Nuclear association of the cytoplasmic tail of MUC1 and [beta]-catenin*

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Running Title: Subcellular distribution of MUC1 CT and [beta]-catenin in human pancreatic cancer

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The abbreviations used are: MUC1 CT MUC1 cytoplasmic tail, APC adenomatous polyposis coli tumor suppressor, LEF-1 lymphoid-enhancing factor I, Tcf T-cell transcription factors, GSK-3[beta] glycogen synthase kinase 3[beta], mAb, monoclonal antibody, RT room temperature, PBS phosphate-buffered saline, PAGE polyacrylamide gel electrophoresis

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ABSTRACT

MUC1, an integral membrane mucin associated with the metastatic phenotype, is overexpressed by most human carcinoma cells. The MUC1 cytoplasmic tail (CT) is postulated to function in morphogenetic signal transduction via interactions with Grb2/Sos, cSrc, and β-catenin. We investigated intracellular trafficking of the MUC1 CT, using epitope-tagged constructs that were overexpressed in human pancreatic cancer cell lines S2-013 and Panc-1. The MUC1 CT was detected at the inner cell surface, in the cytosol and in the nucleus of cells overexpressing MUC1. Fragments of the MUC1 CT were associated with β-catenin in both cytoplasm and nuclei. Overexpression of MUC1 increased steady state levels of nuclear β-catenin, but decreased nuclear levels of plakoglobin (γ-catenin). There was no detectable association between plakoglobin and the MUC1 CT. Co-immunoprecipitation experiments revealed that the cytoplasmic and nuclear association of MUC1 CT and β-catenin was not affected by disruption of Ca^{2+}-dependent intercellular cadherin interactions. These results demonstrate nuclear localization of fragments of MUC1 CT in association with β-catenin, and raise the possibility that overexpression of the MUC1 CT stabilizes β-catenin and enhances levels of nuclear β-catenin during disruption of cadherin mediated cell-cell adhesion.
INTRODUCTION

Human MUC1 is a large, type I transmembrane protein normally expressed on the apical surface of ductal epithelia (1). Full-length MUC1 is synthesized as a single polypeptide chain, which undergoes an early proteolytic cleavage (probably in the endoplasmic reticulum) creating two subunits that remain associated during its post-translational processing and transport to the cell surface (2). The larger of the two fragments contains most of the extracellular domain, including the signal sequence and a tandem repeat domain (3-5). The smaller subunit contains a short extracellular domain, transmembrane domain and cytoplasmic tail (CT), which are highly conserved across species (88% identity with murine transmembrane and CT sequence) (6). The function of MUC1 is partially elucidated for normal and transformed cells. The extracellular fragment plays a significant role in configuring the adhesive and anti-adhesive properties of cells and is believed to contribute to the establishment of molecular structures that protect the cell surface in the relatively harsh environment encountered by different ductal epithelia (7). However, the function of the intracellular portion of the MUC1 cytoplasmic tail (CT) is not known. Indirect evidence suggests that the MUC1 CT is involved in signal transduction, as it contains potential docking sites for Grb2/Sos and catenin, and can be phosphorylated by GSK-3, c-Src, EGFR and PKC- (7-16). This, together with the general transmembrane structure of MUC1 molecule, suggests a potential role in morphogenetic signaling; however, little is known about mechanisms by which the MUC1 CT functions in this capacity and nothing has been reported with regard to the relationship between the extracellular and intracellular functions of MUC1.
Previous studies reported that the cytoplasmic domain of MUC1 interacts with β-catenin at a serine-rich motif, which is similar to binding sites found in E-cadherin and the adenomatous polyposis coli (APC) tumor suppressor (10,11). β-catenin and its closely related homologue γ-catenin (plakoglobin), play important roles in cell-cell adhesion and transcriptional regulation (17). Both have signaling activity as members of the Wnt pathway (18). Binding of Wnt to the Frizzled receptor and the subsequent inhibition of GSK-3β kinase activity on β-catenin result in the translocation of β-catenin from the cytosol to the nucleus, where β-catenin binds to the N-terminus of LEF-1/TCF, forming a tertiary complex that is involved in transcriptional transactivation (19-23). Deregulation of β-catenin turnover, which results in its nuclear accumulation and enhanced activity as a transcription factor, is associated with tumorigenesis (24-28). Other studies indicate that GSK-3β and EGFR regulate interactions between β-catenin and MUC1 (11,12,14). To date, the biological consequences of the interaction between MUC1 CT and β-catenin remain poorly understood.

In the present study, we investigated the subcellular localization of MUC1 CT and β-catenin in the human pancreatic cancer cell lines S2-013 and Panc-1. Our results indicated that fragments of MUC1 CT were distributed on the inside of the plasma membrane, in the cytoplasm, and in the nucleus. In addition, co-immunoprecipitation experiments revealed an association between the MUC1 CT and β-catenin in both cytoplasm and nuclei of cells overexpressing MUC1. Steady state levels of nuclear β-catenin were significantly increased by overexpression of MUC1. The steady state levels of nuclear β-catenin and the fragment of MUC1 CT were not affected by disruption of intercellular E-cadherin interactions (by Ca²⁺ withdrawal) in cells overexpressing MUC1;
however, the levels of nuclear $\beta$-catenin were dramatically reduced (presumably by the APC-$\beta$-catenin degradation pathway (29)) upon chelation of extracellular Ca$^{2+}$ in cells expressing low-levels of MUC1. We also determined that overexpression of MUC1 decreased the steady state levels of nuclear $\gamma$-catenin (plakoglobin); however, no direct association between MUC1 CT and $\gamma$-catenin was detected in our cell lines. The results show that a fragment of the MUC1 CT undergoes cytosol-to-nucleus trafficking in association with $\beta$-catenin, and suggest the possibility that the MUC1 CT stabilizes $\beta$-catenin and thereby influences its transcriptional coactivator function. The findings also suggest that overexpression of the MUC1 CT had opposite effects on the nuclear levels of plakoglobin and $\beta$-catenin.
EXPERIMENTAL PROCEDURES

Materials. The human pancreatic cancer cell line Panc-1 was originally obtained from the American Type Culture Collections (Rockville, MD). The S2-013 cell line is a cloned subline of human pancreatic tumor cell line (SUIT-2), which was derived from a liver metastasis (30). Armenian Hamster mAb CT-2 against MUC1 CT was kindly provided by Dr. Sandra Gendler, Mayo Clinic Scottsdale, AZ. Murine mAb anti-β-catenin and anti-E-cadherin were purchased from Transduction Laboratory Co. Murine mAbs M2-anti-Flag, anti-β-actin, and M2-conjugated agarose beads were purchased from Sigma. Mouse mAb 11E4 binds to the N-terminus of plakoglobin. Mouse mAb anti-β-actin and goat polyclonal Ab anti-histone H2B (sc-8650) were purchased from Sigma and Santa Cruz, respectively. The secondary antibodies used for immunostaining in confocal microscopy, including FITC-conjugated Goat anti-mouse IgG1, TxRed-conjugated AffiniPure Goat anti-Armenian hamster IgG(H+L), and CyTMS-conjugated Affininpure Goat Anti-Armenian Hamster IgG(H+L) were purchased from Jackson ImmunoResearch Laboratories Inc.

