Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 288-295

STIMULATED PLATELETS RELEASE AMYLOID β-PROTEIN PRECURSOR

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Received June 1, 1990

Human platelets can be stimulated by thrombin or ionomycin to secrete soluble truncated amyloid β-protein precursor and particulate membrane fragments which contain C-terminal and N-terminal immunoreactive amyloid β-protein precursor. This suggests a possible circulating source of β-protein in serum which may play a role in the formation of amyloid deposits. The release of soluble amyloid β-protein precursor could be involved in normal platelet physiology.

Alzheimer's disease (AD) is characterized by extracellular deposits of amyloid β or A4 protein fibrils in senile plaque cores and vessel walls in the brain (1,2). Preamyloid deposits of the same β-protein appear to occur in large numbers early in the disease process (3–5). Amyloid β-protein precursor (APP) represents a family of transmembrane glycoproteins of ~Mr 90–135 kD which contain the ~4 kD amyloid β-protein and part of this β-protein is included in the transmembrane sequence (6–10). The mechanism by which this transmembrane β-protein is removed from cells and forms extracellular deposits is currently unknown. The N-terminal part of APP is normally constitutively released by cultured cells, which involves truncation of the C-terminal region (11–13). The normal cleavage site is within the β-protein sequence (14) which should prevent the release of intact β-protein. There is also evidence for lysosomal degradation of both soluble (C-terminal truncated) APP, which may represent reinternalized APP, and full-length C-terminal immunoreactive

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ABBREVIATIONS: AD, Alzheimer's disease; APP, Amyloid β-protein precursor; CNS, central nervous system; APPX-Y, peptide at location X-Y in APP695; CHO, Chinese hamster ovary; BSA, Bovine serum albumin.

0006-291X/90 $1.50

APP (15). APP is almost ubiquitously expressed and there are several potential cellular sources of β-protein deposits in the brain. Until recently, amyloid deposits had been found only in the CNS in AD suggesting a possible neuronal or glial origin. However, the association of amyloid deposits with vessel walls (1) and the occurrence of similar vascular β-protein immunoreactive preamyloid deposits in skin and intestines in AD have raised the possibility of a circulating source for β-protein (16). Indeed, the C-terminus of APP has been previously detected by radioimmunoassay in human serum (5), although the size of this protein and therefore the inclusion of β-protein within it were not determined. The possible mechanisms by which C-terminal APP immunoreactive material may be externalized and released into serum and other extracellular compartments and the presence of the β/A4 sequence in this C-terminal APP immunoreactive material remain to be explored. Here we report that stimulated platelets release membrane fragments containing full-length C-terminal and N-terminal immunoreactive APP which should contain intact β/A4 sequence. If platelet vesicle release occurs *in vivo*, this type of mechanism may help account for circulating β-protein, and may be a source of β-protein in amyloid deposits. If it occurs only *in vitro*, it may still account for the presence of C-terminal APP epitopes in serum.

MATERIALS AND METHODS

Antisera and Immunoblotting. The preparation of a rabbit polyclonal antiserum to a synthetic peptide representing APP695 (6) residues 175–186 ("anti-GID") has been previously described and extensively charac-terized (13,15,17). Using identical methodology, a rabbit polyclonal antiserum was prepared to the synthetic peptide *N'*-Lys–Lys–Lys–Gln–Tyr–Thr–Ser–Ile–His–His–Gly–Val–Val–Glu–Cys-C' representing residues 649–662 of the Kang sequence for APP695 with C-terminal addition for coupling to rabbit serum albumin with *m*-maleimidobenzoyl-*N*-hydroxy-succinidimide as previously described (17). The antibody was affinity purified on an agarose–peptide column (17). The specificity of this C-terminal antiserum was verified by testing the preimmune, immune, absorbed and affinity purified antisera on Western blots of homogenates from parent Chinese hamster ovary (CHO) cells and CHO cells transfected with the full-length cDNA for APP770 under the control of a metallothionein promoter (the generous gift of Dr. Sangram S. Sisodia) which had been induced to overexpress APP with 100 nm $CdCl_2$ added to the culture medium for 24 hr prior to harvesting. Gel electrophoresis and Western blotting were carried out as previously described (17) using the N-terminal anti-APP 175–186 serum at 1:2,000 dilution and the C-terminal anti-APP 649–662 at 1:1,000 dilution.

