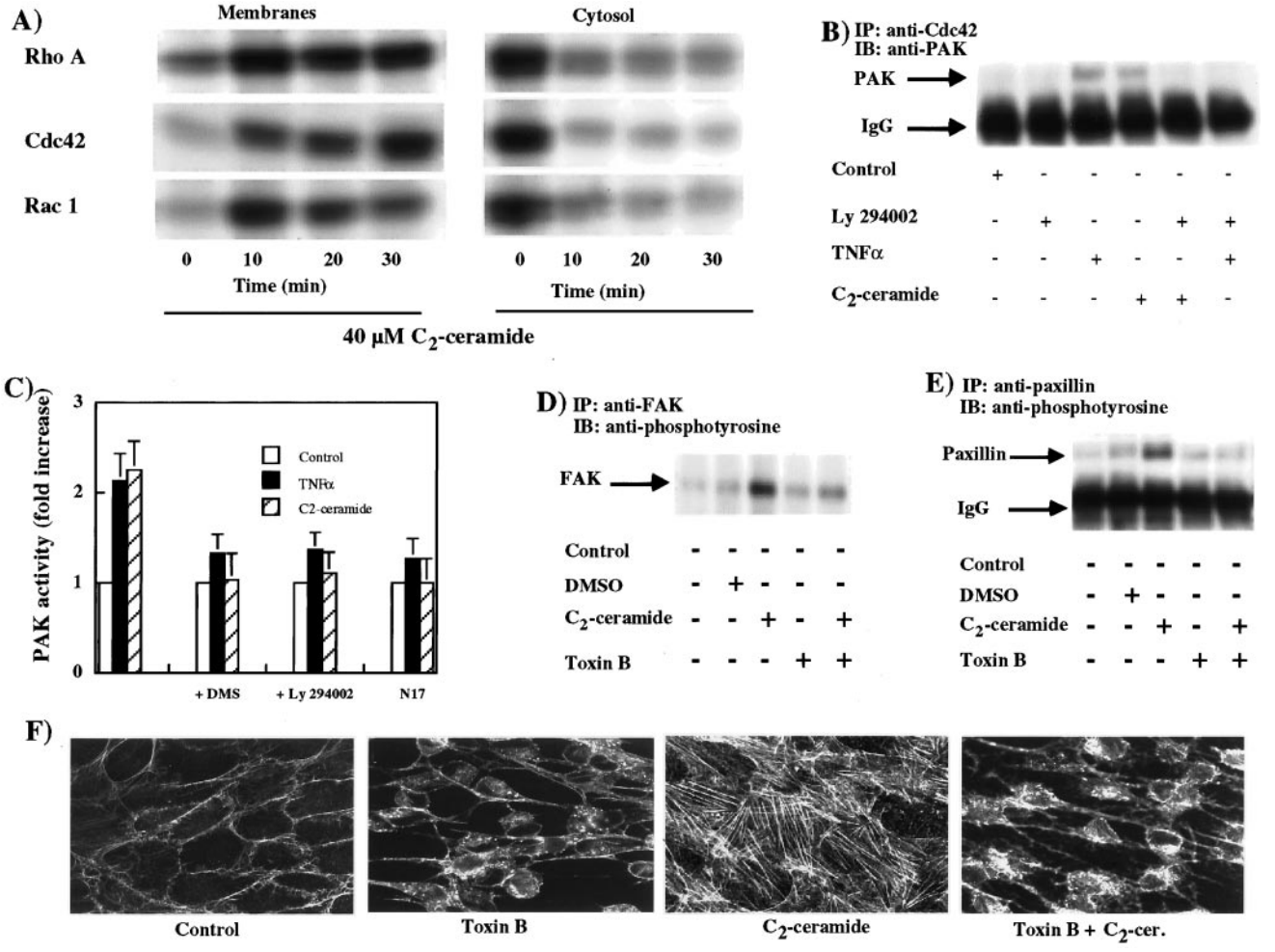

Figure 5. TNF-α and C_2-ceramide treatment increases membrane association of Rho family GTPases, association of Cdc42 with PAK, and phosphorylation of FAK and paxillin. (A) Time courses for the translocations of Cdc42, Rac-1, and RhoA to membranes after treatment with 40 μM C_2-ceramide. The results were reproduced in three independent experiments. (B) Physical association of Cdc42 with PAK after treatment of Rat2 fibroblasts with 10 ng/ml TNF-α or 40 μM C_2-ceramide for various times in absence or presence of PI 3-kinase inhibitor, 20 μM Ly 294002. (C) PAK activation after treatment of fibroblasts with TNF-α and C_2-ceramide for 30 min in the presence or absence of DMS, Ly 294002, or N17 Ras. Results are means ± ranges from two independent experiments. (D and E) Rat2 fibroblasts were treated with or without 0.5 ng/ml toxin B for 2 h in the presence or absence of 40 μM C_2-ceramide. The tyrosine phosphorylations of FAK and paxillin were measured with the use of either anti-FAK or anti-paxillin for precipitation and anti-phosphotyrosine for immunoblotting. The blots in this figure were reproduced in a further experiment. (F) Effect of toxin B on ceramide-induced stress fiber formation in Rat2 fibroblasts.

