The Golgi Protein RCAS1 Controls Cell Surface Expression of Tumor-associated O-Linked Glycan Antigens*

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Tumor immunology has received a large impetus from the identification of tumor-associated antigens. Among them, a monoclonal antibody, 22.1.1, was instrumental in defining a novel tumor-associated antigen that was termed “receptor binding cancer antigen expressed on SiSo cells” (RCAS1). RCAS1 was proposed to induce growth arrest and apoptosis on activated immune cells, mediated by a putative death receptor. Structurally, RCAS1 was predicted to exist as a type II transmembrane protein and in a soluble form. Here, we analyzed occurrence, membrane topology, and subcellular localization of the RCAS1-encoded protein. RCAS1 was shown to be a ubiquitously expressed type III transmembrane protein with a Golgi-predominant localization. Monoclonal antibody 22.1.1 failed to recognize RCAS1, as demonstrated by confocal microscopy. Instead, we showed that the cognate 22.1.1 epitope is identical with the tumor-associated O-linked glycan Tn (N-acetyl-D-galactosamine, GalNAc). Overexpression of RCAS1 in cell lines that are negative for 22.1.1 surface staining led to the generation of Tn and the closely related TF (Thomsen-Friedenreich, Galβ1–3GalNAc) antigen, thus providing a functional link to the generation of the 22.1.1 epitope. We suggest that RCAS1 modulates surface expression of tumor-associated, normally cryptic O-linked glycan structures and contributes indirectly to the antigenicity of tumor cells.

Tumor-associated antigens are structures that allow the cellular or humoral immune system to recognize and eliminate malignant cells. Aberrant carbohydrate epitopes occur on cell surfaces of essentially all types of experimental and human cancers. Among the large number of different tumor-associated glycan antigens that have been described so far (1, 2), the O-linked blood group precursor structures TF1 (Thomsen-Friedenreich) and Tn have gained much interest, because these epitopes are broadly expressed on tumor cells of diverse origin (3). Tn (N-acetyl-D-galactosamine, GalNAc) and TF (Galβ1–3GalNAc) result from incomplete O-glycan synthesis and are normally occluded from the immune system by glycan extension and sialylation (3). However, the mechanisms underlying the occurrence of these truncated glycans in carcinomas are still poorly understood. It has been suggested that an abnormal distribution of glycosyltransferases throughout the exocytic pathway and an altered expression of gene products involved in the assembly of glycans might contribute to the generation of the TF and Tn antigens (1).

Recently, a novel tumor-associated antigen that was attributed an important role in tumor immune interactions, RCAS1, was described (4, 5). Initially, RCAS1 was defined by the 22.1.1 monoclonal antibody (mAb), which was raised by immunization of mice with the human uterine cervical adenocarcinoma cell line SiSo (4). Cell surface staining with mAb 22.1.1 was shown immunohistochemically in a large number of different tumor tissues, and in some tumor entities 22.1.1 staining correlated well with poor clinical prognosis (6–8). A soluble form of the 22.1.1 antigen was purified from the supernatant of cultured SiSo cells (4).

Expression cloning led to the identification of a cDNA apparently encoding the 22.1.1 antigen. The gene product was termed “receptor binding cancer antigen expressed on SiSo cells” (RCAS1) and is identical with the estrogen-responsive protein EBAG9 (5, 9, 10).

Recombinantly expressed RCAS1 was shown to bind to a yet unidentified receptor, which was predominantly expressed on activated immune cells. Cell culture supernatant from SiSo cells inhibited proliferation of activated T cells and induced apoptotic cell death in receptor bearing cells. Therefore, RCAS1 was introduced as a new death receptor ligand involved in tumor immune escape (5). Moreover, it has been suggested that RCAS1 is involved in down-regulation of the maternal immune response during pregnancy (11). Matsumishita et al. (12) demonstrated that RCAS1 expression by bone marrow macrophages is important in the regulation of erythropoiesis by induction of apoptosis in erythroid progenitor cells, thereby extending the role of RCAS1 to a general cell death–inducing system according to the Fas/Fas ligand system (13).

Although RCAS1 seems to be highly conserved in phylogeny (14) and was demonstrated to have important physiological functions, little is known about the gene product itself. Data
bank screens revealed that there are no homologies to any known genes or proteins. According to sequence predictions, RCAS1 has been postulated to be a type II transmembrane (TM) protein with an N-terminal TM region (amino acids 8–27) and an extracellular C-terminal coiled-coil region (amino acids 179–206). Data available on the molecular mass are confusing. Calculations attribute RCAS1 a molecular mass of about 24 kDa, whereas in SDS-PAGE the gene product migrates around 32 kDa (14). According to Sonoda et al. (4, 5), mAb 22.1.1 immunoprecipitates generated a 78-kDa signal in SDS-PAGE gels under reducing and non-reducing conditions. A homodimerization via the C-terminal coiled-coil region was suggested (4, 5).

