

Recombinant Human Mullerian Inhibiting Substance Inhibits Epidermal Growth Factor Receptor Tyrosine Kinase

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Autophosphorylation of the epidermal growth factor (EGF) receptor in A-431 cells and plasma membrane fractions was inhibited by partially purified recombinant human Mullerian Inhibiting Substance (MIS). Immunoprecipitation of the EGF receptor using anti-EGF receptor or anti-phosphotyrosine antibodies, and phosphoamino acid analysis of this receptor, demonstrated that MIS specifically inhibited EGF-induced tyrosine phosphorylation. Inhibition of EGF receptor autophosphorylation by MIS in membrane preparations was not affected by increasing concentrations of EGF, manganese or [γ -(32)P] ATP. Thus, it is unlikely that MIS competes for EGF binding sites or sequesters substrate. Immunoabsorption of MIS with anti-human MIS antibody blocked the MIS inhibition of EGF receptor autophosphorylation, indicating that the inhibition was due to MIS. Our data suggest that MIS regulates the activity of the EGF receptor tyrosine kinase in A-431 cells.

KEYWORDS: Human Mullerian Inhibiting Substance, epidermal growth factor receptor, tyrosine kinase

INTRODUCTION

Mullerian Inhibiting Substance (MIS) is a 140-kDa glycoprotein dimer produced by Sertoli cells from both the fetal and neonatal testes (Blanchard and Josso, 1974; Budzik et al., 1983; Donahoe et al., 1977c) and the granulosa cells of the ovary (Takahashi et al., 1976). In the male embryo, MIS causes regression of the Mullerian duct, preventing formation of the Fallopian tubes, uterus, and upper third of the vagina (Donahoe et al., 1987); in the more mature female, MIS purified by ion-exchange and dye-affinity chromatography inhibits oocyte meiosis (Takahashi et al., 1986; Ueno et al., 1988).

We have shown previously that the ability of MIS to cause regression of the embryonic Mullerian duct in the organ culture assay (Picon, 1969) is blocked by epidermal growth factor (EGF) (Hutson et al., 1984) but not by other growth factors known to stimulate tyrosine kinase (Budzik et al., 1985). Con-

versely, testicular extracts and semipurified preparations of naturally occurring bovine MIS inhibited both EGF-induced phosphorylation of the EGF receptor in A-431 membranes and colony formation in soft agar (Coughlin et al., 1987). The effect of bovine MIS on EGF receptor phosphorylation was not due to an alteration in the number of EGF receptor binding sites in A-431 membranes and was independent of intrinsic phosphatase activity (Coughlin et al., 1987). These observations strongly linked MIS activity to inhibition of EGF receptor autophosphorylation at the cellular level.

The cDNA and genomic clones for bovine and human MIS were recently obtained, and human genomic MIS was expressed in Chinese hamster ovary cells (Cate et al., 1986). MIS purified from conditioned media of these cells caused regression of the Mullerian duct in the organ culture bioassay (Cate et al., 1986). In this study, we show that recombinant human MIS inhibits autophosphorylation of the EGF receptor in intact A-431 cells and in membrane preparations. The data suggest that MIS is a naturally occurring inhibitor of EGF receptor tyrosine kinase.

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METHODS

Materials

Dulbecco's Modified Eagle's medium, Connaught Medical Research Laboratory medium, methionine- and phosphate-free Roswell Park Memorial Institute-1640 medium, alpha-Modified Eagle's medium, and fetal calf serum were obtained from Gibco, New York. Receptor-grade EGF was purchased from Collaborative Research, Massachusetts. Mouse monoclonal anti-EGF receptor antibody was purchased from Amersham, Illinois. Affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate (western blotting grade) was acquired from BioRad, California, and pansorbin was purchased from Calbiochem, California. All remaining reagents were of analytical grade and obtained from Sigma, Missouri.

Membrane Phosphorylation Assay

EGF receptor autophosphorylation assays were performed in A-431 membranes at 4°C as previously described (Carpenter *et al.*, 1978, 1979). Plasma membranes obtained from A-431 cells in the S phase of the cell cycle were purified by methods also previously described (Cassell and Glasser, 1982; Thom *et al.*, 1977). A-431 membranes (5 µg) were incubated without or with EGF (26 nM) in a solution containing 20 mM Hepes [4-(2-hydroxyethyl)-1-(piperazine ethane sulfonic acid)], 1 mM manganese chloride, and 2 mM bovine serum albumin. After a 10-min incubation, the reaction was initiated by the addition of 0.025 mCi [γ -³²P]ATP at a concentration of 0.136 µM. The effect of MIS on EGF receptor autophosphorylation was evaluated by including preparations of human recombinant MIS at concentrations ranging from 3 pM–250 nM in the incubation mixture prior to the addition of EGF and [γ -³²P]ATP. All MIS samples were dialyzed against 8 liters of 20 mM Hepes, pH 7.4, containing 0.001% NP-40 (ethylphenyl polyethylenglycol) for 24 hr. Equiprotein fractions derived from wild-type Chinese hamster ovarian cell media were similarly dialyzed and used in these experiments as negative controls for the MIS-containing preparations. MIS, purified by serial ion-exchange and dye-affinity chromatography employing an elution buffer containing 10 mM AMP in the purification protocol (Budzik *et al.*, 1983, 1985; Cate *et al.*, 1986), was determined to have an AMP concentration of 1.42 µM by using [³H(G)]-AMP in the purification scheme. The final

AMP concentration after the addition of MIS to the membrane phosphorylation assay was 0.568 µM. AMP was thus tested to determine whether this nucleotide, at a concentration ranging from 4 pM to 40 µM, competes for [γ -³²P]ATP incorporation into the EGF receptor, thereby leading to a reduction in EGF receptor autophosphorylation by Cerenkov counts or autoradiography. The concentrations of EGF, manganese chloride, and [γ -³²P]ATP were also varied in separate experiments to assess the reversibility of the MIS effect by these substrates.

All reactions had a final volume of 62.5 µl and were stopped after 10 min by the addition of Laemmli sample buffer (Laemmli, 1970). Labeled proteins were separated by sodium dodecyl sulfate (SDS)-gel electrophoresis under reduced conditions on 7.5% polyacrylamide gels (Laemmli, 1970), and the location of the phosphorylated EGF receptor was identified by autoradiography.

