the role of phosphorylation has been extensively studied (for example, the EGF and insulin receptors). EGF is transcytosed from the basolateral to the apical surface in MDCK cells (*24*) and it is tempting to speculate that phosphorylation of its receptor may have a role in this process. Of course, phosphorylation alone is not sufficient to cause all proteins to be transcytosed. For instance, the tranferrin receptor is phosphorylated, but after endocytosis is recycled exclusively to the basolateral surface of MDCK cells (*3*). Many other membrane proteins are transcytosed from the basolateral to apical surface, including apical proteins in both hepatocytes and enterocytes (*23, 25*). It is not currently known whether such proteins are phosphorylated, although potential phosphorylation sites exist in the cytoplasmic domains of both sucrase isomaltase and dipeptidylpeptidase IV (*26*).

In neonatal rat small intestine, the IgG-Fc receptor transcytoses maternal IgG from the apical to basolateral domain (*27*). Similarly, an isoform of the macrophage-lymphocyte Fc receptor has been shown to undergo transcytosis from the apical to basolateral surface of MDCK cells (*28*). Whether phosphorylation of these receptors is required for entry into the apical to basolateral pathway remains to be determined.

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Protease Nexin-II (Amyloid β-Protein Precursor): A Platelet α-Granule Protein

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Protease nexin-II (PN-II) [amyloid β-protein precursor (APP)] and the amyloid β-protein are major constituents of neuritic plaques and cerebrovascular deposits in individuals with Alzheimer's disease and Down syndrome. Both the brain and the circulation have been implicated as sources of these molecules, although they have not been detected in blood. Human platelets have now been found to contain relatively large amounts of PN-II/APP. Platelet PN-II/APP was localized in platelet α-granules and was secreted upon platelet activation. Because PN-II/APP is a potent protease inhibitor and possesses growth factor activity, these results implicate PN-II/APP in wound repair. In certain disease states, alterations in platelet release and processing and clearance of PN-II/APP and its derived fragments could lead to pathological accumulation of these proteins.

THE AMYLOID β-PROTEIN IS A 4.2-kD peptide that is deposited in neuritic plaques and the cerebrovasculature in Alzheimer's disease, Down syndrome, and to a lesser extent in normal aging (*1*). It is derived from a large precursor protein, the amyloid β-protein precursor (APP) (*2*), which can be translated from at least three alternatively spliced mRNAs, two of which contain an insert encoding a Kunitz-type protease inhibitor domain (*3*). The secreted form of APP containing the Kunitz-type inhibitor domain is protease nexin-II (PN-II) (*4, 5*). PN-II effectively inhibits chymotrypsin and trypsin (*4, 6*). In addition, it inhibits two proteases associated with growth factors: the epidermal growth factor binding protein (EGF BP) and the γ subunit of nerve growth factor (*6, 7*). The proteolytic events that lead to the formation and deposition of the amyloid β-protein

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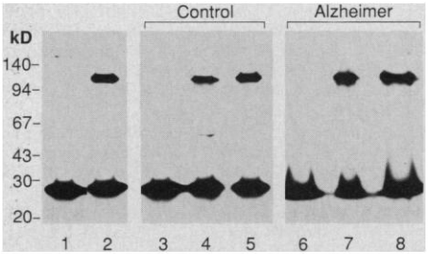

Fig. 1. ^{125}I-labeled EGF BP–PN-II/APP complex formation in platelet lysates. Platelet-poor plasma and platelets were prepared from freshly collected blood obtained from control and Alzheimer's disease patients (*24*). Ten-milliliter aliquots of plasma were diluted with a 20-ml solution of 20 m*M* potassium phosphate and 0.2*M* NaCl, *p*H 7.4. As a control, purified PN-II was added to some plasma aliquots at a final concentration of 0.5 n*M*. All plasma samples were then passed through 1-ml DEAE-Sepharose columns; the columns were then washed with 25 ml of the buffer. Adsorbed proteins were eluted from the columns with 20 m*M* potassium phosphate and 1*M* NaCl, *p*H 7.4. Platelets were resuspended to a final concentration of 0.5×10^9 to 1.0×10^9 per milliliter and solubilized by incubation with 0.5% Triton X-100 for 30 min at room temperature. Aliquots of the DEAE-Sepharose eluates and platelet lysates were then incubated with 40 ng of ^{125}I-labeled EGF BP (1.75×10^6 cpm/pmol) for 15 min at 37°C. An equal volume of SDS-sample buffer was added, and the samples were subjected to nonreducing 10% SDS-PAGE (*23*). Completed gels were dried and autoradiograms were prepared. Lane 1, EGF BP alone; lane 2, EGF BP + purified PN-II; lanes 3 and 6, EGF BP + plasma; lanes 4 and 7, EGF BP + platelet lysates; lanes 5 and 8, EGF BP + (plasma + PN-II).