Recently expressed RCAS1-GST fusion protein was shown to bind a putative receptor, whereas a truncated version of RCAS1-GST lacking the postulated TM region did not show biological activity. However, full-length RCAS1 is poorly soluble in aqueous solutions because of its hydrophobic TM domain. This raises the question how RCAS1 acquires solubility and functional effects in an aqueous solution.

Here we studied the biochemical properties of RCAS1 and its subcellular localization in two human cell lines, HEK293 and MCF7. We show that RCAS1 is a Golgi resident protein with a cytoplasmic orientation and, unexpectedly, is not recognized by the 22.1.1 mAb. Instead, the 22.1.1 mAb recognizes the TN glycan antigen, which appears upon RCAS1 overexpression on the cell surface of HEK293 cells.

EXPERIMENTAL PROCEDURES

Antibodies—A polyclonal anti-RCAS1 serum was raised by immunization of a rabbit with repeated subcutaneous injections of 500 μg of purified GST-RCAS1 fusion protein. For this purpose, an RCAS1-cDNA fragment coding for the amino acids 30–213 was generated by PCR using the primers 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′ and 5′-GGGGTACCCACCATGGAAGATGTCGGGAGGACGG-3′ (containing BamHI) and 5′-CCGGGATTCTGGGCTGAAAGTTACTTTACCGTCAC-3′ (EcoRI) for in-frame ligation into pGEX-2T (Amersham Biosciences, Braunschweig, Germany). Recombinant GST-RCAS1 fusion protein was expressed in Escherichia coli DH5α and affinity-purified using glutathione-Sepharose (Amersham Biosciences). Correct cloning was confirmed by DNA and partial protein sequencing. The rabbit antiserum was affinity-purified and immuno blotting and immunofluorescence were performed using the primers 5′-CCGGGATTCTGGGCTGAAAGTTACTTTACCGTCAC-3′ (EcoRI) and 5′-CCGGGATTCTGGGCTGAAAGTTACTTTACCGTCAC-3′ (EcoRI site). Primers were as follows: 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′ and 5′-GGGGTACCCACCATGGAAGATGTCGGGAGGACGG-3′; RCAS1 30–213, 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′ and 5′-GGGGTACCCACCATGGAAGATGTCGGGAGGACGG-3′; RCAS1 Δ1–27, 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′ and 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′; RCAS1 Δ30–213, 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′ and 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′; RCAS1 Δ1–163, 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′ and 5′-GGGGTACCCACCATGAGATGTCGGGAGGACGG-3′. Protein A affinity chromatography. The 22.1.1 mAb was purchased from MoBiTec, Göttingen, Germany, and an IgM isotype control was from BD Pharmingen, San Diego, CA. Supernatants from hybridoma clones 2F4 (kindly provided by H. Clausen, Copenhagen) and 2A7 (16) were used for the recognition of the TN and TF antigens, respectively. mAb AM-75 recognizing the RCAS1 protein was obtained from Onogene, San Diego, CA.

Expression Plasmid Constructs—RCAS1 cDNA was a kind gift of T. Watanabe, Kyushu University, Fukuoka, Japan. All restriction enz ymes were purchased from New England BioLabs (Beverly, MA), and T4 DNA ligase and Pwo polymerase for PCR amplifications were from Roche Applied Science, Mannheim, Germany. For transfection studies, plasmids were cotransfected with the indicated RCAS1 expression constructs and β-galactosyl transferase-YFP (1.4-GT-YFP, Clonetech). Cells were grown on coated cover slips for 48 h and washed once with phosphate-buffered saline (PBS), and cover slips were transferred into a self-made humidified chamber. Cells were covered with 1 ml of PBS containing 0.05% trypsin blue for plasma membrane staining (17) and immediately subjected to microscopic analysis on a Zeiss LSM510 inverted laser scanning microscope. Fluorescence signals were detected using the following configurations: GFP: λex = 488 nm, BP λem = 496–517 nm; YFP: λex = 514 nm, BP λem = 517.7–646 nm. Trypan blue fluorescence was detected on a separate channel (λex = 514 nm, λem ≥ 690 nm). For optimal visualization of overlapping signals, YFP-fluores cence was changed to red, and trypan blue fluorescence was changed to light blue using the Zeiss LSM image browser software.

For the detection of the 22.1.1 antigen, RCAS1-GFP-transfected cells were grown on coverslips for 48 h, then fixed with PBS containing 5% paraformaldehyde for 15 min, followed by permeabilization with PBS containing 0.1% Triton X-100 for 10 min. Cells were stained with the FITC-conjugated goat anti-rabbit IgG and anti-mouse antibody (Dako, Hamburg, Germany). Nuclei were stained with DAPI. PE and DAPI signals were recorded with λex = 543 nm, BP λem = 560–646 nm and λex = 364 nm, BP λem = 385–470 nm wavelengths, respectively.

For the detection of endogenous RCAS1 in MCF7 cells, cells were grown on coverslips, washed with PBS, and fixed with methanol. RCAS1 antisera, control serum, or mAb AM-75 were diluted 1:100 in PBS containing 10% normal goat serum and incubated overnight at 4 °C. Slides were rinsed, and bound antibodies were detected with a biotinylated goat anti-rabbit or goat anti-mouse antibody (Dako) and streptavidin-conjugated Alexa Fluor™ 488 or Alexa Fluor™ 568 (Molecular Probes, Eugene, OR). Signals were visualized on a Leica DM IRBE microscope equipped with a Zeiss Axiovid digital camera. All images were processed in Corel Photo Paint.

