We have shown previously that cytoskeletal reorganization (CSR) induced by pharmacological reagents such as colchicine or cytochalasins can up-regulate the urokinase-type plasminogen activator (uPA) gene via the Ras/Erk signaling pathway. In this present study using the small interfering RNA technique, we have found that ShcA adapter proteins play a rather active role in CSR-induced Erk activation, contrary to their mostly redundant role in other signaling pathways, e.g. growth factor-induced Erk activation, where Grb2 can bind directly to the receptor tyrosine kinase and activate Erk in the absence of ShcA. ShcA knockdown abolished CSR-induced activation of both Erk and the uPA promoter. Expression of small interfering RNA-escaping silent mutants of p52 or p46 but not p66 ShcA isoform efficiently rescued CSR-induced Erk activation. Moreover, we have shown that phosphorylation of either Tyr-239/Tyr-240 or Tyr-313 in p52ShcA can mediate CSR-induced Erk activation equally well. In a quest for molecules upstream of ShcA in this signaling, we found that CSR-induced ShcA tyrosine phosphorylation, its association with Grb2, Erk activation, and uPA gene expression were all dependent on Rho kinase, p38 mitogen-activated protein kinase, and Src. In summary, we have found a novel, non-redundant role for ShcA in contrast to its redundant role in many other signaling pathways.

The Shc family of adaptor/docking proteins is an important component of signaling pathways induced by various extracellular signals, such as growth factors, cytokines, and integrins, linked mainly to Ras activation (1–3). Upon ligand binding, activated receptor tyrosine kinases recruit and phosphorylate Shc proteins, which in turn recruit downstream signaling molecules. The most conspicuous of these is Grb2, which is constitutively complexed with Ras GTP exchange factor Sos. Thus, by activating Erk, one of the effector downstream molecules of Ras, Shc is involved in processes such as cell proliferation and differentiation (4–6).

There are three members of the Shc family, ShcA, ShcB, and ShcC, encoded by three different genes (7). ShcA is ubiquitously expressed, whereas ShcB and ShcC are expressed predominantly in the neuronal system (7–9). ShcA is expressed in three isoforms, p46Shc, p52Shc, and p66Shc, derived from a single gene through differential usage of transcription/translation initiation sites and alternative splicing, which differ in their amino-terminal sequence. The three proteins share an amino-terminal phosphotyrosine binding, a carboxyl-terminal Src homology 2, and a central CH1 domain (7, 10). p66Shc has an additional amino-terminal collagen homology-like domain denoted as CH2. Although all three isoforms of ShcA contain three conserved tyrosine residues (Tyr-239/Tyr-240 and Tyr-317) within the CH1 domain that are phosphorylated by activated tyrosine kinases and serve as docking sites for the Grb2-Sos complex (3, 11), we have reported recently that they differ from each other with respect to their serine/threonine phosphorylation patterns (12). Ubiquitous expression of p52Shc/p46Shc and the presence of p66Shc in most cells other than those of hematopoietic lineage suggest a distinct biological role for each isoform (13).

ShcA gene knockout in mice results in death at day 11.5 of embryogenesis, demonstrating an important role for ShcA in development (14). Using two different genetic approaches, inducible expression of a phosphorylation-defective mutant of Shc in transgenic mice and conditional knockout of Shc in thymocytes, Zhang et al. (15) have shown that Shc plays an essential and non-redundant role in T cell development. Some studies have suggested isoform-specific functions. Overexpression of p52Shc/p46Shc exerted a positive effect on growth factor-induced c-fos promoter activity, but p66Shc expression showed a negative effect (13). Isoform-specific gene knockout of p66Shc increased mouse longevity, most likely through suppressing oxidative stress-induced apoptosis (16). Although expression of the largest isoform is not essential for development (16), the benefit to the organism of this particular isoform remains to be elucidated. Likewise, it is not clear how important ShcA is in the regulation of growth factor-induced Ras/Erk signaling. In several systems, Grb2 has been shown to be recruited directly to the activated receptor tyrosine kinases, leading to Ras activation (17–21). In line with this, growth factor-induced Erk activation seems to proceed normally in ShcA+/− fibroblasts (14). In this system, however, ShcA seemed to be required for sensitizing cells and giving full induction of Erk activity at low concentrations of growth factor (14). Based on these observations, it has been suggested that p52Shc/p46Shc act as amplifiers of receptor tyrosine kinase-mediated signaling in pathways leading to Ras activation and involving a Grb2-Sos complex (10).

