Activation of the Protein Kinase Akt/PKB by the Formation of E-cadherin-mediated Cell-Cell Junctions

EVIDENCE FOR THE ASSOCIATION OF PHOSPHATIDYLINOSITOL 3-KINASE WITH THE E-CADHERIN ADHESION COMPLEX*

Salvatore Pece, Mario Chiariello, Cristina Murga, and J. Silvio Gutkind‡

From the Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892-4330

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E-cadherins are surface adhesion molecules localized at the level of adherens junctions, which play a major role in cell adhesiveness by mediating calcium-dependent homotypic interactions at sites of cell-cell contacts. Recently, E-cadherins have been also implicated in a number of biological processes, including cell growth and differentiation, cell recognition, and sorting during developmental morphogenesis, as well as in aggregation-dependent cell survival. As phosphatidylinositol (PI) 3-kinase and Akt play a critical role in survival pathways in response to both growth factors and extracellular stimuli, these observations prompted us to explore whether E-cadherins could affect intracellular molecules regulating the activity of the PI 3-kinase/Akt signaling cascade. Using Madin-Darby canine kidney cells as a model system, we show here that engagement of E-cadherins in homotypic calcium-dependent cell-cell interactions results in a rapid PI 3-kinase-dependent activation of Akt and the subsequent translocation of Akt to the nucleus. Moreover, we demonstrate that the activation of PI 3-kinase in response to cell-cell contact formation involves the phosphorylation of PI3-kinase in tyrosine residues, and the concomitant recruitment of PI3-kinase to E-cadherin-containing protein complexes. These findings indicate that E-cadherins can initiate outside-in signal transducing pathways that regulate the activity of PI 3-kinase and Akt, thus providing a novel molecular mechanism whereby the interaction among neighboring cells and their adhesion status may ultimately control the fate of epithelial cells.

The maintenance of structural and functional integrity of epithelia requires highly dynamic cell-to-cell and cell-to-matrix interactions, which are mediated by adhesion mechanisms involving different types of cell-surface receptors. Among them, cadherins and integrins play a major role, as they are able to recognize and interact with other cell adhesion receptors on neighboring cells or with proteins of the extracellular matrix, respectively (1–3). E-cadherins belong to the family of integral membrane glycoproteins promoting homotypic calcium-dependent cell-cell interactions and are well characterized adhesion receptors found within adherens-type junctions in epithelia. The extracellular domain of E-cadherins is able to mediate per se calcium-dependent homotypic interactions at sites of cell-cell contacts, while its highly conserved intracytoplasmic tail is involved in the strengthening of the homotypic adhesions by binding a set of related proteins called catenins which, in turn, link the complex to the actin cytoskeleton and elicit certain nuclear responses (4, 5). Recently, the dynamic aspects of cell adhesion and its relationship to physiological and pathophysiological events have been intensively investigated. They include cell growth and differentiation, cell recognition and sorting during developmental morphogenesis (reviewed in Ref. 2), and a role in certain pathological processes, including the correlation between loss of E-cadherins at the level of cell surface and enhanced cell invasiveness in vitro (6–9) and tumor progression in vivo (10, 11).

Several lines of evidence indicate that the E-cadherin-mediated adhesion system is subject to regulation from the cytoplasmic side in response to intracellular events (9, 12–15). In contrast, the generation of signals at the level of adherens junctions as a consequence of E-cadherin engagement has been thus far poorly investigated, although newly available evidence suggest that E-cadherins may participate in transducing outside-in signals (16). Of interest, it has been reported recently that E-cadherins can mediate aggregation-dependent cell survival in a variety of experimental settings (17–19). As the Akt kinase is an integral component of survival pathways utilized by both growth factors and extracellular stimuli (20–23), these observations prompted us to investigate whether E-cadherins could affect the activity of signaling molecules controlling Akt function. In this study, we used an in vitro model for the disruption and subsequent re-formation of E-cadherin-dependent interactions in epithelial MDCK cells to explore the possibility that E-cadherin-mediated cellular aggregation could result in Akt activation. We provide evidence that engagement of E-cadherins in homotypic adhesion with neighboring cells promotes a remarkable PI 3-kinase-dependent increase in the state of activation of Akt and the rapid translocation of Akt to the nucleus. We also demonstrate that engagement of E-cadherins is necessary and sufficient for the induction of Akt activity upon adherens junction assembly, and co-immunoprecipitation experiments demonstrate a physical association between PI 3-kinase and E-cadherin-containing multiprotein complexes in response to cell-cell contact formation, thus providing a likely mechanism for Akt activation. Overall, these findings indicate that E-cadherins may initiate outside-in signal transducing pathways, thus supporting an active role for E-cadherins in the control of key early post-aggregation events.

