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Characterization of a focal adhesion protein, Hic-5, that shares extensive homology with paxillin

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SUMMARY

Paxillin is a focal adhesion scaffolding protein which was originally identified as a substrate of the oncogenic tyrosine kinase, v-src. Paxillin has been proposed to be involved in regulation of focal adhesion dynamics. Two alternatively spliced mouse paxillin cDNAs were cloned and in the process, a paxillin-related protein, Hic-5, was also identified. Cloning and characterization of Hic-5 indicates that this protein shares extensive homology with paxillin. Although Hic-5 was originally characterized as a TGF-β-inducible gene and proposed to be a transcription factor involved in senescence, the studies here demonstrate that Hic-5 is localized to focal adhesion in REF52 cells and can interact with the focal adhesion proteins, Fak, Frnk, and vinculin. In addition, like paxillin, Hic-5 can bind to a negative regulator of Src PTKs, csk but does not bind to the adaptor protein Crk. Like paxillin, localization of this protein to focal adhesions is mediated primarily by the LIM domains; however, sequences outside the LIM domains also play a minor role in focal adhesion targeting. These results suggest that Hic-5 like paxillin could be involved in regulation of focal adhesion dynamics and raise the possibility that Hic-5 and paxillin could have overlapping or opposing functions in the overall regulation of cell growth and differentiation.

Key words: Paxillin, Integrin, Focal adhesion, LIM domain, Fak, Vinculin

INTRODUCTION

Adhesion of cells to the extracellular matrix is mediated by a class of heterodimeric receptors called integrins. Engagement of these receptors initiates a cascade of signaling events including changes in protein phosphorylation, alterations in the cytoskeletal architecture and changes in gene expression. These events allow integrins to regulate cell survival, migration, growth, and differentiation (Clark and Brugge, 1995; Schwartz et al., 1995). Although a variety of cytoplasmic molecules that mediate integrin signal transduction have been identified a subgroup of these proteins are found in the specialized structures which mediate a cell's attachment to the extracellular matrix, focal adhesions. Focal adhesion proteins have been divided into two groups, structural proteins and regulatory proteins. The structural proteins are fairly abundant and include proteins such as tensin, vinculin and α-actinin. The regulatory proteins are less abundant and have been proposed to be involved in regulating focal adhesion dynamics. Examples of regulatory proteins include focal adhesion kinase (FAK), p130CAS, and paxillin. Many of these proteins serve as scaffolding molecules and thus serve as signaling nucleation centers by providing binding sites for different proteins (Jockusch et al., 1995).

Paxillin is one such scaffolding protein which provides binding sites for cytoplasmic tyrosine kinases, adaptor proteins, and structural proteins. Paxillin is a 68 kDa phosphoprotein which was originally identified as a tyrosine phosphorylated protein in chick embryo fibroblasts infected with the retrovirus, Rous Sarcoma Virus. Paxillin has leucine rich motifs (LD repeats) in the N-terminal half, tyrosine phosphorylation sites, proline rich sequences, and four LIM domains in the C-terminal half of the molecule (Turner, 1994).

There are five LD repeats in the N-terminal-half of paxillin and three of these LD repeats are likely to be important for paxillin's interaction with the focal adhesion tyrosine kinase, Fak and the structural focal adhesion protein, vinculin (Brown et al., 1996). The LD repeats have also been shown to be important for paxillin's interaction with the papillomavirus E6 protein (Tong and Howley, 1997; Tong et al., 1997; Vande Pol et al., 1998).

Paxillin has both tyrosine and serine phosphorylation sites (Bellis et al., 1995, 1997; De Nichilo and Yamada, 1996; Schaller and Parsons, 1995). At least three tyrosine phosphorylation sites are present and these include Y31, Y40, and Y118 (Bellis et al., 1995; Schaller and Parsons, 1995). The tyrosine phosphorylation sites are important for mediating paxillin's interactions with the SH2 domains of the cytoplasmic

tyrosine kinases Src and Csk and the adaptor protein Crk (Birge et al., 1993; Sabe et al., 1994; Schaller and Parsons 1995). The proline rich stretches also are involved in mediating binding of Src and Crk via the SH3 domains of these molecules (Birge et al., 1993; Weng et al., 1993) While the precise roles of these interactions are unclear, the ability of these proteins to communicate with other signaling pathways suggests that they may link paxillin to different signal transduction cascades. For example, Crk is able to interact with a Ras activator, SOS, which exchanges GDP for GTP (Okada and Pessin, 1996). Thus, binding of paxillin to Crk and recruitment of SOS to focal adhesions could regulate Ras activation.

LIM domains are cysteine rich regions composed of two zinc fingers. These domains were originally identified in homeodomain proteins, but have been subsequently found in both cytoplasmic and nuclear proteins. LIM domains mediate protein-protein interactions. This includes binding to other LIM domains as well as different protein motifs (Dawid et al., 1995; Schmeichel and Beckerle, 1994). Two of the LIM domains of paxillin are important for mediating paxillin localization to the focal adhesions (LIM 2 and 3), but the protein(s) that these LIM domains interact with have not been identified (Brown et al., 1996). Thus, paxillin has multiple binding domains which target paxillin to its proper subcellular localization and provide docking sites to link this focal adhesion protein to a variety of signaling pathways.

As indicated above, paxillin is tyrosine phosphorylated in v-src transformed cells (Glenney and Zokas, 1989). Subsequent studies have shown that this protein is also tyrosine phosphorylated in BCR-Abl transformed cells and becomes tyrosine phosphorylated in response to a variety of extracellular stimuli including engagement of integrins, G-protein-coupled receptors, and receptor tyrosine kinases (Salgia et al., 1995a; Turner, 1994). Paxillin is also subject to serine/threonine phosphorylation in response to engagement of certain integrin receptors (Bellis et al., 1997; De Nichilo and Yamada, 1996) While this protein functions in many different signaling pathways, the precise role of paxillin in focal adhesion dynamics or other signaling events is uncertain. One potential approach to understanding paxillin function is the use of embryonic stem cell technology and homologous recombination to generate paxillin deficient cells. For this purpose, a mouse paxillin cDNA was isolated. In the process of isolating this gene, a paxillin-related gene was also identified which was previously called *hic-5* (Shibanuma et al., 1994).

