The role of the platelet cytoskeleton in platelet function

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1. Introduction

Platelets are anucleate circulating blood cells. Activation of platelets is an important part of the complex mechanism of thrombosis and haemostasis. In an inactive state they circulate in blood and have the shape of disks with a smooth surface. Platelets are activated in the body when they come into contact with areas of endothelial damage or by activation of the coagulation cascade. Activated platelets change in shape, release their granule contents and form aggregates by sticking together. These shape changes may include conversion to a spherical shape, extension of pseudopodia or flattening on a surface during spreading - depending on the type of stimulus. The cytoskeleton is primarily responsible for regulating platelet shape [Fox 2001]. The platelet cytoskeleton is responsible for binding and positioning signaling molecules. Some parts of the cytoskeleton can bind signaling molecules in the unstimulated platelet, keeping them at specific submembranous locations where they can be triggered when the platelet is activated. Activated platelets bind to the plasma protein fibrinogen and this may result into a platelet aggregate. Next the platelet aggregate is stabilized by contraction of actin and myosin fragments in the platelets, leading to clot retraction. This physiological process allows platelet aggregation, contraction or spreading at sites of injury to prevent blood loss. In case of inappropriate or excessive platelet activation, however, this process results in blood flow defects due to thrombotic complications. The most common arterial thrombotic diseases are acute myocardial infarction and stroke. In addition, activated platelets are involved in inflammatory processes by expressing and releasing growth factors, chemokines and cytokines that attract and activate leukocytes and endothelial cells. Via these processes, platelets may be involved in inflammatory arthritis, adult respiratory distress syndrome, and tumor growth/metastasis. The role of the cytoskeleton in inflammatory processes remains to be delineated. A better understanding of how the cytoskeleton is regulated in platelets will provide insight in the regulation of platelet deformation, platelet adhesion, aggregation and clot retraction. In this review an overview of platelet physiology in relation to the major components of the cytoskeleton will be discussed. It includes mechanisms of platelet cytoskeleton reorganization upon stimulation and in resting platelets. Furthermore, important signaling molecules that are now known to be involved in inducing cytoskeletal reorganizations in platelets will be discussed.

2. Biological function platelet cytoskeleton

Accumulation of platelets at the site of vascular injury play a major role in preventing blood loss after injury. However, platelets are also responsible for the formation of pathogenic thrombi. For example acute coronary syndromes (ACS), peripheral artery disease (PAD) and transient ischemic attack (TIA).

The platelet cytoskeleton plays a key role in preventing blood loss after an injury and abnormal cytoskeletal function in platelets may result in thrombi formation. Wound healing and formation of thrombi begins with activation, adhesion, migration and aggregation of platelets. The cytoskeleton plays a prominent role in the all these processes. For example migration through tissues and endothelial barriers is a complex series of events requiring major dynamic rearrangements of the cytoskeleton [Adrian J et al.2009]. Chapter five will give a clear overview of different ways of platelet activation and the crucial role of the cytoskeleton in spreading and aggregation of platelets which finally leads to clot retraction.

3. Major components of the cytoskeleton

The most abundant cytoskeletal proteins are microtubules, actin microfilaments and intermediary filaments. Here the intermediary filaments will not be discussed. Electron microscopy studies have shown interconnections between cytoskeletal components and other proteins in intact platelets [Escolar et al. 1987, Nachmias 1983]. In this chapter the microtubuli and actin filaments will be discussed.

A. Microtubuli

The microtubuli form the submembranous skeleton in contrast to the network of long interconnecting actin filaments that are mainly located in the cytoplasm. Early studies showed depolymerization and repolymerization of microtubule filaments in activated platelets [Fox 2001]. However, little is known about the mechanism in which organizations of these microtubules are maintained in unstimulated platelets or about regulation of depolymerization and repolymerization in activated platelets.

Different studies showed that microtubules can interact with signaling molecules and play a role in the regulation of the integrin-induced signaling in platelets [Danowski 1989, Cook et al. 1998, Kaverina et al. 1998, Pletjushkina et al. 1998]. Microtubules may play an important role in the regulating of spatial interconnections and activation of signaling molecules in platelets. More research is needed to get better insight in the relation of the microtubules in relation to the cytoskeleton.

B. Actin

More is known about the organization, composition and function of the actin microfilaments of the cytoskeleton. Actin filaments contain a “barbed end” and a “pointed end”. They can add monomers on both ends. However this occurs more rapidly at the barbed end in contrast to the pointed ends [Pollard et al. 1986]. The dissociation of monomers is more rapidly from the pointed ends. This process is regulated by the integrated interaction of actin monomers and filaments with a variety of proteins that can break filaments or inhibit actin polymerization (i.e. gelsolin, CAPZ, adseverin, tensin and adusin) and proteins that prevent depolymerization of filaments or induce polymerization (i.e. gelsolin,

profilin, ARP 2/3, WASP and VASP). All together this will result in reorganization of the platelet cytoskeleton which will be discussed in chapter four and five in more detail.

Actin filaments and associated proteins remain insoluble and most of the actin filaments are cross-linked networks and can sediment at 15,600 X g. These actin filament networks contain different proteins (Table 1) that can interact with actin filament networks inducing cross-linking or regulating their association with other proteins.

Actin filaments of unstimulated platelets that are located proximal to the plasma membrane glycoprotein can develop a much higher g-force of 100,000 X g [Fox et al. 1988]. These membrane actin filaments are crucial components of the cytoskeleton, because it lines the membrane, mediates linkage to the membrane and recruits signaling molecules. The membrane actin filaments that are associated with the cell membrane can co-sediment with several proteins and signaling molecules. These co-sediment proteins can cross-link actin filaments or regulate their interactions with the membrane. Thereby these proteins may also bind signaling molecules. Together with actin filaments, these proteins play a main role in regulating the overall signaling pathways and the directing shape of the platelet. All proteins are capable of binding membrane proteins and link the membrane skeleton to the membrane.

