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Heparan Sulfate regulates the antiangiogenic activity of EMAP II at acidic pH

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Running title: Heparan sulfate and pH regulate EMAP II

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EMAP II, endothelial monocyte-activating polypeptide II; EC, endothelial cell; BAEC, bovine aorta endothelial cell; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; ECM, extracellular matrix; HS, heparan sulfate; PBS, phosphate buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbant assay; MFI, mean fluorescent intensity

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Abstract

Endothelial monocyte-activating polypeptide-II (EMAP II) is an antiangiogenic factor for rapidly growing endothelial cells, which is released from tumor cells under the physiological stress such as hypoxia. We have previously shown that the interaction between EMAP II and the α-subunit of ATP synthase (α-ATP synthase) can play a regulatory function for the growth of endothelial cells. In the current study, we found that EMAP II-α-ATP synthase interaction could be inhibited by excess heparin whereas the interaction could be enhanced by a low concentration of heparin. Both EMAP II and α-ATP synthase could specifically interact with heparin and this interaction was increased in the acidic condition. In addition, EMAP II and α-ATP synthase were found to contain the heparin binding motifs determined by analysis using site-directed mutant forms. In endothelial cells, the binding of EMAP II to cells was dramatically enhanced and α-ATP synthase could associate with heparan sulfate at acidic pH. The inhibitory effect of EMAP II on the growth of cultured endothelial cells was also significantly enhanced at acidic pH. Analysis using mutant EMAP II proteins demonstrated that heparan sulfate was essential for the enhanced binding and EMAP II function to endothelial cells at acidic pH. Furthermore, the enhanced inhibitory effects of EMAP II could be abrogated by excess heparin or heparinase treatment. In the endothelial cell, heparan sulfate may regulate the function of EMAP II released from the tumor cell in hypoxic condition.

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Introduction

Endothelial monocyte-activating polypeptide-II (EMAP II) is a novel molecule, first identified from cell growth medium conditioned by murine methylcholanthrene A-induced (meth A) fibrosarcoma cells, with pleiotrophic activities toward endothelial cells (ECs), monocytes/macrophages, and neutrophils (Kao *et al*, 1992, 1994a, 1994b). EMAP II is structurally and functionally identical to the C-terminal domain of p43 that is associated with the mammalian multisynthase tRNA synthase complex (Quevillon et al., 1997; Shalak et al., 2001; Ko et al., 2001). It has been shown that EMAP II was released from the cells not only undergoing apoptosis (Knies et al., 1998) but also in response to cellular stress such as exposure to hypoxia, or treatment with certain chemotherapeutic agents (Barnett et al., 2000; Matschurat et al., 2003). EMAP II induces a procoagulant activity on the surface of ECs, increases expression of TNF-R1 (Berger et al., 2000a), and is chemotactic for neutrophils and monocytes, suggesting that EMAP II is a proinflammatory cytokine (Kao et al, 1994b). More recently, EMAP II has been shown to induce lymphocyte apoptosis after secretion from colorectal cancer cells without any stimuli (Murray et al., 2004).

EMAP II also has an antiangiogenic property that targets rapidly growing vascular beds (Berger et al., 2000b). EMAP II induces proliferation inhibition and apoptosis of growing

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cultured capillary endothelium, which is magnified by concomitant hypoxia (Schwarz et al., 1999). Our previous report has shown that EMAP II binds with the α subunit of ATP synthase (α-ATP synthase) on the surface of EC and the interaction between EMAP II and α-ATP synthase can play a regulatory function for the growth of ECs (Chang et al., 2002). In general, ATP synthase is present in the inner membrane of mitochondria and play an important role for the energy metabolism synthesizing ATP (Stock et al., 2000). Recent reports suggested that ATP synthase was also expressed in the plasma membrane and the ectopic ATP synthase functions in the unexpected manner. For instance, cell surface ATP synthase is a cellular receptor for HDL in hepatocytes (Martinez et al., 2003). In addition, ATP synthase in the surface of ECs is a cellular receptor for angiostatin (Moser et al., 1999) as well as EMAP II (Chang et al., 2002).

Tumor tissues have been shown to be hypoxic and acidic (Tannock et al., 1972; Yamagata et al., 1998). Nevertheless, ECs in the tumor tissues survive fairly well under these conditions (Burbridge et al., 1999). In fact, hypoxia is one of the major signals that induce angiogenesis. Fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) are the major regulators of angiogenesis (Veikkola et al., 2000). In addition, it has been shown that EMAP II can be secreted from tumor cells under hypoxic condition (Barnett et al., 2000)

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suggesting that hypoxia can also lead to release the antiangiogenic factor from tumor cells. Nevertheless, there is little understanding of how EMAP II functions at acidic pH following hypoxia in the tumor tissue although the antiangiogenic function of EMAP II in the cell culture system at neutral pH has been studied intensively. Moreover, the antiangiogenic activity of EMAP II is in contradiction to the fact that acidic tumor tissue under hypoxia needs more blood vessels to enhance blood supply.

In this study, we investigated the regulation of heparin or HS in the interaction between EMAP II and α-ATP synthase. Accidentally, we found that the interaction between EMAP II and α-ATP synthase was inhibited by heparinized serum. Basic amino acid clusters, which may serve as a binding site for heparin, were predicted from the amino acid sequence of both of EMAP II and α-ATP synthase suggesting that the interaction between EMAP II and α-ATP synthase could be modulated by heparin or HS. The binding assay indicated that heparin could bind with both EMAP II and α-ATP synthase. Interestingly, low concentrations of heparin and acidic pH increased the interaction between EMAP II-α-ATP synthase while excess heparin at neutral pH inhibited it. Binding of EMAP II to ECs was also increased at acidic pH which was mainly ascribable to the binding of EMAP II to HS. Furthermore, α-ATP synthase could interact with HS as well as EMAP II on the cell surface suggesting the

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possibility that a tertiary complex of HS-EMAP II-α-ATP synthase might be formed at acidic pH. Finally, we described a role of HS for the function of EMAP II through α-ATP synthase of EC at acidic pH.

