Human Enhancer of Filamentation 1, a Novel p130^{cas}-Like Docking Protein, Associates with Focal Adhesion Kinase and Induces Pseudohyphal Growth in *Saccharomyces cerevisiae*

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Budding in *Saccharomyces cerevisiae* follows a genetically programmed pattern of cell division which can be regulated by external signals. On the basis of the known functional conservation between a number of mammalian oncogenes and antioncogenes with genes in the yeast budding pathway, we used enhancement of pseudohyphal budding in *S. cerevisiae* by human proteins expressed from a HeLa cDNA library as a morphological screen to identify candidate genes that coordinate cellular signaling and morphology. In this report, we describe the isolation and characterization of human enhancer of filamentation 1 (HEF1), an SH3-domain-containing protein that is similar in structure to p130^{cas}, a recently identified docking protein that is a substrate for phosphorylation by a number of oncogenic tyrosine kinases. In contrast to p130^{cas}, the expression of HEF1 appears to be tissue specific. Further, whereas p130^{cas} is localized predominantly at focal adhesions, immunofluorescence indicates that HEF1 localizes to both the cell periphery and the cell nucleus and is differently localized in fibroblasts and epithelial cells, suggesting a more complex role in cell signalling. Through immunoprecipitation and two-hybrid analysis, we demonstrate a direct physical interaction between HEF1 and p130^{cas}, as well as an interaction of the SH3 domain of HEF1 with two discrete proline-rich regions of focal adhesion kinase. Finally, we demonstrate that as with p130^{cas}, transformation with the oncogene *v-abl* results in an increase in tyrosine phosphorylation on HEF1, mediated by a direct association between HEF1 and *v-Abl*. We anticipate that HEF1 may prove to be an important linking element between extracellular signalling and regulation of the cytoskeleton.

A common feature of cancerous transformation in cells is the alteration of cell shape. Early support for the idea that such shape changes may directly contribute to the development of a cancerous phenotype was provided by the observation that simply altering the shape of nontransformed mammalian cultured cells can profoundly alter their pattern of response to growth signals (12). Mounting evidence suggests that such a mechanism may be extremely important for many types of cancer. Loss of function of the antioncogene NF2, a member of a family of proteins responsible for organizing cytoskeleton-membrane attachments (47, 54), leads to the development of nervous system tumors. Rho class GTPases, including Rho, Rac, and Cdc42hs, which were initially defined as factors controlling cell morphology and actin cytoskeleton (44, 45), can be oncogenic when deregulated (25, 36). Conversely, proteins such as Dbp or Vav, possessing exchange activity for these GTPases, were originally isolated as oncogene products (19, 22). Significantly, a recent study has shown that contact between integrin and its receptor triggers the rapid aggregation of a large number of signaling kinases and cytoskeletal components to a focal adhesion, suggesting that the processes of targeted intracellular localization and activation of the kinase pathways are inseparable (38).

Budding in the yeast *Saccharomyces cerevisiae* is a genetically controlled process involving issues of cell polarity, morphological regulation, and response to external environmental signals

(reviewed in reference 27). Defined components of the *S. cerevisiae* budding control pathway include a number of genes related to mammalian cytoskeletal/oncogenic signalling, including *CDC42* (a homolog of *CDC42hs*) (5), *CDC24* (related to a group of oncogenes including *vav*, *bcr*, *dbp*, and *Tiam-1* [1, 21, 25, 46]), and *BUD1/RSR1* (5, 9, 10) (a homolog of the Ras family GTPase gene *Krev-1/rap1A*, an antioncogene [26, 48]). Further, a number of genes originally identified as important for budding control, such as *BEM1* or *CDC42*, have recently been found to additionally interact with mitogen-activated protein kinase cascades, such as the *STE20-STE11-STE7* cascade required for mating (31, 51). We were interested in isolating and characterizing additional genes responsible for coordinating cell signalling and cell morphology in higher eukaryotes. Previously, successful use has been made of complementation strategies in *S. cerevisiae* to identify molecules important for cell cycle control in mammalian cells (30, 32). In this manner, we hypothesized that human genes that could specifically affect the budding pattern in *S. cerevisiae* might include cytoskeletal and oncogenic regulatory proteins.

Diploid yeast cells grown on low-nitrogen medium convert their budding pattern from vegetative to pseudohyphal (15). Conversion to pseudohyphal growth causes alteration of polarity of cell division, regulation of cell morphology, and alteration of cell cycle control (28). Mechanistically, formation of pseudohyphae is stimulated by Ras overexpression, is dependent on mating type (and was subsequently shown to require members of the STE mitogen-activated protein kinase cascade [33]), and requires intact budding control genes (e.g., *BUD1*). Microcolonies of yeast cells growing pseudohyphally are readily distinguishable from vegetatively growing cells under a

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low-power microscope. On the basis of these properties, we performed a screen for human genes capable of enhancing or constitutively inducing pseudohyphal growth. HEF1 (human enhancer of filamentation 1) possesses the most dramatic phenotype of the cDNAs obtained in this screen. Characterization of HEF1 reveals that it is a novel protein that is similar to the recently described docking protein p130^{cas} (49) and may play a central role in coordinating tyrosine kinase-based signalling and cellular morphology.

MATERIALS AND METHODS

Isolation of HEF1-C. HEF1-C was isolated by transforming a HeLa cDNA library constructed in the TRP1⁺ vector pJG4-4 (a gift of J. Gyuris), with inserts expressed as native proteins under the control of the galactose-inducible *GAL1* promoter, into *S. cerevisiae* CGx74 (*MATa* α *trp1*/*trp1*; a gift of C. Gimeno). TRP⁺ transformants were plated to SLAGR medium, and ~140,000 colonies were visually screened with a Wild dissecting microscope at $\times 50$ amplification to identify colonies that are forming pseudohyphae more extensively than background. cDNAs were isolated and retransformed to naive CGx74: those that reproducibly generated enhanced pseudohyphae were sequenced. The 900-bp cDNA encoding a 182-amino-acid open reading frame corresponding to the C terminus of HEF1 (HEF1-C) possessed the most dramatic phenotype of cDNAs obtained in this screen.

