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Proteolysis of the Docking Protein HEF1 and Implications for Focal Adhesion Dynamics

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The dynamic regulation of focal adhesions is implicated in cellular processes of proliferation, differentiation, migration, and apoptosis. The focal adhesion-associated docking protein HEF1 is cleaved by caspases during both mitosis and apoptosis. Common to both of these cellular processes is the loss of focal adhesions, transiently during mitosis and permanently during apoptosis. The proteolytic processing of HEF1 during both mitosis and apoptosis therefore posits a general role for HEF1 as a sensor of altered adhesion states. In this study, we find that HEF1 undergoes proteolytic processing specifically in response to cellular detachment, while HEF1 proteolysis is prevented by specific integrin receptor ligation and focal adhesion formation. We show that overexpression of a C-terminal caspase-derived 28-kDa HEF1 peptide causes cellular rounding that is demonstrably separable from apoptosis. Mutation of the divergent helix-loop-helix motif found in 28-kDa HEF1 significantly reduces the induction of apoptosis by this peptide, while deletion of the amino-terminal 28 amino acids of 28-kDa HEF1 completely abrogates the induction of apoptosis. Conversely, these mutations have no effect on the rounding induced by 28-kDa HEF1. Finally, we detect a novel focal adhesion targeting domain located in the C terminus of HEF1 and show that this activity is necessary for HEF1-induced cell spreading. Together, these data suggest that proteolytic and other posttranslational modifications of HEF1 in response to loss of adhesion serve to modulate the disassembly of focal adhesions.

Focal adhesions possess a dual function as points of structural linkage between the extracellular matrix (ECM), transmembrane integrin receptors, and the internal cytoskeleton and as sensors of the extracellular environment that transduce signals controlling cellular decisions to proliferate, differentiate, or undergo apoptosis. Dynamic regulation of focal adhesion components is required for a number of different cellular functions. For example, at the approach to the mitotic phase of the cell cycle, cells round up and decrease adhesion to the ECM, with replacement of attachments synchronized to the process of cytokinesis and reentry into G_1. During migration, cells must rapidly break down and reform adhesions with the ECM (31). The formation of novel integrin/ECM interactions can specify cellular differentiation by activating specific signaling cascades, culminating in the induction of differentiation-promoting transcription factors and in parallel enforcing removal from the cell cycle (7). The importance of attachments for normal function of primary cells is emphasized by the fact that in many cell types, sustained loss of adhesion is a sufficient stimulus to induce apoptosis, in a process known as anoikis (13). Hence, one frequent effect of oncogenic transformation is the circumvention of the adhesion-viability coupling, leading to acquisition by cancer cells of the ability to grow in an anchorage-independent manner (50). Based on these diverse biological roles, there has been considerable research directed at elucidating the role of focal adhesions in integrin-mediated adhesion (49). Presently, the issue of how modulation of focal adhesion components may differentially signal to the nucleus in cases of transient versus permanent loss of adhesion is of particular interest.

One mechanism that seems likely to play an important role in communicating cellular adhesion status to the nucleus is the transient or permanent posttranslational modification of focal adhesion components. A particularly well-studied example is that of focal adhesion kinase (FAK). The in vitro data suggesting that regulation of FAK activity controls apoptosis (15, 22, 57) has an intriguing in vivo corollary in the observation that FAK is cleaved by caspases during the process of terminal detachment occurring in apoptosis (36, 55). Caspase cleavage separates two FAK functional domains, the kinase domain and the C-terminal focal adhesion targeting (FAT) domain. In normally growing adherent cells, exogenously expressed FAK C-terminal peptides corresponding to the caspase cleavage products act as dominant negatives on the full-length FAK molecule by inhibiting phosphorylation of FAK (16). The fact that this peptide causes cell rounding (36, 58, 59) and apoptosis (3, 58) suggests that the FAK cleavage products produced in apoptosis may play an active role in advancing the process of cell death by promoting focal adhesion disassembly. In particular, an interaction between FAK and one or more molecules which interact with the polyproline motif is essential for prevention of apoptosis (3). To date, only Cas proteins and Graf (49) have been identified as interacting with the FAK polyproline motifs, potentially implicating them in this control process. Finally, during more transient disruptions of cell attachment, such as occur in cell cycle progression, phosphorylation of FAK on serine and threonine residues has been identified and proposed to prevent the interaction of FAK with other signaling molecules during the cell rounding that accompanies mitosis (60). In sum, these results indicate that discrete modes of

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FAK modulation can result in either reversible or irreversible focal adhesion loss.

The Cas family proteins, p130Cas (47), Efs/Sin (2, 25), and HEF1/CasL (32, 41), are a family of docking proteins which, among other roles, serve as important intermediates in creating signaling complexes at sites of focal adhesion (46). These proteins have a conserved overall domain structure consisting of an amino-terminal Src homology 3 (SH3) domain utilized for interaction with polyproline-containing partners, a substrate domain containing multiple tyrosine residues that upon phosphorylation mediate interaction with SH2 domain-containing proteins, and a highly conserved C terminus that has been proposed to mediate homo- and heterodimerization among the family members (46) and may mediate interaction with other molecules (48). Cas proteins can localize to focal adhesions via interactions between their amino-terminal SH3 domains and a polyproline stretch in the C terminus of FAK (43). However, it has further been reported that a region in the C terminus of p130Cas contributes to the localization of p130Cas at focal adhesions in transformed cells (43). FAK coexpression with a truncation of p130Cas lacking the SH3 domain results in apoptosis (3). Combined with the report that mutation of the FAK polyproline motif mediating interaction with Cas proteins generates an apoptotic response, it appears that regulation of the FAK-Cas association is essential for cellular viability.

Finally, recent observations that a number of adhesion complex components undergo caspase-mediated cleavage during apoptosis suggest that this may be a common mechanism for the down-regulation of adhesion-mediated signals and may actively promote the progression of cell death. Caspase cleavage targets at adhesion complexes include adherens junctions proteins (5, 20), Cbl and Src (56), and the Cas proteins p130Cas (5, 28, 33) and HEF1 (33). Strikingly, HEF1 is not only cleaved during apoptosis but also at mitosis in a reaction targeting two distinct caspase-sensitive sites (a DLVD motif at amino acids [aa] 360 to 363 and a DDYD motif at aa 627 to 630), and the processing of the peptides released following caspase cleavage differs in mitosis and apoptosis. Caspase cleavage of HEF1 in mitosis produces a stable 55-kDa N-terminal peptide that localizes to the mitotic spindle (35) and unstable 65- and 28-kDa C-terminal peptides that are rapidly degraded (33, 35). In apoptosis, the 28-kDa peptide is stabilized and is proposed to contribute to the apoptotic progression, based on the identification of a potent activity in inducing cell death associated with exogenous production of the species in normally growing cells (33). At present, the pathways controlling the alternative processing of HEF1 in mitosis and apoptosis are unknown, as is the method of 28-kDa HEF1 in inducing its phenotypes. However, one particularly interesting possibility is that the promotion of cell death by this species may be integrally associated with induction of cellular detachment.

Considering the role of HEF1 at focal adhesions (46), cleavage of HEF1 during mitosis (35) and apoptosis (33), and the induction of apoptosis in cell lines constructed to overexpress inducible HEF1 (33), we have explored the control of HEF1 proteolysis in response to changes in cellular adhesive status. Additionally, we have probed the relationship between cell-rounding effects and apoptosis related to 28-kDa HEF1 expression. We find that HEF1 is dephosphorylated and proteolytically cleaved following culture of MCF7 cells in serum-free medium (SFM), under conditions that inhibit cell attachment. HEF1 is protected from this cleavage by attachment to solid support prior to serum removal and by stimulation of integrin receptors via binding to either fibronectin or laminin attached to a solid matrix but not by treatment of suspended cells with soluble integrin ligands. Cleavage of HEF1 is preceded by loss of hyperphosphorylated HEF1. HEF1 cleavage is demonstrated to occur in the absence of detectable apoptosis, therefore separating the two processes. Further, we show here that cell rounding induced by overexpression of the 28-kDa HEF1 occurs even in the presence of the apoptosis inhibitor z-VAD-fmk, providing additional support for the idea that HEF1-dependent rounding and death are separable. Finally, we demonstrate that discrete mutations of the 28-kDa peptide can be generated which differently impact rounding and apoptosis. Mutation of a predicted divergent helix-loop-helix (dHLH) domain in the C terminus of HEF1 (33) significantly reduces apoptosis induction, while deletion of the amino-terminal 28-aa residues of 28-kDa HEF1 completely abrogates apoptosis induction, yet none of these mutations affect rounding. We further show that the region of 28-kDa HEF1 that stimulates rounding is located in the C-terminal end of the peptide. We identify a novel focal adhesion localizing activity in the C-terminal region corresponding to 28-kDa HEF1; deletion of this region prevents HEF1-mediated cell spreading. Based on these findings, we propose that regulation of the posttranslational modification of HEF1 results in production of a complex mixture of HEF1 isoforms which collaborate to control cell morphology, attachment, and viability.

