Biochemical Journal (2016) **473** 2893–2909
DOI: 10.1042/BCJ20160198

PORTLAND PRESS

Research Article

Isoform-specific function of calpains in cell adhesion disruption: studies in postlactational mammary gland and breast cancer

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Cleavage of adhesion proteins is the first step for physiological clearance of undesired cells during postlactational regression of the mammary gland, but also for cell migration in pathological states such as breast cancer. The intracellular Ca^{2+}-dependent proteases, calpains (CAPNs), are known to cleave adhesion proteins. The isoform-specific function of CAPN1 and CAPN2 was explored and compared in two models of cell adhesion disruption: mice mammary gland during weaning-induced involution and breast cancer cell lines according to tumor subtype classification. In both models, E-cadherin, β-catenin, p-120, and talin-1 were cleaved as assessed by western blot analysis. Both CAPNs were able to cleave adhesion proteins from lactating mammary gland *in vitro*. Nevertheless, CAPN2 was the only isoform found to co-localize with E-cadherin in cell junctions at the peak of lactation. CAPN2/E-cadherin *in vivo* interaction, analyzed by proximity ligation assay, was dramatically increased during involution. Calpain inhibitor administration prevented the cytosolic accumulation of truncated E-cadherin cleaved by CAPN2. Conversely, in breast cancer cells, CAPN2 was restricted to the nuclear compartment. The isoform-specific expression of CAPNs and CAPN activity was dependent on the breast cancer subtype. However, CAPN1 and CAPN2 knockdown cells showed that cleavage of adhesion proteins and cell migration was mediated by CAPN1, independently of the breast cancer cell line used. Data presented here suggest that the subcellular distribution of CAPN1 and CAPN2 is a major issue in target-substrate recognition; therefore, it determines the isoform-specific role of CAPNs during disruption of cell adhesion in either a physiological or a pathological context.

Introduction

During each pregnancy/lactation cycle, the mammary gland suffers a process of regression known as involution, where epithelial cell death is coupled to tissue remodeling [1,2]. In multicellular organisms, the different ways of apoptotic and nonapoptotic cell death provide important mechanisms to assure removal of undesired defective cells or tissue remodeling during growth and development. Cell detachment from extracellular matrix (ECM) is a key event triggering cell death during both development and postlactational regression of mammary gland. Importantly, disruption of cell adhesion is the initiating step not only for apoptotic and nonapoptotic cell removal [2–4], but also for cell migration and epithelial-to-mesenchymal transition during breast tumor progression [4–6].

The prominent role of calpains in the proteolytic processing of adhesion proteins to promote cell migration and metastasis has been extensively documented [7–13]. Calpains (EC 3.4.22.18; clan CA; family C2) are neutral Ca^{2+}-dependent cysteine proteases involved in the limited processing of

Received: 8 March 2016
Revised: 24 June 2016
Accepted: 11 July 2016

Accepted Manuscript online: 11 July 2016
Version of Record published: 12 September 2016

Biochemical Journal (2016) **473** 2893–2909
DOI: 10.1042/BCJ20160198

proteins. Among the calpain family members, calpain-1 (CAPN1) and calpain-2 (CAPN2) are the most ubiquitously expressed isoforms. Both calpains are heterodimers, composed of a different catalytic subunit (80 kDa), and a common regulatory subunit (30 kDa), known as calpain-4 (CAPN4) [14,15]. Although they are highly specific for the recognition of substrates, the basis for this recognition is not completely understood, and redundant functions are usually described for both calpains [14]. However, the pattern of gene expression and subcellular localization of CAPNs is highly dependent on cell context [15–20]. Differences in the localization or activity of both CAPNs have not been studied in breast tumors, and although CAPNs are good targets for cancer therapy, there is no evidence for the role of a particular CAPN isoform. Reports about the isoform-specific function of CAPNs in cell migration use cultured cells that do not resemble the polarized structure of mammary epithelium [8,10,13,17,21].

The mammary gland during the pregnancy/lactation cycle has been used as a unique approach to investigate the early events during breast tumor progression [1,22]. During the early and reversible phase of involution, luminal cells detach from the ECM and are shed into the lumen of alveoli where they are cleared by different mechanisms. In the second irreversible phase of involution, the remaining epithelial cells massively die, mammary tissue is extensively remodeled, and the fat pad is repopulated with adipocytes [1,2].

We recently showed that CAPN localization/functions are subjected to the dynamic changes suffered by mammary epithelia during involution. CAPN1 was found to mediate mitochondrial and lysosomal cell death of mammary epithelia at sequential time points of early involution [18]. Its later nuclear localization in epithelial cells and adipocytes promoted both nuclei destabilization of epithelial cells and adipocyte differentiation during the second stage of involution [17]. These data suggest that although both CAPNs show almost indistinguishable substrate specificity, their differential localization at a subcellular compartment, or cell type might govern the rules for substrate recognition.

Here, we explored the isoform-specific role of CAPNs in a physiological and pathological model of cell detachment. Combining analysis in both models, such as mammary gland involution and cultured breast cancer cells, we obtained evidence supporting the notion that the subcellular distribution of isoform-specific CAPNs is dependent on the biological context. In addition, this distribution seems to condition the targets of CAPN1 or CAPN2 and therefore, their specific functions. We identified CAPN2 as the membrane-associated isoform involved in cell detachment of epithelial cells during the early phase of involution. Conversely, CAPN2 was restricted to the nuclear compartment in breast cancer cells and CAPN1 was found to be the main isoform mediating migration of these cells independently of the cancer subtype.

Experimental

Materials

Primary antibodies against CAPN1 (–COOH terminal domain, rabbit; ab39170), CAPN1 (–NH_2 terminal domain; ab28257), CAPN1 (mouse; ab3589), δ-catenin p-120 (ab11508), and β-Catenin (ab6301) were purchased from Abcam. Other antibodies used were α-Tubulin (sc-5286; Santa Cruz), α-CAPN2 for western blot (3372-100; BioVision), α-CAPN2 for immunofluorescence (2539; Cell signaling), α-Talin-1 (T3287; Sigma-Aldrich), and α-E-Cadherin (610182; BD Biosciences). Recombinant proteins and calpain inhibitors were all from Calbiochem: recombinant CAPN2 (208718), Calpain Inhibitor VI (208745), Calpeptin (03-34-0051), and ALLN (*N*-acetyl-L-leucyl-L-leucyl-norleucinal; 208719), except recombinant CAPN1 (C-6108) that was obtained from Sigma.

Animals and tissue extraction

C57BL/6 mice were obtained from Taconic (Ejby, Denmark). Virgin female (10 weeks old) were mated and males were subsequently removed at mid-gestation. Following parturition, litters were maintained with at least seven pups. Then, at the peak of lactation (days 9–11), mice were divided into different groups: control lactating mice and weaned mice whose pups were removed 10 days after delivery to initiate involution. Weaning took place for 6, 24, 48, and 72 h before sacrifice.

Calpeptin inhibitor (i.p. 40 mg/kg) was administered to 10-day lactating mice right after weaning of the pups. Mice received calpeptin or vehicle every 12 h during 3 days. Mice were killed by a single dose of sodium pentobarbital (i.p. 60 mg/kg). Inguinal mammary glands were removed and quickly freeze-clamped in liquid nitrogen or fixed in formaldehyde for histological studies. All the animals were given food and water *ad libitum* and housed in a controlled environment (12 h light/12 h dark cycle). Experimental protocols were approved by

the Research Committee of the School of Medicine (University of Valencia, Valencia, Spain). Mice were cared for and handled attending to the National Institutes of Health guidelines and the Guiding Principles for Research Involving Animals and Humans approved by the Council of the American Physiological Society.

Cell culture

Human breast cancer cell lines were purchased from American Type Culture Collection. Cell lines from luminal, basal, or the recently classified claudin-low subtypes were used in the present study as representative models of breast cancer heterogeneity [23]. Two different luminal cell lines were chosen according to their human epidermal growth factor receptor2 (HER2) status: MCF-7 (estrogen receptor (ER)+; progesterone receptor (PgR)+; HER2−) and BT474 (ER+; PgR+; HER2+). Two triple-negative cell lines, either basal or claudin-low, were also studied: MDA-MB468 (ER−; PgR−; HER2−) and MDA-MB-231 (ER−, PgR−, HER2−).

Cell lines were maintained in DMEM (Gibco) supplemented with 10% FBS, 1% penicillin/streptomycin (K952, Amresco), and L-glutamine (G7513, Sigma). Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2.

