Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions

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Proteins secreted by activated platelets can adhere to the vessel wall and promote the development of atherosclerosis and thrombosis. Despite this biologic significance, however, the complement of proteins comprising the platelet releasate is largely unknown. Using a proteomics approach, we have identified more than 300 proteins released by human platelets following thrombin activation. Many of the proteins identified were not previously attributed to platelets, including secretogranin III, a potential monocyte chemoattractant precursor; cyclophilin A, a vascular smooth muscle cell growth factor; calumenin, an inhibitor of the vitamin K epoxide reductase-warfarin interaction, as well as proteins of unknown function that map to expressed sequence tags. Secretogranin III, cyclophilin A, and calumenin were confirmed to localize in platelets and to be released upon activation. Furthermore, while absent in normal vasculature, they were identified in human atherosclerotic lesions. Therefore, these and other proteins released from platelets may contribute to atherosclerosis and to the thrombosis that complicates the disease. Moreover, as soluble extracellular proteins, they may prove suitable as novel therapeutic targets. (Blood. 2004;103:2096-2104)

Introduction

Atherosclerosis is a chronic inflammatory disease influenced by circulating cells, including platelets.[1] Endothelial dysfunction, an early event in this process, leads to platelet adhesion, which in turn leads to several steps in the development of atherosclerosis, including leukocyte infiltration.[2] Furthermore, inhibition of platelet adhesion reduces leukocyte accumulation and attenuates the progression of atherosclerotic lesions in the cholesterol-fed apolipoprotein E-deficient ($ApoE^{-/-}$) mouse.[3] Platelets may mediate such effects through products released following adhesion and activation. Indeed, platelet-derived chemokines such as platelet factor 4 (PF4) are found in atherosclerotic plaques where they express biologic activities that may contribute to several aspects of the disease.[3,4]

Platelets contain a number of preformed, morphologically distinguishable storage granules—α-granules, dense granules, and lysosomes—the contents of which are released upon platelet activation.[5] Platelets also release 2 distinct membrane vesicle populations during activation: cell surface–derived microvesicles and exosomes of endosomal origin.[6] Microvesicles have a protein content similar to the activated plasma membrane and have procoagulant and inflammatory functions.[7] On the other hand, exosomes are released following fusion of subclasses of α-granules and multivesicular bodies (MVBs) and their function remains unknown.[6] Exosomes are secreted by a multitude of other cells including those of hematopoietic lineage, such as cytotoxic T cells,[8] antigen-presenting B cells,[9] and dendritic cells,[10] where they play an immunoregulatory role.[11] Proteomic analysis of exosomes secreted from various cell types has revealed the presence of ubiquitous proteins such as tubulin, actin, and actin-binding proteins, as well as cell type–specific proteins.[12,13]

Secreted platelet proteins act in an autocrine or paracrine fashion to modulate cell signaling. Several of the proteins (eg, growth arrest specific gene 6 [GAS-6], factor V) are prothrombotic, whereas others (eg, platelet-derived growth factor [PDGF]), regulate cell proliferation.[14] Platelets also release several immune modulators such as platelet basic protein whose proteolytic product is neutrophil-activating peptide 2 (NAP-2),[15] in addition to adhesion proteins such as platelet endothelial cell adhesion molecule (PECAM) that may support leukocyte migration.[16] Thus, the platelet releasate contains factors of major significance in the development of atherothrombosis. Here, we used a comprehensive proteomics approach to isolate, separate, and identify the contents of the platelet releasate, a fraction highly enriched for platelet granular and exosomal contents, and we demonstrate the presence of several of these proteins in human atherosclerotic lesions.

Patients, materials, and methods

Platelet aggregation and isolation of supernatant fraction

Washed platelets were prepared and aggregations performed with 0.5 U/mL thrombin, as previously described.[17] Following aggregation, platelets were

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removed by centrifuging sequentially twice at 1000*g* for 10 minutes and harvesting the supernatant. The supernatant was further subjected to ultracentrifugation for 1 hour at 4°C at 50 000g_{max} using a 50.4 Ti rotor (Beckman Instruments, Fullerton, CA) to remove microvesicles. The purity of the releasate fraction was confirmed by the absence of platelet membrane–specific protein α_{IIb} and the signaling protein focal adhesion kinase (FAK; results not shown).

SDS-PAGE and Western blotting

The methods for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were as previously described.[17] Protein concentration was measured according to Bradford, and the same amount of protein (40 μg) loaded onto each well. For total platelet lysate this was equivalent to about 1.5×10^7 platelets and for releasate about 8×10^8 platelets. The primary monoclonal antibody to thrombospondin clone p10 (1:1000 dilution) and polyclonal antigoat (C-19) antibody to secretogranin III were purchased from Chemicon International (Hampshire, United Kingdom) and Santa Cruz Biotechnology (Heidelberg, Germany), respectively. The polyclonal antirabbit antibody to calumenin was a kind gift from Dr Reidar Wallin (Wake Forest University, Winston-Salem, NC). The polyclonal antirabbit antibody against cyclophilin A was from Upstate Biotechnology (Milton Keynes, United Kingdom). Antimouse, antigoat, and antirabbit secondary horseradish peroxidase (HRP) antibodies were obtained from Pierce (Rockford, IL). Fluorescence was detected using West Pico Supersignal chemiluminescent substrate (Pierce).

Two-dimensional electrophoresis

Two-dimensional electrophoresis (2-DE) was performed as previously delineated,[17] with the following minor modifications. A total of 400 μg released protein ($\sim 8 \times 10^9$ platelets) was focused for 100 000 volt hours (Vh) on 18-cm, pI 3-10 immobiline dry strips using the Multiphor Isoelectric Focusing Unit (Amersham Biosciences, Buckinghamshire, United Kingdom) and then sealed on top of 10% polyacrylamide gels (20×20 cm). Protein spots were visualized using a G-250 colloidal Coomassie blue dye (Sigma, Dublin, Ireland).

