Regulation of adherens junction protein p120<sup>ctn</sup> by 10 nM CCK precedes actin breakdown in rat pancreatic acini

J. Leser, M. F. Beil, O. A. Musa, G. Adler, and M. P. Lutz
Department of Internal Medicine I, University of Ulm, 89070 Ulm, Germany

ACUTE PANCREATITIS is an inflammatory disorder that is initiated through damage of acinar cells (18). Its initial morphological hallmarks are intracellular disruption of the apical actin web, disturbance of regulated apical secretion that results in the intracellular colocalization of digestive and lysosomal enzymes within cytoplasmatic vacuoles, and rapid distension of the extracellular space with accumulation of activated digestive enzymes (16). Premature activation of proteases within the intracellular vacuoles can explain part of the cellular damage and may be sufficient for subsequent degradation of intracellular proteins, including cytoskeletal structures (12, 14). Other potential mechanisms responsible for actin disassembly include the regulation of actin-binding proteins such as gelsolin or β-thymosin (20) or the disruption of membrane-bound actin anchoring systems.

One of the known actin anchoring protein complexes are the adherens junctions that physically connect neighboring cells. In polarized epithelial cells, the cell-cell contacts of adjacent cells via adherens junctions are mediated by Ca<sup>2+</sup>-dependent homophilic interaction of the transmembrane protein E-cadherin, a member of the cadherin protein family, which also includes R-cadherin, N-cadherin, L1-cadherin, and neural cell adhesion molecule (1). E-cadherin contains a single transmembrane domain, five extracellular domain repeats with highly conserved Ca<sup>2+</sup> binding motifs, and an intracellular actin microfilament connecting domain (21). The intracellular domain of E-cadherin serves as anchor for the epithelial cell cytoskeleton through interaction with several proteins, most of them part of the cadherin protein family. β-Catenin and γ-catenin interact directly with E-cadherin, whereas α-catenin mediates attachment of this complex to the microfilament system (1). Another catenin, p120<sup>ctn</sup>, binds to the cytoplasmatic juxtamembrane region of E-cadherin in close proximity to the binding site for β- and γ-catenin (22). p120<sup>ctn</sup> was originally identified in fibroblasts as the putative substrate of the activated tyrosine kinase Src (3), and this phosphorylation, together with the tyrosine phosphorylation of β- and γ-catenin, may be the key event for the disruption of adherens junctions (2, 25). p120<sup>ctn</sup> is also phosphorylated in response to mitogenic stimulation of breast cancer cells with epidermal growth factor (11). Binding of p120<sup>ctn</sup> promotes lateral clustering of cadherin molecules and thus strengthens cell-cell adhesion (27), an effect that may be blocked by hyperphosphorylation during mitosis (11).

Recently, we (17) examined for the first time the expression of E-cadherin and β-catenin in the adult pancreas and demonstrated their restriction to acinar and duct cells, in which they were colocalized at adherens junctions. Induction of pancreatitis by supramaximal secretory concentrations of caerulein, a well-characterized experimental model of acute edematous pancreatitis, led to dissociation, internalization, and degradation of both proteins within 2 h. Reassembly of E-cadherin and β-catenin occurred within 12 h after the start of supramaximal caerulein infusion. The
regulation of the E-cadherin-β-catenin assembly was not addressed.

We have now identified the additional adherens junction proteins α-catenin and p120<sup>ctn</sup> in pancreatic acinar cells and can confirm their colocalization with E-cadherin and the terminal actin web. Stimulation of isolated acinar cells with CCK, a well-established system to examine secretagogue effects on acinar cell integrity (10), led to increased tyrosine phosphorylation of p120<sup>ctn</sup> without changing the integrity of the adherens junction protein complex. Our data indicate that CCK may regulate actin assembly through adhesion protein phosphorylation.

