Reduction of Epidermal Growth Factor Receptor Phosphorylation by Activated Mullerian Inhibiting Substance Is Vanadate-Sensitive

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The carboxy-terminal domain of recombinant human Mullerian inhibiting substance (MIS) inhibits cellular proliferation in vitro and decreases epidermal growth factor (EGF)-dependent phosphorylation of the EGF receptor. Proteolytically cleaved and undissociated MIS is more potent than carboxy-terminal MIS alone, supporting a functional role for the amino-terminal region of the molecule. MIS does not block EGF binding to the EGF receptor, thus, MIS reduction of EGF receptor phosphorylation must occur distal to receptor ligand binding. The effect of proteolytically cleaved MIS on reduction of EGF receptor phosphorylation in membrane preparations is decreased by a specific phosphatase inhibitor, vanadate, thus implicating a membrane phosphatase in this MIS action at the EGF receptor.

MULLERIAN inhibiting substance (MIS), a 140-kd glycoprotein homodimer secreted by Sertoli cells of the fetal testis[1-3] and granulosa cells of the adult ovary,[4,5] induces Mullerian duct regression, inhibits granulosa luteal cell growth,[6] arrests oocyte meiosis,[7,8] and inhibits growth of several human tumor cell lines in vitro and in vivo.[9-13] Each of these MIS activities can be counteracted by epidermal growth factor (EGF).[8,11,14-16] The carboxy-terminal domain of MIS, plasmin cleaved from holo-MIS at the monobasic cleavage site, arginine 427–serine 428, induces Mullerian duct regression and inhibits proliferation of A431 cells, a human vulvar tumor line.[17,18] Site-directed mutagenesis of serine 428 to threonine yields a noncleavable mutant unable to induce Mullerian duct regression[19]; conversely, a more easily cleaved MIS variant created by changing serine 428 to arginine is more potent in blocking tumor metastases in vivo.[18] These data support the conclusion that MIS, like other members of the transforming growth factor-beta (TGF-β) family of growth and differentiation factors, requires proteolytic processing to generate its bioactive moiety. Partially purified bovine* and recombinant human† MISs block EGF receptor autophosphorylation, predominately at tyrosine residues[16,20,21]; this response is completely abolished by preabsorption with polyclonal anti-MIS antibodies.[20,21] In contrast, MIS purified to homogeneity does not reduce EGF receptor tyrosine kinase activity,[16,20,21] although it induces Mullerian duct regression in the embryonic urogenital ridge bioassay. The most likely reason for these findings is that partially purified MIS contains MIS-cleavage products, whereas homogeneous preparations of MIS consist of holo-uncleaved MIS. Reduction of phosphorylation by partially purified MIS is observed when whole cells or membrane preparations are coincubated with EGF and MIS in the presence of radiolabeled adenosine triphosphate. However, MIS does not significantly alter phosphorylation of prelabeled membrane preparations, suggesting that MIS does not contain intrinsic phosphatase activity or that activated EGF receptor is resistant to MIS effects.[16] The present study was initiated to determine whether reduction of EGF receptor phosphorylation in vitro requires MIS proteolytic activation, and to investigate further the mechanism of MIS regulation of EGF receptor phosphorylation.

This study analyzes the effects of activated forms of MIS, including carboxy-terminal MIS and plasmin-cleaved MIS, on EGF receptor autophosphorylation and A431 cell cycle progression. Evidence is presented that implicates a membrane phosphatase sensitive to vanadate (a group 5B transitional metal that inhibits tyrosine phosphatases) as a mediator of these responses. Activated MIS therefore appears to stimulate a tyrosine phosphatase that decreases EGF receptor phosphorylation rather than preventing binding to the EGF receptor or, as previously suggested, inhibiting the kinase activity of the EGF receptor.

