Evidence That Atypical Protein Kinase C-λ and Atypical Protein Kinase C-ζ Participate in Ras-mediated Reorganization of the F-actin Cytoskeleton

Florian Überall,* Karina Hellbert,* Sonja Kampfer,* Karl Maly,* Andreas Villunger,* Martin Spitaler,* James Mwanjewe,* Gabriele Baier-Bitterlich,* Gottfried Baier,‡ and Hans H. Grunicke*

*Institute of Medical Chemistry and Biochemistry and ‡Institute of Medical Biology and Human Genetics, University of Innsbruck, A-6020 Innsbruck, Austria

Abstract. Expression of transforming Ha-Ras L61 in NIH3T3 cells causes profound morphological alterations which include a disassembly of actin stress fibers. The Ras-induced dissolution of actin stress fibers is blocked by the specific PKC inhibitor GF109203X at concentrations which inhibit the activity of the atypical aPKC isotypes λ and ζ, whereas lower concentrations of the inhibitor which block conventional and novel PKC isotypes are ineffective. Coexpression of transforming Ha-Ras L61 with kinase-defective, dominant-negative (DN) mutants of aPKC-λ and aPKC-ζ, as well as antisense constructs encoding RNA-directed against isotype-specific 5′ sequences of the corresponding mRNA, abrogates the Ha-Ras–induced reorganization of the actin cytoskeleton. Expression of a kinase-defective, DN mutant of cPKC-α was unable to counteract Ras with regard to the dissolution of actin stress fibers. Transfection of cells with constructs encoding constitutively active (CA) mutants of atypical aPKC-λ and aPKC-ζ lead to a disassembly of stress fibers independent of oncogenic Ha-Ras. Coexpression of (DN) Rac-1 N17 and addition of the phosphatidylinositol 3′-kinase (PI3K) inhibitors wortmannin and LY294002 are in agreement with a tentative model suggesting that, in the signaling pathway from Ha-Ras to the cytoskeleton aPKC-λ acts upstream of PI3K and Rac-1, whereas aPKC-ζ functions downstream of PI3K and Rac-1.

This model is supported by studies demonstrating that cotransfection with plasmids encoding L61Ras and either aPKC-λ or aPKC-ζ results in a stimulation of the kinase activity of both enzymes. Furthermore, the Ras-mediated activation of PKC-ζ was abrogated by coexpression of DN Rac-1 N17.

Key words: Ha-Ras L61 • atypical PKC • F-actin • Rac-1 • PI3K

Expression of transforming Ha-Ras leads to profound morphological alterations which include a disassembly of F-actin stress fibers (Bar-Sagi and Feramisco, 1986; Ridley and Hall, 1992; Prendergast and Gibbs, 1993; Dartsch et al., 1994). Similar effects have been described in Src-transformed cells (Felice et al., 1990; Chang et al., 1995). Ras has been shown to induce changes in cytoskeletal actin through members of the Rho family (Ridley et al., 1992; Rodriguez-Viciana et al., 1994). This family comprises the RhoA, RhoB, and RhoC proteins, Rac-1 and Rac-2, TC10, two CDC42Hs proteins (also known as G25K), RhoG (Hall, 1990; Shinjo et al., 1990; Ridley and Hall, 1992; Ridley, 1995), and Rnd1 and Rnd-3/RhoE (Nobes et al., 1998). Expression of a dominant-negative (DN)1 mutant of Rac-1 (Asn-17 Rac1) has been shown to inhibit focus formation and tumorigenesis induced by oncogenic Ras (Qui et al., 1995a,b; Prendergast et al., 1995). Similar findings have been described after expression of a DN Asn-19 RhoB (Prendergast et al., 1995). Expression of Asn-19 RhoB did not interfere with foci formation by Raf-1, indicating that the Ras-RhoB pathway is independent of Raf-1 (Prendergast et al., 1995). The compound SCH 51344 has been described as a suppressor of Ha-Ras–mediated transformation (Kumar et al., 1995) by antagonizing the actin fiber reorganization without in-

1. Abbreviations used in this paper: a, atypical; c, conventional; CA, constitutively active; DN, dominant-negative; GFP, green fluorescence protein; n, novel; PI3K, phosphatidylinositol-3′ kinase; PKC, protein kinase C; PVDF, polyvinylene difluoride.
terference with the growth factor-induced activation of MEK, p44ERK1, or p90RSK (Kumar et al., 1995). The data obtained with the DN Rac and Rho mutants and SCH 51344 reverting transformation by Ras emphasize the significance of the Ras-induced reorganization of the actin cytoskeleton for the transformation by the oncogene.