CAPN knockdown by small interfering RNA

Cells were transiently transfected with 30 nM *Capn1*small interfering RNA (esiRNA) (EHU032581-50UG), CAPN2 (EHU025391-50UG), or Universal Negative Control #1 siRNA (SIC001), all of them purchased from Sigma. MDA-MD-231 and MDA-MB-468 were transfected following the forward transfection protocol and using Lipofectamine 3000 (L3000008, Life Technologies) as Transfection reagent, whereas MCF-7 and BT-474 were reverse-transfected with Lipofectamine RNAiMAX (13778075, Life Technologies). The transfection reaction was carried out for 24 h. Dilutions of esiRNA and Lipofectamine were performed in Opti-MEM following the manufacturer's instructions. esiRNA transfection efficiency was analyzed by real-time quantitative PCR (RT-qPCR) at 48 and 72 h after transfection and by western blot at 72 h after transfection.

Wound-healing assay

MB-231 cells (16×10^4) were plated in six-well plates, cultured under standard conditions for further 24 h, and transfected as described above. MCF-7 cells (6×10^5) were simultaneously plated and reverse-transfected in six-well plates. Twenty-four hours after transfection, both cell lines were starved and 24 h later, confluent monolayers were scratched with a 1 ml pipette tip to induce a wound. The wounded edges were imaged using an inverted microscope Nikon Eclipse Ti (×10 magnification). Images were collected at 48 h (MCF-7) or 28 h (MB-231) after scratch. The areas of five representative wounds for each condition were analyzed and quantified as the percentage of cells that migrated to the wound area after scratch.

Transwell migration assay

Cell migration was analyzed by transwell assay, using 'Fluorimetric QCM 24-well (8 μm) Chemotaxis Cell Migration Assay' (ECM509, Millipore) and following the manufacturer's instructions. In brief, MCF-7 and MB-231 cells were transfected as described for wound-healing experiments. Before starting transwell assays, transfected cells were cultured for 48 h under standard conditions and then starved for 24 h. Cells were suspended in serum-free medium (DMEM 5% BSA) and placed in the upper chambers of Transwell plates (12×10^4 cells/well). DMEM 10% FBS was used as a chemoattractant in the lower chambers. Cell migration was measured by fluorescence using the CyQUANT® GR Dye after 8 and 24 h incubation for MCF-7 and MB-231, respectively.

Immunofluorescence analysis

For immunohistochemistry, 5 μm sections from control and involuting mammary gland were analyzed as previously described [18]. Sections were incubated overnight at 4°C with primary antibodies (CAPN1, CAPN2, or E-cadherin). Secondary fluorescent antibodies Alexa Fluor 488 anti-rabbit IgG (A11008, Thermo Fisher) or Cy3 anti-mouse IgG (C2181, Sigma-Aldrich) were used for detection.

Breast cancer cells were cultured onto 13 mm Ø borosilicate coverglass (VWR 631-0149) and immunostained as described. In brief, cells were incubated with CAPN1 (ab3589, Abcam) or CAPN2 (#2539, Cell Signaling) overnight at 4°C. Cy3 anti-mouse IgG (CAPN1) or Alexa Fluor 488 anti-rabbit IgG (CAPN2) secondary antibodies were used for detection. Nuclei were counterstained with Hoechst 33342 (Invitrogen). Pictures were acquired on an Leica TCS-SP 2 confocal microscope.

Biochemical Journal (2016) **473** 2893–2909
DOI: 10.1042/BCJ20160198

Duolink proximity ligation assay

In situ CAPN1/E-cadherin or CAPN2/E-cadherin interaction in mammary gland was detected using the Duolink® *In Situ* based on proximity ligation assay (PLA) from Sigma-Aldrich. Mammary gland tissue sections from lactating (0 h) and weaning mice (24 and 72 h involution) were fixed and permeabilized in antigen-blocking solution as described previously [24]. The calpain-mediated cleavage of E-cadherin was also analyzed in 72 h weaning samples from mice treated with calpeptin or vehicle (DMSO). Following permeabilization, tissue sections were incubated with CAPN1 or CAPN2 and E-cadherin-specific antibodies (each from different host species). PLA probe ligation and amplification were performed following the manufacturer's instructions. Finally, samples were covered with Duolink *In Situ* Mounting Medium with DAPI (for nuclei staining) and analyzed in a confocal microscope Leica TCS-SP 2.

Protein extraction and immunoblotting

Total protein was extracted in the presence of protease inhibitors as described recently [17]. Equal amounts of protein (15 μg for mammary gland extracts and 20 μg for breast cancer extracts) were separated, subjected to SDS–PAGE, and electroblotted onto nitrocellulose membranes (Protran®, Whatman). The specific proteins were detected using the indicated primary antibodies and HRP-conjugated secondary antibody (DAKO). Blots were developed by enhanced chemiluminescence reaction (ECL Detection Kit, GE Healthcare, Uppsala, Sweden). Equal loading was confirmed by reprobing the blot against α-tubulin or β-actin and by Ponceau Red staining.

Calpain activity

CAPN activity was measured as described previously using a calpain activity assay kit (QIA-120, Calbiochem, Billerica, MA, USA) and according to the manufacturer's instructions [17,18]. In brief, total protein extracts from human breast cancer cell lines were solubilized in cell lysis buffer (CytoBuster™ Protein Extraction Reagent). Samples (in the presence or absence of inhibition buffer containing BAPTA) and standards were then incubated during 15 min in a 96-well plate with activation buffer (containing Ca^{2+} and TCEP reducing agent) and the substrate (Suc Leu-Leu-Val-Tyr-AMC) provided in the kit. Fluorescence was measured using a fluorescence plate reader at an excitation wavelength of ∼360–380 nm and an emission wavelength of ∼440–460 nm. CAPN activity was determined as the difference between the activity obtained using the CAPN-inhibition buffer (BAPTA) and that detected with the activation buffer.

RT-qPCR analysis

Total RNA from breast cancer cell lines was extracted by TRIzol® (Invitrogen) followed by additional column purification (RNeasy, Qiagen, Hilden, Germany). RNA quantity and purity were determined using the NanoDrop ND-2000 (NanoDrop Technologies). RNA (1 μg) was reverse-transcribed to cDNA using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, California, USA). cDNA products were amplified by qPCR using the GeneAmp Fast PCR Master Mix (Applied Biosystems). All reactions were carried out in triplicate. RT-qPCR was run in the 7900HT Fast Real-Time PCR System. Pre-developed TaqMan primers specific for *Capn1*, *Capn2*, and 18S were purchased from Applied Biosystems. Results were normalized according to 18S quantification in the same sample reaction. The threshold cycle (C_t) was determined, and the relative gene expression was expressed as follows:

$$\text{Relative amount} = 2^{-\Delta(\Delta C_t)},$$

where $C_t = C_t(\text{target}) - C_t(18S)$ and $\Delta(\Delta C_t) = \Delta C_t$ (breast cancer cell line) $- \Delta C_t$(MCF-7).

Statistics

Statistical significance was estimated with one-sample Student's *t*-test. Experiments described in Figure 5, a one-way ANOVA was used for statistical analysis. Significant differences were determined by a Tukey–Kramer test. Different superscript letters indicate significant differences. The letter 'a' always represents the lowest value within the group. Differences were considered significant at least at $P < 0.05$. Independent experiments were conducted with a minimum of three replicates per condition to allow for statistical comparison.

