Signal Transduction from N-cadherin Increases Bcl-2

REGULATION OF THE PHOSPHATIDYLINOSITOL 3-KINASE/Akt PATHWAY BY HOMOPHILIC ADHESION AND ACTIN CYTOSKELETAL ORGANIZATION*

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Associated with the metastatic progression of epithelial tumors is the dynamic regulation of cadherins. Whereas E-cadherin is expressed in most epithelium and carcinomas, recent studies suggest that the up-regulation of other cadherin subtypes in carcinomas, such as N-cadherin, may function in cancer progression. We demonstrate that a signal transduction cascade links the N-cadherin-catenin adhesion complex to up-regulation of the anti-apoptotic protein Bcl-2. In suspension, aggregates of DU-145 cells, an E-cadherin expressing human prostate carcinoma line, survive loss of integrin-dependent adhesion by a different anti-apoptotic signaling pathway than the N-cadherin expressing lines PC3 and PC3N. N-cadherin intercellular adhesion mediates a 3.5-fold increase in Bcl-2 protein expression, whereas the level of the proapoptotic protein Bax remains constant. Only N-cadherin ligation in PC3 cells, which express both N-cadherin and E-cadherin, is sufficient to induce activation of Akt/protein kinase B. N-cadherin homophilic ligation initiates phosphatidylinositol 3-kinase-dependent activation of Akt resulting in Akt phosphorylation of Bad on serine 136. Following N-cadherin homophilic adhesion phosphatidylinositol 3-kinase was identified in immunoprecipitates of the N-cadherin-catenin complex. The recruitment of phosphatidylinositol 3-kinase to the adhesion complex is dependent on ligation of N-cadherin and an organized actin cytoskeleton because cytochalasin D blocks the recruitment. We propose that N-cadherin homophilic adhesion can initiate anti-apoptotic signaling, which enhances the Akt cell survival pathway in metastatic cancer.

Cadherins are transmembrane cell-cell adhesion receptors, which have important functional roles in mediating cell segregation during embryonic development and in maintenance of adult tissue integrity (1). Functional cell adhesion by cadherin subtypes requires both the coordinated homophilic binding of the extracellular domain and the cadherin cytoplasmic domain with the actin cytoskeleton (2). Calcium binds to the cadherin extracellular domain inducing a conformation that initiates and stabilizes the homophilic binding of cadherin subtypes on adjacent cells (3, 4). The conserved cadherin cytoplasmic domain is associated with a lateral network of actin filaments through the catenins (5). Either β-catenin or γ-catenin binds the complex to α-catenin, which either interacts directly with peripheral actin filaments, or indirectly through actin-associated proteins, such as vinculin or α-actinin (6, 7). In addition, p120<sup>ctn</sup> binds to a separate site in the juxta membrane region of cadherins and controls the strength of cadherin-mediated adhesion (8).

Although the classical cadherin subtypes and the catenins lack enzymatic activity, they can associate with kinases and phosphatases, such as Fer and PTP1B, in adheren junctions (9, 10). Homophilic E-cadherin adhesion initiates the activation of phosphatidylinositol 3-kinase (PI 3-kinase)<sup>1</sup> followed by downstream activation of Akt/protein kinase B (11, 12). The serine/threonine kinase Akt is an important regulator of metabolic pathways, as well as, apoptotic pathways. Akt is activated in response to diverse extracellular stimuli, such as EGF and other growth factors and integrin adhesion to matrix (13). Akt kinase activity is regulated by PI-3 kinase at the plasma membrane through the production of phosphatidylinositol 3,4-biphosphate and phosphatidylinositol 3,4,5-triphosphate, which bind to the pleckstrin homology domain of Akt altering its conformation. For full activation, Akt requires phosphorylation on threonine 308 (Thr-308) and serine 473 (Ser-473) by 3-phosphoinositide-dependent kinase 1 (14) or the phosphoinositide-dependent kinase 2/integrin-linked kinase (15). Phosphorylation of Thr-308 occurs when Akt is recruited to the membrane, but is not sufficient for activation of kinase activity, which requires phosphorylation of Ser-473 (16). Activated Akt can phosphorylate several substrates that result in suppression of apoptosis (13).

Upon exposure to apoptotic signals, it is proposed that cell fate is determined by the relative balance between pro- and anti-apoptotic protein interactions of the Bcl-2 family (17). Members of this family include the pro-apoptotic proteins Bad, Bik, Bid, and the anti-apoptotic cell survival proteins Bcl-2 and Bcl-xL (18). Whereas homodimers of Bcl-2 in the mitochondrial membrane prevent the activation of caspase-9, heterodimerization of Bcl-2 and Bad induces the activation of caspase-9 and initiates an irreversible pathway (19). One mechanism by which Akt prevents apoptosis is through the phosphorylation of the pro-apoptotic protein Bad. Akt phosphorylates Bad on Ser-

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1 The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; GST, glutathione S-transferase; PARP, poly(ADP-ribose)polymerase; HA, hemagglutinin; EGF, epidermal growth factor; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium salt; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.
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136, which inhibits the heterodimer formation of Bad and Bcl-2 in the mitochondrial membrane (20). Phosphorylated Bad is sequestered in the cytoplasm by interacting with 14-3-3 scaffolding proteins, which in turn suppresses apoptosis (21).

