Intestinal HT-29 cells with dysfunction of E-cadherin show increased pp60src activity and tyrosine phosphorylation of p120-catenin

Anouchka SKOUDY, Maria del Mont LLOSAS and Antonio GARCÍA DE HERREROS*

Unitat de Biologia Cel.lular i Molecular, Institut Municipal d'Investigació Mèdica, Universitat Autònoma de Barcelona, C/ Dr. Aiguader, 80, 08003 Barcelona, Spain

1. HT-29 M6 cells are a subpopulation of HT-29 cells that, contrarily to the parental cells, establish tight cell contacts and differentiate. Cell-to-cell contacts in HT-29 M6 cells are also regulated by protein kinase C; addition of the phorbol ester phorbol 12-myristate 13-acetate (PMA) decreases the homotypic contacts of these cells. We show here that HT-29 cells or HT-29 M6 cells treated with PMA contain lower levels of functional E-cadherin, determined by analysing the association of this protein with the cytoskeleton. No significant differences in the localization of α-, β-, or p120-catenins were detected under the three different conditions. 2. Dysfunction of E-cadherin can be reversed by incubation of HT-29 cells with the tyrosine kinase inhibitor herbimycin A. On the other hand an augmentation of c-*src* activity in HT-29 cells or HT-29 M6 cells treated with PMA was observed with respect to control HT-29 M6 cells. The phosphorylation status of catenins was also investigated; in HT-29 or in HT-29 M6 cells treated with PMA, dysfunction of E-cadherin was accompanied by an increased phosphorylation of p120-catenin and by an elevated association of this protein to E-cadherin. These results suggest a role for pp60src and the pp60src substrate p120-catenin in the control of E-cadherin function in HT-29 cells.

INTRODUCTION

In epithelial cells, a key role in the establishment of cellular contacts is played by the protein E-cadherin [1,2]. Activity of E-cadherin requires its association with the cytoskeleton through its cytosolic C-terminus [3]. Several cytosolic proteins (denominated α-, β-, γ- or p120-catenins) interact with E-cadherin; these proteins are involved in linking E-cadherin to the cytoskeleton and modulating its activity [4,5]. In the last five years subpopulations of intestinal HT-29 cells capable of differentiating in culture to goblet or absorptive cells have been isolated [6,7]. One of these cell lines, the mucus-secreting HT-29 M6 cells [6,8], has been extensively studied in our laboratory; at subconfluency these cells form compact colonies with tighter cell contacts than the parental HT-29 cells [6]. We have reported that cell-to-cell adhesion in HT-29 M6 cells can be disturbed by the addition of activators of protein kinase C (PK-C) such as the phorbol ester phorbol 12-myristate 13-acetate (PMA) [9]. This loss in cell-to-cell contacts is accompanied by the rapid internalization of E-cadherin as shown by immunofluorescence with a specific monoclonal antibody (mAb) [9]. Our goal in this article has been to study the molecular basis responsible for the difference in cell-to-cell adhesion observed between HT-29 and HT-29 M6 cells, or in the latter cells after treatment with the phorbol ester.

EXPERIMENTAL

Reagents

Leupeptin, aprotinin, α-casein, PMA, sodium vanadate and phenylarsine oxide (PAO) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein A–agarose beads and PMSF were from Boehringer (Mannheim, Germany). [γ-^{32}P]ATP was from Amersham (Amersham, Bucks., U.K.). All other chemicals used were commercial products of the highest grade available. Herbimycin A (Hb) was from Calbiochem (San Diego, CA, U.S.A.).

Antibodies

Mouse mAb anti-pp60src (327) was obtained from Oncogene Science (Manhasset, NY, U.S.A.). Anti-(E-cadherin) mAbs HECD1 (from mouse) and ECCD-2 (from rat) were kind gifts from Dr. Amparo Cano (Universidad Autónoma, Madrid, Spain). Mouse anti-phosphotyrosine mAb (4G10) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Mouse mAbs against α-, β- or p120-catenins were from Transduction Laboratories (Lexington, KY, U.S.A.).

Cell culture

HT-29 and HT-29 M6 cells were supplied by Dr. Alain Zweibaum (INSERM, Villejuif, France). The isolation and characteristics of the HT-29 M6 cell line, a subpopulation of the HT-29 cells isolated by selection with 10^{-6} M methotrexate, have been exhaustively described [6,8]. Cells were seeded at a density of 2×10^4 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO_2/95% air. Culture medium was changed every other day to avoid nutrient depletion. Experiments were always performed in cells 4–5 days after plating, when they were 40–70% confluent.