Isolation of full-length HEF1 and partial MEF1 sequences. The 900-bp HEF1 cDNA isolated in the pseudohyphal enhancement screen was radiolabelled by random priming in a reaction incorporating [32 P]dCTP and used to probe a placentar cDNA library cloned in λ gt11 (a gift of J. Chernoff). A longer, 2.2-kb HEF1 clone obtained in this screen was used as a basis for 5' rapid amplification of DNA ends (RACE) with a kit from Clontech containing RACE-ready cDNA prepared from human kidney. Multiple independent clones were obtained by this means, with several possessing identical 5' endpoints located 164 bp upstream of the ATG assigned as initiating translation. Sequence alignment was done with the University of Wisconsin Genetics Computer Group package of programs, and homology searches were performed with the BLAST algorithm (3). For the MEF clone, [32 P]dCTP-labeled HEF1 cDNA was used to probe a genomic library derived from B6 inbred mice cloned in λ mbda DASH (17). A cross-hybridizing clone, mHEF1, yielded an insert encoding an exon corresponding to the novel C-terminal domain fragment of HEF1.

Northern analysis. The 2.2-kb HEF1 cDNA obtained from the λ gt11 library was labeled with [32 P]dCTP by random priming and used to probe a Northern (RNA) blot containing 2 μ g of human mRNA from tissues as noted (MTN1; Clontech) per lane. The blot was stripped by immersion in boiling 0.5% sodium dodecyl sulfate (SDS) for 10 min and reprobed with a similarly labeled 2.0-kb actin cDNA to control for equivalent load.

Interaction trap/two-hybrid analysis. All experiments described were performed with reagents and techniques detailed previously (16). A fragment of p130Cas comparable to the HEF1-C domain was obtained by PCR with primers based on published sequences (forward sequence 5'-GTGCGACAGGGAA AAGGCCAACTGGAG-3' and reverse sequence 5'-GGCGGCAGCCAGCTG GCCCAGGACACGG-3', with flanking restriction sites), and confirmed by sequencing both strands. For analysis of dimerization, the HEF1-C fragment and the comparable p130^{cas}-C fragment were cloned into the vector pEG202 to synthesize LexA-HEF1-C and LexA-p130^{cas}-C, respectively, and into the vector pJG4-5 to synthesize activation domain fused proteins AD-HEF1-C and AD-p130^{cas}-C, respectively. AD-Ras, used as a negative control, contains the full-length Ras protein cloned in the vector pJG4-5 (a gift of A. Makris). Twelve separate independent colonies were used for each pair of constructs in analysis of interactions. β -Galactosidase activities were calculated as in reference 37.

For library screening, an *EcoRI-XhoI*-ended fragment encoding amino acids 1 to 129 of HEF1 (spanning the SH3 domain) was cloned in the vector pEG202 to create a LexA fused bait. Strain EGY191 and *lacZ* reporter plasmid pJK103 have been described previously (13). The library used was derived from HeLa cDNA expressed in the vector pJG4-5 and has been described previously (20).

Production of GST and MBP fusion proteins. The HEF1-C and p130^{cas}-C fragments were cloned as *EcoRI-XhoI*-ended fragments into the vector pGEX (Pharmacia) to produce glutathione-S-transferase (GST) fusions and into pMal-c2 (New England Biolabs) to produce maltose-binding protein (MBP) fusions. Constructs were transformed into the protease-deficient bacterial strain CAG456, cultures were induced with isopropyl- β -D-thiogalactopyranoside (IPTG), and lysates were made as recommended by vector suppliers. Individual fusion proteins were purified by affinity chromatography with columns of glutathione-conjugated Sepharose beads (GST fusions) or amylose resin (MBP fusions). For immunoprecipitations, ~700 ng of each construct was diluted into 500 μ l of buffer A (described below). Following this, anti-GST antibodies (Sigma) and protein A-Sepharose were added for precipitation and 25% of the precipitate was used for SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Antibodies. HEF1-directed antiserum was generated in rabbits against two multiantigen peptides (8) derived from HEF1. The amino acid sequence EQEIT

KPVENDISK (one-letter code) was used to generate the antibody α -HEF1-CT, specific for the HEF1 C-terminal region. The amino acid sequence EPPAET SEKANPOER was used to generate the antibody α -HEF1-SB, specific for the HEF1 SH2-binding-site-containing region. Anti-HEF1 antisera were affinity purified against the appropriate cognate peptide. This was done by coupling the peptides to an Affi-Gel 15 column (Bio-Rad) and eluting the antibodies as specified by the manufacturer. Affinity-purified antibodies were used at a dilution of 1:100 for Western blotting (immunoblotting). Mouse monoclonal antibodies to phosphotyrosine, p130^{cas}, and human focal adhesion kinase (FAK) were purchased from Transduction Laboratories, and paxillin ascites fluid was a gift of M. Brown and C. Turner. Additional polyclonal antibodies to p130^{cas}, B (residues 318 to 486, derived from the SH2-binding-site domain), and F (residues 670 to 896, derived from the carboxy-terminal domain) were a generous gift of A. Bouton and T. Parsons. Polyclonal antibodies to Abl were purchased from Santa Cruz Biotechnology (K-12). Primary antisera were used at a dilution of 1:1,000 for Western blots. The secondary antibody for visualization of the Western blots was goat anti-rabbit or goat anti-mouse antibody conjugated to alkaline phosphatase (Sigma) or conjugated to horseradish peroxidase for detection by enhanced chemiluminescence (Amersham). For specificity testing of α -HEF1-CT, the HEF1-C, p130^{cas}-C, and HEF1-SH3 domain fragments were cloned as *EcoRI-XhoI*-ended fragments into the TRP1 vector pJG4-6 (a gift of J. Gyuris), which expresses proteins under the control of the *GAL1* inducible promoter as fusions to the HA epitope tag. These constructs were transformed into yeast strain EGY48 (16), expression was induced with galactose, and protein lysates were made as described previously (24).