Among the alterations that facilitate prostate cancer progression are changes in cell adhesion molecule expression along with the increased resistance to apoptosis (22). E-cadherin is essential for adhesion of glandular epithelial cells to each other, and homophilic cell-cell adhesion results in the formation of adheren junctions, which in turn regulates differentiation, growth, and survival within the tissue microenvironment (23, 24). Decreased expression of E-cadherin is an important factor in the regulation of carcinoma invasiveness and metastasis (25).

The dysregulation of cell adhesion in prostate cancer metastasis is, in part, facilitated by the gain in expression of nonepithelial cadherins and members of the integrin family (26). N-cadherin is up-regulated in dedifferentiated, invasive prostate carcinomas (27, 28) and N-cadherin expression is reported to induce a motile scattered phenotype in breast cancer cells (29) and squamous cell carcinomas (30).

Evidence indicates that disruption of cadherin adhesion can initiate apoptosis in both normal and cancer cells (31, 32) similar to the inhibition of integrin adhesion to extracellular matrix (33). Several studies have partly delineated the molecular mechanisms that link integrin-mediated signal transduction pathways to the intracellular apoptotic machinery, but the mechanism whereby cadherin adhesion contributes to cell survival is poorly understood. To test the importance of cadherin subtypes in cell survival, we examined the homophilic ligation of cadherins on the phosphorylation and activation of Akt. Increased Akt activity has been implicated in prostate tumor progression androgen independence (34). We demonstrate that the anti-apoptotic effects of N-cadherin in prostate carcinomas depend on its ability to recruit PI 3-kinase to the N-cadherin-catenin-actin complex and results in an increased Bcl-2/Bax protein level. Overexpression of Bcl-2 has been observed in androgen-independent prostate adenocarcinomas, which correlated with the presence of metastases (35, 36). Our findings suggest that the gain of N-cadherin expression by prostate carcinomas in advanced stages of metastasis may be an inhibitor of apoptosis through stabilization of Bcl-2.

EXPERIMENTAL PROCEDURES

Cell Culture—PC3 (American Type Culture Collection; ATCC), PC3N (27), and DU-145 human prostate carcinoma cell lines (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and penicillin/streptomycin at 37 °C in 5% CO2 atmosphere at constant humidity. PC3N cells, which only express N-cadherin, were grown to 75% confluence, deprived of serum for 24 h, and detached from the culture surface expression of cadherin subtypes. Cells were dissociated thoroughly to single cells by trituration 10 times with a Pasteur pipette. After centrifugation the cells were suspended in final volume of 0.5 ml in calcium-free suspension modified Eagle's medium (Invitrogen) in the absence of serum, and 106 cells were maintained in suspension on 25 μl/ml poly-2-hydroxyethyl methacrylate (Sigma) coated 2-cm2 culture dishes to prevent cell attachment. Cells were compared in the presence of Ca2+ (1.8 mM) to induce cell-cell aggregation or without Ca2+ to prevent cell-cell adhesion. The number of cells per multicellular aggregate (n = 3) was counted as the mean of three independent experiments. At a number of single cells, A = number of cells, the number of multicellular aggregates (number of cellular aggregates) was determined to be ~8400 DU-145 cells per aggregate and 4000 PC3N cells per aggregate. At various times, the cells were washed once in phosphate-buffered saline (PBS) and the cell pellet was lysed in 2× SDS sample buffer, equivalent protein was separated on a 7% SDS-PAGE and analyzed for PARP cleavage by immunoblotting.

To analyze DNA integrity after incubation in suspension culture (39),
cells were washed in PBS and lysed with 500 μg/ml proteinase K in 25 mM Tris- HCl, pH 8.0, 100 mM NaCl, 10 μM EDTA, and 0.5% SDS. Lysates were extracted three times with phenol/chloroform (1:1), treated with RNase A, and separated on a 1.5% agarose gel containing ethidium bromide.