MALDI-TOF mass spectrometry

In-gel digestion and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was carried out as earlier outlined.[17,18] Spectra were analyzed by searching with the publicly available search algorithm Mascot (http://www.matrixscience.com/cgi/search_form.pl?FORMVER = 2&SEARCH = PMF) using the National Center for Biotechnology Information (NCBI) and SwissProt databases.[19]

Multidimensional protein identification technology and liquid chromatography-ion trap MS

Approximately 300 μg protein ($\sim 6 \times 10^9$ platelets) was precipitated overnight with 5 volumes of ice-cold acetone followed by centrifugation at 21 000*g* for 20 minutes. The pellet was solubilized in 8 M urea, 50 mM Tris (tris(hydroxymethyl)aminomethane)–HCl, pH 8.5, at 37°C for 2 hours and reduced by addition of 1 mM dithiothreitol (DTT) for 1 hour at room temperature, followed by carboxyamidomethylation with 5 mM iodoacetamide for 1 hour at 37°C. The samples were diluted to 4 M urea with 50 mM ammonium bicarbonate, pH 8.5, and digested with a 1:150 molar ratio of endoproteinase Lys-C at 37°C overnight. The next day, the mixtures were further diluted to 2 M urea with 50 mM ammonium bicarbonate, pH 8.5, supplemented with calcium chloride to a final concentration of 1 mM, and incubated overnight with Poroszyme-immobilized trypsin beads at 30°C with rotating. The resulting peptide mixtures were solid-phase extracted with SPEC-Plus PT C18 cartridges (Ansys Diagnostics; Lake Forest, CA) according to the manufacturer's instructions and stored at −80°C until further use.

A fully automated 7-cycle, 14-hour multidimensional protein identification technology (MudPIT) chromatographic procedure was set up essentially as described.[20,21] Briefly, a high-performance liquid chromatography (HPLC) quaternary pump was interfaced with an LCQ DECA XP ion trap tandem mass spectrometer (ThermoFinnigan, San Jose, CA). A 150-μm internal diameter fused silica capillary microcolumn (Polymicro Technologies, Phoenix, AZ) was pulled to a fine tip using a P-2000 laser puller (Sutter Instruments, Novato, CA) and packed with 10-cm of 5-μm Zorbax Eclipse XDB-C18 resin (Agilent Technologies, Mississauga, ON, Canada) and then with 6 cm of 5-μm Partisphere strong cation exchange resin (Whatman, Clifton, NJ). Samples were loaded manually onto separate columns using a pressure vessel and chromatography was performed as described.[22] The SEQUEST algorithm was used to identify proteins from tandem mass spectra.[23] The ion state, XCorr, and DCn criteria to yield less than 1% false-positive identification were used.[24]

Affymetrix RNA analysis

Total RNA was isolated from highly purified platelet preparations, pooled, and hybridized to the Affymetrix HG-U95Av2 array, according to the manufacturer's instructions. Only samples that were positive by polymerase chain reaction (PCR) for platelet-specific proteins (GPIIb, GPIIIa and the low abundance CD151) and were negative for white cell markers (CD53 and δ chain of the T-cell antigen receptor–associated T3 complex) were pooled for analysis. Average difference values were scaled and transformed in accordance with the Gene Expression Atlas (http://expression.gnf.org/cgi-bin/index.cgi).[25] For comparison to proteomic data, Affymetrix probe sets were mapped to UniGene clusters (build 157, November 2002).

Confocal microscopy

Glass slides were coated with 20 μg/mL fibrinogen in buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20) at 37°C for 2 hours and subsequently blocked with 1% bovine serum albumin (BSA) for 1 hour. The platelets were resuspended in a modified Tyrode buffer (130 mM NaCl, 10 mM trisodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 10 mM Tris, pH 7.4) at a concentration of 20 to 30×10^5/μL and allowed to adhere to the blocked slides for the indicated time, prior to fixation for 7 minutes in ice-cold methanol. Slides were then permeabilized in ice-cold acetone for 2 minutes and blocked with normal goat serum or BSA at room temperature for 30 minutes. The slides were incubated with primary antibody (antibodies as for Western blotting) for 45 minutes and then a fluorescent secondary antibody (Alexa 488–conjugated goat antirabbit or mouse antigoat) for 10 minutes. These slides were visualized using an argon laser at 488 nm. For dual-stained images, the slides were incubated with anti-CD41 monoclonal antibody (IgG1; clone SZ22; Beckman Coulter, Buckinghamshire, United Kingdom) for an additional 45 minutes and then a fluorescent Alexa 546–conjugated goat antimouse secondary for 10 minutes. Control procedures included unstained cells to allow for autofluorescence, secondary antibody only, and primary antibodies with 2 differentially labeled secondaries to check for nonspecific fluorescence. All images were acquired using a Zeiss LSM 510 Confocal Microscope (Carl Zeiss, Jena, Germany).

Immunohistochemistry

Samples of arterial tissue were obtained from patients with atherosclerosis at the time of surgery for carotid or peripheral vascular disease (n = 5) and

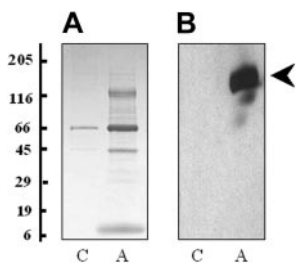

Figure 1. Proteins released by platelets following thrombin activation. (A) The proteins from the supernatant of nonactivated (C) and thrombin-activated platelets (A) were solubilized in SDS reducing buffer, separated by one-dimensional SDS-PAGE (4%-20% gradient gel) and stained with colloidal Coomassie blue. (B) A duplicate of the gel was probed with a monoclonal antibody to thrombospondin (clone p10). Molecular weight markers are indicated.

Table 1. Proteins released from thrombin-activated platelets identified by MALDI-TOF MS

Spot no.	Protein identity	Accession no.	Molecular weight, M_r	Isoelectric point, pI	Sequence coverage, %	MOWSE score
1	Apo A1 fragment	CAA00975	28 061	5.27	59	232
2	Apo A1 fragment	CAA00975	28 061	5.27	46	125
3	14-3-3 protein ζ/δ	1QIBA	26 297	4.99	38	116
4	TMP4-ALK fusion oncoprotein type 2	Q9HBZ0	27 570	4.77	29	102
5	Haptoglobin	AAC27432	38 722	6.14	25	91
6	Haptoglobin	AAC27432	38 722	6.14	36	151
7	Haptoglobin	AAC27432	38 722	6.14	30	137
8	Haptoglobin	AAC27432	38 722	6.14	36	140
9	Actin	CAA27396	39 446	5.78	54	158
10	Osteonectin	O08953	35 129	4.81	37	127
11	Osteonectin	O08953	35 129	4.81	37	119
12	Thrombospondin	CAA32889	133 261	4.71	15	111
13	α_1-antitrypsin	CAA00206	44 291	5.36	21	120
14	α_1-antitrypsin	CAA00206	44 291	5.36	21	109
15	α_1-antitrypsin	CAA00206	44 291	5.36	21	120
16	Serum albumin	CAA00298	68 588	5.67	30	194
17	Serum albumin	1AO6A	676 090	5.63	27	161
18	Serum albumin	CAA00298	68 588	5.67	27	159
19	Albumin	CAA01216	68 425	5.67	29	154
20	Serum albumin	1AO6A	67 690	5.63	25	164
21	Serum albumin	1AO6A	67 690	5.63	17	90
22	Albumin	AA64922	53 416	5.69	33	150