Platelet Preparation. Washed human platelets were prepared by differential centrifugation and washing in the absence of apyrase but

otherwise as described by Mustard et al. (18). Platelets were isolated from acid citrate/ dextrose treated platelet rich plasma obtained from healthy normal volunteers who had not received medications, in particular aspirin, for 2 weeks prior to donation.

Platelet Stimulation. Freshly prepared human platelets (approximately 3 x 10^9 per 100 µl in Eppendorf tubes) in Tyrode's buffer with heparin, and with or without BSA, were incubated with buffer alone or with 10 U/ml of human α-thrombin or 50 nM ionomycin (Calbiochem) at 37°C. No decline in platelet viability as indexed by trypan blue exclusion or morphology was evident during the course of this incubation. Platelets were pelleted by centrifugation at 1200 x g for 15 min at 22°C and the supernatants were taken as "platelet releasates." Releasates were either pelleted at 100,000 x g for 1 hr in a Beckman TLA-100 rotor or brought up to 1x Laemmli sample buffer and electrophoresed and immunoblotted for APP.

RESULTS AND DISCUSSION

As shown in Fig. 1, the new C-terminal anti-APP 649–662 antiserum recognizes the overexpressed Mr~110 kD band in the membrane pellet of CHO cells transfected with full-length APP770 cDNA. The same band is recognized by the N-terminal GID anti-APP 175–186 antiserum whose specificity we have previously validated (13,15,17). The secreted form of amyloid precursor in conditioned medium from transfected cells is recognized by the N-terminal, but not the C-terminal APP antisera. Higher concentrations of C-terminal antiserum and longer exposure times failed to reveal any C-terminal immunoreactive staining in the conditioned medium. Preimmune antisera controls are negative and affinity-purified C-terminal antibodies only recognize the full set of ~Mr 120 kD N-terminal immunoreactive bands.

Homogenates of freshly harvested platelets reveal two N-terminal immunoreactive bands which can be absorbed out by incubation with excess free APP 175–186 peptide (Fig. 2A). After stimulation by either ionomycin or thrombin, platelets release N-terminal immunoreactive material which can be pelleted at 100,000 x g for 1 hr (Fig. 2B). The observation that stimulated platelets released pelletable APP immunoreactive material under conditions where membranous vesicles or microparticles are known to be released prompted an examination with C-terminal anti-APP serum.

Thrombin-stimulated platelets release two N-terminal immunoreactive bands, but only the upper band is stained by the C-terminal anti-APP serum, indicating that the lower band probably contains C-terminal truncated APP (Fig. 3). Figure 3 also demonstrates that thrombin-

