Journal of Cell Science 111, 2181-2188 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS9779

Recruitment of the LIM protein hic-5 to focal contacts of human platelets

Jörg Hagmann[1,*], Marianne Grob[1], Arkadiusz Welman[1], Gijsbert van Willigen[2] and Max M. Burger[1]

[1]Friedrich Miescher-Institute, Basel, Switzerland
[2]Department of Haematology, University of Utrecht, The Netherlands
*Author for correspondence

Accepted 29 May; published on WWW 15 July 1998

SUMMARY

Platelets are anuclear, membrane-bounded fragments derived from megakaryocytes which, upon stimulation, assemble an actin skeleton including stress fibres and focal contacts. The focal contacts resemble those of tissue culture cells. However, they lack paxillin, a conspicuous component of these organelles. We found that instead of paxillin, platelets contain a related protein with a molecular mass of 55 kDa that crossreacts with a monoclonal antibody against paxillin. The gene for the 55 kDa protein was cloned from a bone marrow cDNA library and turned out to be identical to a recently discovered gene encoding hic-5. Like paxillin, hic-5 is a cytoskeletal protein containing four carboxy-terminal LIM domains and LD motifs in the amino-terminal half. The LIM domains of both hic-5 and paxillin are capable of targetting green fluorescent protein to focal contacts. In addition, GST-hic-5 precipitates the focal adhesion kinase pp125^{FAK} and talin from platelet extracts. Only trace amounts of hic-5 occur in DAMI cells, a megakaryocytic cell line, and in megakaryocytes cultured from CD34^{+} cells obtained from umbilical cord blood. However, RT-polymerase chain reactions performed with RNA obtained from platelets gave a positive result when primers specific for hic-5 were used, but were negative with paxillin-specific primers, indicating that a switch from paxillin expression to hic-5 expression must occur late in the maturation of megakaryocytes into platelets.

Key words: Platelet, Hic-5, paxillin, Focal contact, Cytoskeleton

INTRODUCTION

Focal contacts (focal adhesions) anchor stress fibers of tissue culture cells in the plasma membrane. They consist of integrins, which provide the link with the extracellular matrix, and a large number of proteins, such as vinculin, α-actinin, talin, VASP, zyxin and paxillin, the enzymes calpain, protein kinase C and focal adhesion kinase (pp125^{FAK}), and members of the src kinase family (reviewed by Jokusch et al., 1995; Yamada and Geiger, 1997). Although many focal contact components have been well characterized, and some of the binding interactions between them have been established in vitro, it is still neither known how the whole structure is built, nor how its formation and disassembly are regulated.

Focal contacts transmit tension to the extracellular matrix and to whole tissues. Tension, however, is not only extended to the neighbourhood of a cell, but also to intracellular structures, notably the nucleus (Maniotis et al., 1997). This opens two possibilities for focal adhesion-mediated signal transduction: directly through mechanical forces tugging at the nucleus, and indirectly through mediators bound or released from focal contacts and shuttling between them and the nucleus. Proteins associated with focal contacts might therefore exert regulatory functions by either modulating the strength of the adhesion or by taking part in messenger transfer mechanisms.

Recently, the focal adhesion kinase pp125^{FAK} and its substrate paxillin have attracted a lot of interest: activation of pp125^{FAK}, usually coupled to tyrosine phosphorylation of paxillin, has been correlated with the turnover of focal contacts in several experimental systems (reviewed by Turner, 1994; Clark and Brugge, 1995; Craig and Johnson, 1996; Otey, 1996; Parsons, 1996). Paxillin is a 68 kDa protein composed of an amino-terminal domain with binding sites for pp125^{FAK} and vinculin, and of a carboxy-terminal domain containing four LIM domains (Turner et al., 1990; Turner and Miller, 1994; Wood et al., 1994; Brown et al., 1996). Although paxillin is an in vitro and possibly also an in vivo substrate for pp125^{FAK} (Bellis et al., 1995; Schaller and Parsons, 1995), the roles of the kinase and of its substrates are far from clear: cells lacking pp125^{FAK} are less motile, have more focal contacts, and express more paxillin than wild-type cells. Moreover, paxillin is tyrosine-phosphorylated despite the absence of pp125^{FAK} presumably by an alternative tyrosine kinase (Ilic et al., 1995; see also Wilson et al., 1995).

Platelets are membrane-bounded cellular fragments derived from megakaryocytes. Their two most important functions are secretion of a large number of pharmacologically active substances and the polymerisation of an actin/myosin–based cytoskeletal network which provides platelet aggregates with the mechanism of contraction necessary in haemostasis and woundhealing. Although the morphological patterns, including focal contacts, observed in platelets spread on glass or other flat surfaces are artificial, they may be taken as a model for the

situation in a thrombus where the proteins found in focal contacts anchor actin filaments in the plasma membrane and connect platelets through integrins and fibrin bridges to neighbouring platelets. Many proteins involved in the organization of stress fibres and focal contacts in tissue culture cells have also been found in platelets (Rosenfeld et al., 1985; Nachmias and Golla, 1991; Hagmann and Burger, 1992; Hagmann, 1993), including $pp125^{FAK}$ which is phosphorylated and activated by the interaction of the integrin αIIbβ3 ligands (Shattil et al., 1994). However, in the present work we report that paxillin is absent from platelets. Whereas cell-cell contacts (adherens junctions) differ from focal contacts in some respects, no consistent differences have been reported for different cell types. The lack of paxillin in platelet focal contacts thus represents the first such difference. We also present evidence that the role of paxillin in platelets may be performed by a related protein, hic-5. Hic-5 is a 55 kDa protein that shares many structural properties with paxillin: four carboxy-terminal LIM domains that target the molecule to the focal contacts and LD motifs in the amino-terminal half (Brown et al., 1996) containing binding sites for $pp125^{FAK}$ and talin. We also demonstrate that the switch from paxillin-expression to hic-5 expression must occur late in the maturation of megakaryocytes, the precursors of platelets.

MATERIALS AND METHODS

Human platelets and tissue culture cells

Citrated blood was obtained from the blood bank (Kantonsspital, Basel). Platelets were isolated as described (Hagmann, 1993) and resuspended at 5×10^7 platelets/μl in Hepes-bufferred Tyrode's solution. For spreading experiments, 3-5 μl of the platelet suspension were dropped on top of a coverslip immersed in Hepes-bufferred Tyrode's solution containing 2 mM $CaCl_2$ and incubated at 37°C for 20-30 minutes.

