Quantitative proteomics of the integrin adhesome show a myosin II-dependent recruitment of LIM domain proteins

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A characteristic of integrins is their ability to transfer chemical and mechanical signals across the plasma membrane. Force generated by myosin II makes cells able to sense substrate stiffness and induce maturation of nascent adhesions into focal adhesions. In this paper, we present a comprehensive proteomic analysis of nascent and mature adhesions. The purification of integrin adhesion complexes combined with quantitative mass spectrometry enabled the identification and quantification of known and new adhesion-associated proteins. Furthermore, blocking adhesion maturation with the myosin II inhibitor blebbistatin markedly impaired the recruitment of LIM domain proteins to integrin adhesion sites. This suggests a common recruitment mechanism for a whole class of adhesion-associated proteins, involving myosin II and the zinc-finger-type LIM domain.

Keywords: integrin adhesome; quantitative proteomics; focal adhesion; myosin-II; LIM domain

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INTRODUCTION

Integrins are a key family of cell-adhesion receptors that link the extracellular matrix (ECM) to the F-actin cytoskeleton (Hynes, 2002). The coupling of the ECM to F-actin is important, not only for transducing forces from the cytoskeleton to the extracellular environment, but also for translating external forces into biochemical signals within cells. As integrin cytoplasmic domains lack actin-binding sites and catalytic activity, they regulate signalling and F-actin network remodelling by recruiting

cytoskeletal and adaptor proteins, as well as several enzymes, to integrin adhesion sites. A total of 180 proteins—collectively known as the adhesome—have been found at these sites so far (Zaidel-Bar *et al*, 2007; Zaidel-Bar & Geiger, 2010).

The composition of the adhesome is sensitive to mechanical cues, which can change the composition and size of the adhesome and induce the maturation of newly formed nascent adhesions into focal adhesions (Zamir & Geiger, 2001; Pasapera *et al*, 2010). However, the quantitative and qualitative changes to the myosin II-driven protein profile during adhesion maturation have not been systematically studied.

In this paper, we report a comprehensive and unbiased analysis of the molecular composition of focal adhesions and nascent adhesions, the maturation of which was blocked by blebbistatin-mediated myosin II inhibition. First, we established a new method for purifying focal adhesions and nascent adhesions formed on fibronectin-coated culture plates, and then we performed quantitative mass spectrometry and bioinformatic network analysis.

RESULTS AND DISCUSSION

Purification of the integrin proximal subproteome

The isolation and analysis of membrane-associated signalling networks, such as integrin adhesion complexes, has been difficult because of the transient nature and low affinity of many molecular interactions within these signalling hubs. A recently published protocol used cell-permeant chemical crosslinkers to improve the recovery of integrin adhesion-associated proteins from cells bound to fibronectin-coated microbeads (Humphries *et al*, 2009).

We modified this strategy and crosslinked integrin adhesion complexes to fibronectin-coated plastic dishes in the absence or presence of mechanical tension. In our initial experiments, we treated fibroblasts isolated from a 4-week-old male mouse kidney for 5, 15 or 30 min with the cell permeant and reversible crosslinkers dithiobis[succinimidyl propionate] and 1,4-di-[3'-(2'-pyridyldithio)-propionamido]butane, which crosslink lysine and cysteine residues, respectively. Cells were then lysed and subjected to a stringent, high shear flow water wash to remove

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non-crosslinked material. A 5 min treatment with the crosslinkers was sufficient to preserve the focal adhesion localization of the canonical adhesome protein paxillin after cell lysis with a SDS-containing buffer. By contrast, transferrin receptor (CD71)—which is found at the plasma membrane but is not enriched in integrin adhesions (Zaidel-Bar *et al*, 2007)—was less efficiently preserved. Crosslinking for 30 min was required for strong non-specific crosslinking of CD71 to fibronectin (Fig 1A,B). The stringent shear flow water wash procedure was essential to increase the ratio of paxillin signal to CD71 (Fig 1C).

