**Cell-Cell Dissociation upon Epithelial Cell Scattering Requires a Step Mediated by the Proteasome**

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During development, tissue repair, and tumor metastasis, both cell-cell dissociation and cell migration occur and appear to be intimately linked, such as during epithelial “scattering.” Here we show that cell-cell dissociation during scattering induced by hepatocyte growth factor (HGF) or activation of the temperature-sensitive v-Src tyrosine kinase in MDCK cells can be blocked by inhibiting the proteasome with lactacystin and MG132. Although both proteins of the tight junction and the adherens junction redistributed during cell scattering, proteasome inhibitors largely prevented this process, resulting in the stabilization of Triton X-100-insoluble tight junction proteins as well as adherens junction proteins at sites of cell-cell contact. Proteasome inhibition also led to a decrease of E-cadherin turnover in 35S-labeled cells. In addition, proteasome inhibition partly preserved cell polarity, as determined by the subcellular distribution of Na\(^+\),K\(^+\)-ATPase (basolateral marker) and gp135 (apical marker), and the structure of the subcortical actin ring, both of which are normally disrupted during scattering. However, cells were able to establish focal contacts, and single cell migration toward HGF was unaffected by proteasome inhibition in quantitative assays, indicating that cell-cell dissociation during scattering occurs independently of anchorage-dependent cell migration. Thus, a proteasome-dependent step during scattering induced by HGF and pp60\(^{v-Src}\) appears to be essential for cell-cell dissociation, disassembly of junctional components, and (at least indirectly) it also plays a role in the loss of protein polarity.

In largely epithelial tissues such as kidney and intestine, both the permeability barrier and the polarized sorting of proteins and lipids are intimately linked to the formation of intercellular junctions, such as adherens junctions (AJs)\(^1\) and tight junctions (TJs)\(^1,\,2\). In general, the structure and function of these intercellular junctions depends upon transmembrane proteins (e.g. E-cadherin, occludin/claudins) that are linked to nonmembrane proteins on the cytosolic face, which in turn are associated with the actin-based cytoskeleton (3–5). Under steady-state conditions, the assembly and maintenance of these intercellular junctions appear to be tightly regulated (3, 6, 7). However, during development, cell division, inflammation, and tissue repair, as well as invasion and metastasis of tumor cells, these structures are disassembled and sometimes internalized as cells diminish their contacts and become motile (2, 8, 9). Little is known of the molecular basis of junction disassembly and reassembly during the alterations in cell-cell interactions and motility that characterize these highly dynamic states of tissue remodeling, regeneration, and transdifferentiation, although in models such as the “calcium switch,” a variety of signaling molecules, including protein kinase Cs, calcium, and GTP binding proteins have been implicated in junctional reassembly (10–13).

A key question concerning cell dissociation during cell movement is the metabolic fate of components within junctional complexes. One well studied experimental system in which this issue is clearly important is “scattering.” Epithelial cell scattering requires the attenuation or dissolution of cell-cell adhesion before cell-cell dissociation and motility (14). To perform an analysis that might be applicable to a broader context, we employed two different systems: scattering induced by hepatocyte growth factor (HGF) and scattering resulting from the activation of temperature-sensitive v-Src tyrosine kinase (pp60\(^{v-Src}\)) (15).