Swiss 3T3 cells were maintained in DME supplemented with 10% newborn-calf serum. They were transfected using lipofectamine (Gibco BRL, Gaithersburg, MD, USA) for 5 hours according to the manufacturer's instructions.

Homogenates of MRC-5 (lung fibroblast) cells, HUT12 (chemically transformed diploid fibroblast) cells, and MDAH-172 (Li-Fraumeni syndrome; human) cells were gifts from Dr R. Chiquet, FMI, Basel. The megakaryoblastic cell line DAMI was cultured as described by Vuurst et al. (1997b). The cells were matured in the presence of 10 nM phorbol ester. Details of this procedure have been described elsewhere. $CD34^+$ cells were isolated from cord blood and megakaryocytes were cultured by incubating these cells for 10 days in the presence of thrombopoietin as described by Vuurst et al. (1997b).

Polyacrylamide gel electrophoresis and western blotting

SDS-PAGE was performed as described by Laemmli (1970). The polyacrylamide concentration was 7.5%. The proteins were blotted onto PVDF membranes (Millipore), incubated with the first antibody and a horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary IgG (Dako, Glostrup, Denmark) and detected with the ECL western-blot detection reagent from Amersham Life Science.

Microscopy and image processing

Platelets immunostained as previously described (Hagmann, 1993) or live Swiss 3T3 cells transfected with EGFP-fusion constructs were observed by either confocal microscopy or video-microscopy.

An inverted Leica microscope equipped with the TCS confocal system and an Ar/Kr laser was used to obtain images from fixed and immunostained platelets through a ×100 objective with a numerical aperture of 1.4. Stacks of images were analysed using the Imaris programme (Bitplane AG, Zürich, Switzerland) implemented on a Silicon Graphics computer (Indy).

Live cells were observed under an inverted microscope (Axiovert 35, Zeiss) equipped with the Endow EGFP filter set (Chroma Technology Corp., Brattleboro, Vt, USA) and a ×100 objective, NA 1.3. Images were recorded with a cooled slow-scanning CCD camera (MicroMAX, Princeton Instruments, Quakerbridge Road Trenton, NJ, USA) controlled by the image processing programme 'Metamorph' (Universal Imaging, West Chester, PA).

Antibodies

A polyclonal antibody against the carboxy-terminal peptide CLRPLTKGSFQERASKPY of hic-5 coupled to key hole limpet hemocyanin was raised in a rabbit as described (Shibanuma et al., 1994). The IgG fraction obtained from the serum was not further purified.

GST-hic-5 fusion proteins purified from transformed *E. coli* (JM 109 or BL 21(DE3)pLys S) bacteria were used to raise a polyclonal antibody against hic-5 in rabbits. The antiserum was diluted 1:5,000 for western blots; 25 μl of antiserum was used to precipitate 1 mg of platelet protein. IgGs specific for hic-5 were affinity-purified from the IgG fraction using a column of GST-hic-5 coupled to Sepharose CL-4B (Pharmacia, Uppsala, Sweden). The solution of 1 mg/ml was used for immunofluorescence at a dilution of 1:100.

His-tagged paxillin was expressed in BL21(DE3)pLYS S. The fusion protein which was found in inclusion bodies was separated on a preparative SDS-PAGE gel, cut out and used directly for immunising rabbits.

Monoclonal anti-paxillin antibody was obtained from Transduction Laboratories, Lexington, KY (cat. no. P13520). It was diluted 1:200 in PBS for immunofluorescence and 1:5,000 for western blots.

Screening of expression library

The human bone marrow 5′-stretch plus cDNA library, cloned in λgt11 (Clontech, Cat. No. HL5005b), was plated together with the host strain Y 1090r– to yield about 8,000-10,000 plaques per 150 mm LB-agar plate. Nitrocellulose filters, saturated with 10 mM IPTG, were placed on top of the agar to induce protein expression and to immobilise the gene product. Positive plaques were detected using a monoclonal anti-paxillin antibody, a goat anti-mouse secondary antibody coupled to alkaline phosphatase and BCIP/NBT (5-bromo-4-chloro-3-indolyl-1-phosphate/nitro blue tetrazolium, Boehringer Mannheim) as the colour substrate. Three rounds of screening were performed.

The λ phage DNA from immunopositive clones was isolated using the LambdaTrap kit (Clontech). Inserts were excised with *Eco*RI, ligated into a Bluescript vector and amplified in *E. coli* (XL-1 Blue).

DNA sequencing was carried out on a ABI 377 DNA sequencer (Perkin Elmer) using rhodamine terminators. The complete sequence of hic-5 was determined on both strands by generating overlapping sequences with sequence specific primers.

Oligonucleotides and PCR reactions

The following oligonucleotides were used for obtaining the various PCR products: Hic-5 was cloned into the *Hin*dIII site in front of EGFP using the PCR primers CCC AAG CTT CCG GCC ATG TCA CGG TTA GGG GCT CCA (amino terminus) and CCC AAG CTT GCC GAA GAG CTT CAG GAA GCA (carboxy terminus). Note that a Kozak sequence was inserted between the *Hin*dIII restriction site and the start codon. The primers for the four LIM domains of hic-5 to be inserted into the *Bsr*GI site preceding the stop codon of EGFP were: ACG AGC TGT ACA AGG GCC TCT GTG GCT CCT GCA ATA AAC C (amino terminus, including two amino acids preceding the first LIM domain cystein) and TTA CTT GTA CAT CAG CCG AAG

AGC TTC AGG AAG CAA GGC (carboxy terminus). GST-hic-5 was obtained with the help of the primers CCG GAA TTC ATG TCA CGG TTA GGG GCT CCA (amino terminus) and CCG GAA TTC TCA GCC GAA GAG CTT CAG GAA (carboxy terminus) containing *Eco*RI restriction sites. The following PCR primers were used for obtaining paxillin sequences to be inserted into the *Hin*dIII restriction site preceding EGFP: CCC AAG CTT CCG GCC ATG GAC GAC CTC GAC GCC CTG (amino terminus of whole paxillin); CCC AAG CTT GCA GAA GAG CTT GAG GAA GCA (carboxy terminus of whole paxillin); CCC AAG CTT GCA GAC TCC TTT GGC GAC TGT (carboxy terminus of the amino-terminal domain without the LIM domains); CCC AAG CTT CCG GCC ATG GGG GCC TGC AAG AAG CCC ATC (amino terminus of the four LIM domains).

