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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The objectives of this grant are: (1) examine how mts1 expression alters directed cellular motility *in vitro*; (2) generate myosin-IIA antibodies that mimic mts1 binding to examine how the regulation of myosin-IIA affects directed motility *in vitro*; and (3) utilize an intravital imaging system to evaluate the impact of mts1 expression on metastasis in live animal models. We have established high expressing MTC-GFP-mts1 and MTLn3-GFP-mts1 cell lines and are now poised to begin our intravital imaging studies, which will visualize the motile behavior of mts1 expressing tumor cells within the primary tumor, during intravasation and extravastion *in situ*. Importantly, these analyses will allow us to determine how mts1 expression impacts on the motile processes associated with invasion and metastasis *in vivo* and identify those steps in the metastatic cascade affected by mts1 expression. These cell lines will also be used in an *in vitro* assay to evaluate the effects of mts1 expression on chemoattractant-stimulated motility. This will allow us to obtain comprehensive behavioral phenotype and will identify which aspects of directed motility are sensitive to the expression of mts1. We have established a quantitative glutathione-Sepharose pull-down assay for mapping the mts1 binding site on the myosin-IIA rod. Our analysis with a GST fusion of residues 1900-1961 indicates that the entire mts1 binding site is contained within the C-terminal 62 residues of the myosin-IIA heavy chain and further suggests that mts1 binds a linear sequence as previously proposed by us. This assay will be used to further narrow and define the mts1 binding domain for the production of a myosin-IIA antibody that mimics the effects of mts1 binding on myosin-II assembly and activity.

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Introduction

Mts1, a member of the S100 family of Ca^{2+}-binding proteins, exhibits a strong causal link with breast cancer metastasis. Expression of mts1 in nonmetastatic breast tumor cells confers a metastatic phenotype, whereas in metastatic cells, a reduction in mts1 expression suppresses metastatic potential. Recent work has demonstrated that mts1 displays Ca^{2+}-dependent interactions with nonmuscle myosin-II. This binding interaction is consistent with the observation that mts1 expression levels correlate strongly with the motility of tumor cells and suggests a direct link between the actomyosin cytoskeleton and the regulation of metastasis-associated motility by mts1. The objectives of this grant are: (1) examine how mts1 expression alters directed cellular motility *in vitro*; (2) generate myosin-IIA antibodies that mimic mts1 binding to examine how the regulation of myosin-IIA affects directed motility *in vitro*; and (3) utilize an intravital imaging system to evaluate the impact of mts1 expression on metastasis in live animal models. These studies will provide important new information on mts1-mediated regulation of tumor cell motility, invasion and metastasis.

Body

Objective 1: We used the mammalian expression vector pcDNA3.1 (Invitrogen), which contains a hygromycin resistance cassette, to overexpress mts1 in MTC and MTLn3 rat mammary adenocarcinoma cells that already stably express GFP. Immunoblots demonstrated that a mass pool of hygromycin resistant MTC-GFP colonies highly overexpressed mts1; however, individual MTC-GFP clones displayed only low mts1 expression levels both in the presence of hygromycin and following growth for 1 month in the absence of hygromycin. To achieve higher mts1 expression levels, we switched to the mammalian expression vector pIREShyg2 (Clontech), which contains an internal ribosomal entry site. This permits the gene of interest and the selectable marker to be translated from a single mRNA, thus a high does of antibiotic will select for cells expressing high levels of mts1. Individual MTC-GFP clones displayed high mts1 expression levels on immunoblots; however, after growth for 1 month without antibiotic, the clones either lost their ability to express mts1 or displayed greatly reduced mts1 expression. The requirement of maintaining mts1 expression in the absence of antibiotic must be stressed as these cell lines will be injected into live animals for intravital imaging studies (Objective 3). Since palpable primary tumors are first detected 14-25 days post-injection and secondary tumors in the lymph nodes and lungs are observed within 42 days, MTC-GFP-mts1 cells must maintain high mts1 expression levels for at least 2 months without antibiotic. To achieve high mts1 expression levels, we used the vector pLHCX (Clontech) and retroviral infection, which allows the mts1 gene to integrate into the host genome, resulting in reliable, heritable expression, without variability or loss of expression due to loss of the construct. MTC-GFP and MTLn3-GFP cells infected with retroviral mts1/pLHCX display high levels of mts1 expression that was unchanged following growth for 2 months without antibiotic.

