The Human Platelet Membrane Proteome Reveals Several New Potential Membrane Proteins*§

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We present the first focused proteome study on human platelet membranes. Due to the removal of highly abundant cytoskeletal proteins a wide spectrum of known platelet membrane proteins and several new and hypothetical proteins were accessible. In contrast to other proteome studies we focused on prefractionation and purification of membranes from human platelets according to published protocols to reduce sample complexity and enrich interesting membrane proteins. Subsequently protein separation by common one-dimensional SDS-PAGE as well as the combined benzylidimethyl-n-hexadecylammonium chloride/SDS separation technique was performed prior to mass spectrometry analysis by nano-LC-ESI-MS/MS. We demonstrate that the application of both separation systems in parallel is required for maximization of protein tagging out of a complex sample. Furthermore the identification of several potential membrane proteins in human platelets yields new potential targets in functional platelet research. Molecular & Cellular Proteomics 4:1754–1761, 2005.

Because of its sensitivity and high throughput capabilities proteomics has become an important method in protein research for the analysis of complex protein samples. This in turn represents the basis for functional characterization and exploration of the biological relevance of proteins and complete protein networks. Platelets represent an optimal field for proteome research because of their anucleate nature, which renders genomic techniques inappropriate. Additionally, platelets are of major relevance to a broad spectrum of cardiovascular diseases including coronary heart disease, myocardial infarction, and stroke (1, 2). Membrane proteins and receptors act as signal acceptors, mediators, enhancers, and multipliers and therefore work generally as key molecules in cellular functions in many cases. This is expressed in the fact that about 50% of current small molecule drugs target plasma membrane receptors as well as other membrane proteins (3). Therefore, proteomics represents a very promising technology for the comprehensive analysis of platelets and platelet membrane proteins to discover new potential membrane receptors or other relevant proteins that could be potential new drug targets as well as missing links for a basic understanding of platelet function. However, only a few studies engaging the human platelet proteome have been performed so far (4–6). All these studies have in common that they are focused on high resolution 2D-PAGE as the primary separation technique. Unfortunately this technique is known to be unsuitable for hydrophobic proteins (7) because the IEF requires the usage of zwitter- or non-ionic detergents, which are normally weak. In contrast, ionic detergents provide much better solubilization capabilities but are not applicable for IEF (8). Therefore, hydrophobic proteins cannot be solubilized or tend to precipitate when reaching their pI as this represents their point of lowest solubility (9). Although several approaches have been applied to overcome these obstacles no standard application has been established yet (10). As a consequence in all platelet proteome studies based on 2D-PAGE, membrane proteins such as receptors are almost completely missing apart from the high abundant glycoproteins, e.g. GPIIb/IIIa and GPIb, which could be identified in these studies (5, 6). Furthermore, dynamic range is another important aspect with regard to the achievable proteome coverage. Platelets comprise an extensive dynamic cytoskeleton that is required for both stabilization of cell shape in the blood stream and shape change following activation (11, 12). As a major dynamic protein actin is highly abundant in platelets; other cytoskeletal components, e.g. myosin, tubulin, or actin-binding proteins, are present as well. Along with thrombospondin they constitute the predominant majority of the total protein amount in platelets, representing the major drawback of complex analyses because all separation and identification techniques are

* The abbreviations used are: 2D, two-dimensional; 1D, one-dimensional; 16-BAC, benzylidimethyl-n-hexadecylammonium chloride; GPCR, G-protein-coupled receptor; TMD, transmembrane domain; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; GP, glycoprotein.

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restricted with regard to dynamic range. Protein staining methods have a dynamic range between ~50 for Coomassie Brilliant Blue™, 1,000 for silver staining procedures, and up to 10,000 for fluorescent dyes (13, 14). In correlation, chromatographic separation is limited in binding capacity and dynamic range, too. Consequently, in complex mixtures proteins expressed at low levels cannot be analyzed extensively in the presence of high abundance proteins. To handle these limitations prefractionation is a useful and inevitable approach for the reduction of sample complexity thus leading to a facilitated access to the subproteome of interest (15, 16). Subcellular fractionation grants the advantage that nearly all proteins that are not localized to the organelle of interest are removed or at least significantly depleted.

