The cancer antigen CA125 represents a novel counter receptor for galectin-1

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Summary

CA125 is an ovarian cancer antigen whose recently elucidated primary structure suggests that CA125 is a giant mucin-like glycoprotein present on the cell surface of tumor cells. Here, we establish a functional link between CA125 and β-galactoside-binding, cell-surface lectins, which are components of the extracellular matrix implicated in the regulation of cell adhesion, apoptosis, cell proliferation and tumor progression. On the basis of mass spectrometry and immunological analyses, we find that CA125 is a counter receptor for galectin-1, as both soluble and membrane-associated fragments of CA125 derived from HeLa cell lysates are shown to bind specifically to human galectin-1 with high efficiency. This interaction is demonstrated (1) to depend on β-galactose-terminated, O-linked oligosaccharide chains of CA125, (2) to be preferential for galectin-1 versus galectin-3 and (3) to be regulated by the cellular background in which CA125 is expressed. Despite lacking a conventional signal peptide, a CA125 C-terminal fragment of 1148 amino acids, representing less than 10% of the full-length protein, retains the ability to integrate into secretory membranes such as the endoplasmic reticulum (ER) and the Golgi, and is targeted to the plasma membrane by conventional secretory transport. As demonstrated by a novel assay that reconstitutes non-conventional secretion of galectin-1 based on fluorescence-activated cell sorting (FACS), we find that tumor-derived HeLa cells expressing endogenous CA125 present more than ten times as much galectin-1 on their surface compared with non-tumor-derived, CA125-deficient CHO cells. Intriguingly, both the galectin-1 expression level and the cell-surface binding capacity for galectin-1 are shown to be similar in CHO and HeLa cells, suggesting that CA125 might be a factor involved in the regulation of galectin-1 export to the cell surface.

Key words: CA125, Galectin, Counter receptor, Nonclassical export, Cell-surface expression, Tumor antigen

Introduction

CA125 is a tumor antigen that was defined by the monoclonal antibody (mAb) OC125 more than 20 years ago (Bast et al., 1981). On the basis of an immunological serum assay (Bast et al., 1983; Bon et al., 1996), the CA125 antigen has been defined as a marker primarily for ovarian carcinoma (Davis et al., 1986; Lloyd and Yin, 2001). Although CA125 is expressed both by normal and tumor cells (O'Brien et al., 1986; Zurawski et al., 1988; Hardardottir et al., 1990; Nap et al., 1996), cell-surface expression and release of soluble proteolytic fragments of CA125 into the extracellular space (Lloyd and Yin, 2001) appear to be associated with the conversion from benign to cancer cells (Meyer and Rustin, 2000). Consistently, CA125 has been shown to accumulate in the serum of cancer patients bearing ovarian as well as other carcinomas (Bast et al., 1983; Bon et al., 1996). However, the biological function of CA125 remains to be established.

Only recently was the primary structure of CA125 elucidated, demonstrating that CA125 represents a giant mucin-like glycoprotein (O'Brien et al., 2001; Yin and Lloyd, 2001). On this basis, CA125 has been termed Muc16 to reflect the nature of CA125 as a new member of the protein family of mucins (Yin and Lloyd, 2001). Full-length CA125 contains more than 11,000 amino acids that form the proteinaceous core structure (O'Brien et al., 2001; Yin and Lloyd, 2001). CA125 is both N- and O-glycosylated in its N-terminal extracellular domain (Zurawski et al., 1988; Nagata et al., 1991; Lloyd et al., 1997; Lloyd and Yin, 2001), which is composed of a stalk domain next to the transmembrane span, more than 60 repeat structures (each of which consists of 156 amino acids) and an N-terminal extension (O'Brien et al., 2001; Yin and Lloyd, 2001). Towards the C-terminus, CA125 contains a putative transmembrane span and a short cytoplasmic tail (O'Brien et al., 2001). The release of soluble fragments of CA125 into the extracellular space appears to be triggered by serine/threonin- and/or tyrosine-dependent phosphorylation within the cytoplasmic domain (Fendrick et al., 1997; Lloyd and Yin, 2001).

Tumor-specific cell-surface expression concomitant with the release of extracellular fragments suggests a role for CA125 in the regulation of cell proliferation and/or tumor progression. Owing to its nature as a glycoprotein, potential ligands of CA125 include lectins of the extracellular matrix (ECM) such as the β-galactoside-specific family of galectins (Perillo et al., 1998; Rabinovich et al., 2002). Intriguingly, galectin-1 and galectin-3 expression is upregulated in various cancer-derived cell lines compared with benign tissue (Iurisci et al., 2000; Lahm et al., 2001; Lloyd, 2001; Armstrong et al., 2002).

Moreover, one member of this family, galectin-3, has been reported to represent a ligand of a colon cancer mucin (Bresalier et al., 1996).

At the molecular level, the best-characterized members of this protein family are galectin-1 and -3 (Hughes, 1997; Hughes, 1999). Owing to its dimeric character, cell-surface recruitment of galectin-1 is thought to affect the conformation and oligomeric status of glycosylated protein domains by forming intra- or intermolecular bridges that, in turn, might exert a cellular response (Perillo et al., 1998). Biological counter receptors for galectin-1 include laminin, fibronectin, lamp 1 and 2, GM1 glycolipid (reviewed by Perillo et al., 1998) as well as cell-type-specific molecules such as the T-cell glycoproteins CD43 and CD45 (Perillo et al., 1995; Nguyen et al., 2001). The pattern of oligosaccharide chains presented on the cell surface of individual cells is likely to influence the way galectin-1 interacts with their surface. Therefore, the way a particular cell type responds to galectin-1 might also be regulated by variations in the activity of glycosyl transferases and/or glycosidases (Perillo et al., 1998). Known cellular responses to the cell-surface recruitment of galectin-1 include a change in proliferation activity, regulation of cell survival and regulation of cell adhesion. Interestingly, depending both on the cellular context and its local concentration, galectin-1 exerts both inhibitory and stimulatory effects on these processes (Perillo et al., 1998).

Galectin export from mammalian cells has been shown to occur in a nonclassical manner independent of the function of the endoplasmic reticulum (ER) and the Golgi (Cooper and Barondes, 1990; Cleves et al., 1996). Consistently, galectins lack a conventional signal peptide for translocation into the ER (Cleves, 1997; Hughes, 1999). The balance between cytoplasmic and extracellular populations appears to be tightly regulated (Hughes, 1999). For example, galectin-1 export from muscle cells is developmentally regulated as increased export is observed upon differentiation from myoblasts to myotubes (Cooper and Barondes, 1990). Moreover, it has been reported that galectin-1 externalization can be triggered upon differentiation of K562 leukemia-derived cells induced by erythropoietin (Lutomski et al., 1997).

In the current study, we identify CA125 as a novel counter receptor for galectin-1. Using affinity chromatography and mass spectrometry, as well as immunological analyses, galectin-1 is shown to bind specifically to CA125 in a direct manner. A comparison with the second most-abundant family member, galectin-3, indicates that CA125 exhibits specificity towards galectin-1. A C-terminal fragment of CA125, CA125-C-TERM (defined by NCBI clone AK024365) retains the ability to integrate into secretory membranes and, like full-length CA125, is shown to be transported to the cell surface. Cell-surface delivery of CA125-C-TERM is demonstrated to occur by ER/Golgi-dependent vesicular transport. CA125-C-TERM is found to bind to galectin-1 with twofold higher efficiency compared with galectin-3 when expressed in HeLa cells, and with sevenfold higher efficiency compared with galectin-3 when expressed in CHO cells. These results demonstrate that CA125 represents a novel counter receptor for galectins and has binding characteristics that can be regulated by the cellular background in which it is expressed.

In order to investigate the functional significance of the interaction reported, we compared tumor-derived, CA125-expressing HeLa cells with non-tumor-derived, CA125-deficient CHO cells with regard to various galectin-1 parameters. Although we find that galectin-1 expression and cell-surface binding capacity for galectin-1 is similar in HeLa and CHO cells, we demonstrate that HeLa cells contain more than ten times as much cell-surface galectin-1 compared with CHO cells. Our results suggest that CA125 might allow tumor cells to interact differentially with the ECM in a galectin-1-dependent manner.

Materials and Methods

Recombinant proteins and antibodies

GST–galectin-1 and GST–galectin-3 fusion constructs were cloned using the vector pGEX-2T (Amersham). For this purpose, appropriate PCR products were generated using the IMAGE clones 2666528 and 2419761 as a source for the ORFs (ORF) of human galectin-1 and -3, respectively. The corresponding proteins were expressed in *Escherichia coli* BL21(DE3) cells (Novagen). Protein purification was achieved by affinity chromatography using GSH-sepharose (Amersham) according to standard procedures. An N-terminal fragment of CA125-C-TERM (defined by the NCBI clone AK024365) that corresponds to amino acids 1-356 (CA125-C-TERM$_{1\text{-}356}$) was cloned into the vector pIVEX 2.4b Nde (Roche) in order to express a His$_6$-tagged version of the protein in vitro employing the rapid translation system (Roche). Homogenous preparations of GST–galectin-1 and His$_6$-CA125-C-TERM$_{1\text{-}356}$, respectively, were used to generate polyclonal antisera in rabbits. Anti-galectin-1 antibodies were affinity-purified from the corresponding rabbit serum in two steps: the serum was first incubated with GST beads to remove anti-GST antibodies, followed by affinity purification of anti-galectin-1 antibodies on GST–galectin-1 beads. Anti-galectin-1 antibodies were eluted from the GST–galectin-1 affinity matrix under acidic conditions according to standard procedures. Affinity-purified anti-GST antibodies were eluted under acidic conditions from GST beads obtained in step 1.

The mAb anti-CA125 antibody OC125 was purchased from Zymed. Secondary antibodies used for western blotting were from Bio-Rad, those used for FACS sorting and indirect immunofluorescence confocal microscopy were from Molecular Probes.