MATERIALS AND METHODS

Preparation of Plasmin-Cleaved MIS

Immunoaffinity-purified holo–recombinant human MIS was prepared as described previously[22]; 1.5-mL aliquots in 20 mmol/L HEPES, pH 7.4, and 0.001% Nonidet P-40 were cleaved with 100 μL plasmin-bound beads for 48 hours with shaking at room temperature according to the protocol of Catlin et al.[23] Cleavage was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 15% resolving gels run under reducing conditions. Plasmin-cleaved MIS was concentrated by centrifugation in a Centricon-10 filter (Amicon, Beverly, MA) to a final volume of 200 μL (400 μg/mL protein) and stored at 4°C until used.

*Serial column chromatography purification of bovine MIS involves lectin-affinity and ion-exchange columns (nonimmunoaffinity), and the penultimate fraction (called DG3) contains less than 10% bovine proteins that are non-MIS proteins, in addition to the bioactive MIS proteins.

†Conditioned media from transfected Chinese hamster ovary cells containing the human MIS gene were purified to near-homogeneity using both serial ion-exchange and dye-affinity chromatography, as well as immunoaffinity chromatography using a mouse monoclonal antihuman MIS antibody.

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Preparation of Carboxy-Terminal MIS

Immunoaffinity-purified holo-rhMIS was cleaved with plasmin (EC 3.4.21.7; Sigma, St Louis, MO) at a 25:1 MIS to plasmin ratio (wt/wt) for 2 hours at room temperature, and placed on a P-100 polyacrylamide column (Bio-Rad, Fullerton, CA) equilibrated with 1.0 mol/L acetic acid and 20 mmol/L HEPES, pH 3.0, as previously reported.[17] The column was eluted in 0.54-mL fractions and analyzed for protein concentration.[24] Carboxy-terminal MIS was concentrated by lyophilization and dissolved in 20 mmol/L HEPES, pH 7.4 (1 mg/mL protein) or by centrifugation over Centricon-10 filters and dilution in HEPES buffer.

Isolation of A431 Cell Plasma Membranes

A431 plasma membranes were prepared at 4°C under calcium-free conditions.[16,21] Cells were maintained in alpha-minimal essential medium (α-MEM) containing 2X essential amino acids and 10% female fetal bovine serum (which is devoid of MIS). Before membrane isolation, cells were passed 1:2, cultured for 48 hours, split 1:6, grown to 85% confluency, and washed with harvesting solution (150 mmol/L NaCl and 20 mmol/L HEPES, pH 7.4). Cells were scraped in 6 mL harvesting solution, centrifuged at 500 × *g* for 5 minutes (1,500 rpm; Damon IEC), resuspended in an equal volume of harvesting solution, lysed by adding the cell suspension drop by drop to 100 vol stirring 20 mmol/L boric acid and 0.2 mmol/L EDTA, pH 10.2, and stirred for 10 minutes. Cytoplasmic proteins were coagulated with 10 vol 0.5-mol/L boric acid, pH 10.2, stirred for 5 minutes, and then removed by filtration through 900-μm mesh and centrifugation at 450 × *g* for 5 minutes (2,200 rpm; Beckman J2-21, Fullerton, CA). Partially purified membranes were collected by centrifuging the supernatant at 11,000 rpm for 30 minutes (Beckman J2-21) and resuspending in 6 mL 20-mmol/L HEPES, pH 7.4. The membrane/HEPES mixture was layered onto 35% sucrose and centrifuged at 24,000 × *g* for 60 minutes (15,000 rpm, SW 41 rotor, Beckman L8-55). The interfacial plasma membrane layer was then centrifuged at 24,000 × *g* for 10 minutes (15,000 rpm, 50.2 Ti rotor, Beckman L8-55), and the pellet was resuspended in 20 mmol/L HEPES, pH 7.4 (300 μg/mL protein concentration).