Peroxidase Oxidation—Sodium periodate oxidation of carbohydrates was carried out essentially as described previously (18). Cells were grown on coverslips and fixed with 5% (v/v) paraformaldehyde in PBS for 15 min. After washing with PBS and acetate buffer (50 mM sodium acetate, pH 4.5), cover slips were incubated with 20 mM periodate in acetate buffer for 30 min at RT and processed for immunostaining with the indicated antibodies. Bound antibodies were visualized with a peroxidase-conjugated goat anti-mouse antibody. Nuclei were stained with DAPI, and cells were analyzed using a Leica DM IRBE microscope and an Axiovid digital camera.

Subcellular Fractionation and High Salt Treatment—2 × 10^6 cells were washed with PBS, resuspended in 10 mM Tris, pH 7.4, 250 mM sucrose, 1 mM PMSF, 1.5 μg/ml aprotinin, and homogenized in a Dounce homogenizer by 20 strokes with a tight fitting pestle. After nuclei were pelleted by centrifugation at 250 × g for 10 min, a postnuclear supernatant was ultracentrifuged at 100,000 × g for 1 h. Crude membrane pellets were washed with either 10 mM Tris, pH 7.4, 1 mM KCl, 100 mM NaCl, or 100 mM Tris and 100 mM KCl. Pellets were resuspended in a 250 mM sucrose, 1 mM EDTA and gently homogenized by five strokes in a Dounce homogenizer with a tight fitting pestle. Nuclei were pelleted at 250 × g for 10 min and...
supernatant was mock treated or treated with 60 μg/ml proteinase K for 1, 5, and 15 min. The reaction was stopped by the addition of 5 mM PMFS for 5 min. 2 × rSB was added, and samples were boiled for 10 min and subjected to SDS-PAGE.

Cell Surface Biotinylation—2 × 10^7 cells were washed twice in PBS before cell surface biotinylation was performed for 30 min at room temperature with 1 mg of sulfo-N-hydroxysulfo succinimide–LC-biotin (Pierce, Rockford, IL) in 2 ml of PBS at pH 7.4. Cells were washed three times in PBS containing 10 mM lysine and lysed in 1 ml of 50 mM Tris, pH 7.4, 5 mM MgCl₂, 0.5% Nonidet P-40, 1 mM PMFS, 1.5 μg/ml aprotinin for 30 min at 4 °C. Nuclei were pelleted for 10 min at 13,000 rpm, and streptavidin-Sepharose (Amersham Biosciences) was added to the supernatant for 1 h under agitation at 4 °C. Streptavidin-Sepharose beads were washed five times with ice-cold PBS, before biotinylated surface proteins were recovered from the beads by boiling in 100 μl of rSB for 5 min. Proteins were subjected to SDS-PAGE.

In Vitro Transcription, Translation, and Protease Protection Assay—In vitro transcriptions and translations were performed in the presence of [³²P]methionine according to the manufacturer’s instructions using the PROMega Riboprobe kit (PROMega, Mannheim, Germany) and the PROMega Flexi rabbit reticulocyte system. Where indicated, canine pancreatic microsomal membranes (Promega) were added to the reaction. Reaction products were analyzed directly by SDS-PAGE, or further processed in a protease protection assay essentially as described (20). Briefly, microsomes were resuspended in ice-cold PBS and treated or mock treated with 4 μg/ml proteinase K (MERCK, Darmstadt, Germany) for 15 min on ice. Microsomes were pelleted immediately for 10 min at 13,000 rpm at 4 °C in a microcentrifuge, followed by one washing step with ice-cold PBS containing 1 mM PMFS and 1.5 μg/ml aprotinin. Pellets were dissolved in rSB and boiled for 5 min. Endo-β-N-acetylglucosaminidase H (Endo H, New England BioLabs) digests were performed as described by the manufacturer.

Get Electrophoresis and Immunoblotting—Cell lysates were separated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (0.45-μm pore size, Schleicher & Schuell, Dassel, Germany) using a Bio-Rad tank blot chamber. Membranes were incubated with the first antibodies for 60 min in PBS containing 0.05% Tween 20 (PBS-T) and 1% nonfat dry milk, followed by horseradish peroxidase-labeled secondary antibodies for 60 min in PBS containing 0.05% Tween 20 and 1% nonfat dry milk. Bound antibodies were visualized by chemiluminescence (ECL Western Blot Detection kit, Amersham Biosciences, Braunschweig, Germany).

