Biochimie 95 (2013) 1252–1257

Contents lists available at SciVerse ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Regulation of β-catenin stabilization in human platelets

Sharda Kumari, Debabrata Dash*

Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, UP, India

ARTICLE INFO

Article history:
Received 29 October 2012
Accepted 30 January 2013
Available online 9 February 2013

Keywords:
Platelet aggregation
Wnt signalling
Protein kinase C
Proteasome
Calpain
Thrombin

ABSTRACT

The Wnt/β-catenin pathway controls developmental processes and homeostasis; however, abnormal activation of this pathway has been linked to several human diseases. Recent reports have demonstrated regulation of platelet function by canonical and non-canonical Wnt signalling. Platelet aggregation plays a crucial role in haemostasis and thrombosis. Here we report for the first time that, induction of sustained aggregation of platelets by a strong agonist in the presence of calcium was associated with nearly complete proteolysis of β-catenin, which was abrogated upon depletion of calcium from platelet suspension. β-catenin cleavage was disallowed in absence of aggregation, thus implicating integrin $\alpha_{IIb}\beta_3$ engagement in β-catenin proteolysis. Degradation of β-catenin was blocked partially by inhibitors of either proteasome or calpain and completely when cells were exposed to both the inhibitors. Protein kinase C inhibition, too, abolished β-catenin degradation. Thus activities of proteasome, calpain and protein kinase C regulate stabilization of β-catenin in aggregated human platelets.

1. Introduction

The Wnt pathway controls developmental processes and homeostasis but abnormal activation of this pathway has been linked to many human diseases [1]. β-catenin is a multifunctional protein serving both as a structural component of cadherin cell adhesion complex and a signalling component of the Wnt pathway in regulating embryogenesis and tumorigenesis. Presence of β-catenin in cytoplasm or nucleus is minimized because of its rapid degradation by proteasome, facilitated by the multiprotein destruction complex consisting of axin, glycogen synthase kinase-3β (GSK-3β) and adenomatous polyposis coli (APC). Within this complex β-catenin is sequentially phosphorylated by casein kinase Iα (CKIα) followed by GSK-3β. Phosphorylated β-catenin is then recognized by the F-box protein β-TrCP, which is a component of E3 ligase complex, and targeted for proteasomal cleavage. In the presence of Wnt, dishevelled (Dvl) negatively regulates phosphorylation of β-catenin, preventing its degradation and leading to its cytosolic stabilization and transcription enhancing activity [1–4]. In another report thrombin has been shown to induce GSK-3β phosphorylation in human umbilical vein endothelial cells contributing to stabilization of cytosolic β-catenin Ref. [5]. Recently, Protein kinase C (PKC) isoforms have also been reported to contribute to phosphorylation as well as degradation of β-catenin [6,7].

A key role for cytosolic β-catenin is to link adhesion receptors of the cadherin family to the actin cytoskeleton. In non-stimulated cells β-catenin is largely associated with cadherin [8,9]. Earlier report has demonstrated the presence of cadherin in human blood platelets and recently cadherin 6 (CDH 6) has been implicated in platelet adhesion [10,11]. It is important to keep the activation of this pathway tightly controlled, because mutations in APC or β-catenin, which result in stabilization of β-catenin, are detected in different types of cancer [12].

Recent reports have demonstrated that platelets express several components of Wnt/β-catenin signalling pathway regulated by both canonical and non-canonical Wnt signalling [13,14]. Responsiveness of platelets towards Wnt ligand was established from the fact that recombinant Wnt3a, one of the canonical Wnt, inhibited platelet aggregation [13], whereas Wnt5a, a typical non-canonical Wnt, stimulated platelet aggregation [14]. Platelet activation is critical for haemostasis as well as pathological thrombus formation. Understanding the regulation of platelet activity is thus fundamental to comprehending thrombotic disorders and developing therapeutic strategies [15].

Here we report for the first time that stabilization of β-catenin in platelets is determined by the activation state of cells. Sustained aggregation of platelets for 15 min in the presence of extracellular calcium was associated with nearly complete proteolysis of β-catenin. Phosphorylation of β-catenin by PKC but not by GSK-3β directed it towards proteolysis, which was regulated by peptidase activities of calpain as well as proteasome. Thus, stabilization of β-catenin is determined by different signal mediators in activated platelets.

* Corresponding author. Tel.: +91 9336910665; fax: +91 542 2367568.
E-mail address: ddass@satyam.net.in (D. Dash).

0300-9084/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.biochi.2013.01.021

2. Material and methods

2.1. Materials

Mouse monoclonal antibody against β-catenin was procured from BD Transduction Laboratories. Anti-β-actin antibody, A23187, LiCl, aspirin, apyrase, RGDS and thrombin were obtained from Sigma. Ro-31-8220, PS1, ALLN, calpeptin, MG132, BAPTA-AM and Wortmanin were from Calbiochem. Horseradish peroxidase (HRP)-labelled anti-mouse secondary antibody was the product of Santa Crutz. Abciximab (Reopro) was purchased from Eli lilly. Reagents for electrophoresis and $CaCl_2$ were purchased from Merck India. All other reagents were of analytical grade. Milli-Q grade deionised water (Millipore) was used for preparation of solutions.

