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Characterization of Vasoactive Intestinal Peptide Receptors on Human Megakaryocytes and Platelets

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Vasoactive intestinal peptide receptor I (VIPRI) expression was examined in megakaryocytes using reverse transcriptase-polymerase chain reaction (RT-PCR). VIPRI protein was characterized in platelet membranes using covalent crosslinking techniques. Human megakaryocytes were isolated from suspension cultures of cord blood and adult bone marrow mononuclear cells using a murine monoclonal antibody to human platelet glycoprotein IIB/IIIA (CD41) and immunomagnetic beads. RT-PCR primers were constructed for the VIP, VIPRI, and VIPRII genes as well as for megakaryocyte specific genes, *c-mpl* and platelet factor 4 (PF-4). VIP, VIPRI, *c-mpl*, and PF-4 were coexpressed in megakaryocyte mRNA. Southern blot analysis confirmed the expression of VIPRI.

^{125}I-VIP was covalently cross-linked to human platelet membranes using the homobifunctional reagent disuccinimidyl suberate, followed by polyacrylamide gel electrophoresis and autoradiography. A ^{125}I-VIP-protein complex of M_r = 50,000 was identified. Labeling of the M_r = 50,000 component was completely abolished by unlabeled VIP, but not by peptide histidine methionine or growth hormone releasing factor, indicating specific binding of VIP to the platelet membranes. Taken together, these results suggest that VIP may have direct effects on megakaryocytopoiesis and support our earlier observations of VIP modulation of platelet aggregation.

VASOACTIVE intestinal peptide (VIP) is a 28 amino acid neuropeptide that is widely distributed in mammalian tissues.[1] The name, VIP, was coined to describe its potent vasodilatory action in lung tissue.[2] VIP functions as a neurotransmitter, a hormone, and also as an immune regulator,[3] but, to our knowledge, a possible role in hematopoiesis has not been explored.

Neuropeptide modulation of the immune response has been well-documented.[4,5] The effects of VIP, somatostatin, and substance P on immune functions are mediated via specific, high affinity receptors on both T and B lymphocytes as well as on monocytes.[6-9] VIP modulates lymphocyte proliferation, IgA production, natural killer cell cytotoxicity, and lymphocyte migration.[4] Substance P is a comitogen for T lymphocytes and also enhances Ig synthesis.[10,11] Both effects are mediated through modulation of cytokine production.[12,13] Substance P also stimulates synthesis and release of interleukins-1 and -6 (IL-1, IL-6) as well as tumor necrosis factor in human monocytes.[14] In contrast, somatostatin exerts a negative regulatory role on lymphocyte proliferation, Ig synthesis, and cytokine production.[4,8,15]

More recently, neuropeptides have been shown to regulate hematopoiesis.[14,16] Substance P regulates hematopoiesis by inducing the release of IL-3 and granulocyte-macrophage colony stimulating factor from bone marrow (BM) mononuclear cells.[14] A specific substance P receptor on BM cells was suggested by the effect at low substance P concentrations (10^{-11} mol/L) and inhibition of the effects by substance P antagonists.

Neuropeptide modulation of platelet function and megakaryocytopoiesis is largely unexplored. Neuropeptide tyrosine (Neuropeptide Y, NPY) has been identified in both rat and human megakaryocytes,[17,18] but NPY receptors have not been identified on BM cells. VIP has been shown to elevate cAMP and inhibit platelet aggregation in vitro.[19] These functional studies suggest that platelets or megakaryocytes express neuropeptide receptors, but this hypothesis has not been directly tested at the molecular level. In a previous study, we have shown that human platelet membranes possess high affinity VIP receptors that modulate platelet aggregation.[20] The present study was designed to examine VIPRI and VIPRII gene expression in megakaryocytes using reverse transcriptase-polymerase chain reaction (RT-PCR) and to characterize the structure of VIPR protein using covalent cross-linking methods.

MATERIALS AND METHODS

Human subjects. Human use approval was obtained from the Institutional Review Boards of The Ohio State University Hospitals and Columbus Children's Hospital. Venous cord blood was drawn from the umbilical vessels of placentas of normal, full-term infants immediately after delivery. Peripheral blood (PB) (100 mL) or BM (10 mL) was obtained from healthy adult donors after appropriate informed consent.