Whole-Cell Phosphorylation Assay

Phosphorylation of intact A-431 cells was carried out as previously described (Kasuga *et al.*, 1985). Cells were subcultured every other day for at least 3 cycles to assure that a maximum number of cells were in the S phase of the cell cycle, and then transferred as the last sub-culture to 100-mm² tissue culture plates containing 20 ml of Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum. Twelve hours before each experiment, the culture medium was changed to serum-free media. Approximately 10 million cells/100 mm diameter plate approaching confluency were labeled with ³²P_i-orthophosphate (0.5 mCi/ml) for 3 hr in 5 ml of serum-free, methionine- and phosphorus-free Roswell Park Memorial Institute-1640 medium. The cells were incubated for 1 hr at 37°C in the absence or presence of human recombinant MIS (25–100 nM). Cells not treated with MIS received equiprotein fractions derived from purification of Chinese hamster ovarian cell wild-type conditioned media, or MIS buffer composed of 20 mM Hepes and 0.001% NP-40, pH 7.4. Cells were subsequently treated without or with EGF at a concentration of 100 nM for 1 min. The phosphorylation reaction was terminated as previously described (Kasuga *et al.*, 1985), and the Triton X-100 solubilized proteins were incubated with 10 µg phosphotyrosine antibody (Pang *et al.*, 1985) or 5 µg EGF receptor antibody. The precipitated proteins were separated by SDS-gel electrophoresis under reducing condi-

tions (Laemmli, 1970). The location of the radio-labeled phosphorylated proteins was determined by autoradiography.

Phosphoamino Acid Analysis

EGF receptor phosphoamino acid analysis was performed as previously described (Cooper et al., 1983; Pang et al., 1985). Briefly, the gel fragment containing the ^{32}P -labeled EGF receptor was cut out from the dried polyacrylamide gel obtained from the whole-cell assay, washed for 12 hr at 37°C with 20% methanol, and incubated with 100 $\mu\text{g}/\text{ml}$ of trypsin for 24 hr. The eluted peptides were hydrolyzed in 6 N HCl for 2 hr at 110°C. The resultant phosphoamino acids, together with unlabeled standards, were separated by high-voltage electrophoresis on a thin-layer cellulose plate and subjected to autoradiography. The phosphoamino acid standards were visualized by reaction with ninhydrin spray.

Purification of Recombinant Human MIS

Dihydrofolate reductase deficient Chinese hamster ovarian cells transfected with a plasmid containing a linear construct of both the human MIS gene (pBG 311 hmis) and the dihydrofolate reductase gene (pSV2DHFR) were grown to confluence in either serum-containing (10% female fetal calf serum) or serum-free selective media (Cate et al., 1986). MIS was purified from serum-containing conditioned media by salt extraction of protein, followed by serial ion-exchange and dye-affinity chromatography using Matrex Gel Green A (Amicon) (Budzik et al., 1983, 1985; Cate et al., 1986). After adapting Chinese hamster ovarian clones to serum-free conditions and scaling up production, larger volumes of media were collected and purified by Q-Sepharose (Pharmacia) ion-exchange chromatography (personal communication R. B. Pepinsky, Biogen Research Corporation, Cambridge, MA) and dye-affinity chromatography (Cate et al., 1987). Conditioned media from wild-type nontransfected Chinese hamster ovarian cells were purified in an identical manner at each level of purification to produce preparations lacking MIS. In addition, MIS was purified to near homogeneity from serum-free conditioned medium by immunoaffinity chromatography using a mouse monoclonal anti-human MIS antibody (monoclonal anti-human MIS antibody provided by Biogen research Corporation, Cambridge, MA). MIS was eluted from the immunoaffinity column using 2 M sodium thio-

cyanate at a pH of 6.3. After immunopurification, MIS was desalted on a Sephadex G-25 column (Pharmacia) pre-equilibrated in 0.15 M sodium chloride, 10% glucose, and 0.02 M Hepes, pH 7.5 (Picard and Josso, 1984; Shima et al., 1984).

Proteins of each preparation were measured by the method of Lowry et al. (1951), analyzed on Coomassie Brilliant Blue R-250-stained polyacrylamide gels and in an enzyme-linked immunoassay (P. L. Hudson, manuscript in preparation), and further screened by western blot analysis (Towbin et al., 1979) using a rabbit polyclonal antibody raised to the 140-kDa MIS band eluted from a nonreduced polyacrylamide gel. The total protein concentrations of the MIS samples purified under serum-free conditions by conventional chromatography approximated 0.5 mg/ml, and they contained the 70-kDa band characteristic of MIS under reduced conditions as confirmed by western blot analysis. The MIS-containing bands, purified by immunoaffinity chromatography, represented approximately 90% of the protein present on the gel by comparative analysis of the stained protein bands. MIS purified from serum-containing media by serial ion-exchange and dye-affinity chromatography, on the other hand, represented approximately 10% of the protein in the gel. These preparations were tested in the organ culture assay to detect biological activity (Donahoe et al., 1977a,b; Picon, 1969). All MIS-containing preparations caused regression of the Mullerian duct at concentrations ranging from 0.1–100 nM (Fig. 1B, 38 MIS samples tested). In contrast, equiprotein purification fractions from Chinese hamster ovarian wild-type cell media did not cause regression of the Mullerian duct (Fig. 1A, 3 wild-type preparations tested).