For all proteomics experiments, we crosslinked cells for 5 min. To distinguish integrin-mediated recruitment of proteins from a non-specific background, we plated cells on fibronectin- or poly-L-lysine (PLL)-coated plastic dishes (Fig 1D). Cell attachment and spreading on PLL is independent of integrin and occurs through electrostatic interactions (Schottelndreier *et al*, 1999), whereas integrin–fibronectin bonds induce the formation of actomyosin contractile fibres and focal adhesions (Fig 2A). Moreover, inhibition of myosin II by treatment of cells with blebbistatin for 30 min (Choi *et al*, 2008; Pasapera *et al*, 2010) led to the disappearance of contractile stress fibres and focal adhesions and to the relative enrichment of nascent adhesions in the lamellipodium (Fig 2A).

Myosin II regulates protein recruitment to adhesion sites

Purified adhesion fractions (for details, see Methods section) of untreated or blebbistatin-treated cells plated on fibronectin- or PLL-coated dishes were analysed by quantitative mass spectrometry and bioinformatic analysis (experimental workflow in Fig 1D). An unpaired, multiclass statistical analysis (supplementary information online) for the median mass intensities ($n=5$) identified 770 proteins that were significantly different in at least one of the tested conditions (supplementary File S1 online). Next, we analysed the median mass intensities by using unsupervised hierarchical average linkage clustering (Fig 2B). In total, we identified and quantified 2,118 proteins, which were assigned to 16 distinct clusters (labelled A–P). Five clusters (C/D/E/F/G) representing 890 proteins showed enriched mass intensities on fibronectin compared with PLL. Comparison of the five clusters with the remaining clusters using an unbiased enrichment analysis for gene ontology terms, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and PFAM (protein family) and INTERPRO protein domains, led to the identification of significantly enriched annotations such as focal adhesion, actin cytoskeleton, adherens junction, basolateral plasma membrane and stress fibre within the clusters C–G. Furthermore, we noted a significant enrichment of proteins containing an RNA recognition motif and proteins containing the zinc-finger-type LIM (Lin11, Isi-1, Mec-3) domain (supplementary Table S1 online).

The recruitment of ribosomes and RNA-binding proteins to integrin adhesions has been reported previously (Chicurel *et al*, 1998; de Hoog *et al*, 2004) and might reflect the need for localized translation of a specific subset of protein complexes in the cell periphery, as is observed in the axons and dendrites of neurons (Tcherkezian *et al*, 2010). Two canonical integrin adhesion-associated proteins, paxillin and vinculin, were shown to interact with the RNA-binding proteins raver-1 and poly-(A)-binding protein 1, respectively (Huttelmaier *et al*, 2001; Woods *et al*, 2002). Moreover, messenger RNAs for cytoskeletal proteins such as β -actin are actively transported to and translated at the cell

periphery, close to integrin adhesion sites (Lawrence & Singer, 1986; Huttelmaier *et al*, 2005).

Overall, 180 integrin adhesion-associated proteins were compiled to the adhesome network on the basis of a meta-study of the cell-adhesion literature (Zaidel-Bar *et al*, 2007; Zaidel-Bar & Geiger, 2010). Although this probably overestimates the number of proteins in a single cell type cultured under specific conditions, we detected 87 of these adhesome proteins in one experimental setup. This result differs from that of a recent study that only identified 37 out of the 180 adhesome components on fibronectin-coated microbeads (Humphries *et al*, 2009). Overall, 64 out of the 87 known focal adhesion components found in our study were enriched on fibronectin, compared with PLL (C–G). A total of 40 of these proteins were reduced by blebbistatin treatment (D/F/G), whereas the levels of fibronectin-binding integrins as well as integrin tail-interacting proteins—including talin, kindlin and filamin—did not significantly change after blebbistatin treatment (C/E). The intensities of seven adhesome proteins were enhanced with blebbistatin (A/B), which might reflect an increased proximity of these proteins to integrin, on myosin II inhibition (Fig 2C). Interestingly, we could recover 562 (approximately 80%) of the 708 proteins that have been identified using fibronectin-coated microbeads (Humphries *et al*, 2009). Considering that the two studies were carried out with different cellular systems and methodologies, 80% is a high overlap of protein recovery.