EGF Receptor Phosphorylation Assays

Phosphorylation assays on A431 plasma membranes were performed as described previously.[21] Membranes (5 μg) were incubated for 10 minutes on ice with or without MIS and 26 nmol/L EGF (receptor grade; Collaborative Research, Bedford, MA) in 20 mmol/L HEPES, pH 7.4, 1 mmol/L $MnCl_2$, and 2 nmol/L bovine serum albumin (BSA). Reactions were initiated with 0.015 mCi γ-^{32}P-labeled adenosine triphosphate (final reaction volume, 62.5 μL) and terminated with sample buffer. Samples were heated (90°C for 10 minutes), and labeled proteins were separated by SDS-PAGE under reducing conditions on 7.5% polyacrylamide gels. ^{32}P incorporation into the 170-kd EGF receptor was detected by autoradiography (5-minute exposure to Kodak XRP film, Rochester, NY) and quantified by a Betascope blot imager (Betagen, Waltham, MA). Additional phosphorylation assays were performed with 20 μmol/L sodium orthovanadate and 500 nmol/L okadaic acid.[25]

A431 Cell Antiproliferation Assays

Monolayer cell culture assays were performed as described for 96-well plate studies.[17] The 24-well plate studies were completed in a similar manner, except that cells were maintained in 1-mL cultures and the total culture time was lengthened to 10 days. Control and experimental additions to the cells were performed on days 0, 1, and 2 for small plates and on days 1, 4, and 7 for larger plates. Plated cell numbers ranged from 5 to 7 × 10^3 cells per well, and counts were performed on a Coulter Counter Model ZF (Hialeah, FL). Experiments were performed in triplicate, and three samples of each treatment were analyzed for cell number.

Cell Cycle Analyses

Cell cycle analyses were performed as described previously.[17] Cell counts were often less than 1 × 10^6, and thus nuclei were isolated to provide accurate data. Cells were suspended in 3.4 mmol/L citrate, 1.25 mmol/L trypsin, 1.5 mmol/L spermine, 0.1% Nonidet P-40, and 0.5 mmol/L Tris hydrochloride, pH 7.6, for 10 minutes. Cells, plus a trypsin inhibitor and RNase, were incubated with propidium iodide and spermine for 30 minutes at 4°C. Nuclei were analyzed using the Cellfit program on a Becton Dickinson (San Jose, CA) FACSCAN with a 488-nm argon laser; 5,000 events were counted before the data were evaluated, and samples were repeated in triplicate.

Confocal Microscopy Studies

A431 cells at 85% confluency were plated 36 hours before the experiment at densities of 5 × 10^4 and 5 × 10^5 cells per milliliter on glass cover slips. Leiden chambers (Medical Systems, Greenvale, NY) or Nunclon four-well multidish plates (NUNC, Naperville, IL) were used for incubations. Cover slips were washed twice with α-MEM/0.1% BSA and incubated at 4°C for 45 minutes in the dark with 33 nmol/L tetramethyl rhodamine isothiocyanate–conjugated EGF([EGF-RITC] Molecular Probes) with or without a 150-fold molar excess of unconjugated EGF (tissue culture grade; Molecular Probes) or a 200-fold molar excess of unconjugated plasmin-cleaved MIS, and then washed six times with α-MEM/0.1% BSA, rinsed with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde in PBS for 20 to 30 minutes at 4°C. Cover slips were washed three times in PBS and placed cell-side down on each slide with *N*-propyl gallate (8:2 mixture of glycerol/PBS) and secured with nail polish. Samples were examined using a Bio-Rad MRC 600 Scanning Confocal Imaging System attached to a Zeiss Axiovert 35 inverted microscope (Thornwood, NY). Fluorescence was excited using the 568-nm line of the argon-krypton mixed-gas laser. The confocal parameters of aperture, frames accumulated, gain, black level, and scan rate were the same for each sample.