MATERIALS AND METHODS

Cell lines, antibodies, and materials. Except where indicated, MCF7 breast carcinoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. The anti-HEF1 rabbit polyclonal antibody has been previously described (anti-HEF1-SB) (32). Other antibodies used include anti-p130Cas (here labeled anti-HEF1/2, as the antibody cross-reacts with the carboxy-terminal domain of both HEF1 and p130Cas; see Fig. 4B); antipaxillin antibodies from Transduction Laboratories (San Diego, Calif.); antiphosphotyrosine clone 4G10 from Santa Cruz (Santa Cruz, Calif); anti-β1 integrin antibody clone P4C10 from Gibco BRL (Rockville, Md.); rhodamine-conjugated goat anti-mouse antibodies from Molecular Probes (Eugene, Oreg.); and anti-mouse immunoglobulin and anti-rabbit immunoglobulin horseradish peroxidase conjugates from Amersham Pharmacia Biotech (Piscataway, N.J.). Fibronectin, poly(2-hydroxyethyl methacrylate) (poly-HEMA), laminin, poly-L-lysine, protein A-Sepharose, and 4′,6′-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, Mo.). Transfection reagent Lt1 was obtained from Mirus (Madison, Wis.).

Expression constructs. The vector pEGFP-C4 (Clontech, Palo Alto, Calif.) was used to create fusion protein expression constructs consisting of green fluorescent protein (GFP) fused to the amino terminus of HEF1 peptides (see Fig. 4A). Expression constructs encoding full-length HEF1 (pGFP.HEF1, aa 1 to 834) and the 28-kDa HEF1 peptide (pGFP.28, aa 626 to 834) have been previously described (33). Additional HEF1 mutant constructs containing lysine-to-proline amino acid substitutions in the first helix (pGFP.H1 [aa 626 to 834, L722P]) and in the second helix (pGFP.H2 [aa 626 to 834, L751P]) and encoding peptides corresponding to truncated forms of 28-kDa HEF1 (pGFP.M_{654} [aa 654 to 834], pGFP.28ΔCT [aa 626 to 693], pGFP.M_{654}ΔCT [aa 654 to 693], and pGFP.28ΔNH2 [aa 695 to 834]) were prepared by subcloning *Eco*RI-*Xho*I cDNA inserts from previously described clones (34) into *Eco*RI-*Xho*I-digested pEGFP-C4 vector. The construct containing mutations in both helices, pGFP.H1H2, was created using pGFP.H1 as the template and standard protocols for mutational PCR to create the second mutation. The presence of both mutations was confirmed by DNA sequence analysis and the resulting PCR

fragment, constructed to contain 5′ *Eco*RI and 3′ *Xho*I sites, was ligated with *Eco*RI-*Xho*I-digested pEGFP-C4 DNA. Finally, a mutant construct in which the C-terminal region corresponding to the 28-kDa peptide was deleted (pGFP.HEF1ΔCT, aa 1 to 653) was created by carrying out PCR on a full-length HEF1 template with primers that correspond to the start site of HEF1 and the sequence located at $KELLIKENI_{653}$ followed by an in-frame stop codon. Again, PCR was designed to create 5′ *Eco*RI and 3′ *Xho*I sites. The PCR fragment was therefore ligated with *Eco*RI-*Xho*I-digested pEGFP, and the resulting clones were confirmed by DNA sequence analysis. Expression by constructs of proteins of an appropriate molecular weight was confirmed by Western blotting. Relative levels of fusion protein expression were determined by visual examination with a fluorescence microscope and by scoring GFP-positive cells for fluorescence (scale, 1+ to 4+, with 4+ representing strongest signal). Values reported represent the percentage of cells scored with each level of fluorescence.

Plating cells on different extracellular matrices. To test the effects of integrin receptor ligation, cells were plated on tissue culture dishes coated with different ECM components. Dishes (100-mm diameter) were coated with 2 μg of fibronectin/cm^2 diluted in 5 ml of phosphate-buffered saline (PBS), 2.5 μg of laminin/cm^2 diluted in 5 ml of PBS, and 7 μg of poly-L-lysine/cm^2 in 5 ml of distilled water. After 5 min, plates were rinsed with distilled water, 3 μg of anti-β1 integrin antibodies/cm^2 kept in 1 ml of PBS at 4°C overnight, and 80 μg/cm^2 poly-HEMA diluted in 5 ml of 95% ethanol. All coated plates were air dried overnight and rinsed twice with PBS before use. For suspension cultures, 5 ml of media was supplemented with either 150 μg of fibronectin or 200 μg of laminin. Prior to plating, MCF7 cells were grown to ~80% confluence. Cells were then detached by treatment with 3 mM EDTA in PBS and collected by centrifugation. Pelleted cells were resuspended in either DMEM containing 10% FBS or in unsupplemented DMEM (SFM), and ECM components were added as indicated.

Immunoprecipitation. Cell lysates (250-μg aliquots) were incubated with 2 μg of antipaxillin antibodies for 2 h at 4°C. Next, 20 μl of a 50% slurry of protein A-Sepharose prepared in A-PTY buffer (50 mM HEPES [pH 7.5], 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, and 10 mM $Na_4P_2O_7$ supplemented immediately before use with 1 mM phenylmethylsulfonyl fluoride, 0.01-mg/ml aprotinin, 0.01-mg/ml leupeptin, and 1 mM Na_3VO_4) was added, and reaction mixtures were incubated overnight at 4°C. On the following day, protein A-Sepharose beads were collected by centrifugation at 20,000 × *g* for 15 s and were washed three times with A-PTY lysis buffer, and immunoprecipitated proteins were released from the beads by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer for 10 min.

Preparation of cell lysates and Western blot analysis. Cell lysates were prepared by extraction with A-PTY buffer. For adherent cultures, only those cells adhering to the plate were extracted. For cells grown on poly-HEMA-coated dishes, suspended cells were collected by centrifugation of the media and were then extracted with A-PTY buffer. Total protein concentrations were determined using the bicinchoninic acid protein determination kit (Pierce), and equivalent concentrations of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots were probed with anti-HEF1 antibodies at a dilution of 1/100, and all other antibodies were used at the manufacturer's recommended dilutions. Chemiluminescence detection was performed as described elsewhere (35). We note that expression of each HEF1-derived construct was confirmed by Western analysis and that each construct was shown to produce a protein of the predicted size. Because of the differing capacity of the constructs to induce apoptosis, resulting in very different apparent transfection efficiencies, it was difficult to effectively normalize expression levels between populations.

Transfection, caspase inhibition, and immunofluorescence. MCF7 cells for transfection were plated onto microscope coverslips in 6-well tissue culture dishes 24 h prior to transfection. Cells were then transfected with plasmid DNA (at a concentration of 0.1 μg/μl) in OptiMEM (Mirus) as described by the manufacturer. The caspase inhibitor z-VAD-fmk was added at a concentration of 25 μM following aspiration of the transfection complexes and remained in the culture medium for the duration of the incubation period. Approximately 18 h after a change to DMEM plus 10% FBS, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS with 0.5% bovine serum albumin, and stained with 0.1 μg of DAPI/ml in PBS with 0.5% bovine serum albumin. Detection of focal adhesions by immunofluorescence analysis was carried out on MCF7 cells grown on coverslips for 24 h in the presence of serum or SFM as indicated. Cells were fixed and permeabilized as above and were then stained with antipaxillin antibodies (1/800 dilution). Bound antibodies were detected by probing with rhodamine-labeled anti-mouse secondary antibodies. Images were prepared using a fluorescence microscope (Nikon TE800) and charge-coupled device camera or by using the Bio-Rad 600 laser scanning confocal microscope.