**METHODS**

Materials and animals. Soybean trypsin inhibitor was from Boehringer (Mannheim, Germany), and collagenase was from Worthington (Cell Systems, Hamburg, Germany). Polyclonal antibodies against β-catenin and α-catenin were from Sigma (St. Louis, MO). Monoclonal antibodies against E-cadherin, γ-catenin, and p120<sup>ctn</sup> were purchased from Transduction Laboratories (Lexington, KY). Oregon Green 488 phalloidin was from Molecular Probes (Eugene, OR). Peroxidase-conjugated affinity-purified rabbit anti-mouse IgG and peroxidase-conjugated affinity-purified goat anti-rabbit IgG were from Diana (Hamburg, Germany). Enhanced chemiluminescence reagents were obtained from Pierce (Rockford, IL). Sulfated CCK-8 was from Bachem (Bubendorf, Switzerland). Essential and nonessential amino acids were purchased from GIBCO (Gaithersburg, MD). Male Wistar rats (150–200 g) were bred at the animal care and treatment facility of the University of Ulm.

Preparation of isolated rat pancreatic acini. The preparation of isolated rat pancreatic acini was performed essentially as described previously (19). Isolated acini were washed twice in oxygenated Krebs-Ringer-HEPES buffer consisting of 104 mM NaCl, 5 mM KCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 0.2% (wt/vol) BSA, 0.01% (wt/vol) soybean trypsin inhibitor, 10 mM glucose, and 25 mM HEPES, pH 7.4 with NaOH, supplemented with minimal essential amino acid solution and glutamine. Cell viability, as assessed by trypan blue exclusion, exceeded 95%. To verify the responsiveness and physiological integrity of the preparation, isolated acini were stimulated with CCK as described previously. The secretion of amylase was <5% of total cellular amylase under basal conditions, with a biphasic dose-response curve and maximum secretion of >25% of the total between 10<sup>−10</sup> and 3 × 10<sup>−10</sup> M CCK after 30 min. All preincubation and incubation steps were carried out at 37°C.

Immunocytochemistry. Acini were stimulated with the indicated concentrations of CCK or buffer control. After an excess volume of ice-cold Krebs-Ringer-HEPES buffer was added, cells were pelleted at 100 g for 2 min, were placed on SuperFrost microscope slides, were allowed to adhere to the slides for 5 min, and were then fixed using 4% formaldehyde for 10 min on ice. The slides were washed once in PBS, permeablixalized with 0.5% Triton X-100 in PBS, and then washed three times in PBS. Blocking of nonspecific binding with PBS-3% BSA was performed for 40 min at room temperature. Primary antibodies were added for 60 min in PBS-0.1% BSA. The slides were washed three times in PBS and incubated with Alexa488-coupled goat anti-mouse IgG together with Cy3-coupled goat anti-rabbit IgG and/or with Oregon Green 488 phalloidin (0.2 µM) for 1 h in the dark. Cells were embedded in Mowiol (Calbiochem, Bad Soden, Germany) and coverslipped. Signal distribution was analyzed using a confocal laser scanning microscope (TCS 4D, Leica, Heidelberg, Germany).

Isolation of E-cadherin-β-catenin complexes. To examine the integrity of E-cadherin-β-catenin complexes, isolated pancreatic acini were equilibrated for 20 min at 37°C. CCK was added for indicated time periods, and the incubation was terminated by suspending the acini in an excess volume of ice-cold Krebs-Ringer-HEPES buffer. The acini were then pelleted by centrifugation at 300 g (4°C). Cellular protein was extracted by trituration in lysis buffer consisting of 50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 50 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.5% NP-40, and 0.1% Triton X-100, supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 10 µg/ml aprotinin) and 0.5 mM sodium orthovanadate. The lysate was cleared by centrifugation at 10,000 g at 4°C, and the protein content of the supernatant was measured using the Bradford method (Biorad, Munich, Germany). Two hundred micrograms of protein extract were incubated with five micrograms of E-cadherin antibody in lysis buffer for 1 h. Complexes were pelleted after additional incubation with affinity-purified rabbit anti-mouse IgG precoated to protein A agarose. After three washes in lysis buffer, precipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel-resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA). Membranes were incubated overnight in blocking buffer [50 mM Tris·HCl, pH 7.8, 100 mM NaCl, 0.5% Tween 20, 2% (wt/vol) BSA]. The membranes were then incubated for 1 h with primary antibodies in blocking buffer. After three washes with Tris-buffered saline-0.1% Tween 20, antigen-antibody complexes were visualized using peroxidase-conjugated secondary antibody and the enhanced chemiluminescence system by exposure to Kodak X-OMAT AR films for 30 s to 2 min. Quantitation was performed densitometrically using Phoretix 1D gel analysis software (Phoretix, Newcastle upon Tyne, UK).