Protein identifications were generated from the MASCOT database. The validity of the matches was quantified using MOWSE probability score. The percentage of the protein sequence matched by the generated peptides (the sequence coverage) was also documented.

fixed in formal saline for immunohistochemistry analysis.[26] The study was approved by the Irish Medicines Board and the Ethics Committee of Beaumont Hospital, Dublin, and all patients gave written informed consent. All patients were undergoing surgical revascularization for peripheral vascular disease or carotid endarterectomy. Normal arterial sections were obtained from young individuals who had no gross or microscopic evidence of atherosclerosis. Sections (5 μm) were stained with hematoxylin and eosin and for proteins of interest, as described previously.[26] Primary antibodies were as for Western blotting and confocal microscopy. Antirabbit PF4 polyclonal antibody (Ab1488P) was from Chemicon International (Temecula, CA). Monoclonal anti-α smooth muscle actin (mouse IgG2a isotype) was purchased from Sigma Aldrich (Tallaght, Ireland).[26]

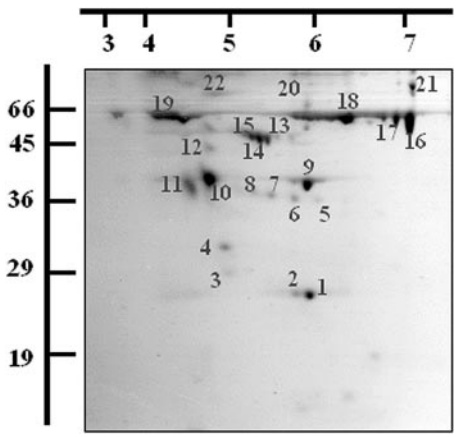

Figure 2. Two-dimensional electrophoresis of the releasate fraction from thrombin-activated platelets. A total of 400μg of the releasate fraction from thrombin-activated platelets was separated by 2-DE and stained with Coomassie blue dye. Spots were excised and digested with trypsin and the resulting peptides were analyzed by MALDI-TOF MS. A representative gel is shown and the proteins identified are listed (see Table 1). Molecular weight markers and pI values are indicated.

Results

Platelets were isolated by differential centrifugation and stimulated with 0.5 U/mL thrombin for 3 minutes to achieve maximum release of all granule contents (α, dense, and lysosomal).[27] It is expected that the secretion profile of platelets would change depending on the agonist, although this may be more quantitative than qualitative. To visualize the proteins released from thrombin-activated platelets, the releasate fraction was harvested by centrifugation and

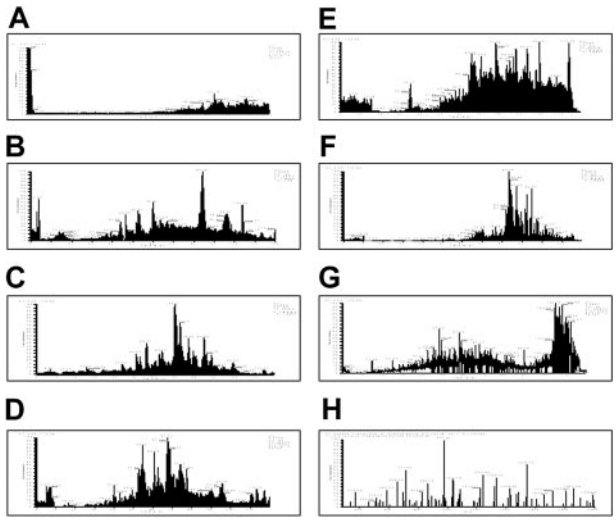

Figure 3. Seven-step MudPIT. (A-G) The releasate from thrombin-activated platelets was digested with trypsin and the resulting peptides were separated using strong cation exchange and reverse-phase chromatography before introduction into an ion trap mass spectrometer. This figure displays the resulting chromatograms from the 2-dimensional-LC tandem MS. Chromatograms in panels A to G represent 7 successive salt elutions from an HPLC column. (H) A representative tandem MS spectrum is shown for a peptide from thrombospondin that was identified using SEQUEST.

separated using one-dimensional SDS-PAGE. A considerably higher concentration of protein was found in the thrombin-activated sample, particularly between the molecular weights of 5 and 15 kDa, 30 and 50 kDa, and 120 and 180 kDa (Figure 1A). Western blot analysis demonstrated thrombospondin (p10), a known secreted protein, only in the activated releasate lane (Figure 1B). Flow cytometry for P-selectin (CD62), an α-granule marker protein, showed a 3-fold increase in platelet expression following treatment with thrombin, indicative of α-granule membrane fusion and exocytosis. Western blotting for the signaling protein FAK and the membrane protein α_{IIb} were performed on platelet lysates and platelet supernatant fractions. These proteins were not found in the platelet supernatant fractions indicating that platelet cell lysis had not occurred and microvesicles were not present in the preparation (data not shown).

Twenty-two protein spots were excised and digested with trypsin following 2-dimensional (2-D) gel electrophoresis of the activated platelet releasate and staining with Coomassie blue. The resulting peptides were analyzed by MALDI-TOF MS and the proteins identified using Mascot. The identifications were accepted if they represented the highest-ranking hit, had MOWSE scores over 64, and if the sequence coverage was at least 15% to 30% (depending on protein size).[28] The protein identities, sequence coverage, and MOWSE probability scores obtained for the each of the 22 spots are detailed in Table 1 and Figure 2. Nine different proteins were identified from the 22 spots, several being isomeric forms of the same protein (Figure 2).