RESULTS

Plasmin cleavage of MIS generates three major protein products that are evident on PAGE (Fig 1). The 55-, 34-, and 12.5-kd MIS protein fragments produced are sensitive to disulfide-bond reduction and dimerize in the absence of β-mercaptoethanol. Incorporation of ^{32}P into proteins in isolated A431 plasma membranes was measured in the presence of MIS and/or EGF. EGF increases the degree of phosphorylation of the 170-kd EGF receptor over basal levels, as seen on PAGE, and purified 25-kd carboxy-terminal MIS dimer reduces this activity (Fig 2A). Plasmin-cleaved MIS causes a greater reduction of EGF-stimulated phosphorylation than purified carboxy-terminal MIS (Fig 2A). The additional bands at approximately 145 and 90 kd are likely partially cleaved EGF receptors from the membrane isolation process.[26] When quantified by Betascope image analysis, plasmin-cleaved MIS at 400-nmol/L concentrations produces 111.1% ± 5.4% reduction of EGF receptor phosphorylation (n = 5) relative to control incubations, whereas equimolar carboxy-terminal MIS produces only

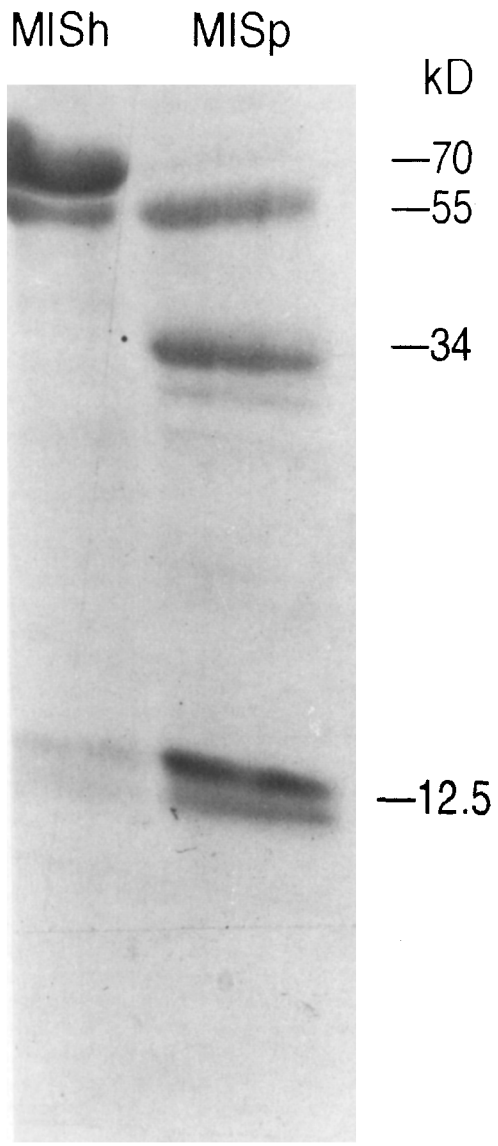

Fig 1. Fifteen percent polyacrylamide gel of holo MIS (MISh) and plasmin-cleaved MIS (MISp) run under reducing conditions and stained with Coomassie blue. MISh lane shows the predominant 70-kd band and cleavage products at 55 and 12.5 kd. The MISp lane contains the 12.5-kd reduced MISc and 55-kd amino terminal MIS; the 32-kd band is a shortened 55-kd cleavage product.

66.2% ± 26% reduction (n = 5; $P < .03$). The reduction of autophosphorylation by carboxy-terminal MIS strongly suggests a dose-dependent relationship (Fig 2B). Given the variation in the data, a clear dose-dependency is not demonstrated. In contrast, uncleaved holo-MIS has no effect on EGF-stimulated phosphorylation (n = 10; Fig 2A).

Carboxy-terminal MIS has been previously shown to inhibit cell growth[17]; we further studied the antiproliferative action of carboxy-terminal MIS by analyzing A431 cell cycle progression. Carboxy-terminal MIS halts synchronized A431 cells in the G1 phase of the cell cycle (Table 1). The effects of plasmin-cleaved, undissociated MIS on cellular proliferation were not known before these studies. Therefore, plasmin-cleaved MIS was incubated with A431 cells in monolayer cultures. At concentrations of 92.8 nmol/L, plasmin-cleaved MIS inhibits cell proliferation (Fig 3) by 54.31% ± 7.07% as compared with the vehicle control. TGF-β and EGF have been shown to be additive in their antiproliferative effects on mink lung cells, and thus we decided to examine the effects of EGF and MIS on proliferation of A431 cells. Co-addition of EGF and plasmin-cleaved MIS was found to be additive, that is, A431 proliferation was almost completely blocked (1.25% relative to control growth of 100%; Fig 3).

