MUC1 Initiates Src-CrkL-Rac1/Cdc42–Mediated Actin Cytoskeletal Protrusive Motility after Ligating Intercellular Adhesion Molecule-1

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Abstract
MUC1, a transmembrane glycoprotein of the mucin family, when aberrantly expressed on breast cancer cells is correlated with increased lymph node metastases. We have previously shown that MUC1 binds intercellular adhesion molecule-1 (ICAM-1) on surrounding accessory cells and facilitates transendothelial migration of MUC1-bearing cells. Nevertheless, the underlying molecular mechanism is still obscure. In the present study, we used a novel assay of actin cytoskeletal reorganization to show that by ligating ICAM-1, MUC1 triggers Rac1- and Cdc42-dependent actin cytoskeletal protrusive activity preferentially at the heterotypic cell-cell contact sites. Further, we show that these MUC1/ICAM-1 interaction–initiated lamellipodial and filopodial protrusions require Src family kinase and CT10 regulator of kinase like (CrkL) accompanied by the rapid formation of a Src-CrkL signaling complex at the MUC1 cytoplasmic domain. Through inhibition of Src kinase activity, we further revealed that Src is required for recruiting CrkL to the MUC1 cytoplasmic domain as well as mediating the observed actin cytoskeleton dynamics. These findings suggest a novel MUC1-Src-CrkL-Rac1/Cdc42 signaling cascade following ICAM-1 ligation, through which MUC1 regulates cytoskeletal reorganization and directed cell motility during cell migration. (Mol Cancer Res 2008;6(4):555–67)

Introduction
The development of metastases in distant vital organs such as the lungs, liver, and brain is the principal cause of death in breast cancer patients. Metastasis requires increased cell motility, which is a tightly orchestrated process of numerous molecular events, characterized by directed cytoskeletal rearrangements. Thus, clarifying the mechanism underlying the hyperactivated cytoskeletal reorganization in cancer cells may suggest targets for effective therapeutic strategies to reduce breast cancer mortality.

MUC1 (also called DF3, CA15-3, or epsialin) is a transmembrane heterodimeric glycoprotein normally restricted to the apical surface of mammary epithelium (1, 2). However, the apical polarization is frequently lost in breast cancers, substituted by highly overexpressed MUC1 either throughout the cytosol or circumferentially around the plasma membrane (3, 4). Clinically, this aberrant expression of MUC1 in breast cancer cells is associated with a poor prognosis and increased lymph node metastases (4). We have previously reported that MUC1 binds intercellular adhesion molecule-1 (ICAM-1; refs. 5, 6), an adhesion molecule normally involved in the firm arrest and the subsequent extravasation of leukocytes through the vascular endothelium during inflammation (7). Analogous to the involvement of ICAM-1 in the extravasation of leukocytes, our recent transwell studies suggested that the MUC1/ICAM-1 interaction also facilitates in vitro trans-endothelial migration of MUC1-bearing cells through a monolayer of ICAM-1–expressing cells (8). This is significant, as the adhesion of tumor cells to endothelium and the subsequent exit from vasculatures of secondary organs are thought to be critical steps in metastasis (9, 10). However, the fundamental mechanism(s) of MUC1/ICAM-1–potentiated cell transmigration is largely unknown.

MUC1 contains a large extracellular domain composed of a variable number of tandem repeats of a 20-amino-acid sequence, which has multiple O-glycosylation sites and represents the binding site for the extracellular domain-1 of ICAM-1 on adjacent cells (5, 11). The 72-amino-acid cytoplasmic domain of MUC1 (MUC1-CD) contains seven highly overexpressed Tyr residues, four of which are confirmed phosphorylation sites and construct potential docking motifs for SH2 or SH3 domain–containing proteins, such as Src family kinases and growth factor receptor binding protein 2 (12, 13). Thus far, epidermal growth factor receptor (14, 15), PKC家族 (16), and Src (14, 17, 18) have been found to physically associate with and phosphorylate MUC1-CD. This indicates that MUC1, although without intrinsic kinase activity (19), could function as a scaffold protein in signal transduction. Supporting this, we
have shown that MUC1 can initiate an intracellular calcium signal in response to ICAM-1 ligation (20). Calcium-based signaling is frequently implicated in actin cytoskeletal “treadmilling” dynamics and cell migration (21). Also, the signal mediators Src family kinase and PLCγ involved in the MUC1/ICAM-1–induced calcium oscillation (20) could play crucial roles in directing cell migration (22, 23). All these findings imply that, in addition to mediating cell adhesion, the MUC1/ICAM-1 interaction may play a signaling role in cell motility.

Rho family small GTPases (Rac1, Cdc42, and RhoA) are central regulators of actin-based cell motility (24, 25). Once activated in the GTP-bound forms, Rac1 and Cdc42 can consequently interact with the downstream effectors, Arp2/3 activators of WAVE/WASP family, to initiate membrane ruffling and formation of membrane lamellipodial/filopodial protrusions (26), whereas RhoA is frequently involved in cellular contraction and focal adhesion dynamics (27). Acting upstream of Rho GTPases, the adaptor protein CrkL has been identified as a crucial mediator of cell migration through the association with guanine nucleotide exchange factors, such as Dock180, for catalyzing the GDP/GTP exchange and Rho GTPase activation (28, 29). In response to migratory cues, like integrin engagement, CrkL can be recruited to focal contacts where Src forms a signaling complex with focal adhesion kinase (FAK) and phosphorylates FAK-associated p130CAS or paxillin on the conserved Y-x-x-P binding motifs for the CrkL-SH2 domain (23, 30), thereby facilitating Rho GTPase–mediated cytoskeletal rearrangements and cell migration (30).