Immunoabsorption of MIS

The mouse monoclonal anti-human MIS antibody, raised against the intact 140-kDa MIS dimer electroeluted from nonreduced polyacrylamide gels, was purified by affinity chromatography on immobilized Protein A (Pierce Chemical). This antibody and normal mouse serum were dialyzed against 8 liters of 20 mM Hepes and 0.001% NP-40, pH 7.4, for 24 hr. Then, 50 μl anti-human MIS antibody (13 μg), normal mouse serum (13 μg), or dialysate buffer were plated into separate Falcon plate wells and allowed to incubate overnight at 4°C. The wells were then rinsed with deionized water and filled with 10% gamma globulin-free horse serum to block

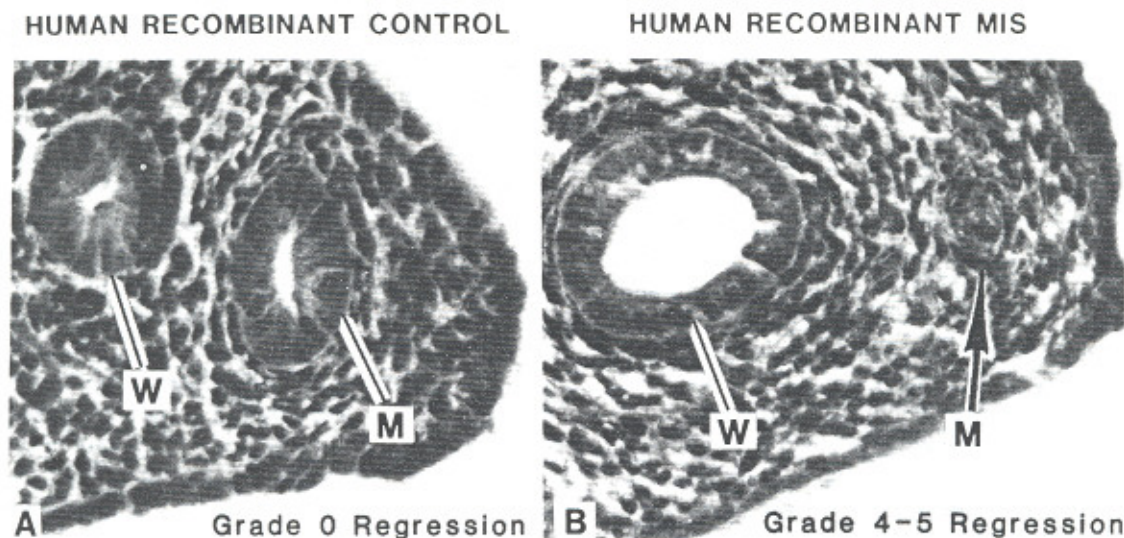


FIGURE 1. Human recombinant MIS preparations were purified both from serum-containing and serum-free media obtained from Chinese hamster ovarian cells transfected with the MIS gene. A representative MIS sample caused essentially complete regression (grade 4-5) of the female fetal rat Mullerian duct (B) at a concentration of 100 nM. In contrast, an equiprotein purification fraction derived from Chinese hamster ovarian wild-type media did not cause regression of the Mullerian duct (A). M, Mullerian duct; W, Wolffian duct.

nonspecific binding sites. After a 1 hr room temperature incubation, the gamma globulin-free horse serum was aspirated, and the wells rinsed with deionized water. MIS (36 pg), purified from serum-containing media obtained from CHO cells transfected with the MIS gene, was then placed into each pretreated Falcon plate well for 4 hr at 4°C, following which the fractions were removed and tested in the membrane phosphorylation assay for their ability to inhibit EGF receptor autophosphorylation. The results are expressed as the percent incorporation of ^{32}P into the treated EGF receptor relative to the EGF-stimulated control, as measured by Cerenkov counts, after cutting the autoradiographically identified receptor from the polyacrylamide gels.

In separate experiments, MIS (25 μg), purified by the ion-exchange and dye-affinity chromatography protocols discussed above and causing potent inhibition of EGF receptor autophosphorylation, was applied onto the immunoaffinity column containing 5 mg monoclonal anti-human MIS antibody; the flow through (unbound protein fraction) was then assayed for MIS by enzyme-linked immunoassay and tested for its ability to inhibit EGF receptor autophosphorylation in A-431 plasma membranes.

Enzyme-Linked Immunoassay

Mouse monoclonal anti-human MIS antibody was used to bind MIS into microtiter wells. Rabbit poly-

clonal anti-human MIS antibody was used as a secondary antibody to detect bound MIS. Using a goat anti-rabbit IgG peroxidase conjugate to recognize the antigen-antibody complexes, a colorimetric standard curve was constructed for the quantitative MIS can be detected (Hudson *et al.*, in preparation).

Organ Culture Bioassay

Intact 14-day female rat urogenital ridges were co-incubated for 72 hr with Conaught Medical Research Laboratory media (supplemented with 10% fetal calf serum and 1% penicillin/streptomycin) containing individual fractions purified from media of MIS gene-containing or wild-type Chinese hamster ovarian cell cultures. A 1-mm³ section of a 14-day rat testis was used as a positive control in this assay. Slides of hematoxylin and eosin stained tissue were prepared, and Mullerian duct regression graded on a 0-5 scale, with 0=no regression and 5=complete regression (Donahoe *et al.*, 1977a,b; Picon, 1969).

RESULTS

Membrane Phosphorylation Assay

EGF stimulated the phosphorylation of its 170-kDa

receptor in membranes of A-431 cells (Fig. 2A,B: +EGF). Human recombinant MIS preparations, purified from serum-containing media and recognized by western blot analysis, enzyme-linked immunoassay, and by organ culture bioassay, inhibited EGF-induced autophosphorylation of its receptor at concentrations ranging from 3 pM to 100 nM (Fig. 2A: +EGF +MIS, representative example of one of the 5 MIS preparations tested).

preparations tested). The MIS-directed inhibition of EGF receptor autophosphorylation was not affected by varying concentrations of EGF, manganese, or [γ - 32 P]ATP (Fig. 3A-C, one MIS preparation tested 2 times). AMP at concentrations ranging from 4 pM to 40 μ M did not interfere with EGF receptor autophosphorylation ($n=3$, figure not shown).

