Biochemical and Biophysical Research Communications 439 (2013) 203–208

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

EpCAM associates with endoplasmic reticulum aminopeptidase 2 (ERAP2) in breast cancer cells

Salah-Eldin Gadalla [a], Karin Öjemalm [b], Patricia Lara Vasquez [b], IngMarie Nilsson [b], Christer Ericsson [a], Jian Zhao [a], Monica Nistér [a,*]

[a] Department of Oncology-Pathology, Karolinska Institutet, Cancer Center Karolinska, R8:04 Karolinska University Hospital, Solna, SE-171 76 Stockholm, Sweden
[b] Department of Biochemistry and Biophysics, Center for Biomembrane Research, Arrhenius Laboratories of Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 13 August 2013
Available online 26 August 2013

Keywords:
ERAP2
EpCAM
Co-localization
Cleavage
Glycosylation

ABSTRACT

Epithelial cell adhesion molecule (EpCAM) is an epithelial and cancer cell "marker" and there is a cumulative and growing evidence of its signaling role. Its importance has been recognized as part of the breast cancer stem cell phenotype, the tumorigenic breast cancer stem cell is EpCAM$^+$. In spite of its complex functions in normal cell development and cancer, relatively little is known about EpCAM-interacting proteins. We used breast cancer cell lines and performed EpCAM co-immunoprecipitation followed by mass spectrometry in search for novel potentially interacting proteins. The endoplasmic reticulum aminopeptidase 2 (ERAP2) was found to co-precipitate with EpCAM and to co-localize in the cytoplasm/ER and the plasma membrane. ERAP2 is a proteolytic enzyme set in the endoplasmic reticulum (ER) where it plays a central role in the trimming of peptides for presentation by MHC class I molecules. Expression of EpCAM and ERAP2 *in vitro* in the presence of dog pancreas rough microsomes (ER vesicles) confirmed N-linked glycosylation, processing in ER and the size of EpCAM. The association between ERAP2 and EpCAM is a unique and novel finding that provides new ideas on EpCAM processing and on how antigen presentation may be regulated in cancer.

1. Introduction

Breast cancer is the most common cancer in women and the leading cause of female cancer deaths [1]. In 2003 it was shown that tumorigenic breast cancer cells are EpCAM$^+$, CD44$^+$ and CD24$^{low/-}$ [2]. Epithelial cell adhesion molecule (EpCAM) [3], also known as KS1/4, EGP40, or GA733-2 (Supplementary Reference), is an epithelial and cancer cell protein [4]. It is a type I integral membrane protein not structurally related to the major families of cell adhesion molecules (CAMs) [5] (and references therein). EpCAM functions partly via homotypic protein interactions mediating cell-to-cell adhesion of epithelial cells [3,6]. It is a Ca^{2+} independent cell-CAM that has an N-terminal extracellular domain (ectodomain; EpEx) composed of two epidermal growth factor (EGF)-like domains and a cysteine poor region, followed by the transmembrane domain (TM) and a short cytoplasmic tail of 26 amino acids [6–8].

EpCAM is abundantly present in cancerous epithelium, and its subcellular distribution changes from a basolateral localization in normal tissue to a dispersed membrane, cytoplasmic and nuclear pattern in cancer cells [9]. In mammary gland, enhanced levels of EpCAM are associated with poor prognosis especially in node-positive invasive breast carcinoma. EpCAM is also a potential predictive marker of sensitivity to adjuvant hormonal and/or cytotoxic treatments [10,11].

In addition to EpCAMs adhesive function there is cumulative evidence of its signaling role [9,12]. Outside-in signaling of EpCAM involves proteolytic cleavage, shedding of EpEx, and surface-to-nucleus translocation of the cytoplasmic tail (intracellular domain; EpICD). EpICD accumulates in the cell nucleus [9,12] where it induces a proliferative signaling activity [13]. In this process, ADAM17 (TACE) and γ-secretase interact with EpCAM and cleave off the EpICD that becomes free to associate with FHL2, β-catenin, and LEF-1, which are all members of the Wnt signaling pathway. EpCAM signaling is also modified by its presence in tetraspanin-enriched microdomains (TEMs), in complex with tetraspanin CD9, CD44 variants and claudin7. Further, EpCAM interacts with the actin cytoskeleton via α-actinin [7], and a PDZ-domain has been identified in its C-terminal end [5,12,14].

Based on the importance of EpCAM in cancer, we have searched for novel EpCAM interacting proteins in human breast cancer cells, and show here that the endoplasmic reticulum aminopeptidase 2 (ERAP2) [15] associates with EpCAM. Aminopeptidases are

* Corresponding author. Fax: +46 8 3210 47.
E-mail addresses: Salah.Eldin.Gadalla@ki.se (S.-E. Gadalla), Karin.geiger@dbb.su.se (K. Öjemalm), Patricia.lara@dbb.su.se (P.L. Vasquez), Ingmarie.nilsson@dbb.su.se (I. Nilsson), Christer.Ericsson@ki.se (C. Ericsson), Jian.Zhao@ki.se (J. Zhao), Monica.Nister@ki.se (M. Nistér).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.08.059

essential for protein maturation, for activation, modulation, and degradation of bioactive peptides, and for determining protein stability [16]. The major histocompatibility complex (MHC) class I molecules rely on amino peptidase such as ERAP1 and ERAP2 for the antigenic trimming of N-terminally extended precursors present in the ER [17,18].