We then used multidimensional liquid chromatography (LC) coupled with electrospray MS to further characterize the platelet releasate. The released protein fraction from thrombin-activated platelets was digested with trypsin and the resulting peptides loaded under pressure onto a nanocapillary column containing both strong cation exchange and reverse-phase materials.[29] Seven successive salt elutions and HPLC cycles were used to separate the peptides (Figure 3A-G). The thrombin-activated releasates of pooled donors were analyzed using identical cycle conditions in 3 independent experiments. More than 300 proteins were identified, with 81 observed in 2 or 3 experiments (Table 2). Seventy percent of these 81 were identified in all 3 experiments. Reproducibility of identification for a repeat analysis of a single sample was about 80%. This suggests that about 20% of the variation in the results arises from missed identifications due to saturation of the MudPIT analysis, whereas about 10% may represent donor-to-donor variation.

Thirty-seven percent of the proteins identified were previously reported to be released from platelets including thrombospondin,[30] PF4,[31] osteonectin,[32] metalloproteinase inhibitor 1,[33] and transforming growth factor.[34] Another 35% are known to be released from other secretory cells. These include cofilin, profilin, 14-3-3 ζ and actin from dendritic cells,[12] peptidyl-prolyl-*cis* isomerase (cyclophilin A) from smooth muscle cells,[35] phosphoglycerate kinase from fibrosarcoma cells,[36] and β_2-microglobulin[37] and vitamin D–binding protein[38] from the liver. The remaining proteins are not known to be released from any cell type with several mapping to expressed sequence tags (ESTs) of unknown function (Table 2). Additionally, 75 of the 81 released proteins were matched to UniGene clusters, 68 of which had corresponding Affymetrix probe sets. Of these 68 array-comparable proteins, messages for 46 (68%) were detected in the platelet mRNA (J.P.M. et al, manuscript submitted; Table 2).

We focused on 3 proteins, secretogranin III (SgIII), cyclophilin A, and calumenin, which are not known to be present in or released from platelets. SgIII (Figure 4C), cyclophilin A (Figure 4B), and calumenin (Figure 4A) were found by Western blot in resting (PC) and thrombin-activated platelet lysates (PA), as well as in the supernatant of thrombin-activated platelets (RA; Figure 4). Neither SgIII nor calumenin was detected in a crude leukocyte lysate (white blood cells [WBCs]), although cyclophilin A was present in low amounts (Figure 4). These 3 proteins were identified in P-selectin–positive platelets by flow cytometry (data not shown) and in $CD41^+$ platelets adhering to fibrinogen-coated slides using confocal microscopy (Figure 5B). No staining was observed in platelets stained for secondary antibody only (Figure 5C).

Sections of arterial tissue from 5 patients with atherosclerosis were examined using immunohistochemistry.[26] The results from a representative patient are shown (Figure 6). Low-power hematoxylin and eosin staining of normal (A) and atherosclerotic plaque (H) are provided for orientation of these sections. Atherosclerotic but not normal artery stained for the platelet-specific proteins PF4 (Figure 6J) and CD41 (Figure 6I). SgIII (Figure 6L) and calumenin (Figure 6M) were also expressed widely in the plaque, whereas vascular smooth muscle cells in the lesion, identified by staining for actin (Figure 6G,N), stained for cyclophilin A (Figure 6K). None of the proteins were found in normal artery (Figure 6B-F).

Discussion

We identified more than 300 proteins in the platelet releasate; a fraction highly enriched for platelet granular and exosomal contents. This list included proteins of relatively low (eg, thrombospondin) and high (eg, platelet glycoprotein V) isoelectric point, as well as low (eg, PF4) and high (eg, von Willebrand factor) molecular mass. Of the 81 proteins observed in 2 or 3 repeat experiments, 30 (37%) are known to be released from platelets including multimerin, thrombospondin, and PF4. Integral membrane proteins, such as α_{IIb} and known signaling proteins, were not represented, suggesting that the identified fraction is relatively specific and enriched for secreted and exosomal proteins.

Fifty-one (63%) of the proteins identified were not known to be released from platelets. A possible explanation for the presence of these proteins is contamination of the releasate with intracellular platelet proteins released by platelet lysis. However, the absence of major signaling proteins argues against this. Furthermore, 28 (35%) of the identified proteins are proteins that are released by other secretory cells (Table 2). For example, a number of the platelet releasate proteins identified, including tubulin and adenylyl cyclase–associated protein (CAP 1) are found in macrophage phagosomes.[39] Other proteins identified in the releasate fraction may be involved in the process of exocytosis, for example, calmodulin.[40] Furthermore, neither vesicle-associated membrane protein (VAMP) nor syntaxins, which are present in high amounts in platelets,[41] appear in the releasate.

Twenty-three (28%) proteins in the releasate are not reported to be released from any cell type. Several map to ESTs of unknown function. However, of 81 proteins found consistently in the platelet releasate, 46 were detected at the mRNA level in platelets (J.P.M. et al, manuscript submitted; Table 2). Remarkably, 18 of the 50 most abundant platelet messages were represented in the releasate. Protein synthesis in the platelet is limited[42] and the platelet transcriptome may largely reflect that of the parent megakaryocyte.[43] The messages for many secreted proteins may, therefore, be transcribed in the megakaryocyte and passed to the daughter

Table 2. Summary of 81 proteins from the thrombin-activated platelet releasate identified using MudPIT