remain obscure. However, cross-reactivity of secreted forms of APP (identical to PN-II) with antibodies to the amyloid β-protein has been reported, indicating that at least part of the amyloid β-protein is present in PN-II and that secreted PN-II might be a source of amyloid β-protein (*8*). APP mRNA is expressed and PN-II/APP and the amyloid β-protein can be localized by immunohistochemistry in normal brain and in neuritic plaques and cerebrovascular deposits of individuals with Alzheimer's disease and Down syndrome (*1–4*). Deposition of the amyloid β-protein at sites of cerebrovascular malformations led to the suggestion that abnormalities of blood vessel walls might lead to its deposition from the circulation (*9*). In addition, the amyloid β-protein is deposited in non-neural tissues in Alzheimer's disease, a finding that suggested a circulating source of the amyloid β-protein (*10*). However, evidence for PN-II/APP or amyloid β-protein in blood has been lacking.

We searched for a circulating source of PN-II/APP by screening different fractions of whole blood with functional and immunochemical assays. The functional assay was based on the ability of PN-II/APP to form SDS-stable complexes with EGF BP that can be detected after electrophoresis (*6, 7*). Fractionated plasma prepared from fresh blood of a control subject and a patient with Alzheimer's disease did not contain detectable PN-II/APP as judged by this assay (Fig. 1, lanes 3 and 6). Before assaying the plasma, it was fractionated over a DEAE-Sepharose anion-exchange column to concentrate and enrich for PN-II (*4, 6*). This step also removed the large amounts of albumin, immunoglobulins, and other endogenous protease inhibitors. Purified PN-II (0.5 n*M*) added to an equivalent amount of plasma and fractionated by the same procedure could be detected (Fig. 1, lanes 5 and 8). In contrast, platelet lysates prepared from these same blood samples contained PN-II/APP as judged by this functional assay (Fig. 1, lanes 4 and 7).

In support of these findings, protein immunoblots showed the absence of PN-II/APP immunoreactivity in the fractionated plasma samples with the use of a mouse monoclonal antibody (MAb) P2-1 to PN-II that recognizes an amino terminal epitope (*4*) (Fig. 2A) or with affinity-purified rabbit polyclonal immunoglobulin G (IgG) raised against the entire PN-II protein (Fig. 2B). In these experiments the plasma was fractionated as in Fig. 1 for the functional assays. Protein immunoblotting of unfractionated plasma samples also failed to reveal immunoreactivity for PN-II/APP or fragments of it. In contrast, both antibodies to PN-II recognized PN-II/APP in the platelet lysates. Total PN-II/APP in platelet lysates from four different normal individuals was 160 ± 21 ng per 10^8 platelets. MAb P2-1 was also used to effectively immunopurify PN-II/APP from platelet lysates to apparent homogeneity (Fig. 2C). Immunopurified platelet PN-II/APP was recognized in protein immunoblots by rabbit polyclonal antiserum to a synthetic fragment of amyloid β-protein (amino acids 1–38) (*10*) (Fig. 2D), demonstrating that at least part of the amyloid β-protein is present in platelet PN-II/APP.

In order to elucidate the mechanism by which platelets can make PN-II/APP available, freshly washed platelets were treated with collagen or thrombin, physiologic platelet agonists that trigger secretion of platelet granule constituents. Activation of platelets with either collagen or thrombin resulted in secretion of approximately 46 and 53%, respectively, of total PN-II/APP (Table 1). Similarly, low-affinity platelet factor 4, an α-granule constituent (*11*), and adenine nucleotides, dense granule constituents (*12*), were also secreted after treatment of platelets with either agonist. The cytosolic marker lactate dehydrogenase was not detected, demonstrating that the PN-II/APP or the granule markers did not result from platelet lysis. Treatment of the platelets with metabolic inhibitors before activation by either agonist blocked the secretion of PN-II/APP and the granule markers, consistent with findings that platelet activation and granule secretion are active processes.