PCR reactions (35 cycles) were performed on a Cyclogene Thermal Cycler (Techne, Cambridge, UK).

Construction of expression plasmids containing EGFP fusion sequences

The sequences of paxillin, hic-5 and fragments thereof were cloned in pβactin-EGFP developed in the laboratory of Dr A. Matus, Friedrich Miescher-Institute, Basle. pβactin-EGFP is identical to the vector described by Ludin et al. (1996), except that EGFP (Clontech) was used instead of GFP. Either the *Hin*dIII site in the polylinker at the 5′-end of the EGFP sequence or the *Bsr*GI site immediately preceding the 3′-terminal stop codon of EGFP were used in cloning. cDNA sequences for the construction of gene fusion were obtained by PCR for paxillin on a human placental cDNA library (Dr F. Bachmann, FMI) or on recombinant λ phage DNA containing the sequence for hic-5 (see above).

Binding of platelet extract proteins to GST-hic-5

GST-hic-5 fusion protein or GST alone was coupled to glutathione Sepharose and incubated with platelet extracts as described by Mazaki et al. (1997). Bound proteins were western blotted and probed with antibodies against $pp125^{FAK}$, vinculin (Sigma, hVin-1) or talin (Hagmann et al., 1992).

Platelet RNA and reverse transcription

RNA from human platelets was isolated as described by Willigen et al. (1995). It was free of leucocyte RNA. First strand cDNA was prepared using 'Superscript', reverse transcriptase (Gibco, Life Technologies). 2 µl of the resulting mixture were used as a template for PCR. Two sets of primers were used to obtain hic-5 sequences: CCATTCTCCTCTTCC and CAGAGCCGTCACCACT-TGCCCAGCAAT (500 base pairs from the middle) and AGTGCTACTTTGAGCGCTTCTC and GCCGAAGAGCT-TCAGGAAGCAAGG (552 base pairs from the C-terminal end). The PCR products were inserted into the pCRII-TOPO vector and cloned using the TA-cloning kit (Invitrogen, Carlsbad, CA) prior to DNA sequencing.

RESULTS

Extracts of human platelets were separated by SDS-PAGE, transferred to immobilon, and probed with a monoclonal antibody against paxillin. Fig. 1A shows that, even in lanes overloaded with platelet extract, no band was detected corresponding to a molecular mass of 68 kDa, the molecular

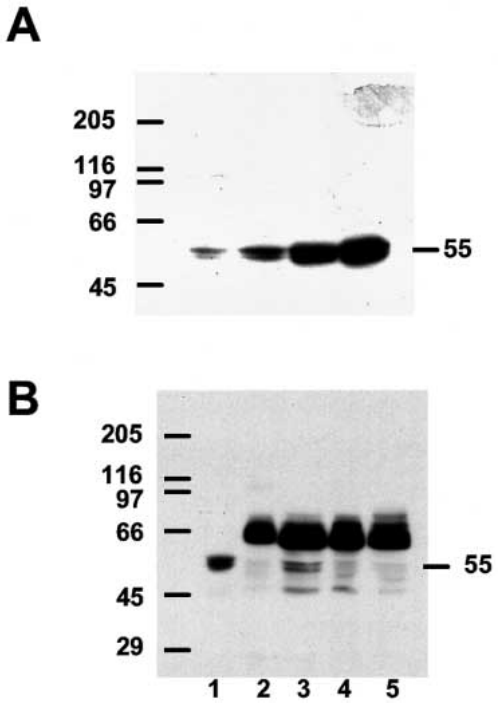

Fig. 1. Immunoblots of cell homogenates decorated with anti-paxillin antibody. (A) Increasing amounts of platelet homogenate (10-100 µg of protein). (B) Lane 1: platelets; lane 2: Swiss 3T3 cells; lane 3: MRC-5 cells; lane 4: HUT-12 cells; lane 5: MDAH-172 cells. 50 µg of homogenate protein was loaded per lane. All homogenates were prepared by dissolving the cells directly in sample buffer. Molecular mass values are given as kDa.

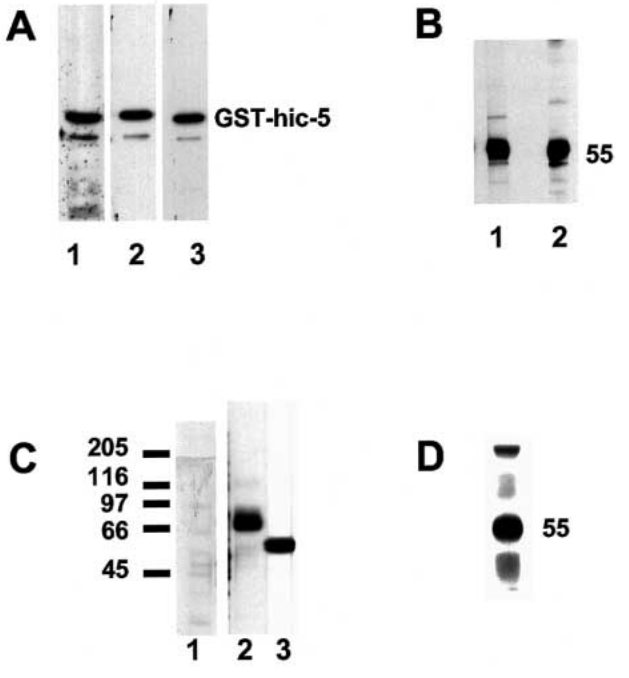

Fig. 2. Western blots of (A) extracts of bacteria expressing GST-hic-5 decorated with anti-GST-hic-5 (lane 1), an antibody raised against a carboxy-terminal peptide (lane 2), and anti-paxillin (lane 3); (B) platelet extracts decorated with anti-GST-hic-5 (lane 1) and anti-paxillin (lane 2); (C) 50 µg (protein) of Swiss 3T3 homogenate (1 and 2) or platelet homogenate (3) probed with anti-hic-5 antibody (1) or anti-paxillin (2 and 3); (D) immunoprecipitate of platelet extract with anti-GST-hic-5, decorated with anti-paxillin (the high molecular mass band is IgG). Molecular mass values are given as kDa.

mass of paxillin. Instead, a conspicuous doublet with an apparent molecular mass of 55 kDa was seen. Four arbitrarily chosen cell lines which all gave a strong signal at 68 kDa, corresponding to paxillin, contained at most trace amounts of the 55 kDa protein (Fig. 1B). The 55 kDa doublet was the only protein picked out by the monoclonal anti-paxillin antibody in western blots of platelet homogenates.