Biochemical Journal (2016) **473** 2893–2909
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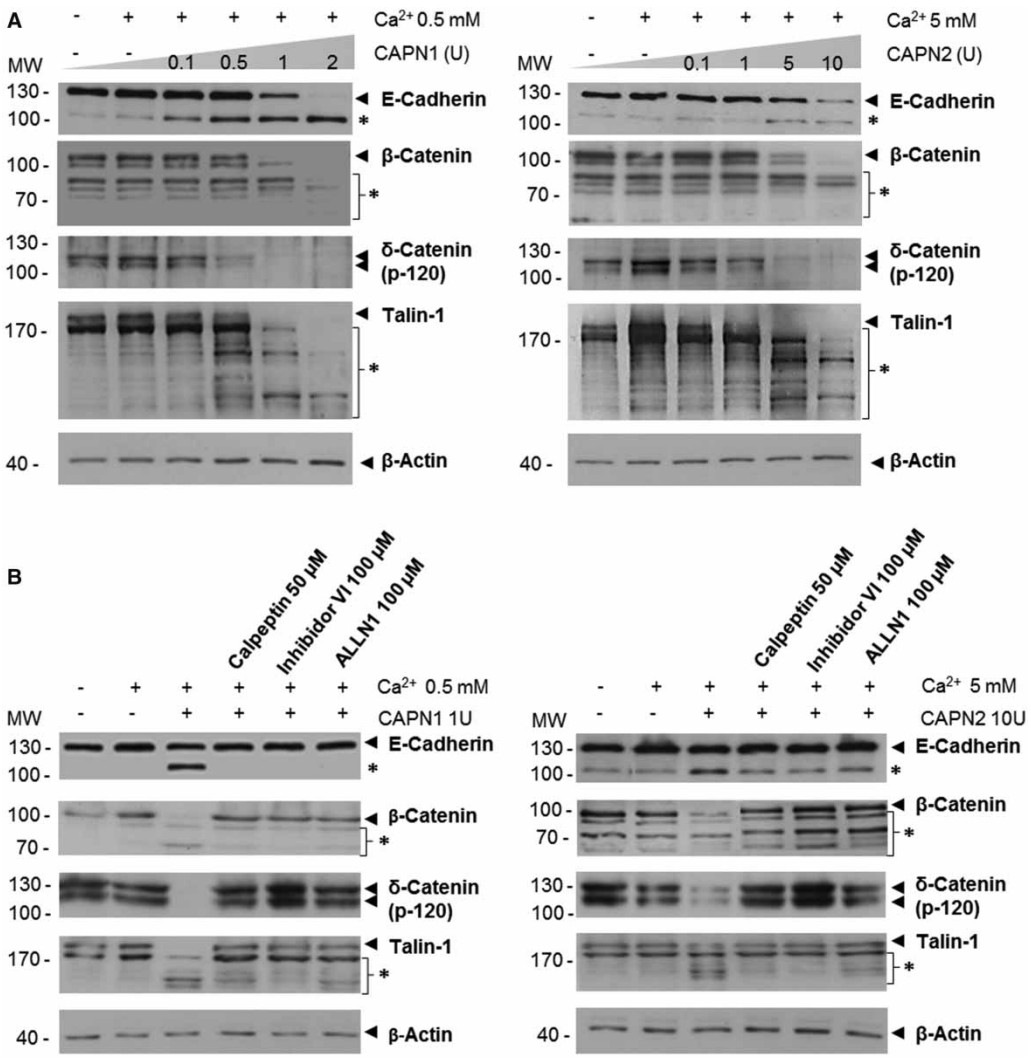

Figure 1. Adhesion proteins from lactating mammary gland as targets of CAPN1 and CAPN2 *in vitro*.
(**A**) Calpain-mediated cleavage of E-cadherin, β-catenin, δ-catenin (p-120), and talin-1 was analyzed by western blot in protein extracts from lactating mammary gland. Protein samples were incubated with increasing concentrations of either recombinant CAPN1 (left panel) or CAPN2 (right panel) in the presence of calcium. (**B**) Cleavage of adhesion proteins by recombinant CAPN1 (left) and CAPN2 (right) in the presence of specific protease inhibitors, calpeptin, calpain inhibitor VI, and ALLN, was analyzed by western blot in proteins extracts from lactating mammary gland. Protein cleavage was analyzed by either detection of truncated fragments (asterisks) or disappearance of full proteins (arrow heads). Equal loading was assessed by the analysis of β-actin expression. Representative blots are shown ($n \geq 3$). Predicted cleavage sites are shown in Supplementary Figure S1.

Results

Adhesion proteins as physiological targets of CAPN1 and CAPN2

A cell-free assay with recombinant CAPNs and extracts from lactating mammary acini was conducted to understand which of these ubiquitously expressed proteases, CAPN1 or CAPN2, is involved in cell detachment of physiological mammary epithelia *in vivo*. Proteolysis of E-cadherin, catenin p-120 and β-catenin was analyzed by western blot as a measure of adherens junction disruption, whereas loss of focal adhesion was analyzed by means of talin cleavage. These *in vitro* studies clearly showed that both focal and adherens junctions from lactating samples were cleaved when extracts were incubated with increasing concentrations of recombinant CAPN1 or CAPN2 in the presence of calcium (Figure 1A). In the absence of CAPN and/or Ca^{2+}, no

Biochemical Journal (2016) 473 2893–2909
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cleavage was observed. Furthermore, calpain inhibitors blocked cleavage of adhesion proteins induced by recombinant CAPNs (Figure 1B), demonstrating that both CAPNs are able to specifically recognize and cleave adhesion proteins from mammary gland epithelia.

Subcellular distribution of specific CAPN isoforms in mammary epithelial cells

The context-dependent subcellular distribution of a CAPN isoform might specify its target-substrate to undertake a given biological function. Consequently, the subcellular distribution of both CAPNs was analyzed in lactating mammary gland tissue sections and breast cancer cells. To detect possible differences among breast cancer subtypes according to their HER2 status and basal/luminal origin, a cell line from each subtype was used.

As shown in Figure 2A, CAPN1 immunostaining was diffusely detected in epithelial cells from lactating mammary tissue. CAPN2 was mainly localized at the cell-to-cell adhesion interface of basal cells. CAPN1 resulted distributed in the cytosol and nuclei of breast cancer cells (Figure 2B). CAPN2, barely detected in the cytosolic compartment, was found to be mainly localized in nuclei of all the cell lines tested. Importantly, no membrane-associated CAPN2 was identified in breast cancer cells. Although apparently expressing different CAPN levels, no difference in CAPN distribution was observed among the cell lines.

These data suggest that although the breast cancer subtype does not condition the subcellular localization of these CAPNs, CAPN2 subcellular distribution in mammary epithelial cells is dependent on cell context.

Isoform-specific colocalization of CAPN with E-cadherin during mammary gland involution

CAPN2 seemed to have a polarized basal distribution in mammary acini at the peak of lactation. Nevertheless, epithelial cells are shed into the lumen only during the time course of involution. To explore the role of CAPN2 on cell adhesion in a physiological model of cell–cell disruption, tissue sections from postlactation mammary gland were immunostained with antibodies against CAPN1 or CAPN2 and E-cadherin and their subcellular distribution was analyzed.

Co-localization of CAPN2 and E-cadherin was detected at the peak of lactation and during the time course of involution (Figure 3A). As described previously [25] at 72 h involution, E-cadherin seemed to be re-localized to the cytoplasmic fraction of epithelial cells. Interestingly, we did not observe an increased co-localization of CAPN2/E-cadherin during involution but instead, a clear redistribution of CAPN2 to the apical side of luminal cells (24 h) and thereafter to the cytosolic compartment (72 h), co-localizing at both time points with E-cadherin. On the other hand, no redistribution of CAPN1 was observed at lactation or 72 h involution (Figure 3B).

These results point out to CAPN2 as the most probable CAPN involved in cell detachment in the physiological model of mammary gland involution.

Cleavage of adhesion proteins in physiological and tumoral models of cell adhesion disruption

To examine the functional role of CAPN2 under physiological conditions, cleavage of adherens and focal adhesion proteins was studied by western blot in extracts from mammary gland during the time course of involution. In agreement with immunohistochemical data, E-cadherin was disrupted during the first phase of involution (Figure 4A). Indeed, although starting at 6 h after weaning, the proteolytic processing of E-cadherin was not completed until the next 48–72 h of involution. The same proteolytic pattern was observed for other proteins from the cell–cell interaction (catenin p-120 and β-catenin) or focal adhesion complexes (talin-1). Our *in vivo* data strongly support a correlation between the proteolytic processing of adhesion proteins and the expression and membrane localization of CAPN2 in mammary gland after weaning [17,18].

Breast cancer cell lines in agreement with their metastatic potential both adherens junctions and focal adhesion proteins were cleaved in luminal cells (Figure 4B). As described recently, triple-negative MB-231 and MB-468 cells do not establish adherens junctions [23,26] and consequently, in triple-negative cell lines only talin-1, a focal adhesion protein, was cleaved.