Flow Cytometric Analysis—After blocking in FACS buffer containing 10% normal goat serum, primary antibodies were added to the cells at a dilution of 1:200 for 1 h on ice. Cells were washed twice and incubated for 15 min with PE-conjugated goat anti-mouse secondary antibody. For the detection of 22.1.1, Tn, or TF surface staining of GFP fusion protein-expressing cells, a biotinylated goat anti-mouse secondary antibody or IgM isotype control, diluted 1:200 in PBS containing 1% bovine serum albumin for 20 min at 4 °C. Plates were washed and incubated with streptavidin–FITC conjugate. Bound antibodies were visualized by incubation with 0.05% 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]dimannoium salt (6) (Roche Applied Science), 0.05% H₂O₂ in 50 mM citrate buffer, pH 4.0, and analyzed with a Bio-Rad Microplate Reader at 405 nm.

RESULTS

The RCAS1-encoded Gene Product Is a Cytoplasmically Oriented Membrane Protein—The N terminus of the RCAS1-encoded gene product contains a stretch of hydrophobic amino acids. It has been suggested that this region constitutes a TM region (5). However, this prediction is not supported by all available algorithms. TMHMM2.0 (21), HMMTOP2.0 (22), and PRED-TMR2.0 (23) do not predict a TM region, and SignalP version 2.0 predicts a signal peptide for the first 21 amino acids (24).

To explore the membrane association, signal peptide cleavage, and membrane orientation of RCAS1, we employed a cell-free translation system in the presence of canine pancreatic microsomes that provides a functional environment for membrane insertion. RCAS1 was found to be associated with microsomal membranes without an apparent cleavage of a signal peptide (Fig. 1A). In SDS-PAGE the full-length form migration is nearly identical to that of RCAS1 as obtained from translation without microsomes. The shift in molecular weight shown in Fig. 1A is unlikely to account for a signal peptide cleavage. On average, signal peptides in eukaryotes are 15–50 amino acids long and are usually located at the N terminus (25). Therefore, the shift expected for signal peptide cleavage would be at least 2 kDa. The signal obtained in the presence of microsomal membranes reflects a doublet that could also be observed in immunoblots from cell extracts using the polyclonal anti-RCAS1 serum (Fig. 1E). The doublet might indicate modifications or conformational changes that occur at the endoplasmic reticulum (ER) membrane. Because an N-linked glycosylation motif was absent from the RCAS1 polypeptide sequence and RCAS1 was not Endo H-sensitive (Fig. 1B), we could exclude that the occurrence of a doublet was due to glycosylation modifications.

RCAS1 was suggested to be a type II TM protein with a large C-terminal luminal/extracellular domain and a short N-terminal cytoplasmic part (5). The orientation of the RCAS1 gene product in microsomal membranes was probed by protease protection assays. After translation, addition of proteinase K to the microsomes led to proteolytic cleavage of cytoplasmic protein domains, whereas luminal regions were protected (Fig. 1C). As a control for a protein with a large luminal part and a short cytoplasmic tail, we made use of the human MHC class I heavy chain molecule HLA-A3. Proteinase K treatment effectuated the complete digestion of RCAS1, whereas the large luminal part of HLA-A3 remained protected from proteolytic cleavage (Fig. 1C).

Results from in vitro experiments were confirmed in cell lines. We performed protease protection assays in the mAβ 22.1.1-negative human embryonal kidney cell line HEK293 and in the mAβ 22.1.1-positive mammary carcinoma cell line MCF7. Whole cells were homogenized under conditions that leave intracellular compartments intact, and homogenates were exposed to proteinase K. This treatment leads to the degradation of the cytoplasmic parts of organelle-associated proteins (19). Whereas the ER-resident intracellular chaperone calreticulin and the large luminal part of the MHC class I heavy chain remained unaffected, RCAS1 was subject to complete degradation in both cell lines (Fig. 1D). A potential intraluminal/TM N terminus of about 30 amino acids was not resolved by the gel system employed.

Two alternatives might account for the membrane orientation of RCAS1. First, the non-polar region at the N terminus of RCAS1 constitutes a TM segment indicative of a type III TM protein with a reverse signal anchor, according to the classification of Spiess (26). Type III TM proteins possess a large cytoplasmically oriented C terminus and a single TM-spanning region at the N terminus that serves as non-cleaved membrane targeting signal. Second, a peripheral membrane association could be considered. To explore if RCAS1 might be an integral membrane protein, crude cell membranes from MCF7 and HEK293 cells were prepared and washed with 1 M KCl, 10 mM EDTA, 100 mM Na₂CO₃, or 4 mM urea. In general, high salt treatment releases soluble and peripheral membrane proteins, whereas integral membrane proteins remain inserted in the
lipid bilayer. RCAS1 could not be detected in the cytosolic fractions but was recovered from untreated and high salt treated postnuclear membranes (Fig. 1E). Detergent treatment with Triton X-100 allowed the solubilization of membrane-bound RCAS1 (Fig. 1E). Taken together, we conclude that RCAS1 is a type III TM protein with its large C terminus localized in the cytoplasm.

