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Interaction of the C-terminal Domain of p43 and the α Subunit of ATP Synthase

ITS FUNCTIONAL IMPLICATION IN ENDOTHELIAL CELL PROLIFERATION*

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Human p43 is associated with macromolecular tRNA synthase complex and known as a precursor of endothelial monocyte-activating polypeptide II (EMAP II). Interestingly, p43 is also secreted to induce proinflammatory genes. Although p43 itself seems to be a cytokine working at physiological conditions, most of the functional studies have been obtained with its C-terminal equivalent, EMAP II. To gain an insight into the working mechanism of p43/EMAP II, we used EMAP II and searched for an interacting cell surface molecule. The level of EMAP II-binding molecule(s) was significantly increased in serum-starved tumor cells. Thus, the EMAP II-binding molecule was isolated from the membrane of the serum-starved CEM cell. The isolated protein was determined to be the α subunit of ATP synthase. The interaction of EMAP II and α-ATP synthase was confirmed by enzyme-linked immunosorbent assay and *in vitro* pull down assays and blocked with the antibodies raised against EMAP II and α-ATP synthase. The binding of EMAP II to the surface of serum-starved cells was inhibited in the presence of soluble α-ATP synthase. EMAP II inhibited the growth of endothelial cells, and this effect was relieved by soluble α-ATP synthase. Anti-α-ATP synthase antibody also showed an inhibitory effect on the proliferation of endothelial cells mimicking the activity of EMAP II. These results suggest the potential interaction of p43/EMAP II with α-ATP synthase and its role in the proliferation of endothelial cells.

Endothelial monocyte-activating polypeptide II (EMAP II)[1] is structurally and functionally equivalent to the C-terminal domain of p43 that is associated with a macromolecular tRNA synthase complex (1). EMAP II has been first found in the culture medium of murine methylcholanthrene A-induced fibrosarcoma cells based on its capacity to induce activation of tissue factor in human umbilical vein endothelial cells (HUVECs) (2). Murine methylcholanthrene A fibrosarcomas exhibit spontaneous vascular insufficiency manifested by a heterogeneous pattern of thrombohemorrhage and central necrosis, providing an ideal starting point for isolation of tumor-derived mediators that perturb the vasculature (3, 4). The purified EMAP II possesses a wide range of activities toward endothelial cells (ECs), neutrophils, and monocyte/macrophages *in vitro* (5, 6). In addition to the induction of tissue factor-dependent coagulation on ECs and monocytes, EMAP II up-regulates endothelial E-selectin and P-selectin expression and induces the release of von Willebrand factor. It is also chemotactic for neutrophils and monocytes and induces release of myeloperoxidase activity from neutrophil. *In vivo*, local injection of EMAP II into a mouse footpad evokes an acute inflammatory response characterized by edema and neutrophil-rich infiltrates. EMAP II is cleaved from p43 at the amino acid motif ASTD by caspase-7 activated in apoptotic cells (7–9). In addition, EMAP II mRNA is most abundant at the sites of tissue undergoing apoptosis in mouse embryo and human tissue (10, 11). Therefore, p43/EMAP II could be a good candidate for the chemokine recruiting leukocytes and monocytes to cells undergoing apoptosis to remove apoptotic corps.

EMAP II also has an antiangiogenic property that targets rapidly growing vascular beds. EMAP II is capable of sensitizing tumors resistant to the anti-tumor effects of tumor necrosis factor (12–14) because it induces the expression of tumor necrosis factor-R1 in the ECs (15). In addition, EMAP II inhibited EC proliferation, vasculogenesis, and neovessel formation (16). EMAP II also suppresses the growth of primary and metastatic tumors by anti-angiogenic properties without toxicity to normal organ (17). Exposure of growing cultured capillary endothelium to EMAP II induces apoptosis (18), which is magnified by concomitant hypoxia. These effects on endothelium implied that EMAP II might play an important role in regulating tumor vasculature. However, little is known about the molecular mechanism(s) by which EMAP II functions to regulate endothelial cell behavior.

Although we have recently shown that p43, not EMAP II, may be a real cytokine at physiological conditions (19), EMAP II was functionally equivalent to p43 in all the aspects we have tested (data not shown). In addition, EMAP II is easier to purify than p43 because it is produced at higher yield and is more soluble. Thus, we used EMAP II in most of the experiments in this work for convenience. We found that the EMAP II-binding protein on the serum-starved cells was the α subunit of ATP synthase (α-ATP synthase). Interaction of EMAP II

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[1] The abbreviations used are: EMAP II, endothelial monocyte-activating polypeptide II; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; BAEC, bovine aorta endothelial cell; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum.