**Calcium Switch Assay** (4)—Confluent cultures were deprived of serum overnight, and N-cadherin-mediated cell-cell contacts were disrupted by treatment with 4 mM EDTA in Dulbecco’s modified Eagle’s medium for 40 min at 37 °C. The calcium-free medium was removed and N-cadherin adherent junctions were allowed to re-establish by addition of Dulbecco’s modified Eagle’s medium, which contains 1.8 mM [Ca2+]o and 1 mM [Ca2+]i. In the calcium switch experiments the time of re-addition of Ca2+ was considered as 0 min. Following calcium restoration at 37 °C, cells were harvested and lysed in 2× SDS sample buffer containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin, 20 mM NaF, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonlfyl fluoride. In individual experiments, a N-cadherin monoclonal antibody (GC4), wortmannin (50 nM), Tocris Cookson, Inc., Ballwin, MO, LY294002 (Calbiochem, San Diego, CA), or cytochalasin D (Sigma) were included in the medium for pretreatment of cells.

**Antibody Immobilization**—Bacteriolytic 60-mm Petri dishes were coated for 2 h at 37 °C with goat anti-mouse Fab fragments (50 μg/ml) in 100 mM NaHCO3, pH 9.6 (final volume 1.5 ml). Plates were rinsed and then incubated overnight with monoclonal antibodies specific for N-cadherin, E-cadherin (10 μg/ml) at 4 °C and blocked with heat-denatured 1% BSA in PBS, pH 7.4. Serum-starved cells were harvested with EDTA treatment, washed in calcium/magnesium-free PBS, and resuspended in 2 ml of serum-free medium without calcium (suspension modified Eagle’s medium). Single cells (106) were permitted to adhere to the antibody-coated dishes at 37 °C or poly-l-lysine (Sigma)-coated dishes as control after which nonadherent cells were removed with washing. Attached cells were lysed, and subjected to immunoblot analyses as described above.

**Immunoprecipitation and GST-Bad Pull Down**—For immunoprecipitation, cells were lysed on ice for 10 min in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 2 mM sodium vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin. Equivalent amounts of protein (500 μg) were precleared and immunoprecipitated from the lysates, washed with lysis buffer followed by S1 buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 2 mM EDTA, 1.5% Triton X-100, 0.5% deoxycholate, 0.2% SDS) (40). Samples were then resuspended in 2× SDS sample buffer and boiled in the presence of 2-mercaptoethanol (Sigma), separated by SDS-PAGE, transferred to nitrocellulose overnight at 4 °C, and proteins were detected as described above.

For Akt co-precipitation with Bad, cell extracts from confluent cells were prepared as described above. Preclearing was first carried out with agarose-conjugated GST at 4 °C for 1 h. Supernatants containing 500 μg of proteins were then incubated with 0.3 μg of purified GST fusion protein containing full-length Bad for 4 h at 4 °C. The GST-Bad-Sepharose beads were washed five times with 1 ml of lysis buffer, and the precipitated proteins were boiled in SDS sample buffer. The samples were separated on SDS-PAGE and transferred onto nitrocellulose membrane.

**Immunofluorescence Microscopy**—Cells were grown on glass coverslips to confluence, fixed for 5 min in 4% paraformaldehyde in PBS. Cells were then permeabilized with 0.5% Triton X-100 in 10 mM PIPES, pH 6.8, 50 mM NaCl, 3 mM MgCl2, 0.3 μl sucrose for 5 min at 4 °C. After washing with PBS, cells were blocked with 2% bovine serum albumin and 1% goat serum, and incubated with primary antibody for 1 h at 25 °C. Following washing with 0.1% BSA in PBS and incubation with Cy3-conjugated anti-mouse IgG or fluorescein isothiocyanate-conjugated anti-rabbit, cell nuclei were stained with bis-benzimide (Molecular Probes, Inc., Eugene, OR) in PBS for 5 min, washed, and mounted in 2% n-propyl gallate, 90% glycerol, pH 8.0. Immunofluorescent samples were examined under laser scanning confocal microscope, LSM 410, equipped with helium, neon, and argon lasers (Zeiss) using the appropriate filters. Images were processed using Adobe Photoshop.