Immunoprecipitation. The *v-abl*-transformed cell line used for immunoprecipitation was generated in the laboratory of Gary Kruh. Cell lysates were prepared as follows, with all steps done at 4°C, the cells were washed twice in 1 $\times$ phosphate-buffered saline (PBS) and then scraped into buffer A (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.5], 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 10 mM Na₂P₂O₇, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 0.01 mg of aprotinin per ml, 0.01 mg of leupeptin per ml). The lysates were then centrifuged in an Eppendorf Microfuge for 10 min at 10,000 rpm. Following this, the supernatant was removed and used in the immunoprecipitation experiments. Anti-HEF1 antiserum (10 μ l), 3 μ g each of anti-p130^{cas}, FAK, and Abl, and 3 μ l each of the polyclonal antisera B and F were incubated with 0.5 mg of cell lysate and 30 μ l of 50% protein A-Sepharose beads (Sigma) overnight at 4°C. The beads were washed four times in 500 μ l of buffer A and analyzed by immunoblotting. SDS-PAGE gels of different resolution, either 6% (15-cm plates) or 10% (9-cm plates, minigel apparatus [Bio-Rad]), were used to analyze the products of the immunoprecipitation.

Immunofluorescence. Cells were plated on coverslips 24 h before being processed for immunofluorescence. All steps were carried out at room temperature. Coverslips were washed twice in 1 $\times$ PBS and then fixed in 3.5% paraformaldehyde for 7 min. The cells were permeabilized for 5 min in buffer B (0.1 M Tris [pH 7.5], 1.5 M NaCl, 1% bovine serum albumin) with 0.2% Triton X-100. The coverslips were then washed for 5 min in buffer B. The primary antibody incubations were done for 1 h at room temperature. The antibody dilutions were made in buffer B and were identical to those used for immunoblotting. The coverslips were washed for 5 min in buffer B-Triton X-100 and then for 5 min in buffer B alone. Secondary antibody (either fluorescein isothiocyanate-conjugated anti-mouse antibody or biotinylated anti-rabbit antibody [Vector Laboratories]) was diluted in buffer B and incubated with the slides for 1 h. The cells were washed twice for 5 min each in buffer B, and those that had biotin-conjugated secondaries were incubated with Texas red streptavidin (Vector Laboratories) for 15 min. Then, the cells were washed twice with buffer B and mounted with anti-fade mounting medium (Vector Laboratories).

Microscopy. JG4-6 constructs expressing HEF1-C, p130^{cas}-C, or vector alone were transformed into CGx74 or CG189 yeast cells as indicated in Results. For photography, the cells were grown as described previously (28) and pictures were taken by using differential interference contrast optics with a Nikon microscope. Immunofluorescent pictures were taken with a Nikon fluorescence microscope at $\times 60$.

Nucleotide sequence accession number. The HEF1 nucleotide and protein sequences have been deposited in GenBank and have been assigned accession number L43281.

RESULTS

Isolation of HEF1. A HeLa cell cDNA expression library under the control of a galactose-inducible promoter was transformed into the diploid yeast strain CGx74, and ~140,000 transformants were examined to identify cDNAs that induced pseudohyphal growth. Expression of a 900-bp partial cDNA fragment encoding the carboxy-terminal 182 amino acids of HEF1 (HEF1-C) in CGx74 diploid yeast cells strongly enhances pseudohyphal growth on low-nitrogen SLAGR medium (Fig. 1B) and also induces a partial pseudohyphal phenotype

FIG. 1. Induction of pseudohyphae by HEF1-C. *S. cerevisiae* CGx74 cells were transformed with the pJG4-6 vector expressing the HEF1 C-terminal fragment isolated in library screening (B and E) or with a comparable segment of p130^{cas} (C and F) or the pJG4-6 vector control (A and D), and plated on either low-nitrogen SLAGR medium (like SLAD but with 2% galactose plus 1% raffinose as a carbon source [28]) (A to C) or complete defined minimal medium with 2% galactose (D to F). Isogenic haploid *S. cerevisiae* CG189 cells were transformed with either pJG4-6 expressing HEF1-C (H) or vector (G) alone and plated on SLAGR plates. Thin slices of agar were mounted on slides, and photographs were taken using with differential interference contrast optics after 48 h.

on complete minimal medium (Fig. 1E), in contrast to control yeast cells, which form pseudohyphae only in response to nitrogen limitation (15) (Fig. 1A and D). Expression of HEF1-C has no effect on the morphology of the parental haploid strain CG189 (Fig. 1G and H). These results suggested that HEF1-C selectively interacts with diploid cell-specific components that control cell morphology (15), rather than functioning as a general promoter of cell elongation.