The cytoskeleton, consisting of actin, microtubule, and intermediate filaments, is a highly organized architectural entity that regulates cell shape and size and is associated with diverse functions depending on cell type (22). Cells respond to changes in cytoskeletal networks by inducing expression of specific genes through specific signaling cascades (23–29). Of particular interest among the signaling molecules activated by cy-
endogenous gene. Using this approach, we have determined the signaling molecules upstream of ShcA in Cytoskeletal Reorganization-induced Signaling.

We have shown previously that, as is usually the case, a gene of interest involves, where various signaling molecules co-localize, including ShcA (25). ShcA tyrosine phosphorylation and its association with focal adhesion kinase (FAK) and Src after CSR were also observed, suggesting a role for these proteins in CSR-induced Erk activation and subsequent UPA induction (28). However, it has not been demonstrated that ShcA is essential to this process as Grb2 can be recruited directly to FAK (34). Also undermined is the possible relative contribution of each ShcA isoform toward Erk activation.

FAS (31) has been rather difficult to study the role of individual isoforms in a clear background (i.e., without the influence of other isoforms) when, as is usually the case, a gene of interest is expressed in multiple isoforms in a given cell. Recently, we developed a system, termed knockdown-in (35), in which isoform-specific down-regulation and expression are achieved utilizing siRNA-mediated RNA interference. In this method, a specific isoform is expressed from an expression vector with silent mutations within the region where siRNA targets the endogenous gene. Using this approach, we have determined the dispensability of ShcA for CSR-induced Erk activation. We show that either p52Shc or p66Shc alone is enough to mediate Erk activation after CSR but that p66Shc does not play a role in this regulation, and we show that phosphorylation of either Tyr-313 (equivalent to Tyr-317 in human ShcA) or Tyr-239/Tyr-240 is required for either pathway. We also show signaling molecules upstream of this pathway in CSR-induced Erk signaling.

MATERIALS AND METHODS

Materials—Cytocidalin D (CytD) was purchased from Sigma. Luciferin was from Chemie Brunschwig AG. TPA, horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies, ECL reagents, and protein A- and G-Sepharose were from Amersham Biosciences. Anti-ShcA polyclonal and antiphospho-ShcA Tyr-416 polyclonal antibodies were obtained from Transduction Laboratories. Antiphospho-Erk, antiphospho-p38 MAP kinase, and antiphospho-Src (Tyr-416) polyclonal antibodies were from Cell Signaling, and anti-Erk polyclonal and antiphospho-p38 MAP kinase polyclonal antibodies were obtained from Santa Cruz. Mouse monoclonal antibodies against hemagglutinin (HA; 12CA5) and against phosphotyrosine (4G10) were purified on a protein A-Sepharose column. Anti-Src mouse monoclonal antibody (clone 327) was a gift from Dr. Kurt Ballmer. SB203580 and CPG77675 were kindly provided by E. Blum (Novartis AG).

Cells and Transfections—LLC-PK1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (AMIMED, Allschwil, Switzerland), 0.2 mg/ml streptomycin, and 50 units/ml penicillin at 37 °C in a humidified incubator with 5% CO2. For serum starvation, cells were incubated in Dulbecco's modified Eagle's medium containing 0.1% fetal calf serum. Cells were transfected with siRNA as described previously (35). Briefly, a day before transfection, cells (106/well) were plated in 6-well plates in medium without antibiotics, and the next morning siRNAs were transfected into cells using the LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions, with 10 μl of 20 μM siRNA and 5 μl of transfection reagent/well.

Analysis of Reporter Gene Expression—One day after siRNA transfection, cells were replated in 6-well plates. On the next day, cells were co-transfected with a reporter plasmid and the Renilla control plasmid using the calcium phosphate precipitation method (Amersham Biosciences). After 6 h of transfection, cells were starved for 16 h followed by treatment with CytD for 6 h, and luciferase expression was measured as described previously (36) and normalized against Renilla expression.

Stable Cell Lines—Stable cell lines were generated as described previously (12). Briefly, a plasmid encoding mouse wild-type p46Shc, p52Shc, or p66Shc mutant p66Shc S36A, p66Shc T29A, p66Shc S36A/T29A, p52Shc Y313F, or p52Shc Y239F/Y240F was co-transfected with the plasmid pX343 (37) expressing a hygromycin resistance gene in LLC-PK1 cells, at a ratio of 9:1 by the calcium phosphate method. All shA-encoding plasmids contained two silent mutations at the site of targeting siRNA (see below). Stable cell lines were selected by culturing cells with 3 mg/ml hygromycin B. Clones were isolated and screened for ShcA expression by immunoblotting with anti-HA antibody. The parent cell line transfected with pcDNA3 plus pX343 was used as a control.