LIM domain proteins are potential tension sensors

The clusters D/F/G represented 358 proteins, the median mass intensities of which were enhanced on fibronectin compared with PLL and were reduced after blebbistatin treatment (Fig 2B, supplementary File S1 online). The LIM domain proteins pinch1, limd1, fh12, fh13, hic5, lpp, zyxin, migfilin, trip6, eplin, csrp1, csrp2, lasp1, paxillin, pdlim1, pdlim2, pdlim4, pdlim5, pdlim7, testin and ablim1 were significantly enriched in clusters D/F/G compared with all other clusters, and showed the strongest mass intensity reductions (supplementary Table S2 online and supplementary File S1 online). In agreement with these findings, a paired, two-class statistical analysis (for details, see Methods section) identified 24 proteins—including most LIM domain proteins—with significantly reduced mass intensities after blebbistatin treatment (supplementary File S1 online).

To show known protein–protein interactions (PPI) and subcomplexes within the adhesion-associated subproteome, we performed PPI network analysis with the identified proteins using PPIs derived from various databases (for details, see Methods section). In the network in Fig 3 (supplementary Files S2, S3 online), we placed three integrin- α and three integrin- β chains in the centre and the known direct and indirect integrin interactors in the inner and outer network rings, respectively. To visualize changes in stoichiometry relative to integrin- β 1 on inhibition of myosin II, we calculated the log2 ratios of the mass intensities, normalized them to the integrin- β 1 ratio and colour-coded the network nodes accordingly. Despite their clear enrichment on fibronectin compared with PLL (red colour; supplementary Fig S1 online), the levels of many proteins within the network were either unaffected by blebbistatin (white colour) or even increased relative to integrin- β 1 (blue colour). A possible explanation for this increase could be that the blue node proteins have important roles in the early phases of adhesion. The red nodes represent those proteins that were highly sensitive to

Fig 1 | Experimental workflow. (A) Immunostaining of paxillin and CD71 after treatment of cells with crosslinkers for 5, 15 and 30 min. DNA was stained with DAPI (blue). (B) Crosslinked cells were lysed, washed with high shear flow water jetting and immunostained as in A. (C) The integrated total fluorescence intensities of CD71 and paxillin staining were quantified using Metamorph software ($n > 15$ cells) and ratios were calculated. (D) Experimental workflow for identification and quantification of the myosin II-dependent adhesion-associated subproteome. DAPI, 4,6-diamidino-2-phenylindole; LC-MS/MS, liquid chromatography-mass spectrometry; PAGE, polyacrylamide gel electrophoresis; TIRFM, total internal reflection microscopy.

◀ **Fig 2** | Myosin II-dependent and -independent protein recruitment to integrin adhesions. (A) Cells were seeded on FN- or PLL-coated glass coverslips for 45 min in the absence or presence of blebbistatin, fixed and stained for nuclei (blue), actin (red) and paxillin (green). (B) Overall, 2,118 proteins were identified, quantified and grouped into clusters labelled A–P. Clusters D, F and G show FN enrichment and intensity reduction by blebbistatin and are marked with an asterisk. FN-enriched clusters C–G are shown with a black bar. (C) The depicted proteins were previously associated with integrin adhesions. A–P refer to the hierarchical cluster analysis in B. FN, fibronectin; PLL, poly-L-lysine.

Fig 3 | Hierarchical PPI network around fibronectin-bound integrins. Integrin subunits are in the centre and the inner and outer circles show their direct and indirect interactors, respectively. Black lines between nodes indicate high confidence PPI, red arrows indicate activating and blue lines indicate inhibiting interactions. The nodes were labelled with gene symbols and colour-coded according to the log₂ ratio of control over blebbistatin-treated cells (median; $n = 5$). Red nodes were reduced relative to integrin- $\beta 1$ on addition of blebbistatin. The LIM symbol marks proteins with LIM domains. FN, fibronectin; PPI, protein-protein interaction.

blebbistatin for localization. These were almost exclusively LIM domain proteins.