The detailed molecular mechanism by which Ras affects the actin cytoskeleton via Rac and Rho is still insufficiently understood. Microinjection of recombinant, activated RhoA induces actin stress fibers in the absence of added growth factors (Paterson et al., 1990). Serum-induced stress fiber formation can be blocked by the Clostridium botulinum exoenzyme C3, an inhibitor of Rho (Rubin et al., 1988; Aktories et al., 1989; Chardin et al., 1989). Actin stress fibers are linked to integrins at the inner surface of the plasma membrane through a multimeric protein complex called focal adhesion (Burridge et al., 1988). Evidence for an implication of enzymes of the protein kinase C (PKC) family in focal adhesion formation has been reported (Chun and Jacobson, 1992; Vuori and Ruoslahti, 1993; Mogi et al., 1995). Activation of PKC isoenzymes causes a stimulation of cell attachment, spreading, and enhanced tyrosine phosphorylation of focal adhesion kinase, pp125 FAK, a constituent of the focal adhesion complex (Smith-Sinnett et al., 1993). FAK is tyrosine phosphorylated and its tyrosine kinase activity enhanced upon integrin-mediated interaction with the extracellular matrix (Guan et al., 1991; Kornberg et al., 1992; Zachary and Rozengurt, 1992). Enhanced tyrosine phosphorylation of FAK is also observed after exposure to several growth factors (Burridge et al., 1992; Sinnett-Smith et al., 1993; Rankin and Rozengurt, 1994). Thus, FAK may represent a point of convergence where growth factor-induced signals meet signals from activated integrins (Zachary and Rozengurt, 1992).

Stimulation of cells by some growth factors like platelet derived growth factor (PDGF), epidermal growth factor (EGF), or insulin has been shown to induce a reorganization of actin filaments by mediating actin polymerization at the plasma membrane, where actin filaments form a compact meshwork resulting in the formation of membrane ruffles and lamellipodia (Mellström et al., 1988; Ridley and Hall, 1992; Rankin and Rozengurt, 1994). Actin filament organization underlying membrane ruffles appears to be mediated by Rac as microinjection of a DN Asn-17 Rac-1 inhibits PDGF-induced membrane ruffles, whereas the constitutively active (CA) Val-12 Rac-1 induces membrane ruffling and the formation of focal complexes (Ridley and Hall, 1992; Qiu et al., 1995a,b). Evidence for an implication of a LIM kinase catalyzed phosphorylation of coflin in Rac-mediated reorganization of actin cytoskeleton has been presented (Arber et al., 1998; Yang et al., 1998). An additional form of actin filament organization is found in microspikes and filopodia where small bundles of actin filaments are attached to focal complexes at the tips of the filopodia (Nobes and Hall, 1995). Actin filament organization in filopodia appears to be regulated by CDC 42Hs (Nobes and Hall, 1995). CDC 42, Rac-1, and Rho-induced focal complexes are morphologically distinct but share a variety of constituents like vinculin, paxillin, and pp125 FAK (Nobes and Hall, 1995). Evidence for a hierarchical relationship between CDC 42, Rac and Rho, in which activation of CDC 42 leads to a sequential activation of Rac and Rho has been presented (Nobes and Hall, 1995). The detailed mechanisms, however, regulating the assembly and the spatial organization of the different structures of actin filaments are still insufficiently understood. In view of the similarities between Rho- and Rac-induced focal complexes and the well-documented implication of PKC in the assembly of integrin-containing focal adhesions, a similar role of representatives of the PKC family in the formation of Rac-regulated focal complexes appears conceivable. An implication of enzymes of the PKC family in cytoskeleton organization is supported by a series of published observations (for review see Keenan and Keleher, 1998). The interleukin (IL)-2-mediated alteration of the cytoskeleton has recently been demonstrated to require atypical αPKC-ζ (Gomez et al., 1997). Transforming Ras has been shown to activate PKC (Morris et al., 1989). Evidence for an implication of atypical αPKC-λ in the v-Ras-mediated activation and nuclear translocation of mitogen-activated protein kinase has been presented (Bjorkoy et al., 1997). Induction of c-fos by oncogenic Ras has recently been shown to require the coordinated activities of PKC-λ, PKC-ε, and PKC-ζ (Kamper et al., 1998). However, whether the Ras-mediated reorganization of the actin cytoskeleton is PKC-dependent, which PKC isotypes are involved, and what their function in the Ras-mediated restructuring of the cytoskeleton is, has remained unclear.