1 h was blocked in cells expressing N19 Rho. We have not yet established the hierachical activation of Rho family G proteins by ceramides, but our results are compatible with the activation of Cdc42 and Rac being upstream of RhoA as described for TNF-α (Puls *et al.*, 1999). Furthermore, Kim *et al.* (1999) showed that TNF-α causes sequential activation of PI 3-K and Rac. We showed that TNF-α activates Rho family G proteins partly through ceramides production, activation of Ras (unpublished results), and PI 3-K.

Wang and Bitar (1998) showed translocation of RhoA to membranes in colonic smooth muscles treated with ceramides for 30 s to 4 min and concluded that RhoA translocation was upstream of PKC and $pp60^{src}$. Our results demonstrate that the ceramide effect on Rho in Rat2 fibroblasts is downstream of Ras, PI 3-K, and SK (Figure 8). This observation is compatible with work by Kim and Kim (1998) who showed that ceramides stimulate Rac-dependent activation of phospholipase A_2 and the *c-fos* serum response element in Rat2 fibroblasts. These authors did not elucidate the signaling pathways upstream or downstream of Rac. Ceramides also induce Rac1-dependent apoptosis after 48 h in NIH 3T3 cells (Embade *et al.*, 2000). However, these long-term effects depended upon protein synthesis and therefore differed from the present studies.

We showed that ceramides blocked the translocations of RhoA, ARF, and Cdc42 to membranes in HL60 cells treated with *N*-formylmethionylleucylphenylalanine and thus phospholipase D1 activation (Abousalham *et al.*, 1997). The present work demonstrates that ceramides on their own may activate small G proteins, although they can interfere