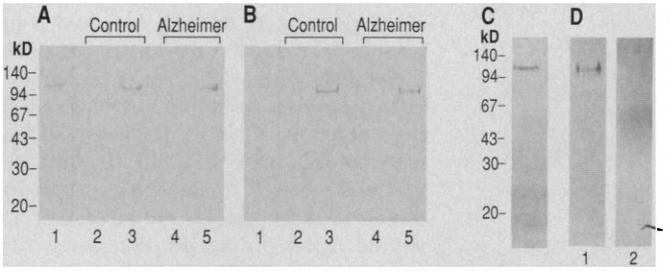

Fig. 2. Protein immunoblots of PN-II/APP in plasma and platelet lysates. (**A**) Protein immunoblot with mouse MAb P2-1 to PN-II (*4*). Samples of plasma and platelets from control and Alzheimer's disease patients were prepared as in Fig. 1. Samples were subjected to 10% SDS-PAGE, followed by electroelution onto nitrocellulose membranes for 2.5 hours at 0.4 A in a Transblot unit (Bio-Rad Laboratories). The membranes were gently agitated overnight at 25°C in tris-buffered saline (TBS) (50 m*M* tris-HCl, 150 m*M* NaCl, *p*H 7.5) containing 0.25% gelatin to block unoccupied sites, and then incubated with mouse MAb P2-1 hybridoma culture supernatant for 1 hour at 37°C with gentle agitation. After several washes with TBS containing 0.05% Tween 20, bound mouse MAb P2-1 was detected with a biotinylated sheep antibody to mouse IgG (Amersham) and a streptavidin–horseradish peroxidase complex. To develop the protein immunoblots, 17 m*M* 4-chloro-1-naphthol in 16 ml of ice-cold methanol was added to 80 ml of ice-cold TBS, followed by addition of 64 μl of H_2O_2. Lane 1, purified human fibroblast PN-II; lanes 2 and 4, fractionated plasma; lanes 3 and 5, platelet lysates. (**B**) Protein immunoblot with affinity-purified rabbit polyclonal IgG to PN-II. Protein immunoblots were performed as in (A) except that affinity-purified rabbit polyclonal IgG (5 μg/ml) was used as the primary antibody. Bound rabbit antibody was detected with a biotinylated donkey antibody to rabbit IgG (Amersham) and a streptavidin–horseradish peroxidase complex. Lane designations are as in (A). (**C**) Immunopurified PN-II/APP from platelets (*26*). Purified platelet PN-II/APP (5 μg) was subjected to 10% nonreducing SDS-PAGE, followed by staining with Coomassie Brilliant Blue. (**D**) Protein immunoblot of purified platelet PN-II/APP with rabbit polyclonal antiserum to synthetic amyloid β-protein (1–38). Purified platelet PN-II/APP (2 μg) was subjected to protein immunoblotting as in (A) except that rabbit polyclonal antiserum (1:200) was used as the primary antibody. Bound rabbit antibody was detected as in (B). Lane 1, rabbit polyclonal antiserum to synthetic amyloid β-protein (1–38). Lane 2, as in lane 1, absorbed with synthetic amyloid β-protein (1–38).

Because our results suggested that PN-II/APP was a platelet granule protein, we ascertained its specific subcellular localization. Differential centrifugation of platelet lysates resulted in the isolation of four fractions. Approximately 53% of the PN-II/APP was recovered in the granule fraction F2 (Fig. 3A). Similar results were obtained for platelet fibrinogen and low-affinity platelet factor 4, known α-granule proteins (*11*). This granule preparation was further fractionated by sucrose density ultracentrifugation. This process showed that approximately 80% of the PN-II/APP activity in the granule fraction F2 was recovered in fraction c, which is enriched for platelet α-granules (Fig. 3B). Similar results were obtained for fibrinogen and low-affinity platelet factor 4. In contrast, serotonin, a known dense granule constituent, was recovered almost exclusively in fraction d, a pellet of the sucrose gradient.

Table 1. Secretion of PN-II/APP from platelets by collagen or thrombin. The preparation and activation of platelets by collagen or thrombin was as described (*21*). Fresh platelets washed by albumin density gradient centrifugation and gel filtration were incubated for 30 min at 37°C in the absence or presence of the combined metabolic inhibitors antimycin A (15 μg/ml). 2-deoxy-D-glucose (30 m*M*), and D-gluconic acid δ-lactone (10 m*M*). The platelets were then placed into a cuvette in a 37°C water bath with stirring at 1200 rpm, and collagen (20 μg/ml) or α-thrombin (1 U/ml) was added. After 10 min, the samples were centrifuged at 12,000*g* for 4 min and supernatants were collected. Aliquots were removed and quantitated for PN-II/APP by incubation with ^{125}I-labeled EGF BP and analysis of complex formation by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. Autroradiograms were aligned with dried gels; ^{125}I-labeled EGF BP–PN-II/APP complexes were located, excised, and quantitated in a γ counter. Aliquots were also quantitated for platelet markers: total adenosine diphosphate/adenosine triphosphate (ADP/ATP) secretion was measured as in (*12*); low-affinity platelet factor 4 (LA-PF4) secretion was quantitated as in (*22*), and cytosolic lactate dehydrogenase (LDH) loss was assessed as in (*23*). Values represent the percentage secreted, which was determined from the ratio of agonist-treated sample to the total amount present in detergent lysate after nonstimulated platelet supernatants were subtracted from both. Values expressed are the mean ± SEM of three experiments, each consisting of combined platelets from two donors.