A full-length HEF1 cDNA of ~3.7 kb was isolated by screening a placental library and using 5' RACE (see Materials and Methods). This cDNA was found to encode a single continuous open reading frame of 835 amino acids (Fig. 2A), with 164 bp of 5' untranslated sequence in the longest clones obtained and an in-frame stop codon located 36 bp upstream of the predicted translational start site (29a). The predicted HEF1 protein contains an amino-terminal SH3 domain and an adjacent domain containing multiple SH2-binding motifs (52, 53). Database analysis revealed that the amino acid sequence of HEF1 is 64% similar to that of p130^{cas} (49), a protein recently characterized in rats and mice. However, HEF1 is a distinct gene from p130^{cas} rather than a human homolog. A screen of a murine genomic library with the HEF1 cDNA led to isolation of an exon corresponding to HEF1-C that encoded

a mouse protein sequence essentially identical to HEF1 on the amino acid level in a region that was only 74% similar to rat p130^{cas} (Fig. 2B and C). Overall, the similarity between HEF1 and p130^{cas} is most pronounced in the SH3 domain and the carboxy-terminal region, while the domain containing the SH2-binding motifs is more divergent.

The p130^{cas} protein was first identified as a species that becomes highly phosphorylated in mammalian cells following transformation by the oncoproteins Src and Crk and has been shown to be an *in vitro* substrate for Abl and Src kinases (14, 42, 49). This protein has recently become the focus of considerable interest, because the great abundance of SH2 consensus recognition sites on p130^{cas} has suggested that this protein may function as a general docking protein for input of growth-regulatory signals (49) and because antisense studies have suggested that p130^{cas} function may be an obligate intermediate in transformation by oncogenes such as *ras* and *src* (4). While the majority of the putative SH2-binding sites in p130^{cas} match the consensus for the Crk SH2 domain, the amino acids flanking the tyrosine residues in HEF1 are more diverse, suggesting a potentially broader range of associating proteins (Fig. 2D).

Tissue-specific expression of HEF1. Northern blot analysis of human tissues (Fig. 3), revealed that HEF1 is expressed as

FIG. 2. Comparison of HEF1 with p130^{cas}. (A) HEF1 sequence alignment with the rat p130^{cas} protein. Identical amino acid residues are boxed. The solid circle marks the site of the initial methionine in the truncated clone of HEF1 (HEF1-C) isolated in the screen for enhancers of pseudohyphae. The thick underline marks the conserved SH3 domain. Tyrosines are marked with asterisks. Thin lines denote the site of the two peptide sequences used to generate antibodies HEF1-SB and HEF1-CT. (B) Conservation of HEF1-C with a murine genomic clone (MEF1) indicates that the HEF1 gene represents a distinct gene from that encoding the rat p130^{cas}, rather than a human homolog. Comparison of these sequences shows a much closer relationship to HEF1 (96% identity over 78 amino acids) than to p130^{cas} (47% over 78 amino acids) in the HEF1-C region. (C) Comparison of HEF1 and p130^{cas} identity and similarity. The bar depicts the various regions of HEF1 and p130^{cas}, while the originally isolated HEF1-C is shown as a bent arrow. (D) Conservation of putative tyrosine-containing SH2-binding sites present in HEF1 and p130^{cas}. SH2 domains that are predicted to recognize these motifs (52, 53) are indicated in italics, those in parentheses represent near matches to the known motifs, and the question marks depict motifs conserved between HEF1 and p130^{cas} for which a binding partner is not predicted on the basis of current information.

D

Tyrosines and flanking sequences, HEF1 and p130^{Cas}

HEF

1.	MK	Y	KNL	
2.	RAL	Y	DNV	<i>fgr</i> (src, fes)
3.	QKL	Y	QVP	?
4.	DTI	Y	QVP	?
5.	PPS	Y	QNQ	<i>grb2</i>
6.	QGI	Y	QVP	?
7.	EQE	Y	QVP	?
8.	GHG	Y	VYE	?
9.	GYV	Y	EYP	(abl, vav, src)
10.	VYE	Y	PSR	
11.	PSR	Y	QKD	
12.	KDV	Y	DIP	<i>crk</i>
13.	QGV	Y	DIP	<i>crk</i>
14.	QGV	Y	DIP	<i>crk</i>
15.	KGV	Y	AIP	<i>abl</i>
16.	EKD	Y	DFP	<i>crk</i>
17.	EGV	Y	DIP	<i>crk</i>
18.	HVK	Y	NCD	
19.	NDA	Y	DVP	<i>crk</i>
20.	DGV	Y	DVP	<i>crk</i>
21.	WRC	Y	GYM	
22.	CYG	Y	MER	(vav)
23.	LKE	Y	LHF	
24.	MDD	Y	DYV	(src, fes)
25.	DYD	Y	VHL	
26.	LCF	Y	YDQ	
27.	CFY	Y	DQC	
28.	ALH	Y	PST	

p130^{Cas}

1.	MK	Y	LNV	
2.	KAL	Y	DNV	<i>fgr</i> (src, fes)
3.	VGM	Y	DKK	
4.	ASQ	Y	SPM	
5.	PTA	Y	QPQ	
6.	DNV	Y	LVP	
7.	QGL	Y	QAP	
8.	TDL	Y	QVP	?
9.	QDI	Y	QVP	?
10.	HDI	Y	QVP	?
11.	GQG	Y	VYE	?
12.	GYV	Y	EAS	
13.	QDE	Y	DTP	<i>crk</i>
14.	QDI	Y	DVP	<i>crk</i>
15.	PNQ	Y	GQE	
16.	QEV	Y	DTP	<i>crk</i>
17.	LDV	Y	DVP	<i>crk</i>
18.	HSV	Y	DVP	<i>crk</i>
19.	EET	Y	DVP	<i>crk</i>
20.	EDV	Y	DVP	<i>crk</i>
21.	PDL	Y	DVP	<i>crk</i>
22.	GTL	Y	DVP	<i>crk</i>
23.	DGV	Y	AVP	<i>abl</i>
24.	EDV	Y	QTL	
25.	DGQ	Y	ENS	<i>3BP2</i> (abl, src)
26.	MED	Y	DYV	(src, fes)
27.	DYD	Y	VHL	
28.	LLF	Y	LEQ	(vav)
29.	VTH	Y	SNL	
30.	ALQ	Y	PSP	