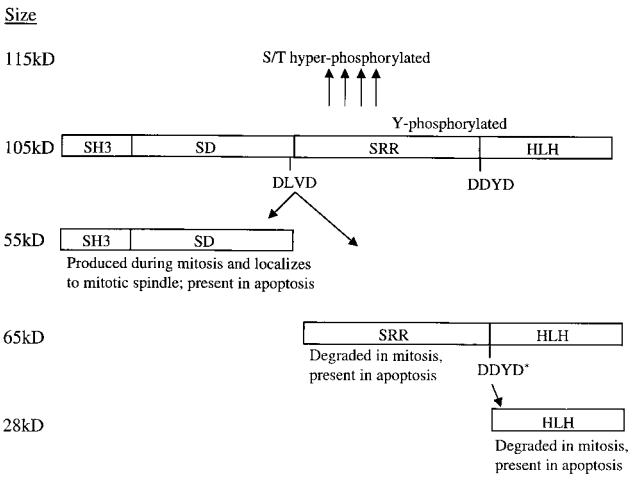


FIG. 1. Posttranslational modifications of HEF1 produce multiple HEF1 isoforms. Shown is a schematic representation of the HEF1 isoforms, based on work presented elsewhere (32, 34, 35). Indicated domains of HEF1 include an SH3 domain, a substrate binding domain (SD) that includes numerous tyrosine residues that upon phosphorylation mediate interaction with SH2 proteins, a serine-rich region (SRR), and a conserved carboxy terminus encompassing a dHLH domain. The antibody anti-HEF1 reacts with an epitope in the SD, while the antibody anti-HEF1/2 reacts with an epitope in the carboxy-terminal dHLH domain. *, it is not known whether cleavage to produce 65-kDa HEF1 must precede production of 28-kDa HEF1 or whether 28-kDa HEF1 can be produced by cleavage from other, full-length HEF1 isoforms. Molecular masses are shown at left.

Analysis of cell rounding and apoptosis. Images of GFP-positive cells were captured with a charge-coupled device camera attached to a fluorescent microscope (Nikon TE800) under the 20× objective. The area (reported in pixels) of imaged cells was calculated for an average of 100 cells per transfection, which was carried out in triplicate for each construct, using ISee software (Inovision) to outline the perimeter of cells and calculate the area within the perimeter. Data are expressed as the mean ± the standard error of the mean. To determine the rate of apoptosis, transfected cells stained with DAPI were viewed under the 40× objective of a fluorescence microscope (Nikon TE800). Transfected cells, indicated by green fluorescence, were then scored for the presence of apoptotic nuclei, evidenced by small, condensed nuclei brightly stained with DAPI. The number of apoptotic nuclei is expressed as a percentage of the total nuclei scored; approximately 200 GFP-positive cells were scored per transfection. Values shown are the mean from six separate transfections ± standard error of the mean. Statistical significance between means was calculated using the Student *t* test.

RESULTS

HEF1 undergoes cleavage and is differentially phosphorylated in the absence of focal adhesions. Full-length HEF1 exists during interphase as two isoforms that are approximately 105 and 115 kDa and localizes to focal adhesions in adherent cells. The 115-kDa HEF1 protein is a hyperphosphorylated form of the 105-kDa protein (35), with the hyperphosphorylation induced by integrin engagement and other stimuli (46). In contrast to this profile of HEF1 expression in interphase cells, it has previously been shown that endogenous HEF1 is cleaved by a caspase-like activity during mitosis (35), as well as during apoptosis induced by tumor necrosis factor alpha treatment of MCF7 cells or induced by antibody cross-linking of WEHI 231 B-cells (33), at two caspase consensus sites, DLVD (aa 360 to 363) and DDYD (aa 627 to 630) (Fig. 1). During both mitosis

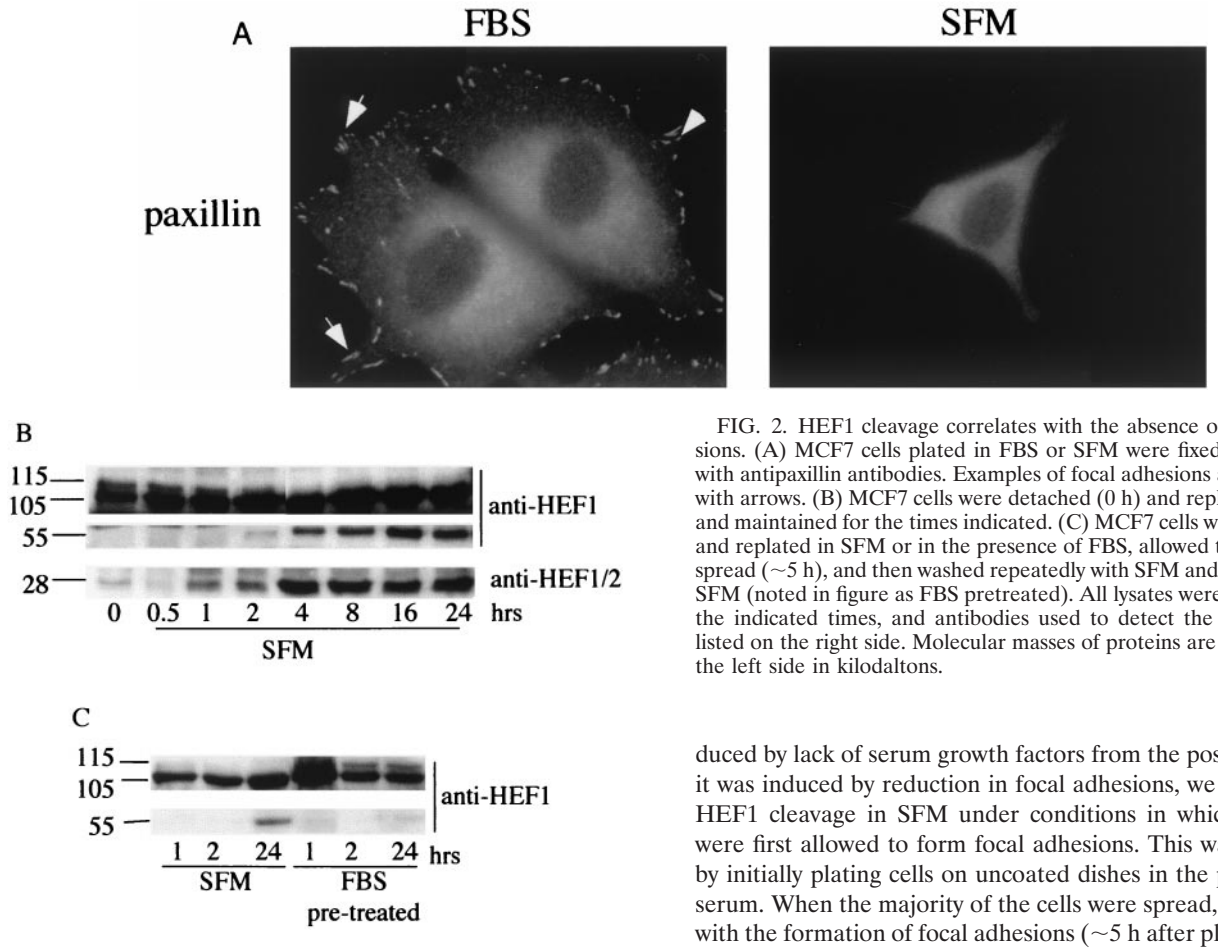


FIG. 2. HEF1 cleavage correlates with the absence of focal adhesions. (A) MCF7 cells plated in FBS or SFM were fixed and probed with antipaxillin antibodies. Examples of focal adhesions are indicated with arrows. (B) MCF7 cells were detached (0 h) and replated in SFM and maintained for the times indicated. (C) MCF7 cells were detached and replated in SFM or in the presence of FBS, allowed to attach and spread (~5 h), and then washed repeatedly with SFM and incubated in SFM (noted in figure as FBS pretreated). All lysates were extracted at the indicated times, and antibodies used to detect the proteins are listed on the right side. Molecular masses of proteins are indicated on the left side in kilodaltons.

and apoptosis, cells round up and decrease contact with the ECM (that is, have reduced focal adhesions), potentially indicating a convergent control mechanism in these two processes. This suggests that HEF1 may be cleaved under conditions where there is focal adhesion disassembly, functioning as an indicator or mediator of the cellular detachment process. To date, the processing of HEF1 in response to transient cellular detachment has been unexamined.