To obtain a better representation of hydrophobic proteins the application of stronger detergents in subsequent separations is mandatory. Common SDS-PAGE is a well established technique but has the disadvantage of inferior resolution when separating complex protein mixtures. Recently 16-BAC/SDS-PAGE has gained broad application for the separation of membrane and hydrophobic proteins (17, 18). This technique is based on the consecutive separation of proteins by means of electrophoretic mobility using two different detergents, the cationic 16-BAC in the first and the anionic SDS in the second dimension (19). In comparison to 2D-PAGE the resolution is reduced but still much better than in one-dimensional gel systems. By using a two-dimensional separation technique the proteins are focused within spots, which increases their local concentration compared with one-dimensional gels where proteins are localized in broad bands. Additionally, improved resolution reduces the number of different proteins in single spots, leading to a better accessibility of low abundance proteins in the sample.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Neuraminidase (Type X) from Clostridium perfringens and Triton X-114 were purchased from Sigma. Protease inhibitor mixture Complete Mini™ was bought from Roche Applied Science. Sequencing grade modified trypsin was obtained from Promega, Mannheim, Germany. All other chemicals and HPLC solvents were acquired from Merck KGaA.

**Platelet Preparation**—With the approval of the ethics commission of the University of Wuerzburg fresh platelet concentrates with a total amount of approximately 10^13 cells were used as samples. The preparation protocol was performed according to Authi (20). Briefly, plasma was centrifuged twice at 200 × g and 20 °C for 15 min to remove residual red and white blood cells. Afterward the suspension was acidified with citrate buffer (0.3 m citric acid) to pH 6.4, and subsequently platelets were pelleted at 1,200 × g and 20 °C for 10 min. The cells were reconstituted in neuraminidase treatment buffer (152 mM NaCl, 3 mM EDTA, 10 mM Hepes, 4.17 mM KCl, pH 6.4) and incubated for 20 min with 0.05 unit/ml neuraminidase at 37 °C. After treatment platelets were rebuffed to pH 7.2 and washed twice with Hepes washing buffer (152 mM NaCl, 3 mM EDTA, 10 mM Hepes, 4.17 mM KCl, pH 7.2). Sedimented platelets were resuspended in ice-cold sonication buffer (0.34 m sorbitol, 10 mM Hepes, one tablet Complete Mini in 50 ml of buffer, 0.3 unit/ml aprotinin, 1 mM DTT, 200 μM PMSF) for subsequent lysis.

**Platelet Lysis and Membrane Preparation**—Disintegration of cells was performed by ultrasonication (Bandelin Sonoplus, Berlin, Germany) for 20 s at 70% maximum power. Lysate was separated from cell debris by centrifugation at 1,500 × g and 4 °C. Precleared lysate was applied onto a discontinuous sorbitol gradient (3.5, 1.8, and 1.0 m in Hepes washing buffer) and centrifuged in a Beckman-Coulter SW 41Ti rotor at 36,000 rpm for 1.5 h at 4 °C. The crude membrane sample was collected at the 1.0 and 1.8 M interface. Membrane fragments were sedimented by an additional centrifugation step with a TLA 120.2 rotor at 100,000 rpm and 4 °C. Afterward the crude membrane pellet was suspended in ice-cold sodium carbonate buffer (100 mM, pH 11.5), stirred on ice for 1 h, and pelleted by centrifugation as described before. The carbonate-extracted pellet was suspended in Triton X-114 and extracted with Hepes washing buffer at 4 °C for 1 h. After incubation for 15 min at 37 °C the organic and aqueous phase were separated (21, 22). Proteins solubilized in the organic phase were precipitated with TCA/acetone according to Jiang et al. (23).

**Protein Separation**—Proteins were reduced with DTT and solubilized with lithium dodecylsulfate sample buffer (Invitrogen). Samples were separated on 10% precast BisTris gels (NuPAGE™, Invitrogen). 16-BAC/SDS-PAGE separation was performed as described previously (24). Briefly, 16-BAC gels were cast in glass tubes (1-mm inner diameter, 15-cm length). First dimension separation was accomplished in a tube gel IEF apparatus (Model 175 tube cell, Bio-Rad). Afterward gels were rebuffered for 20 min in 100 mM Tris, pH 6.8, and incubated for 15 min in reducing SDS sample buffer before application onto 12.5% SDS-Tris-glycine gels (20 × 24 cm). Gels were silver-stained using a protocol according to Mortz et al. (25) that is compatible with mass spectrometry.

**Immunodetection of G-protein-coupled Receptors**—Protein samples were separated by gel electrophoresis and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences) with the Novex X-Blot cell (Invitrogen) according to the manufacturer’s instructions. Detection of P2Y12 was performed with the ECL Advance detection kit (Amersham Biosciences) with a 1:4,000-fold dilution of the polyclonal anti-P2Y12 antibody (Acris, Karlsruhe, Germany) and a dilution of 1:8,000 of a secondary anti-rabbit horseradish peroxidase-conjugated anti-IgG antibody (Sigma). X-ray films were exposed to the immunoblot for 1 min and developed with the X-Omat 1000 x-ray developer (Eastman Kodak Co.).