We now show that cell-cell dissociation during scattering induced by HGF or pp60\(^{v-Src}\) in MDCK epithelial cells can be blocked by inhibiting the proteasome without affecting cell migration, resulting in stabilization of junctional proteins at sites of cell-cell contact. We conclude that the proteasome plays an essential role as a regulator of cell-cell dissociation during cell movement.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Antibodies—**MDCK cells (type II) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum at 37 °C in an atmosphere of 95% air/5% CO\(_2\). Temperature-sensitive v-Src MDCK kindly provided by Drs. J. Behrens and W. Birchmeier (Max Delbrück Center for Molecular Biology, Germany) were maintained in DMEM containing 10% fetal calf serum at 40 °C. Cell permeable protease inhibitors were purchased from Biomol (lactacystin, MG132, ALLN, E64 and calpeptin) and Sigma (chloroquine and primaquine). HGF was kindly supplied by Dr. T. Nakamura (Osaka University, Osaka, Japan). Anti-ZO-1 rat monoclonal antibody (R40.76) and anti-ZO-2 rabbit polyclonal antibody (kindly supplied by Dr. D. Goodenough, Harvard University), anti-occludin polyclonal antibody (Transduction Laboratories, Lexington, KY), anti-paxillin mouse monoclonal antibody (by Dr. K. Matlin, Harvard University), anti-gp135 mouse monoclonal antibody (by Dr. D. Goodenough, Harvard University), anti-Na\(^+\),K\(^+\)-ATPase \(\beta\) subunit mouse monoclonal antibody (by Dr. K. Matlin, Harvard University), anti-catenin mouse monoclonal antibody, anti-\(\alpha\)-catenin rabbit polyclonal antibody (Sigma), and anti-\(\beta\)-catenin and anti-paxillin mouse monoclonal antibodies (Transduction Laboratories, Lexington, KY) were used.
Preparation of Cell Lysate, Co-immunoprecipitation, and Western Immunoblotting—Cells were lysed with a modified RIPA buffer (1% Triton X-100, 0.5% deoxycholate, 0.2% SDS, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1.5 mM MgCl2, 2 mM EGTA, a protease inhibitor mixture consisting of 1 mM phenylmethylsulfonyl fluoride/aprotinin and 20 μg/ml each leupeptin, pepstatin A, and antipain) on ice, and insoluble materials were removed by centrifugation at 4 °C for 30 min at 14,000 × g. Protein content was determined using the BCA protein assay reagent kit (Pierce), and 50 μg/lane of total protein was separated by SDS-PAGE. For co-immunoprecipitation experiments, cells were lysed with a buffer containing 1% Triton X-100, 1% deoxycholate, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1.5 mM MgCl2, 2 mM EGTA, plus the protease inhibitor mixture (TN buffer) for 30 min at 4 °C, and insoluble materials were separated by centrifugation at 4 °C for 30 min at 14,000 × g. The supernatant containing 1 mg of protein was clarified and incubated either with anti-ZO-1 or anti-E-cadherin antibody on a rocking platform at 4 °C overnight. The immune complexes were collected either with anti-rat IgG-Sepharose (Organon Teknika, West Chester, PA) or protein A-Sepharose beads (Amersham Pharmacia Biotech) for 30 min at 4 °C. The beads were washed three times with the lysis buffer, resuspended in 2× sample buffer, and boiled for 5 min. Immunoprecipitated proteins were then analyzed by SDS-PAGE and visualized by Western immunoblotting using horseradish peroxidase-conjugated secondary antibodies (Jackson Labs, West Grove, PA) and the ECL kit (Pierce) as described (11, 16).

Triton X-100 Extraction Assay—Cells were scraped into CSK-A buffer (0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 300 mM sucrose, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate) plus the protease inhibitor mixture and extracted for 20 min at 4 °C on a gently rocking platform. The extract (E-fraction) was separated by centrifugation (14,000 × g) for 10 min at 4 °C, and the residue (R-fraction) was dissolved in 2× sample buffer. Equal volumes of both fractions were separated by SDS-PAGE and subjected to Western immunoblot. Blots were quantified with NIH image software as described previously, and samples were examined using a laser scanning confocal system (Bio-Rad MRC 1024) (17).