FIG. 2.—Continued

two predominant transcripts of approximately 3.8 and 5.4 kb. Although present in all tissues examined, these transcripts are detected at significantly higher levels in kidney, lung, and placenta tissue (as much as 80-fold greater than in tissues such as brain tissue). This is in notable contrast to the more uniform distribution and single transcript reported for p130^{Cas} (49). While we have not yet characterized the 5.4-kb transcript, we believe that it is unlikely to represent further 5' sequence of HEF1, because our current clone aligns with p130^{Cas} through its amino-terminal start and because repeated efforts at 5' RACE did not yield additional upstream coding sequence. Given the nearly identical tissue distribution of the 3.8- and 5.4-kb transcripts, the larger transcript most probably represents internal splice variants and/or additional 3' untranslated sequence. Two other minor hybridizing species, migrating at 8.0 kb in lung tissue and 1.2 kb in liver tissue, were detected with HEF1 probe. These may represent alternatively spliced HEF1 transcripts or further HEF1/p130^{Cas}-related genes.

Similar function of the HEF1-C/p130^{Cas}-C domain in cell polarization and dimerization. The conservation between HEF1 and p130^{Cas} and the method of our isolation of HEF1 suggest a model in which the interaction of tyrosine kinases and other proteins with the HEF1 and p130^{Cas} SH3 domains and SH2-binding sites might regulate the function of their carboxy-terminal domains, which are direct effectors of the cytoskeletal changes occurring in oncogenic transformation. If so, the carboxy-terminal region of p130^{Cas} should similarly enhance pseudohyphal growth. Expression of the carboxy-terminal fragment of rat p130^{Cas} (p130^{Cas}-C) in CGx74 indeed resulted in enhanced pseudohyphal formation, although to a somewhat lesser degree than did expression of HEF1-C (Fig. 1C and F). No enhancement of pseudohyphal growth by HEF1

or p130^{Cas} was observed in yeast cells grown on plates containing 2% glucose as the carbon source, conditions that are non-inducing for the *GAL1* promoter from which the proteins were expressed (29a).

Structural predictions for the carboxy-terminal domain of HEF1 suggested a continuous helical region (3) that might

FIG. 3. HEF1 RNA levels are highest in placenta, lung, and kidney tissue. Quantitation of the ³²P signal with a Fuji image analyzer indicates a greater than 80-fold signal difference between high- and low-expressing tissues.

FIG. 4. HEF1 and p130^{cas} functional relationship, and HEF1 dimerization. (A) The specificity of the anti-HEF1 antiserum α -HEF1-CT was tested with hemagglutinin (HA)-tagged fusion proteins produced in *S. cerevisiae*. Duplicate lysates of *S. cerevisiae* containing HA-tagging vector (pJG4-6) or this vector expressing the HEF1 carboxy-terminal region (HEF1-C), the comparable region of p130^{cas} (p130cas), or the SH3 domain of HEF1 (HEF1 SH3) were visualized by using antibody to HEF1 (α -HEF1-CT) or to the HA epitope tag (α -HA). (B) GST- and MBP-fused HEF1-C were purified and subsequently immunoprecipitated with antibodies against GST (Sigma). The left-hand lane contains affinity column-purified MBP-HEF1-C. The next lane contains affinity column-purified GST-HEF1-C. The three right-hand lanes contain immunoprecipitates of MBP-HEF1-C, GST-HEF1-C, and MBP-HEF1-C and GST-HEF1-C. HEF1 fusion proteins were visualized with the HEF1-CT antiserum. (C) NIH 3T3 cell lysates were used for immunoprecipitation with protein A-Sepharose (lane 1) or with antibodies to HEF1 (α -HEF1-SB) (lane 2) or p130^{cas} (lanes 3 and 5). Duplicate samples of immunoprecipitate were then resolved on 6% polyacrylamide SDS-PAGE gels, and proteins were visualized with antibody to p130^{cas} or to HEF1. α -HEF1-CT yielded similar results but with much lower signal strength (data not shown).

mediate oligomerization with other proteins. This was tested by an interaction trap/two-hybrid approach (16), which showed that LexA-HEF1-C both homodimerized with activation domain-HEF1-C and heterodimerized with the corresponding activation domain-p130^{cas}-C protein (346 and 340 β -galactosidase units, respectively) but did not interact with the nonspecific control protein Ras or the activation domain fusion vector pJG4-5 (39 and 45 β -galactosidase units, respectively), with the values reflecting the intrinsic ability of LexA-HEF1-C to weakly activate transcription. To confirm HEF1-C-mediated self-association, we used a peptide-directed antiserum specific for HEF1, α -HEF1-CT (Fig. 4A), to visualize two coimmunoprecipitated HEF1-C fusion proteins expressed *in vitro* (Fig. 4B). The HEF1-C domain was purified as a fusion protein to MBP and to GST. Coimmunoprecipitation of MBP-HEF1-C through its association with GST-HEF1-C confirmed the association of the two fusion proteins.