Plasmids—Construction of expression vectors for HA-tagged mouse p46Shc, p52Shc, and p66Shc mutant p66Shc S36A, p66Shc T29A, p66Shc S36A/T29A, p52Shc Y313F, or p52Shc Y239F/Y240F was co-transfected with the plasmid pX343 (37) expressing a hygromycin resistance gene in LLC-PK1 cells, at a ratio of 9:1 by the calcium phosphate method. All shA-encoding plasmids contained two silent mutations at the site of targeting siRNA (see below).

Stable cell lines were generated as described previously (35). Tyr-239/Tyr-240 in p52Shc (equivalent to Tyr-317 in human p52Shc) were mutated to Phe with the Quick Site mutagenesis kit (Stratagene) using overlapping oligonucleotides 5'-CCT GAC CAT CAG TTC TCT AAT GTC TCC GGA AAG GAA C-3' and 5'-TGG AAA GTC ATT GAA GGG GTC TGG CTC GGA GGA GGA GCA GAG GAA C-3' as well as 5'-GAT GAC CCC TCT TTT GTC AAT CCC CAG AAT CTA GAC G-3' and 5'-CTG GAT GTT GAC AAA GGA GGG GTC ATC GAA GAG G-3', respectively. Similarly, Ser-36 and Thr-29 in p66Shc were mutated to Ala using 5'-GGG TCT GCC TCT GCC CCG GAG GAG GGG GGG GGG GGT CCG CAG CCG CAG GAA GGA GGA GGA GGA GCA GAG GAA C-3' and 5'-CTG GAT GTT GAC AAA GGA GGG GTC ATC GAA GAG GGA GGA GGA GCA GAG GAA C-3', respectively. Similarly, Ser-36 and Thr-29 in p66Shc were mutated to Ala using 5'-GGG TCT GCC TCT GCC CCG GAG GAG GGG GGG GGT CCG CAG CCG CAG GAA GGA GGA GGA GGA GCA GAG GAA C-3' and 5'-CTG GAT GTT GAC AAA GGA GGG GTC ATC GAA GAG GGA GGA GGA GCA GAG GAA C-3', respectively. Similarly, Ser-36 and Thr-29 in p66Shc were mutated to Ala using 5'-GGG TCT GCC TCT GCC CCG GAG GAG GGG GGG GGT CCG CAG CCG CAG GAA GGA GGA GGA GGA GCA GAG GAA C-3' and 5'-CTG GAT GTT GAC AAA GGA GGG GTC ATC GAA GAG GGA GGA GGA GCA GAG GAA C-3', respectively. Similarly, Ser-36 and Thr-29 in p66Shc were mutated to Ala using 5'-GGG TCT GCC TCT GCC CCG GAG GAG GGG GGG GGT CCG CAG CCG CAG GAA GGA GGA GGA GGA GCA GAG GAA C-3' and 5'-CTG GAT GTT GAC AAA GGA GGG GTC ATC GAA GAG GGA GGA GGA GCA GAG GAA C-3', respectively.

An siRNA corresponding to nucleotides 753–773 of the firefly luciferase gene (697–717) was designed as described previously (38). An siRNA corresponding to nucleotides 753–773 of the firefly luciferase gene (697–717) was designed as described previously (38).

Immunoprecipitation and Western Blot Analysis—LLC-PK1 cells were transfected with siRNA as described above. One day after siRNA transfection, cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (AMIMED, Allschwil, Switzerland). They were transfected using LipofectAMINE (Invitrogen) according to the manufacturer's instructions with 10 μl of 20 μM siRNA and 5 μl of transfection reagent/well.

Erk Kinase Assay—LLC-PK1 cells were transfected with siRNA as described above. On the second day after transfection, cells were transfected with HA-Erk2 plasmid using LipofectAMINE according to the manufacturer's instructions in a serum-free medium; 5 h later serum was added to 10%. After 10 h of transfection, cells were starved with 0.1% fetal calf serum for 1 h. Cells were transfected with CytD and lysed, and HA-Erk2 was immunoprecipitated as described above. The immunoprecipitated HA-Erk2 was used for a kinase assay as described previously (39).