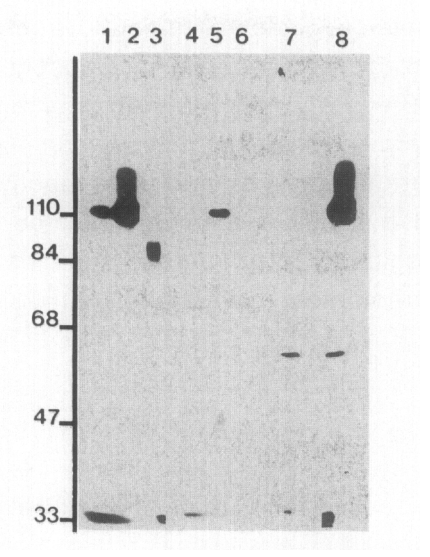

Fig. 1: A Western blot from an 8% SDS–PAGE gel illustrating the specificity of the antisera employed in this study. Confluent CHO cells and CHO cells transfected with APP770 cDNA under the control of a metallothionein promoter were grown overnight in serum-free MCDB medium supplemented with 100 nm $CdCl_2$ and 100 μm $ZnCl_2$. Conditioned medium and cells were harvested, electrophoresed, blotted, and developed with antisera and I^{125} Protein A as previously described (15). Lanes 1, 4: CHO cell homogenate; Lanes 2, 5, 7, 8: homogenate from CHO cells expressing APP770 cDNA; Lanes 3, 6: conditioned medium from CHO cells expressing APP770 cDNA. Lanes 1–3, anti-APP 175–186 sera at 1:2,000 dilution; Lanes 4–6, anti-APP 649–662 sera at 1:1,000 dilution; Lane 7, preimmune sera (1:1,000) for anti-APP 649–662; Lane 8, a 1:100 dilution of affinity-purified anti-APP 649–662. Antisera are used at the same dilutions in subsequent experiments. Note that both antisera recognize the APP overexpressed in APP770-transfected cell homogenates, but only the N-terminal APP 175–186 recognizes the released APP in the conditioned medium.

stimulated platelet releasates contain C-terminal immuno-reactive APP, suggesting that the full-length membrane spanning APP is released from stimulated platelets. This suggests the release of intact APP containing the entire β/A4 sequence capable of participating in the formation of preamyloid deposits. In Fig. 3 we show that the C-terminal and N-terminal immunoreactive bands are pelletable with a high-speed spin (100,000 x g) and extractable with detergent as one would expect from a membrane protein. A lower N-terminal APP immunoreactive band is present in the supernatant of the platelet releasate.

These results are consistent with the well-established calcium-mediated release of membrane vesicles from platelets after stimulation

with thrombin, collagen, or calcium ionophore (19–22). These vesicles have been characterized with respect to phospholipid content (20) and shown to contain a variety of platelet membrane and cytoskeletal proteins, notably the prothrombinase enzyme complex of factors VaXa and actin (21–23). Platelet-derived vesicles are readily isolated from normal serum or plasma and available evidence indicates that they are probably not an artifact of isolation procedures, but instead occur in normal physiological and pathological conditions (21–23). These vesicles are