**In-gel Digestion and Peptide Extraction**—Sample preparation was performed according to the protocol of Shevchenko et al. (26). Briefly, samples were washed two times alternating with 50 mM ammonium hydrogen carbonate buffer and 25 mM ammonium hydrogen carbonate buffer with 50% acetonitrile. Proteins were reduced with 10 mM DTT for 30 min at 56 °C and subsequently alkylated by incubation with 20 mM iodoacetamide at room temperature for 30 min. Again samples were washed as described before. Gel pieces were shrunk in a SpeedVac (Thermo Electron, Dreieich, Germany) and rehydrated with 12.5 ng of trypsin in 50 mM ammonium hydrogen carbonate buffer. Digestion was performed by incubation at 37 °C overnight. The resulting peptides were extracted by application of 15 μl of 5% formic acid for 10 min.

**LC-MS Analysis**—For reversed phase separation 0.1% formic acid as solvent A and 0.1% formic acid with 84% acetonitrile as solvent B were used. Separation was performed on the Ultimate nano-HPLC system (Dionex, Idstein, Germany) consisting of an autosampler, a loading pump, and a nano-HPLC gradient pump combined with a nanospray source consisting of a Vydac 180 nm × 3 mm, 1.9 μm column, which was coupled to an Agilent 1100 nanoLC for reversed-phase separation. For peptide separation a 150 cm × 75 μm, 3 μm particle size, and 100-Å pore size). Peptides were preconcentrated onto a 300-μm × inner diameter C18 PepMap column of 1-mm length at a flow rate of 25 μl/min. Separation was performed with a flow rate of 250 nl/min using a binary gradient starting at 5% solvent B rising to...
50% in 30 min. After elution the column was rinsed with 95% solvent B for 10 min and subsequently equilibrated with 5% solvent B for 20 min. Peptides were directly eluted into an ESI mass spectrometer. For mass spectrometric analysis an ESI ion trap LCQ™ Deca XP Plus (Thermo Electron, Dreieich, Germany) and an ESI-Q-TOF QStar XL or an ESI linear ion trap QTrap 4000 (both Applied Biosystems, Darmstadt, Germany) were used. MS acquisition duty cycle was set up with a 1-s survey scan and three dependent scans (each −1 s) with the ion trap mass spectrometers or 2-s survey scans and two dependent scans, each 2 s, for the ESI-Q-TOF mass spectrometer.

**Data Interpretation**—Mass spectra were transformed into peak lists in dta or mgf format using the two in-house software solutions wiff2dta (27) and raw2dta, respectively. We applied the default values for generating mgf or dta files. Generated data were processed in parallel with the search algorithms Sequest™ (28) (Version 27) and Mascot™ (29) (Version 1.8). For sequence alignment the human National Center for Biotechnology Information (NCBI) subdatabase from December 2004 was used. As fixed modification carbamidomethylation of cysteine residues was used, and as variable modification oxidation of methionine residues was selected. As filter criteria for Mascot algorithm the minimum score was set to 35 for each peptide. Only protein hits that were identified with these parameters by both algorithms and had at minimum two identified peptides were accepted. Additionally all significant hits were revised manually. Afterward the NCBI gene indices were transformed to the corresponding Swiss-Prot accession numbers to avoid redundancies and improve lucidity.

**RESULTS**

**Membrane Preparation**—Preparation and lysis of the platelet samples was adapted from Authi (20), but subsequent preparation and purification of membranes and membrane proteins, respectively, were changed. Meanwhile another method for platelet membrane preparation according to Kinoshita et al. (30) based on binding of membranes onto polylysine-tagged beads was also tested but turned out to be unsuitable for the enrichment of pure platelet membrane proteins (data not shown). In contrast the original protocol based on ultrasonication lysis and preclearing on sorbitol gradients facilitated additional purification steps. Although this lysis method is not very effective, sufficient amounts of sample could be obtained from the preparation. Besides the band between 1.0 and 1.8 M sorbitol, smaller debris and partially broken cells were detected at higher densities. These two fractions were discarded to prevent contamination with higher amounts of cytoskeletal proteins. After isolation of a crude membrane pellet a significant reduction of cytoskeletal proteins was visually detected after SDS-PAGE separation (Fig. 1). A subsequent carbonate extraction was applied to convert vesicular membranes into β-sheets, thus releasing the incorporated soluble proteins (21). Although the effect of sodium carbonate extraction could not be directly observed on SDS-PAGE band patterns, it was integrated in the preparation protocol to ensure removal of vesicle-incorporated proteins. By solubilization of hydrophobic proteins with Triton X-114 and subsequent extraction with an aqueous buffer the concentration of high molecular weight membrane proteins and receptors, respectively, could be increased. However, protein recovery from the detergent phase is limited to a few methods, for instance dialysis or precipitation. In this study TCA/acetone precipitation provided good yields, but a loss of proteins cannot be fully excluded.