Megakaryocyte culture and isolation of megakaryocyte mRNA. Cord blood and adult BM were collected and diluted in calcium and magnesium-free phospate-buffered saline (PBS) solution. Diluted cells were layered over an equal volume of isotonic Percoll gradient solution (Pharmacia, Uppsala, Sweden) adjusted to a density of 1.077 g/mL. Gradients were centrifuged at 400*g* for 30 minutes at 22°C and buoyant mononuclear cells (MNC) were washed twice in PBS. The MNC were gently underlayed with fetal bovine serum (FBS) (Hyclone Sterile Systems, Logan, UT) and centrifuged, 10 minutes, 100*g* to remove platelets.[21] The cells were washed with Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO, Grand Island, NY), resuspended in IMDM containing 10% human AB serum, and placed in 100-mm plastic Petri dish for 1 hour at 37°C, 5% CO_2 for monocyte depletion. Nonadherent (NA) cells at 1.0×10^6 NA cells/mL were placed in IMDM supplemented with 5% human AB plasma, IL-3 (5 ng/mL), c-kit ligand (20 ng/mL), and 5% postirradiated canine serum at 37°C and 5% CO_2.

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Table 1. Sequence of Primers and Expected Size of RT-PCR Products

	Sense Primer	Antisense Primer	Expected Size of RT-PCR Products (bp)
PF-4	(nucleotide −2)5′-TAGTAGCTAACTCTCCAAAAG 3′	(nucleotide 312)5′-TAGTAGCTAACTCTCCAAAAG 3′	315
c-mpl	(nucleotide 843)5′-TGGAGATGCAGTGGCACTTG 3′	(nucleotide 1048)5′-AGAACTGTGGGGTCTGTAGT 3′	206
VIPRI	(nucleotide 563)5′-CTTCTGGTCGCCACAGCTATCCTG 3′	(nucleotide 1096)5′-ACTGCTGTCACTCTTCCTGATATC 3′	534
VIPRII	(nucleotide 372)5′-CGTCACGGTGCCCTGCCCAAAAGT 3′	(nucleotide 835)5′-GCCCCTCACAGCAGCCAGAAGA 3′	464
VIP	(nucleotide 301)5′-GCCAGGCATGCTGATGGAGTTTTC 3′	(nucleotide 531)5′-CCTCTTTCCATTCAGAATTGAGTT 3′	231
CD4	(nucleotide 1009)5′-GTGAACCTGGTGGTGATGAGAGC 3′	(nucleotide 1446)5′-GGGGCTACATGTCTTCTGAAACCGGTG 3′	438
CD8	(nucleotide 776)5′-TTTCGGCGAGATACGTCTAACCCTGTGC 3′	(nucleotide 1154)5′-TTTAGCCTCCCCCTTTGTAAAACGGGCG 3′	379
CD11a	(nucleotide 1680)5′-TCAACGGCGATGGGCTGGTAGAC 3′	(nucleotide 2086)5′-GCGGCCTTGGAACTGGGGGTAG 3′	407

Megakaryocyte precursors were isolated by immunomagnetic beads on day 7 of suspension culture. Cells were exposed to 10 μg (10 μg/10^7 cells) of murine antihuman CD41a (platelet glycoprotein IIb/IIIa) monoclonal antibody (AMAC, Westbrook, ME) on a rotator for 30 minutes. After two washes, 10 μL of magnetic beads (4 beads/target cell) coated with goat antimouse IgG (Dynal, Great Neck, NY) were added to the cells and incubated on a shaker for 30 minutes at 4°C. The cells, rosetted with immunomagnetic beads, were collected with the magnet, and washed twice with PBS. Cytospins were prepared and stained for glycoprotein IIb/IIIa by immunoperoxidase technique[22] to confirm the purity of megakaryocytes. The mRNA was isolated from cell pellets using the Micro-Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA).

A human megakaryoblastic cell line (CMK) established from the PB of a patient with acute megakaryoblastic leukemia was kindly provided by Dr T. Sato.[23,24] CMK cells were cultured in a liquid culture system with IMDM, supplemented with 10% FBS; media was changed every 3 to 4 days.

RT-PCR. RT-PCR was performed using a commercial kit (Perkin Elmer, Norwalk, CT). Megakaryocyte mRNA was reverse transcribed using random hexamers as primers followed by 40 PCR amplification cycles (94°C for 1 minutes, primer annealing at 60°C for 2 minutes, extension at 72°C for 3 minutes) using a Temp-Tronic thermal cycler (Thermolyne, Dubuque, IA). Primers specific for the *c-mpl,* PF-4, VIP, VIPRI, VIPRII, and CD11a (leukocyte function-associated antigen) are shown in Table 1.[25-31] Primers for CD4 and CD8 were purchased from Clontech (Palo Alto, CA).