Cell dissociation assay

This assay was performed as described elsewhere [10] with some minor modifications. Cells (approx. 1×10^6 cells per 6-cm-diam. dish) were treated with 2 ml of trypsin (0.05%) in Puck's–EDTA medium ($NaHCO_3$, 4 mM; NaCl, 136 mM; KCl, 4 mM; EDTA, 1 mM; glucose, 1 mg/ml; Phenol Red, 0.005 mg/ml) (TE treatment) or in Puck's–EDTA medium supplemented with $CaCl_2$ (5 mM) (TC treatment) for 20 min at 37 °C, and dissociated by pipetting 20 times. The extent of dissociation of the cells was

Abbreviations used: FBS, fetal bovine serum; Hb, herbimycin A; mAb, monoclonal antibody; PAO, phenylarsine oxide; PK-C, protein kinase C; TBS, Tris-buffered saline; PMA, phorbol 12-myristate 13-acetate.

* To whom correspondence should be addressed.

represented by the index N_{TC}/N_{TE}, where N_{TC} and N_{TE} are the total particle counts after the TC and TE treatments, respectively.

Cell extraction and immunoblotting

Soluble and cytoskeletal fractions were prepared essentially as described by Nelson and co-workers [11]. Cells were rinsed in PBS plus 1 mM $CaCl_2$ and homogenized in CSK buffer (50 mM NaCl, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 300 mM sucrose) supplemented with 1 mM PMSF, 10 μg/ml leupeptin, 0.5 mM sodium vanadate and 20 μM PAO for 10 min at 4 °C with gentle rocking. After centrifugation in a microfuge for 10 min at 4 °C, the supernatant constituted the Triton-soluble fraction. The pellet was triturated in the same volume of SDS buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS) and boiled at 100 °C for 10 min. After centrifugation for 10 min in a microfuge, this supernatant constituted the Triton-insoluble fraction. This fraction usually contained 5-fold less protein than the Triton-soluble fraction, as determined with the Bio-Rad (DC) assay; this ratio was not significantly altered under any of the conditions studied (varied from 4.5 to 5.1). Therefore, in order to analyse equivalent amounts of these fractions, 70 μg of Triton-soluble and 15 μg of the Triton-insoluble fractions were routinely analysed. Cell fractions were separated by PAGE in the presence of SDS (SDS/PAGE), transferred electrophoretically to nitrocellulose and incubated for 1 h with the indicated mAbs (1 μg/ml). After washing, membranes were incubated with peroxidase-conjugated goat anti-(mouse IgG) antibodies (Dakopatts, Copenhagen, Denmark), and reacting antigens were visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham). Autoradiograms were quantified by scanning densitometry (Hoefer GS-300 Scanning Densitometer).

pp60src kinase assay

The assay was performed as described in [12] with minor modifications. Cells were lysed in a modified RIPA buffer (20 mM Mops, pH 7.0, 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 0.5 mM sodium vanadate and 20 μM PAO. Lysates were centrifuged at 13000 ***g*** in a microfuge for 5 min at 4 °C and supernatants were divided into aliquots and stored at −40 °C until required for use. Samples (400 μg) of protein were immunoprecipitated with mAb 327 (2 μg/ml) at 4 °C for 12 h. After adding a rabbit anti-(mouse IgG) antibody (Zymed Laboratories, San Francisco, CA, U.S.A.) for 1 h, immunocomplexes were collected by adding Protein A–agarose for a further hour and spinning in a microfuge. Pellets were extensively washed in RIPA buffer plus 0.5 mM sodium vanadate and resuspended in 25 μl of kinase buffer (20 mM Mops, pH 7.0, 5 mM $MgCl_2$) containing 5 μM [γ-^{32}P]ATP (8000 c.p.m./pmol). After 10 min at 23 °C, the reaction was stopped by adding Laemmli's sample buffer and proteins were separated by SDS/PAGE, gels were dried and exposed for 12 h. When casein phosphorylation was analysed, 2 μg of α-casein was added to the kinase buffer described above.

