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An Acidic Extracellular pH Disrupts Adherens Junctions in HepG2 Cells by Src Kinases-Dependent Modification of E-Cadherin

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ABSTRACT

We have previously shown that culturing HepG2 cells in pH 6.6 culture medium increases the c-Src-dependent tyrosine phosphorylation of β-catenin and induces disassembly of adherens junctions (AJs). Here, we investigated the upstream mechanism leading to this pH 6.6-induced modification of E-cadherin. In control cells cultured at pH 7.4, E-cadherin staining was linear and continuous at cell–cell contact sites. Culturing cells at pH 6.6 was not cytotoxic, and resulted in weak and discontinuous junctional E-cadherin staining, consistent with the decreased levels of E-cadherin in membrane fractions. pH 6.6 treatment activated c-Src and Fyn kinase and induced tyrosine phosphorylation of p120 catenin (p120ctn) and E-cadherin. Inhibition of Src family kinases by PP2 attenuated the pH 6.6-induced tyrosine phosphorylation of E-cadherin and p120ctn, and prevented the loss of these proteins from AJs. In addition, E-cadherin was bound to Hakai and ubiquitinated. Furthermore, pH 6.6-induced detachment of E-cadherin from AJs was blocked by pretreatment with MG132 or NH_4Cl, indicating the involvement of ubiquitin-proteasomal/lysosomal degradation of E-cadherin. An early loss of p120ctn prior to E-cadherin detachment from AJs was noted, concomitant with a decreased association between p120ctn and E-cadherin at pH 6.6. PP2 pretreatment prevented the dissociation of these two proteins. In conclusion, pH 6.6 activated Src kinases, resulting in tyrosine phosphorylation of E-cadherin and p120ctn and a weakening of the association of E-cadherin with p120ctn and contributing to the instability of E-cadherin at AJs. J. Cell. Biochem. 108: 851–859, 2009.

KEY WORDS: EXTRACELLULAR ACIDIC PH; ADHERENS JUNCTION; E-CADHERIN; p120 CATENIN; c-Src; FYN KINASE

An acidic extracellular pH (pHe) is a feature of the tumor microenvironment [Tannock and Rotin, 1989; Webb et al., 1999] and has been associated with the invasive behavior of some types of tumor cells [Kato et al., 1992]. Several factors, including type IV gelatinase, metalloprotease-2, metalloprotease-9, cathepsins B and C, and angiogenic factors, have been reported to contribute to this acidic pHe-mediated cell invasion [Rofstad et al., 2006]. However, most studies have focused on the upregulation of metalloproteases under these circumstances.

Disruption of the adherens junction (AJ) is often associated with epithelial mesenchymal transition and cell invasiveness [Murai et al., 2004]. AJs, composed of E-cadherin and catenin (ctn), are responsible for cell–cell contact. At the AJ, β-ctn binds to the C-terminus of E-cadherin and indirectly links to the actin cytoskeleton via α-catenin (α-ctn) [Takeichi, 1990; Takeichi et al., 1992]. The ctn family consists of α-, β-, γ-, and p120 ctn (p120ctn) [Anastasiadis and Reynolds, 2000]. The integrity of the AJ depends on the stable interactions between the molecules of the cadherin/ctn complex. Expression of cell adhesion molecules is regulated by transcriptional and posttranslational controls. The latter include cadherin endocytosis, cadherin/ctn phosphorylation and dephosphorylation by protein kinases and phosphatases, and the assembly of junctional actin filaments [Akhtar and Hotchin, 2001; Le et al., 2002]. Downregulation of E-cadherin is associated with increased migration and invasiveness of tumor cells

Abbreviations used: AJ, adherens junction; ctn, catenin; DAPI, 4′,6-diamidino-2-phenylindole dilactate; IgG-HC, immunoglobulin heavy chain; IgG-LC, immunoglobulin light chain; MTT, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; p120ctn, p120 catenin; pHe, extracellular pH; PI, propidium iodide; RIPA, radioimmunoprecipitation; siRNA, small interfering RNA; TBS, Tris-buffered saline.

Shih-Horng Huang and Seu-Mei Wang contributed equally to this work.

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[Lu et al., 2003; Guarino, 2007; Herzig et al., 2007]. Tyrosine phosphorylation of E-cadherin results in endocytosis of E-cadherin, disruption of cell–cell contacts, and cell dispersion [Takeichi et al., 1994; Daniel and Reynolds, 1997]. Fujita et al. [2002] reported that c-Src activation induces endocytosis of E-cadherin by ubiquitination of Hakai-bound E-cadherin [Fujita et al., 2002]. Whether this regulatory mechanism is involved in acidic pH-induced AJ disassembly deserves further study.

P120ctn consists of multiple isoforms generated by alternative splicing [Anastasiadis and Reynolds, 2000]. By binding to the juxtamembrane domain of E-cadherin, p120ctn stabilizes E-cadherin at the cell membrane [Reynolds et al., 1994], and small interfering RNA (siRNA) knockdown of p120ctn accelerates cadherin degradation and reduces cell adhesion [Davis et al., 2003]. P120ctn is phosphorylated by Src kinase and receptor-tyrosine kinases [Downing and Reynolds, 1991; Reynolds et al., 1992, 1994] and by serine/threonine kinases, such as protein kinase C (PKC) [Mariner et al., 2001; Xia et al., 2003]. We have previously shown that an acidic pHe activates c-Src [Chen et al., 2008], but it is not known whether it induces tyrosine phosphorylation of p120ctn.

In HepG2 cells, an acidic pHe induces β-ctn tyrosine phosphorylation by c-Src kinase and reduces the binding of β-ctn to E-cadherin and subsequently disrupts AJs [Chen et al., 2008]. However, whether E-cadherin and p120ctn in HepG2 cells are affected by an acidic pHe remains to be explored. We hypothesized that an acidic pHe might disrupt AJs by modifying E-cadherin and p120ctn. In this study, we used acidic growth medium to mimic an acidic microenvironment to study the posttranslational modification of E-cadherin and p120ctn.

MATERIALS AND METHODS

CELL CULTURE

The human hepatocellular carcinoma cell line HepG2 (ATCC, HB8065) was purchased from the American Type Culture Collection (Rockville, MD) and was cultured in growth medium (DMEM containing non-essential amino acids, 1 mM sodium pyruvate, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal calf serum; all from Gibco, Rockville, MD) in a 5% CO_2 humidified atmosphere at 37°C. Cells (2×10^5 cells/ml) were plated either in 35 mm culture dishes or on coverslips in culture dishes. For the preparation of acidic growth medium, growth medium was adjusted to pH 6.6 by addition of 1.2 N HCl.

INHIBITORS

The Src family kinase inhibitor PP2 and the ubiquitin-proteosome inhibitor MG132 were purchased from BioMol Lab (Plymouth Meeting, PA). NH_4Cl was from Sigma (St. Louis, MO).