In the present study, we investigated the potential role of MUC1 in the regulation of actin-based cell motility after ligation ICAM-1. Our data revealed that the MUC1/ICAM-1 interaction triggers Rho GTPase Rac1- and Cdc42-dependent actin cytoskeletal reorganization and increased cell protrusive motility. We also showed that these MUC1-induced cytoskeletal dynamics require Src and CrkL, both of which are recruited to MUC1-CD in response to ICAM-1 ligation. Further, using selective inhibition of Src kinase activity, we show that Src functions upstream of CrkL in these Rho GTPase-mediated cytoskeletal rearrangements. These results suggest a novel MUC1-initiated Src-CrkL-Rac1/Cdc42 promigratory signaling, which may represent a crucial mechanism underlying the MUC1/ICAM-1 interaction in promoting breast cancer metastasis.

**Results**

**MUC1 Induces Dramatic Actin Cytoskeletal Rearrangements and Protrusive Motility in the Presence of ICAM-1**

To investigate if MUC1 initiates actin-based cell motility in response to ICAM-1 ligation, phosphorylated enhanced green fluorescent protein (pEGFP)-actin expression vector was transfected into three breast cancer cell lines (T47D, MCF-7, and Hs578T) and three MUC1-transfected 293T cell sublines (SYM25, SYM33, and SYM3), which differed in their MUC1 expression levels (Fig. 1A). Then, using time-lapse confocal microscopy, actin cytoskeletal dynamics were examined in these pEGFP-actin–transfected cells in the presence or absence of human ICAM-1 expressed on NIH3T3 transfectants (Fig. 1B). We found that stable expression of exogenous MUC1 on 293T cells did not elicit obvious cell membrane and cytoskeletal changes in monoculture (data not shown). However, dramatic actin cytoskeletal reorganization and membrane ruffling were provoked in the MUC1-positive breast cancer cells and 293T transfectants within 4 minutes after contact with ICAM-1–transfected NIH3T3 cells. These initial membrane dynamics rapidly developed into continually extending lamellipodial/filopodial protrusions, which preferentially occurred at the heterotypic cell-cell contact sites (Fig. 1C, first and third lanes). These protrusions were highly motile, as they frequently migrated out of the focus-plane and were accompanied by forward motion of the cell body (Fig. 1C, third lane, far right). In marked contrast, if either the plated cells lacked MUC1 or the NIH3T3 transfectants lacked ICAM-1, the breast cancer cells and 293T transfectants exhibited only a low degree of membrane ruffling and minor protrusions, and these membrane events were always transient and ceased within minutes (Fig. 1C, second and fourth lanes; Supplementary Fig. S1), indicating that the presence of both MUC1 and ICAM-1 in this system is essential for the augmented cytoskeletal rearrangements.

To quantitatively analyze the protrusive dynamics of the actin cytoskeleton, a novel method was developed in this study for quantifying the proportion (%) of actin voxel intensity sum (AVIS) in membrane protrusions compared with the whole cell (Fig. 2A–F). Compared with conventional indices based on either the number or area of protrusions, this technique is more sensitive to cytoskeletal changes because it compensates for the volume of each protrusion (Supplementary Fig. S2). Further, by tracing the proportion of AVIS in protrusions over time, we found that the cytoskeletal protrusive activity seems to be a wave-like dynamic process with MUC1-positive cells showing increased but varying amplitude in the presence of ICAM-1, compared with their controls (Fig. 2G). Because there were wide cell-to-cell asynchronization and variation in these dynamic cytoskeletal responses, a quantifiable index—actin cytoskeletal reorganization factor (ACRF), which is defined as the average of the AVIS proportion changes in protrusions during the 90-minute assay (Fig. 2H), was established and used to represent the level of actin cytoskeletal rearrangements in each experiment. For each experimental condition, ACRFs were obtained from four to nine independent trials and the average ACRF value was subsequently calculated to facilitate comparisons between different experimental conditions. It was shown that significantly higher levels of cytoskeletal rearrangements were initiated exclusively in the MUC1-positive breast cancer T47D and MCF-7 cells (Fig. 3A), as well as in 293T SYM25 and SYM33 cells (Fig. 3B) in response to NIH3T3-ICAM-1 stimulation, compared with NIH3T3-Mock stimulation or in the MUC1-negative Hs578T and 293T SYM3 cells (Fig. 3A and B). These findings suggest that MUC1 may initiate actin cytoskeletal reorganization and protrusive motility in the presence of ICAM-1.

To exclude the possible bias due to the unrelated differences in breast cancer cell lines or the 293T SYM subclone selection, we generated a Flp-In T-REx 293 MUC1-inducible expression system (Fig. 3C). Time-lapse confocal microscopy showed results similar to those seen previously, in that the Flp-In T-REx 293 MUC1+ cells exhibited significantly (~3-fold) higher levels of cytoskeletal protrusive motility in response to NIH3T3-ICAM-1 stimulation compared with NIH3T3-Mock.
stimulation (Fig. 3D). On the other hand, Flp-In T-REx 293 MUC1-cells showed only minor levels of cytoskeletal protrusive dynamics regardless of the presence or absence of ICAM-1 on the contacting NIH3T3 transfectants (Fig. 3D). These results substantiate that MUC1 is the key molecule that induces these increased actin cytoskeletal rearrangements upon ICAM-1 stimulation.