Immunofluorescence—Cells were transfected with siRNA as described above.
concentrations of CytD for 30 min. Cells were lysed, and 12 μg of total cellular proteins were resolved by SDS-PAGE (top) and analyzed by Western blotting using anti-Erk or antiphospho-Erk antibodies. Alternatively, 300 μg of total cellular proteins were immunoprecipitated (IP) with anti-Grb2 antibodies, resolved by SDS-PAGE (bottom), and probed with antiphosphotyrosine, anti-Shc, and anti-Grb2 antibodies. B, time course of CytD-induced Erk activation and ShcA tyrosine phosphorylation. Cells were starved and stimulated with CytD (3 μM) for the times indicated. Erk activation, ShcA tyrosine phosphorylation, and Grb2 association with ShcA were analyzed as described under A.

As shown above, and 2 days later they were starved in 0.1% FBS for 16 h and stimulated with CytD for 30 min. Cells were then washed with PBS, fixed in 1 ml of prewarmed 3% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 min, blocked with 5% normal goat serum for 20 min, and incubated with a polyclonal antiphospho-Erk (1:100) antibody in PBS containing 1% goat serum for 2 h. Cells were washed twice with PBS (10 min/wash), incubated with secondary Alexa-fluor anti-rabbit goat antibody (1:100, Molecular Probes) and phallolidin (1:100) for 40 min, and washed three times with PBS (10 min/wash). To visualize nuclei, DAPI (4',6-diamidino-2-phenylindole, 1:5000) was added during the last wash. Coverslips were mounted on glass slides with Fluoromount (Serva). Fluorescence was visualized with a Zeiss Axioplan 2 fluorescence microscope, and all images were captured at ×600 magnification.

RESULTS

CSR-induced ShcA Tyrosine Phosphorylation, Grb2 Association, and Erk Activation—LLC-PK1 cells treated with varying concentrations of CytD were analyzed for ShcA tyrosine phosphorylation, its association with Grb2, and Erk phosphorylation. As shown in Fig. 1A, CytD induced all these events at concentrations as low as 0.25 μM and exhibited optimal effects at 1 μM. In a kinetic analysis, Erk phosphorylation was observed after 15 min of CytD treatment, with a maximum at 30 min. This level was maintained for 1 h of treatment but declined slowly thereafter. Enhanced Erk phosphorylation was detected even after 4 h of treatment (Fig. 1B). Tyrosine phosphorylation of ShcA and its association with Grb2 occurred as early as 5 min, reached a maximum at 15 min, and stayed at high levels until 4 h after treatment (Fig. 1B).

ShcA Is Essential for CSR-induced Erk Activation—ShcA has been considered an important component of receptor tyrosine kinase-mediated signaling leading to Erk activation. However, based on results with ShcA−/− fibroblasts, it has been suggested recently that ShcA proteins are dispensable but act as amplifiers in growth factor-induced signaling (10). Although we showed previously that CytD induces ShcA tyrosine phosphorylation just like growth factor treatment (28), the role of ShcA in CSR-induced Erk activation was not determined. Here, we used siRNA to address this question and found that knockdown of ShcA completely abolished CSR-induced Erk activation (Fig. 2A). The suppression of Erk activation was not due to a nonspecific effect of RNA interference on the Ras/Erk signaling pathway as TPA- and growth factor-induced Erk activation was not affected (Fig. 2A and data not shown). The result suggests an essential role for ShcA in CSR-induced Erk activation. To be sure that inhibition of CSR-induced Erk activation by ShcA siRNA is a genuine effect of RNA interference, we performed rescue experiments using a modified knockdown-in protocol (35). In this experiment, we first prepared stable LLC-PK1 cell lines expressing ShcA isoforms from vectors with silent mutations at the targeting site of siRNA and then down-regulated endogenous ShcA by transfecting with ShcA siRNA. As shown in Fig. 2B, knockdown of ShcA in control cells completely abolished CSR-induced Erk phosphorylation/activation. This could be rescued effectively by expressing silent mutants of either p52Shc (Fig. 2, B and C) or p46Shc (Fig. 2C) but not of p66Shc (Fig. 2B) as shown by Western blot analysis and in vitro Erk kinase assays. Further analysis showed, in accordance with these results, that CSR-induced Erk localization to the nucleus was suppressed by transfection with Shc siRNA and that this suppression could be overcome in cells expressing silent mutants of p46Shc and p52Shc but not of p66Shc (Fig. 2D). Thus, CSR-induced phosphorylation, activation, and nuclear localization of Erk are strictly dependent on the presence of either of the two shorter isoforms of ShcA.