CYTOTOXICITY ASSAY

HepG2 cells were plated in 24-well plate and incubated in growth medium for 24 h before cell viability assay. Cells were incubated in pH 7.4 or 6.6 for 3 and 5 h. After two washes with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4), 500 μl of growth medium containing 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well for 4 h reaction. The reaction medium was then removed and the cells were lysed in 500 μl DMSO per well, and the absorbance was measured at 590 nm on a spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

IMMUNOFLUORESCENCE STAINING

HepG2 cells grown on coverslips were washed briefly in PBS. For E-cadherin staining, the cells were fixed in cold acetone for 10 min at −20°C. After a brief wash with PBS, they were incubated overnight at 4°C with mouse monoclonal anti-human E-cadherin antibodies (BD Biosciences, San Jose, CA) diluted 1:50 in PBS. After a PBS wash, bound antibodies were detected by incubation for 1 h at 37°C with FITC-conjugated goat anti-mouse IgG antibodies (Sigma) diluted 1:50 in PBS. The cells were counterstained for 15 min with 1 μg/ml of 4′,6-diamidino-2-phenylindole dilactate (DAPI; Sigma) in 0.9% NaCl. After washes in 0.9% NaCl, the coverslips were mounted in a mixture of 2% *n*-propyl gallate and 60% glycerol in 0.1 M phosphate-buffered saline, pH 8.0, and sealed with nail polish. Immunofluorescence images were examined using a Zeiss Axiophot microscope (Carl Zeiss, Oberkocheu, Germany) equipped with epifluorescence, and the images were captured and digitized using a Nikon D1X digital camera (Nikon, Tokyo, Japan).

PREPARATION OF WHOLE CELL HOMOGENATES AND MEMBRANE FRACTIONS

All steps were at 4°C. HepG2 cells were suspended in 20 mM HEPES, 0.28 M sucrose, 50 mM NaCl, 20 mM sodium pyrophosphate, 2 mM EDTA, 2 mM PMSF, 1 mM NaF, 2 mM Na_3VO_4, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, and 1 μg/ml of pepstatin, pH 7.4, and ultrasonicated for 2 × 10 s. In some studies, the cell homogenate was used, while in others, membrane fractions were prepared by centrifuging the homogenate at 1,000*g* for 10 min to remove nuclei, then centrifuging the supernatant at 16,000*g* for 45 min. The membrane pellets were dissolved in radioimmunoprecipitation (RIPA) buffer (50 mM Tris–HCl, 0.25% Na deoxycholate, 150 mM NaCl, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml of aprotinin, leupeptin, and pepstatin, pH 7.4) containing 1% Nonidet-40.

WESTERN BLOTTING

Gel electrophoresis and immunoblotting were performed as described previously [Towbin et al., 1979; Fritz et al., 1989] using a 10% resolving gel and a 3.5% stacking gel. After various treatments, HepG2 cells were homogenized or fractionated into membrane and cytosolic fractions. An equal volume of sample buffer was added and the mixture heated at 90°C for 3 min. Proteins (50 μg per lane) were electrophoresed and transferred to a nitrocellulose membrane. Strips from the membrane were blocked with 5% non-fat milk in Tris-buffered saline (TBS: 150 mM NaCl, 50 mM Tris, pH 8.2) containing 0.1% Tween-20 and incubated overnight at 4°C with primary antibodies in TBS containing 0.1% Tween 20 and 5% non-fat milk. For detection of internal standards (GADPH or ATPase), rabbit anti-GADPH (Abcam, Cambridgeshire, UK) or rabbit anti-ATPase antibodies (Santa Cruz Biotechnology Inc., CA) was used. After washes with TBS-Tween, the strips were incubated with alkaline phosphatase-conjugated secondary

antibodies in TBS-Tween and positive bands visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as chromogen. Densitometry was performed using Gel pro 3.1 (Media Cybernetics, Silver Spring, MD). The density of the band in the control sample was defined as 100% and the density of the band in the test sample expressed as a percentage of this value. All experiments were performed at least three times, and the values are expressed as the mean ± SD.

IMMUNOPRECIPITATION

After treatment, HepG2 cells were lysed by ultrasonication in RIPA buffer containing 0.1% SDS and the lysate centrifuged at 16,000*g* for 10 min at 4°C. The supernatants were incubated for 24 h at 4°C on a shaker with mouse anti-human E-cadherin antibody (BD Biosciences) or anti-phosphotyrosine antibody (Zymed Lab, South San Francisco, CA) (1 μg antibody for 400 μg of protein). Prewashed aliquots of protein G-Sepharose beads (50 μl of a 50% suspension in RIPA buffer) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were added to the mixture for 1 h at 4°C on a shaker. After centrifugation at 16,000*g* for 1 min, the immunoprecipitates were washed, dissolved in 30 μl of reducing SDS sample buffer, and analyzed by Western blotting with mouse monoclonal antibodies against phosphotyrosine (Zymed), human E-cadherin, uniquibitin,

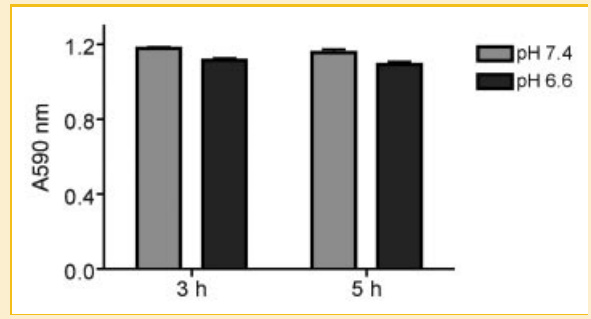

Fig. 1. Effect of acidic culture medium on cytotoxicity. HepG2 cells were incubated for 3 and 5 h in culture medium adjusted to pH 7.4 or 6.6, then assayed for viability by the MTT test. The results are expressed as the mean absorbance at 590 nm ± SD, n = 3.

