Identification of a New Immunoglobulin Superfamily Protein Expressed in Blood Vessels with a Heparin-binding Consensus Sequence

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ABSTRACT

A novel immunoglobulin-type protein expressed in blood vessels has been identified. The cDNA for AAMP (angio-associated, migratory cell protein) was first isolated from a human melanoma cell line during a search for motility-associated cell surface proteins. Upon analysis of the tissue distribution of AAMP, it was found to be expressed strongly in endothelial cells, cytrophoblasts, and poorly differentiated colon adenocarcinoma cells found in lymphatics. The sequence of AAMP predicts a protein (Mr, 49,000) with distant identity (25%) to known proteins. It contains immunoglobulin-like domains (one with multiple homologies to deleted in colon carcinoma (DCC) protein), the WD40 repeat motif, and a heparin-binding consensus sequence. A 1.6-kilobase mRNA transcript of AAMP is detected in tissue culture cell lines and tissues. Affinity-purified polyclonal antibodies, anti-recombinant AAMP, and anti-peptide 189 (AAMP derived) recognize a Mr, 52,000 protein in human tissue and cellular extracts. The protein size is in keeping with the mRNA and predicted sequence. The AAMP-derived peptide, P189, contains a heparin-binding domain (dissociation constant, 14 pmol) and mediates heparin-sensitive cell adhesion. The shared expression of AAMP in endothelial cells, cytrophoblasts, and tumor cells implies a common function in migrating cells.

INTRODUCTION

Molecules that mediate cell-cell and cell-substrate interactions include members of the immunoglobulin superfamily. These contain immunoglobulin-like domains that share evolutionary homology and function primarily in recognition and binding processes on cell surfaces (1–5). Most are cell surface proteins, a few are intracellular, and some are secreted (4). Immunoglobulin superfamily proteins and the mechanisms involved in their regulation of migratory cells are of special interest. These proteins include those that help mediate endothelial cell interactions with lymphocytes, monocytes, neutrophils, and tumor cells that result in adhesion and transendothelial migration (6–9). Activation of T cells (10, 11) and the processes of cell adhesion and migration in the nervous system also involve immunoglobulin superfamily members (3). The binding mechanisms of some immunoglobulin superfamily proteins that participate in the above processes, including PECAM, NCAM, CD4, as well as other adhesive proteins such as CD44, involve glycosaminoglycans (heparin, chondroitin sulfate, and hyaluronan, in these examples) (10, 12–18). Some immunoglobulin superfamily members are multifunctional and participate in both cell binding and signaling (4, 19). Determining the identity and characteristics of new proteins that participate in cellular interactions involving migrating cells and endothelium should help elucidate mechanisms involved in biological processes such as inflammation, wound healing, and metastases of tumor cells.

We have identified and sequenced cDNA from a human melanoma cell library that encodes a new protein, called AAMP, that shares characteristics with immunoglobulin superfamily proteins and contains a heparin-binding consensus sequence. Immunoblotting and immunohistochemistry using affinity-purified antibodies with specificity for rAAMP and for a derived peptide, P189, were used to identify the native protein in human cultured cells and tissues. In addition, the heparin-binding capacity of peptide 189 was determined in order to evaluate the potential role of this epitope in cell adhesion.

MATERIALS AND METHODS

cDNA Library Screening. A human melanoma A2058 cDNA expression library (Ag11) was screened with an antibody, 1AA3AA, suspected to inhibit cell motility.

1AA3AA Monoclonal Antibody Preparation. Mouse hybridoma clones were generated with A2058 melanoma cells using the myeloma cell line X63.Ag8.653 (a generous gift of R. P. Siraganian, National Institute of Dental Research, NIH, Bethesda, MD) (20–22). They were screened for inhibition of melanoma chemotaxis (23) to autotaxin (24), type IV collagen (Collaborative Biomedical Products, Bedford, MA), and laminin (a gift of S. Aznavorian, National Cancer Institute, NIH, Bethesda, MD).

dNA Sequencing. The Ag11 phage insert, AAMP, from the A2058 cDNA library, was subcloned into Bluescript plasmid (Stratagene, La Jolla, CA) for double-stranded DNA sequencing (both strands; Ref. 25). PCR products generated from the amino terminal region of AAMP cDNA (obtained with human brain mRNA; Clontech, Palo Alto, CA) were sequenced with Taq polymerase (Perkin-Elmer Roche Molecular Systems, Branchburg, NJ) both directly and as subcloned cDNA (26–28).