Involvement of ShcA Tyrosine Phosphorylation in CSR-induced Erk Activation—ShcA is phosphorylated by receptor or non-receptor tyrosine kinases on three tyrosine residues, Tyr-317 (Tyr-313 in mouse) and the twin Tyr-239/Tyr-240 (11). Because phosphorylation at these residues has been attributed to different functions of ShcA (40, 41), we looked at the relative importance of these tyrosine residues in CSR-mediated Erk activation by a rescue experiment similar to that described above. Stable cells lines expressing silent mutants of p52Shc Y313F, p52Shc Y239F/Y240F, and p52Shc Y239F/Y240F/Y313F (3YF) were prepared. Suppression of CSR-induced Erk activation by ShcA siRNA was rescued by the expression of either p52Shc Y313F or p52Shc Y239F/Y240F/Y313F (3YF) (Fig. 3A) by not by the triple tyrosine mutant p52Shc 3YF (Fig. 3B). These results indicate that CytD treatment can induce ShcA tyrosine phosphorylation at either of these residues, which contributes equally well to the mediation of Erk activation. To confirm this, we examined tyrosine phosphorylation of these mutants and their association with Grb2. As shown in Fig. 3C, CytD treatment induced tyrosine phosphorylation of the p52Shc WT, Y313F, and Y239F/Y240F mutants but not of 3YF. This tyrosine phosphorylation pattern correlated with the ability to associate with Grb2 (Fig. 3C, bottom).

ShcC Can Replace ShcA in CSR-induced Erk Activation—Two other members of Shc family, ShcC and ShcB, play an
adaptor role similar to ShcA in the neural system (42), and their importance in neural development has been well demonstrated in vivo (43). ShcC, however, was found to be less efficient than ShcA in mediating nerve growth factor-induced Erk activation in PC12 cells because it has only one high affinity binding site for Grb2 compared with two such sites in ShcA (44). Therefore, we investigated whether ShcC is as efficient as ShcA in mediating CSR-induced Erk activation in our non-

FIG. 2. Shc indispensability in CytD-mediated Erk activation. A, ShcA knockdown blocked CytD-induced Erk activation. Cells were transfected with buffer, Shc or control siRNA as described under “Materials and Methods,” starved for 14 h, and left untreated or treated with 3 μM CytD or 100 ng/ml TPA for 30 or 10 min, respectively. Equal amounts of total cellular proteins were resolved by SDS-PAGE, and Western blot analysis was performed using specific antibodies against Erk, phospho-Erk, and Shc. unstim., unstimulated. B, rescue of CytD-induced Erk activation by p52Shc. Western blot analysis. Stable cell lines expressing empty vector or silent mutants of HA-p52Shc or HA-p66Shc were prepared. Cells were transfected with Shc siRNA, control siRNA, or buffer alone. After starvation for 14 h, cells were treated with 3 μM CytD and collected. Erk activation and Shc knockdown were analyzed by Western blot using specific antibodies. sm, silent mutant. C, rescue of CytD-mediated Erk activation by p46Shc and p52Shc; in vitro kinase assay. Cells were transfected with siRNAs as described above, and HA-Erk2 was transfected 2 days later as described under “Materials and Methods.” After starvation for 14 h, cells were stimulated with 3 μM CytD for 30 min and lysed. HA-Erk2 was immunoprecipitated (IP) using anti-HA antibodies. The immunoprecipitates were incubated with myelin basic protein (MBP) in the presence of [γ-32P]ATP, resolved by SDS-PAGE, and autoradiographed. HA-Erk2 was also analyzed by Western blotting using anti-Erk and antiphospho-Erk antibodies. D, rescue of CytD-mediated Erk activation by p46Shc and p52Shc; indirect immunofluorescence. The stable cell lines indicated were transfected with siRNA, starved, and stimulated with CytD as described above. Cells were fixed and permeabilized, and the actin cytoskeleton and phospho-Erk were visualized by indirect immunofluorescence using phalloidin and antiphospho-Erk antibodies, respectively, as described under “Materials and Methods.”
neuronal LLC-PK1 cell system. For this we prepared stable cell lines expressing either of the mouse ShcC isoforms p55ShcC and p69ShcC (45) and addressed the question by further rescue experiments. As shown in Fig. 4A, both isoforms of ShcC were able to rescue ShcA siRNA-suppressed CSR-induced Erk activation. Interestingly, in contrast to p66ShcA (46), the p69ShcC isoform did rescue Erk activation (Fig. 4A, cf. Fig. 2B).