**MUC1/ICAM-1 Interaction Initiates the Increased Actin Cytoskeletal Reorganization**

To further confirm that this MUC1-initiated cytoskeletal reorganization is specifically induced by the ligation of ICAM-1, anti–ICAM-1 monoclonal antibody (mAb; 18E3D or 164B) blockade was introduced before the cytoskeletal reorganization assays. Our previous work has shown that mAb 18E3D, but not 164B, targets the MUC1 ligation site on ICAM-1, thereby blocking the MUC1/ICAM-1 interaction (6). By stimulating breast cancer cells (T47D and Hs578T) and MUC1-transfected 293T cells (SYM25 and SYM3) with NIH3T3-ICAM-1 cells that had been preincubated with either 18E3D or 164B mAb, we found that the anti–ICAM-1 mAb 18E3D significantly (>70%) blocked MUC1-initiated actin cytoskeletal rearrangements in the T47D cells, but had no effect on the MUC1-negative Hs578T cells (Fig. 4A). Conversely, the anti–ICAM-1 mAb 164 B pretreatment did not show a significant abrogating effect on the MUC1-induced cytoskeletal reorganization when compared with the T47D cells stimulated with nonpretreated NIH3T3-ICAM-1 cells (Fig. 4A). Similar results were also obtained in 293T SYM25 and SYM3 cells where 18E3D, but not 164B, abrogated the MUC1-induced actin cytoskeletal

**FIGURE 1.** Cytoskeletal reorganization is initiated in MUC1-positive cells in response to ICAM-1 stimulation. **A.** MUC1 expression levels were examined in breast cancer cell lines (T47D, MCF-7, and Hs578T) and MUC1-transfected 293T sublines (SYM25, SYM33, and SYM3). The MUC1 heterodimer dissociates under reducing conditions into a large extracellular domain (~200 kDa) and a smaller cytoplasmic domain (15-30 kDa). Tubulin was used as a loading control. IB, immunoblotting. **B.** ICAM-1 expression levels were examined in NIH3T3-ICAM-1 and NIH3T3-Mock transfectants. **C.** All the cell lines, indicated in A, were transfected with pEGFP-actin. As representatives to be shown here, T47D (green cells in the first and second lanes) and SYM25 (green cells in the third and fourth lanes) cells were plated on MatTek microwell dishes and then subjected to cytoskeletal reorganization assays upon stimulation by NIH3T3-ICAM-1 (gray cells in the first and third lanes) and NIH3T3-Mock (gray cells in the second and fourth lanes) transfectants. White arrows, membrane ruffling; red arrows, membrane lamellipodial-filopodial protrusions. The time “0 min” represents the moment of dropping NIH3T3 transfectants, and the images at each time point are three-dimensionally reconstructed Z-stacks, including all planes of these living cells. Bars, 5 μm.
protrusive motility in the presence of ICAM-1 (Fig. 4B). Collectively, we conclude that MUC1 initiates these increased actin cytoskeletal rearrangements and motile membrane protrusions by ligating ICAM-1.

**Rho GTPases Rac1 and Cdc42 Are Required for the MUC1/ICAM-1 Interaction–Induced Cytoskeletal Rearrangements**

Rho family small GTPases Rac1, Cdc42, and RhoA are crucial regulators in directing actin cytoskeletal reorganization and formation of motile lamellipodial or filopodial protrusions (24, 25). As the data presented above show that the MUC1/ICAM-1 interaction initiates actin-based cell protrusive motility, we speculated that these Rho GTPases may be involved. To test this hypothesis, T47D and 293T SYM25 cells were microinjected with the Rac1-T17N, Cdc42-T17N, or RhoA-T19N expression plasmid, which confers the dominant negative phenotype of the corresponding Rho GTPase, using Dextran-Alexa 568 as an injection tracer (Fig. 5A). Time-lapse confocal microscopy revealed that coexpression of the dominant

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**FIGURE 2.** Quantitative analysis of cytoskeletal protrusive motility. A, pEGFP-actin cytoskeleton was three-dimensionally reconstructed from Z-stack images at each time point in the surpass mode of Imaris. B, The three-dimensional actin cytoskeleton image was surface-rendered by setting a background threshold, allowing the quantitation of the AVIS of the whole cell. C, The cell body was defined at each plane of the cell image in the Z-series. D, A contoured surface of the cell body was generated. E, The voxels inside the contoured surface were set to zero. F, AVIS in protrusions was obtained. G, The cytoskeletal dynamics were tracked by tracing the proportion of AVIS in protrusions relative to whole-cell AVIS every 10 min over the trial period. H, Using a 293T SYM25 cell stimulated with NIH3T3-ICAM-1 as an example, ACRF represents the average of the changes of the AVIS in protrusions during the cytoskeletal reorganization assays. Bars, 5 μm.
negative Rac1-T17N, as well as Cdc42-T17N, led to complete inhibition of the MUC1/ICAM-1 interaction–induced cytoskeletal protrusive motility (Fig. 5B and C). Moreover, the initial membrane ruffling and cell movement were also considerably abrogated in both of these cell lines. In contrast, coexpression of dominant negative RhoA-T19N had no obvious inhibitory effect on the MUC1/ICAM-1–induced cytoskeletal activity compared with the control cells injected only with Dextran-Alexa 568 (Fig. 5B and C). Further, to confirm these live imaging results, Rac1, Cdc42, and Rho activation assays were carried out. As shown in Fig. 5D, in contrast to Rho, both Rac1 and Cdc42 were significantly activated in T47D and 293T SYM25 cells following NIH3T3-ICAM-1 stimulation, compared with the control cells that were serum starved and stimulated with NIH3T3-Mock transfectants. Therefore, we conclude that the Rho family small GTPases Rac1 and Cdc42, but not RhoA, are required for mediating the MUC1/ICAM-1 ligation–initiated actin cytoskeletal rearrangements and protrusive motility.