Biochemical Journal (2016) **473** 2893–2909
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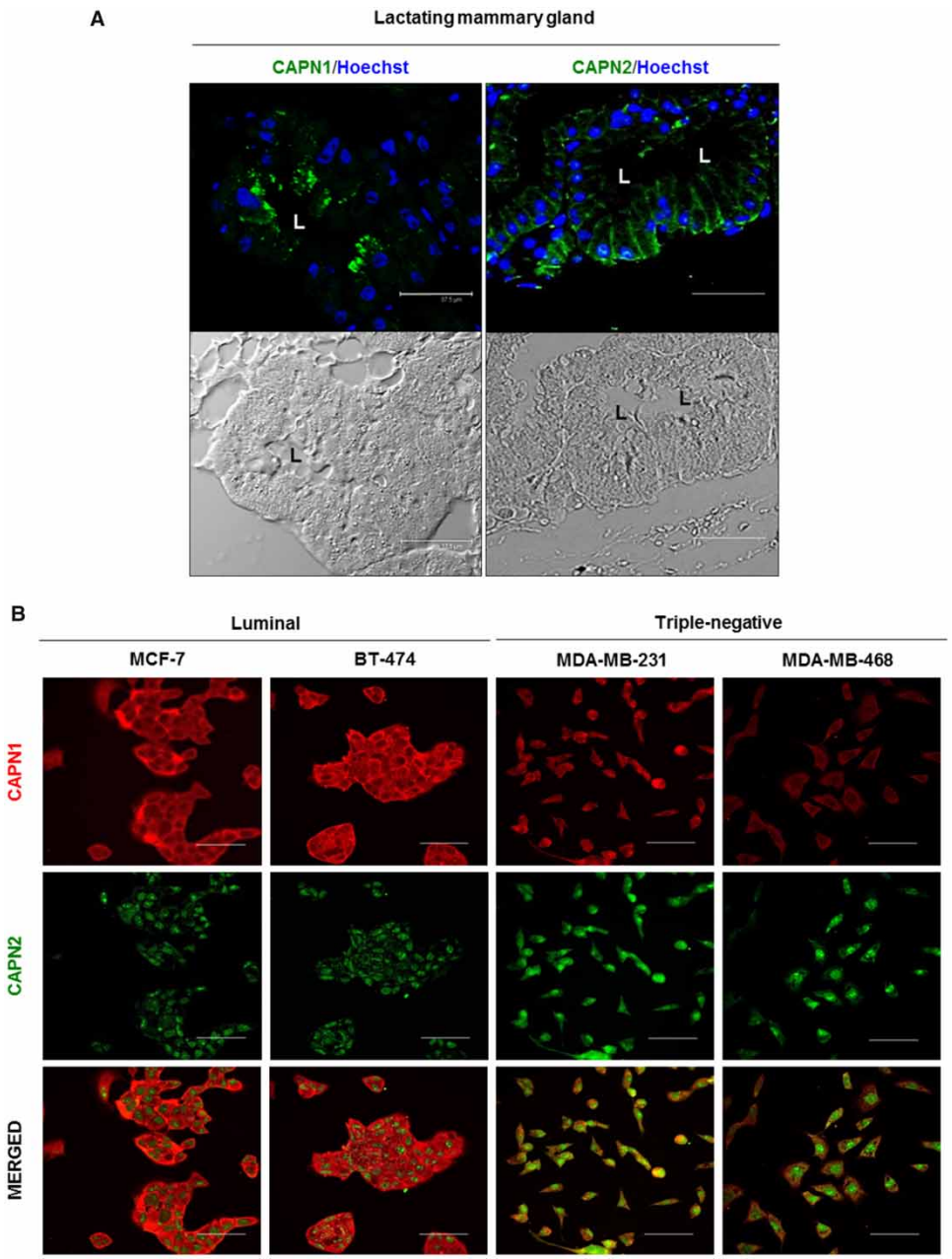

Figure 2. Subcellular distribution of CAPN1 and CAPN2 in lactating mammary gland and breast cancer cell lines.
(**A**) Representative immunofluorescence staining of CAPN1 and CAPN2 (green) in tissue sections from mice mammary gland at the peak of lactation. Cell nuclei were counterstained by Hoechst 33342 (blue). Bright field images were included simultaneously to visualize the exactly localization of immunofluorescence in mammary gland structure. L, lumen. Scale bars, 37.5 μm. (**B**) Immunostaining of CAPN1 (red) and CAPN2 (green) in MCF-7, BT-474, MDA-MB-231, and MDA-MB-468 breast cancer cell lines. Scale bars, 70 μm. Representative images are shown ($n \geq 3$).

Identification of the CAPN isoform catalytically active in different subtypes of breast cancer cells

To establish a putative correlation between a specific CAPN isoform and the proteolytic processing of adhesion proteins in breast cancer cells, an exhaustive analysis of CAPN expression and enzymatic activity was performed in all cell lines.

Biochemical Journal (2016) **473** 2893–2909
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Expression of CAPN1 and CAPN2 was measured by RT-qPCR and western blot in cell lines cultured under standard conditions. As shown in Figure 5A,B, CAPN1 and CAPN2 were differentially expressed in the four cell lines according to either HER2 status or the triple-negative subtype. MCF-7 expressed higher CAPN1 mRNA and protein levels than the other cell lines studied. On the other hand, CAPN2 expression was much higher in triple-negative than in luminal cells (Figure 5A,B). Consequently, CAPN1/CAPN2 ratio was dramatically higher in luminal than in triple-negative cells (Figure 5B, right panel).

However, higher levels of CAPN expression do not necessarily mean higher enzymatic activity. We analyzed enzymatic activity in the different cell lines by molecular analysis of well-known mechanisms thought to overcome Ca^{2+} requirements and to facilitate calpain activation [27,28]: (i) autolysis of the anchor helix of CAPN1, but not of CAPN2; (ii) cleavage and dissociation of the small regulatory subunit CAPN4; and (iii) binding to calpastatin, the endogenous enzymatic inhibitor of both CAPNs.

As seen in Figure 5B, the absence of CAPN1 amino-terminal (Nt) domain analyzed by western blot indicates a complete autolysis of CAPN1 anchor helix in luminal cells indicating its activation. CAPN4 protein levels were equally abundant in all the cell lines tested. Calpastatin protein levels resulted to be significantly higher in triple-negative cells than in luminal cells. Moreover, calpastatin was not even detected in the more aggressive luminal phenotype HER2 cells (BT-474).

These results suggest that luminal cells show not only higher CAPN1/CAPN2 expression ratio, but also increased CAPN1 activity when compared with triple-negative cells. Calpain activity analyzed by enzymatic assay further confirmed the higher calpain activity in luminal than in triple-negative cell lines (Figure 5C), a pattern that strongly resembled CAPN1 expression.

Functional role of isoform-specific CAPNs in breast tumor cells

To dissect the role of each calpain isoform on cell adhesion of breast tumors, CAPN1 or CAPN2 was knocked down in the four cell lines and cleavage of adhesion proteins was studied by western blot (Figure 6A). In addition, CAPN1 and CAPN2 mRNA levels were analyzed by RT-qPCR to confirm the specificity of siRNA knockdown experiments (Supplementary Figure S2)

Transfection of luminal cells with CAPN1 siRNA prevented cleavage of both focal and adherens junction proteins. Although adherens junctions are known to be disrupted in MB-231 and MB-468 cells [26], cleavage of focal adhesion in these triple-negative cell lines was also prevented by CAPN1 knockdown. Conversely, knockdown of CAPN2 showed no apparent effect on adhesion proteins in either luminal or triple-negative cell lines. These results suggest that independently of the tumor subtype, CAPN1 is the preferential calpain involved in the regulation of cell adhesion of breast cancer cell lines.

The biological role of calpains was also investigated in CAPN1 and CAPN2 knocked down breast tumor cell lines. MCF-7 and MB-231 were selected as representative cell lines from both subtypes of breast tumors, and cell migration was analyzed by wound-healing assay. Wound-healing assays (Figure 6B) indicate that CAPN1 down-regulation decreased the migration of MCF-7 or MB-231 cells by 50% compared with control cells. Conversely, cell migration was not affected by CAPN2 knockdown (Supplementary Figure S3A). Transwell migration assays (Supplementary Figure S3B) further confirmed the conclusion that independently of the breast tumor subtype, CAPN1 enhances migration of tumor cells.

Interaction of CAPN2/E-cadherin in physiological mammary gland involution

Once discerned the isoform-specific role of CAPN in breast cancer migration, we sought to elucidate the functional role of CAPN2 in the proteolytic processing of adhesion proteins *in vivo*. To examine whether CAPN2 disrupts membrane localization of E-cadherin in physiological conditions, the interaction between both proteins was analyzed by the sensitive *in situ* Duolink PLA in tissue sections from mammary gland after weaning. As shown in Figure 7A, CAPN2/E-cadherin interaction was barely detected at the peak of lactation, but dramatically increased as the involution progressed (quantification of PLA data shown in Supplementary FigureS4A). Conversely, no CAPN1/E-cadherin interaction was detected (Supplementary Figure S4B).