Confocal microscopy imaging was used to determine whether MIS interferes with ligand binding at the EGF receptor. A431 cells incubated with EGF-RITC demonstrated high-intensity fluorescent labeling of cell surfaces. This fluorescent staining was effectively competed by a 150-fold molar excess of unconjugated EGF (Fig 4). Coincubation of EGF-RITC with a 200-fold molar excess of plasmin-cleaved MIS did not result in reduced fluorescent staining of A431 cells, indicating that plasmin-cleaved MIS does not compete with EGF for binding to its receptor.

Previous studies demonstrated an absence of intrinsic phosphatase activity in A431 membranes isolated under calcium-free conditions.[21] The question of phosphatase action associated with the MIS effect on EGF phosphorylation was further addressed using vanadate and okadaic acid. Addition of vanadate to the phosphorylation assays increased basal and EGF-stimulated phosphorylation (Fig 5), indicating that one or more membrane-associated phosphatases are involved in EGF receptor phosphorylation. Vanadate reduces plasmin-cleaved MIS–mediated reduction of EGF receptor autophosphorylation (Fig 5), implying that MIS acts through a phosphatase-regulated pathway, most probably a tyrosine-specific phosphatase. Serine or threonine phosphatases are likely not involved in this pathway, since okadaic acid, an inhibitor of these enzymes, failed to change basal or EGF-stimulated phosphorylation ($P < .95$, n = 4, and $P < .4$, n = 4, respectively; data not shown).

DISCUSSION

Activated MIS, as a homogenous preparation of carboxy-terminal MIS or as plasmin-cleaved MIS containing carboxy- and amino-terminal fragments in noncovalent association, reduces EGF receptor autophosphorylation in A431 membranes in a dose-dependent manner and inhibits cell proliferation in this same cell line by halting cell cycle progression at G1. Activated MIS does not interfere with EGF binding to the EGF receptor. These MIS effects occur at doses similar to those required for regression of Mullerian ducts in vitro.[17] The antagonistic effect of carboxy-terminal MIS on EGF receptor signaling is analogous to that of the hepatocyte glycoprotein, pp63, which blocks insulin receptor tyrosine kinase activity and inhibits insulin-augmented growth of rat hepatoma cells.[27] The increased specific activity of plasmin-cleaved MIS relative to homogeneous carboxy-terminal MIS suggests that the amino-

terminal domain of MIS augments the function of the carboxy terminus. In fact, Wilson et al[28] showed that a 10-fold molar excess of amino-terminal MIS added to isolated carboxy-terminal MIS enhanced its specific activity as an inhibitor of aromatase in fetal rat ovaries. The amino-terminal fragment may maintain the configuration of the MIS carboxy-terminus for binding to the receptor, as outlined by Klagsbrun and Baird[29] for basic fibroblast growth factor. In this context, amino terminus refers to all but the last 109 carboxy-terminal amino acids of MIS. Since holo-MIS is inactive in A431 plasma membrane preparations and monolayer cell cultures but does function in the Mullerian duct organ culture system, an as yet unidentified protease present in the urogenital ridge but absent from A431 cells is presumed to activate holo-MIS.

Table 1. Distribution by Cell Cycle Phase (%)

Parameter	G1	S	G2/M
Density arrest	93.5	2.4	4.2
Control	37.5	62.1	2.2
MISc	83.6	15.3*	1.1

NOTE. MISc inhibits cell cycle progression of A431 cells in monolayer culture. After growing cells to 100% confluency, they were analyzed by flow cytometry (density arrest) and subcultured in vehicle (control) or 540 nmol/L MISc before analysis at 24 hours.

Abbreviation: MISc, carboxy-terminal MIS.

*$P < .0001$; n = 3.