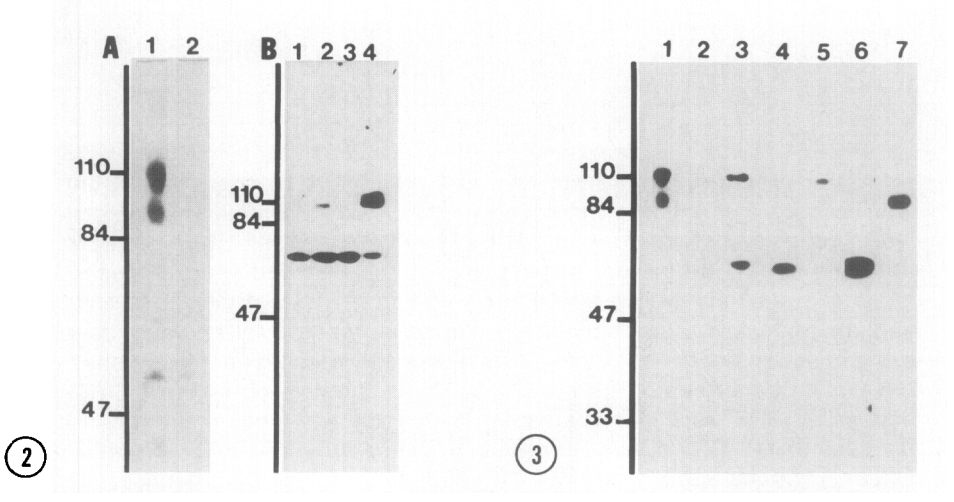

Fig. 2: A. Platelet homogenates were run on an 8% gel and immunoblotted with anti-APP 175–186 (Lane 1) and with anti-APP 175–186 absorbed with 10 μg/ml APP 175–186 peptide (Lane 2). B. Platelets were stimulated with 50 nM ionomycin for 30 min or 10 U/ml α-thrombin for 1 hr in the presence of Tyrodes buffer with BSA and pelleted (see Methods). The supernatants (platelet releasates) were centrifuged at 100,000 x g to bring down membranous vesicles in the releasate. The pelleted material was electrophoresed on an 8–16% minigel and immunoblotted with anti-APP 175–186. Lane 1: control platelet releasate pellet (30 min); Lane 2: ionomycin platelet releasate pellet (30 min); Lane 3: control platelet releasate pellet (60 min); Lane 4: thrombin platelet releasate pellet (60 min). The band at 68 kD present in the controls represents a nonspecific interaction with the large amounts of BSA present in these samples.

Fig. 3: A Western blot of platelet releasate fractions prepared with a 60-min incubation with thrombin in Tyrodes buffer without BSA. Lanes 1–4, platelet releasates with anti-APP 175–186 (Lane 1); absorbed anti-APP 175–186 (Lane 2); anti-APP 649–662; preimmune for anti-APP 649–662. A platelet releasate fraction was pelleted at 100k and the pellet was then extracted with 1% Triton and pelleted again at 100k. The material released by detergent from the first 100k pellet is shown with anti-APP 649–662 in Lane 5. The supernatant from the first 100,000 x g centrifugation is shown with anti-APP 649–662 in lane 6 and with anti-APP 175–186 in Lane 7. Again, the bands at 68 kD represent nonspecific staining of residual from the large amounts of BSA in the original platelet preparation.

thought to make a substantial contribution to the physiologicaly active prothrombinase activity which plays a positive feedback role in the local generation of thrombin (21). Factor Xa is a serine protease which can be inhibited by the Kunitz protease inhibitor bearing forms of APP (24). Thus APP with the inhibitor (protease nexin II) could inhibit thrombin generation, possibly as part of a negative feedback loop. Our antisera against the Kunitz protease inhibitor domain label the released APP band, but also several other bands (not shown).

The observation of full-length, C-terminal immunoreactive APP in platelet releasates has several potentially important implications. First, it suggests a possible serum source for intact, circulating β–protein and a possible explanation for the C-terminal immunoreactive APP found in serum (5). Second, it is particularly intriguing in view of the recent report that AD brain contains markedly reduced free levels of a potent and specific thrombin inhibitor, protease nexin I (25). In the absence of inhibition by protease nexin I in AD brain, local generation of thrombin activity could stimulate APP release from platelets. Third, the calcium-mediated release of membrane vesicles from platelets is also induced by the complement attack complex (C5b-9) (21) and this complement component has been identified immunologically as a neuritic plaque component in AD (26). Finally, the calcium-mediated release of C-terminal immunoreactive vesicles might be enhanced in familial AD since there is evidence for abnormal platelet membrane systems in familial AD (27,28) and abnormal responses to calcium in familial AD cells in culture (29).

The calcium-mediated release of C-terminal immunoreactive APP might occur in other cells as even kidney tubular cells have been shown to exhibit this type of exocytotic, "blebbing" response (30). Given the close biological parallels which have lead to the use of platelets as model systems for neuronal synaptosomes (31), one might even anticipate that neurons could be induced to release similar vesicles containing intact β-protein, possibly in response to calcium influx associated with excitotoxic stimulation or even thrombin, given the presence of thrombin receptors on neurons (32,33).

We have previously described two routes for APP traffic, secretion and lysosomal degradation. The release of membranous microparticles from activated platelets is a well-established phenomenon *in vitro* and is also thought to occur *in vivo* because of the presence of platelet-derived microparticles in serum and plasma. This provides an example of a novel pathway for the export of APP molecules which should include the amyloidogenic domain. While this is without doubt normally a minor

pathway, it may become significant over a lifetime or in pathological situations. Finally, the stimulated release of the C-terminal truncated, soluble APP may play a role in normal platelet physiology and the regulation of coagulation.

Acknowledgments This work was supported by grants from the California State Dept. of Health Services Alzheimer Disease Program Contract (G.M.C.) and from the NIA (P50-AG05131).

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