To effectively study HEF1 interactions with other proteins in a physiological context and to contrast these interactions with those of p130^{cas}, we proceeded to establish conditions and reagents to study HEF1 in cultured mammalian cells. Two potential hindrances were cross-reaction of antibodies developed against p130^{cas} with the highly related HEF1 protein and the possibility that the α -HEF1-CT peptide-specific antiserum cross-reacted with additional cellular proteins. To preclude these problems, we first developed α -HEF1-SB, a second peptide-specific antibody targeted against a 15-amino-acid sequence in the SH2-binding-site-containing region of HEF1 that was highly divergent from p130^{cas} (Fig. 2A). We then used tests similar to those described above (Fig. 4A) to demonstrate that this antiserum was specific for HEF1 (29a). We then used the α -HEF1-SB antibody to HEF1 and the p130^{cas}-specific antisera to perform sequential immunoprecipitation and Western blot visualization of NIH 3T3 cell lysates. By this approach, we found that through the use of low-percentage SDS-PAGE gels coupled with long runs to resolve higher-molecular-weight species, we were able to distinguish one doublet migrating at $\sim$ 112 to 127 kDa, specifically recognized by the HEF1 specific antibody and by three independent antibodies generated to parts of the p130^{cas} protein, and an additional species migrating at $\sim$ 130 kDa, recognized only by the antibodies to the p130^{cas} protein (Fig. 4C). From these results, we concluded that the HEF1 antibodies were specific for the two lower species, which were presumably endogenous HEF1, whereas the p130^{cas}-specific antibodies cross-reacted with both proteins.

Phosphorylation of HEF1 following v-abl transformation, and intracellular association of HEF1 with v-Abl. To determine whether endogenous HEF1 associated with any of the known partners of p130^{cas}, we first used the antiserum α -HEF1-CT, specific for the HEF1 carboxy terminus (Fig. 4A), to immunoprecipitate HEF1 from normal versus v-abl-transformed NIH 3T3 cells (Fig. 5). A probe of these immunoprecipitates with antibody to phosphotyrosine revealed a species migrating at $\sim$ 130 to 140 kDa that was observed only in v-abl-transformed fibroblasts. This association was specific for HEF1, because it was eliminated in the presence of excess HEF1 peptide.

To support the idea that this species represented HEF1 phosphorylated by v-Abl, we used antibodies to v-Abl to perform immunoprecipitations from NIH 3T3 cells or NIH 3T3 cells transformed with v-abl. These were run out on a long-distance low-percentage SDS-PAGE gel and probed with the α -HEF1-SB antibody to identify HEF1 (Fig. 5B). We were clearly able to co-precipitate HEF1 with antibody to v-Abl in

FIG. 5. HEF1 associates with and is phosphorylated by Abl in vivo. (A) The anti-HEF1 antiserum α -HEF1-CT was used for immunoprecipitation from NIH 3T3 cells and NIH 3T3 cells transformed with *v-abl* in the presence or absence of HEF1 peptide (Hp) block. A Western blot of the immunoprecipitates was probed with anti-phosphotyrosine (Anti-Ptyr) antibodies, revealing a protein species of ca. 130 kDa specific to *v-abl*-transformed cells. (B) Lysates of NIH 3T3 cells (lanes N) or NIH3T3 cells transformed with *v-Abl* (lanes A) were used for immunoprecipitation either with protein A-beads (lanes 1) or with antibodies to *v-Abl* (lanes 2). Immunoprecipitates were then reprobed with antibodies to HEF1 (α -HEF1-SB).

v-abl-transformed cells, these antibodies failed to immunoprecipitate HEF1 in normal NIH 3T3 cells.

FAK associates with HEF1 in the yeast two-hybrid system and in cultured cells. As a further investigation of HEF1 signaling partners, interaction trap/two-hybrid screens were conducted with the HEF1 SH3 domain as a bait. Of the isolated clones, 75% consisted of a small protein of uncharacterized function containing a carboxy-terminal polyproline region possessing significant SH3-binding-site consensus (2). The remaining 25% (5 of 20) were partial cDNAs of FAK (GenBank accession number, L13616) (50), which included the domain required for FAK localization to focal adhesions (23). The five cDNAs represented two alternatively spliced forms of FAK, each possessing a single polyproline motif for SH3 binding, with two clones (clones 1A and 1B) containing a motif at amino acids 705 to 725, and the remainder containing a motif at amino acids 859 to 886 (clones 2A to 2C) (Fig. 6A). Subsequent tests to confirm the specificity of FAK interaction indicated that both FAK polyproline domains interacted strongly with the SH3 domain of HEF1 but not at all with the SH3 domain of the Abl-related protein Arg (29) or the nonspecific control protein RPB7 (Table 1).

To confirm the association of HEF1 and FAK in cells, we again performed immunoprecipitations from *v-abl*-transformed NIH 3T3 cells with antibodies to HEF1, p130^{cas}, and FAK. Subsequent visualization of precipitated proteins with antibody to FAK indicated that substantial quantities of FAK were associated with HEF1 (Fig. 6B).

HEF1 localizes to the cell periphery and to the nucleus. Previous immunofluorescence studies of p130^{cas} have localized this protein to focal adhesions and stress fibers in some cell

types (40), although some data suggest that some p130^{cas} may also be found in the nucleus or cytosol under particular growth conditions (49). To check whether these previous results were affected by cross-reactivity of p130^{cas}-directed antibody with HEF1 and to establish the intracellular localization of HEF1, we used the α -HEF1-SB antibody to visualize HEF1 in *v-Crk*-transformed 3Y1 cells and in HeLa cells (Fig. 7).

This analysis suggested that HEF1 possesses a more complex pattern of intracellular localization than does p130^{cas}. In 3Y1 fibroblasts transformed with *v-Crk*, predominant staining of p130^{cas} was at focal adhesions (Fig. 7B). In contrast, HEF1 staining was strongly nuclear and in some cells appeared to be present in the Golgi apparatus; additional, much weaker staining was observed in a pattern similar to p130^{cas} at focal adhesions but was difficult to photograph because of the intensity of nuclear staining (Fig. 7C). In HeLa cells, while nuclear staining was still present, it was also possible to readily observe large focal structures at the cell periphery. Although these were to some degree coincident with paxillin staining (a focal adhesion marker protein [Fig. 7E]), they were not identical but formed larger clustered structures located primarily in lamellipodia (Fig. 7F). In contrast, repeated attempts to stain HeLa cells with p130^{cas} yielded only a faint, diffuse staining pattern throughout the cell (29a). Finally, as controls, we have determined that the staining patterns of α -HEF1-SB antiserum are blockable by inclusion of the HEF1-SB peptide in primary antibody incubations, and similar patterns of HEF1 localization were obtained with the second HEF1-specific antibody α -HEF1-CT, although background staining was higher when this antibody was used (29a).