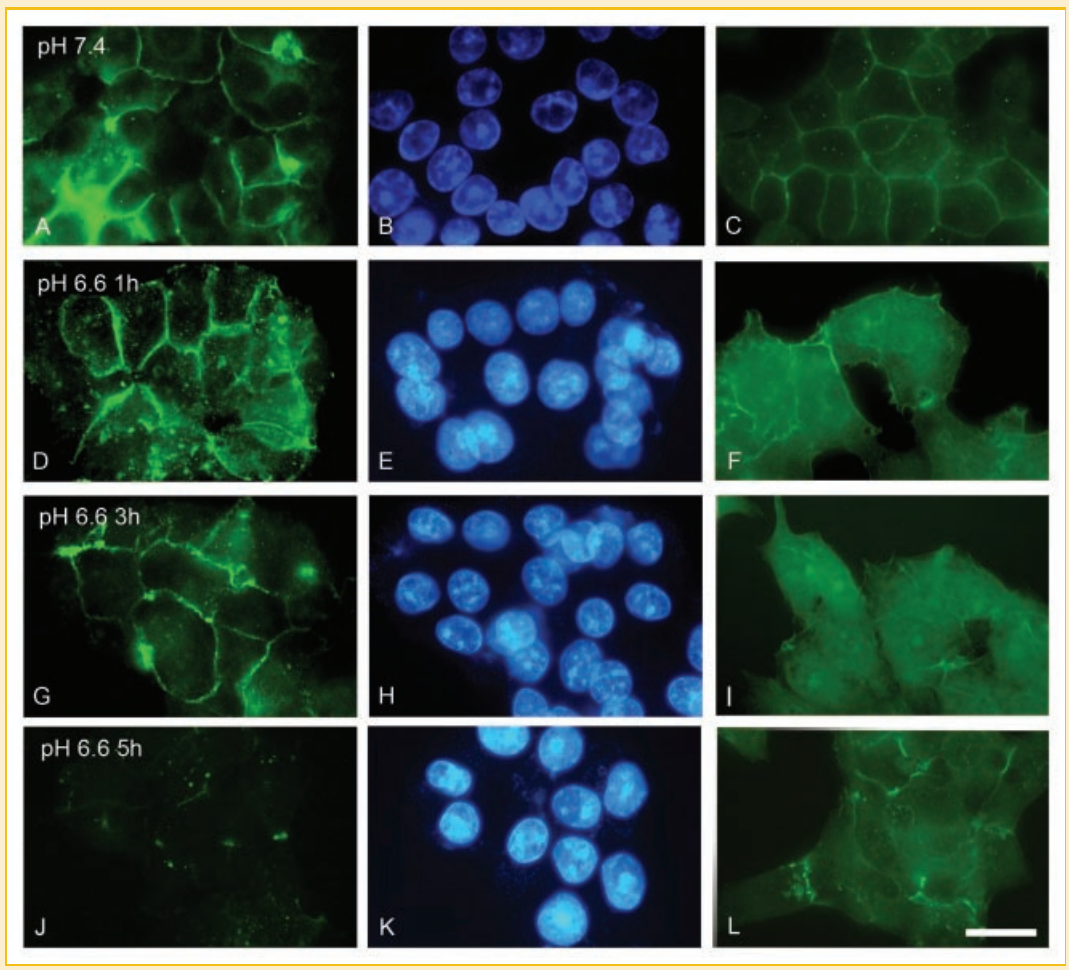

Fig. 2. Distributional change of E-cadherin and p120ctn in HepG2 cells at pH 6.6. HepG2 cells were incubated in culture media at pH 7.4 (A–C) for 5 h or at pH 6.6 for 1 h (D–F), 3 h (G–I), and 5 h (J–L). All were immunostained for E-cadherin (A,D,G,J) or p120ctn (C,F,I,L) and counterstained with DAPI (B,E,H,K). Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Hakai or human p120ctn (BD Biosciences, San Jose, CA), followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin G antibodies (Promega Corporation, Madison, WI).

STATISTICAL ANALYSIS

All results are expressed as the mean ± SD. Statistical differences between means were evaluated using Student's *t*-test. Differences were considered significant if $P < 0.05$.

RESULTS

ACIDIC CULTURE MEDIUM DECREASES E-CADHERIN STAINING AT CELL–CELL CONTACT SITES

We first examined whether pH 6.6 caused cell cytotoxicity. MTT assay showed that cells incubated in pH 6.6 culture medium for 3 and 5 h did not affect cell viability (Fig. 1). As previously stated by Chen et al., [2008], prodium iodide vital staining and 4′,6-diamidino-2-phenylindole dilactate staining showed very few apoptotic or necrotic cells after 24 h incubation at pH 6.6, the percentages being comparable to those in the pH 7.4 group [Chen et al., 2008].

A time course study on the distributional change of E-cadherin was carried out. The cells were counterstained with DAPI to show the location of the nuclei in pH 7.4-treated cells (Fig. 2B) and pH 6.6-treated cells (Fig. 2E,H,K). HepG2 cells grown in pH 7.4 culture medium showed linear, continuous staining of E-cadherin at cell junctions (Fig. 2A,B). Intracellular punctates positive for E-cadherin were increased after 1 h of pH 6.6 treatment (Fig. 2D,E). A noticeable decrease in E-cadherin immunoreactivity at AJ was detected after 3 h of pH 6.6 treatment (Fig. 2G,H). E-cadherin staining became faint after 5 h of pH 6.6 treatment (Fig. 2J,K).

ROLE OF c-Src IN THE PH 6.6-INDUCED DOWNREGULATION OF E-CADHERIN

We next examined the role of Src family kinases in the pH 6.6-induced downregulation of E-cadherin at AJs. Phosphorylation of Src (Tyr416) family kinases was increased at 30 min and remained high up to at least 90 min of pH 6.6 treatment (Fig. 3A). Immunoprecipitation and Western blotting showed that both c-Src kinase and Fyn kinase were activated by tyrosine phosphorylation in response to pH 6.6, and pharmacological inhibition of Src kinases by PP2 prevented pH 6.6-induced activation of both kinases (Fig. 3B). Furthermore, PP2 pretreatment prevented the pH 6.6-induced loss of E-cadherin from AJs, as the intensity of E-cadherin immunostaining in the PP2 + pH 6.6 group was comparable to that in the pH 7.4 group (Fig. 4A). In agreement with the immunofluorescence data, E-cadherin levels in the membrane fraction were reduced at pH 6.6 compared to pH 7.4, and this effect was prevented by PP2 pretreatment (Fig. 4B). To examine whether pH 6.6 induced tyrosine phosphorylation of E-cadherin, cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and the amounts of E-cadherin in the immunoprecipitates were analyzed. Figure 5A shows a significant increase in tyrosine phosphorylation of E-cadherin after 1 h of pH 6.6 treatment, and this pH 6.6-induced

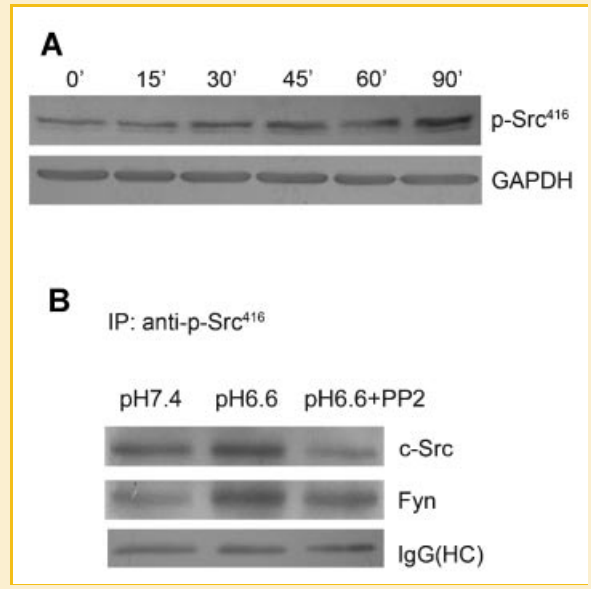

Fig. 3. pH 6.6 induces c-Src activation. A: HepG2 cells were cultured at pH 6.6 for 0, 15, 30, 45, 60, or 90 min, then the cell homogenates were analyzed for phosphorylated Src kinase (Tyr416) and GADPH (loading control) on Western blots. B: Cells were cultured at pH 7.4 or pH 6.6 for 1 h or were pretreated for 30 min with 10 μM PP2 at pH 7.4 for 30 min, then at pH 6.6 for 1 h, then the cell lysates were immunoprecipitated with anti-phosphorylated Src kinase (Tyr416), and the resulting immunoprecipitates analyzed for c-Src kinase, Fyn kinase and mouse IgG heavy chain (IgG-HC) (loading control). The results shown in (A) and (B) are representative of those obtained in two separate experiments.