After this multistep purification protocol, an increase of membrane proteins could be observed, whereas the amount of cytoskeletal proteins was significantly reduced. However, a complete removal of cytosolic proteins could not be achieved, and further soluble proteins from other cell compartments were detected as well (see Supplemental Table 1).

**Protein Separation**—Although mass spectrometry represents a very sensitive and versatile method for protein identification, it is mandatory to reduce sample complexity prior to analysis as much as possible. The introduction of additional separation dimensions enables access to low abundance proteins and increases the number of detected and identified peptides per protein. For these reasons we established various protein separation techniques and combined them with the segregation of the resulting peptides by reversed phase chromatography after proteolytic digest. On the one hand, common 1D-SDS-PAGE is a very popular and robust system and has only a few restrictions concerning the separation of proteins in a wide molecular mass range between 5 and 250 kDa. On the other hand, it lacks sufficient resolution for the discrete separation of several hundreds of proteins. Therefore, even a single gel slice of 1-mm width from a complex sample may contain several dozens of proteins rendering it impossible to identify all components by LC-MS/MS due to the limited dynamic range. Nevertheless, SDS-PAGE is a val-

![Fig. 1. A, SDS-PAGE separation of different purification stages of platelet membrane proteins. m, Mark12 protein marker (Invitrogen); a, complete platelet lysate; b, crude membrane fraction after density gradient centrifugation; c, purified membranes after carbonate extraction; d, hydrophilic phase of differential Triton X-114 extraction; e, hydrophobic fraction of Triton X-114 extraction after TCA/acetone precipitation. Note the intensity ratio between the high molecular mass band of GPIIb/IIIa and the 40 kDa-band of Actin. The red box indicates the region where the corresponding signals from the immunoblot (B) are localized. B, anti-P2Y12 immunoblot. The different signal intensities show a significant increase from the complete cell lysate to the purified membrane protein sample after Triton X-114 extraction. A direct identification by mass spectrometry was not possible due the co-localization with actin.](image-url)
Protein Identification—Protein identification by mass spectrometry requires complete and detailed protein databases generated from genome sequencing projects. In general, the NCBI or Swiss-Prot databases are used as data sources for these purposes. In our case, we decided to use the human NCBI subdatabase for the search and subsequently transformed the NCBI accession numbers to Swiss-Prot identifiers to reduce redundancy. Because the two search algorithms Sequest and Mascot use either statistical or determining tests for spectra evaluation and benchmarking, we decided to rely on both algorithms in parallel and accepted only positive identifications if both algorithms identified the same protein.

Although the majority of identifications are unambiguously correct, in some cases spectra were assigned to false-positive hits, or a protein was identified with only a single peptide. For these reasons we applied filter criteria to ensure that questionable protein identifications were not considered in our result list. Most proteins were identified by multiple peptides, and additionally we revised all peptide spectra manually to remove false-positive hits. To increase the reliability of the identification we repeated the analysis with independently prepared samples. In general, it could be observed that several well known membrane proteins, e.g. GPIb/IIa, had very high signal intensities and could be identified with very high sequence coverages.

Altogether we identified 297 different species (see Supplemental Table 1). We extracted the localization and functional information from the Swiss-Prot database for all identified proteins and arranged them according to their subcellular localization. In some cases either no localization data could be obtained or localization data were not exactly determined. These proteins were automatically arranged in the group "unknown/no information." Based on these data we identified 83 plasma membrane proteins (27.8%). Additionally, we found 48 membrane proteins that are localized in other cellular compartments such as mitochondria, endoplasmic reticulum, and vesicles. After manual revision of the complete list we assigned an additional 24 proteins as membrane-related or -associated, e.g. G-proteins. Furthermore, we analyzed several hypothetical and putative receptors from the group with no localization information by using the algorithms SOSUI (31) and TMHMM Version 2.0 (32) for the calculation of putative transmembrane domains. For 19 proteins with no defined localization the algorithms predicted one or more TMDs, whereas nine of them are also marked as hypothetical proteins. To test the reliability of these computational tools we calculated the predicted values for all proteins in the protein list. In general, both algorithms seem to be able to classify potential membrane proteins correctly. However, the numbers of calculated TMDs differed several times. For instance, the protein potassium-transporting ATPase α chain 2 (P54707) has seven TMDs according to the SOSUI algorithm, whereas TMHMM predicted only one. Thus, the absolute number of predicted membrane domains should be regarded very carefully. About 17% of all identifications were classified as cytosolic proteins. Many of these are known cytoskeletal or cytoskeleton-associated proteins, such as actin, myosin, and filamin, with high abundance and presence in multiple isoforms. Because several of these proteins interact with membrane proteins either directly or via adaptors the complete removal seems to be infeasible by this approach. But still the overall majority of these proteins were depleted compared with the initial amounts in complete cell lysates. Because this preparation approach does not focus on plasma membrane proteins exclusively, a noticeable amount of other membranous proteins also is present in the prepared samples. However, in some cases a discrete classification of the definite localization of these proteins cannot be performed because of the dynamic changes in the cell. Although we could not identify known G-protein-coupled receptors on platelets by mass spectrometry we were able to detect P2Y12 via immunoblotting.
The comparison of signal intensities from the complete cell lysate with purified membrane proteins indicates enrichment of this protein as well. In the case of P2Y12 the signal is co-located with the actin band. Therefore, the identification by LC-MS/MS seems to have failed due to suppression effects. Additionally GPCRs with their seven-TMD structure are not well accessible by digestion with trypsin because the hydrophobic regions normally do not contain any basic amino acids, leading to very long tryptic peptides, which cannot be analyzed classically by reversed phase separation with C18 phases. In further studies we will have to evaluate the influence of the digestion with other proteases to obtain more appropriate peptides from hydrophobic proteins.

