A Mammalian Severin Replaces Gelsolin in Transformed Epithelial Cells

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ABSTRACT

A persisting paradox in cytoskeletal regulation of cell motility is the loss of the actin filament fragmenting protein, gelsolin, in transformed epithelial cells that have gained the ability to migrate. Either actin filament severing does not occur during motility of carcinoma cells or a novel fragmentation protein is expressed during transformation. Using an antibody specific for severin, the M, 40,000 actin filament severing protein from Dictyostelium discoideum amoebae, we have identified a mammalian form of severin in murine LL/2 carcinoma cells lacking gelsolin. Mammalian severin (M-severin) isolated from LL/2-derived Lewis lung carcinoma tumors severed F-actin in a calcium-dependent manner, mimicking the function of Dictyostelium severin. M-severin preferentially localized to the cleavage furrow of dividing LL/2 cells and to the actin-rich cortex of migratory LL/2 cells, known sites of active actin cytoskeleton rearrangement. The mammalian severing protein was fully expressed in transformed LL/2 epithelial cells but went undetected in normal mouse muscle, liver, spleen, or kidney. Normal mouse lung tissue contained minute amounts of M-severin, attributed to motile cells in pulmonary connective tissue. In striking contrast to M-severin, gelsolin was highly expressed in normal lung but disappeared in transformed LL/2 carcinoma cells. Based on prior observations of a functional role for actin filament fragmentation in cell migration, the simultaneous induction of M-severin and loss of gelsolin during epithelial transformation suggests that replacement of gelsolin by M-severin may function to actin filament rearrangements necessary for active cell migration in invasive or metastatic carcinoma. Induction of M-severin in an invasive tumor was directly observed in human colon adenocarcinoma by cytowmunochemistry with antibodies directed against severin isolated from both Dictyostelium amoebae and Lewis lung carcinoma cells. Because normal colon epithelium from the same patient did not express M-severin, it may serve as a sensitive marker for detection and staging of epithelial tumors.

INTRODUCTION

In malignant carcinoma tumors, the invasion of transformed epithelial cells into the underlying connective tissue occurs by cell migration (1–3). Metastasis of carcinoma tumors also involves cell migration from the primary tumor site into blood vessels by diapedesis through the vessel endothelium (2). Migration of metastatic tumor cells was clearly described by Waldeyer in 1872 as amoeboid movement (4), a form of cell motility that requires coordinated mobilization and remodeling of the actin cytoskeleton by actin-binding proteins (5–10). An initial step in cortical actin cytoskeleton rearrangement includes site-specific actin polymerization onto actin filament ends that have been generated by severing or uncapping of existing filaments (11). Two families of actin filament fragmenting/capping proteins are presently recognized, the severin/gelsolin family containing shared 125-amino acid repeat domains (12–16), and the actin depolymerization factor family of ADF (17), depactin (18), destrin (19), and actophorin (20). Severin from Dictyostelium amoebae (21, 22) and fragmin in Physarum slime molds (23) are the earliest phylogenetic examples of actin filament fragmenting proteins. The parallel actin severing protein in mammalian cells is gelsolin, an M, 80,000 protein derived from duplication of the ancestral severin gene (24). A cytoplasmic gelsolin is expressed in epithelial cells, fibroblasts, and leukocytes, and secreted plasma gelsolin is present in blood (5, 6, 25). In gelsolin, it is the conservation of severin amino acid sequences that accounts for the actin filament severing activity (13, 26, 27).

Gelsolin is implicated in mammalian cell motility by the demonstration that increased expression of gelsolin in fibroblasts by gene transfection proportionally enhances the rate of migration (28). ABP* 120 has also been implicated in cell motility by functional phenotype analysis (29, 30). Paradoxically, despite the heightened migratory behavior of invasive tumor cells, gelsolin is extensively down-regulated during transformation of mammary epithelium and fibroblasts (31, 32). If migration of transformed epithelial cells utilizes similar cytoskeletal mechanisms as other motile mammalian cells to achieve pseudopodial extension (33), then an alternate F-actin severing activity should be present. We therefore analyzed lysates of highly motile and transformed epithelial LL/2 cells together with their resultant LLC tumors for the presence of severin, the ancestral actin filament fragmentation protein prominent in Dictyostelium amoebae. The results indicate that both LL/2 cells and their derived tumors contain a M-severin. Moreover, whereas gelsolin is dominantly expressed in normal lung epithelium, M-severin appears to become expressed during transformation to replace gelsolin in LL/2 cells and tumors. Furthermore, M-severin expression appears to be a general feature of motile and/or transformed epithelial cells but not of nonmotile cells of muscle, liver, or normal epithelium. It is this specificity for motile cells that makes M-severin an interesting candidate for marking invasive carcinoma tumors. Consequently, we further show that invasive human colon adenocarcinoma tumors contain abundant levels of M-severin, whereas normal colon epithelium from the same patient does not express the protein.