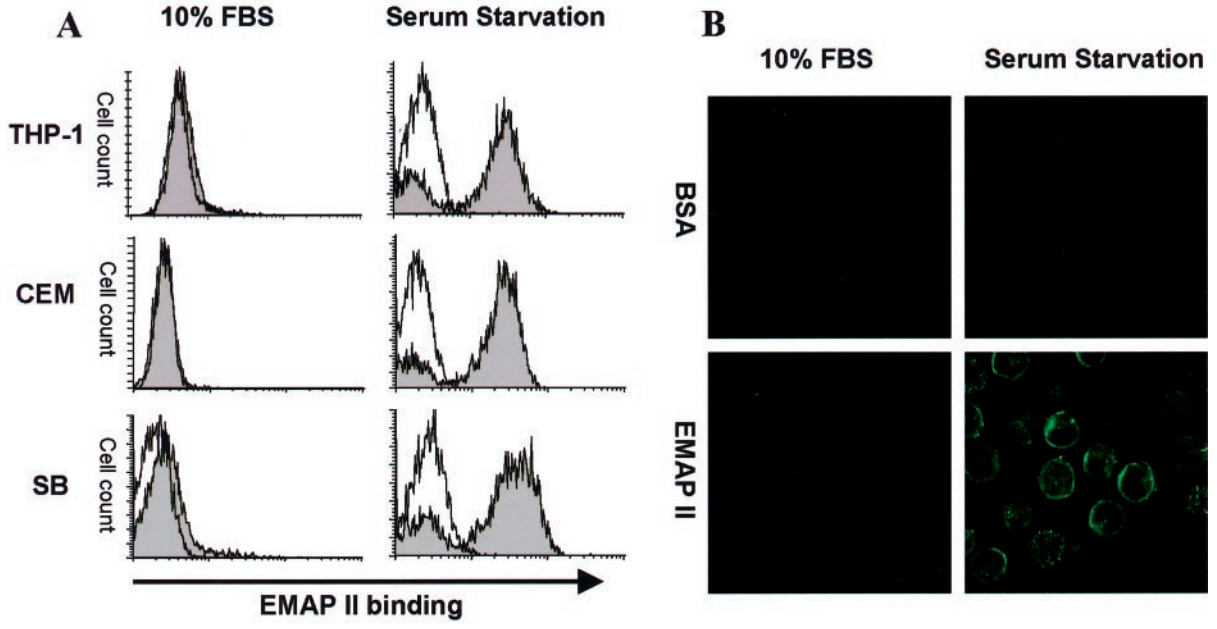

FIG. 1. **Binding of EMAP II to the serum-starved cells.** The binding of EMAP II was determined by flow cytometry (*A*) and confocal microscopy (*B*). *A*, THP-1, CEM, and SB cells were stained with 5 μg/ml biotin-conjugated EMAP II (*shaded*) or BSA (*unshaded*) as a control. Representatives of the analyzed cells are displayed. Binding of EMAP II was increased the most in cells cultured in the serum-free medium. *B*, the serum-starved CEM cells for 24 h were stained with 5 μg/ml biotin-conjugated EMAP II or BSA in the nonpermeabilized condition. The staining with EMAP II was mainly detected on the surface of CEM cells cultured in the serum-free medium.

with α-ATP synthase was confirmed using ELISA, pull down assays, flow cytometry, confocal microscopy, and competitive cellular binding. Furthermore, proliferation assays suggested that both EMAP II and anti-α-ATP synthase antibodies inhibit EC proliferation, and the inhibitory effect of EMAP II on the EC proliferation was abrogated in the presence of soluble α-ATP synthase. These results imply that EMAP II binding to the α-ATP synthase may be involved in the inhibition of the EC proliferation.

MATERIALS AND METHODS

Cell Culture—CEM, SB, and THP-1 were cultured in RPMI 1640 supplemented with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum (Invitrogen) and antibiotics at 37 °C in 5% CO_2. Bovine aorta endothelial cells (BAECs) were maintained in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and retained for up to six passages. Serum-starved (quiescent) cells were prepared by culturing in the medium containing 0.2% fetal bovine serum for 1–2 days.

Flow Cytometry—The cells were washed in cold PBS with 1% BSA and 0.1% sodium azide and then incubated with 5 μg/ml biotin-conjugated EMAP II, 10 μg/ml biotin-conjugated p43, and 50 μg/ml polyclonal antibodies containing anti-α-ATP synthase antibodies for 1 h at 4 °C. Binding inhibition experiments were performed by staining with biotin-conjugated EMAP II or p43, which was preincubated with an excess of recombinant α-ATP synthase (10 μg/ml). After two additional washes, FITC-avidin (Pierce) or FITC-conjugated goat anti-mouse IgG (Sigma) were added to each sample and incubated on ice for 30 min in the dark. After the last wash, the cells were resuspended in PBS, and viable cells from each sample were analyzed on a PASIIIi flow cytometer (Partec Gmbh).

Confocal Microscopy—The serum-starved CEM cells were suspended in PBS and adhered to poly-L-lysine-coated glass coverslips. Cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. After the fixed cells were washed with PBS, the cells were blocked with 1% BSA (Sigma) in PBS for 30 min. The cells were then incubated with 5 μg/ml biotin-conjugated EMAP II in PBS containing 0.5% BSA for 1 h 30 min. Control staining were performed with 10 μg/ml biotin-conjugated BSA. Binding inhibition experiments were performed by staining with 5 μg/ml biotin-conjugated EMAP II, which was preincubated with an excess of recombinant α-ATP synthase (10 μg/ml). After washing with PBS, the cells were incubated with the FITC-conjugated avidin (Pierce) for 1 h in the dark. The stained cells were examined using DM IRB/E confocal microscopy (Leica Microsystems).