Hic-5 was originally identified as a TGF-β-inducible gene in the mouse osteoblastic cell line, MC3T3 (Shibanuma et al., 1994). Hic-5 has been proposed to be a transcription factor based on the

A

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  1 MDDLDALLADLESTTSHISKRPVFLSEEPPYSYPTGNHTYQEIAVPPPV  49
 50 PPPPSSEALNGTVLDPLDQWQPSGSRYAHQQPPSPLPVYSSSAKNSSAS  98
 99 NTQDGVGSLCSRAGEEEHVYSFPNKQKSAEPSPTVMSSSLGSNLSELDR 147
148 LLLELNAVQHSPPGFPADEAESSPPLPGALSPLYGIPENNTPLGGKAGP 196
197 LVKEKPKRNGGRGLEDVRPSVESLLDELESSVPSPVPAITVNQGEMSSP 245
246 QRVTSSQQQTRISASSATRELDELMASLSDFK*FMAQGKTGSSSPPGGL 293
294 SKPGSQLDSMLGSLQSDLNKLGVATVAKGVCGACKKPIAGQVVTAMGKT 342
343 WHPEHFVCTHCQEEIGSRNFFERDGQPYCEKDYHSLFSPRCYYCNGPIL 391
392 DKVVTALDRTWHPEHFFCAQCGAFFGPEGFHEKDGKAYCRKDYFDMFAP 440
441 KCGGCARAILENYISALNTLWHPECFVCRECFTPFVNGSFFEHDGHPYC 489
490 EVHYHERRGSLCSGCQKPITGRCITAMAKKFHPEHFVCAFCLKQLNKGT 538
539 FKEQNDKPYCQSCFVKLFC                               557
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***Beta insert:** MQGLEQRVDGERPWAASWPPSSRQSSPEGQDEGG

B

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      1                                     *          *      ________               60
Mouse MDDLDALLAD LESTTSHISK RPVFLSEEPP YSYPTGNHTY QEIAVPPPVP PPPSSEALNG
Human MDDLDALLAD LESTTSHISK RPVFLSEEtP YSYPTGNHTY QEIAVPPPVP PPPSSEALNG
Chick MDDLDALLAD LESTTSHISK RPVFLtEEtP YSYPTGNHTY QEIAVPPPVP PPPSSEALNG

     61                       _____                                    *           120
      TVLDPLDQWQ PSGSRYAHQQ PPSPLPVYSS SAKNSSASNT QDGVGSLCSR AGEEEHVYSF
      TiLDPLDQWQ PSGSRfiHQQ PqSssPVYgS SAKtSSASNp QDsVGSpCSR vGEEEHVYSF
      TViDPLDQWQ PSvSRYgHQQ PqSqsPiYSS SAKsSSASvp rDGlsSpsSR AsEEEHVYSF

    121                                                    __________              179
      PNKQKSAEPS PTVMSSSLGS NLSELDRLLL ELNAVQHSPP -GFPADEAES SPPLPGALSP
      PNKQKSAEPS PTVMStSLGS NLSELDRLLL ELNAVQHnPP -GFPADEAnS SPPLPGALSP
      PNKQKSAEPS PTmtStSLGS NLSELDRLLL ELNAVQHnPP sGFsADEvsr SPsLPnvtgP

    180                                                                            239
      LYGIPENNTP LGGKAGPLVK EKPKRNGGRG LEDVRPSVES LLDELESSVP SPVPAITVNQ
      LYGvPEtNsP LGGKAGPLtK EKPKRNGGRG LEDVRPSVES LLDELESSVP SPVPAITVNQ
      hYvIPEssSs aGGKAaPptK EKPKRNGGRG iEDVRPSVES LLDELESSVP SPVPAITVsQ

    240                                                   _________                298
      GEMSSPQRVT SSQQQTRISA SSATRELDEL MASLSDFKFM AQGKTG-SSS PPGGLSKPGS
      GEMSSPQRVT STQQQTRISA SSATRELDEL MASLSDFKFM AQGKTG-SSS PPGGPPKPGS
      GEVSSPQRVN ASQQQTRISA SSATRELDEL MASLSDFKFM AQGKAGgSSS PPSTTPKPGS

    299                                                                            358
      QLDSMLGSLQ SDLNKLGVAT VAKGVCGACK KPIAGQVVTA MGKTWHPEHF VCTHCQEEIG
      QLDSMLGSLQ SDLNKLGVAT VAKGVCGACK KPIAGQVVTA MGKTWHPEHF VCTHCQEEIG
      QLDTMLGSLQ SDLNKLGVAT VAKGVCGACK KPIAGQVVTA MGKTWHPEHF VCTHCQEEIG

    359                                                                            418
      SRNFFERDGQ PYCEKDYHSL FSPRCYYCNG PILDKVVTAL DRTWHPEHFF CAQCGAFFGP
      SRNFFERDGQ PYCEKDYHNL FSPRCYYCNG PILDKVVTAL DRTWHPEHFF CAQCGAFFGP
      SRNFFERDGQ PYCEKDYHNL FSPRCYYCNG PILDKVVTAL DRTWHPEHFF CAQCGVFFGP

    419                                                                            478
      EGFHEKDGKA YCRKDYFDMF APKCGGCARA ILENYISALN TLWHPECFVC RECFTPFVNG
      EGFHEKDGKA YCRKDYFDMF APKCGGCARA ILENYISALN TLWHPECFVC RECFTPFVNG
      EGFHEKDGKA YCRKDYFDMF APKCGGCARA ILENYISALN TLWHPECFVC RECFTPFING

    479                                                                            538
      SFFEHDGHPY CEVHYHERRG SLCSGCQKPI TGRCITAMAK KFHPEHFVCA FCLKQLNKGT
      SFFEHDGQPY CEVHYHERRG SLCSGCQKPI TGRCITAMAK KFHPEHFVCA FCLKQLNKGT
      SFFEHDGQPY CEVHYHERRG SLCSGCQKPI TGRCITAMGK KFHPEHFVCA FCLKQLNKGT