In culture, the metastatic MTLn3 cells have a rounded morphology, whereas the nonmetastatic MTC cells are highly polarized. In the majority of MTC-GFP clones that express high levels of mts1, the cells lost their polarized morphology and became rounded similar to the MTLn3 cells. These observations suggest that mts1 expression significantly affects cell shape, consistent with its proposed role in regulating the actomyosin cytoskeleton.

Objective 2: To map the mts1 binding site on the myosin rod, we generated GST fusions of subfragments of the myosin-IIA rod and measured the calcium-dependent binding of mts1 to the subfragments in a glutathione-Sepharose pull-down assay. Mts1 binds to residues 1900-1961, which encompasses the C-terminal 62 residues of the myosin-IIA heavy chain with a K_d of 5.9 μM. This value is comparable to the equilibrium dissociation constant (5.3 μM) obtained with a 74 kDa fragment of the myosin-IIA rod. These data indicate that the entire mts1 binding site is

contained within the C-terminal 62 residues of the myosin-IIA heavy chain and further suggest that mts1 binds a linear sequence as previously proposed by us. Initial studies with a GST fusion of residues 1909-1940 demonstrate that mts1 displays calcium-dependent binding to this shorter myosin-IIA fragment. Quantitative binding studies with this fragment and the production of smaller fragments (10-15 residues in length) are in progress.

Objective 3: MTC-GFP cells that were stably transfected with the pcDNA3.1/mts1 construct and which express low levels of mts1 were injected into the mammary fat pad of Fisher 344 rats or SCID mice and allowed to grow for 6 weeks. The cells formed tumors that were similar to those observed with the parental MTC-GFP cells that do not express mts1. Although metastases were not detected in rats, in SCID mice, we observed metastases in the lungs. These observations suggest that in these immune compromised animals, low levels of mts1 expression can induce metastases. We are now using our new MTC-GFP-mts1 cells lines, which highly express mts1, in a spontaneous metastasis assay in Fisher 344 rats, to evaluate whether high levels of mts1 expression can induce the formation of metastases in animals that are not immune compromised. The MTLn3-GFP-mts1 cells are also being analyzed in the spontaneous metastasis assay to determine if mts1 expression can convert a moderately metastatic cell line into a highly metastatic cell line.

Key Research Accomplishments

- Development of MTC-GFP-mts1 and MTLn3-GFP-mts1 cell lines.
- Development of a quantitative GST pull-down assay for defining the mts1 binding site on the myosin-IIA heavy chain.

Reportable Outcomes

- Development of MTC-GFP-mts1 and MTLn3-GFP-mts1 cell lines.
- Abstract for the annual American Society for Cell Biology meeting held December 2001.
- Abstract for the annual American Society for Cell Biology meeting held December 2002 (will be submitted August 1).
- Submitted NIH R01 proposal entitled "Mts1 Metastasis Factor: Mechanism of Tumor Cell Motility" June 1.

Conclusions

We have established high expressing MTC-GFP-mts1 and MTLn3-GFP-mts1 cell lines and are now poised to begin our intravital imaging studies, which will visualize the motile behavior of mts1 expressing tumor cells within the primary tumor, during intravasation and extravastion *in situ*. Importantly, these analyses will allow us to determine how mts1 expression impacts on the motile processes associated with invasion and metastasis *in vivo* and identify those steps in the metastatic cascade affected by mts1 expression. These cell lines will also be used in an *in vitro* assay to evaluate the effects of mts1 expression on chemoattractant-stimulated motility. This will allow us to obtain comprehensive behavioral phenotype and will identify which aspects of directed motility are sensitive to the expression of mts1.

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References
Not applicable

Appendices
Not applicable