Markers	Washed platelets		Metabolic inhibitor–treated platelets	
	Collagen (%)	Thrombin (%)	Collagen (%)	Thrombin (%)
PN-II	46.3 ± 7.7	53.7 ± 7.6	0	0
ADP/ATP	45 ± 16	68 ± 11	1.3 ± 1.3	0
LA-PF4	52 ± 11	68 ± 6.2	0	0.4 ± 0.4
LDH	1.3 ± 1.3	0	0	0

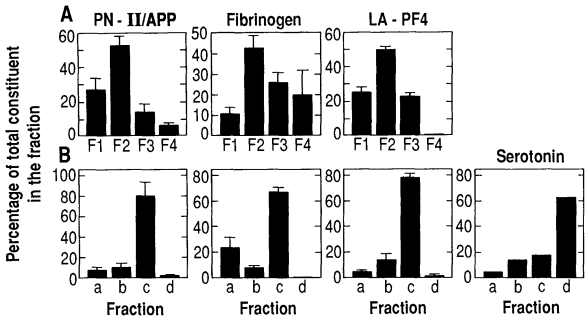

Fig. 3. Platelet subcellular fractionation and localization of PN-II/APP. Platelet subcellular fractionation was performed as described (*21, 27*). (**A**) Platelet subcellular fractionation by differential centrifugation. Data are percentage of each marker ± SEM found within each fraction of the differential centrifugation. A platelet pellet was prepared from 450 ml of blood (*21*), resuspended in a 25-ml solution of 10 m*M* Hepes, 1 m*M* EDTA, and 0.25*M* sucrose and homogenized twice in a French pressure cell. Differential centrifugation of the homogenate (*21*) resulted in four fractions: F1, a 1,000*g* pellet that contained whole platelets and large platelet fragments; F2, a 12,000*g* pellet that contained mitochondria and granules; F3, a 100,000*g* pellet containing membranes; and F4, a 100,000*g* supernatant containing cytosolic material. Aliquots of each fraction were quantitated for PN-II/APP, as in Table 1, and for the α-granule markers fibrinogen (*28*) and low-affinity platelet factor 4 (LA-PF4) (*22*). Only preparations that had a recovery of ≥60% for total protein and each constituent were used in the analysis. The relative specific amounts of each marker in the preparation were calculated from the ratio of the specific amount of marker in the fraction (amount of antigen or activity per milligram of total protein) to the specific amount of the marker in the total platelet lysate as in (*29*). The product of the relative specific amount of marker and percentage of total protein indicates the percentage of marker found within the fraction. (**B**) Platelet subcellular fractionation by sucrose density ultracentrifugation. The granule fraction F2 was further fractionated on a sucrose step gradient that increased from 0.8*M* to 2.0*M* in 0.2*M* increments. Centrifugation resulted in four major fractions: a, material which did not enter the gradient; b, lysosomal granule constituents; c, α-granule constituents; and d, a pellet that consists of dense granules. PN-II/APP, fibrinogen, and LA-PF4 were quantitated as in (A). Serotonin was assayed as in (*30*). The data were calculated as in (A), and the results were plotted as in (A). Each bar graph, except for the serotonin, is the mean ± SEM of three experiments. Data for serotonin are the mean of two experiments.

Our results show that PN-II/APP does not circulate freely in plasma but that it is contained in the α-granules of platelets. On platelet activation, PN-II/APP is secreted along with other platelet α-granule constituents. Our findings suggest a physiological function for PN-II/APP, particularly in light of reports that it possesses growth factor activity (*13, 14*). For example, at wound sites, platelets aggregate and secrete the contents of their storage granules, which include growth factors (*15*). Wound sites also contain elevated levels of proteases, which mediate formation of the fibrin clot, participate in tissue repair by stimulating cell division and cell migration, and influence inflammatory responses (*16*). In fact, PN-II/APP is a potent inhibitor of the intrinsic blood coagulation factor Xla (*17, 18*). Thus, PN-II/APP, which has both protease inhibitor activity and growth factor activity, might be involved in the events that lead to tissue repair. Our results also indicate a pathophysiologic mechanism for the deposition of cerebrovascular and peripheral perivascular PN-II/APP and amyloid β-protein (*9, 10, 19*). In these situations, subtle changes in the vessel walls of the vasculature, as may occur in certain malformations (*9*), may expose sites that could activate platelets and cause them to secrete their granule contents, including PN-II/APP and derived fragments. Platelets from individuals with Alzheimer's disease have alterations in their plasma membranes (*20*). Perhaps this contributes to increased platelet adherence to the vasculature and secretion of PN-II/APP. Thus, possible sources of PN-II/APP and the amyloid β-protein include both the circulation and extravascular tissues. Aberrant proteolytic processing of PN-II/APP or alterations in its clearance might result in the deposition and accumulation of PN-II/APP, the amyloid β-protein, and possibly other fragments in certain disease states.