    539                   557
      FKEQNDKPYC QSCFVKLFC
      FKEQNDKPYC QNCFLKLFC
      FKEQNDKPYC QNCFLKLFC
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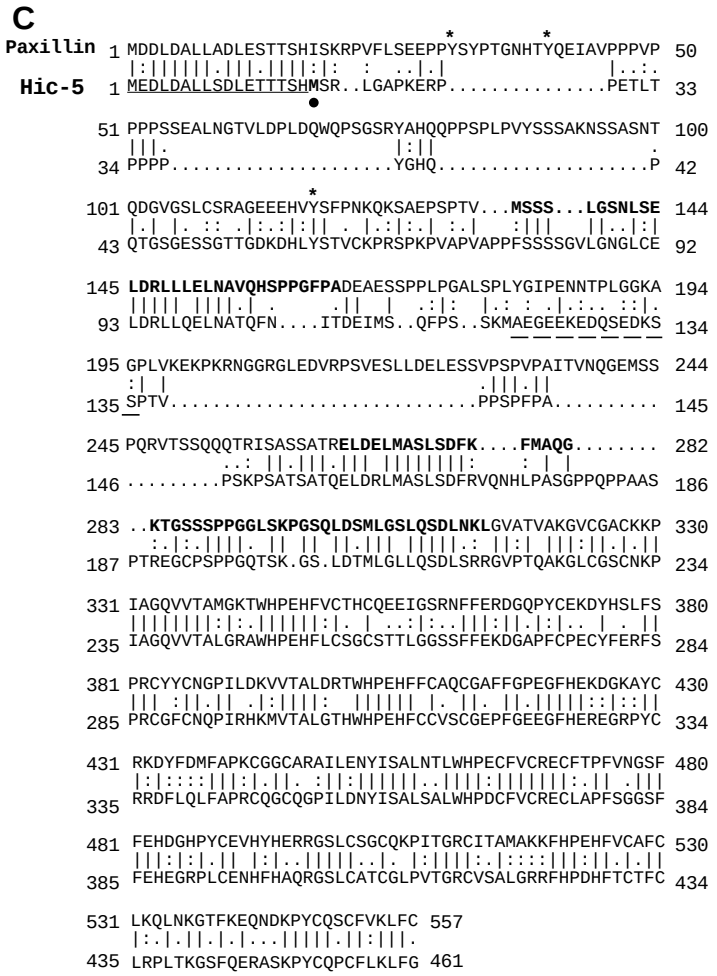
Fig. 1. Sequence of mouse paxillin isoforms and mouse Hic-5. (A) The amino acid sequence of the alpha paxillin isoform is shown. The * denotes the position of the 34 aa insert present in the beta isoform and the sequence of the insert is listed below the alpha sequence. (B) An alignment of the mouse (upper), human (middle) and chicken (lower) paxillin proteins is shown. Dashes represent a gap in the sequence. Small bold letters indicate differences while capital letters indicate identity. Mapped tyrosine phosphorylation sites are indicated by an asterisk. Potential SH3 binding motifs are indicated by a solid line. C. Alignment of the mouse Hic-5 (lower) and paxillin (upper) proteins. '|' represents amino acid identity and ':' or '.' represents amino acid similarity. '●' denotes the initiator methionine of the original Hic-5 clone. '*' represent tyrosine phosphorylation sites identified in paxillin. Sequences in bold are the amino acids in paxillin that have been shown to bind Fak and vinculin. The dashed line is the peptide sequence of Hic-5 used to make the Hic-5 specific antibody. (D) A diagram showing the overall domain structures of paxillin and Hic-5. The tyrosines denoted are known sites of phosphorylation in paxillin.



presence of the LIM domains and immunolocalization studies (Shibanuma et al., 1997). Additionally, Hic-5 induces senescence when overexpressed in human diploid fibroblasts, and hic-5 expression is lost in *k-ras*-transformed cells and in several human tumor cell lines. These results have led to the proposal that this protein may be involved in growth arrest or cell senescence (Shibanuma et al., 1994). The studies presented here demonstrate that Hic-5 is closely related to the focal adhesion protein, paxillin and that Hic-5, like paxillin, is localized to focal adhesions and interacts with Fak, FRNK, and vinculin. Hic-5 does not bind to the adaptor protein Crk but does bind to the negative regulator of Src PTKs, Csk. Taken together, these results raise the possibility that paxillin and Hic-5 may modulate each others activities by competing for focal adhesion binding sites.

MATERIALS AND METHODS

cDNAs

For cloning of the mouse paxillin cDNAs, a λ YES (Stratagene) library constructed with ES cell cDNAs (kindly provided by Dr Zhi Chen, University of Michigan) ws screened at low stringency with a full length chicken paxillin cDNA by standard methods (Sambrook et al., 1989). Two partial paxillin cDNAs were obtained and these were used to re-screen the library at higher stringency. Positives were sequenced with forward and reverse primers using an ABI automated sequencer. For cloning of the mouse hic-5 cDNA, a hic-5 cDNA was isolated from a kidney RT PCR reaction (kindly provided by Dr Brian Zambrowicz, Lexigen) (Zambrowicz et al., 1997) using primers that were generated to the 5′ untranslated region of hic-5 (acatcccctccttcccaaactc) and the 3′ region of hic-5. An internal primer was used to confirm the identity of the clone. This cDNA was then used to probe an E12.5 cDNA λ YES cDNA library (kindly provided by Dr Zhi Chen, University of Michigan) using standard methods (Sambrook et al., 1989). Resulting clones were fully sequenced with both forward and reverse primers on an ABI automated sequencer.

GST fusion proteins

The paxillin, crk, and csk fusion proteins are described previously (Birge et al., 1992; Songyang et al., 1994; Turner and Miller 1994). For generation of GST-Hic-5, a *Nco*I-*Stu*I fragment (encodes aa 1-226), was cloned into the *Stu*I site of pGEX2TK. Junctions were confirmed by sequencing. Fusion protein was made as previously described (Weng et al., 1993).

GFP fusion proteins

The green fluorescent protein (GFP) contains a modified GFP protein kindly provided by Dr Akira Imamoto (University of Chicago) (Chalfie et al., 1994). The fusions of paxillin and HIC-5 with GFP were made by PCR amplification of the mouse paxillin or HIC-5 cDNAs. All primers contained an *Xho*I linker for cloning into the GFP vector. All of the GFP proteins are N-terminal fusions. For generation of the NH2 Hic-5 GFP fusion the forward primer used was ‘cccgctcgagcggaccgccatggaggacctggat’ and the reverse primer was ‘ccgctcgagcggggctccacagacccctttggc’. The NH2 paxillin construct was made using the 5′ primer, ‘ccgctcgagcggggaccagccatggacgacctc’ and the 3′ primer, ‘ccgctcgagcggggctccacagacccctttggc’. For generation of the Hic-5 LIM GFP fusion, the forward primer used was ‘ccgctcgagcggctcagccgtcgtggtgtcccc’ and the reverse primer used was ‘ccgctcgagcggtcagccgaagagcttcaggaa’. The paxillin-LIM GFP was made using the forward primer, ccgctcgagcggctgaacaagctaggggtcgcc’ and the reverse primer, ‘ccgctcgagcgggcaggtggtagggacaagaga’. All products were cloned into the pbluescript vector (Stratagene) and sequenced before cloning them into the GFP vector.

Binding assays

Binding assays were done as previously described (Brown et al., 1996). For binding assays with paxillin or Hic-5 fusion proteins, chicken gizzard lysates were prepared by lysing the tissue in lysis buffer (1% NP40, 100 mM NaCl, 1 mM EDTA, 1 mM vanadate, 1% Trasylol and 100 μM leupeptin) and then spun at 100000 ***g*** for 20

minutes at 4°C. Cell lysates were incubated at 4°C with 5 μg of each GST fusion protein coupled to glutathione beads. Beads were washed 5 times with lysis buffer and bound proteins eluted by boiling in sample buffer. A similar procedure was used for binding assays with Crk and csk fusion proteins except that lysates were prepared from REF52 cells expressing $pp60^{v\text{-}src}$. Eluted proteins were separated on an 8% gel using SDS-PAGE.