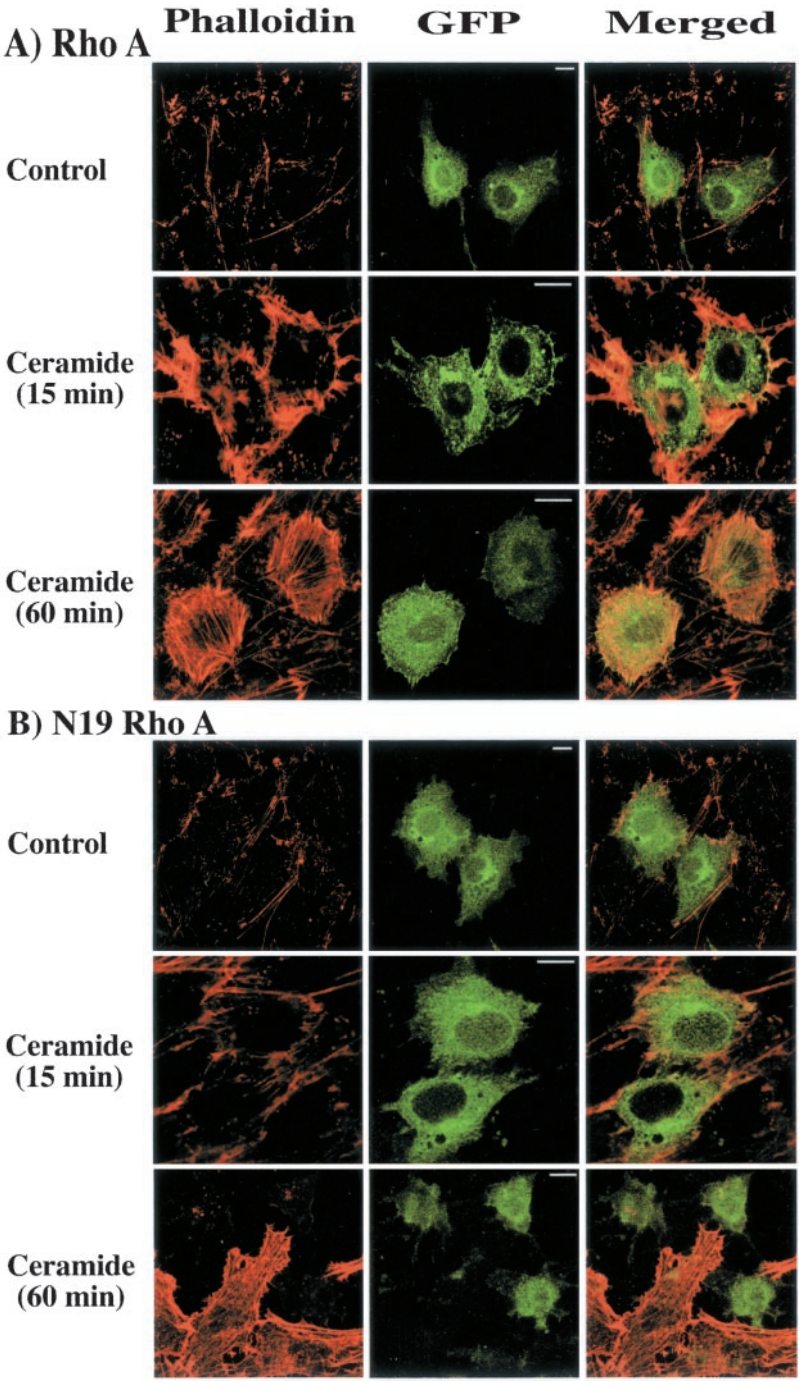

Figure 6. Role of RhoA in C_2-ceramide-induced stress fiber formation. This figure shows cytoskeletal rearrangement in fibroblasts transiently transfected with wild-type RhoA or inactive N19 RhoA, both attached to GFP, in the presence of 40 μM C_2-ceramide for 15 min or for 1 h. Transfected cells were identified by the green fluorescence of GFP. Actin stress fibers were labeled with the use of Texas Red-conjugated phalloidin and colocalization with GFP is shown in yellow in the merged images. The size bar in the central panels represents 10 nm. The controls are from untreated cells at 15 min and the images were not significantly different for untreated cells incubated for 60 min (our unpublished results). The results were reproduced in three independent experiments and similar results were obtained with 10 ng/ml TNF-α (our unpublished results).

with G protein activation through another agonist. For example, ceramides block insulin-stimulated glucose uptake in 3T3 L1 adipocytes (Wang *et al.*, 1998) and L6 myocytes (Hajduch *et al.*, 2001). These effects are downstream of PI 3-K and involve inhibition of protein kinase B (Akt). However, in the absence of insulin, ceramides stimulate glucose uptake through increased PI 3-K activity and increased synthesis of GLUT1 (Wang *et al.*, 1998).

Our work shows that sphingomyelinase and C_2-ceramide stimulate cytoskeletal changes through Ras and PI 3-K. However, TNF-α increases stress fibers by additional signaling mechanisms because the tyrosine phosphorylations of FAK and paxillin were only decreased by 89 and 66%, respectively, in fibroblasts expressing N17 Ras and the equivalent inhibitions by Ly 294002 were each ~60%. SK activation by TNF-α was decreased by ~35% in fibroblasts

