Role of Tyrosine Kinase Csk in G Protein-coupled Receptor- and Receptor Tyrosine Kinase-induced Fibroblast Cell Migration*

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Tyrosine kinase Csk is essential for mouse embryonic development. Csk knock-out mice died at early stages of embryogenesis (around embryonic day 10). The molecular mechanism for this defect is not completely understood. Here we report that Csk deficiency in mouse embryonic fibroblast cells blocked cell migration induced by lysophosphatidic acid through G protein-coupled receptors, by platelet-derived growth factor and epidermal growth factor through receptor tyrosine kinases, and by serum. Re-expression of Csk in these Csk-deficient cells rescued the migratory phenotype. Furthermore, deletion of Csk did not interfere with Rac activation and lamellipodia formation, but impaired the focal adhesions. Our data demonstrate a critical role for Csk in cell migration.

Protein-tyrosine kinase Csk (C-terminal Src kinase) was originally purified as a kinase capable of phosphorylating Src and other Src family kinases at their C-terminal tyrosine residues (1, 2). This phosphorylation suppresses the kinase activity of Src-family tyrosine kinases (such as c-Src, Fyn, and Lyn). Therefore, most physiological studies regarding Csk function have been focused on Csk as a negative regulator of Src-family tyrosine kinases. Csk is ubiquitously expressed in mammalian cells and is evolutionarily conserved from the early diverged metazoan Hydra to humans (3). The Csk−/− mouse embryos exhibited defects in neurulation, an inability to complete the turning process, and a failure of the allantois to connect with the chorion, preventing the formation of the umbilical cord and placenta (4, 5). The Csk-deficient mouse embryos died around embryonic day 10. Elegant mouse genetic studies revealed that Src−/−/Csk−/− mouse embryos showed partial rescue of Csk−/− phenotypes (6). However, Src−/−/Csk−/− mouse embryos still died around embryonic days 10–11, implying a Src-dependent and -independent function for Csk (6). Indeed, cellular and biochemical studies suggested that Csk also interacts with other proteins, such as protein-tyrosine phosphatase (PTP)2-PEST (7). Because of the embryonic lethal phenotype, the mechanisms that regulate adhesion formation and disassembly remain largely unknown.

Previously, we and others have shown a critical role of Csk in regulating actin cytoskeletal reorganization. However, a potential role of Csk in controlling cell migration has not been investigated. Here we have shown that deficiency of Csk blocked the MEF cell migration induced by various stimuli including lysophosphatidic acid (LPA) through its G protein-coupled receptors, by platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) through their receptor tyrosine kinases, and by serum. Hence, Csk plays a general regulatory role in cell migration. Although Csk is generally considered to play a negative role in cellular signaling, our data demonstrate a positive role for Csk in cell migration. Furthermore, we have shown that Csk deficiency led to impaired focal adhesions, thus providing a possible mechanism by which Csk controls cell migration.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The MEF, Csk−/−, Csk−/−/Csk, Csk−/−/Csk(D314N), and Csk−/−/Csk(R318A) cells were described before (11). MEF cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, streptomycin, and penicillin. PDGF-BB was from Sigma. LPA was from Cayman Chemical Company. Src-family kinase inhibitor PP2 was from Calbiochem.

Fluorescence Microscopy—Preparation of samples for fluorescence microscopy was performed as described previously (11). Cells were plated onto gelatin-coated glass coverslips. Cells were then fixed with 3.7% formaldehyde, washed three times with PBS, permeabilized with 0.1% Triton X-100 for 5 min, and then washed with PBS three times. To block nonspecific binding, the cells were incubated with a solution of PBS containing 1% bovine serum albumin for 30 min and then incubated with primary antibody diluted in 1% bovine serum albumin in PBS for 1 h. Anti-vinculin antibody (Sigma) was used at a 1:1000 dilution. Alexa Fluor 488-conjugated phallolidin (Molecular Probes) was used to visualize actin. After incubation with primary antibody, cells were washed three times with PBS and incubated with rhodamine-conjugated anti-mouse antibody (Molecu-
lar Probes). The coverslips were then fixed onto slides and imaged using a Zeiss fluorescence microscope.

**In Vitro Wound-healing Cell Migration Assay**—Cell migration assays were performed as described previously (12, 13). Cells were allowed to form a confluent monolayer in a 24-well plate coated with gelatin before wounding. The wound was made by scraping a conventional pipette tip across the monolayer. The migration was induced by adding medium supplemented with or without 10% FBS. For MEF cells, it typically took 8–10 h for the wound to close. When the wound for the positive control closed, cells were fixed with 3.7% formaldehyde and stained with crystal violet staining solution.