Cell culture. The Panc-1 and S2-013 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum. Cells were incubated at 37°C in 5% CO2, and passaged at 80–90% confluence using 0.05% trypsin with 0.53 mM EDTA (Gibco BRL, Gaithersburg, MD). Cells were grown in 225 cm2 flasks for collecting cell lysates, or on 18-mm glass coverslips for confocal microscopy.

Expression of Epitope-tagged MUC1 deletion constructs and generation of
**transfectant cell lines:** Flag epitope-tagged MUC1 cDNA constructs were used in which 66 amino acids of the cytoplasmic tail (MUC1 CT3), or the tandem repeat (MUC1 [TR]) were deleted and subcloned into the expression vector pHb-Apr1-neo, as described previously (31). Panc-1 and S2-013 cells were transfected with plasmid DNA using the lipofectin method (Gibco BRL, Gaithersburg, MD). Cells were plated in six-well culture plates. At pre-confluence, cells were incubated with 10 µl of Lipofectin reagent and 10 µg of circular plasmid DNA. Cells carrying integrated MUC1 constructs were selected by culture in 600µg/ml G418 (Gibco BRL, Gaithersburg, MD), and single clones were selected with cloning cylinders and expanded for screening. Expression of FLAG epitope-tagged MUC1 isoforms in each transfectant was evaluated by immunofluorescence with anti-Flag mAb M2 and by immunoblotting with M2.

**Immunofluorescence microscopy.** S2-013 and Panc-1 cells were cultured on glass coverslips (Fisherbrand Microscope cover glass: 12-545-100 18CIR-1) at 4.5x10^5/well for 12 hours. All subsequent steps were performed at 25°C. Cells were washed once in serum-free DMEM for 5 mins followed by fixation for 15 mins in PBS with 4% paraformaldehyde and 120 mM sucrose. Residual paraformaldehyde was neutralized with 0.1M glycine in PBS for 15 min. For immunofluorescence, cells were permeabilized for 15 minutes by incubation in PBS with 0.1% Triton X-100. After washing with PBS, cells were incubated for 2 hours with primary antibodies diluted in DMEM media. Cells were washed 3 times with PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated Goat anti-mouse IgG1 (Southern Biotech) or indodicarbocyanine (Cy5™)-conjugated Goat-anti-Armenian Hamster IgG (Jackson ImmunoResearch) for 1 hour. Propidium iodide (PI) was used as the nuclear dye at a 1:500 dilution with 0.1mM
RNAase for triple-color confocal laser scanning microscopy analysis (32). After washing with PBS for 5 mins, and with serum-free DMEM for another 5 mins, cells were mounted in fluoromount-G (Southern Biotech). Isotype controls included mouse myeloma IgG1 (Zymed), and CT-2 antibody blocked with CT-1 peptide (SSLSYTNPAVAATSANL, Dr. Gendler, Mayo Clinic, AZ) to confirm binding specificity. Cells were analyzed on a Zeiss LSM 410 dual beam laser confocal scanning microscope.

**Preparation of cell lysates.** Panc-1 and S2-013 cells (~90% confluent) were lysed on ice for 30 mins in ice-cold lysis buffer (10mM Tris-HCL pH8.0 containing 150mM NaCl, 1mM PMSF and 1%Triton X-100). Insoluble material was pelleted at 10,000g for 15 mins at 4°C. Lysates were diluted in the lysis buffer with protease-inhibitor sets [10ug/ml leupeptin, 10ug/ml pepstatin, 1ug/ml Pefabloe SC, 1ug/ml aprotinin, and 0.1mM EDTA (Roche Diagnostics GmbH, Mannheim, Germany)] to a total protein concentration of 1.5 ug/ul and stored at -80°C for future analysis. Protein content of the lysates was determined by using the Bio-Rad protein assay (BioRad) with bovine serum albumin as standard.

**Cellular fractionation.** Preparation of membrane/cytoplasmic extracts and nuclear extracts was as previously described (33-35). Panc-1 and S2-013 cells were cultured on 225 cm² flasks to 90% confluence. All steps were performed on ice unless otherwise indicated. Cells were washed with PBS twice at 4°C, and placed in 1.5 ml NPB extraction buffer for 2 hours [NPB buffer contains 10mM HEPES pH7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5% NP-40, 0.5 mM DTT, 0.5 mM PMSF, and a protease inhibitor cocktail containing 10ug/ml leupeptin, 10 ug/ml pepstatin, 1 ug/ml Pefabloe SC, 1 ug/ml aprotinin, and 0.1 mM EDTA (Roche Diagnostics GmbH, Mannheim, Germany). Cells
were scraped into NPB solution and disrupted with a glass Dounce Homogenizer (40–50 strokes with a pestle). The disruption was monitored by light microscopy after staining with 0.4% Trypan Blue Solution (Sigma Chemical Co.). The homogenate was subjected to centrifugation at 13,000 g for 1 hour. The supernatants were designated “membrane/cytoplasmic extract” and the pellets were designated “nuclear extract” following purification steps described below.

**Membrane/cytoplasmic extracts.** The supernatants were dialyzed against Dialysis Buffer containing 20mM HEPES pH7.9, 20% glycerol, 0.1M KCl, 0.2mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10ug/ml leupeptin, 10ug/ml pepstatin, 1ug/ml Pefablo SC and 1ug/ml aprotinin, with 3,500 Dalton Dialysis Cassettes (Pierce, IL) at 4°C for overnight. The dialyzed solution was stored at -80°C as “membrane/cytoplasmic extracts” until further use.