ALIGN Sequence Comparisons. Immunoglobulin domains (20 residues beyond each predicted cysteine bond) of immunoglobulin superfamily members were ALIGN matched with AAMP (29) using standard parameters. Scores of 3.1, 4.3, and 5.2 SD indicate probabilities of 10−3, 10−5, and 10−7, respectively, that unrelated domains show similarity by chance (30, 31).

Recombinant AAMP Purification. AAMP insert was subcloned into pGEX-1AT EcoRI/BAP plasmid (Pharmacia, Uppsala, Sweden) for production of recombinant AAMP from glutathione S-transferase in Escherichia coli according to the manufacturer’s instructions. Bacteria were induced with isopropyl-β-D-thiogalactopyranoside, sonicated, and centrifuged. Supernates were loaded onto glutathione Sepharose columns (Pharmacia), washed, and thrombin (Sigma Chemical Co., St. Louis, MO) digested for purification of recombinant AAMP from glutathione S-transferase. Digestion (1 h) occurred at a thrombin-sensitive site in AAMP, as 14.

Peptide Preparation. AAMP-derived peptides and variants were synthesized on a Biosearch model 9600 synthesizer (Fullerton, CA) using standard Merrifield solid phase synthesis protocols and t-butoxycarbonyl chemistry and were analyzed by reverse-phase HPLC. To generate peptides from recombinant AAMP, it was reduced and alkylated (N-isopropylidaoacetamide), digested with trypsin, and subjected to HPLC chromatography (32).

Peptide Sequencing. Amino acid sequence analysis was performed using a Protein/Beckman 2000 on-line sequencer (Fullerton, CA) using standard program no. 1. Phenylthiohydantoin amino acid analysis was carried out on a Beckman System Gold system (Fullerton, CA) using a modified sodium acetate gradient program and a Hewlett-Packard narrow bore C18 column.

Polycyclonic Antibody Preparations. Polyclonal antipeptide antibodies specific for AAMP (generated in rabbits using peptides conjugated to bovine albumin) were affinity purified on columns of peptide covalently attached to Affi-Gel 10 beads (Bio-Rad, Richmond CA). Polyclonal anti-recombinant AAMP antibodies were generated in rabbits against recombinant AAMP.
Anti-rAAMP was affinity purified on columns of Affi-Gel 10 beads with recombinant AAMP covalently attached.

**Human Tissue and Cell Lysate Preparations for Immunoblotting.** Human tissues were homogenized in 3% SDS and 4% β-mercaptoethanol buffer at 100°C. Whole cell lysates of A2058 melanoma cells (passed fewer than 20 times) and bovine endothelial cells (passage 6), aortic and corneal (gifts of N. H. Guo and J. Kaiser, both from the National Cancer Institute, NIH, Bethesda, MD), were prepared in 0.5% NP40 buffer.

**Immunoblot Preparation.** Tissue and cell lysates electrophoresed in 10% SDS polyacrylamide gels were blotted and reacted with either affinity-purified rabbit IgG at 100°C. Whole cell lysates of A2058 melanoma cells (passaged fewer than 20 times) and bovine endothelial cells (passage 6), aortic and corneal (gifts of N. H. Guo and J. Kaiser, both from the National Cancer Institute, NIH, Bethesda, MD), were prepared in 0.5% NP40 buffer.

**Northern Blots.** Total RNA from melanoma cells, Jurkat lymphoma cells, human brain, liver, lung, breast, kidney, and placenta and rat liver tissues was extracted (33, 34) and enriched for polyadenylate RNA (35). Total RNA from normal and malignant human gastric and breast tissues was isolated (36). All samples were electrophoresed in formaldehyde 1% agarose gels and blotted. DNA probes (AAMP and glyceraldehyde-3-phosphate dehydrogenase) were labeled with random priming. Hybridization for melanoma cell RNA blots was according to Church (37). All other blots were hybridized overnight at 42°C.

**Immunohistochemistry.** Routine deparaffinized sections of human non-malignant brain, adrenal gland, uterine, and placenta, hyperplastic endometrium, and poorly differentiated colon adenocarcinoma were microwaved for antigen retrieval (38) in 1.5 liters of 10 mM sodium citrate, pH 6.0, at the highest setting for 40 min. Vectastain Elite ABC kits (rabbit and mouse; Vector, Burlingame, CA) were used. Affinity-purified rabbit anti-rAAMP and anti-P189 (0.25—1 μg/ml) were test antibodies. Mouse anti-von Willebrand Factor (1:35; Dako, Carpinteria, CA), rabbit anti-human choricorn gonadotrophin (1:200; Dako), and mouse anti-glial fibrillary acid protein (1:100; Dako) were positive controls. Rabbit IgG (1 μg/ml) and goat anti-rabbit (1:200) were negative controls. Sections were counterstained in hematoxylin.