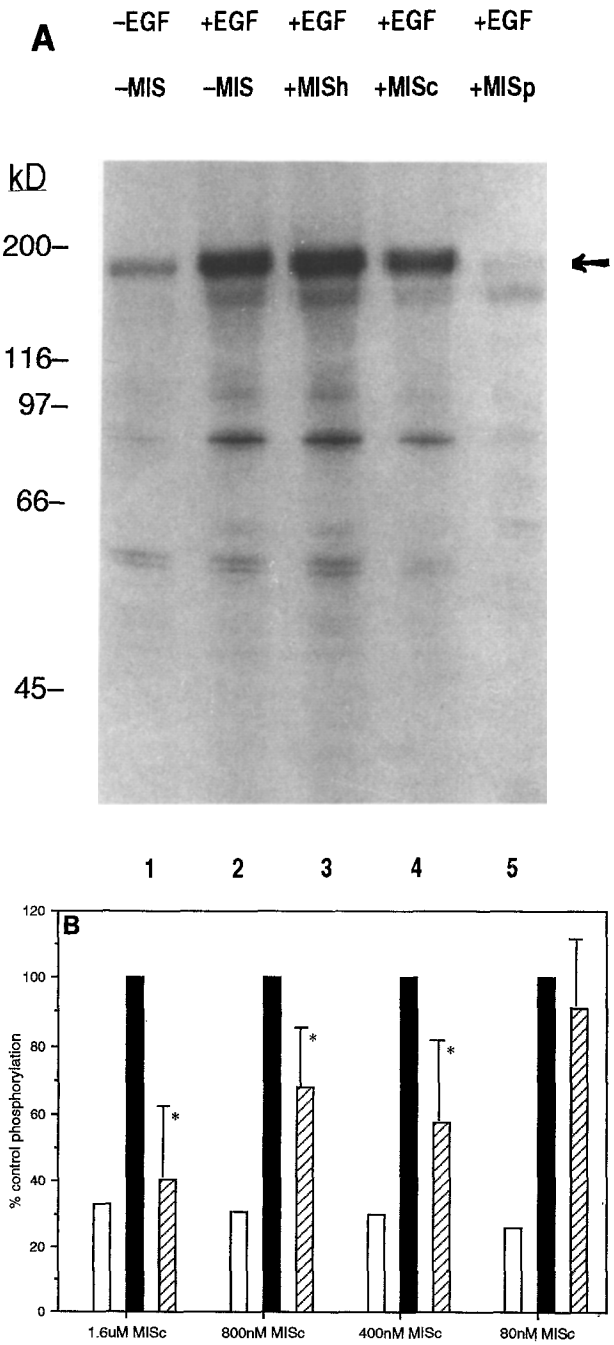

Fig 2. (A) Autoradiograph of a 7.5% polyacrylamide gel comparing 400 nmol/L concentrations of holo MIS (MISh), carboxy-terminal MIS (MISc), and plasmin-cleaved MIS (MISp) on ^{32}P incorporation into the 170-kd EGF receptor band (arrow). Lane 1: basal phosphorylation; lane 2: EGF-stimulated phosphorylation (maximal EGF stimulation set at 100%); lane 3: no change in EGF-stimulated phosphorylation with addition of MISh; lane 4: 50% reduction with MISc; lane 5: >100% reduction of EGF stimulated phosphorylation with MISp. Equal protein loading was confirmed by Coomassie blue staining. (B) Dose-response of MISc reduction of EGF receptor phosphorylation as percent change from EGF-stimulated phosphorylation. (□) Basal phosphorylation (−EGF, −MISc); (■) EGF-stimulated phosphorylation (+EGF, −MISc); (▨) EGF-stimulated phosphorylation in the presence of MISc (+EGF, +MISc). EGF-stimulated phosphorylation decreases in response to increasing MISc concentrations (*400 mmol/L, 66.2% reduction, $P < .005$, n = 5; 800 nmol/L, 46.1% reduction, $P < .00001$, n = 14; 1.6 μmol/L 88.3% reduction, $P < .04$, n = 5). Fourteen percent reduction of autophosphorylation by 80 nmol/L MISc was not statistically significant ($P < .43$, n = 4).