In this paper evidence is presented for an implication of the two atypical PKC-λ, and PKC-ζ isotypes in the Ras-mediated reorganization of the actin cytoskeleton. The data support a tentative model for a signaling pathway in which αPKC-λ acts downstream of Ras but upstream of phosphatidylinositol-3′-kinase (PI3K) and Rac-1, whereas αPKC-ζ functions downstream of Rac-1.

Materials and Methods

Reagents and Plasmids

Dulbecco’s modified Eagle’s medium (DME) and restriction enzymes for molecular biological approaches were purchased from Boehringer Mannheim. Fetal calf serum and l-glutamine were obtained from BioWhittaker. Leupeptin, myelin basic protein (M-1891), tetramethylrhodamine-isothiocyanate (TRITC)-labeled phalloidin, and aprotinin are products from Sigma. Lipofectin transfection reagents and Opti-Mem I medium were purchased from Life Technologies. [γ-32P]ATP (10 mCi/ml, 3000 Ci/mmol) and Hyperfilm-MP were obtained from Amersham. PCR primers used for subcloning strategy were obtained from ARK Scientific.

Subcloning strategy and oligonucleotides used for antisense constructs had been described elsewhere (Kamper et al., 1998). Orientation of insertion was determined by restriction analysis and sequencing. Subcloning strategy, mutagenic primers, as well as selection primers for the generation of kinase-defective, DN, as well as CA mutants of PKC isotypes had been described elsewhere (Baier-Bitterlich et al., 1996; Überall et al., 1997; Kamper et al., 1998). All cDNAs for PKC isotypes, green fluorescence protein (GFP), and the cDNA for Rac-1 N17 were subcloned into the expression vector pEF-1neo. GF109203X, LY294002, wortmannin, and Pansorbin beads were obtained from Calbiochem-Novabiochem.

Cell Culture and Transient Transfection Procedures

NIH3T3 fibroblasts were kept at logarithmic growth phase in DME supplemented with 10% heat-inactivated fetal calf serum, lysophosphatidic acid (100 ng/ml), and 2 mM l-glutamine in a humidified atmosphere containing 5% CO2. To obtain transient transfectants, NIH3T3 cells (5 × 10⁶ cells per well) were seeded in 100-mm-diam wells containing circular glass.
coatings (eight per well) and were transfected for 8 h with 1 μg pEF-1neoGFP expression plasmid, 1.5 μg pSR-xII L61 Ha-Ras (encoding a constitutively active Ras leucine L61 mutant), and 20 μg of plasmids encoding for kinase-defective, DN cPKC-ζ K368R, atypical aPKC-λ K275W, and aPKC-ζ K275W mutants. Alternatively, NIH3T3 fibroblasts were cotransfected with plasmids encoding pEF-1neoGFP plus a CA aPKC-λ A119E, (CA) aPKC-ζ A119E, and (CA) cPKC-α A25E mutants, or vector controls (pEF-1neo), respectively.

**Fluorescence Imaging**

48 h after the transfection procedure cells were washed twice with PBS (140 mM NaCl, 2.7 mM KCl, 4.6 mM NaHPO₄, 12 H₂O, 1.3 mM NaH₂PO₄, 2 H₂O), fixed with 3.5% formaldehyde (wt/vol in PBS) at room temperature and after extensive washing, permeabilized for 5 min with ice-cold acetone. After permeabilizing, the coverslips were washed twice with PBS and incubated for 1 h with FACS® buffer (500 ml PBS, 1 g sodium azide, and 4% fetal calf serum). For visualizing the F-actin structure, assembly cells fixed onto glass coverslips were overlaid with 100 μl (0.1 μl/ml) TRITC-conjugated phalloidin per coverslip for 1 h. Afterwards the stained cells were washed six times with PBS and with distilled water and mounted in Mowiol containing 0.1% (wt/vol) p-phenyleneediamine.

Mounted cells were viewed on an Olympus BX50 fluorescence microscope, and images of green fluorescence-positive cells were done by using a RGB-mode video real-color camera (Optronics Engineering DEI-470). Image processing was carried out with the image processing software MetaMorph (Princeton Instruments). The green and red fluorescence images were recorded separately by changing the excitation wavelength (from 480 to 550 nm), exported into Adobe Photoshop, and then printed on a color laser copier system (Agfa 707).

