MUC1 Initiates a Calcium Signal after Ligation by Intercellular Adhesion Molecule-1*

Jennifer J. Rahni, Qiang Shen, Brian K. Mah, and Judith C. Hugh

From the Department of Laboratory Medicine and Pathology, Cross Cancer Institute, University of Alberta, Edmonton, Alberta T6G 1Z2, Canada

The MUC1 mucin is normally restricted to the apical surface of breast epithelial cells. In tumors, it is frequently overexpressed and underglycosylated. The MUC1 peptide core mediates firm adhesion of tumor cells to adjacent cells via binding to intercellular adhesion molecule-1 (ICAM-1). There is increasing evidence that MUC1 is involved in signaling, with current reports focusing on phosphorylation of the MUC1 cytoplasmic tail after indirect or artificial modes of stimulation. ICAM-1 is the only known direct ligand of the MUC1 extracellular domain. The data presented herein show that MUC1 expressed on the surface of breast cancer cell lines or transfected 293T cells can initiate a calcium-based oscillatory signal on contact with ICAM-1-transfected NIH 3T3 cells, and we present a novel method of quantifying and comparing calcium oscillations. The MUC1-induced signal appears to be distinct from those previously described, and may involve a Src family kinase, phosphoinositol 3-kinase, phospholipase C, and lipid rafts, but not mitogen-activated protein kinase. As calcium signaling has been associated with cytoskeletal change and motility, it is possible that the functions of MUC1 include heterotypic cell-cell adhesion followed by a calcium-based promigratory signal within tumor cells, thus facilitating metastasis.

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‡ These authors contributed equally to this work.
§ To whom correspondence should be addressed: Dept. of Laboratory Medicine and Pathology, Cross Cancer Inst., 11560 University Ave., Edmonton, Alberta T6G 1Z2, Canada. Tel.: 780-432-8450; Fax: 780-432-8455; E-mail: judith.hugh@cancerboard.ab.ca.

The abbreviations used are: EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; ICAM-1, intercellular adhesion molecule-1; FBS, fetal bovine serum; YFP, yellow fluorescent protein; EYFP, enhanced cyan fluorescent protein; ICAM-1, intercellular adhesion molecule-1; ROI, regions of interest; MOPC, mineral oil plasmacytoma; PBS, phosphate-buffered saline; PI, phosphoinositol; CD, cytoplasmic domain; PLC, phospholipase C; PP2, (3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; 2-APB, aminoethoxydiphenylborane (MAPK)/extracellular signal-related kinase (ERK) pathway. Our laboratory has previously identified intercellular adhesion molecule-1 (ICAM-1) as a natural, endogenous ligand for MUC1 (13, 14). ICAM-1 is currently the only reported direct ligand for the MUC1 extracellular domain, and binding promotes adhesion of tumor cells to a simulated vessel wall construct under fluid flow conditions (15). In this report, we demonstrate a novel signaling paradigm, in which MUC1 participates in initiating intracellular calcium oscillations after direct stimulation by ICAM-1. As calcium signaling has previously been implicated in cytoskeletal remodeling and motility (16), we hypothesize that this signal is involved in tumor cell migration. Thus, the MUC1/ICAM-1 interaction may facilitate extravasation of tumor cells after mediating binding to the blood vessel wall.

EXPERIMENTAL PROCEDURES

Reagents—The CT2 antibody against the MUC1 cytoplasmic domain and the pC1Neo TR+ FLAG plasmid carrying the MUC1 gene were generously provided by Dr. Sandra Gendler, Mayo Clinic, Scottsdale, AZ. The MUC1 gene was PCR amplified from the plasmid and inserted into the Clontech pEYFP-N1 plasmid at the BamHI/NcoI cut sites. A synthetic MUC1-specific signal sequence, TCGACTAGGCTATAGCA-CGGGACCACCTTCTTCTCTGCTGCTCCTCACAGTGCTTCACAGTGCTTACAGTGTTCAGC, made by the DNA Core Services Laboratory, Biochemistry Department, University of Alberta, was inserted into the multiple cloning site. Flu-3 was from Molecular Probes. Pluronic F-127, U-73122, U-73343, methyl-p-cyclodextrin, nystatin, wortmannin, MOPC C31 mouse IgG1 isotype control antibody, anti-tubulin B-5-1-2 antibody, and gelatin were from Sigma. PD98059 was from Calbiochem. PP2 and 2-APB were from Tocris. B27.29 antibody against the MUC1 extracellular domain was a gift from Biomira, Inc. ICR5 antibody against human ICAM-3 was a gift of ICOS Corp. Goat anti-mouse phycoerythrin secondary antibody was purchased from Southern Biotechnology Associates, Inc. Goat anti-mouse or anti-Armenian hamster peroxidase-conjugated antibodies were purchased from Jackson ImmunoResearch. ECL Plus was from Amersham Biosciences. FBS and culture media were from Invitrogen.