Catenins phosphorylation

Cells, incubated with PMA for different times, were supplemented with sodium vanadate (0.5 mM) 10 min before preparing the extracts in order to inhibit phosphotyrosine phosphatases. Extracts were made in modified RIPA buffer supplemented with 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and 0.5 mM sodium vanadate. After centrifugation, 400 μg of extract was incubated with 5 μg of mAb 4G10 in a final volume of 1 ml for 12 h at 4 °C. Immunocomplexes were collected as described above, extensively washed in RIPA buffer plus inhibitors and solubilized in Laemmli's sample buffer. After SDS/PAGE, the presence of p120-, α- or β-catenins was determined by blotting with specific mAbs.

p120-catenin–E-cadherin association

These experiments were performed essentially as described by Burridge and co-workers [13]. Cell extracts were prepared in RIPA buffer (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 0.5 mM sodium vanadate and 20 μM PAO. After clarification in a microfuge for 10 min at 13000 ***g***, 500 μl of lysate (approx. 600 μg of protein) was immunoprecipitated with anti-(E-cadherin) ECCD-2 antibody (1/100) for 2 h at 4 °C. Rabbit anti-(rat IgG) antibodies (Dakopatts) and Protein A–agarose were added and immunocomplexes were precipitated as described above and subjected to SDS/PAGE. The presence of p120-catenins, phosphotyrosine or E-cadherin was determined by Western blot. When necessary, the blots were stripped with 62 mM Tris/HCl, pH 6.8, containing 2% SDS and 100 mM 2-mercaptoethanol for 1 h at 60 °C [13]. The blots were washed in Tris-buffered saline overnight and the removal of the antibody was determined by incubating with the corresponding peroxidase-labelled secondary antibody before reprobing with a new primary antibody.

RESULTS

Decreased cell-to-cell contacts correlate with a lower association of E-cadherin with the cytoskeleton

Observed under a phase-contrast microscope, HT-29 M6 cells show a more compact appearance than the parental HT-29 cells with tighter colonies, which suggests that cell–cell adhesiveness is different in the two subpopulations. We have quantified this difference using the aggregation assay described by Nagafuchi and co-workers [10]. The index N_{TC}/N_{TE}, inversely proportional to cell adhesion, was 2.8-fold higher in HT-29 than in HT-29 M6 cells, when both cell lines were grown for the same length of time after seeding to reach the same confluent density (Table 1). This lower index reflects the fact that HT-29 M6 cells possess tighter cell–cell contacts than HT-29 cells. In HT-29 M6, as in other epithelial cell lines, cellular contacts can be modulated by addition of the phorbol ester PMA, a PK-C activator. This compound profoundly alters the morphology of the cultures, causing the

Table 1 Decreased cell-to-cell association in HT-29 cells or in HT-29 M6 cells in the presence of PMA

HT-29 and HT-29 M6 cells were seeded at the same density and grown for 5 days in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum as described in the Experimental section. HT-29 M6 cells were treated with PMA (200 nM) for 6 h before the analyses were performed. Cells in duplicate were treated with trypsin in the presence of Ca^{2+} (TC treatment) or EDTA (TE treatment) and the cell dissociation index N_{TC}/N_{TE} was determined. The Table shows the mean ± S.D. of five experiments performed.

Cells	N_{TC}/N_{TE} index
HT-29	0.232 ± 0.133
HT-29 M6	0.084 ± 0.053
HT-29 M6 + PMA	0.344 ± 0.087

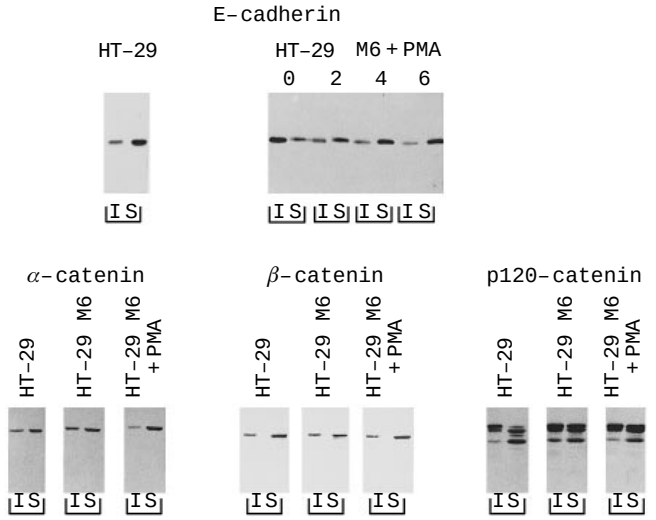

Figure 1 Association with the cytoskeleton of E-cadherin and catenins in HT-29 cells, or HT-29 M6 cells treated with PMA