**Heparin-binding Assays.** Peptides were solubilized (2 mg/ml) by boiling in 50% DMSO/DPBS for 10 min. P189 and P357 (6.25 μg/ml) were immobilized on CovaLink plates (Nunc, Naperville, IL; Ref. 39). CovaLink Buffer without DPBS was used for washes and overnight incubation following peptide linkage. Following buffer removal, tritiated heparin (4 μg/well (0.7 mCi/mg; NEN Dupont, Wilmington, DE), alone, and with unlabeled heparin sodium (0.4—40 μg/well; Sigma) was added in DPBS for 4 h at 27°C ± 2°C. The wells were rinsed, separated, and counted. The predominant heparin molecular weight, tritiated and unlabeled, was M, 17,000—20,000. There were 176 and 143 USP units/mg in unlabeled and tritiated heparin, respectively.

**Cell Binding by P189 and Its Variants.** Peptides were attached to CovaLink plates as described above. For testing the heparin sensitivity of cell binding by P189 and its scrambled variant, P350, the peptide-coated wells (6.25 μg/ml) were incubated with varying amounts of heparin sodium (Lyphomed, Deerfield, IL) for 1 h (27°C ± 2°C). Washes removed unbound heparin before A2058 melanoma cells (100,000/well) were added, suspended in 0.1% BSA/DMEM (diluted 1:2 with DPBS). Following 1-h incubations (37°C) and removal of unattached cells, the remaining cells were stained with Diff Quik (Baxter Healthcare, McGaw Park, IL). Plates were photographed, scanned, and read with densitometry (one densitometer unit represents approximately 84 cells). Cell binding by peptides, with and without the heparin-binding consensus sequence, was performed in 0.1% BSA/DMEM. The Student t test was used to test for difference. The group of peptides with the RXRXRX motif, P189 (RRLRMRSESESE), P358 (RRLRMQOQOSQ), P359 (RRGRRGSESE), and P360 (RRLRMRMEAEAA) was compared to those without it, P350 (MRSRERESRSL), P357 (QOLQOMESSE), and P369 (RRLRMEESE).

**RESULTS**

**AAMP cDNA Sequence.** The screening antibody, 1AA3AA, reacted with three cDNA clones in the human melanoma A2058 cell expression library. They were the same size and cross-hybridized. One selected for sequencing, AAMP, contains a 1335 bp reading frame (aa 8—452; Fig. 1). Amino terminal fragments generated from human brain mRNA with gene-specific, nested primers using 5'-RACE PCR contain sequence that codes for known sequence of AAMP and several additional 5' protein residues consistent with an open reading frame. Four subcloned PCR products code for aa 2–7, followed by a "G" in the 5' direction. One of these products contains a complete AUG codon at this site, consistent with an initiating methionine. Several other products that also code for aa 2–7 show alternative sequence (<40 bp) replacing the AUG codon at this location that is currently being characterized. The reactivity of the anti-peptide antibody generated to P189 (aa 14—25) with the natural protein (Fig. 2) indicates that coding message for protein is present in the amino terminal region of AAMP. A Kozak sequence (40) has not been found. Twenty-two peptides obtained as digestion products from recombinant AAMP were sequenced to confirm that the cDNA sequencing of the A2058 library clone predicted the protein sequence. Recombinant protein was reduced, alkylated, and digested with cyanogen bromide and trypsin or with trypsin alone. These peptide sequence runs, which averaged eight residues in length, located the corresponding peptides with their amino termini at the following residue positions: 15, 121, 134, 139, 153, 178, 199, 204, 221, 228, 241, 248, 259, 264, 273, 304, 312, 402, 410, 416, 429, and 444. Polycyclonal antibodies (anti-P189 and anti-P279), generated against peptides derived from AAMP at positions 14 and 352, reacted with recombinant protein encoded by AAMP cDNA, also confirming the cDNA sequencing. Excess peptides, P189 and P279 (5000X molar concentrations), competitively inhibited these reactions appropriately.