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Materials and Methods

Cell Culture

HepG2, Chinese hamster ovary (CHO-K1) and Jurkat T cell were obtained from the American Type Culture Collection (Manassas, VA). HepG2 and Jurkat T cell were cultured in RPMI 1640 (BioWhittaker, Walkersvile, MD) supplemented with 10% fetal bovine serum (BioWhittaker) and 1% penicillin/streptomycin (BioWhittaker) at 37ºC in 5% CO_2. CHO-K1 was cultured in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Bovine aorta endothelial cell (BAEC) was a gift from Dr. Sunghoon Kim at Seoul National University (Seoul, Korea). BAEC were maintained in DMEM supplemented with 20% fetal bovine serum and 1% penicillin/ streptomycin. For experiments, BAEC were used at confluence from passages 5 to 9.

Expression and purification of recombinant protein

Expression and purification of recombinant EMAP II from pET28a-EMAP II have been described previously (Chang et al., 2002). pET28a-EMAP II was used as a template to introduce single to triple point mutations by appropriate pairs of overlapping oligonucleotides by PCR. The primer pairs were 5'-catatgtctaagccaatagatgtttcc-3' and 5'-

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gagataatactcccatcatctttgcagg-3' for Em73 (71-73aa: KMR→KMM) and 5'-aaggagctgaatcctatgaagatgatttgggagcag- 3' and 5'-ctcgagttatttgattccactgttgctcatggt-3' for Em121 (121-123aa: KKK→MKM). The double mutant form of EMAP II, Em73,121 (71-73aa: KMR→KMM and 121-123aa: KKK→MKM) was also constructed. The primer pair used for the preparation of mutant α-ATP synthase, αATPs-m167 (167-171aa: KTRRR→MTRMM) was 5'-tacgcgtcatggaaccaattggac-3' and 5'- aacgcgtatgatggttggtctgaaagcccccggtatc- 3'. pET24d-α-ATP synthase (generously gifted by Dr. Pizzo, Moser et al., 1999), pET28a-Em73, pET28a-Em121, pET28a-Em73,121 and pET28a-αATPs-m167 were introduced into competent *Escherichia coli* BL21 (DE3) and induced with IPTG. The His-tagged proteins of α-ATP synthase, Em73, Em121, Em73,121 and αATPs-m167 were purified using nickel-affinity chromatography following the manufacturer's instruction (Invitrogen, Carlsbad, CA).

ELISA

Modulation of the interaction between EMAP II and α-ATP synthase by heparin was determined by ELISA. Briefly, 96well-microtiter plate (Maxisorp F96; Nunc) were coated with 200ng/well recombinant α-ATP synthase in 50mM Carbonate buffer (pH9.6) and incubated overnight at 4ºC. After washing with PBS, the remaining sites were blocked with PBS containing 1%BSA (Sigma, St. Louis, MO) for 30min at room temperature. For inhibition

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studies, coated α-ATP synthase were pre-incubated with serial-diluted sera or increasing amounts of heparin (GibcoBRL, Gaithersburg, MD) for 30min before adding of EMAP II and then incubated with 250ng/ml biotin conjugated EMAP II for 2hr at room temperature. For cross-binding by heparin, coated α-ATP synthase were pre-incubated with or without 250mU/ml heparin and after washing out to remove unbound heparin, incubated with biotin conjugated EMAP II. For heparin binding studies, each of native and mutant form of EMAP II or α-ATP synthase were coated with 200ng/well in 50mM Carbonate buffer (pH 9.6). After blocking with 1%BSA/PBS, serial-diluted biotin conjugated heparin-BSA were added and incubated for 1hr at room temperature. The plates were washed and incubated with alkaline phosphatase conjugated streptavidin (Pierce, Rockford, IL) diluted in PBS/0.1%BSA/Tween20 (1:2000) for 1hr at room temperature. The plates were washed and then 100μl of phosphatase substrate (*p*-nitrophenyl-phosphate in a carbonate buffer, pH 9.6) was added to each well. The absorbance was read at 405nm (reference wavelength 490nm) using an Emax microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

Flow Cytometry

BAECs were harvested, washed with cold PBS and then incubated with 20μg/ml fluorescein isothiocyanate (FITC) conjugated EMAP II or Em73,121 for 1hr at 4ºC in PBS containing

1%BSA and 0.1% sodium azide (pH 7.5 or pH 6.5). Binding inhibition experiments by heparin were performed by staining with 20ug/ml FITC conjugated EMAP II after pre-incubation with various concentrations of heparin. For double inhibition by heparin and α-ATP synthase, BAEC, HepG2, Jurkat T cell and CHO-K1 were pre-incubated with both of and either 10U/ml heparin or 80μg/ml soluble recombinant α-ATP synthase and then stained with 20ug/ml FITC conjugated EMAP II. After the last wash, the cells were resuspended in 200ul PBS and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and a CellQuest Pro software (BD Biosciences, San Jose, CA).

Immunoprecipitation

BAECs were incubated with 20ug/ml EMAP II for 1hr at 4ºC under either pH 7.5 or pH 6.5 medium, washed with cold PBS and cell lysates were prepared using CytoBuster protein extraction reagent (Novagen, San Diego, CA) containing protease inhibitor cocktail (Roche, Indianapolis, IN). For co-immunoprecipitation, 500ul lysate of BAEC were incubated with agarose conjugated anti-syndecan-1antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for binding to HS and Ni^{+}-agarose (Invitrogen) for binding to his-tagged EMAP II for 3hr at 4ºC. The beads were then washed extensively with binding buffer (10mM Tris.HCl, pH 8.0, 140mM NaCl, 0.025% NaN3) containing 0.1% Triton X-100, resuspended in SDS-PAGE buffer, boiled

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for 5min. The supernatants were resolved on 12% SDS-PAGE, transferred to a PVDF membrane and blocked with 5% skim milk in TBST (10 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Tween) for 1 hr. Precipitation of HS and EMAP II were confirmed by probing with anti-syndecan-1 antibody (Santa Cruz Biotechnology) and anti-his-tag antibody (Qiagen, Chatsworth, CA) in the TBST containing 0.1% skim milk, respectively. For the co-precipitation of α-ATP synthase, transferred membrane was incubated with polyclonal α-ATP synthase antibody (Chang et al., 2002) in the TBST containing 0.1% skim milk. After being washed four times with TBST, they were incubated with HRP-conjugated secondary antibodies for 1 hr and developed on film using an ECL substrate (Santa Cruz Biotechnology), according to the manufacturer's instructions.