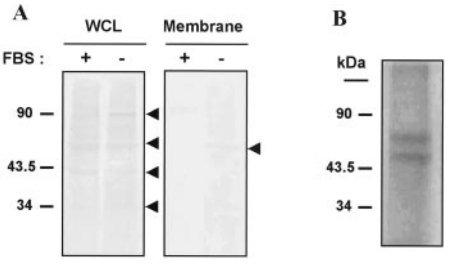

FIG. 2. **Affinity purification of the EMAP II-binding proteins from the serum-starved CEM.** *A*, whole cell lysates and plasma membranes were prepared from the CEM cells cultured for 24 h in the serum-free medium or normal growth medium, which were reacted with EMAP II-Sepharose. The proteins eluted from the precipitants were resolved by SDS-PAGE, blotted onto membrane, and detected by horseradish peroxidase-conjugated streptavidin. Precipitates from whole cell lysates revealed four proteins of approximate molecular masses of 34, 43, 55, and 90 kDa whereas a protein with approximate molecular mass of 55 kDa was precipitated from the plasma membrane fraction. Molecular mass markers are indicated on the *left side*. *B*, the EMAP II-binding proteins were purified by subjecting the serum-starved CEM plasma membranes to affinity chromatography on EMAP II-Sepharose. Two bands with approximate molecular masses between 50 and 60 kDa were obtained, and amino acid sequences of the proteins were analyzed.

Affinity Precipitation and Purification of EMAP II-binding Molecules—CEM cells were harvested, washed with ice-cold PBS, and suspended in PBS (pH 8.0). Biotin labeling was performed by incubating cell suspensions with 0.5 mg/ml biotin for 1 h at room temperature. Total lysates from *N*-hydroxysuccinimidobiotin (Pierce)-labeled CEM were prepared by lysing the cells in ice-cold lysis buffer (1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.025% sodium azide, 1 mM iodoacetamide, 0.2 unit/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Roche Molecular Biochemicals)) and pelleting to remove cellular debris by centrifuging at 12,000 rpm for 30 min. Plasma membrane extracts from sulfo-*N*-hydroxysuccinimide-LC-biotin (Pierce)-labeled CEM were prepared by ultracentrifugation (20). After preclearing, each sample was incubated with EMAP II-Sepharose for 2 h at 4 °C and washed five times in lysis buffer. After extraction of precipitated proteins by boiling, the samples were electrophoresed by SDS-PAGE and transferred onto nitrocellulose membranes (Gelman). The membranes were blocked for 1 h in 3%

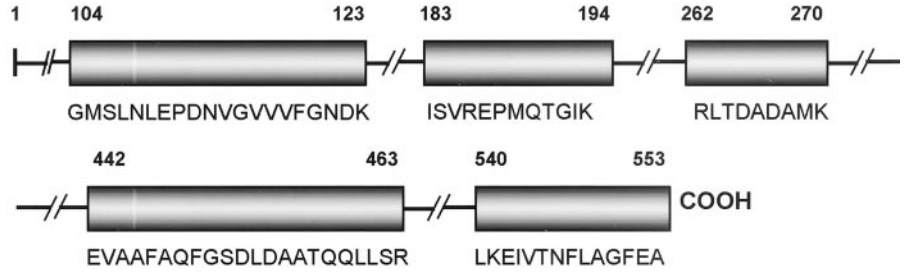

FIG. 3. **Amino acid sequences of the purified EMAP II-binding protein.** Amino acid sequences of fragments resulting from a tryptic digest of the EMAP II-binding protein purified from plasma membrane of the serum-starved CEM are noted. The EMAP II-binding protein was identified as the α subunit of human ATP synthase with a molecular mass of 55 kDa.

skimmed milk and subsequently probed with horseradish peroxidase-conjugated avidin (Amersham Biosciences, Inc.). The precipitated proteins were visualized using a DAB detection kit according to the manufacturer's instruction (DAKO). For purification of EMAP II-binding protein(s), membrane extracts from the serum-starved CEM were incubated with a EMAP II-Sepharose column and eluted with 20 mM glycine (pH 2.5). The eluates were concentrated by Speed-vac, electrophoresed on SDS-PAGE, and stained with Coomassie Brilliant Blue. The resulting band were excised tightly, washed twice with 50% acetonitrile, and digested. The amino acid sequences of digested peptides were analyzed by MS/MS spectrometry at the Harvard Microchemistry facility.

Expression of the α-Subunit of ATP Synthase and Purification of Polyclonal Antibodies—The cDNA encoding the full-length human α-ATP synthase, EMAP II, and p43 were produced by reverse transcription-PCR from total RNA of the CEM cells as a template. The cDNAs were amplified by PCR between the two specific oligonucleotides. The primers used were 5′-ACATATGCTGCCGTGCGCGTTG and 5′-TCTCGAGAGCTTCAAATCCAGCC for α-ATP synthase, 5′-ACATATGGCAAATAATGATGC and 5′-TCTCGAGTTATTTGATTCCAC for p43, and 5′-ACATATGGACTCTAAGCCAATAGAT and the same primer of p43 downstream for EMAP II. The PCR products were cleaved with *Nde*I and *Xho*I designed into each of the primers and inserted into the same site of pET28a encoding the His-tail (Novagen). The resulting plasmids were introduced into *Escherichia coli* BL21 (DE3) and induced with IPTG. The His-tagged proteins of α-ATP synthase, EMAP II, and p43 were purified using nickel affinity chromatography following the manufacturer's instructions (Invitrogen). Each purified protein was used to raise specific polyclonal mouse antibodies. After the third immunization, polyclonal anti-EMAP II antibodies were purified from mouse antiserum using an EMAP II-Sepharose column. The total IgG containing polyclonal anti-α-ATP synthase antibodies was purified from the mouse α-ATP synthase antiserum by a protein A affinity chromatography according to the manufacturer's protocol (Amersham Biosciences, Inc.), and the antibody specificity was confirmed by immunoblotting of the proteins extracted from the starved CEM cells.