**AAMP Protein Expression.** Anti-recombinant AAMP reacts with a M, 52,000 protein in immunoblots of extracts from human cells and tissues. These include A2058 melanoma cells, bovine endothelial cells (aortic and corneal), activated T cells, and the following tissues: metastatic melanoma, adult liver, skin, kidney, heart, lung, lymph node, and skeletal muscle. AAMP was also detected in blots of bovine brain tissue. Anti-P189, raised against a synthetic peptide whose sequence was derived from AAMP, also reacted with AAMP in A2058 melanoma cells, activated T cells, and brain tissue (human and bovine). Examples of these blots are shown in Fig. 2A. The reactivity of the protein with anti-P189 can be competed with 5000X molar concentration of peptide 189 (Fig. 2B). Reactivity with anti-recombinant AAMP is competed also (2100X excess molar concentration). Reactivity of the M, 52,000 protein in cells and tissues on immunoblots with both antibodies that have specificity for AAMP (recombinant protein and a derived peptide) indicate that the M, 52,000 protein must be the native form of AAMP. Its molecular weight is very close to that predicted by sequencing (M, 49,000) and corresponds with the 1.6-Kb message. The original screening antibody, 1AA3AA, reacts with two bands, one of which (the smaller) corresponds to AAMP in size (data not shown).

**AAMP mRNA Expression.** The AAMP mRNA transcript in A2058 melanoma cell blots is 1.6 Kb (Fig. 3), consistent with the AAMP cDNA (1787 bp). Northern blots of other human cells and tissues show that the message of AAMP is also expressed in cultured human breast cancer cells (MCF7 and MDA231), phorbol myristate acetate/phytohemagglutinin-stimulated T cells and monocytes, Jurkat lymphoma cells, fetal brain, lung, liver, and kidney tissues, and adult heart, lung, brain, liver, breast, pancreas, kidney, placental, stomach mucosal, gastric adenocarcinoma, and breast carcinoma tissues. Examples of the message in RNA blots of human tissues are shown in Fig. 4.

**AAMP Homologies.** Since early nucleic acid searches indicated a relationship between AAMP and CD4, an immunoglobulin superfamily member, the sequence of AAMP was analyzed for the presence of immunoglobulin-like domains. The amino acid region 94—303 contains predicted β sheets and turns consistent with immunoglobulin
Fig. 1. The nucleotide sequence of AAMP cDNA with its predicted amino acid sequence. Base pairs 22-1787 are from a clone isolated from an Agt11 expression library of human A2058 melanoma cells. The first 21 bp are from PCR-generated amino terminal products obtained from human brain tissue. Nucleotide residues are numbered beginning at the 5' end. Amino acid sequence numbering begins with the initiating methionine. The putative heparin binding site, aa 14-18, is underlined with " " . The acidic region, aa 42-102, is underlined with " " . Cysteine pairs, 114 and 148 and 234 and 283, predicted by immunoglobulin domain homology to most likely form disulfide bonds, are marked "<>". The carboxy terminal boundaries of the WD40 repeats are indicated by brackets "[ ]" around the last pair of amino acid residues in each repeat. The potential transmembrane region, aa 341-362, is underlined, " " . The polyadenylation site at nucleic acid residues, 1744-1750, is in parentheses.
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Fig. 2. Immunoblots of cultured human cells and tissues probed with polyclonal AAMP-specific antibodies. The immunoreactive Mr 52,000 protein is the putative AAMP protein (see text). A. Lanes 1 and 2 are nonreduced NP40 whole cell lysates of human A2058 melanoma cells (117 µg/lane) and bovine aortic endothelial cells (41 µg/lane), respectively, reacted with anti-rAAMP (2 µg/ml). Lanes 4–11 and 13 are human tissue SDS lysates (reduced) from brain, skin, kidney, melanoma, lung, lymph node, liver, heart, and brain, respectively, also reacted with anti-rAAMP (1 µg/ml). Lane 15 shows the immunoreactivity of AAMP in reduced brain SDS lysate with anti-P189 (1 µg/ml). Lanes 4–11, 13, and 15 were loaded with 40, 26.5, and 26.5 µg protein, respectively. Anti-rAAMP was generated with affinity-purified rAAMP (see text). Anti-P189 was generated to a synthetic peptide derived from the sequence of AAMP. Both antibodies were produced in rabbits and were affinity purified. Lanes 3, 12, and 14 contain protein standards with apparent molecular weights of 215,000, 105,100, 69,800, 43,300, and 28,300 in vertical descending order. The molecular weight of AAMP is unaffected by the presence or absence of reducing agents. B. Lanes 1 and 6 show competition of antibodies' binding to AAMP for anti-P189 (0.5 µg/ml) and anti-rAAMP (2 µg/ml), respectively. Competition was 5000-fold for anti-P189 and 2100-fold for anti-rAAMP on a molar basis using P189 and rAAMP, respectively. Lanes 3 and 5 were reacted with antibodies only. Brain lysate (40 µg each) was loaded in Lanes 1 and 3, and A2058 melanoma cell lysate (117 µg each) was loaded in Lanes 5 and 6. Lanes 2 and 4 contain protein standards (M, 215,000, 100,000, 69,000, 43,000, 28,000 and 206,000, 105,000, 71,000, 44,000, and 28,000, respectively). Gels were 10% polyacrylamide with SDS present.