**Boyden Chamber Cell Migration Assay**—MEF cells (5 × 10^4) suspended in DMEM (or in DMEM + 0.5% FBS when PDGF, EGF, or LPA were present in the lower chamber) were added to the upper chamber of an insert (6.5 mm diameter, 8-μm pore size, Becton Dickinson), and the insert was placed in a 24-well dish containing DMEM with or without 10% FBS, 20 ng/ml PDGF, 25 ng/ml EGF, or 10 μg/ml LPA. In the case of C3 toxin treatment, 0.5 μg/ml was added to both the upper and lower chamber. Migration assays were carried out for 4 h, and cells were fixed with 3.7% formaldehyde. Cells were stained with crystal violet staining solution, and cells on the upper side of the insert were removed with a cotton swab. Three randomly selected fields (10× objective) were photographed, and the cells that had migrated were counted. The migration was expressed as a percentage of migrated cells in positive control MEF cells. Percentage was calculated with the following formula: \( P = \frac{100 \times (M - M_r)}{M_w} \), where \( P \) is the percentage of migrated cells, \( M \) is the number of migrated cells, \( M_r \) is the number of migrated cells in negative controls (DMEM only), and \( M_w \) is the number of migrated cells in positive controls.

**Western Blots**—Western blots were performed as previously described (14). Whole cell extracts were prepared as follows. Confluent cells were harvested from 10-cm plates, washed twice with cold phosphate-buffered saline, and pellets were resuspended in 0.8 ml of lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.03 mg/ml leupeptin). Resuspended pellets were sonicated, centrifuged at 5000 rpm for 5 min at 4 °C to remove insoluble material, and the supernatant was saved as the whole cell extract. Protein concentrations were measured by Bradford assay, and equal amounts of protein transfections were measured by Bradford assay, and equal amounts of protein were loaded onto a gel. After SDS-PAGE, protein samples were transferred to nitrocellulose filters. Membrane filters were incubated in 1× Tris-buffered saline, 5% milk for 1 h, and then incubated in primary antibody diluted 1:1000 in 5% milk/Tween solution for 1 h. After extensive washing, the bead was cleaved with thrombin overnight at 4 °C. The supernatant was collected and treated with 1 mM phenylmethylsulfonyl fluoride. The purified protein was applied to cells at a final concentration of 0.5 μM.

**Rho and Rac Activity Assays**—Cells were treated with 20 μM LPA (for Rho assay) or 20 ng/ml PDGF (for Rac assay) for 10 min and then washed with PBS. Cells were then lysed in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Thirty μg of GST-PBD (for Rac assay) or GST-RBD (for Rho assay) attached to beads were added to the cell lysates. Samples were incubated at 4 °C for 60 min, and then the beads were washed three times with lysis buffer. SDS sample buffer was added to the beads, and the samples were boiled at 90 °C for 10 min and run on 12% SDS-polyacrylamide gels. Rac and Rho were visualized by Western blot using anti-Rac antibody (clone 23A8, Upstate) and anti-Rho antibody (clone 26C4, Santa Cruz).

**Statistical Analysis**—Data are expressed as mean ± S.D. from three experiments and analyzed by one-way analysis of variance followed by Dunnett’s multiple comparison test with significance defined as \( p < 0.05 \).

**RESULTS**

**Deficiency of Csk Blocks MEF Cell Migration**—To investigate a possible role of Csk in cell migration, we have used two approaches to compare the migration of Csk-deficient cells and wild-type cells. One approach is the qualitative in vitro wound-healing assay, the other the quantitative Boyden chamber assay (12, 13). For the wound-healing assay, wild-type and Csk−/− MEF cells were grown to confluence. A wound (small scratch) was made in the middle of the tissue culture plate with a pipette tip. After ~12 h in the presence of serum, wild-type MEF cells migrated and covered the wound, yet Csk−/− cells did not migrate (Fig. 1). Therefore, serum-induced migration of Csk−/− cells was markedly reduced compared with the migration of wild-type MEF cells. These results were confirmed with Boyden chamber assays (Fig. 2A). To determine whether this failure of serum-induced migration of Csk−/− cells was the result of an absence of Csk, we re-expressed wild-type Csk in Csk−/− cells. As shown in Figs. 1 and 2A, Csk expression restored the migration of Csk−/− cells in response to serum. Furthermore, the Csk−/−/Csk rescue cell line expressed Csk at a lower level than the wild-type MEF cells as revealed by Western blot (Fig. 2B), indicating that the Csk protein level was not unphysiologically high in Csk−/−/Csk cells.