Cell Proliferation Assay

BAECs were seeded into 96-well tissue culture plates at a density of 10,000cells/well in the complete medium and then changed with the medium depleted of fetal bovine serum overnight to allow the cells to become quiescent. BAECs were treated with various concentrations (12.5-100nM) of EMAP II or mutant forms of it diluted with a fresh medium containing 20% fetal bovine serum (pH 7.5 or pH 6.5). Cell density was measured after 24hr by using the CellTiter 96 Aqueous Assay kit (Promega, Madison, WI). The absorbance of formazan was

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quantitated on an Emax microplate reader at a wavelength of 490nm according to the manufacturer's instructions. The absorbance values were used to calculate the percent inhibition of the cell proliferation. For inhibition of EMAP II function by excess heparin, BAECs were treated with 50nM EMAP II in the presence of excess heparin (12.5-400U/ml) at pH 7.5 or pH 6.5. For HS depletion, BAECs were treated with 0.5U/ml heparinase I (Sigma) and heparinase III (Sigma) for 2hr at 37ºC. After extensive washing with PBS, BAECs were treated with 50nM EMAP II at pH 7.5 or pH 6.5.

Statistics

Comparative statistical analyses were performed using the Student's *t*- test. Each experiment was repeated at least twice.

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Results

Exogenous Heparin Inhibits the Interaction between EMAP II and α-ATP synthase

Our previous study demonstrated that EMAP II interacted with α-ATP synthase in the specific manner (Chang et al., 2002). In this study, we surprisingly found that naïve murine and human serum as well as antiserum against α-ATP synthase inhibited the interaction of EMAP II with α-ATP synthase (Figure 1A). Inhibitory capacity of naïve serum was slightly lower than but comparable to antiserum against α-ATP synthase. Moreover, 50% inhibition was achieved in the highly diluted sample of naïve serum (1:12800) suggesting that an unknown factor inhibiting the interaction might be present in these sera. Therefore, we wished to identify the factor inhibiting the interaction between EMAP II and α-ATP synthase. Several angiogenic factors and inhibitors are known to bind with heparin which is closely associated with angiogenesis and antiangiogenesis. On the other hand, the blood samples are generally treated with excess heparin to prevent clotting. To check heparin as a candidate for the inhibition factor, we tested whether the interaction between EMAP II and α-ATP synthase was inhibited by non-heparinized serum. The inhibition capacity of non-heparinized serum was significantly decreased (Figure 1B). Moreover, exogenous heparin could completely inhibit the interaction at high concentrations (1000-62.5mU/ml) (Figure1C). These results suggested that the inhibition factor of EMAP II-α-ATP synthase interaction could be heparin.

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Heparin binds with EMAP II and α-ATP synthase

Modulation of EMAP II-α-ATP synthase interaction by heparin suggested the possibility that heparin could bind with EMAP II and/or α-ATP synthase. To test this, we evaluated the binding of heparin to EMAP II or α-ATP synthase. Heparin bound with both EMAP II (Figure 2A) and α-ATP synthase (Figure 2C) in a concentration dependent manner and the binding was saturable at high concentration. When heparin binding motif was searched from the amino acid sequences of EMAP II and α-ATP synthase, EMAP II and α-ATP synthase were found to contain two and one basic heparin binding motif, respectively. In the subsequent studies, we performed site directed mutagenesis changing critical arginine or lysine residues to methionine residues. In order to map binding sites, we mutated the putative heparin binding motif of EMAP II gene either individually or in double combination and expressed recombinant mutant forms of EMAP II including Em73 (71-73aa: KMR→KMM), Em121 (121-123aa: KKK→MKM) and Em73,121 (71-73aa: KMR→KMM and 121-123aa: KKK→MKM). Binding of heparin with Em73 and Em121 was partially reduced while binding with Em73,121 was dramatically abolished (Figure 2A). However, binding capacities of mutant EMAP II proteins to α-ATP synthase were similar to that of native EMAP II (Figure 2B). Similarly, heparin binding to the mutant form of α-ATP synthase, αATPs-m167 (167-171aa:

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KTRRR→MTRMM) was dramatically reduced (Figure 2C) although the interaction between αATPs-m167 and EMAP II (Figure 2D) was not changed. These results suggested that heparin binding motifs of EMAP II and α-ATP synthase were critical for heparin binding whereas not for the interaction between EMAP II and α-ATP synthase. When taken together, the data demonstrated that heparin could regulate the interaction between EMAP II and α-ATP synthase by binding with both EMAP II and α-ATP synthase.

Interaction between EMAP II and α-ATP synthase increases by heparin and acidic pH

Hypoxia is a potent inducer of the release and processing of biologically active EMAP II from tumor cells (Barnett et al., 2000). Hypoxic conditions lead to reduced extracellular pH. Several reports proposed that the interaction of heparin and heparin binding molecules such as VEGF could be increased at acidic pH (Goerges et al., 2003). Thus, we first considered the possibility that heparin binding to EMAP II or α-ATP synthase could be altered by pH. As expected based on previous reports, heparin binding to each of EMAP II and α-ATP synthase also increased at pH 6.5 (Figure 3*A* and *B*). In contrast with the inhibition of the α-ATP synthase-EMAP II interaction by excess heparin (Figure 1*C*), removal of unbound heparin to α-ATP synthase before adding of EMAP II could increased EMAP II-α-ATP synthase interaction and the increased binding was more potentiated by acidic pH (Figure 4*A*). In consistent with

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this observation, low concentrations (4-0.25mU/ml) of heparin slightly enhanced the interaction between EMAP II and α-ATP synthase (Figure 1*C*). However, the interaction between Em73,121 and αATPs-m167 was increased by neither acidic pH nor the addition of heparin (Figure 4*B*). These results suggested that the interaction between EMAP II and α-ATP synthase could be further enhanced by cross-linking of heparin under acidic conditions.