Subcellular Localization of RCAS1—Applying the mAb 22.1.1 to immunohistochemistry, RCAS1 was reported to be localized to intracellular compartments and to the plasma membrane. However, the subcellular localization was reported to vary among tissues and degree of malignancy of the tumors explored (27, 28). When we employed our polyclonal serum for the detection of RCAS1 in acetone-fixed MCF7 cells, a perinuclear reticular staining pattern characteristic of the Golgi complex in these cells was seen (Fig. 2, A and B). To further resolve the subcellular localization of the RCAS1-encoded gene product, confocal laser scanning microscopy on living cells was carried out. The 22.1.1-negative cell line HEK293 and the 22.1.1-positive cell line MCF7 were used. The RCAS1 antiserum stains perinuclear structures but not plasma membranes. A and B, MCF7 cells were grown on coverslips, fixed with acetone, and stained with normal rabbit serum (A) or anti-RCAS1 polyclonal serum (B) as described under “Experimental Procedures.” Nuclei were counterstained with DAPI. C-H, colocalization of RCAS1-GFP and the epitope recognized by our polyclonal serum and mAb AM-75 directed against the RCAS1 gene product were probed in RCAS1-GFP-transfected MCF7 cells. GFP signals (C and F) and signals from the RCAS1 (D) antisera as well as from mAb AM-75 (G) share an identical subcellular distribution. Nuclei were counterstained with DAPI. Merged images are shown on the right (E and H). Scale bars, 10 μm.

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line MCF7 were chosen for direct comparison. Both cells lines were transiently cotransfected with RCAS1-GFP and the Golgi marker 1,4-GT-YFP. After 48 h, plasma membranes were stained with trypan blue immediately followed by confocal microscopic analysis. Comparison of RCAS1-GFP with 1,4-GT-YFP revealed a considerable overlap of the fluorescence signals. RCAS1-GFP signals could not be detected at the plasma membranes or at any other subcellular localization in either of the two cell lines analyzed (shown for MCF7, Fig. 3, A–D). RCAS1-GFP and endogenous RCAS1, as detected by our polyclonal serum (Fig. 2, C and D) and by the commercially available mAb AM-75 directed against the RCAS1 gene product (Fig. 2, F–H), shared the same Golgi predominant expression pattern.

The N-terminal Transmembrane Domain and the C-terminal Coiled-coil Region Determine Organelle Association—To elucidate the polypeptide domains responsible for the Golgi localization of RCAS1, four deletion variants of RCAS1 were constructed as GFP fusion proteins (Fig. 3). Subcellular distribution patterns of these mutants were analyzed in HEK293 and MCF7 cells using confocal microscopy. Deletion of the C-terminal coiled-coil region did not alter the subcellular localization, compared with wild-type RCAS1 (Fig. 3, A–D and E–H), whereas deletion of the N-terminal 27 amino acids led to a cytosolic staining pattern, as would be expected if a functional membrane anchor was deleted (Fig. 3, M–P). The same staining pattern could be observed when the coiled-coil domain was fused directly to GFP (Fig. 3, Q–T). Scale bar, 20 μm.

**Fig. 3.** RCAS1-GFP localizes to the Golgi complex. MCF7 cells were transiently cotransfected with 1,4-GT-YFP as Golgi marker (B, F, J, N, and R) and with five different RCAS1-GFP fusion protein deletion variants, respectively (A, E, I, M, and Q). Deletion variants are schematically depicted on the left. TM, transmembrane region (amino acids 8–27); CC, coiled-coil region (amino acids 179–206). Plasma membranes were stained with trypan blue prior to microscopy (C, G, K, O, and S). For optimal visualization of overlapping signals, YFP fluorescence color was changed to red and trypan blue fluorescence color was changed to light blue. Overlays are shown in the right panel (D, H, L, P, and T).
Although MCF7 cells were strongly positive, essentially no surface staining was observed in HEK293 cells. The 22.1.1 antigen, but not the RCAS1-encoded gene product, is localized at the plasma membrane. A, HEK293 and MCF7 cells were surface-labeled with a membrane-impermeable biotinylation reagent. Cells were homogenized and incubated with streptavidin-agarose to recover surface-biotinylated proteins. Unbound (S) and streptavidin-bound fractions (P) were resolved by SDS-PAGE and analyzed by immunoblot employing the RCAS1 antisera. In both cell lines, RCAS1 was found in the intracellular fraction only. MHC class I heavy chain served as marker for cell surface deposition. B, HEK293 and MCF7 cells were stained with the mAb 22.1.1 and analyzed by flow cytometry. Although MCF7 cells were strongly positive, essentially no surface staining was observed in HEK293 cells.

To our surprise, when we employed confocal microscopy, we failed to detect full-length RCAS1-GFP or any of the deletion variants at the plasma membrane of mAb 22.1.1-positive MCF7 cells (Fig. 3, A–D). Lack of RCAS1-encoded antigen expression on the cell surfaces of HEK293 and MCF7 cells could also be confirmed biochemically in non-transfected cells. HEK293 and MCF7 cells were biotinylated, and surface proteins were recovered using streptavidin beads. Intracellular, non-biotinylated proteins were precipitated with trichloroacetic acid. Both pools, surface and intracellular proteins, were analyzed by immunoblot with the anti-RCAS1 serum (Fig. 4A). The anti MHC class I heavy chain mAb HC10 served as control for a plasma membrane glycoprotein. In our hands, the IgM mAb 22.1.1 was only applicable to immunocytochemistry and ELISA. Therefore, 22.1.1 surface expression was assessed by flow cytometric analysis in parallel. MCF7 cells were strongly stained with mAb 22.1.1, and HEK293 cells were essentially negative (Fig. 4B). In contrast to this observation, but in agreement with the topology and subcellular distribution of the RCAS1 gene product as demonstrated before (Figs. 1–3), neither cell line exhibited RCAS1 surface expression after surface biotinylation (Fig. 4A).