MATERIALS AND METHODS

Propagation of Lewis Lung Adenocarcinoma Tumors. C57 B1 mice (Charles River Breeding Laboratories) were provided free access to standard laboratory chow and water. To generate tumors, approximately $10^6$ mouse LLC cells (LL/2; American Type Culture Collection, CRL 1642) were injected s.c. into 15-g C57 B1 females, and the tumors were allowed to grow for 2 weeks before passage. Under light pentobarbitol anesthesia (Membual; 75 mg/kg body weight), a dorsal incision was made, and approximately 3 mm$^3$ of viable tumor cortex was implanted s.c. Tumors were passaged at least three times before use. Animals were sacrificed by cervical dislocation, and tumors were removed and stored at −80°C until use. All animal protocols were approved by the Animal Care and Use Committees of Cornell University Medical College.

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3 Supported in part by NIH Grant GM32458, Biomedical Research Support Grant 507-RR 05396, an Irma T. Hirsch Career Scientist Award (to J.D.P.), and an American Heart Association Medical Student Research Fellowship Award (to W.J.B.). 4 The abbreviations used are: ABP, actin-binding protein; M-severin, mammalian severin; LLC, Lewis lung carcinoma; HAP, hydroxylapatite.
Actin Filament Severing Assays. Rabbit muscle F-actin was used as a substrate for M-severin. Fractions to be assayed for severing activity were added to 0.1 mg/ml F-actin in buffer F [10 mM triethanolamine (pH 7.5), 0.2 mM DTT, 50 mM KCl, 2 mM MgCl₂, and 1 mM ATP] containing either 0.1 mM CaCl₂ (+ Ca⁴⁺) or 2 mM EGTA (–Ca⁴⁺). Mixtures were incubated for 10 min at 25°C. Aliquots (10 μl) of the reaction mixture were placed on parlodion, carbon-coated grids and stained for 1 min with 0.2% of filtered 1% uranyl acetate. Stained grids were blotted on the edge with filter paper, air-dried, and viewed in a JEOL 2000 electron microscope at 80 kV accelerating voltage. To quantitate severing efficiency, mixtures resulting from severing assays were centrifuged at 50,000 × g for 15 min to differentially sediment intact actin filaments. Resulting supernatant (actin monomers + fragments) and pelleted (actin filament) fractions were resolved by SDS-PAGE, and the actin and severin content was assayed by gel scanning densitometry (Hoeffer, San Francisco, CA).

Purification of M-Severin. Isolation of M-severin from Lewis lung adenocarcinoma tumors followed the purification method previously established for Dictyostelium severin (21, 22), with a slight modification. Tumor burdens of 15–20% of total body weight were excised, rinsed with 5 mM triethanolamine buffer (pH 7.5), and stored at −80°C until use. All isolation steps were carried out at 4°C or on ice. For each preparation, approximately 50 g of tumor tissue were thawed, minced, and added to 3 volumes (w/v) of cold lysis buffer [10 mM triethanolamine (pH 7.5), 60 mM sodium pyrophosphate, 30% (w/v) sucrose, and 0.4 mM DTT]. Phenylmethylsulfonyl fluoride in ethanol was added to a final concentration of 1 mM, and the suspension was immediately sonicated on ice with three 30-s bursts (Heat Systems W-220F sonicator at 30 MHz power). The cell lysate was centrifuged at 25,000 × g for 30 min, and the supernatant fraction was centrifuged at 150,000 × g for 90 min. Total protein concentration was determined for the high-speed supernatant fraction (36), and the fraction was diluted to 5 mg/ml with cold lysis buffer. Triethanolamine (1 mM; pH 7.5) was added to obtain a final concentration of 50 mM. Solid ammonium sulfate was incrementally added to obtain 60% saturation at 0°C, and the mixture was stirred on ice for 1 h. After centrifugation at 25,000 × g for 30 min, the resulting supernatant fraction was brought to 80% saturation on 3 M ammonium sulfate was added to a final concentration of 1 M, and the suspension was immediately pelleted for 90 min. Total protein concentration was calculated using the method of Bradford (40) using bovine plasma albumin as a standard. The resulting supernatant fraction was recentrifuged at 150,000 × g for 15 min, and the supernatant was stored on ice until use.