The 55 kDa protein crossreacting with anti-paxillin could have been a breakdown product of paxillin. We tested this hypothesis and excluded it by the following experiments: (a) extracts of platelets (containing the 55 kDa protein) and of fibroblasts (containing paxillin) were partially digested with V8 protease, separated on SDS-PAGE, and western-blotted with anti-paxillin. No bands common to both preparations were found (data not shown). (b) An antibody against a GST-paxillin fusion protein was raised in a rabbit. The resulting polyclonal antibody recognized the 68 kDa form of paxillin in tissue culture cells, but not the 55 kDa protein found in platelets (data not shown).

Since the 55 kDa protein was not derived from paxillin, we screened a human bone marrow cDNA expression library with the monoclonal antibody against paxillin in a search for crossreacting proteins. Ten clones were obtained, and after sequencing, five of them turned out to be paxillin itself; one of the remaining clones was almost identical with the mouse gene hic-5. It attracted our attention because hic-5 encodes a protein of 55 kDa, and because, like paxillin, it contains four C-terminal LIM domains (Shibanuma et al., 1994). The fully sequenced

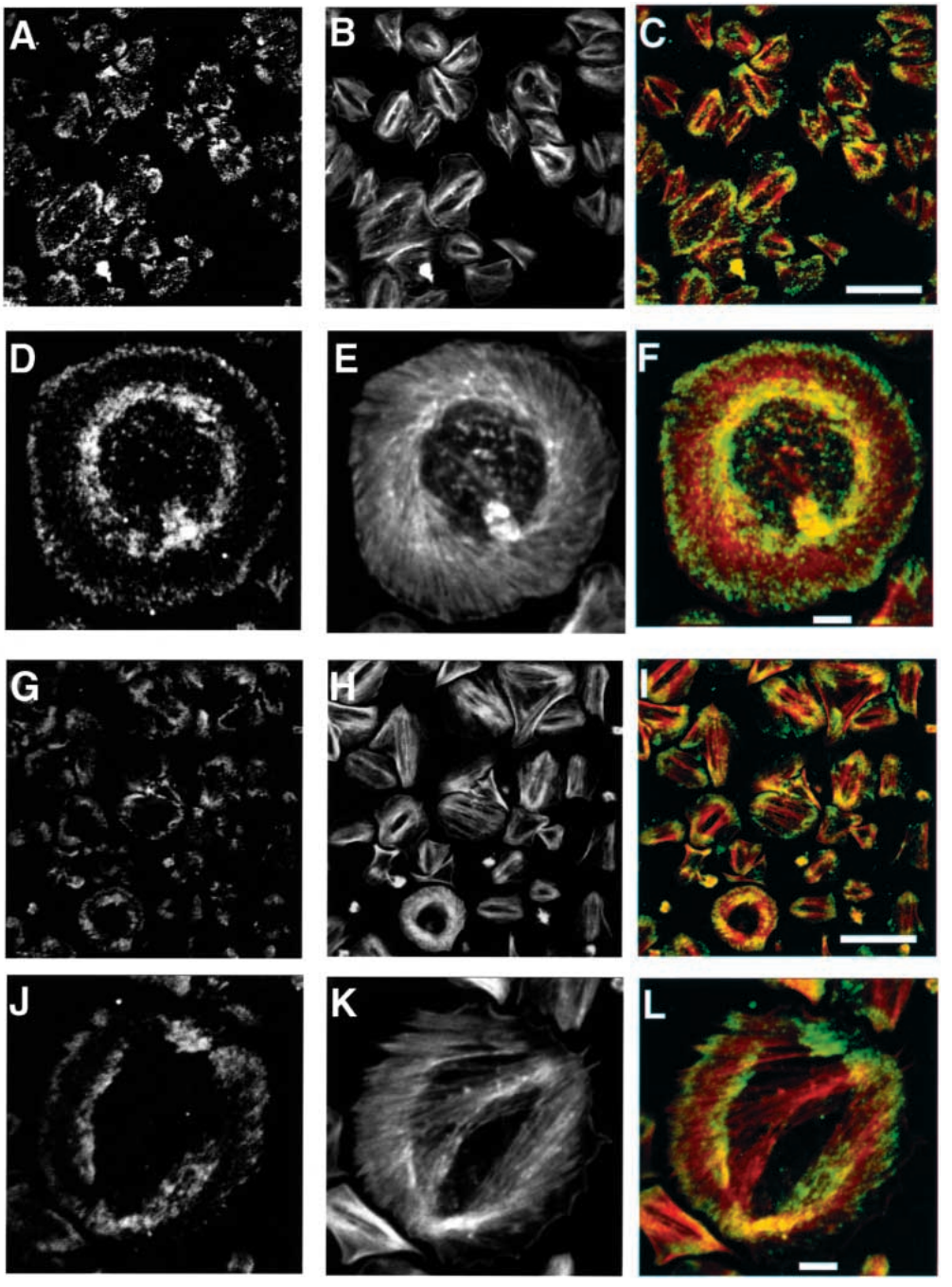

Fig. 3. Immunostaining of spread platelets. (A and D) Anti-paxillin; (G and J) anti-hic-5; (B,E,H,K) phalloidin; (C and F) anti-paxillin (green) and phalloidin (red); (I and L) anti-hic-5 (green) and phalloidin (red). (D-F and J-L) show large single platelets. Bars: 10 μm (C and I); 2.5 μm (F and L).