In light of the known cross talk between signals derived through integrin receptors and growth factor receptors (17), we chose to examine HEF1 posttranslational modifications, including phosphorylation and cleavage status, under conditions that allow the discrimination between these two signaling pathways. Initially, cleavage of endogenous HEF1 was assessed in MCF7 cells plated on uncoated tissue culture dishes in SFM. MCF7 cells plated under these conditions attached loosely but did not spread and form focal adhesions, evidenced by a rounded, light-refractory appearance under phase microscopy and by reduction in paxillin-positive focal adhesions as determined by immunofluorescence microscopy (Fig. 2A). Proteolysis of HEF1 was next examined by Western blot analysis of lysates from cells grown in SFM. HEF1 cleavage peptides are detected at 4 h after plating in SFM and continue to be detectable for at least 24 h (Fig. 2B), thereby confirming the proteolytic processing of HEF1 under SFM conditions.

To discriminate the possibility that HEF1 cleavage was induced by lack of serum growth factors from the possibility that it was induced by reduction in focal adhesions, we next tested HEF1 cleavage in SFM under conditions in which the cells were first allowed to form focal adhesions. This was achieved by initially plating cells on uncoated dishes in the presence of serum. When the majority of the cells were spread, correlating with the formation of focal adhesions (~5 h after plating), they were washed repeatedly with SFM and were then incubated in SFM for the indicated times. In contrast to control cells plated directly in SFM, there is no production of HEF1 cleavage products in the population of cells that were allowed to first form focal adhesions and were then grown in SFM (Fig. 2C, FBS pretreated). The absence of HEF1 cleavage products is maintained throughout continued incubation of preattached cells in SFM for up to 72 h (results not shown). We note that the serum-pretreated cells remained attached and spread on the surface of the tissue culture plate despite the absence of serum. Thus, prevention of HEF1 cleavage correlates specifically with the presence of multiple focal adhesions and a spread phenotype, rather than with the continued presence of serum in growth medium.

Separately, examination of MCF7 cell lysates shows an altered pattern of HEF1 phosphorylation in response to SFM. Lysates prepared immediately upon detachment from the tissue culture dish display both the 105- and 115-kDa isoforms (Fig. 2B, time zero). Following growth under SFM conditions, there is an early loss of hyperphosphorylated 115-kDa HEF1 (~2 h after plating), while the 105-kDa HEF1 form is detectable up to 24 h after treatment (Fig. 2B). Notably, loss of hyperphosphorylated 115-kDa HEF1 preceded cleavage (Fig. 2B). In contrast, there is maintenance of the hyperphosphorylated HEF1 115-kDa form in the cells that were allowed to first form focal adhesions by pretreatment with FBS (Fig. 2C). These results suggest that in the absence of focal adhesions,

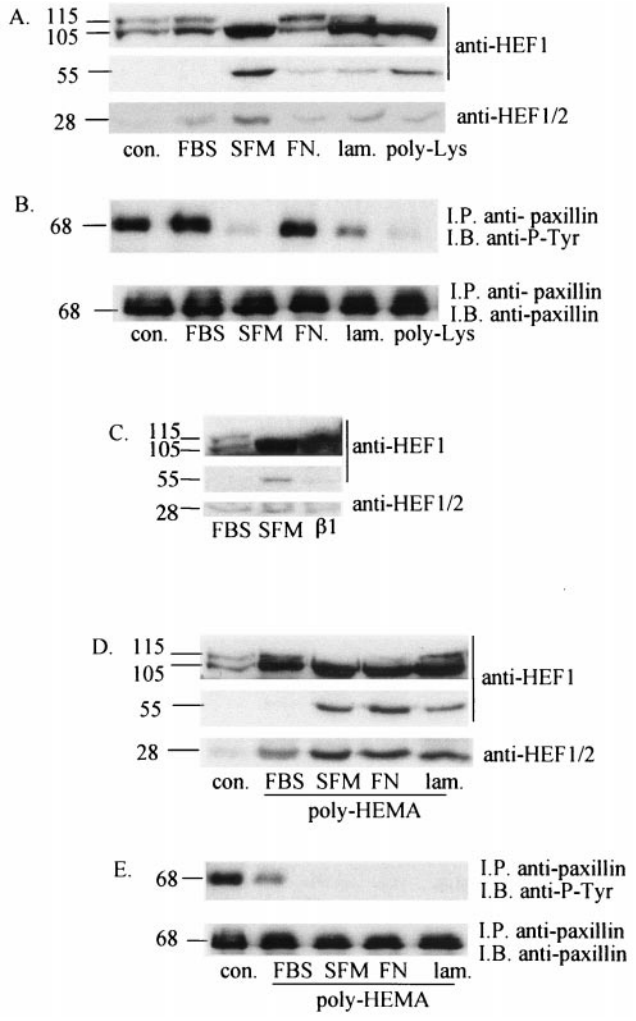


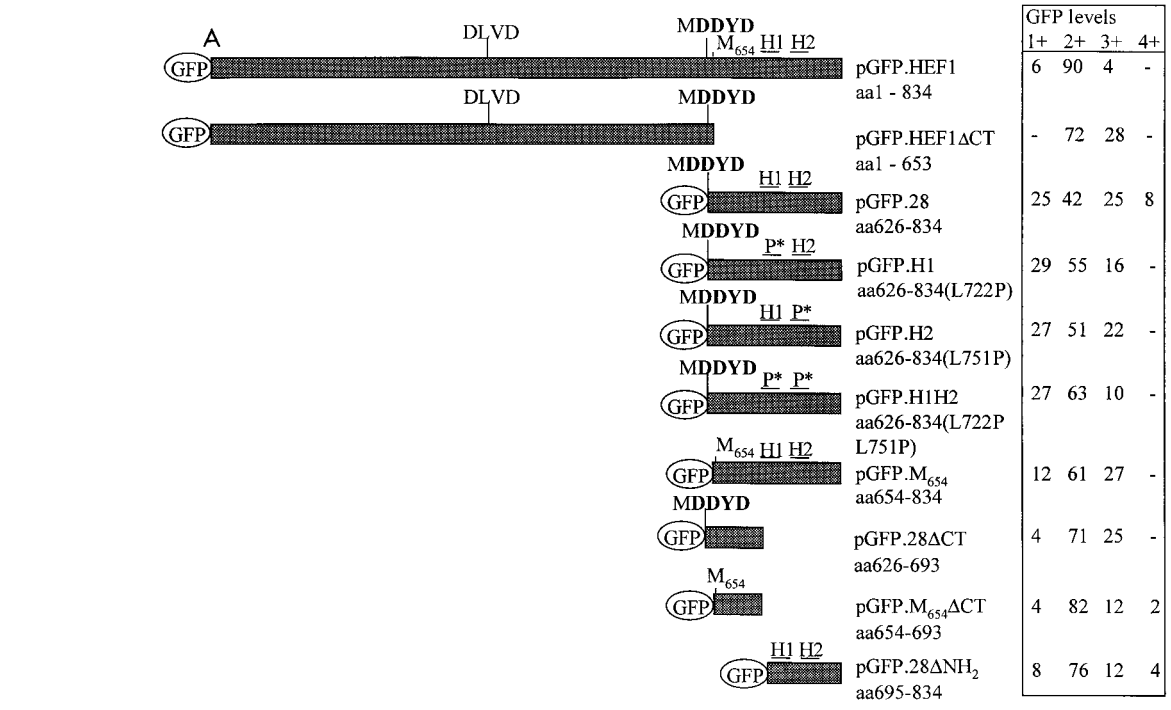
FIG. 3. Integrin receptor ligation prevents HEF1 cleavage. (A) MCF7 cells were plated as follows: untreated control (con.), in FBS, in SFM, and in SFM on plates coated with fibronectin (FN), laminin (lam.), and polylysine (poly-Lys). (B) Antipaxillin immunoprecipitates (I.P.) of cell lysates shown in panel A. P-Tyr, phosphotyrosine. (C) MCF7 cells were plated in SFM onto dishes coated with anti-β1 integrin antibody (β1) or onto uncoated dishes in the presence (FBS) or absence (SFM) of serum as indicated. (D) MCF7 cells were plated on dishes coated with poly-HEMA to prevent attachment, in FBS, SFM, and in SFM plus FN or lam. Extracts were also prepared from control cells grown on uncoated dishes in FBS (con.). (E) Antipaxillin immunoprecipitates of cell lysates shown in panel C. All cells were grown under indicated conditions for 24 h prior to extraction. Immunoblots were probed with antibodies indicated on the right side (I.B.). Molecular masses of proteins are indicated on the left side.

there is firstly a loss of HEF1 hyperphosphorylation followed by cleavage of HEF1. While it is possible that preferential cleavage of the hyperphosphorylated HEF1 form accounts for the early loss of this species, this seems unlikely, given that at 2 h after plating in SFM there is no detectable 115-kDa HEF1, while few or no corresponding HEF1 cleavage products are detectable (Fig. 2B and C). It is likely, therefore, that the 105-kDa HEF1 form may represent the preferred caspase substrate.