LIM domains consist of two tandem-arranged zinc-fingers that mediate PPIs in many subcellular compartments, including focal adhesions (Kadmas & Beckerle, 2004). The myosin II-dependent recruitment of such a large number of LIM domain proteins to

focal adhesions was striking and novel. A possible explanation for this phenomenon is that LIM domains might be biosensors of mechanical tension at integrin adhesion sites. This hypothesis is supported by the observations that recruitment of the LIM protein zyxin to focal adhesions and stress fibres is induced by cell stretching (Hirata *et al*, 2008; Colombelli *et al*, 2009) and that the

Fig 4 | Differential dependence of migfilin and kindlin 2 on myosin II analysed by TIRFM. (A) Selected subnetwork. (B) The log₂ ratio of protein intensities from control compared with blebbistatin-treated cells analysed by mass spectrometry ($n = 5$). (C–E) Fibroblasts expressing (C) vasp-cherry, (D) migfilin-cherry and (E) kindlin 2-cherry monitored over 40 min (30 s time laps) by TIRFM. For acquiring a bleaching, baseline cells were either left untreated for 40 min, or 10 min followed by blebbistatin treatment for the remaining 30 min. Total integrated fluorescence intensities of (C) vasp-cherry (blebbistatin, $n = 6$; untreated, $n = 4$), (D) migfilin-cherry (blebbistatin, $n = 11$; untreated, $n = 5$) and (E) kindlin 2-cherry (blebbistatin, $n = 8$; untreated, $n = 3$) in the TIRFM field were quantified using the Metamorph program. (F) Fluorescence intensity values of blebbistatin-treated migfilin-cherry and kindlin 2-cherry cells were corrected for the bleaching and overlaid. TIRFM, total internal reflection microscopy.

muscle LIM protein (MLP) functions in stretch sensing at muscle Z-discs (Knoll *et al*, 2002).

How can mechanical tension generate LIM protein-binding sites? Partial protein unfolding by mechanical tension was shown to generate new binding sites for some adhesome proteins (Sawada *et al*, 2006; del Rio *et al*, 2009). It is also conceivable that LIM domains function in a cooperative manner by binding to a combination of focal adhesion proteins. Indeed, most LIM domain proteins found in our study contain serial LIM domains. Moreover, it has been shown that zyxin, lpp and migfilin require more than one LIM domain for focal-adhesion targeting (Nix *et al*, 2001; Petit *et al*, 2003; Gkretsi *et al*, 2005). A defined spatial arrangement of serial LIM domains might promote binding to a polymeric backbone such as F-actin/ α -actinin, the LIM-binding sites of which might only become available under mechanical tension. Several observations support this hypothesis. First, a change in the length of the linker sequence between LIM1 and LIM2 of lpp abrogated its focal adhesion localization (Petit *et al*, 2003). Second, several LIM domain proteins can bind to F-actin (Kadmas & Beckerle, 2004). Third, the enrichment of LIM domain

proteins in focal adhesions compared with F-actin stress fibres might be due to different levels of force-induced strain along actin stress fibres, which are highest close to focal adhesions (Lu *et al*, 2008; Colombelli *et al*, 2009). Further studies are needed to identify the putative strain-dependent docking structure and to characterize the precise myosin II-dependent recruitment mechanism of LIM domain proteins to focal adhesions.

Rapid loss of migfilin from blebbistatin-treated adhesions

To substantiate and validate our findings, we analysed a PPI subnetwork in cultured fibroblasts consisting of blebbistatin-sensitive and -insensitive nodes (Fig 4A). The chosen subnetwork consists of migfilin and its binding partners kindlin 2, filamin (Tu *et al*, 2003) and vasp (Zhang *et al*, 2006). Migfilin is thought to act as a molecular switch that regulates integrin activation and the dynamics of the ECM-actin link (Ithychanda *et al*, 2009). Migfilin binds to kindlin 2 with its carboxy-terminal LIM domains (Tu *et al*, 2003) and to filamin and vasp with its amino-terminal domains (Zhang *et al*, 2006; Lad *et al*, 2008). Only the mass intensities of migfilin and vasp, but not of kindlin 2 or filamin, were reduced by