Western blots

Immunoblot analysis was performed as described previously (Weng et al., 1993). Briefly, following SDS-PAGE analysis, proteins were immobilized on nitrocellulose filters. Filters were blocked in 5% milk in TBST (10 mM Tris, pH 7.4, 100 mM NaCl, 1% Tween-20). They were then incubated with paxillin 165 (Turner et al., 1990), Hic-5, Fak (BC3, kindly provided by Tom Parsons, University of Virginia), vinculin antibodies (Vin 11-5, Sigma), or myc (9E10) antibody (Evans et al., 1985). Following four washes in TBST, immunoblots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000; Jackson Immunochemicals). Filters were then washed four times in TBST and developed by chemiluminescence (NEN Dupont).

Immunoprecipitations

REF52 cells were transfected with vector or myc-tagged Hic-5 using Lipofectamine according to the manufacturer's recommendations (Gibco-BRL). 293T cells were transfected by standard calcium phosphate precipation (Chen and Okayama, 1987). 48 hours after transfection, lysates were prepared as described above. Cell lysates were normalized for protein levels and then incubated with Protein A purified Fak antibody (kindly provided by Tom Parsons, University of Virginia) control IgG (Rabbit anti-mouse, Jackson Immunochemicals), or a Hic5 specific antibody followed by Protein A Sepharose. Beads were washed 5 times with lysis buffer and bound protein eluted by boiling in sample buffer. Eluted proteins were separated on an 8% gel using SDS-PAGE.

Immunofluorescence

Immunofluorescence was done as described previously (Thomas et al., 1995). Briefly, cells were fixed in freshly prepared 4% paraformaldehyde for 10 minutes, permeablized for 2 minutes with 2% Triton rinsed 3 times 5 minutes in PBS and then blocked for 10 minutes with 2% BSA in PBS. Coverslips were incubated with Hic-5 antibody (1:50), vinculin antibody (1:300), or paxillin antibody (Transduction Laboratories) for 45 minutes, washed 3 times 10 minutes in PBS and then incubated with fluorescently tagged secondary antibodies at a 1:400 dilution (Jackson Immunochemicals). In some cases, cells were also co-stained with rhodamine-conjugated phalloidin (Molecular Probes). Cells were washed 3 times 10 minutes in PBS and then mounted onto glass slides using Mowiol. For experiments with the GFP fusions, REF52 cells were transiently transfected using LIPOfectmine according to the recommended protocol (Gibco-BRL) or Fugene (Boehringer Mannheim Biochemicals). 12-16 hours following transfection, cells were plated on fibronectin-coated coverslips overnight. Cells were then fixed and mounted or in some cases, stained with vinculin antibody before mounting. Cells were visualized on a Nikon immunofluorescence microscope and images processed using a Photometrics digital camera and phase3 imaging software (Phase3 Imaging).

RESULTS

Isolation and characterization of the paxillin and hic-5 cDNAs

A chicken paxillin probe was used to screen a mouse embryonic stem (ES) cell cDNA library (Turner and Miller, 1994). A partial cDNA was obtained and used to reprobe the ES cell library. Two full length clones were isolated and several partial clones encompassing both the 5′ and 3′ ends of paxillin. One of the full length clones is the mouse homolog of the human alpha isoform of paxillin and the second clone is the beta isoform which was originally identified in HeLa cells (Mazaki et al., 1997; Salgia et al., 1995b) (Fig. 1A). A gamma isoform may also exist but has not been identified yet in the mouse. An alignment of the mouse, chicken, and human alpha paxillin proteins is depicted in Fig. 1B. Paxillin appears to be conserved throughout evolution as a blast search revealed a potential homologue in *Caenorhabditis elegans* and *Drosophila melanogaster*. The murine paxillin protein has a 91% and 96% amino acid identity, respectively, with chicken and human paxillin. This identity includes the LD motifs, LIM domains and the three known tyrosine phosphorylation sites. Not all of the proline rich regions, which can mediate interactions with SH3 containing proteins, are completely conserved between species (e.g. 81-86).

A blast search using the mouse paxillin protein sequence revealed a highly related protein, Hic-5. The homology between these proteins was not restricted to the carboxyl LIM domains, but included sequences in the amino terminal half (Fig. 1C). To begin to understand the functional relationship between paxillin and hic-5, a hic-5 cDNA was isolated from a mouse embryonic day 12.5 (E12.5) cDNA library. One full length clone and three partial overlapping clones were obtained and sequenced. The full length hic-5 clone and paxillin clone were used to map the mouse chromosomal locations using the 94 N2 DNAs from the Jackson Laboratory BSS cross, (C57BL/6jEi × SPRET/Ei)F1 × SPRET/Ei backcross panel (Rowe et al., 1994). The allele type data was compared to over 2000 other loci previously mapped in the cross and linkage was found to markers on the distal end of Chromosome 7 for Hic-5. Similar studies were also done to map the paxillin locus which resides on Chromosome 5 at offset 71.7. There are no obvious mouse mutants which map to these regions. The Hic-5 locus has been given the name pxnr (paxillin-related) and the paxillin locus is designated pxn. The data are available from the Jackson Laboratory Backcross DNA Panel Mapping Resource web site (http://www.jax.org/resources/documents/cmdata).

The sequence of the full length hic-5 cDNA was identical to the original hic-5 clone except at the 5′ end. In particular an additional 17 amino acids was encoded by the cDNA isolated from the E12.5 cDNA library. This difference could be due to alternative splicing since we were able to isolate the cDNA cloned by Shibanuma et al. (1994) by RT PCR using a primer that is unique to this clone.

The sequences surrounding the potential new initiator methionine provide a strong consensus Kozak sequence suggesting that this is, in fact, a true translational start site. In addition, in vitro transcription/translation of the cDNA produced a 51 kDa protein, the predicted molecular mass of Hic-5 (data not shown). These 17 amino acids contain the first LD repeat and extends the homology between paxillin and Hic-5 (Fig. 1C). Interestingly, this LD repeat has been shown to be critical for mediating paxillin's interaction with the bovine papillomavirus protein E6 (Tong et al., 1997; Vande Pol et al., 1998).