**Nuclear extracts.** The pellets were purified for nuclear extracts as previously described (36). Pellets were rinsed with NPB buffer containing 0.5%NP-40. The nuclei were resuspended in Nuclear Lysis Buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 420mM NaCl, 1.5 mM MgCl_2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 ug/ml leupeptin, 10 ug/ml pepstatin, 1 ug/ml Pefablo SC and 1 ug/ml aprotinin and extracted for 1 hour. Resuspended pellets were disrupted with the Dounce Homogenizer (20–30 strokes with pestle B). The homogenate was subjected to centrifugation at 4°C. The supernatant was dialyzed against Dialysis Buffer using the Microdialyzer System with 1000 Dalton dialyzer membranes (Pierce, IL) at 4°C overnight. The dialyzed extracts were stored at -80°C as “nuclear extract” for future analysis.
**Immunoprecipitation and immunoblot analysis.** Equal amounts of protein from cytoplasmic or nuclear extracts were incubated with the following mAbs: anti β-catenin, anti-E-cadherin, CT-2 anti-MUC1 CT, M2-antiFlag or a mouse IgG control. The immune complexes were precipitated with protein G-agarose beads (Sigma) overnight at 4°C. After washing 3 times with NET buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.1% NP-40, bound material were eluted from the immunoprecipitates in reducing SDS-PAGE loading buffer containing 10% SDS, 1 M Tris-HCl pH 6.8, 50% glycerol, 10% 2-ME, 2% Bromphenol Blue at 100°C for 10 mins.

Lysates and immunoprecipitated proteins were resolved on 6% or 10% denaturing polyacrylamide gels (with 3% polyacrylamide stacking gels), transferred to polyvinylidene difluoride membranes electrophoretically, and blocked in 5% dry milk in TBS (0.9% NaCl, 10 mM Tris, pH 7.4, 0.5% MgCl₂) at 4°C overnight. Primary antibodies were diluted 1:1000 or 1:500 in blotto (5% nonfat dry milk in TBS). Incubations were for 1 h at room temperature and were followed by three 10-min washes with TBS. Horseradish peroxidase-conjugated secondary antibodies were diluted 1:20,000 in TBS, and incubated for 1 h at room temperature. Second antibody incubations were followed by three washes as described above. ECL reagents were applied as per the manufacturer's instructions, and the membranes were exposed to ECL-sensitive film (Amersham Life Sciences) (31).

**Depletion of extracellular calcium (Ca²⁺) by EDTA:** S2-013 and Panc-1 cells were cultured on glass coverslips or flasks for 12 hours in DMEM+ 5% FBS until they achieved a monolayer with 90% confluence and established cell-cell adhesions. For immunoprecipitation, cells were washed twice with Ca²⁺-free PBS, incubated in serum-
free DMEM with 10mM EDTA for 3-6 hours and evaluated every 15 minutes for loss of cell adhesion and release from the monolayer. Upon release from the monolayer, suspended cells were collected by centrifugation and used for the preparation of cell extracts as described above.
RESULTS

Transfection of Cell lines and Expression of MUC1 deletion constructs. Two cell lines were chosen to evaluate epitope-tagged MUC1 constructs. The first, Panc-1, is a poorly differentiated human pancreatic adenocarcinoma cell line that expresses low levels of endogenous MUC1 and relatively low levels of other glycoproteins (37). The second, S2-013, is a moderately differentiated human pancreatic tumor cell line that is known to express O-glycosylated mucin-like proteins, including low levels of endogenous MUC1 (38).

Schematics of proteins encoded by the cDNA deletion constructs used in this study are shown in Figure 1A. DNA encoding the FLAG epitope was added to different MUC1 cDNA constructs, as described previously (4,31). This insertion yields recombinant product with the epitope tag on the extracellular domain of the protein. The addition of the epitope tag facilitates identification and analysis of the extracellular domain. Insertion of the FLAG epitope at this position is unlikely to interfere with biological function of the protein because the amino acid sequence of this region is poorly conserved among different species (6,39,40). The membrane-associated and cytoplasmic moieties of MUC1 are detected with a monoclonal antibody (CT2) against an epitope near the carboxyl terminus of the cytoplasmic tail.

FLAG tagged constructs encoding MUC1 with the tandem repeat deleted [MUC1F(ΔTR)], cytoplasmic tail deleted [MUC1F.CT3], or full-length [MUC1F] were stably expressed in S2-013 and Panc-1 human pancreatic cancer cells using the expression vector pHb-Apr1-neo, as described in ‘Experimental Procedures’ (31,41,42). Expression of recombinant MUC1 was evaluated by immunofluorescence with mAb M2.
(anti-FLAG) (data not shown) and by immunoprecipitation followed by immunoblotting with M2 (Figure 1B). Panc-1.MUC1F or S2-013.MUC1F cells expressed a full-length MUC1F isoform of approximately 250 kDa. The extracellular domain of the cytoplasmic tail deleted MUC1F.CT3 protein demonstrated mobility on SDS-PAGE slightly different than full-length MUC1F, for unknown reasons. Deletion of the cytoplasmic tail in the MUC1F.CT3 molecule does not affect its trafficking to the cell surface and is not predicted to affect the length of the large extracellular domain fragment (43). Unpublished studies from our group have shown that similar carbohydrate structures are expressed on both the full length and CT3 forms (data not shown), though it is not known if the extracellular domains are glycosylated to the same density. Similar to previous studies (2,4), the tandem repeat domain deleted MUC1F(DTR) protein trafficked to the cell surface and was detected as a broad band with an apparent mobility of 60 kDa.

Cytoplasmic and nuclear distribution of fragments of MUC1 CT. Nuclear and membrane/cytoplasmic extracts were prepared from Panc-1.MUC1F, Panc-1.NEO, S2-013.MUC1F, and S2-013.NEO cells by Dounce homogenization of cells incubated in hypotonic buffer (as described in the Experimental Procedures). Nuclear extracts from washed nuclei were shown not to be contaminated by membrane/cytoplasmic proteins (44). Contamination of membrane/cytoplasmic MUC1 in nuclear extracts was evaluated by performing immunoprecipitation and immunoblotting with mAb M2. The FLAG-epitope tagged extracellular domain of MUC1 should only be expressed in the endoplasmic reticulum, Golgi and at the cell surface. Nuclear extracts from the MUC1F transfected Panc-1 or S2-013 cells showed no detectable levels of the FLAG epitope tagged extracellular domain of MUC1, whereas membrane/cytoplasmic extracts showed
significant levels (Figure 2). In addition, we evaluated nuclear extracts by immunoblotting with mAb HMFG-2, which binds to the tandem repeat, to confirm that the extracellular domain was not detected in these fractions (in case the FLAG epitope was blocked or cleaved). The results showed that the extracellular domain was not contained in nuclear fractions (data not shown).