Regarding the different protein datasets obtained from the analysis of the two gel systems, we found 233 proteins from the 16-BAC/SDS gels compared with 140 hits from 1D SDS-PAGE. 75 proteins, which equal 25%, were identified out of both gel systems, leading to the assumption that each gel system is preferably suited for different proteins and produces different protein subsets. One possible explanation for this result could be deviation in sample treatment. Protein samples were heated in lithium dodecylsulfate sample buffer at 80 °C for common SDS-PAGE, which could result in precipitation of several proteins, whereas for the 16-BAC gels the samples were incubated in the 16-BAC sample buffer at 60 °C. Additionally, the enhanced resolution and consequentially better protein separation provide higher sensitivity. For this reason glycoprotein VI (33) with an estimated copy number of about 1,000 per cell and the tetraspanin receptor tetraspan net-5 (34) were exclusively identified after separation with the 16-BAC/SDS system (Fig. 3). Comparing these results with the protein identification of SDS-PAGE-separated samples, both approaches seem to be complementing each other because several potentially interesting proteins such as G6b-A (35) and some hypothetical proteins (see Table I) were only identified by the SDS-PAGE separation technique. To benchmark these results with state-of-the-art proteome studies we merged three 2D PAGE-based platelet proteome studies into a single non-redundant protein dataset corresponding to 408 unique protein species and classified them in the same way we did with our own data (Fig. 4). After classification, we assigned ~10% of all proteins to be membrane-related compared with 57% in our proteome study. However, the usage of classical 2D PAGE grants better access to soluble proteins, which is represented by the fact that about 50% of all identified proteins are localized in the cytosol. Hence both approaches should be taken as complementary proteome studies that have both their advantages and disadvantages.

**DISCUSSION**

We present an extensive proteome approach that is focused on membrane proteins in human platelets. The newly identified candidates may prove to be important for the research on functions of platelets in general as well as in pathological events. The comparison between the published 2D PAGE-based proteome studies and our prefractonation approach with compatible protein separation methods demonstrated that we were able to identify 6 times more membrane-relevant proteins. The amount of plasma membrane proteins with 83 unique identifications represents the largest subpopulation. Besides the known platelet membrane proteins such as GPIb/IIa or the GPIb-IX-V complex, also tetraspans and some ion channels were detected. Additionally, we identified membrane-associated proteins, for instance G-proteins; a glycosylphosphatidylinositol-anchored protein named major prion protein (P04156); junctional adhesion proteins; major histocompatibility complex proteins; and several other transmembrane proteins that are involved in protein processing.
trafficking, vesicular fusion, and signal transduction. Briefly, the complete spectrum of known membrane protein classes was detected, demonstrating the suitability of this approach for a comprehensive purification and enrichment of all membrane protein classes. We identified several proteins from other intracellular compartments as well. However, the majority of these proteins were also assigned as membrane-localized, representing about 16% of all hits with several known organelle membrane proteins such as ATPases in the inner membrane of mitochondria or the dolichyl-diphosphooligosaccharide glycosyltransferases in the endoplasmic reticulum. Regarding the group of cytosolic and cytoskeletal proteins, respectively, we achieved a significant decrease from 54 to 17% compared with the classical 2D-PAGE proteome studies that used complete cell lysates (see Fig. 4). Furthermore, different cytoskeletal proteins, such as actin, myosin, and tubulin, were identified in several isoforms, which virtually increased the amount of this group. In fact several cytoskeletal proteins, e.g. filamin, are connected directly or via adapter proteins to the membrane or receptors, forming the so-called submembranous cytoskeleton (36–38). Additionally Qingqi et al. (39) showed that actin is covalently coupled to the membrane itself, rendering it nearly impossible to deplete this protein and its interaction partners completely. Nevertheless, the significant reduction seems to be sufficient for the access to low abundance membrane proteins.