Fig. 3. Northern blot of human melanoma A2058 cells probed with AAMP cDNA. Lanes 1 and 2 contain total (41 µg) and polyadenylate-enriched A2058 RNA(2.2 µg), respectively. The 1.6-kb message of AAMP is seen in both lanes. The origin is indicated by dotted lines. Positions of the RNA standards are shown.

Fig. 4. Northern blot demonstrating the widespread tissue distribution of AAMP's mRNA. All RNA was from normal human tissues. Lane 1: brain, adult. Lane 2: brain, fetal. Lane 3: liver, adult. Lane 4: liver, fetal. Lane 5: placental. Lane 6: breast. Two µg of polyadenylate-enriched RNA were loaded in each lane. A. AAMP, 1.6-kb message. B. Glyceraldehyde-3-phosphate dehydrogenase, standard.
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Fig. 5. ALIGN scores for immunoglobulin superfamily members with the immunoglobulin-like domains of AAMP. Immunoglobulin-like domains (in parentheses) of the immunoglobulin superfamily members listed were compared with potential immunoglobulin superfamily members: NCAM, deleted in colon carcinoma (DCC) protein, neural-glial cell adhesion molecule (NgCAM; Ref. 59), myelin-associated glycoprotein superfamily members: NCAM, deleted in colon carcinoma (DCC; Ref. 42). Identical and/or similar amino acid residues are boxed together. Similar amino acids are grouped as follows: S.T; R.K: Q,N; E,D; F,W,Y; and L,M,I,V.

**Heparin Binding.** Peptide 189, derived from the amino terminal region of AAMP, contains the sequence RRLRRM, which resembles a consensus sequence for heparan sulfate binding, -XBBXBX- (B's shares a similar arrangement of WD40 repeats, contains a domain similar to the immunoglobulin-type domains of AAMP (ALIGN scores of 6.58 and 6.79 SD), and its entire sequence shows overall similarity with AAMP (ALIGN score of 10.68 SD; 97 runs).

AAMP has one potential transmembrane region, aa 341–362, predicted by the method of Rao and Argos (50). A large, negatively charged region, aa 42–102, including a run of seven glutamic acid residues, is present near the amino terminal end (Fig. 1). The isoelectric point (calculated from sequence data) is 4.14. AAMP has an instability index of 35.9 and is thus predicted to be a stable protein (index < 40; Ref. 51).

**Immunohistochemistry.** In all human tissues tested (brain, placenta, periaortic connective tissue, colon, and uterus), anti-rAAMP and anti-P189 strongly stain endothelial cells in all vessel types seen, compared to controls. Vessels in brain, uterine wall, and colon are shown (Fig. 7, A, B, D, G, and H). Anti-rAAMP also strongly stains small groups of poorly differentiated colon adenocarcinoma cells found in lymphatics near the primary tumor and subpopulations of mononuclear inflammatory cells (Fig. 7, G and H). Cytotrophoblasts stain strongly and syncytiotrophoblasts less strongly (Fig. 7, J and K). Surface staining of glandular cells in the uterine endometrium is seen with both anti-rAAMP and anti-P189 (Fig. 7, M and N). Some central nervous system neurons and their processes show moderate staining.

**WD40 motif**

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**Fig. 5.** ALIGN scores for immunoglobulin superfamily members with the immunoglobulin-like domains of AAMP. Immunoglobulin-like domains (in parentheses) of the immunoglobulin superfamily relationships (30, 31). Immunoglobulin-like AAMP domains (specified above the columns), and the resulting scores in standard deviations (SD) are listed. Scores greater than 3.00 SD (outlined) indicate significant evolutionary immunoglobulin superfamily relationships (30, 31). Immunoglobulin superfamily members: NCAM, deleted in colon carcinoma (DCC; Ref. 58). Identical and/or similar amino acid residues are boxed together. Similar amino acids are grouped as follows: S.T; R.K; Q,N; E,D; F,W,Y; and L,M,I,V.