Southern blot. Southern blot analysis[32] was used to confirm the identity of the VIPRI cDNAs. RT-PCR products were separated on an ethidium bromide stained 1% agarose gel; the gel was washed twice in 0.4 N NaOH, 1.5 mol/L NaCL for 15 minutes, once in 1.5 mol/L NaCL, 0.5 mol/L Tris-HCL, pH 7.4 for 15 minutes, once with 10 × SSC (1.5 mol/L sodium chloride, 0.15 mol/L sodium citrate) for 10 minutes at room temperature. Nucleic acids were transferred to nylon membrane and cross-linked to the membrane using a UV Stratalinker (Stratagene, La Jolla, CA). Nonspecific binding of the DNA probe was blocked by incubating the membrane with 100 μg/mL salmon sperm DNA at 42°C for 4 hours. The membrane was hybridized overnight with a ^{32}P-labeled 534 bp human VIPRI or VIPRII probe (generated by RT-PCR amplification from HT29 human colonic cell mRNA and verified by DNA sequencing). The membrane was then washed twice in 2 × SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature and twice in 0.1 × SSC, 0.5% SDS at 65°C. Autoradiography was performed using Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

Preparation of platelet membranes. Five hundred milliliters of PB anticoagulated with 14% citrate-phosphate-dextrose was centrifuged 1,000*g* for 3.5 minutes according to method of Baenziger and Majerus.[33] The resulting platelet-rich plasma (PRP) was immediately passed through an Imugard IG500 filter (Terumo, Tokyo, Japan) to remove leukocytes. After filtration, the platelet/leukocyte ratio was ≥2,000:1, and platelet/erythrocyte ratio was ≥20:1. The resulting PRP (100 cc) was centrifuged 120*g,* 12 minutes, 25°. The pellet containing leukocytes and erythrocytes was discarded; PRP was centrifuged at 1,200*g,* 15 minutes; and the supernatant was discarded. The platelet pellet was washed in washing buffer (5.5 mmol/L glucose, 113.7 mmol/L NaCl, 3.2 mmol/L K_2HPO_4, 4.3 mmol/L Na_2HPO_4, 24.4 mmol/L NaH_2HPO_4, 2.94 mmol/L EDTA, pH 7.4) and recovered by centrifugation, 1,200*g,* 15 minutes. Platelets were resuspended in washing buffer and centrifuged twice at 80*g,* 8 minutes. The purified PRP was then centrifuged, 1,400*g,* 10 minutes and the final supernatant discarded. Platelets containing no detectable leukocytes or erythrocytes were suspended in 2 mL of cold lysing buffer (10 mmol/L Tris, 5 mmol/L EDTA, pH 7.4) and disrupted on ice with a polytron at a setting of "8" for 1 minute. The crude homogenate was diluted to 40 mL with lysing buffer and centrifuged at 26,000*g* for 30 minutes at 4°C. The membrane pellet was suspended in 4 mL of Buffer A (20 mmol/L Hepes, 2 mmol/L $MgCl_2$, 5 mmol/L EDTA, 1 mmol/L 2-mercaptoethanol, 150 mmol/L NaCl, 50 μg/m phenylmethylsulfonylfluoride pH 7.4). Protein was determined by the method of Lowry et al[34]; membrane protein was aliquoted and stored at −80°C until use.