Role of Src Family Kinase, CrkL, and PLC in MUC1/ICAM-1 Interaction–Initiated Cytoskeletal Rearrangements

We then investigated the potential mediators downstream of MUC1 in this Rac1- and Cdc42-dependent cytoskeletal promigratory responses. The adaptor protein CrkL, as a downstream mediator of Src, plays a crucial role in the spatial-temporal regulation of Rho GTPase Racl-Cdc42 activation and cell migration (30). Our previous work has shown that Src family kinase and PLC are involved in the MUC1/ICAM-1 interaction–initiated calcium signal (20). These findings suggest that CrkL may cooperate with Src in this MUC1/ICAM-1 interaction–induced actin cytoskeletal protrusive motility. In addition, PLC-mediated cleavage of PtdIns(4,5)P2 regulates the actin cytoskeleton modulating proteins, profilin, gelsolin, and cofilin, which together with Ins(1,4,5)P3-mediated Ca2+ signaling could result in directional actin cytoskeletal rearrangements at the migrating leading edge (21).

To investigate the role of Src family kinase and PLC in the MUC1/ICAM-1 interaction–induced cytoskeletal dynamics, T47D cells, as well as 293T SYM25 cells, were pretreated with the pharmaceutical inhibitors PP2 (Src family kinase inhibitor), U-73122 (PLC inhibitor), or U-73343 (inactive analogue of U-73122) and then compared with untreated control cells in the cytoskeletal reorganization assays. To characterize the role of CrkL in this MUC1/ICAM-1–induced cytoskeletal protrusive motility, T47D cells and 293T SYM25 cells were transfected with Cy3-labeled CrkL small interfering RNA (siRNA; 100 nmol/L) before the cytoskeletal reorganization assays (Fig. 6A). As shown in Fig. 6B, compared with the mock transfections, the CrkL protein levels were efficiently reduced by ~50% in both T47D and 293T SYM25 cells at 24 hours.
posttransfection and ~70% after 48 hours posttransfection, using ImageJ as the quantitative analyses software with tubulin as a loading control (Fig. 6B; Supplementary Fig. S3). In contrast to CrkL, the CrkI, CrkII, and Src expression levels remained similar at all time points (Fig. 6B; Supplementary Fig. S3), indicating that the down-regulation by CrkL siRNA was specific for CrkL. In the ensuing time-lapse confocal microscopy experiments, we found that the MUC1/ICAM-1 interaction–induced cytoskeletal protrusive motility was substantially (>75%) abrogated in breast cancer T47D cells under the conditions with selective inhibition of either Src or CrkL (Fig. 6C). However, inhibition of PLC activity using U-73122 exhibited only a modest decrease in the MUC1/ICAM-1–induced cytoskeletal dynamics, which was not statistically significant when compared with the inactive analogue U-73343–pretreated cells (Fig. 6C). Similar results were also observed in 293T SYM25 cells (Fig. 6D). These results show that Src and CrkL mediate the Rac1- and Cdc42-dependent cytoskeletal rearrangements in response to MUC1/ICAM-1 ligation.

Src Functions Upstream of CrkL in the MUC1/ICAM-1 Interaction–Induced Promigratory Signaling

To further clarify the signaling mechanism underlying the MUC1/ICAM-1 ligation–induced cytoskeletal rearrangements, we investigated the possible interaction(s) between MUC1 and these signaling mediators after ICAM-1 ligation. In this study, breast cancer T47D cells and 293T SYM25 cells were serum starved and then stimulated with either NIH3T3-ICAM-1 or NIH3T3-Mock transfectants for 10 seconds or 1 minute, followed by MUC1 immunoprecipitation using anti-MUC1 mAb B27.29. The immunoprecipitates were then analyzed by SDS-PAGE and sequential immunoblotting for tyrosine-phosphorylated proteins, Src, CrkL, and MUC1. Using the whole-cell lysates as positive controls, we found that there was always a basal level of association of Src with MUC1 under all conditions (Fig. 7A). However, in both T47D and 293T SYM25 cells that were stimulated with NIH3T3-ICAM-1 transfectants, the anti-MUC1 mAb B27.29 coimmunoprecipitate increased amounts of associated Src compared with the NIH3T3-Mock–stimulated and serum-starved controls (Fig. 7A; Supplementary Fig. S4A). Similar results were also obtained when probing the membranes for CrkL. In contrast to the negligible levels observed in controls, CrkL was significantly increased in MUC1 immunoprecipitates within 10 seconds of stimulation by ICAM-1 (Fig. 7A; Supplementary Fig. S4A). These ICAM-1–induced increased associations of MUC1 with Src and CrkL were further confirmed in the reciprocal immunoprecipitations using anti-Src antibody (Fig. 7B; Supplementary Fig. S4B) and anti-CrkL antibody (Fig. 7C; Supplementary Fig. S4C). Taken together, these results suggest that MUC1/ICAM-1 ligation recruits Src and CrkL to MUC1-CD where they form a signaling complex crucial for actin-based cell motility.