The distribution of the MUC1 CT in membrane/cytoplasmic and nuclear extracts from Panc-1.MUC1F, Panc-1.NEO, S2-013.MUC1F or S2-013.NEO cells was investigated by immunoblotting with mAb CT-2 (raised against the last 17 amino acids on the C-terminal cytoplasmic tail of MUC1, precise epitope not known). The results indicated that fragments of MUC1 CT were detected in a broad band at 26-30 kDa from cytosolic lysates and a narrower band of approximately 26 kDa in nuclear extracts of cells Panc-1.MUC1F and S2-013.MUC1F cells. Lower but detectable amounts of endogenous MUC1 CT were detected in cytosol and nuclei from Panc-1.NEO or S2-013.NEO cells (Figure 2A). β-actin (42 kDa) was used as an internal reference for protein loading in each lane (Figure 2). Membranes were also reprobed with a mAb against histone 2B as a second reference for protein loading. Consistent with previous reports (45), one or two forms of histone 2B protein were identified at around 15 kDa (the bottom panel of Figure 2). These results led us to hypothesize that a post-translational cleavage of the MUC1 CT released fragments from membrane-associated forms, which subsequently were distributed in the cytoplasm and the nucleus.

*Nuclear levels of β-catenin are enhanced by overexpression of MUC1.* Previous studies showed that the cytoplasmic tail of human MUC1 directly binds to β-catenin in ZR-15-7 human breast cancer cells (10,13). Thus, we evaluated the association of MUC1
with β-catenin in the pancreatic tumor cell lines S2-013 and Panc-1, and sought to
determine if the fragment of the MUC1 CT detected in nuclear extracts was associated
with β-catenin.

We first evaluated levels of β-catenin in cytosol and nuclei of transfected and
control cell lines. Notably, steady state levels of nuclear β-catenin were significantly
increased in cells overexpressing recombinant full length MUC1F, as compared to NEO
controls expressing lower levels of endogenous MUC1 (Figure 3). This result suggested
that overexpression of MUC1 in Panc-1 or S2-013 cells resulted in enhanced nuclear
levels of β-catenin.

The association between MUC1 and β-catenin in Panc-1 and S2-013 cells was
investigated by performing co-immunoprecipitations and immunoblots using mAbs M2
(extracellular domain), CT-2 (cytoplasmic tail) or anti-β-catenin. M2
immunoprecipitates from lysates of cells transfected with MUC1F, MUC1F.CT3,
MUC1F(ΔTR) were immunoblotted with anti-β-catenin. In reciprocal experiments, we
immunoprecipitated with mAb anti-β-catenin and immunoblotted with mAb M2 anti-
FLAG. Results, shown in Figure 4, demonstrated that full-length MUC1 protein
(containing both subunits) was associated with β-catenin in both S2-013 and Panc-1 cells.
There was no detectable association between β-catenin and cytoplasmic-tail-deleted
MUC1F.CT3, as expected since the predicted binding site is deleted. The tandem-repeat-
deleted MUC1F (ΔTR) showed little (S2013) or no (Panc-1) detectable association with
β-catenin, which was somewhat surprising, given that the deletion was in the
extracellular portion of MUC1 that is no longer covalently attached to the
transmembrane/cytoplasmic tail domains. One interpretation of this finding is that some
aspect of the conformation and/or function of the extracellular tandem repeat of MUC1 contributes to the ability of the intracellular cytoplasmic tail to interact with (and potentially signal through) β-catenin.

To further evaluate the association between intracellular fragments of the MUC1 CT and β-catenin, nuclear and cytosolic extracts were prepared as described in ‘Experimental Procedures’. Reciprocal co-immunoprecipitations with mAbs CT-2 (against the cytoplasmic tail of MUC1) and anti-β-catenin were performed on membrane/cytoplasmic and nuclear extracts of transfected Panc-1 and S2-013 cells. A 92 kDa form of β-catenin was co-immunoprecipitated with the MUC1 CT from both membrane/cytoplasmic and nuclear extracts of Panc-1.MUC1F and S2-013.MUC1F cells. There was no detectable nuclear β-catenin in CT2 immunoprecipitates of MUC1 CT from control (NEO) cells expressing low-levels of endogenous MUC1 (Figure 4B). Additional experiments showed no detectable β-catenin in immunoprecipitates with CT-2 from either membrane/cytoplasmic or nuclear extracts of Panc-1.MUC1F.CT3 or S2-013.MUC1F.CT3 cells expressing the cytoplasmic tail-deleted MUC1 (data not shown). Reciprocal experiments in which anti-β-catenin immunoprecipitates were immunoblotted with CT-2 were positive for MUC1 CT in cells overexpressing full-length MUC1. These results show that there is an association in cytosol and nuclei between fragments of the MUC1 CT and β-catenin in Panc-1 or S2-013 cells overexpressing full-length MUC1. Given that overexpression of MUC1 enhanced levels of nuclear β-catenin (Figure 3), we hypothesized that the association between β-catenin and fragments of the MUC1 CT facilitated the cytosol-to-nuclear translocation of β-catenin, and contributed to its nuclear accumulation.
The subcellular localization of MUC1 CT and β-catenin were further investigated by confocal microscopy. As shown in Figure 5, the cytoplasmic tail of MUC1 was localized to the membrane-associated cytoplasmic area, cytosol, and nucleus of S2-013.MUC1F or Panc-1.MUC1F cells (Figure 5A,B II & V). β-catenin was mainly localized in the cytosol and the nucleus, and was also observed at cell-cell junctions. Triple overlays of staining patterns for MUC1 CT, β-catenin and nuclei in Panc-1.MUC1F or S2-013.MUC1F cells revealed that colocalization of MUC1 CT and β-catenin was primarily in the apical membrane, and a small amount was detected in nuclei, as illustrated by the arrows in Figure 5. Previous studies reported that MUC1 exists as an integral membrane-associated protein after post-translational modifications (3,46). These data provide morphological evidence of both cytoplasmic and nuclear localization for the cytoplasmic tail of MUC1, and support the hypothesis that fragments of the MUC1 CT, which we predict to be released by a proteolytic cleavage, undergo cytosol-to-nucleus trafficking in association with other protein factors that translocate to nuclei, including β-catenin.