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† To whom correspondence should be addressed: Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health, 9000 Rockville Pike, Bldg. 30, Rm. 211, Bethesda, MD 20892-4330. Tel.: 301-496-6259; Fax: 301-402-0823; E-mail: gutkind@nih.gov.

1 The abbreviations used are: MDCK, Madin-Darby canine kidney; PI 3-kinase, phosphatidylinositol 3-kinase; Tyr(P), phosphotyrosine; PI3P, phosphatidylinositol 3-phosphate; PBS, phosphate-buffered saline; HA, hemagglutinin.

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Experimental Procedures

Cell Culture and Expression Plasmids—An expression vector for hemagglutinin-(HA) tagged Akt (pCEFL-HA-Akt) has been reported elsewhere (22). Early passage MDCK cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transfections, cells were grown at 70% confluence in 60-mm cell culture dishes and transfected with 4 μg of HA-tagged PKB/Akt cDNA by the calcium phosphate precipitation technique and selected with 500 μg/ml G418. Transfected clones were maintained in 500 μg/ml G418 to provide selection pressure.

Antibodies and Immunologic Detection Methods—Antibodies specific for E-cadherins, purchased from Transduction Laboratories (Lexington, KY), were used for immunoprecipitation and Western blotting. Antibodies to the extracellular domain of E-cadherin molecule (DECMA-1 clone, Sigma) were used for immunostaining and for antibody inhibition experiments. An anti-HA-specific monoclonal antibody (HA11, Babco, Richmond, CA) and a goat polyclonal anti-Akt antibody (C20, Santa Cruz Biotechnologies, Inc.) were used to detect ectopic and endogenously expressed Akt, respectively. Antibodies against the p85 regulatory subunit of PI 3-kinase and anti-phosphotyrosine (anti-Tyr(P), G410 clone) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-mouse and anti-rabbit secondary antibodies coupled to horseradish peroxidase were from Cappel Laboratories (Durham, NC). Co-immunoprecipitation and Western blotting experiments were performed as described previously (22).

Akt and PI 3-Kinase Assays—MDCK cells were serum-starved overnight in the presence of 10 mM Hepes, and E-cadherin-mediated cell-to-cell contacts were disrupted by treatment with EGTA to a final concentration of 4 mM for 30–40 min at 37 °C. Thereafter, intercellular interactions were allowed to re-establish in the presence of fresh, calcium-containing medium (final concentration CaCl₂, ~1.8 mM) (24). At different time points after calcium restoration, cells were harvested, lysed on ice, and assayed for Akt activation as described under “Experimental Procedures.” Autoradiograms showing the time course of Akt activity correspond to representative experiments. Similar results were obtained in three to five independent experiments. Data represent the average (mean ± S.D.) of three to five independent experiments, expressed as fold increase with respect to untreated cells (control).

Fig. 1. E-cadherin-mediated cellular aggregation induces Akt activation. A, MDCK cell monolayers were left untreated (panel a) or treated with 4 mM EGTA (panel b). The EGTA-containing medium was then replaced with serum-free, calcium-containing medium for 5 min (panel c) and 30 min (panel d). Cells were fixed and stained with anti-E-cadherin-specific antibodies. B, MDCK cells (panel a) and MDCK cells expressing an epitope-tagged Akt (panel b) were serum-starved and treated with 4 mM EGTA for 30–40 min and then calcium was restored for 5–60 min, as indicated. As controls, starved cells were left untreated (control) or stimulated with 100 nM EGF for 5 min. After calcium restoration, lysates were immunoprecipitated with anti-Akt or anti-HA antibodies and used for Akt kinase reaction as described under “Experimental Procedures.” Autoradiograms showing the time course of Akt activity correspond to representative experiments. Similar results were obtained in three to five independent experiments. Data represent the average (mean ± S.D.) of three to five independent experiments, expressed as fold increase with respect to untreated cells (control). C, MDCK cells were stably transfected with HA-Akt, as described above. Following starvation, cells were left untreated (panel a), treated with 4 mM EGTA (panel b), or restored in calcium-containing medium for 30 min after EGTA treatment (panel c). Cells exposed to 100 nM EGF for 5 min were used as positive control (panel d). After stimulation, cells were fixed, permeabilized, stained with anti-HA monoclonal antibodies followed by a fluorescein isothiocyanate-conjugated secondary antibodies and prepared for microscope analysis at a × 63 magnification.
E-cadherin-mediated Activation of PI 3-Kinase/Akt Pathway

