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Csk, a Critical Link of G Protein Signals to Actin Cytoskeletal Reorganization

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Summary

Heterotrimeric G proteins can signal to reorganize the actin cytoskeleton, but the mechanism is unclear. Here we report that, in tyrosine kinase Csk-deficient mouse embryonic fibroblast cells, G protein ($G\beta\gamma$, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$)-induced, and G protein-coupled receptor-induced, actin stress fiber formation was completely blocked. Reintroduction of Csk into Csk-deficent cells restored the G protein-induced actin stress fiber formation. Chemical rescue experiments with catalytic mutants of Csk demonstrated that the catalytic activity of Csk was required for this process. Furthermore, we uncovered that $G\beta\gamma$ can both translocate Csk to the plasma membrane and directly increase Csk kinase activity. Our genetic and biochemical studies demonstrate that Csk plays a critical role in mediating G protein signals to actin cytoskeletal reorganization.

Introduction

Heterotrimeric ($\alpha\beta\gamma$) G proteins are major cellular signal transducers in transmembrane signaling processes (Gilman, 1987). Based on sequence homologies and functional similarities of their α subunits, these G proteins are currently grouped into four families: G_s, G_i, G_q, and $G_{12/13}$ (Simon et al., 1991). Both α and $\beta\gamma$ subunits are able to directly relay signals from membrane receptors (named G protein-coupled receptors) to activate downstream effector molecules such as enzymes to regulate diverse physiological functions and cellular events (Clapham and Neer, 1997). One unresolved question in G protein signaling is how G protein-coupled receptor signals are transmitted to the reorganization of the actin cytoskeleton, leading to changes in cell motility, cell shape, and other dynamic cellular processes (Sah et al., 2000). From pharmacological experiments with tyrosine kinase inhibitors, it is known that tyrosine kinase activity is required for G protein-mediated actin cytoskeletal reorganization (Gohla et al., 1999; Katoh et al., 1998; Kranenburg et al., 1999; Mao et al., 1998; Nobes et al., 1995; Ridley and Hall, 1994). The identities of the involved tyrosine kinases are unknown.

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Protein tyrosine kinase Csk (*C*-terminal *S*rc *k*inase) was originally purified as a kinase capable of phosphorylating Src and other Src family kinases at their C-terminal tyrosine residues (Okada et al., 1991; Nada et al., 1991). Csk plays an important role in keeping the basal activity of c-Src at a low level, although it could not prevent c-Src activation through conformational changes (Ma et al., 2000; Moarefi et al., 1997). Csk is ubiquitously expressed in mammalian cells and is evolutionarily conserved from early-diverging metazoan Hydra to humans (Miller et al., 2000). Mouse embryos deficient in Csk exhibited developmental defects, including an inability to "turn," impaired neural tube formation, and failure of the allantois to connect with the chorion (Imamoto and Soriano, 1993; Nada et al., 1993). The Csk-deficient mouse embryos died around day 10 post gestation. Elegant mouse genetic studies revealed that $Src^{-/-}Csk^{-/-}$ mouse embryos showed partial rescue of $Csk^{-/-}$ phenotypes (Thomas et al., 1995). However, $Src^{-/-}Csk^{-/-}$ mouse embryos still died around E10–E11, implying a Src-dependent and -independent function for Csk (Thomas et al., 1995). Indeed, cellular and biochemical studies suggested that Csk also interacts with other proteins, such as protein tyrosine phosphatases CD45 and PTP (Cloutier and Veillette, 1996; Thomas and Brown, 1999). Little is known about how Csk activity is regulated, although it is believed that membrane translocation of Csk accompanies Csk activation.

Rho family small GTPases have been demonstrated to play important regulatory roles in actin cytoskeletal reorganization (Hall, 1998; Ridley, 2001). There are at least 20 Rho family GTPase proteins in the human genome. RhoA, Rac1, and Cdc42 have been the most widely studied members. RhoA induces actin-myosin-based contractility, leading to the formation of stress fibers in many types of adherent cells and/or cell retraction. Both Rac and Cdc42 stimulate actin polymerization: Rac induces broad plasma membrane extensions known as lamellipodia and membrane ruffles, and Cdc42 induces the extension of finger-like plasma membrane extensions called filopodia, or microspikes (Ridley and Hall, 1992; Ridley et al., 1992). Stress fibers are bundles of actin and myosin II filaments. Currently it is believed that two of the several direct downstream effectors of Rho, namely, ROCK (a serine/threonine protein kinase) and Dia1 (a profilin binding protein), cooperate to mediate Rho-induced stress fiber formation (Watanabe et al., 1999). Dia1, likely acting through profilin, promotes extension of actin filaments by recruiting actin monomers. Meanwhile, ROCK, by inhibiting myosin light chain (MLC) phosphatase, promotes accumulation of phosphorylated MLC. Phosphorylated myosin II can assemble into myosin filaments, which, together with actin filaments, form stress fibers. Like heterotrimeric G proteins, Rho family GTPases cycle between an active

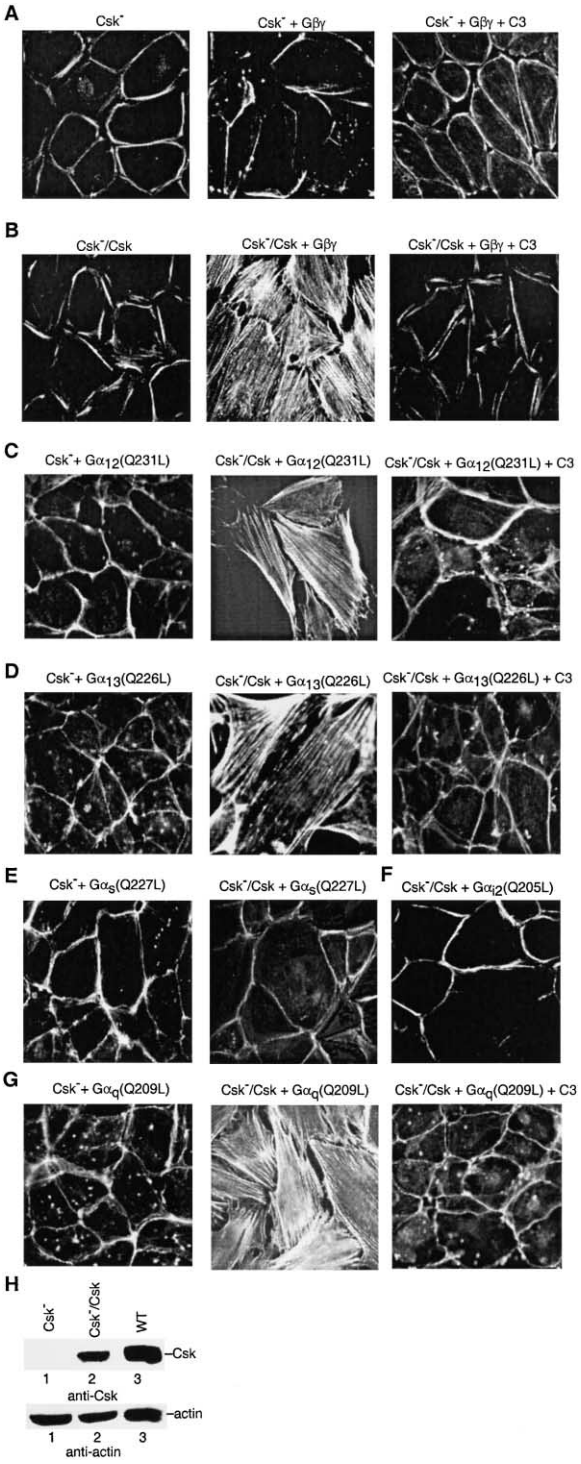

Figure 1. G Protein-Induced Actin Stress Fiber Formation Requires Csk

(A) In serum-starved Csk⁻ cells, actin was stained with phalloidin-rhodamine (1.6% ± 0.8% of cells had stress fibers). $G\beta_1\gamma_2$ was expressed by retroviral infection (5.2% ± 0.3% of cells had stress fibers). Some cells were also treated with a membrane-permeable C3 toxin.

(B) Csk⁻/Csk cells were stained in the absence of serum (12% ± 1.6% of cells had stress fibers). Expression of Gβγ in Csk⁻/Csk cells significantly increased the density of actin stress fiber formation in quiescent embryonic fibroblasts (79% ± 4.6% of cells with stress fibers).