Association between MUC1 CT and β-catenin is unaffected by disruption of cadherin mediated cell-cell adhesion. It has been proposed that MUC1 plays a role in modulating cell-cell adhesion (4), and that MUC1 competes with E-cadherin for binding to β-catenin (11). We investigated whether the subcellular distribution and association of MUC1 CT and β-catenin was affected by disruption of cadherin mediated cell-cell adhesion. EDTA was used to chelate extracellular Ca\(^{2+}\) and thereby disrupt the Ca\(^{2+}\)-dependent cell adhesion mediated by cadherin complexes (47). Depletion of extracellular Ca\(^{2+}\) by EDTA causes disruption of Ca\(^{2+}\)-dependent cell adhesion and leads to co-
internalization of E-cadherin and β-catenin (48,49). Disruption of Ca\(^{2+}\)-dependent cell-cell adhesion may also result in the degradation of β-catenin through the adenomatous polyposis coli (APC)-mediated ubiquitination-proteasome pathway (50,51). Our immunoblot analyses demonstrated that the levels of cytoplasmic and nuclear MUC1 CT and β-catenin were not affected by EDTA treatment in Panc-1.MUC1F or S2-013.MUC1F cells, whereas EDTA treatment significantly reduced the levels of nuclear β-catenin in Panc-1.NEO or S2-013.NEO cells (Figure 6). Thus, overexpression of MUC1 may contribute to maintenance of nuclear levels of β-catenin during loss of Ca\(^{2+}\)-dependent cell-cell adhesion. It is also noteworthy that EDTA treatment decreased the expression levels of β-actin. Consistent with previous reports that EDTA chelation of extracellular Ca\(^{2+}\) induced phosphorylation of the NH2-terminal region of histone H2B (45,52) and blocked antibody binding, data in Figure 6 indicated that the EDTA treatment decreased detectable steady state levels of histone 2B.

Maintenance of cytosolic and nuclear β-catenin levels in Panc-1.MUC1F and S2-013.MUC1F cells after EDTA treatment raised the possibility that overexpression of MUC1 stabilized β-catenin and enhanced its nuclear accumulation during disruption of cell adhesion. We investigated whether the association between MUC1 CT fragments and β-catenin remained during disruption of Ca\(^{2+}\)-dependent cell-cell adhesion by performing immunoprecipitation with mAb CT-2 and immunoblotting with anti-β-catenin. The results showed that β-catenin (92 kDa) was associated with the MUC1 CT in both the cytoplasm and nuclei of Panc-1.MUC1F cells and S2-013.MUC1F cells after EDTA treatment (Figure 6B). However, detectable association between β-catenin and MUC1 CT was reduced in Panc-1 or S2-013 NEO cells (Figure 6B). Parallel immunoprecipitations
of cellular extracts with or without EDTA treatment were undertaken with mAbs against 
\[\beta\]-catenin, followed by immunoblotting with anti-E-cadherin. The results indicated that 
disruption of Ca\(^{2+}\)-dependent cell adhesion abolished the association between E-cadherin 
and \[\beta\]-catenin (Figure 6B, right panels).

*Decrease in nuclear plakoglobin induced by overexpression of MUC1.* We sought to determine if fragments of the MUC1 CT associated with another member of the 
armadillo family, plakoglobin (\[\beta\]-catenin). Elevation of cellular plakoglobin levels in 
carcinoma cells may be associated with early stages of oncogenic signaling (53).

Cytoplasmic extracts and nuclear extracts from transfected Panc-1 and S2-013 
cells were immunoblotted with mAb CT-2 or the anti-plakoglobin mAb 11E4 (Figure 7A). Overexpression of MUC1 did not affect the total cellular levels of plakoglobin, but nuclear levels of plakoglobin were decreased about 25% in cells overexpressing MUC1 
as compared to the NEO control cells (Figure 7A Middle panel). It was notable that 
nuclear forms of plakoglobin showed slightly slower mobilities in SDS-PAGE as 
compared to cytoplasmic/membrane forms, suggesting that post-translational 
modifications of plakoglobin may be associated with either nuclear or 
cytoplasmic/membrane localization, though we do not know the nature of this 
modification in these cells. CT-2 immunoprecipitates from membrane/cytoplasmic 
extacts and nuclear extracts of transfected Panc-1 or S2-013 cells were immunoblotted 
with 11E4. Results indicated there was no detectable association between the MUC1 CT 
and plakoglobin (Figure 7B). This suggested that overexpression of the MUC1 CT 
decreased nuclear levels of plakoglobin by an indirect mechanism. These data indicate 
that overexpression of MUC1 has opposite effects on the levels of nuclear plakoglobin
and nuclear β-catenin (decreased and increased respectively). Our results indirectly support the hypothesis that the overall function of plakoglobin is not completely redundant with that of β-catenin (54).
DISCUSSION

MUC1, a cell surface associated mucin, is synthesized as a single polypeptide that undergoes an early proteolytic cleavage event in the extracellular juxtamembrane region, creating a heterodimer that remains associated during its post-translational processing in the Golgi and expression on the cell surface (3,5,46). MUC1 has been shown to influence the cellular surface binding properties during cell adhesion (4). Previous reports that the cytoplasmic tail (CT) of MUC1 associates with β-catenin, Grb2/Sos, and c-Src (7-16), together with evidence that the extracellular domain of MUC1 associates with different adhesion molecules, including intercellular adhesion molecule (ICAM-1) and selectins (4,55-60), suggest that MUC1 plays an important role in morphogenetic signal transduction; however, its precise role has not been elucidated.

In this report, we present evidence [immunoprecipitation (Figure 2) and immunofluorescence analysis (Figure 5A.V&5B.V)] of localization of fragment(s) of MUC1 CT in cytosol and nuclei. The nuclear fragments of MUC1 CT showed faster mobility in SDS-PAGE than those from cell membrane/cytoplasm. These data lead us to hypothesize that a proteolytic cleavage event releases a fragment of the MUC1 CT that trafficks to the nucleus. It is not possible to predict a site of cleavage based on the mobility of nuclear MUC1 CT presented here (because of poor resolution of the SDS-PAGE gels used in these experiments). We are attempting to precisely define the nature of the nuclear MUC1 fragments by proteomics analysis. In support of these results, there is a recent report of nuclear localization of the MUC1 CT following stimulation of a myeloma cell line with IL-7 (61,62), and for the record it should be noted that
observations of nuclear MUC1 CT have been discussed at international meetings during the past 4 years (Hollingsworth, M., personal communication).

The detection of fragments of MUC1 CT in the nucleus is of interest given its sequence conservation across species, (6) and the fact that it contains putative docking sites for different kinases and proteins involved in signal transduction (7-16). It was also notable that overexpression of MUC1 was correlated with increased levels of nuclear β-catenin (Figure 3).