The localization and activation of CrkL, the SH2-SH3-SH3 adaptor protein, is controlled by protein tyrosine kinase–mediated phosphorylation, which creates the SH2 domain binding motifs for recruiting CrkL (31). The MUC1-CD has seven tyrosine residues, four of which are established phosphorylation sites of protein tyrosine kinases, including Src (12, 18, 32). Therefore, we postulate that Src kinase activity may be required for recruiting CrkL to the MUC1-CD in the ICAM-1 ligation–induced promigratory signaling. To address this, coimmunoprecipitation experiments were done on the Src kinase inhibitor PP2-pretreated T47D and 293T SYM25 cells using anti-MUC1 mAb B27.29. The resultant immunoprecipitates were then examined for Src, CrkL, MUC1, and tyrosine-phosphorylated proteins. We found that, compared with the non–PP2-pretreated controls, which showed significantly increased tyrosine phosphorylation of the MUC1-CD following MUC1/ICAM-1 interaction, no obvious MUC1-CD tyrosine phosphorylation was observed in the PP2-pretreated T47D and 293T SYM25 cells under all experimental conditions (Fig. 8A). Correspondingly, there was no increase in Src and CrkL in the MUC1 immunoprecipitates of the PP2-pretreated cells following ICAM-1 stimulation (Fig. 8B). Further, PP2 pretreatment
significantly decreased transmigration of ICAM-1–stimulated T47D cells in a transwell assay (Supplementary Fig. S5). Based on all the evidence above, we conclude that Src kinase activity is required for recruiting CrkL to MUC1-CD, where Src functions upstream of CrkL in the Rho GTPases Rac1- and Cdc42-mediated cytoskeletal protrusive motility.

Discussion

MUC1, as the fundamental member of the mucin family glycoproteins, has long been associated with mammary tumorigenesis and malignant progression. In addition to the potential roles that have been suggested for MUC1 in tumor cell proliferation, scattering, and survival (15, 33-35), we were the first to report that MUC1 was a proadhesive molecule capable of mediating heterotypic cell-cell adhesion by binding ICAM-1 on accessory cells (5, 6). Further, we showed that increased transendothelial migration could be obtained under conditions of increased MUC1/ICAM-1 interaction (8). As ICAM-1 is present throughout the entire expected migratory track of a transiting cell and is essential in mediating cell migration in leukocytes (7, 36), a crucial role is suggested for MUC1/ICAM-1 interaction during breast cancer metastasis. However, the molecular mechanism(s) underlying the promigratory property of MUC1 remained elusive.

In this study using a novel assay of actin cytoskeletal reorganization, we show that MUC1 initiates dramatic cytoskeletal rearrangements and increased protrusive activity in response to ICAM-1 ligation. Human breast cancer is not a homogeneous disease. There are at least four genetic subtypes defined by hierarchical analysis of gene expression (37). T47D and MCF-7 differ from Hs578T in that they are positive for the estrogen receptor and are representative of one of the two luminal subtypes of breast cancer, which together account for 70% of breast cancers. Luminal A is very indolent whereas Luminal B tumors show a progressive decline in survival, eventually being equivalent to the estrogen receptor–negative, HER2, and basal subtypes (38). Because T47D and MCF-7 were isolated from metastatic disease, they most likely represent Luminal B tumors, the single most common genetic
variant. Therefore, our results using cells that have an inherently slower motility and clinical progression may be particularly relevant to a large proportion of human breast cancers in which the ability of these cells to metastasize is low and perhaps dependent on a recognition phenomenon. The rapid adverse clinical course of the estrogen receptor–negative tumors, as exemplified in vitro by the Hs578Ts, probably indicates a different mechanism of interaction with the host vasculature that is not ICAM-1 dependent. In support of this, we did not find a difference in protrusions when the Hs578Ts were exposed to ICAM-1–expressing cells.

Because the enhanced cytoskeletal promigratory responses are only induced in the MUC1-expressing cells and exclusively abrogated by the anti–ICAM-1 antibody that specifically blocks the MUC1/ICAM-1 ligation, an initiating role of the MUC1/ICAM-1 interaction is shown for relaying the promigratory signaling into cells and inducing the dynamic cytoskeletal rearrangements. These findings based on our previous studies showing that the MUC1/ICAM-1 interaction mediates cell adhesion (6) provide the first evidence that MUC1, by ligating ICAM-1, initiates actin-based cell protrusive motility. Because cancer metastatic spread is characterized by increased tumor cell motility, this result represents a crucial step for fully understanding the potential role of MUC1 in breast cancer metastasis. During metastasis, tumor cells lose their original tissue contacts, invade the extracellular matrix, transit via the lymphatic/blood system, and adhere to and then extravasate at the secondary metastatic site. Most recently, consistent with our previous transendothelial migration study (8), we found that MUC1, by ligating ICAM-1 on stromal fibroblasts, also potentiates cell invadopodial protrusions and stromal invasion (data not shown). This implies that tumor cells may use MUC1

![Image](https://example.com/image.png)

**FIGURE 6.** Src family kinase and CrkL are required for the MUC1/ICAM-1–induced cytoskeletal promigratory motility. **A.** Following CrkL siRNA transfection, the successfully transfected cells were identified by the red fluorescence of the siRNA-tagged Cy3. Bars, 5 μm. **B.** Using mock transfection (no siRNA) as a control, the expression levels of CrkL protein were significantly knocked down by CrkL siRNA (~50%) 24 h posttransfection in both T47D and 293T SYM25 cells, and the levels of CrkL were further reduced (~70%) after 48 h of transfection. The knockdown was specific for CrkL as protein levels of CrkII and CrkI were not significantly altered (see Supplementary Fig. S3 for quantification). Tubulin and Src were used as loading controls. The following cytoskeletal reorganization assays on T47D (C) and 293T SYM25 (D) cells subsequent to selectively inhibiting Src family kinase (PP2), CrkL, and PLC (U-73122) using the inactive analogue U-73343 as a control revealed that Src family kinase and CrkL are required for the MUC1/ICAM-1–induced cytoskeletal promigratory motility. Columns, average ACRF from at least three independent experiments; bars, SE. *, P < 0.05.
as an aberrantly up-regulated adhesion receptor to facilitate cell–extracellular matrix interactions, thereby initiating actin-based cell invasive motility.