Recombinant human MIS purified to near homogeneity by immunoaffinity chromatography, and

HUMAN RECOMBINANT MIS INHIBITS EGF RECEPTOR AUTOPHOSPHORYLATION

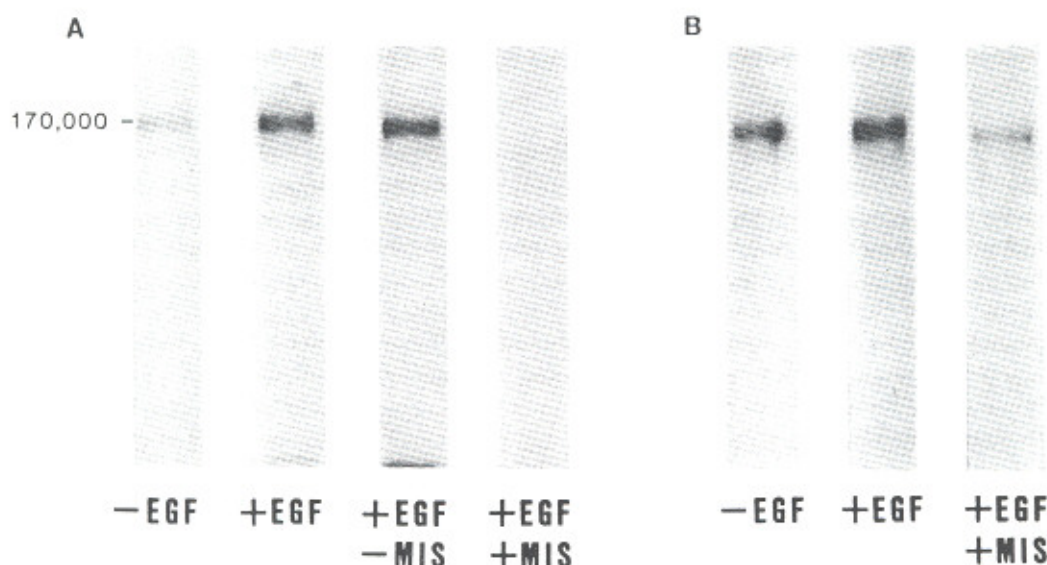


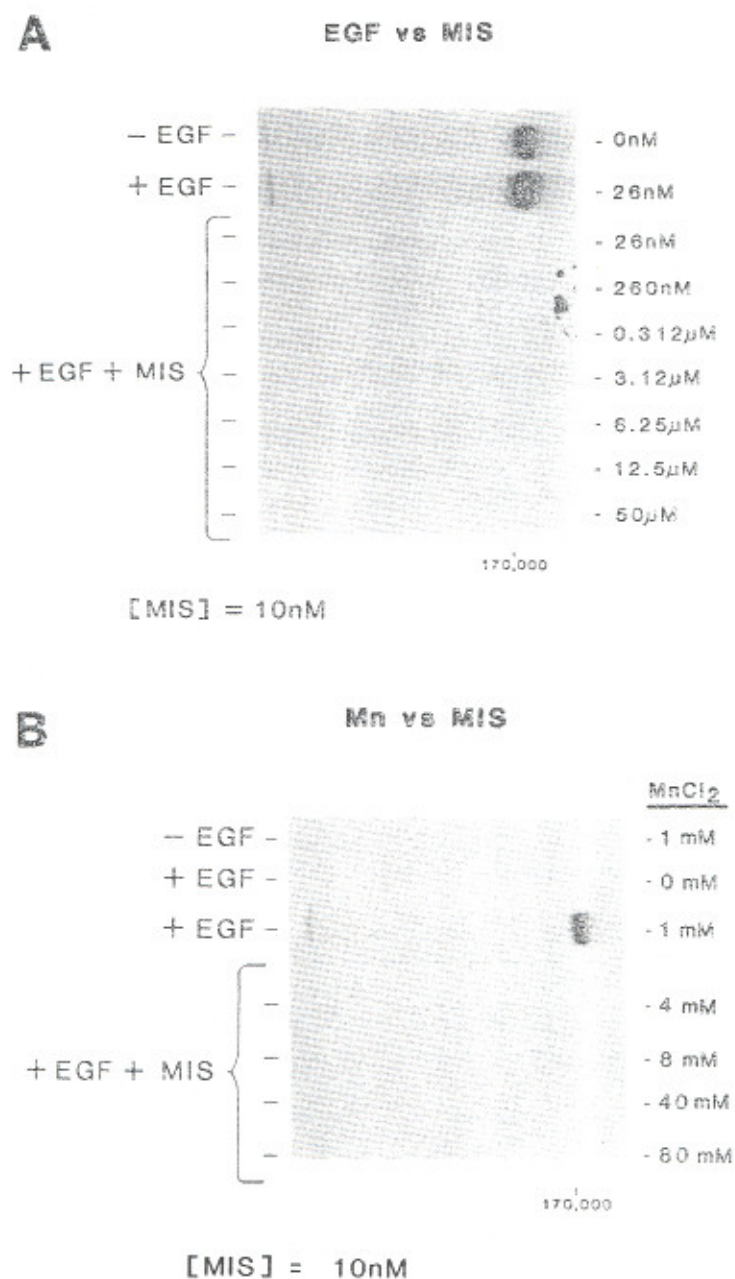
FIGURE 2. (A). Human recombinant MIS (+EGF+MIS) purified from serum-containing conditioned media inhibited EGF-stimulated 32 P-incorporation into the 170-kDa EGF receptor (+EGF) in A-431 membranes at a concentration of 70 nM. An equiprotein purification fraction lacking biological activity (+EGF-MIS) did not inhibit 32 P-incorporation into the EGF receptor. (-EGF) represents basal EGF receptor phosphorylation. Autoradiogram: 30-min exposure. (B). Human recombinant MIS (+EGF+MIS) purified from serum-free conditioned media inhibited EGF-stimulated 32 P-incorporation into the 170 kDa EGF receptor (+EGF) in A-431 membranes at a concentration of 100 nM. Basal EGF receptor phosphorylation is shown (-EGF). Autoradiogram: 30-min exposure.