GTP-bound conformation and an inactive GDP-bound form. Thus, their activity is controlled positively by guanine nucleotide exchange factors (GEFs) and negatively by GTPase-activating proteins (GAPs) (Zheng, 2001). Upon extracellular stimulation, GEFs promote the intrinsic exchange rate of GDP for GTP and, consequently, activate Rho family GTPases. GAPs promote the hydrolysis of bound GTP to GDP and, consequently, lead to inactivation. However, little is known about the signaling molecules relaying membrane receptor messages to Rho-GEFs and/or GAPs.

Here we show that tyrosine kinase Csk is essential for G protein-mediated stress fiber formation. In Csk-deficient mouse embryonic fibroblast cells, stress fiber formation induced by G proteins Gβγ, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$ and G protein-coupled receptors was completely blocked. Reintroduction of Csk into Csk-deficent cells restored the G protein-induced stress fiber formation. Chemical rescue experiments with catalytic mutants of Csk demonstrated that the catalytic activity of Csk was required for its function in this process. To further reveal the biochemical mechanism by which G proteins regulate the kinase activity of Csk, we uncovered that Gβγ can directly interact with Csk. Gβγ is able to both translocate Csk to the plasma membrane and directly increase Csk kinase activity. Downstream of Csk exist Src-dependent and -independent signaling pathways to stress fiber formation. These studies demonstrate that Csk is a critical signal transducer for relaying G protein signals to actin cytoskeletal reorganization.

Results

Critical Role of Csk in Mediating G Protein Signals to Actin Stress Fiber Formation

To investigate the possible role of tyrosine kinase Csk in G protein signaling to the actin cytoskeleton, we used a molecular genetic approach to examine the G protein-induced actin stress fiber formation in Csk-deficient cells (Csk⁻). These Csk⁻ mouse embryonic fibroblast cells were produced from Csk knockout mice (Nada et al., 1994). To obtain an efficient introduction of ectopic genes for G proteins into these fibroblast cells, we used retroviral vectors with IRES (internal ribosome entry site)-linked green fluorescent protein (GFP). The efficiency of the infection was confirmed by GFP expression to be almost 100%. As shown in Figures 1A and 1B, in the absence of serum, both Csk⁻ and Csk⁻/Csk (wild-type Csk stably reintroduced back into Csk-deficient cells) cells had very few stress fibers (Figures 1A and

(C, D, and G) Constitutively active mutants of human $G\alpha_{12}$Q231L, $G\alpha_{13}$Q226L, or $G\alpha_q$Q209L were expressed by retroviral infection in Csk⁻/Csk cells (81% ± 2.5%, 74% ± 4.9%, or 76% ± 8.5% of cells, respectively, had stress fibers) and in Csk⁻ cells (3.5% ± 1.3%, 3.8% ± 2.8%, or 4.3% ± 0.9% of cells, respectively, had stress fibers).

(E and F) Expression of $G\alpha_s$Q227L and $G\alpha_i$Q205L in Csk⁻/Csk cells (10% ± 4.3% and 10% ± 6.1% of cells, respectively, had stress fibers).

(H) Anti-Csk Western blot of normalized Csk⁻, Csk⁻/Csk, and wild-type 3T3 cell lysates (WT). Data are representative of four separate sets of experiments.

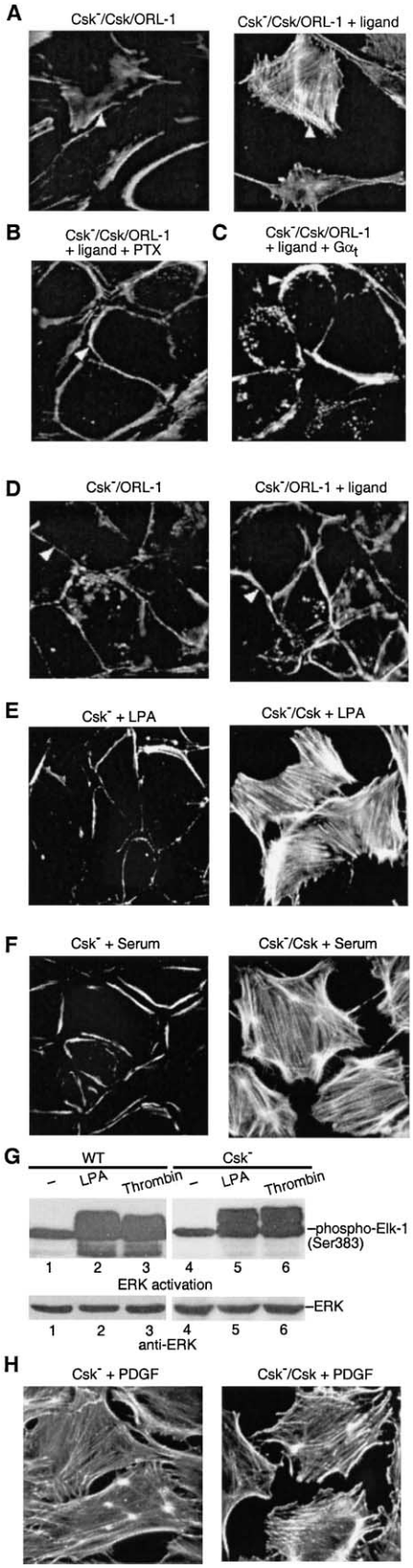

Figure 2. G Protein-Coupled Receptor-Induced Stress Fiber Formation Depends on Csk

1B); instead, these cells showed accumulation of actin in cortical bundles around the plasma membrane, as shown by staining of actin with phalloidin-rhodamine. Expression of Gβγ in Csk⁻/Csk cells significantly increased the density of actin stress fiber formation in quiescent embryonic fibroblasts (Figure 1B). However, expression of Gβγ in Csk⁻ cells did not induce actin stress fiber formation (Figure 1A). We further examined all four families of Gα subunits. Constitutively active mutants of human $G\alpha_{12}$Q231L, $G\alpha_{13}$Q226L, or $G\alpha_q$Q209L induced actin stress fiber formation in Csk⁻/Csk cells (Figures 1C, 1D, and 1G). Induction by each of these mutants was blocked by Csk deficiency (Figures 1C, 1D, and 1G). On the other hand, constitutively active mutants of human $G\alpha_s$Q227L and $G\alpha_i$Q205L did not induce stress fiber formation in either Csk⁻ or Csk⁻/Csk cells (Figures 1E and 1F). Wild-type 3T3 fibroblast cells behaved similarly to Csk⁻/Csk cells (Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/2/6/733/DC1). A Western blot (Figure 1H) showed not only that Csk⁻ cells indeed lack Csk but also that the stable Csk⁻/Csk cells express Csk at a lower level then wild-type 3T3 fibroblast cells, indicating that these Csk⁻/Csk cells did not have an unphysiological level of Csk. These data clearly demonstrate that Csk is critical for G protein-induced actin stress fiber formation in mouse embryonic fibroblast cells.

G Protein-Coupled Receptors Induce Stress Fiber Formation, Depending on Csk

Next we investigated the possible role of Csk in stress fiber formation induced by G protein-coupled receptors. Chemokine receptors can induce cell chemotaxis, and these Gi-coupled chemokine receptors use Gβγ subunits to mediate this cellular event (Premack and Schall, 1996). Indeed, Gi-coupled opioid receptors have been shown to act through Gβγ to induce cell chemotaxis (Arai et al., 1997; Neptune and Bourne, 1997). Therefore, we tested whether stress fiber formation in mouse embryonic fibroblast cells induced by ORL-1, a Gi-coupled nociceptin receptor, requires Csk (Figure 2). We tagged ORL-1 with GFP for easy observation of transfected cells (Figures 2A–2D, arrowheads). As shown in Figure

(A) Opiate GPCR ORL-1 was tagged with GFP for easy observation of transfected cells (indicated by arrows). Stimulation of Csk⁻/Csk cells transiently transfected with ORL-1 with ORL-1 agonist nociceptin (10 nM for 5 min) induced stress fiber formation.
(B) Cells were pretreated with PTX.
(C) Overexpression of $G\alpha_t$ by cotransfection of $G\alpha_t$ and ORL-1 was done with a 10:1 ratio of plasmid DNAs.
(D) Stimulation of Csk⁻ cells transfected with ORL-1 with nociceptin.
(E) Csk⁻/Csk and Csk⁻ cells were treated with 15 ng/ml LPA for 5 min (89% ± 2.8% and 9.1% ± 4.1% of cells, respectively, had stress fibers).
(F) Quiescent Csk⁻/Csk and Csk⁻ cells were treated with 10% serum for 5 min (78% ± 3% and 5.1% ± 3.3% of cells, respectively, had stress fibers).
(G) Activation of ERK MAP kinase in wild-type 3T3 cells (WT) and Csk⁻ cells by LPA (15 ng/ml for 5 min) and thrombin (for 5 min).
(H) Csk⁻ and Csk⁻/Csk cells were treated with 10 ng/ml platelet-derived growth factor for 30 min (77% ± 3.5% and 86% ± 3.5% of cells, respectively, had stress fibers). Data are representative of three separate sets of experiments.