In sum, these results suggest that HEF1 cleavage is a consequence of focal adhesion reduction and that under these conditions there is an accumulation of the 105-kDa HEF1 isoform and of the caspase cleavage products.

Integrin receptor ligation prevents HEF1 cleavage. It has been demonstrated that both HEF1 (40) and p130Cas (45) undergo phosphorylation in response to integrin receptor ligation. Therefore, in light of the above data suggesting that the cleavage of HEF1 correlates with absence of the hyperphosphorylated form and focal adhesions, we decided to test whether integrin receptor stimulation could prevent the cleavage of HEF1. In a fashion similar to the experiments reported by Nojima et al. (45), employing SFM conditions facilitates adding back individual matrix components to assess the contribution of integrin receptor ligation to HEF1 proteolysis (45). Since MCF7 cells are epithelial, the effects of fibronectin and laminin, two matrix components reported to be bound by the integrin receptors on epithelial cells (53) and shown to stimulate HEF1 (39, 40) and p130Cas (45) phosphorylation, were studied. MCF7 cells were therefore plated in SFM on tissue culture dishes coated with fibronectin and laminin or on control plates coated with polylysine, which allows cell adherence via a non-integrin-mediated mechanism, and on uncoated plates, as described in the preceding section.

Growth of cells on either fibronectin or laminin reduced the production of HEF1 cleavage products compared with that of cells grown on control uncoated and polylysine-coated dishes (Fig. 3A). The detection of HEF1 cleavage products in cells grown on polylysine-coated plates indicates that the observed protective effects of fibronectin and laminin do not simply reflect nonspecific protection brought about by cellular adhesion but instead indicate that the protective effects are due to specific integrin receptor ligation. Additionally, following the observation that maintenance of hyperphosphorylated HEF1 correlates with absence of HEF1 cleavage, cell lysates were examined to determine whether this was also true following specific integrin receptor ligation. Under each treatment condition showing reduced HEF1 cleavage, there is maintenance of the 115-kDa HEF1 hyperphosphorylated form, albeit to a lesser extent in the cells grown on laminin than in those grown on fibronectin (Fig. 3A). To confirm that those cells displaying reduced HEF1 cleavage and maintenance of the 115-kDa hyperphosphorylated HEF1 isoform did indeed have focal adhesions, we assayed for phosphorylation status of paxillin, as paxillin phosphorylation has been shown to correlate with focal adhesion formation (9). In all treatments where there is little HEF1 cleavage, there is strong paxillin tyrosine phosphorylation (Fig. 3B). Conversely, under those conditions in which HEF1 cleavage products are detected, namely on uncoated dishes in SFM conditions and on dishes coated with polylysine, there is little detectable tyrosine phosphorylation in the immunoprecipitated paxillin (Fig. 3B). The lower levels of 115-kDa HEF1 in the cells grown on laminin additionally correlate with a lower level of paxillin tyrosine phosphorylation in these cells. Therefore, integrin receptor ligation by binding to either fibronectin or laminin and by formation of focal adhesions as detected by paxillin phosphorylation appears to prevent the cleavage of HEF1 in the absence of serum. The fact that cleavage occurs in cells grown on polylysine suggests that the protection is specific to integrin ligation, rather than a by-product of non-integrin-mediated adhesion. To further con-



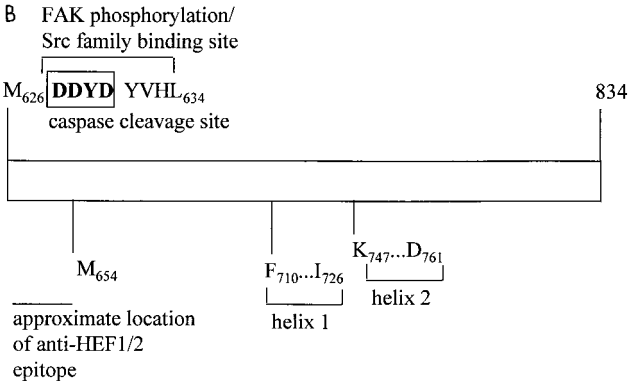


FIG. 4. HEF1 derivatives and sequence elements. (A) Schematic representation of GFP-HEF1 fusion constructs. Indicated are positions of the caspase cleavage sites (DLVD and DDYD), the first (H1) and second (H2) helices of the predicted dHLH motif, positions of leucine-to-proline mutations in these helices (P*), and the methionine residues (M immediately preceding the caspase cleavage site [MDDYD] at aa 626 and downstream at aa 654). Clone names and lengths in amino acids corresponding to the position in the wild-type sequence are indicated on the right. Relative expression of GFP fluorescence (scored in a range from 1+ to 4+, with 4+ reflecting the highest level of expression) is indicated for each construct in the right panel. Numbers represent the percentage of total cells scored exhibiting each level of fluorescence. =, no transfectants scored with the indicated levels of GFP fluorescence. (B) Detailed map of the cDNA sequence encoding 28-kDa HEF1. Shown is the caspase cleavage site, DDYD, which additionally constitutes part of the site of FAK phosphorylation and Src protein binding site. The approximate location of the anti-HEF1/2 antibody epitope is located between the two methionine residues, and the helices of the predicted helix-loop-helix motif are shown.

firm that the observed effects were due to integrin-mediated interactions with the ECM, we next stimulated the integrin receptors with immobilized anti-β1 integrin antibody, previously reported to cause receptor aggregation and activation (42). Plating MCF7 cells in SFM on immobilized anti-β1 integrin antibodies reduced the production of HEF1 cleavage products compared with that of cells grown on uncoated dishes in SFM (Fig. 3C), confirming that protection against HEF1 cleavage is integrin mediated.

It is clear that occupancy and clustering of integrin receptors can be functionally separated (42). From the above results it is not possible to determine whether HEF1 cleavage is prevented by simple receptor occupancy or whether it in fact requires the integrin receptor clustering that accompanies the formation of focal adhesions. To address this question, we treated cells in suspension with soluble matrix proteins, thereby preventing the formation of focal adhesions but allowing integrin receptor and ligand interaction. MCF7 cells were grown in dishes coated with poly-HEMA to prevent adhesion using established protocols (1, 13, 24); in media containing FBS; in SFM; or in SFM plus either fibronectin or laminin. Focusing on the 55- and 28-kDa HEF1 species to which we have previously ascribed mitotic (35) or apoptotic (33) functions, we determined that HEF1 is not cleaved to 55-kDa HEF1 in control cells grown in suspension in FBS-containing media, although we note that there is 28-kDa HEF1 evident under these conditions (Fig. 3D). In contrast, 55- and 28-kDa HEF1 cleavage products are detected in suspension cultures grown in SFM, despite the addition of soluble fibronectin and laminin (Fig. 3D). Again, we assessed levels of paxillin tyrosine phosphorylation and found corresponding loss of paxillin tyrosine phosphorylation in treatments resulting in production of the HEF1 cleavage products (Fig. 3E). We note that there is also some tyrosine phosphorylation of paxillin from cells grown in suspension in FBS, correlating with the lack of cleavage products detected in