**RESULTS**

**Ca2+—dependent Cell Adhesion Inhibits Apoptosis during Loss of ECM Attachment**—Prostate carcinomas require signals from integrin-matrix adhesion and cell-cell adhesion for cell survival (41). Previous studies had indicated that E-cadherin homophilic cell-cell adhesion was sufficient to regulate cell cycle progression and cell survival during anchorage-independent growth (32, 42). To further examine the mechanism of cadherin adhesion in anti-apoptotic signal transduction, human prostate carcinoma cell lines were cultured in serum-free anchorage-independent conditions. By varying the concentration of calcium in the culture medium, DU-145 and PC3N cells were cultured in suspension as single cells in low Ca2+ and normal Mg2+ containing medium, or allowed to form multicellular aggregates in the presence of a normal physiological concentration of Ca2+ and Mg2+ (Fig. 1A). Apoptosis in the suspension cultures was assessed at different time points by immunoblot analysis examining nuclear PARP proteolytic cleavage (Fig. 1B). PARP is cleaved by caspase 3 between Asp-213 and Gly-214 to produce two fragments of apparent molecular weights of 29,000 and 85,000 (43). When cultured as single cells in the absence of integrin-mediated adhesion, PARP cleavage was detected by the appearance of an 85-kDa fragment at 12 h in both DU-145 (lane c) and PC3N cells (lane i). At 24 h the level of the 85-kDa proteolytic fragment of PARP in DU-145 (lane e) and PC3N (lane k) cells was increased and determined by densitometry to represent ~40% cleavage of PARP (Fig. 1B). Ca2+-dependent multicellular aggregates of PC3N cells (lane i) in suspension did not undergo PARP cleavage, whereas multicellular aggregates of DU-145 cells showed reduced PARP proteolytic cleavage (18.6%; lane f) compared with single DU-145 cells (42.0%; lane e). Substrate-attached cells showed no PARP cleavage when subjected to low calcium medium (data not shown). Analysis of genomic DNA from PC3N prostate cells maintained in suspension following detachment from the substrate was used to confirm the initiation of apoptosis (Fig. 1C). Nucleosomal DNA ladders were detectable in suspension cultures of single cells in low calcium maintained for 24 h on nonadhesive substrates. Multicellular aggregates of PC3N cells in suspension in normal calcium showed no apoptotic DNA laddering. These results suggest that in the absence of integrin-mediated adhesion and cell survival factors, Ca2+-dependent PC3N multicellular aggregates are able to induce an anti-apoptotic pathway.

Because Bcl-2 plays a central role in prostate carcinoma cell survival, we determined the level of Bcl-2 expression in the suspension cultures. Bcl-2 protein levels were down-regulated in suspension cultures of single PC3N cells and no detectable expression of Bcl-2 was detected in DU-145 cells (Fig. 1B). Immunoblot analysis showed that Bcl-2 expression levels were increased 2-fold in multicellular aggregates of PC3N cells. We further explored the involvement of activated PI 3-kinase in regulating the Bcl-2 level in PC3N multicellular aggregates using biochemical inhibitors. PC3N multicellular aggregates treated with 50 nM wortmannin or 20 μM LY294002 (data not shown), inhibitors of PI 3-kinase, showed PARP cleavage to be similar to that found in single cells in suspension. Cleavage of PARP was not observed in PC3N multicellular aggregates treated with a solvent control. These results suggest the involvement of Ca2+-dependent cell-cell adhesion in the activation of PI 3-kinase, which can regulate the cellular level of Bcl-2 (13).

**N-cadherin Engagement Increases the Bcl-2/Bax Protein Ratio**—Because the protein kinase Akt is both a critical regulator of cell survival and one of the downstream substrates activated by PI 3-kinase signaling, we evaluated whether formation of Ca2+-dependent cell-cell adhesion in adherent PC3N cells could affect the activation state of Akt by assessing phosphorylation of Akt on Ser-473 using a phospho-specific antibody (Fig. 2A). Cadherin homophilic adhesion is Ca2+-dependent and is disrupted when extracellular calcium is reduced. In a calcium switch assay restoration of calcium re-establishes the adherens junction cell-cell contacts (3, 4). Dissociation of cell-cell contacts reduced the basal phosphorylation level of Akt on
Ser-473 as compared with control cells in normal calcium (Fig. 2A). Engagement of cell-cell adhesion by calcium restoration (1.8 mM) induced Akt Ser-473 phosphorylation in a time-dependent manner (~3-fold at 30 min). When PC3N cells form multicellular aggregates in the presence of Ca$^{2+}$ they display an induction of Akt Ser-473 phosphorylation in a time-depend-
subjected to a Ca\textsuperscript{2+} switch experiment and analyzed by immunoblotting for Bad Ser-136 phosphorylation and total Bad protein expression. Transfection of wild type Akt or the Akt kinase-deficient construct was detected by a monoclonal antibody against HA. B, co-precipitation of Akt and Bad from PC3N lysates after a Ca\textsuperscript{2+} switch assay. GST-Bad precipitates and immunoblots of Akt-phosphorylated Ser-473 and endogenous Akt. Relative densities of Akt Ser-473 phosphorylation were normalized to endogenous Akt and further corrected to the 0-h protein lysate. The data represented are the mean of three independent experiments.

ent manner, and no tyrosine phosphorylation of the focal adhesion kinase was detected (data not shown). These observations suggest that cadherin-mediated cell-cell contacts trigger a signal transduction pathway involving PI 3-kinase and Akt that is independent of integrin-mediated signaling pathways.

