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Differential Translocation of Protein Kinase C ϵ during HeLa Cell Adhesion to a Gelatin Substratum*

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Jang-Soo Chun‡, Mahn-Joon Ha§, and Bruce S. Jacobson¶‖

From the ‡Department of Biology, Kyungpook National University, Taegu 702-701, Korea, the §Laboratory of Medical Genetics, Institute for Medical Science, Ajou University, Suwon 441-749, Korea, and the ¶Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01003

The spreading of HeLa cells, following attachment to a collagen or gelatin substratum, requires the activation of protein kinase C (PKC). Membrane-bound PKC was previously shown to be activated during cell attachment and in response to the activation of a series of lipid second messengers turned on by the ligation of β1-integrin collagen receptors. HeLa cells express the α, γ, ϵ, ζ, λ, and ι isozymes of PKC as determined by Western blotting with specific antibodies. Only PKCϵ redistributed from the cytosol to the membrane during cell adhesion. Most of the PKCϵ in cells that were in suspension was in the cytosolic fraction. During cell attachment to a gelatin matrix, all of the PKCϵ moved out of the cytosol, with most going to the membrane fraction. After the cells became fully spread, PKCϵ began to reappear in the cytosol. Translocation of PKCϵ was not observed during the adhesion of cells to culture dishes where cells nonspecifically attach but do not spread. The conventional PKCα and -γ isozymes were translocated from the cytosol to the membrane only when phorbol ester was present at a concentration that increases the rate and extent of cell spreading. Under normal conditions, *i.e.* in the absence of phorbol ester, PKCϵ appears to be the PKC isozyme responsible for the regulation of HeLa cell adhesion to the extracellular matrix.

The attachment and spreading of cells on an extracellular matrix (ECM)[1] regulate a number of biological processes such as cell motility, proliferation, and differentiation. The attachment of cells to a particular ECM component is mediated by specific cell surface receptors such as integrins (1, 2). The ECM receptors provide a linkage between the ECM and the cytosol by interacting with cytoskeletal proteins on the cytoplasmic side of the plasma membrane (3, 4). In addition, there is increasing evidence that during cell attachment the multivalent ECM components cluster cell surface receptors to give rise to a variety of second messengers within the cells (4, 5). Signaling molecules that have been shown to be activated by integrins include $pp125^{FAK}$ tyrosine kinase (6, 7), protein kinase C (PKC) (8, 9), and mitogen-activated protein kinase (10, 11). Also activated are G proteins (12), ion transporters (13), and the lipid modifying enzymes phospholipase C, phospholipase A_2, and lipoxygenase that produce, respectively, the lipid second messengers diacylglycerol, arachidonic acid, and hydroxyeicosatetraenoic acid (14–17).

HeLa cells attach to a variety of substrata, but subsequent spreading is specific to a collagen or gelatin substratum (18). The spreading of HeLa cells on a gelatin substratum is initiated by the clustering of collagen receptors, including β1 integrins that activate phospholipase A_2 to produce arachidonic acid (16, 17). The released arachidonic acid is further metabolized by lipoxygenase to produce metabolites that induce the production of diacylglycerol. Diacylglycerol production is correlated to an increase in membrane-bound PKC activity that occurs during the attachment phase of cell adhesion and prior to cell spreading. Inhibition of PKC blocks cell spreading, and the activation of PKC enhances it, indicating that PKC activity is required for cell spreading (8). Furthermore, PKC activation with phorbol ester overcomes the inhibition of cell spreading that is induced by blocking either arachidonic acid release or by lipoxygenase metabolite formation, indicating that PKC is a downstream second messenger in the regulation of HeLa cell spreading (8, 17). To date, it is not known which PKC isozymes are involved in adhesion of cells to the ECM.

Protein kinase C is a family of related serine and/or threonine protein kinases that appears to play an important role in a variety of cellular responses. The multiple PKC isozymes may have different physiological roles as PKC isozymes display both common features and substantial differences in primary structure, enzymatic activities, tissue and intracellular distribution, and cofactor requirements (19, 20). This study was performed to identify the PKC isozymes involved in the regulation of HeLa cell adhesion to a gelatin substratum. The data obtained indicate that among the PKCα, -γ, -ϵ, -ζ, -λ, and -ι isozymes expressed in HeLa cells, only the PKCϵ isozyme is activated during cell substratum adhesion as determined by the translocation of cytosolic PKC to the membrane fraction.