Galectin-1 affinity matrix and binding experiments employing subcellular fractions from HeLa cells

To conduct affinity purification of galectin-1-interacting proteins, GST–galectin-1 and GST–galectin-3 fusion proteins, as well as GST as a control, were expressed in *E. coli* BL21(DE3) cells. Cells were resuspended in PBS containing 1 mM DTT, 0.1% (w/v) Triton X-100, 10% (w/v) glycerol and protease inhibitor tabs (Roche), followed by homogenization using a cell disruptor (Avestin). A 100,000 $\boldsymbol{g}_{av}$ supernatant was obtained and incubated with glutathione beads for 2 hours at 4°C on a rotating wheel. Following extensive washing using homogenization buffer (see above), 250 μl of beads containing 250 μg of coupled protein were used per binding experiment.

S-HeLa cells (ATCC CCL-2.2) were cultured in spinner flasks according to standard procedures. Typically, cultures were grown to a density of about 6-7×10^5 cells per ml. Cells were collected by centrifugation and resuspended in HeLa homogenization buffer (25 mM Tris, pH 7.5; 130 mM KCl; protease inhibitor tabs) at 1 g cells per ml. Following cell breakage using a Balch homogenizer (Balch and Rothman, 1985), the homogenate was sequentially centrifuged twice at 1000 $\boldsymbol{g}_{av}$ and twice at 3500 $\boldsymbol{g}_{av}$. The resulting supernatant was subjected to centrifugation at 100,000 $\boldsymbol{g}_{av}$. Following separation of supernatant and sediment, the soluble fraction was diluted with PBS

(supplemented with 1 mM DTT and protease inhibitor tabs) to give a final protein concentration of about 0.25 mg/ml. Typically, when starting with 5 g of cells, the soluble fraction was adjusted to a final volume of 50 ml. The corresponding sediment was then resuspended in 50 ml of PBS supplemented with 1 mM DTT, protease inhibitor tabs and 1% (w/v) NP-40 (Roche). Per experimental condition, 25 ml of the soluble or the membrane fraction, respectively, were incubated with 250 μl of GSH beads containing about 250 μg GST–galectin-1, GST–galectin-3 or GST, respectively. Bound proteins were eluted sequentially with 100 mM lactose and 25 mM glutathione, respectively. Further details are given in the corresponding figure legends.

Protein identification employing MALDI-TOF mass spectrometry

In order to identify individual proteins eluted from the galectin-1 affinity matrix, the eluates were separated on 10% Novex Bis-Tris gels (Invitrogen) followed by protein staining using the SilverQuest system (Invitrogen). After excision of gel pieces containing individual proteins, in-gel trypsin digestion allowed extraction of tryptic peptides. Proteins were identified based on the masses of the peptides obtained in this way by employing MALDI-TOF mass spectrometry (Wilm et al., 1996).

Retroviral transduction of MCAT-expressing HeLa and CHO cells

In order to transduce target cells with the cDNA of CA125-C-TERM, the ORF was cloned into the vector pFB (Stratagene). This vector promotes constitutive expression of the cDNA in question. Retroviral particles were generated by conventional transfection of HEK293T cells employing the VPack vector system (Stratagene). In this context, an envelope protein with an ecotropic host range was used, encoded by the vector pVPack-eco (Stratagene). Retroviral particles harvested from the medium of triple-transfected HEK293T cells were added to the medium of the target cells. As target cells, HeLa and CHO cells were used that stably express the murine cation amino acid transporter MCAT-1 (Albritton et al., 1989; Davey et al., 1997) and a doxicycline-dependent transactivator (Urlinger et al., 2000). These cell lines were designated $HeLa_{MCAT\text{-}TAM2}$ and $CHO_{MCAT\text{-}TAM2}$, respectively (Engling et al., 2002).

FACS

$HeLa_{MCAT\text{-}TAM2}$ and $CHO_{MCAT\text{-}TAM2}$ were cultured according to standard procedures. Further details are given in the corresponding figure legends. In order to detach the cells from the culture plates without using protease-based protocols, cell-dissociation buffer (Life Technologies) was used to generate a cell suspension devoid of cell aggregates. Cell-surface antigens were detected with the primary antibodies indicated, followed by decoration with secondary antibodies coupled to either Alexa-488 or allophycocyanine (APC; Molecular Probes), respectively. Antibody incubations were performed on a rotating wheel for 1 hour at 4°C (primary antibody) and 30 minutes at 4°C (secondary antibody), respectively. Wash procedures were carried out by sedimenting the cells at 200 ***g*** for 3 minutes at 4°C. Where indicated, propidium iodide (1 μg/ml) was added prior to the FACS analysis in order to detect damaged cells.

Flow cytometric measurements were performed using a Becton Dickinson FACSCalibur system. Autofluorescence was determined by measuring trypsinized cells that were otherwise treated identically compared with the positive controls. Alexa-488-derived and APC-derived fluorescence can be measured simultaneously on a FACSCalibur two-laser system without the need of channel compensation.

Confocal microscopy

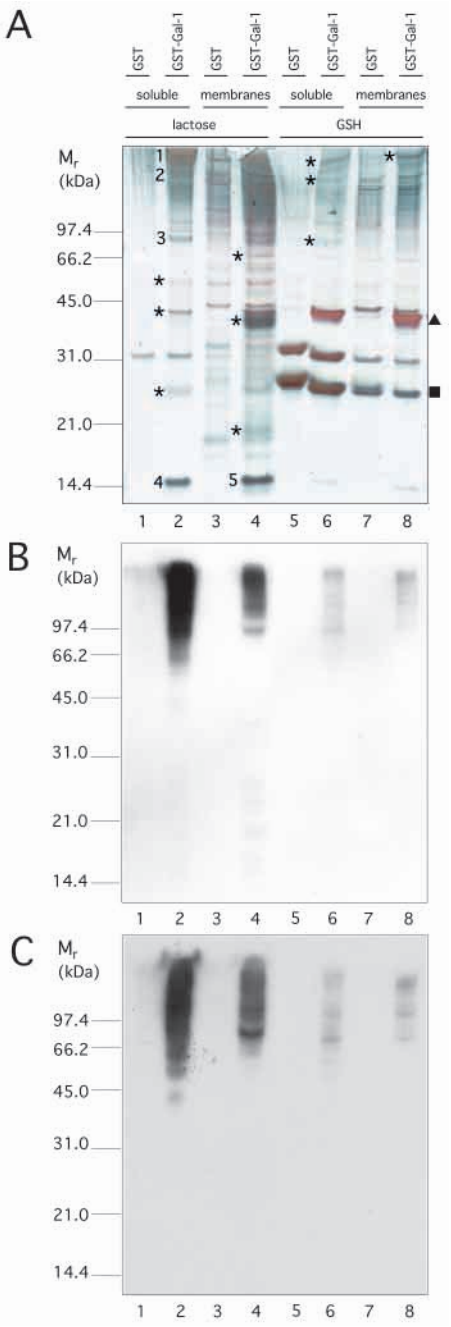
Cells were grown on glass cover slips to about 75% confluency. Following a wash with PBS, the cells were further processed by paraformaldehyde fixation (3% w/v, 20 minutes at 4°C), with or without permeabilization, employing 0.5% (w/v) Triton X-100. Antibody processing was achieved as indicated in the corresponding figure legends. For double-staining procedures, Alexa-488- and Alexa-546-coupled secondary antibodies (Molecular Probes) were used in all experiments. The specimens were mounted in Fluoromount G (Southern Biotechnology Associates) and viewed with a Zeiss LSM 510 confocal microscope.

Results

Identification of CA125 as a galectin-1 counter receptor

To search for ligands of human galectin-1, we attached a recombinant GST–galectin-1 fusion protein to glutathione sepharose beads in order to prepare an affinity matrix. As a source for galectin-1-binding proteins, HeLa cells were fractionated into a soluble and a membrane fraction (see above). Following dilution to about 0.25 mg/ml of total protein, these fractions were incubated with the galectin-1 affinity matrix. Bound proteins were eluted sequentially using lactose (Fig. 1A, lanes 1-4) and glutathione (Fig. 1A, lanes 5-8). This procedure allowed us to collect proteins that interact with the galectin-1 matrix in a galactose-dependent manner, followed by the elution of proteins bound to the matrix by a galactose-independent mechanism. As shown in Fig. 1A, several specifically bound proteins (some of which have been labeled with an asterisk) could be identified in lanes 2 (GST–galectin-1 matrix; soluble HeLa fraction; elution with lactose), 4 (GST–galectin-1 matrix; HeLa membrane fraction; elution with lactose), 6 (GST–galectin-1 matrix, soluble HeLa fraction, elution with glutathione) and 8 (GST–galectin-1 matrix, HeLa membrane fraction, elution with glutathione) when compared with the corresponding GST control matrices (lanes 1, 3, 5 and 7). GST (■; lanes 5 and 7) and GST–galectin-1 (▲; lanes 6 and 8) were co-eluted upon treatment with glutathione. Using mass spectrometry, protein band #2 was identified as a chondroitin sulfate proteoglycan, band #3 was identified as the cell adhesion molecule L1-CAM, and bands 4 and 5 were identified as galectin-1. These results suggest that recombinant galectin-1 and HeLa-derived galectin-1 form a dimer that is disassembled in the presence of lactose.