HT-29 and HT-29 M6 cells were grown in Dulbecco's modified Eagle's medium plus 10% (v/v) fetal bovine serum for 5 days after seeding; PMA (200 nM) was then added to the HT-29 M6 medium for the indicated times (h). Triton X-100-soluble (S) and insoluble (I) fractions were prepared as described and analysed for the presence of E-cadherin, α-, β- or p120-catenins. Samples (70 μg) of the Triton-soluble fraction and (15 μg) of the Triton-insoluble fraction were subjected to SDS/PAGE and Western blotting with specific mAbs; the molecular masses of the unique bands detected with E-cadherin, α-catenin and β-catenin were, respectively, 120, 105 or 95 kDa. In the case of p120-catenin, the bands detected presented molecular masses of 120, 115, 105 (faint) and 95 kDa. In the analysis of catenins the panel HT-29 M6 + PMA corresponds to cells treated with this phorbol ester for 6 h. The Figure shows the result of one representative experiment of five performed in the case of E-cadherin, and three, for the catenins.

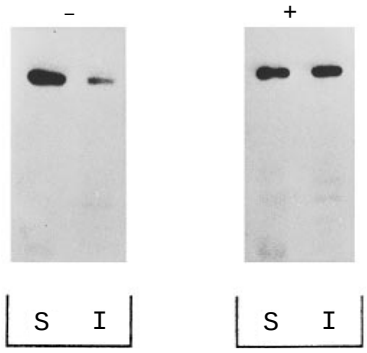

Figure 2 Hb increases the association of E-cadherin with the cytoskeleton

HT-29 cells were grown for 5 days after seeding; Hb (2 μg/ml) was added to the cultures indicated (+) for the last 24 h. Triton-soluble (S) or insoluble (I) fractions were prepared and analysed as indicated in Figure 1. A representative assay of three performed is shown.

rapid scattering of the colonies and the loss of intercellular contacts [9]. Using the aggregation assay described above we have determined that after 6 h of incubation with PMA, the cell dissociation index was increased 4.1-fold (Table 1).

Therefore, we can define two conditions in which cell aggregation is diminished (HT-29 with respect to HT-29 M6 cells, or the latter cells treated with or without PMA). We have determined that, under these two conditions, the activity of E-cadherin is also altered. As mentioned elsewhere [3], E-cadherin is a protein that plays a key role in the establishment and maintenance of homotypic contacts in epithelial cells; the correct function of E-cadherin requires its association with the cytoskeleton. For this reason, the localization of E-cadherin has been widely used to determine its level of activity [4,10,11,14,15]. Membrane and cytoskeletal fractions of HT-29 M6 cells were prepared following the method of Nelson and co-workers [11] and E-cadherin levels were determined by Western blotting with the mAb HECD-1. As observed in Figure 1, E-cadherin was mostly found in the cytoskeletal fraction in HT-29 M6 cells; scanning of the autoradiograms showed that the proportion of E-cadherin in this fraction varied between 60 and 80% at this day of culture (in five different experiments). After incubation with PMA, E-cadherin levels decreased in the cytoskeleton fraction and increased in the soluble fraction; this translocation was detected after 2 h and was completed after 6 h of incubation with PMA (Figure 1). In HT-29 cells, the proportion of E-cadherin associated with the cytoskeleton (10–25%) was lower than in HT-29 M6 cells, although the total levels of the protein were similar (Figure 1). These experiments indicate that the loss of cell adhesion observed in HT-29 cells or in HT-29 M6 cells treated with PMA correlates with the inactivation of E-cadherin and its dissociation from the cytoskeleton.

The localization of the catenins, proteins that mediate the binding of E-cadherin to the cytoskeleton, was studied using specific mAbs. α-Catenin was found in the two fractions studied in similar proportions, without significant differences in HT-29 and HT-29 M6 cells; addition of PMA decreased slightly the amount of cytoskeleton-associated protein (Figure 1). The distribution of β-catenin was not different in the three experimental conditions studied (Figure 1). In contrast to the mAbs specific for α- and β-catenins, the anti-(p120-catenin) mAb detected four bands of molecular masses 120, 115, 105 and 95 kDa. Four different isoforms of this molecule with very similar molecular masses to those found here have been described [5]. Isoforms 1A (120 kDa) and 1B (115 kDa) were the most commonly represented in HT-29 and HT-29 M6 cells, although a significant amount of isoform 2B (105 kDa) was detected in the cytoskeleton (Figure 1). Addition of PMA did not alter the distribution of the p120-catenin isoforms.