**Measurement of Total F-actin Fiber Length**

Total F-actin fiber length was calculated after digitalizing TRITC-phalloidin stained F-actin by using the Meta Morph image processing software. The edges of the cells were detected by the aid of a convolution kernel comparing the brightness of the neighboring pixels. After thresholding and separating from the background specimen, fiber lengths were determined and grouped as the percentage of the mean fiber length compared with the fiber length of mock-transfected fibroblasts.

**Partial Purification of 6× His-tagged PKC Isotypes and PKC In Vitro Assays**

African green monkey kidney fibroblasts (COS-1, 10⁶/100-mm dish) were transfected with 15 μg of circular plasmid DNA per dish by lipofectin reagents, as described by the manufacturer. 48 h posttransfection, cells were lysed in 1 ml lysis buffer (150 mM NaCl, 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid [HEPES, pH 7.5], 1% Nonidet P-40 [vol/vol], 50 μg/ml each aprotinin and leupeptin, and 1 mM phenyl-methylsulfonyl fluoride). Lysates were purified by using a Ni²⁺-resin batch procedure, and equal amounts of recombinant PKC isoforms were subjected to an enzymatic PKC assay as described elsewhere (Baier-Bitterlich et al., 1997), and as shown in Fig. 2 C, exerts a similar effect on Ras-induced depolymerization of actin stress fibers. For these experiments NIH3T3 cells were transiently cotransfected with vectors encoding Ha-Ras L61 and kinase-defective, as well as PKC-ζ-specific Antisense Constructs

As shown in Fig. 3, B and D, expression of aPKC-λ K275W as well as PKC-ζ K275W mutants, which contain an inactive ATP-binding site, is able to revert the Ras-induced depolymerization of actin stress fibers. For these experiments NIH3T3 cells were transiently cotransfected with vectors encoding Ha-Ras L61 and kinase-defective,
DN aPKC-\(\lambda\) K275W or aPKC-\(\zeta\) K275W mutants, respectively, using a green fluorescence protein expression vector as a transfection marker. Ras-induced reorganization of F-actin cytoskeleton was not affected by an expression of a kinase-defective, DN mutant of PKC-\(\alpha\) K368R (Fig. 3 F). Expression levels of cPKC-\(\alpha\) K368R, aPKC-\(\lambda\) K275R, and aPKC-\(\zeta\) K275R were found to be in a similar range (data not shown).

The effects observed after expression of the DN versions of PKC-\(\lambda\) and PKC-\(\zeta\) were checked by PKC-\(\lambda\)– and PKC-\(\zeta\)–specific antisense constructs. For this purpose, cells were transfected with constructs encoding isotype-specific 5’ sequences targeted to the corresponding mRNA. As described previously, generation of these antisense RNA sequences leads to an almost complete depletion of the endogenous aPKC-\(\lambda\) and -\(\zeta\) (Kampfer et al., 1998).

Fig. 3, C and E demonstrate that isotype-specific depletion of aPKC-\(\lambda\) and aPKC-\(\zeta\) yields the same results as expression of DN mutants of these isotypes. In agreement with the results obtained with the DN PKC-\(\alpha\) mutant, PKC-\(\alpha\) antisense did not affect the Ras-mediated alterations of F-actin organization (Fig. 3 G). Neither cPKC-\(\alpha\) nor aPKC-\(\lambda\)/-\(\zeta\) sense constructs did affect the Ras-mediated disassembly of F-actin fibers (data not shown).

A quantitative evaluation of Ras-mediated stress fiber depolymerization and the effects of (DN) aPKC-\(\lambda\) K275W, (DN) aPKC-\(\zeta\) K275W, aPKC-\(\lambda\), and aPKC-\(\zeta\) antisense on Ras-induced actin fiber organization was performed by measuring F-actin fiber length with the aid of the MetaMorph image processing software (Fig. 4, A and B).

The data shown in Figs. 3 and 4 clearly indicate that the Ras-mediated alteration of actin cytoskeleton is antagonized by an isotype-specific inhibition or depletion of aPKC-\(\lambda\) and aPKC-\(\zeta\). These findings strongly suggest that the Ras-mediated reorganization of the actin cytoskeleton is mediated by these two atypical aPKC isotypes.

**Expression of CA Mutants of Atypical aPKC-\(\lambda\) and aPKC-\(\zeta\) Mimic the Effect of Transforming Ras L61 on Actin Cytoskeleton**

It has previously been demonstrated that substitution of an alanine by a glutamate within the pseudosubstrate domain of PKC isotypes generates CA mutants with reduced cofactor requirements. Biochemical and biological properties of these mutants had been described in preceding publications (Baier-Bitterlich et al., 1996; Überall et al., 1997; Kampfer et al., 1998).