ELISA for Binding Assay—Binding of EMAP II to α-ATP synthase was determined by ELISA. Briefly, 96-well microtiter plates (Maxisorp F96; Nunc) were coated with 200 ng/well recombinant α-ATP synthase or heat-inactivated form in 50 mM carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After washing with PBS, the remaining sites were blocked with PBS containing 1% BSA (Sigma) for 30 min at room temperature. Binding studies were performed with increasing amounts of biotin-conjugated EMAP II, p43, and heat-inactivated forms of them (0–8 μg/ml) for 2 h at room temperature. For binding inhibition studies with antibodies, biotin-labeled EMAP II (250 ng/ml) containing a range of concentrations of anti-EMAP II antibodies was applied to microtiter wells coated with α-ATP synthase. In the case of antibody directed against α-ATP synthase, coated α-ATP synthase was incubated with serial diluted polyclonal anti-α-ATP synthase antibodies before adding biotin-labeled EMAP II (250 ng/ml). The plates were washed and incubated with alkaline phosphatase-conjugated streptavidin (Pierce) diluted in PBS, 0.1% BSA, Tween 20 (1:2000) for 1 h at room temperature. The plates were washed, and then 100 μl of phosphatase substrate (*p*-nitrophenyl phosphate in a carbonate buffer, pH 9.6) was added to each well. The absorbance was read at 405 nm using an Emax microplate reader (Molecular Devices).

Pull Down Assay—Equal amounts of recombinant proteins prepared from *E. coli* were incubated with protein A-Sepharose beads in binding buffer (10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 0.025% NaN_3) with or without polyclonal antibodies containing anti-α-ATP synthase antibodies at 4 °C for 3 h. The beads were then washed extensively with binding buffer containing 0.1% Triton X-100, resuspended in SDS-PAGE buffer, boiled for 5 min, and resolved on 12% SDS-PAGE, followed by Coomassie Brilliant Blue staining. The presence of α-ATP synthase, EMAP II, and p43 was subsequently confirmed by Western blotting with their specific antibodies.

Cell Proliferation Assay—BAECs were seeded into 96-well tissue culture plates at a density of 10,000 cells/well in medium depleted of fetal bovine serum overnight to allow the cells to become quiescent. Fresh medium containing 20% fetal bovine serum was added to the wells along with 0.25 μM EMAP II, 0.25 μM p43, 0.25 μM α-ATP synthase, and 6.25, 25, and 100 μg/ml polyclonal antibodies containing anti-α-ATP synthase antibodies. Cell density was measured after 24 h by using the CellTiter 96 aqueous assay kit (Promega). The absorbance of formazan was quantitated on an Emax microplate reader at a wavelength of 490 nm according to the manufacturer's instructions (Promega). The absorbance values were used to calculate the percent proliferation of the cells.

RESULTS

EMAP II Binds to the Surface of the Serum-starved Cells—To understand the working mechanism of p43, it is important to identify the cell surface molecule that can associate with p43. In the experiments, we decided to use EMAP II instead of p43 because EMAP II is functionally equivalent to p43 but shows higher solubility. We first searched for the optimal condition for the binding of EMAP II to the cell with high efficiency. To find such a condition, we applied various treatments (lipopolysaccharide, DNA damaging agents, cytokine treatment, and serum starvation) to the THP-1 cells and analyzed the cell binding of EMAP II by flow cytometry. Among them, serum starvation dramatically increased binding of EMAP II to the THP-1 compared with the cells grown in medium containing 10% FBS (Fig. 1*A*, *upper*). Increased EMAP II binding by serum starvation was further investigated in the different cells to confirm whether the result was a monocyte-specific response. Starvation-induced EMAP II binding was also detected in all of the human T cell line (CEM, Fig. 1*A*, *middle*), human B cell line (SB, Fig. 1*A*, *lower*), hepatocellular carcinoma line (Chang), breast cancer cell line (SK-BR-3), and RAW 264.7 (data not shown). These data suggested that starvation universally increases the binding of EMAP II on the cell surface. To further confirm the possibility, the binding of EMAP II on the cell surface was analyzed by confocal microscopy using biotin-conjugated EMAP II. The staining with EMAP II was mainly detected on the surface of CEM cells cultured in the serum-free medium, confirming that the binding of EMAP II was increased on the cell surface by serum starvation (Fig. 1*B*).

The EMAP II Binding Molecule Was Identified as the α Subunit of ATP Synthase—We suspected that the enhanced EMAP II binding to the serum-starved cells might result from the increase of cell surface molecules that can associate with EMAP II. We thus used the serum starvation as an experimental condition to isolate the EMAP II-binding surface protein(s). The cell surface molecules responsible for the binding of EMAP II to the serum-starved CEM were precipitated with EMAP II-linked Sepharose. Four proteins with approximate molecular masses of 34, 43, 55, and 90 kDa were precipitated from the whole cell lysates (Fig. 2*A*, *left*). However, only one protein with an approximate molecular mass of 55 kDa was eluted from the precipitates of plasma membrane fraction obtained from the serum-starved CEM cells (Fig. 2*A*, *right*). These results sug-