One role of Akt kinase activity in promoting cell survival is its ability to regulate the cellular level of Bcl-2. Cell survival is favored by a high Bcl-2/Bax protein ratio (44). To examine whether N-cadherin ligation could regulate the Bcl-2/Bax ratio, we again utilized the calcium switch assay. As shown in Fig. 2, A and B, the relative phosphorylation of Akt Ser-473 was elevated in a time-dependent manner following restoration of N-cadherin intercellular adhesion. Phosphorylation of Akt Ser-473 was maximal at 2 h showing a 14-fold increase. Analyses of equivalent proteins from the same cellular lysates were then used to measure the steady state protein level of Bcl-2 and Bax by immunoblotting. In Fig. 2C, the relative Bcl-2/Bax protein ratio increased upon N-cadherin engagement with approximately a 2.5-fold induction after 2 h. The protein level of Bax remained constant and the level of Bcl-2 increased upon N-cadherin engagement.

One mechanism by which Akt activation increases the Bcl-2/Bax ratio is through the phosphorylation of the pro-apoptotic protein Bad on Ser-136 (20). The Bcl-2 protein level has been shown to increase when Bad is phosphorylated on Ser-136. This increase in Akt Ser-473 phosphorylation was not seen in samples maintained in low calcium medium (Fig. 3B, lane b). In addition, Akt was not affinity precipitated in cell lysates with control GST-lamin (Fig. 3B, lane a). Together, these data support the role of N-cadherin engagement in inducing BAD phosphorylation mediated by increased Akt kinase activity.

Expression of the Akt kinase-deficient construct in PC3N cells prevented the N-cadherin-mediated induction of phosphorylation of Bad on Ser-136 (Fig. 3A, lane h). However, expressing N-cadherin engagement Bad Ser-136 phosphorylation was readily detected in cells containing the transfected wild type Akt expression construct (Fig. 3A, lane g). Thus, Bad Ser-136 phosphorylation induced by N-cadherin-mediated cell-cell adhesion is dependent on Akt kinase activity. These findings demonstrate that assembly of the N-cadherin-mediated adherens junction induces a distinct cell-cell adhesion-dependent signal transduction pathway.

To determine the phosphorylation state of Akt interacting with Bad upon N-cadherin engagement, we performed a GST-Bad pull-down assay. Following N-cadherin engagement, cell lysates were prepared at different time points, and incubated with an excess of GST-Bad-Sepharose, which was followed by immunoblotting for Akt Ser-473 phosphorylation. In all samples an equivalent amount of Akt was affinity precipitated with GST-Bad (Fig. 3B). Approximately a 17-fold phosphorylation of Akt on Ser-473 was affinity purified with GST-Bad following 1 h after reformation of the adherens junction that is comparable with the EGFP control (Fig. 3B, compare lanes c and f). This increase in Akt Ser-473 phosphorylation was not seen in samples maintained in low calcium medium (Fig. 3B, lane b).

Ligation of N-cadherin Elicits Recruitment of PI 3-Kinase to the Adhesion Complex—To confirm that Akt activation was because of N-cadherin homophilic engagement and not calcium manipulation, we utilized a N-cadherin neutralizing monoclonal antibody. As shown in Fig. 4A, pretreatment of cells with an antibody that inhibits N-cadherin-mediated formation of cell-cell contacts led to suppression of Akt Ser-473 phosphorylation upon adherens junction reformation. The N-cadherin
monoclonal antibody did not suppress EGF-stimulated phosphorylation of Akt. This indicates that in the calcium switch assay, N-cadherin ligation is responsible for Akt Ser-473 phosphorylation and that the soluble anti-N-cadherin antibody functions as an antagonist. To confirm that PI 3-kinase is required for the activation of Akt kinase activity in response to N-cadherin ligation, cells were pretreated with 50 nM wortmannin for 30 min prior to calcium restoration in the presence or absence of neutralizing N-cadherin antibody. Wortmannin completely suppressed Akt Ser-473 phosphorylation following N-cadherin ligation and in EGF-stimulated cells.

Because N-cadherin-mediated activation of Akt is dependent upon PI 3-kinase activity, we explored whether PI 3-kinase was recruited to the N-cadherin/H18528 catenin complex upon engagement of cell-cell adhesion. After restoration of the adherens junction, cell lysates were immunoprecipitated with an antibody to N-cadherin and immunoblotted with antibodies to the p85 regulatory and p110 catalytic subunits of PI 3-kinase. The p85 and p110 subunits of PI 3-kinase associated with the N-cadherin complex in a time-dependent manner reaching a maximal level at 15 min following N-cadherin adhesion (Fig. 4B). Together with our studies of PI 3-kinase inhibition, these results suggest that engagement of N-cadherin forms a novel complex with PI 3-kinase at the plasma membrane that results in the activation of Akt.