RESULTS AND DISCUSSION

Proteasome Inhibition Blocks Cell Scattering Induced by HGF and pp60SV—MDCK cells (at the “cell island” stage) were pretreated with either potent proteasome inhibitors (lactacystin and MG132), neutral cysteine protease inhibitors (ALLN and E64), a calpain inhibitor (calpeptin), or inhibitors of lysosomal proteolysis (chloroquine and primaquine) for 30 min. Cells were then subjected to scattering conditions by adding either 20 ng/ml HGF or, in the case of the cells with temperature-sensitive pp60SV, inducing a temperature shift from the nonpermissive (40 °C) to permissive temperature (35 °C). Of the inhibitors tested, only the proteasome inhibitors could markedly inhibit cell-cell dissociation in MDCK cells during scattering driven by HGF or pp60SV (Fig. 1). Both MG132 and the highly specific agent lactacystin, which inhibit the proteasome through different mechanisms, gave similar results. ALLN showed a weak inhibitory effect on this cell-cell dissociation consistent with the compound’s weak inhibition of the proteasome (20), whereas neither E64 nor lysosomal inhibitors affected scattering (data not shown). We have previously demonstrated that comparable concentrations of MG132 and ALLN inhibit the degradation of short and long-lived proteins in...
MDCK cells in a manner consistent with inhibition of the proteasome (20). Moreover, we have shown that reduction of the aldehyde group in MG132, which is key moiety of this molecule necessary for proteasome inhibition, markedly diminishes its ability to inhibit protein degradation in MDCK cells, indicating that the action of this agent is largely on the proteasome (20).

Notably, the shape of the cell island after proteasome inhibition under scattering conditions was different from the normal cellular island: 1) cells at the margin appeared to spread and the supernatant was subjected to immunoprecipitation (IP) with anti-ZO-1 or anti-E-cadherin antibody. The immune complexes were separated by 7% SDS-PAGE and probed with antibodies specific for ZO-1, ZO-2, occludin, E-cadherin, α-catenin, and β-catenin. The arrow indicates a ubiquitinated form of β-catenin. B, MDCK cells were either treated with 20 ng/ml HGF (type II) or transferred to nonpermissive temperature (ts-v-Src) in the presence or absence of 0.5 (type II) or 2 (ts-v-Src) μM lactacystin overnight and lysed with TN buffer. The supernatant was subjected to immunoprecipitation (IP) with anti-ZO-1 or anti-E-cadherin antibody. The immune complexes were separated by 7% SDS-PAGE and probed with the indicated antibody.

FIG. 2. Total levels of TJ as well as AJ proteins and stoichiometry of ZO-1 and E-cadherin containing complexes after treatment with HGF in the presence or absence of various protease inhibitors. A, type II MDCK cells at a cellular island stage were treated with 20 ng/ml HGF in the presence or absence of cell permeable protease inhibitors (MG, 0.5 μM MG132, lac, 0.5 μM lactacystin; AN, 10 μM ALLN; cal, 20 μM calpeptin; E64, 25 μM E64; chl, 50 μM chloroquine). Equal amounts of RIPA buffer solubilized protein (50 μg/lane) were separated by 7% SDS-PAGE and probed with antibodies specific for ZO-1, ZO-2, occludin, E-cadherin, α-catenin, and β-catenin. The arrow indicates a ubiquitinated form of β-catenin. B, MDCK cells were either treated with 20 ng/ml HGF (type II) or transferred to nonpermissive temperature (ts-v-Src) in the presence or absence of 0.5 (type II) or 2 (ts-v-Src) μM lactacystin overnight and lysed with TN buffer. The supernatant was subjected to immunoprecipitation (IP) with anti-ZO-1 or anti-E-cadherin antibody. The immune complexes were separated by 7% SDS-PAGE and probed with the indicated antibody.

The Total Amounts of TJ as Well as AJ Proteins Are Minimally Changed during Scattering in the Presence or Absence of Protease Inhibitors—To analyze the blockage of scattering by proteasome inhibitors biochemically, we sought to examine the overall amounts of TJ as well as AJ proteins in MDCK cells in a manner consistent with inhibition of the proteasome (20). Moreover, we have shown that reduction of the aldehyde group in MG132, which is key moiety of this molecule necessary for proteasome inhibition, markedly diminishes its ability to inhibit protein degradation in MDCK cells, indicating that the action of this agent is largely on the proteasome (20).