Using selective inhibition of Src family kinase, CrkL, and PLC, we found that Src family kinase and CrkL are required for this MUC1/ICAM-1 ligation–initiated cytoskeletal rearrangements and cell protrusive motility. In contrast to the MUC1/ICAM-1–induced calcium oscillatory signaling, inhibition of PLC activity caused only a minor decrease in the MUC1-induced cytoskeletal promigratory activities in response to ICAM-1 ligation. This result indicates that, although the PLC-mediated activation of actin cytoskeleton-regulatory proteins (e.g., profilin, gelsolin, cofilin) and calcium-based signaling have been implicated in cytoskeleton reorganization as well as cell migration (21, 22), the regulatory role of PLC in the MUC1/ICAM-1–induced cytoskeletal dynamics may be merely auxiliary or redundant for Src and CrkL. Therefore, we speculate that this MUC1/ICAM-1–induced cytoskeletal protractive activity does not share the same molecular mechanism underlying the previously reported calcium signaling following MUC1/ICAM-1 interaction. There are several lines of evidence suggesting that MUC1 modulates transcriptional activities via nuclear factor-κB and nuclear factor-AT (39, 40). Considering that nuclear factor-κB and nuclear factor-AT are efficiently activated by intercellular calcium oscillations (41) and frequently implicated in malignant cell survival (42), the MUC1/ICAM-1–initiated calcium signaling may have an antiapoptotic effect that is parallel to the MUC1/ICAM-1–induced invasive motility.

This study revealed that Src is a critical regulator of this actin-based cell motility. In response to the MUC1/ICAM-1 interaction, Src is shown to be recruited to the MUC1-CD where it forms a signaling complex with CrkL. Because the MUC1-CD lacks intrinsic kinase activity, its association with the Src non–receptor tyrosine kinase is crucial for understanding the initial signaling events downstream of ICAM-1 ligation. There is evidence suggesting that the intracellular targeting of Src is a kinase-independent mechanism that is determined principally by interactions involving the SH3 domain with modulation by the SH2 domain (43). Supporting this, previous studies by Li et al. (18) showed that Src interacts with MUC1 via its NH2-terminal SH3 domain and is able to phosphorylate the tyrosine residues of MUC1-CD to generate conserved binding sites for SH2-containing proteins. In this report, we
found that there was always a minor level of association between Src and MUC1 in both breast cancer cells and MUC1-transfected 293T cells. Subsequent to MUC1/ICAM-1 interaction, the MUC1-Src association was significantly increased and accompanied by increased MUC1-CD phosphorylation and CrkL recruitment. Further, using inhibition of Src kinase activity, we revealed that Src functions upstream of CrkL in the MUC1/ICAM-1–induced promigratory signaling.

The adaptor protein CrkL is usually localized and activated by recruitment to a tyrosine-phosphorylated “Y-x-x-P” motif via its SH2 domain (23, 30). For example, in the integrin-mediated cell migration, Src interacts directly with FAK to form a “Src-FAK signaling complex” at the newly formed focal contacts, whereSrc can subsequently phosphorylate the FAK-associated p130CAS/paxillin at the conserved binding sites for CrkL thereby initiating the Rho GTPase Rac1 and Cdc42-mediated cell motility (23, 44). In this study, FAK was not associated with the MUC1-CD in the MUC1 immunoprecipitates, regardless of the presence or absence of ICAM-1 stimulation (data not shown). The MUC1-CD contains two highly conserved Y-x-x-P sequences (Y35VPP38 and Y60TNP63), which represent potential docking sites for the SH2 domain of the adaptor protein CrkL. Therefore, we propose that in response to MUC1/ICAM-1 ligation, Src-mediated phosphorylation of MUC1-CD serves to directly recruit CrkL and induce the actin-based cell motility in a FAK-independent manner.

It is well established that the Rho GTPases (Rac1, Cdc42, and RhoA) are key effectors of actin cytoskeleton protrusion machinery (25). In this study, using coexpression of the respective dominant negative Rho GTPase, as well as Rho GTPases (Rac1, Cdc42, and Rho) activation assays, we revealed that Rac1 and Cdc42, but not RhoA, mediate the MUC1/ICAM-1–induced cytoskeletal protrusive motility. This is significant, as the regulatory role of CrkL in actin-based cell motility is mediated by Rho GTPase Rac1 and Cdc42 (30, 45).

In summary, this study presents evidence showing that MUC1, by interacting with ICAM-1 on the accessory cells, initiates actin-based cell invasive motility, which is mediated by Src, CrkL, as well as Rho GTPases Rac1 and Cdc42. In addition, we showed that Src functions upstream of CrkL in this Rho GTPase Rac1– and Cdc42–mediated cytoskeletal rearrangements, suggesting a novel MUC1-initiated Src-CrkL-Rac1/Cdc42 promigratory signaling pathway in response to ICAM-1 ligation. This work builds on our previous studies and provides not only functional evidence that implicates MUC1/ICAM-1 ligation as a key molecular event in the aggressive tumor phenotype, but also an insight into the underlying molecular mechanisms that tumor cells may use to promote tumor metastasis.