Name	Accession no.	Known platelet protein	Known to be released / exocytosed	Function	mRNA rank in platelets
PROTEINS IN THE PLATELET RELEASATE KNOWN TO BE RELEASED FROM PLATELETS					
Thrombospondin 1	TSP1_HUMAN	Yes	From platelet α-granules	On secretion, can bind $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, and GPIV. Can potentiate aggregation by complexing with fibrinogen and becoming incorporated into fibrin clots.	213
Fibrinogen α chain	FIBA_HUMAN	Yes	From platelet α-granules	Cofactor in platelet aggregation. Endocytosed into platelets from plasma.	632
Fibrinogen γ chain	FIBG_HUMAN	Yes	From platelet α-granules	Cofactor in platelet aggregation. Endocytosed into platelets from plasma.	—
Platelet basic protein	SZ07_HUMAN	Yes	From platelet α-granules	Proteolytic cleavage yields the chemokines β-thromboglobulin and neutophil-activating peptide (NAP) 2.	23
PF4	PLF4_HUMAN	Yes	From platelet α-granules	Platelet-specific chemokine with neutrophil-activating properties.	8
Serum albumin	ALBU_HUMAN	Yes	From platelet α-granules	Major plasma protein secreted from the liver into the blood. Endocytosed into platelets from plasma.	—
Endothelial cell multimerin	ECM_HUMAN	Yes	From platelet α-granules	Carrier protein for platelet factor V/Vα.	—
SPARC (osteonectin)	SPRC_MOUSE	Yes	From platelet α-granules	On secretion, forms a specific complex with thrombospondin.	14
α-actinin I	AAC1_HUMAN	Yes	From platelet α-granules	Actin-binding and actinin cross-linking protein found in platelet α-granules. Interacts with thrombospondin on the platelet surface.	171
α_1-antitrypsin	A1AT_HUMAN	Yes	From platelet α-granules	Acute phase protein, similar to complement, inhibits proteinases.	—
Fibrinogen β chain	FIBB_HUMAN	Yes	From platelet α-granules	Cofactor in platelet aggregation. Endocytosed into platelets from plasma.	—
Factor V	FA5_HUMAN	Yes	From platelet α-granules	Cofactor that participates with factor Xa to activate prothrombin to thrombin.	—
Secretory granule proteoglycan core protein	PGSG_HUMAN	Yes	From platelet α-granules	Function unknown. Associates and coreleased with inflammatory mediators such as PF4.	343
Thymosin β-4	TYB4_MOUSE	Yes	From platelets α-granules	G actin–binding protein. Functions as an antimicrobial peptide when secreted.	5
Fructose biphosphate aldolase	ALFA_MOUSE	Yes	From platelets and exosomes from dendritic cells	Glycolytic enzymes that convert fructose 1,6-bis phosphate to glyceraldehydes-3-phosphate and dihydroxy acetone phosphate.	79
Clusterin	CLUS_HUMAN	Yes	From platelet α-granules	Not clear. Possibly platelet-derived apolipoprotein J participates in short-term wound repair and chronic pathogenic processes at vascular interface.	1
Coagulation factor XIIIA chain	F13A_HUMAN	Yes	From platelet α-granules	Coagulation protein involved in the formation of the fibrin clots.	19
Metalloproteinase inhibitor 1	TIM1_HUMAN	Yes	From platelet α-granules	Interacts with metalloproteinases and inactivates them. Stimulates growth and differentiation of erythroid progenitors, dependent on disulfide bonds.	112
Platelet glycoprotein V	GPV_HUMAN	Yes	Cleaved from platelet surface	Part of the GPIb-IX-V complex on the platelet surface. Cleaved by the protease thrombin during thrombin-induced platelet activation.	—
von Willebrand factor	VWF_HUMAN	Yes	From platelet α-granules	Binds GPIb-IX-V.	2465
Amyloid β-A4 protein (protease nexin II	A4_HUMAN	Yes	From platelet α-granules	Exhibits potent protease inhibitor and growth factor activity. May play a role in coagulation by inhibiting factors XIa and IXa.	379
Latent transforming growth factor β (TGF-β)- binding protein isoform 1S	LTBS_HUMAN	Yes	From platelet α-granules	Subunit of the TGF-β1 complex secreted from platelets.	—
α-Actinin 2	AAC2_MOUSE	Yes	From platelet α-granules	Actin-binding and actinin cross-linking protein found in platelet α-granules. Interacts with thrombospondin on the platelet surface.	—
Latent TGF-β–binding protein 1L	O88349	Yes	From platelet α-granules	Subunit of the TGF-β1 complex secreted from platelets.	617
Proactivator polypeptide	SAP_HUMAN	Yes	From lysosomes	Activator proteins for sphingolipid hydrolases (saposins) that stimulate the hydrolysis of sphingolipids by lysosomal enzymes.	147
Platelet glycoprotein 1b α chain	GPBA_HUMAN	Yes	Cleaved from platelet surface (glycocalicin)	Surface membrane protein of platelets that participates in formation of platelet plug by binding A1 domain of von Willebrand factor.	26
Vitamin K–dependent protein S	PRTS_HUMAN	Yes	From platelet α-granules	Cofactor to protein C in the degradation of coagulation factors Va and VIIIA.	1810
PF4 variant	PF4V_HUMAN	Yes	From platelet α-granules	Platelet-specific chemokines with neutrophil-activating properties.	346
α_2-macroglobulin	A2MG_HUMAN	Yes	From platelet α-granules	Acute phase protein, similar to compliment, inhibits proteinases.	*
α-actinin 4	AAC4_HUMAN	Yes	From platelet α-granules	Actin-binding and actinin cross-linking protein found in platelet α-granules. Interacts with thrombospondin on the platelet surface.	467
SECRETORY PROTEINS IN THE PLATELET RELEASATE NOT PREVIOUSLY IDENTIFIED IN PLATELETS					
Vitamin D–binding protein	VTDB-HUMAN	No	From liver to plasma	Carries vitamin D sterols. Prevents actin polymerization. Has T lymphocyte surface association.	—
β_2-microglobulin	B2MG_HUMAN	No	Exosomes from dendritic cells, B cells, enterocytes, tumor cells, and T cells	Is the β chain of the major histocompatibility complex (MHC) class I molecule.	3
Hemoglobin α chain	HBA_HUMAN	No	Exosomes from dendritic cells and phagosomes in macrophages	Oxygen transport. Potentiates platelet aggregation through thromboxane receptor.	21
Plasminogen	PLMN_HUMAN	Yes	From kidney into plasma	Dissolves fibrin in blood clots, proteolytic factor in tissue remodeling, tumor invasion, and inflammation.	—
Serotransferrin	TRFE_HUMAN	Yes	From liver into plasma	Precursor to macromolecular activators of phagocytosis (MAPP), which enhance leukocyte phagocytosis via the FcγRII receptor.	—
Pyruvate kinase, M2 isozyme	KPY2_MOUSE	Yes	B-cell exosomes	Involved in final stage of glycolysis. Presented as an autoantigen by dendritic cells.	61
Actin, aortic smooth muscle	ACTA_HUMAN	Yes	Exosomes from B cells, dendritic cells, enterocytes, and mastocytes	Major cytoskeletal protein.	—
Actin	ACTB_HUMAN	Yes	Exosomes from B cells, dendritic cells, enterocytes, and mastocytes	Major cytoskeletal protein. External function unknown.	11
14-3-3 protein ζ/δ	143Z_MOUSE	Yes	Exosomes from dendritic cells and phagosomes in macrophages	External function unknown. Involved intracellularly in signal transduction, however, may have a role in regulating exocytosis.	63
Hemopexin	HEMO_HUMAN	No	From liver to plasma	Haem-binding protein with metalloproteinase domains.	—
Hemoglobin β chain	HBB_HUMAN	No	From liver to plasma and phagosomes from macrophages	Oxygen transport.	9
Peptidyl-prolyl-cis isomerase A (cyclophilin A)	CYPH_MOUSE	No	From smooth muscle cells	Cellular protein with isomerase activity. Secreted vascular smooth muscle cell growth factor.	116
Calumenin	CALU_MOUSE	No	From many cells, including fibroblast and COS cells	An inhibitor of the vitamin K epoxide reductase-warfarin interaction.	1816