Antibody immobilization assays have been reported to cluster cell surface adhesion receptors, thereby acting as agonists and eliciting downstream signal transduction pathways (45). To further examine whether the cadherin ligation is sufficient to promote Akt phosphorylation on Ser-473, single cells in serum-free conditions were allowed to attach to a substrate consisting of an immobilized N-cadherin extracellular domain monoclonal antibody. The clustering of N-cadherin has been shown to localize actin filaments to the plasma membrane and induce localized tyrosine phosphorylation (46). As shown in Fig. 4C, the phosphorylation of Akt on Ser-473 expression was determined to be 10-fold higher in PC3N cells adherent to the immobilized N-cadherin antibody as compared with nonadherent carcinoma cells seeded on a nonadhesive substrate of an immobilized nonimmune mouse IgG. Pretreatment with wortmannin prior to attachment to immobilized N-cadherin monoclonal antibodies suppressed Akt phosphorylation on Ser-473. Because PC3N cells do not express E-cadherin, there was no adhesion to immobilized E-cadherin antibody. These results further confirm that N-cadherin ligation induces Akt phosphorylation on Ser-473.

Because the human prostate carcinoma line PC3 expresses both E-cadherin and N-cadherin (27), we examined whether both cadherin subtypes could activate Akt. PC3 cells attached to both immobilized antibodies recognizing the extracellular domain of E-cadherin and N-cadherin in similar numbers (data not shown). Both antibodies to E-cadherin and N-cadherin
have previously been shown to inhibit cadherin-dependent cell-cell contact (47, 48). As shown in Fig. 4D, the PC3 cells that attached to the N-cadherin antibody also expressed E-cadherin, and PC3 cells that had attached to the E-cadherin antibody express N-cadherin. As expected, the PC3 cells attached to immobilized anti-N-cadherin showed activation of Akt by phosphorylation on Ser-473. The activation of Akt on immobilized anti-N-cadherin was PI 3-kinase-dependent as shown by pretreatment of PC3 cells with wortmannin. In contrast, the PC3 cells attached to immobilized anti-E-cadherin showed negligible phosphorylation of Akt on Ser-473. These results suggest that in the PC3 prostate carcinoma cell line N-cadherin ligation preferentially activates a PI 3-kinase/Akt signal transduction pathway.

Cytoskeletal Organization Is Necessary for Formation of the N-cadherin-Catenin Complex and Signaling Activity—Functional adhesion of the N-cadherin-catenin complex requires association with the actin cytoskeleton (5, 49), although previous studies have shown that initial clustering of the cadherin extracellular domains by homophilic binding is independent of a direct attachment to the actin cytoskeleton (5, 8). This initial contact of cadherins is remodeled and strengthened by association with the actin cytoskeleton (49, 50). We investigated whether the association of N-cadherin-catenin with filamentous actin was necessary for stimulating phosphorylation of Akt on Ser-473. As shown in Fig. 5A, disruption of the actin cytoskeletal integrity by pretreatment with cytochalasin D suppressed Akt Ser-473 phosphorylation following calcium restoration. We used an additional approach to ligate N-cadherin that does not depend on the cell-cell adhesion induced by the Ca^{2+} switch assay. In Fig. 5B, cells treated with cytochalasin D were able to bind and adhere to the N-cadherin-immobilized antibodies, but were not able to induce Akt Ser-473 phosphorylation similar to cells seeded on poly-L-lysine- or BSA-coated dishes. However, cells treated with a solvent control adhere to the immobilized N-cadherin antibodies and induce a 10-fold phosphorylation of Akt on Ser-473. This indicates that a functional actin cytoskeleton is necessary for Akt activation induced by N-cadherin ligation. In contrast, activation of Akt by addition of 100 nM EGF was not inhibited by pretreatment with cytochalasin D (data not shown).

Because the actin-binding protein α-catenin has been deleted in PC3N cells (51), we investigated whether vinculin is associated with the N-cadherin-catenin complex. Immunoprecipitation of N-cadherin from PC3N cells as shown in Fig. 6A indicates that in addition to β-catenin and p120ctn, vinculin was also detected in the N-cadherin-catenin complex. Co-precipitation of N-cadherin with vinculin was verified in immunoprecipitations with a vinculin antibody. N-cadherin was found in the anti-vinculin immunoprecipitations associated with both β-catenin and p120ctn (Fig. 6A). These results suggest that vinculin is present with the N-cadherin-catenin complex in prostate carcinoma cells similar to what has been observed for E-cadherin in breast cancer cell lines (6), and this interaction may mediate N-cadherin-catenin binding to the actin cytoskeleton.