2. Materials and methods

2.1. Breast cancer cell lines and cell culture

The breast cancer cell lines MCF-7, ZR-75-1 and MC2 are ER-$\alpha^{+/high}$ EpCAM$^{+/high}$, while MDA-MB-231 and HS-578T are ER-$^{-/low}$ EpCAM$^{-/low}$ [19,20]. All cell lines were grown in D-MEM with L-glutamine and 10% fetal calf serum, supplied with sodium pyruvate (GIBCO) and penicillin/streptomycin (SIGMA).

2.2. Antibodies

Information is provided in Supplementary Materials and Methods.

2.3. Reciprocal co-immunoprecipitation followed by Western blotting

Standard procedures were used (see Supplementary Materials and Methods). Briefly, cells were lysed in RIPA buffer (0.1% SDS), and immunoprecipitation was performed with the anti-human EpCAM mouse monoclonal antibody (C-10, Santa Cruz) alternatively with the anti-ERAP2 goat polyclonal antibody (R&D systems; AF3830). Precipitated proteins were separated by electrophoresis in 10% SDS-PAGE and total cell extracts were run in parallel. Proteins were identified using Western blotting.

2.4. Immunofluorescence microscopy

Breast cancer cell lines were cultured on cover slips, washed with PBS and fixed in 96% ethanol for 15 min at room temperature. After washing, cells were permeabilized with 0.5% Triton X100 in PBS for 15 min, incubated with 10% BSA in PBS for 1 h at 4 °C, washed in PBS and incubated overnight at 4 °C with the appropriate primary antibodies [the mouse monoclonal antibody against human EpCAM N-terminal domain (Santa Cruz, C-10) and the goat polyclonal antibody against ERAP2 (R&D systems, AF3830)] followed by washing and incubation with the corresponding FITC-respectively TexasRed-conjugated secondary antibodies (1:1000 dilution, 1 h, 4 °C). The nuclei were stained with DAPI in Vectashield mounting media (Vector Lab). Digital pictures were acquired by Axioplan 2 imaging (Carl Zeiss) using Axiovision v4 software.

2.5. Tunicamycin treatment

Tunicamycin specifically inhibits the asparagine-linked N-glycosylation of proteins. Tunicamycin was dissolved in DMSO (dimethyl sulfoxide) (1 mg/ml). In order to optimize glycosylation inhibition three different concentrations, 1, 5 and 10 μg/ml, were used, while control cells received equivalent amounts of DMSO. The viability of cells was determined by trypan blue exclusion. Cells were analyzed during 24 h to guarantee viability. The cell lysates were analyzed by Western blotting, using a rabbit polyclonal antibody against a synthetic peptide derived from within residue 250 to the C-terminus of human EpCAM (Abcam).

2.6. Expression of EpCAM and ERAP2 in presence of dog pancreas rough microsomes

The EpCAM cDNA (GA733-2) (kind gift from Prof. Dorothee Herlyn, Wistar Institute of Anatomy and Biology) and the ERAP2