continued

Table 2. Summary of 81 proteins from the thrombin-activated platelet releasate identified using MudPIT (*continued*)

Name	Accession no.	Known platelet protein	Known to be released / exocytosed	Function	mRNA rank in platelets
Adenylyl cyclase–associated protein 1 (CAP 1)	CAP1_MOUSE	No	Phagosomes from macrophages	Contains a WH2 actin-binding domain (as β-thymosin 4). Known to regulate actin dynamics. May mediate endocytosis.	174
Tubulin	TBA1_HUMAN	Yes	Exosomes from dendritic cells and phagosomes from macrophages	Cytoskeletal protein involved in microtubule formation.	33
Apolipoprotein A-1	APA1_HUMAN	Yes	From liver to plasma, from monocytes and exosomes of dendritic cells	Role in high-density lipoprotein binding to platelets.	—
Compliment C3	CO3_HUMAN	No	From liver cells and monocytes	Activator of the compliment system. Cleaved to α, β, and γ chains normally prior to secretion and is a mediator of the local inflammatory response.	*
Transthyretin	TTHY_HUMAN	No	From choroid plexus into cerebrospinal fluid (CSF)	Thyroid hormone–binding protein secreted from the choroid plexus and the liver into CSF and plasma, respectively.	—
Cofilin	COF1_MOUSE	Yes	Exosome from dendritic cells	Actin demolymerization/regulation in cytoplasm.	31
Profilin	PRO1_MOUSE	Yes	Exosome from dendritic cells	Actin demolymerization/regulation in cytoplasm.	70
Secretogranin III	SG3_MOUSE	No	From neuronal cells	Unclear; possibly involved in secretory granule biogenesis. May be cleaved into active inflammatory peptide like secretogranin II.	*
Phosphoglycerate kinase	PGK1_MOUSE	Yes	From tumor cells	Glycotic enzyme. Secreted from tumor cells and involved in angiogenesis.	378
α-IB glycoprotein	AIBG_HUMAN	No	From many cell including white blood cells	Found in plasma, not clear, possibly involved in cell recognition as a new member of the immunoglobulin family.	*
Compliment C4 precursor	CO4_HUMAN	No	From many cells including white blood cells	Activator of the compliment system. Cleaved normally prior to secretion, its products mediate the local inflammatory response.	—
Prothrombin	THRB_HUMAN	No	From liver to plasma	Converts fibrinogen to fibrin and activates coagulation factors including factor V.	2143
Glyceraldehyde 3-phosphate dehydrogenase	G3P2_HUMAN	Yes	From B-cell exosomes and phagosomes from macrophages	Mitochondrial enzyme involved in glycolysis. May catalyze membrane fusion.	60
α_1-acid glycoprotein	A1AH_HUMAN	No	From liver to plasma	Modulates activity of the immune system during the acute phase reaction. Binds platelet surface.	—
Gelsolin	GELS_HUMAN	Yes	Secreted isoform released from liver and adipocytes	Two isoforms, a cytoplasmic actin modulating protein and a secreted isoform involved in the inflammatory response.	438
PROTEINS IN THE PLATELET RELEASATE NOT PREVIOUSLY REPORTED TO BE RELEASED FROM ANY CELL					
Calmodulin	CALM_HUMAN	Yes	No evidence	Known to regulate calcium-dependent acrosomal exocytosis in neuroendocrine cells.	55
Pleckstrin	PLEK_HUMAN	Yes	No evidence	A substrate for protein kinase C, its phosphorylation is important for platelet secretion.	532
Nidogen	NIDO_HUMAN	Yes	No evidence	Glycoprotein found in basement membranes, interacts with laminin, collagon, and integrin on neutrophils.	—
Fibrinogen-type protein	Q8VCM7	No	No evidence	Similar to fibrinogen.	—
Rho GDP-dissociation inhibitor 2	GDIS_MOUSE	Yes	No evidence	Regulates the GDP/GTP exchange reaction of Rho proteins. Regulates platelet aggregation. Involved in exocytosis in mast cells.	97
Rho GTPase activating protein	Q92512	Yes	No evidence	Promotes the intrinsic GTP hydrolysis activity of Rho family proteins. Involved in regulating myosin phosphorylation in platelets.	*
Transgelin	TAG2_HUMAN	No	No evidence	Actin-binding protein. Loss of transgelin expression important in early tumor progression. May serve as a diagnostic marker for breast and colon cancer.	7
Vinculin	VINC_HUMAN	Yes	No evidence	Actin-binding protein.	44
WD-repeat protein	WDR1_HUMAN	No	No evidence	Actin-binding protein.	127
Superoxide dismutase (SOD)	SODC_HUMAN	Yes	No evidence	Important enzyme in cellular oxygen metabolism, role for SOD-1 in inflammation.	1730
78-kDa glucose-related protein	GR78_MOUSE	No	No evidence	Chaperone in the endoplasmic reticulum (ER) involved in inhibition of secreted coagulation factors thus reducing prothrombotic potential of cell.	—
Bromodomain and PHD finger-containing protein 3 (fragment)	BRF3_HUMAN	No	No evidence	Unknown.	*
Titin	Q8WZ42	Yes	No evidence	Anchoring protein of actinomyosin filaments. Role in secretion of myostatin.	—
Similar to hepatocellular carcinoma-associated antigen 59	Q99JW3	No	No evidence	Tumor marker.	—
FKSG30	Q9BYX7	No	No evidence	Actin-binding protein.	*
RNA-binding protein	Q9UQ35	No	No evidence	RNA-binding protein.	—
Hypothetical protein	Q9BTV9	No	No evidence	Unknown.	1744
Intracellular hyaluronan-binding protein p57	Q9JKS5	No	No evidence	Unknown.	—
Hypothetical protein	Y586_HUMAN	No	No evidence	Unknown.	*
Filamin fragment (hypothetical 54-kDa protein)	Q99KQ2	Yes	No evidence	Unknown.	—
Filamin	FLNA_HUMAN	Yes	No evidence	Actin-binding protein. Essential for GP1b-α anchorage at high shear. Substrate for caspase-3.	43
Talin	TALI_HUMAN	Yes	No evidence	Actin-binding protein that binds to integrin-β3 domain.	17
Zyxin	ZYX_HUMAN	Yes	No evidence	Associates with the actin cytoskeleton near adhesion plaques. Binds α actinin and VASP.	145