In addition to these known interaction partners of galectin-1, we identified 16 tryptic peptides from protein band #1 whose masses were consistent with corresponding tryptic fragments of a potential ORF defined by cDNA clone AK024365 (NCBI database; Fig. 1D, boxed sequences indicate peptides identified by mass spectrometry). In order to verify whether band #1 is the product of a gene corresponding to AK024365, we generated a polyclonal antiserum against an artificial recombinant protein corresponding to the N-terminal part (amino acids 1-356; M_r: ~39 kDa) of AK024365. As shown in Fig. 1B, immunoreactive material with a broad high-molecular-weight migration behavior was detected in lanes 2, 4, 6 and 8, which correspond to the various eluates (see above) of the GST–galectin-1 affinity matrix. No signal could be observed under control conditions. Binding of immunoreactive material to galectin-1 appeared to be mediated by a galactose–lectin interaction as more than 90% (determined by



D
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LFTINFTITNLRYEENMHHPGSRKFNTTER
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RNEPLTGNSDLPFWAVIFIGLAGLLGLITC
LICGVLVTTRRRKKEGEYNVQQQCPGYYQS
HLDLEDLQ

Fig. 1. Identification of CA125 as a counter receptor of galectin-1. (A) Affinity purification of galectin-1-interacting proteins. Both soluble (lanes 1, 2, 5, 6) and membrane (lanes 3, 4, 7, 8) fractions of HeLa cells were incubated with either GST–galectin-1 beads (lanes 2, 4, 6, 8) or GST beads as a control (lanes 1, 3, 5, 7). Bound proteins were eluted sequentially with lactose (lanes 1-4) and glutathione (lanes 5-8), followed by separation on Novex NuPage 10% Bis-Tris gels. Protein bands were visualized using SilverQuest. (B) Immunoblot analysis of the proteins eluted from the GST–galectin-1 and GST matrices, respectively. The fractions were loaded in the same order as shown in panel A. The anti-CA125 antibody OC125 was used as primary antibody followed by detection by electrochemiluminescence. (C) Immunoblot analysis as shown in panel B employing an anti-CA125-C-TERM$_{1-356}$ antiserum for the detection of CA125-derived fragments. (D) Amino acid sequence of CA125-C-TERM. Boxed sequences indicate tryptic peptides derived from band 1 (panel A, lane 2) as identified by mass spectrometry.

a quantitative analysis using Bio-Rad® QuantityOne® Software) eluted upon treatment of the affinity matrix with lactose. About 80% of the total immunoreactive material was recovered from the soluble fraction, with the remaining population derived from the membrane fraction.

More recently, the AK024365 ORF was found to represent a C-terminal fragment of 1148 amino acids in length of a giant mucin-like glycoprotein (O'Brien et al., 2001; Yin and Lloyd, 2001). Molecular cloning of the corresponding gene revealed that this new mucin is identical to the ovarian cancer antigen CA125, a putative integral membrane protein present on the cell surface of tumor cells that has originally been defined by the mAb OC125 (Bast et al., 1981). Therefore, we analyzed the various eluates from the galectin-1 affinity matrix with regard to immunoreactivity based on OC125. As shown in Fig. 1C, the pattern of immunoreactive bands detected with OC125 is strikingly similar to the pattern detected with the polyclonal anti-AK024365 antiserum (from now on referred to as anti-CA125-C-TERM$_{1-356}$ antiserum) described above (Fig. 1B). Since CA125 was reported to represent an integral membrane protein with a single membrane span that is cleaved in the extracellular domain in order to release soluble fragments, we conclude that the pattern of immunoreactive bands eluted from the galectin affinity matrix represents both soluble and membrane-anchored fragments of CA125. From now on, the 1148 amino acid, C-terminal part of CA125 (defined by cDNA clone AK024365) will be termed CA125-C-TERM.

Specificity of CA125-mediated galectin binding

In order to analyze whether CA125 preferentially binds to certain β-galactoside-specific lectins, we compared CA125 binding efficiency for galectin-1 with that for galectin-3, the second most prominent member of this protein family of lectins (Barondes et al., 1994; Perillo et al., 1998; Hughes, 1999; Rabinovich et al., 2002). As demonstrated in Fig. 2, HeLa-derived fragments of CA125 bind to galectin-1 twice as efficiently compared with galectin-3 (Fig. 2A,B; compare lanes 1 and 2 as well as 3 and 4, respectively). This difference is significant because comparable amounts of galectin-1 and galectin-3 fusion proteins were used (Fig. 2A, compare lanes 9 and 10) and because the total patterns of lactose-eluted molecules reveal proteins that specifically bind to galectin-1 (labeled with ●), galectin-3 (labeled with ▲), as well as proteins that bind equally to both galectin-1 and galectin-3 (labeled with ■). This demonstrates that differential binding efficiencies can be detected under the experimental conditions applied (Fig. 2C).

When CA125-C-TERM was expressed in both HeLa and CHO cells by retroviral transduction, a more defined protein band was observed (Fig. 3). Interestingly, CA125-C-TERM retains the ability of full-length CA125 to bind galectin-1, an observation consistent with the fact that the translation product of this construct contains both the stalk domain of CA125 [which is supposed to represent the part of the full-length CA125 molecule that contains the majority of N-linked sugars (O'Brien et al., 2001)] and almost three CA125 repeat structures that are heavily O-glycosylated (O'Brien et al., 2001). Intriguingly, CA125-C-TERM expressed in HeLa cells exhibits the same characteristics as endogenous full-length CA125 with regard to galectin interactions as it binds galectin-1 about twice as efficiently as galectin-3 (Fig. 3). By contrast, CA125-C-TERM expressed in CHO cells binds galectin-1 more than seven times as efficiently as galectin-3 (Fig. 3). These results demonstrate that, besides N- and/or O-linked sugar moieties of CA125, the proteinaceous core structure of CA125 contributes to the specificity of galectin recruitment. Moreover, we have established cell-type-dependent galectin-binding characteristics of CA125.

In order to provide evidence for a direct interaction between CA125-C-TERM and galectin-1, crosslinking experiments were conducted where CA125-C-TERM bound to GST–galectin-1 beads was treated with the crosslinking reagent disuccinimidyl glutarate (DSG, Pierce) (Fig. 3C). Following elution with SDS sample buffer, crosslinking products (labeled with a square bracket) with an apparent molecular mass of about 160-180 kDa (corresponding to the approximate molecular mass of CA125-C-TERM plus GST–galectin-1 in a 1:1 complex) can be detected that react with both anti-galectin-1 and anti-CA125 antibodies. The products display a smear-like appearance as expected for a glycoprotein-containing crosslinking product and can only be observed in the presence of DSG. Larger crosslinking products (>180 kDa), which would be indicative of an indirect interaction of CA125-C-TERM with the galectin-1 affinity matrix, cannot be observed.

CA125-C-TERM binding to galectin-1 largely depends on O-linked β-galactose-terminated oligosaccharide chains

To characterize further the molecular mechanism of galectin-1

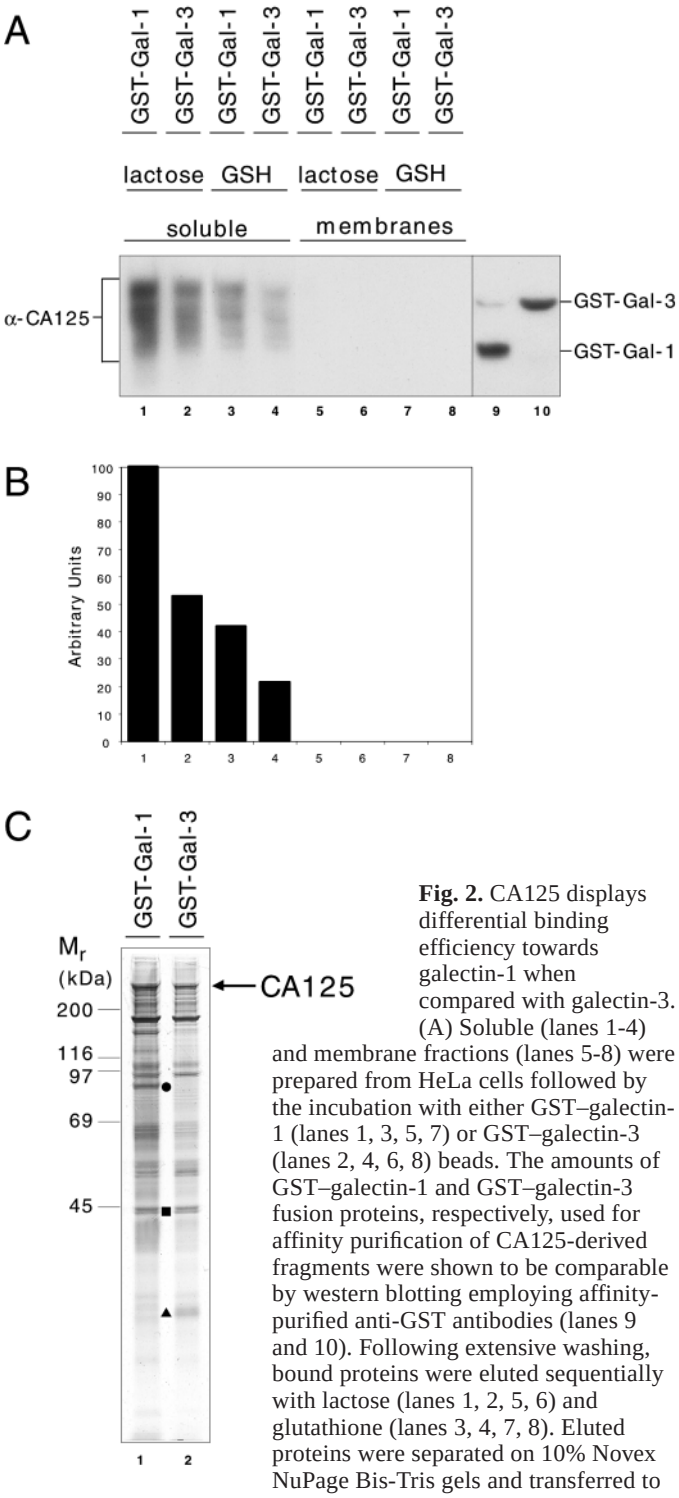


Fig. 2. CA125 displays differential binding efficiency towards galectin-1 when compared with galectin-3. (A) Soluble (lanes 1-4) and membrane fractions (lanes 5-8) were prepared from HeLa cells followed by the incubation with either GST–galectin-1 (lanes 1, 3, 5, 7) or GST–galectin-3 (lanes 2, 4, 6, 8) beads. The amounts of GST–galectin-1 and GST–galectin-3 fusion proteins, respectively, used for affinity purification of CA125-derived fragments were shown to be comparable by western blotting employing affinity-purified anti-GST antibodies (lanes 9 and 10). Following extensive washing, bound proteins were eluted sequentially with lactose (lanes 1, 2, 5, 6) and glutathione (lanes 3, 4, 7, 8). Eluted proteins were separated on 10% Novex NuPage Bis-Tris gels and transferred to a blotting membrane. Electrochemiluminescence detection of CA125-derived fragments was performed employing the mAb OC125. (B) Quantitative analysis of CA125-derived fragments in the fractions shown in panel A employing Bio-Rad® QuantityOne® Software. (C) Total protein pattern of lactose-eluted proteins derived from the galectin-1 matrix (lane 1) and the galectin-3 matrix (lane 2). Eluted proteins were separated on NuPage Bis-Tris gels followed by silver staining according to standard procedures. Labels indicate examples for proteins that preferentially bind to galectin-1 (●), galectin-3 (▲) or proteins that equally bind galectin-1 and galectin-3 (■).

