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Development of an efficiently cleaved, bioactive, highly pure FLAG-tagged recombinant human Mullerian Inhibiting Substance

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Abstract

Mullerian Inhibiting Substance (MIS), a member of the TGF- β family, causes regression of the Mullerian duct in male embryos, after binding to Mullerian Inhibiting Substance Receptor II (MISRII). It has also been extensively demonstrated that it can inhibit proliferation of various cancer cell lines such as ovarian, prostate, and breast cancer *in vitro* and *in vivo*. Hence, the availability of a recombinant, epitope tagged, bioactive MIS is important for the selection of patients for treatment and for probing novel molecular targets for MIS in various tissues. To this end, we have expressed a recombinant, internally FLAG-tagged form of hMIS with the tag (DYKDDDDK) immediately after the cleavage site (427–428) of MIS at the C-terminus with a modified dibasic cleavage motif sequence. We show that this construct results in a highly pure, endogenously processed (cleaved) FLAG MIS, that causes complete regression of the Mullerian Duct in an organ culture assay. In addition, purified FLAG MIS was able to bind and affinity purify both transfected and endogenous MIS type II receptor. The availability of this fully functional, epitope tagged form of MIS should facilitate scale-up for preclinical and clinical use and should also be used for the study of MIS binding proteins and for tracking in pharmacokinetic studies.

Keywords

Mullerian Inhibiting Substance (MIS); Purification; FLAG tag; Bioactivity

Disclosure

TDP, JT, DTM, PKD and DV have a pending patent application for filing.

Introduction

Mullerian Inhibiting Substance (MIS)¹, also known as anti-Mullerian hormone, is a 140-kDa disulfide-linked homodimer glycoprotein member of the TGF β superfamily. The human MIS gene is located on chromosome 19 [1], and its expression is sexually dimorphic. In males, MIS expression begins at 9 weeks gestation in the fetal testes and continues at high levels until puberty, when expression levels fall dramatically. In females, MIS is produced only postnatally in granulosa cells from prepuberty through menopause at levels similar to adult males, after which expression ceases [2,3]. Recent evidence suggests the protein is also a product of motor neurons [4]. In male fetuses MIS causes regression of the Mullerian ducts, the precursors to the Fallopian tubes, uterus, cervix and upper third of the vagina [5].

Holo-human MIS is cleaved into its N- and C-terminal domains most likely by means of furin or a related prohormone convertase PC5 [6], expressed in the gonads. Cleavage occurs primarily at a kex-like site characterized by R⁻⁴XXR⁻¹ with a serine in the +1 site, which makes the MIS cleavage site monobasic. The purified C-terminal domain is the biologically active moiety [7] and cleavage is required for biological activity [8]. A secondary cleavage site, whose significance is unknown [9], is observed less frequently at residues 229–230. Non-cleavable mutants of MIS are not biologically active [10] and mutations in the human gene that truncate the carboxyterminal domain lead to persistent Mullerian duct syndrome [11]. The role of the amino-terminal domain *in vivo* may be to assist in protein folding and to facilitate delivery of the C-terminal peptide to its receptor. In one study addition of the N-terminal peptide was shown to enhance the biological activity of the C-terminal moiety [8] *in vitro*, but the mechanism was unclear. The cleavage of recombinant MIS expressed by CHO cells [9] is minimal, thus cleavage with an exogenous serine protease such as plasmin [7] is required to enhance bioactivity.

MIS exerts its biologic effect after binding to a heterodimer of type I and type II single transmembrane spanning serine threonine kinase receptors, leading to cross phosphorylation of the GS box kinase domain of the type I receptor by the type II receptor [12–14]. Subsequently, SMAD 1, 5 and 8 are activated [15] which, together with SMAD 4, regulate gene transcription [12,15]. Only one MIS receptor type II (MISRII) gene has been identified in mice, rats, rabbits [16] where in humans it's gene localizes to chromosome 12. It is a 65-kDa protein which has been detected in embryonic and adult Mullerian structures, breast tissue, prostatic tissue, the gonads, motor neurons, and brain. In the fetus, mesoepithelial cells expressing MISRII in the coelomic epithelium covering the urogenital ridge migrate into and become part of the mesenchymal cells surrounding the Mullerian duct epithelium [15]. Expression is also detected in the gonads [17–19] as well as in the ovarian coelomic epithelium [20]. Type I MIS receptors have been identified in mammals, with activin receptor-like kinase (ALK) 2 and 3 being the most likely candidates [12,13], depending upon animal species and the tissue examined.