tyrosine phosphorylation of E-cadherin was Src family kinase-dependent, as it was greatly attenuated by PP2 pretreatment (Fig. 5A). Tyrosine phosphorylation of E-cadherin by v-Src promotes the interaction with Hakai, a c-Cbl-like ubiquitin ligase, which has a high affinity for tyrosine phosphorylated E-cadherin, and the binding of Hakai with E-cadherin triggers the ubiquitination of E-cadherin [Fujita et al., 2002]. We then examined the binding of Hakai with these E-cadherin molecules. As expected, the amounts of Hakai bound to the E-cadherin increased by 50% 1 h after pH 6.6 treatment (Fig. 5B). We further evaluated the levels of E-cadherin ubiquitination. The E-cadherin-bound ubiquitin analyzed by co-immunopreipitation was increased 1 h after pH 6.6 treatment (Fig. 5C).

We had used MG132, a cell-permeable proteasome inhibitor, which reduces the degradation of ubiquitin-conjugated proteins [Holecek et al., 2006], to examine its effect in preventing the loss of E-cadherin from cell junctions induced by pH6.6. Figure 6A shows the continuous and linear staining of E-cadherin at cell contacts in pH 7.4-treated group, in contrast to the weak and diffuse E-cadherin staining in pH 6.6-treated groups (Fig. 6B). Pretreatment with MG132 could block pH 6.6-induced detachment of E-cadherin from cell junctions (Fig. 6C). In addition, pretreatment with NH_4Cl, a lysosome inhibitor, abrogated pH 6.6-induced loss of E-cadherin from cell–cell junctions (Fig. 6D). These results indicated that pH 6.6 reduced the levels of junctional E-cadherin through ubiquitin-proteasome/lysosome pathway.

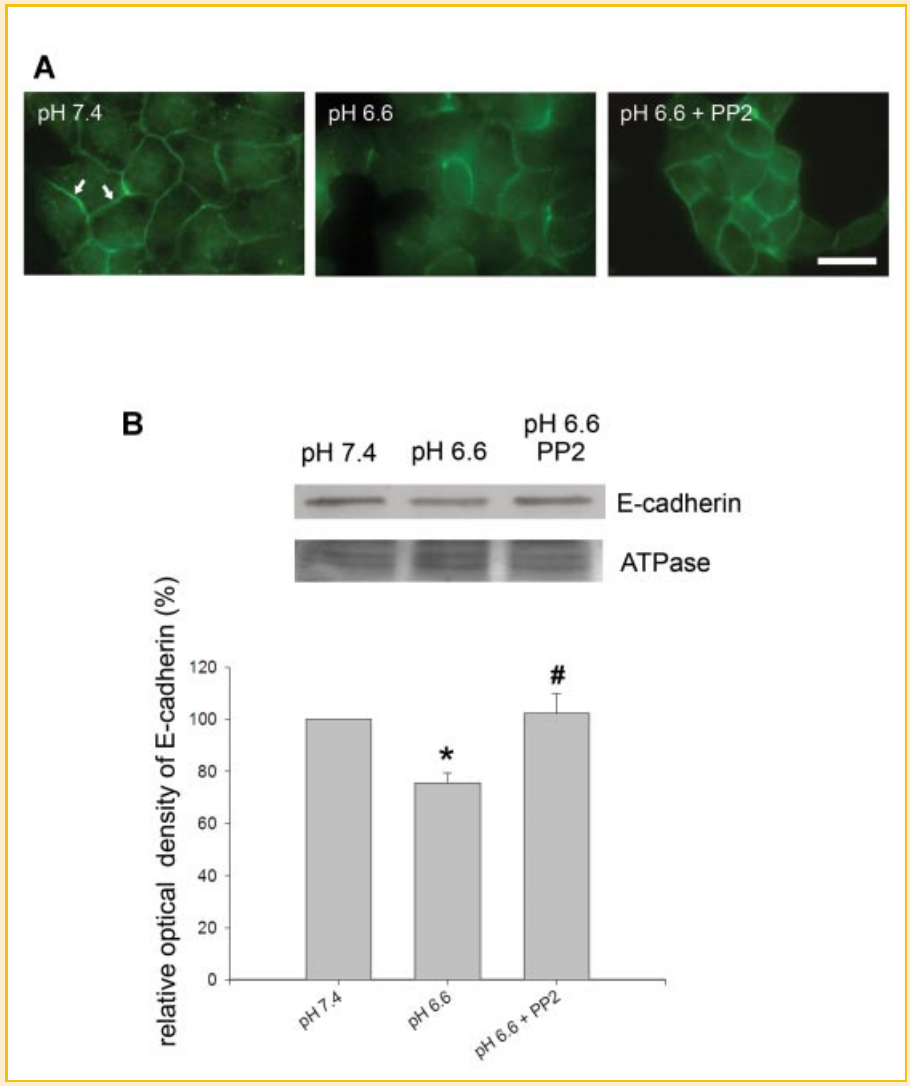

Fig. 4. pH 6.6 induces a Src kinase–dependent reduction in junctional E-cadherin levels. A: HepG2 cells were incubated in culture media for 5 h at pH 7.4 or 6.6 or pretreated for 30 min with 10 μM PP2 at pH 7.4, then at pH 6.6 in the presence of PP2 for 5 h (pH 6.6 + PP2), then immunostained for E-cadherin. The arrows indicate E-cadherin at cell contacts. Bar = 20 μm. B: Cells were incubated for 5 h in culture medium at pH 7.4 or 6.6 or pretreated for 30 min with 10 μM PP2 at pH 7.4, then at pH 6.6 in the continued presence of PP2 (pH 6.6 + PP2) for 5 h. The membrane fractions were collected and analyzed for E-cadherin and Na^+/K^+ ATPase (internal control) by Western blotting. The upper panel shows a typical result and the lower panel the mean ± SD for the densitometric scans of three blots expressed as a percentage of the pH 7.4 result. $^*P < 0.05$, compared to pH 7.4; $^\#P < 0.05$, compared to pH 6.6. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

c-Src ACTIVATION INDUCES TYROSINE PHOSPHORYLATION OF p120ctn

The association of p120ctn with the juxtamembrane domain of E-cadherin is thought to stabilize E-cadherin at AJs [Reynolds et al., 1992, 1994]. We hypothesized that the pH 6.6-induced loss of E-cadherin might partially result from inadequate protection of E-cadherin by p120ctn. A time-course study on the distribution of E-cadherin and p120ctn was therefore carried out. At pH 7.4, the cells displayed a continuous, linear distribution of both E-cadherin (Fig. 2A) and p120ctn (Fig. 2C) at cell–cell contact sites. The loss of p120ctn staining from AJs in response to pH 6.6 treatment consistently preceded that of E-cadherin. As early as 1 h at pH 6.6, p120ctn staining at cell–cell contact sites was reduced and discontinuous (Fig. 2F), while E-cadherin staining remained intense and continuous (Fig. 2D). At 3 h, no p120ctn staining at cell–cell contacts was seen, while E-cadherin staining was seen as discontinuous dots (Fig. 2G,I). At 5 h, no staining of p120ctn staining or E-cadherin was seen at cell–cell contacts (Fig. 2J,L). The diffuse cytoplasmic p120ctn staining increased with time of pH 6.6 treatment.