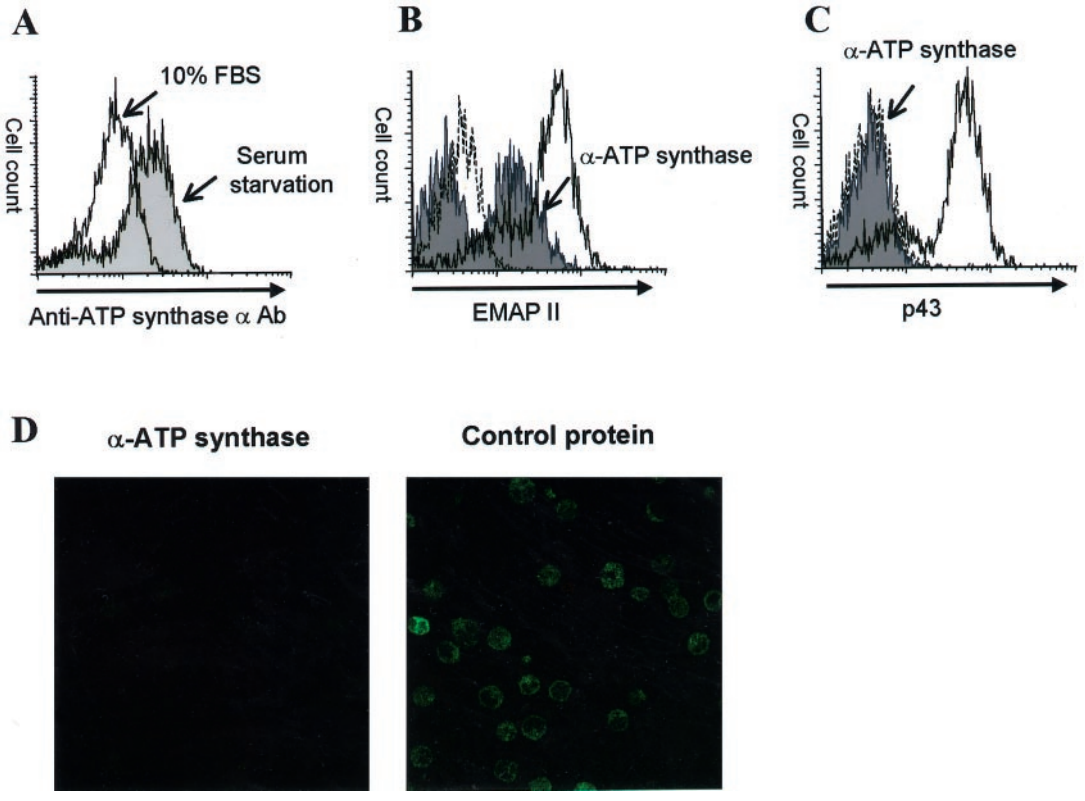

FIG. 4. **Inhibition of EMAP II binding to the starved CEM by soluble α-ATP synthase.** Inhibition of EMAP II binding to the starved cells was determined by flow cytometry and confocal laser microscopy. *A*, expression of α-ATP synthase on the surface of the serum-starved CEM cells was determined by immunostaining with antibody raised against α-ATP synthase. There was a significant increase in the binding of antibody to the starved CEM cells compared with the CEM grown in 10% FBS medium. The serum-starved CEM was stained with EMAP II (*B*) or p43 (*C*) containing 10 μg/ml α-ATP synthase (*shaded histogram*) or the same dose of control protein (*solid line*). *Dashed line* represents avidin-FITC only. Binding of EMAP II and p43 to the starved CEM cells was decreased by soluble recombinant α-ATP synthase. Unrelated control protein showed no inhibition. *D*, binding of EMAP II (5 μg/ml) to the starved CEM cells was completely inhibited by preincubation with soluble recombinant α-ATP synthase (10 μg/ml).

gested that the 55-kDa protein could be one of the EMAP II-binding proteins on the cell surface.

To identify the EMAP II-binding protein, affinity purification was performed by subjecting the serum-starved CEM plasma membranes to an EMAP II-Sepharose column. Two bands with molecular masses between 50 and 60 kDa were purified from the column (Fig. 2*B*). The amino acid sequences of the peptides digested from the affinity-purified protein were analyzed by MS/MS spectrometry. The EMAP II-binding membrane protein was identified as the α subunit of ATP synthase with a molecular mass of 55 kDa (Fig. 3). The expression of α/β subunits of ATP synthase has been reported on the plasma membrane of HUVEC and several tumor lines in the previous studies (21, 22). Therefore, we surmised that the EMAP II-binding protein on the surface of the starved CEM could be the α subunit of ATP synthase.

EMAP II Specifically Interacts with the α-Subunit of Human ATP Synthase—We first cloned the α-ATP synthase gene from the total RNA of CEM cells and then expressed recombinant human α-ATP synthase in *E. coli*. To investigate the surface expression of α-ATP synthase, mouse polyclonal antibodies specific to the human α-ATP synthase were prepared using a protein A-Sepharose column. Their antigenic specificity was determined by immunoblotting of the proteins extracted from the starved CEM cells. The antibodies showed the specificity to their antigen (data not shown). To determine the surface expression of α-ATP synthase, CEM cells were analyzed by flow cytometry using this polyclonal antibody. α-ATP synthase was detected only in the serum-starved cells but not in the cells grown in 10% FBS medium (Fig. 4*A*). In addition, the cell surface localization was further investigated by nonpermeabilized confocal microscopy, and α-ATP synthase was detected in the serum-starved cell surface (data not shown). These results showed that the α subunit of ATP synthase could be expressed on the surface of cells cultured under serum-free conditions.