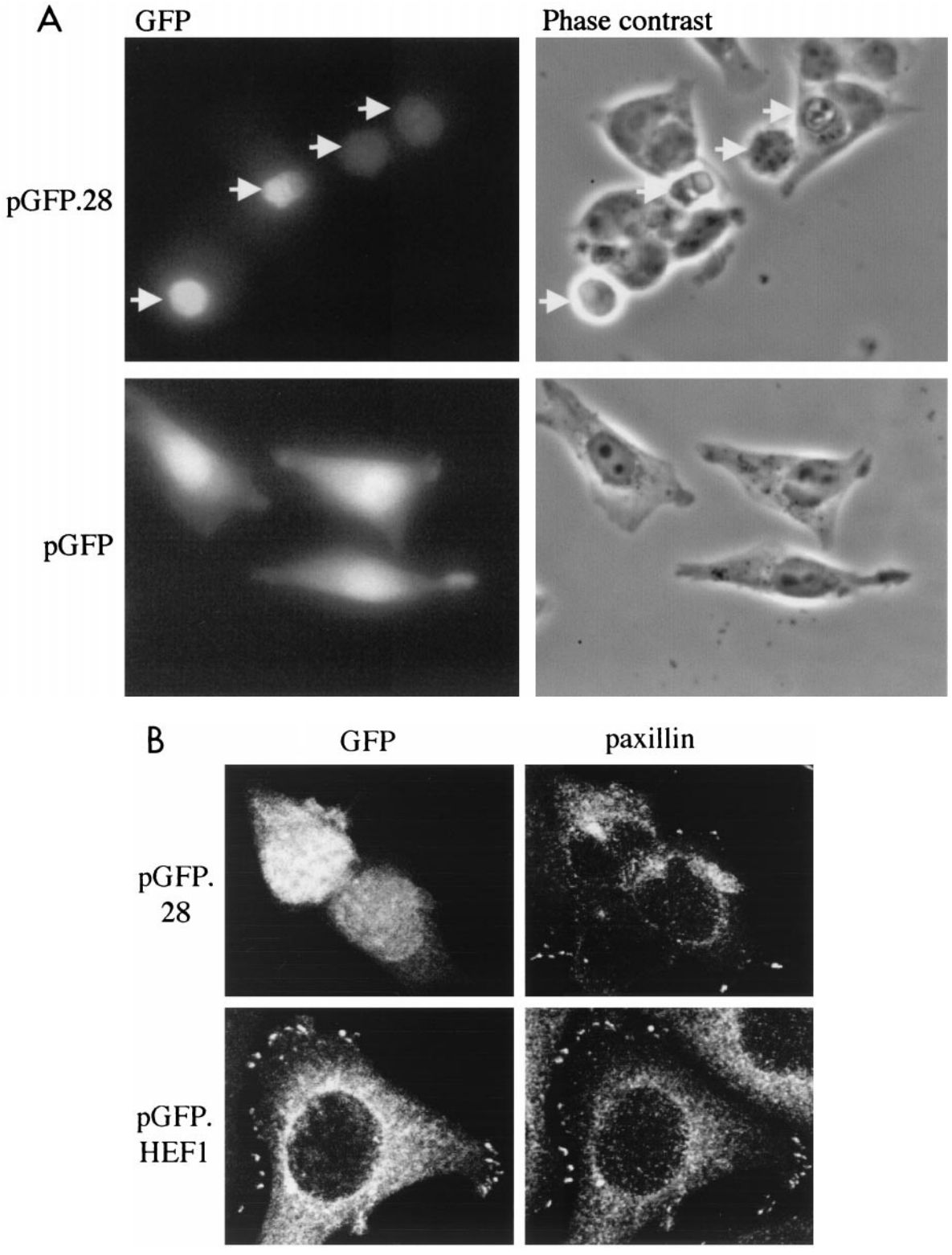


FIG. 5. The 28-kDa HEF1 causes cell rounding separable from apoptosis. (A) MCF7 cells transfected with pGFP.28 or pGFP vector alone. Shown are fluorescence (GFP) and phase contrast images of transfected cells. pGFP.28-transfected cells are indicated with arrows. (B) MCF7 cells transfected with either pGFP.28 or pGFP.HEF1 were coimmunostained with antipaxillin antibodies. Note the reduced number of paxillin-positive focal adhesions in the cells transfected with pGFP.28. (C) MCF7 cells were transfected with pGFP.28, pGFP.28 in the presence of z-VAD-fmk, or pGFP. The cell area (in pixels) of GFP-positive transfectants was calculated as described in Materials and Methods. As the pGFP.28 transfectants are round, the quantitation of a reduced area compared to the area of vector control transfectants is referred to as "rounding." (D) The same transfectants were assessed for apoptosis by examining the DAPI-stained nuclei of GFP-positive cells. Numbers of apoptotic nuclei are expressed as a percentage of the total nuclei examined, and data points were calculated as described in Materials and Methods.

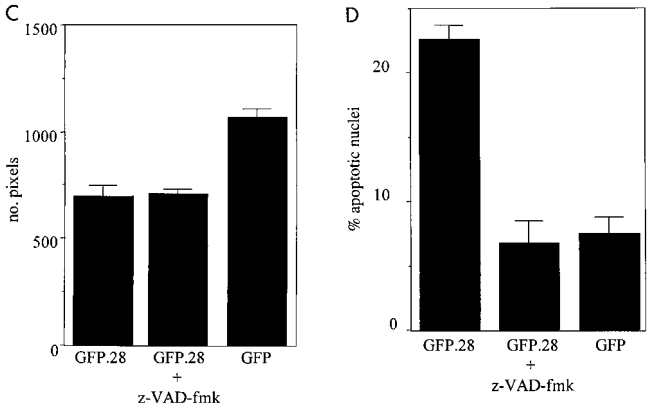


FIG. 5—*Continued.*

these lysates. Prevention of HEF1 cleavage in suspension cultures grown in the presence of serum may be stimulated by growth factors present in the serum. Alternatively, there may be cooperation between ligand binding of the integrin receptors to soluble ECM factors in the serum and binding to serum-derived growth factors. Interestingly, a recent report has suggested that survival signaling from the ECM may operate through distinct pathways to survival signals derived from serum (3). Finally, although a formal possibility for the failure of soluble fibronectin to control HEF1 cleavage in cells grown in plates coated with poly-HEMA is the failure of this fibronectin to bind the MCF7 cells, this is unlikely for several reasons. In particular, previous studies have noted that soluble fibronectin binding to suspended cells occurs significantly at concentrations of 2.5 μg/ml (24); the present studies were performed in significant excess of this quantity. Together, these results indicate that under SFM conditions, integrin-ligand binding is not sufficient to prevent the cleavage of HEF1; rather, integrin clustering and the formation of focal adhesions are required.

Finally, we have previously shown that HEF1 is cleaved during cellular apoptosis (33). As maintenance of cells in SFM in the absence of integrin ligation may be a proapoptotic stimulus (23), it was important to determine whether or not all cases of HEF1 cleavage were accompanied by apoptosis. To assess apoptosis, MCF7 cells grown on ECM-coated coverslips were stained with DAPI and the numbers of apoptotic nuclei were assessed. Cultures were fixed and stained 24 h after plating, and no significant levels of apoptotic nuclei were detected under any plating condition (percentages of apoptotic nuclei: SFM [2.5% ± 0.8%], FBS [1.4% ± 0.5%], fibronectin [1.9% ± 0.4%], laminin [2.3% ± 1.2%], and poly-L-lysine [3.0% ± 0.7%]), suggesting that the cells were not undergoing apoptosis. This correlates with our observations that cells grown under SFM conditions for 24 h can be stimulated to grow upon the readdition of FBS (results not shown). The observation that HEF1 is cleaved to different extents on surfaces coated with fibronectin and laminin versus cells grown in FBS and cells grown on dishes coated with polylysine (Fig. 3A) in the absence of apoptosis suggests that HEF1 cleavage may be separable from apoptosis. Together these data support the speculation that HEF1 cleavage is not simply a reflection of the apoptotic status of the cell but may independently be correlated with the adhesion status of the cell. We note that the continued abundance of the full-length HEF1 form (p105) (see, for example, Fig. 2B) in these experiments, in contrast to the loss of full-length HEF1 observed in mitosis and apoptosis (33, 35), further suggests that the cells remain in active growth, continuing to synthesize full-length HEF1 to replace a limited population cleaved in response to detachment from matrix. These results indicate that in the case of deadhesion, HEF1 exists as a mixed population of full-length and truncated protein within the cell, in contrast to the situation observed in apoptosis.