EXPERIMENTAL PROCEDURES

Cell Culture, Substratum Preparation, and Adhesion Assay—HeLa-S3 cells were grown in suspension culture to midlog phase (2–4 × 10^5 cells/ml) in RPMI 1640 medium supplemented with 5% calf serum. The cells used for attachment and spreading studies were harvested by centrifugation, washed twice, and resuspended in RPMI 1640 medium. The cells were seeded in 35- or 60-mm culture dishes covalently coated with type I gelatin as described previously (8, 18) and incubated for 30 min at 37 °C prior to scoring for the percent of cells spread unless otherwise indicated. The percent of cells spread was calculated from the number of spread cells/total number of cells × 100 from ~200–300 cells in several microscopic fields of view using phase-contrast microscopy (17).

Cell Fractionation—For the separation of PKC isozymes between the cytosol and the membrane, HeLa cells, either kept in suspension or plated on gelatin for the indicated periods, were scraped into buffer A

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‖ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Massachusetts, GRC Tower B, Amherst, MA 01003. Tel.: 413-545-2048; Fax: 413-545-3291.

[1] The abbreviations used are: ECM, extracellular matrix; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

(20 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, and a mixture of protease inhibitors including 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). The cells were briefly sonicated and centrifuged at 100,000 *g* for 1 h to sediment all membranes and the insoluble cytoskeletal components, *e.g.* actin filaments and microtubules (21, 22). The supernatant was designated as a cytosolic fraction. The membrane proteins in the pellet were extracted with buffer B (20 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and protease inhibitors) on ice for 30 min (21, 22). Following centrifugation, the supernatant was saved as a detergent-soluble membrane fraction. The pellet containing the nondenaturing detergent-resistant cell cytoskeleton (21, 22) was dissolved with buffer C (20 mM Tris-HCl, pH 7.5, containing 1% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and protease inhibitors) and designated as a particulate fraction.

Western Blot Analysis of the Protein Kinase C Isozymes—Proteins (25 μg) from cytosolic, membrane, or particulate fractions were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 3% nonfat dry milk in Tris-buffered saline. PKC isozymes such as α, β, γ, ε, θ, λ, and ι were detected with isozyme-specific anti-PKC monoclonal antibodies (Transduction Laboratories, Lexington, KY), while δ and ζ isozymes were detected with polyclonal antibodies (Life Technologies, Inc.). The PKC antibodies that were bound to the proteins on the cellulose nitrate sheets were detected with goat anti-rabbit or anti-mouse IgG conjugated with peroxidase using the ECL system of Amersham Life Sciences Inc.

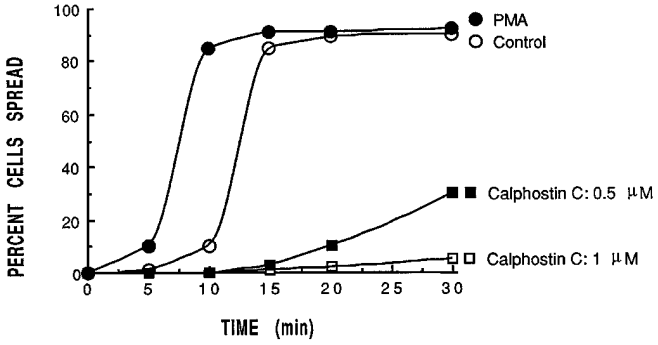

FIG. 1. **Requirement of PKC activity for HeLa cell spreading to a gelatin substratum.** Suspension HeLa cells were pretreated with vehicle alone (*Control*) or calphostin C (0.5 or 1 μM), a specific inhibitor of PKC, for 5 min, and plated on a gelatin substratum. Alternatively, the cells were plated on gelatin-coated culture dishes in the presence of PMA (1 μM). The cells were incubated at 37 °C for the indicated periods and scored for the percent of cells spread.