Yamamoto et al. (10) reported that MUC1 interacts with β-catenin at an SXXXXXSSL site on the C-terminal region in ZR-75-1 human breast cancer cells. In agreement, we found the cytoplasmic tail of MUC1 to be associated with β-catenin in Panc-1 and S2-013 human pancreatic tumor cells (Figure 4A). It is of some interest that there was reduced association between the MUC1 CT and β-catenin in cells expressing the tandem repeat deleted MUC1F(DTR) protein. Given that the tandem repeat binds to different adhesion molecules, the fact that deletion of the tandem repeat significantly reduces detectable association with β-catenin suggests that there is a functional link between the extracellular tandem repeat and signaling through the cytoplasmic tail. In a recent report (63), we showed that alterations in MUC1, specifically deletion of either the cytoplasmic tail or tandem repeat, resulted in an increased propensity of S2-013 tumor cells to invade vessels and metastasize to lymph nodes compared to a cell line overexpressing full length MUC1. Analysis of gene expression by DNA microarrays found that numerous genes were differentially expressed upon overexpression of full length MUC1, which were not differentially expressed by cells overexpressing MUC1 with tandem repeat or cytoplasmic tail deleted. There were very few differences in gene
expression patterns among cells overexpressing MUC1 isoforms lacking the tandem repeat or the cytoplasmic tail. Those results further support the hypothesized functional link between the cytoplasmic tail and tandem repeat domain of MUC1, and support the hypothesis that an important function of the molecule is morphogenetic signal transduction that is mediated in part by the cytoplasmic tail.

\[\beta\]-catenin and the MUC1 CT were coimmunoprecipitated from both cytosol and nuclei of Panc-1.MUC1F and S2-013.MUC1F (Figure 4B). This leads us to posit that association with the cytoplasmic tail of MUC1 stabilizes \[\beta\]-catenin and thereby contributes to the observed enhancement in accumulation of nuclear \[\beta\]-catenin in cells overexpressing MUC1 (Figure 3).

What are the complexes that contain MUC1 and \[\beta\]-catenin in the membrane/cytosol fraction and the nucleus? \[\beta\]-catenin has previously been found to be associated with: presenilin in the ER; E-cadherin at the cell surface in cell-cell junctions; EGFR at the cell surface; APC/GSK-3\[\beta\]/Axin degradation complex in the cytosol; and LEF-1/TCF transcriptional complex in the nucleus. The function of the complex between \[\beta\]-catenin and presenilin in the ER is not completely understood, but presenilin contributes to turnover of \[\beta\]-catenin (64), perhaps through pathways that regulate ubiquitination (64), and it is unlikely that this association would contribute to the increased nuclear localization of \[\beta\]-catenin observed here. However, presenilin also contributes to the intramembrane cleavage of Notch at sites other than the ER (65,66). Notch is a morphogenetic signaling molecule that bears overall structural and biological similarities to MUC1, in that it is a heavily O-glycosylated cell surface heterodimer, whose cytoplasmic tail is released as part of its signaling function (65). Thus, the
potential association of presenilin with MUC1 and/or β-catenin should be investigated in future studies.

EGFR and MUC1 have previously been co-immunoprecipitated from human and mouse breast cancer samples and EGFR has been shown to phosphorylate MUC1 (12), though the nature of the association and the regulation of phosphorylation has not been clearly defined. EGFR and β-catenin have also been shown to be associated in some breast cancer samples (67). Thus, it is possible that MUC1-EGFR-β-catenin are in a complex at the cell surface of the pancreatic tumor cell lines evaluated here; however, we have not yet directly addressed that question. In any case, an association of MUC1-EGFR-β-catenin at the cell surface does not directly explain the nuclear localization of MUC1 CT and its association in the nucleus with β-catenin, as EGFR has not been reported to be localized in the nucleus. Nonetheless, it will be important to determine events upstream of the localization of the MUC1-β-catenin complex in the nucleus. It is possible that nuclear MUC1 CT-β-catenin originates from a MUC1-EGFR-β-catenin complex at the cell surface in response to ligand binding events that result in cleavage of the MUC1 CT, a hypothesis that will be further explored in future studies.

In results not shown here, we evaluated expression of the adenomatous polyposis coli (APC) gene product, a factor that may contribute to β-catenin degradation, in transfected Panc-1 and S2-013 cells. Expression of APC proteins at 300 kDa, 180 kDa and 80 kDa were observed in Panc-1 and S2-013 MUC1F cells (unpublished data), showing that APC is expressed and turned over (68), though it is not known whether APC is mutated in either of these cell lines. There was no detectable association between APC and MUC1 CT (data not shown). This result indirectly supports the hypothesis that
the cytoplasmic tail of MUC1 functions as a positive factor for stabilizing cytoplasmic
and nuclear $\beta$-catenin and suggests that the MUC1- $\beta$-catenin complex is not localized to
the APC/GSK-3$\beta$/Axin degradation complex in the cytosol.

We sought to determine if the MUC1 CT-$\beta$-catenin complex was associated with
E-cadherin, and if the nuclear localization of MUC1 and $\beta$-catenin were influenced by
disruption of E-cadherin-mediated cell-cell adhesion. MUC1 was not found in association
with E-cadherin when evaluated by immunoprecipitation (data not shown), whereas we
detected the co-localization and an association between $\beta$-catenin and E-cadherin at the
cell surface by immunofluorescence (data not shown) and by co-immunoprecipitation
(Figure 6). EDTA was used to disrupt cadherin interactions at the cell surface by
removing Ca$^{2+}$ from cell culture media. The association between E-cadherin and $\beta$-
catenin was abolished after EDTA treatment. The fate of $\beta$-catenin upon release from
cadherin is complex, and significantly influenced by other factors in the cell such as the
status of the adenomatous polyposis coli (APC) gene product (69), or the status of GSK-
3$\beta$ regulated Wnt signaling pathway (70). Our data indicated that removing Ca$^{2+}$ from the
media enhanced the nuclear localization distribution of the MUC1 CT and maintained
levels of nuclear $\beta$-catenin, as detected by confocal microscopy (unpublished data) and
immunoprecipitation (Figure 6).

The relative intensity of the reactive bands on immunoblots and the
immunofluorescence staining patterns (Figure 4, Figure 5, Figure 6, and data not shown
from three dimensional reconstructions of sequential Z-sections of confocal images)
suggest that only a fraction of the total detectable MUC1 CT and $\beta$-catenin were
associated in the cytosol and nucleus. One interpretation of these data is that the cytosol-
to-nuclear translocation of the fragment of MUC1 CT and β-catenin represents one of multiple pathways of signal transduction for these two factors. This led us to hypothesize that the MUC1 CT is associated with other factors in the cytoplasm and nucleus.