We examined the group of unknown localized proteins by analyzing the sequences with different algorithms to evaluate their putative localization and probable function. In Table I we extracted a short excerpt of four new potential receptors or membrane proteins that were analyzed with the SMART (40, 41) algorithm available on line and the Pfam (42) toolset. In two cases a protein module could be identified that gave us a first assumption about the potential function of these proteins. For G6b-A an Ig domain was found indicating the potential function of an Ig-like receptor. The hypothetical protein Q9BSJ8 contains five C2 domains like several membrane proteins that are involved in signal transduction. For the re-

| Accession | Protein Name         | Potential protein modules | Potential function                          | Graphical scheme adapted from SMART |
|-----------|----------------------|---------------------------|---------------------------------------------|-------------------------------------|
| Q96A91    | G6b-A protein        | IG-Domain                 | IG-like surface receptor                    | ![IG](image)                        |
| Q96VC6    | Hypothetical protein | 4 TMD domains             | similar to mitsugumin 23 family, Tetraspanin? | ![Image](image)                     |
| Q96578    | KIAA0747 protein     | 5 C2 Domains              | involved in signal transduction?            | ![Image](image)                     |
| Q96SU4    | Hypothetical protein | 2 TMD                     | similar to TMEM40 protein (Q9WWA1)          | ![Image](image)                     |

![Fig. 4. Comparison of the protein distribution between published 2D PAGE data and the focused membrane proteome approach.](image)

Three major publications on platelet proteomics were merged in a non-redundant dataset of 408 different proteins. Afterward the proteins were grouped according to their localization derived from the Swiss-Prot database. This was also done in the same way with the protein list from Supplemental Table 1. Both datasets represent huge differences in their distribution. Although the common 2D-PAGE approach is useful for the analysis of soluble proteins, the membrane preparation approach enables the identification of many different membrane-related proteins.
remaining four proteins the SMART algorithm could not detect any known protein module. Additionally we used the BLAST algorithm to analyze the proteins for homologues. Although we found homologues for two additional proteins, the function was not known for these proteins either. Regarding the complete list of identified proteins (Supplemental Table 1) only some of the known GPCRs are absent. To test whether the isolation protocol is inappropriate to purify these proteins we performed an immunoblot, which detected the P2Y12 receptor (Fig. 1B). The intensity signals showed an increase in the concentration of this particular receptor by each applied purification step. The reason why identification by mass spectrometry was not yet possible can be explained by two facts. First the GPCR itself has a seven-transmembrane motif that yield no utilizable peptides for C18 reversed phase HPLC separation after tryptic digest. Therefore, only a very small number of applicable tryptic peptides are available for these proteins. Second the expression rate of GPCRs is quite low, which is noted in Table II where G-protein-coupled receptors on platelet membranes are present with only a few hundred copies per cell. Due to the limited dynamic range and sample complexity it is very difficult to obtain good quality MS spectra for these proteins. This can also be observed in the case of P2Y12, which is co-localized with the residual actin band on the 1D-SDS-PAGE separation (see Fig. 1). Further studies are in progress to assess whether an improved separation resolution will be sufficient to provide the access to this membrane protein class. Additionally, application of different proteases such as chymotrypsin, Glu-C, or enolase will produce different peptides that have a better capability for MS analysis.

**TABLE II**

| Receptor            | Copies/platelet | Ref. | Remarks                      |
|---------------------|-----------------|------|------------------------------|
| Integrin Ibllia     | 500,000–800,000 | 46   |                              |
| Integrin αvβ3       | A few hundreds  | 46   |                              |
| Integrin α2β1       | 2,000–4,000     | 46,47| Binds collagen               |
| GPVI                | −1,000          | 46,47|                              |
| CD 36               | 20,000          | 46,47|                              |
| GPIb-IX-V complex   | −60,000         | 46   | Leucine-rich repeat family   |
| GPCR thrombin PAR1  | −2,500          | 46   |                              |
| GPCR P2Y1           | −300            | 48   |                              |
| GPCR P2Y12          | −600            | 48   |                              |
| GPCR TxA2           | −1,700          | 49   |                              |
| P-selectin (CD62P)  | −10,000         | 50   | On activated platelets       |