Overall, there is 57% amino acid identity and 72% similarity

between Hic-5 and paxillin. As indicated above the homology between these proteins is not restricted to the carboxyl LIM domains, but also includes sequences in the amino-terminal half. In particular, four of the five LD repeats as well as sequences surrounding these motifs are conserved between paxillin and Hic-5. Two of these repeats lie within sequences that are required for paxillin's interaction with the focal adhesion proteins, Fak and vinculin (Brown et al., 1996). Paxillin also has SH2 and SH3 binding motifs in the amino-terminal half (phosphotyrosine and proline rich sequences, respectively) (Schlessinger, 1994). These sites mediate interactions with signaling molecules such as Crk and Src (Birge et al., 1993; Schaller and Parsons, 1995; Turner, 1994; Weng et al., 1993). Only one of the mapped tyrosine phosphorylation sites in paxillin is conserved in Hic-5 (Y118) but the overall SH2 binding motif is not conserved. Thus while Hic-5 contains proline-rich sequences and potential tyrosine phosphorylation sites, it is unlikely that Hic-5 will interact with Src or Crk since residues which are critical for interaction with the SH2 or SH3 domains of these signaling proteins are not conserved between paxillin and Hic-5 (Rickles et al., 1994; Songyang et al., 1993). Thus, paxillin and Hic-5 may have both overlapping and unique binding partners.

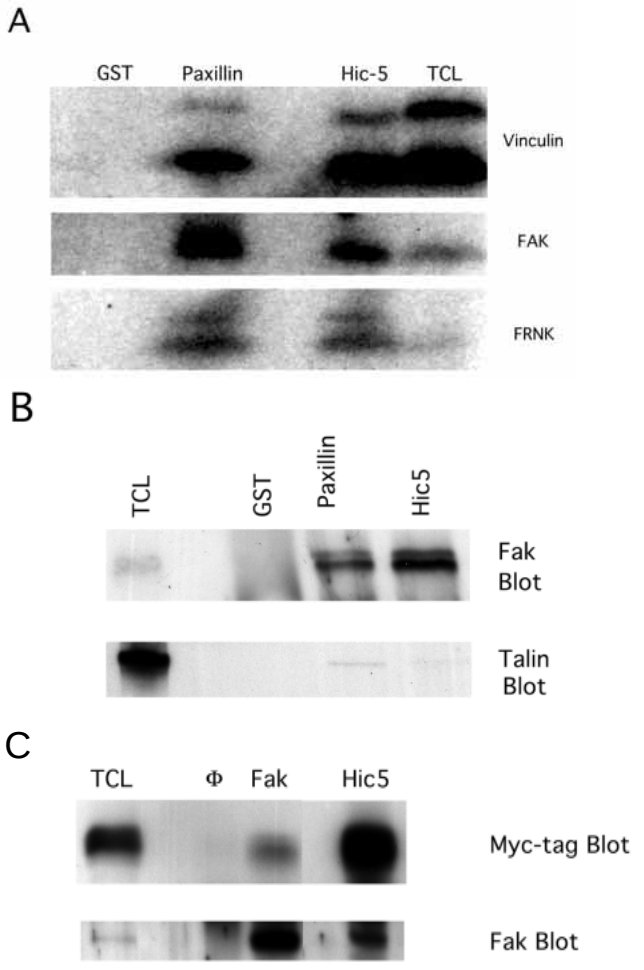


Fig. 2. Hic-5 can interact with Fak, Frnk and vinculin. (A) GST, GST-paxillin N-terminal (aa 54-331) or GST-Hic-5 N-terminal (aa 1-226) fusion proteins were used in binding assays and the bound proteins probed with antibodies to Fak, vinculin or Frnk. A total cell lysate (TCL) has also been included. (B) GST Hic-5 N-terminal fusion protein was used in a binding assay and the bound proteins probed with antibodies to Fak or Talin. (C) Cell lysates from 293T cells transfected with myc-tagged Hic-5 were incubated with rabbit Ig (Φ), Fak antibody, or Hic-5 antibody and the bound proteins probed with the myc-tag antibody, 9E10 or Fak.

Interaction of Hic-5 with Fak and vinculin

Hic-5 was originally proposed to be a nuclear protein and potential transcription factor (Shibanuma et al., 1994, 1997). Given the overall homology with paxillin, it was of interest to determine whether Hic-5 may play a role in focal adhesions. To address this question, GST fusion proteins were generated to determine if Hic-5, like paxillin, could interact with Fak, the Fak-related protein, FRNK, and vinculin (Hildebrand et al., 1995; Turner and Miller, 1994). FRNK is an alternatively spliced form of Fak that contains the paxillin binding site (Hildebrand et al., 1995; Schaller et al., 1993). GST fusion proteins containing amino acids 1-226 of Hic-5 were incubated with chicken gizzard lysates or REF52 lysates and the precipitated proteins were separated by SDS-PAGE and immobilized on nitrocellulose filters. Immunoblotting with antibodies to Fak showed that Hic-5 could associate with Fak and FRNK. Immunoblotting with antibodies to vinculin showed that Hic-5, like paxillin, also bound to vinculin and metavinculin (upper band, Fig. 2A) (Turner and Miller, 1994). In contrast, under conditions where Fak bound to the paxillin or Hic-5 fusion proteins, Talin binding was essentially undetectable (Fig. 2B).

To determine if Hic-5 can bind to Fak in vivo, 293T cells were transfected with a myc-tagged full length Hic-5. Lysates were incubated with Protein A purified Fak antibody, purified rabbit IgG, or Hic-5 specific antibody. The precipitated proteins were separated by SDS-PAGE and immobilized on nitrocellulose. The immunoblots were incubated with the myc-tag antibody or with a Fak antibody (Fig. 2C). The myc-tagged Hic-5 was detected only in cells transfected with this construct (data not shown) and only in lysates precipitated with the Fak antibody or Hic-5 antibody. In addition, a significant amount of Fak could also be co-precipitated with the Hic-5 antibody. Thus, consistent with the in vitro binding data, Hic-5 can associate with Fak in vivo.

Generation of a Hic-5 specific antibody and localization of Hic-5 in vivo

While these studies suggest that Hic-5 is localized to the cytoplasm and potentially focal adhesions, Shibanuma et al. (1997) have suggested that the subcellular localization of Hic-5 is nuclear. The Hic-5 specific antibody was used to stain REF52 cells. This antibody recognizes the GST fusion protein described above and a 50-51 kDa doublet present in REF52 cells but does not recognize paxillin (data not shown, Fig. 3A).

To determine the subcellular localization of Hic-5 in REF52 cells, cells were co-stained with the Hic-5 specific antibody and phalloidin or the Hic-5 antibody and a paxillin antibody. Hic-5 is localized to focal adhesions in REF52 cells (Fig. 3B and C). No nuclear staining was observed under these conditions. Co-staining of REF52 cells with paxillin and Hic-5 antibodies revealed that Hic-5 and paxillin co-localize in focal adhesions. In addition, myc-tagged Hic-5 also localizes to focal adhesions (data not shown).