The diffuse and punctate fluorescence staining in the cytosolic compartment during involution seems to be the product from CAPN2-mediated cleavage of E-cadherin, which was gradually accumulated in the cytoplasm. In PLA assays, a fluorescent signal is generated only when the plus and minus probes attached to each antibody are bound together [24]. This proximity could be achieved when E-cadherin is bound to the active center of CAPN2; therefore, calpain-mediated cleavage of E-cadherin would result in fluorescence increase along the

Biochemical Journal (2016) **473** 2893–2909
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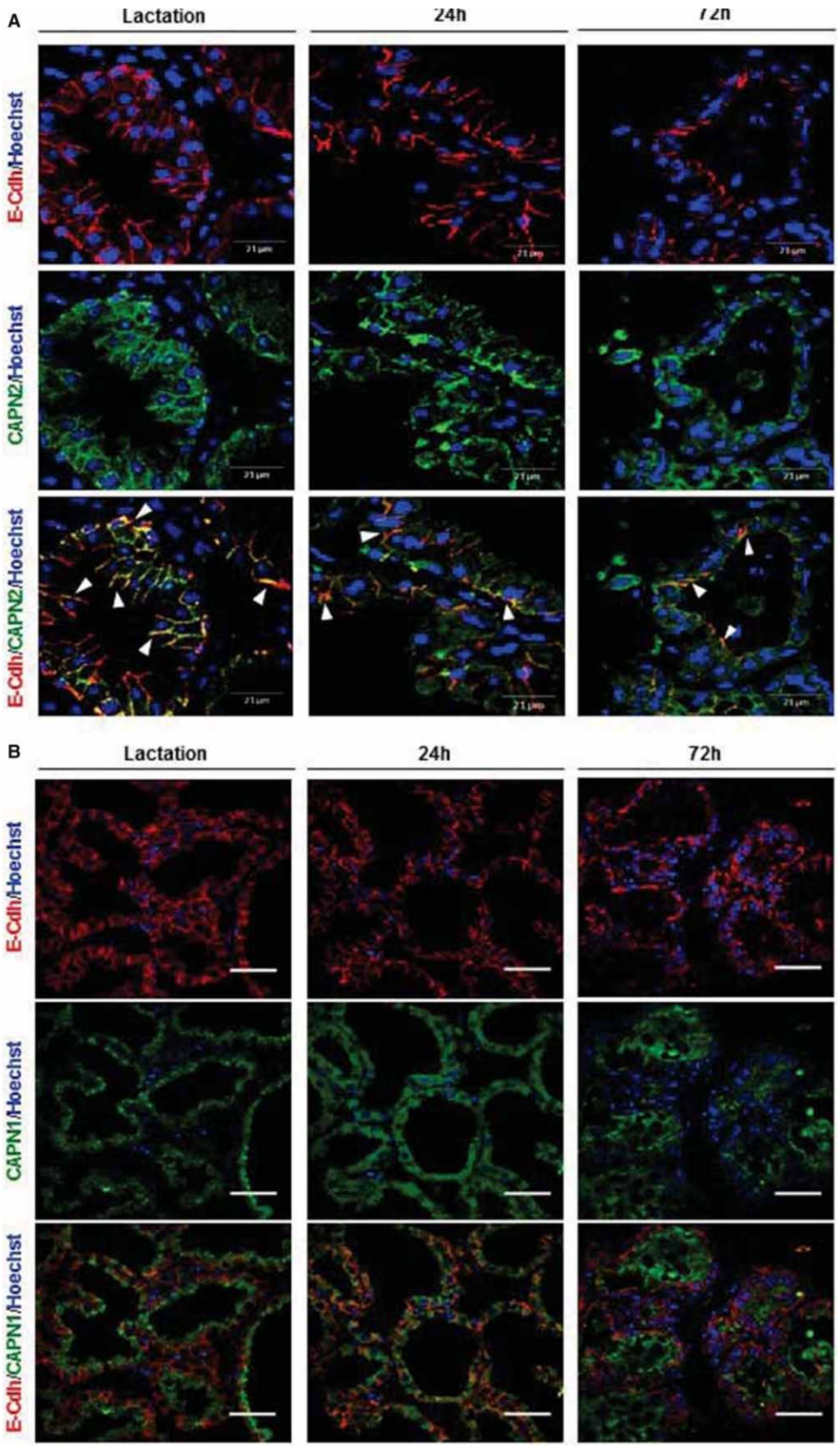

Figure 3. CAPN/E-cadherin co-localization during the time course of mammary gland involution.

(**A**) Tissue sections from mammary gland at the peak of lactation and after 24 and 72 h of involution were immunostained with antibodies against E-cadherin (red) or CAPN2 (green). Nuclei were counterstained with Hoechst 33342 (blue). Arrowheads in the merged images show CAPN2/E-cadherin co-localization (orange). Scale bar, 21 μm. (**B**) The same analysis as above was carried out for E-cadherin (red) and CAPN1 (green). No CAPN1/E-cadherin co-localization was found. Scale bar, 78 μm. Colored words in the artwork define the color staining for each protein.

Biochemical Journal (2016) **473** 2893–2909
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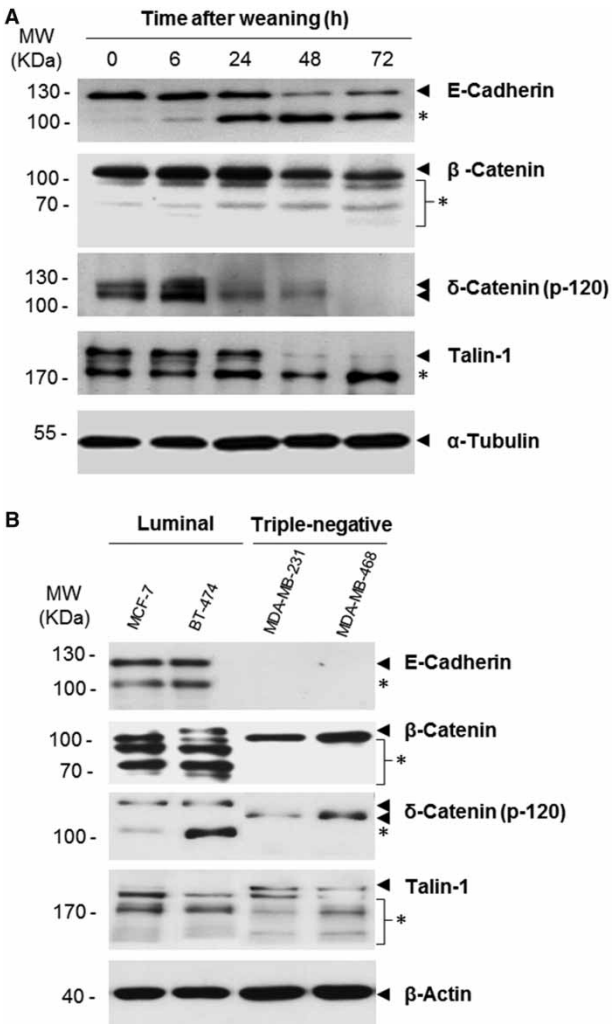

Figure 4. Cleavage of adhesion proteins in physiological and pathological mammary cells.

(**A**) Cleavage of E-cadherin, β-catenin, δ-catenin (p-120), and talin-1 was analyzed by western blot in protein samples from mammary gland at the peak of lactation (0 h), and during the time course of involution (6, 24, 48, and 72 h). α-Tubulin was used as a loading control. (**B**) Cleavage of adhesion proteins was analyzed by western blot in luminal (MCF-7 and BT-474) and triple-negative (MDA-MD-231 and MDA-MD-468) breast cancer cell lines as above. β-Actin was used as a loading control. Protein cleavage was analyzed by either detection of truncated fragments (asterisks) or disappearance of full proteins (arrow heads). Representative immunoblots are shown ($n \geq 3$).

time course of mammary gland involution. In agreement with this, CAPN2/E-cadherin interaction at 72 h after weaning was prevented by calpeptin, the specific competitive inhibitor of calpain (Figure 7B, upper panels and Supplementary Figure S4C). CAPN1/E-cadherin interaction was not detected by PLA assay at 72 h involution, in either DMSO or calpetin-treated mice (Figure 7B, middle panel).