P120ctn is a substrate for c-Src kinase [Reynolds et al., 1992, 1994]. P120ctn isoforms 1 and 2 are expressed in equal amounts in HepG2 cells [Mo and Reynolds, 1996]. Both isoforms were tyrosine-phosphorylated at pH 6.6 (Fig. 7A). Isoform 1 is found in highly motile fibroblasts and macrophages [Reynolds et al., 1994] and is responsible for the increase in cell motility induced by epidermal

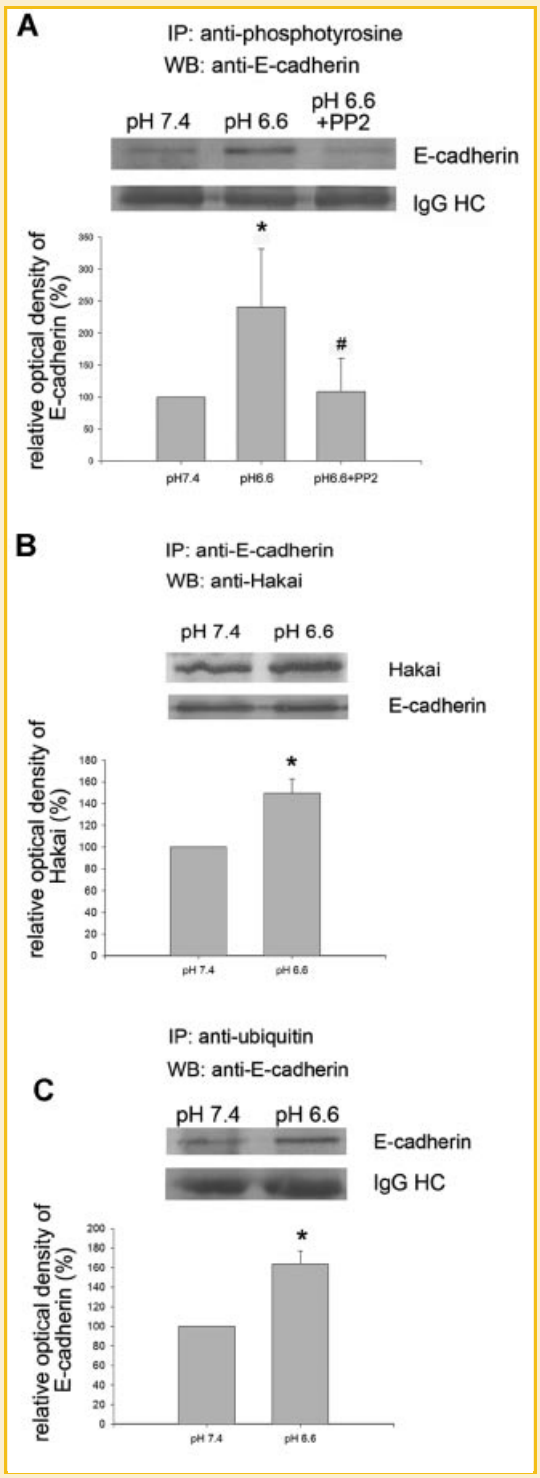

Fig. 5. pH 6.6 induces tyrosine phosphorylation and ubiquitination of E-cadherin. The cells were cultured for 1 h at pH 7.4 or 6.6 Whole cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (A), anti-E-cadherin antibodies (B) or anti-ubiquitin (C), and the immunoprecipitates analyzed for E-cadherin (A.C), Hakai (B) or IgG-HC (loading control). Pretreatment with 10 μM PP2 (pH 6.6 + PP2) prevents pH 6.6-induced tyrosine phosphorylation of E-cadherin. A representative gel is shown and the results for three experiments are shown in the lower panel expressed as a percentage of the pH 7.4 result. $^{*}P < 0.05$, compared to pH 7.4. $^{\#}P < 0.05$, compared to pH 6.6.

growth factor and hepatocyte growth factor [Cozzolino et al., 2003]. To study the impact of tyrosine phosphorylation of p120ctn on the association with E-cadherin, we performed immunoprecipitation with anti-p120ctn antibodies and analyzed the immunoprecipitates for E-cadherin by blotting. The amount of E-cadherin associated with p120ctn was drastically reduced after 1 h of pH 6.6 treatment, and PP2 pretreatment prevented this effect (Fig. 7B). Thus, the weakening of the binding of p120ctn to E-cadherin at pH 6.6 might contribute to the early loss of p120ctn from AJs and accelerate the destabilization of E-cadherin.

DISCUSSION

Loss of E-cadherin function can result from transcriptional or post-translational processes. Previous study has shown that tyrosine phosphorylation of E-cadherin by v-Src promotes the interaction with Hakai [Fujita et al., 2002]. In this study, we showed that pH 6.6 induced the activation of c-Src kinase and Fyn kinase and tyrosine phosphorylation of E-cadherin, which was subsequently bound to Hakai and ubiquitin. The modified E-cadherin has been proposed to enter endosomal compartments, targeted to lysosomes or recycling [Pece and Gutkind, 2002]. In our results, degradation of E-cadherin from cell junctions induced by pH 6.6 condition was both proteasome- and lysosome-dependent (Fig. 6). In mouse enterocytes, the degradation of E-cadherin from cell–cell contacts upon anoikis is mediated by both proteasome- and lysosme-dependent pathways [Fouquet et al., 2004].

The increased numbers of punctuate structures positive for E-cadherin in the cytoplasm might represent the processing of E-cadherin, which might account for the decrease in the junctional E-cadherin at pH 6.6. Thus, modifications of E-cadherin by c-Src kinase contribute to the disruption of AJs in HepG2 cells in an acidic environment.

The Src family kinase members, c-Src and Fyn, regulate the interaction between cadherin and ctns (α-ctn, β-ctn, and p120-ctn) by tyrosine phosphorylation of both cadherin and catenin. c-Src kinase and Fyn kinase can phosphorylate E-cadherin, β-ctn, and p120-ctn [Roura et al., 1999; Lilien et al., 2002; Piedra et al., 2003]. Different sites of tyrosine phospshorylation on p120ctn have opposite effects on RhoA binding. c-Src phosphorylates p120ctn at Tyr217 or Tyr228 and stimulates p120ctn and RhoA binding, whereas Fyn kinase phosphorylates p120ctn at Tyr112 and inhibits the interaction between p120ctn and RhoA [Castano et al., 2007]. Fyn kinase exerts a dominant effect, since simultaneous phosphorylation of p120ctn by Src and Fyn diminished the amount of RhoA associated to p120ctn [Castano et al., 2007]. Tyrosine phosphorylation of p120ctn by either Fyn kinase or c-Src promotes an increased association between p120ctn and E-cadherin [Castano et al., 2007]. In this study, however, we observed a decreased association between E-cadherin and p120ctn when both c-Src kinase and Fyn kinase were activated by pH 6.6. It is possible that pH 6.6 treatment triggers other signaling pathways which regulate the interaction between p120ctn and E-cadherin.