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26. Approximately ten units of outdated platelets were extensively washed and pelleted by centrifugation. They were then resuspended in a 200-ml solution of 20 m*M* potassium phosphate and 0.15*M* NaCl, *p*H 7.4, containing 1% Triton X-100, 5 m*M* EDTA, 500 μ*M* phenylmethanesulfonyl fluoride, 10 μ*M* chymostatin, and 10 μ*M* tosyl phenyl chloromethyl ketone and solubilized with a Polytron (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 10,000*g* for 30 min at 4°C; the resulting supernatant was subjected to 40 to 80% ammonium sulfate precipitation. The 80% pellet was resuspended in a 50-ml solution of 20 m*M* potassium phosphate and 0.2*M* NaCl, *p*H 7.4, dialyzed against the same buffer and chromatographed over a 10-ml DEAE-Sepharose column. The adsorbed protein was eluted with 20 m*M* potassium phosphate and 1*M* NaCl, *p*H 7.4. The eluate was directly applied to a 3-ml immunoaffinity column of MAb P2-1–Sepharose, washed and eluted with 0.2*M* glycine-HCl and 0.15*M* NaCl, *p*H 2.7, followed by immediate neutralization with 2*M* tris-HCl, *p*H 8.0.
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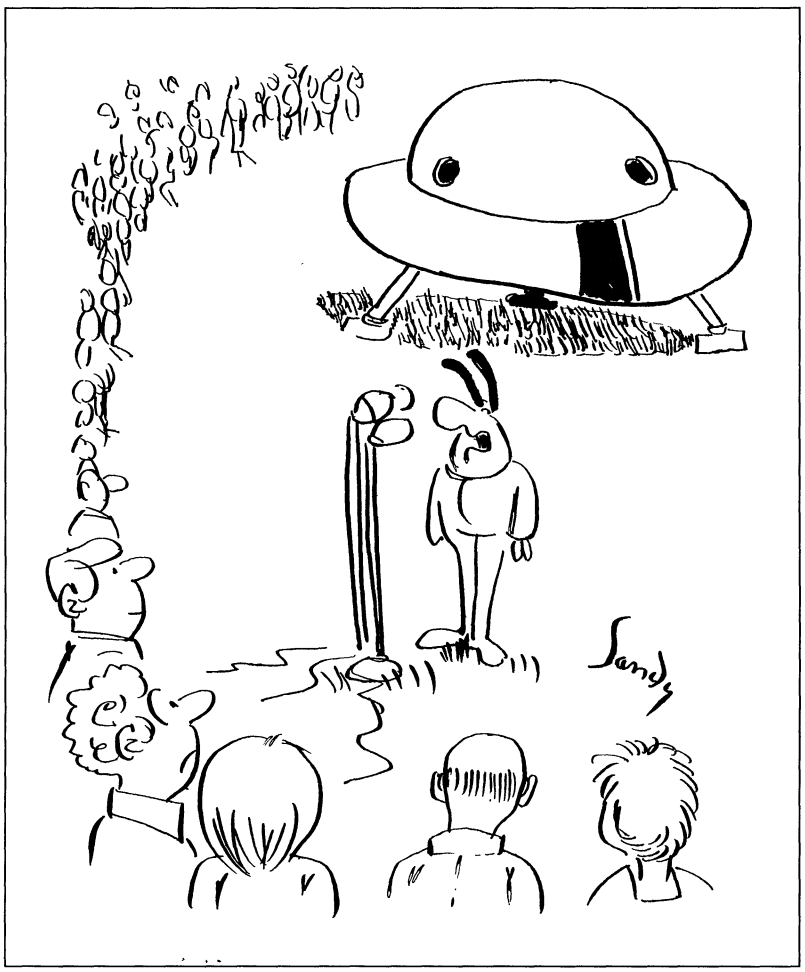

" Unaccustomed as I am to public speaking - "