**IP: E-Cadherin**

**WB: β-Catenin**

**WB: α-Catenin**

**WB: E-Cadherin**

<table>
<thead>
<tr>
<th>15 min.</th>
<th>30 min.</th>
<th>60 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10&lt;sup&gt;−8&lt;/sup&gt; M CCK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Complex formation of catenins with E-cadherin in CCK-treated acinar cells. Acini were stimulated for indicated time periods with supramaximal secretory concentration (10<sup>−8</sup> M) of CCK. E-cadherin was immunoprecipitated using monoclonal anti-E-cadherin antibodies, and associated proteins were analyzed by SDS-PAGE of immunoprecipitates and immunoblots with α- and β-catenin antibodies. Membranes were stripped and were immunostained for a second time with anti-E-cadherin antibodies. IP, immunoprecipitation; WB, Western blotting.
RESULTS

Adherens junctions in pancreatic acinar cells. Adherens junctions are protein structures that connect neighboring epithelial cells and act internally as one of the anchors for the actin cytoskeleton (1). We previously demonstrated colocalization and association of E-cadherin and β-catenin in the intact pancreas, thus defining two proteins of the adherens junction complex (17). Here we examined the distribution of adherens junctions and defined additional component proteins in isolated pancreatic acinar cells. Immunoprecipitation of E-cadherin was used to demonstrate association of α-catenin and β-catenin with E-cadherin (Fig. 1). Immunocytochemistry and immunoprecipitation identified another catenin, p120ctn, as part of this complex (Fig. 2). All four proteins were localized to the lateral plasma membrane and were colocalized by double-staining immunofluorescence, thus providing all the necessary components of functional actin-binding adherens junctions (Figs. 2A and 3). Similar to our results in intact pancreatic tissue, E-cadherin and β-catenin were colocalized with the terminal actin web at a well-defined region of the lateral plasma membrane where the apical actin web terminates and where it seems to attach to the plasma membrane and connect to the actin web of the neighboring cell.

CCK-dependent tyrosine phosphorylation of p120ctn. One of the recognized regulatory mechanisms of adherens junction composition and function is the phosphorylation of p120ctn and β- and γ-catenin (2, 3, 11, 25). Treatment of acinar cells with CCK stimulated rapid and reversible tyrosine phosphorylation of p120ctn. Phosphorylation of p120ctn was concentration dependent, with its maximum at 10^{-8} M CCK. Maximal phosphorylation was observed within 5 min and decreased thereafter to reach baseline levels after 10 min (Fig. 4A). Under the same conditions, we did not detect phosphorylation of β-catenin or γ-catenin either under basal conditions or after stimulation with CCK (data not shown). Preincubation with the tyrosine kinase inhibitor PP1 (15, 24) reduced overall tyrosine phosphorylation, including phosphorylation of p120ctn, and inhibited the CCK-induced breakdown of the apical actin web (Fig. 4, B and C).