RESULTS

We have previously shown that the activity of PKC in the membrane fraction of HeLa cells plated on a gelatin substratum is transiently increased with a time course where the greatest increase in activity occurred during the cell attachment phase of cell adhesion and prior to cell spreading (8, 17). Once cell spreading had ceased, the membrane-associated PKC activity decreased but without a return of activity to the cytosol. Treatment of cells with calphostin C, a specific inhibitor of PKC, blocks cell spreading (Fig. 1). In contrast, PKC-activating phorbol ester PMA (phorbol 12-myristate 13-acetate) enhances the rate of cell spreading (Fig. 1), as well as the extent to which the cells spread (Fig. 2). The results are consistent with our previous report (8), indicating that PKC activity is required for HeLa cell spreading on a gelatin substratum. However, which isozymes of PKC are responsible for inducing cell spreading has not been determined.

The expression of the different PKC isozymes in HeLa cells was determined by Western blotting with isozyme-specific antibodies. All of the antibodies recognized an immunoreactive band from the lysate of mouse brain as a positive control (Fig. 3). The antibodies against the PKCα, -γ, -ε, -ζ, -λ, or -ι isozymes also detected immunoreactive protein from HeLa cell lysates, indicating that these isozymes are expressed in HeLa cells. However, PKCβ, -δ, and -θ subspecies do not appear to be expressed at significant levels since the corresponding antibodies failed to detect any proteins from the HeLa cell lysate (Fig. 3). Maximally loading the gel lanes with 60 μg of protein and exposing the gels until the background made it questionable as to whether a minor band was present did not reveal a band indicative of PKAβ, and very faint bands were seen with anti-δ and anti-θ PKC (data not shown). At this time, we did not pursue a study of the translocation of the PKCδ and -θ isozymes because of the questionable nature of their existence in HeLa cells; however, it should not be ruled out that PKCδ and PKCθ are not in HeLa cells since they might be there albeit at very, very low levels compared with those of the other PKC isozymes found.

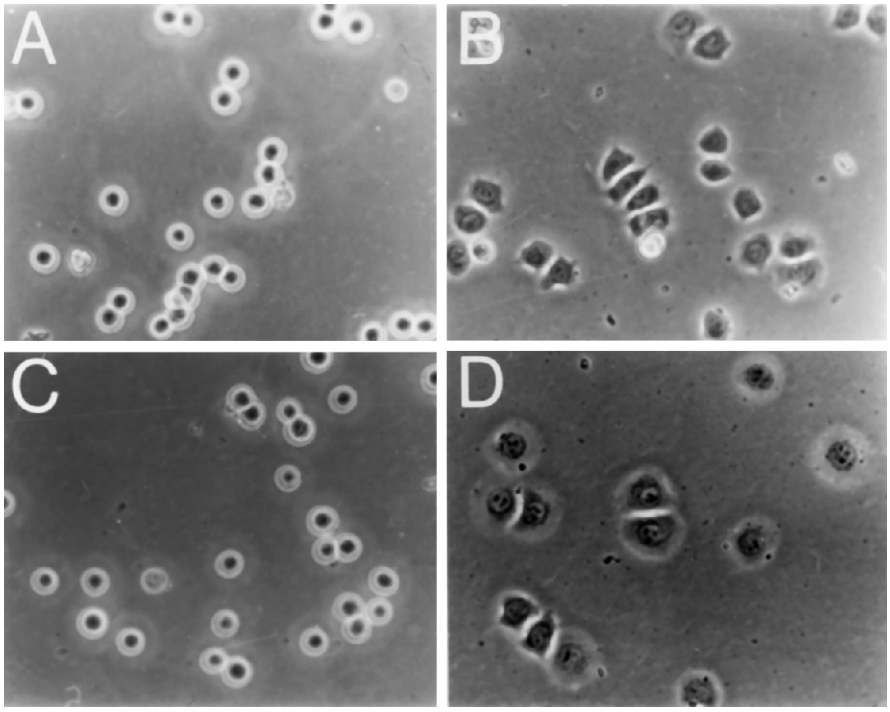

FIG. 2. **Morphology of HeLa cells plated on a gelatin substratum.** HeLa cells were plated on gelatin-coated culture dishes at 37 °C for 5 min to allow attachment (*A*) or for 30 min in the absence (*B*) or presence (*D*) of 0.4 μM PMA to allow spreading. Alternatively, the cells were pretreated with 1 μM calphostin C for 5 min and plated on gelatin for 30 min (*C*). The photographs were taken at ×200 magnification.