The stability of E-cadherin depends on its association with p120ctn, since p120ctn knockdown by siRNA results in complete

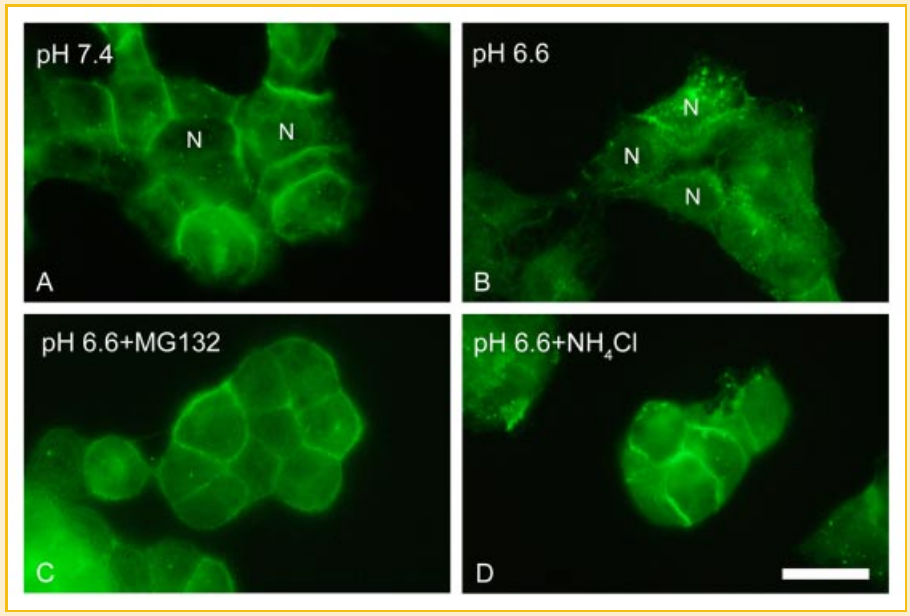

Fig. 6. pH 6.6 induces the detachment of E-cadherin from junctional complex by ubiquitin-proteosomal or lysosomal degradation. HepG2 cells were incubated in culture media for 5 h at pH 7.4 (A) or 6.6 (B) or pretreated for 30 min with 25 μM MG132 or 20 mM NH4Cl at pH 7.4, then at pH 6.6 for 5 h in the presence of MG132 (C, pH 6.6 + MG132) or 20 mM NH4Cl (D, pH6.6 + NH4Cl), then immunostained for E-cadherin. N, nuclei. Bar = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

loss of cell–cell adhesion [Davis et al., 2003; Xiao et al., 2003]. By binding to the cytoplasmic tail of cadherin, p120ctn prevents the interaction of cadherin with the endocytic membrane trafficking machinery [Davis et al., 2003; Hoshino et al., 2005; Xiao et al., 2007]. Consistent with the E-cadherin-protective role of p120ctn, E-cadherin levels are dramatically decreased in p120ctn-null SW48 colon carcinoma cells [Ireton et al., 2002]. Here, we detected tyrosine phosphorylation of p120ctn concomitant with the loss of p120ctn from AJs after 1 h of pH 6.6 treatment, suggesting that tyrosine phosphorylation of p120ctn caused this distributional change of p120ctn. Detachment of p120ctn occurred prior to E-cadherin detachment, and this observation correlated with the decreased binding of p120ctn to E-cadherin after 1 h of pH 6.6 treatment. Failure of p120ctn to protect E-cadherin might contribute to the degradation of E-cadherin from AJs by internalization. Our results provide support for a role of p120ctn in stabilizing junctional E-cadherin.

The role of tyrosine phosphorylation of p120ctn in maintaining the integrity of AJs remains obscure. P120ctn tyrosine phosphorylation is absent until transiently induced by various stimuli [Mariner et al., 2001, 2004]. Epidermal growth factor (EGF) receptor-induced tyrosine phosphorylation of p120ctn in BEL-704 hepatoma cells results in translocation of p120ctn to the nucleus and decreased cell adhesion [Huang et al., 2003]. In vitro tyrosine phosphorylation of p120ctn increases the binding of the Fer/Fyn-p120ctn complex to E-cadherin [Piedra et al., 2003]. Tyrosine phosphorylation of p120ctn correlates with an increased affinity for cadherins, both in vivo [Roura et al., 1999] and in vitro [Calautti et al., 1998; Kinch et al., 1995; Piedra et al., 2003]. However, other investigators reported that no significant change in the p120ctn/E-cadherin association was found, even when p120ctn was heavily tyrosine-phosphorylated [Reynolds et al., 1994; Shibamoto et al., 1994; Papkoff, 1997]. Moreover, tyrosine-phosphorylated p120ctn in v-Src-transfected L cells has been shown to dissociate from the cadherin complex, probably due to a conformational change in tyrosine residues in p120ctn [Ozawa and Ohkubo, 2001]. On the other hand, modification of cadherin and the catenin complex affects the localization of p120ctn at AJs. Tyrosine phosphorylation of VE-cadherin can lead to dissociation of p120ctn from AJs [Fujita et al., 2002; Potter et al., 2005; Tricaud et al., 2005], while tyrosine phosphorylation of β-ctn or p120ctn is known to disrupt the p120ctn/E-cadherin interaction [Ezaki et al., 2007]. pH 6.6 treatment was shown to increase tyrosine phosphorylation of β-ctn in our previous study [Chen et al., 2008] and to increase tyrosine phosphorylation of E-cadherin in this study. The amount of β-ctn-associated E-cadherin is decreased by ~20%, as reported in our previous study [Chen et al., 2008]. In this study, we observed that, when both E-cadherin and p120ctn were tyrosine-phosphorylated, the association between these two molecules became weak. P120ctn at AJ is properly phosphorylated at Ser/Thr [Fukumoto et al., 2008]. Serine/threonine phosphorylation of p120ctn could occur in response to PKC activation and vascular endothelial growth factor [Xia et al., 2003]. PKC activation by PMA dephosphorylates p120ctn at Ser/Thr residues and redistributes p120ctn to the cytoplasm [Xia et al., 2004]. Whether acidic pHe affects p120 ctn Ser/Thr phosphorylation to cause the detachment of p120ctn from AJs awaits for elucidation.