Notably, the shape of the cell island after proteasome inhibition under scattering conditions was different from the normal cellular island: 1) cells at the margin appeared to spread and the supernatant was subjected to immunoprecipitation (IP) with anti-ZO-1 or anti-E-cadherin antibody. The immune complexes were separated by 7% SDS-PAGE and probed with antibodies specific for ZO-1, ZO-2, occludin, E-cadherin, α-catenin, and β-catenin. The arrow indicates a ubiquitinated form of β-catenin. B, MDCK cells were either treated with 20 ng/ml HGF (type II) or transferred to nonpermissive temperature (ts-v-Src) in the presence or absence of 0.5 (type II) or 2 (ts-v-Src) μM lactacystin overnight and lysed with TN buffer. The supernatant was subjected to immunoprecipitation (IP) with anti-ZO-1 or anti-E-cadherin antibody. The immune complexes were separated by 7% SDS-PAGE and probed with the indicated antibody.

FIG. 3. Proteasome inhibition stabilizes junctional proteins at the cell-cell adhesion site under scattering conditions. Type II MDCK cells grown on collagen-coated coverslips were extracted with CSK-A buffer, fixed in 1% paraformaldehyde, and doubly stained with anti-occludin (a and c) and anti-ZO-1 (b and d) or with anti-E-cadherin (e and g) and α-catenin (f and h) antibody. Then samples were observed through a confocal microscope. 20 ng/ml HGF alone, a, b, e, and f; 20 ng/ml HGF plus 0.5 μM lactacystin (lac), c, d, g, and h. Bar, 75 μm. during scattering induced by HGF in MDCK cells. The most notable change was an increase of β-catenin, in which case less mobile forms accumulated in the presence of MG132, lactacystin, or ALLN; however, this increase was also found in MDCK cells treated with lactacystin alone, indicating that this change in β-catenin is not specific to the blockage of cell-cell dissociation during scattering induced by HGF in MDCK cells (Fig. 2A). Because β-catenin is known to be degraded by the proteasome through its ubiquitination (21, 22), this increase is likely to be due to an accumulation of ubiquitinated β-catenin by proteasome inhibitors. In addition, a slight increase (<20%) in levels of total α-catenin, E-cadherin, and occludin was also observed after proteasome inhibition (Fig. 2A). Similar results were obtained with temperature-sensitive v-Src MDCK cells treated with proteasome inhibitors at either nonpermissive or permissive temperatures (data not shown).

To study the stoichiometry of the individual components of the ZO-1 and E-cadherin containing complexes during scattering in the presence or absence of lactacystin, MDCK cells were lysed with TN buffer to preserve protein-protein interactions, and the supernatant was subjected to immunoprecipitation with anti-ZO-1 or anti-E-cadherin antibody, followed by immunoblotting (Fig. 2B). No significant differences in the amounts of co-precipitating proteins in the ZO-1 or E-cadherin containing complex were observed in scattered cells induced by both HGF and pp60v-src in the presence or absence of lactacystin. Although less mobile forms of β-catenin coprecipitated with E-cadherin, this was also found in cells treated with lactacystin alone (data not shown).
Proteasome Inhibitors Block Cell-Cell Dissociation

Fig. 4. A, proteasome inhibition stabilizes TJ proteins in the Triton X-100-insoluble fraction. Type II MDCK cells were treated with 20 ng/ml HGF in the presence or absence of various protease inhibitors (MG132, 0.5 μM MG132; lac, 0.5 μM lactacystin; ALLN, 10 μM ALLN; E64, 25 μM E64). After overnight incubation, cells were extracted with CSK-A buffer, and the insoluble materials were dissolved in 2X sample buffer. Both soluble (E, extract) and insoluble (R, residue) fractions were analyzed by Western blot with indicated antibodies. The arrowhead indicates a higher molecular weight form of occludin. B and C, blots were quantified with National Institutes of Health image software. The measurements were expressed as percentages of total density of both fractions and represent the means ± S.E. MG, MG132, lac, lactacystin; AN, ALLN; E64, E64.