Calcium Oscillation Assay—3-cm glass-bottomed dishes were purchased from MatTek. The glass bottoms were coated with 100 μl of FBS (for untransfected cell lines) or a solution of 0.1% (w/v) gelatin in water (for 293T cells). 100 μl of untransfected cell suspension at 1 × 10^6/ml or MUC1-transfected 293T cells at 5 × 10^5/ml were plated onto coated cover glasses and allowed to equilibrate overnight. This generally resulted in ~60% cell confluence the next day. The medium was aspirated from plated cells, and a 1:1 mixture of 5 ml Fluor-3 in MeSO:20% (w/v) Pluronic F-127 in MeSO, diluted 1:1000 in Dulbecco’s modified Eagle’s medium + 10% FBS was pipetted over the cells. The plated cells were then incubated with Fluor-3 for 1 h at 37°C, 5% CO2. Where indicated,
cells were then incubated with 10 μM PD98059, PP2, U-73122, or U-73343, 15 mM methyl-β-cyclodextrin, 75 μg/ml nystatin, 2 μM wortmannin, or 100 μM 2-APB for 30 min at 37 °C, 5% CO₂. Cells were washed once in 37 °C imaging buffer (152 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES, 5.6 mM glucose, pH 7.2 (17)), then left in imaging buffer at 37 °C for 30–45 min until imaged. For the antibody blockade experiments, this buffer contained either B27.29 (MUC1 block) or ICR5 (irrelevant block) at 120 μg/ml for 293T MUC1 transfectants and either B27.29 or MOPC C31 (irrelevant block) at 60 μg/ml for T47D cells. NIH 3T3 ICAM-1 or mock transfectants were trypsinized and resuspended in imaging buffer at ~1.2 × 10⁶/ml. Immediately before photographing, the imaging buffer was decanted, and if blocking antibodies were used, the cells were gently rinsed with warmed imaging buffer. The MatTek dish was then placed in a microscope stage warmer set to 37 °C on a Zeiss AxioScope Digital Imaging Microscope. Using Metamorph software (Universal Imaging Corp.), a DIC image was recorded, then 60 images at 3-s intervals were recorded under the fluorescein isothiocyanate filter. 100 μl of NIH 3T3 cell suspension was added to the plated cells immediately after the first fluorescein isothiocyanate image was taken so that the calcium flux response of the plated cells was recorded in the remaining 59 images. A final DIC image was taken at the end of the time course to ensure that all plated cells had been covered by the NIH 3T3 cells. In the “reverse” model, NIH 3T3 mock- or ICAM-1-transfected cells were plated on the dish and grown to confluence. MCF-7 cells were loaded with Fluo-3 while in tissue culture flasks, then washed, trypsinized, and resuspended in imaging buffer at ~5 × 10⁵/ml. MCF-7 cells were then added to the NIH 3T3 cells during imaging.

**Data Analysis**—Using Metamorph software, the images taken for each test condition were built into a stack, and 40 random cells per condition were circled (circled areas = regions of interest or ROI). The changes in average fluorescence intensity for each ROI were graphed over time and exported to MS Excel. Each condition was repeated at least three times, n = a minimum of 120. MS Excel was used to plot the data and calculate the “oscillation factors,” which are defined as the number of oscillation cycles multiplied by the “amplitude factor” for each ROI. The number of oscillation cycles was counted manually from the plotted data. The amplitude factors were calculated by plotting an Excel “LOGEST” trend line (y = intercept × slope) through the oscillatory portion of the data, then calculating the absolute value of the difference between the actual plotted data and the trend line for each data point; the sum of these differences was defined as the amplitude factor (Fig. 1D).