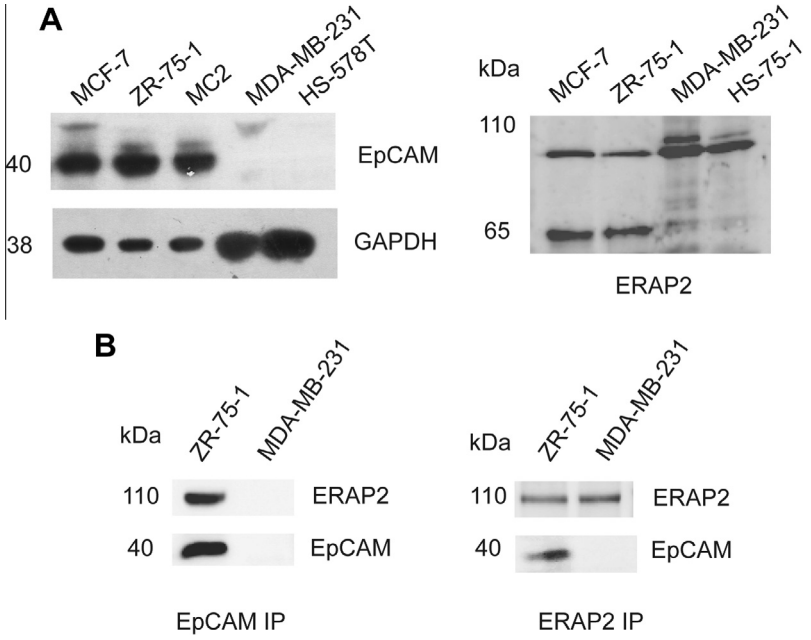

Fig. 1. EpCAM and ERAP2 proteins in breast cancer cell lines. (A) Western blot analysis of EpCAM and ERAP2. A major band of 40 kDa was observed in the ER-α^+ cells MCF-7, ZR-75-1, and MC2, but not in ER-α^- cells (left panel). An equal amount of ERAP2 at 110 kDa was found in all tested cell lines. In addition, a band of 65 kDa was present in MCF-7 and ZR-75-1 (right panel). (B) EpCAM and ERAP2 reciprocal co-immunoprecipitation followed by Western blotting. EpCAM was detected in ZR-75-1 at 40 kDa, but not in MDA-MB-231 by EpCAM immunoprecipitation, and Western blotting with anti-ERAP2 antibody showed a band at 110 kDa in the EpCAM$^+$ but not in the EpCAM$^-$ cell line (left panel). Consistently, ERAP2 was observed at 110 kDa in both cell lines by ERAP2 immunoprecipitation, and Western blotting using anti-EpCAM antibody showed a band at 40 kDa in the EpCAM$^+$ ZR-75-1 cells but not in MDA-MB-231 (right panel).