P120ctn can act upstream of the Rho family GTPase and plays a regulatory role in cell motility [Grosheva et al., 2001]. Mislocalization of p120ctn to the cytoplasm promotes cell motility, as

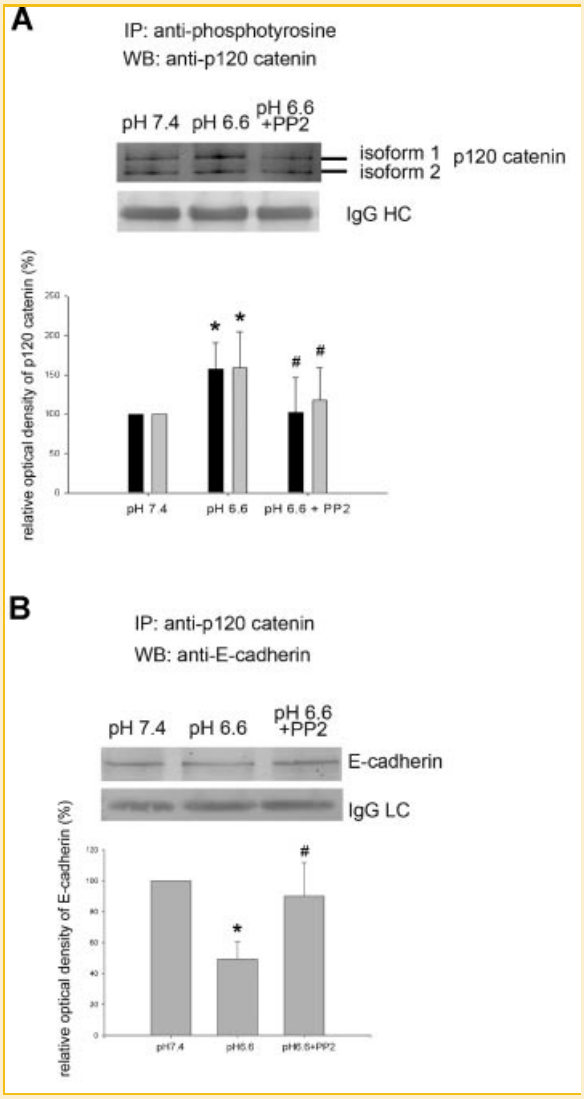

Fig. 7. pH 6.6 induces tyrosine phosphorylation of p120ctn and decreases its association with E-cadherin. A: Cells were cultured at pH 7.4 or pH 6.6 for 1 h or pretreated for 30 min with 10 μM PP2 at pH 7.4, then at pH 6.6 in the presence of PP2 for 1 h (pH 6.6 + PP2), then whole cell lysates were incubated with anti-phosphotyrosine antibody, followed by immobilized protein G-agrose, and the immunoprecipitates analyzed for p120ctn or IgG-HC. The upper panel shows a typical result and the lower panel the mean ± SD for the band density in three separate experiments expressed as a percentage of the pH 7.4 value. Black column, isoform 1. Gray column, isoform 2. $^{*}P < 0.05$, compared to pH 7.4. $^{\#}P < 0.05$, compared to pH 6.6. B: Cells were cultured at pH 7.4 or pH 6.6 for 1 h or pretreated for 30 min with 10 μM PP2 at pH 7.4, then at pH 6.6 in the presence of PP2 for 1 h (pH 6.6 + PP2), then whole cell lysates were incubated with anti-p120ctn antibody, followed by immobilized protein G-agarose, and the immunoprecipitates analyzed for E-cadherin or IgG-LC. The results show are typical of those obtained in four separate experiments. $^{*}P < 0.05$, compared to pH 7.4. $^{\#}P < 0.01$, compared to pH 6.6.

cytoplasmic p120ctn inhibits RhoA activity [Anastasiadis and Reynolds, 2000; Anastasiadis, 2007] and activates Rac [Noren et al., 2000; Grosheva et al., 2001], which favors cell migration. Overexpression of p120ctn in fibroblasts and epithelial cells induces pronounced changes in cell motility, a result of a significant increase in Cdc42 and Rac activity [Grosheva et al., 2001]. All four p120ctn isoforms are able to activate Rac [Yanagisawa et al., 2008]. In this study, we observed loss of p120ctn from AJs and increased diffuse cytoplasmic p120ctn staining at pH 6.6. A high migratory and invasive behavior of HepG2 cells is seen at pH 6.6 [Chen et al., 2008] and the interaction of cytoplasmic p120ctn with Rac might be involved in this invasive property of HepG2 cells.

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REFERENCES

Akhtar N, Hotchin NA. 2001. RAC1 regulates adherens junctions through endocytosis of E-cadherin. Mol Biol Cell 12:847–862.

Anastasiadis PZ. 2007. p120-ctn: A nexus for contextual signaling via Rho GTPases. Biochim Biophys Acta 1773:34–46.

Anastasiadis PZ, Reynolds AB. 2000. The p120 catenin family: Complex roles in adhesion, signaling and cancer. J Cell Sci 113(Pt 8): 1319–1334.

Calautti E, Cabodi S, Stein PL, Hatzfeld M, Kedersha N, Paolo Dotto G. 1998. Tyrosine phosphorylation and src family kinases control keratinocyte cell–cell adhesion. J Cell Biol 141:1449–1465.

Castano J, Solanas G, Casagolda D, Raurell I, Villagrasa P, Bustelo XR, Garcia de Herreros A, Dunach M. 2007. Specific phosphorylation of p120-catenin regulatory domain differently modulates its binding to RhoA. Mol Cell Biol 27:1745–1757.

Chen KH, Tung PY, Wu JC, Chen Y, Chen PC, Huang SH, Wang SM. 2008. An acidic extracellular pH induces Src kinase-dependent loss of beta-catenin from the adherens junction. Cancer Lett 267:37–48.

Cozzolino M, Stagni V, Spinardi L, Campioni N, Fiorentini C, Salvati E, Alema S, Salvatore AM. 2003. p120 Catenin is required for growth factor-dependent cell motility and scattering in epithelial cells. Mol Biol Cell 14:1964–1977.

Daniel JM, Reynolds AB. 1997. Tyrosine phosphorylation and cadherin/catenin function. Bioessays 19:883–891.

Davis MA, Ireton RC, Reynolds AB. 2003. A core function for p120-catenin in cadherin turnover. J Cell Biol 163:525–534.

Downing JR, Reynolds AB. 1991. PDGF, CSF-1, and EGF induce tyrosine phosphorylation of p120, a pp60src transformation-associated substrate. Oncogene 6:607–613.

Ezaki T, Guo RJ, Li H, Reynolds AB, Lynch JP. 2007. The homeodomain transcription factors Cdx1 and Cdx2 induce E-cadherin adhesion activity by reducing beta- and p120-catenin tyrosine phosphorylation. Am J Physiol Gastrointest Liver Physiol 293:G54–G65.

Fouquet S, Lugo-Martinez VH, Faussat AM, Renaud F, Cardot P, Chambaz J, Pincon-Raymond M, Thenet S. 2004. Early loss of E-cadherin from cell–cell contacts is involved in the onset of Anoikis in enterocytes. J Biol Chem 279:43061–43069.

Fritz JD, Swartz DR, Greaser ML. 1989. Factors affecting polyacrylamide gel electrophoresis and electroblotting of high-molecular-weight myofibrillar proteins. Anal Biochem 180:205–210.

Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, Sommer T, Birchmeier W. 2002. Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. Nat Cell Biol 4:222–231.

Fukumoto Y, Shintani Y, Reynolds AB, Johnson KR, Wheelock MJ. 2008. The regulatory or phosphorylation domain of p120 catenin controls E-cadherin dynamics at the plasma membrane. Exp Cell Res 314:52–67.