Covalent cross-linking of ^{125}I-VIP to platelet membrane proteins. Cross-linking of ^{125}I-VIP to platelet membranes was performed according to the method of Wood and O'Dorisio.[35] Platelet membrane protein (200 μg) was incubated with 300pmol/L ^{125}I-VIP at equilibrium (45 minutes at 17°C) in the absence or presence of 1 μmol/L unlabeled VIP. Membranes were centrifuged 48,000*g* for 10 minutes to remove unbound VIP. Membrane-bound ^{125}I-VIP was incubated with 1 mmol/L disuccinimidyl suberate (DSS) for 20 minutes at room temperature. Glycine (final concentration 40 mmol/L) was added to quench unreacted crosslinker, and samples were centrifuged to regain the membrane pellet. Platelet membrane pellets were solubilized in 0.3 mL lysis buffer (9 mol/L Urea, 2% Nonidet P-40, 100 mmol/L dithiothreitol [DTT]), and mixed vigorously. Then 0.3 mL treatment buffer (20% glycerol, 80 mmol/L DTT, 4% SDS, trace of bromphenol blue) was added and mixed vigorously again. Cross-linked platelet membranes were kept in −80°C until electrophoresis. After thawing, 70 μL samples were electrophoresed according to Laemmli.[36] The separating gel was 10% acrylamide, 0.375 mol/L Tris-HCl, pH 8.8, and 0.1% SDS. The acrylamide:bisacrylamide (Bio-Rad Laboratories, Richmond, CA) ratio was 36.5:1, and the gel thickness 1.5 mm. The tank buffer was 0.025 mol/L Tris, pH 8.3, 0.192 mol/L glycine, 0.1% SDS. Electrophoresis was performed at 20 mA constant current for 30 minutes and then at 60 mA until the tracking dye approached the bottom of the gel. Gels were fixed in a fixing solution (9% acetic acid, 50% methanol) for 2 hours. Staining was performed in 0.2% Coomassie brilliant blue, 9% acetic acid, 50% methanol for 1 hour. Fixed and stained gels were destained in a solution of 9% acetic acid, 50% methanol overnight, and dried onto Whatman No. 1 filter papers (Whatman International, Ltd, Kent, UK). Autoradiography was performed at −80°C with Kodak XAR-5 film (Eastman Kodak) and a Du Pont Lightning Plus intensifying screen (Du Pont Co, Wilmington, DE) for 7 to 10 days. Molecular

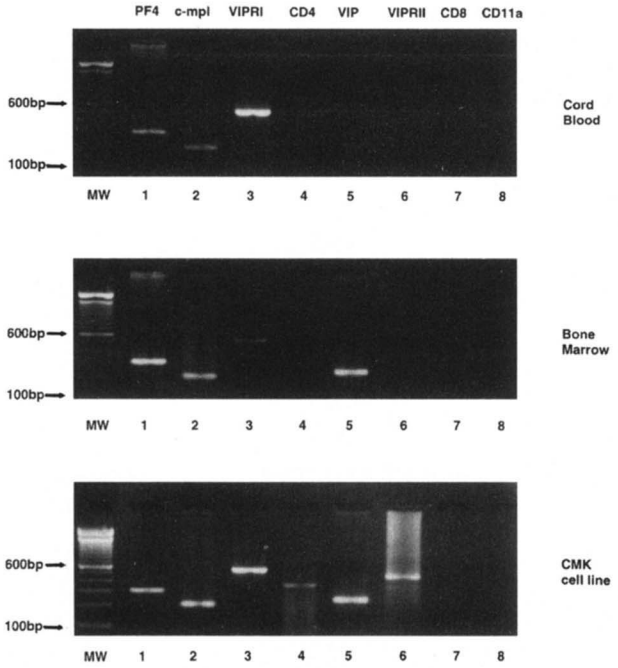

Fig 1. VIPRI expression in megakaryocytes. RT-PCR was performed on 100 ng mRNA isolated from cord blood megakaryocytes (top), BM megakaryocytes (middle), or CMK cells (bottom panel). RT-PCR products electrophoresed in a 3% agarose gel; sizes of MW marker (100-bp ladder) are shown as base pairs at the left side of each panel; lane 1, PF4; lane 2, c-mpl; lane 3, VIPRI; lane 4, CD4; lane 5, VIP; lane 6, VIPRII; lane 7, CD8; lane 8, CD11a.

weights were evaluated by comparison of mobilities with those of the standard proteins electrophoresed in different lanes on the same gel.

RESULTS

VIPR expression by megakaryocytes. Nonadherent cells were procured from suspension cultures of adult BM. Immunomagnetic bead selection of megakaryocyte precursors was performed using a low concentration of anti-IIB/IIIA antibody (10 μg/10^7 cells) and a low bead to target cell ratio (4:1). After immunobead selection, $1.2 \pm 0.2 \times 10^5$ megakaryocytes of 98% purity, as confirmed by immunoperoxidase staining for glycoprotein IIb/IIIa, were isolated from $3.8 \pm 0.2 \times 10^8$ adult BM cells ($\pm$ SD, n = 3). Using similar techniques, $1.5 \pm 0.7 \times 10^5$ megakaryocytes (>98% purity) were isolated from $5.2 \pm 2.5 \times 10^8$ cord blood cells (n = 6).