Human recombinant MIS purified in the absence of serum by Q-Sepharose and dye-affinity chromatography also inhibited EGF receptor autophosphorylation at concentrations ranging from 10–100 nM (Fig. 2B: +EGF+MIS, an example of one of the 3 MIS preparations tested). In contrast, biologically inactive fractions purified from media of confluent wild-type CHO cells, and lacking MIS by western blot analysis and enzyme-linked immunoassay, did not inhibit EGF receptor phosphorylation (Fig. 2A: +EGF-MIS, an example of one of the 3 wild-type

recognized in the three separate identification assays described above, caused no inhibition of EGF-induced autophosphorylation of its receptor at concentrations ranging from 10 to 250 nM in A-431 membranes (3 MIS preparations tested, figure not shown). To rule out the possibility that a protein other than MIS was responsible for the inhibition of EGF receptor autophosphorylation, MIS was immunodepleted from less pure but active MIS preparations derived from serum-containing conditioned media. MIS incubated with immobilized

mouse monoclonal anti-human MIS antibody lost its ability to inhibit EGF receptor autophosphorylation; in contrast, the same MIS preparation incubated with normal mouse serum resulted in a 42% inhibition of EGF-stimulated autophosphorylation of its receptor in plasma membranes, attesting to the specificity of the inhibition of EGF receptor autophosphorylation as due to MIS in less purified MIS preparations (Fig. 4, one MIS preparation tested 2 times). The flow through fraction, obtained after

applying MIS to the immunoaffinity column but before its elution with sodium thiocyanate, did not inhibit EGF receptor autophosphorylation (Fig. 5, an example of one of 2 preparations tested). Because the flow-through fraction did not have any detectable MIS by enzyme-linked immunoassay, the experiment suggested that the MIS retained by the anti-human MIS antibody on the immunoaffinity column was responsible for inhibiting EGF receptor autophosphorylation.



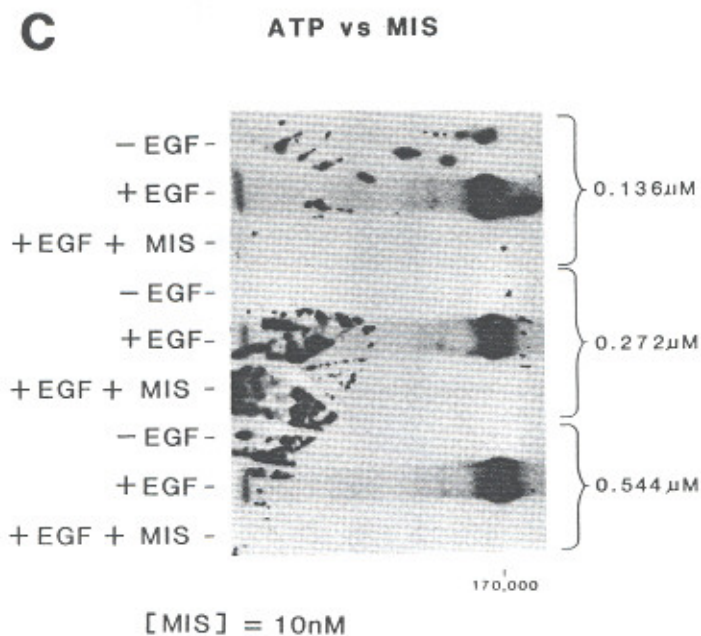


FIGURE 3. Human recombinant MIS (+EGF+MIS) purified from serum-containing conditioned media inhibited EGF-stimulated 32 P-incorporation into the 170-kDa EGF receptor (+EGF) in A-431 membranes at a concentration of 10 nM (A-C). The inhibition of EGF receptor autophosphorylation was not affected by varying concentrations of EGF, manganese chloride, or ATP (A-C). (-EGF) represents basal EGF receptor phosphorylation in each experiment. Autoradiogram; 30-min exposure.

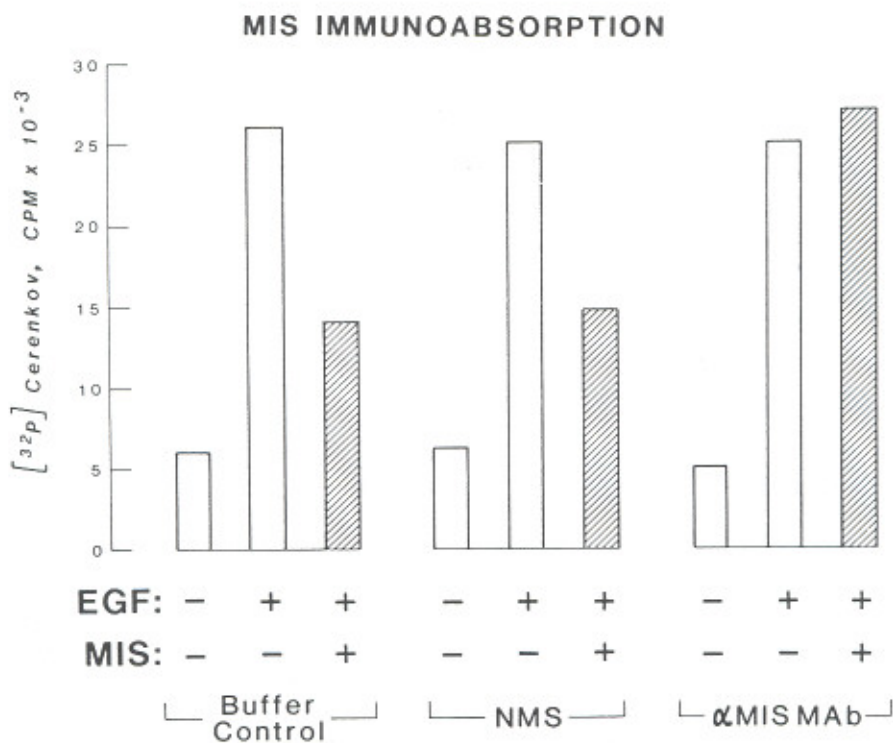


FIGURE 4. MIS purified from serum-containing conditioned media was preincubated with antibody buffer control, normal mouse serum (NMS), or monoclonal anti-human MIS antibody (α MISMAB), and then tested in the membrane phosphorylation assay. MIS (+EGF+MIS) preincubated with buffer control and normal mouse serum inhibited EGF-stimulated autophosphorylation of its receptor (+EGF-MIS). In contrast, MIS (+EGF+MIS) preincubated with monoclonal anti-human MIS antibody lost its ability to inhibit EGF-stimulated autophosphorylation of its receptor. Basal EGF receptor phosphorylation is shown (-EGF-MIS).