**Fig. 6.** A. Homology of the immunoglobulin-like domain of AAMP (defined by cysteines 234 and 283) with the immunoglobulin-like domains (I, III, and IV) of deleted in colon carcinoma (DCC; Ref. 42). Identical and/or similar amino acid residues are boxed together. Similar amino acids are grouped as follows: S.T; R.K; Q,N; E,D; F,W,Y; and L,M,I,V. Note that seven identities and four additional similarities are conserved in all domains. Gaps introduced to optimize alignments are shown as dashes. The significant ALIGN score listed in Fig. 5 [the domain of AAMP compared with DCC (IV)] indicates that there is an evolutionary relationship. B, the WD40 repeat motif found in AAMP is shown. This motif, indicated at the bottom, is found in β transducin and other proteins (45–49, 54). Residues in parentheses are interchangeable. The identities of AAMP with the motif are boxed. The first four repeats are contiguous. The fifth and sixth repeats are also contiguous. Gaps are as described above. The functional significance of the motif is not known, but there is speculation that it may be involved in protein-protein recognition (54).
Fig. 7. Immunoperoxidase staining of human tissues with anti-recombinant AAMP and anti-P189. Anti-rAAMP (1 μg/ml) stains endothelial cells (veins and capillaries) as seen in sections of brain (A and B). It also stains endothelial cells in the uterine wall (D) and in the colon (G and H). In the colon, venules are shown (with red cells), and lymphatics (containing tumor and mononuclear inflammatory cells and lined with discontinuous endothelial cells) are shown in (G) and (H). Arterioles are seen in the upper portion of (G). Endothelial cells stained with anti-von Willebrand Factor (positive control) are seen in (E). The small groups of poorly differentiated colon adenocarcinoma tumor cells present in the lymphatics stain strongly positive with anti-rAAMP (1 μg/ml). Cytotrophoblasts stain strongly, and syncytiotrophoblasts stain moderately in sections of placenta (J and K). The concentration of anti-rAAMP is 0.25 μg/ml in (J) and 1 μg/ml in (K). Anti-rAAMP (1 μg/ml) and anti-P189 (1 μg/ml) stain endometrial glandular cell surfaces in (M) and (N), respectively. Staining with rabbit IgG (1 μg/ml) used as a negative control is seen in (C), (F), (I), (L), and (O). Paraffin-embedded tissues were prepared routinely, deparaffinized, immersed in buffer, and heated in a microwave oven for "antigen retrieval" (38) prior to staining. Anti-rAAMP and anti-P189 were generated as polyclonal antibodies in rabbits and were affinity purified using pure antigenic recombinant protein and peptide. Goat anti-rabbit secondary antibody conjugated with horseradish peroxidase was reacted with diaminobenzidine and peroxidase. Sections were counterstained with hematoxylin. The bars shown on (C), (F), (I), (L), and (O) indicate the magnifications (100 μm) for their rows.
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are basic and X's are hydrophobic residues; Ref. 52). Solubilized P189, attached to CovaLink plates (0.3 μg/well) binds heparin strongly with a dissociation constant, \( K_d \), of 14 pmol (Fig. 8A) according to Scatchard analysis (53) and is saturable (Fig. 8B). A peptide variant of P189 that lacked the heparin-binding consensus sequence, P357 (sequence in “Materials and Methods”), showed no consistent heparin binding. This was also seen with plates prepared with no peptide. Heparin binding for P189 was at background levels (no peptide present on the plates) when competing unlabeled heparin (1–10X) was added. The presence of a heparin-binding epitope in recombinant fusion AAMP protein was confirmed qualitatively by comparing its binding of tritiated heparin to that of a control protein, BSA. Recombinant fusion AAMP protein (approximately 15 μg), gel purified away from bacterial proteins, and transblotted to Immobilon-P bound tritiated heparin (10.6 μCi/ml) 2.2 ± 0.4 times more (six assays) and was competed (77% reduced) with an equal amount of unlabeled heparin (three assays).