HT-29 cells with E-cadherin dysfunction display higher c-*src* tyrosine kinase activity

Several results have suggested that the product of the proto-oncogene c-*src* (pp60src) plays a role in regulation of cell-to-cell contacts. Rapid loss of cellular contacts has been observed in Madin–Darby canine kidney (MDCK) epithelial cells after transformation with the v-*src* gene; these effects are probably due to the inactivation of E-cadherin since this protein is internalized after activation of this protein kinase [16]. These results prompted us to analyse whether pp60src could be involved in the control of E-cadherin function in HT-29 cells.

Hb is a potent and specific tyrosine kinase inhibitor [17] which is widely used to selectively block pp60src activity in several systems, among them HT-29 cells [18]. As shown in Figure 2, addition of Hb (2 μg/ml) to HT-29 cells increased the amount of E-cadherin associated with the cytoskeleton. This result suggested a role for pp60src, or another kinase sensitive to this drug, in the regulation of E-cadherin activity.

pp60src activity was also measured in HT-29 cells. Extracts were prepared in RIPA buffer as described in the Experimental section in order to solubilize the cytoskeleton-associated pp60src,

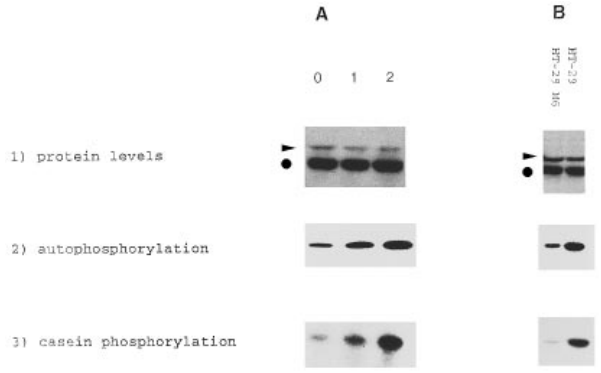

Figure 3 HT-29 M6 cells show lower pp60src protein kinase activity than HT-29 M6 cells treated with PMA or parental HT-29 cells

Cells were seeded as described in the Experimental section and cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%) for 5 days; PMA (200 nM) was then added for the indicated times (in h). Cell extracts were prepared and immunoprecipitated with mAb 327, and total pp60src levels were determined by Western blot with the same mAb (1), or the autokinase (2) and casein kinase (3) activities were measured. In (**A**), the effect of an incubation with PMA (time in h) of HT-29 M6 cells is shown; in (**B**), HT-29 and HT-29 M6 cells were compared. The Figure shows a representative experiment of three performed. Western blots of pp60src or autokinase reaction were analysed using 8% polyacrylamide gels; casein kinase in 12% gels. Only one band of 60 kDa or 25 kDa was detected in the experiments of autophosphorylation and casein phosphorylation respectively. The arrowhead in 1 corresponds to pp60src mobility, the dot to IgGs.

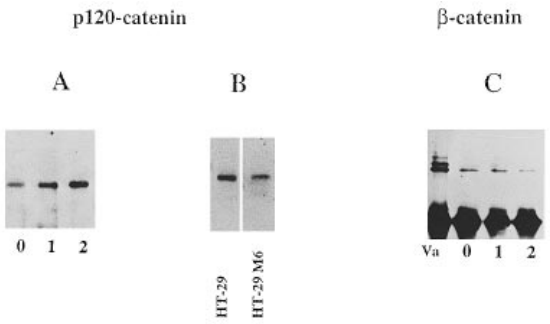

Figure 4 PMA increases the tyrosine phosphorylation of p120-catenin but not β-catenin

HT-29 and HT-29 M6 cells were grown for 5 days and incubated with PMA (200 nM) for the indicated times (in h). Medium was supplemented with sodium vanadate (0.5 mM) 10 min before preparing the extracts. Immunoprecipitation was performed as described with anti-phosphotyrosine mAb 4G10; immunocomplexes were analysed by SDS/PAGE and Western blot with mAbs against p120-catenin (**A** and **B**) or β-catenin (**C**). No phosphorylation was detected with either of the two mAbs when the immunoprecipitation was performed with an irrelevant antibody. The migration of the phosphorylated band corresponded to 120 and 95 kDa for p120- and β-catenin, respectively. The strong signal detected around 55 kDa in the blot of β-catenin corresponded to IgGs. Va indicates an extract prepared from cells treated with sodium vanadate (0.5 mM) for 12 h, that was used as positive control. The slow-migrating bands that appear after incubation with Va probably correspond to hyperphosphorylated forms of this protein, as described in other cases [26].