2A, stimulation of ORL-1-transfected Csk⁻/Csk cells with ORL-1 agonist nociceptin induced stress fiber formation. Pretreatment of these cells with pertussis toxin (PTX) blocked the stress fiber formation, confirming that ORL-1 signals through PTX-sensitive Gi-family G proteins (Figure 2B). Overexpression of $G\alpha_t$, which sequesters free $G\beta\gamma$ (Arai et al., 1997; Neptune and Bourne, 1997), prevented the stress fiber formation induced by ORL-1 stimulation (Figure 2C), indicating that $G\beta\gamma$ is essential for this signaling process. Furthermore, stimulation of Csk⁻ cells expressing ORL-1-GFP with agonist nociceptin did not induce stress fiber formation, suggesting that Csk deficiency blocked this signaling event (Figure 2D). Hence, G protein-coupled receptor ORL-1 requires Csk to signal to the actin cytoskeleton.

We further investigated the possible requirement of Csk for lysophosphatidic acid (LPA) receptor-induced actin stress fiber formation. LPA works through G protein-coupled Edg receptors to induce stress fiber formation in fibroblast cells (Gohla et al., 1999). As shown in Figure 2E, LPA induced actin stress fiber formation in Csk⁻/Csk cells but not in Csk⁻ cells, demonstrating that LPA receptors also require Csk to induce stress fiber formation in mouse embryonic fibroblast cells. Moreover, addition of serum to the quiescent embryonic fibroblasts induced stress fiber formation in Csk⁻/Csk cells but not in Csk⁻ cells, indicating that Csk plays a critical role in serum-induced actin stress fiber formation (Figure 2F). The active component in serum that induces stress fiber formation is believed to be phospholipids such as LPA working through G protein-coupled receptors (Ridley and Hall, 1994).

In order to allay concerns that Csk⁻ cells might have gross signaling defects, we examined whether, in Csk⁻ cells, G protein-coupled receptors could activate the mitogen-activated protein kinase (MAPK) pathway and whether platelet-derived growth factor (PDGF) could induce stress fiber formation. LPA and thrombin, through their G protein-coupled receptors, have been previously shown to activate the ERK MAPK in fibroblast cells (van Corven et al., 1993). As shown in Figure 2G, both LPA and thrombin increased the activity of ERK in Csk⁻ cells as well as in wild-type fibroblast cells. These data suggest that not all G protein-mediated signaling pathways are blocked by Csk deficiency. Furthermore, as initially reported, PDGF, EGF, and insulin induced distinct responses in fibroblast cells different from that of serum (or LPA) (Ridley and Hall, 1992). PDGF, EGF, and insulin induced a rapid formation of membrane ruffles (through Rac). Stress fibers formed at a later stage (30 min), however, and the number and thickness of fibers never reached the density observed following serum addition (for 5 min) (Ridley and Hall, 1992). As shown in Figure 2H, PDGF was still able to stimulate stress fiber formation in Csk⁻ cells as well as in Csk⁻/Csk cells. The same was true for EGF (data not shown). These data imply that Csk is specifically required for relaying G protein signals to actin stress fiber formation in mouse embryonic fibroblast cells.

The Catalytic Activity of Csk Is Needed for Stress Fiber Formation

To investigate whether the catalytic activity of Csk is needed for the stress fiber formation induced by G proteins and G protein-coupled receptors, we performed chemical rescue experiments. We generated stable cell lines of Csk⁻ cells expressing Csk(D314N) or Csk(R318A) mutants. These two mutations at the catalytic loop of Csk have impaired catalytic activity by comparison with the wild-type Csk kinase (Williams et al., 2000). The catalytic activity of Csk(R318A), but not that of Csk(D314N), can be rescued by several exogenous small molecules, including imidazole (Williams et al., 2000). In both Csk⁻/Csk(R318A) and Csk⁻/Csk(D314N) cells, the addition of serum or overexpression of $G\beta\gamma$ did not induce stress fiber formation (Figures 3A–3C and data not shown), consistent with the impaired in vitro kinase activity of these two mutants of Csk. Upon addition of imidazole and serum, Csk⁻/Csk(R318A) cells, but neither Csk⁻ cells nor Csk⁻/Csk(D314N) cells, showed induced stress fiber formation (Figures 3A–3C). Since only Csk(R318A), but not Csk(D314N), catalytic activity could be rescued by imidazole, these chemical rescue experiments demonstrate that the catalytic activity of Csk is required for stress fiber formation in fibroblast cells.

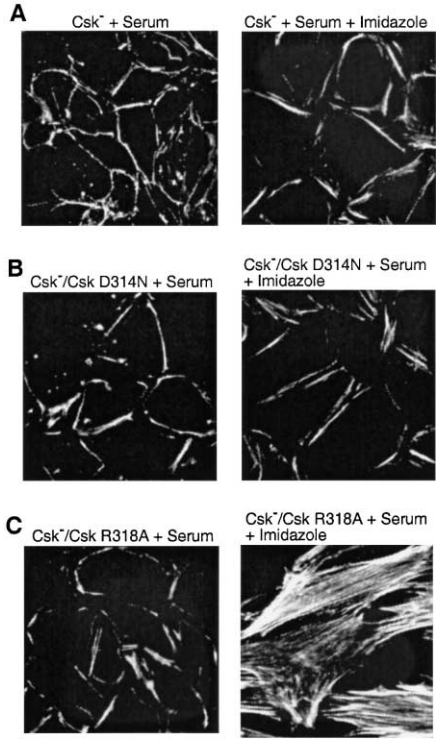

Figure 3. The Catalytic Activity of Csk Is Needed for the Stress Fiber Formation

(A) Csk⁻ cells treated with serum or serum together with imidazole.
(B) Csk⁻ cells stably transfected with unrescuable Csk(D314N) and treated with serum or serum together with imidazole (6.1% ± 0.8% or 4.5% ± 1.5% of cells, respectively, had stress fibers).
(C) Csk⁻ cells stably expressing rescuable Csk(R318A) and treated with serum (4.7% ± 1.2% of cells had stress fibers). Addition of imidazole rescued stress fiber formation (85% ± 0.7% of cells had stress fibers). Data are representative of three separate sets of experiments.

Csk Acts Upstream of Rho

The Rho GTPase controls actin stress fiber formation (Hall, 1998; Ridley and Hall, 1992). Therefore, we examined whether Csk acts upstream or downstream of Rho

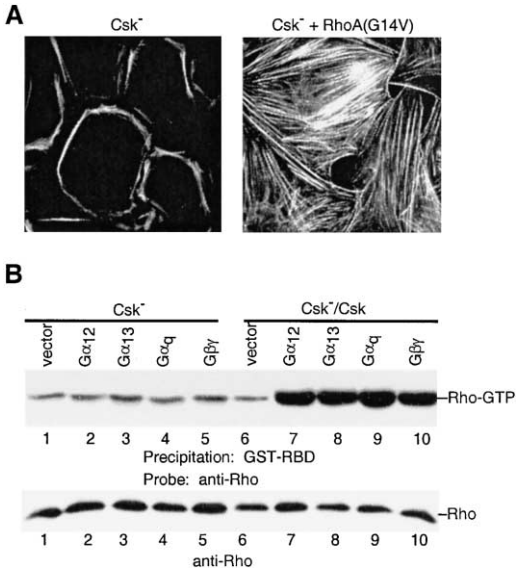

Figure 4. Csk Acts Upstream of Rho

(A) Retroviral expression of activated RhoA(G14V) mutant in quiescent Csk⁻ cells.