Binding of EMAP II to ECs increases at acidic pH

ECs constitutively express HS as well as α-ATP synthase in the cell surface (Moser et al., 1999; Goerges et al., 2003). Since HS can be a cellular counterpart of heparin, we evaluated whether EMAP II interacted with HS as well as α-ATP synthase on the surface of EC. The binding of EMAP II to ECs increased as the extracellular pH was decreased from 7.5 to 6.5 (Figure 5*A*) but OVA protein used as a control didn't bind to EC in any pH (data not shown). Unlabeled EMAP II competed with FITC-labeled EMAP II for the binding to ECs at pH 6.5 suggesting that EMAP II binding to ECs at pH 6.5 was specific (Figure 5*B*). In addition, the enhanced EMAP II binding at pH 6.5 was dramatically inhibited by excess heparin (10-100U/ml) in a concentration dependent manner while the binding at pH 7.5 was not inhibited by heparin (Figure 5*C*). Furthermore, binding of Em73,121 to EC at pH 6.5 was decreased compared to that of EMAP II although there was no difference at pH 7.5 (Figure 5*D*). These results

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suggested that the enhanced binding of EMAP II to ECs at acidic pH was mainly ascribable to the binding of EMAP II to HS. It has been shown that HepG2 as well as BAEC express constitutively both HS and α/β-ATP synthase in the cell surface (Martinez et al., 2003). In BAEC and HepG2, EMAP II binding at pH 6.5 was significantly blocked by soluble α-ATP synthase or heparin and more dramatically by both of them. Similar results could be obtained in Jurkat T cells having β-ATP synthase in the lipid raft (von Haller et al., 2001) and CHO K1 cell unknown for the presence of surface ATP synthase complex (Figure 5*E*).

To further define the binding of EMAP II to ECs, binding curves were obtained from flow cytometry data using FITC conjugated EMAP II (Figure 6*A*). After subtraction of the nonspecific interactions, the binding was found to be a concentration dependent and saturable at high concentrations. Scatchard plot analysis at pH 7.5 showed a single binding slope with a binding affinity Kd of 245nM (Bmax 28.79) which was probably involved in the EMAP II binding to surface α-ATP synthase. Unexpectedly, scatchard plot analysis at pH 6.5 also showed a single binding slope with a lower binding affinity Kd of 2469nM (Bmax 1493) although we hypothesized that BAEC had two binding sites for EMAP II including HS and α-ATP synthase at acidic condition. On the basis of these data, we predicted that HS-α-ATP synthase complex might be a binding receptor for EMAP II under acidic conditions. Alternatively, HS might be a major binding site with low binding affinity to EMAP II at pH 6.5

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whereas α-ATP synthase, a high affinity receptor, might play a minor role for EMAP II binding because of very low frequency of α-ATP synthase compared to abundant HS in the surface of ECs. To exploit the interaction among them in the EC surface, we performed immunoprecipitation against HS and EMAP II from BAEC lysates after binding of EMAP II at pH 7.5 or pH 6.5 (Figure 6*B*). The immunoprecipitation of HS was independent on pH. EMAP II was more precipitated at pH 6.5 than pH 7.5 supporting that binding of EMAP II to BAEC increased at pH 6.5. To check the co-precipitation of α-ATP synthase, western blotting of each precipitant using polyclonal anti-α-ATP synthase antibody was performed. α-ATP synthase was co-precipitated with EMAP II at pH 7.5 in consistent with our previous report (Chang et al., 2002) and more co-precipitation was shown at pH 6.5. Furthermore, α-ATP synthase was co-precipitated with HS at pH 6.5 but not at pH 7.5. These data suggested that the EMAP II-α-ATP synthase interaction and the HS-α-ATP synthase interaction were increased at acidic pH. In addition, these data suggested the possibility that ternary complexes of HS-EMAP II-α-ATP synthase might be formed at acidic pH. Taken together, these results supported our hypothesis that after EMAP II was secreted from the cells under oxidative stress, it could interact with both HS and α-ATP synthase on ECs at acidic environment following hypoxia.

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HS can regulate the function of EMAP II on the EC at acidic pH

To define the role of HS for the antiangiogenesis at an acidic condition, we tested the EC proliferation using the MTT assay. BAEC could grow fairly well at pH 6.5 and the proliferation of BAEC at pH 6.5 was similar to that at pH 7.5 (Figure 7A). Percent inhibition of BAEC proliferation by EMAP II was significantly increased at pH 6.5 (Figure 7*B*). However, inhibition by Em73,121 was not increased at pH 6.5 compared to that at pH 7.5 (Figure 7*C*). In addition, the inhibitory capacity of Em73,121 was significantly decreased compared to that of EMAP II at pH 6.5 although inhibitory capacities between EMAP II and Em73,121 had little difference at pH 7.5. On the basis of these data, we predicted that the enhanced effect of EMAP II at pH 6.5 could be ascribable to the enhanced binding of EMAP II to ECs which resulted from the enhanced interaction among EMAP II-HS-α-ATP synthase at an acidic condition. To evaluate which of two heparin binding motifs was more important for the enhanced effect at acidic pH, the inhibition of BAEC proliferation by mutant series of EMAP II was analyzed. The inhibition by Em73 was slightly but not significantly reduced compared with EMAP II whereas inhibition by Em121 and Em73,121 were significantly reduced (Figure 7*D*). Thus, the data suggested that KKK (121-123aa)-HS binding region was more important for the function of EMAP II at acidic pH. To confirm whether the enhanced effect of EMAP II was resulted from the regulation of HS at acidic pH, we tested whether exogenous heparin could

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inhibit the enhanced effect of EMAP II at pH 6.5. Although high concentrations of heparin were required, the inhibition of BAEC proliferation by EMAP II was abrogated by heparin at pH 6.5 (Figure 7*E*). In contrast, exogenous heparin had no effect at pH 7.5. To provide further evidence, we tested anti-proliferative effects of EMAP II after HS depletion. The enhanced effects of EMAP II on ECs at pH 6.5 were significantly inhibited by Heparinase I and heparinase III treatment (Figure 7*F*). These data suggested that HS could modulate the function of EMAP II at acidic pH. Collectively, these results suggested that the effect of EMAP II on ECs could increase as the extracellular pH decreased and this was ascribable to the interaction of EMAP II with HS at an acidic condition.