immunoprecipitated with the c-*src*-specific mAb 327, and pp60src autophosphorylation activity or its activity towards an exogenous substrate (casein) was determined. As shown in Figure 3(A), PMA augmentated both the autophosphorylation activity of pp60src [2.6-fold ± 0.2 (S.E.M.), as quantified by densitometry] and the kinase activity towards an exogenous substrate (α-casein) (7.5-fold ± 0.8). Maximum increase was obtained after 2 h of incubation with the phorbol ester, coincident with the beginning of E-cadherin translocation (see Figure 1). Using the same assays, we also determined that an elevated pp60src activity was present in HT-29 cells with respect to control HT-29 M6 cells (Figure 3B). The increases were estimated to be 3.5-fold (± 0.6) (autophosphorylation activity) and 6.2-fold (± 1.2) (casein kinase activity). No differences in the total amount of immunoprecipitated pp60src were detected by Western blot under any of the conditions studied (Figure 3).

PMA increases the phosphorylation of tyrosine in p120-catenin but not in β-catenin

It has been shown that two catenins are phosphorylated on tyrosine after activation of v-*src* [16,19]; these results have suggested a role for these two proteins (p120- and β-catenin) in the regulation of E-cadherin activity.

We have examined whether the phosphorylation of some of the catenins was altered by PMA. Cell extracts, prepared after incubation of the cells with the phorbol ester, were precipitated with the anti-phosphotyrosine mAb 4G10 and the immunocomplexes analysed with mAb specific for p120-, α-, or β-catenin. As observed in Figure 4, PMA enhanced the phosphotyrosine content of p120-catenin after 2 h of treatment with the phorbol ester (Figure 4A). The phosphorylated band showed a molecular mass of 115–120 kDa, suggesting that this corresponds to isoform 1A or 1B. In comparison, no significant increases were detected in β-catenin phosphorylation over the background level (Figure 4C). As positive control of this assay, tyrosine phosphorylation of β-catenin was stimulated by a long-term (12 h) incubation with sodium vanadate. As expected, the extent of phosphorylation of p120-catenin was also greater in HT-29 than in HT-29 M6 cells (Figure 4B). No phosphorylation of α-catenin was detected under any of the conditions analysed (results not shown).

Dysfunction of E-cadherin correlates with a higher degree of association of this protein with p120-catenin

We also examined whether PMA modified the association of E-cadherin with catenins. Total cell extracts were prepared, immunoprecipitated with anti-(E-cadherin) mAb ECCD-2, and immunocomplexes were analysed by Western blotting with antibodies against E-cadherin, p120-catenin or phosphotyrosine. This method has been used to analyse the interaction of E-cadherin with catenins since these proteins co-immunoprecipitate. In HT-29 cells or in HT-29 M6 cells treated with PMA, p120-catenin associated with E-cadherin was found in a greater amount than in control HT-29 M6 cells (Figure 5). Analysis of tyrosine phosphorylation in these complexes revealed a prominent band that co-migrates with p120-catenin in HT-29 M6 cells plus PMA or in HT-29 cells, and other minor bands of 105 and 95 kDa. Another band of approx. 70 kDa was also heavily phosphorylated on tyrosine, apparently to a higher extent in HT-29 cells than under any other condition studied. At the present moment we do not know whether this band corresponds to a degradation product of p120-catenin or to an as yet undescribed protein with affinity for E-cadherin.

DISCUSSION

In this work we have quantified the differences in cell aggregation of HT-29 cells under conditions in which they are or are not able to differentiate. When compared with HT-29 M6 cells, the parental population, or HT-29 M6 cells treated with the phorbol ester PMA, showed decreased cellular adhesiveness. This property can be explained by a dysfunction of E-cadherin, since in

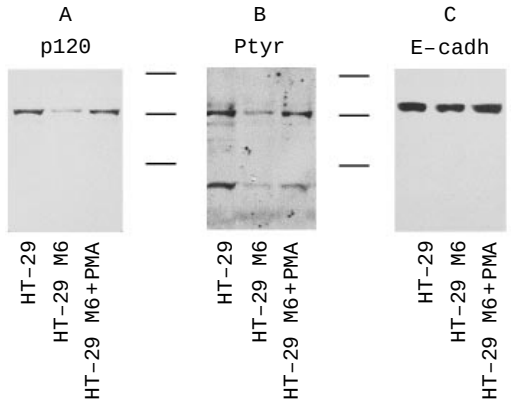

Figure 5 Tyrosine phosphorylation of p120-catenin increases its association with E-cadherin