(B) Rho activation assay: after normalization of cell lysates from the conditions shown by anti-Rho Western blot, the lysates were probed with GST-Rhotekin, which only binds active Rho-GTP. Anti-Rho Western analysis showed relative Rho activity. Data are representative of three separate sets of experiments.

in G protein-induced actin stress fiber formation in mouse embryonic fibroblast cells. Treatment of Csk⁻/Csk cells with the *Clostridium botulinum* C3 exoenzyme, a toxin that specifically inactivates Rho proteins by irreversibly ADP-ribosylating Asn-41 in its effector region, blocked $G\beta\gamma$-, $G\alpha_{12}$-, $G\alpha_{13}$-, and $G\alpha_q$-induced actin stress fiber formation (Figures 1B, 1C, 1D, and 1G). This confirms that G protein-initiated actin stress fiber formation in mouse embryonic fibroblast cells is Rho dependent. To study whether Csk acts upstream or downstream of Rho, we examined the actin stress fiber formation induced by constitutively active Rho [RhoA(G14V) mutant] in Csk⁻ cells. As shown in Figure 4A, activated human RhoA(G14V) mutant was still able to induce stress fiber formation in Csk⁻ cells. This suggests that Csk likely works upstream of Rho, though they could possibly work in parallel.

To more directly measure the requirement of Csk for G protein stimulation of Rho, we employed a Rho activation assay whereby only GTP-bound, active Rho (Rho-GTP) can be pulled down by its effector (such as Rhotekin), providing a measure of the active Rho in cells (Sander et al., 1999). Utilizing a GST fusion protein containing the Rho binding domain (RBD) of Rhotekin (Ren et al., 2001), cell lysates from Csk⁻ and Csk⁻/Csk cells infected with retroviruses carrying constitutively active mutants of $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, or $G\beta\gamma$ were probed, and active Rho was pulled down. As shown in Figure 4B, quiescent Csk⁻ and Csk⁻/Csk cells infected with empty retroviral vectors had similar Rho activity. However, when various G proteins were expressed in these two cell types, the role of Csk in Rho activation became clear. $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, or $G\beta\gamma$ that was able to stimulate stress fiber formation in Csk⁻/Csk cells increased Rho activity only in cells expressing Csk (Figure 4B). $G\alpha_s$ and $G\alpha_i$ failed to increase Rho activity in Csk⁻ and Csk⁻/Csk cells (data not shown). These data confirm that Csk is required for heterotrimeric G protein signaling to Rho.

$G\beta\gamma$, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$ Translocate and Activate Csk in Cells

The Csk dependence of G protein signaling to the actin cytoskeleton indicates that G protein signals should affect Csk kinase activity. Since Csk is mainly located in the cytoplasm and one of its targets, Src, is on the membrane, it has been proposed that activation of Csk is accompanied by its membrane translocation. Indeed, a transmembrane protein, Cbp, was recently shown to be able to anchor Csk on the membrane in order to phosphorylate Src (Kawabuchi et al., 2000). To examine the possible effect of G proteins on Csk membrane translocation, we generated a Csk fusion construct with COOH-terminal GFP in order to visualize Csk in live cells (Figure 5). Csk-GFP is catalytically active (Figure 5G). We employed fluorescence microscopy to view the effect of G proteins on the in vivo subcellular localization of Csk. HEK-293 cells were transfected with Csk-GFP fusion constructs with or without G proteins (with a G protein/Csk-GFP plasmid DNA ratio of 10:1). GFP alone was used as a negative control. As seen in Figure 5A, Csk-GFP fusion proteins were uniformly distributed in HEK-293 cells. Coexpression of Csk-GFP and $G\beta\gamma$ (FLAG-tagged $G\beta_1$ and HA-tagged $G\gamma_2$) led to the subcellular redistribution of Csk (Figure 5A). $G\beta\gamma$ was mainly on plasma membrane, as stained by anti-FLAG antibody (Figure 5A). Csk-GFP could also be redistributed by the stimulation of Gi-coupled m2 muscarinic acetylcholine receptor (mAChR) (Figure 5B). This effect was blocked by sequestration of $G\beta\gamma$ by coexpression of transducin $G\alpha_t$ (Figure 5B). Similarly, coexpression of constitutively active mutants of $G\alpha_{12}$, $G\alpha_{13}$, or $G\alpha_q$, but not $G\alpha_s$, translocated Csk-GFP from the cytoplasm to the membrane (Figures 5C–5F). Furthermore, immunoprecipitation kinase assays from whole-cell lysates showed that $G\beta\gamma$, $G\alpha_{12}$, $G\alpha_{13}$, or $G\alpha_q$, but not $G\alpha_s$, increased the kinase activity of Csk in cells (Figure 5G). In addition, G protein-coupled m2 mAChR also stimulated Csk activity (Figure 5G). This stimulation was reduced by coexpression of $G\alpha_t$, demonstrating that the receptor, acting through $G\beta\gamma$, stimulated Csk. Moreover, expression of the Csk-GFP construct in Csk⁻ cells rescued LPA-induced stress fiber formation, consistent with the notion that Csk-GFP is catalytically active (Figure 5H). Hence, G proteins can translocate and activate Csk in cells. These data also imply that the direct substrate(s) of Csk in this cytoskeletal reorganization pathway is likely on or associated with the membrane.

$G\beta\gamma$ Directly Interacts with Csk

To explore further the biochemical regulatory mechanism of Csk by G proteins, we asked whether any of these G protein subunits could directly interact with Csk. We were able to show that purified $G\beta\gamma$ (but not purified $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$) could directly interact with purified Csk (Figure 6A and data not shown). As shown in Figure 6A, purified $G\beta\gamma$ (attached to Ni-NTA beads) interacted with purified Csk, but not with boiled Csk. To study the interaction of Csk and $G\beta\gamma$ in cells, we performed

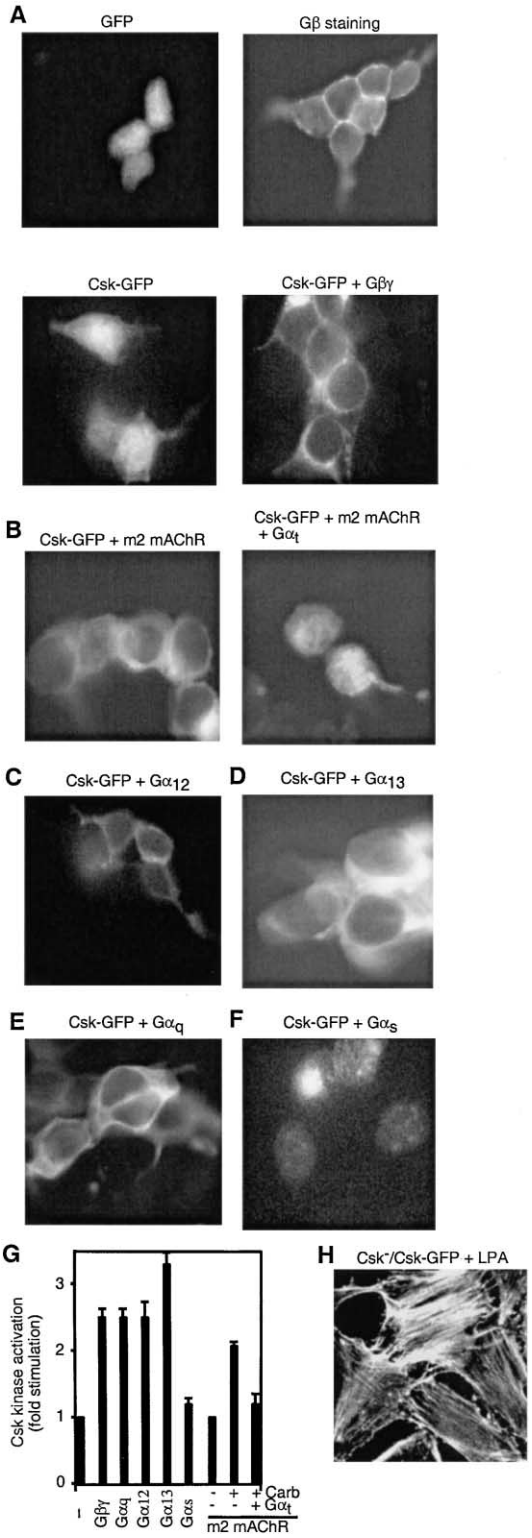

Figure 5. Gβγ, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$ Translocate and Activate Csk in Quiescent HEK-293 Cells

(A) Uniform cellular distribution of transfected GFP was used as control. Gβγ was localized by immunohistochemical staining of Gβ. Csk was localized by transfection of Csk-GFP in the presence or absence of Gβγ.