The growth response of A431 cells to EGF is a function of specific culture conditions. EGF stimulates growth of A431 cells in vivo or in soft agar, but in monolayer culture these cells exhibit their classic biphasic dose-response to EGF receptor tyrosine kinase activity,[30] ie, increased proliferation at low EGF concentrations and inhibition of proliferation at high EGF doses. Since EGF stimulates and MIS reduces EGF receptor tyrosine phosphorylation, we expected EGF to counteract the effects of MIS on A431 cell proliferation. However, co-addition of EGF and plasmin-cleaved MIS is additive in growth inhibition, since A431 proliferation is almost completely blocked in this setting. Similar inhibition of A431 cell proliferation is seen when TGF-β and EGF are added together; however, the mechanisms of TGF-β and MIS must be different, given that TGF-β stimulates rather than reduces EGF-induced receptor autophosphorylation.[31] Clarification of the differences in these molecular mechanisms awaits further study.

The current working model for MIS action proposes that the MIS carboxy-terminal fragment binds to the MIS receptor(s),[23] consisting of type I[32] and type II[33] serine/threonine kinase receptors. The putative type II receptor, apparently a homologous serine/threonine kinase,[33] is required for binding, whereas signal transduction occurs

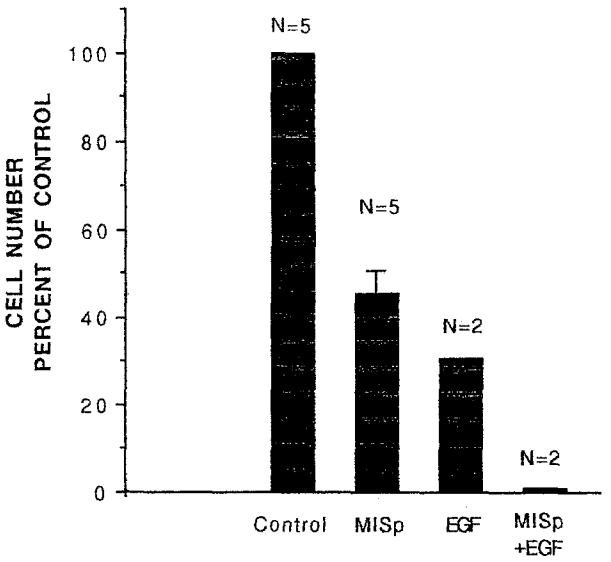

Fig 3. Growth of A431 cells subcultured after density arrest and incubated with buffer control (n = 5), 92.8 nmol/L plasmin-cleaved MIS (MISp) (n = 5), 3.3 nmol/L EGF (n = 2), or both factors (n = 2). Experimental data are compared with the growth of control cultures.

