Platelet Ubiquitylation—It's Everywhere

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A decade ago, the use of omics—the global analysis of biological or molecular systems—in vascular biology was highlighted in a major review by Rüegg et al in *Thrombosis and Haemostasis*.[1] The relative value of these new emerging techniques was considered complementary and synergistic to traditional, more focused scientific approaches, notwithstanding the large amount of data generated by omics, and already significant contributions to developments in the vascular biology field.[1] In recent years, applications of omics alone or in combination has continued to lead to diverse new discoveries, including unique identification of proteomes of vascular cells associated with particular disease states, markers of prognosis and/or identification of novel signalling or other pathways.[2–6] In one interesting example, the combined analysis of proteome, phosphoproteome and proteolytic cleavage was used to identify altered platelet functions in the human Scott syndrome, a rare congenital bleeding disorder associated with impaired Ca^{2+}-signalling and dysfunctional platelet pro-coagulant activity.[6] With regard to human platelets, not only the proteome, but also phosphoproteomics, secretomes and sheddomes have been reported,[7–10] the latter using particular agonists or treatments to induce phosphorylation, secretion or shedding, respectively. In this issue of *Thrombosis and Haemostasis*, new studies by Unsworth et al[11] examine the human platelet ubiquitome, the ubiquitous post-translational modification of proteins involving covalent attachment of ubiquitin protein to lysine residues, in resting and activated platelets to not only demonstrate the range and scope of ubiquitylation in human platelets, but also to implicate ubiquitylation as a regulatory component of signalling pathways downstream of platelet-specific receptor, glycoprotein (GP) VI.

For detailed omics analysis of proteins, there must by now be in the order of several hundred different types of post-translational modifications at various sites in proteins from different species. Analysis of individual modifications in resting or activated cells or systems is therefore likely to lead to the acquisition of large datasets, raising questions regarding how to use these data, for example, in terms of understanding specific regulatory pathways and/or cellular functions. There are also likely advantages or disadvantages depending on the systems used, such as cultured cells, model systems or fresh human platelets. In general, data and interpretation may require considerations of factors related to (1) sample type and source including numbers of replicates and/or donors; (2) sample preparation including treatments, timing, extraction methods, any inhibitors used or other conditions potentially affecting protein modifications/interactions; and (3) detection by mass spectrometry or other means, including data acquisition and analysis. All these factors could potentially influence the extent of false-positive or false-negative discovery rates for protein modifications or sub-set omics, modified protein substrates or extent/dynamics of binding partner interactions (interactomes).

The current findings of Unsworth et al[11] compare the ubiquitome in resting washed human platelets from a total of three healthy donors per experiment, and in platelets activated by the GPVI-specific ligand, cross-linked collagen-related peptide (CRP-XL). Briefly, following isolation, washed platelet samples with or without activation were then detergent-lysed, reduced and alkylated, trypsin-digested, acidified and desalted, lyophilized and immunoprecipitated with anti-Gly-Gly motif antibody beads (that recognize the characteristic tag of Gly-Gly ligated to ubiquitylated Lys), eluted and desalted, solvent-precipitated and ultimately subjected to tandem mass spectrometry (for full details and conditions, see Unsworth et al[11]). The approach identified more than a thousand ubiquitylated peptides derived from several hundred different proteins, and more than half of these peptides showed increased ubiquitylation, by greater than twofold, following stimulation of platelets with CRP-XL. Multiple sites of ubiquitylation were identified on proteins, including Syk (with 17 ubiquitylation sites, all of

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which increased following activation), as well as filamin and integrin sub-units, consistent with other experimental evidence obtained using proteasome or deubiquitylase inhibitors, and together supporting a role for ubiquitylation in platelet activation/function.[11]

But what is the specific function of ubiquitylation of platelet proteins, how is the extent of ubiquitylation regulated and how does ubiquitylation control particular signalling pathways and cellular functions? Ubiquitin consists of 76 amino acid residues and can be ligated to Lys residues in proteins, and also contains Lys residues enabling complex polymerization patterns. Ubiquitylation is regulated by different forms of E1, E2 and E3 ligases, and by deubiquitylating enzymes. Ubiquitylation of a protein can affect its recognition by other proteins, which may target the protein for proteasomal degradation, or control protein complexes involved in receptor-mediated intracellular signalling. In this regard, these recent findings[11] are revealing new knowledge of how platelet GPVI signalling may be regulated in human platelets in health and disease, with implications for improved understanding of mechanisms as well as potential therapeutic targeting.

GPVI is a member of the immunoglobulin (Ig) superfamily, with two extracellular Ig domains, a short mucin-like region, a transmembrane domain and a cytoplasmic tail.[12] GPVI forms a non-covalent complex with the FcRγ chain, essential for GPVI surface expression, and following engagement by multivalent ligands collagen or fibrin, or CRP-XL, dimerization/clustering leads to activation of Syk kinase-dependent signalling pathways and platelet activation/secretion involved in haemostasis–thrombosis, wound healing and maintaining vascular integrity.[12,13] Recent evidence also suggests a role for platelet GPVI and Syk-related pathways in tumour metastasis (reviewed in Schlesinger[14]). In addition, the cytoplasmic domain of GPVI contains a membrane-proximal positively charged sequence that constitutes a binding site for calmodulin, which regulates metalloproteolytic ectodomain shedding.[12] This same sequence also binds to tumour necrosis factor receptor-associated factor 4 (TRAF4), a scaffold protein that can interact with redox-regulating enzymes, and GPVI engagement also leads to Syk-dependent and Syk-independent generation of reactive oxygen species.[15,16] Interestingly, regulation of ubiquitin enzymes in the transforming growth factor-β pathway in other cells has a recognized role for TRAF family proteins (reviewed in Iyengar[17]), while it was recently shown that TRAF4-mediated ubiquitylation of the high-affinity nerve growth factor receptor, tropomyosin receptor kinase A, also plays a functional role in regulating cancer metastasis.[18] While TRAF4 was not identified as an ubiquitylation target in platelets, there were reportedly three ubiquitylation sites on calmodulin.[11] The GPVI cytoplasmic domain also contains a proline-rich sequence that binds Fyn/Lyn kinases, interacting with Syk associated with an immunoreceptor tyrosine-based activation motif in the cytoplasmic domain of FcRγ. In terms of the GPVI/FcRγ/Syk signalling pathway, the study reveals several ubiquitylation sites in resting platelets on FcRγ, Lyn and additional downstream components of this pathway, whereas treatment with GPVI ligand dramatically increased overall ubiquitylation, in particular in Syk, with up to 17 sites showing up-regulation of ubiquitylation following CRP-XL treatment.

In summary, the new data[11] highlight in comprehensive detail the changes in resting platelet ubiquitylation following activation via GPVI, and identifies specific sites in Syk and other components of Syk signalling pathways that are subject to increased ubiquitylation following activation via GPVI, and so could be developed as specific modulators of GPVI function. Given the history of development and application of omics technologies to vascular systems from past decades to present,[1–11] it will be of interest to see what further advances in this field are possible in future years. In this case, the mechanisms for the regulation of ubiquitylation, precise consequences on phosphorylation and signalling and down-regulation following activation in healthy or diseased human platelets will be of further interest to evaluate in future, not to mention the array of other ubiquitylated proteins identified including amyloid-related proteins, which may share common mechanisms of proteolytic regulation with GPVI.

Conflict of Interest
None declared.

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