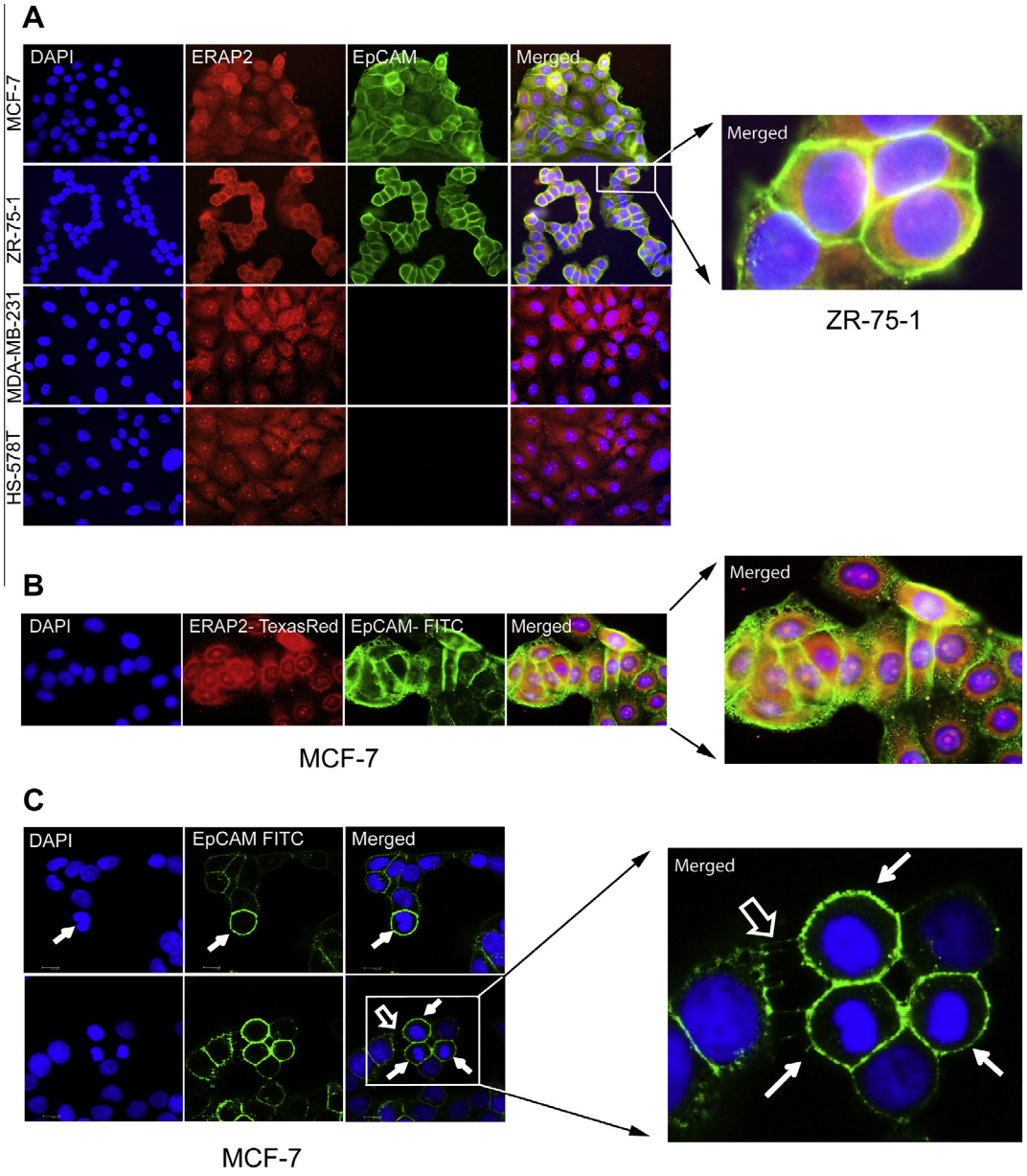

Fig. 2. EpCAM and ERAP2 co-localization by double immunofluorescence staining. (A) Double immunofluorescence staining of EpCAM and ERAP2. MCF-7 and ZR-75-1 cells showed both membranous and cytoplasmic staining of EpCAM (green), while there was no staining in MDA-MB-231 and HS-578T. ERAP2 (red) was detected in the cytoplasm and also associated with the plasma membrane of MCF-7 and ZR-75-1 cells (blow up view). In contrast, ERAP2 was mainly present in the cytoplasm of MDA-MB-231 and HS-578T cells. The cytoplasmic ERAP2 staining pattern is consistent with localization in ER. Transient co-localization (yellow) of ERAP2 and EpCAM both in the ER and in the plasma membrane is suggested by these results. (B) Co-localization of ERAP2 and EpCAM. High magnification of area in (A) illustrates the co-localization (yellow) in MCF-7 cells. (C) Localization of EpCAM in dividing MCF-7 cells. MCF-7 cells were co-stained with DAPI (nuclei; blue) and anti-EpCAM antibodies (green). We observed an enhanced EpCAM membrane staining, but virtually no cytoplasmic staining in actively dividing cells (arrows).

cDNA (kind gift from Prof. Peter van Endert, Université Paris Descartes) were both introduced into the pGEM1 vector [21,22] and verified by sequencing at Eurofins MWG Operon (Ebersberg, Germany).

The TNT SP6 Quick coupled transcription/translation system (Promega) was used to synthesize EpCAM and ERAP2 in presence of dog pancreas rough microsomes (RM). 150–200 ng pGEM1-EpCAM or pGEM1-ERAP2 plasmid, 5 μCi L-[^{35}S]methionine (Perkin Elmer), and 1 μl dog pancreas RM [23] were added to 10 μl reticulocyte lysate and incubated for 90 min at 30 °C [24,25]. The samples were then subjected to SDS-PAGE and protein bands were visualized using a Fuji FLA-3000 phosphoimager (Fujifilm).

3. Results

3.1. Identification of ERAP2 as an EpCAM associated protein

In an initial search for EpCAM-interacting proteins, gels with EpCAM co-immunoprecipitates from breast cancer cells were stained with Colloidal Blue dye. When selecting differentially stained bands for mass spectrometry analysis we chose those bands that were present in ZR-75-1 (EpCAM$^{+/high}$) but not in MDA-MB-231 (EpCAM$^{-/low}$), to enhance the chance of identifying EpCAM-interacting proteins. EpCAM was identified at 40 kDa by mass spectrometry, only present in precipitates from ZR-75-1 but

not in precipitates from MDA-MB-231 and so served as internal control (not shown). This preliminary analysis indicated that ERAP2 could be co-precipitated with EpCAM. The single-pass type II membrane protein ERAP2 is an aminopeptidase set in the ER, that plays a central role in antigen peptide trimming afterwards presented by MHC class I molecules [17,18]. We chose to study this unexpected association further and confirmed the co-localization of ERAP2 and EpCAM.

First, we performed Western blot analysis of total cell lysates for a panel of breast cancer cell lines. EpCAM was detected at 40 kDa in MCF-7, ZR-75-1, and MC2, but not in MDA-MB-231 and HS-578T (Fig. 1A, left panel) and ERAP2 was detected at 110 kDa in all the cell lines. An additional band for ERAP2 was found at 65 kDa in MCF-7 and ZR-75-1 (Fig. 1A, right panel). Next, to confirm that ERAP2 can interact with EpCAM, we performed reciprocal co-immunoprecipitation followed by Western blotting. ERAP2 was detected at 110 kDa when EpCAM immunoprecipitates from ZR-75-1 cells were analyzed (Fig. 1B, left panel), but not in immunoprecipitates from the negative control cell line MDA-MB-231. Likewise, EpCAM was detected at 40 kDa in ERAP2 immunoprecipitates from ZR-75-1 cells but not in immunoprecipitates from MDA-MB-231 (Fig. 1B, right panel).