Cell Binding by P189 and Its Variants. P189 and its variants that contain the RRXRRX sequence also bind A2058 melanoma cells (CovaLink assays). Cell binding to P189-coated plates was pronounced and heparin sensitive (Fig. 9). In contrast, plates prepared with P350, the scrambled version of P189 with dispersed basic residues, showed only slight cell binding with no competition by heparin at most concentrations (Fig. 9A). The cell binding of P189 was 2.95 times that of P350 with no heparin present. The morphology of cells in culture bound to P189 appears similar to that of the few cells that bound to P350 (Fig. 10). Peptides were solubilized and immobilized on linker arms 2 nm above the plate surfaces. Some cells were able to spread slightly with both peptides, and this is shown for P189 (Fig. 10C). The pictures in Fig. 10 are higher magnification photographs of the bottom wells (no heparin present) in Fig. 9A. In additional assays, peptide variants containing the RRXRRX motif, P189, P358, P359, and P360, showed comparable cell binding (no heparin), with results ranging from 66,000 ± 6,000 to 72,000 ± 11,000 cells/well. Peptides

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**Fig. 8.** Heparin binding mediated by the derived peptide of AAMP, P189. Solubilized P189 (0.3 μg/well) was attached to CovaLink plates and incubated with tritiated heparin, 4 μg/well, alone, and with fresh unlabeled heparin (0.4–40 μg/well) of the same molecular weight. Following incubation and washing, the wells were counted to determine their tritiated heparin content. cpm from multiple wells (3–5) for each amount of competing unlabeled heparin were averaged. Calculations using amounts of tritiated heparin displaced provided the amounts of unlabeled heparin bound/well as the concentration of unlabeled heparin increased. A, a Scatchard plot was generated (54) showing strong heparin binding with a dissociation constant, \( K_d \), of 14 pmol. B, the heparin binding of P189 is saturable as shown.

**Fig. 9.** The inhibition by heparin of cell binding by P189. Increasing concentrations of heparin were added in wash solutions to CovaLink wells coated with P189 and P350 (scrambled version of P189). These peptides had been previously solubilized and attached to the plates. Unbound heparin was removed prior to addition of A2058 melanoma cell suspensions. Following a 1-h 37°C incubation, unbound cells were removed, and the cells remaining on the plates were stained. A, the stained cells bound by P189 are seen in the first two lanes labeled 3 and 7. Those bound by P350 are in Lanes 6 and 8. The bottom wells were never exposed to heparin, and the others in ascending order were exposed to 0.5, 1, 5, 10, 25, 50, and 100 units of heparin in a wash prior to the addition of cells. B, densities of stained cells bound by P189 (O) and P350 (•) (computer scanned) were quantitated by image analysis. Each densitometry unit represents approximately 84 cells.
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Fig. 10. Magnified views of the wells with no heparin from Fig. 9A are shown to illustrate cell morphology with exposure to immobilized P189 and its scrambled version. P350. A, peptide 350. B, peptide 189. C, closer view of P189-exposed cells. P189 is oriented on linker arms (2 nm above the plate surfaces) so that its heparin-binding site is available. More cells bind to P189 compared to the control peptide. P350, but the morphology of the bound cells does not appear to differ significantly for the two peptides.

The tissue and cellular distributions of AAMP seen on RNA blots (1.6-kb message; Fig. 4) and on immunoblots (Mr 52,000 band identified by both anti-recombinant AAMP and anti-AAMP derived peptide; Fig. 2) were compared. Correspondence of the widespread distribution seen only for the Mr 52,000 protein with the broad mRNA distribution also supports its identity as the AAMP protein. The Mr 52,000 band is also the only prominent band that reacts with both anti-rAAMP and anti-P189 on immunoblots (Fig. 2). Also, it is almost the same size as predicted by the sequence of its cDNA clone (Mr 49,000). Although the broad distribution of AAMP in tissues could be attributed to its presence in endothelial cells, it also appears in other cell types, including many with migratory potential in human tissues (A2058 melanoma cells from a brain metastasis, metastatic melanoma tissue, and activated T cells on immunoblots, and in the following cells with immunohistochemistry: cytotrophoblasts, invasive poorly differentiated colon adenocarcinoma cells in lymphatics, mononuclear inflammatory cells, and some central nervous system neurons). RNA isolated from several types of human cells in tissue culture (melanoma, breast cancer, lymphoma, monocytes, and T cells) contained AAMP mRNA as shown on Northern blots.