binding to CA125-C-TERM, we conducted interaction studies using cell lysates derived from CA125-C-TERM-expressing CHO cells grown in the presence of tunicamycin (Fig. 4). Under control conditions (Fig. 4A, lanes 1-3), about 40% of CA125-C-TERM could be recovered on GST–galectin-1 beads as calculated based on the input amount shown in lane 1 of Fig. 4. This value was set to 100% (Fig. 4B) and compared with the galectin-1-binding efficiency of CA125-C-TERM derived from tunicamycin-treated cells (Fig. 4A, lanes 4-6). As depicted in Fig. 4B, binding efficiency was reduced to about 65% compared with control conditions. When CA125-C-TERM was expressed in $CHO_{clone\ 13}$ cells that are incapable of translocating UDP-galactose into the lumen of the Golgi and, therefore, neither form galactosylated glycoproteins nor galactosylated glycolipids (Deutscher and Hirschberg, 1986), the binding capacity of CA125-C-TERM to GST–galectin-1 was almost abolished (Fig. 4A, lanes 14-16) at about 10% residual binding efficiency (Fig. 4B). It is of note that antigenicity towards OC125 and/or the expression level in both tunicamycin-treated $CHO_{MCAT\text{-}TAM2}$ cells and $CHO_{clone\ 13}$ cells was observed to be lowered compared with untreated $CHO_{MCAT\text{-}TAM2}$ cells. Under all experimental conditions, CA125-C-TERM binding to galectin-1 was established to be specific (Fig. 4B).

In order to analyze whether CA125-C-TERM binding to galectin-1 depends on O-linked galactose-terminated oligosaccharide chains in vivo, we studied binding of exogenously added recombinant GST–galectin-1 to untreated $CHO_{MCAT\text{-}TAM2}$ cells, tunicamycin-treated $CHO_{MCAT\text{-}TAM2}$ cells and $CHO_{clone\ 13}$ cells using FACS (Fig. 5). As expected, untreated $CHO_{MCAT\text{-}TAM2}$ cells displayed a high galectin-1-binding activity (Fig. 5, dark-blue curve), which was not saturated under the conditions used. This binding activity was significantly reduced when cells were pre-treated with tunicamycin (Fig. 5, red curve). As expected, GST–galectin-1 binding to the cell surface was almost abolished in $CHO_{clone\ 13}$ cells (Fig. 5, dark green curve), which allowed us to determine whether expression of CA125-C-TERM under these conditions (i.e. in a background deficient for galactosylation of glycoproteins and glycolipids) is capable of binding galectin-1. As demonstrated in Fig. 5, CA125-C-TERM cell-surface expression (data not shown, see also next section) does not alter cell-surface binding capacity for galectin-1 (Fig. 5, compare the dark green and the light green curves), demonstrating that galectin-1 binding to CA125-C-TERM requires galactosylation. The combined data shown in Figs 3, 4 and 5 suggest that the interaction between CA125-C-TERM and galectin-1 is direct.

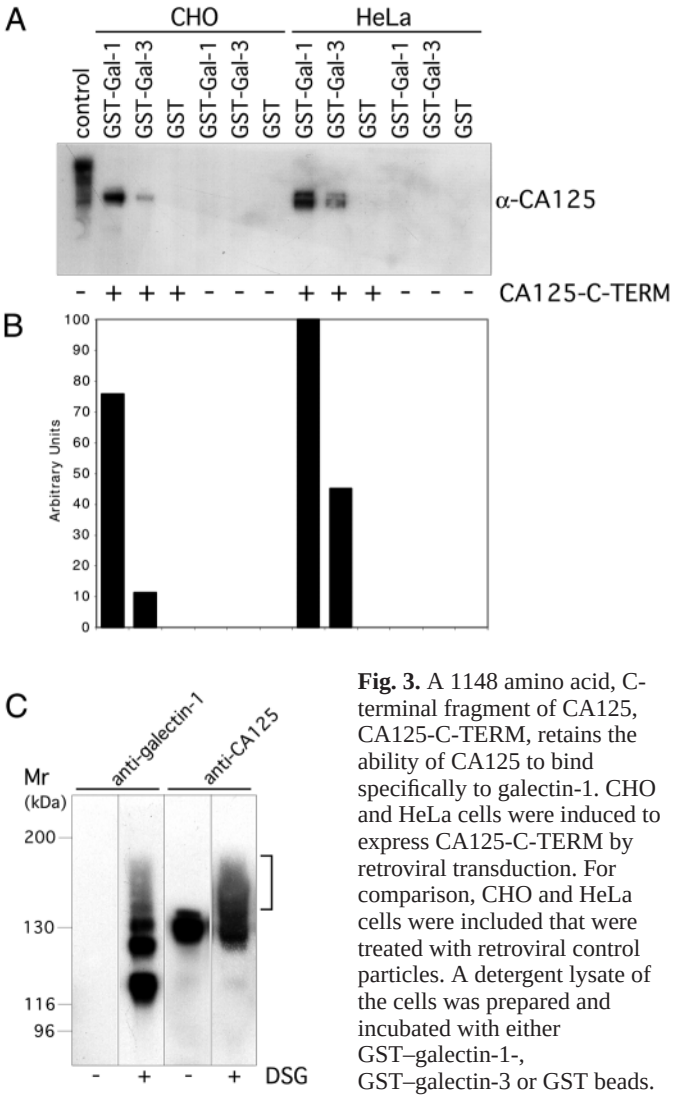
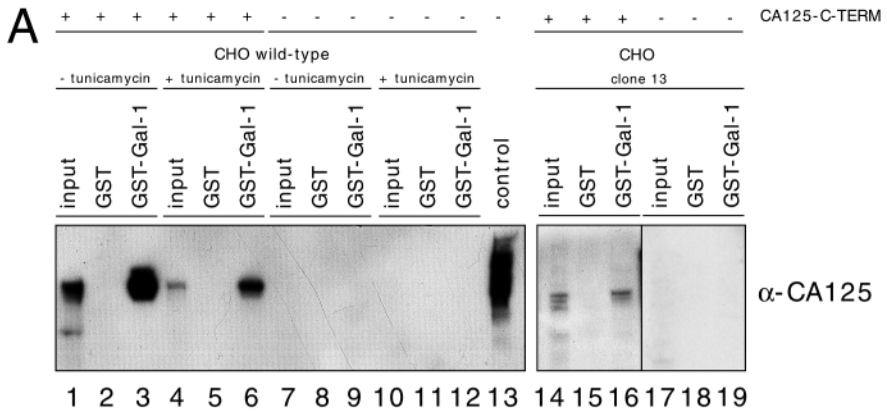


Fig. 3. A 1148 amino acid, C-terminal fragment of CA125, CA125-C-TERM, retains the ability of CA125 to bind specifically to galectin-1. CHO and HeLa cells were induced to express CA125-C-TERM by retroviral transduction. For comparison, CHO and HeLa cells were included that were treated with retroviral control particles. A detergent lysate of the cells was prepared and incubated with either GST–galectin-1-, GST–galectin-3 or GST beads. Following extensive washing, the beads were treated with lactose. Eluted proteins were separated on 10% Novex NuPage Bis-Tris gels followed by transfer to a blotting membrane. CA125-C-TERM was then detected by OC125 staining using electrochemiluminescence (A). For comparison, the pattern of CA125-derived fragments isolated from HeLa cells is shown in the leftmost lane (control). (B) The intensity of CA125-C-TERM-derived bands was quantified using Bio-Rad® QuantityOne® software. (C) A crosslinking experiment is shown employing disuccinimidyl glutarate (DSG). CA125-C-TERM-expressing CHO cells were lysed with detergent followed by incubation of the cell-free supernatant with GST–galectin-1 beads. After extensive washing, DSG was added at a final concentration of 0.5 mM. Crosslinking products were eluted with SDS sample buffer and analysed by SDS-PAGE and western blotting employing affinity-purified anti-galectin-1 and monoclonal anti-CA125 antibodies. The square bracket indicates crosslinking products with an apparent molecular mass of about 160-180 kDa positive for galectin-1 and CA125. In the range of 120-130 kDa, other galectin-1-containing crosslinking products are observed.