To see whether the same result is obtained with the EMAP II precursor, p43, we repeated the experiment with p43. Similar to EMAP II, p43 bound only to the serum-starved CEM cells but not in the 10% FBS (data not shown). To determine the interaction of EMAP II and p43 with α-ATP synthase, binding of EMAP II or p43 to the recombinant α-ATP synthase was measured by ELISA. EMAP II and p43 bound to the recombinant α-ATP synthase in a dose-dependent manner, and their bindings were saturable at high concentrations (Fig. 5*A*). However, the heat-inactivated EMAP II and p43 lost the binding activity, indicating that the native conformation of EMAP II and p43 is important in their binding to α-ATP synthase. On the other hand, the interaction of EMAP II and p43 with heat-inactivated α-ATP synthase was decreased slightly, suggesting that the α-ATP synthase is heat-stable or the conformation is less critical for the interaction with EMAP II and p43 (Fig. 5*B*). In contrast, EMAP II, p43, or α-ATP synthase did not bind to any other recombinant proteins we tested (data not shown). We further confirmed the interaction of EMAP II or p43 with α-ATP synthase by pull down assays. The α-ATP synthase mixed with either EMAP II or p43 was incubated with anti-α-ATP synthase antibody and precipitated with protein A-Sepharose. The proteins in the precipitates were detected by Coomassie Brilliant