Integrity of E-cadherin-β-catenin complexes after CCK treatment. E-cadherin-β-catenin complexes dissociate and both proteins degrade during experimental acute hyperstimulation pancreatitis, and a role for acinar cell damage was proposed (17). To examine whether this indeed could be a direct effect of CCK or is secondary to other cellular events, we stimulated isolated rat pancreatic acini with concentrations of CCK known to induce cell injury. Stimulation with 10^{-8} M CCK did not change the cellular distribution of E-cadherin, of the catenin p120ctn (Fig. 2A), or of α- and β-catenin. Similarly, E-cadherin and p120ctn remained colocalized during stimulation with cell-damaging concentrations of CCK (10 nM). Immunoprecipitation was used to confirm the integrity of adherent junction complexes during up to 2 h of 10^{-8} M CCK treatment. As illustrated in Fig. 1, 10^{-8} M CCK had no effect on the association of E-cadherin to either β-catenin or α-catenin. In control experiments, which were designed...
to examine whether cadherin-catenin complexes could be regulated in acinar cells, chelation of calcium in the incubation medium was sufficient to inhibit β-catenin binding to E-cadherin (Fig. 5).

**DISCUSSION**

Supraphysiological doses of CCK or of its homologue caerulein initiate acute edematous pancreatitis in the rat (16). In isolated acinar cells, a similar cell injury is stimulated by supramaximal secretory concentrations of CCK (23). The cellular mechanisms that mediate these events are incompletely understood. One of the central events appears to be the breakdown of the apical actin filament system, which is required for regulated exocytosis (20). In fact, depolymerization of filamentous actin by actin monomer binding proteins or by cytochalasin D blocks regulated secretion (20) and inhibits CCK-induced tyrosine phosphorylation of the actin-associated proteins paxillin, p125FAK, and p130cas (7, 8). However, regulatory mechanisms for the balance of actin assembly and disassembly in vivo in acinar cells are largely unknown. They likely include actin cross-linking proteins or actin anchoring complexes at the plasma membrane resembling focal adhesion protein complexes or adherens junctions. At least some component proteins of both types of actin anchoring complexes are expressed in acinar cells and are capable of binding filamentous actin (17, 19).

Similar to other epithelial cell types, neighboring acinar cells are closely connected by adherens junctions, which we have shown to be composed of E-cadherin and β-catenin (6, 17). During the course of acute rodent pancreatitis, these complexes rapidly disassemble within <2 h and might be the permissive factor for extracellular edema formation and/or intracellular actin disruption. The details of their regulation in acinar cells are unknown. In this work, we identify p120ctn and α-catenin as additional adherens junction proteins in acinar cells. Both proteins communoprecipitate in a complex with E-cadherin and β-catenin and colocalize in intact cells with the actin filament system and with the other components of adherens junctions. This not only establishes all necessary components for functional adherens junctions but also strongly indicates a potential regulatory function for actin assembly.

**METHODS**

**Fig. 4.** CCK-stimulated tyrosine phosphorylation of p120ctn in pancreatic acini. A: acini were stimulated with 10^{-8} M CCK for indicated time periods. Tyrosine phosphorylated proteins were immunoprecipitated using monoclonal anti-phosphotyrosine antibodies (clone PY20) and were analyzed by SDS-PAGE and immunoblotting with p120ctn antibodies. B: acini were stimulated for 5 min with 10^{-8} M CCK with or without preincubation with 100 µM PP1. Total cellular extracts were analyzed by SDS-PAGE and immunoblotting using anti-phosphotyrosine antibodies. C: acini were preincubated in 100 µM PP1 or buffer control. Cells were then treated with 10^{-8} M CCK and fixed with formaldehyde. F-actin was visualized using Oregon Green-conjugated phalloidin and laser confocal scanning microscopy.

**Fig. 5.** Dissociation of E-cadherin-β-catenin complexes in calcium-free medium. Acini were preincubated in normal or calcium-free Krebs-Ringer-HEPES (KRH) buffer. Indicated cells were stimulated for 30 min with 10^{-8} M CCK. Immunoprecipitation and analysis of E-cadherin-associated β-catenin were performed as described in METHODS.