Despite lacking a signal peptide, CA125-C-TERM is transported to the cell surface of CHO and HeLa cells

Endogenous CA125 is expressed on the cell surface of tumor cells (Bast et al., 1981). However, based on available primary structure information (O'Brien et al., 2001; Yin and Lloyd, 2001), an obvious signal peptide is not present at the N-terminus of either full-length CA125 or CA125-C-TERM. In order to initiate studies on the molecular mechanism of CA125 cell-surface expression, we first asked whether the C-terminal fragment of CA125 (CA125-C-TERM) used in this study is also able to reach the cell surface. CA125-C-TERM was expressed in $MCAT^{+}$ CHO and HeLa cells (Engling et al., 2002) using retroviral transduction. Cell-surface expression was assessed by a FACS analysis using the monoclonal anti-CA125 antibody OC125 (Fig. 6). Autofluorescence of CHO



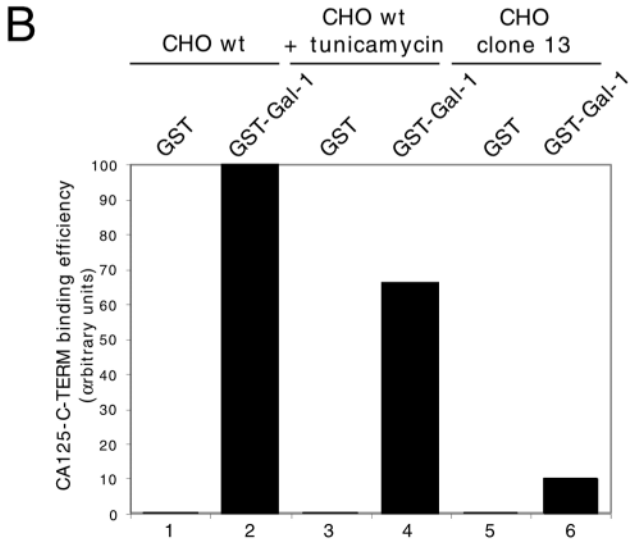


Fig. 4. Galectin-1 binding to CA125-C-TERM largely depends on O-linked β-galactose-terminated oligosaccharide chains. $CHO_{MCAT\text{-}TAM2}$ (wild-type background with regard to galactosylation of both proteins and lipids) and $CHO_{clone\ 13}$ cells (deficient with regard to galactosylation of both proteins and lipids; (Deutscher and Hirschberg, 1986) stably expressing CA125-C-TERM were used to prepare cell-free detergent lysates followed by incubation with GST and GST–galectin-1 beads, respectively. Where indicated, $CHO_{MCAT\text{-}TAM2}$ were treated with 10 μg/ml tunicamycin for 18 hours at 37°C prior to cell lysis. In each experiment, the CA125-C-TERM signal derived from 0.2% of the input was compared with 4% of the material bound to either GST or GST–galectin-1 beads. Protein samples were separated on NuPage Bis-Tris gels followed by CA125-C-TERM immunoblotting employing the mAb OC125. (A) Lysates derived from CA125-C-TERM-expressing $CHO_{MCAT\text{-}TAM2}$ cells (lanes 1-3), CA125-C-TERM-expressing $CHO_{MCAT\text{-}TAM2}$ cells treated with tunicamycin (lanes 4-6), CA125-C-TERM-deficient $CHO_{MCAT\text{-}TAM2}$ cells (lanes 7-9), CA125-C-TERM-deficient $CHO_{MCAT\text{-}TAM2}$ cells treated with tunicamycin (lanes 10-12), CA125-C-TERM-expressing $CHO_{clone\ 13}$ cells (lanes 14-16) and CA125-C-TERM-deficient $CHO_{clone\ 13}$ cells (lanes 17-19). In lane 13, HeLa-derived CA125 eluted from GST–galectin-1 beads is shown as a control. (B) Quantification of the results shown in panel A. On the basis of the input signal (0.2% of starting material; panel A, lane 1), about 40% of CA125-C-TERM present in the cell lysate is recovered on GST–galectin-1 beads under the conditions used (panel A, lane 3, 4% of eluate), based on quantification employing Bio-Rad® QuantityOne® software. This value was set to 100% binding efficiency and compared with CA125-C-TERM–galectin-1 binding efficiencies measured with lysates either derived from tunicamycin-treated $CHO_{MCAT\text{-}TAM2}$ cells or from $CHO_{clone\ 13}$ cells. The results shown represent mean values of two independent experiments.

(Fig. 6A) and HeLa cells (Fig. 6B) was determined using trypsin-treated cells (red curves). Whereas CHO cells treated with retroviral control particles did not present endogenous CA125 on their cell surface (A, green curve), HeLa cells treated under identical conditions did contain small but significant amounts of endogenous CA125 on their surface (B, green curve). Upon retroviral expression of CA125-C-TERM, cell-surface staining strongly increased for both CHO and HeLa cells (Fig. 6A,B; blue curves). In the case of CHO cells, retroviral transduction with CA125-C-TERM was more than 90% efficient whereas about 60% of HeLa cells were found to be transduced under the conditions applied. The vast majority of this signal disappeared when cells were treated with trypsin prior to the FACS analysis (data not shown). Therefore, despite lacking a conventional signal peptide at the N-terminus, CA125-C-TERM is transported to the cell surface.

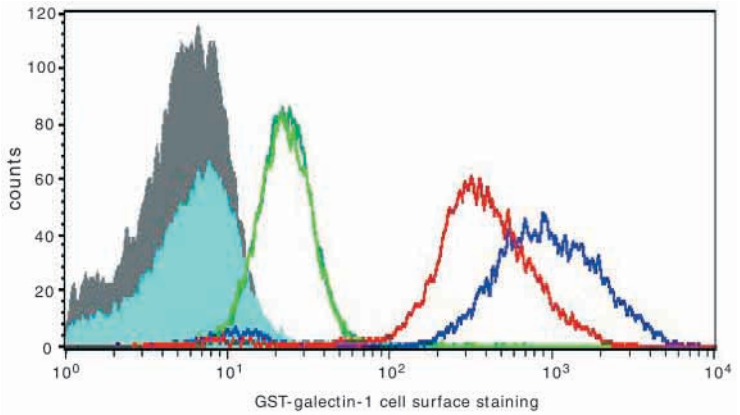


Fig. 5. CA125-C-TERM cell-surface expression in $CHO_{clone\ 13}$ cells does not result in increased binding capacity for exogenously added galectin-1. CA125-C-TERM-expressing and CA125-C-TERM-deficient $CHO_{MCAT\text{-}TAM2}$ and $CHO_{clone\ 13}$ cells were grown to 70% confluency. Where indicated, cells were treated with 10 μg/ml tunicamycin for 18 hours at 37°C. Cells were then dissociated from the culture plates followed by incubation with 40 μg/ml recombinant GST–galectin-1 for 30 minutes at room temperature. Following labeling with affinity-purified anti-galectin-1 antibodies under native conditions, the various samples were analyzed for cell-surface-bound recombinant galectin-1 using FACS. Autofluorescence (filled light-blue curve: $CHO_{MCAT\text{-}TAM2}$; filled gray curve: $CHO_{clone\ 13}$) was determined based on cells not treated with antibodies. Untreated $CHO_{MCAT\text{-}TAM2}$ cells are shown in dark blue. Tunicamycin-treated $CHO_{MCAT\text{-}TAM2}$ cells are shown in red. $CHO_{clone\ 13}$ cells are shown in dark green (CA125-C-TERM-expressing) and light green (CA125-C-TERM-deficient), respectively.

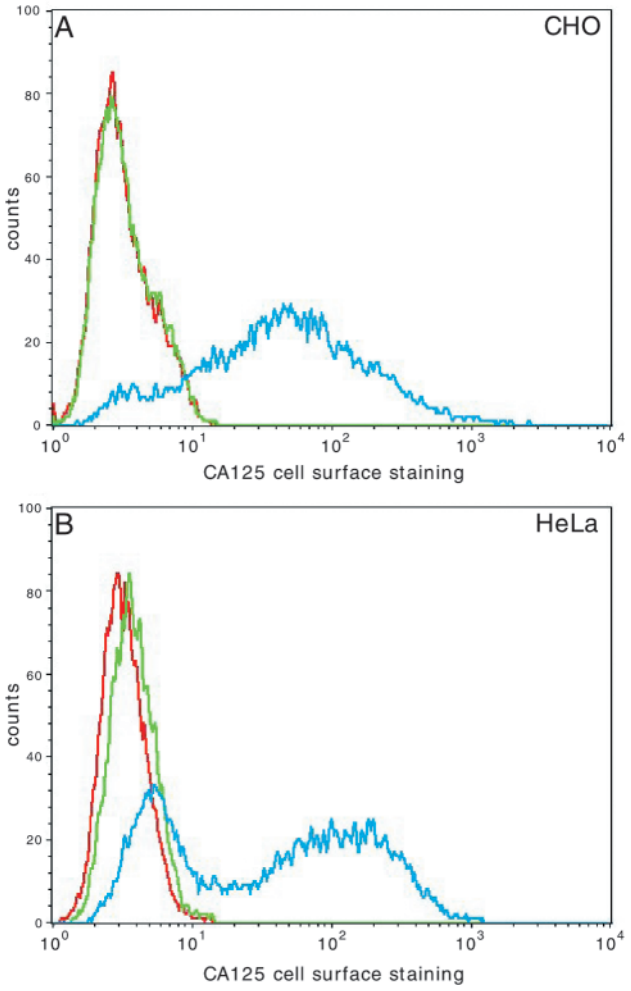


Fig. 6. CA125-C-TERM is transported to the cell surface of both CHO and HeLa cells as determined by FACS. $CHO_{MCAT\text{-}TAM2}$ (A) and $HeLa_{MCAT\text{-}TAM2}$ (B) cells, respectively, were transduced with retroviral particles encoding CA125-C-TERM. Following 3 days of incubation at 37°C, cells were dissociated from the culture plates using a protease-free buffer and processed with anti-CA125 antibodies (OC125). Primary antibodies were detected with anti-mouse antibodies coupled to Alexa-488. CA125 cell-surface localization was analyzed by FACS. Autofluorescence was determined with trypsin-treated cells (red curves). Non-transduced cells prepared in the absence of trypsin are indicated by green curves. CA125-C-TERM-transduced cells prepared in the absence of trypsin are indicated by blue curves.