The C-terminal 28-kDa peptide of HEF1 causes cell rounding in cells blocked for apoptosis. We have previously shown that exogenous expression of the 28-kDa HEF1 cleavage product efficiently induces apoptosis (33). The conclusion that cleavage of endogenous HEF1 could be segregated from an apoptotic program led us to investigate functions of this peptide in control of adhesion versus apoptosis. We first examined MCF7 cells transiently transfected with a cDNA construct encoding the 28-kDa HEF1 peptide, pGFP.28 (Fig. 4A and B). During scrutiny of transfected cells, it was noted that at 18 h following transfection, pGFP.28 transfectants appear smaller and more rounded than cells transfected with the GFP vector alone (Fig. 5A). Further, paxillin costaining of these cells demonstrated that they had a reduced number of focal adhesions when compared with pGFP.HEF1-transfected cells (Fig. 5B) and vector control-transfected cells (data not shown). In order to allow quantitation of the rounding induced by transfected cDNAs, images of GFP-positive cells were captured and computer-assisted determination of the area (expressed in pixels) of the two-dimensional cell images was performed. Calculation of the cell image area confirmed that cells transfected with 28-kDa HEF1 are rounder, with 67% of the mean area of cells transfected with vector alone (Fig. 5C). We confirmed that pGFP.28 was inducing apoptosis in these cells, by DAPI staining followed by quantitation under UV light of shrunken and condensed apoptotic nuclei (Fig. 5D).

To determine whether pGFP.28-induced rounding was simply a by-product of apoptosis or whether pGFP.28 action in promoting rounding could be separated from its role in cell death induction, we performed the rounding analysis in the presence of the cell-permeating caspase inhibitor z-VAD-fmk, a treatment reported to prevent apoptosis in MCF7 cells (26). As shown, there is no change in pGFP.28-induced rounding in the presence or absence of the z-VAD-fmk inhibitor (Fig. 5C). Treatment with the z-VAD-fmk caspase inhibitor reduced the percentage of apoptotic nuclei to the same level as is observed in vector control-transfected cells, indicating efficacy of the inhibitor (Fig. 5D). Intriguingly, previous reports have noted that fibroblasts transfected with the C-terminal FAT domain of FAK (also fused to GFP) experienced similar cell rounding, even in the presence of z-VAD-fmk (23), supporting the idea that the process is generally attributable to focal adhesion loss. Thus, it appears that while p28 HEF1 causes induction of rounding and apoptosis, the rounding phenotype is separable and is likely a direct consequence of HEF1 action. The rounded appearance of the cells implies a loss of focal adhesions, a belief which is supported by the reduced staining with paxillin in these cells, and raises the possibility that 28-kDa HEF1 may be contributing to the process of apoptosis by

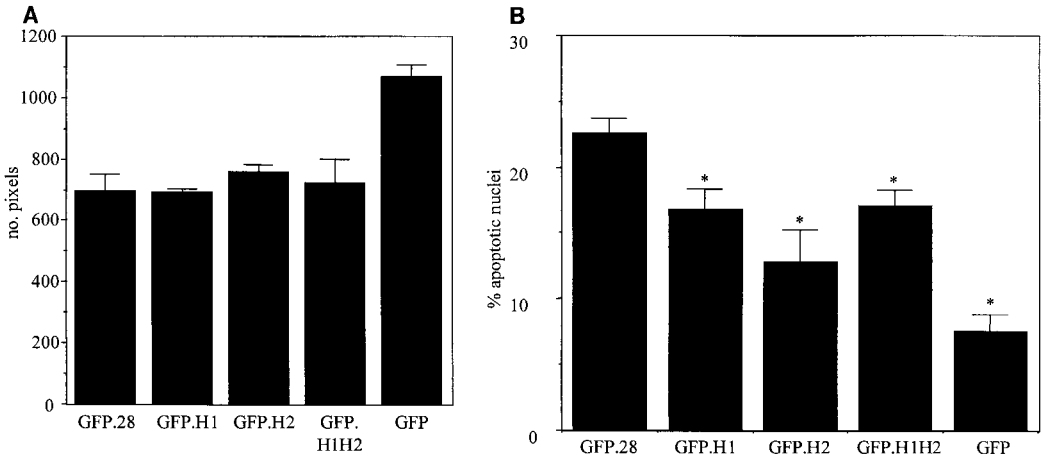


FIG. 6. The dHLH motif is dispensable for rounding but plays a role in apoptosis. (A) Cell surface area of MCF7 cells transfected with the indicated dHLH mutant constructs (Fig. 4A) was calculated (in pixels) as described in Materials and Methods. (B) MCF7 cells transfected with the dHLH mutant constructs were assessed for apoptosis by examining the DAPI-stained nuclei of GFP-positive cells. The number of apoptotic nuclei is expressed as a percentage of the total nuclei examined, and data points are calculated as described in Materials and Methods. Bars marked with an asterisk represent values that are significantly different from values for pGFP.28 transfectants ($P < 0.05$) as determined using Student's *t* test. Note that the values for GFP.H1, GFP.H2, and GFP.H1H2 were calculated to have no significant difference from each other ($P < 0.05$).

stimulating loss of focal adhesions, for example, by preventing the formation of signaling complexes at focal adhesions, as has been suggested for FAK peptides (3).

p28 mutations that differentially affect apoptosis and rounding. The sequence encompassed within the 28-kDa region of HEF1 (aa 631 to 834) is particularly highly conserved among all members of the Cas protein family (32; see Fig. 11) and hence appears likely to be required for essential functions of Cas proteins. Recently we characterized a sequence motif within the 28-kDa HEF1 peptide that resembles a dHLH motif (34). This motif, which is conserved with p130Cas, mediates homo- and heterodimerization between the Cas family proteins HEF1 and p130Cas and also between HEF1 and a limited number of HLH-containing transcription factors (34). The dimerization activity conferred by this motif could be inhibited by single amino acid substitutions that disrupted the two putative helices, suggesting a specific and potentially important biological function. The role of the dHLH sequence in rounding and/or apoptosis was examined by assaying cells transfected with constructs carrying mutations in the motif. These included pGFP.H1, containing an L722P mutation in the first helix; pGFP.H2, containing an L751P mutation in the second helix; and pGFP.H1H2, containing both mutations. MCF7 cells transfected with the dHLH mutant constructs versus pGFP.28 were first assessed for rounding. All constructs promoted rounding to a comparable degree, with scored areas of 694.4 ± 53.1 (pGFP.28); 691 ± 11.5 (pGFP.H1); 756 ± 25.2 (pGFP.H2); and 722 ± 75.8 (pGFP.H1H2) (Fig. 6A), indicating that an intact dHLH does not appear to be essential for 28-kDa HEF1-induced cell rounding.

Next, the effect of the dHLH mutations on apoptosis induction was calculated (Fig. 6B). Interestingly, all mutants showed significantly (*t* test; $P < 0.05$) decreased apoptosis when compared with pGFP.28 transfectants. The frequency of pycnotic

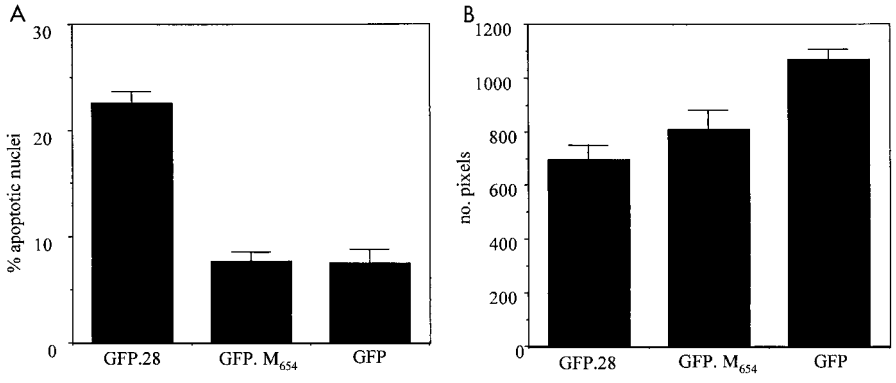


FIG. 7. Deletion of the N terminus of 28-kDa HEF1 abrogates apoptosis but has no effect on rounding. (A) Assessment of apoptosis in pGFP.M_{654} transfectants, a construct that is deleted for the N terminus of 28-kDa HEF1 (Fig. 4A), compared with pGFP.28 and pGFP transfectants. The number of apoptotic nuclei is expressed as a percentage of the total nuclei examined, and data points are calculated as described in Materials and Methods. (B) Cell area (in pixels) of the pGFP.M_{654}, pGFP.28, and pGFP transfectants is calculated as described in Materials and Methods.