If Ha-Ras uses atypical PKC isotypes for the reorganization of the actin cytoskeleton as suggested by the data shown in Figs. 3 and 4, expression of CA aPKC-\(\lambda\) and (CA) aPKC-\(\zeta\) mutants should affect actin stress fibers like transforming Ha-Ras. As shown in Fig. 5, B and D, this is indeed the case. A CA mutant of PKC-\(\alpha\) A25E did not show any significant effect on stress fiber rearrangements (data not shown).

Stress fibers reappear after treatment of the aPKC-\(\lambda\) A119E- or aPKC-\(\zeta\) A119E-expressing cells with the PKC inhibitor GF109203X demonstrating that the alterations of the actin cytoskeleton in cells expressing the constitutively active versions of these atypical PKC isotypes are indeed caused by a PKC activity (Fig. 5, C and E).

**Evidence That Atypical aPKC-\(\lambda\) Acts Upstream and aPKC-\(\zeta\) Acts Downstream of Rac-1**

An early event after expression of oncogenic Ras is the generation of membrane ruffles and a reorganization of the actin cytoskeleton, i.e., stress fibers disappear whereas F-actin accumulates at the cell periphery (Bar-Sagi and Feramisco, 1986; Ridley et al., 1992; Rodríguez-Viciana et al., 1994). Evidence for an implication of Rac-1 in Ras-mediated reorganization of the actin cytoskeleton has been pre-
The data described so far suggest that the atypical PKC isotypes \( \lambda \) and \( \zeta \) are also involved in this process. It appeared interesting, therefore, to investigate whether they act within the Ras/Rac pathway and if they do, whether their position within this pathway can be identified.

As should be expected, DN N17 Rac-1 inhibits the Ras-mediated disassembly of stress fibers (Fig. 6, A and B). To obtain some information whether PKC-\( \lambda \) and PKC-\( \zeta \) cooperate with Rac-1 in the same pathway, cells were cotransfected with combinations of either CA aPKC-\( \lambda \) A119E and N17 Rac-1 or CA aPKC-\( \zeta \) A119E and N17 Rac-1.

As shown in Fig. 6 C, N17 Rac-1 is able to overcome stress fiber disassembly induced by CA aPKC-\( \lambda \) A119E (compare with Fig. 5 for the effect of aPKC-\( \lambda \) A119E in the absence of N17 Rac-1), indicating that aPKC-\( \lambda \) acts upstream of Rac-1. Stress fiber disassembly by aPKC-\( \zeta \) A119E, however, is not affected by N17 Rac-1 (Fig. 6 D), suggesting that aPKC-\( \zeta \) acts either downstream or independent of Rac-1. A quantitative evaluation of the stress fiber alterations shown in Fig. 6 is presented in Fig. 4 B and Fig. 10.

**Effects of Transforming Ras and CA Rac on aPKC-\( \lambda \) and aPKC-\( \zeta \)**

The data presented so far suggest that Ras mediates the effects on the cytoskeleton via a pathway containing aPKC-\( \lambda \)-Rac-1 and aPKC-\( \zeta \). If this model is correct, Ras should activate aPKC-\( \lambda \) and aPKC-\( \zeta \) whereas Rac should be able to stimulate aPKC-\( \zeta \). Unfortunately, this question could not be addressed in NIH3T3 cells due to the low transfection efficiencies in this cell type. Therefore, these studies were performed with COS cells. As shown in Fig. 7 A, cotransfection of COS cells with plasmids encoding Ras L61 and 6× His-tagged aPKC-\( \lambda \) leads to a significant activation of the kinase activity of aPKC-\( \lambda \). Coexpression of Ras L61 and aPKC-\( \zeta \) results in a marked stimulation of aPKC-\( \zeta \) as demonstrated in Fig. 7 B. Furthermore, cotransfection of a plasmid encoding CA V12Rac with a construct encoding aPKC-\( \zeta \) also revealed an activation of...
aPKC-ζ by Rac (Fig. 7 C). Surprisingly, V12Rac in addition to aPKC-ζ also activated aPKC-λ (data not shown). However, this finding is not in conflict with data or models presented so far. Possible interpretations for this effect will be presented in the Discussion. Our conclusion that Ras activates aPKC-ζ by a Rac-1–dependent mechanism is supported by the fact that expression of DN N17Rac blocks Ras-mediated stimulation of aPKC-ζ (Fig. 7 D). N17Rac does not inhibit Ras-mediated activation of PKC-λ (data not shown).