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Discussion

In ECs, surface α-ATP synthase can interact with EMAP II (Chang et al., 2002) or angiostatin (Moser et al., 1999) and their interactions lead to EC apoptosis. In this study, we first reported that the interaction between EMAP II and α-ATP synthase could be regulated by heparin or HS. Each of EMAP II and α-ATP synthase could interact with heparin and was found to contain the heparin binding sites evidenced by analysis using the site directed mutant forms (Figure 2). One of the best studied model systems for protein-HS interactions is the FGF family and recent observations have demonstrated that FGF-FGFR-HSGAG ternary complexes can be formed since HS also interacts directly with FGFRs as well as FGF and these interactions potentiate FGF binding to FGFR (Yayon et al., 1991; Fannon et al., 2000). Likewise, it has been shown that VEGF binding to VEGF receptors is dependent on HS (Tessler et al., 1994). However, several antiangiogenic factors are known to bind to heparin, but their biological meanings are less well established. Although endostatin, the well-known antiangiogenic factor, is extensively studied regarding the interaction between endostatin and HS and endostatin inhibits angiogenesis by binding to HS as a low affinity coreceptor (Ricard-Blum et al., 2004), the importance of heparin binding for the antiangiogenic activity of endostatin also remains to be elucidated. Our data demonstrated that HS as well as heparin in the cellular level could interact directly with both of EMAP II and α-ATP synthase and enhance the interaction between

EMAP II and α-ATP synthase. On the basis of these data, we proposed the possibility that the interaction among them might form the HS-EMAP II-α-ATP synthase complexes on the EC surface at an acidic condition and this complex via cross-interaction of HS could enhance EMAP II binding to its functional receptor, α-ATP synthase. This idea was supported since the interaction between EMAP II and α-ATP synthase was increased by a low dose of heparin at the molecular level. Thus, when taken together, the data clearly showed that HS in the ECs could potentiate the binding of EMAP II to α-ATP synthase and the effects of EMAP II on ECs. On the other hand, p43, the precursor of EMAP II contains also many putative heparin binding motifs in the N-terminal region as well as EMAP II region. Thus, it may be possible that p43 also interacts with HS and the binding of p43 to ECs increases by p43-HS interaction.

Our studies suggested that HS was involved in the function of EMAP II on ECs at acidic pH but not at neutral pH. Low extracellular pH is a common feature of solid tumors (Tannock et al., 1972; Yamagata et al., 1998). ECs are exposed to this environment while undergoing angiogenesis under many pathological and physiological conditions. However, it has been shown that ECs are protected from apoptosis in an acidic environment (D'Arcangelo et al., 2000). On the other hands, it has also been found that the antiangiogenic activity of angiostatin on EC is enhanced in culture when the microenvironmental extracellular pH is

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reduced to levels similar to that of many tumors (Wahl et al., 2002). These results provide an evidence for the importance of pH in the growth of ECs. Hypoxia in the growing tumor, which results in the acidic microenvironment, is a major factor not only for releasing angiogenic factor such as VEGF but also for releasing EMAP II from tumor cells. ECs in the tumor vasculature can be exposed to EMAP II released from tumor cells under the hypoxic and acidic microenvironment. In our results, the inhibitory effects of BAEC proliferation by EMAP II were increased at acidic pH. These results were supported by the previous report that EMAP II–induced apoptosis of ECs was enhanced in the hypoxic condition (Schwarz et al., 1999). Taken together, the antiangiogenic activity of EMAP II might be enhanced by hypoxia. However, this is contradictory to the fact that hypoxia induces the expression of angiogenic factors and inhibits EC apoptosis since tumors under hypoxia need more blood vessels to enhance blood supply. In this regard, we have speculated why the tumor cells release EMAP II under hypoxic condition.

In general, HS are expressed in most tissues and are major components of cell surfaces and ECM (Bernfield et al., 1999). HS can act as suppressors or activators of angiogenesis (Nugent et al., 2000; Esko et al., 2001; Turnbull et al., 2001). HS on EC surface may localize heparin binding proteins whereas HS in the other cell or ECM may act as sites for heparin binding

protein storage, sequestering them from their receptors on EC surface. The enhanced inhibitory effects of EMAP II by HS at an acidic condition were obtained from in vitro EC culture system. In the tumor tissue, these acidic locations would not be adjacent to the existing vasculature; hence there would be little ECs in the immediate environment of the secreted EMAP II. Thus, EMAP II released under a hypoxic condition binds to HS in the non-endothelial cells or ECM and can be sequestered. When tissues are sufficient for blood supply by angiogenesis and pH increases from acidic to neutral condition, the sequestered EMAP II can release from ECM and exert its effects on its target cells. Therefore, the function of EMAP II under physiological conditions may be regulated via modulation of the binding affinity between HS-EMAP II.

Our data demonstrated that the interaction of EMAP II and α-ATP synthase to HS could be regulated by extracellular pH and modulate the antiangiogenic effects of EMAP II. EMAP II also has a biological activity in T lymphocytes and monocytes as well as ECs (Murray et al., 2004). T cells (von Haller et al., 2001) and monocytes (Li et al., 2003) have been found to express both of HS and ATP synthase. The investigation regarding the involvement of HS and α-ATP synthase in T cell apoptosis or monocyte chemotaxis will be interesting. Moreover, recent results suggest that cell-surface HS are not uniformly distributed but instead

appear to be localized to cholesterol-rich lipid raft domains (Tkachenko et al., 2002) and HS can modulate FGF-2 binding through a lipid raft-mediated mechanism (Chu et al., 2004). In addition, endostatin associates with lipid raft and heparinase treatment of cells prevents the recruitment of endostatin to the lipid rafts (Wickströ et al., 2003). Since ATP synthase in T cell and monocyte has been found to be present in lipid raft, it remains to be investigated whether HS can regulate the lipid raft localization of ATP synthase.

In fact, the differences in the antiangiogenic effects of EMAP II between pH7.5 and pH6.5 (Figure 7) were relatively small even though they are statistically significant. However, physiological pH under hypoxic condition can be more acidic. The previous report showed that the extracellular space within malignant tissues has been measured to be as low as pH 5.8 (Wike-Hooley et al., 1984). We observed that the binding of EMAP II could be more increased when the pH decreased more than pH 6.0 (data not shown). Therefore, the antiangiogenic acticity of EMAP II might be more potent under physiological hypoxia than normal oxidative condition. Meanwhile, blood vessels in tumor tissues are under balance between various angiogenic and antiangiogenic factor. The regulation mechanisms of vascular system are regarded as a very complicated process. Furthermore, the relation between the antiangiogenesis and hypoxia (acidic pH) are remained to be found. We thought that our

results could be a clue to unveil the puzzling network.