blebbistatin (Fig 4B). By using total internal reflection microscopy, we confirmed this finding by showing that blebbistatin perfusion (50 μ M) induced a rapid reduction in the levels of cherry-tagged migfilin and vasp in mature focal adhesions of cells plated for 24 h on fibronectin, that resulted in a complete loss of migfilin 10 min after myosin II inhibition (Fig 4C,D; supplementary Movies S2, S4 online). In contrast to migfilin, residual vasp remained in the cell leading edge, in agreement with previous studies (Bear *et al*, 2002). In untreated cells, we observed a slow and steady reduction of cherry-tagged migfilin and vasp from focal adhesions due to photobleaching (Fig 4C,D,F; supplementary Movies S1, S3 online). Cherry-tagged kindlin 2 localization in focal adhesions was significantly less affected by blebbistatin than by its binding partner migfilin (Fig 4E,F; supplementary Movies S5, S6 online). After correcting for photobleaching, the total integrated kindlin 2 intensity was reduced to 40% after 10 min of treatment and then remained constant for the remaining 20 min (Fig 4F). This 60% reduction of kindlin 2 intensity is probably due to the overall reduction in adhesion size after myosin II inhibition. As the mass intensity ratios were normalized to integrin- β 1 ratios, we normalized for the changing adhesion area in those experiments. Newly forming nascent adhesions positive for kindlin 2 were observed after blebbistatin treatment, indicating that kindlin 2 is recruited independently of myosin II (supplementary Movie S6 online). By contrast, migfilin was absent in nascent adhesions of blebbistatin-treated cells but present in focal adhesions of untreated cells (supplementary Fig S2 online). Using paxillin-green fluorescent protein/migfilin-cherry double-transfected cells, we observed that the loss of migfilin from focal adhesions after myosin II inhibition occurred before the disassembly of the adhesion site (supplementary Fig S3; supplementary Movies S7, S8 online). We conclude that the dynamic localization behaviour of vasp, migfilin and kindlin 2 after myosin II inhibition confirms the results obtained by mass spectrometry analysis.

Our study shows that a chemical crosslinking strategy combined with quantitative mass spectrometry can be used to characterize the molecular composition of transmembrane receptor complexes under various conditions. The finding that recruitment of almost all LIM domain-containing focal adhesion proteins was dependent on myosin II activity opens the possibility that targeting a tension-specific LIM domain-binding motif might block the recruitment of a whole class of integrin adhesion-associated proteins.

METHODS

Enrichment of the integrin adhesion proximal subproteome.

Cells were starved of serum for 2 h and then collected using trypsin/EDTA. Detached cells were washed twice, seeded in serum-free DMEM at a density of 7×10^6 cells on pre-coated 10 cm dishes and incubated for 45 min. Dishes were coated with 10 μ g/ml fibronectin in PBS at 4 °C overnight or with 0.01% poly-L-lysine (PLL) in PBS at room temperature for 1 h, followed by blocking with 1% BSA in PBS for 1 h at room temperature. Treatment with blebbistatin (100 μ M) was carried out 15 min after seeding for the remaining 30 min. Subsequently, the plates were washed once with PBS (1 mM $MgCl_2^{2+}$ and 1 mM $CaCl_2^{2+}$) and then incubated for 5 min at room temperature with PBS (+Ca/+Mg) containing 0.5 mM dithiobis[succinimidyl propionate] and 0.05 mM 1,4-di-[3'-(2'-pyridyl)dithio]-propionamido]

butane. The crosslink reaction was quenched with 1 M Tris-HCl, pH 7.5. Crosslinked cells were lysed for 30 min with radio-immunoprecipitation assay (RIPA) lysis buffer (25 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% TX100; 0.2% SDS; 0.5% sodium deoxycholic acid; protease inhibitors) on ice. Thereafter, plates were subjected to a high-pressure water stream for 1 min to effectively remove all non-crosslinked material. The remaining crosslinked complexes were eluted by reduction with dithiothreitol buffer (25 mM Tris-HCl, pH 7.5; 10 mM NaCl; 0.1% SDS; 100 mM dithiothreitol) for 1 h at 60 °C. Eluted proteins were stained with GlycoBlue reagent (Ambion #AM9515) and precipitated with 4 $\times$ volume of acetone at -20 °C overnight. Precipitated proteins were pelleted by centrifugation, reconstituted in 1 $\times$ Laemmli buffer, boiled for 5 min and separated by SDS-polyacrylamide gel electrophoresis on a 4–15% gradient gel. The gel was stained with Coomassie using the GelCode Blue Safe Protein Stain reagent (Thermo #1860957) and used for mass analysis.

Cell culture, mass spectrometry, bioinformatic analysis, immunofluorescence and total internal reflection microscopy. Detailed Methods, the complete proteomics data set, PPI network files and supplementary movies are available as supplementary information.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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