3.2. EpCAM/ERAP2 co-localization in breast cancer cells

Double immunofluorescence staining of EpCAM and ERAP2 was performed to detect co-localization of the two proteins. MCF-7 and ZR-75-1 cells showed a strong membranous and cytoplasmic EpCAM staining (Fig. 2A). Especially strong plasma membrane staining was found in mitotic cells, forming bridges between cells (Fig. 2C). In contrast, EpCAM was not visualized in MDA-MB-231 and HS-578T cells (Fig. 2A). ERAP2 staining was equally strong in all tested breast cancer cell lines, observed as both cytoplasmic and plasma membrane staining in the EpCAM$^{+/high}$ MCF-7 and ZR-75-1 cells, while mainly as cytoplasmic staining in the EpCAM$^{-/low}$ MDA-MB-231 and HS-578T cells (Fig. 2A). The

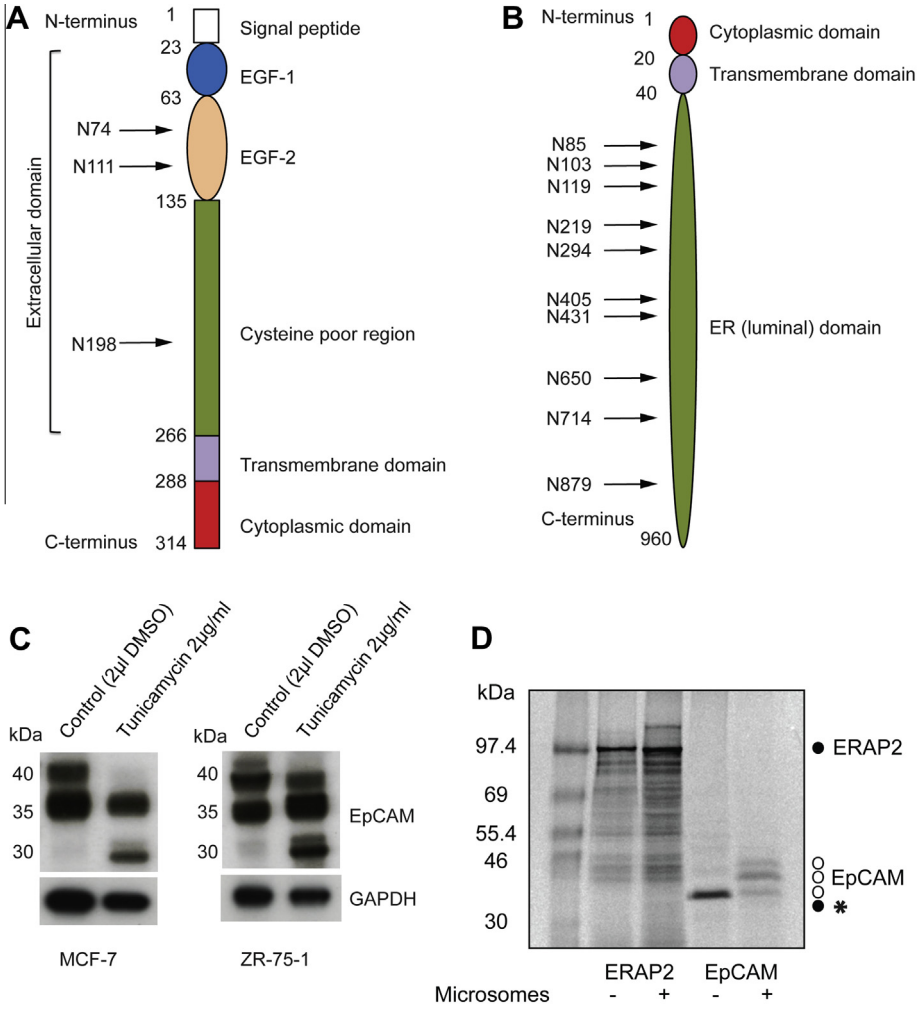

Fig. 3. EpCAM and ERAP2 domain structure and potential sites for N-linked glycosylation; tests for glycosylation in breast cancer cells and microsomes. (A) Schematic drawing of EpCAM. The EpCAM extracellular domain is composed of two EGF-like repeats (EGF-1 and EGF-2) and a cysteine poor region. The protein is attached to the plasma membrane via a single pass TM domain and there is a short C-terminal cytoplasmic domain, the EpICD. Arrows indicate N-linked glycosylation sites (schematic drawing based on NCBI protein). (B) Schematic drawing of ERAP2. ERAP2 lacks a signal peptide, it has a short cytoplasmic N-terminal domain, a TM domain and a long ER luminal C-terminal domain. Arrows indicate potential N-linked glycosylation sites (schematic drawing based on NCBI protein). (C) Tunicamycin treatment of breast cancer cells. When MCF-7 cells were treated with tunicamycin the 40 kDa band of EpCAM was no longer observed. The same type of effect was seen in the ZR-75-1 cells, but the effect was considerably less. In addition, tunicamycin treatment resulted in the appearance of an additional protein (double) band, at ~30 kDa. (D) EpCAM and ERAP2 expression in dog pancreas microsomes. Expression of EpCAM and ERAP2 proteins from the respective plasmids using an *in vitro* assay generated a single product of ~40 kDa respectively ~100 kDa (−), while when expressed using dog pancreas microsomes (+) a cleaved product and three N-glycosylated forms of EpCAM appeared, where the lowermost product was at 35 kDa. Note also that ERAP2 was migrated as two products, one at 100 kDa and the other at 110–120 kDa when microsomes were used. Non-glycosylated and cleaved form is indicated by filled circle respectively star, and glycosylated form by unfilled circle.

membranous ERAP2 formed demarcating lines between cells, while the cytoplasmic ERAP2 formed a punctate or reticulated pattern consistent with ER localization.