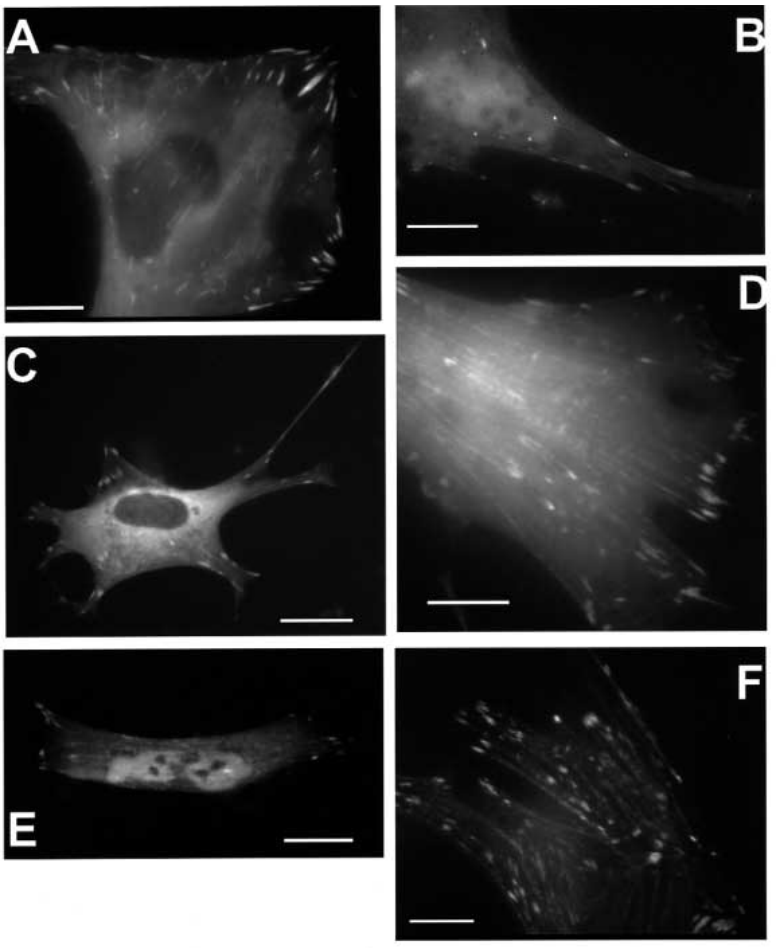

Fig. 4. Live Swiss 3T3 cells expressing paxillin-EGFP (A), paxillin$_{Lim}$-EGFP (B), hic-5-EGFP (C and D), and EGFP-hic-5$_{LIM}$ (E and F). Bars: 20 μm (C and D); 10 μm in all other panels.

human protein was different from the mouse protein in 42 out of 442 amino acids. Most differences occurred in the amino half of the sequence, whereas the carboxy half containing the LIM domains was highly conserved. Unexpectedly, the monoclonal antibody against paxillin is directed against the amino-terminal domain of paxillin, not the domain including the LIM domains common to both paxillin and hic-5. This was demonstrated by western blots of extracts of neuroblastoma cells transfected with the following constructs: EGFP alone, paxillin-EGFP, paxillin minus LIM-domains-EGFP, and LIM domains-EGFP. The blots were probed with anti-paxillin and anti-EGFP. The anti-paxillin antibody recognised constructs containing the amino-terminal part of paxillin only (not shown). Thus, there must also be (a) common structure(s) in the amino-terminal domain (see Discussion).

In order to demonstrate that the 55 kDa protein in platelets was indeed hic-5, we raised a polyclonal rabbit antibody against a GST-hic-5 fusion protein. The antibody recognises both the GST-hic-5 fusion protein expressed in bacteria and the 55 kDa protein of platelets, but not the 68 kDa paxillin in tissue culture cells (Figs 2 and 6). The same result was obtained with a polyclonal antibody raised against the peptide CLRPLTKGSFQERASKPY derived from the carboxy-terminal end of hic-5 (Fig. 2A, lane 2). This peptide had been used as an antigen by others (Shibanuma et al., 1994). As expected, anti-paxillin recognises the GST-hic-5 fusion protein (Fig. 2A, lane 3). No corresponding band was found in bacteria expressing GST only (not shown). Finally, when the 55 kDa protein was immunoprecipitated from platelet extracts with the anti-GST-hic-5 antibody, it was recognised by the anti-paxillin antibody (Fig. 2D). Preimmune serum failed to precipitate hic-5 (not shown).

Upon attachment to glass surfaces, platelets spread and develop an extensive system of stress fibers and vinculin-positive focal contacts (Rosenfeld et al., 1985; Nachmias and Golla, 1991; Hagmann and Burger, 1992; Hagmann, 1993). Focal contacts were also strongly immuno-stained with a monoclonal antibody against paxillin (Fig. 3A, and green structures in C). The antibody stained only the ends of the stress fibers, as is clearly visible in Fig. 3D-F showing a single, big platelet at higher magnification (green structures: antipaxillin; red structures: polymerized actin). Thus, hic-5, the 55 kDa protein that cross-reacts with the anti-paxillin antibody is localised in focal contacts of platelets. Identical results were

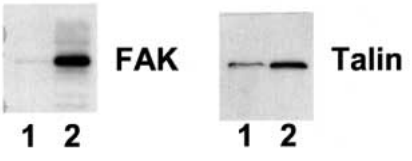

Fig. 5. Platelet extracts precipitated with GST beads (lanes 1) and GST-hic-5 beads (lanes 2) and probed with anti-pp125^{FAK} and anti-talin.

Fig. 6. Western blots of CD34$^+$ cells (stem cells) (lane 1), glycoprotein Ib-positive cells (mature megakaryocytes) (lane 2), DAMI cells (lane 3), DAMI cells stimulated with PMA (lane 4) and platelets (lane 5) decorated with anti-hic-5 antibody.

obtained with the antibody raised against GST-hic-5 and purified on a GST-hic-5 column (Fig. 3G-L).

It was originally reported that hic-5 is localized in the nucleus of tissue culture cells (Shibanuma et al., 1997). In a more recent paper, however, it was shown that, like paxillin, hic-5 is concentrated in focal contacts of cultured cells (Matsuya et al., 1998). In order to show that hic-5 contains a sequence capable of targeting proteins to focal contacts, we transfected Swiss 3T3 cells with a vector containing fusion genes consisting of paxillin or hic-5 and EGFP. Examination of live cells showed that the fusion proteins were found in focal contacts (Fig. 4A,C,D). The carboxy-terminal part containing the LIM domains was responsible for the targetting to focal contacts: a construct consisting of the four LIM domains of paxillin or hic-5 fused to EGFP brought the fluorescent protein into focal contacts and, to a lesser degree, into stress fibers (Fig. 4B,E,F). Fusion proteins containing paxillin or hic-5 sequences lacking the LIM domains were found in the cytoplasm only (data not shown). Whereas constructs containing the complete sequences were excluded from the nucleus, the LIM domains tagged with EGFP were found concentrated in the nucleus. But because EGFP alone also shows nuclear localization, short peptides attached to EGFP might be nonspecifically dragged along.