Average yields of mRNA total obtained from adult BM and cord blood megakaryocytes were 2.8 ± 0.2 and 3.1 ± 0.2 μg, respectively. Expression of message for *c-mpl* and PF-4 was confirmed in megakaryocyte RNA by RT-PCR. The 206 bp product for *c-mpl* and 320 bp product for PF-4 were shown in BM, cord blood, and CMK RNA (Fig 1) confirming the identity of the cells as megakaryocytic. No expression of CD4, CD8, or CD11a was observed in either BM or cord blood derived megakaryocyte preparations, indicating the complete removal of both lymphocytes and myeloid cells using this immunobead purification method. Coexpression of VIP and VIPRI transcripts in megakaryocyte mRNA was shown in 3 BM and 6 cord blood megakaryocyte cultures with RT-PCR using primers specific for a 231 base sequence of VIP and a 534 base sequence of the human VIPRI (Fig 1 and Table 2). Both sets of primers crossed one or more introns; the lack of the 991 bp genomic VIP product indicates the purity of the RNA without significant DNA contamination. The predicted 2998 bp VIPRI product from genomic DNA would not likely be amplified under these RT-PCR conditions.

Neither BM nor cord blood megakaryocytes expressed VIPRII, CD4, CD8, or CD11a; however, the megakaryocytic cell line, CMK, expressed VIP, VIPRI, VIPRII, and CD4 (Fig 1). Autoradiograms of Southern blot analysis confirmed identification of mRNA for the human VIPRI in megakaryocyte RNA from both adult BM and cord blood (Fig 2).

Characterization of platelet VIPR. Having shown the mRNA for VIPRI in megakaryocytes, we wanted to confirm translation of the mRNA into functional receptor protein. Platelets were chosen for these studies for two reasons: (1) platelets do not synthesize mRNA; hence, platelet proteins reflect gene transcription in megakaryocytes; and (2) platelets are easily isolated in high purity with sufficient quantity of membrane for crosslinking studies.

Platelets were incubated with ^{125}I-VIP in the presence of the cross-linking agent, DSS; the platelets were lysed, and the cellular proteins separated on polyacrylamide gels. As shown in the top panel of Fig 3, multiple platelet membrane proteins stain with Coomassie blue. In contrast, the autoradiogram from such a gel (lower panel of Fig 3) shows that ^{125}I-VIP cross-links to a single radioactive band at an approximate molecular weight of 50,000 Da (lanes 1 and 3) which is completely abolished in the presence of 1 μmol/L unlabeled VIP (lanes 2 and 4). Some radioactivity was observed at the top of the gel in all lanes, indicating either solubilization of membrane protein was not complete or aggregation occurred among proteins; unreacted ^{125}I-VIP was seen at the dye front in all lanes.

For further analysis of the platelet VIPR, the reducing agent, DTT, was omitted from the lysis and treatment buffers as shown in Fig 4. Bovine serum albumin (BSA) contains intrachain S-S bonds; in the presence of DTT, its globular structure has been disrupted causing DTT treated BSA to

Table 2. Expression of Genes in Adult BM Megakaryocytes, Cord Blood Megakaryocytes, and CMK Cells

	PF-4	c-mpl	VIPRI	VIPRII	VIP	CD4	CD8	CD11a
CMK	+	+	+	+	+	+	–	–
BM1	+	+	+	–	+	–	–	–
BM2	+	+	+	–	+	–	–	–
BM3	+	+	+	–	+	–	–	–
CB1	+	+	+	–	+	–	–	–
CB2	+	+	+	–	+	–	–	–
CB3	+	+	+	–	+	–	–	–
CB4	+	+	+	–	+	–	–	–
CB5	+	+	+	–	+	–	–	–
CB6	+	+	+	–	–	–	–	–

Abbreviation: CB, cord blood.