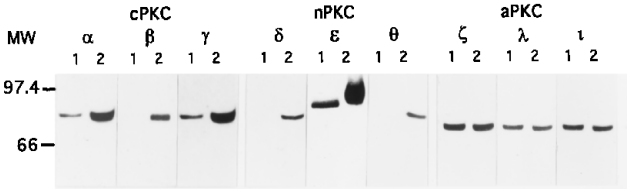

FIG. 3. **Expression of PKC isozymes in HeLa cells.** Total cell lysates (25 μg) extracted from either suspension HeLa cells (*lane 1*) or mouse brain (*lane 2*) were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and detected with PKC isozyme-specific antibodies. *cPKC*, conventional PKC isozymes; *nPKC*, novel PKC isozymes; and *aPKC*, atypical PKC isozymes.

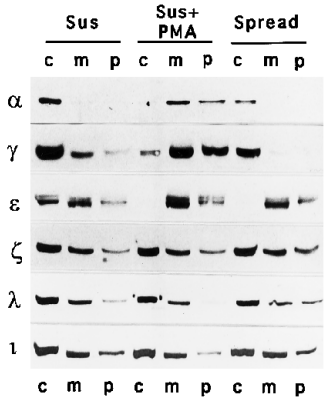

FIG. 4. **Distribution of PKC isozymes in HeLa cells.** Suspension HeLa cells were treated with vehicle alone (*Sus*) or 1 μM PMA for 10 min (*Sus + PMA*). Alternatively, the cells were plated on gelatin for 30 min to allow spreading (*Spread*). The cells were fractionated, and the lysates (25 μg) were used for immunoblotting with PKC isozyme-specific antibodies. *c*, Cytosolic fraction; *m*, membrane fraction; and *p*, particulate fraction.

The relative distribution of the PKC isozymes among the cytosolic, membrane, and particulate fractions was determined in cells that were either kept in suspension or spread on gelatin (Fig. 4). In suspension cells, PKCα was detected exclusively in the cytosolic fraction. The other PKC isozymes were mostly in the cytosolic fraction but with various amounts in the membrane fraction and even smaller amounts in the particulate fraction (Fig. 4).

To further assess the function of the various PKC isozymes in HeLa cells, we determined whether cell spreading on a gelatin matrix or treating cells with the conventional PMA induced the translocation of the HeLa cell PKC isozymes from the cytosol to the membrane. In an effort to clearly demonstrate any changes in the relative amounts of each PKC isozyme in the cytosolic, membrane, and particulate fractions in response to cell spreading or PMA treatment, equal amounts of protein from cells that were either treated with PMA or allowed to spread were loaded on the same gels in lanes next to each other and immunoblotted under identical conditions. The treatment of suspension cells with 1 μM PMA induced the association of PKCα, -γ, and -ε primarily with the membrane fraction with concurrent losses from the cytosolic fraction. The distribution of PKCζ, -λ, and -ι appears to be unchanged upon PMA treatment (Fig. 4). PMA at 1 μM was not cytotoxic as indicated by trypan blue exclusion and confirmed the observation that this concentration of PMA increased the rate and extent of cell spreading on a gelatin matrix. The inactive ester of PMA, phorbol 20-oxo-20-deoxy 12-myristate 13-acetate (αPMA), was also evaluated as a control and found not to induce any change in the distribution of the PKCα, -γ, -ε, and -ζ isozymes among the cytosolic, membrane, and particulate fractions. When HeLa cells were allowed to spread on a gelatin substratum, the relative amounts of PKCα, -γ, -ζ, -λ, and -ι were not changed. However, the levels of PKCε in the cytosolic fraction were significantly reduced with a concomitant increase occurring in the membrane fraction (Fig. 4). The results suggest that PKCε, among the isozymes expressed in HeLa cells, is specifically translocated to the membrane fraction during cell spreading.