In addition to its well established role in the regression of Mullerian ducts, MIS inhibits the proliferation of various human cancer cell lines *in vitro* and *in vivo*. The cell lines showing inhibition were derived from ovarian [21,22], cervical [23], endometrial [24], prostate [25] and breast cancers [26]. Toxicity has not been observed *in vivo* even when high concentrations of MIS are maintained systemically in rodents or in human patients with tumors secreting MIS for prolonged periods of time [21,27]. These findings of relatively restricted receptor expression, anti-proliferative activity against cancer cells expressing the MIS RI and RII, and its apparent non-toxicity, taken together, make MIS an ideal reagent for

¹Abbreviations used: MIS, Mullerian Inhibiting Substance; MISRII, Mullerian Inhibiting Substance Receptor II; ALK, activin receptor-like kinase; CHO, Chinese Hamster Ovary; HEK, Human Embryonic Kidney; TBS, Tris Buffered Saline.

use in combination with existing chemotherapeutic drugs for the treatment of ovarian cancer, which are known to become resistant to these conventional agents. With the goal of creating a therapeutic biologic agent, we initially scaled-up MIS production of the native sequence using recombinant technologies, but high concentrations were required to observe an anti-proliferative effect [9].

Most recently we engineered changes to the native human sequence to increase endogenous cleavage and thus the potency of MIS and to insert a tag to facilitate its purification. Furthermore, there is an unmet need to have a form of bioactive MIS that is labeled for use in receptor and other binding studies that will be very important both for the selection of patients for treatment and for addressing molecular mechanistic questions regarding the interaction of MIS in various receptor bearing tissues. In addition, the labeled ligand will be essential to determine if another receptor or other binding proteins exist in various tissues. We report the first production of an internally epitope tagged MIS that retains full bioactivity in the Mullerian duct regression assay. We chose to use the FLAG tag because of the availability of high quality reagents used for its detection and purification. Moreover, we engineered a FLAG-tagged MIS cDNA with a more efficient cleavage site at the carboxyterminal end of the N-terminal domain, thereby eliminating the need for exogenous cleavage. This molecule can be used both as therapeutic and as probing molecule.

Materials and methods

Plasmid constructs (Fig. 1)

A pcDNA3.1(+) expression vector that contained the unlabeled full-length human MIS sequence between HindIII and XbaI sites was used for site-directed mutagenesis. The cleavage site of the wild-type construct was modified, as previously described [10], with an arginine instead of the serine right after the cleavage at the position 428 (N...RAQR/R...C instead of N...RAQR/S...C, the slash denotes the site of primary proteolytic cleavage of holo MIS). This modification enhanced endogenous cleavage compared to the wild-type sequence. Primers containing the FLAG epitope (DYKDDDDK) were designed as follows: (nucleotides in bold are encoding the FLAG epitope) Forward primer 5'-GGT CGG GCA CAG CGC CGC **GAC TAC AAG GAC GAC GAC AAG** GCG GGG GCC ACC GCC GCC-3', Reverse 5'-GGC GGC GGT GGC CCC CGC **CTT GTC GTC GTC GTC CTT GTA GTC** GCG GCG CTG TGC CCG ACC-3'. In addition, we also designed another construct with the FLAG tag located at the same position but with different cleavage site to determine if we could improve endogenous processing [6] (N...RAQR/R was changed to N...RARR/S...). Forward primer 5'-CGC GGG CCG GGT CGG GCA CGC CGC AGC GAC TAC AAG GAC G-3', Reverse 5'-CGT CCT TGT AGT CGC TGC GGC GTG CCC GAC CCG GCC CGC G-3'. PCR for both constructs was performed in two stages [28]. In stage one, two extension reactions containing 50 ng template with 2.5 U of Pfu Turbo (Stratagene) for 10 cycles was performed in separate tubes; one containing the forward primer and the other containing the reverse primer. This first stage was done to avoid primer-dimer formation and to allow for optimization of annealing temperatures for the two different primers. In the second stage, 25 µl from each tube were combined, 2.5 U of Pfu Turbo was added and PCR was performed for 18 cycles. For the RAQR/RFLAG construct the PCR conditions were stage 1, 95 °C preheating for 1 min, 10 cycles with 95 °C for 1 min, 59 °C for the forward primer and 55 °C for the reverse primer for 1.5 min, and 68 °C for 7 min (1 min/kb of plasmid); DMSO at a final concentration of 10% was added to both reactions to reduce secondary structure of the GC rich primers. Stage 2 was performed with preheating at 95 °C for 1 min, 18 cycles at 95 °C for 1 min, 59 °C for 1.5 min and 68 °C for 7 min. The conditions for the RARR/SFLAG construct were stage 1, 95 °C preheating for 1 min, 10 cycles at 95 °C for 1 min, 63 °C for the forward primer and 58 °C