Materials and Methods

Antibodies and Reagents

B27.29 mouse mAb against the MUC1-ECD was a gift from Biomira, Inc. CT2 Armenian hamster mAb against MUC1-CD was generously provided by Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ). Mouse anti-human ICAM-1 mAbs, 18E3D and 164B, were from ICOS Corporation. Mouse mAbs against Src (clone GD11) and CrkL were purchased from Upstate Cell Signaling Solutions. Mouse anti-Crk mAb was from BD Transduction Laboratories. Mouse anti-CrkII mAb and anti-phosphotyrosine mAb PY99 were purchased from Santa Cruz Biotechnology.

FIGURE 8. Src kinase activity is required for recruiting CrkL to MUC1-CD following MUC1/ICAM-1 interaction. Subsequent to the inhibition of Src kinase activity using PP2, coimmunoprecipitation (IP) experiments were done on T47D and 293T SYM25 cells following stimulation with NIH3T3-Mock or NIH3T3-ICAM-1 cells for 10 s and 1 min. A. By reprobing MUC1 itself as the loading control, the phosphorylation of MUC1-CD (red arrows) was significantly reduced in PP2-pretreated T47D cells and 293T SYM25 cells following MUC1/ICAM-1 interaction, compared with the non-PP2–pretreated controls. B. The recruitment of CrkL to MUC1-CD was also significantly abrogated in PP2-pretreated T47D cells and 293T SYM25 cells following MUC1/ICAM-1 interaction.
Biotechnology, Inc. The goat anti-mouse and anti-Armenian hamster horseradish peroxidase–conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. MOPC 31C mouse IgG1 and B-5-1-2 anti-tubulin mAb were purchased from Sigma-Aldrich. Protein G–agarose was purchased from Roche Diagnostics. ECL Plus Western Blotting detection reagents were purchased from GE Healthcare (Amersham Biosciences). U-73122, U-73343, protease inhibitor cocktail, phosphatase inhibitor cocktail 2, and gelatin were from Sigma-Aldrich. PP2 was obtained from Tocris. Dextran-Alexa Fluor 568 was purchased from Molecular Probes. DMEM, fetal bovine serum (FBS), G418, blasticidin S, hygromycin B, and tetracycline were purchased from Invitrogen, Inc.

Expression Plasmids

pEGFP-actin plasmid was obtained from Clontech Laboratories, Inc. The pcDNA5/FRT/TO-MUC1 plasmid was constructed by releasing the MUC1 insert from the pcDNA1-hMUC1-TR+ plasmid (kindly provided by Dr. Sandra Gendler) with NotI, and ligated into a dephosphorylated, NotI digested pcDNA5/FRT/TO plasmid (Invitrogen). Digestion with KpnI was used to check correct directionality of the MUC1 insert, which has a KpnI restriction site close to the 3′ end. The pOG44 Flp recombinase expression plasmid was purchased from Invitrogen. The dominant negative Rac1-T17N, Cdc42-T17N, and RhoA-T19N plasmids were kindly provided by cDNA Resource Center, University of Missouri-Rolla (Rolla, MO).

Cell Culture and Transfections

Human breast cancer cells T47D, MCF-7, and Hs578T were from the American Type Culture Collection and were maintained in DMEM containing 10% FBS. The MUC1-transfected 293T SYM3, SYM25, and SYM33 sublines, as previously described (20), were maintained in DMEM containing 10% FBS and 200 μg/mL G418. Human ICAM-1–transfected NIH3T3 cells and the mock-transfected counterparts were a generous gift of Dr. Ken Dimock (University of Ottawa, Ottawa, ON, Canada) and were maintained in DMEM containing 10% FBS and 2 μg/mL blasticidin S. To generate the Flp-In T-REx 293 MUC1-inducible expression system, the Flp-In T-REx 293 parental cells (Invitrogen) were cotransfected with pcDNA5/FRT/TO-MUC1 plasmids and pOG44 plasmids using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. The generated Flp-In T-REx 293 MUC1 cells were maintained in DMEM containing 10% FBS, 15 μg/mL blasticidin S, and 150 μg/mL hygromycin B. As a result, MUC1 expression can be induced or inhibited in this system by tetracycline-responsive regulation.

Cytoskeletal Reorganization Assays

Thirty-five–millimeter glass-bottomed microwell dishes (MatTek Corp.) were coated with 100 μL of FBS or 0.1% (w/v) gelatin as described previously (20). One hundred microliters of pEGFP-actin transfected breast cancer cells, 293T SYM sublines, or Flp-In T-REx 293 MUC1 cells at 5 × 10⁵/mL were plated on the precoated dishes and equilibrated overnight. The cells were then washed once with 37°C imaging buffer [152 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L MgCl₂, 1.8 mmol/L CaCl₂, 10 mmol/L HEPES, 5.6 mmol/L glucose (pH 7.2); refs. 20, 46], and left in imaging buffer at 37°C for 30 min until imaged. Meanwhile, NIH3T3-ICAM-1 or NIH3T3-Mock transfectants were trypsinized, rinsed once with PBS, and resuspended in imaging buffer at ~1 × 10⁶/mL.