The biochemical properties of RCAS1, as specified by its membrane orientation and subcellular distribution, were found to be identical in HEK293 and MCF7 cells. In conclusion, our failure to detect surface expression of RCAS1 in cell lines that were stained with mAb 22.1.1 on their surfaces raised the concern of whether the 22.1.1 mAb recognizes the RCAS1 gene product, as defined by the published cDNA sequence. To further resolve this discrepancy, MCF7 cells were transfected with RCAS1-GFP fusion protein, grown on coverslips for 48 h, fixed, permeabilized, and stained with 22.1.1 mAb or IgM isotype control. Confocal microscopy images suggested that RCAS1 was not recognized by the 22.1.1 mAb (Fig. 5, A–F). We failed to observe a significant overlap between RCAS1-GFP and the 22.1.1 mAb-defined antigen. Instead, 22.1.1 mAb showed reactivity for vesicle-like structures mainly localized in the vicinity of the plasma membrane. Results from flow cytometry, antibody feeding experiments (data not shown), and the observation that the 22.1.1 antigen is shed into the cell culture supernatant (29) are indicative of a close relationship between the 22.1.1-positive vesicles and the cell surface. More specifically, our observation from confocal microscopy suggests that the 22.1.1-defined antigen might be stored in secretory vesicles prior to plasma membrane fusion and surface release.

The 22.1.1 Antibody Recognizes the Tn Antigen—The mAb 22.1.1 exhibits a broad tumor-specific reactivity, suggesting that a common tumor-associated antigen might be recognized. It has been appreciated that cancer cells of diverse histological origin often exhibit abnormal O-linked glycoprotein structures that might elicit humoral or cellular immune responses. In addition, such humoral immune responses, as obtained from cancer patients or from mice immunized with tumor cell lines, are predominantly of the IgM type.

Overexpression of RCAS1 Renders HEK293 Cells Positive for 22.1.1 Surface Staining—Based on its membrane topology and predominant Golgi localization, it appears unlikely that RCAS1 serves as soluble or plasma membrane-bound receptor ligand. We therefore revisited the data on RCAS1 expression cloning and transfected HEK293 cells with different amounts of RCAS1 expression plasmid, followed by flow cytometry using mAb 22.1.1. Depending on the amount of RCAS1 cDNA transfected, permeabilized and non-permeabilized HEK293 cells stained positively for 22.1.1 mAb (Fig. 6A). To analyze the localization of the 22.1.1 mAb-defined antigen in RCAS1-overexpressing HEK293 cells, cells were transfected with RCAS1-GFP, grown on coverslips, stained with 22.1.1 or isotype controls, and analyzed by confocal microscopy. The subcellular localization of the 22.1.1 antigen was indistinguishable from that in MCF7 cells (Fig. 5, G–I). In agreement with our data from MCF7 cells, there was essentially no overlap between fluorescence signals from RCAS1-GFP and the 22.1.1-defined antigen. Transfection of the GFP mock control failed to induce 22.1.1 expression (Fig. 5, M–O).

The relevance of the major domains of RCAS1 for 22.1.1 staining was analyzed by flow cytometry after transfection of RCAS1-GFP deletion variants. The percentage of 22.1.1-positive cells was calculated as the ratio of 22.1.1 and GFP-positive cells to total GFP-positive cells. Identical region settings were used in all experiments. Fig. 6B shows mean values of five independent experiments. An average of 20% of transfected cells were stained with the 22.1.1 mAb when full-length RCAS1 or the Δ coiled-coil variant (Δ179–213-GFP) were transfected. Transfection of other variants did not lead to a significant 22.1.1 epitope up-regulation. We conclude that the coiled-coil domain did not contribute to the generation of the 22.1.1 antigen.
an inhibitor of O-linked glycosylation. Benzyl-α-GalNAc treatment facilitates incomplete O-linked glycosylation due to inhibition of sialyltransferases and galactosyltransferases, resulting in premature O-linked glycan structures like terminal GalNAc (Tn antigen) or Galβ1-3GalNAc (TF antigen) that are often found on tumor cells (30–32). Although benzyl-α-GalNAc treatment of MCF7 cells had no influence on 22.1.1 staining, HEK293 and HeLa cells were rendered 22.1.1-positive (shown for HEK293, Fig. 7). Simultaneous overexpression of RCAS1-GFP led to an additional effect on mAb 22.1.1 surface staining, as determined by flow cytometry.