To further confirm the association of vinculin with the N-cadherin complex, we performed immunolocalization studies to determine the localization of N-cadherin and vinculin. N-cadherin immunofluorescence showed strong localization at cell-cell contacts as anticipated (Fig. 6B, arrow). When cells were co-immunolabeled with vinculin and N-cadherin, two patterns for vinculin localization were observed. One pattern was similar to that for N-cadherin at cell-cell contacts, and merging of the two confocal images revealed identical patterns of co-localization (data not shown). In addition, vinculin but not N-cadherin was also detected in focal adhesion sites that were associated with stress fibers (Fig. 6B, arrowhead). In summary, the interaction and localization of vinculin with N-cadherin-mediated cell-cell contacts can replace α-catenin in the complex and may play a role in linking the N-cadherin-catenin complex to the peripheral actin cytoskeleton.

Because a functional actin cytoskeleton is required for Akt phosphorylation on Ser-473 upon N-cadherin ligation, we investigated whether the recruitment of the p85 subunit of PI 3-kinase to the N-cadherin-catenin complex was also dependent upon actin cytoskeleton. Cells were pretreated with 1 μM cytochalasin D or Me_{6}SO as solvent control prior to adherens junction reformation. Immunoblot analysis of N-cadherin immunoprecipitates showed that the p85 regulatory subunit of PI 3-kinase was not able to associate with the N-cadherin-catenin complex in cells treated with cytochalasin D (Fig. 6C). However, the vinculin interaction with the N-cadherin-catenin complex was not disrupted by cytochalasin D.
The studies presented here suggest new insights into the function and interaction of the cadherin subtypes, E-cadherin and N-cadherin. Our data indicates that N-cadherin has dual functional roles in the regulation of apoptosis and in homophilic cell-cell adhesion. Genetic studies have also demonstrated a dual role for N-cadherin in null mouse embryos where populations of neural cells in the homozygous null embryos were apoptotic and myocardium was disorganized but not apoptotic (52). We found that N-cadherin homophilic adhesion in prostate carcinoma cells is linked to Akt signaling and inhibition of the mitochondrial apoptotic pathway. Members of the Bcl-2 family of proteins affect mitochondrial function and regulate the release of apoptosis-activating factors (18). Anti-apoptotic Bcl-2 family members act to preserve mitochondrial integrity by suppressing the release of cytochrome c. The clustering of N-cadherin extracellular domains between adjacent PC3N cells leads to organization of actin cytoskeletal structure and provides specific outside-in signals that regulate the steady state level of Bcl-2. Inhibition of homophilic binding of the N-cadherin extracellular domain correlates with decreased cellular levels of activated Akt and Bcl-2 protein expression. Cadherin engagement between adjacent cells has an essential role in the recruitment of the actin cytoskeleton to the adherens junction complex and regulation of actin polymerization (12, 49, 50). We identified that formation of the adherens junction complex leads to the recruitment and activation of PI 3-kinase and phosphorylation of Akt. Subsequent activation of the PI 3-kinase/Akt pathway leads to phosphorylation of Bad at Ser-136 and stabilization of Bcl-2. The cytoprotective effects of Bcl-2 are in part through the inhibition of formation of Bax homodimers in the mitochondrial outer membrane (53).

In previous studies (11, 12) E-cadherin ligation has been implicated in the regulation of PI 3-kinase and the localized production of phosphatidylinositol 3,4,5-triphosphate that initiates phosphorylation of Akt. Thus, both E-cadherin and N-cadherin have the potential to activate Akt signaling pathways. In a prostate carcinoma cell line, which expresses both E-cadherin and N-cadherin, our results using an antibody immobilization assay to ligate cadherin subtypes with specific anti-
bodies show that only N-cadherin leads to phosphorylation of Akt. Ligation of E-cadherin in PC-3 cells under the same conditions failed to activate Akt (Ser-473 phosphorylation). In prostate carcinomas, E-cadherin adhesion can prevent apoptosis in suspension, however, this is through a survival mechanism controlling retinoblastoma activation and G1 arrest (41). Results from studies by Laure et al. (54) indicate that cadherin subtypes are directly involved in the differentiation of different tissue types suggesting that cadherins influence specific signal transduction pathways regulating gene expression activity. Taken together these results suggest that the outside-in signaling of cadherin subtypes depends on their expression in a cellular context.