If p69ShcC could mediate CSR-induced Erk activation, why could p66ShcA not? Serine/threonine phosphorylation of p66ShcA has been attributed to its inability to associate with activated EGF receptor and thus to mediate Erk activation (46). We showed previously that Thr-29 and Ser-36 in the CH2 domain of p66ShcA are phosphorylated upon TPA and growth factor treatment (12). To see whether possible serine/threonine phosphorylation of p66ShcA is responsible for its inability to mediate CSR-induced Erk activation, we mutated these serine and threonine residues and made stable cell lines expressing the double mutant p66ShcA S36A/T29A. As shown in Fig. 4B, this double mutant was still unable to rescue Erk activation, indicating that it is not the serine/threonine phosphorylation of p66ShcA that prevents it from mediating CSR-induced Erk activation. Because tyrosine phosphorylation is necessary for Shc to recruit the downstream Grb2-Sos complex, we next sought the difference in tyrosine phosphorylation between p66ShcA and p69ShcC. As shown in Fig. 4C, EGF induced tyrosine phosphorylation in both p66ShcA (with either wild type or the S36A/T29A mutant) and p69ShcC, but CSR induced tyrosine phosphorylation only in p69ShcC. These results suggest that tyrosine phosphorylation is important for Erk activation and that the CH2 domain of p66ShcA specifically interferes with the CSR-induced mechanism responsible for ShcA tyrosine phosphorylation.

Involvement of Src, p38 MAP Kinase, and Rho Kinase Upstream of ShcA in CSR-induced Erk Activation—Src and p38 MAP kinase have been shown to be involved in Erk activation and gene expression induced by microtubule or actin filament-disrupting drugs (32, 47). In addition, we have shown that a dominant negative mutant of Src inhibits CSR-induced uPA promoter activation in LLC-PK1 cells (28). Consequently, we examined whether Src and p38 MAP kinase are involved in CSR-induced ShcA tyrosine phosphorylation and Erk activation. As shown in Fig. 5A, CGP77675 and SB203580, Src-specific and p38 MAP kinase-specific inhibitors (48, 49), respectively, attenuated CSR-induced Erk activation. The two inhibitors also inhibited ShcA tyrosine phosphorylation and its association with Grb2 (Fig. 5B), suggesting that both Src and p38 MAP kinase act upstream of ShcA in CSR-induced Erk activation. Rho proteins are important for regulating the actin filament network, and their inhibition by C3 exoenzyme has been shown to increase EGF receptor tyrosine phosphorylation and Erk activation (50). Therefore, it is possible that Rho proteins mediate the effect of CSR on ShcA tyrosine phosphorylation and Erk activation.

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cytoskeleton, and RhoA has been shown to be activated after treatment with CytD in Swiss 3T3 cells (50). Therefore, we tested the involvement of Rho kinase in Erk activation using a specific Rho kinase inhibitor, Y27632 (49). Treatment with Y27632 completely inhibited both Erk activation and ShcA tyrosine phosphorylation (Fig. 5C), indicating a potential role for Rho kinase in CSR-induced signaling.

Src, p38 MAP Kinase, and RhoA in CSR-induced Erk Activation—We examined whether these molecules are actually activated by CSR and, if so, how they are related to each other. Fig. 6A shows that CSR enhanced Src activation more than 2-fold (top), p38 MAP kinase 1.8-fold (middle), as measured by their phosphorylation status, and RhoA 3-fold (bottom), as measured by RhoA bound to GST-RBD as described previously (50). Interrelationships between the kinases were assessed by the effects of specific inhibitors. Note that these inhibitors inhibit the enzymatic activities of the target kinases and should not inhibit their own phosphorylation unless they are autophosphorylated directly or indirectly. CSR-induced Src phosphorylation was attenuated by its own inhibitor CGP77675, Rho kinase inhibitor Y27632, and the p38 MAP kinase inhibitor SB203580 (Fig. 6B), suggesting that Src is involved in its own activation and that Rho kinase and p38 MAP kinase are upstream of Src. However, activation of p38 MAP kinase was also inhibited by Src inhibitor, indicating a possible interdependence between these two molecules for activation (Fig. 6C). SB203580 also inhibited p38 MAP kinase activation, suggesting also the involvement of p38 MAP kinase autophosphorylation, directly or indirectly, as for Src in response to CSR. Rho kinase activation was not inhibited by Src and p38 MAP kinase inhibitors, showing that Rho is upstream of both Src and p38 MAP kinases (data not shown). We also showed previously that CytD causes Shc-FAK and Src-FAK association (28). Therefore, we asked whether Shc-FAK association is also affected by inhibition of these upstream kinases. As shown in Fig. 6D, treatments with inhibitors of Rho kinase, p38 MAP kinase, and Src all blocked Shc association with FAK.