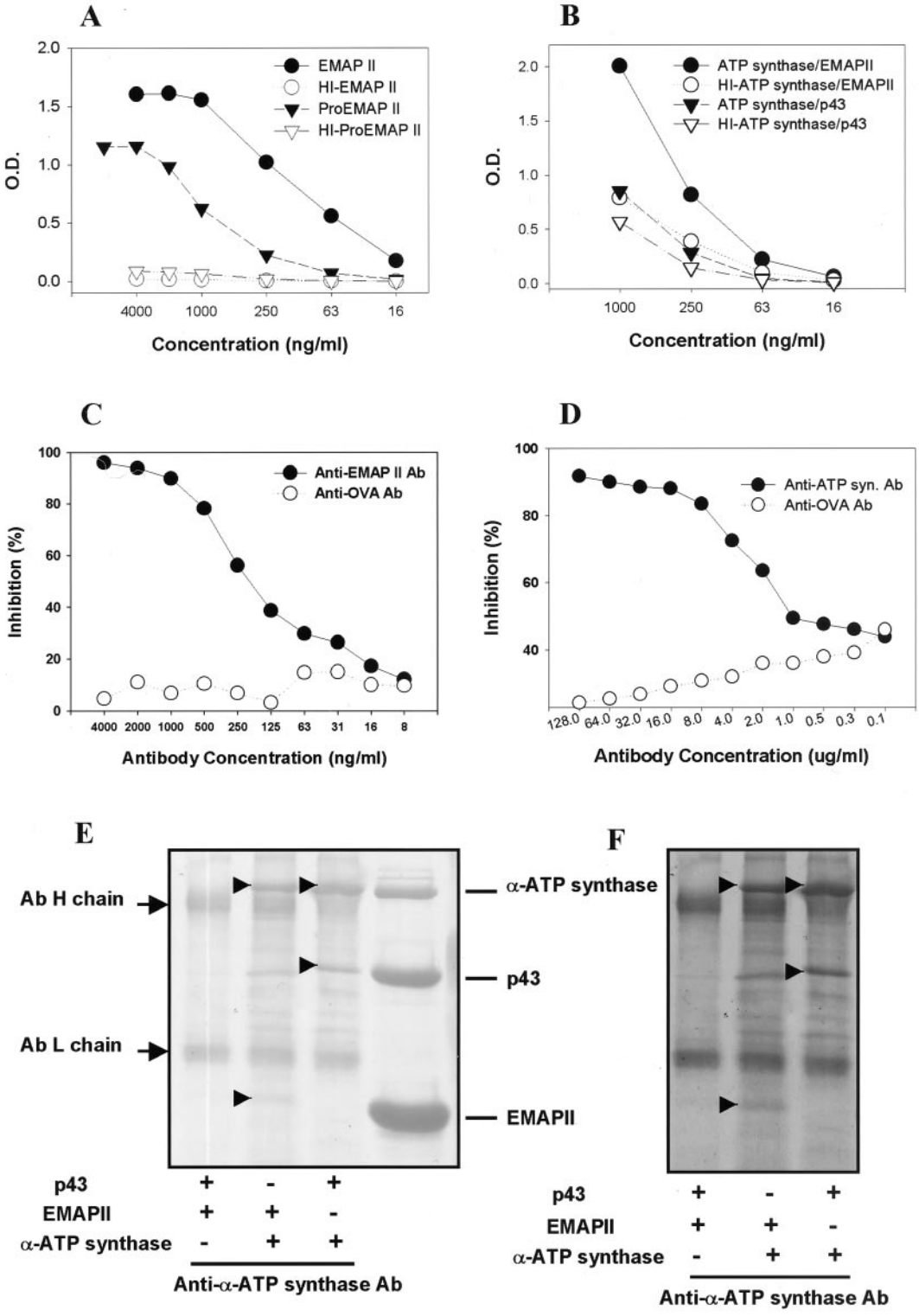

FIG. 5. **Binding of EMAP II to α-ATP synthase and inhibition of their binding in the presence of the antibody against α-ATP synthase or EMAP II.** Interaction of EMAP II and p43 with α-ATP synthase was determined by ELISA (*A–D*) and pull down assay (*E–F*). *A*, biotin-conjugated EMAP II or p43 was added to microtiter wells coated with α-ATP synthase. Bound EMAP II and p43 were detected with alkaline phosphatase-conjugated streptavidin. EMAP II and p43 were bound with α-ATP synthase in a dose-dependent manner. However, heat-inactivated forms of them were not bound to α-ATP synthase. *B*, heat-inactivated α-ATP synthase was incubated with biotin-conjugated EMAP II or p43. Binding of heat-inactivated α-ATP synthase with EMAP II or p43 was decreased by 50%. *C*, biotin-labeled EMAP II (250 ng/ml) containing a range of concentrations of anti-EMAP II antibody applied to microtiter wells coated with α-ATP synthase. Binding of EMAP II to α-ATP synthase was inhibited by polyclonal anti-EMAP II antibody in a dose-dependent manner. Unrelated polyclonal antiovalbumin (*OVA*) antibody was used as a control. *D*, coated α-ATP synthase was incubated with serial diluted polyclonal antibodies containing anti-α-ATP synthase antibody before adding biotin-labeled EMAP II (250 ng/ml). Binding of EMAP II to α-ATP synthase was inhibited in a dose-dependent manner in the presence of increasing concentrations of polyclonal antibody containing anti-α-ATP synthase antibody. *E*, indicated ligand mixtures were incubated with anti-α-ATP synthase antibody and then precipitated by protein A-Sepharose. Proteins bound to the antibody-bead complex were eluted, after which they were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining. The precipitates of α-ATP synthase with EMAP II or p43 were detected. *F*, the presence of α-ATP synthase, EMAP II, and p43 was subsequently confirmed by Western blotting with their specific antibodies.

Blue staining (Fig. 5*E*), and the presence of α-ATP synthase, EMAP II, and p43 was subsequently confirmed by Western blotting with their specific antibodies (Fig. 5*F*). EMAP II or p43 was not precipitated when mock IgG was used (data not shown). We then evaluated whether the antibodies directed against EMAP II and α-ATP synthase can inhibit the binding of EMAP II to α-ATP synthase. The polyclonal antibodies raised against EMAP II completely inhibited the binding of EMAP II to α-ATP synthase (Fig. 5*C*). Although a high concentration was required for the inhibition, binding of EMAP II to α-ATP synthase was blocked by the polyclonal antibodies containing anti-α-ATP synthase antibody (Fig. 5*D*). High concentrations required for the inhibition may be due to a low proportion of anti-α-ATP synthase antibody in the polyclonal antibodies because the antibodies were purified using a protein A-Sepharose column. These data also support that EMAP II bound to α-ATP synthase in a specific manner.

To provide further evidence that α-ATP synthase is responsible for the cell surface binding of EMAP II, we analyzed the binding of EMAP II to the starved cells in the presence of soluble α-ATP synthase or polyclonal antibodies containing anti-α-ATP synthase antibody by flow cytometry and confocal microscopy. Soluble α-ATP synthase blocked binding of EMAP II to the starved CEM cells significantly (Fig. 4, *B* and *D*), whereas polyclonal antibodies containing anti-α-ATP synthase antibody inhibit slightly (data not shown). In contrast, an unrelated control protein or control antibody showed no inhibition. Similar results were obtained when binding of p43 to the starved CEM in the presence of soluble α-ATP synthase was analyzed by flow cytometry (Fig. 4*C*), implying the possibility that EMAP II and p43 share the binding molecule.

The Antiproliferative Effect of EMAP II on EC Is Blocked by Soluble α-ATP Synthase—Previous studies showed that EMAP II inhibits EC proliferation and induces EC apoptosis (15) and that α and β subunits of ATP synthase colocalize on the surface of HUVECs (23). We investigated whether EMAP II also binds to the EC surface and α-ATP synthase is expressed in the EC surface (data not shown). HUVECs and BAECs without serum starvation were stained with either EMAP II or anti-α-ATP synthase antibody when analyzed by confocal microscopy and flow cytometry of the non-permeabilized cells. This result is supported by the fact that human and bovine ATP synthase are highly homologous (98.5%), differing only by eight amino acid residues in the mature α-chain. The staining pattern of α-ATP synthase was similar to the previous report (23).

To gain insight into the function of EMAP II through α-ATP synthase, we tested whether EMAP II and p43 can inhibit the growth of BAECs. EMAP II and p43 inhibited BAEC proliferation by 40 and 50%, respectively (Table I). In contrast, soluble α-ATP synthase exhibited no effect on proliferation. Furthermore, the polyclonal antibodies containing anti-α-ATP synthase antibody inhibited the BAEC proliferation in a concentration-dependent manner, suggesting that the antibodies directed against α-ATP synthase mimic the antiangiogenic effect of EMAP II in the ECs. An unrelated polyclonal antibody had no effect on inhibition (Table I). Similar results were obtained from the experiments with HUVEC (data not shown).

To determine whether the antiproliferative effects of EMAP II were mediated by ATP synthase binding, cell proliferation assays were performed in the presence of soluble α-ATP synthase. The inhibitory effects of EMAP II on BAEC proliferation were abrogated by 30% in the presence of soluble α-ATP synthase at low concentrations (Fig. 6). These data showed that the binding of EMAP II or p43 to α-ATP synthase may function as a mechanism for the inhibition of the EC growth.