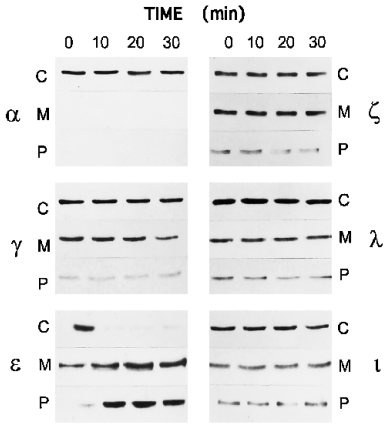

FIG. 5. **Translocation of PKCε during HeLa cell adhesion to a gelatin substratum.** HeLa cells were either kept in suspension (0 min) or plated on a gelatin substratum for the indicated periods. Cells were fractionated, and the lysates (25 μg) were used for immunoblotting with PKC isozyme-specific antibodies. *C*, cytosolic fraction; *M*, membrane fraction; and *P*, particulate fraction. Immunoblots for the particulate fractions were exposed for longer periods of time than the membrane fractions to more clearly demonstrate whether any changes in the isozymes during the time of spreading took place.

To further evaluate which of the PKC isozymes is responsible for the regulation of HeLa cell adhesion to a gelatin substratum, cells that were either kept in suspension or plated on gelatin and allowed to spread for various time intervals were fractionated and analyzed for the PKC isozymes. The distribution of the PKCα, -γ, -ζ, -λ, and -ι isozymes between the cytosolic and membrane fractions appears to be unchanged during cell to substratum adhesion (Fig. 5). PKCε was the only isozyme that showed a significant reduction in the cytosolic fraction upon attachment to a gelatin substratum with corresponding increases in the membrane and particulate fractions (Fig. 5). Translocation of PKCε was not observed during the adhesion of HeLa cells to sulfonated or polylysine-coated culture dishes where cells nonspecifically attach but do not spread (data not shown). Since the activation of PKC involves the stable association of cytosolic PKC with the membrane, the results suggest that PKCε is specifically activated to regulate HeLa cell adhesion to a gelatin substratum.

DISCUSSION

The involvement of PKC in cell to ECM adhesion has long been suggested from the observation that PKC-activating phorbol ester enhances the adhesion of various cells to the ECM and that inhibitors of PKC prevent the adhesion (23–25). Direct evidence indicating that endogenous PKC activity modulates attachment and spreading of cells to an ECM comes from the adhesion of Chinese hamster ovary cells to a fibronectin substratum (9) and of HeLa cells to a collagen substratum (8). In both cell lines, PKC activity in a membrane fraction is transiently increased, and the inhibition of endogenous PKC activity blocks adhesion of the cells. While the above indicates that PKC is involved in regulating cell adhesion to an extracellular matrix, it is not known which isozymes are involved and whether the same isozymes function when phorbol ester is present.

In the experiments reported here, only PKCε was found to parallel the translocation of total PKC enzymatic activity from cytosol to membrane as previously seen (8, 17). Before the cells attached to a gelatin matrix, no detectable PKC activity was observed in the membrane fraction. Upon cell attachment to a gelatin matrix, essentially all of the PKC enzymatic activity translocated from the cytosol to the membrane and occurred before the cells began to spread. When the cells finished spreading, the PKC activity in the membrane fraction began to decrease. Fig. 5 indicates that PKCε, as determined by immunoblotting, also follows a similar pattern of translocation as the activity measurements did (8, 17). PKCε was primarily found in the cytosolic fraction of cells prior to its attachment to a gelatin matrix. Within the first 10 min of attachment, PKCε was totally lost from the cytosolic fraction and could be accounted for in the membrane and particulate fractions. As with the previous PKC enzymatic activity measurements (17), PKCε began to diminish in the membrane fraction when the cells finished spreading. The above strongly supports the conclusion that PKCε and not the other PKC isozymes is involved in HeLa cell attachment and spreading on a gelatin matrix. Interestingly, small amounts of the PKC isozymes detected in HeLa cells other than PKCα were observed in the membrane and particulate fractions of cells in suspension. It is likely, however, that they were either enzymatically inactive or that their activity was insufficient to induce cell spreading. This is because the total PKC enzymatic activity in the membrane and particulate fraction as previously found (8, 17) was insignificant unless the cells had attached to the gelatin substratum and begun to spread.