According to the calpain-mediated cleavage of E-cadherin, mislocalization and loss of E-cadherin at cell–cell junctions were observed at 72 h weaning by immunofluorescence, and prevented in calpeptin-treated mice (Figure 7B, lower panels). In addition, proteolysis of other adhesion proteins was also prevented by calpeptin as shown by western blot analysis (Figure 7C).

Altogether these results suggest that although CAPN2 is the critical isoform involved in the disruption of cell adhesion during mammary gland involution, during tumor progression there is a molecular switch from CAPN2 to CAPN1 that is essential for the cleavage of adhesion proteins and cell migration.

Biochemical Journal (2016) **473** 2893–2909
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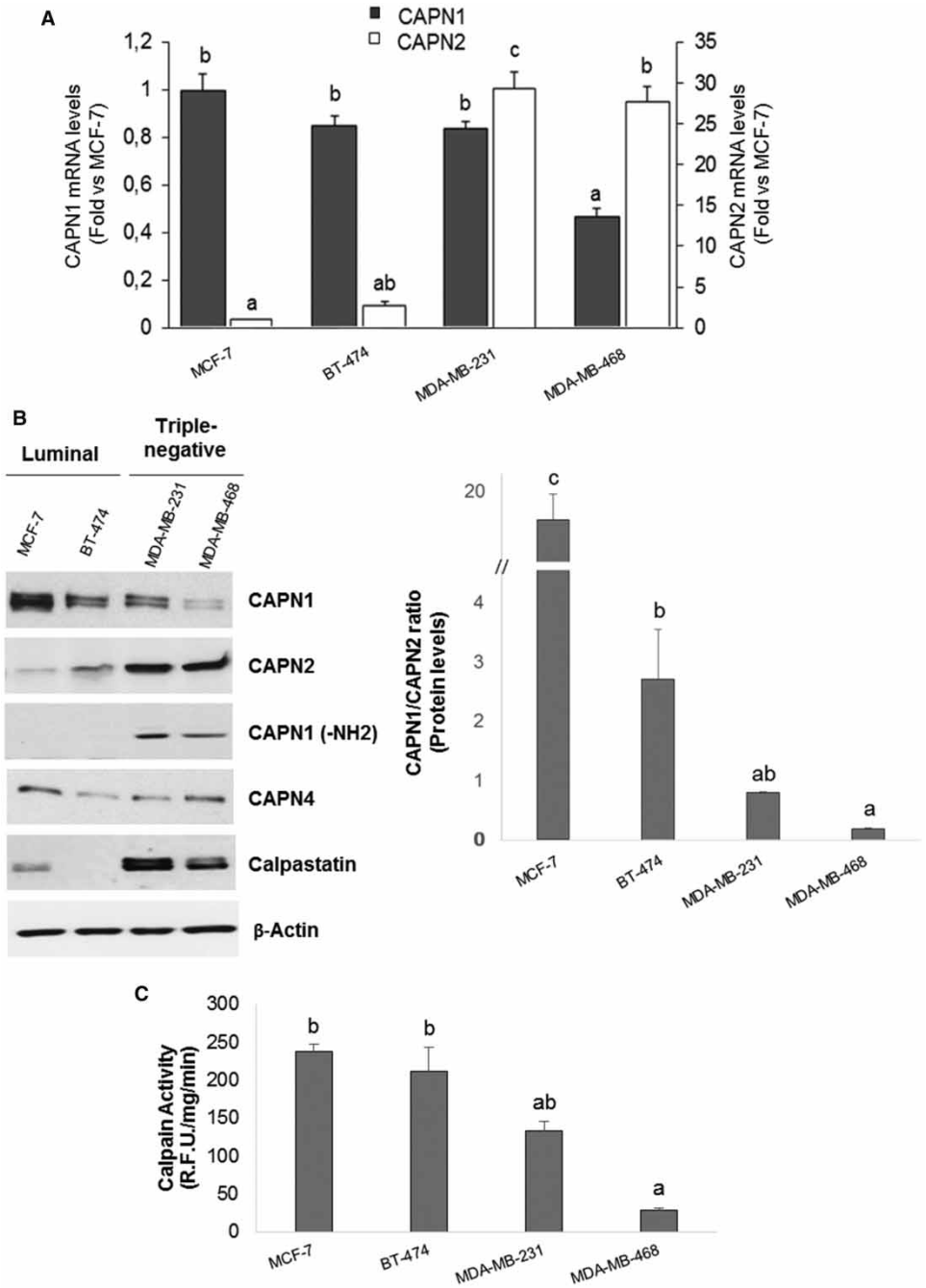

Figure 5. Isoform-specific expression and activity of calpains in breast cancer cells.

(**A**) CAPN1 (black bars) and CAPN2 (white bars) mRNA levels in MCF-7, BT-474, MDA-MB-231, and MDA-MB-468 cell lines were analyzed by quantitative real-time PCR. Data were normalized according to 18S mRNA, analyzed and quantified in the same sample reaction. Expression levels are shown as fold vs. MCF-7. Results ($n \geq 4$) are means ± SEM. (**B**) CAPN1, CAPN2, Nt-CAPN1, CAPN4, and calpastatin total protein levels from breast cancer cell lines were analyzed by western blot (left panel). β-Actin was used as a loading control. Expression of CAPN1 and CAPN2 was quantified and plotted as the ratio of CAPN1/CAPN2. Data are represented as means ± SEM ($n \geq 3$) (right panel). (**C**) Calpain enzymatic activity in breast cancer cell lines was measured by a fluorogenic assay where Suc-LLY-AFC was used as a calpain substrate. Results are shown as mean ± SEM ($n = 3$). ANOVA was performed for the statistical analysis, where different superscript letters indicate significant differences, $P < 0.05$; the letter 'a' always represents the lowest value within the group.