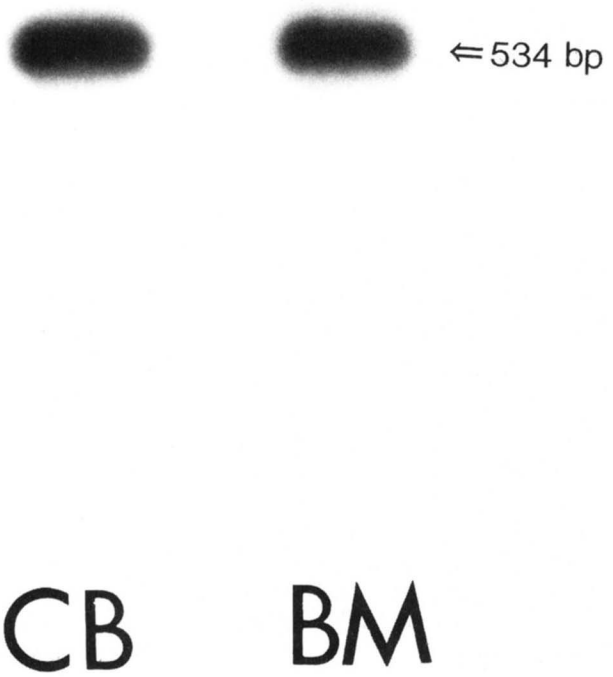

Fig 2. Southern blot analysis of VIP receptor on megakaryocytes. RT-PCR products for VIP receptor electrophoresed in a 1% agarose gel and transferred to nylon membrane. Nylon membrane was hybridized with the ^{32}P-labeled 534 bp DNA human VIPRI DNA probe. Bands at 534 bp show the presence of VIPRI on cord blood, adult BM megakaryocytes.

migrate more slowly. The mobility of the M_r = 50,000, ^{125}I-VIP labeled band was unchanged in the absence of DTT, implying that the human platelet VIPR does not have disulfide bonds. A nonspecific band (not abolished with unlabeled VIP), was observed around 31,000 Da in the presence (Figs 3 and 4), but not in the absence (Fig 4), of DTT; this may be because of increased electrostatic interaction between VIP, a strongly basic peptide, and acidic membrane proteins in the presence of DTT. There were no other major radioactive bands detected in this gel, which included molecular weights between M_r = 10,000 and 100,000.

The hormone specificity of ^{125}I-VIP cross-linking to the M_r = 50,000 species is shown in Fig 5. Visual inspection of the autoradiogram shows the total disappearance of the labeling of the M_r = 50,000 component with 1 μmol/L VIP, whereas 1 μmol/L Peptide Histidine Methionine (PHM) and 1 μmol/L Growth Hormone Releasing Factor (GHRF), show less effect on abolishing of the M_r = 50,000 labeled component. The order of potency of neuropeptides in inhibiting ^{125}I-VIP cross-linking to the M_r = 50,000 band is VIP > GHRF > PHM. This is in agreement with their estimated dissociation constant (K_D) values.[20] These results imply that the M_r = 50,000 cross-linked species is an integral component of the platelet VIPR.

DISCUSSION

Human VIPRI cDNA has been cloned from HT29 colonic epithelial cells, human lung, and kidney tissues.[27,28] Couvineau et al[37] reported two human VIP receptor cDNA clones isolated from a jejunal epithelial cell cDNA library, which differ slightly from that characterized in human HT29 colonic epithelial cells. Using RT-PCR, we were able to clearly identify expression of message for the VIPRI in megakaryo-

