Using proteomics to identify potential therapeutic targets in platelets

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Abstract
Proteomics has provided powerful new insights into the complex events of the anucleate platelet and has revealed many potential protein targets in the search for suitable agents for thrombotic disease. In the present study, we summarize recent proteomic approaches to analyse specific platelet subproteomes, such as the platelet releasate, the platelet phosphotyrosine proteome and characterization of the proteins associated with membrane lipid rafts.

The human ‘proteome’ (the protein complement of the genome) is a highly complex and dynamic entity with an estimated up to one million different protein components generated from 30 000 expressed human genes [1,2]. This, along with the fact that the dynamic range of protein concentration in cells varies by estimates of up to eight orders of magnitude, makes the global identification of the human proteome a daunting task. Proteomic methods have now emerged, however, which can provide a comprehensive overview of all biological processes by cataloguing, quantifying and comparing, with respect to function and interaction partners, the thousands of components of biological networks and mechanisms. This is achieved by the combination of high-resolution protein separation techniques with MS and modern sequence database-mining tools, to permit the routine identification of hundreds or even thousands of proteins in a single experiment [3].

Protein separation technologies can be broadly described as either gel-based such as 2-DE (two-dimensional electrophoresis) and non-gel-based methodologies such as multidimensional chromatography. 2-DE uses isoelectric focusing and gel electrophoresis to separate solubilized proteins by charge and molecular mass [4], whereas the most common multidimensional chromatographic method separates peptides by charge and hydrophobicity using ion-exchange and reverse-phase chromatography [5]. This method, unlike 2-DE, is amenable to automation and detects proteins not well represented by 2-DE, such as transmembrane and basic proteins. Recent advances have made MS an unsurpassed protein identification technique, due to the accuracy of mass detection, the detection sensitivity, the capacity to deal simultaneously with mixtures of multiple proteins and the fact that it can be automated, making it high throughput. MS instruments range from the MALDI–TOF (matrix-assisted laser-desorption ionization-time-of-flight) mass spectrometer, which can provide precise peptide masses, to complex tandem MS machines that also yield partial amino acid sequence information for unambiguous protein identification [6,7].

The incorporation of proteomics into functional biochemical and biological investigations has proved to be a powerful tool when applied to the complex events of the anucleate platelet, from adhesion and activation to secretion and aggregation. For an in-depth review of these studies, see [8,9]. In the present study, we summarize recent proteomic approaches to analyse specific platelet subproteomes such as the platelet releasate, the platelet phosphotyrosine proteome and characterization of the proteins associated with membrane lipid rafts.

Characterization of the platelet releasate
Platelets contain a number of preformed, morphologically distinguishable storage granules: α-granules (some containing exosomes of endosomal origin [10]), dense granules and lysosomes; the contents of these are released into the external milieu upon platelet activation [11]. Many of the proteins released in this manner act in an autocrine or paracrine fashion to modulate cell signalling, including the prothrombotic protein GAS-6 (growth arrest-specific gene 6) and the immune modulator, platelet basic protein [12,13]. Furthermore, certain chemokines released by activated platelets such as platelet factor-4 have been found in atherosclerotic plaques and, through macrophage differentiation, may contribute to the inflammation that is a hallmark of the disease [14]. Therefore, since the platelet releasate contains factors that conceivably contribute to the development of atherothrombosis, we recently identified, using a proteomics approach, more than 300 proteins that were released by human platelets after thrombin activation [15].

After verification that the supernatant fraction of thrombin-activated platelets contained known secreted proteins but contained no membrane proteins, such as αvβ3, the releasate fraction was separated by 2-DE; then, 22 Coomassie Blue-stained protein spots were excised, proteolytically digested and identified using MALDI–TOF MS. The identified proteins included the known platelet-secreted proteins

Key words: MS, membrane lipid raft, platelet phosphoproteome, platelet releasate, platelet, proteomics.

Abbreviations used: 2-DE, two-dimensional electrophoresis; DRM, detergent-resistant membrane; GP, glycoprotein; MALDI–TOF, matrix-assisted laser-desorption ionization-time-of-flight; NMAHCH-IA, non-muscle myosin heavy-chain type IIA; VWF, von Willebrand factor.