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**REFERENCES**

1. White, J. G. (1994) Platelets and atherosclerosis. *Eur. J. Clin. Investig.* 24, Suppl. 1, 25-29
2. Meade, T. W. (1992) Thrombosis and cardiovascular disease. *Ann. Epidemiol.* 2, 353–364
3. Hopkins, A. L., and Groom, C. R. (2002) The druggable genome. *Nat. Rev. Drug Discov.* 1, 727–730
4. Marcus, K., Immier, D., Stormberger, J., and Meyer, H. E. (2000) Identification of platelet proteins separated by two-dimensional gel electrophoresis and analyzed by matrix assisted laser desorption/ionization-time of flight-mass spectrometry and detection of tyrosine-phosphorylated proteins. *Electrophoresis* 21, 2622–2636
5. O’Neill, E. E., Brock, C. J., von Kriegsheim, A. F., Pearce, A. C., Dwek, R. A., Watson, S. P., and Hebestreit, H. F. (2002) Towards complete analysis of the platelet proteome. *Proteomics* 2, 288–305
6. Garcia, A., Prabhakar, S., Brock, C. J., Pearce, A. C., Dwek, R. A., Watson, S. P., Hebestreit, H. F., and Zitzmann, N. (2004) Extensive analysis of the human platelet proteome by two-dimensional gel electrophoresis and mass spectrometry. *Proteomics* 4, 656–668
7. Santini, V., Rabilloud, T., Doumas, P., Rouquie, D., Mansion, M., Kieffer, S., Garin, J., and Rossignol, M. (1999) Towards the recovery of hydrophobic proteins on two-dimensional electrophoresis gels. *Electrophoresis* 20, 705–711
8. Molloy, M. P. (2000) Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. *Anal. Biochem.* 280, 1–10
9. Adessi, C., Miege, C., Abrioux, C., and Rabilloud, T. (1997) Two-dimensional electrophoresis of membrane proteins: a current challenge for immobilized pH gradients. *Electrophoresis* 18, 127–135
10. Santini, V., Molloy, M., and Rabilloud, T. (2000) Membrane proteins and proteomics: un amour impossible? *Electrophoresis* 21, 1054–1070