Eighty-one proteins were identified using MudPIT from the thrombin-stimulated platelet supernatant fraction. Spectra were identified using the SEQUEST program and a composite mouse and human database (NCBI July 2002 release) in 3 replicate experiments. Information on their functions and whether they are secretory proteins are provided. Also indicated is whether these proteins have a corresponding platelet mRNA. The rank of abundance of the message is denoted numerically in the last column.

— indicates levels below the threshold for detection on the Affymetrix microarray; *, not present on Affymetrix microarray.

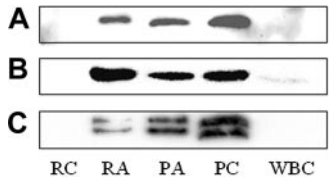

Figure 4. Western blot for calumenin, cyclophilin A, and secretogranin III. The presence of calumenin (A), cyclophilin 4 (B), and secretogranin III (C) was confirmed in lysates from control (platelet control, PC) and thrombin-activated (platelet activated, PA) platelets, as well as the thrombin-activated releasate (releasate activated, RA). These proteins were not found in the supernatant from unactivated platelets (releasate control, RC). In addition, SgIII and calumenin were not detected in a crude leukocyte lysate (WBC), although cyclophilin 4 was present in low amounts.

platelet cells. Other proteins released that do not have a corresponding mRNA may be endocytosed by platelets, for instance, fibrinogen[44] and albumin.[45] Although the detection of hemoglobin messages in platelet RNA might point to contamination of the platelet preparations, our platelet RNA results are in close agreement with those of Gnatenko et al,[46] whose comparison of the transcriptomes from platelets, erythrocytes, and whole blood suggests that mRNAs for hemoglobin are present in platelets.

A number of cytoskeletal and actin-binding proteins were found in the thrombin-activated releasate. Changes in the actin cytoskeleton on platelet activation play an important role in granule movement and exocytosis and many ubiquitous actin-binding proteins interact with endosomes and lysosomes.[47] Moreover, α-actinin and thymosin-β4 are known to be released from platelet α-granules,[48] where they bind thrombospondin and exhibit antimicrobial activity, respectively.[49] Many of the other actin-binding proteins identified are released from other cells including profilin, cofilin, actin, and tubulin from exosomes of dendritic cells.[12] Interestingly, the widely expressed intermediate filament protein vimentin is secreted by activated macrophages, where it is involved in bacterial killing and the generation of oxidative metabolites.[50]

Five mitochondrial proteins were identified including pyruvate kinase, fructose biphosphate aldolase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and superoxide dismutase. The most straightforward explanation for these proteins would be mitochondrial contamination; however, 2 of the most abundant mitochondrial proteins, fumarate hydratase and aconitase, are not present in our preparation.[51] Although there are no reports of superoxide dismutase secretion, pyruvate kinase and GAPDH are released from B cells,[13] fructose biphosphate aldolase is released from dendritic cells,[12] and phosphoglycerate kinase is released from tumor cells.[36]

Three proteins, SgIII, cyclophilin A, and calumenin, not previously recognized to be present in or released by platelets, were examined further because they are of potential interest in the pathogenesis of atherosclerosis. These 3 proteins were localized to platelets by Western blotting, confocal microscopy, flow cytometry, and, for cyclophilin A and calumenin, by microarray analysis of mRNA. Although absent from normal artery, these 3 proteins were found in human atherosclerotic plaque. These lesions also stained for CD41 and PF4, 2 platelet-specific proteins. We have previously demonstrated CD41 staining as a marker for platelets in the atherosclerosis of the ApoE$^{-/-}$ model.[52] In addition, PF4 and oxidized low-density lipoprotein (LDL) have been found to colocalize in atherosclerotic lesions, especially in macrophage-derived foam cells, as PF4 binds to oxidized LDL and may contribute to its uptake.[53]

SgIII is a member of the chromogranin family of acidic secretory proteins, previously shown only to be localized to storage vesicles of neuronal and endocrine cells.[54,55] Secreteogranin II, a close homolog also found in our platelet releasate (n = 1; see the online data supplement), is present in neuroendocrine storage vesicles and is the precursor of the neuropeptide secretoneurin, which has a tissue distribution and function similar to the proinflammatory neuropeptides, substance P and neuropeptide Y. Secretoneurin stimulates monocyte adhesion to the vessel wall followed by their transendothelial migration.[56] Whether cleavage products of SgIII play a similar role is unknown.