In the current study, we proposed the regulation of EMAP II-induced antiangiogenesis by HS at acidic pH. In ECs, HS may regulate the function of EMAP II released from the tumor cell in hypoxic conditions (acidic condition). These observations provide a biological mechanism which plausibly explains the regulation mechanism of antiangiogenesis via HS and pH. Furthermore, these findings provide a novel insight in understanding the complex network of tumor cell and vasculature system.

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Footnotes

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Legends for figures

Figure 1. Inhibition of the interaction between EMAP II and α-ATP synthase by heparin

Modulation of the interaction between EMAP II and α-ATP synthase by heparinized sera or exogenous heparin was determined by ELISA. The coated α-ATP synthase was preincubated with serial-diluted sera (A and B) or increasing amounts of heparin (C) for 30min before adding of 250ng/ml biotin conjugated EMAP II. The bound EMAP II was probed with alkaline phosphatase conjugated streptavidin and then detected with phosphatase substrate. The data were shown as a percent inhibition calculated from the absorbance at 405nm. (A) Naive murine and human sera as well as murine antiserum could inhibit the interaction between EMAP II and α-ATP synthase. (B) The inhibition by heparin-free serum was reduced compared to heparinized serum. (C) Excess heparin could inhibit the interaction between EMAP II and α-ATP synthase in a concentration dependent manner.

Figure 2. Heparin Binding Motif of EMAP II and α-ATP synthase

Recombinant proteins mutated in the putative heparin binding motif, Em73, Em121, Em73, 121 and αATPs-m167 were expressed. These mutant forms were tested for the binding activity to heparin (A and C) and their counterpart protein (B and D). To test the heparin binding activity of mutated sites, native and mutant forms of EMAP II (A) or α-ATP synthase (C) were

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incubated with serial-diluted biotin conjugated heparin-BSA (1-4000ng/ml). (B) To check the binding activities of mutant forms of EMAP II to α-ATP synthase, various concentrations of biotin conjugated naive or mutant EMAP II proteins were incubated with coated α-ATP synthase. (D) To check the binding activity of the mutant form of α-ATP synthase to EMAP II, coated naive or mutant form of α-ATP synthase were incubated with various concentrations of biotin conjugated EMAP II. The binding of biotin conjugated heparin-BSA or biotin conjugated EMAP II were detected with alkaline phosphatase conjugated streptavidin.

Figure 3. Increased binding of Heparin at acidic pH

To test whether heparin binding to EMAP II or α-ATP synthase can be affected by pH, we performed the heparin binding assay at two different pH using ELISA. Heparin binding to α-ATP synthase (A) or EMAP II (B) was increased at pH 6.5 compared with heparin binding at pH7.5. *, $p<0.01$, **, $p<0.001$ (Student's t-test).

Figure 4. Increased binding between EMAP II and α-ATP synthase by Heparin and acidic pH

To test whether the interaction between EMAP II and α-ATP synthase may be affected by crosslinking of heparin, we performed the binding assay using ELISA. (A) For cross-binding by

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heparin, coated α-ATP synthase were pre-incubated with (closed circle, closed triangle) or without 250mU/ml heparin (open circle, open triangle) in pH 7.5 (circle) or pH 6.5 (triangle) and after washing out to remove unbound heparin, incubated with biotin conjugated EMAP II. The α-ATP synthase-EMAP II binding was enhanced by both of heparin and acidic pH. (B) The interaction between Em73,121 and mutant α-ATPs-m167 was increased by neither acidic pH nor heparin.

Figure 5. Increased EMAP II binding to BAECs at acidic pH

EMAP II binding to ECs was measured using flow cytometry. (A) EMAP II binding to BAEC was dramatically increased at pH 6.5 and (B) the increased EMAP II binding was inhibited by unlabeled EMAP II. (C) EMAP II binding to BAECs was inhibited in presence of excess heparin in a concentration dependent manner at pH 6.5 while the binding was independent of heparin at pH 7.5. (D) Em73,121 binding to BAEC was more reduced than EMAP II binding at pH 6.5 although the binding of EMAP II and Em73,121 at pH 7.5 were similar. (D) For double inhibition by heparin and α-ATP synthase, BAEC, HepG2, Jurkat T cell and CHO-K1 were preincubated with both and either 10U/ml heparin or 80μg/ml soluble α-ATP synthase and then stained with 20ug/ml FITC conjugated EMAP II. All of BAEC, HepG2, Jurkat T cell and CHO-K1 cells were inhibited by both of heparin and α-ATP synthase at pH 6.5.

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Figure 6. Binding analysis of EMAP II to ECs and HS-α-ATP synthase interaction on the EC surface

(A) Binding assay was performed in BAECs, with various concentrations of FITC conjugated EMAP II at pH 7.5 and pH 6.5 using flow cytometry. The inserts show Scatchard plots of the specific binding. (B) For co-immunoprecipitation, lysates of BAECs which were incubated with 20ug/ml EMAP II for 1hr at 4ºC at either pH 7.5 or pH 6.5, were precipitated with agarose conjugated anti-syndecan-1antibody for binding to HS or Ni^{+}-agarose for binding to his-tagged EMAP II. Precipitation of HS and EMAP II were confirmed by probing with anti-syndecan-1 antibody and anti-his-tag antibody, respectively. The co-precipitation of α-ATP synthase was detected by western blotting using polyclonal α-ATP synthase antibody.

Figure 7. Regulation of EMAP II induced antiangiogenesis by HS at acidic pH

The proliferation of BAECs was measured by MTT assay. (A) The growth of BAEC at pH 7.5 and pH 6.5. The data were expressed as a percent proliferation ± SE with respect to the cell at pH 7.5. (B) BAECs were treated with EMAP II at either pH 7.5 (black) or pH 6.5 (white). Percent inhibition of BAEC proliferation by EMAP II was significantly increased at the pH 6.5. (C) BAECs were treated with EMAP II or Em73,121 at either pH7.5 or pH 6.5. Inhibitory

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effects of BAEC proliferation by Em73,121 was not increased at the pH 6.5. (D) BAECs were treated with naive or mutant forms of EMAP II at pH 6.5. The proliferation inhibition by Em121 and Em73,121 was significantly reduced compared to EMAP II. In A-C, the data were expressed as a percent inhibition ± SE of cell proliferation with respect to non-treated cells. (E) BAECs were treated with 50nM EMAP II in the presence of excess heparin at pH 7.5 or pH 6.5. Data were expressed as percent inhibition ± SE of EMAP II induced antiproliferative effects by heparin with respect to 50nM EMAP II treated cell and non-treated cells. (F) BAECs were treated with heparinase I+III, washed extensively with PBS and then incubated with 50nM EMAP II. The data were expressed as a percent inhibition ± SE of cell proliferation with respect to non-treated cells. *,$p<0.01$, **, $p<0.001$, ***, $p<0.0001$ (Student's t-test).