(B) Csk-GFP localization upon coexpression and stimulation of Gi-coupled m2 muscarinic acetylcholine receptor (mAChR) with carbachol (100 μM). This effect was blocked by coexpression of transducin $G\alpha_t$.

(C–F) Localization of Csk upon coexpression of $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, and $G\alpha_s$.

(G) Immunoprecipitation kinase assays: Csk-GFP was pulled down from lysates prepared from the listed conditions with anti-GFP antibody.

(H) Csk^- cells stably expressing Csk-GFP were stimulated with LPA (15 ng/ml for 5 min) and stained with phalloidin-rhodamine. Data are representative of three experiments.

coimmunoprecipitation experiments. As shown in Figure 6B, stimulation of mAChRs in NG 108 cells showed that anti-Csk antibody coprecipitated Gβγ, establishing their in vivo interaction (Figure 6B). To further identify the interacting domain on Csk for Gβγ, we generated and purified GST-fusion proteins of the SH3, SH2, SH3SH2, and catalytic domains of Csk (Sondhi and Cole, 1999) (Figure 6C). The catalytic domain, but neither a SH3, SH2, nor a SH3SH2 domain of Csk, interacted with Gβγ from NG108 cell extracts (Figure 6C, lanes 1–5) or purified Gβγ (Figure 6C, lanes 6–9). The interaction of Gβγ with the catalytic domain of Csk did not depend on the phosphorylating activity of Csk, since GST-Csk(D314N) and GST-Csk(R318A) bound to Gβγ as well as GST-Csk (Figure 6D). Furthermore, the association between the catalytic domain of Csk and Gβγ could be blocked by the addition of $G\alpha_o$-GDP, which sequesters Gβγ (Figure 6E). Thus, Gβγ interacts specifically with the catalytic domain of Csk.

To investigate whether the direct interaction of Gβγ with the catalytic domain of Csk has any effect on Csk kinase activity, we performed enzymatic studies of purified Csk in the absence or presence of purified Gβγ. This was assayed with a substrate peptide polymer of glutamate and tyrosine residues, poly(Glu,Tyr) (Sondhi and Cole, 1999). As shown in Figure 6F, Gβγ increased the kinase activity of Csk modestly (~2-fold), but reproducibly. This direct stimulation of Csk kinase activity by Gβγ was also observed when purified c-Src was used as substrate for Csk (data not shown). Similar experiments using purified activated mutants of $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$ failed to produce any direct stimulation of Csk activity in vitro (data not shown). These data demonstrate that Gβγ subunits can directly increase the catalytic activity of Csk and suggest that the in vivo cellular stimulation of Csk by Gα subunits must come about by an indirect association through intermediate signaling molecules.

To further demonstrate the direct interaction of Gβγ and Csk in living cells by another approach, we used bioluminescence resonance energy transfer (BRET) technology (Angers et al., 2000). BRET uses a bioluminescent *Renilla* luciferase (*R*Luc) fused to one protein of interest and GFP fused to another protein of interest. A specific interaction between the two proteins brings *R*Luc and GFP close enough for resonance energy transfer to occur upon addition of luciferase substrate. We expressed Gβ-*R*Luc, Gγ, and Csk-GFP in HEK-293 cells for BRET assays (Figure 6G). A fusion construct covalently linking *R*Luc to GFP (pBRET+) was used as a positive control. Cells transfected with Gβ-*R*Luc, Gγ, and Src-GFP, *R*Luc and Csk-GFP, or *R*Luc and GFP were used as specificity or negative controls (Figure

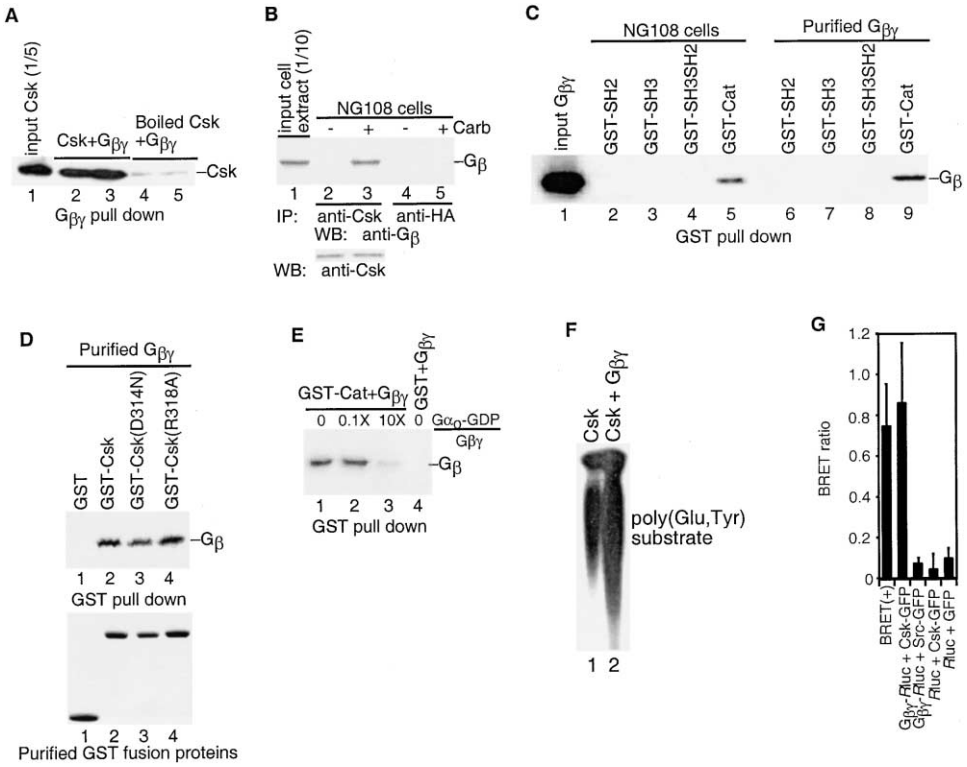

Figure 6. Gβγ Directly Interacts with Csk

(A) As shown by anti-Csk Western blot, purified Gβγ bound to Ni-NTA beads pulled down purified Csk, but not boiled Csk.

(B) Coimmunoprecipitation experiments with NG108 cell extract showed that anti-Csk antibody precipitated Gβγ bound to Csk after stimulation of mAChR with carbachol. A control antibody (anti-HA) did not co-immunoprecipitate Gβγ under the same experimental conditions.

(C) GST-fusion proteins of the catalytic domain, but neither the SH3, SH2, nor SH3SH2 domains of Csk, interacted with Gβγ from cell extracts (lanes 1–5) or purified Gβγ (lanes 6–9).

(D) GST pull down analysis showed that the Csk mutants [Csk(D314N) and Csk(R318A)] used in Figure 3 bound to Gβγ as well as wild-type Csk.

(E) The association between the purified catalytic domain of Csk and purified Gβγ could be reduced by the addition of purified $G\alpha_o$-GDP.

(F) In an in vitro kinase assay with [γ-^{32}P]ATP and a polymer of glutamate and tyrosine as substrate, addition of purified $G\beta_1\gamma_2$ increased the kinase activity of purified Csk.

(G) Bioluminescence resonance energy transfer (BRET) assay. HEK-293 cells were transfected with various constructs. A substrate for luciferase was added and the BRET ratio was measured in a luminometer. Data are representative of three experiments.

6G). After addition of the cell permeable *R*Luc substrate coelenterazine, BRET occurred in cells expressing the positive control BRET+ as well as in cells expressing Gβ-*R*Luc, Gγ, and Csk-GFP, but not in cells expressing Gβ-*R*Luc, Gγ, and Src-GFP, *R*Luc and Csk-GFP, or *R*Luc and GFP (Figure 6G). These BRET data confirmed the direct interaction of Gβγ and Csk in living intact cells.