Evidence suggests that following cellular attachment the suppression of apoptosis is not mediated solely by integrin-mediated adhesion to extracellular matrix but, rather, by the ability of the cells to spread and adopt an optimal cell shape (55). Cadherin-mediated cell-cell adhesion is an additional mechanism to control cell shape and organization of the cytoskeleton. Formation of intercellular adherens junctions is a dynamic process that involves actin reorganization and polymerization initiated by cadherin adhesion (50, 56). Cadherin homophilic binding initiates clustering of the extracellular domains, and interaction of the cytoplasmic domain with the actin cytoskeleton is an important mechanism to strengthen the weak forces provided by homophilic binding (57). Association of the N-cadherin-catenin-vinculin complex in PC3 cells with the peripheral actin cytoskeleton provides sites for attachment of the cytoskeleton at the membrane, which are important for the development of mechanical stress during epithelial polarization and control of cell shape (56). This cadherin-mediated interaction also transmits signals from the plasma membrane to the actin cytoskeleton, ranging from activation of the Rho family of small GTPases (58–60) to production of phosphoinositol 3,4,5-triphosphate (61), which can regulate actin cytoskeleton organization. Homophilic E-cadherin ligation activates the GTPases Cdc42 and Rac, which regulates actin cytoskeletal rearrangement and can modulate cadherin function (58, 62). The PI 3-kinase product phosphoinositol 3,4,5-triphosphate is required for the activation and recruitment of exchange factors for the GTPases such as Tiam-1, which is required for epithelial adherens junction formation (64). The survival signals initiated by integrin adhesion activate specific pathways that converge on the activation of Akt (65), in addition to the activation of other molecular mechanisms that inhibit apoptosis (66). Thus, our results suggest an overlap in the cell survival signal transduction cascades that are stimulated by integrin adhesion to extracellular matrix, and intercellular adhesion by N-cadherin.

Following homophilic ligation of N-cadherin, our observations show that an intact actin cytoskeleton with the N-cadherin-catenin complex recruits PI 3-kinase. In addition to the remodeling of the actin cytoskeleton at the membrane by N-cadherin engagement, Lambert et al. (46) demonstrated that local accumulation of N-cadherin signaling complexes result in tyrosine phosphorylation of proteins in the adhesion complex. These tyrosine-phosphorylated proteins apparently serve as binding sites for the two Src homology 2 domains of the p85 regulatory subunit of PI 3-kinase to the cadherin-catenin complex. The major role of the p85 Src homology 2 domain is to mediate tyrosine kinase-dependent regulation of PI 3-kinase by increasing the catalytic activity of the p110 and recruitment of PI 3-kinase to the signaling complex (67). Binding of the p85 subunit of PI 3-kinase has been identified to tyrosine-phosphorylated cytosolic and cadherin-associated β-catenin (68). The activation and autophosphorylation of EGF receptor by EGF elicits remodeling of actin (69), which initiates the formation of lamellipodia (70) and requires the activity of PI 3-kinase. Distinct signaling properties for the class I isoforms of the p110 catalytic subunit of PI 3-kinase have been demonstrated (71). EGF-stimulated lamellipodia extension was shown to be a function of the α isoform and not the β isoform of the catalytic subunit (70), although both p110 catalytic subunits are capable of leading to downstream Akt activation (72). The differential utilization of the p110 isoforms could reflect the specific signaling properties that distinguish between the N-cadherin and EGF receptor signal transduction complexes.

Because the protein and lipid phosphatase PTEN is a negative regulator of the PI 3-kinase pathway and is lost in advanced prostate cancer (73), tumor progression is likely to be associated with increased responsiveness to extracellular signals, such as from cadherin adhesion that activates the PI 3-kinase pathway. Our studies show that the level of Bcl-2 protein expression is enhanced in PTEN negative prostate carcinoma cell lines that form N-cadherin-mediated adherens junctions. The association of PI 3-kinase with the N-cadherin-catenin complex was transient even though PC3 cells are deficient in PTEN. Tyrosine phosphatases, such as PTP1B (10), are also associated with the N-cadherin-catenin adhesion complex and potentially function to reduce PI 3-kinase binding. We observed that following N-cadherin ligation, Akt translocated from the plasma membrane to the cytoplasm and then to the nucleus (data not shown), and returned to a basal level of phosphorylation by 24 h suggesting action of a phosphatase, such as PP2A.

Altogether our results suggest a potential mechanism where N-cadherin adhesion can contribute to the regulation of apoptosis in carcinoma cells. Several studies have shown that regulation of apoptosis by the Bcl-2 family of proteins involves regulation of the mitochondrial outer membrane integrity (18). In the unphosphorylated state Bad can form heterodimers with anti-apoptotic Bcl-2 homologs and promotes cell death by allowing Bax-Bax homo-oligomers in the mitochondrial membrane. Monomeric Bax is predominantly cytoplasmic in cells under normal conditions, but translocates to the outer mitochondrial membrane in response to apoptotic signals (63). During apoptosis the activation of caspases is a critical step and may contribute to the loss of anti-apoptotic signaling from cadherins because many of the substrates of caspases have been shown to be components of adherens junctions including plakoglobin, β-catenin, and E-cadherin (24, 74). Thus these new insights into the function of the cadherin survival signaling pathway may provide new approaches for the therapeutic treatment of uncontrolled growth and metastasis of prostate cancer cells.

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