CA125-C-TERM is transported to the cell surface via the ER/Golgi-dependent secretory pathway

To analyze whether CA125-C-TERM enters the classical (i.e. ER/Golgi-dependent) secretory route or whether it, in a similar manner to the galectins, makes use of a so-far-uncharacterized nonclassical secretory pathway we determined its subcellular distribution in permeabilized CHO and HeLa cells using confocal microscopy (Figs 7, 8). In nonpermeabilized cells (Fig. 7A-D), CA125-C-TERM was detected on the cell surface of both CHO (B) and HeLa (D) cells. Specificity of the observed staining pattern was established using retroviral control particles (A and B, respectively). CA125-C-TERM cell-surface staining was found not to be homogenous but rather appeared in subdomains with significant parts of the plasma membrane not stained at all.

Permeabilization of HeLa cells prior to anti-CA125 antibody treatment revealed that CA125-C-TERM expression results in its incorporation into membranes of the classical secretory pathway (Figs 7, 8). At low magnification, intracellular CA125-C-TERM could be detected in a perinuclear region (Fig. 7E-H), where it was colocalized with the Golgi marker p27 (Füllekrug et al., 1999; Jenne et al., 2002). Again, this signal was established to be specific as it could not be observed when cells were treated with retroviral control particles (Fig. 7E). Additionally, high-resolution confocal microscopy revealed CA125-C-TERM$^+$ staining of the nuclear envelope (Fig. 8B,D), which is indicative for ER localization. This was confirmed by double-labeling experiments using antibodies directed against the ER marker calreticulin (Fig. 8A) (Sönnichsen et al., 1994). Whereas most of the calreticulin staining was found to be ER associated, only low amounts of CA125-C-TERM were found in the ER compared with high amounts in the Golgi (Fig. 8, compare A and B). These results indicate that, following insertion into the ER membrane, CA125-C-TERM is efficiently transported in an anterograde direction from the ER to the Golgi. In order to rule out the possibility that CA125-C-TERM$^+$ perinuclear structures represent endosomal compartments localized at the microtubule organizing center, we treated CA125-C-TERM-expressing HeLa cells with brefeldin A, a drug that disrupts the Golgi apparatus and, therefore, inhibits biosynthetic secretory transport (Lippincott-Schwartz et al., 1989; Orci et al., 1991). As shown in Fig. 8F, the compact perinuclear staining of CA125-C-TERM (Fig. 8D) disappears following brefeldin A treatment. The resulting staining pattern matches brefeldin A-induced redistribution of an established marker protein of the cis-Golgi, the KDEL receptor (Fig. 8C,E) (Lewis and Pelham, 1990; Lewis and Pelham, 1992; Füllekrug et al., 1997). These results provide proof for the presence of CA125-C-TERM in the ER and the Golgi apparatus.

In order to characterize functionally the mode of intracellular transport of CA125-C-TERM, we conducted in vivo cell-surface expression experiments in the presence or absence of brefeldin A based on FACS (Fig. 9). CA125-C-TERM-expressing HeLa cells were grown to about 70% confluency, followed by incubation for 90 minutes in the presence of brefeldin A. The cells were then trypsinized to remove pre-existing cell-surface CA125-C-TERM, spread onto new culture plates at the same cell density and were then further incubated in the presence or absence of brefeldin A for 4 hours at 37°C. As a control, cells were applied to the same protocol without adding brefeldin A at any time point of the experiment. The amount of CA125-C-TERM transported to the cell surface within 4 hours in the absence of brefeldin A was set to 100% (Fig. 9A, light green curve; Fig. 9B, lane 2). When compared with the level of cell-surface CA125-C-TERM under steady-state conditions (Fig. 9A, red curve; Fig. 9B, lane 1), more than 50% of the cell-surface population recovers after trypsinization within 4 hours of incubation (Fig. 9B, lane 2). When cells were treated with brefeldin A before trypsinization, followed by incubation for 4 hours in the absence of brefeldin A, the level of cell-surface CA125-C-

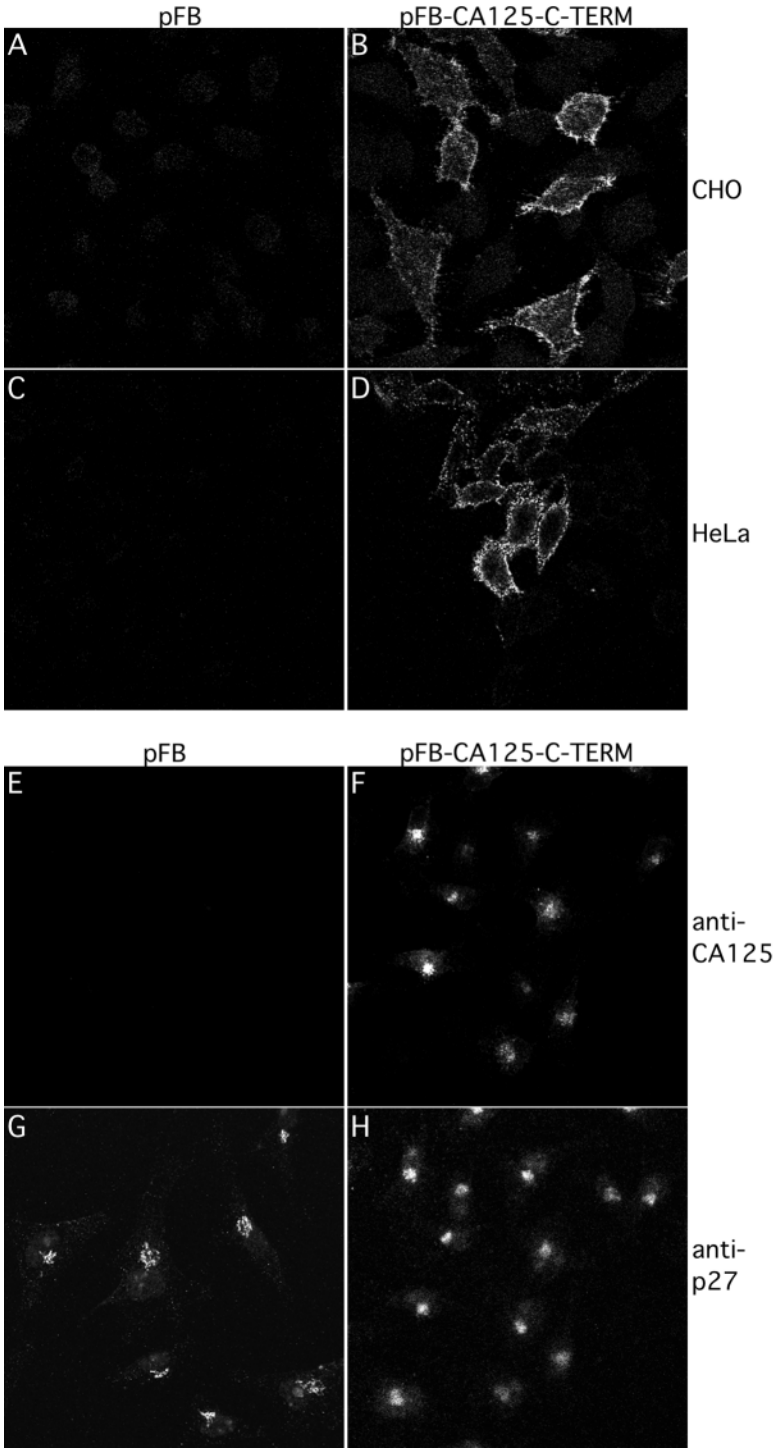


Fig. 7. CA125-C-TERM is transported to the cell surface of both CHO and HeLa cells as determined by confocal microscopy. $CHO_{MCAT\text{-}TAM2}$ and $HeLa_{MCAT\text{-}TAM2}$ cells, respectively, were grown on glass cover slips followed by transduction with retroviral particles encoding CA125-C-TERM or with retroviral control particles that lack a cDNA insert in the viral genome. After 3 days of incubation at 37°C, the cells were fixed with paraform aldehyde. Specimens shown in A-D represent $CHO_{MCAT\text{-}TAM2}$ cells that were not permeabilized to visualize exclusively cell-surface-localized CA125-C-TERM. Specimens shown in E-H represent Triton X-100-permeabilized $HeLa_{MCAT\text{-}TAM2}$ cells to detect intracellular CA125-C-TERM. CA125-C-TERM was visualized with the mAb OC125 (A, B, E-H). The Golgi marker p27 was detected with a polyclonal rabbit antiserum directed against a synthetic peptide that corresponds to the cytoplasmic tail of p27 (C and D) (Jenne et al., 2002). Double staining was performed using secondary antibodies coupled to Alexa-488 and Alexa-546, respectively. Specimens were analyzed with a Zeiss LSM510 confocal microscope.

TERM was reduced by about 60% (Fig. 9A, dark green curve; Fig. 9B, lane 3). When cells were treated with brefeldin A throughout the course of the experiment, cell-surface transport of CA125-C-TERM was reduced by about 90% (Fig. 9A, blue curve; Fig. 9B, lane 4).

These data combined with the morphological analysis presented in Fig. 8 establish that CA125-C-TERM is transported to the cell surface via conventional secretory transport involving the ER and the Golgi apparatus.

Correlation of endogenous CA125 expression with increased cell-surface expression of endogenous galectin-1 in CHO and HeLa cells

Our observation that CHO cells do not express detectable amounts of endogenous CA125 as opposed to HeLa cells (Fig. 6) is consistent with the fact that CHO cells are not derived from a tumor (Puck et al., 1958), whereas HeLa cells were isolated from a cervix carcinoma (Gey et al., 1952). Therefore, we were interested to compare CA125-deficient CHO cells with CA125-expressing HeLa cells for various parameters with regard to galectin-1. Using FACS analysis to analyze CHO and HeLa cells for the amount of cell-surface expression of galectin-1, we found that HeLa cells contain more than ten times as much galectin-1 on their surface compared with CHO cells (Fig. 10). For this purpose, autofluorescence of CHO and HeLa cells was determined with trypsin-treated cells and adjusted to the same value for both cell lines (Fig. 10A,B; red curves). Employing an affinity-purified anti-galectin-1 rabbit antiserum, a relatively small but significant population of endogenous galectin-1 (A; green curve) could be detected on the surface of CHO cells when the cells were not treated with trypsin prior to the FACS analysis. This observation is consistent with various studies that demonstrated cell-surface expression of endogenous galectin-1 in CHO cells (Cho and Cummings, 1995; Lutomski et al., 1997). However, HeLa cells that do express endogenous CA125 in appreciable amounts contain more than ten times the amount of endogenous galectin-1 on their surface (B; blue curve) compared with CA125-deficient CHO cells.