The analysis indicated a co-localization of EpCAM and ERAP2 both in the ER and in the plasma membrane (Fig. 2A, B and blow up view). The presence of ERAP2 in/near the plasma membrane was an unexpected finding.

3.3. EpCAM potential N-glycosylation sites; glycosylated form in breast cancer cells

There are three potential sites for N-linked glycosylation of EpCAM as indicated in Fig. 3A (N74, N111 and N198), all located in the extra cellular domain of the protein (Fig. 3A, Fig. S1) and previously shown to be glycosylated [26,27]. We therefore examined if EpCAM is glycosylated in the breast cancer cells and *in vitro* using dog pancreas microsomes. Treatment of EpCAM$^+$ breast cancer cells with tunicamycin showed that EpCAM is glycosylated in these cells. The slowest migrating band of EpCAM (at 40 kDa; Fig. 1A, left panel) is a glycosylated form, since its size was decreased to 35 kDa upon tunicamycin treatment (Fig. 3C). In addition, this inhibition of N-glycosylation seemed to be causing a proteolytic effect, as new EpCAM products in the form of a double band around 30 kDa appeared in both MCF-7 and ZR-75-1 (Fig. 3C). However, the size of the EpCAM$^+$ cell pool was unchanged in tunicamycin treated cell cultures relative to control cultures as shown by flow cytometry (Fig. S3).

3.4. Expression of EpCAM and ERAP2 in vitro confirms N-linked glycosylation, signal peptidase cleavage and the size of EpCAM

To confirm glycosylation and signal peptidase cleavage, we expressed both EpCAM and ERAP2 using an *in vitro* transcription/translation assay supplemented with dog pancreas RMs (ER vesicles) [22]. EpCAM was expressed as a single protein product of the molecular weight ~40 kDa when transcribed/translated *in vitro* from the pGEM1-EpCAM plasmid (Fig. 3D). When supplemented with RMs the single EpCAM protein product migrated in the SDS–PAGE as four products, probably due to signal peptide cleavage by signal peptidase (SPase) [28,29] and N-linked glycosylation (N74, N111 and N198) by the oligosaccharyl transferase (OST) enzyme (Fig. 3A, D, Fig. S1) [24,30]. The glycosylation efficiency and the number of glycosylation sites of EpCAM were confirmed using endoglycosidase H treatment of the translated product. Additionally, the size of the cleaved and un-glycosylated product of EpCAM was determined to the size around 35 kDa (Fig. S4). ERAP2 was detected at 100 kDa when expressed *in vitro* (Fig. 3D). In the presence of ER vesicles an additional weak product appeared at 110–120 kDa (Fig. 3D) that may be due to glycosylation of some of the ten potential N-linked glycosylation sites (N85, N103, N119, N219, N294, N405, N431, N650, N714 and N879) located in the C-terminal, luminal part of the protein (Fig. 3B, Fig. S2). ERAP2 seems not to be cleaved by SPase, but glycosylated by the OST complex.

4. Discussion

In this study we show for the first time an association between the breast cancer stem cell and adhesion molecule EpCAM and the proteolytic enzyme ERAP2. The observation was made in the epithelial breast cancer cell lines ZR-75-1 and MCF-7, known to express a large amount of EpCAM. We first hypothesized that EpCAM is a candidate for proteolytic cleavage by ERAP2. Enzymatic analysis performed by others showed that ERAP2 preferentially hydrolyzes the basic residues arginine and lysine [17]. We searched the entire EpCAM protein amino acid sequence, and noticed that there are many hypothetical sites for proteolytic cleavage by ERAP2, and postulate that ERAP2 might act on these residues to trim the EpCAM N-terminal extracellular domain. Since ERAP2 is localized in the cytosol and ER with its major C-terminal part and active site in the ER lumen (Fig. S2) and the latter communicates with the cell membrane there is at least a hypothetical possibility of EpCAM proteolytic cleavage by ERAP2. Such a transient cytoplasmic/ER and plasma membrane co-localization of the two proteins in EpCAM$^+$ cells is supported by the double immunofluorescence staining (Fig. 2A, B) and by the fact that both are N-glycosylated proteins. Although ERAPs normally reside in the ER and their function is to trim short peptides for antigen presentation, ERAP1 has been reported to facilitate cleavage of cytokine receptors and to co-localize with TNFRI [31] ([16] and references therein).