We therefore examined the possibility that the MUC1 CT fragment associates with factors other than β-catenin. Plakoglobin was evaluated, as it is a homologue of β-catenin and displays a cytosol-to-nucleus trafficking pathway similar to β-catenin (71). Steady state levels of nuclear plakoglobin were significantly decreased upon overexpression of MUC1 (Figure 7). We detected no direct interactions between plakoglobin and the MUC1 CT. It was also notable that overexpression of the cytoplasmic tail of MUC1 had different (opposite) effects on levels of nuclear β-catenin and nuclear plakoglobin. Previous studies reported that levels of nuclear plakoglobin were directly correlated with activity of c-Myc and other oncogenes (52). Thus, the effects on global gene expression that result from overexpression of MUC1, decreases in levels of nuclear plakoglobin and increases in levels of nuclear β-catenin, which have been investigated in a preliminary manner (63), should be explored in depth in the future.

In conclusion, we postulate that one role of the MUC1 CT in morphogenetic signal transduction is to modulate the functional activity of nuclear β-catenin as a transcriptional factor. This possibility is being explored in ongoing experiments that evaluate LEF-1/TCF-β-catenin responsive transcriptional activity during overexpression of MUC1 in human pancreatic cancer cells. Other studies in progress include proteomic analysis to determine the proteolytic cleavage site on the cytoplasmic tail of MUC1 and the sequence of the nuclear MUC1 CT fragment. We are also endeavoring to identify
other protein factors that are associated with the cytoplasmic or nuclear fragments of
MUC1 CT.

In summary, we report that under conditions of overexpression, fragments of the
MUC1 cytoplasmic tail are released and transported to the nucleus in association with β-
catenin, but not plakoglobin (γ-catenin) or APC. This supports the hypothesis that MUC1
plays a role in catenin-related morphogenetic signal transduction, and suggests a
mechanism for the role of MUC1 in carcinogenesis and metastasis that should be further
explored.
(Page 16) ¹,² Y.F. Wen, T.C. Caffrey, M.A. Hollingsworth, unpublished data.

(PAGE21,22)³,⁴ Y.F. Wen, T.C. Caffrey, M.A. Hollingsworth, unpublished data.

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Figure Legends:

Figure 1. Expression of recombinant MUC1 isoforms. A. Schematic representation of epitope-tagged MUC1 constructs. The full-length MUC1 construct includes a FLAG epitope, tandem repeat domain (black box), transmembrane domain (shaded box), and cytoplasmic tail (red box). Sixty-six (out of sixty-nine) amino acids in the cytoplasmic tail of MUC1F were deleted in the MUC1F.CT3 forms. The tandem repeat domain of MUC1F was deleted in MUC1F.(D)TR). B. Transfected MUC1 deletion constructs were expressed in the Panc-1 or S2-013 cells. Cell lysates (250 μg total protein/well) from each transfectant of Panc-1 or S2-013 cells were immunoprecipitated and immunoblotted with M2 anti-flag mAb against the FLAG epitope present on the recombinant proteins.

Figure 2. Subcellular expression of MUC1 CT and β-catenin in Panc-1 and S2-013 cells. Panc-1 and S2-013 MUC1F cells were fractionated into membrane/cytoplasmic and nuclear extracts as described in Experimental Procedures. Membrane/cytoplasmic and nuclear extracts from Panc-1 or S2-013 MUC1F, and Panc-1 or S2-013 NEO cells were immunoprecipitated (IP) and immunoblotted (IB) using a mAb against the FLAG epitope (M2) to examine the membrane/cytoplasmic contamination of nuclear extracts (Top panel). Membrane/cytoplasmic or nuclear extracts (200 μg sample protein each) were subjected to 10% SDS-PAGE, followed by IB with CT-2 mAb against the C-terminus 17-amino-acid cytoplasmic tail of MUC1 (Middle panel). The housekeeping gene protein product β-actin was used as an internal reference for protein loading per lane, as described previously (72) (Bottom panel), and same membrane was reprobed with mAb anti-histone 2B as a second reference for protein loading.
Figure 3. Enhancement of nuclear β-catenin by overexpression of MUC1. Cell lysates, membrane/cytoplasmic extracts, and nuclear extracts were subjected to 10% SDS-PAGE and analyzed by IB with an anti-β-catenin mAb. The membrane was reprobed with mAbs against β-actin, and histone 2B as internal controls for immunoblot analysis. Results shown are representative of at least three independent experiments.

Figure 4. Association between MUC1 CT and β-catenin in cytosol and nuclei. A. The cytoplasmic tail of MUC1 is required for association with β-catenin. M2 anti-FLAG immunoprecipitates from cell lysates of Panc-1 or S2-013 transfected cells overexpressing MUC1 deletion constructs were immunoblotted with anti-β-catenin (Top panel). Reciprocal experiments (immunoprecipitations with anti-β-catenin and immunoblotting with M2 anti-FLAG) were also performed (bottom panel). B. Subcellular association of MUC1 CT and β-catenin. The Panc-1 and S2-013 cells were fractioned into membrane/cytoplasmic and nuclear extracts as described in Experimental Procedures. CT-2 anti-MUC1 CT immunoprecipitates from cell lysates, membrane/cytoplasmic and nuclear extracts of Panc-1 or S2-013 MUC1F or NEO cells were immunoblotted with anti-β-catenin. Mouse IgG was used as a control for immunoprecipitation.

Figure 5. Subcellular localization of MUC1 CT and β-catenin in Panc-1 and S2-013 MUC1F cells. Confocal microscopy was used to determine the intracellular distribution of MUC1 CT and β-catenin in Panc-1 MUC1F (A) and S2-013 MUC1F (B). Cells grown to 80% confluence on coverslips were fixed and permeablized with Trixton-X100 before
incubation with mAbs CT-2, anti-[β]-catenin and the nuclear dye Propidium Iodide (PI). Fluorescence signals from each scan were acquired sequentially. mAb anti-[β]-catenin was identified with FITC-conjugated secondary antibody and visualized as green color (I), mAb CT-2 (anti-MUC1 CT) was identified as blue color by using a Cy5\textsuperscript{TM}-conjugated secondary antibody (II), and nuclei were stained with PI and visualized as red color (III). Overlay of BLUE and GREEN shown as WHITE color demonstrated the subcellular co-localization of MUC1 CT and [β]-catenin, which was mainly distributed in the membrane-associated cytoplasmic area (IV). The colocalization of BLUE and RED, shown as PURPLE (indicated by arrow), demonstrated nuclear distribution of the cytoplasmic tail of MUC1 (V). Triple-merger of BLUE, GREEN and RED, shown as PINK-WHITE, demonstrated the distribution of a small amount of MUC1 CT and [β]-catenin in the nucleus (VI). These images were examined with a Zeiss LSM 410 laser scanning microscopy Bar = 20μM; 100X magnification; results shown here represent 3 to 4 individual cell scanning observations.