We next investigated whether this effect was due to (1) different total galectin-1 expression levels, (2) different cell-surface binding capacities for galectin-1 or (3) different regulation of galectin-1 export in CHO and HeLa cells, respectively. As shown by a western blot analysis (Fig. 1C), similar signals for galectin-1 were obtained from CHO and HeLa cells when the amount of SDS-lysed cells was titrated

(20,000, 50,000 and 150,000 cells, respectively) and analyzed with affinity-purified anti-galectin-1 antibodies. Thus, CHO and HeLa cells do not differ to a significant extent in the total amount of galectin-1 expression. Cell-surface binding capacity for galectin-1 was analyzed using FACS by titrating increasing amounts of a recombinant GST–galectin-1 fusion protein into cultures of CHO and HeLa cells, respectively. The total binding capacity for galectin-1 was found to exceed the amount of endogenous galectin-1 present on the cell surface of CHO and HeLa cells by a factor of more than 50-fold, with CHO cells being the cell type with an even higher galectin-1-binding capacity compared with HeLa cells (data not shown). Therefore, the strikingly different amounts of endogenous cell-surface galectin-1 on CHO versus HeLa cells (Fig. 6A,B) cannot be due to a lower galectin-1-binding capacity of CHO cells.

On the basis of these experimental observations, we conclude that CA125-expressing HeLa cells possess a more active galectin-1 export pathway than CA125-deficient CHO cells. These results are discussed in the following section with regard to the origin of HeLa and CHO cells as tumor- and non-tumor-derived cell lines, respectively.

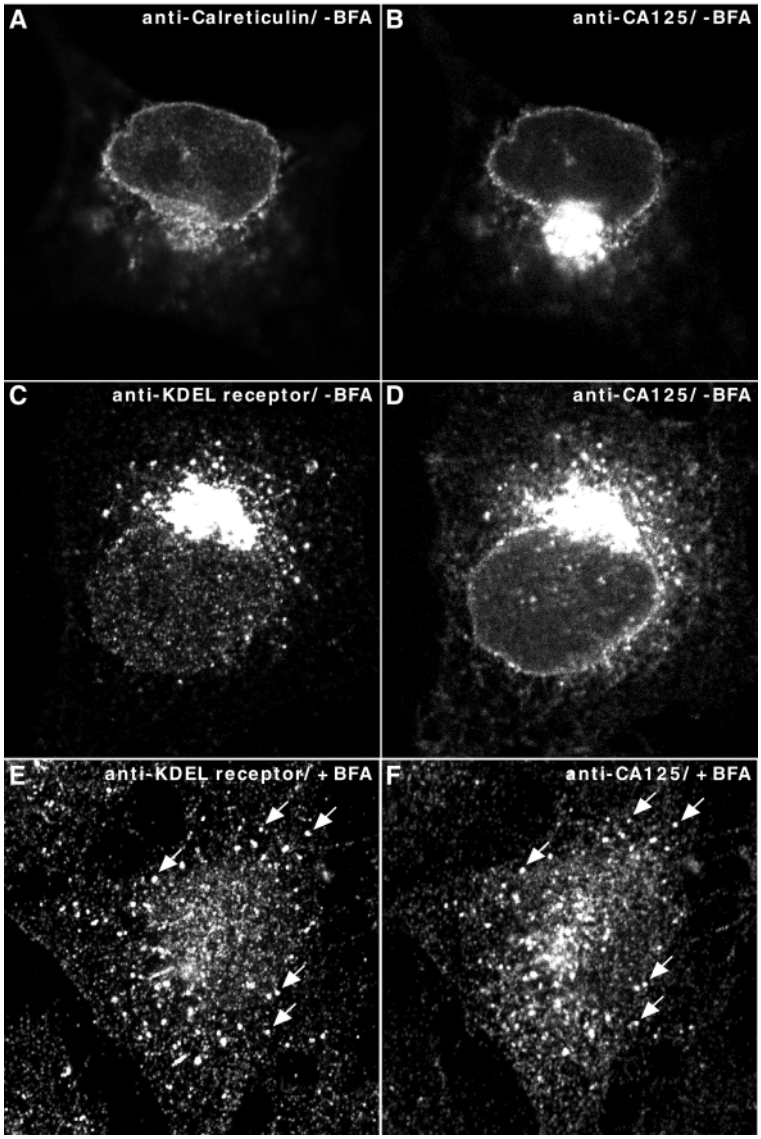


Fig. 8. Intracellular CA125-C-TERM is localized to organelles of the classical ER/Golgi-dependent secretory pathway. $HeLa_{MCAT\text{-}TAM2}$ cells stably expressing CA125-C-TERM were grown on glass cover slips. At about 70% confluency, cells were treated with brefeldin A (5 μg/ml) for 60 minutes or were left untreated as a control. Following fixation with paraform aldehyde, cells were treated with Triton X-100 to allow intracellular staining of antigens using antibodies directed against calreticulin, the KDEL receptor and CA125. Double staining was performed using secondary antibodies coupled to Alexa-488 and Alexa-546, respectively. Specimens were analyzed with a Zeiss LSM510 confocal microscope. (A) Anti-calreticulin, not treated with brefeldin A; (B) anti-CA125, not treated with brefeldin A; (C) anti-KDEL receptor, not treated with brefeldin A; (D) anti-CA125, not treated with brefeldin A; (E) anti-KDEL receptor, treated with brefeldin A; (F) anti-CA125, treated with brefeldin A.

Discussion

The molecular identity and biological function of the CA125 cancer antigen has remained elusive since it was discovered by Bast and colleagues using the ovarian cell line OVCA433 as an immunogen for the generation of mAbs (Bast et al., 1981). From immunological studies, CA125 is known to be present on the cell surface of ovarian cancer cells; however, it has also been found in other carcinomas and, to a limited extent, in normal secretory tissues (O'Brien et al., 1986; Zurawski et al., 1988; Hardardottir et al., 1990). The results of the current study may provide the first insight into a potential biological function of CA125 as we establish a link to a family of β-galactoside-specific lectins of the ECM, the galectins (Barondes et al., 1994; Perillo et al., 1998; Hughes, 1999; Rabinovich et al., 2002). This observation might be of significant biomedical importance as galectins themselves are tumor markers involved in the regulation of cell proliferation and tumor progression (Perillo et al., 1998). In this context, CA125 cell-surface expression by tumor tissue might effect cell attachment to the ECM in a galectin-1-dependent manner.

Recently, two research groups independently succeeded in cloning the gene that encodes CA125 (O'Brien et al., 2001; Yin and Lloyd, 2001), showing that CA125 is a giant mucin-like glycoprotein that consists of more than 11,000 amino acids. Full-length CA125 is suggested to represent a type I transmembrane protein with a single membrane-spanning domain close to the C-terminus. The extracellular domain contains repeat structures that are likely to be heavily O-glycosylated (O'Brien et al., 2001). Besides its putative nature as an integral membrane protein, soluble fragments of CA125 have been observed (Fendrick et al., 1997; Lloyd and Yin, 2001). Apparently, phosphorylation of the cytoplasmic domain causes extracellular cleavage of the N-terminal domain, which results in the release of soluble fragments into the extracellular space (Fendrick et al., 1997; Lloyd and Yin, 2001).

In the current study, we conducted protein–protein

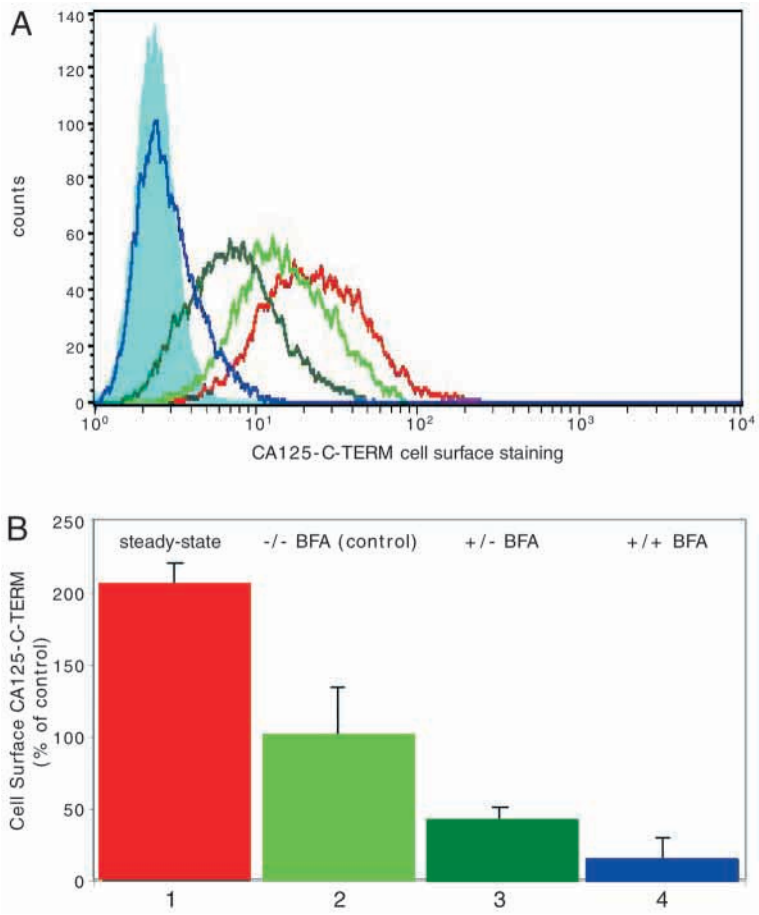


Fig. 9. CA125-C-TERM is transported to the cell surface by the classical ER/Golgi-dependent secretory pathway. $HeLa_{MCAT\text{-}TAM2}$ cells stably expressing CA125-C-TERM were grown to 70% confluency. Where indicated, brefeldin A was added to the medium at 5 μg/ml. Following incubation for 90 minutes at 37°C, cells were trypsinized to remove cell-surface CA125-C-TERM and spread onto new culture plates at 70% confluency. The culture was then continued for 4 hours at 37°C in the presence or absence of brefeldin A, as indicated. CA125-C-TERM transported to the cell surface within this time period was quantified by FACS using the mAb OC125. (A) FACS histograms. Autofluorescence was determined by analyzing cells that were not treated with antibodies (light blue curve, filled). The red curve represents cells under steady-state conditions. The light green curve represents cells that were not treated with brefeldin A. Cells that were grown for 90 minutes in the presence of brefeldin A, followed by incubation for 4 hours in its absence, are shown in dark green. Cells that were incubated with brefeldin A over the whole course of the experiment are shown in dark blue. (B) Statistical analysis of four independent experiments. The colors of the bars correspond to the conditions detailed above.