In tissue culture cells, paxillin interacts with the cytoskeletal proteins vinculin, talin, pp125^{FAK}, and CAKb (Brown et al., 1996; Mazaki et al., 1997; Matsuya et al., 1998). We examined whether or not GST-hic-5 coupled to beads was capable of precipitating three of these proteins after incubation with platelet extracts. When we probed the precipitate with antibodies against pp125^{FAK}, talin, or vinculin, we found a clear precipitate of pp125^{FAK} and a weaker, but specific reaction with talin (Fig. 5). On the other hand, two attempts using different incubation conditions and buffers revealed no specific interaction with vinculin (not shown).

Paxillin has been found in megakaryocyte-like cell lines (Hiregowdara et al., 1997). In platelets, however, as demonstrated in this work, no detectable paxillin remained. We tried to establish whether or not megakaryocytes and related cell lines also expressed hic-5. Extracts of the following cell types were probed with the polyclonal antibody against hic-5: CD34$^+$ cells (stem cells) obtained from cord blood, mature human megakaryocytes (glycoprotein Ib-positive cells in the megakaryocyte culture), DAMI cells, a megakaryocyte-like cell line, and DAMI cells induced to differentiate in the presence of phorbol ester. Whereas no hic-5 could be detected in megakaryocyte extracts, the other samples contained very small amounts only (Fig. 6), as is typical for most cell lines (Shibanuma et al., 1994). Interestingly, DAMI cells induced to differentiate in the presence of phorbol myristate acetate expressed less hic-5 than untreated cells.

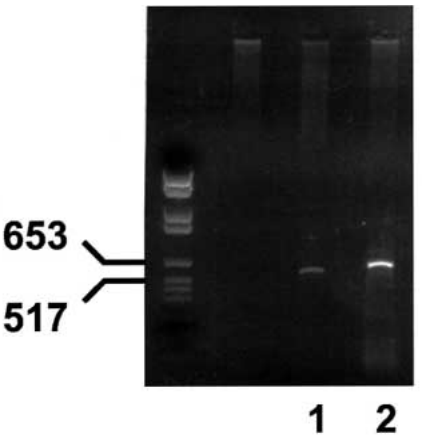

Fig. 7. PCRs of two sequences of human hic-5. Lane 1, 500 bp in the central part of hic-5; lane 2, the carboxy-terminal 552 bp. The sizes of two standards are indicated in kDa (molecular size standards # VI, Boehringer Mannheim).

Finally, we tried to establish whether or not, among the RNA carried over into platelets, sequences encoding hic-5 and paxillin could be detected. Fig. 7 shows that PCR reactions performed on cDNA obtained from platelet RNA by reverse transcription readily picked up two different sequences from the carboxy-terminal and the central part of hic-5. The sequences were cloned and their complete identity with human hic-5 was confirmed by sequence analysis (not shown). In contrast, using the same conditions, no signal was picked up when primers for two partial sequences of paxillin, which gave a positive result with a library derived from human placenta, were used (not shown).

DISCUSSION

We have presented data showing that, while paxillin is absent from human platelets, a related LIM protein, hic-5, is abundant and, like paxillin, is targeted to focal contacts. Hic-5 interacts with pp125^{FAK} and talin of platelet extracts and appears to be expressed late during the maturation of megakaryocytes.

Although speculations about possible regulatory functions of focal contact-associated proteins abound, little firm evidence is available. This also holds for paxillin which, as a substrate for the tyrosine kinase pp125^{FAK}, has been proposed as an adaptor molecule recruiting other proteins, such as Src, Crk, Csk and Lyn, to focal contacts (reviewed by Turner, 1994; Parsons, 1996; Hanks and Polte, 1997; Yamada and Geiger,

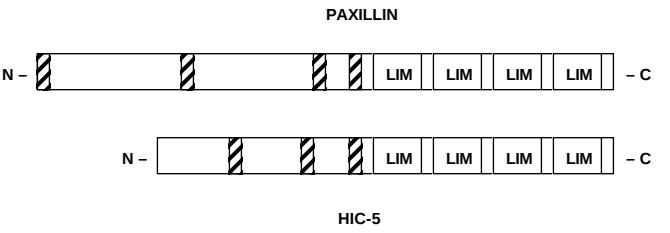

Fig. 8. Schemes of paxillin and hic-5 showing the positions of the LIM domains and of the LD motifs (hatched boxes).

1997). Binding of these proteins is mediated by their SH2 and SH3 binding motifs. In addition, paxillin interacts with vinculin and with pp125^{FAK} through its LD motifs (Brown et al., 1996). Finally, localisation of paxillin in focal contacts is ensured by the carboxy-terminal LIM domains (Brown et al., 1996, see also Fig. 5E). It is now well established that pp125^{FAK} is not needed for the assembly of focal contacts (Ilic et al., 1995; Wilson et al., 1995). But this result can not be extrapolated to its substrate paxillin, because in the cells of pp125^{FAK}-deficient mice which possess an increased number of focal contacts the level of tyrosine-phosphorylation of paxillin is not reduced when compared with control cells. Possibly (a) different kinase(s) can assume the role of pp125^{FAK}. In short, the ability of paxillin to interact with a large number of focal contact proteins is consistent with the hypothesis that it stabilises focal contacts by forming multiple crosslinks.

The assembled actin cytoskeleton of activated platelets may be regarded as a model system for stress fibers and focal contacts in other cell types (Jokusch et al., 1995). No consistent differences between the focal contacts of platelets and tissue culture cells have been reported so far. Platelet talin, unlike talin derived from chicken gizzard and porcine stomach, is not *O*-glycosylated, but the functional significance of this modification is unclear (Hagmann et al., 1992). The finding that platelets lack paxillin is therefore of considerable interest. Given the central position of paxillin in the network of interacting focal contact proteins and its wide occurrence in nucleated cells, how can platelets assemble and maintain their focal contacts without paxillin? In the present work we show that platelets contain hic-5, another member of the paxillin family. The evidence we presented may be summarised as follows: (i) a monoclonal antibody against paxillin recognises a 55 kDa protein in platelet homogenates; (ii) this 55 kDa protein is localised in focal contacts of spread platelets; (iii) screening of a bone marrow library revealed that the anti-paxillin antibody crossreacts with hic-5; (iv) two antibodies raised against hic-5 recognise the platelet 55 kDa protein, but not paxillin; (v) EGFP is targetted to focal contacts of tissue culture cellls by hic-5 and by the four LIM domains of hic-5. We therefore suggest that in human platelets the functions of paxillin are assumed by hic-5.