The possible reactivity of 22.1.1 with one of the known tumor-associated carbohydrate antigens or closely related structures was investigated by ELISA (Fig. 8A). It was evident that the mAb 22.1.1 showed strong binding toward Tn. Similar structures, like sialylated Tn or TF, were not recognized. Carbohydrate specificity was also confirmed by periodate oxidation of fixed MCF7 cells. Treatment with periodate destroys terminal saccharide rings with vicinal OH groups, thereby abolishing immunological recognition (18). Fig. 8B shows that the 22.1.1 signal is extinguished after periodate treatment. An established Tn-specific mAb, 5F4, was used as a control. For additional proof of antibody specificity, we transiently transfected HEK293 cells with RCAS1-GFP or GFP alone and performed flow cytometry with the 22.1.1 mAb and the 5F4 mAb. Staining with both antibodies exhibited an identical pattern in dot blot diagrams (Fig. 8C). From these data we conclude that overexpression of the RCAS1-cDNA contributes indirectly to the generation of the Tn antigen. Interestingly, overexpression of RCAS1 also led to the enhanced surface expression of the closely related TF antigen as analyzed by flow cytometry using the mAb GA7 (Fig. 8C). We note that an RCAS1 mutant with a deletion of a potential O-linked glycan acceptor site at the N terminus (Thr4→Ala) had identical effects on the generation of the 22.1.1 epitope, compared with wild-type RCAS1 (data not shown).

**DISCUSSION**

It was suggested that the RCAS1-encoded antigen is expressed as type II TM protein on the plasma membrane of tumor cells, where it might serve as a ligand for a putative death receptor (5). Expression studies of this antigen were largely performed with the 22.1.1 mAb, employing paraformaldehyde or paraffin-fixed tissue sections. However, when this technique was applied to fixed MCF7 cells or to gastric carcinoma, some discrepancies on the subcellular localization of the cognate tumor-associated antigen were discovered. The cognate 22.1.1 antigen was also found intracellularly as perinuclear, cytoplasmic staining or in vesicle-like structures (29).

The subcellular localization and membrane topology of the RCAS1-encoded gene product were the subject of this study. Employing RCAS1 transfectants and GFP-tagged truncation variants derived thereof, we found the RCAS1-encoded gene product to be localized to the Golgi complex. Protease protection assays revealed the overall membrane topology of RCAS1 with a predominant C terminus facing toward the cytoplasm. Such type III TM protein orientation would leave the RCAS1-encoded gene product with an extremely short span of seven amino acids encompassing the N terminus. This structural characteristic imposes the problem of how the 22.1.1 mAb can recognize such extracellular domain in surface staining. Second, the short N terminus at the outer site of a cell obviously would limit the capacity of the RCAS1-encoded molecule to interact with the postulated death receptor on immune cells (5).

To resolve these discrepancies, we compared 22.1.1 mAb staining with the subcellular localization of the RCAS1-GFP-tagged constructs. In addition, we made use of our polyclonal RCAS1-antiserum raised against recombinant RCAS1 fusion protein. Confocal microscopic analysis revealed that 22.1.1 failed to recognize the RCAS1-GFP constructs in diverse cell lines. Although the mAb exhibited a plasma membrane predominant staining pattern in MCF7 cells, RCAS1-GFP signals were obtained from the Golgi region, exclusively. Although the addition of a GFP tag might alter some properties of a native
molecule, we consider it unlikely that in our case the endogenous RCAS1 behaves differently from its tagged derivatives. This could be concluded from the staining experiments of MCF7 cells with the polyclonal anti-RCAS1 serum (Fig. 2). Furthermore, GFP-tagged constructs of RCAS1 as well as the wild-type sequence-encoded antigen behaved identically in the generation of the 22.1.1 epitope. From our deletion mutant studies we conclude that the transmembrane domain of RCAS1

**Fig. 6.** Overexpression of full-length RCAS1 or the coiled-coil deletion variant confers HEK293 cells reactivity with the 22.1.1 mAb. A, 2 x 10^6 cells HEK293 cells were electroporated with 5, 10, 20, or 40 μg of pcDNA3.1(+)-RCAS1 or pcDNA3.1(+) alone as mock control. 22.1.1 surface expression was analyzed by flow cytometry. Open curves, RCAS1; filled curves, mock control. No significant difference could be detected between surface staining (upper panel) and staining of permeabilized cells (lower panel). B, the relevance of the different RCAS1-GFP deletion variants for mAb 22.1.1 surface staining was analyzed by flow cytometry. Data shown represent the percentage of cells with 22.1.1 surface reactivity in relation to the total number of GFP-positive cells. The diagram shows mean values of five independent experiments ± S.D. FACS region settings were identical in all experiments.