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Figure 1

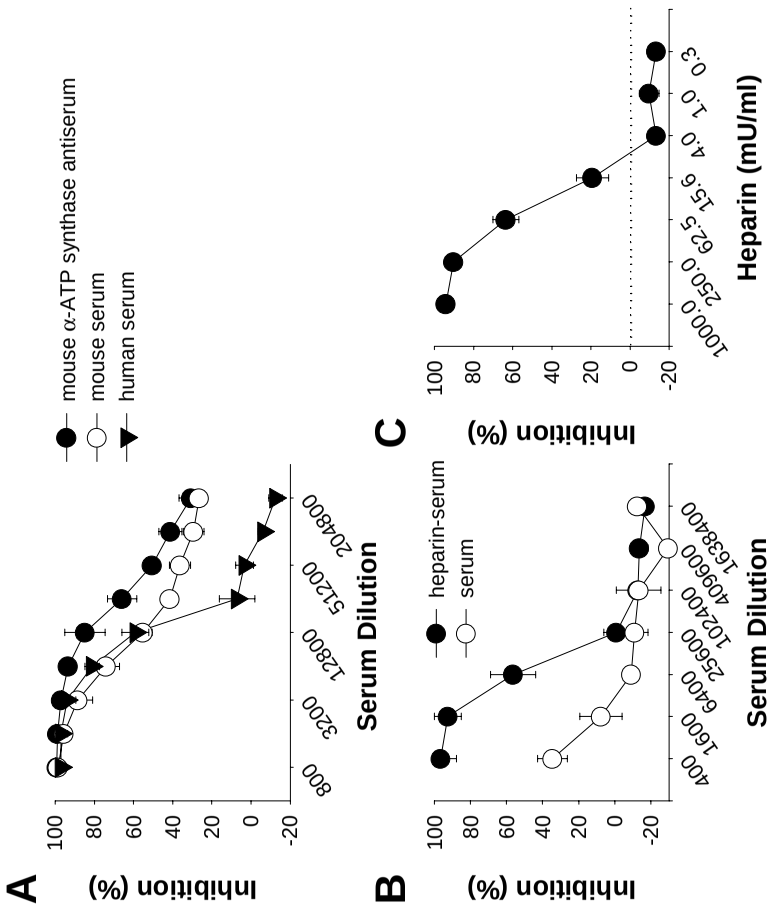

Figure 2

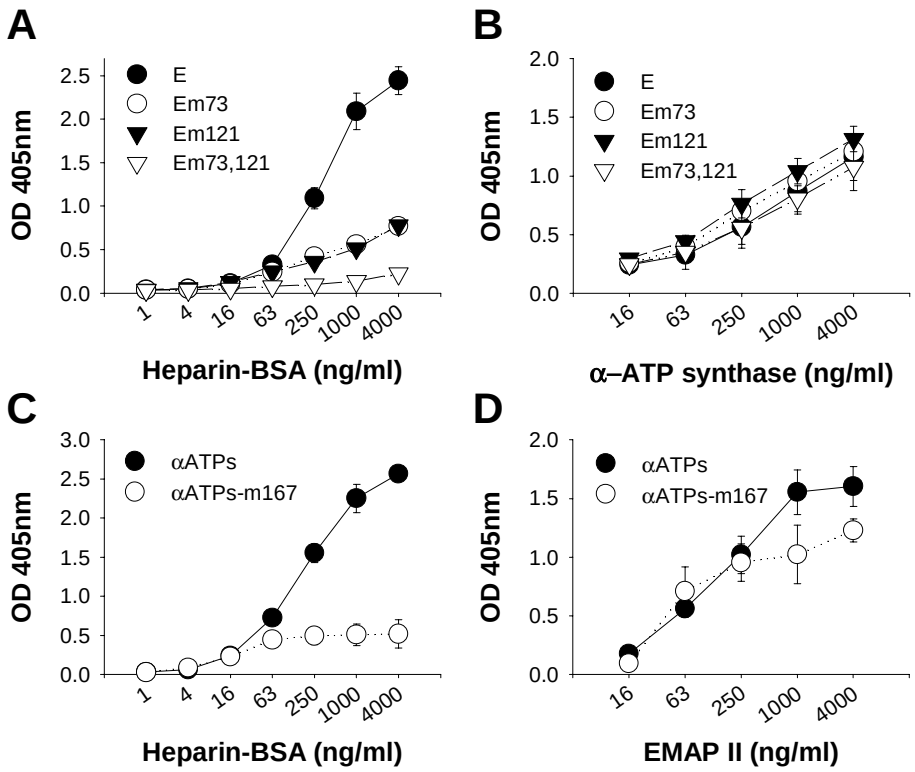

Figure 3

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Figure 4

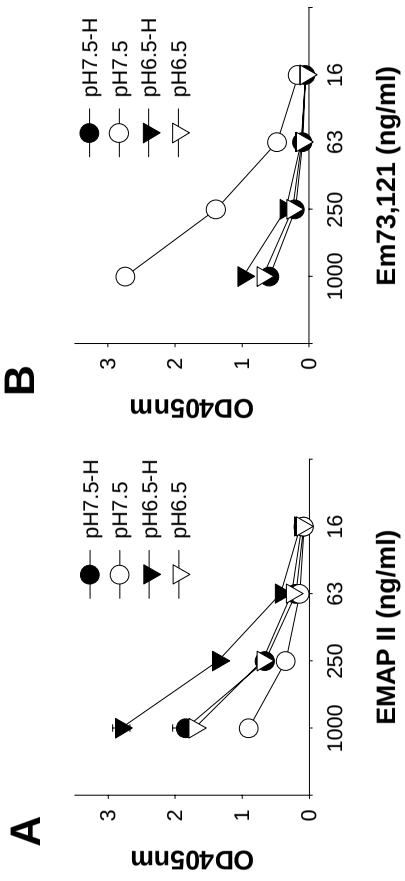