for the reverse primer for 1.5 min, and 68 °C for 7 min. DMSO was again added at a final concentration of 8% to both reactions to minimize the secondary structure of the GC rich primers. Stage 2 was performed by preheating at 95 °C for 1 min, 18 cycles with 95 °C for 1 min, 63 °C for 1 minute, and 68 °C for 7 min. Following the PCR the parent template was destroyed by incubation with 10 U DpnI endonuclease (Stratagene) that cleaves the methylated parental strands. The products of the PCR were transformed into XL-1 Blue (Stratagene) competent cells. DNA was sequenced to verify proper construct sequence.

FLAG MIS purification

FLAG MIS was isolated from serum containing media of Chinese Hamster Ovary (CHO) cells and Human Embryonic Kidney (HEK 293) cells, each stably transfected with both linear constructs (described above). These cells, amplified by 500 µg/ml G418 (SIGMA) selection, were grown to confluency in T 175 cm² flasks in DMEM/Ham's F12 (1:1) medium (Invitrogen), supplemented with 5% fetal bovine serum (Invitrogen), 2 nM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) in 5% CO₂, 37 °C. Media were collected every 3–4 days, filtered to discard dead cells and collected in new tubes. The cells were maintained at 100% confluency in culture no more than 10 days. Subsequently, the media were incubated overnight with anti-FLAG agarose beads (SIGMA, 20 µl/50 ml media). Then, the beads were washed extensively (7×) with cold 1× TBS (SIGMA) and FLAG MIS was eluted with 25 µg of 3× FLAG peptide (SIGMA)/20 µl of agarose beads in 1× Tris Buffered Saline (TBS) at 4 °C overnight. After the overnight elution the beads with the eluate were briefly centrifuged at 13,000 rpm and the supernatant containing the FLAG MIS was collected and stored at –20 °C for subsequent use.

Enzyme treatments

FLAG MIS was cleaved with 0.4 IU/ml human plasmin (SIGMA) while bound to the anti-FLAG agarose beads. Briefly, the anti-FLAG agarose beads were washed twice with cleavage reaction buffer (5 mM MgCl₂, 2.5 mM CaCl₂ and 10 mM KCl in 1× TBS). Then, the beads were incubated with 0.4 IU/ml of human plasmin in cleavage reaction buffer for 1 h at room temperature. Subsequently, the beads were washed twice more with 1× TBS and FLAG MIS was eluted with the 3× FLAG peptide as described above and stored for subsequent use at –20 °C.

Bioassay of FLAG MIS

The standard organ culture bioassay for MIS was performed as described previously [29]. Briefly, 14.5-day-old female fetal rat urogenital ridges were placed on agar coated stainless steel above fortified Cambridge Medical Research Laboratories (CMRL) 1066 media containing female fetal calf serum. Only female fetal calf serum was used to avoid contamination with bovine MIS. Testosterone, (1 nM) was added to aid the development of the Wolffian duct and to permit morphologic comparison of the regressing Mullerian duct to the Wolffian duct. After incubation for 72 h in humidified 5% CO₂ at 37 °C, the specimens were fixed in 15% formalin and embedded in paraffin, and 8-µm sections of the cephalic end were stained with hematoxylin and eosin. The sections were then scored from 0 (no regression) to 5 (complete regression), by two experienced observers. The FLAG peptide preparation used to elute the FLAG MIS protein preparations from the FLAG agarose beads was used as a negative control in the regression bioassay.