Different cell proliferation rates of wild-type MEF cells and Csk−/− cells might affect their migratory rates in wound-healing assays because these assay were performed over ~12 h (the chamber assay was performed over ~4 h). Thus, we examined the cell proliferation of wild-type MEF cells, Csk−/− cells, and Csk−/−/Csk rescue cells. As shown in Fig. 2C, proliferation of Csk−/−/Csk cells was slower than that of MEF or Csk−/− cells, thus, the faster rate of migration exhibited by Csk−/−/Csk cells is not because of a faster proliferation rate. Also, Csk−/− cells proliferate at a faster rate than MEF or Csk−/−/Csk cells, indicating that the lack of migration exhibited by these cells cannot be attributed to a slower rate of proliferation (Fig. 2C).

Because we used serum here as an inducer and serum contains various factors, it is likely that Csk plays a general role in controlling cell migration. Indeed, LPA, PDGF, and EGF all failed to induce Csk−/− cells to migrate, whereas they induced Csk−/−/Csk cell migration (Fig. 2D). LPA acts through G protein-coupled receptors, whereas PDGF and EGF work on receptor tyrosine kinases. These results demonstrate that a...
deficiency of Csk blocks cell migration induced by various factors and that Csk plays a general role in controlling cell migration.

The Kinase Activity of Csk Is Required for Cell Migration—Tyrosine kinases have other structural domains in addition to their catalytic kinase domains (16). Some of their physiological functions are mediated by other structural domains, independent of their kinase activity. To investigate whether the kinase activity of Csk is required for cell migration, we made use of two kinase-dead mutations of Csk (CskR318A and CskD314N).
Csk in Fibroblast Cell Migration

FIGURE 3. The catalytic activity of Csk is required for its role in cell migration. A, in vitro wound-healing assay showed that serum-induced migration of MEF cells, but not Csk−/−, Csk−/−/ Csk(R318A), and Csk−/−/Csk(D314N) cells. B, Boyden chamber assay showed that serum induced migration of MEF cells, but not Csk−/−, Csk−/−/ Csk(R318A), and Csk−/−/Csk(D314N) cells. C. Src family tyrosine kinases are involved in Csk-regulated cell migration. In Csk−/− cells, lower concentrations of PP2 increased the migration of Csk−/− cells, whereas higher concentrations of PP2 inhibited cell migration. The PP2 data are shown in two different types of plots. The left graph is for easy visualization of the increased and decreased cell migration. The right plot is the dose-response curve. Data represent mean ± S.D. of three experiments. *p < 0.05.

These mutations at the catalytic loop of Csk reduce the catalytic activity of the protein (11, 17, 18). As assayed with both wound-healing and chamber assays, stable lines of Csk−/− cells expressing CskR318A or CskD314N displayed impaired migration compared with Csk−/−/Csk and MEF cells (Fig. 3, A and B). The level of Csk mutant proteins in each of these cell lines is shown in Fig. 2B. Hence, these data demonstrate that the kinase activity of Csk is required for its role in cell migration.

The best studied physiological substrates for Csk are Src family tyrosine kinases. Regulation of actin cytoskeletal organization by Csk has been genetically demonstrated through Src family kinase-dependent as well as -independent pathways (6, 11). Therefore, we examined whether Src family tyrosine kinases are involved in Csk-regulated cell migration. In Csk−/− cells, the specific activity of Src is high. To study whether this higher Src activity contributed to the cell migration defect in Csk−/− cells, we treated Csk−/− cells with Src family tyrosine kinase inhibitor PP2 (Fig. 3C). The underlying assumption is that this higher Src activity might be detrimental to cell migration even though normal levels of Src activity or regulation might be required for cell migration. As shown in Fig. 3C, low concentrations of PP2 increased Csk−/− cell migration, suggesting that higher activity of Src family kinases is at least partly responsible for the Csk−/− phenotype of defective cell migration. On the other hand, higher concentrations of PP2 inhibited cell migration. Hence, these data demonstrate that Src family tyrosine kinases are involved in Csk regulation of fibroblast cell migration.