Localization of paxillin to focal adhesions is mediated by the 2nd and third LIM domains (Brown et al., 1996). To

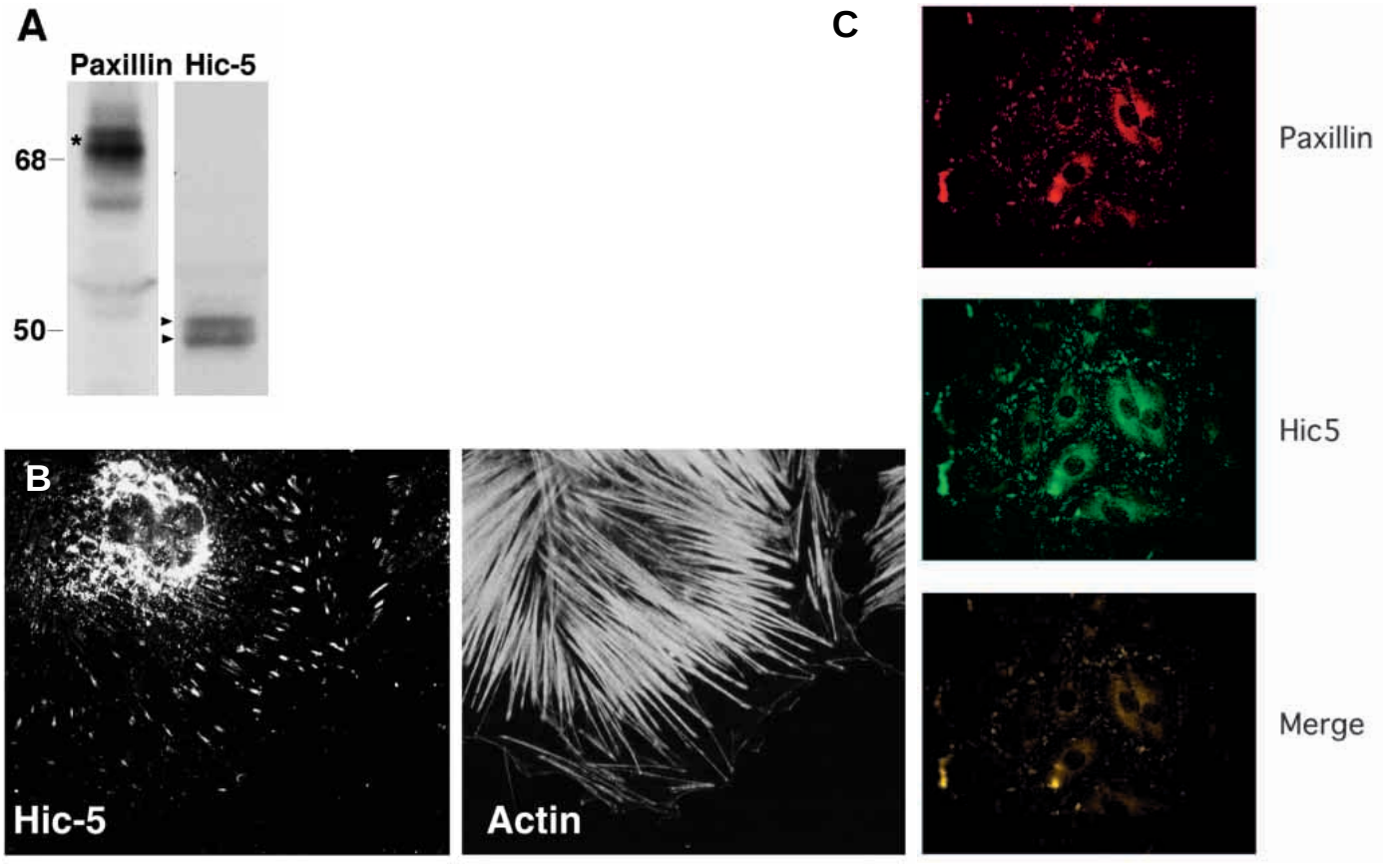


Fig. 3. Expression of Hic-5 in REF52 cells and localization to focal adhesions. (A) REF52 cell lysates immobilized on nitrocellulose were probed with a Hic-5 specific or paxillin-specific antibody. Paxillin is a broad doublet and is indicated by the asterisk. Hic-5 is a doublet and is indicated by the two arrowheads. The sequence to which the antibody was made is indicated by the dashed line in C. (B) REF52 cells plated overnight on fibronectin-coated coverslips were co-stained with the Hic-5 specific antibody and phalloidin. (C) REF52 cells plated overnight on fibronectin-coated coverslips were co-stained with the Hic-5 specific antibody and a paxillin antibody. Focal adhesions are clearly stained with the Hic-5 specific antibody.

determine which domains of Hic-5 are important for focal adhesion localization, green fluorescence protein (GFP) was fused to an N-terminal fragment, containing the Fak and vinculin binding sites, or a C-terminal fragment containing the four LIM domains. REF52 cells were transiently transfected with the GFP constructs and, following fixation, localization of the fusion proteins was assessed (Fig. 4). Cells expressing GFP show a punctate perinuclear staining as well as diffuse cytoplasmic localization. 80% of cells expressing the N-terminal Hic-5 fusion (GFP-NH2) show a similar pattern of staining as cells transfected with the vector control (Fig. 4A, NH2*). 20% of the cells, however, show some focal adhesion staining (Fig. 4A,B GFP-NH2). This was confirmed by co-staining with vinculin (Fig. 4B). Similar results are also seen with the paxillin N-terminal-GFP fusion (data not shown). In contrast, no focal adhesion staining is observed with the GFP control. Interestingly, GFP-NH2 co-localized with vinculin in the membrane cytoskeleton at potential focal contact sites (Fig. 4B).

Consistent with previous studies on paxillin, GFP fused to the Hic-5 LIM domains or paxillin LIM domains localized to focal adhesions (Brown et al., 1996) (Fig. 4A,B). In addition, the GFP-LIM domain fusions localized along stress fibers in some cells. Interestingly, there was also nuclear localization of both the Hic-5 and paxillin LIM domains. This was confirmed by co-staining with DAPI (data not shown). Nuclear localization was not observed with either the vector control or N-terminal-GFP fusion. These results suggest that the primary sequences important for localization of Hic-5 to focal adhesions reside in the LIM domains. Based on the homology between Hic-5 and paxillin, this is likely to be mediated by the 2nd and third LIM domains (Brown et al., 1996). In addition, the N-terminal region of Hic-5 may also play a minor role in focal adhesion localization since the GFP-N-terminal fusion could also localize to focal adhesions in some cells. These results support the binding studies and indicate that Hic-5 can localize to focal adhesions in vivo. The nuclear localization of the LIM domains, raises the possibility that paxillin and Hic-5 could reside in the nucleus; however, additional studies are clearly necessary to prove this hypothesis.