**Figure 6. Subcellular association of MUC1 CT and [β]-catenin is stabilized by overexpression of MUC1 during disruption of Ca\textsuperscript{2+}-dependent cell adhesion. A.**

**Subcellular expression of MUC1 CT and [β]-catenin after EDTA treatment.** After 10mM EDTA treatment for 3 hours, cell lysates and subcellular extracts of Panc-1.MUC1F, S2-013.MUC1F, or Panc1.NEO, or S2013.NEO cells were prepared as described in Experimental Procedures. EDTA-treated lysates or membrane/cytoplasmic and nuclear extracts from Panc-1 or S2-013 MUC1F, and Panc-1 or S2-013 NEO cells were immunoblotted (IB) using mAb CT-2 to examine the subcellular expression of MUC1 CT.
(Top panel). Other sets of EDTA-treated cell lysates, membrane/cytoplasmic, or nuclear extracts (200ug protein each) were resolved by 10% SDS-PAGE and immunoblotted with mAb anti-[[-]-catenin to examine the subcellular expression of [-]-catenin (Middle panel). The housekeeping gene protein product [[-]-actin and histone H2B were used as internal references for protein (Bottom panel).

B. Subcellular association of MUC1 CT and [-]-catenin after EDTA-treatment. Anti-MUC1CT CT-2 immunoprecipitates from EDTA-treated membrane/cytoplasmic and nuclear extracts of Panc-1 or S2-013 MUC1F or NEO cells were immunoblotted with mAb anti-[[-]-catenin. Mouse IgG was used as a control for immunoprecipitation (Left panel). Another set of EDTA-treated cell lysates, membrane/cytoplasmic, or nuclear extracts with the same amount of sample protein (200ug each) were immunoprecipitated with anti-[-]-catenin and immunoblotted with mAb anti-E-cadherin to examine disruption of the cadherin-catenin complex by EDTA-treatment. Mouse IgG was used as a control for immunoprecipitation (Middle panel). Control experiments included one set of (EDTA-untreated) cell lysates, membrane/cytoplasmic or nuclear extracts with the same amount of sample protein (200ug each), immunoprecipitated with anti-[-]-catenin and immunoblotted with mAb anti-E-cadherin to demonstrate that a 120 kDa form of E-cadherin was associated with [-]-catenin in cell lysate and membrane/cytoplasmic extracts. Mouse IgG was used as a control for immunoprecipitation (Right panel).

Figure 7. Decrease in nuclear plakoglobin in cells overexpressing MUC1. A.
Detection of plakoglobin in membrane/cytoplasmic and nuclear extracts. Cytoplasmic and nuclear extracts from Panc-1.MUC1F, S2-013.MUC1F, and Panc-1.NEO or S2-
013.NEO cells were immunoblotted (IB) using mAb CT-2 to examine the subcellular expression of MUC1 CT (Top panel). Cytoplasmic or nuclear extracts (200 ug sample protein each) were resolved by 10% SDS-PAGE and immunoblotted with mAb 11E4 (anti-plakoglobin) to examine the subcellular expression of plakoglobin (Middle panel). The housekeeping gene protein product β-actin and histone H2B were used as internal references for protein loading (Bottom panel). Results shown here were representative of two independent experiments.

B. Plakoglobin is not associated with MUC1 CT. Anti-MUC1CT CT-2 immunoprecipitates from cytoplasmic and nuclear extracts of Panc-1.MUC1F, S2-013.MUC1F, Panc-1.NEO or S2013.NEO cells were immunoblotted with mAb 11E4 anti-plakoglobin. Mouse IgG was used as a control for immunoprecipitation, and lysates of each cell was loaded for direct immunoblotting with 11E4 as markers for plakoglobin expression.
Fig. 1A

Signal Sequence
FLAG Epitope
Tandem Repeat Domain

Signal Sequence
FLAG Epitope
Tandem Repeat Domain

Deletion of Tandem-Repeat Domain

Transmembrane Domain
Cytoplasmic Tail (69 amino acids)

MUC1F

Transmembrane Domain
66-aa deletion in cytoplasmic tail

MUC1F. CT3

Transmembrane Domain
Cytoplasmic Tail (69 amino acids)

MUC1F(ΔTR)
Fig 1B

Panc-1

S2-013

IP/IB: M2 anti-Flag

kDa

MUC1F
NEO
MUC1F.CT3
MUC1FΔTR

kDa

MUC1F
NEO
MUC1F.CT3
MUC1FΔTR
Fig 4

A

**Panc-1**

| IP: M2-antiflag | IB: anti-β-cat |
|-----------------|----------------|
| kDa             |                |
| 105             |                |
| 75              |                |

**S2-013**

| IP: anti-β-cat | IB: M2-antiflag |
|----------------|-----------------|
| KDa            |                 |
| 250            |                 |
| 160            |                 |
| 105            |                 |
| 75             |                 |
| 50             |                 |
Fig. 5B  
S2-013 MUC1F cells

β-cat  
MUC1 CT  
Nucleus-Pl

MUC1 CT/β-cats  
MUC1 CT/nucleus  
MUC1 CT/β-cats/nucleus
Fig 6

B

IP: CT-2 anti-MUC1CT
IB: anti-β-catenin

+EDTA

IP: CT-2 anti-MUC1CT
IB: anti-β-catenin

-EDTA

IP: anti-β-catenin
IB: anti-E-cad

+EDTA

IP: anti-β-catenin
IB: anti-E-cad

-EDTA

S2-013 NEO

kDa
105
75

S2-013 MUC1F

kDa
105
75

Panc-1 NEO

kDa
105
75

Panc-1 MUC1F

kDa
105
75
Fig 7
B

**S2-013 MUC1F**
- IP: CT-2 anti-MUC1
- CT
- IB: 11E4
- IgG+Cell lysate
- Nuclear extract
- Membrane/Cytoplasmic extract
- IB: 11E4+Cell lysate

**Panc-1 MUC1F**
- IP: CT-2 anti-MUC1
- CT
- IB: 11E4
- IgG+Cell lysate
- Nuclear extract
- Membrane/Cytoplasmic extract
- IB: 11E4+Cell lysate

kDa

75
Nuclear association of the cytoplasmic tail of MUC1 and β-catenin
Yunfei Wen, Thomas C. Caffrey, Margaret J. Wheelock, Keith R. Johnson and Michael A. Hollingsworth

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