Cell extracts were prepared in RIPA buffer and immunoprecipitated with the anti-(E-cadherin) mAb ECCD-2. After collecting, the immunocomplexes were subjected to SDS/PAGE (8% polyacrylamide) and analysed, succesively, with mAbs against p120-catenin, phosphotyrosine and E-cadherin (mAb HECD-1). Between incubations with two different primary antibodies, blots were stripped as described and the removal of the antibody verified. The bars indicated the migration of the prestained molecular mass markers: from top to bottom, myosin (205 kDa), β-galactosidase (115 kDa) and BSA (80 kDa), respectively.

both conditions the association of this protein with the cytoskeleton is greatly reduced. This association is required for the protein to be functional and is mediated by several proteins, collectively called catenins. In our studies we do not detect extensive changes in the association with the cytoskeleton of any other protein except E-cadherin; the only other effect observed, on α-catenin, was much smaller. E-cadherin dysfunction was accompanied by an increase in the tyrosine phosphorylation of one of these catenins, p120-catenin, and increased association of this protein with E-cadherin. Side-by-side SDS/PAGE of tyrosine-phosphorylated p120-catenin and the different isoforms of this protein present in the cell extract indicated that it corresponds to isoform 1, probably to isoform 1A.

p120-catenin was initially characterized as a major v-*src* substrate [19]. Cloning of the gene revealed the presence in this molecule of an 'arm' domain, also present in β-catenin and plakoglobin [20,21]. It has been shown that p120-catenin associates with E-cadherin through the same C-terminal site as other catenins [5,15]. Several authors have suggested that p120-catenin, which can be phosphorylated not only in response to v-*src* activation but also by growth factors [22], might play a critical role in the regulation of E-cadherin-mediated cell adhesion. Recent data from Burridge and co-workers [13] demonstrate that transformation by the oncogene *ras* of a mammary cell line decreases the association with the cytoskeleton of E-cadherin and raises the phosphorylation on tyrosine of p120-catenin; these changes are accompanied by the formation of complexes between this protein and E-cadherin. Our results with HT-29 cells are in complete agreement with these data and further support a role for p120-catenin in the control of cell-to-cell contacts.

In our cell system, loss of E-cadherin function was accompanied by increased pp60src activity. An inverse relationship between these two parameters was reinforced by the fact that incubation with Hb, an inhibitor of the pp60src, causes the contrary effect, i.e. higher association of E-cadherin with the cytoskeleton. Stimulation of pp60src was observed rapidly after treatment with PMA; to our knowledge, this is the first work that reports a stimulation of this protein kinase by PMA in epithelial cells, although the possible involvement of pp60src in a signal pathway triggered by other PK-C activators has been proposed [23]. It is noteworthy that not only p120-catenin but also β-catenin has been shown to be a substrate of the v-*src* product [15,18,24]. However, we have only detected changes in the phosphotyrosine content of p120-catenin, although β-catenin was exhaustively studied as well. It should be pointed out that in the cellular systems used by other groups (transformation with temperature-sensitive v-*src* genes) [15,24], the increases in the activity of this tyrosine kinase were much higher than in our HT-29 M6 cells after treatment with PMA. Therefore it is possible that the augmentation in pp60src activity is not high enough to induce an elevation in the tyrosine phosphorylation of β-catenin.

As mentioned in the Introduction, HT-29 M6 cells are a subpopulation of HT-29 cells isolated by their resistance to methotrexate, that, contrarily to parental cells, show ability to differentiate [8]. This ability of HT-29 M6 cells is affected by incubation with PMA; in addition to modifying the cellular adhesiveness of these cells, this phorbol ester blocks the appearance of markers characteristic of a mature goblet cell [25]. We have shown here that HT-29 cells unable to differentiate show a dysfunction of E-cadherin. It has been reported in several epithelial cells that inactivation of E-cadherin prevents the establishment of correct cell-to-cell contacts, an event essential for the polarization and differentiation of epithelial cells. Our results suggest a difference in activity in HT-29 and HT-29 M6 cells of a signal transduction pathway regulating the association of E-cadherin with the cytoskeleton, in which pp60src plays a role. This pathway, and its relationship to goblet cell differentiation, is currently being studied in our laboratory.