Figure 3. Reversion of Ras-induced disassembly of F-actin cytoskeleton by kinase-defective, DN as well as PKC isotype-specific antisense constructs. Shown are representative fluorescence images of fibroblasts transiently expressing Ha-Ras L61 (A–G). (A) Ha-Ras L61 alone, or (B) together with DN aPKC-λ; (C) antisense aPKC-λ; (D) DN aPKC-ζ; (E) antisense aPKC-ζ; (F) DN cPKC-α; and (G) antisense cPKC-α. 48 h posttransfection, cells were fixed and visualized as described in Materials and Methods. Representative cells of at least three different experiments are shown for all panels. The corresponding sense constructs were analyzed in parallel (data not shown). Stacks of images were exported into Adobe Photoshop and printed on a color laser copier system.
Effect of PI3K Inhibitors on Actin Cytoskeleton
Reorganization Induced by Oncogenic Ras or CA Mutants of aPKC-λ and aPKC-ζ

PI3K has been shown to be implicated in the Ras-induced reorganization of actin cytoskeleton (Rodriguez-Viciana et al., 1994). In agreement with these findings, treatment of Ras-expressing cells with the PI3K inhibitors wortmannin or LY294002 counteracts the effects of Ras on the actin cytoskeleton (Fig. 8). Both inhibitors also antagonize the disassembly of actin stress fibers induced by constitutively active aPKC-λ A119E, whereas the cytoskeletal reorganization mediated by the aPKC-ζ A119E mutant was not affected (Fig. 9). These data suggest that aPKC-λ acts upstream of PI3K whereas aPKC-ζ functions either downstream or independent of PI3K. A quantitative evaluation of the effects of the PI3K inhibitors is presented in Fig. 4 B and Fig. 10.

Discussion

The data presented here demonstrate that transforming Ha-Ras uses atypical aPKC-λ and aPKC-ζ for the rearrangement of actin cytoskeleton. This conclusion is based on the observation that (a) the Ha-Ras-induced dissolution of actin stress fibers is blocked by the specific PKC inhibitor GF109203X at concentrations which inhibit the activity of the atypical aPKC isotypes λ and ζ, (b) Coexpression of transforming Ha-Ras L61 with kinase-defective, DN mutants of aPKC-λ and aPKC-ζ, as well as antisense constructs encoding RNA directed against iso- type-specific 5’ sequences of the corresponding mRNAs, abrogate the Ha-Ras–induced reorganization of the actin cytoskeleton. (c) Finally, transfection of cells with constructs encoding CA mutants of atypical aPKC-λ and aPKC-ζ mimic the effect of oncogenic Ha-Ras on actin cytoskeleton reorganization.

With regard to the effects of the PKC inhibitor GF109203X, it may appear surprising that concentrations of the inhibitor which, in vitro, reduce the activity of aPKC-λ and aPKC-ζ to ~50% cause an almost complete reversal of the Ras-mediated disassembly of F-actin stress fibers. It should be emphasized, however, that neither the biological activators nor the intracellular substrates of atypical PKC isozymes have been sufficiently identified. Thus, the reaction mixtures used for the determination of the enzyme activity of the two kinases certainly differ from the in vivo conditions and this may affect the sensitivity to the inhibitor. Furthermore, both aPKC-λ and aPKC-ζ are affected by the inhibitor to about the same extent, i.e., ~50%. Since both enzymes are required for the Ras-mediated restructuring of actin cytoskeleton, the simultaneous inhibition of both enzymes may result in an additive effect. Finally, the intracellular concentration of the inhibitor is not known. It is possible, therefore, that locally higher concentrations than 6 μM have been achieved.

Expression of a kinase-defective, DN mutant of PKC-α or PKC-α depletion by intracellular generation of PKC-α antisense RNA did not affect the Ras-induced alterations of the actin cytoskeleton. In a previous paper we had demonstrated that the phosphorylation of MARCKS in NIH3T3 cells under biological conditions is mediated by PKC-α and that the phorbol ester-induced phosphorylation of MARCKS is markedly inhibited by expression of the kinase-defective, DN PKC-α K368R mutant (Überall et al., 1997). Thus, the inability of PKC-α K368R to interfere with the Ras-induced effects on actin cytoskeleton is not explained by an insufficient intracellular expression level. Furthermore, the phosphorylation of MARCKS, which has been discussed as an important regulator of actin organization (Rosen et al., 1990), does not appear to be involved in the Ras-induced reorganization of the actin cytoskeleton.