Membrane cytoskeletal related proteins and signaling molecules that are now known to be involved in inducing cytoskeletal reorganizations in platelets are described below. Suggesting that there exist different components of the membrane skeleton. With the focus on how they are assembled in the unstimulated, aggregating and spreading platelets. Some of the main proteins will be discussed.

Cytoskeletal Related Proteins and signaling molecules					
$A_{IIb}\beta_3$	FcY receptors	Laminin	P-selectin	Skelemin	α-thrombin
ABP	Fibrinogen	Layilin	PtdIns(3,4)P2	Spectrin	α2β1, α5β1, α6β1, αVβ3, αLβ2, αMβ2
Actin	Filamin	Mac-1	$P21^{ras}GAP$	Syntrophin	
ankyrin	Gelsolin	Moesin	$Pp60^{c\text{-}src}$, $Pp60^{c\text{-}fyn}$, $Pp62^{c\text{-}yes}$, $Pp72^{syk}$, $Pp54/58^{c\text{-}yes}$	Talin	
ARP2/3	Glycophorin	Myosin	P125FAK, P65FAK	Titin	
Calmodulin	Glycoprotein Ibα	Myosine light chain kinase	Rab1b	VASP	
Calpain	GP Ib-IX	PI(4,5)P2	RAC	Vav	
CDC42	GRB2	PI5-k, PI3-k	Rho	Vinculin	
DRP	HSP27	Protein kinase C	ROK	Von willebrand factor	
Dystrophin	Integrin β1A, β1d, β2, β3, β5, β7	Protein 4.1	Shc	WASP	

Table 1. Proteins and signaling molecules sedimenting from platelets.

1. Actin-binding protein (ABP)

ABP is a dimer of approximately 250 kDa. Three cDNAs from three different genes together with other variants, resulting from alternative splicing have been described [Gorlin et al. 1990, Takafuta et al. 1998, Xu et al. 1998]. ABP-280/filamin1 is the most common ABP in platelets. ABP contains a dimerization domain at the carboxy-terminal and an actin-binding site at the amino-terminal domain. ABP mediates cross-linking of actin filaments and is responsible for the attachment of the membrane skeleton to the membrane. Glycoprotein Ibα is the most important site for attachment ABP to the membrane and the binding domain is located within repeat 17-19 [Andrews et al. 1991, Andrews et al. 1992, Meyer et al. 1997]. This glycoprotein Ibα also interacts with von Willebrand factor at the injured vessel wall, which results in platelet adhesion and simultaneously triggers intracellular signaling events (i.e. elevation of intracellular calcium and activation of multiple protein kinase pathways), that results in integrin activation and thrombus formation. Thereby, glycoprotein Ibα can bind P-selectin, α-thrombin and Mac-1 at the extracellular N-terminal 282 binding site residues [Wang et al. 2010]. Beside glycoprotein Ibα, many other unidentified glycoproteins have been recovered in Glycoprotein Ib-IX-ABP immunoprecipitates. Beside glycoprotein Ibα, platelet ABP can interact with other membrane proteins (i.e. tissue factors and the FcY receptors) [Ohta et al. 1991, Ott et al. 1998]. Lastly, before repeats 24, an insertion of 35 amino acids was found, the hinge region, for respectively cleavage ABP by Calpain [Wang et al. 2010, Washington RW et al. 2008, Uribe R et al. 2009].

2. Calpain

Platelets are the only cells in which calpain directly become active as a consequence of stimulation. Calpain is a calcium dependent protease that is located in the cytosol of activated, aggregating and spreading platelets [Fox et al. 1985, Gorlin et al. 1990]. Calpains are optimally active at neutral pH and are highly expressed in human platelets. They have a regulatory role in both early platelet activation (i.e. platelet secretion, aggregation, spreading and cytoskeletal remodeling) and late platelet events in activated platelets (i.e. platelet mediated fibrin clot reaction). Beside ABP, Calpain has different binding substrates; Glycoprotein Ibα, integrin β3, talin, spectrin and signaling proteins (Ilk and Fak). Activation of Calpain results in selective hydrolysis of these proteins [Wang et al. 2010, Fox et al. 1990].

3. Spectrin

Each spectrin molecule is a tetramer consisting of α and β subunits [Dubreuil 2006]. It is known that spectrin is cleaved by calpain during platelet aggregation [Fox et al. 1987] and plays an important role in allowing integrin-induced changes in cell shape.

Spectrin is involved in cross-linking the actin filaments in red blood cells and consists of two subunits of respectively 240 kDa and 220 kDa and form a tetramer of ~180 nm in length. Different gene

products and alternatively spliced spectrin forms have been observed. Today it is not known which gene products exist in platelets. However, two spectrin subunits of 240 kDa and 235 kDa have been observed in platelets. This platelet spectrin has been shown to be different from those of red blood spectrin but is similar to nonerythroid spectrin [Fox et al. 2001].

Glycophorin and adaptor protein ankyrin can bind spectrin at the anion exchanger band 3. Spectrin tetramers are linked to the plasma membrane via protein 4.1 that binds to glycophorin and ankyrin. Protein 4.1 belongs to the superfamily of proteins with a FERN domain and are associated with the junctions between spectrin and actin, that is involved in mediating network formation [Dubreuil 2006]. Both protein 4.1, glycophorin and ankyrin have been indentified in platelets by Western blotting and have many different gene products and alternatively splices forms [Bennet 1979, Davies and Cohen 1985]. As with spectrin the forms present in platelets are not known yet.