Deficiency of Csk Does Not Affect Rac Activation and Lamellipodia Formation—We have previously shown that Csk acts upstream of Rho in actin stress fiber formation induced by serum, LPA, and various G proteins (11). Here we have confirmed that Csk is indeed required for LPA-induced activation of Rho (Fig. 4A). To measure Rho activity in Csk−/− cells and Csk−/−/Csk cells, a Rho activation assay (GST-RBD pull-down) was performed. Rho activation was increased in Csk−/−/Csk cells in response to LPA. However, in Csk−/− cells, there was no increase (Fig. 4A). This Csk signaling through Rho to control actin cytoskeletal reorganization could provide a mechanism by which Csk regulates cell migration. Therefore, we examined whether Rho is
involved in MEF cell migration. *Clostridium botulinum* C3 exoenzyme is a specific inhibitor of Rho that works by irreversible ADP-ribosylation of Asn-41 in its effector region (19). We previously used this C3 toxin to block serum-induced Rho activation and actin stress fiber formation in MEF cells (11). Treatment with C3 toxin had no effect on the migration of Csk−/−/Csk and MEF cells (Fig. 2A), indicating that Rho activity is not required for fibroblast cell migration. This was consistent with our finding that inhibition of Rho had no effect on fibroblast cell migration and inhibition of Rho kinase (ROCK) actually increased the migration of fibroblast cells (13). In fast migrating cells such as macrophages and neutrophils, Rho and Rho kinase appear to be required for cell polarization and migration. In slow migrating cells such as fibroblasts, Rho kinase appears to inhibit migration (20).

Next, we investigated the role of Csk in Rac activation in fibroblast cells. An important step in cell migration is the activation of Rac and inhibition of Rho kinase appears to inhibit migration (20). Toward understanding the underlying mechanism, we found that Csk has no effect on PDGF-induced Rac activation and lamellipodia formation. However, Csk−/− cells displayed larger focal adhesions, consistent with impairment in focal adhesion turnover. Thus, Csk might critically control the cycling steps of cell migration, namely the detachment of adhesions and cell body contraction.

How does Csk control the repetitive turnover of focal adhesions during cell migration? We propose that Csk functions as a thermometer switch (Fig. 6). In our proposed model, extracellular signals such as integrin activate Src (probably through FAK). Src then phosphorylates p130Cas. Phosphorylated p130Cas forms a complex with Crk, in turn leading to p130Cas-Crk-Dock180 complex formation. In this complex, Dock180 catalyzes the activation of Rac, leading to the formation of focal complexes. As the number of focal adhesions increases, more paxillin is accumulated at the focal complexes. Therefore, more Csk will be recruited to focal adhesions by paxillin. Csk will in turn inhibit Src activity and at the same time activate the tyrosine phosphatase PTP-PEST, starting the focal adhesion disassembly process. When the numbers of focal adhesions decrease, less paxillin and, hence less Csk, will be present at the focal complex. This will allow new focal complexes to form. This cycling of Csk association with and dissociation from the focal complex regulates the repetitive turnover of focal adhesions during cell migration.

In essence, this model is based on extensive published data. We have incorporated a major regulatory role for Csk in this focal adhesion turnover. A role for the Src family tyrosine kinases in focal adhesion turnover is supported by published data (24, 25). In Csk−/− MEF cells, tyrosine phosphorylation of several focal adhesion proteins such as cortactin, tensin, FAK, and paxillin was increased (6, 26). Although cortactin and tensin hyperphosphorylation is Src-dependent, FAK and paxillin hyperphosphorylation is dependent on both Src and Fyn. In addition, Src tyrosine kinase has been shown to phosphorylate p130Cas to regulate p130Cas interaction with Crk (27). Furthermore, Csk−/− cells had reduced adhesion-induced tyrosine phosphorylation of p130Cas and Csk−/− cells showed enhanced phosphorylation of p130Cas (28). Src
Paxillin directly binds Csk and PTP-PEST (39–41). We are performing experiments to test this model. Hopefully, our study on the mechanism by which Csk regulates cell migration will shed light on the mechanism of cell migration and cancer metastasis and provide possible targets for chemotherapeutic drugs and markers to help clinicians assess tumor aggressiveness.