The Ras-induced reorganization of actin cytoskeleton

Überall et al. PKC in Ras-mediated Reorganization of Actin Cytoskeleton

Figure 4. Quantitative evaluation of total fiber length. Effects of kinase-defective, DN PKC-isotypes. Total F-actin fiber length was calculated after digitalizing TRITC-phalloidin stained F-actin by using the MetaMorph image processing software S/N 3542A. The edges of the cells were detected by the aid of a convolution kernel. This means that the brightness of the neighboring pixels were compared. After thresholding and separating from the background specimen, fiber lengths were calculated and expressed as the means of total fiber length compared with the fiber length of mock-transfected fibroblasts. Shown are morphological alterations of Ras-mediated stress fiber disassembly under the influence of (A) (DN) aPKC-λ K275W, antisense aPKC-λ, (DN) aPKC-ζ, antisense aPKC-ζ, and (B) (DN) N17 Ras-1, and the PI3K inhibitors wortmannin and LY294002. Bars indicate means (± SEM) of at least three independent experiments with ~60–75 GFP-positive cells which were separately analyzed per coverslip.
has been shown to be mediated by Rac-1 and PI3K (Jone-
son and Bar-Sagi, 1997). The data presented here suggest
that αPKC-ζ functions either downstream or independent of
Rac-1. This conclusion is based on the following findings:
(a) the effects of the constitutively active mutant of αPKC-λ
which mimics Ras with regard to the alterations of actin
cytoskeleton are inhibited by the PI3K blockers wortman-
nin and LY294002 and also by expression of DN N17 Rac-1;
and (b) expression of N17 Rac-1 does not interfere with
the disassembly of actin stress fibers induced by the CA
αPKC-ζ A119E mutant. The sequence Ras-αPKC-λ–PI3K-
Rac-1 would be in accordance with the model suggested
by Rodriguez-Viciana et al. (1994), who propose a path-
way in which the Ras-mediated activation of Rac-1 is me-
diated by PI3K. Our findings would add atypical αPKC-λ
as an upstream element of this sequence, a model consist-
tent with data demonstrating a physical interaction of
αPKC-λ with p21ras (Diaz-Meco et al., 1994).

The studies presented here demonstrate that in intact
cells, Ras is capable of activating αPKC-λ. Furthermore,
it is shown here that in addition to the stimulation of

Figure 5. CA PKC mutants mimic the Ras-mediated disassem-
bly of F-actin. Potency of the PKC-specific inhibitor GF109203X
to revert the disassembly of actin fibers induced by CA mutants.
Shown are representative fluorescence images of fibroblasts transiently expressing constitutively active (CA) PKC mutants
alone (B and D), or in the presence of 6 μM GF109203X (C and
E). (A) Control, (B) (CA) atypical αPKC-λ A119E; (C) plus
GF109203X, (D) (CA) αPKC-ζ A119E; (E) plus GF109203X.
48 h posttransfection, cells were fixed and visualized as de-
scribed in Materials and Methods. Representative cells of at
least three different experiments are shown for all panels. Stacks
of images were exported into Adobe Photoshop and printed as
described above.
structures are incompletely understood, it can be only speculated with regard to the function of aPKC-λ or aPKC-ζ in this pathway. The disassembly of actin stress fibers has been correlated to the accumulation of inactive, GDP-charged RhoA (Qui et al., 1995a,b). Activation of RhoA by an exchange of the GDP by a GTP was found to stimulate stress fiber formation (Ridley and Hall, 1992). These findings suggest that the Ras-induced disassembly of actin stress fibers is the result of a conversion of the active, GTP-charged Rho to the inactive GDP-bound form. Models for a biochemical linkage between the Ras and Rho proteins have been proposed (Boguski et al., 1992; Nobes and Hall, 1994; Chant and Stowers, 1995). In one simple model, the Ras/Rho pathway includes p120GAP, the Ras GTPase-activating protein which may also act as a Ras effector (Chant and Stowers, 1995) and p190, a p120GAP-associated protein which exhibits a Rho-specific

Figure 6. Evidence that atypical aPKC-λ acts upstream and aPKC-ζ acts downstream of Rac-1. Shown are representative fluorescence images of fibroblasts transiently expressing Ha-Ras L61 (A and B). (A) Ha-Ras L61 alone, or (B) together with DN Rac-1 N17; (C) CA aPKC-λ A119E together with (DN) Rac-1 N17; or (D) CA aPKC-ζ A119E together with (DN) Rac-1 N17. 48 h posttransfection, cells were fixed and visualized as described in Materials and Methods. Representative cells of at least three different experiments are shown for all panels. Stacks of images were exported into Adobe Photoshop and printed on a color laser copier system.