We thank Drs. F.X. Real, A. Cano and A. Zweibaum for providing cells and reagents and the members of the Unitat de Biologia Cellular i Molecular of the I.M.I.M. for their advice and support. We especially appreciate the suggestions of Dr. Myriam Fabre during the early part of this work. We thank Mercè Figueras for her help with the scanning of the autoradiograms. This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología to A.G.H. (SAL91-0837 and SAF94-1008). A.S. and M.M.L. were supported by predoctoral fellowships from the I.M.I.M. and Ministerio de Educación, respectively.

REFERENCES

1 Gumbiner, B. and Simons, K. (1986) J. Cell Biol. **102**, 457–468
2 Takeichi, M. (1990) Annu. Rev. Biochem. **59**, 237–252
3 Ozawa, M., Baribault, H. and Kemler, R. (1989) EMBO J. **8**, 1711–1717
4 Kemler, R. (1993) Trends Genet. **9**, 317–321
5 Reynolds, A. B., Daniel, J., McCrea, P. D., Wheelock, W., Wu, J. and Zhang, Z. (1994) Mol. Cell. Biol. **14**, 8333–8342
6 Lesuffleur, T., Barbat, A., Dussaulx, E. and Zweibaum, A. (1990) Cancer Res. **58**, 6334–6443
7 Hafez, M. M., Infante, D., Winawer, S. and Friedman, E. (1990) Cell Growth Differ. **1**, 617–626
8 Lesuffleur, T., Barbat, A., Luccioni, C., Beaumatin, J., Clair, M., Kornowski, A., Dussaulx, E., Dutrillaux, B. and Zweibaum, A. (1991). J. Cell Biol. **115**, 1409–1418
9 Fabre, M. and García de Herreros, A. (1993) J. Cell Sci. **106**, 513–522
10 Nagafuchi, A., Ishihara, S. and Tsukita, S. (1994) J. Cell Biol. **127**, 235–245
11 Hinck, L., Nathke, I. S., Papkoff, J. and Nelson, W. J. (1994) J. Cell Biol. **125**, 1327–1340
12 Rosen, N., Bolen, J. B., Scwartz, A. S., Cohen, P., DeSeau, V. and Israel, M. (1986) J. Biol. Chem. **261**, 13754–13759
13 Kinch, M. S., Clark, G. J., Der, C. J. and Burridge, K. (1995) J. Cell Biol. **130**, 461–471
14 Shore, E. and Nelson, W. J. (1991) J. Biol. Chem. **266**, 19672–19680
15 Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Miyazawa, K., Kitamura, N., Johnson, K. R., Wheelock, M. J., Matsuyoshi, N., Takeichi, M. and Ito, F. (1995) J. Cell Biol. **128**, 949–957
16 Behrens, J., Vakaet, L., Winterhage, E., Van Roy, F., Mareel, M. M. and Birchmeier, W. (1993) J. Cell Biol. **120**, 757–766

17 Uehara, Y., Murukami, Y., Sugimoto, Y. and Mizuno, S. (1989) Cancer Res. **49**, 780–785
18 Garcia, R., Parikh, N. V., Saya, H. and Gallick, G. E. (1991) Oncogene **6**, 1983–1989
19 Reynolds, A. B., Roesel, D. J., Kanner, S. B. and Parsons, J. T. (1989) Mol. Cell. Biol. **9**, 629–638
20 Reynolds, A. B., Herbert, L., Cleveland, J. L., Berg, S. T. and Gaut, J. R. (1992) Oncogene **7**, 2439–2445
21 Peifer, M., Berg, S. and Reynolds, A. B. (1994) Cell **76**, 789–791
22 Downing, J. R. and Reynolds, A. B. (1991) Oncogene **6**, 607–613
23 Lee, H., Hsu, S., Winawer, S. and Friedman, E. (1993) J. Biol. Chem. **268**, 8181–8187
24 Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. (1993) EMBO J. **12**, 307–314
25 García de Herreros, A., Fabre, M., Batlle, E., Balagué, C. and Real, F. X. (1993) J. Cell Sci. **105**, 1165–1172
26 Herrera, R., Lebwohl, D., García de Herreros, A., Kallen, R. G. and Rosen, O. M. (1988) J. Biol. Chem. **263**, 5560–5568

Received 8 November 1995/28 February 1996; accepted 13 March 1996