Using epithelial MDCK cells, we first evaluated whether adherens junction assembly could affect the state of activation of endogenous Akt. As epithelial cells require de novo formation of tight homophilic cell-cell attachments to form homotypic boundaries among epithelial cells, we first used a simple method to study the adhesive properties of surface molecules. Using epithelial MDCK cells, we first evaluated whether adherens junction assembly could affect the state of activation of endogenous Akt. As epithelial cells require de novo formation of tight homophilic cell-cell attachments to form homotypic boundaries among epithelial cells, we first used a simple method to study the adhesive properties of surface molecules.

RESULTS AND DISCUSSION

E-cadherin-mediated Adherens Junction Formation Elicits Akt/PKB Activation—Using epithelial MDCK cells, we first evaluated whether adherens junction assembly could affect the state of activation of endogenous Akt. As epithelial cells require Ca²⁺ to form homophilic cell-cell adhesions, a simple method to study the adhesive properties of surface molecules involves the disruption of Ca²⁺-dependent homotypic boundaries among cells by EGTA-treatment and the re-establishment of cell-cell contacts by the subsequent restoration of Ca²⁺ ions. Whereas control cells present a typical pattern of E-cadherin immunostaining at the level of cell-cell contacts (Fig. 1A, panel a), in cells treated with the calcium chelator, EGTA, E-cadherins appear to be diffusely distributed (Fig. 1A, panel b). In this case, the loss of E-cadherins at the level of cell-cell contacts is most likely due to their redistribution throughout the cell surface rather than to the internalization of E-cadherins, as reported for other types of cadherins (26). After restoration of calcium, de novo formation of adherens junctions could be observed as early as 5–10 min (Fig. 1A, panel c), and the process appeared to be almost complete after 30–40 min (Fig. 1A, panel d).

To explore the possibility that E-cadherin engagement leads to Akt activation, MDCK cells were grown as long confluent cultures by maintaining cells in complete medium for at least 24–48 h after reaching confluence, to optimize cell-cell contacts as well as to minimize the influence of integrin-extracellular matrix interactions (27). Cells were then serum-starved for 18–24 h and cell-cell contacts disrupted by the treatment with EGTA (4 mM) for 30–40 min. Subsequently, adherens junctions were allowed to re-form in the presence of fresh calcium-containing medium, and, at the indicated time points, cells were lysed and assayed for Akt kinase activity. As shown in Fig. 1B, under these experimental conditions, the reassembly of adherens junctions induced a remarkable elevation of Akt kinase activity. Kinetic studies demonstrated a rapid increase in Akt...
activity as early as 15 min after calcium restoration, with a peak at 30–40 min followed by a decrease over a 1-h time course. Stable expression of a HA-tagged Akt in transfected MDCK cells revealed to be a useful tool to enhance the detectability of Akt activation upon E-cadherin engagement. Kinetics of activation of ectopically expressed Akt (Fig. 1B, panel b) was similar to that observed for the endogenous enzymatic activity (Fig. 1B, panel a), thus suggesting that endogenous levels of E-cadherins and Akt are sufficient to sustain a potent Akt activity following adherens junction assembly under physiological conditions.

Activation of Akt following E-cadherin Engagement Leads to Akt Translocation to the Nucleus—Following activation by a PI 3-kinase-mediated pathway, Akt has been shown previously to translocate to the nucleus (28), where it participates in the regulation gene expression (29). On the basis of these observations, we examined whether E-cadherin engagement could lead to a change in the subcellular distribution of an ectopically expressed Akt. Serum-starved cells stimulated with EGF (100 nM) for 5–10 min were used as a positive control (Fig. 1C, panel d). Whereas Akt appeared to be diffusely distributed in the cytoplasm of untreated and EGTA-treated cells (Fig. 1C, panels a and b), respectively, calcium restoration caused a marked translocation of Akt to the nucleus (Fig. 1C, panel c).