TABLE I

Inhibition of BAEC proliferation in the presence of EMAP II, p43, and anti α-ATP synthase antibody

BAEC were plated at a density of 10,000 cells/well and incubated with media containing 0.25 μM EMAP II, p43, α-ATP synthase, and 100, 25, and 6.25 μg/ml polyclonal antibodies containing anti-α-ATP synthase antibody. Cell proliferation was measured by MTS assay according to the manufacturer's instructions. Unrelated polyclonal antibody was used as control.

Treatment	% inhibition ± S.E.
EMAP II (0.25 μM)	40.0 ± 4.53
p43 (0.25 μM)	50.8 ± 3.12
α-ATP synthase (0.25 μM)	−6.2 ± 1.25
Anti-α-ATP synthase antibody (μg/ml)	
100	59.1 ± 7.11
25	44.7 ± 6.78
6.25	19.2 ± 4.15
Control antibody (μg/ml)	
100	3.8 ± 1.05
25	7.3 ± 0.95
6.25	5.8 ± 0.10

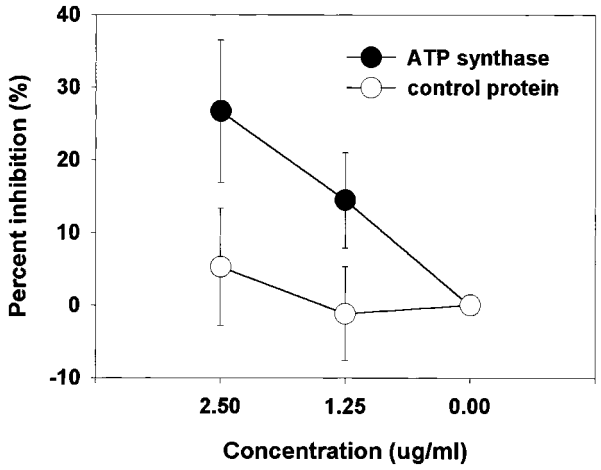

FIG. 6. **Reversal of EC proliferation inhibition of EMAP II by soluble α-ATP synthase.** BAECs were treated with media containing 0.25 μM EMAP II. Soluble α-ATP synthase was added concomitantly at a final concentration of 1.25–2.5 μg/ml. Cell proliferation was measured by a methanethiosulfonate assay according to the manufacturer's instructions. Anti-proliferative response of EMAP II in the ECs was inhibited by soluble α-ATP synthase in a dose-dependent manner. *Error bars* represent S.E. Representative data are shown; $n = 3$.

DISCUSSION

Here we showed that the EMAP II binding was significantly increased in the serum-starved tumor cells (Fig. 1). The EMAP II-binding protein purified from the serum-starved cell membrane was identified as the α subunit of ATP synthase (Figs. 3 and 4*A*). The interaction of EMAP II with α-ATP synthase was inhibited by their specific antibodies (Fig. 5, *C* and *D*) and the soluble recombinant α-ATP synthase (Fig. 4*B*), suggesting that their interaction is specific. To gain an insight into the function of EMAP II through α-ATP synthase, we investigated whether α-ATP synthase is involved in the inhibition of the EC proliferation by EMAP II. We first confirmed that α-ATP synthase was expressed on the surface of ECs (data not shown) and EMAP II inhibited the EC proliferation (Table I) as previously described (16, 17, 21, 23). Furthermore, the inhibitory effect of EMAP II on the EC proliferation was blocked by the soluble α-ATP synthase (Fig. 6). Interestingly, α-ATP synthase polyclonal antibody inhibited the EC proliferation, mimicking the effect of EMAP II (Table I). Collectively, these results suggest that the interaction of EMAP II with α-ATP synthase may inhibit the proliferation of ECs.

We found that the expression of the EMAP II binding molecule, α-ATP synthase, is increased on the surface of the serum-

starved cells and that the α-ATP synthase on the serum-starved cells specifically interacted with EMAP II (Fig. 4). However, we do not know the physiological meaning for the increase of the surface interaction between EMAP II and α-ATP synthase on serum-starved conditions.

In this report, we propose that the function of EMAP II on ECs might be exerted through α-ATP synthase on the surface of ECs (Fig. 6). The possible molecular mechanism of the EMAP II function through ATP synthase on the EC can be similar to that of angiostatin. In the previous studies, ATP synthase was already identified as the binding target of angiostatin that is an anti-angiogenic factor (21, 23). ATP synthase is composed of the F_1 portion of multiple subunits ($\alpha_3\beta_3\gamma\delta\epsilon$) that acts as the catalytic site for ATP synthesis and hydrolysis and the F_0 portion that acts as the proton channel (24). All of the components forming the core catalytic complex of F_1-ATP synthase are present on the external EC surface. F_1-F_0-ATP synthase constitutes the major EC-binding site for angiostatin, which is active in ATP synthesis and inhibits the EC proliferation by blocking conformational changes of the enzyme complex required for ATP synthesis or hydrolysis (21, 23, 25–27). Conclusively, angiostatin inhibits vascularization by suppression of ATP metabolism on the EC surface, which, in turn, may regulate vascular physiology. Although our studies showed that EMAP II interacts with α-ATP synthase, a functional test remains to be performed whether EMAP II directly inhibits the activity of ATP synthase and whether EMAP II competes with angiostatin for the binding to ATP synthase.

The results of this work suggest that the interaction of EMAP II with α-ATP synthase may function as a mechanism for the inhibition of EC growth and play a regulatory role in the tumor vasculature. Although p43/EMAP II was found to interact with ATP synthase, we do not exclude the possibility that it may have other interacting surface molecules working at other physiological conditions.

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