Cyclophilins are peptidyl-propyl *cis-trans*-isomerases that act intracellularly both as catalysts and chaperones in protein folding[57] and have extracellular signaling functions such as the induction of chemotaxis and adhesion of memory CD4 cells.[58] Recently, cyclophilin A was found to be secreted by vascular smooth muscle cells in response to oxidative stress, where it acted in an autocrine manner to stimulate extracellular signal-regulated kinase (ERK1/2)

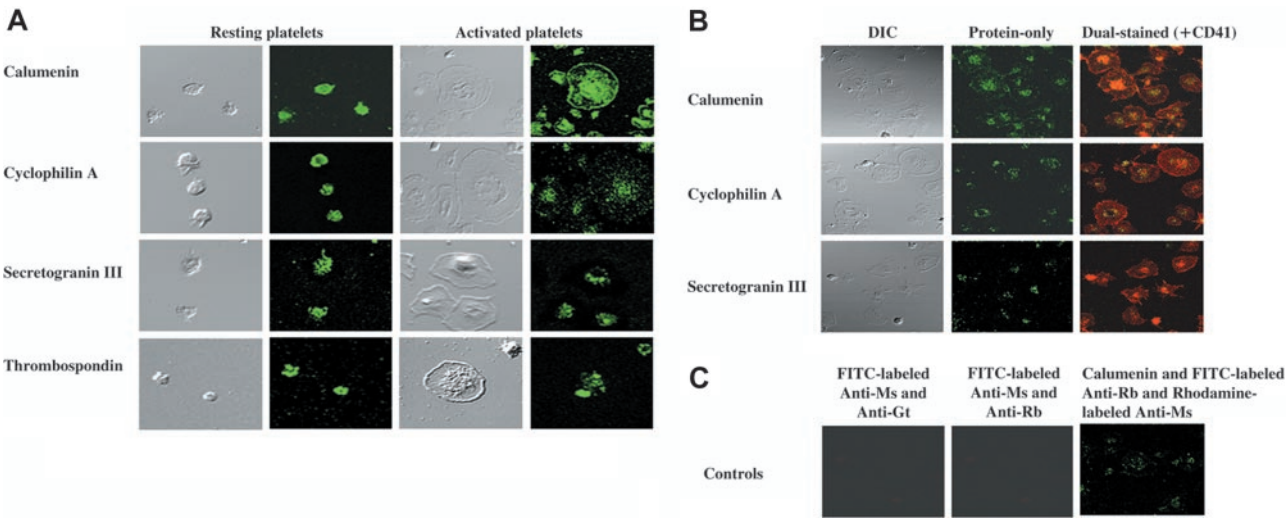

Figure 5. Confocal microscopy for calumenin, cyclophilin A, and secretogranin III. (A) Platelets were adhered to fibrinogen-coated slides for 5 (platelets resting) and 60 minutes (platelets activated and spread). A granular staining pattern similar to thrombospondin was observed for cyclophilin A and secretogranin III at both time points, whereas a more diffuse pattern was observed for calumenin. (B) Activated platelets labeled with CD41 and a rhodamine-labeled secondary antibody, and dual stained with antibodies to secretogranin III, cyclophilin A, and calumenin (labeled with fluorescein isothiocyanate [FITC]–conjugated secondary antibodies). (C) Secondary antibodies alone. Original magnification × 63.

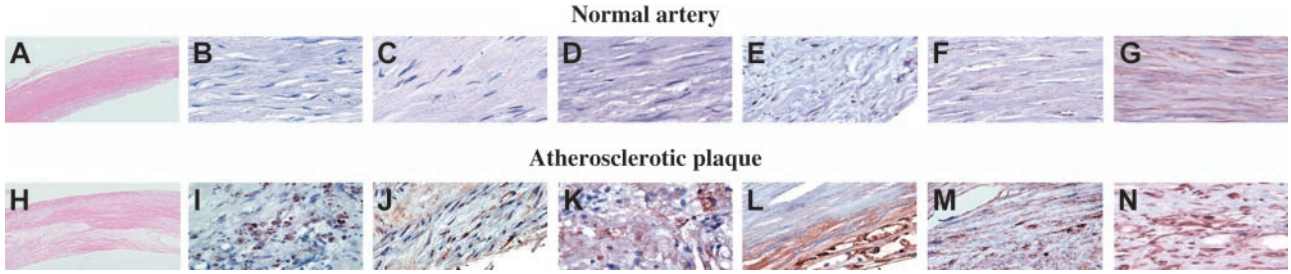

Figure 6. Immunohistochemistry of normal and atherosclerotic tissue. Immunohistochemistry for each of the 3 proteins was performed on sections of arterial tissue from 5 patients with atherosclerosis. The results from one representative patient are shown. Low-power hematoxylin and eosin staining of normal (A) and atherosclerotic plaque (H). Platelet incorporation into the plaques was demonstrated by staining for the platelet-specific proteins PF4 (J) and CD41 (I). Secretogranin III (L) and calumenin (M) were widely expressed in the plaque, whereas vascular smooth muscle cells in the lesion, identified by smooth muscle actin staining (G,N), stained for cyclophilin A (K). No staining for secretogranin III, calumenin, or cyclophilin A was observed in sections of normal artery (B-F). Original magnification × 2.5 (A, H); × 63 (B-G, I-N).

activation and vascular smooth muscle proliferation.[35] Therefore, cyclophilin A released from activated platelets may stimulate the migration and proliferation of smooth muscle cells, a process implicated in the development of atherosclerosis.

The third protein, calumenin, belongs to the CREC family of calcium-binding proteins and was recently found to be secreted from early melanosomes, which are closely related to platelet dense granules.[59] Calumenin has a chaperone function in the endoplasmic reticulum, but little is known about its extracellular function.[60] It has, however, been shown to bind to serum amyloid P component, which is also released from platelets (Table 2).[61] Calumenin inhibits the activity of vitamin K–dependent γ-carboxylation[62] responsible for the activation of coagulation factors and proteins such as matrix Gla protein (MGP).[63] Both calumenin and warfarin target the enzyme vitamin K epoxide reductase (VKOR), an integral membrane complex, which converts vitamin K to its hydroquinone form, a cofactor for the enzyme γ-carboxylase, thus inhibiting the γ-carboxylation.[62] Mice deficient in MGP, which inhibits bone morphogenetic protein activity, develop complete ossification of the aorta, presumably as a result of unopposed osteogenic activity on vascular mesenchyme.[64] Because MGP function requires γ-carboxylation in the aortic vessel wall, warfarin treatment and, indeed, presumably, calumenin deposition in atherosclerotic plaques, may promote vascular calcification by blocking vitamin K–dependent γ-carboxylation and hence MGP activity.[65]

In conclusion, platelet adhesion contributes to the development of atherosclerosis, possibly through proteins released from platelets on activation. SgIII, cyclophilin A, and calumenin are potential candidates given their known biologic activity, and such extracellular platelet proteins may prove suitable as therapeutic targets. Indeed, inhibition of platelet-derived proteins, such as CD40 ligand, reduces the development of atherosclerosis in mice.[66] Thus, the targeting of selected secreted platelet proteins may provide a novel means of modifying atherosclerosis without the risk associated with direct inhibition of platelet adhesion.

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