Proteasome Inhibitors Stabilize TJ as Well as AJ Proteins in the Triton X-100-insoluble Pool—Although the immunoblot analysis shown in Fig. 2 indicated that the overall protein amount of TJ as well as AJ proteins is not markedly changed in MDCK cells treated with HGF and the activation of pp60v-Src in the presence of proteasome inhibitors, this did not exclude the possibility that the subcellular localization and the cytoskeletal association of TJ and AJ proteins is affected by the treatment. To examine this possibility, we employed a Triton X-100 extraction assay in the cells (17). After overnight incubation with proteasome inhibitors under scattering conditions driven by HGF, cells were extracted with CSK-A buffer, which contains 0.5% Triton X-100, and processed for indirect immunofluorescence (Fig. 3). Indirect immunofluorescence revealed that after proteasome inhibition under scattering conditions, Triton X-100-insoluble ZO-1 was clearly detectable at the cell-cell contact site of HGF-stimulated MDCK cells treated with lactacystin (Fig. 3d), as was the case with a integral membrane TJ protein, occludin (Fig. 3c) (23); these staining patterns are similar to untreated cells (data not shown) (17, 24). In addition, after proteasome inhibition under scattering conditions, the transmembrane AJ protein, E-cadherin (Fig. 3g), also colocalized with α-catenin (Fig. 3h), which links E-cadherin to the actin cytoskeleton via β-catenin at the lateral border of the cells (3). In marked contrast, in the absence of proteasome inhibition, the normal junctional localization of both TJ and AJ proteins, was disrupted, and these proteins lost their association with cytoskeletal elements in scattered cells, judging from immunocytochemistry of Triton X-100-insoluble junctional proteins (Fig. 3, a, b, e, and f), consistent with results of others (25). Similar results were obtained in temperature-sensitive v-Src MDCK cells (data not shown).

Immunoblot analyses of Triton X-100-soluble and -insoluble ("cytoskeletal") fractions were also consistent with the notion that proteasome inhibitors stabilized associations of TJ proteins with the cytoskeletal fraction (Fig. 4a). A significant portion of occludin and ZO-1 remained in the Triton X-100-insoluble pool in the presence of inhibitors, whereas TJ proteins became Triton X-100 soluble after scattering in the absence of the inhibitors (Fig. 4b). The changes in solubility of these proteins became obvious after ~4 h of HGF treatment or the activation of temperature-sensitive pp60v-Src and correlated well with cell-cell dissociation observed by light microscopy (data not shown). Moreover, the amount of a higher molecular weight form of occludin in the Triton X-100-insoluble pool, which appears to be localized at the TJ (Fig. 3c) (17, 24), was increased by the inhibitors. The alteration of Triton X-100 extractabilities of ZO-2, E-cadherin, and α-catenin was less striking than ZO-1, occludin, and β-catenin (Fig. 4, b and c). The absolute increase in Triton X-100-insoluble β-catenin may be partly due to the total increase (Fig. 2); however, it is possible that the blockage of catenin degradation partly explains the inhibition of cell-cell dissociation upon scattering, because in addition to their key role in stabilizing the AJ (3), catenins may play a role in the stabilization of the AJ (26, 27).

Thus, these results suggest that this blockage of cell-cell dissociation by proteasome inhibitors might stabilize not only
Fig. 6. Proteasome inhibition does not block rearrangement of focal contacts and chemotaxis induced by HGF. A, cells were incubated with 20 ng/ml HGF (panels b and c) in the presence (panel c) or absence (panels a and b) of 0.5 μM lactacystin overnight and processed for indirect immunostaining with anti-paxillin antibody. The arrows indicate accumulations of paxillin at the cellular protrusions after HGF treatment. Bar, 30 μm. B, MDCK cell migration toward 20 ng/ml HGF was assayed with the modified Boyden chamber technique in the presence or absence of protease inhibitors. P.I., protease inhibitor; lac, 5 μM lactacystin; E64, 25 μM E64; cal, 20 μM calpeptin; chl, 50 μM chloroquine. The results were expressed as fold increase in migrated cell number compared with that in the absence of HGF and represent the mean values ± S.D. of triplicate samples.