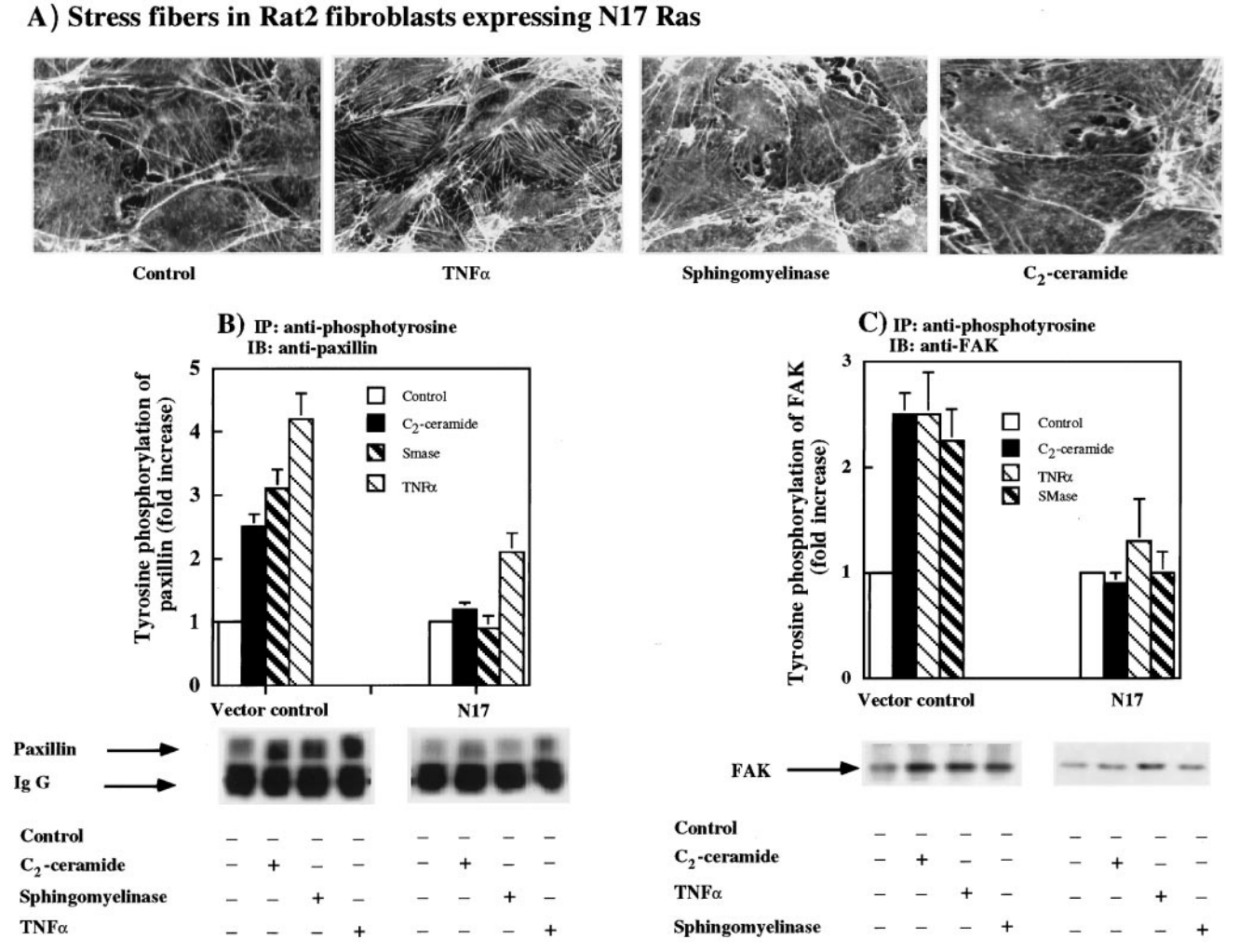

Figure 7. Role of Ras-GTP in TNF-α-, sphingomyelinase- and C_2-ceramide-induced stress fiber formation and tyrosine phosphorylation of paxillin and FAK. Rat2 fibroblasts stably expressing N17 Ras were treated with or without 10 ng/ml TNF-α, 0.1 unit/ml sphingomyelinase or 40 μM C_2-ceramide for 1 h and stress fibers were visualized. The results were reproduced in two further independent experiments. These images can be compared directly with the results for Rat2 fibroblasts shown in Figure 1A, or to those for fibroblasts transduced with the empty vector (our unpublished results). (B and C) Expression of N17 Ras inhibits the TNF-α-, sphingomyelinase-, and C_2-ceramide-induced tyrosine phosphorylation of paxillin and FAK compared with fibroblasts transduced with the empty vector. Results are means ± SEM for three independent experiments.

expressing N17 Ras and by ~48% by Ly 294002. Also, TNF-α-induced activation of PI 3-K was only inhibited by ~70% in fibroblasts expressing N17 Ras (Hanna *et al.*, 1999). In contrast, the effects of sphingomyelinase and C_2-ceramide on PI 3-K, SK, FAK, paxillin, and stress fibers were almost completely abolished by N17 Ras, or treatment with Ly 294002. These combined results show that ceramide is responsible for ~60–70% of the TNF-α-induced stress fiber formation. Pretreatment of Rat2 fibroblasts with desipramine and perhexiline to inhibit sphingomyelinase (Albouz *et al.*, 1981; Harada-Shiba *et al.*, 1998) resulted in complete inhibition of TNF-α-induced activation of PAK and tyrosine phosphorylation of FAK (our unpublished results). This implies that the TNF-α effects are dependent on ceramide accumulation.