Antibodies. A rabbit polyclonal antibody raised against purified Dictyostelium severin (36) was isolated by chromatography through a Zeta Chrom 60 Disk (Cuno, Inc.). Severin-specific IgG (Anti-DdSev) was subsequently affinity-purified using purified Dictyostelium severin cross-linked to a CNBr-activated Sepharose 4B column (37). The antibody Anti-MSev was raised in rabbits by s.c. injection of purified M-severin from LLC tumors. Injection of 2 μg of protein in complete Freund’s adjuvant at each of six dorsal sites was followed by an equivalent challenge inoculation after 2 weeks and bleedings at 2-week intervals. Positive sera were stored at −20°C. A monoclonal antibody to human plasma gelosin showing specificity to an epitope on the M₅, 47,000 nonsevering chymotrypsin-like protein (38) was purchased from Sigma (G4896).

RESULTS

Immunological Detection of Severin in LLC Tumors. The requirement for cell migration in epithelial malignancy prompted a survey for severin in LL/2 cell LLC tumors. As seen in Fig. 1, an antibody against Dictyostelium severin (Anti-DdSev Ref. 36)
specifically detected a Mr 40,000 protein in both Dictyostelium and tumor cell lysates. Antibody avidity was 1000-fold greater for Dictyostelium severin, with positive Western blots obtained with 1 ng/ml Anti-DdSev compared to 1 µg/ml for the Mr 40,000 protein in tumor lysates.

Isolation of Severin from Tumors. To establish functional identity, the Mr 40,000 tumor protein was purified from LLC tumors by previously established methods to isolate severin from Dictyostelium amoebae (Refs. 21 and 22; Fig. 2). Isolation utilized ammonium sulfate fractionation of a clarified tumor lysate, followed by DEAE and HAP chromatography. Purification was followed by both Ca^{2+}-activated actin filament severing activity and immunoblots with Anti-DdSev (Fig. 2). Final HAP chromatography produced a purified Mr 40,000 protein with Ca^{2+}-activated severing activity and cross-reactivity to Anti-DdSev. The average yield of severin was 0.36 mg/50 g of tumor, representing 0.03% of total lysate protein. Like Dictyostelium severin, the tumor protein was completely soluble in 80% ammonium sulfate and eluted from HAP in 0.3 M KCl to give a pure product. However, the isolated M-severin was not biochemically identical to Dictyostelium severin, because M-severin showed a moderate affinity to DEAE at pH 7.5 compared to no affinity for Dictyostelium severin.

Functional Activity of M-Severin. The actin filament fragmenting activity of purified tumor-derived severin was assayed by electron microscopy and differential sedimentation of various stoichiometric mixtures of severin and F-actin in the presence or absence of 50 µM Ca^{2+}. Actin filaments remained intact in severin:actin mixtures in the absence of Ca^{2+} (presence of 2 mM EGTA; Fig. 3a) but were rapidly fragmented upon the addition of CaCl_{2}. Average fragment length was 30 nm, c, a 1:20 molar ratio of M-severin to F-actin in CaCl_{2} produced shorter fragments with an average length of 10.5 nm.
M-SEVERIN REPLACES GELSOLIN IN TRANSFORMED EPITHELIAL CELLS

![Image](5352) 5 Unpublished observations.

Fig. 4. Selective expression of M-severin in tumor tissue. Lane 1, molecular weight markers. Total protein (10 µg) from muscle (Lane 2), liver (Lane 4), lung (Lane 6), and LLC tumor (Lane 8), respectively, from tumor-bearing animals. Western blots of muscle (Lane 3), liver (Lane 5), lung (Lane 7), total LLC tumor (Lane 9), tumor cortex (Lane 10), and tumor core (Lane 11) probed with 5 µg/ml Anti-DdSev. M-severin was specifically detected in LLC tumors.

ments and 28 subunits in 1:20 fragments, indicating a stoichiometric rather than catalytic fragmenting activity by M-severin. Isolated M-severin did not induce coalignment, bundling, or cross-linking of actin filaments, suggesting an exclusive fragmentation and capping activity. Based on close similarities in size, immunological cross-reactivity, purification properties, and functional activity, M-severin has been identified as the mammalian homologue of *Dictyostelium* severin.