MIS IMMUNOABSORPTION

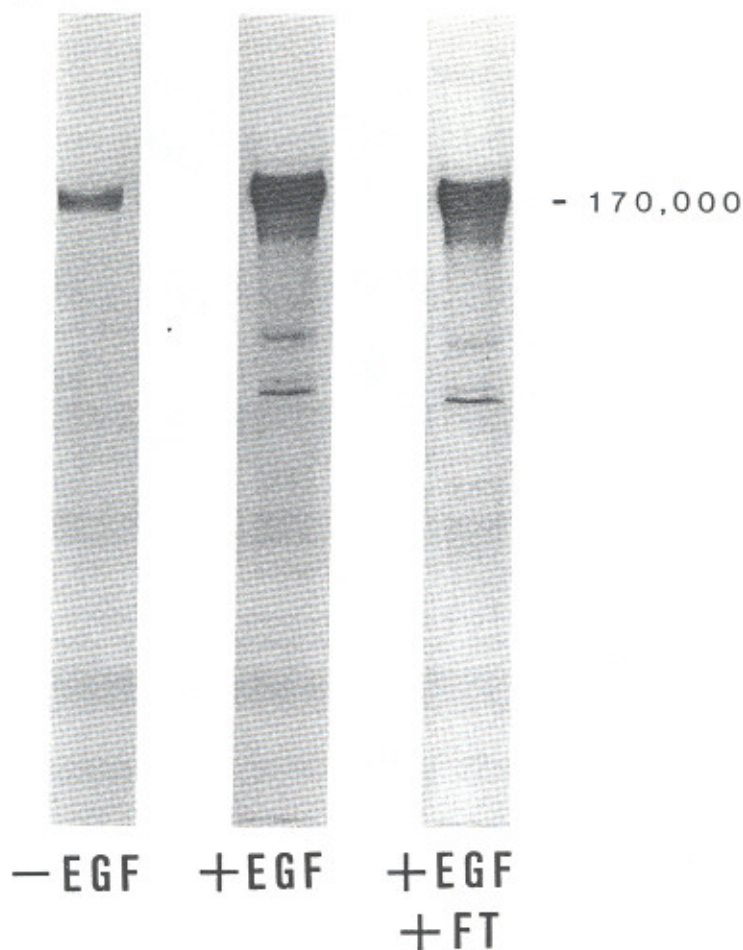


FIGURE 5. The flow-through fraction (FT), obtained from the immunoaffinity column used to purify MIS, did not inhibit EGF-stimulated ^{32}P -incorporation into the 170-kDa EGF receptor (+EGF) in A-431 membranes. Basal EGF receptor phosphorylation is shown (-EGF). Autoradiogram: 30-min exposure.

Effect of MIS on EGF Receptor Autophosphorylation in Intact Cells

In the absence of EGF there was no detectable phosphorylation of the EGF receptor in ^{32}P -labeled A-431 cells (Fig. 6A: lane 1). As predicted, EGF-stimulated autophosphorylation of the 170-kDa EGF receptor (Fig. 6A: lane 2); immunoprecipitation of this receptor using anti-phosphotyrosine antibody revealed that a large component of the phosphorylation occurred at tyrosine residues (Fig. 6B: lane 2). Partially purified MIS inhibited EGF-stimulated phosphorylation of the EGF receptor in intact cells (Fig. 6A: lane 4, an example of one of 5 MIS preparations tested). This inhibition was due to de-

creases in tyrosine phosphorylation because no phosphotyrosine residues were recognized on the EGF receptor by the phosphotyrosine antibody (Fig. 6B: lane 4, an example of one of three MIS preparations tested). Phosphoamino acid analysis of the EGF receptor immunoprecipitated by the anti-EGF receptor antibody further confirmed that MIS inhibited EGF-induced tyrosine phosphorylation of its receptor (Fig. 7, a representation example of one of 2 MIS preparations tested). Cells treated with MIS alone did not significantly alter basal EGF receptor phosphorylation. As in the membrane system, MIS eluted from the anti-human MIS antibody failed to inhibit EGF receptor autophosphorylation in intact A-431 cells (2 MIS preparations tested, figure not shown).