Hic-5 and paxillin share structural features, notably four carboxy-terminal LIM domains and LD domains in the amino-terminal part of the protein (Fig. 8). The LIM domains consist of two loops of 16-23 amino acids, each one organized by four cysteine residues (which in certain positions might be replaced by histidine, aspartic acid or glutamic acid) complexed with zinc (reviewed by Sánchez-García and Rabbitts, 1994; Gill, 1995). The domains occur in a large number of different proteins: homeodomain proteins, signalling proteins, 'LIM-only' proteins and cytoskeletal proteins (Pomiès et al., 1997; Schmeichel and Beckerle, 1997). It has been suggested that they provide binding interfaces for other proteins. The importance of the LIM domains for the function of hic-5 is underlined by the fact that the amino acid sequence of the human version is almost identical to the mouse sequence. LD motifs, on the other hand, have only recently been discovered (Brown et al., 1996). It is possible that the monoclonal antibody we used is directed against the LD domains, because it doesn't bind to the other related sequence, the LIM domains. The LD domains of the human and mouse forms of hic-5 are identical, with the exception of an asparagine residue at position 77 of the human sequence which replaces an aspartic acid in the mouse protein. The domain of paxillin containing the LD motifs binds vinculin and pp125^{FAK} (Brown et al., 1996). We found that GST-hic-5 coupled to glutathione Sepharose could precipitate talin and pp125^{FAK} from platelet extracts (Fig. 5). At present we are not sure whether or not our inability to specifically precipitate vinculin is real or due to the experimental conditions. Other investigators have observed that the binding of vinculin is weak when compared to that of pp125^{FAK} (Mazaki et al., 1997).

Shibanuma et al. (1997) reported that hic-5 is localised in the nucleus of tissue culture cells. More recently, hic-5 was also discovered in focal contacts, as was to be expected, given the structural similarities between hic-5 and paxillin. In platelets, which lack nuclei, we found hic-5 to be concentrated in focal contacts (Fig. 3; some of the platelets display the handed whorls previously described (Hagmann, 1993). Note that here the actin filaments are radiating counterclockwise because an inverted microscope was used). Because focal contacts are also home to paxillin in tissue culture cells, and because the LIM domains of paxillin (in particular the third of the four domains) are essential for targetting paxillin to focal contacts, we tried to establish whether or not this also held for hic-5. The approach we chose was to fuse the LIM domains of paxillin and of hic-5 to EGFP, transfect Swiss 3T3 cells, and study the localisation of the fusion protein. The LIM domains of both proteins did direct the fluorescent tag to the focal contacts (Fig. 4). Fusion proteins of EGFP with the amino-terminal halves of paxillin and hic-5 were localised to the cytoplasm, as was EGFP alone (not shown). The cytoskeletal target of the LIM domains remains to be established. Among the LIM-proteins that are bound to the cytoskeleton, no common targetting mechanism exists. For example the actinin-associated LIM protein (ALP) binds via an amino-terminal PDZ domain to α-actinin and the Z lines of skeletal muscle (Xia et al., 1997), whereas actin-binding LIM (abLIM) interacts directly with actin through a carboxy-terminal domain related to dematin (Roof et al., 1997).

Overexpression of hic-5 in tissue culture cells has a cytostatic effect (Shibanuma et al., 1997). In agreement with this observation, the protein is downregulated in transformed cells (Shibanuma et al., 1994). That hic-5 is prominent in platelets might therefore not come as a surprise, platelets being the anuclear endproducts of the megakaryocytic line of development. Just when the switch from paxillin- to hic-5-expression occurs is not clear yet. It is presumably a very late event, since mature megakaryoctes, like megakaryocytic cell lines, express at most only trace amounts of hic-5 (Fig. 6). Proteins associated with focal contacts might regulate adhesive strength or have some signalling function. Signalling could be mediated mechanically (Maniotis et al., 1997) or by the shuttling of messenger molecules between focal contacts and the nucleus (see Introduction). Because platelets lack nuclei, the latter two mechanisms are not required. A first possible explanation for the replacement of paxillin by hic-5 in platelets could therefore be that hic-5 is specialised in regulating adhesive strength and has lost its signalling functions. The requirements of cell division might offer a second explanation: In dividing cells the actin cytoskeleton consisting of stress

fibers and focal contacts undergoes periodical disassembly and reformation. Cell division-related disassembly does not occur in tissue culture cells overexpressing hic-5 (Shibanuma et al., 1997) and it obviously does not occur in platelets whose actin network, once formed, is maintained until they die and are cleared from the circulatory system. Hic-5 might therefore render focal contacts particularly stable. To test these hypotheses is the aim of our present work.

We thank Drs P. Caroni, S. Kaech, U. Müller and E. Oakley for their helpful discussions. This work was supported by a grant to J.Hagmann from the Swiss Cancer League (KFS 363-9-1996).

REFERENCES

Bellis, S. L., Miller, J. T. and Turner, C. E. (1995). Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. *J. Biol. Chem.* **270**, 17437-17441.

Brown, M. C., Perrotta, J. A. and Turner, C. E. (1996). Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J. Cell Biol.* **135**, 1109-1123.

Clark, E. A. and Brugge, J. S. (1995). Integrins and signal transduction pathways: the road taken. *Science* **268**, 233-239.

Craig, S. W. and Johnson, R. P. (1996). Assembly of focal adhesions: progress, paradigms, and portents. *Curr. Opin. Cell Biol.* **8**, 74-85.

Gill, G. N. (1995). The enigma of LIM domains. *Curr. Biol. Struct.* **3**, 85-1289.

Hagmann, J. and Burger, M. M. (1992). Phosphorylation of vinculin in human platelets spreading on a solid surface. *J. Cell. Biochem.* **50**, 237-244.

Hagmann, J., Grob, M. and Burger, M. M. (1992). The cytoskeletal protein talin is O-glycosylated. *J. Biol. Chem.* **267**, 14424-14428.

Hagmann, J. (1993). Pattern formation and handedness in the cytoskeleton of human platelets. *Proc. Nat. Acad. Sci. USA* **90**, 3280-3283.

Hanks, S. K. and Polte, T. R. (1997). Signaling through focal adhesion kinase. *BioEssays* **19**, 137-145.