Csk Signaling through Src-Dependent and -Independent Pathways to Stress Fiber Formation

The above data provide biochemical and cellular mechanisms by which G proteins signal through Csk to reorganize the actin cytoskeleton. To investigate the possible signaling molecules downstream of Csk, we asked whether Src family tyrosine kinases are involved in these G protein signaling pathways. We used Src family tyrosine kinase-deficient mouse embryonic fibroblast cells (Klinghoffer et al., 1999; Ma et al., 2000). Deletion of the ubiquitously expressed Src family tyrosine kinases Src, Yes, and Fyn in mice led to embryonic lethality. Embryonic fibroblast cells (SYF cells) from these knockout mouse embryos are devoid of all known Src family kinase activity (Klinghoffer et al., 1999). As shown in Figure 7, G proteins Gβγ, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$ could still induce actin stress fiber formation in SYF cells, implying the existence of Src-independent pathway(s) for Csk signaling to actin stress fiber formation. However, we noticed that the density of stress fibers induced by G proteins in SYF cells was less than that in Csk$^-$/Csk or wild-type fibroblast cells. Thus, G protein-induced actin stress fiber formation is at least partially Src family tyrosine kinase independent.

Discussion

Requirement of Tyrosine Kinase Activity in Linking G Protein Signaling to Actin Cytoskeletal Reorganization

Since the finding that lysophosphatidic acid (LPA) in serum induces actin stress fiber formation in cells through its G protein-coupled receptors and Rho GTPase (Ridley and Hall, 1992; Ridley and Hall, 1994),

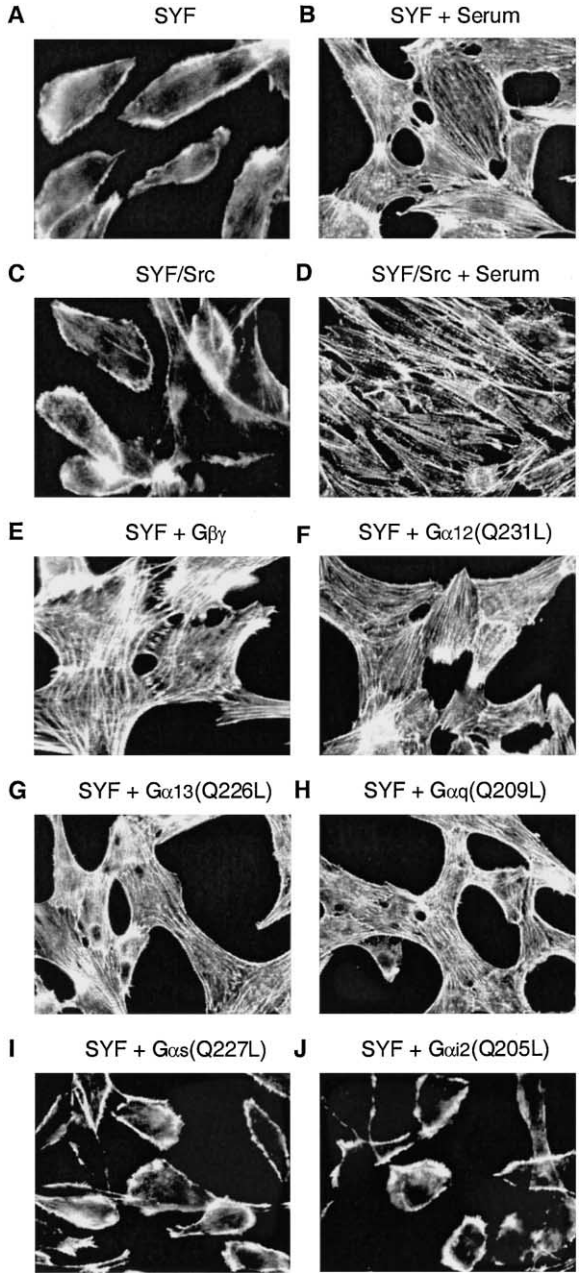

Figure 7. G Protein-Induced Actin Stress Fiber Formation in SYF Cells

(A) Actin staining with phalloidin-rhodamine of serum-starved SYF cells showed cortical staining (5.5% ± 0.9% of cells had stress fibers).

(B) SYF cells in the presence of serum (83% ± 1% of cells with stress fibers).

(C) Serum-starved SYF/Src cells showed cortical actin staining (6.7% ± 2% had stress fibers).

(D) SYF/Src cells in the presence of serum (76% ± 1% of cells with stress fibers).

(E) Retroviral expression of Gβγ in serum-starved SYF cells (91% ± 3% of cells had stress fibers).

(F–H) Retroviral expression of $G\alpha_{12}$Q231L, $G\alpha_{13}$Q226L, and $G\alpha_q$Q209L in serum-starved SYF cells (90% ± 2%, 88% ± 5%, or 93% ± 3% of cells, respectively, had stress fibers).

(I and J) Expression of $G\alpha_s$Q227L and $G\alpha_i$Q205L in SYF cells (11% ± 6% and 7% ± 3% of cells, respectively, had stress fibers). Data are representative of four separate sets of experiments.

one of the challenges in the field has been to determine how G proteins are connected to Rho-family GTPases. Elegant microinjection experiments demonstrated that a constitutively active RhoA mutant induced actin stress fiber formation in serum-starved Swiss 3T3 cells, whereas inactivation of Rho prevented serum-induced actin stress fiber formation (for reviews, see Hall, 1998; Sah et al., 2000). Subsequently, a variety of GPCR agonists have been shown to be able to induce actin stress fiber formation in a Rho-dependent manner (Sah et al., 2000). Initial experiments with expression of individual activated Gα subunits in Swiss 3T3 cells revealed that the $G\alpha_{12}$ family members ($G\alpha_{12}$ and $G\alpha_{13}$), but neither $G\alpha_i$ nor $G\alpha_q$ families of G proteins, could induce actin stress fiber formation (Buhl et al., 1995). This finding drew attention to the connection between $G\alpha_{12}$ family members and Rho. This connection was further strengthened when $G\alpha_{12}$ and $G\alpha_{13}$ were shown to directly bind to the RGS (*r*egulator of *G* protein *s*ignaling) domain present in p115 Rho-GEF/Lsc, a guanine-nucleotide exchange factor for Rho (Hart et al., 1998; Kozasa et al., 1998). This RGS domain could accelerate the GTP hydrolysis of $G\alpha_{12}$ and $G\alpha_{13}$, thus terminating $G\alpha_{12}$ and $G\alpha_{13}$ signaling (Kozasa et al., 1998). It was also initially reported that $G\alpha_{13}$, but not $G\alpha_{12}$, could moderately increase the exchange activity of p115 Rho-GEF/Lsc (Hart et al., 1998). However, it was recently noted that p115 Rho-GEF has a very high basal exchange activity when purified by comparison with that in cells and that immunoprecipitated p115 Rho-GEF (without $G\alpha_{13}$ present in the complex) from stimulated cells remained highly active, indicating that a possible posttranslational modification might be involved in p115 Rho-GEF activation (Wells et al., 2001). Indeed, in cells, activation of Rho by G protein-coupled receptors is often sensitive to tyrosine kinase inhibitors (Gohla et al., 1999; Katoh et al., 1998; Kranenburg et al., 1999; Mao et al., 1998; Nobes et al., 1995; Ridley and Hall, 1994), demonstrating a role for tyrosine kinase activity in linking G protein-coupled receptors to the cytoskeleton. For example, tyrosine kinase inhibitors blocked LPA-induced, but not constitutively active Rho-induced, actin stress fiber formation in Swiss 3T3 cells, implying that a tyrosine kinase(s) acts upstream of Rho (Nobes et al., 1995). Also, $G\alpha_{13}$- and $G\alpha_q$-induced Rho-dependent morphological changes in differentiated PC12 neuronal cells were sensitive to tyrosine kinase inhibitors (Katoh et al., 1998). Moreover, Gβγ subunits as well as $G\alpha_{12}$ have been shown to induce actin stress fiber formation in HeLa cells in a tyrosine kinase activity-dependent manner (Ueda et al., 2000). The tyrosine kinases involved remain to be identified.