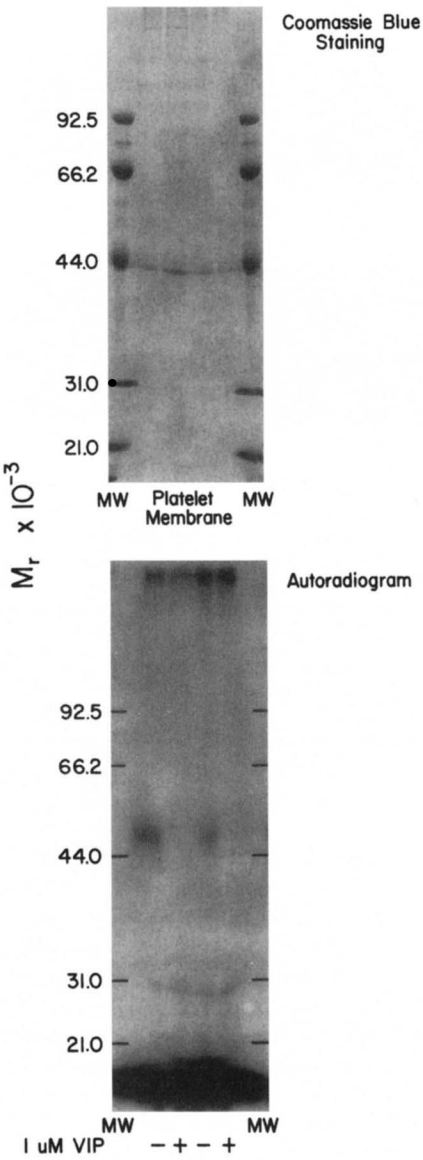

Fig 3. Cross-linking of ^{125}I-VIP to platelet membranes using DSS. Two hundred–microgram platelet membranes were incubated with 300 pmol/L ^{125}I-VIP at equilibrium, washed free of unbound VIP and treated with 1 mmol/L cross-linker (DSS), followed by SDS-polyacrylamide-gel electrophoresis. Top (Coomassie Blue Staining): Four lanes between two molecular weight markers indicate solubilization of multiple platelet membrane proteins between 10,000 and 100,000 daltons. Bottom (Corresponding Autoradiogram): Lanes 1 and 3 (from left to right order) received ^{125}I-VIP, and lanes 2 and 4 received ^{125}I-VIP + 1 μmol/L unlabeled VIP. Lanes 1 and 3 show the ^{125}I-VIP receptor complex at an approximate molecular weight of 50,000 that was absent when 1 μmol/L unlabeled VIP was present (lanes 2 and 4). Cross-linking to a nonspecific band around 31,000 was not competitively inhibited by unlabeled VIP.

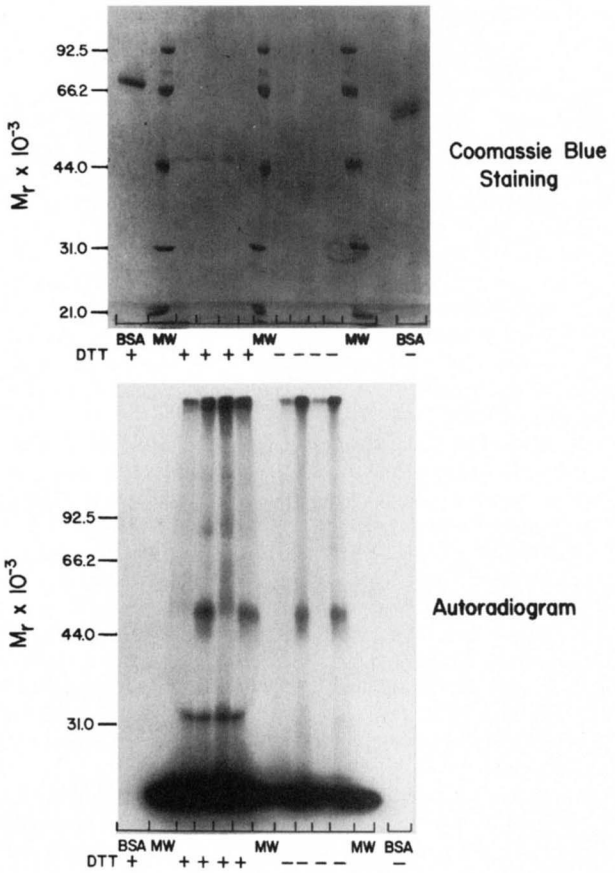

Fig 4. Effect of DTT on crosslinked membrane protein. Two hundred–microgram platelet membranes were incubated with ^{125}I-VIP at equilibrium, membrane bound ^{125}I-VIP was separated from free by centrifugation and treated with 1 mmol/L cross-linker (DSS) in the absence or presence of DTT. Top (Coomassie Blue Staining): First lane, BSA with DTT; three lanes marked MW, molecular weight markers; four lanes marked +, platelet membranes with DTT; four lanes marked −, platelet membranes without DTT; last lane, BSA without DTT. Bottom (Corresponding Autoradiogram): Four lanes marked +, platelet membranes with DTT; four lanes marked −, platelet membranes without DTT. Lanes 1, 3, 5, and 7 received ^{125}I-VIP + 1 μmol/L unlabeled VIP. Lanes 2, 4, 6, and 8 received only ^{125}I-VIP.

cytes isolated from cultures of human cord blood and adult BM; Southern blot analysis confirmed the presence of VIPRI mRNA. Genes specific to the megakaryocyte lineage were also tested. The proto-oncogene *c-mpl* encodes the receptor for a new cytokine, thrombopoietin, which specifically regulates megakaryocytopoiesis.[25] PF-4 is a platelet-specific protein synthesized in the maturing megakaryocytes and packaged into the platelet α granules.[38,39] PF-4 may be involved in neutralizing heparin-like molecules in the plasma[40] and, thus, may be a negative regulator of megakaryocytopoiesis.[41] Whenever blood is exposed to negatively charged surfaces, coagulation, and fibrinolysis are activated simultaneously. Thus, our observation that the coagulation activator, PF-4, and a coagulation inhibitor mechanism, VIPRI, are coexpressed in megakaryocytes is not surprising. Such coexpression may allow for more complete control over a delicate coagulation/anticoagulation balance.