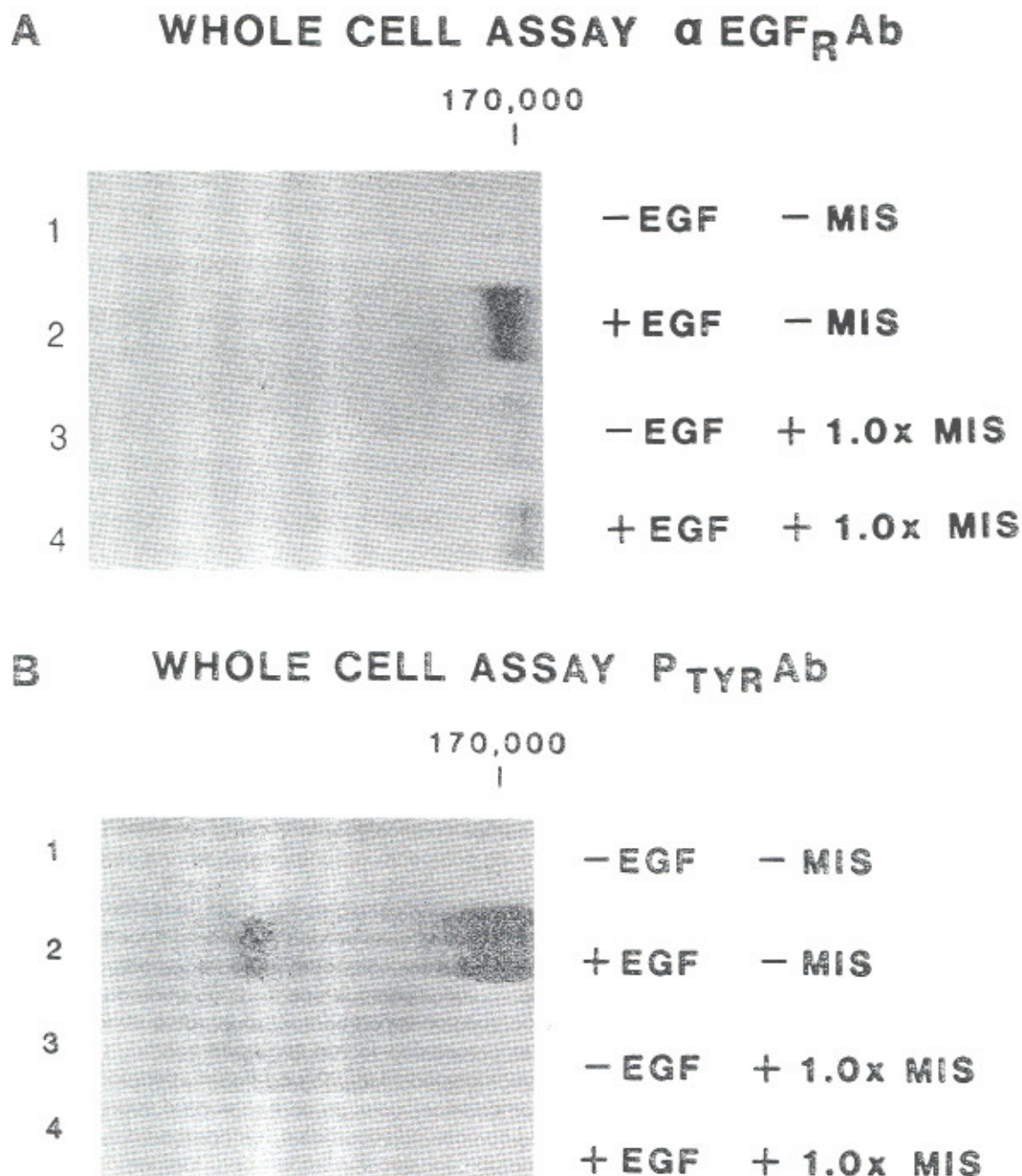


FIGURE 6. (A). The EGF receptor was immunoprecipitated from solubilized cell extracts using anti-EGF receptor antibody and then subjected to gel electrophoresis and autoradiography. In the absence of EGF and MIS (-EGF-MIS), there was no detectable phosphorylation of the 170-kDa EGF receptor. EGF (+EGF-MIS) resulted in stimulated autophosphorylation of its receptor. Human recombinant MIS (+EGF+1.0xMIS), purified from serum-containing conditioned media, inhibited EGF-induced autophosphorylation of its receptor at a concentration of 50 nM. MIS alone (-EGF+1.0xMIS) caused a minimal increase in EGF receptor phosphorylation compared with basal phosphorylation (-EGF-MIS). 7.5% SDS-polyacrylamide gel; autoradiogram: 3-hr exposure. (B). The EGF receptor was immunoprecipitated from solubilized A-431 cell extracts using anti-phosphotyrosine antibody. In the absence of EGF and MIS (-EGF-MIS) there occurred no tyrosine phosphorylation at the EGF receptor. EGF (+EGF-MIS) resulted in stimulated autophosphorylation of tyrosine residues at its receptor. Human recombinant MIS (+EGF+1.0xMIS), purified from serum-containing conditioned media, inhibited EGF-stimulated autophosphorylation of tyrosine residues at its receptor at a concentration of 50 nM. MIS alone (-EGF+1.0xMIS) caused no change in EGF receptor tyrosine phosphorylation compared with basal phosphorylation (-EGF-MIS). Autoradiogram: 3-hr exposure.

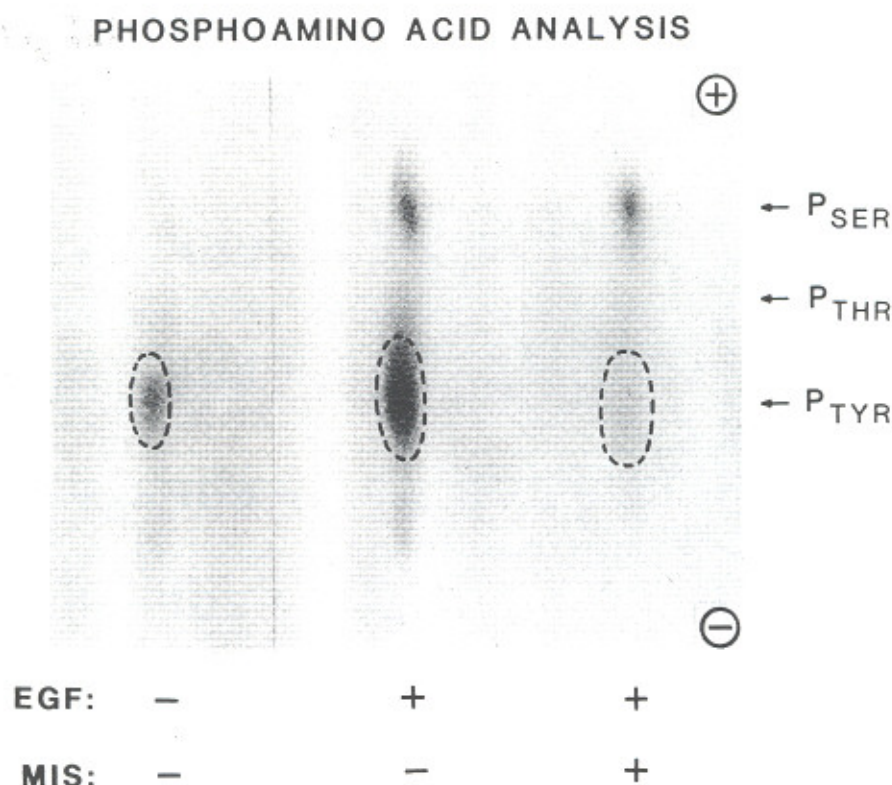


FIGURE 7. The EGF receptor was immunoprecipitated with anti-EGF receptor antibody and subjected to phosphoamino acid analysis. In the absence of EGF and MIS (-EGF-MIS) there was minimal phosphorylation of tyrosine. In the presence of EGF (+EGF-MIS) there occurred phosphorylation of tyrosine and serine residues at the EGF receptor. MIS (+EGF+MIS) resulted in inhibition of EGF-stimulated tyrosine phosphorylation without affecting serine phosphorylation at a concentration of 50 nM. Phosphoserine, P_{SER}; phosphothreonine, P_{THR}; phosphotyrosine, P_{TYR}. Autoradiogram: 10-day exposure.

DISCUSSION

The discovery of an inhibitory interaction between EGF and MIS in the embryonic organ culture assay led to a series of studies on the mechanism of MIS action in membrane preparations (Budzik *et al.*, 1985; Coughlin *et al.*, 1987; Hutson *et al.*, 1984). The subsequent successful expression of MIS from Chinese hamster ovarian cells transfected with a human MIS gene construct provided larger quantities of MIS (Cate *et al.*, 1986), and allowed the extension of MIS mechanism studies from a static membrane assay to one involving intact cells.