The present work was designed to elucidate signaling pathways by which TNF-α induces cytoskeletal rearrangement (Figure 8). We demonstrated the role of ceramide formation in this process with the use of a cell-permeable ceramide and sphingomyelinase. Ceramide formation in fibroblasts stimulates a tyrosine kinase activity (Hanna *et al.*, 1999) such as $pp60^{c\text{-}src}$ (Su *et al.*, 1999), resulting in Ras-GTP formation (Hanna *et al.*, 1999). Treating Rat2 fibroblasts with TNF-α, sphingomyelinase, or ceramide causes PI 3-K to interact physically with Ras-GTP (Hanna *et al.*, 1999) and phosphorylated FAK (Figure 3C), resulting in a synergistic activation of PI 3-K (Rodriguez-Viciana *et al.*, 1996). TNF-α-induced formation of Ras-GTP and activation of PI 3-K then causes SK activation, an effect also mimicked by C_2-ceramide. SK activation is compatible with increased cell divi-

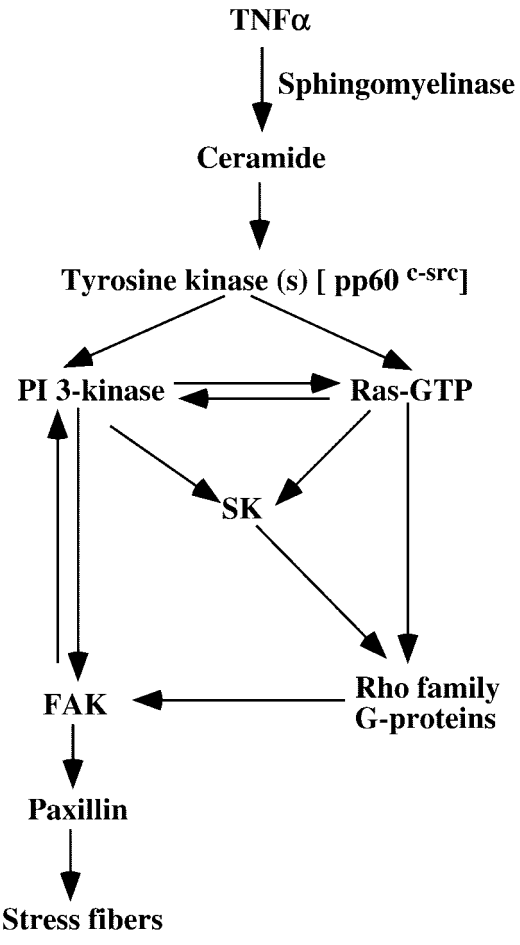

Figure 8. Proposed scheme for the signaling pathway of TNF-α leading to stress fiber formation. The figure illustrates how ceramide formation can contribute to the signaling pathways whereby TNF-α induces the formation of stress fibers. The ceramide-mediated pathway depends upon the formation of Ras-GTP and the activations of PI 3-K and SK. In addition, TNF-α also induces the tyrosine phosphorylations of FAK and paxillin and stress fiber formation independently of the activation of Ras and PI 3-K.

sion rather than apoptosis when fibroblasts are treated with TNF-α or ceramides. Activation of PI 3-K and SK also stimulates PAK and activation of Rho family G proteins followed by tyrosine phosphorylation of paxillin and actin polymerization. Our results provide novel information that ceramide production accounts for ~60 to 70% of the TNF-α-induced signal that leads to stress fiber formation. A comprehensive description of the signaling pathway is provided and this involves ceramide-induced activation of SK. This process of cytoskeletal rearrangement by TNF-α through ceramide production participates in many activities such as cell motility, cell survival, and cytokinesis.

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