Critical Role of Csk in G Protein Signaling to Actin Cytoskeletal Reorganization

We have demonstrated here that tyrosine kinase Csk is required to link G protein signals to actin stress fiber formation in mouse embryonic fibroblast cells. In Csk-deficient cells, neither $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, nor Gβγ could induce the formation of actin stress fibers. These defects were due to Csk deficiency, since reintroduction of Csk into Csk-deficient cells restored the ability of $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, and Gβγ to induce stress fiber formation. This genetic evidence demonstrates an essential role for Csk

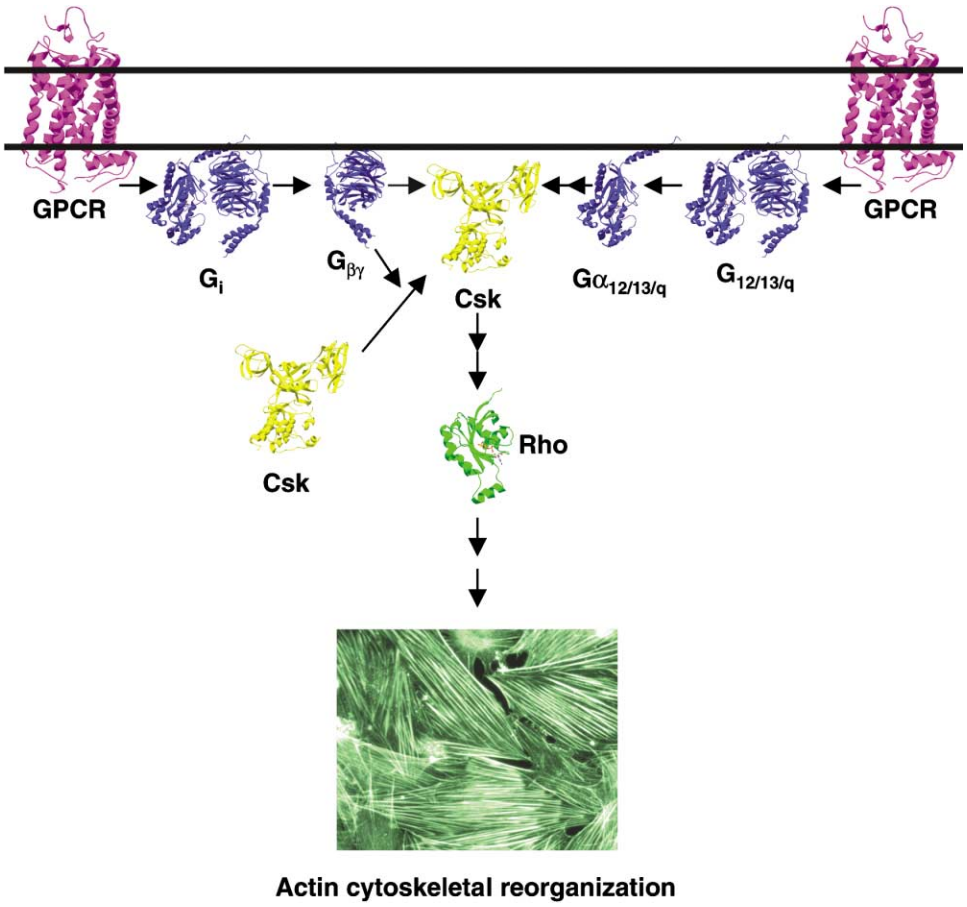

Figure 8. Csk Is Critical in G Protein Signaling to Actin Cytoskeletal Reorganization

Activation of G protein-coupled receptors led to activation of Gi proteins, which release Gβγ subunits. Gβγ can directly recruit Csk to the plasma membrane and activates Csk. On the other hand, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$, after their activation by G protein-coupled receptors, activate Csk through intermediate signaling molecules. Csk works through Rho small GTPases to induce actin cytoskeletal reorganization.

in linking G protein signals to actin stress fiber formation. We have also shown that Csk is a direct effector of Gβγ, since Gβγ directly binds to Csk, translocates Csk to the plasma membrane, and increases the kinase activity of Csk (Figure 8).

Possible Signaling Components Downstream of Csk

What is downstream of Csk in this G protein-Rho signaling pathway? Csk was originally purified as a tyrosine kinase that phosphorylates the tyrosine residue of the C-terminal tail of c-Src (Nada et al., 1991). In Src family tyrosine kinase knockout mouse embryonic fibroblast cells, the actin cytoskeleton was disrupted (Thomas et al., 1995). Expression of activated Src in fibroblasts alters cell morphology and reduces adhesion to the extracellular matrix (Thomas and Brugge, 1997). Furthermore, Src/Fyn double knockout mice exhibit defects in axon guidance and fasciculation (Morse et al., 1998).

Downstream of Src, p190 RhoGAP, a GTPase-activating protein for Rho, has been shown to be phosphorylated and activated by Src (Arthur et al., 2000; Brouns et al., 2001; Chang et al., 1995; Haskell et al., 2001). p190 RhoGAP was first observed as the major p120 RasGAP-associated protein in Src-transformed fibroblast cells (Ellis et al., 1990; Settleman et al., 1992a). p190 RhoGAP was found to be a prominent Src substrate in fibroblasts and contains an amino-terminal GTPase domain and a carboxy-terminal RhoGAP domain (Settleman et al., 1992a, 1992b). Recent mouse genetic studies demonstrated that p190 RhoGAP is the principle substrate for Src in the mouse brain as well (Brouns et al., 2001). Src regulation of p190 RhoGAP controls axon outgrowth, guidance, and fasciculation (Brouns et al., 2001). Indeed, integrin, cell adhesion molecules, and epidermal growth factor (EGF) have been shown to employ this Src/p190 RhoGAP signaling pathway to inhibit Rho activity (Arthur et al., 2000; Chang et al., 1995; Haskell et al., 2001). We are investigating whether G proteins work through Csk to c-Src to p190 RhoGAP to relieve the suppression on Rho activity, thus leading to stress fiber formation in mouse embryonic fibroblast cells. This mechanism of G protein relieving suppression on Rho would be akin to the action of another G protein, Gαt (transducin), which relieves the sup-

pression on cGMP-specific phosphodiesterase (Stryer, 1986).

Our data with the SYF cells suggest that G protein-induced stress fiber formation is only partially dependent on Src family tyrosine kinases in mouse embryonic fibroblast cells, implying the existence of other signaling pathways. In addition to RhoGAP proteins, Rho activity is also controlled by guanine-nucleotide exchange factors (RhoGEFs). Both $G\alpha_{12}$ and $G\alpha_{13}$ could interact with p115 RhoGEF, and $G\alpha_{13}$ could increase the activity of p115 RhoGEF (Hart et al., 1998; Kozasa et al., 1998). $G\beta\gamma$ could directly interact with several RhoGEFs (Cdc24 in yeast; Dbl, Ost, and Kalirin in mammalian cells), although no effect on the exchange activity had been observed yet from these direct interactions (Nern and Arkowitz, 1998; Nishida et al., 1999; Zhao et al., 1995). In addition, some RhoGEFs have been shown to be regulated by protein tyrosine phosphorylation (Aghazadeh et al., 2000). It is possible that heterotrimeric G proteins could control the activity of Rho by increasing the activity of positive regulator RhoGEF and/or by decreasing the activity of the negative regulator RhoGAP.

Experimental Procedures

Retrovirus Production

Retroviruses were produced in Phoenix-E-type cells. Human $G\beta_1$, $G\gamma_2$, $G\alpha_{12}$Q231L, $G\alpha_{13}$Q226L, $G\alpha_q$Q209L, $G\alpha_s$Q227L, and $G\alpha_i$Q205L (from Guthrie cDNA Resource Center) were each cloned separately into pBMN-IRES-GFP retroviral vector. Each clone was then transfected into Phoenix-E cells, a packaging cell line. After incubation for 48 hr in growth media, the media containing secreted retroviruses was harvested. That freshly harvested media was then applied to desired cells overnight in the presence of polybrene (Sigma). The cells were then washed and incubated with serum-free media for 48 hr before microscopy. The empty retrovirus was produced by transfecting Phoenix-E cells with empty pBMN-IRES-GFP vector.