Biologically active human recombinant MIS, purified from serum-containing media of transfected Chinese hamster ovarian cells, resulted in complete inhibition of EGF-induced autophosphorylation of its receptor in A-431 membranes (5 separate MIS preparations tested). Similarly, equal concentrations of biologically active human recombinant MIS purified under serum-free conditions blocked EGF-

induced phosphorylation of its receptor (3 separate MIS preparations tested). The inhibition caused by MIS was not reversed by increasing concentrations of EGF, manganese, or ATP, making it unlikely that MIS competes for EGF binding sites or sequesters these substrates. In intact A-431 cells, antibodies directed against the EGF receptor or phosphotyrosine residues, as well as phosphoamino acid analysis of the EGF receptor, demonstrated that MIS specifically inhibited tyrosine phosphorylation. AMP at concentrations ranging from 4 μ M to 40 μ M did not inhibit EGF receptor autophosphorylation; this observation excluded the possibility of AMP, which is present in the MIS preparations at a concentration of 1.42 μ M, as a cause of diminished EGF receptor autophosphorylation in these assays.

EGF receptor binding affinity for its ligand (EGF) is reduced by protein kinase C phosphorylation of EGF receptor-threonine-654 resulting in decreased EGF receptor tyrosine kinase activity (Bertics *et al.*, 1985). The action of MIS on EGF receptor tyrosine

kinase could be explained by MIS activation of this calcium-phospholipid-dependent protein kinase C (King and Cooper, 1986). It is unlikely that MIS decreases tyrosine kinase activity by stimulating protein kinase C, however, since MIS inhibition of EGF receptor autophosphorylation occurred in plasma membrane fractions in the absence of calcium; moreover, phosphoamino acid analysis of the EGF receptor immunoprecipitated from intact cells did not detect phosphothreonine residues. The results obtained from both intact cells and membrane preparations of A-431 cells thus suggest that MIS inhibits EGF-stimulated phosphorylation of the EGF receptor by modulating tyrosine kinase activity via an alternate mechanism.

MIS purified to near homogeneity by immunoaffinity chromatography, and active in the organ culture assay, failed to inhibit EGF receptor autophosphorylation (3 separate MIS preparations tested). It is interesting to note that MIS prepared in a similar fashion (Tsafiri et al., 1988) failed to cause the inhibition of meiosis of the rat oocyte observed with MIS obtained by ion-exchange techniques (Takahashi et al., 1986; Ueno et al., 1988). The latter observation raised the possibility that a contaminant copurifying with MIS was actually responsible for the observed inhibitory effects on EGF receptor autophosphorylation. To rule out this possibility, biologically active MIS partially purified from serum-containing media was immunoabsorbed with a highly specific monoclonal anti-human MIS antibody, and the samples retested in the membrane phosphorylation assay. MIS preabsorbed with monoclonal anti-human MIS antibody lost its ability to inhibit EGF receptor autophosphorylation, whereas MIS pretreated with normal mouse serum continued to inhibit EGF-induced phosphorylation of its receptor. Flow-through fractions lacking MIS by immunoassay, obtained by immunoaffinity column chromatography purification of MIS, also failed to inhibit EGF receptor autophosphorylation (1 preparation tested). The ability of monoclonal anti-human MIS antibody to block the MIS inhibition of EGF receptor autophosphorylation, the failure of the flow-through fractions obtained from the immunoaffinity column to block EGF receptor autophosphorylation, and the lack of inhibitory activity of control preparations derived from Chinese hamster ovarian wild-type media (3 separate preparations tested) provide compelling evidence that the inhibition of EGF receptor autophosphorylation in A-431 membranes is specific for

MIS. Furthermore, all of the preparations of MIS that inhibited EGF receptor autophosphorylation also caused Mullerian duct regression in the standard bioassay. Therefore, inhibition EGF receptor tyrosine kinase activity was never observed in the absence of regression activity. The fact that EGF receptor autophosphorylation can also be inhibited by naturally occurring bovine MIS, purified from a totally different source, further supports this specificity (Coughlin et al., 1987).

The finding that the ability of MIS purified by ion-exchange and dye- or carbohydrate-affinity chromatography to inhibit EGF receptor autophosphorylation is lost when MIS is purified by immunoaffinity chromatography requires further explanation. It is possible that a necessary cofactor for MIS activity may be depleted during immunopurification or that a change in MIS configuration might occur as a result of the low pH and high sodium thiocyanate concentrations (Dandliker et al., 1967) used in its elution from the immunoaffinity column. Since the organ culture bioassay is a 3-dimensional system requiring 10% fetal calf serum to maintain the integrity of the urogenital ridge for 3 days at 37°C, it mimics *in vivo* conditions more closely than other reconstituted membrane and single-cell experiments used in this study. The more physiological conditions in the organ culture assay may renature and/or cleave the hormone to explain why pure MIS causes regression of the Mullerian duct since mesenchymal-epithelial cell interactions, and either enzyme or cofactor production, can take place (Pepinsky et al., 1988). In the cell culture and isolated membrane preparations, on the other hand, MIS must be fully active upon its addition to observe the inhibition of EGF receptor autophosphorylation. These possibilities are currently being tested in the laboratory. An understanding of the conditions required to recapitulate the *in vivo* environment will increase our knowledge of MIS action.

It is our hope that these studies will eventually lead to a better understanding of the molecular mechanism of action of MIS. At the present time, accumulated evidence with bovine MIS (Budzik et al., 1985; Coughlin et al., 1987; Donahoe et al., 1987; Hutson et al., 1984), and now recombinant human MIS, reinforces the hypothesis that MIS inhibits EGF receptor tyrosine kinase (Coughlin et al., 1987). As such, MIS may serve as an agent to reverse the transforming properties of oncogenes such as *erbB-2* and *neu*, whose products mimic the EGF receptor

tyrosine kinase system (DiFiore *et al.*, 1987). In support of this hypothesis, we have shown that A-431 cells which overexpress EGF receptors are growth inhibited in soft agarose in the presence of bovine MIS (Coughlin *et al.*, 1987).

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