Ligand assisted precipitation of the MISRII

COS 7 cells were transfected with an MISRII expression vector in 6-well plates. Briefly, Eugene HD reagent was mixed with 3 µg of purified endotoxin-free plasmid in a ratio 3:1, respectively, in serum free media, incubated at room temperature for 25 min and then added

to the cells. An empty vector was used for control. Two days later protein was extracted in lysis buffer with the added protease inhibitors. In addition, endogenous MISRII protein was also extracted from MOVCAR 7 cells (mouse ovarian cancer cell line created with the MISRII promoter driving the T antigen). The lysates were spun down at 14,000g for 10 min and the supernatants were stored at -20 °C. The next day, the lysates were incubated overnight at 4 °C with FLAG MIS immobilized on the FLAG agarose beads and FLAG agarose beads alone. Then, the beads were washed 3 times with 1× TBS and bound proteins were eluted with the 3× FLAG peptide as described above, before running on SDS PAGE under reducing conditions as described below.

Electrophoresis and Western blotting

Samples were reduced with 2.5% 2-mercaptoethanol and boiled at 90 °C for 10 min and run on 4–12% Bis–Tris gels at 200 V with MES running buffer (Invitrogen). Gels were stained with GelCode blue stain (Pierce) for 10 min and destained with dH₂O for 20 min.

For Western blot analysis gels were transferred onto PVDF membranes, previously equilibrated in transfer buffer containing 10% methanol (V/V), at 25 V for 45 min and at 35 V for another 45 min. Membranes were blocked with 1× TBS containing 3% dry milk for 30 min at room temperature and probed with the mouse monoclonal anti-FLAG M2 antibody (SIGMA) (1:500 in blocking buffer for 30 min at room temperature) or a rabbit polyclonal antibody against MIS (MGH 4, 1:1000 in blocking buffer for 2 h at room temperature) previously developed in our laboratory [3]. For Western analysis of the immunoprecipitated MISRII, membranes were blocked with 1× TBS Tween 0.1% with 5% dry milk for 1 h at room temperature and probed with a rabbit monoclonal antibody against the MISRII developed in a collaboration between our laboratory and Cell Signaling Technology (1:1000 in 1× TBS Tween 0.1% with 5% BSA, overnight at 4 °C). Thereafter the blots were incubated with horseradish peroxidase conjugated secondary antibody (1:30,000 for anti-FLAG and 1:100,000 for MGH and anti-MISRII in blocking buffer for 30 min). Blots were washed three times with 1× TBS Tween 0.05%, then developed by enhanced chemiluminescence (ECL, Perkin-Elmer) and exposure onto Fuji RX film.

Results and discussion

Two different human recombinant MIS constructs were made. Both had a mutation at the 427/428 cleavage site. The first one had the S428 mutated to arginine (S → R428; RAQR/RFLAG) and the second construct had the Q426 mutated to arginine (Q → R427; RARR/SFLAG). Both constructs had the eight amino acids that comprise the FLAG sequence inserted between amino acid 428 and 429 (Fig. 1). We designed these constructs in order to have the active C-terminus moiety tagged even after cleavage and avoid interference with its activity. The mutations of the amino acids around the cleavage site were performed in order to have a construct that is not cleaved (S → R428; RAQR/RFLAG) and a construct that is cleaved endogenously in an efficient manner (Q → R427; RARR/SFLAG).