There is also evidence that spectrin may spatially regulate the ability of activating αIIbβ3 and the way spectrin is involved in integrin activation by creating microdomains, containing integrin and other proteins that are involved in signaling transduction. Furthermore it is known that spectrin is involved in direct binding of signaling molecules [Fox et al. 1996].

An interesting feature related to platelets is the potential role in regulating activation induced shape change. Calpain is involved in cleaving spectrin at the β-subunit that results in two fragments of 140 kDa and 150 kDa or two fragments of 125 kDa and 165 kDa. The last mentioned cleavage will result in a loss of ability of spectrin to crosslink actin [Harris et al. 1989, Harris et al. 1990]. However cleavage will only occur at the presents of calmodulin.

Defects in components of the network i.e. spectrin, ankyrin, and protein 4.1 are associated with cell shape and membrane abnormalities [Dubreuil 2006].

4. Dystrophin and Dystrophin-related protein (DRP)

DRP is a polypeptide of approximately 395 kDa and is 80% homologous to Dystrophin. The DNA and amino-acid sequences of DRP and dystrophin show a similar overall structure for a putative actin binding domain in the first 250 amino-acids, a long region containing multiple repeats followed by a cysteine-rich domain. There are already many isoforms and alternatively spliced forms known of both DRP and dystrophin [Tinsley et al. 1992, Winder et al. 1995, Lumeng et al. 1999]. Dystrophin is encoded by the DMD gene and are members of a spectrin superfamily whose main function is to bind membrane glycoproteins and cytoplasmic proteins including the actin network [Cerecedo D et al. 2006]. Dystrophin can also bind to the extracellular matrix laminin, which has been described as a G-protein-coupled receptor. It is already known that laminin is involved in cell differentiation, migration and adhesion of cells.

Both DRP and dystrophin contain an amino-terminal domain that can interact with actin filaments and a carboxy terminal domain that can interact with dystroglycan – one of the dystrophin-associated glycoproteins [Stone et al. 2005]. Dystrophin and DRP gene products are associated with proteins,

such as dystroglycans, dystrobrevins, sarcoglycans and syntrophins, forming the named dystrophin-associated protein complex (DAPC) – linking the extracellular matrix and actin cytoskeleton. Recently, the presence of the DAPC together with dystrophins participate in shape changes, adhesion process and aggregation in human platelets [Cerecedo D et al. 2006].

Also for platelets, this DRP protein co-isolates with the membrane skeleton. As with ABP and spectrin, DRP undergoes covalent modifications in activated platelets for example after cleaving DRP by calpain following integrin-induced signaling [Earnest et al. 1995]. Dystrophin isoform 71 is re-distributed with talin and vinculin upon binding of adhesive extracellular ligands to integrin $A_{IIb}\beta_3$. Furthermore, it is known that the feasible role of dystrophin 71 isoforms gene products are involved in mediating the formation of focal adhesion and stress fibers [Cerecedo D et al. 2006].

There are several large insertions/deletions and point mutations in dystrophin that are involved in Duchenne muscular dystrophy (DMD). DMD is a fatal X-linked recessive disorder and can lead to a total loss of dystrophin protein function. This can result in fatal complications like respiratory failure an cardiac failure with cardiomyopathy and cardiac conduction abnormalities. To improve the quality of life for these patients an early surgical fusion of the spine is needed. Investigators have noted that these DMD patients bleed more during spinal surgery in contrast to other patients with other underlying disorders. This is probably related as result from impaired primary haemostasis. No association was found between DMD and clotting factor deficiencies. Indeed, previous studies show platelet dysfunctions. This dysfunction was explained by a glycoprotein IV, syntrophin, dystrophin 71 deficiency or absence in platelets. Absent or decreased expression correlates with significant Gs upregulation and a higher cAMP level after Gs stimulation. Recent studies explore the role of the G-protein signaling pathway in the platelet function of DMD. These studies investigate the way Gs signaling (a heterotrimeric G-protein subunit that activates cAMP- dependent pathway) in platelets can result in an increased bleeding affinity, because the cAMP inhibits platelet function. This increased bleeding affinity was caused by platelet hypersensitivity to prostacyclin – lipid molecule, that is released from injured vessel walls. This prostacyclin increases cAMP via Gs stimulation and finally results in platelet dysfunction and a haemorrhagic tendency during surgery [Labarque V et al. 2008]. Furthermore, DMD platelets show a slower respond to collagen with an extensive shape change. So DMD platelets have a disorganized cytoskeleton and manifest Gs hyperactivity and reduced and reduced platelet collagen reactivity which partly contributes to the increased bleeding during surgery [Labarque et al. 2008].

5. Talin

Today two different talin isoforms are known. Talin 1 is expressed ubiquitously and talin 2 is namely expressed in brain and striated muscles [Earnest JP et al. 1995, Petrich et al. 2007, Nieswandt et al. 2007]. Talin 1, approximately 235 kDa contains an amino-terminal globular domain (47 kDa) and a carboxy-terminal domain (190 kDa). These domains are separated by a Calpain cleavage site during platelet aggregation [Rees et al. 1990]. This could play an important role in regulating the cytoskeletal reorganizations [Fox et al. 1985]. Talin 1 was indentified in platelets and comprises 3-8% of total platelet proteins [Kaverina I et al. 1998, Petrich et al. 2007]. The amino-terminal domain is highly homolog to the amino-terminal domain of Moesin and protein 4.1 and can bind to several transmembrane receptors/proteins (i.e. β1A, β1d, β2, β3, β5, β7-integrin subunits or layilin) [Horwitz et al. 1986, Arpin et al. 1994, Petrich et al. 2007]. The carboxy-terminal end of talin can bind to the actin cytoskeleton and vinculin with the help of PI(4,5)P2 – phospholipid component of cell membranes [Burridge et al. 1984, Muguruma et al. 1990, Nuckolls et al. 1990, Gilmore et al. 1993]. This PI(4,5)P2 is generated during platelet activation and may influence the regulation of talin upon platelet activation.