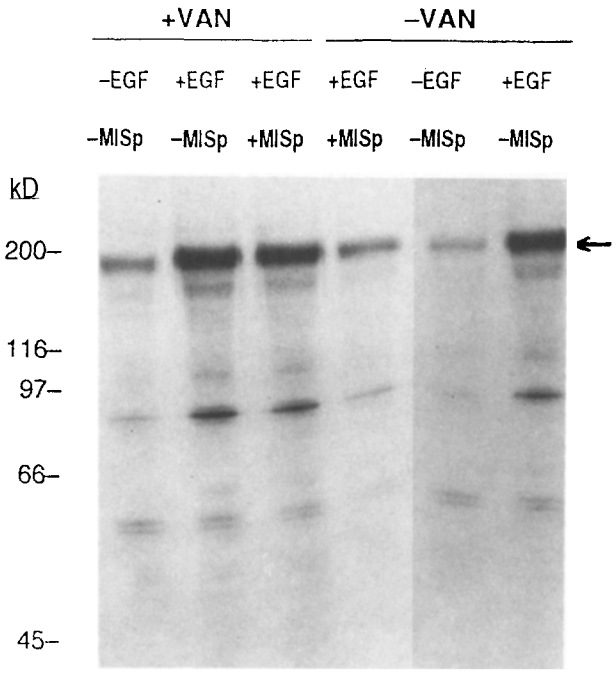

Fig 5. Autoradiograph of a polyacrylamide gel containing phosphorylation assays with and without vanadate. With vanadate (+VAN), basal phosphorylation (−EGF, −plasmin-cleaved MIS [MISp]) is enhanced, +71.5% ± 11.3%, $P < .01$, n = 3, and EGF-stimulated phosphorylation (+EGF, −MISp) is enhanced, +40.2% ± 6.7%, $P < .01$, n = 3. MISp reduction of autophosphorylation (−VAN, +EGF, +MISp *v* −VAN, +EGF, −MISp) is blocked by vanadate (+VAN, +EGF, +MISp *v* +VAN, +EGF, −MISp), −110% ± 9.7% *v* −12% ± 4%, $P < .02$, n = 3.

through the type I partner,[34] leading to gene activation and/or inhibition. One possible mechanism is that the MIS-mediated phosphatase action at the EGF receptor occurs after type I/type II receptor binding by ligand. Alternatively but less likely, vanadate may bind to and inactivate MIS.

MIS reduction of EGF-mediated stimulation has implications for reversing growth factor pathway oncogenesis in MIS receptor–positive tumors of any type.[20,22,35] The finding that a tyrosine protein phosphatase inhibitor such as vanadate and not a serine/threonine phosphatase inhibitor such as okadaic acid prevents the MIS-mediated reduction of phosphorylation suggests that blockade of a receptor tyrosine kinase signal transduction pathway, perhaps by activating a phosphatase, may be a strategy to explore for controlling abnormal cellular growth. Gruppuso et al[36] described similar findings wherein TGF-β–induced growth arrest of human keratinocytes was associated with protein phosphatase activation. Whether the MIS receptor complex directly activates the membrane phosphatase that blocks EGF action or the downstream transducers of MIS are co-opted for this activity is the focus of ongoing research. However, in light of this model that includes a serine/threonine kinase receptor for MIS, it was predicted that okadaic acid would enhance MIS activity by preserving receptor-dependent phosphorylation. Since this was not the case, the role of MIS receptor kinase activity in signaling is being reevaluated.

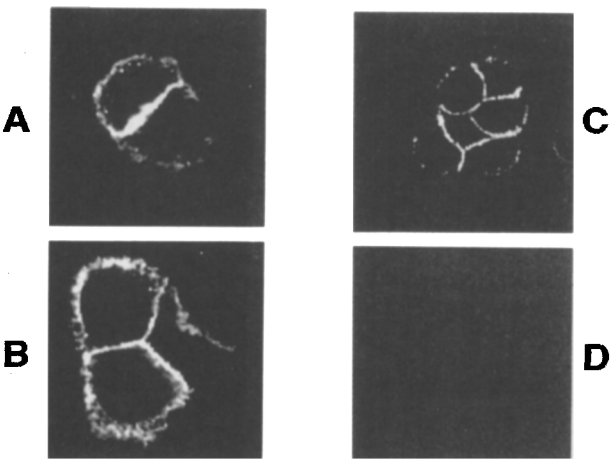

Fig 4. Fluorescence confocal microscopy demonstrating that plasmin-cleaved MIS (MISp) does not interfere with EGF binding. (A, C) A431 cells incubated with 33 nmol/L rhodamine conjugated EGF (EGF-RITC), which binds at surface receptors. (B) Incubation with EGF-RITC and 200-fold molar excess of MISp (+EGF-RITC, +MISp) or (D) EGF-RITC coincubated with a 150-fold molar excess of unconjugated EGF (+EGF-RITC, +EGF), n = 3. Fluorescence intensity of cells labeled with EGF-RITC is not altered by excess MISp, demonstrating that MIS does not compete with EGF binding to its receptor. Molar excesses of unconjugated EGF effectively compete for binding of labeled EGF.

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