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Figure 5

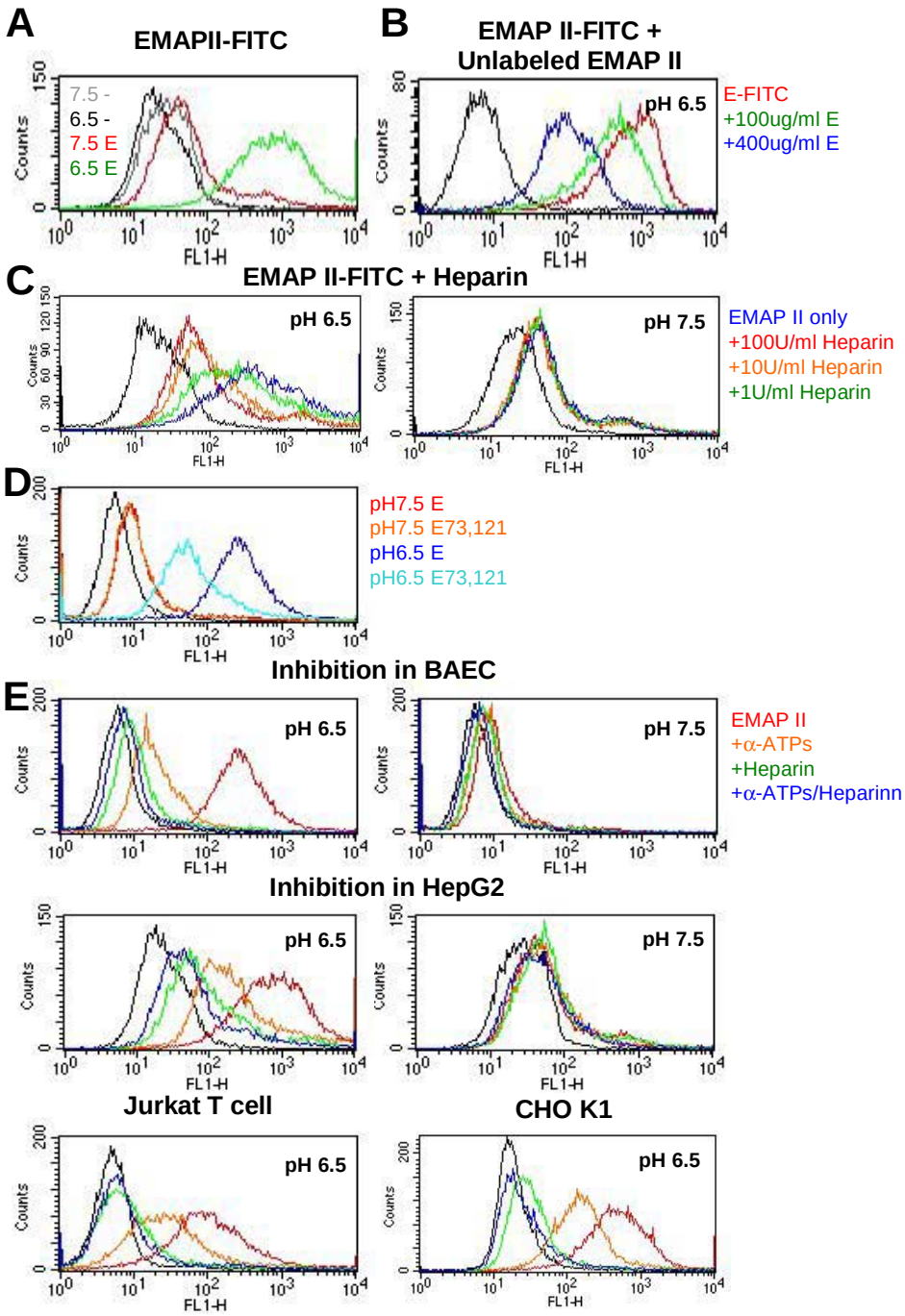
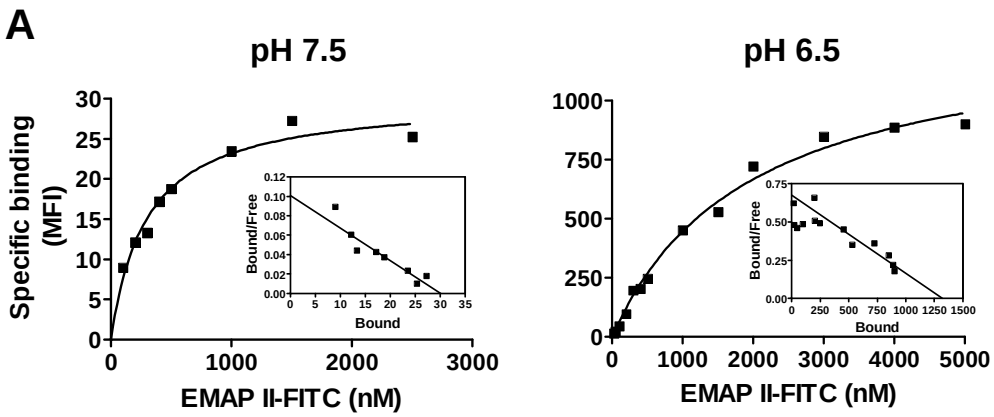


Figure 6



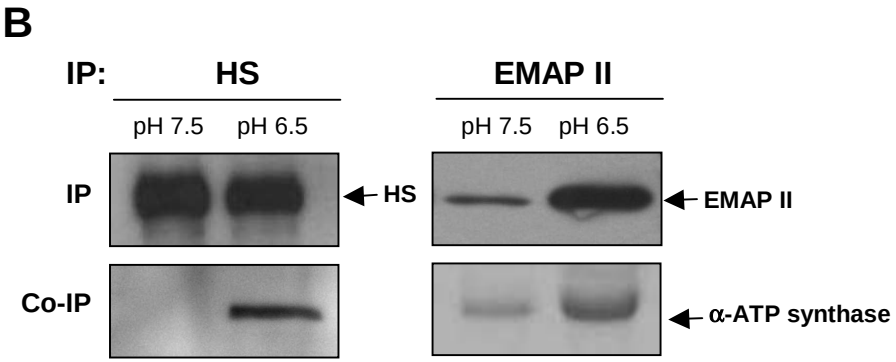


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Figure 7