Platelet talin 1 is essential for hemostasis because it is required for the function and activation of platelet α2β1 and αIIbβ3 integrins. Several experiment demonstrate that this is done by talin via inside-out activation of platelet integrins in hemostasis and thrombosis [Petrich et al. 2007]. Thus talin serves as an important link between integrins and actin [Nieswandt et al. 2007] and is essential for the function of certain integrins and for integrin activation during cyoskeletal reorganization.

6. Moesin

Moesin, radix and ezrin are all family of the ERM proteins and are for ~75% identical in their amino acid sequence. These closely related proteins of ~80 kDa support cell surface projections by forming oligomeric head-to-tail structures linking the underlying cytoskeleton with the plasma membrane [Tsukita et al. 1997]. The amino-terminal domain is associated with the plasma membrane and the carboxy-terminal domain with the actin cytoskeleton of the cell [Yonemura et al. 1998]. The amino-terminal domain of each of the ERM proteins is highly conserved. However, moesin is the only ERM protein member that has been shown in platelets [Shcherbina A et al. 1999]. Moesin is similar to protein 4.1, merlin (tumor suppressor) and talin. These proteins can all link the plasma membrane to actin and can be cleaved by calpain [Bretscher 1989, Nakamura et al. 1995, Shuster and Herman 1995]. The membrane interaction of these proteins can be increased with the presence of PI(4,5)P2 [Hirao et al. 1996]. Further functions of moesin in platelets needs further study.

7. Skelemin

Skelemin is a ~195 kDa protein that contains five fibronectin $(FN)_2$ type III domain like motifs and seven the immunoglobulin superfamily C2 (IgC) like domain motifs [Price 1987, Price and Gomer

1993]. Skelemin is involved in maintaining the organization of actin and myosin filaments in skeletal muscles. Skelemin is namely involved in the M-band where it is connected to titin and links the tails of the myosin heavy chain. Skelemin and titin are also detected in platelets [Kazmierski et al. 1996]. Repeats 4 and 5 show a conserved Ig-fold and can interact with αIIbβ3 cytoplasmic tails. Specific regions between IgC of skelemin and the integrin β3 cytoplasmic tail have been observed. Additionally a skelemin binding site in the membrane-proximal region of αIIb cytoplasmic tail was observed. Previous studies also suggest that IgC-like repeats are involved in binding to integrin β3 cytoplasmic tail. This way skelemin can regulate integrin-mediated signaling and cell spreading. So skelemin IgC domains form a complex with both integrin β3 cytoplasmic tail and integrin α_{IIb} cytoplasmic tail. These data give a molecular insight into how skelemin can interact with integrins and regulate intergin- mediated signaling and cell spreading. Further studies are needed to investigate the relation of skelemin in linking integrins to the cytoskeleton [Deshmukh et al. 2007].

8. Myosin

Myosin was first identified in skeletal muscle and consists of two light chains of 20 kDa and one heavy chain of ~ 200 kDa that contains ATPase activity and an actin-binding site. Today this myosin is now known as conventional myosin or myosin II. All other types of myosin are called non-conventional. Both conventional and non-conventional types of myosine contain an ATPase domain in the amino-terminal end of the heavy chain, however they differ in the carboxy-terminal end of the heavy chain. Non-conventional myosin occurs in platelets and only contains one light chain, this is often calmodulin. Class I and VII non- conventional myosins have a carboxy-terminal domain that contains a sequence that is similar to the membrane-binding sequence of the ERM family of proteins. There is a possibility that these myosins regulate contraction by interacting with membrane proteins. But until know there is no clear evidence. Furthermore some of these non-conventional myosins (IX myosin I and IX) can bind different signaling molecules (i.e. RhoGAP- a GTPase activating protein for Rho). Another difference between conventional and non-conventional myosins is the smaller size of the heavy chain of the non-conventional myosin. It could be possible that non-conventional myosin is involved at integrin complexes in lamellipodia of the spreading platelets.

Platelet shape change is the earliest event in the activation by physiological stimuli and it is accompanied by reorganization of the cytoskeleton and formation of actin filaments. The 20 kDa light chain of non-conventional myosin (MLC 20) is involved in platelet shape change after phosphorylation [Fox JEB et al. 1993, Kiss E et al.2002]. This phosphorylation are balanced by the activity of protein kinases and phosphatases. The so called cell-permeable inhibitor toxins (CL-A) that inhibits Thr-specific protein phosphatases can induce shape change in platelets. This can even be done without the help of physiological stimuli [Kurisaki T et al. 1995, Kiss E et al.2002]. Today there are

two major toxin sensitive enzymes known in platelets named protein phosphatase type 1 (PP1) and type 2A (PP2A). In resting platelets PP1 is presented in the cytoskeleton, cytosol and membrane cytoskeleton. PP2A was only presented in cytosol and membrane cytoskeleton of platelets [Muranyi A et al. 1998, Kiss E et al.2002]. Furthermore controlling myosin phosphorylation in platelets involves activation of RhoA and may be involved in cytoskeletal rearrangements in platelets [Suzuki Y et al. 1999, Kiss E et al.2002].