Biochemical Journal (2016) **473** 2893–2909
DOI: 10.1042/BCJ20160198

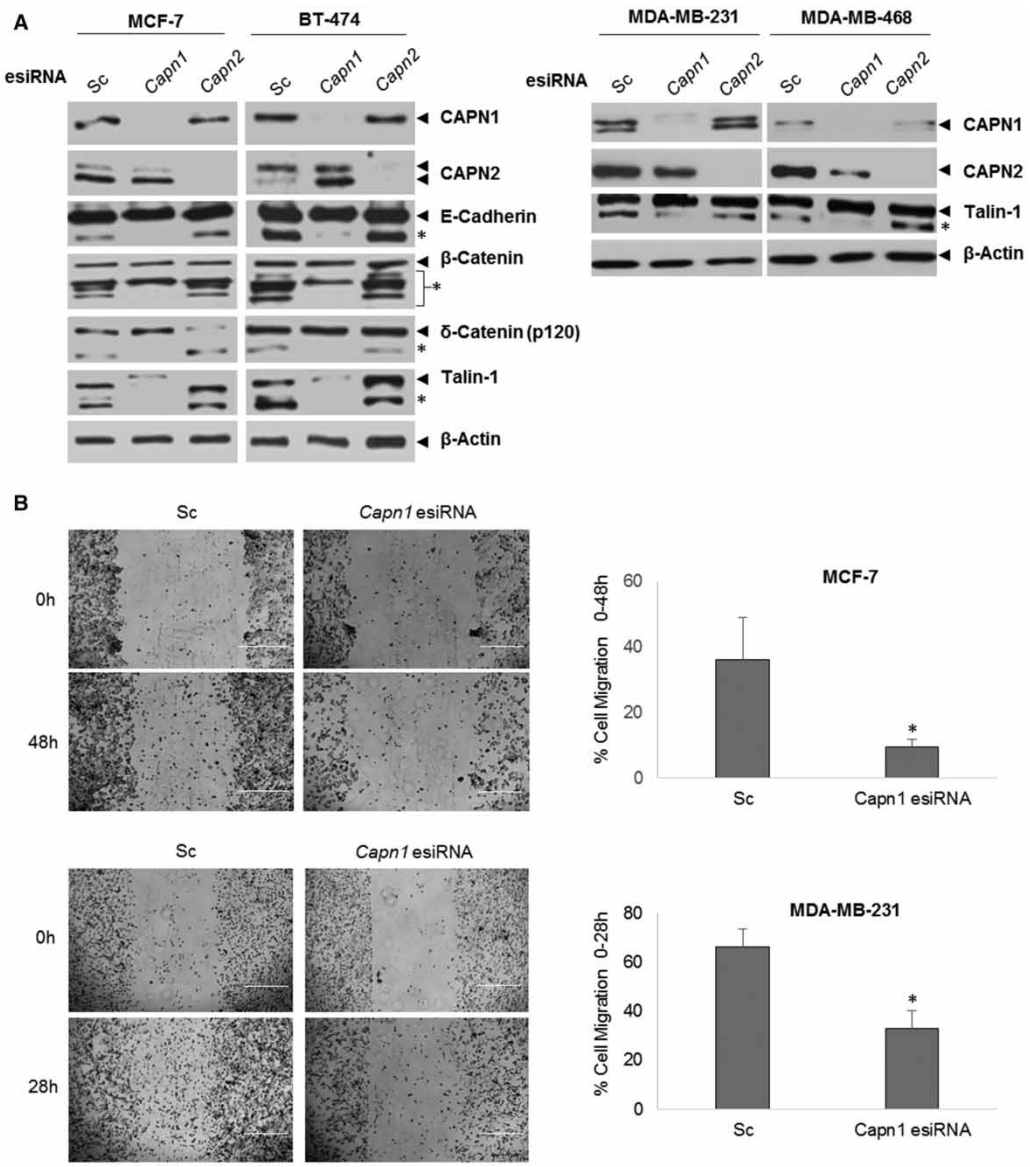

Figure 6. Functional role of CAPN1 and CAPN2 on disruption of adhesion proteins and migration of breast cancer cells.
(**A**) Breast cancer cell lines were transfected with Scramble (Sc), Capn1 or Capn2 siRNAs, and cleavage of E-cadherin, β-catenin, δ-catenin (p-120), and talin-1 in knockdown cells was analyzed by western blot. CAPN1 and CAPN2 protein levels were analyzed to confirm the efficiency of knockdown experiments. Protein cleavage was analyzed by either detection of truncated fragments (asterisks) or disappearance of full proteins (arrow heads). (**B**) Cell migration in Capn1-knockdown cells (MCF-7 and MDA-MB-231) was analyzed by wound-healing assay (left panels). Graphs show the percentage of cells that migrated to the wound area after 48 h (MCF-7) or 28 h (MDA-MB-231). Data ($n \geq 3$) are means ± SEM. Student's *t*-test was performed for the statistical analysis, $^*P < 0.01$ vs. scramble (Sc). Scale bar, 500 μm.

Discussion

Postlactational regression, or involution, of the mammary gland is one of the best physiological models to study *in vivo* the molecular events leading to the effective clearance of secretory epithelial cells that are no longer needed. Upon cessation of lactation, 80% of the mammary epithelium is removed. Epithelial cell death and tissue remodeling will return the gland to a pre-pregnant state for the next pregnancy/lactation cycle [1,2,17,18,22].

Biochemical Journal (2016) **473** 2893–2909
DOI: 10.1042/BCJ20160198

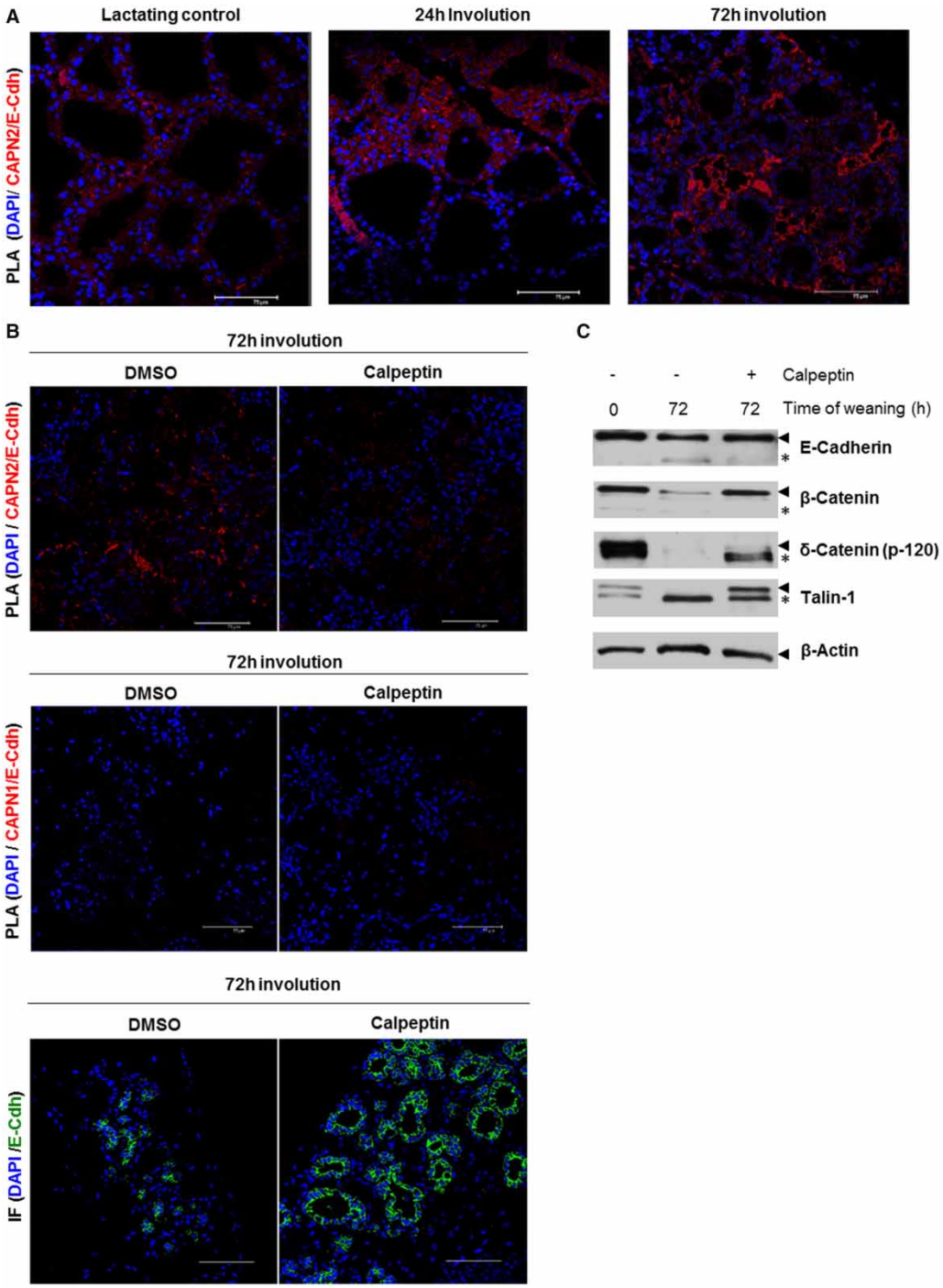

Figure 7. *In vivo* CAPN2-mediated cleavage of E-cadherin during mammary gland involution.

(**A**) CAPN2/E-cadherin *in vivo* interaction (red) was analyzed by PLA in tissue sections from mammary gland during the time course of involution (0, 24, and 72 h). (**B**) CAPN2/E-cadherin interaction (red) analyzed by PLA in tissue sections from DMSO (vehicle) or calpeptin-treated mammary gland at 72 h weaning (upper panel). No interaction was observed for CAPN1/ E-cadherin by PLA (middle panel). Lower panel shows the immunofluorescence staining of E-cadherin in the same experiments (green). Scale bar, 75 μm. Nuclei were stained with DAPI (blue). (**C**) Cleavage of E-cadherin, β-catenin, δ-catenin (p-120), and talin-1 at 0 and 72 h weaning in DMSO or calpeptin-treated mice was assessed by western blot. β-Actin was used as a loading control. Representative immunoblots are shown ($n \geq 3$).

Biochemical Journal (2016) **473** 2893–2909
DOI: 10.1042/BCJ20160198

Cleavage of adhesion-protein complexes plays an important role in the regulation of epithelial homeostasis that encompasses the effective clearance of undesired cells [3,4]. Here, we show the time-dependent cleavage of adhesion proteins during the time course of involution, all of the substrates that can be equally recognized *in vitro* by CAPN1 and CAPN2. Nevertheless, our data suggest that CAPN2 has a prominent role in the proteolytic cleavage of adhesion proteins in weaning-induced mammary gland involution. In agreement with the important role played by calpains in cell adhesion disruption, many reports have linked calpain activity to cleavage of adhesion complexes [7–13,21,29]. We have previously reported the dual role of CAPN1: inducing mitochondrial, lysosomal and nuclear membrane destabilization of epithelial cells and participating in the epigenetic program of adipocyte differentiation during mammary gland involution [17,18]. Now, we propose a role for CAPN2 in epithelial cell detachment during mammary gland involution. We have demonstrated that only CAPN2 co-localizes with E-cadherin at the peak of lactation. Moreover, as can be deduced from the experiments in calpeptin-treated mice analyzed by PLA, E-cadherin was specifically bound to and cleaved by CAPN2 during involution.