The increase in PKC enzymatic activity in HeLa cell membranes (8) that appears to occur as a consequence of PKCε translocation from the cytosol during cell attachment to a gelatin matrix must result in the activation of the cytoskeletal machinery in order for cell spreading to occur (18). Preliminary work by us (data not shown) indicates that activation of PKC induces both the polymerization of actin and the up-regulation of β_1-integrin receptors from intracellular vesicles to the cell surface in a microtubule-dependent manner. This implies that PKC might associate with both the microfilament- and microtubule-based cytoskeleton. To evaluate this possibility, we analyzed for the association of the HeLa cell PKC isozymes in three subcellular fractions (cytosolic, membrane, and particulate fractions) during cell adhesion to a gelatin matrix (see "Experimental Procedures"). Based upon previous work (21, 22), the particulate fraction contains the cytoskeletal components. The only PKC isozyme that was found to translocate from the cytosolic fraction to the particulate (cytoskeletal) fraction during attachment and spreading on a gelatin matrix was the ε isozyme (Fig. 5). Several experiments indicate that the amount of PKCε translocated to the particulate (cytoskeletal) fraction was less than that translocated to the membrane fraction. Interestingly, all of the PKCs in HeLa cells except the α isozyme had small but significant amounts associated with the particulate fraction. However, only the ε isozyme exhibited an increase in the particulate (cytoskeletal) fraction during adhesion of the cells to gelatin.

Many of the PKC isozymes exhibit individual characteristics (19, 20). Presently, 11 isozymes of PKC have been identified in mammalian tissues. These isozymes can be divided into four groups based on activation mechanisms: Ca^{2+}-dependent classical or conventional PKCα, -β, and -γ; Ca^{2+}-independent novel PKCδ, -ε, -θ, and -η; atypical PKCζ, -λ, and -ι; and PKCμ. In this study, we have shown that, among the expressed multiple PKC isozymes, PKCε is specifically activated during the attachment and spreading of HeLa cells on a gelatin substratum, as determined by the translocation of cytosolic PKC to the membrane fraction. Interestingly, other isozymes also appear to be able to function in HeLa cell spreading but only in response to the presence of an exogenously supplied activator such as the phorbol ester PMA. Both the α and γ isozymes in HeLa cells translocate from the cytosol to the membrane when the cells are in the presence of PMA (Fig. 4). Since all of the ε isozyme is translocated to the membrane and particulate fractions during HeLa cell adhesion to a gelatin matrix (Fig. 5), it is likely that the increase in rate and extent of cell spreading when PMA is present (Figs. 1 and 2) are due to the translocation of the α and γ isozymes.

PKCε, like all conventional and novel PKC isozymes, requires phosphatidylserine and diacylglycerol for binding to the membrane and activation. However, PKCε, unlike the conventional PKCs, does not require elevation of Ca^{2+} for its activation (19, 20). The activation mechanism of PKCε is consistent with the signaling events observed during HeLa cell adhesion to a gelatin substratum. The level of diacylglycerol increases upon attachment and before spreading of HeLa cells, but the level of cytosolic free calcium does not change during cell attachment or cell spreading (8, 17). The absence of an increase in intracellular calcium also explains why PKCα and -γ are not translocated to the membrane during cell attachment and spreading, while the ε isozyme is translocated.

It is generally accepted that the multiple PKC isozymes are responsible for different specialized physiological processes, and many cell types express the multiple PKC isozymes (19, 20). Among the multiple isozymes, the selective activation of PKCε has been reported in some cases such as insulin-mediated stimulation of PKCε in fetal chick neurons (26) and nerve growth factor-mediated activation in PC-12 cells (27). In this study, we have also shown that the selective activation of PKCε is most likely responsible for the regulation of HeLa cell adhesion to a gelatin matrix. It should be noted that the activation of PKCε is in part a downstream response to the clustering of β_1-integrin collagen receptors by the gelatin matrix (16). Currently, the action of PKCε in the regulation of adhesion of HeLa cell to ECM is not clarified. However, the activated PKC appears to regulate HeLa cell adhesion by dual pathways. One is through the formation of F-actin that is essential for cell spreading, and the other is by delivering integrin β1 to the cell surface where they can bind to the ECM molecules for optimum adhesion of cells.[2]

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