4. Relation platelet integrins and cytoskeleton

Platelets adhere to sites of injury and aggregate, this way preventing excessive bleeding. Stable platelet adhesion to the injured blood vessel and aggregation in turn depend on integrin adhesion receptors [Petrich et al. 2007]. These integrin receptors are responsible for connection between cells and their surrounding tissue. Today at least 24 different integrins are known in vertebrates. All integrin (except for α4) subunits consist of a short cytoplasmic tail, a transmembraneous region and a large extracellular domain. Integrins have two major functions. First, integrins play an important role in the cell signaling; they are involved in both outside-in signaling as well as inside-out signaling. Second, integrins are often connected with other proteins and have the function to mediate cell-cell, cell-matrix and cell- pathogen interaction and communication. Here we will focus on the role of integrins in platelets, and how they are involved in coupling the extra cellular matrix outside a cell to the cytoskeleton inside the cell [Hynes RO 1992, Hynes RO 2002]. Cytoskeleton forces exerted on the extra cellular matrix and neighboring cells is called focal adhesion. These extra cellular matrix-cytoskeleton connections are formed by direct or indirect association of integrin β-tails with actin-binding proteins, such as talin, α-actinin and filamin. The change from focal complexes into focal adhesion is accompanied by the actin network together with associated actin proteins result into stress fibers. This process is essential for the start of platelet aggregation [Cerecedo D et al. 2006].

Resting platelets express integrins α2β1, α5β1, α6β1, αvβ3, and αIIbβ3 [Faull RJ et al. 1994]. In addition to these, αLβ2 and αMβ2 are expressed on activated platelets [Philippeaux MM et al. 1996]. Platelet integrins consist of α and β -heterodimeric transmembrane receptors that mediates the connection and are expressed in a low affinity state in resting platelets, which is mainly the case in circulating platelets. There have been eighteen different α-subunits and 8 different β-subunits identified so far. After stimulation through agonist receptors (e.g. ADP, collagen, or thrombin), the platelet lead to complex biological effects including activation of integrins [Petrich et al. 2007]. Until now, it is known that integrins shift to high affinity state and from that moment extracellular adhesive ligands, cellular receptors and the cytoskeleton can bind [Nieswandt et al. 2007]. And so a cellular matrix-cytoskeleton connection can be created. These interactions are most of the time regulated by inside-out signaling. This way, a cell surface receptor (e.g. G protein coupled receptor and protein tyrosine kinase receptor) initiates integrin activation by intracellular signaling. This results in

conformational changes of the integrin α and/or β cytoplasmic domains, which lead to transmembrane and extracellular domains to increase receptor affinity [Watanabe et al. 2008]. This switch to activated state must be done rapidly in platelets because these cells have to be recruited from the blood stream by rapid activation of their integrins. However the underlying molecular events that link agonist receptors to integrin activation are incompletely understood [Petrich et al. 2007].

αIIbβ3 integrin is the most abundant integrin in platelets and has fibrinogen, fibrinonectin and von Willebrand factor as major ligands. The affinity of αIIbβ3 integrin for ligands is highly modulatable and after activation it mediates adhesion, aggregation and spreading on the exposed extracellular matrix of injured vessel walls [Nieswandt et al. 2007]. This αIIbβ3 integrin mainly responsible for cross-linking platelets in fibrinogen, which may result in a blood clot. αIIbβ3 is also involved in mediating local modifications of the cholesterol membrane. This plays an important role in the organization of the membrane-cytokeleton linkage during the late phase of platelet activation, that is crucial for the regulation of blood platelet functions [Bodin et al. 2005].
However recent human studies also show abnormalities in inside-out signaling and this may cause either reduced platelet adhesive function and bleeding [Watanabe et al. 2008]. For example for patients that suffer from the bleeding disorder Glanzmann thromasthenia, the platelet aggregation does not work normal which manifest in a milder bleeding defect due to reduced platelet adhesion to vascular surfaces. In these patients a genetic defect was found in the integrin subunits αIIb or β3.Other studies demonstrated that heightened platelet function may cause thrombosis [Watanabe et al. 2008]. So well integrin function is crucial for hemostasis and thrombosis because integrins mediate both platelet adhesion and aggregation.

5. Platelet activation and cytoskeleton

During activation platelets convert from smooth, nonadherent into a sticky spiculated particle that releases and expresses biologically active substances and can bind the plasma protein fibrinogen. Activation can be caused rapidly by exposure to a agonists. The main important agonists are thrombin, collagen, thromboxane A_2, adinosine diphosphate (ADP), epinephrine and PMA (a phorbol ester, a tumor promoter). Agonists differ in their ability to induce platelet activation and have different receptors to bind, related signaling molecules and activation pathways are involved. For example when you compare thrombin and PMA, they both initiate different series of biological responses, including shape changes and aggregation properties. These responses are involved after reorganization of the cytoskeleton. After thrombin stimulation, the platelet cytoskeleton contained the major proteins actin, myosin and actin-binding protein and three additional proteins of 56 kDa, 80 kDa and 85 kDa. These three additional proteins were induces by a thrombin dose-response relationship. In contrast, after PMA stimulation only actin was induced. The other proteins were not found in the cytoskeletal fraction. Furthermore, the actin polymerizationd and platelet aggregation were induced by a

mechanism dependent on protein kinase C and in case of the thrombin activated platelets, additional cytoskeltal components could make stronger actin polymerization and an increased platelet aggregation was observed [Chen R et al. 1998].