Grosheva I, Shtutman M, Elbaum M, Bershadsky AD. 2001. p120 catenin affects cell motility via modulation of activity of Rho-family GTPases: A link between cell–cell contact formation and regulation of cell locomotion. J Cell Sci 114:695–707.

Guarino M. 2007. Epithelial-mesenchymal transition and tumour invasion. Int J Biochem Cell Biol 39:2153–2160.

Herzig M, Savarese F, Novatchkova M, Semb H, Christofori G. 2007. Tumor progression induced by the loss of E-cadherin independent of beta-catenin/Tcf-mediated Wnt signaling. Oncogene 26:2290–2298.

Holecek M, Muthny T, Kovarik M, Sispera L. 2006. Proteasome inhibitor MG-132 enhances whole-body protein turnover in rat. Biochem Biophys Res Commun 345:38–42.

Hoshino T, Sakisaka T, Baba T, Yamada T, Kimura T, Takai Y. 2005. Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. J Biol Chem 280:24095–24103.

Huang HY, Nong CZ, Guo LX, Xu Z, Zha XL. 2003. p120(ctn) tyrosine phosphorylation are involved in the biologic behavior changes of hepatocellular carcinoma cells. Zhonghua Yi Xue Za Zhi 83:1801–1806.

Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy F, Reynolds AB. 2002. A novel role for p120 catenin in E-cadherin function. J Cell Biol 159:465–476.

Kato Y, Nakayama Y, Umeda M, Miyazaki K. 1992. Induction of 103-kDa gelatinase/type IV collagenase by acidic culture conditions in mouse metastatic melanoma cell lines. J Biol Chem 267:11424–11430.

Kinch MS, Clark GJ, Der CJ, Burridge K. 1995. Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. J Cell Biol 130:461–471.

Le TL, Joseph SR, Yap AS, Stow JL. 2002. Protein kinase C regulates endocytosis and recycling of E-cadherin. Am J Physiol Cell Physiol 283:C489–C499.

Lilien J, Balsamo J, Arregui C, Xu G. 2002. Turn-off, drop-out: Functional state switching of cadherins. Dev Dyn 224:18–29.

Lu Z, Ghosh S, Wang Z, Hunter T. 2003. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell 4:499–515.

Mariner DJ, Anastasiadis P, Keilhack H, Bohmer FD, Wang J, Reynolds AB. 2001. Identification of Src phosphorylation sites in the catenin p120ctn. J Biol Chem 276:28006–28013.

Mariner DJ, Davis MA, Reynolds AB. 2004. EGFR signaling to p120-catenin through phosphorylation at Y228. J Cell Sci 117:1339–1350.

Mo YY, Reynolds AB. 1996. Identification of murine p120 isoforms and heterogeneous expression of p120cas isoforms in human tumor cell lines. Cancer Res 56:2633–2640.

Murai M, Shen X, Huang L, Carpenter WM, Lin CS, Silverman S, Regezi J, Kramer RH. 2004. Overexpression of c-met in oral SCC promotes hepatocyte growth factor-induced disruption of cadherin junctions and invasion. Int J Oncol 25:831–840.

Noren NK, Liu BP, Burridge K, Kreft B. 2000. p120 catenin regulates the actin cytoskeleton via Rho family GTPases. J Cell Biol 150:567–580.

Ozawa M, Ohkubo T. 2001. Tyrosine phosphorylation of p120(ctn) in v-Src transfected L cells depends on its association with E-cadherin and reduces adhesion activity. J Cell Sci 114:503–512.

Papkoff J. 1997. Regulation of complexed and free catenin pools by distinct mechanisms. Differential effects of Wnt-1 and v-Src. J Biol Chem 272:4536–4543.

Pece S, Gutkind JS. 2002. E-cadherin and Hakai: Signalling, remodeling or destruction? Nat Cell Biol 4:E72–74.

Piedra J, Miravet S, Castano J, Palmer HG, Heisterkamp N, Garcia de Herreros A, Dunach M. 2003. p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta-catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction. Mol Cell Biol 23:2287–2297.

Potter MD, Barbero S, Cheresh DA. 2005. Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. J Biol Chem 280:31906–31912.

Reynolds AB, Herbert L, Cleveland JL, Berg ST, Gaut JR. 1992. p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo. Oncogene 7:2439–2445.

Reynolds AB, Daniel J, McCrea PD, Wheelock MJ, Wu J, Zhang Z. 1994. Identification of a new catenin: The tyrosine kinase substrate p120cas associates with E-cadherin complexes. Mol Cell Biol 14:8333–8342.

Rofstad EK, Mathiesen B, Kindem K, Galappathi K. 2006. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. Cancer Res 66:6699–6707.

Roura S, Miravet S, Piedra J, Garcia de Herreros A, Dunach M. 1999. Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. J Biol Chem 274:36734–36740.

Shibamoto S, Hayakawa M, Takeuchi K, Hori T, Oku N, Miyazawa K, Kitamura N, Takeichi M, Ito F. 1994. Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. Cell Adhes Commun 1:295–305.

Takeichi M. 1990. Cadherins: A molecular family important in selective cell–cell adhesion. Annu Rev Biochem 59:237–252.

Takeichi M, Hirano S, Matsuyoshi N, Fujimori T. 1992. Cytoplasmic control of cadherin-mediated cell–cell adhesion. Cold Spring Harb Symp Quant Biol 57:327–334.

Takeichi M, Watabe M, Shibamoto S, Ito F. 1994. Cadherin-dependent organization and disorganization of epithelial architecture. Princess Takamatsu Symp 24:28–37.

Tannock IF, Rotin D. 1989. Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res 49:4373–4384.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Tricaud N, Perrin-Tricaud C, Bruses JL, Rutishauser U. 2005. Adherens junctions in myelinating Schwann cells stabilize Schmidt-Lanterman incisures via recruitment of p120 catenin to E-cadherin. J Neurosci 25:3259–3269.

Webb SD, Sherratt JA, Fish RG. 1999. Alterations in proteolytic activity at low pH and its association with invasion: A theoretical model. Clin Exp Metastasis 17:397–407.

Xia X, Mariner DJ, Reynolds AB. 2003. Adhesion-associated and PKC-modulated changes in serine/threonine phosphorylation of p120-catenin. Biochemistry 42:9195–9204.

Xia X, Brooks J, Campos-Gonzalez R, Reynolds AB. 2004. Serine and threonine phospho-specific antibodies to p120-catenin. Hybrid Hybridomics 23:343–351.

Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V, Kowalczyk AP. 2003. Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. J Cell Biol 163:535–545.

Xiao K, Oas RG, Chiasson CM, Kowalczyk AP. 2007. Role of p120-catenin in cadherin trafficking. Biochim Biophys Acta 1773:8–16.

Yanagisawa M, Huveldt D, Kreinest P, Lohse CM, Cheville JC, Parker AS, Copland JA, Anastasiadis PZ. 2008. A p120 catenin isoform switch affects Rho activity, induces tumor cell invasion, and predicts metastatic disease. J Biol Chem 283:18344–18354.