The reactivity for AAMP in endothelium may provide clues in determining its function. Endothelial cells express several immunoglobulin superfamily proteins such as PECAM, Cell-Cam 105, LFA-3, ICAM-1, ICAM-2, VCAM, and HT7 (4, 10, 55, 56). Endothelial cells also interact with immunoglobulin-type proteins on migratory cells that adhere to the endothelium (lymphocytes, neutrophils, and tumor cells). In general, immunoglobulin-type proteins are known to be part of the complex collection of binding proteins that mediate selective and nonselective adhesion and transendothelial migration of various nonmalignant and malignant circulating cells (6, 55). Studies have shown that endothelial adhesion proteins vary in their expression (constitutive or induced), location (cell surface or intracellular granules that relocate to the surface when induced), type

**DISCUSSION**

AAMP is a newly identified protein found in normal and malignant human tissues that shares sequence homology with two types of proteins, immunoglobulin superfamily members and proteins that contain the WD40 repeat motif. The homologies of the immunoglobulin superfamily members with the AAMP domain defined by cysteines 234 and 283 identify AAMP as an immunoglobulin superfamily member. The immunoglobulin superfamily relatives (Fig. 5) include proteins that either are known or suspected to play a role in cell adhesion (NCAM, NgCAM, MAG, CD2, and DCC; Refs. 3, 4, and 42). Most are in the immunoglobulin superfamily C2 subset. The eleven identities/similarities that three domains in DCC share with one domain in AAMP may constitute a previously unrecognized motif (Fig. 6A). The AAMP domain defined by cysteines 114 and 148 is possibly a second immunoglobulin domain. Other potential domains in AAMP defined by the cysteine pairs, 157 and 226 and 300 and 344, show weaker immunoglobulin domain homology.

The WD40 repeat motif found in β transducin and other proteins is speculated to represent a general protein-protein recognition/binding site (54). Several of these, such as β transducin, PRP4, and Tup1, are parts of large protein complexes (47, 48). This motif is present as six repeats or half repeats in AAMP. Many of the variations in the GH and WD amino acid positions seen in some of the repeats of AAMP can also be found in those of known members (48). The first four WD40 repeats in AAMP are contiguous and involve a large portion of the region containing its immunoglobulin-type domains. The fifth and sixth repeats are in the carboxy terminal region of AAMP. The fifth repeat involves the potential transmembrane region. Of all the repeats present in AAMP, the sixth one shows the strongest homology with the WD40 motif. It is notable that AAMP contains a region that shares homology with both immunoglobulin-type domains and WD40 repeats. These motifs are both thought to contain β strands (30, 31, 46). The potential heparin-binding domain in the amino terminal region of AAMP and the large, negatively charged region (61 amino acids in length) that follows it, precede the region of immunoglobulin-type domains and WD40 repeats.

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of binding (homophilic, heterophilic, and some use bridging molecules), and dependence on calcium (3, 6, 7, 57). Diffuse cellular staining of some cells for AAMP (Fig. 7, J and K) does not rule out its possible participation in these processes.

The domain in AAMP which has the strongest homology with known immunoglobulin domains contains a large region (aa 231–299) that shares local homology with immunoglobulin domains in two endothelial adhesive proteins, HT7 (ALIGN score, 3.43 SD) and PECAM (ALIGN score, 3.63 SD). The cellular distribution of AAMP is similar to that of PECAM (endothelial cells and migratory cells including monocytes, T cells, and tumor cells). PECAM mediates cellular aggregation that is inhibited by heparin and chondroitin sulfate (18). Similarly, the derived peptide of AAMP, P189, also binds cells in a heparin-sensitive manner (Fig. 9). In addition to its normal physiological functions, PECAM participates in intercellular adhesive processes involved in tumor metastasis, such as tumor cell–tumor cell and tumor cell–platelet-endothelial interactions (8). AAMP’s sequence homologies, distribution among endothelial and migratory cells, and potential for heparin-sensitive adhesive functions indicate that it may also participate in adhesion/migration activities.

ACKNOWLEDGMENTS

We thank Reuben P. Siraganian, Cynthia Fischer, Susan M. Mackem, Douglas E. Moul, Mark E. Sobel, and Edward A. Unsworth for advice and technical assistance. William G. Stetler-Stevenson, Anna T. Levy, and Jean E. Maguire allowed us to reprove previously prepared Northern blots (data not shown). Mariana Doval (Washington Hospital Center, Washington, DC), Stephanie Barr (George Washington University, Washington, DC), and Jean Colandrea (St. Agnes Hospital, Baltimore, MD) provided tissue samples.

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