**Western Blotting**—Cells were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 5 μg/ml Sigma protease inhibitor cocktail, 50 mM Tris, pH 7.6 (4)) and subjected to shearing with a 28-gauge needle. Insoluble materials were pelleted, and the supernatant was assessed for protein concentration using the Bio-Rad DC assay kit. Equal amounts of protein for each test sample were loaded onto polyacrylamide gels. Resolved gels were transferred to Immobilon-P or PSQ membranes and probed with B27.29 or CT2 antibody. Specific labeling was visualized with ECL Plus, and imaged using a Typhoon 9400 Variable Mode Imager and ImageQuant 5.2 Software (Amersham Biosciences). Membranes were re-probed for tubulin as a loading control.

**Flow Cytometry**—Cells were trypsinized, washed one time in FBS-containing medium, then divided into three aliquots, each of which was incubated with one of 2% bovine serum albumin, 0.02% Tween 20 in Tris-buffered saline (BTT, unlabeled control), 5 μg/ml MOPC 31C in BTT (isotype control), or 5 μg/ml B27.29 in BTT, on ice for 1 h in a volume of 30 μl. Cells were washed in a 50× volume of cold PBS, then resuspended in 30 μl of BTT (unlabeled control) or 30 μl of phyco-
erythrin secondary, 2 μg/ml in BTT for a further 1 h in the dark on ice.

Cells were again washed in a 50-volume of cold PBS, then resuspended in 300 l of PBS and treated with final concentrations of 120 units/ml DNase I and 4.2 mM MgCl₂ for 15 min at room temperature. Samples were stored at 4 °C in the dark until analyzed by flow cytometry. At least 10,000 events were recorded on the FL2 (phycoerythrin) channel. As the cells were chilled, unfixed, and unpermeabilized, only surface MUC1 was stained.

**Statistical Analysis**—The Newman-Keuls multiple range comparison was used to determine statistical differences in data sets where there were more than two conditions. Otherwise, the Student’s t test was used.
RESULTS

Intracellular Calcium in MUC1-positive Cells Increases and Oscillates on Contact with ICAM-1-positive Cells—293T cells, which express no endogenous MUC1, were transfected with the MUC1 construct shown in Fig. 1A, and a series of subclones designated as SYM were isolated. Fig. 1B (QuickTime movie links) shows that a calcium-based response in Fluo-3-loaded SYM1 cells was more intense and oscillatory when these cells came into contact with ICAM-1-transfected NIH 3T3 cells, as compared with mock-transfected cells. In SYM3 cells, a subclone not expressing MUC1, the calcium-based responses did not have large oscillations regardless of the presence of ICAM-1. Tracings of the fluorescence intensities over time (Fig. 1C) show more clearly the difference in the SYM1 cells responses and also demonstrate that in SYM3 cells the calcium-based responses to the NIH 3T3 ICAM-1 or mock cells are similar to that seen in the SYM1 cells when ICAM-1 was absent.

When observing several cells within a given trial, there were cell-to-cell variations in the oscillation responses, requiring that signals be averaged into oscillation factors (Fig. 1D) to facilitate comparisons between experimental conditions. 40 cells were selected for each trial, chosen randomly on the pre-exposure DIC image, or a summed stack of the fluorescent images captured over the entire time course. The oscillation factors for a total of 120 individual cells could then be averaged and compared with other test conditions. This overcame potential bias in selecting “representative” tracings in a non-synchronized cell population.

Surface Expression of MUC1 Is Required to Induce an ICAM-1-triggered Oscillatory Calcium Signal—A series of human breast cancer cell lines showed a gradient of oscillatory responses when tested in the calcium oscillation assay (Fig. 2A). Two of these cell lines, T47D and MCF-7, displayed statistically significant differences in a t test comparing oscillation factors resulting from contact with NIH 3T3 ICAM-1 or NIH 3T3 mock transfectants (p < 0.0009). A reverse model, in which Fluo-3-loaded MCF-7 cells in suspension were added to adherent NIH 3T3 ICAM-1 or mock transfectants demonstrated that the MCF-7 cells oscillated only on contact with ICAM-1 expressing cells (data not shown); thus the cells did not need to be adherent to respond.

A series of SYM (293T MUC1-transfected) cells expressing differing levels of MUC1 also showed higher oscillatory responses after contact with ICAM-1-expressing cells in comparison with mock transfectants (t test, p < 1.5–14; Fig. 2B). Western blotting for the total cellular levels of both the extracellular and cytoplasmic domains of MUC1 in all tested cell lines suggested that a trend between levels of MUC1 expression and oscillatory response was inconsistent (Fig. 2C); however, flow cytometry analysis without permeabilization showed that although the low oscillating subclone, SYM25, had high total cellular MUC1, surface expression was lower than that observed on SYM1 cells (Fig. 2D). This suggests that only surface MUC1 is able to interact with exogenous ICAM-1 to initiate the calcium signal, and there may be a cell-specific “threshold” of MUC1 expression necessary to elicit a response.