Internal tagging of MIS with FLAG epitope did not interfere with the ability of cells to produce and secrete FLAG MIS (Fig. 2A). Moreover anti-FLAG immunopurification of media from CHO cells transfected with FLAG-tagged or non-tagged MIS selectively brings down FLAG-tagged MIS and did not immunoprecipitate untagged MIS that may be present from the male contributions to mixed sex fetal bovine serum (Fig. 2A). To verify that our cleavage site mutations result in the expected outcomes we transfected our constructs in two different cell lines (CHO and HEK). The (S → R428; RAQR/RFLAG) constructed showed a single 70 kDa band in CHO cells (Fig. 2A and B) while when it was transfected in HEK293 cells we observed a 70 kDa band, a 37 kDa band resulting from a secondary cleavage site at residues 229–230, and a weak signal at 12.5 kDa from the carboxyterminal domain (primary

cleavage site at residues 427–428) (Fig. 2B and C). As judged by Coomassie stain of the gel (and not the western blot) more than 90% of the total RAQR/RFLAG MIS protein in the gel is non-cleaved endogenously. In contrast to the RAQR/RFLAG MIS construct the Q → R427; RARR/SFLAG when expressed in both CHO and HEK cells resulted in more than 60% of the MIS being cleaved endogenously at the primary cleavage site (427–428) and displayed no cleavage at the secondary site (229–230, LLPR/SEPA) (Fig. 2B and C). Subsequent to purification both products were treated with exogenous plasmin. When the RAQR/RFLAG MIS was the substrate, plasmin acted primarily on the secondary cleavage site in RAQR/RFLAG (Fig. 2C). In contrast when the RARR/SFLAG MIS was the substrate, plasmin acted at the primary cleavage site (427/2428) resulting in complete cleavage (Fig. 2C).

To examine if the FLAG-tagged MIS retains its biological activity, we performed Mullerian duct regression assays. Interestingly, the non-cleaved holo RAQR/RFLAG MIS protein expressed in CHO or HEK cells but untreated with plasmin did not exhibit any activity (grade 0), equal to that seen when the urogenital ridge was incubated with fetal ovary. (Fig. 3B and E). In contrast, RARR/SFLAG MIS (containing holo FLAG MIS, the N-terminal peptide and the C-terminal peptide) caused complete regression (grade 5) in the Mullerian duct regression assay, equal to that seen when the urogenital ridge was incubated with fetal testis (Fig. 3D). The purified cleaved protein retained its activity for at least 2 months while stored at –20 °C and we did not observe any obvious degradation of protein.

To test RARR/SFLAG MIS binding to its receptor, MISRII was immunoprecipitated from lysates of COS 7 cells transfected with the human MISRII cDNA (Fig. 4A) and protein from MOVCAR 7 cells (Fig. 4B). For the immunoprecipitation we used RARR/SFLAG MIS immobilized on FLAG agarose beads. The ligand–receptor complex was eluted from the agarose beads with the 3× FLAG peptide without the need to crosslink the ligand–receptor complex. Western analysis was used to detect the MISRII with a rabbit polyclonal antibody against MISRII (Cell Signaling Technology). Immunoreactive bands were not seen with lysate from MISRII transfected cells and MOVCAR 7 cells after the incubation with anti-FLAG agarose beads alone or when lysates from empty vector transfected cells and MEFs (mouse embryonic fibroblasts) were incubated with immobilized RARR/SFLAG MIS or anti-FLAG agarose beads alone.

Thus RARR/SFLAG MIS can be eluted by a single step to produce a highly purified efficiently cleaved preparation with full bioactivity. When scaled-up, this purification of MIS will be suitable for clinical applications; furthermore it will be useful for various binding assays in both clinical and experimental settings. Internal labeling of MIS during translation has proved to be more effective than labeling after purification of the protein as iodination or biotinylation greatly reduced MIS bioactivity. Unpublished observations from our lab suggest that this construct is probably even more bioactive than the wild-type MIS. Inserting the FLAG tag sequence has several other distinct advantages. First, its unique 8 amino acid domain is not present in any other gene (except for a mouse brain phosphatase), thus making the anti-FLAG antibody very specific. Second, the elution of the protein with the 3× FLAG peptide is specific for the FLAG MIS and not other proteins that bind non-specifically to the agarose beads. The decision was made to tag the MIS internally at the carboxy terminus immediately downstream from the cleavage site, in a domain where 3 dimensional modeling predicted instability (data not shown). We hypothesized that labeling at this site was most likely to preserve biologic activity, which proved to be the case. To our knowledge, this is the first demonstration of internally tagged MIS with well preserved bioactivity.