Fluorescence Microscopy

Various mouse embryonic fibroblast cell lines were plated onto coverslips coated with gelatin. These cell lines included Csk^-, Csk^-/Csk, SYF, SYF/c-Src, and wild-type 3T3 cell lines. The Csk^- cells were from $Csk^{-/-}$ mouse embryos (Nada et al., 1994). The Csk^-/Csk cells were Csk^- cells stably transfected with Csk. SYF cells (from ATCC) were established from Src, Yes, and Fyn knockout mouse embryos (Klinghoffer et al., 1999). SYF/c-Src cells were SYF cells stably expressing c-Src (Ma et al., 2000). Control experiments were also done with wild-type 3T3 cell lines established from embryos of wild-type littermate mice. These cells were infected with retroviruses carrying G proteins where indicated. The expression of G proteins was monitored by IRES-linked GFP on the retroviral vector. The cells to which G protein retroviruses were not added were infected with empty retrovirus. In some experiments, Csk^- or Csk^-/Csk cells were transfected (with Effectene kit, Qiagen) with ORL-1-GFP plasmid (with or without 10-fold molar excess of $G\alpha_i$). Pretreatment with PTX (100 ng/ml) was for 12 hr. In some cases, after starvation for 48 hr, cells were treated with serum (10% FBS) for 5 min, LPA (15 ng/ml) for 5 min, and PDGF (10 ng/ml) for 30 min. The infected cells were starved in serum-free media for 48 hr prior to fixation. Cells were then fixed with 3.7% formaldehyde. The fixed cells were then permeabilized in 0.1% Triton X-100 for 5 min. After washing in PBS, phalloidin conjugated to rhodamine (Molecular Probes) in a solution containing PBS, and 1% BSA was added to stain actin. After incubation for 30 min at room temperature, the cells were washed extensively to reduce nonspecific interactions. The coverslips were then fixed onto slides and imaged using a Zeiss fluorescence microscope.

Preparation of Cell Membrane Permeable C3

The C3 exoenzyme was introduced into cells by making a fusion protein of the active domain of C3 and TAT protein of HIV-1 (Sauzeau et al., 2001). The pGEX-KG TAT-C3 clone was a generous gift of Dr. Michael Olson (Institute of Cancer Research, London). Recombinant fusion protein was produced in BL21 *E. Coli*. A one liter culture was grown to an OD_{600} reading of 0.6 and then induced by addition of 0.3 mM IPTG for 3 hr. Cells were flash frozen and then lysed by sonication in TBS with 5 mM $MgCl_2$ and 1 mM DTT. The supernatant was then incubated with glutathione Sepharose for 2 hr at 4°C. After extensive washing, the beads were cleaved with thrombin overnight at 4°C. The supernatant was collected and treated with 1 mM PMSF. The purified protein was applied to cells 6 hr prior to imaging at a final concentration of 0.5 μM.

Chemical Rescue

Three stable Csk^-/Csk mutant cell lines were produced to demonstrate the ability of imidazol to rescue mutant Csk activity. Wild-type human Csk, Csk(D314N), and Csk(R318C) in pcDNA3.1/hyg were transfected into Csk^- cells, and, after selection with hygromycin, stable cell lines were produced. These cells were cultured as in previous experiments, but, prior to fixation, the cells were incubated in media containing 50 mM imidazol (pH 7.5) for 30 min.

Csk Cellular Localization

In order to visualize Csk localization in cells, a Csk-GFP fusion construct was produced. After transfection of HEK-293T cells with Csk-GFP (with or without the indicated G-proteins; with a G protein/Csk-GFP plasmid DNA ratio of 10:1), the cells were transferred to poly(D) lysine-coated coverslips and incubated 48 hr in the absence of serum. For these studies with HEK-293 cells, G proteins in pcDNA3.1+ vector were used. FLAG-tagged $G\beta_1$ and HA-tagged $G\gamma_2$ plasmid DNAs (1:1) were cotransfected with Csk-GFP. After fixation, the coverslips were mounted on slides, and microscopy for GFP signal was performed with a fluorescence microscope.

Protein Purification

Recombinant Csk was purified from *E. Coli* as described (Sondhi and Cole, 1999; Ma et al., 2000). Purified Csk was detected by Coomassie staining and Western blot with anti-Csk antibody (Santa Cruz Biotechnology). The purified protein was desalted into storage buffer (20 mM Tris [pH 8.0] and 10% glycerol). G proteins were purified from SF9 cells as described (Kozasa and Gilman, 1995). The purified protein was concentrated and exchanged into Csk kinase buffer (40 mM Tris [pH 8.0], 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 10 mM DTT) and 5% glycerol for storage.

Kinase Assays

Purified Csk (final concentration 10 nM) was combined with 1.5 μg poly(Glu,Tyr) (Sigma). Purified $G\beta\gamma$ (200 nM) was added. Kinase buffer was added to bring reaction volume to 19 μl. Ten microcuries [γ-^{32}P]ATP (3000 Ci/mol) were added, and the mixture was incubated at 30°C for 20 min. Reactions were stopped by adding SDS-PAGE sample buffer. After incubation at 90°C for 5 min, the substrate was separated on an SDS-PAGE gel, dried, and autoradiographed.

Immunoprecipitation Kinase Assay

Immunoprecipitation kinase assay was performed as described (Ma et al., 2000). Relative Csk activities were shown normalized against the amount of Csk pulled down.

Rho Activation Assay

The Rho activity assay was performed as described (Sander et al., 1999). Various cell lysates were prepared as described above and normalized by Bradford assay, anti-Rho, and anti-actin Western blots. To each lysate was added 20 μg of GST-RBD (Rhotekin Rho binding domain) bound to glutathione beads, which was purified as described (Ren et al., 2001). After incubation at 4°C for 45 min, the beads were washed extensively in cell lysis buffer and loaded onto SDS-PAGE. Anti-Rho (Upstate Biotechnology) Western blot analysis demonstrated the amount of active Rho-GTP in each cell lysate.

MAP Kinase Assay

Cell lysates were produced as described above and normalized by Bradford assay and anti-actin Western blot. ERK MAP kinase assays

were performed using a kit from Cell Signaling Technology. Anti-phosphoERK antibody was used to immunoprecipitate activated ERK. This immunocomplex was used to phosphorylate GST-Elk-1 to measure ERK activity. Phospho-Elk-1 was detected with anti-phospho-Elk-1 antibody.

In Vitro Binding Assay

One microgram of G$\beta\gamma$ attached to Ni-NTA beads was mixed with 1 μg purified Csk in 300 μl binding buffer (20 mM Tris [pH 8.0] and 10 mM DTT), and complexes were allowed to form for 1 hr at room temperature. The beads were washed extensively in binding buffer plus 100 mM NaCl, and then elutions were performed with increasing NaCl concentrations (250–1000 mM). The eluates were run on SDS-PAGE and Western blotted with anti-Csk antibody (Santa Cruz Biotechnology).

GST Pull-Down Assay

GST pull-down assay was done as described (Ma et al., 2000).

Coimmunoprecipitation Assay

One milligram of NG108 cell lysate (after stimulation of endogenous mAChR with 100 uM carbachol for 5 min) was precleared with protein-A/G PLUS-agarose. Either monoclonal anti-Csk antibody (Transduction Laboratory) or control monoclonal anti-HA antibody was added. Immunocomplexes were pulled down with protein-A/G PLUS-agarose and washed extensively in lysis buffer. Complexes were run on SDS-PAGE and Western blotted with anti-Gβ antibody.

Bioluminescence Resonance Energy Transfer (BRET) Assay

The BRET assay was done with the BRET^2 kit purchased from BioSignal Packard (Canada) and performed according to the manufacturer's instructions and as previously described (Angers et al., 2000). Gβ_1 was PCR subcloned into the codon-humanized p*R*Luc fusion vector. Csk or c-Src was PCR subcloned into the codon-humanized pGFP fusion vector. Forty-eight hours after transfection, cells were seeded in triplicate in a 96-well microplate. Cells were washed twice with BRET buffer (Dulbecco's PBS containing $CaCl_2$ [0.1 g/l], $MgCl_2$ [0.1 g/l], and D-glucose [1 g/l]). An equal volume of BRET buffer containing 10 μM coelenterazine (DeepBlueC) was then added, and the microplate was agitated. The emission at 410 nm (for *R*Luc) and 515 nm (for GFP) was immediately acquired using a Fluoroskan Ascent FL (Labsystems) with microplate luminometric detection (with the excitation lamp turned off). The BRET signal was measured by the amount of green light emitted by GFP as compared to the blue light emitted by *R*Luc. The BRET ratio was calculated according to the manufacture's instructions as follows: (Emission at 515 nm – $Background_{515}$)/(Emission at 410 nm – $Background_{410}$), where $Background_{515}$ is the emission at 515 nm and $Background_{410}$ is the emission at 410 nm of a 10 μM solution of coelenterazine prepared in BRET buffer.

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