Akt Activation in Response to Adherens Junction Assembly Requires E-cadherin Engagement—To confirm that Akt activation is not due to the manipulation of calcium levels but to the ability of calcium restoration to mimic the physiological engagement of E-cadherins in cell-cell contacts, we took advantage of the availability of function-perturbing anti-E-cadherin antibodies, DECMA-1 clone, which have been described previously to be effective in blocking E-cadherin mediated adherens junction formation (30). As illustrated in Fig. 2 (A and B, sixth lanes), pretreatment with anti-E-cadherin antibodies, that hinders the formation of adherens junctions, led to a dramatic suppression of Akt activity upon calcium restoration. This suggests that E-cadherin-dependent homophilic interactions among cells are strictly required for induction of Akt activity. In contrast, the presence of anti-E-cadherin antibodies did not affect EGF-induced Akt activation (Fig. 2, A and B, third lanes), thus establishing the specificity of the experimental approach and suggesting that E-cadherin-mediated Akt activation occurs through a growth factor-independent mechanism. Unexpectedly, during the course of dose-response experiments with blocking antibodies, we observed that high antibody dilutions caused a dramatic increase in the activity of Akt (Fig. 2, A and B, eighth lanes). Antibody immobilization experiments, using MDCK cells suspended in 4 mM EDTA and plated on cell culture dishes precoated with different antibody dilutions, confirmed these observations (data not shown). The most straightforward explanation for these seemingly conflicting results relies on the fact that in the presence of a vast excess of DECMA-1 antibodies the majority of the antibody-bound E-cadherins would be expected to remain in a monomeric, inactive form. However, at high antibody dilutions each molecule of antibody would be expected to bind two molecules of E-cadherin, thus causing lateral dimerization and clustering of E-cadherins, which can mimic E-cadherin activation by calcium-dependent homophilic interactions (31, 32). Together, these observations suggest that E-cadherin engagement is necessary and sufficient for Akt activation in response to adherens junction assembly.

Role of PI 3-Kinase in the Activation of Akt in Response to E-cadherin-mediated Cellular Aggregation—The Akt/PKB kinase represents a downstream target of PI 3-kinase in a pathway critical for signaling cell survival in response to several stresses or growth factor deprivation (20–22). We therefore used a PI 3-kinase inhibitor, wortmannin, to address the possible involvement of PI 3-kinase in the induction of Akt kinase activity by adherens junction assembly. As shown in Fig. 3, preincubation of cells with wortmannin (50 nM) for 30 min before addition of calcium completely abolished Akt activation, with Akt kinase levels falling even below the unstimulated background in wild-type as well as in stably transfected MDCK cells. These observations suggested that the functional activity of PI 3-kinase is required for the activation of Akt in response to E-cadherin engagement.

Formation of Adherens Junctions Causes Association of PI 3-Kinase with E-cadherin-containing Protein Complexes—Agonist activation of PI 3-kinase frequently involves the translocation of this enzyme to the plasma membrane where it can gain access to its lipid substrates (reviewed in Ref. 33). Thus, it was tempting to speculate that a similar mechanism may underlie the activation of Akt upon organization of E-cadherin-mediated cell-cell contacts. In preliminary experiments we found that the p85 regulatory subunit of PI 3-kinase was tyrosine-phosphorylated as a function of time after calcium restoration, overlapping with the kinetics of Akt activation (data not shown). Therefore, we decided to evaluate directly the pattern of activation of PI 3-kinase after immunoprecipitation of MDCK cell lysates with anti-Tyr(P) antibodies. Of interest, the profile of PI 3-kinase activity after calcium restoration mirrored that of Akt, as illustrated in Fig. 4A. We next tested the possibility that Akt activation in response to E-cadherin engagement could involve the docking of PI 3-kinase to the adherens junction complex. As shown in Fig. 4B, upon calcium restoration, PI 3-kinase was found to be associated to the E-cadherin immunoprecipitates in a time-dependent manner, as judged by Western blotting with specific anti-p85/PI 3-kinase antibodies.

Taken together, overall results of this study indicate that one of the molecular events resulting from the E-cadherin-mediated cellular aggregation is the rapid activation of a PI 3-kinase/Akt cascade. Although the mechanism whereby E-cadherin engagement stimulates this biochemical route is not fully understood, it likely involves a yet to be identified tyrosine kinase, which, when activated in response to E-cadherin mediated-cellular aggregation, might facilitate the recruitment of PI 3-kinase to E-cadherin-containing complexes at the level of the plasma membrane. Indeed, the recovery of the p85 regulatory subunit of PI 3-kinase in co-immunoprecipitation experiments with anti-E-cadherin antibodies, along with the kinesthetic pattern of PI 3-kinase activity detected in anti-Tyr(P) immunoprecipitates, strongly argue in favor of this hypothesis.