1.760 **Molecular & Cellular Proteomics** 4.11
11. Fox, J. E. (1993) The platelet cytoskeleton. *Thromb. Haemostasis* 70, 884–893
12. Stossel, T. P. (1989) From signal to pseudopod. How cells control cytoplasmic actin assembly. *J. Biol. Chem.* 264, 18261–18264
13. Lopez, M. F., Berggren, K., Chernokalskaya, E., Lazarev, A., Robinson, M., and Patton, W. F. (2000) A comparison of silver stain and SYPRO Ruby Protein Gel Stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. *Electrophoresis* 21, 3673–3683
14. Roegener, J., Lutter, P., Reinhart, R., Bluggel, M., Meyer, H. E., and Anselmetti, D. (2003) Ultrasensitive detection of unstained proteins in acrylamide gels by native UV fluorescence. *Anal. Chem.* 75, 157–159
15. Mortz, E., Krogh, T. N., Vorum, H., and Gorg, A. (2001) Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Anal. Chem.* 73, 4811–4824
16. Stasyk, T., and Huber, L. A. (2004) Zooming in: fractionation strategies in proteomics. *Proteomics* 4, 3704–3716
17. Coughenour, H. D., Spaulding, R. S., and Thompson, C. M. (2004) Tissue-specific vesicle proteome: a comparative study in membrane protein identification. *Proteomics* 4, 3141–3155
18. Guillen, I., Becker, M., Ociepka, K., Friauf, E., and Nothwang, H. G. (2005) A subcellular prefractionation protocol for minute amounts of mammalian cell cultures and tissue. *Proteomics* 5, 35–45
19. Hartinger, J., Stenius, K., Hogemann, D., and Jahn, R. (1996) 16-BAC/SDBS-PAGE: a two-dimensional gel electrophoresis system suitable for the separation of integral membrane proteins. *Anal. Biochem.* 240, 126–133
20. Authi, K. S. (1992) Localization of the [252]PitP binding site on human platelet intracellular membranes isolated by high-voltage free-flow electrophoresis. *FEMS Lett.* 296, 173–176
21. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazaro, P. B. (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93, 97–102
22. Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256, 1604–1607
23. Jiang, L., He, L., and Fountoulakis, M. (2004) Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J. Chromatogr. A* 1023, 317–320
24. Zahedi, R. P., Meisinger, C., and Sickmann, A. (2005) Two-dimensional BAC/SDBS PAGE for membrane proteomes. *Proteomics* 5, 3581–3588
25. Mortz, E., Krogh, T. N., Vorum, H., and Gorg, A. (2001) Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Proteomics* 1, 1359–1363
26. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 68, 850–858
27. Boehm, A. M., Galvin, R. P., and Sickmann, A. (2004) Extractor for ESI quadrupole TOF tandem MS data enabled for high throughput batch processing. *BMC Bioinformatics* 5, 162
28. Yates, J. R., Ill, Eng, J. K., McCormack, A. L., and Schieltz, D. (1995) Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal. Chem.* 67, 1426–1436
29. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20, 3551–3567
30. Kinoshita, T., Nachman, R. L., and Minick, R. (1979) Isolation of human platelet plasma membranes with polylysine beads. *J. Cell Biol.* 82, 688–696
31. Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* 14, 378–379
32. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–580
33. Clermeton, J. M., Polgar, J., Magenat, E., Wells, T. N., and Clermeton, K. J. (1999) The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcεR and the natural killer receptors. *J. Biol. Chem.* 274, 29019–29024
34. Serru, V., Dessen, P., Bouchéix, C., and Rubinstein, E. (2000) Sequence and expression of seven new tetraspans. *Biochim. Biophys. Acta* 1478, 159–163
35. de Vet, E. C., Aguado, B., and Campbell, R. D. (2001) G6b, a novel immunoglobulin superfamily member encoded in the human major histocompatibility complex, interacts with SHP-1 and SHP-2. *J. Biol. Chem.* 276, 42070–42076
36. Bearer, E. L. (1990) Platelet membrane skeleton revealed by quick-freeze deep-etch. *Anat. Rec.* 227, 1–11
37. Fox, J. E., Bycles, J. K., Berndt, M. C., Steffen, P. K., and Anderson, L. K. (1988) Identification of a membrane skeleton in platelets. *J. Cell Biol.* 106, 1525–1538
38. Fox, J. E., Reynolds, C. C., Morow, J. S., and Phillips, D. R. (1987) Spectrin is associated with membrane-bound actin filaments in platelets and is hydrolyzed by the Ca2+-dependent protease during platelet activation. *Blood* 69, 537–545
39. Qingqi, Z., and Stracher, A. (1998) Platelet membrane actin may be partially embedded in lipid bilayer and disulfide linked. *Biochem. Biophys. Res. Commun.* 252, 407–411
40. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. U. S. A.* 95, 5857–5864
41. Letunic, I., Copley, R. R., Schmidt, S., Ciccarelli, F. D., Doerks, T., Schultz, J., Ponting, C. P., and Bork, P. (2004) SMART 4.0: towards genomic data integration. *Nucleic Acids Res.* 32, D142–D144
42. Sonnhammer, E. L., Eddy, S. R., and Durbin, R. (1997) Pfam: a comprehensive database of protein domain families based on seed alignments. *Proteins* 28, 405–420
43. Yi, E. C., Marelli, M., Lee, H., Purvine, S. O., Aebersold, R., Alchison, J. D., and Goodlett, D. R. (2002) Approaching complete peroxisome characterization by gas-phase fractionation. *Electrophoresis* 23, 3205–3216
44. Utleg, A. G., Yi, E. C., Xie, T., Shannon, P., White, J. T., Goodlett, D. R., Hooi, L., and Lin, B. (2003) Proteomic analysis of human prostatomes. *Prostate* 56, 150–161
45. Blond, J., Rodriguez-Galan, M. C., Lucas, D. A., Young, H. A., Issaq, H. J., Veenstra, T. D., and Conrads, T. P. (2004) Proteomic investigation of natural killer cell microsomes using gas-phase fractionation by mass spectrometry. *Biochim. Biophys. Acta* 1698, 87–95
46. Clermeton, J. M. (2002) in Platelets (Michelson, A. D., ed) pp. 65–76, Academic Press, San Diego, CA
47. Siljander, P. R. M., and Fardaneh, R. W. (2002) in Platelets in Thrombotic and Non-Thrombotic Disorders: Pathophysiology, Pharmacology and Therapeutics (Gresele, P., Page, C., Fuster, V., and Vermulen, J., eds) pp. 158–178, Cambridge University Press, Cambridge, UK
48. Gachelin, C., and Cazenave, J. P. (2002) in Platelets in Thrombotic and Non-Thrombotic Disorders: Pathophysiology, Pharmacology and Therapeutics (Gresele, P., Page, C., Fuster, V., and Vermulen, J., eds) pp. 127–139, Cambridge University Press, Cambridge, UK
49. Vezza, R., and Fitzgerald, D. J. (2002) in Platelets in Thrombotic and Non-Thrombotic Disorders: Pathophysiology, Pharmacology and Therapeutics (Gresele, P., Page, C., Fuster, V., and Vermulen, J., eds) pp. 140–157, Cambridge University Press, Cambridge, UK
50. McEver, R. P. (2002) in Platelets (Michelson, A. D., ed) pp. 139–148, Academic Press, San Diego, CA