Hiregowdara, D., Avraham, H., Fu, Y., London, R. and Avraham, S. (1997). Tyrosine phosphorylation of the related adhesion focal tyrosine kinase in megakaryocytes upon stem cell factor and phorbol myristate acetate stimulation and its association with paxillin. *J. Biol. Chem.* **272**, 10804-10810.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T. and Aizawa, S. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377**, 539-544.

Jokusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rüdiger, M., Schlüter, K., Stanke, G. and Winkler, J. (1995). The molecular architecture of focal adhesions. *Annu. Rev. Cell Dev. Biol.* **11**, 379-416.

Laemmli, U. K. (1970). Cleavage of strutural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680-685.

Ludin, B., Doll, T., Meili, R., Kaech, S. and Matus, A. (1996). Application of novel vectors for GFP-tagging of proteins to study microtubule-associated proteins. *Gene* **173**, 107-111.

Maniotis, A. J., Chen, C. S. and Ingber, D. E. (1997). Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc. Nat. Acad. Sci. USA* **94**, 849-854.

Matsuya, M., Sasaki, H., Aoto, H., Mitaka, T., Nagura, K., Ohba, T., Ishino, M., Takahashi, S., Suzuki, R. and Sasaki, T. (1998). Cell adhesion kinase beta forms a complex with a new member, hic-5, of proteins localized at focal adhesions. *J. Biol. Chem.* **273**, 1003-1014.

Mazaki, Y., Hashimoto, S. and Sabe, H. (1997). Monocyte cells and cancer cells express novel paxillin isoforms with different binding properties to focal adhesion proteins. *J. Biol. Chem.* **272**, 7437-7444.

Nachmias, V. T. and Golla, R. (1991). Vinculin in relation to stress fibers in spread platelets. *Cell Motil. Cytoskel.* **20**, 190-202.

Otey, C. A. (1996). pp125^{FAK} in the focal adhesion. *Int. Rev. Cytol.* **167**, 161-183.

Parsons, J. T. (1996). Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins. *Curr. Opin. Cell Biol.* **8**, 146-152.

Pomiès, P., Louis, H. A. and Beckerle, M. C. (1997). CRP1, a LIM domain protein implicated in muscle differentiation, interacts with α-actinin. *J. Cell Biol.* **139**, 157-168.

Roof, D. J., Hayes, A., Adamian, M., Chishti, A. H. and Li, T. (1997). Molecular characterization of abLIM, a novel actin-binding and double zinc finger protein. *J. Cell Biol.* **138**, 575-588.

Rosenfeld, G. C., Hou, D. C., Dingus, J., Meza, I. and Bryan, J. (1985). Isolation and partial characterization of human platelet vinculin. *J. Cell Biol.* **100**, 669-676.

Sánchez-García, I. and Rabbitts, T. H. (1994). The LIM domain: a new structural motif found in zinc-finger-like proteins. *Trends Genet.* **9**, 315-320.

Schaller, M. D. and Parsons, J. T. (1995). pp125^{FAK}-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol. Cell. Biol.* **15**, 2635-2645.

Schmeichel, K. L. and Beckerle, M. C. (1997). Molecular dissection of a LIM domain. *Mol. Biol. Cell* **8**, 219-230.

Shattil, S. J., Haimovich, B., Cunningham, M., Lipfert, L., Parsons, J. T., Ginsberg, M. H. and Brugge, J. S. (1994). Tyrosine phosphorylation of pp125^{FAK} in platelets requires coordinated signaling through integrin and agonist receptors. *J. Biol. Chem.* **269**, 14738-14745.

Shibanuma, M., Mashimo, J., Kuroki, T. and Nose, K. (1994). Characterization of the TGFß1-inducible hic-5 gene that encodes a putative novel zinc finger protein and its possible involvement in cellular senescence. *J. Biol. Chem.* **269**, 26767-26774.

Shibanuma, M., Mochizuki, E., Maniwa, R., Mashimo, J.-I., Nishiya, N., Imai, S.-I., Takano, T., Oshimura, M. and Nose, K. (1997). Induction of senescence-like phenotypes by forced expression of hic-5, which encodes a novel LIM motif protein, in immortalized human fibroblasts. *Mol. Cell. Biol.* **17**, 1224-1235.

Turner, C. E., Glenney, J. R. and Burridge, K. (1990). Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol.* **111**, 1059-1068.

Turner, C. E. (1994). Paxillin: a cytoskeletal target for tyrosine kinases. *BioEssays* **16**, 47-52.

Turner, C. E. and Miller, J. T. (1994). Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and pp125^{FAK}-binding region. *J. Cell Sci.* **107**, 1583-1591.

Vuurst, H. v. d., Heemskerk, J. W. M., Hendriks, M., Willigen, G. v. and Akkerman, J. W. N. (1997a). G16-a mediates thromboxane induced Ca^{2+} signalling in maturing megakaryocytes. *Thromb. Haemost. supplement,* **176**.

Vuurst, H. v. d., Willigen, G. v., Spronsen, A. v., Hendriks, M., Donath, J. and Akkerman, J. W. N. (1997b). Signal transduction through trimeric G proteins in megakaryoblastic cell lines. *Arterioscler. Thromb. Vasc. Biol.* **17**, 1830-1836.

Willigen, G. v., Donath, J., Lapetina, E. G. and Akkerman, J. W. N. (1995). Identification of a-subunits of trimeric GTP-binding proteins in human platelets by RT-PCR. *Biochem. Biophys. Res. Commun.* **214**, 254-262.

Wilson, L., Carrier, M. J. and Kelly, S. (1995). pp125^{FAK} tyrosine kinase activity is not required for the assembly of F-actin stress fibers and focal adhesions in cultured mouse aortic smooth muscle cells. *J. Cell Sci.* **108**, 2381-2391.

Wood, C. K., Turner, C. E., Jackson, P. and Critchley, D. R. (1994). Characterisation of the paxillin-binding site and the C-terminal focal adhesion targeting sequence in vinculin. *J. Cell Sci.* **107**, 709-717.

Xia, H., Winokur, S. T., Kuo, W.-L., Altherr, M. R. and Bredt, D. S. (1997). Actinin-associated LIM protein: identification of a domain interaction between PDZ and spectrin-like repeat motifs. *J. Cell Biol.* **139**, 507-515.

Yamada, K. M. and Geiger, B. (1997). Molecular interactions in cell adhesion complexes. *Curr. Opin. Cell Biol.* **9**, 76-85.