RARR/S FLAG MIS was bioactive whereas RAQR/R FLAG MIS was not when compared to native human MIS or to the previously prepared untagged RAQR/R MIS. It is likely that the presence of the acidic FLAG tag so close to the cleavage site may impair the degree of cleavage, thus causing loss of activity. Moreover, we show that the holo RAQR/RFLAG MIS preparation in CHO (or HEK) cells is not bioactive. We did not observe any endogenous processing with the RAQR/R cleavage site in contrast to what was reported by Kurian et al. [10] when the construct lacked the FLAG tag. On the other hand, the retention of the serine at position 428 and the conversion of the monobasic site to dibasic (Q → R at position 426) makes the endogenous cleavage more efficient and very specific. Furthermore, we showed that FLAG MIS is a powerful tool for binding studies as we were able to immunoprecipitate the endogenous MISRII without crosslinking. In summary, this novel and efficient way of producing highly pure and biologically active internally labeled form of MIS can be used for scale-up for preclinical and clinical use, for the study of MIS binding proteins and for tracking in pharmacokinetic studies.

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Fig 1.

Comparison of the protein sequence of the native human MIS, the RAQR/R modified MIS by Kurian et al. with the FLAG tag and the RARR/S FLAG-tagged MIS. The FLAG tag (red letters) is added in both cases after the first amino acid downstream the cleavage site. The green color denotes the amino acid changed compared to the native human sequence.

Fig 2.

(A) The anti-FLAG agarose beads do not immunoprecipitate untagged MIS that may be present in the serum. RAQR/R FLAG MIS was purified and eluted as described in Materials and methods; in parallel media from CHO cells stably transfected with the RAQR/R untagged MIS treated the same way. The left panel is an immunoblot with the anti-FLAG antibody and the right panel is the same membrane probed with a rabbit polyclonal antibody against MIS developed in our laboratory (3). (B) Comparison of the two FLAG MIS constructs in CHO and HEK cells. FLAG MIS was purified and eluted as described in Materials and methods. Western blotting with the anti-FLAG antibody shows that the RAQR/R FLAG MIS is endogenously cleaved in HEK cells primary at the secondary site while there is no cleavage in CHO cells. On the other hand, both cells similarly process the RARR/S FLAG MIS, with the cleavage occurring exclusively at the primary site (427–428). (C) Comparison of the 2 FLAG-tagged MIS products in HEK cells. Left panel: RAQR/R FLAG MIS (from left to right) FLAG MIS was run on SDS-PAGE under reducing conditions and stained with Coomassie blue; transferred to a PVDF membrane and probed with the anti-FLAG antibody; cleaved while on the beads with plasmin, run on SDS-PAGE under reducing conditions, transferred to PVDF and probed with the anti-FLAG antibody (see Enzyme treatments in Materials and methods). Right panel: RARR/S FLAG MIS (same as for RAQR/R FLAG MIS from left to right). The FLAG MIS preparation with both constructs is highly pure and specific cleavage occurs at the primary site with the RARR/S

FLAG MIS construct. In addition, the N-terminal peptide while not tagged is purified from the media as it is associated with the C-terminal peptide in a non-covalent fashion (Right panel, Coomassie stain image).

Fig 3.

Bioassay of both FLAG MIS preparations. FLAG MIS produced in HEK cells was tested in the rat urogenital ridge culture assay as described in Materials and methods. M, Mullerian duct; W, Wolffian duct. 0 denotes no activity and 5 means complete regression. (A and C) Vehicle buffer controls; (B and D) RAQR/R FLAG MIS and RARR/S FLAG MIS, respectively; (E and F) female ridge grown in the absence of MIS (negative control) and male ridge grown with testes (positive control). We attach next to the bioassay figures the Coomassie stain image and the protein sequence of both MIS species. The RARR/S FLAG MIS, which is efficiently cleaved, causes complete regression of the Mullerian duct while the RAQR/R FLAG MIS remains uncleaved and inactive.

Fig 4.

(A) RARR/S FLAG MIS immunoprecipitates the human MISRII from transfected COS 7 cells with the human MISRII cDNA (see Materials and methods) (B) RARR/S FLAG MIS immunoprecipitates endogenous mouse MISRII from MOVCAR 7 cells without the need of crosslinking (see Materials and methods).