Beside the agonist activation of platelets, platelet activation can also occur as a result of a physical stimulus caused by fluid shear stress that is found at sites of critical atrial narrowing [Fox JEB 2001]. The platelet collagen adhesion receptor glycoprotein VI initiates platelet aggregation at low shear stress while Glycoprotein Ib-IX, that binds von Willebrand factor is involved in platelet aggregation under high shear stress conditions. Beside initiating platelet aggregation both glycoprotein VI and Glycoprotein Ib-IX transmit intracellular signals that lead to elevation of cytosolic Ca^{2+} and up-regulation of integrin αIIbβ3. Recent evidence suggests that for a stable thrombus formation under shear stress both glycoprotein VI and Glycoprotein Ib-IX are required [Arthur et al. 2005].

Stimulation by platelet agonists induces signals within the platelet. During this so-called "inside out signaling" a number of biologically active substances are released. This may result in protein synthesis, granule secretion, shape change and ultimately alters the affinity of the platelet Glycoprotein IIb-IIIa receptor. In the integrin nomenclature glycoprotein IIb/IIIa is called αIIbβ3. This glycoprotein IIb-IIIa fibrinogen receptor has 40.000-80.000 glycoprotein IIb-IIIa complexes per platelet and so is the most abundant platelet receptor. Cytoskeleton rearrangement is also observed during inside out signaling. However the exact mechanism of how the cytoskeleton is involved is not known yet. The cytoskeleton plays also a role in the Glycoprotein IIb-IIIa affinity. RAP1b, a small GTPase probably augments the Glycoprotein IIb-IIIa affinity by modulating its interaction with the cytoskeleton [Fox JEB 2001]. Beside the function as adhesion receptor, Glycoprotein IIb-IIIa also induces a number of intracellular signals. This is called "outside-in signaling" and may cause cytoskeleton rearrangement and pseudopodia formation. For example, this "outside-in signaling" results in tyrosine phosphorylation of a number of platelet proteins. Growth factor bound 2 probably allows its interaction with the signaling proteins growth factor receptor bound 2 (GRB2) as well as the cytoskeletal protein myosin. Phosphatidylinositol(3,4)-bisphosphate ($PtdIns(3,4)P_2$), a minor component of the cell membrane has also been implicated in "outside in signaling" as it is dependent on fibrinogen binding to glycoprotein IIb-IIIa and may play a role in actin assembly [Fox JEB 2001].

The different stimulations and signaling pathways result in various responses which are change in shape, secretion of granules, extension of filopodia, expression of receptors for adhesive ligands and aggregation with other platelets [Fox JEB 1990]. These processes will be discussed in more detail in the next chapters.

A. Organization of the cytoskeleton in unstimulated platelet microtubules to maintain disk shaped structure

The organization of unstimulated platelets differs from that of stimulated platelets. Here, the cytoskeleton situation of unstimulated platelets will be discussed briefly. A more detailed situation of the behavior of stimulated platelets will be discussed in the next chapter.

Actin filaments are located throughout the cytoplasm to connect the membrane skeleton with the plasma membrane. The function of the entire cytoskeleton is to maintain the direct contours of the plasma membrane. In unstimulated platelets, the main function of the cytoskeleton with related proteins is to shape the membrane skeleton and give stability to the platelets [Fox JEB 1993].

B. Reorganization of the cytoskeleton upon platelet adhesion and activation

After activation, platelets undergo rapid changes in the amount of actin that is polymerized into filaments and these filaments get organized. The three major proteins that are involved in the actin polymerization are Arp 2/3, WASP and VASP. Arp2/3 consists of complex of two actin related proteins and five other proteins [Machesky LM et al. 1999]. This complex binds to actin filaments and nucleates polymerization of new filaments, which results in networks of filaments. Furthermore Arp 2/3 was found in lamellopodia and is assumed to be involved in the formation of the networks of submembranous filaments that pushes against the membrane, that can cause membrane extension.

WASP is a proline riche protein of 62 kDa that is defect in the Wiskott-Aldrich syndrome - a bleeding disorder that is characterized by thrombocytopenia and small platelets [Remold-O'Donnell E et al. 1996]. Because of the few filopodia in these patients it was suggested that WASP may regulate the actin polymerization. Untill now there is no clear evidence yet. Furthermore, it is known that WASP contains a site that binds actin. Other studies show binding connections between Arp2/3 and WASP during nucleate actin polymerization. The so called A-motif domain (a cluster of acidic residues) of WASP is responsible for this Arp2/3-WASP binding. Another known domains of WASP are a profilin binding domain, that is also involved in binding SH3-containing signaling molecules. Lastly, there is the EVH domain that is involved in localizing WASP to the membrane and the CRIB domain that binds cdc42 and Rac. Rac is family of the Rho GTPases, a family of small signaling G-proteins. The members of the Rho family have been shown to regulate many aspects of intracellular actin dynamics. Furthermore Rho play a role in cell proliferation, gene expression and apoptosis. Because WASP is localized in the platelet membrane skeleton and is mobilized by αIIbβ3 integrin outside-in signaling recent studies found that WASP function is related to integrin. WASP deficient platelets have functional defects related to integrin outside-in dependent physiological responses. A novel function for WASP in regulating pro-aggregation and pro-coagulation responses of integrin outside-in signaling was created [Shcherbina A et al. 2009].

The last known protein that is involved in localizing actin polymerization is VASP. Especially VASP is involved in localizing actin polymerization to the tips of the filopodia or to integrin signaling sites.

Furthermore it is known that the platelet function is inhibited if VASP is phosphorilated by a cGMP-dependent protein kinase at the Ser-157 site [Horstrup K et al. 1994]. A defect in this cGMP mediated VASP inhibition may lack platelet function.