Association of Hic-5 with csk but not Crk

As indicated above, Hic5 has been proposed to be involved in cell senescence and, in general, thought to negatively regulate cell growth while paxillin may be a positive regulator (Shibanuma et al., 1994; Turner, 1994). Further support for this hypothesis comes from analysis of the aligned sequences. As mentioned above, the previously mapped tyrosine

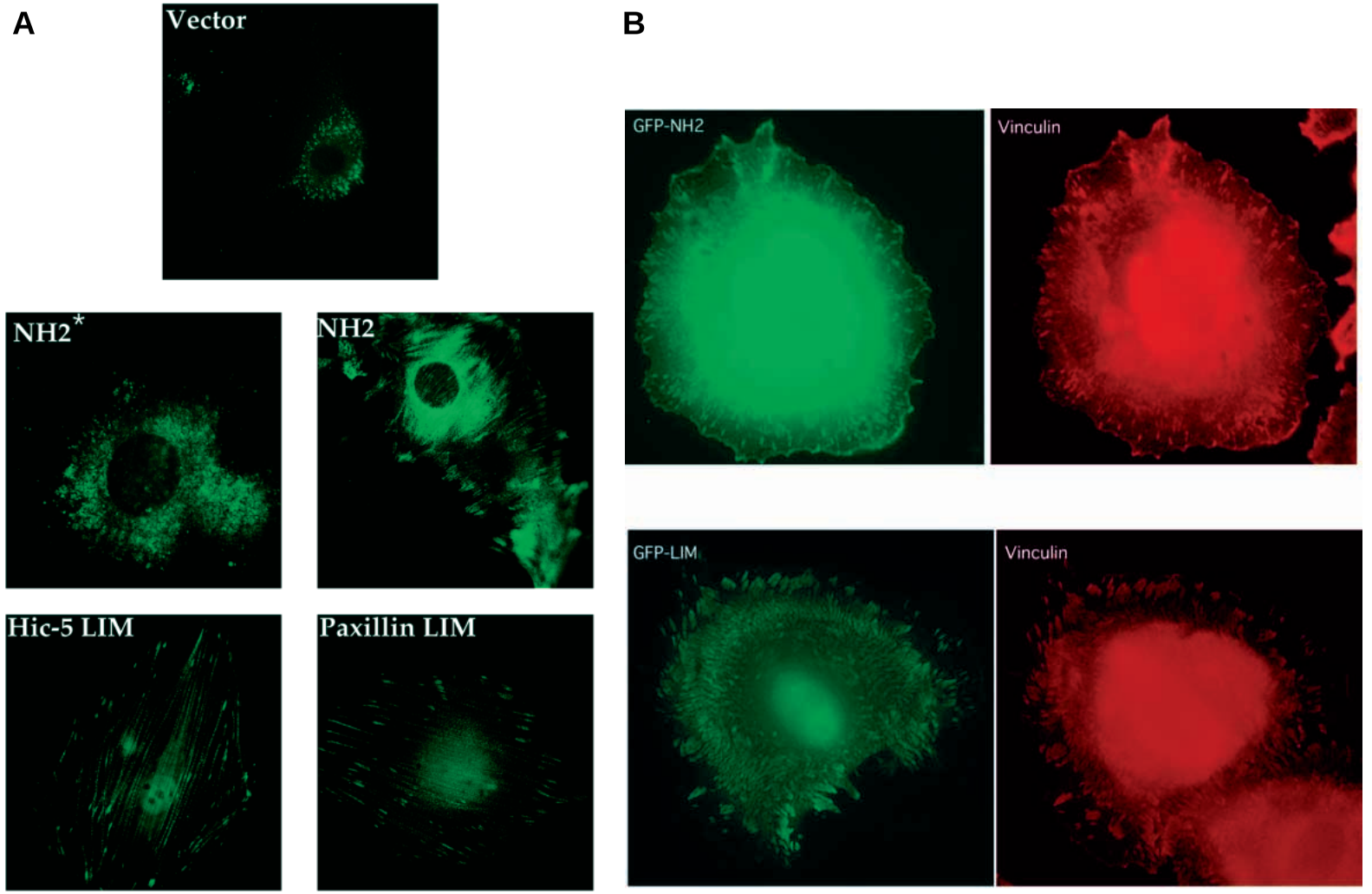


Fig. 4. Localization of GFP fusions. (A and B) GFP fusions were made with the N-terminal (aa 1-230 and aa 1-326, respectively) or C-terminal halves (aa 215-461 and aa 311-557, respectively) of Hic-5 or paxillin. These constructs were transfected into REF52 cells and 12-16 hours later plated onto fibronectin coated coverslips overnight. Cells were then fixed and photographed. NH2* represents what is seen in 80% of cells transfected with the GFP-Hic-5 N-terminal or GFP-paxillin N-terminal fusion protein. GFP-NH2 represents the staining observed in 20% of cells expressing the GFP-N-terminal fusion proteins. In B, cells were co-stained with vinculin. No clear difference in localization in REF52 cells has been observed between the GFP fusions with Hic-5, paxillin alpha or paxillin beta.

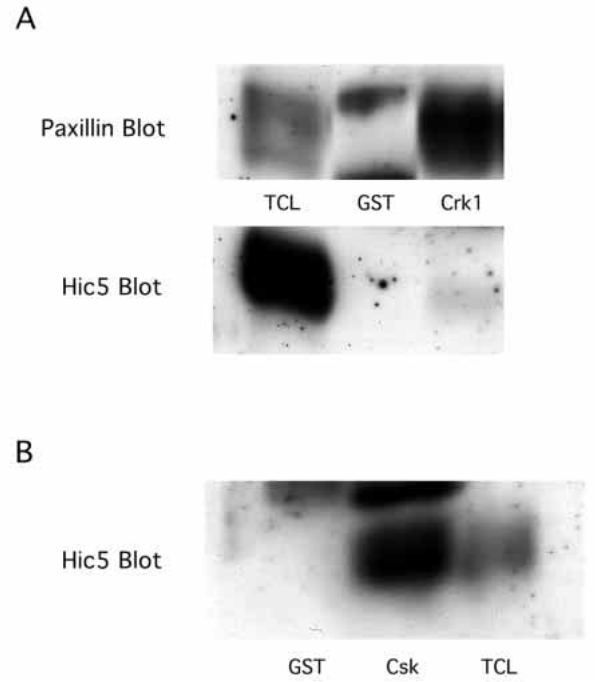


Fig. 5. Association of csk but not Crk with Hic-5. GST (A and B), GST-Crk (A) or GST-Csk (B) were used in binding assays as described in Materials and Methods. Following immobilization on nitrocellulose, the bound proteins were probed with antibodies to Hic-5 (A and B) or paxillin (A). Hic-5 binds to csk but not Crk.

phosphorylation sites in paxillin are poorly conserved in Hic-5. In particular, two sites which would allow binding of the positive signaling proteins, Src (Y40) and Crk (Y31 and Y118) are absent in Hic-5 while the site which would allow binding of the negative regulator, Csk, is present in Hic-5. Binding of paxillin to Crk provides a potential mechanism for paxillin activating the Ras-MAP kinase pathway, since Crk can interact with an exchange factor for Ras, SOS. To determine whether Hic5 can interact with Crk or Csk, GST fusion proteins for Crk and Csk were incubated with REF52 lysates that had been infected with v-src. As shown in Fig. 5, Crk can interact with paxillin but is unable to bind Hic-5. In contrast, Csk can interact with Hic-5 as well as paxillin. These results raise the possibility that Hic-5 may have a negative regulatory function in focal adhesion signaling.