and protein–carbohydrate interaction studies in order to affinity-purify proteins that interact with galectin-1. Tryptic peptides were analyzed by mass spectrometry, which identified one of the proteins shown to interact specifically with galectin-1 as CA125. This conclusion was drawn from the fact that 16 tryptic peptides could be identified as parts of the translation product of cDNA clone AK024365 (NCBI) that, based on sequence information reported by the laboratories of Lloyd and O'Brien (O'Brien et al., 2001; Yin and Lloyd, 2001), encodes the 1148 C-terminal amino acids of CA125 (CA125-C-TERM). These results were confirmed by immunological identification of CA125-derived antigens by both the original anti-CA125 antibody OC125 (Bast et al., 1981) and a rabbit antiserum directed against the N-terminal 356 amino acids of CA125-C-TERM. Since the majority of the material bound to the galectin-1 affinity matrix was elutable with lactose, we concluded that this interaction is galactose dependent. These data were confirmed by experiments demonstrating that the interaction between CA125-C-TERM and galectin-1 both in vitro and in vivo is almost completely abolished when CA125-C-TERM was expressed in a CHO mutant that is incapable of galactosylation of glycoproteins or glycolipids (Deutscher and Hirschberg, 1986). Interestingly, CA125-C-TERM binding to galectin-1 is only partially inhibited when cells were grown in the presence of tunicamycin, a drug that inhibits N-glycosylation. These data establish that the interaction of CA125-C-TERM with galectin-1 largely depends on O-linked β-galactose-terminated oligosaccharide chains. This conclusion appears to be even more significant under physiological conditions for full-length CA125, which is likely to be characterized by a much higher ratio of O- to N-glycosylation compared with CA125-C-TERM.

When we compared the CA125-C-TERM binding efficiency of galectin-1 with that of galectin-3, the second most-abundant member of the galectin family, we found that galectin-1 is the primary ligand for CA125. Thus, the interaction observed does not appear to represent a simple carbohydrate–lectin interaction but rather depends on additional aspects of specificity based on the proteinaceous environment. Moreover, as CA125-C-TERM expressed in CHO cells showed an even higher preference towards binding of galectin-1, we conclude that the cellular background in which CA125 is expressed also has a significant impact on its binding specificity for members of the galectin family. On the basis of experiments presented in this study we, therefore, conclude that CA125 represents a specific galectin counter receptor with galectin-1 as the primary ligand. Since CA125 expressions appears to be largely restricted to tumor cells, it appears likely that tumor cell attachment to the ECM can be modulated in a galectin-1-dependent manner.

CA125-C-TERM encodes about three O-glycosylated repeat structures and the N- and O-glycosylated stalk structure of the extracellular domain, the transmembrane span and the cytoplasmic domain of full-length CA125. As discussed above, these structural features are consistent with our finding that CA125-C-TERM retains binding activity towards galectin-1. Intriguingly, expression of this construct in both CHO- and HeLa cells resulted in CA125-C-TERM cell-surface expression. Besides the lack of an N-terminal signal peptide in both full-length CA125 and CA125-C-TERM, we demonstrate that CA125-C-TERM cell-surface expression is mediated by ER/Golgi-dependent secretory transport. Thus, CA125 represents a classical secretory cargo protein whose molecular mechanism of insertion into the membrane of the ER will be interesting to

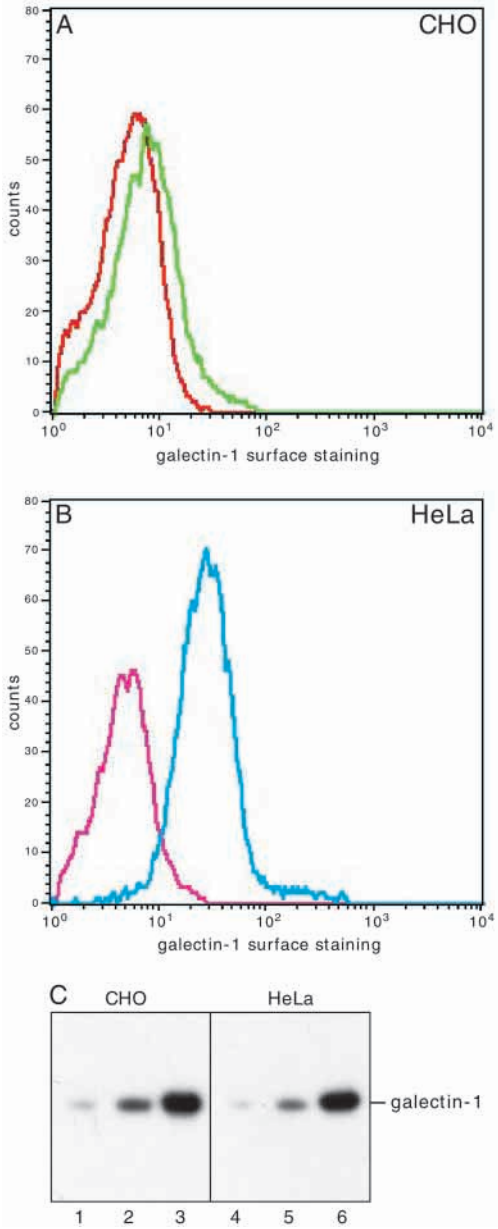


Fig. 10. Correlation of endogenous CA125 expression with cell-surface expression of endogenous galectin-1 in CHO and HeLa cells. CHO (A) and HeLa cells (B) were dissociated from culture plates using a protease-free buffer. Native cells were labeled with affinity-purified anti-galectin antibodies derived from a polyclonal rabbit antiserum. Cell-surface staining was analyzed by FACS using anti-rabbit secondary antibodies coupled to allophycocyanine to detect primary antibodies. Autofluorescence levels (red curves in A and B) were determined with cells treated with trypsin prior to the FACS analysis. Galectin-1 cell-surface levels are indicated in green (CHO; panel A) and blue (HeLa; panel B), respectively. Total galectin-1 expression levels in CHO and HeLa cells, respectively, were analyzed by quantifying galectin-1 in total SDS cell lysates based on a western blot analysis (C). Lanes 1 and 4 represent the material of 20,000 cells, lanes 2 and 5 represent the material of 50,000 cells and lanes 3 and 6 represent the material of 150,000 cells. The results from CHO cells are shown in lanes 1-3, the results from HeLa cells are shown in lanes 4-6. Galectin-1 was detected with an affinity-purified rabbit antiserum directed against recombinant full-length galectin-1.

study in future experiments. As signal-peptide-independent mechanisms of protein insertion into the ER have been described (Kutay et al., 1995), it will also be of interest to analyze how CA125 ER insertion compares with these known processes. Moreover, given its huge size of more than 11,000 amino acids plus mucin-like levels of glycosylation, questions arise about the mode of intracellular transport on its way to the cell surface.

In order to initiate studies investigating the relevance of the interaction reported with regard to a comparison between non-tumor- and tumor-derived cells, we made use of CHO cells, a non-tumor-derived cell line (Puck et al., 1958) and HeLa cells, a cervix carcinoma cell line (Gey et al., 1952; Scherer et al., 1953). While we isolated fragments of endogenous CA125 from the HeLa cell line, we were not able to detect CA125 fragments bound to the galectin-1 affinity matrix when CHO cells were used as starting material (data not shown). This observation is consistent with the detection of endogenous cell-surface CA125 in HeLa cells and the lack of cell-surface CA125 in CHO cells based on flow cytometry. Moreover, these data are in line with studies that suggest that CA125 is expressed primarily in tumor tissue (Bast et al., 1981). Employing a novel in vivo assay that allows us to assess quantitatively galectin-1 export from CHO and HeLa cells by flow cytometry, we have now demonstrated that HeLa cells are characterized by more than tenfold higher levels of cell-surface galectin-1 compared with CHO cells. As we show that CHO and HeLa cells do not differ with regard to total expression levels of galectin-1, as well as cell-surface binding capacity for galectin-1, we conclude that HeLa cells are significantly more active in the non-conventional export of galectin-1 compared with CHO cells.

To this end, the link between CA125 expression by tumor cells concomitant with the increased cell-surface expression of galectin-1 remains correlative and, therefore, future studies must elucidate whether CA125 expression has a direct impact on the non-conventional export route of galectin-1. However, the current study provides the first evidence for a potential functional link between CA125 and galectin-1 which, in the light of the fact that both molecules represent well-characterized tumor markers (Bast et al., 1981; Bast et al., 1983; Bon et al., 1996; Perillo et al., 1998; Rabinovich et al., 2002), might proof to be of significant biomedical importance in the future.

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