2.2. Platelet preparation

Platelets were isolated by differential centrifugation from fresh human blood, as already described [16]. Briefly, Blood from healthy volunteers was collected in citrate–phosphate–dextrose adenine and centrifuged at 180 × *g* for 20 min. Platelet-rich plasma (PRP) was incubated with 1 mM acetylsalicylic acid for 15 min at 37 °C. After addition of ethylenediaminetetraacetic acid (EDTA) (5 mM), platelets were sedimented by centrifugation at 600 × *g* for 10 min. Cells were washed in buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 1 mM ethylene glycol tetraacetic acid (EGTA), supplemented with 5 mM glucose and 0.6 ADPase units of apyrase/ml, pH 6.2. Platelets were finally resuspended in buffer B (pH 7.4), which was the same as buffer A but without EGTA and apyrase. The final cell count was adjusted to $0.5–0.8 \times 10^9$/ml. All steps were carried out under sterile conditions and precautions were taken to maintain the cells in inactivated state. Ethical clearance was obtained in accordance with the guidelines.

2.3. Platelet aggregation

Platelets were stirred (1200 rpm) at 37 °C in a Whole Blood/Optical Lumi-Aggregometer (Chrono-log, model 700-2) for 1 min prior to the addition of agonists. Aggregation was measured as percent change in light transmission, where 100% refers to transmittance through blank sample. Finally, cells were boiled in Laemmli lysis buffer and stored at −20 °C till further analysis.

2.4. Western blotting

Platelet proteins were separated on 10% sodium dodecyl sulphate-polyacrylamide gels and electrophoretically transferred onto PVDF membranes (0.8 mA/cm^2, 1.5 h) in a semi-dry blotter (TE 77 PWR, GE Healthcare India). Membranes were blocked with 5% bovine serum albumin in 10 mM Tris–HCl, 150 mM NaCl, pH 8.0 (TBS) containing 0.05% Tween-20 for 1 h at room temperature. Blots were incubated overnight with monoclonal antibody against β-catenin (1:500) or β-actin (1:1000), followed by HRP-labelled anti-mouse IgG for 2 h. Antibody binding was detected using enhanced chemiluminescence and quantified with Agfa Duoscan T1200 flatbed scanner using GeneTools software (Syngene).

3. Results

3.1. Sustained aggregation of platelets leads to proteolysis of β-catenin in presence of extracellular calcium

Platelet aggregation, the process by which platelets adhere to each other at sites of vascular injury, has long been recognized as critical for haemostatic plug formation and thrombosis [17]. As Wnt signalling is known to modulate platelet activity [13,14], we asked whether platelet aggregation is linked to expression level of β-catenin in cells. When platelets were induced to aggregate by thrombin (1 U/ml) in the presence of calcium (1 mM), there was progressive drop in the amount of β-catenin in time-dependent manner, with substantial fall (by 85%) recorded at 15 min (Fig. 1A). Degradation of β-catenin was completely precluded when thrombin-activated platelets were prevented from aggregation by lack of stirring (Fig. 1A), pre-treatment with abciximab (Reopro), an $\alpha_{IIb}\beta_3$ integrin antagonist, or with the tetrapeptide Arg–Gly–Asp–Ser (RGDS), the competitive inhibitor of fibrinogen binding (Fig. 2A). Addition of abciximab (40 μg/ml) or RGDS (0.5 mM) prevented thrombin-mediated platelet aggregation by about 87% or 83%, respectively (data not shown). As aggregation involves interaction between platelet surface integrins $\alpha_{IIb}\beta_3$ and fibrinogen [18], above data implicated integrin-mediated outside-in signalling in proteolytic cleavage of β-catenin. In order to find out whether observed proteolysis was specific for β-catenin or global in character, platelet proteins following electrophoresis were stained with colloidal Coomassie blue. No change was observed in protein profile between resting and aggregated platelets (data not shown). Degradation of β-catenin was also abrogated when platelets were suspended in a medium without added calcium (Fig. 2C), though there was no change in amplitude of aggregation. As extracellular calcium is known to regulate cytosolic free calcium level in thrombin-stimulated platelets by capacitative entry from the external milieu [19], calcium appeared to play critical role in β-catenin proteolysis.

To assess the role of intracellular Ca^{2+} we pre-incubated platelets with intracellular Ca^{2+} chelator BAPTA-AM (10 μM) that resulted in 83% inhibition of platelet aggregation (data not shown). Under same experimental condition β-catenin degradation was prevented by 61%, thus supporting the role of intracellular calcium in β-catenin degradation (Fig. 2A).

3.2. Expression of β-catenin in human platelets is regulated by proteolytic activities of calpain and proteasome

β-catenin is a known substrate of proteasome in canonical Wnt signalling pathway [20]. β-catenin stability is also determined by the Ca^{2+}–dependent thiol protease calpain [21]. We next explored the factors responsible for downregulation of β-catenin following sustained aggregation of platelets. Proteolysis of β-catenin was blocked partially either by calpeptin (calpain inhibitor, 80 μM) or by MG132 (proteasome inhibitor, 50 μM), and completely when both the inhibitors were employed together (Fig. 3A), thus implicating inputs from both the peptidases in β-catenin destabilization. Neither of inhibitors had any significant effect on platelet aggregation (Fig. 3C). Similar results were obtained when we substituted calpeptin and MG132 with pharmacologically distinct inhibitors of calpain (ALLN, 50 μM) and proteasome (PS1, 50 μM), respectively (not shown).

As calpain is a Ca^{2+}-dependent protease, we next investigated the effect of Ca^{2+} ionophore A23187, which enhances level of cytosolic free calcium, on β-catenin stability. Treatment of platelets with A23187 (1 μM) in presence of extracellular calcium brought about complete degradation of β-catenin (Fig. 3D), consistent with an earlier report [12,21] Calpain activity has been demonstrated to be upstream of proteasome and stimulation of calpain was shown to elicit significant induction in proteasome activity [22]. Thus, it can be surmised that calpain-proteasome axis leads to observed destabilization of β-catenin in a calcium-dependent manner in thrombin-stimulated platelets.