Proteasome Inhibitors Prolong the Half-life of E-cadherin under Scattering Conditions—To gain further insight into the fate of junctional proteins during proteasome inhibition upon cell scattering, we used 35S-labeled temperature-sensitive v-Src MDCK cells because v-Src has less mitogenicity for MDCK cells than HGF (15). During the last 30 min of the labeling period at the nonpermissive temperature, cells were treated with MG132 or vehicle and chased for various lengths of time in normal growth medium at the permissive temperature. Immunoprecipitation from 35S-labeled cells at each time point revealed that E-cadherin is a long-lived protein: the half-life was ~8 h at the permissive temperature (Fig. 5). The proteasome inhibitor, MG132 as well as lactacystin (data not shown), significantly blocked the decrease of radiolabeled E-cadherin at the scattering temperature (Fig. 5B). We cannot exclude the possibility that those chemicals also inhibit cell proliferation (28–30), and thereby radiolabeled E-cadherin might be diluted by newly synthesized E-cadherin molecule in the absence of the inhibitor. Nevertheless, this result supports our hypothesis that proteasomal proteolysis can alter the fate of junctional proteins upon epithelial cell scattering.

Neither Rearrangement of Focal Adhesions nor Single Cell Migration Induced by HGF Is Affected by Proteasome Inhibition—Cell scattering consists of at least two biological responses, which appear to occur simultaneously or synchronously in the cells: 1) cell-cell dissociation, resulting from breaking apart of intercellular junctional complexes, and 2) cell movement, driven by rearrangement of the cytoskeleton and formation of new cell-substratum contacts (focal adhesions). To address the question of whether the proteasomal proteolytic pathway might be involved in the latter step, we examined the immunocytochemical localization of paxillin, a major component of focal adhesions (31), upon cell scattering induced by HGF. As shown in Fig. 6A (panel a), paxillin mainly accumulated along the edge of normal MDCK cells in cellular islands. In scattered (or scattering) cells, paxillin accumulated at cell protrusions and the membrane ruffling site (Fig. 6A, panel b). Of interest was that paxillin also accumulated at cell protrusions of marginal cells in lactacystin-treated MDCK cell islands in a distribution similar to that of scattering cells treated with HGF alone (Fig. 6A, panel c). Moreover, a chemotaxis assay using a modified Boyden chamber technique did not reveal any effect of lactacystin, even at higher concentrations (5 μM), on cell migration toward HGF (Fig. 6B). In agreement with a previous report (32), a calpain inhibitor, calpeptin, partially inhibited cell migration induced by HGF, whereas calpeptin could not block cell scattering induced by HGF and pp60v-Src (data not shown). These data suggest that the proteasomal
proteolytic pathway is not critical for rearrangement of focal adhesions and cytoskeleton induced by HGF as well as cell motility in this context, but it is essential for disruption of cell-cell adhesion and intercellular junctions.

**Proteosome Inhibitors Partially Prevent Loss of Cell Polarity under Scattering Condition**—To analyze cell polarity, we examined the immunocytochemical localization of Na$^+$,K$^+$-ATPase, which is normally present at the basolateral domain of polarized MDCK cells (33), and gp135, a glycoprotein associated with apical microvilli of MDCK cells (34). As shown in Fig. 7A (panel a), the β subunit of Na$^+$,K$^+$-ATPase was clearly localized at the lateral border of control MDCK cells, whereas Na$^+$,K$^+$-ATPase was widely redistributed in scattered cells (Fig. 7A, panel b). In the presence of lactacystin, Na$^+$,K$^+$-ATPase was still found at the lateral border after HGF stimulation; however, this staining at the lateral border of the cells was weaker than in control cells, and faint diffuse intracellular ATPase was still found at the lateral border after HGF stimulation (Fig. 7A, panel c). 

Further examination of the degradation pathway of junctional proteins will lead to a better understanding of how epithelial cell-cell contact is regulated not only under physiological and/or developmental conditions but also in disease states.

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