Requirement of Shc, Rho, and p38 MAP Kinase for CSR-induced uPA Promoter Activation—To examine the biological relevance of the involvement of ShcA and Rho in CSR-induced Erk activation, we examined whether CSR-induced uPA gene expression involved ShcA and Rho. We showed previously that CytD induces uPA gene expression by activating the Ras/Erk signaling pathway (25). Consequently, the involvement of ShcA...
in CSR-induced uPA gene expression was examined by transient transfection assays. CSR induction of uPA promoter activity was completely suppressed by ShcA siRNA pretreatment (Fig. 7A), but the control siRNA also showed some inhibitory effect (about 20%), which could be a nonspecific effect of siRNA on the uPA promoter because we did not notice any such effect on CytD-induced Erk activation. uPA mRNA induction by CytD was partially suppressed by SB203580 (20%) and Y27632 (27%) (Fig. 7B). This indicates that p38 MAP kinase and Rho kinase play a role in CSR-induced uPA gene expression. CSR induction of the uPA promoter assessed by transient transfection assays was efficiently inhibited (71%) by pretreatment with Y27632. This is comparable with the inhibition by PD98059, a potent mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor (Fig. 7C).

DISCUSSION

In this work, we have shown that Shc plays an indispensable role in CSR-induced Erk activation, unlike growth factor-induced signaling, where Shc or Grb2 transduces signaling equally well (20, 21). Knockdown of ShcA by siRNA completely abolished Erk activation after CytD treatment but not after TPA (Fig. 2A) or EGF treatment (data not shown), and this inhibition was suppressed by the expression of silent mutants of different isoforms of ShcA. Moreover, the inhibitory effect of ShcA knockdown on Erk activation and its rescue by either of
the p52Shc/p46Shc isoforms but not by p66Shc were monitored in three different ways to make sure that Erk activity after CSR is really controlled in an ShcA-dependent manner, namely Western blotting using phospho-specific antibodies, in vitro kinase assay, and immunocytochemistry for nuclear localization (Fig. 2, B–D). Because Erk activation is involved in CSR-induced uPA gene expression, we expected and observed uPA gene down-regulation by ShcA knockdown. The inability of p66Shc to rescue CSR-induced Erk activation in ShcA knock-down experiments may correspond to the previous reports showing that p66Shc is a negative regulator of EGF-induced Erk activation and c-fos promoter activation (13, 46). The negative regulatory effect of p66Shc was attributed to its serine phosphorylation because serine-phosphorylated p66Shc associated with Grb2 but was unable to associate with tyrosine-phosphorylated receptor, thus acting in a dominant-interfering manner (46). In our case, however, p66Shc did not act in a dominant-interfering manner; in contrast to EGF stimulation, p66Shc was not phosphorylated on tyrosine residues by CytD treatment and did not compete with other isoforms for Grb2 binding. Furthermore, a serine/threonine mutant of p66Shc in the CH2 domain was also unable to rescue Erk activation, suggesting that p66Shc is not involved, either positively or negatively, in CytD-induced Erk activation (Fig. 4B).

In a quest for signaling molecules that link CSR to ShcA activation/phosphorylation, we identified several essential molecules, including Rho kinase, p38 MAP kinase, Src, and FAK. CSR brought about by CytD or colchicine treatment has been shown to lead to Src activation and subsequent downstream signaling (29, 47, 51). RhoA and p38 MAP kinase have been shown to be required for CSR-induced cyclooxygenase 2 or matrix metalloproteinase gene expression (32, 52). Overexpression of dominant negative RhoA inhibited taxol- and CytD-induced COX2 gene expression (32). Taking into consideration these results and previous reports from our lab (25, 28), the present work has generated a CSR-induced signaling pathway leading to uPA gene expression shown schematically in Fig. 8 and discussed in the following two paragraphs.