**Selective Expression of M-Severin in Transformed Tissues.** M-severin expression was compared in normal and transformed tissues (Fig. 4). M-severin protein was not detected in normal skeletal muscle, liver, or lung tissue taken from tumor-bearing animals. Because LLC tumors derive from pulmonary epithelium, it was of considerable interest to directly assay LLC tumors and normal lung tissue from the same animal for M-severin expression. Tumors showed extensive expression of M-severin (Fig. 4, Lane 9) in both the proliferating tumor cortex and necrotic core (Fig. 4, Lanes 10 and 11), whereas normal lung showed no cross-reactivity with Anti-DdSev (Fig. 4, Lane 7), suggesting M-severin induction in neoplastic C57 B1 mouse lung epithelium.

**Comparative Expression of M-Severin and Gelsolin in Normal and Transformed Epithelial Cells.** Because severin and gelsolin both function as actin filament severing proteins, severin and gelsolin expression patterns were compared in normal lung and LLC tumors. To maximize sensitivity and specificity, a polyclonal antibody (Anti-MSev) was raised against purified M-severin isolated from mouse tumors. High levels of gelsolin were detected in normal lung lysates together with minute amounts of M-severin (Fig. 5, Lung). Because highly motile fibroblasts, macrophages, and neutrophils in pulmonary connective tissue contain M-severin,5 pneumocytes comprising the predominant lung epithelial cell type are not likely to contain the protein. M-severin is immunologically distinct from the NH2-terminal severin-like domain of gelsolin, as evidenced by the lack of cross-reactivity between the Anti-MSev antibody and gelsolin in the lung (Fig. 5, Lanes 6 and 7). The appearance of M-severin in transformed tissues cannot be ascribed to a proteolytic breakdown product of gelsolin. In transformed LL/2 tumor cells, expression of M-severin corresponded to a complete loss of gelsolin (Fig. 5, LL/2). We therefore posit that M-severin replaces gelsolin during epithelial cell transformation.

Anti-M-sev was also used to clone a partial length M-severin cDNA from a P19 carcinomaembryonic cell library (Stratagene). The clone contained a 136-amino acid sequence with 48% homology to *Dictyostelium* severin. The clone allowed an analysis of the expression of M-severin mRNA in three epithelial cell lines (MDCK, LL/2, and P19) having different metastatic potentials. Quantitation of blots by phosphorimaging (Molecular Dynamics) showed that highly metastatic P19 carcinomaembryonic cells expressed approximately 10 times as much M-severin mRNA as weakly metastatic LL/2 cells, which in turn showed 7 times more mRNA than cultured MDCK cells (Fig. 6). M-severin expression in MDCK cell lines was approximately 70-fold less than in P19 cells. The low but detectable basal level of M-severin mRNA expression in MDCK cell cultures may reflect the partially transformed immortalized state of these cells. Consequently, M-severin is not exclusive to LL/2 cells but is expressed in three different transformed epithelial cell types. Furthermore, M-severin mRNA expression showed a strong positive correlation with the metastatic potential of the cell line analyzed.

**Localization of M-Severin.** The intracellular location of M-severin in the actin cytoskeleton of dividing, migratory LL/2 cells was ascertained by confocal microscopy (Fig. 7). In actively dividing cells, M-severin was concentrated in the cleavage furrow and extending cell cortex distal to the furrow (Fig. 7a) and localized to similar cellular regions with F-actin (Fig. 7b). A vertical section through the dividing cell pair shown in Fig. 7a clearly shows high concentrations of M-severin at the leading cell edges and in the cleavage furrow (Fig. 7c). M-severin and F-actin appear in similar intracellular locations and are concentrated in areas actively undergoing actin cytoskeleton re-
Specific Expression of M-Severin in Invasive Colon Adenocarcinoma. That M-severin is expressed in epithelial carcinomas but not normal epithelium was demonstrated by immunohistochemical staining of adenocarcinomas of the colon (Fig. 8), from two different patients. Sections through a surgically resected colon showed that M-severin was not expressed in normal colon epithelium (Fig. 8, c and d, arrows) from the cancer patient. Epithelial cells of normal colonic villi showed no severin staining (Fig. 8, d, arrows), whereas motile connective fibroblasts of the lamina propria underlaying the epithelium contained M-severin (Fig. 8, d, p). In moderately differentiated adenocarcinoma from the same patient, M-severin was abundantly expressed in transformed epithelial cells comprising the tumor (Fig. 8, e and f). Furthermore, advanced stages of undifferentiated adenocarcinoma existing adjacent to moderately differentiated adenocarcinoma heavily expressed M-severin (Fig. 8g, arrow and Fig. 8h), suggesting that the extent of M-severin expression marks advancing stages of epithelial transformation. The striking up-regulation of M-severin in invasive carcinoma follows the paradigm of M-severin expression in motile, dividing cells documented in cell culture and portends a significant potential use of M-severin as a marker for stage-specific diagnosis of carcinoma tumors. Similar detection of epithelial transformation by a M-severin marker has been observed in colon polyps and mammary ductal carcinoma.6–8