Platelets change and behavior after activation depends on the stimuli that are involved. Most of the studies to measure the cytoskeletal reorganization in platelets were performed in the presence of aggregation inhibitors. All studies show an increased amount of actin polymerization after the stimuli. Furthermore in all studies an association was observed between myosin and the cytoskeleton [Fox JEB 2001].

Before aggregation several signaling events occur when platelets are spreading on the extracellular matrix. αIIbβ3 is the major component in this process and after activation it engages ligands which lead to dramatic changes in the cytoskeleton organization [Zaffran Y et al. 2000]. There are various stages of spreading of actin filaments on fibrinogen. First, actin filaments extend into a small number of filopodia. Thereafter membrane extends between the filopodia, this way a more circular spreading is created. At this stage, actine filaments are also presented in punctuate areas at the periphery. In these punctuate regions integrin, vinculin, spectrin, calpain and talin were observed at the periphery of the actin filaments. At these sites of actin complexes, bundles of actin do not terminate. Cultured cell studies show that punctuate areas of integrin and cytoskeletal proteins at the periphery of the cells are comparable to integrin clusters in other cells and which are involved in Rac activation. In the last stage of the platelets spreading, the central circle of actin bundles starting reorganizing into triangles, ovals and rhomboid structures. From this moment these actin bundles start to terminate at the periphery of the platelet. It is typically that this only occurs in only particular places. It is known that these particular places are comparable to the Rho-induced focal adhesion, which occurs when cultured cells have reached a stable spread form [Leng L et al. 1998]. Finally at the last stage, the platelets retract their filopodia, thus retract the clot of fibrin that binds the externally to the developing platelet aggregate. If platelets indeed aggregate then different integrin signaling lead to activation of molecules. For example if calpain get activated it can cleave different cytoskeletal proteins. So many more proteins are involved in aggregate cytoskeleton platelet fractions compared to the platelets that are prevented from aggregating [Fox JEB 1990, Fox JEB 2001]. In the next chapter different signaling molecules that are involved in regulating cytoskeletal reorganization will be discussed.

C. Cytoskeleton reorganization and signaling molecules

Several signaling molecules are associated with the membrane skeleton. Little is known about the exact signaling reaction that induce cytoskeletal organization in platelets. Most likely the cytoskeltal organization are induced as a result of the specific combination of signaling pathways induced under different conditions of activation. Proteins that are involved in actin polymerization, which are

described above are regulated during platelet activation by enzymes and second messengers at specific subcellular locations of the platelets. The major enzymes are tyrosine kinases, serine/threonine kinases, phosphatidylinositol phosphate kinases, Rho family members, and calcium dependent proteins. In this chapter these five enzymes including their produced second messengers will be discussed. Because of the complexity of cross talk and possible combinations of pathways there will be a focus on single signaling pathways of the five enzymes.

1. Tyrosine kinases

Several tyrosine kinases are involved in the organization of the cytoskeleton. Tyrosine kinases can direct phosphorylate cytoskeletal proteins or indirect via phosphorlating signaling molecules or adapters that are involved in inducing interactions of cytoskeletal proteins. The importance of tyrosine kinases in cytoskeletal reorganization is indicated in many studies by the fact that many proteins are phosphorylated by tyrosine kinases in activated platelets. The most important tyrosine kinases in platelets are the Src family members which are pp60c-src, pp60c-fyn, pp62c-yes, pp54/58c-yes, pp72syk and p125FAK- a focal adhesion kinase. It is known that pp60c-src, pp72syk and p125FAK get activated following signaling across the thrombin receptor and αIIbβ3. The activation of pp60c-fyn, pp62c-yes and pp54/58c-yes is until now unknown. Furthermore it is unknown what substrates are involved for tyrosine kinases in platelets in regulating cytoskeletal reorganization [Bertagnolli ME et al. 1999, Satoh K et al. 2000].

2. Serine/threonise kinases

There are many serine/threonine kinases involved in platelets. The most important serine/theronine kinases are protein kinase C, myosin light chain kinase, p65PAK family – family of serine/threonine kinase, MAP kinase – mitogen activated portein kinase and ROK (127-130). Myosin light chain kinase is activated by elevated calcium concentrations and p65PAK and ROK are activated by Rho family members. There are several substrates known for serine/threonine kinases in platelets which are myosin light chain, talin, moesin, ABP and Hsp27. All substrates are involved in regulating the organization of the cytoskeleton in platelets [Zhu Yet al. 1994, Nakashima S et al. 1994].

3. Phosphatidylinositol phosphate kinases

Both Phosphatidylinositol 5-kinase (PI 5-K) and phosphatidylinositol 3- kinase (PI 3-K) are phosphatidylinositol phosphate kinases that are involved in cytoskeletal reorganizations in platelets. PI 5-K is activated by Rac and Rho and leads to the generation of PI(4,5)P2. It is known that PI(4,5)P2 can initiate actin polymerization by releasing gelsolin from the barbed end of actin filaments. PI(4,5)P2 may also be involved in focal adhesion by regulating interactions of vinculin, ERM and

talin. PI 3-K was observed in platelets that were stimulated by thrombin. Another way of activating PI3-K is following integrin activation via cdc42 and Rho. The end product of PI3-K can bind to PH domains and from that moment PH domains can interact with membranes. This way vav – an exchange factor of Rac and Rho, is regulated in platelets [Ma AD et al. 1998]. PH domains were also observed in spectrin. So it is possible that PI3-k end products are involved in regulating interaction of spectrin with the membrane [Pasquet J-M et al. 1999, Fox JEB 2001].