Expression of the VIPRI gene does not, by itself, prove translation of the mRNA into functional receptor protein. Therefore, the receptor protein was characterized in platelet membranes. If we assume that one molecule of VIP (M_r = 3000) is bound per molecule of receptor, the covalent cross-linking experiments indicate that VIP is covalently bound to a platelet membrane protein with an estimated M_r = 47,000 that is in excellent agreement with the predicted 52,000 Da based on the mRNA sequence.[28] The cross-linking of ^{125}I-VIP to this protein is inhibited by 1 μmol/L unlabeled VIP, indicating high affinity binding. Furthermore, the M_r = 47,000 protein is VIP-preferring, because it is not altered by homologous peptides such as PHM and GHRF. The order of potency in inhibiting the cross-linking of ^{125}I-VIP to M_r = 47,000 species is VIP > GHRF > PHM, which is in agreement with the order of calculated K_D.[20] The VIPR in platelets does not appear to have disulfide bonds. The size of VIPR reported for the ligand-binding protein identified by affinity cross-linking ranges from 46,000 to 73,000 Da; splice variants, variation in carbohydrate content, or receptor heterogeneity may account for differences in molecular

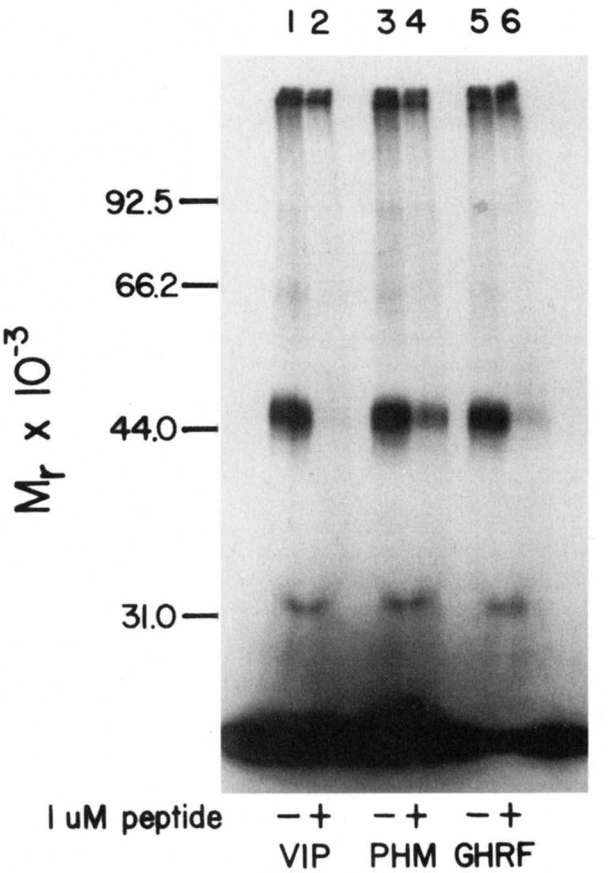

Fig 5. Specificity of cross-linking: ^{125}I-VIP was cross-linked to plasma membrane proteins in the presence or absence of unlabeled VIP, PHM, GHRF. Lanes 1, 3, and 5 show the autoradiographic pattern of membrane with ^{125}I-VIP alone; lane 2 with ^{125}I-VIP + 1 μmol/L unlabeled VIP, lane 4 with ^{125}I-VIP + 1 μ PHM, and lane 6 with ^{125}I-VIP + 1 μmol/L GHRF.

weight of VIPR observed in various tissues.[3,28] The size of the cross-linked VIPR from human platelet membranes is in agreement with previous reports of VIPR identified by cross-linking from lymphoblasts,[35] pancreatic acinar cells,[42] and GH3 pituitary lactotrophs.[43]

Thus, our studies suggest that VIPRI mRNA is transcribed and translated in human megakaryocytes. Additional studies will be needed to investigate the effect of VIP on megakaryocytopoiesis, but identification of VIPR protein in platelet membranes suggests that VIP modulation of platelet aggregation[20] is mediated directly through these specific, high affinity receptors.

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