**IP: phosphotyrosine**

**WB: p120ctn**

control | 2 min. | 5 min. | 10 min.
---|---|---|---
10^{-8} M CCK

- + + 10 nM CCK
- - + 100 µM PP1

**IP: E-Cadherin**

**WB: β-Catenin**

normal KRH

Ca^{++}-free

10^{-8} M CCK
Adherens junctions of neighboring cells consist of a backbone of cross-linked cadherins with intracellular binding sites for the Arm domain-containing proteins β-catenin, γ-catenin, and p120ctn (22). Interaction of the adherens junctions with the actin cytoskeleton occurs through attachment of β-catenin or γ-catenin to the multifunctional α-catenin and through its connection to actin filaments (1). In contrast, p120ctn is not directly involved in the chain of proteins that mediates physical attachment of cadherins to the actin filaments but binds to cadherins in close proximity of β- and γ-catenin binding sites, where it may function as an inhibitory protein for actin binding (4;26). In mesodermal cells, overexpression of p120ctn1B modulates cell motility and orientation, i.e., cellular functions that are linked to an intact cellular cytoskeleton, yet was not sufficient to replace β-catenin function in Wnt signaling, a cellular signaling pathway that is dependent on adherens junctions but independent from actin binding (9). This negative regulatory role of p120ctn seems to be achieved through its tyrosine phosphorylation (5, 11).

Phosphorylation on tyrosine has been observed on β-catenin, γ-catenin, and p120ctn in different cell systems and seems to differentially affect their interaction with other proteins of the adhesion complex. Whereas tyrosine phosphorylation of β- and γ-catenin stabilizes the interaction of cadherin-catenin complexes with the actin cytoskeleton, tyrosine phosphorylation of p120ctn likely destabilizes actin binding (3, 5). In our system, CCK stimulates maximum phosphorylation of p120ctn at supramaximal secretory concentrations without affecting the phosphorylation state of β-catenin. This suggests that regulation of p120ctn by CCK affects the interaction of filamentous actin with adherens junctions. Furthermore, the tyrosine kinase inhibitor PP1 (15, 24) decreased overall tyrosine phosphorylation and reduced the CCK-induced disintegration of the apical actin web. Even though this effect may not be directly related to phosphorylation of p120ctn because tyrosine kinase inhibitors likely are not entirely specific, our data, together with experiments in breast cancer cells in which Src phosphorylation of p120ctn initiated destabilization of actin filaments (11, 13), support a negative regulatory role for p120ctn.

Of note, under conditions in which CCK induced phosphorylation of p120ctn and initiated the destabilization of the acinar cell actin filament system, the backbone of adherens junctions, i.e., E-cadherin with the attached β- and α-catenins, remained intact. Therefore, the dissociation and internalization of cadherin-catenin complexes during experimental CCK-induced acute pancreatitis within 2 h likely are not caused by a direct regulatory role of CCK but rather are secondary, e.g., to mechanical disruption of cell-cell contacts after edema formation, which is further supported by the temporal relationship of actin breakdown and p120ctn phosphorylation, both of which occur within minutes after CCK stimulation.

In summary, we define for the first time a complete adherens junction complex in pancreatic acinar cells with the components necessary for actin binding, i.e., E-cadherin, β-catenin, and α-catenin. Supramaximal secretory CCK was able to regulate one additional component of this complex, p120ctn, which likely is responsible for the actin release from the plasma membrane observed within minutes after secretagogue stimulation.

Present address of O. A. Musa: University of Gezira, Faculty of Medicine, PO Box 20, Medani, Sudan.

Address for reprint requests and other correspondence: M. P. Lutz, Univ. of Ulm, Robert-Koch-Str. 8, 89070 Ulm, Germany (E-mail: manfred.lutz@medizin.uni-ulm.de). Received 2 August 1999; accepted in final form 3 November 1999.

REFERENCES