**Fig. 7.** The 22.1.1 mAb-defined surface epitope is up-regulated by treatment with the O-glycosylation inhibitor benzyl-α-GalNAc. 2 x 10^6 HEK293 cells were transiently transfected with 20 μg of RCAS1-GFP or GFP and treated with 2.5 mM benzyl-α-GalNAc or mock treated (w/o) for 24 h. GFP signals were detected in the FL-1 channel (x-axis, upper panel). 22.1.1 antibody binding and IgM isotype control binding was visualized using Cy5 fluorochrome detected in the FL-4 channel (y-axis, upper panel and histograms in the lower panel). In the upper panel GFP fluorescence is compared with 22.1.1 surface staining in a dot blot diagram. A minor population of RCAS1-GFP-positive cells stained positively for mAb 22.1.1, which could be further enhanced upon addition of benzyl-α-GalNAc. In the lower panel 22.1.1 staining is compared with staining with the IgM isotype control.
is responsible for its localization and sorting in the Golgi complex.

RCAS1 has attracted particular attention because of its suggested role as a tumor-associated antigen. In contrast to the tumor specificity as proposed, mRNA expression was found in all tissues and cell lines examined (5). The human genomic upstream region of RCAS1 contains CpG-rich islands (9, 10). In general, CpG-rich promoter regions are characteristic features of housekeeping genes and promote constitutive basal expression levels (33). The initial observation that RCAS1 antigen expression was missing from normal human tissues is best explained by the method employed, because the mAb 22.1.1 was used for immunostaining. In contrast, using a polyclonal serum for RCAS1 detection in murine tissues, we2 and others (14) confirmed its expression in essentially all murine tissues examined. Likewise, we found RCAS1 expression in all human and murine cell lines examined at the protein level (data not shown), suggesting that RCAS1 is a ubiquitously expressed protein but not tumor-specific. Based on the extensive homology at the protein level where only five amino acids are different, it seems reasonable to suggest that the RCAS1 gene product is expressed in most normal human tissues as well.

When we revisited the data on RCAS1 expression cloning, we could show that RCAS1 overexpression leads to the generation of the antigen recognized by the 22.1.1 mAb, which is identical to the Tn glycan antigen. These results were indicative of an indirect, modulatory role of RCAS1 in the process of Tn antigen generation. Further functional analysis of RCAS1-deletion constructs support our notion that a suggested multimerization of RCAS1, mediated by a coiled-coil domain (5), is not critical for the generation of Tn after RCAS1 overexpression.

To reconcile our cell biological characterization of RCAS1 with the functional results originally reported by Nakashima et al. (5), we would like to point out that the induction of apoptosis in activated T cells was seen upon exposure to SiSo culture supernatant. In our hands, recombinant RCAS1-GST fusion protein is not soluble in aqueous solutions and cannot be purified in the absence of detergent. Moreover, recombinant RCAS1-GST lacking the transmembrane domain failed to bind a putative receptor (5), nor has it any effect on activated T cells.2 We conclude that full-length RCAS1 is not applicable to cell culture experiments, and effects seen on cell viability and integrity are most likely due to residual detergent. RCAS1-induced alterations were not limited to the generation of Tn, a concomitant generation of the TF antigen points to a more general mechanism of interference with the O-glycan extension processes in the Golgi apparatus.

In general, TF and Tn antigens are thought to be linked with cell adhesion, invasion, and metastasis of cancer cells (34). Such glycan epitopes are normally cryptic in healthy and benign tissues, except in early embryonic stages (3).

With respect to its generalized expression pattern, a potential difference in expression and regulation of RCAS1 in benign and tumor tissues warrants further analysis. This could add to

RCAS1-mediated Expression of Tn and TF

the problem if aberrant glycosylation is a result or a prerequisite of initial oncogenic transformation (1, 35).

Several mechanisms for the generation of Tn and TF facilitated by RCAS1-overexpression can be envisaged. Golgi-resident glycan-nucleotide transporters might be affected. There is evidence that transport of nucleotide-activated sugars into the Golgi lumen participates in the regulation of polypeptide glycosylation (36). Massive import of GalNAc-UDP into the Golgi lumen could lead to an excessive basal glycosylation of serine or threonine residues and of glycosphingolipids that cannot further be extended into more complex core structures. A stimulatory effect on such nucleotide-glycan transporters could mimic the observed appearance of the precursor glycan Tn and TF on the cell surface.

Glycosyltransferases are arranged in the Golgi apparatus in a spatial and functional hierarchy. Overexpression of RCAS1 might induce a disturbance of the Golgi architecture, and consequently a dislocation of glycosyltransferases. Interestingly, a spatial and functional hierarchy. Overexpression of RCAS1 and TF on the cell surface.

ther be extended into more complex core structures. A stimulatory effect on such nucleotide-glycan transporters could mimic the observed appearance of the precursor glycan Tn and TF on the cell surface.

An alternative explanation for the modulatory function of RCAS1 on Tn and TF antigen expression would include a role in vesicle trafficking from the Golgi apparatus to the cell surface (32). It could be hypothesized that RCAS1 facilitates the release of premature polypeptides or glycolipids carrying O-linked glycans.

In view of its supposed role as tumor-associated antigen, a more detailed analysis of the modulatory role of RCAS1 in the generation of the tumor-associated Tn and TF glycan structures is required. In particular, it remains unresolved at what level RCAS1 interferes with the secretory pathway for O-linked glycoproteins and if this ubiquitously expressed gene product is related to the malignant transformation of cells.

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