4. Rho family members

Rho is a GTPase and is a member of the Ras superfamily of proteins. The Rho family of proteins, which included Rac1, cdc 42 and Rho (i.e. RhoA) are involved in regulating cytoskeletal scaffolding. All members have different effects on the cytoskeleton. Different studies show that cdc42 is involved in inducing the formation of filopodia, Rac is involved in inducing the formation of lamellipodia and RhoA induces stress fibers formation. All Rho proteins get activated by a variety of exchange factors (f.e. vav) and adaptors. It is known that calpain is activated via integrin induced signaling and is involved in integrin induced activation of Rac and RhoA. Other proteins that may be involved in activating Rho proteins (e.g. p21rasGAP, Grb2, Shc) are also phosphorylated during platelet activation. Activation of Rho family members lead to activation of different downstream targets, which are PI3 -K , WASP, PI5-K and $p65_{PAK.}$ $P65_{PAK}$ can activate a downstream cascade of kinases that leads to phosphorylation of Hsp27 (heat shock protein), that is presented during platelet activation. Both WASP and PI 5-K activation had an important role in regulating actin polymerization in activated platelets. Another function of PI 5-K is the regulating role in interactions between talin and vinculin, which is important in focal adhesion in platelets [Etienne-Manneville S and Hall A 2002].

5. Calcium-dependent proteins

Platelet proteins that are regulated by elevated Ca^{2+} concentrations are calmodulin, calpain and gelsolin. There are many calmodulin-binding proteins present in platelets, nevertheless little is known about the importance of these calmodulin-binding proteins in platelet function. For gelsolin it is known that Ca^{2+} -induced activation of gelsolin is important in regulating actin polymerization. The most important calcium-dependent protein in platelets is calpain. Calpain can cleave several cytoskeletal proteins after integrin receptor stimulation. This make calpain an important signaling molecule in inducing cytoskeletal reorganization. Overexpression of calpain in cultured platelets result in an increase spreading of the platelets and lead to the formation of several cytoskeleton-integrin complexes [Kaverina I et al. 2008]. After using calpain inhibitor in platelet no spreading was

observed. These studies indicate that calpain plays an important role in the earliest step of integrin-induced spreading.

There are two different forms of calpain, which are μ-calpain and m-calpain. Both need different calcium concentrations to induce their activity. μ-calpain is mostly presented in platelets and gets activated when platelets aggregate or when platelets spread on extracellular matrix proteins. Calpain has many different substrates which are talin, spectrin, DRP, ABP, protein 4.1 and the β3 subunit of the integrin. Furthermore different calpain signaling molecules substrates are known. For example protein kinase C, pp60*c-src* and p125FAK. Calpain is also involved in regulating the activity of different kinases that are involved in the generation of lipid second messengers in platelets [Yuan Y et al. 1997].

6. Summery and Conclusion

When blood vessels are injured, haemostatic mechanisms are switched on. In this way excessive bleeding is prevented. Platelets can be activated by multiple agonists that each stimulate a distinct platelet activation pathway. Platelets shape change and behavior after activation depends on the stimuli that are involved. This change is the earliest event in the activation by physiological stimuli and it is accompanied by reorganization of the cytoskeleton. Reorganization of the cytoskeleton in platelets is an important part of the complex mechanism of thrombosis and haemostasis. The main cytoskeletal component is actin. After activation, platelets undergo rapid changes in the amount of actin that is polymerized into filaments and these filaments get organized. The change from focal complexes into focal adhesion is accompanied by the actin network together with associated actin proteins result into stress fibers. This process is essential for the start of platelet aggregation. At the same time there are various stages of spreading of actin filaments on fibrinogen. First, actin filaments extend into a small number of filopodia. Thereafter membrane extends between the filopodia, this way a more circular spreading is created. In the last stage of the platelets spreading, the central circle of actin bundles starting reorganizing into triangles, ovals and rhomboid structures. From this moment these actin bundles start to terminate at the periphery of the platelet. Finally at the last stage, the platelets retract their filopodia, thus retract the clot of fibrin that binds the externally to the developing platelet aggregate.

For the reorganization of the cytoskeleton also other proteins are involved. For example, after activation glycoproteins interact with von Willebrand factor at the injured vessel wall, which results in platelet adhesion and simultaneously triggers intracellular signaling events that results in integrin activation and thrombus formation. For thrombus formation platelets must aggregate. It is known that spectrin is cleaved by calpain during platelet aggregation and plays an important role in allowing integrin-induced changes in cell shape. Also talin plays a crucial role in platelet aggregation. Talin domains are separated by a calpain cleavage site during platelet aggregation. Talin also serves as an

important link between integrins and actin and is essential for the function of certain integrins and for integrin activation during cyoskeletal reorganization.

Integrins play an crucial role in the cell signaling; they are involved in both outside-in signaling as well as inside-out signaling, are often connected with other proteins and have the function to mediate platelet-platelet interaction and communication. αIIbβ3 integrin is the most abundant integrin in platelets. The affinity of αIIbβ3 integrin for ligands is highly modulatable and after activation it mediates adhesion, aggregation and spreading on the exposed extracellular matrix of injured vessel walls. If platelets indeed aggregate then different integrin signaling lead to activation of signaling molecules. The platelet cytoskeleton is responsible for binding and positioning signaling molecules. These signaling molecules are also important for different processes in the cytoskeleton reorganization. Inappropriate or excessive working of these proteins and signaling molecules may result in blood flow defects.

A complete overview of all the proteins and signaling molecules that are involved in platelet function are shown in table 1. After discussing these different protein and signaling molecules it is now clear that tiny anucleate platelets are regulated in a complex way. The underlying processes controlling these platelet functions are complex and many are yet to be discovered.

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