ShcA can be phosphorylated on three conserved tyrosine residues in the CH1 domain, Tyr-239, Tyr-240, and Tyr-317,
which, once phosphorylated, are recognized by the Src homology 2 domain of Grb2. It has been shown that Tyr-239/Tyr-240 and Tyr-317 are phosphorylated by different kinases (53, 54), and they are suggested to have different functions in cellular signaling, being linked to c-Myc and Erk MAP kinase activation, respectively (40). In rescue experiments with tyrosine mutants of p52Shc, we found that both Tyr-239/Tyr-240 and Tyr-313 are phosphorylated in response to CytD and can mediate Erk activation independently of each other. However, as suggested previously, the upstream kinases for these tyrosines may be different (19, 54). Schlaepfer et al. (19) reported that FAK phosphorylated Shc-Tyr-317 in vivo after fibronectin receptor integrin stimulation and in vitro. They also showed in vitro phosphorylation of Tyr-239/Tyr-240 of ShcA by Src. Preferential phosphorylation of Tyr-239/Tyr-240 by Src was also reported by van der Geer et al. (11). Similarly, using the Src family-specific inhibitor SU6656, Blake et al. (54) demonstrated that Tyr-239/Tyr-240 are phosphorylated by Src and Tyr-317 by platelet-derived growth factor receptor. Furthermore, Tyr-239/Tyr-240 phosphorylation was necessary for Src-mediated Myc induction and DNA synthesis. Based on our previous report that CSR induces FAK association with Src and ShcA (28), we showed that FAK phosphorylation rules out the possibility that Src phosphorylates ShcA at Tyr-239/Tyr-240 as well as FAK in the activation loop after activation by CytD treatment. FAK in turn phosphorylates ShcA at Tyr-313 (Fig. 8). The fact that ShcA knockdown completely blocked CytD-induced Erk activation rules out the possibility that Src phosphorylates ShcA at Tyr-925, a binding site for Grb2. Grb2 binding to Tyr-925 in FAK can also mediate fibronectin-induced Erk activation (19), which is not the case for CytD-induced Erk activation.

Interestingly, p38 MAP kinase and Src were mutually dependent for activation, and both activities were required for CSR-induced activation of ShcA as well as FAK. As CSR induces the association of Src and FAK (28), it is also possible that FAK is involved in the mutual dependence on CSR-induced activation. With these observations, we would like to introduce a new concept of “system field,” in which the potential of the field is elevated as a result of mutual activation of its components; the system as a whole acts as an upstream regulator (Fig. 8). The interaction between the components of this system is currently under investigation. In our scheme, the question remains of how CSR is coupled to RhoA activation. As RhoA activation triggers polymerization of actin filaments, disruption of the filaments may induce feedback regulation involving RhoA activation to restore the actin cytoskeleton. The immediate signals induced by CSR still remain to be identified. ShcB and ShcC, the other Shc family members, are involved in receptor tyrosine kinase signaling in the neural system. Mouse ShcC encodes two isoforms, p55ShcC and p69ShcC, which correspond to p52ShcA (p52Shc) and p66ShcC (p66Shc), respectively (45). Recently, Nakamura et al. (44) compared the efficacy of ShcA and ShcC in transducing NGF-induced Erk activation in PC12 cells and found that ShcA was more efficient than ShcC. This was due to the presence of only one high affinity Grb2 binding site in ShcC, compared with two such
sites in ShcA. ShcC, however, had novel tyrosine phosphorylation sites that interacted with Crk in a phosphorylation-dependent manner. Keeping in mind the difference in efficacy of these two Shc family members in growth factor signaling, we also compared the actions of ShcA and ShcC in transducing CSR-induced Erk activation by means of rescue experiments. The efficiency of both isoforms of ShcC in transducing signals for Erk activation was comparable with ShcA. This suggests that although ShcC behaves differently from ShcA in growth factor-induced signaling in the neural system, it could play a role similar to that of ShcA in CSR-induced signaling. It would be interesting to see whether externally caused changes in cell morphology, such as those after injury, induce Erk activation in an ShcC-dependent manner in neural cells. Also interesting is the difference between the longer isoforms of ShcA and ShcC. The two molecules are similar in structure with an additional CH2 domain at their amino termini, but they differ in capacity to mediate CSR-induced signals. p66ShcA did not mediate CSR-induced signaling for Erk activation, whereas p69ShcC did as efficiently as p55Shc/p46Shc. This difference could be attributable to different tyrosine phosphorylation patterns of the two molecules; p66ShcA was tyrosine-phosphorylated by either EGF or CytD treatment, whereas p69ShcC was phosphorylated only by EGF treatment (Fig. 4C). The CH2 domain of p66ShcC likely has some information that suppresses CSR-induced phosphorylation, whether through steric hindrance or association with accessory proteins. Clearly, it does not involve Thr-29 or Ser-36 phosphorylation, which is specific to the p66ShcA isoform. This difference in behavior also suggests that p69ShcC plays a role in the neural system different to that of p66ShcA in other tissues, i.e. to mediate oxidative stress response.

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