For protein stability and stable interactions to occur between proteins, adequate glycosylation may be essential. In an analysis of glycosylation sites in EpCAM, Chong and Speicher showed that there are N-linked but not O-linked carbohydrate groups [26]. Others reported that glycosylation of N198 is essential for protein stability [27]. We therefore confirmed that both EpCAM and ERAP2 are glycosylated proteins and processed in ER. To address *in vitro* whether EpCAM or ERAP2 are substrates for the OST complex and/or the SPase enzyme, we took advantage of an *in vitro* expression system supplemented with dog pancreas RMs. Expression of both EpCAM and ERAP2 in the absence and presence of microsomes resulted in one main product for both EpCAM and ERAP2 (of ~40 kDa respectively ~100 kDa molecular weight and additional larger bands for both proteins) (Fig. 3D). In the case of EpCAM, the most likely explanation for the appearance of three distinct protein products in the microsomes is that those are co-translational modifications due to differential N-glycosylation. To finally confirm this, the EpCAM protein was expressed using RMs and treated with endoglycosidase H. Our results show that the protein was N-linked glycosylated and the molecular size of the un-glycosylated, SPase cleaved EpCAM was confirmed to be around 35 kDa (Fig. S4).

We further treated EpCAM$^+$ breast cancer cells with tunicamycin and saw that the major 40 kDa protein present in human cells is a glycosylated form that decreases upon tunicamycin treatment to around 35 kDa (Fig. 3C). This is similar to the 35 kDa non-glycosylated, cleaved form of EpCAM in the microsomes (Fig. S4). In other experiments we observed a 35 kDa form in the membrane fraction of untreated MCF-7 cells (Fig. S5). Our conclusion is therefore that the N-terminal signal peptide is cleaved off during processing of EpCAM in both microsomes and human cells, and that 35 kDa is the normal size of non-glycosylated EpCAM, in keeping with a report by Munz et al. [27] (see Supplementary Discussion).

An unexpected finding was that of a unique smaller sized EpCAM protein ~30 kDa generated after tunicamycin treatment, possibly indicating that proteolytic processing is enhanced in the non-glycosylated protein (Fig. 3C). A proteolytic cleavage site in EpEx that cleaves off an N-terminal 6 kDa fragment has been reported. This fragment is still attached to the rest of EpEx via disulphide bridges and comprises the binding sites of most EpCAM antibodies available [5]. Involvement of such a cleavage site may explain why the EpCAM$^+$ cell pool, measured by FACS was unchanged after tunicamycin treatment (Fig. S3).

In summary, the association and co-localization of ERAP2 and EpCAM at the surface of breast cancer cells is a unique and novel finding that provides new ideas on EpCAM processing and on how antigen presentation may be regulated in cancer. The presence of EpCAM in the plasma membrane of carcinoma cells may help localize the associated ERAP2 molecule near the surface of these cells. Peptides, properly trimmed by ERAP1 and ERAP2 are

required for MHC class I molecules to appear at the cell surface for antigen presentation [16], and EpCAM can be expressed in antigen presenting cells [32]. Indeed, previously published data provide support for ERAP2 gene expression and its clinical significance in breast cancer (see Supplementary Discussion).

Acknowledgments

The salary of Salah-Eldin Gadalla was provided by the secretariat of higher education in Tripoli, Libya through the Cultural and Student Attachés at the Libyan Embassy in Stockholm. MN was supported by grants from the Swedish Cancer Society, the Swedish Research Council and the Cancer Society in Stockholm. IMN was supported by grants from the Swedish Cancer Society, Magnus Bergvalls Stiftelse, the Swedish Foundation for International Cooperation in Research and Higher Education (STINT), and the Swedish Foundation for Strategic Research (SSF). We thank Dr. Åke Engström for processing samples for mass spectrometry, Dr. Juan Castro for support with FACS analyses, Mrs. Anna-Lena Borg and Prof. Jonas Bergh for providing breast cancer cell lines, and Assoc. Prof. Mikael Lindström for critically reading the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.059.