In addition to E-cadherin, we observed the proteolytic processing of other adhesion proteins during mammary gland involution. The cleavage of these proteins was also dependent on calpain activity, as demonstrated in calpeptin-treated mice. The cytoplasmic cleavage of E-cadherin, β-catenin, or Talin1 by calpain has been already described in other tissues [9,11,13,17,21]. Although most likely these proteins are also the targets of CAPN2, we did not demonstrate the direct role of this isoform in their cleavage. Therefore, we cannot rule out the possibility of a proteolytic destabilization of the adhesion complex as a consequence of E-cadherin cleavage [9,30], or the indirect effect of another target of CAPN.

It is noteworthy to mention that the fragments of E-cadherin remained bound to CAPN2 and accumulated in the cytosol at 72 h of involution. In this sense, it is known that the fate of cytoplasmic fragments of E-cadherin can be the proteasomal degradation or alternatively, the acquisition of a different biological function related to cell proliferation or cell death through the activation of signaling pathways [4,5,30,31]. In the near future, it would be of interest to explore the possible biological activity of E-cadherin fragments associated with CAPN2 in the cytoplasm of epithelial cells during involution.

Altogether these data suggest that although the expression and activity of both CAPN isoforms is increased during involution [17,18], their subcellular localization and proteolytic targets are not the same. In agreement with this, it has been recently reported that CAPN1 and CAPN2 are recruited to different compartments where they play different functions [19,20]. In addition, the idea of 'an interchangeable role for CAPN1 and CAPN2' in cell adhesion is most probably the result of a very heterogeneous number of experimental models reported in the literature. Most of studies do not identify the specific isoform of CAPN involved in cell detachment or use cultured cells from carcinoma origin or even different cell types as experimental models to be compared. Understanding the deregulation of calpains involved in cell detachment during breast tumor progression requires the exhaustive study of their normal counterparts under physiological conditions. Many studies use as experimental model of 'normal cells' the human cell line MCF10A, a predominantly myoepithelial cell line classified as basal subtype [32,33]. In addition, tissue organization is not an issue to be overlooked when studying the physiological disruption of cell contacts, and cultured cells do not resemble the polarized acinar structure of mammary gland.

Cell death induced by detachment from ECM and cell-to-cell contacts functions as a luminal clearing mechanism during mammary gland involution. But, the proteolytic cleavage of adhesion proteins by many proteases is not only the response to physiological stimuli, but also the pathological consequence of cell transformation. Detachment of cells and luminal filling is a hallmark of breast cancer. Deregulated expression or activity of proteases involved in the proteolytic processing of adhesion proteins is known to facilitate metastasis [4,6]. However, data about the isoform-specific role of CAPNs in tumor progression are confusing [34–37]. While low levels of CAPN expression are associated with poor survival in gastric carcinomas [34], high levels of CAPN expression have been associated with breast cancer [35]. A correlation between CAPN1 expression and poor prognosis or even resistance to trastuzumab treatment in breast cancer has been described, but no correlation has been found with the expression of calpastatin, the endogenous inhibitor of both calpains, or with CAPN2 levels [35,36]. It has been argued that discrepancies among reports are most probably caused by the type of analysis performed in these clinical studies and to the different classification of breast tumor subtypes. Breast cancer has been revealed as a heterogeneous disease and choosing the right cancer cell line for breast cancer research is not a minor point [23,32,33]. For that reason, although we are not unaware of possible interspecies differences, we selected cell lines including the most common luminal HER-negative and -positive

subtypes (MCF-7 and BT-474, respectively) and the more aggressive triple-negative basal and claudin-low subtypes (MB-468 and MB-231, respectively).

Interestingly, although there were important differences in mRNA, protein levels, and enzymatic activity of both CAPN isoforms among cell lines, we could demonstrate in CAPN1 and CAPN2 knockdown experiments that CAPN1 was the only isoform involved in the proteolytic cleavage of adhesion proteins of breast cancer, independently of the breast cancer subtype.

An important question regarding the mechanism driving the specific CAPN isoform to the plasma membrane remains to be answered. One could speculate with several hypotheses such as CAPN binding to other proteins that would act as chaperones, or CAPN posttranslational modifications that would favor the protease direct- or indirect-anchoring to plasma membranes. In this sense, CAPN2 binding to membrane lipids seems to be crucial for its activation [38,39]. In addition, phosphorylation of specific amino acids of CAPN2 by protein kinase (PKA) or extracellular regulated kinase (ERK) has been suggested to prevent or induce its stable binding to phosphoinositides, respectively [38–40]. Moreover, it has been proposed that CAPN2 needs to be localized at the plasma membrane to be activated by stimuli triggering the EGFR pathway. Indeed, CAPN2 activation can be prevented by internalization of EGFR or ERK [40], indicating that the EGFR pathway from the cytosolic compartment cannot reach or affect membrane-bound CAPN2. EGFR pathway is one of the most important pathways known to be triggered during mammary gland involution. In addition, it is also the major deregulated pathway in breast cancer. Whether the mutational state of this pathway could drive the isoform fate of CAPN2/CAPN1 in breast cancer remains to be elucidated. However, in the absence of EGF stimulation, inhibition of ERK activation by PD98059 prevents CAPN2 binding to the plasma membrane [38], suggesting that there is an additional substrate-driven ERK-mediated localization of CAPN2. It is noteworthy to mention that CAPN2 is unequally redistributed to the sites of locomotion in cultured cells forced to migrate [21,41]. In this sense, the ECM from postlactational mammary gland is profoundly remodeled; therefore, the redistribution of CAPN2 during mammary gland involution to the luminal side of cells could be the result of an ECM-driven mechanism. On the other hand, we cannot rule out the possibility that culture conditions might affect the switch of the specific CAPN isoform involved in cell adhesion of breast cancer cells that were grown on plastic plates. In the future, a clinical study analyzing not just CAPN1/CAPN2 expression, but rather the isoform direct interaction with E-cadherin in samples from breast cancer patients, would help to elucidate this important question.

In summary, our data point out to an isoform-specific role of calpains in physiological and pathological conditions. While CAPN2 seems to be the isoform involved in the proteolytic processing of adhesion proteins from epithelial cells during physiological mammary gland involution, adhesion junctions are cleaved by CAPN1 in breast cancer cell lines promoting cell migration. CAPN isoform overexpression and high enzymatic activity, frequently analyzed in breast tumors, are important but not sufficient conditions to suggest their invasive potential in breast cancer cells. Importantly, data presented here suggest that the subcellular distribution of CAPN1 and CAPN2 is a major issue in target-substrate recognition and therefore, identifying the mechanisms to drive the selected CAPN to the proper subcellular compartment will be an important question to be explored in the near future.

Abbreviations

ALLN, *N*-acetyl-L-leucyl-L-leucyl-norleucine; CAPN, calpain; ECM, extracellular matrix; ER, Estrogen Receptor; ERK, Extracellular Regulated Kinase; esiRNA, small interfering RNA; HER2, Human Epidermal Growth Factor Receptor2; Nt or NH2, amino-terminal domain; PgR, progesterone receptor; PKA, protein kinase A; PLA, proximity ligation assay; RT-qPCR, real-time quantitative PCR.

Author Contribution

L.R.-F. and I.F.-V. performed the experiments. S.S.O. transfected breast cancer cells. C.G. was in charge of mice and obtained the tissue samples. R.Z., J.R.V. and E.R.G.-T. contributed to the study design and data interpretation and drafted the initial report. All authors contributed to the final draft of the paper.

Funding

The present study was supported by grants from Ministerio de Ciencia e Innovación including FEDER [BFU2013-46434-P to J.R.V. and R.Z.] and Instituto de Salud Carlos III including FEDER [PI12/02394 to E.R.

Biochemical Journal (2016) **473** 2893–2909
DOI: 10.1042/BCJ20160198

G.-T.] and Consellería de Educación [GVPROMETEO 2014/II-055]. L.R.-F. and S.S.O. are funded by Consellería de Educación [GVPROMETEO 2014/II-055] and Ministerio de Educación, Cultura y Deporte [FPU13/04976].

Acknowledgements

We thank Sonia Priego for acquiring confocal microscopy images, and Elisa Alonso-Yuste for tissue histology and Isabel Escoms Doñate for her technical assistance while working in her master thesis.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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