DISCUSSION

In the process of cloning the mouse paxillin cDNA a paxillin-related protein, Hic-5 has been identified. Two other groups have also noted this homology (Brown et al., 1996; Shibanuma et al., 1997). Hic-5 was originally identified as a TGF-β inducible gene and has been suggested to be a transcription factor because of the presence of four LIM domains in the COOH-terminal half and because of its nuclear localization in the MC3T3 osteoblastic cell line (Shibanuma et al., 1994). The studies presented here show that: (1) Hic-5 shares a high degree of homology with the focal adhesion protein, paxillin; (2) Hic-5 can associate with the focal adhesion proteins Fak, FRNK, and vinculin; (3) Hic-5 is localized to focal adhesions in REF52 cells; and (4) localization of Hic-5 to focal adhesions is mediated primarily by the LIM domains although the N-terminal region also plays a minor role. (5) Hic-5 cannot bind to the adaptor protein Crk, but can bind Csk. These results suggest that Hic-5 may play a role in regulation of focal adhesions and raise the question of what is the functional relationship between Hic-5 and paxillin. At least three potential models can be proposed. (1) Hic-5 and paxillin could be redundant; (2) Hic-5 and paxillin could have unique and overlapping functions; (3) Hic-5 and paxillin could regulate each other.

The overall homology between Hic-5 and paxillin suggest that these proteins could have overlapping functions. Although Hic-5 was found in the nucleus in MC3T3 cells, Hic-5 can interact with the focal adhesion proteins Fak and vinculin and is localized to focal adhesions in REF52 cells (Fig. 2 and 3). Like paxillin, this localization is mediated primarily by the LIM domains (Fig. 4). However, the N-terminal half of Hic-5 or paxillin can localize GFP to focal adhesions. Interestingly, the N-terminal domain co-localized with vinculin to the focal contacts. This localization is likely due to the LD motifs which bind to the focal adhesion proteins, Fak and vinculin (Brown et al., 1996). Deletion of these sequences or use of Fak and vinculin deficient cells should help to address this issue.

While the LIM domains of Hic-5 and paxillin clearly localized GFP to the focal adhesions, nuclear localization was also observed. In addition, upon closer examination, constructs containing the four LIM domains exhibited a higher level of nuclear staining than the other constructs (Brown et al., 1996). Precedence for dual localization of LIM domain containing proteins includes the actin filament associated proteins CRP and MLP (Arber and Caroni, 1996). In addition, recent studies on zyxin have also suggested that this LIM domain containing protein can localize to both focal adhesions and the nucleus (Nix and Beckerle, 1997). Since nuclear localization of Hic-5 has been observed (Shibanuma et al., 1997), it is possible that Hic-5, and potentially paxillin, may localize to both focal adhesions and the nucleus depending on the cell type or signals that the cell is receiving. Additional studies on paxillin and Hic-5 should help to distinguish these possibilities.

Paxillin's localization to focal adhesions, its association with the focal adhesion proteins, Fak, FRNK, and vinculin, and its modification in response to integrin engagement has led to the suggestion that paxillin may be involved in focal adhesion dynamics (Jockusch et al., 1995; Turner, 1994). Whether or not Hic-5 is phosphorylated in response to integrin engagement is unclear. Thus far, we have been unable to see any changes in tyrosine phosphorylation of Hic-5 in response to integrin engagement in REF52 cells (S. M. Thomas, unpublished). Since integrin engagement can also induce serine/threonine phosphorylatin of paxillin it is possible that Hic-5 may be modified primarily on these residues in response to integrin engagement (Bellis et al., 1997; De Nichilo and Yamada, 1996). Regardless, the ability of Hic-5 to localize to focal adhesions and to interact with Frnk, Fak and vinculin, suggests that Hic-5 may be involved in regulation of focal adhesion dynamics.

Hic-5's ability to localize to focal adhesions and to interact with Frnk, Fak, and vinculin, suggests that paxillin and Hic-5 may have overlapping or redundant functions. It is interesting

to note, however, that Hic-5 and paxillin are proposed to be involved in opposing biological responses. Hic-5 has been associated with growth arrest and cell senescence since: (1) Hic-5 was originally identified as a TGF-β-inducible gene; (2) overexpression of Hic-5 induces cell senescence; (3) loss of hic-5 is observed in ras-transformed cells; and a number of cell lines derived from different human tumors (Shibanuma et al., 1994). In contrast, paxillin has been associated with cell growth because of: (1) it's identification as a tyrosine phosphorylated protein in *v-src*-, BCR-Abl-, and *v-crk*-transformed cells, and (2) its ability to associate with proteins which are linked to the Ras-MAP kinase pathway (Birge et al., 1993; Glenney and Zokas, 1989; Salgia et al., 1995a). These results raise the possibility that paxillin and Hic-5 may act in opposition to each other and may regulate each others function. Consistent with this model, Hic-5 was unable to bind to a Crk fusion protein in vitro, but could interact with a csk fusion protein. Thus, Hic-5 localization to focal adhesions and association with Fak and vinculin could displace paxillin and prevent paxillin from interacting with proteins such as Crk. Disruption of this interaction, in turn, could alter Ras/MAP kinase activation which could affect cell growth and differentiation. An additional possibility is that Hic-5 and paxillin could have different effects on cell migration. Crk associates with the focal adhesion protein CAS which has been implicated in promoting cell migration. Thus, it is possible that the interaction of paxillin with Crk could be important for regulation of cell movement. If Hic-5 is the predominant protein in focal adhesions then migration might be impaired. It is interesting to note that an example of family members acting in opposing pathways is seen with the focal adhesion protein Fak and the related protein, Pyk-2 (Ilic et al., 1997; Xiong and Parsons, 1997). Although Fak and Pyk2 are closely related proteins, they may function in opposing biological responses in fibroblasts. Fak has been associated with cell survival while overexpression of Pyk2 induces apoptosis in fibroblasts (Xiong and Parsons, 1997). Thus, it is possible that paxillin and Hic-5, while being related by protein sequence may act to regulate each other. This model requires co-expression of these proteins which is clearly observed for paxillin and Hic-5 (Shibanuma et al., 1994; Turner and Miller, 1994) (Fig. 3C). Studies are currently underway to understand the functional relationship between Hic-5 and paxillin.

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