The specificity of MUC1 in regulating the calcium-based signal was demonstrated by pretreating SYM or T47D cells with MUC1-blocking or irrelevant antibodies (Fig. 3). Pretreatment with B27.29, which has previously been shown to block MUC1/ICAM-1 interactions (13, 14), inhibited the oscillatory response, unlike cells pretreated with an irrelevant antibody.

Mediators of the MUC1/ICAM-1 Calcium Signal—SYM1 cells were treated with PD98059 (inhibits MAPK), wortmannin (inhibits PI 3-kinase, MAPK, and myosin light chain kinase), or PP2 (inhibits Src family kinases) and compared with an untreated control in the calcium oscillation assay. Wortmannin and PP2, but not PD98059, could reduce the level of oscillations seen in the presence of both MUC1 and ICAM-1 to levels seen when one or both of the molecules were absent (Fig. 4A). 2-APB (inhibits inositol trisphosphate (IP3)-induced calcium release from the endoplasmic reticulum) and U-73122 (inhibits phospholipase C), but not U-73343 (inactive analogue of U-73122), also abrogated oscillatory responses (Fig. 4B). Disruption of lipid rafts by methyl-β-cyclohexalin and nystatin also inhibited ICAM-1-induced calcium oscillations in the SYM1 cells (Fig. 4C).

DISCUSSION

The MUC1 glycoprotein is found in virtually all breast cancers, with tumor-specific variation in expression levels and cellular distribution. The tumor-specific form of MUC1 is also underglycosylated, exposing the protein core. Previous work in our laboratory (13, 14) has focused on the role of MUC1 in metastasis. We reported that tumor cell MUC1 can bind to endothelial cell ICAM-1 (13, 14) with sufficient strength to withstand shear stresses equivalent to physiologic blood flow (15), suggesting that the MUC1/ICAM-1 interaction could be involved in facilitating the extravasation of blood borne metastases out of the circulatory system. Our current study demonstrates that the MUC1/ICAM-1 interaction can trigger an intracellular oscillatory calcium-based signal in MUC1-expressing epithelial cells, an observation that held true whether MCF-7 cells were adherent or in suspension.

The MUC1/ICAM-1 oscillatory calcium signal appears to differ from the previously described MUC1 cytoplasmic domain (MUC1-CD) phosphotyrosine-based signal(s) (2, 4–6, 8–11, 18, 19), since it is not dependent on the MAPK/ERK pathway (Fig. 4A). Schroeder et al. (9) reported that the MUC1-CD is phosphorylated by EGFR after stimulation with EGF with subsequent activation of the MAPK/ERK pathway. This activation was not associated with proliferation as assayed by proliferating cell nuclear antigen activation (9). In other work, the same group has implicated the MUC1-CD in the disassembly of focal adhesions and increased cellular migration through a transwell membrane (10). Since EGFR activation of MAPK/ERK has also been implicated in migratory processes (16), the MUC1/ICAM-1 calcium signal may function in parallel with MUC1-CD phosphotyrosine-based signaling.

It is significant that we are using ICAM-1 to induce calcium flux, since it is a plausible, physiologically relevant MUC1 ligand, and the oscillatory response is strongly suggestive of a definite signal, as the frequency and amplitude of calcium oscillations encode instructions to activate specific proteins (20). Possible downstream calcium-sensitive targets of this signal may include gelsolin (21), calpain (22), and calmodulin (23), which are involved in cytoskeletal restructuring, focal adhesion disassembly, and cell contraction. Our use of a panel of enzyme-specific inhibitors suggested that this signal is mediated through the classic phospholipase C (PLC)-IP3 pathway (24), and involves Src, which may bridge MUC1 with calcium signal mediators, as Src reportedly physically associates with MUC1 (5) and can be upstream of PI 3-kinase and PLCγ (25–27). The data showing the dependence of this signal on lipid rafts is complementary, as cholesterol depletion has previously been shown to disrupt the function of Src (28) and PLC (29, 30).

Taken together, the evidence suggests that the MUC1/ICAM-1 interaction results in a calcium-based promigratory signal, expanding the repertoire of putative oncogenic actions of MUC1.

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