**DISCUSSION**

The detection of M-severin significantly broadens the occurrence of a protein previously presumed to be expressed only in Dictyostelium amoebae and Physarum slime molds (fragmin). Severin has traditionally been considered to be ancestral to gelsolin, the M, 80,000 F-actin fragmenting protein in mammalian cells, because of extensive sequence homology (15, 13, 42) and because gelsolin is not expressed in Dictyostelium amoebae (38, 43). Expression of M-severin presents the case for evolutionary conservation of a distinct severin gene. The gene product shows strong immunological and functional identity to Dictyostelium severin and shares a common cellular location in the actin-rich cortex. However, M-severin does not derive from a proteolytic breakdown product of gelsolin, because antibodies specific for M-severin do not recognize gelsolin. Two other actin-associated proteins, gCap39 (44–46) and Mbh1 (47), with molecular weights approximating that of M-severin have been described in mammalian...
cells but they do not function as F-actin fragmenting proteins. Based on sequence similarity, MCP, gCap39, Mbh1, gelsolin, villin, and ABP all belong to a family of mammalian actin filament regulatory proteins evolved from a structural motif composed of 120–130 amino acids found in Dictyostelium severin (13, 15, 16, 48). We propose that M-severin itself now be added to the family of actin-regulatory proteins expressed in mammalian cells.

Although severin has been implicated in Dictyostelium cell motility by its Ca$^{2+}$-activated F-actin severing function (21, 22) and restricted localization to extending pseudopods (36), the definitive function of severin in migrating amoebae has not been determined. This is largely due to the inability to produce a nonmotile phenotype in Dictyostelium mutants lacking severin. The precise function of actin fragmentation in highly motile transformed mammalian cells is also problematic because gelsolin, the only fragmentation protein found to date in epithelial cells, is almost completely down-regulated during transformation (31, 32). In fact, a significant number of actin cytoskeleton proteins germaine to cell migration and cytokinesis are extensively down-regulated in proliferating and migrating cancer cells. Tropomyosins (49–52), profilin (53), ABP (53), caldesmon (54), and gelsolin are all substantially diminished or deleted. Especially puzzling has been the disappearance of gelsolin from highly motile transformed human fibroblasts, epithelial cells (31), and human breast carcinoma tissue (32), because enhanced rates of cell migration are known to occur in fibroblasts overexpressing gelsolin (28).

Our demonstration of M-severin induction in transformed epithelial cells not only resolves the apparent paradox of down-regulation of actin filament-regulatory proteins in neoplastic cell types but also provides a natural model system for testing phenotypes resulting from M-severin expression in epithelial cells. Induction of expression of M-severin in normal epithelium and knockout of M-severin in transformed epithelial cells may provide key insights into the functional role of actin filament severing in mammalian cells that has not been possible to define.

Expression of M-severin in LL/2 cells is generalized to other motile mammalian cells and to human carcinoma tumors. In moderately differentiated colon adenocarcinomas, cytotoxicostaining for M-severin is apparent in connective tissue fibroblasts as well as invasive epithelial cells. Western blot and cytotoxicostaining for M-severin has also been obtained from mouse carcinoma tumors, 3T3 fibroblasts, activated lymphocytes, and macrophages (data not shown), leading to our hypothesis that actin cytoskeleton proteins dedicated to motility and cytokinesis are specifically expressed during epithelial cell transformation and leukocyte activation. mRNA expression patterns of M-severin during transformation further demonstrate a correlation between M-severin expression and progressive metastatic potential of epithelial cell lines. Cloning of the full-length cDNA will be required for severin in mammalian cells and its role in the acquisition of motility during epithelial cell transformation.

This work provides the initial observation of the replacement of an actin-regulatory protein in sessile epithelial cells with one of similar function from a highly motile cell type. We posit that alternate cytoskeleton gene expression may constitute a general biological mechanism for enhancing the migratory and proliferative potential of transformed epithelium and leukocytes. This hypothesis is lent credence by our observation that M-severin becomes selectively expressed in transformed, invasive epithelium in adenocarcinomas of the colon. Considering the major role of cell migration in dissemination of transformed cells from primary tumors, it will be of the utmost interest to determine whether induction of M-severin alone or together with other motility-specific actin-regulatory proteins is mandatory for metastasis of carcinomas.
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