Figure 7. Effects of oncogenic Ras and constitutively active Rac on kinase activities of aPKC-ζ and aPKC-λ. Shown are representative immunocomplex kinases assays (B–D) and an in vitro kinase assay (A) of magnetically separated COS-1 cells. The data are designed as bar graphs (top panels) and as corresponding autoradiograms of the myelin basic protein (MBP) assay (bottom panels). Equal amounts of recombinant proteins used in the experiment were employed using a standard Western blotting technique as described by Kampfer et al. (1998). In brief, logarithmically growing cells were transiently cotransfected with (A) Ha-Ras L61 together with aPKC-λ, (B) Ha-Ras L61 together with aPKC-ζ, (C) CA Rac-1 V12 together with aPKC-ζ, and (D) DN Rac-1 N17 together with aPKC-ζ. Concerning magnetic bead separation of positively transfected cells, a truncated CD4 surface marker was cotransfected in B and C. 48 h posttransfection, cells were separated by using magnetic beads as described by the manufacturer and PKC assays were done as described under Materials and Methods. Enzyme activities are expressed as cofactor-independent phosphorylation of (A) a synthetic PKC-a peptide (A25S, for details see Fig. 2 legend) or myelin basic protein (B–D). Computer-assisted calculation of PKC-ζ or PKC-λ activities were done after scanning the corresponding PVDF membranes by using the Scanner Controller Sci System.
The biochemical function of aPKC-α and aPKC-ζ in the Ras/Rac/Rho pathway remains to be elucidated. Evidence for PKC as an important regulator of cytoskeletal functions has been presented by numerous studies (Keenan and Kelleher, 1998). Stress fibers are associated with focal adhesion complexes where cells interact with the extracellular matrix. The interaction with the extracellular matrix is mediated by integrin receptors which are integral components of focal adhesion plaques (Schwartz et al., 1995). Activation of integrin receptors initiates a signaling cascade which has been shown to depend on stress fibers (Hynes, 1992; Rosales et al., 1995; Wu et al., 1995) and to cooperate with growth factor-mediated events (Clark and Brugge, 1995; Schwartz et al., 1995). An implication of PKC in focal adhesion formation and integrin-mediated signaling has been described (Clark and Brugge, 1995; Schwartz et al., 1995). By permitting anchorage-indepen-
dent growth, transforming Ras may override the necessity for cell attachment. Evidence in support of this concept has been presented (Kang and Krauss, 1996). Both Src and Ras have been shown to act as important elements in inte-

![Figure 9](image-url)

**Figure 9.** Evidence that atypical aPKC-λ acts upstream of PI3K and Rac-1, whereas aPKC-ζ functions downstream of PI3K and Rac-1. Shown are representative fluorescence images of fibroblasts transiently expressing (CA) atypical aPKC-λ (A–C). (A) (CA) aPKC-λ alone, or (B) in the presence of 25 nM wortmannin, or (C) 25 μM LY294002; (D) (CA) aPKC-ζ A119E alone, or (E) in the presence of 25 nM wortmannin, or (F) 25 μM LY294002. 48 h post-transfection, cells were fixed and visualized as described in Materials and Methods. Representative cells of at least three different experiments are shown for all panels. Stacks of images were exported into Adobe Photoshop and printed as described above.

![Figure 10](image-url)

**Figure 10.** Quantitative evaluation of total fiber length. Effect of CA aPKC-λ and aPKC-ζ alone, together with (DN) N17Rac, or after treatment with the PI3K inhibitors wortmannin and LY294002. Total F-actin fiber length for all images were calculated as described in Fig. 4. Shown is a quantitative evaluation of total F-actin fiber length from fibroblasts expressing CA aPKC-λ and aPKC-ζ mutants under the influence of (DN) N17 Rac-1, and the PI3K inhibitors wortmannin and LY294002. Final inhibitor concentrations are described above. Bars indicate means (± SEM) of at least three independent experiments with ~70–75 GFP-positive cells analyzed per coverslip.

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