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protein and vitamin K-dependent Protein S (PRTS) were previously reported to be released from platelets, in-
cluding multimerin, amyloid β protein and vitamin K-depen-
dant protein. A further 35% was previously known to be
released from other secretory cells, including β2-micro-
globulin, tubulin and 14-3-3ξ from dendritic cells [16] and
cyclophilin A from smooth-muscle cells [17]. The remaining
proteins have not been released as seen from any cell
type and they include many proteins of unknown function
that were mapped to expressed sequence tags.

Three proteins, secretogranin-III (a potential monocyte
chemoattractant precursor), cyclophilin A (a vascular smooth-
muscle cell growth factor) and calumenin (an inhibitor of
γ-carboxylation by targeting the cofactor vitamin K epoxide
reductase), not previously attributed to platelets, were exam-
ined further since they are of potential interest in the patho-
genesis of atherosclerosis. It was confirmed that these
proteins are localized in platelets and are released upon activation.
Furthermore, although absent from normal vasculature, these
proteins were identified in human atherosclerotic lesions,
which also stained for the platelet-specific proteins integrin
αIIb/β3 and platelet factor-4. Therefore these and other
proteins released from platelets may contribute to athero-
sclerosis and to the thrombosis that complicates the disease.
Indeed, such secreted proteins may prove suitable as therapeu-
tic targets, given their extracellular localization, without
the risk of bleeding that complicates direct inhibition of
platelet activation.

Table 1 | Some of the proteins identified in the thrombin-
activated platelet releasate using multi-dimensional
chromatography coupled with tandem MS
Spectra from the two-dimensional liquid chromatography tandem MS run were identified using the SEQUEST program and a composite mouse and human database (NCBI July 2002 release). The gene name and the number of peptides identified for these proteins are indicated. For the full list of 300 proteins, see [15].

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene name</th>
<th>Matching peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum albumin</td>
<td>ALBU_HUMAN</td>
<td>12</td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td>PLF4_HUMAN</td>
<td>18</td>
</tr>
<tr>
<td>Fibrinogen alpha chain</td>
<td>FIBA_HUMAN</td>
<td>17</td>
</tr>
<tr>
<td>Platelet basic protein</td>
<td>S2D7_HUMAN</td>
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<td>Platelet proteoglycan</td>
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</tr>
<tr>
<td>Thrombospordin</td>
<td>TSP1_HUMAN</td>
<td>37</td>
</tr>
<tr>
<td>SPRC (secreted protein acidic and rich in cysteine)</td>
<td>SPRC_MOUSE</td>
<td>17</td>
</tr>
<tr>
<td>Transforming growth factor</td>
<td>LTBS_HUMAN</td>
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</tr>
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<td>Coagulation factor V</td>
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</tr>
<tr>
<td>Vitamin K-dependent Protein S</td>
<td>PRTS_HUMAN</td>
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</tr>
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<td>Amyloid β protein</td>
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<tr>
<td>Secretogranin III</td>
<td>SG3_MOUSE</td>
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<tr>
<td>Cyclophilin A</td>
<td>CYPH_HUMAN</td>
<td>5</td>
</tr>
<tr>
<td>Calumenin</td>
<td>CALU_HUMAN</td>
<td>4</td>
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</table>

Figure 1 | The phosphotyrosine proteome of human platelets
Characteristic tyrosine-phosphorylated proteomes for (a) resting and (b) 0.1 unit thrombin-activated human platelets were isolated using the monoclonal antibody 4G10 and then separated by 2-DE. pI 3–10 IPhosphorTM strips were used in the first dimension and the gels were silver-stained.