References

[1] D.M. Parkin, F.I. Bray, S.S. Devesa, Cancer burden in the year 2000. The global picture, Eur. J. Cancer 37 (Suppl.8) (2001) S4–S66.
[2] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, et al., Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. USA 100 (2003) 3983–3988.
[3] S.V. Litvinov, M.P. Velders, H.A. Bakker, et al., Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule, J. Cell Biol. 125 (1994) 437–446.
[4] P.A. Baeuerle, O. Gires, EpCAM (CD326) finding its role in cancer, Br. J. Cancer 96 (2007) 417–423.
[5] U. Schnell, V. Cirulli, B.N. Giepmans, EpCAM: structure and function in health and disease, Biochim. Biophys. Acta. 2013 (1828) 1989–2001.
[6] S.V. Litvinov, H.A. Bakker, M.M. Gourevitch, et al., Evidence for a role of the epithelial glycoprotein 40 (Ep-CAM) in epithelial cell–cell adhesion, Cell Adhes. Commun. 2 (1994) 417–428.
[7] M. Balzar, H.A. Bakker, I.H. Briaire-de-Bruijn, et al., Cytoplasmic tail regulates the intercellular adhesion function of the epithelial cell adhesion molecule, Mol. Cell. Biol. 18 (1998) 4833–4843.
[8] M. Balzar, I.H. Briaire-de Bruijn, H.A. Rees-Bakker, et al., Epidermal growth factor-like repeats mediate lateral and reciprocal interactions of Ep-CAM molecules in homophilic adhesions, Mol. Cell. Biol. 21 (2001) 2570–2580.
[9] D. Maetzel, S. Denzel, B. Mack, et al., Nuclear signalling by tumour-associated antigen EpCAM, Nat. Cell Biol. 11 (2009) 162–171.
[10] A.K. Tandon, G.M. Clark, G.C. Chamness, et al., Association of the 323/A3 surface glycoprotein with tumor characteristics and behavior in human breast cancer, Cancer Res. 50 (1990) 3317–3321.
[11] G. Spizzo, P. Went, S. Dirnhofer, et al., Overexpression of epithelial cell adhesion molecule (Ep-CAM) is an independent prognostic marker for reduced survival of patients with epithelial ovarian cancer, Gynecol. Oncol. 103 (2006) 483–488.
[12] G. Carpenter, M. Red Brewer, EpCAM: another surface-to-nucleus missile, Cancer Cell 15 (2009) 165–166.
[13] M. Munz, C. Kieu, B. Mack, et al., The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation, Oncogene 23 (2004) 5748–5758.
[14] M. Munz, P.A. Baeuerle, O. Gires, The emerging role of EpCAM in cancer and stem cell signaling, Cancer Res. 69 (2009) 5627–5629.
[15] T. Tanioka, A. Hattori, S. Masuda, et al., Human leukocyte-derived arginine aminopeptidase. The third member of the oxytocinase subfamily of aminopeptidases, J. Biol. Chem. 278 (2003) 32275–32283.
[16] N. Haroon, R.D. Inman, Endoplasmic reticulum aminopeptidases: biology and pathogenic potential, Nat. Rev. Rheumatol. 6 (2010) 461–467.
[17] L. Saveanu, O. Carroll, V. Lindo, et al., Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum, Nat. Immunol. 6 (2005) 689–697.
[18] T. Serwold, F. Gonzalez, J. Kim, R. Jacob, et al., ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum, Nature 419 (2002) 480–483.
[19] A.M. Sieuwerts, J. Kraan, J. Bolt, et al., Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells, J. Natl. Cancer Inst. 101 (2009) 61–66.
[20] R.M. Neve, K. Chin, J. Fridlyand, et al., A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes, Cancer Cell 10 (2006) 515–527.
[21] M. Kozak, Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems, Mol. Cell. Biol. 9 (1989) 5073–5080.
[22] C. Lundin, R. Nordstrom, K. Wagner, et al., Membrane topology of the human seipin protein, FEBS Lett. 580 (2006) 2281–2284.
[23] P. Walter, G. Blobel, Preparation of microsomal membranes for cotranslational protein translocation, Methods Enzymol. 96 (1983) 84–93.
[24] M. Johansson, I. Nilsson, G. von Heijne, Positively charged amino acids placed next to a signal sequence block protein translocation more efficiently in *Escherichia coli* than in mammalian microsomes, Mol. Gen. Genet. 239 (1993) 251–256.
[25] T. Hessa, H. Kim, K. Bihlmaier, et al., Recognition of transmembrane helices by the endoplasmic reticulum translocon, Nature 433 (2005) 377–381.
[26] J.M. Chong, D.W. Speicher, Determination of disulfide bond assignments and N-glycosylation sites of the human gastrointestinal carcinoma antigen GA733-2 (CO17-1A, EGP, KS1-4, KSA, and Ep-CAM), J. Biol. Chem. 276 (2001) 5804–5813.
[27] M. Munz, K. Fellinger, T. Hofmann, et al., Glycosylation is crucial for stability of tumour and cancer stem cell antigen EpCAM, Front. Biosci. 13 (2008) 5195–5201.
[28] C. Lundin, H. Kim, I. Nilsson, S.H. White, Molecular code for protein insertion in the endoplasmic reticulum membrane is similar for N(in)-C(out) and N(out)-C(in) transmembrane helices, Proc. Natl. Acad. Sci. USA 105 (2008) 15702–15707.
[29] I. Nilsson, P. Whitley, G. von Heijne, The COOH-terminal ends of internal signal and signal-anchor sequences are positioned differently in the ER translocase, J. Cell Biol. 126 (1994) 1127–1132.
[30] M. Aebi, N-linked protein glycosylation in the ER, Biochim. Biophys. Acta. (2013).
[31] X. Cui, F. Hawari, S. Alsaaty, et al., Identification of ARTS-1 as a novel TNFR1-binding protein that promotes TNFR1 ectodomain shedding, J. Clin. Invest. 110 (2002) 515–526.
[32] T.A. Borkowski, A.J. Nelson, A.G. Farr, et al., Expression of gp40, the murine homologue of human epithelial cell adhesion molecule (Ep-CAM), by murine dendritic cells, Eur. J. Immunol. 26 (1996) 110–114.