FIG. 6. HEF1 associates with FAK. (A) Polyproline sites contained within the isolated FAK cDNAs. Motif 1, located at amino acids 712 to 723, is derived from two cDNAs, 1A and 1B, containing residues 704 to 815 of FAK. Motif 2, located at amino acids 867 to 882, is derived from two cDNAs containing residues 782 to 1049 of FAK (2B and 2C) or one cDNA (2A), containing residues 809 to 1049 of FAK. (B) Coimmunoprecipitation of FAK and HEF1. *v-abl*-transformed NIH 3T3 cells were immunoprecipitated with protein A-Sepharose beads (lane 1) or antibody to HEF1 (α -HEF1-CT) (lane 2), p130^{cas} (lane 3), or FAK (lane 4). Antibody to FAK was subsequently used to visualize immunoprecipitated proteins. The species at approximately 125 kDa is FAK.

DISCUSSION

We have used the enhancement of pseudohyphal growth as a screen to identify a novel gene, *HEF1*, and have performed a series of initial functional characterizations of *HEF1*. We have found that the HEF1 protein sequence and domain structure are conserved with the recently described protein p130^{cas}, with both proteins possessing an amino-terminal SH3 domain, a central domain containing multiple potential SH2-binding sites, and a carboxy terminal novel domain. In contrast to p130^{cas}, HEF1 appears to be transcribed in a tissue-specific manner. We demonstrate that the carboxy-terminal domain of p130^{cas} conserves the function of enhancing pseudohyphal growth and show that this region is capable of mediating homo- and heterodimerization between HEF1 and p130^{cas}. HEF1 is hyperphosphorylated in *v-abl*-transformed cells and coprecipitates with *v-Abl*. A two-hybrid library screen with the HEF1 SH3 domain generated only two potential partner proteins, one uncharacterized and the second FAK; the nature of the clones isolated allowed identification of two distinct polyproline-containing sites on FAK permitting interaction with HEF1; and we were able to confirm the association of endogenous FAK and HEF1 in cultured cells by immunoprecipitation. Finally, HEF1 protein localizes in cells to both the

nucleus and the cell periphery, with particular concentration in lamellipodia. In sum, our data suggest that the HEF1 protein may function as an intermediary between signalling and cell polarity controls.

Initially described as a protein becoming hyperphosphorylated following *src* transformation (42), p130^{cas} was later independently identified and cloned as a predominant phosphorylated protein species following *crk*-mediated cellular transformation (49). Recent studies have demonstrated direct phosphorylation of p130^{cas} by Src and Abl (35, 49), while the large number of SH2-binding sites on p130^{cas} suggested that it may be a major intermediate in signalling by still more tyrosine-based oncogenes. The centrality of p130^{cas} function in transformation of some cell types is supported by findings that expression of antisense constructs of p130^{cas} in *ras*- or *src*-transformed NIH 3T3 cells is sufficient to cause cells to revert morphogenetically to a flat cell phenotype (4). A second group of studies have focused on the role of p130^{cas} in control of cell morphology in normal cells. p130^{cas} was found to be phosphorylated during integrin-mediated adhesion to extracellular matrix, along with other cytoskeletal proteins including FAK, paxillin, tensin, and cortactin (40, 55). Immunolocalization revealed that p130^{cas} is situated predominantly at focal adhesions and along adhesion-proximal regions of stress fibers (40). Very recently, a two-hybrid screen with the SH3 domain of FAK isolated p130^{cas} as a major interacting protein (41). Current models hypothesize that p130^{cas} acts together with paxillin, tensin, and talin to coordinate stable connections between the transmembrane protein integrin and the actin cytoskeleton: as such, altered regulation of p130^{cas} function by oncogenic phosphorylation would play a central role in establishing the characteristically altered cell morphologies of normal versus transformed cells.

While the degree of conservation we have observed between p130^{cas} and HEF1 suggests generally similar modes of function for the two proteins, we have observed several differences that may be significant. First, the degree of pseudohyphal conversion induced by the HEF1 C terminus is substantially greater than that induced by the p130^{cas} C terminus (Fig. 1). Second, the level of the HEF1 transcript appears to be specifically elevated in placenta, kidney, lung, and colon tissue (Fig. 3) (29a). These tissues contain large populations of polarized epithelial cells: HEF1 may be required for establishment and maintenance of apical-basal polarity. Third, the HEF1 cDNA specifically lacks polyproline sequences located downstream of the SH3 domain in p130^{cas}. As these polyprolines might in turn represent the binding site for the SH3 domain of yet another interacting protein, this suggests that HEF1 might not be subject to regulation by this hypothetical protein (Fig. 2A).

TABLE 1. Relative interaction strength of the LexA-fused HEF1 SH3 domain with activation-domain-fused FAK clones^a

AD fusion	Strength of LexA fusion ^b		
	HEF1-SH3	Arg-SH3	RBP7
FAK-1A	1,360	6	1
FAK-1B	1,372	6	1
FAK-2A	463	5	1
FAK-2B	1,152	9	1
FAK-2C	821	3	1
Vector	2	3	1

^a Assays were performed as described in the text. LexA-Arg-SH3, LexA-RBP7, and the activation domain fusion vector pJG4-5 were used as nonspecific controls.

^b Results are given as β -galactosidase units.