Analysis of the phosphotyrosine proteome
Signalling cascades, including those in the platelet, are
controlled both positively and negatively by phosphorylation
[18]. To date, many protein and lipid kinases, phosphatases,
phospholipases and GTPases have been shown to be tyro-
sine-phosphorylated during platelet activation and spreading
[18], including ERK2 (extracellular–signal-regulated kinase 2)
and cytosolic tyrosine kinases, e.g. Syk and Src [19–21]. How-
ever, it is probable that additional protein phosphorylation
events contribute to the dynamic changes involved in platelet
activation, and aggregation and proteomics studies are a novel
approach in providing an overview of these phosphorylation
cascades. For a comprehensive review of all the platelet phosphoproteomics studies, see [8,9].

We have used a proteomic strategy to examine tyrosine
phosphorylation upon thrombin activation of platelets, with
the aim of identifying novel signalling events [22]. Dynamic
phosphoryse proteins changes were enriched by immunoprecip-
itation using the monoclonal antibody 4G10, allowing the cap-
ture of relatively low-abundance proteins, and the tyrosine-
phosphorylated proteome from both resting and 0.1 unit
thrombin-activated human platelets was separated by 2-DE (Figure 1). In combination with MALDI–TOF MS and immunoblotting, 67 differences were reproducibly high-
lighted between control and thrombin-activated platelets and
several of these proteins were identified, including FAK, Syk,
mitogen-activated protein kinase (MAPKKK) and activin
Characterization of the proteins associated with membrane lipid rafts

Lipid rafts are specialized membrane regions, consisting of dynamic assemblies of cholesterol and sphingolipids [26]. These domains are rich in dynamic multi-protein complexes [26a] that have been suggested to play a role in the regulation of platelet activation processes, particularly those mediated by GPVI (glycoprotein VI) and GPIb/IX/V [27–31]. Furthermore, several platelet signalling proteins have been shown to be DRM (detergent-resistant membrane)-associated, including CD36 [32], the Src-family kinases Lck and Fyn, the adapter protein LAT (linker for activation of T cells) [33] and, upon binding of VWF (von Willebrand factor), GPIb/IX/V and GPIbα [28].

The standard biochemical method used to isolate rafts and their associated proteins makes use of their insolubility in non-ionic detergents at 4°C. These DRM-associated proteins float in low-density fractions from sucrose gradients [29] and, recently, proteomic studies have identified proteins associated with the DRM fraction of other cells [34–37]. Thus, to elucidate the role of these domains in platelet function, we have undertaken a proteomic profiling of the proteins associated with 1% Triton X-100 DRMs from control and VWF-activated platelets using liquid chromatography coupled with tandem MS.

More than 150 proteins were identified, including characteristic raft proteins such as flotillin-1 and many proteins that were not previously described as DRM-associated, including the Ras GTPase-activating protein 3 and the small GTPase rap 1b. The high level of signalling and trafficking proteins identified, including src (Figure 2) and several proteins novel to the platelet, e.g. rab 5, rab 8 and syntaxin 11, implicates DRMs as concentrating platforms not only in the critical platelet function of activation but also, for the first time, in secretion. Furthermore, platelet activation resulted in the translocation of several proteins into or out of the DRM.
fraction, with proteins recruited upon VWF activation, including GPIbα, glucose transporter 14 and CLP-36 (C-terminal LIM domain protein 36). As an adapter protein, CLP-36 may interact with GPIb/IX/V through another DRM-associated protein, namely α-actinin, thereby facilitating signalling through lipid rafts [38]. Such changes in the proteome of platelet DRM’s upon activation suggest that these dynamic membrane domains may act as concentrating platforms that co-cluster receptors and signalling molecules, thus ensuring efficient, co-ordinated platelet activation and secretory processes. Targeting of proteins recruited to platelet DRM’s upon activation may represent a novel therapeutic strategy in the prevention of thrombosis.

The future
Study of the expression, localization, modification and interactions of platelet proteins, as well as monitoring the changes that could be the cause or a consequence of disease processes, will reveal a wealth of information on platelets and the molecular basis of thrombotic disease. Furthermore, future application to platelet biology and enable new diagnostic and therapeutic strategies for thrombotic disease. The future offers unprecedented opportunities to unlock the mysteries of platelet biology and enable new diagnostic and therapeutic strategies for thrombotic disease.

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References