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REFERENCES

1. Okada, M., Nada, S., Yamanishi, Y., Yamamoto, T., and Nakagawa, H. (1991) J. Biol. Chem. 266, 24249–24252
2. Nada, S., Okada, M., MacAuley, A., Cooper, J. A., and Nakagawa, H. (1991) Nature 351, 69–72
3. Miller, M. A., Malik, I. A., Shenk, M. A., and Steele, R. E. (2000) Oncogene 19, 3925–3930
4. Imamoto, A. and Soriano, P. (1993) Cell 73, 1117–1124
5. Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M., and Aizawa, S. (1993) Cell 73, 1125–1135
6. Thomas, S. M., Soriano, P., and Imamoto, A. (1995) Nature 376, 267–271
7. Davidson, D., Cloutier, J. F., Gregoireff, A., and Veillette, A. (1997) J. Biol. Chem. 272, 23455–23462
8. Lauffenburger, D. A., and Horwitz, A. F. (1996) Cell 84, 359–369
9. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) Science 302, 1704–1709
10. Webb, D. J., Parsons, J. T., and Horwitz, A. F. (2002) Nat. Cell Biol. 4, 97–100
11. Lowry, W. E., Huang, J., Ma, Y. C., Ah, S., Wang, D., Williams, D. M., Okada, M., Cole, P. A., and Huang, Y. X. (2002) Dev. Cell 2, 733–744
12. Shao, D., Chen, L., Nijjaralson, J. T., Gaul, C., Ma, X., Danishefsky, S. J., and Huang, X. Y. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 3772–3776
13. Yang, S., and Huang, X. Y. (2005) J. Biol. Chem. 280, 27130–27137
14. Wang, Y., Kuroskai, T., and Huang, X. Y. (1996) Nature 380, 541–544
15. Sauerwe, V., Le Mellionnec, E., Bertoglio, J., Scalliba, E., Pacaud, P., and Loria, G. (2001) Circ. Res. 88, 1102–1104
16. Hunter, T., and Cooper, J. A. (1985) Annu. Rev. Biochem. 54, 897–930
17. Williams, D. M., Wang, D., and Cole, P. A. (2000) J. Biol. Chem. 275, 38127–38130
18. Williams, D. M., and Cole, P. A. (2002) J. Am. Chem. Soc. 134, 5566–5567
19. Udagawa, T., and McIntyre, B. W. (1996) J. Biol. Chem. 271, 12542–12548
20. Riento, K., and Ridley, A. J. (2002) Nat. Rev. Mol. Cell Biol. 4, 446–456
21. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
22. Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T., and Horwitz, A. F. (2004) Nat. Cell Biol. 6, 154–161
23. Bergman, M., Joukov, V., Virtalan, I., and Alitalo, K. (1995) Mol. Cell Biol. 15, 711–722
24. Thomas, S. M., and Brugge, J. S. (1997) Ann. Rev. Cell Dev. Biol. 13, 513–609
25. Playford, M. P., and Schaller, M. D. (2004) J. Biol. Chem. 279, 9792–9796
26. Nada, S., Okada, M., Aizawa, S., and Nakagawa, H. (1994) Oncogene 9, 3571–3578
27. Goldberg, G. S., Alexander, D. B., Pellicena, P., Zhang, Z. Y., Tsuda, H., and Miller, W. T. (2003) J. Biol. Chem. 278, 46533–46560
28. Fuji, K., Hiroi, H., Aizawa, S., and Rouaslti, E. (1996) Mol. Cell Biol. 16, 2606–2613
29. Li, L., Guiru, D. L., Okura, M., and Imamoto, A. (2003) Mol. Cell Biol. 23, 2883–2892
30. Li, L., Okura, M., and Imamoto, A. (2002) Mol. Cell Biol. 22, 1203–1217
31. Cloutier, J. F., and Veillette, A. (1996) EMBO J. 15, 4909–4918
32. Garton, A. J., Flint, A. J., and Tonks, N. K. (1996) Mol. Cell Biol. 16, 6408–6418
33. Cote, J. F., Charest, A., Wagner, J., and Tremblay, M. L. (1998) Biochemistry 37, 13128–13137
34. Angers-Loustau, A., Cote, J. F., Charest, A., Dwowbenko, D., Spencer, S., Lasky, L. A., and Tremblay, M. L. (1999) J. Biol. Chem. 274, 24479–24484
35. Kiyoshi, E., Hashimoto, Y., Kurata, T., Sugimura, H., and Matsuda, M. (1998) J. Biol. Chem. 273, 24479–24484
36. Kiyoshi, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) Genes Dev. 12, 3331–3336
37. Turner, C. E., Pietras, K. M., Taylor, D. S., and Molloy, C. J. (1995) J. Cell Sci. 108, 333–342
38. Sabe, H., Hata, A., Okada, M., Nakagawa, H., Hanafusa, H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3984–3988
39. Chen, Y., Schneider, G., Cloutier, J. F., Veillette, A., and Schaller, M. D. (1998) J. Biol. Chem. 273, 6474–6481
40. Cote, J. F., Turner, C. E., and Tremblay, M. L. (1999) J. Biol. Chem. 274, 20550–20560