FIG. 7. HEF1 localizes to the cell periphery and to the nucleus. 3Y1 cells transformed with *v-crk* were plated on coverslips and fixed, and immunofluorescence was performed with either control biotin-conjugated secondary antibody (A), antibody to HEF1 (α -HEF1-SB) (B), or antibody to p130^{cas} (C). HeLa cells were plated and fixed, and immunofluorescence was performed with either control biotin-conjugated secondary antibody (D), antibody to HEF1 (α -HEF1-SB) (E) or antibody to paxillin to visualize focal adhesions (F). The diffuse staining in and around the nucleus in HeLa cells with paxillin antibody is due to nonspecific activity of the secondary antibody and is greatly diminished or absent when different preparations of secondary antibody are used.

Fourth, while assignment of a polyproline site on FAK as a specific target of the p130^{cas} SH3 domain is in general agreement with our results (41), the HEF1 SH3 domain clearly can interact with near-comparable affinity with two distinct FAK polyproline sites (Fig. 6A; Table 1). Whether this reflects a difference in the constructions used for the two screens or an

actual difference between the specificity of interaction of HEF1 and p130^{cas} with FAK remains to be determined. Fifth, immunohistochemical staining of fibroblasts and epithelial cells highlights the nonidentical intracellular localization of these two proteins. While both HEF1 and p130^{cas} are clearly present in peripheral focal structures, HEF1 is also abundant

in the nucleus. The peripheral staining of HEF1 is not exactly identical to that of p130^{cas}; stained structures are larger and appear to be enriched in lamellipodia. HEF1 peripheral staining is more pronounced in HeLa cells and other epithelial cell lines than it is in v-*crk*-transformed 3Y1 fibroblasts and other fibroblast lines such as NIH 3T3 cells (Fig. 7) (29a), again suggesting a possible epithelial cell-specific function. In this context, there have been a number of recent reports on Rac and Rho, two small GTPases originally defined by studies in fibroblasts as regulators of membrane ruffles and stress fibers, respectively (44, 45). Subsequent work has suggested that the genes encoding these proteins may function somewhat differently in epithelial cells than in fibroblasts (39, 43), depending on the interaction with different signaling-pathway components present in these diverse cell types.

Our data suggest the possibility that HEF1 and p130^{cas} will homodimerize and heterodimerize in vivo. In this work, we have demonstrated the association of the carboxy-terminal regions of these proteins by the two-hybrid approach and by coimmunoprecipitation of bacterially expressed GST and MBP fusion proteins. At minimum, this suggests that one function, although probably not the only function, of this carboxy-terminal domain may be to act as a dimerization motif. However, use of the α -HEF1-SB antibody to precipitate endogenous HEF1 followed by visualization with antibody to p130^{cas} did not reveal coprecipitation of these proteins in vivo (Fig. 5). Whether this reflects nondimerization under physiological conditions or lack of sensitivity of the assay to detect the association of endogenous proteins remains to be determined. Our intracellular localization of HEF1 and p130^{cas} indicates that the proteins share some cellular locations, making association in vivo a possibility. At minimum, these results imply that homodimerization and potentially higher-order oligomerization are possible for HEF1 and p130^{cas}, thus increasing the number of associated proteins that may be present in a single extended signaling complex. Further, these data suggest that reevaluation of studies on p130^{cas} expression and modification might be useful, because three separate antibodies generated against p130^{cas} cross-reacted with HEF1 and the two proteins migrated with nearly identical molecular weights, making it likely that the species immunoprecipitated may include some proportion of HEF1.

Is the ability of the HEF1 C-terminal domain to induce pseudohyphae in *S. cerevisiae* likely to be relevant, based on the identification of HEF1 as a p130^{cas}-related protein? Studies in progress to overexpress the HEF1-C domain and full-length HEF1 protein in mammalian cells will provide a more definitive answer to this question. However, a number of recent studies have highlighted multiple areas of conservation between the control of mammalian and yeast cytoskeletal architecture (reviewed in references 11, 27, and 56). Budding in *S. cerevisiae* requires asymmetric formation of actin filaments, and some genes whose mutation leads to defective actin cytoskeletal formation are known to be homologs of or related to mammalian focal adhesion proteins (reviewed in reference 56). Significantly, conversion from vegetative to pseudohyphal budding has been shown to be accompanied by constitutive maintenance of a polarized actin cytoskeleton ending in an actin cap at the bud tip (28).

While many classes of proteins, including kinases, phosphatases, GTPases, and other regulatory proteins, have been shown to contribute to pseudohyphal growth (6, 7, 15, 24, 33, 34), current data are most compatible with the view that HEF1, together with p130^{cas}, define a new family of docking proteins that, through multiple associations with signaling molecules, coordinate actin cytoskeletal regulation. In this hypothetical

model, the HEF1-C domain acts as an effector module to enhance actin cytoskeletal polarization while the amino-terminal SH3 domain would anchor FAK to the complex. The extensive SH2-binding domain would negatively regulate the function of HEF1-C while unphosphorylated; phosphorylation on tyrosine due to receipt of growth signals from phosphotyrosine kinases such as Src and Abl would induce HEF1-C to stimulate cell polarization. Such a process might well be important for the acquisition of invasive characteristics by transformed malignant cells. In this context, it is interesting that *Rap1/Krev-1* (26), a functionally conserved human homolog of the yeast *BUD1/RSR1* gene (5, 48), was recently identified as a target for the Crk-associated factor C3G (18), implying cross talk between Crk-directed tyrosine kinase signalling and Rap1-dependent morphological control that might well involve proteins such as p130^{cas} and HEF1. Whatever the mechanism of function, identification of HEF1 by means of a screen for enhancement of pseudohyphal formation in *S. cerevisiae* validates this approach for identifying important mammalian growth regulatory proteins and reemphasizes the parallels between growth control systems in *S. cerevisiae* and higher eukaryotes.

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