As PI 3-kinases are known to play a central role in a number of cellular processes, including mitogenic signaling and cell survival, cytoskeletal remodeling, as well as metabolic control and vesicular trafficking (reviewed in Ref. 33), our present findings may have broad implications to the understanding of epithelial cell biology. In this scenario, E-cadherins might function as "relationship molecules" between the extracellular and the intracellular environment, initiating the transduction of intracellular signaling pathways stimulating the PI 3-kinase/Akt cascade. This might provide an interesting mechanism whereby the adhesion status of the cells may control the cell fate, including cell survival or death by apoptosis, as well as other critical events occurring during the stepwise organization of the epithelium.

REFERENCES
1. Takeichi, M. (1991) Science 251, 1451–1455
2. Gumbiner, B. M. (1996) Cell 84, 345–357
3. Kemler, R. (1993) Trends Genet. 9, 317–321
4. Ozawa, M., and Kemler, R. (1992) J. Cell Biol. 116, 989–996
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5. Aberle, H., Schwartz, H., and Kemler, R. (1996) J. Cell. Biochem. 61, 514–523
6. Birchmeier, W., and Behrens, J. (1994) Biochim. Biophys. Acta 1198, 11–26
7. Simard, D., and Nahs, I. R. (1996) Biochem. Biophys. Res. Commun. 219, 122–127
8. Vermeulen, S. J., Bruyneel, E. A., Bracke, M. E., De Bruyne, G. K., Vennekens, K. M., Vleminkx, K. L., Berz, G. J., van Roy, F. M., and Mareel, M. M. (1995) Cancer Res. 55, 4722–4728
9. Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M., and Birchmeier, W. (1993) J. Cell Biol. 120, 757–766
10. Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G. (1998) Nature 392, 190–193
11. Toyoyama, H., Nuruiki, K., Ogawa, H., Yanagi, M., Matsumoto, H., Nishijima, H., Shimotakahara, T., Aikou, T., and Ozawa, M. (1999) Oncol. Rep. 6, 81–85
12. Zheng, C., Kinch, M. S., and Burridge, K. (1997) Mol. Biol. Cell 8, 2329–2344
13. Kinch, M. S., Clark, G. J., Der, C. J., and Burridge, K. (1995) J. Cell Biol. 130, 461–471
14. Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S., and Takeichi, M. (1992) J. Cell Biol. 118, 703–714
15. Sou, T. S., and Nelson, W. J. (1998) J. Cell Biol. 142, 85–100
16. Bullions, L. C., and Levine, A. J. (1998) Curr. Opin. Oncol. 10, 81–87
17. Kantak, S. S., and Kramer, B. H. (1998) J. Biol. Chem. 273, 16953–16961
18. Miller, J. R., and Moon, R. T. (1996) Genes Dev. 10, 2527–2539
19. Day, M. L., Zhao, X., Vallorosi, C. J., Putzi, M., Powell, C. T., Lin, C., and Day, K. C. (1999) J. Biol. Chem. 274, 9656–9664
20. Marte, B. M., and Downward, J. (1997) Trends Biochem. Sci. 22, 355–358
21. Khalik, G., Klippel, A., and Weber, M. J. (1997) Mol. Cell. Biol. 17, 1595–1606
22. Murza, C., Laguigne, L., Wetzker, R., Cuadrado, A., and Gutkind, J. S. (1998) J. Biol. Chem. 273, 19080–19085
23. Downward, J. (1998) Curr. Opin. Cell Biol. 10, 262–267
24. Volberg, T., Geiger, B., Kartenbeck, J., and Franke, W. W. (1986) J. Cell Biol. 102, 1832–1842
25. Gutkind, J. S., Lecal, P. M., and Robbins, K. C. (1990) Mol. Cell. Biol. 10, 3806–3809
26. Lampugnani, M. G., Corada, M., Caveda, L., Breviario, F., Ayalaon, O., Geiger, B., and Dejana, E. (1995) J. Cell Biol. 129, 203–217
27. Kinch, M. S., Petch, L., Zhong, C., and Burridge, K. (1997) Cell Adhes. Commun. 4, 425–437
28. Andjelkovic, M., Alesso, D. R., Meier, R., Fernandez, A., Lainh, N. J., Frech, M., Cron, P., Cohen, P., Lucco, J. M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 31515–31524
29. Du, K., and Montminy, M. (1998) J. Biol. Chem. 273, 32377–32379
30. Vestweber, D., and Kemler, R. (1985) EMBO J. 4, 3393–3398
31. Ozawa, M., and Kemler, R. (1998) J. Cell Biol. 142, 1605–1613
32. Brieher, W. M., Yap, A. S., and Gumbiner, B. M. (1996) J. Cell Biol. 133, 487–496
33. Wymann, M. P., and Pirola, L. (1998) Biochim. Biophys. Acta 1436, 127–150