Then, the MatTek dish was placed in a 37°C microscope stage heater on a Zeiss 2-photon confocal microscope (Zeiss NLO 510), and a representative cell was focused with a Plan Neofluar ×40/1.3 oil immersion lens. The EGFp was excited using a 488 nm laser line of a 25 mW Argon excitation source (for the dominant negative Rho GTPase–microinjected cells, a 543 nm laser line of a 1 mW HeNe laser was used for excitation of the Alexa Fluor 568 signal) with the intensity output set to 25%. Eight-bit images were collected with a 1.60-μm dwell time per pixel. The pinhole was set to 1 Airy unit and images were collected at 0.11 μm/pixel resolution. The gain and offset were set to expand the signal from the cell being imaged over the 255 gray value scale of the 8-bit data range. Then, Z-stacks (~0.45-μm intervals from cell bottom to top) were acquired every 2 min for a total of 45 min, recorded over the 1.5-h experiment under both differential interference contrast microscopy and a 505-nm-long pass filter (EGFP). Six hundred microliters of NIH3T3-ICAM-1 or NIH3T3-Mock cell suspension were gently added onto the plated cells immediately after the first image was taken. For the ICAM-1 blockade experiments, NIH3T3-ICAM-1–transfectants were incubated with anti–ICAM-1 mAb 164-B (20 μg/mL) or mAb 18E3D (20 μg/mL) for 30 min at 37°C before adding onto the plated cells and commencing cell imaging. In the pharmaceutical inhibitory experiments, the plated cells were preincubated with PP2 (10 μM), U-73122 (10 μM), or U-73343 (10 μM) in imaging buffer for 30 min at 37°C, followed by NIH3T3 cells stimulation and cell imaging. For the selective CrkL inhibitory experiments, the plated cells were transfected with Cy3-labeled CrkL siRNA duplexes for 48 h before the stimulation by NIH3T3-ICAM-1 or NIH3T3-Mock transfectants.

Quantitative Analysis of Cytoskeletal Reorganization

Under the Expert mode of LSM software, the image stacks of Z-series were retrieved at every fifth time point (i.e., every 10 min) from time zero (i.e., the first image Z-stack). The analyses of actin cytoskeletal rearrangements were done using Imaris software (Bitplane AG, version 4.2). First, the three-dimensional time-lapse images were reconstructed. Then, in the Surpass mode of Imaris software, the differential interference contrast microscopic channel was deleted and the three-dimensional EGFP images at each time point were surface rendered by setting a threshold value, which subtracted the nonspecific intensity values of background and generated surface objects enclosing the voxels with intensity values above the threshold. The isosurface was then used as a mask to measure the whole-cell pEGFP-AVIS at each time point and the resultant data were exported to MS Excel with the built-in statistical function of the software. Then, the cell body was manually contoured in each slice of the imaging stacks. Further, by masking the channel with the contoured surface (i.e., cell body) and setting the voxels inside the contoured surface to zero, the AVIS in protrusions was obtained in the same way.
as for the whole-cell pEGFP-actin. Thus, the cytoskeletal rearrangements can be tracked by tracing the proportion (%) of AVIS in the protrusions compared with that in the whole cell over time. The ACRF was calculated as the average of the AVIS changes in protrusions, during the course of the entire cytoskeletal reorganization assay, thereby numerically representing the level of cytoskeletal dynamics under each experimental condition (Fig. 2).

**Microinjection of Single Cells**

Microinjection was done on a Zeiss Axiovert 100M fluorescent microscope by using an Eppendorf microinjection system. The pEGFP-actin transfected T47D or SYM25 cells were plated as described previously for the actin cytoskeletal reorganization assays. Rac1-T17N, Cdc42-T17N, or RhoA-T19N plasmid (100 μg/mL) was mixed with Dextran-Alexa Fluor 568 in sterilized PBS. Then, the mixture was spun down at 14,000 × g for 30 min at 4°C, and the supernatant was taken for microinjection. Following microinjection, the cells were rinsed once with PBS and then incubated with fresh medium for another 8 to 12 h before the actin cytoskeleton reorganization assay. The successfully injected cells were identified by the fluorescence of Alexa Fluor 568 using a Cy3 filter set.

**Rho Family Small GTPase Activation Assay**

The intracellular activities of Rho family GTPases Rac1, Cdc42, and RhoA were examined using Rac1, Cdc42, and Rho activation assay kits, respectively (Upstate Biotechnology) according to the manufacturer’s protocols. In brief, breast cancer T47D cells and 293T SYM25 cells were grown in 10-cm cell culture dishes to ~75% confluence. Then, the cells were serum starved and stimulated with NIH3T3-Mock or ICAM-1 transfectants for 4 min.

Coimmunoprecipitation and Western Blot Analysis

Following treatment under each experimental condition, cells were harvested in lysis buffer [50 mmol/L Tris (pH 7.6), 0.5% NP40, 100 mmol/L NaCl, 0.5 mmol/L EDTA, freshly supplemented with 0.5% protease inhibitor cocktail and phosphatase inhibitor cocktail 2] and the insoluble components were pelleted at 14,000 × g for 1 min. The protein concentrations of the supernatants were then determined using a Bio-Rad detergent-compatible protein assay kit (Bio-Rad) and equal amounts (1.8 mg) of proteins were incubated with specific antibodies for 2 h at 4°C with gentle agitation. The incubation continued with addition of protein G-agarose, rinsed once with lysis buffer, for 6 h at 4°C with end-to-end agitation. The immunocomplexes were then pelleted and washed extensively with lysis buffer. Subsequently, the immunocomplexes were separated by SDS-PAGE, transferred to an Immobilon-P membrane (Millipore Corp.), and immunoblotted with appropriate primary antibodies. After washing and incubating with horseradish peroxidase–conjugated secondary antibodies, the proteins of interest were visualized using ECL Plus detection system and Kodak BioMax MR Film. The films were then scanned and processed with ImageJ software (NIH) for quantification. Where indicated, the membrane was stripped and reprobed with other antibodies.

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**References**


MUC1 Initiates Src-CrkL-Rac1/Cdc42–Mediated Actin Cytoskeletal Protrusive Motility after Ligating Intercellular Adhesion Molecule-1

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