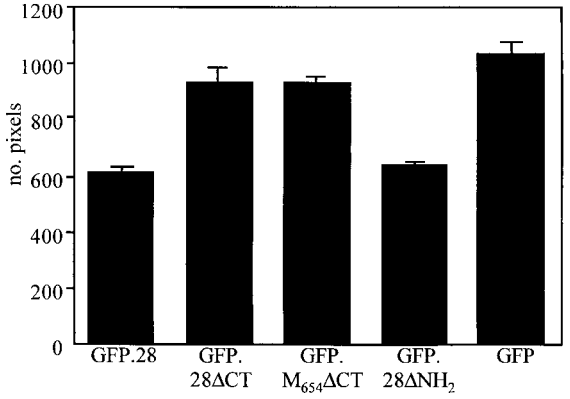


FIG. 8. Truncated derivatives of the 28-kDa C-terminal peptides do not confer rounding. MCF7 cells were transfected with the indicated constructs, and area was calculated (in pixels) as described in Materials and Methods.

nuclei in GFP-positive cells observed following DAPI staining was 22.6 ± 1.1 for intact pGFP.28 versus 16.8 ± 1.5 (pGFP.H1), 12.8 ± 2.4 (pGFP.H2), and 17.0 ± 1.2 (pGFP.H1H2). Importantly, mutation of the dHLH did not reduce apoptosis to the basal level observed in cells transfected with pGFP vector alone (7.5 ± 1.3). These results indicate that an intact dHLH contributes to apoptosis but also suggests that other regions located in the 28-kDa HEF1 molecule promote this process.

In earlier experiments characterizing the HEF1 C terminus, it was proposed that there is a critical domain located at the extreme N terminus of the 28-kDa sequence, between residues 626 and 654 (34). In consideration of the above data suggesting that disrupting the dHLH only partially inhibits apoptosis induction, we next examined the effects of expression of a clone that has the putative critical region deleted, pGFP.M_{654} (Fig. 4A). While this clone contains neither the predicted DDYD caspase cleavage site required to generate p28 nor the reported site for FAK phosphorylation of HEF1 (52), it retains the dHLH motif. Quantitation of apoptotic nuclei in pGFP.M_{654} transfectants shows that deletion of the region immediately preceding M_{654} completely abrogates the induction of apoptosis, reducing the levels of apoptotic nuclei to those observed in cells transfected with pGFP vector alone (Fig. 7A). Examination of cells transfected with pGFP.M_{654} for cell rounding demonstrates that GFP.M_{654} transfection causes cell rounding equivalent to that seen with pGFP.28 (Fig. 7B). Consequently the region of 28-kDa HEF1 encompassed by pGFP.M_{654} is sufficient to induce cell rounding, while the area upstream of M_{654} is apparently dispensable for cell rounding. Together these results implicate the region immediately upstream of M_{654} as required for apoptosis induction and the region downstream of M_{654} as sufficient to induce rounding.

Finally, to map the minimal determinant within the HEF1 C terminus necessary to induce rounding, we analyzed a series of HEF1/p28 derivatives previously described in reference 34. The constructs tested represent a deletion of the C terminus of 28-kDa HEF1 (pGFP.28ΔCT), a deletion of both the C terminus and the extreme N terminus (pGFP.M_{654}ΔCT), and a deletion of the N terminus (pGFP.28ΔNH2) (see Fig. 4, and Materials and Methods). In contrast to the results obtained

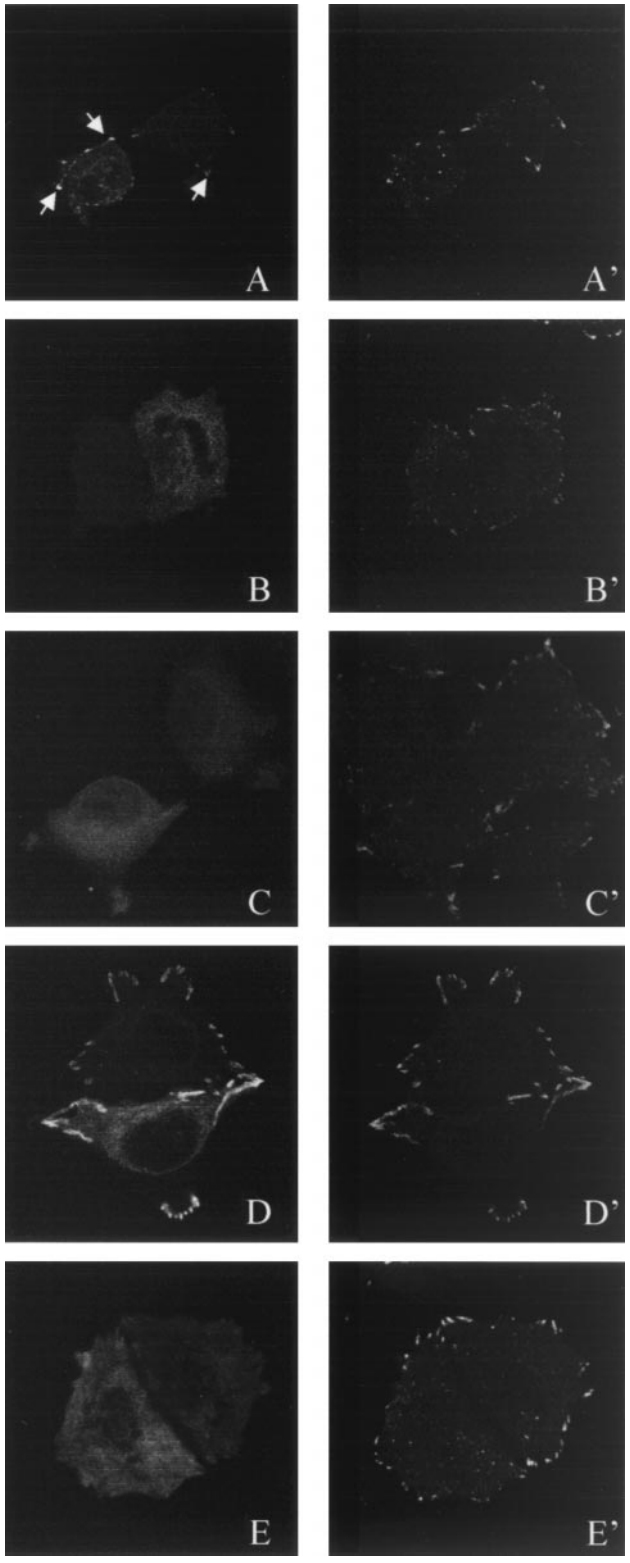


FIG. 9. The C terminus of HEF1 contains a FAT domain. Shown are confocal sections of MCF7 cells transfected with pGFP.M_{654} (A), pGFP.H1 (B), pGFP.H2 (C), pGFP.HEF1 (D), and pGFP.HEF1ΔCT (E). The same cell sections are shown immunostained with paxillin antibodies (A′ to E′, respectively). GFP-fused proteins localized to the small remaining focal adhesions in cells transfected with pGFP.M_{654} are indicated with arrows (A).

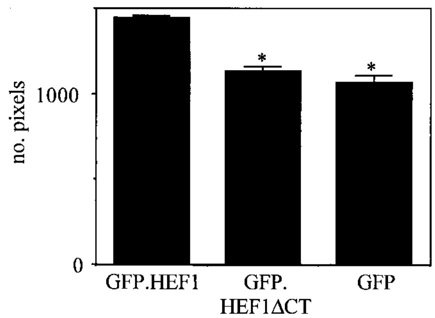


FIG. 10. Deletion of the C terminus prevents HEF1-mediated increases in cell area. MCF7 cells were transfected with pGFP.HEF1, pGFP.HEF1ΔCT, and pGFP, and cell surface area was calculated (in pixels) as described in Materials and Methods. Bars marked with an asterisk represent values that are significantly different ($P < 0.05$) from values obtained with pGFP.HEF1, as determined using Student's *t* test.

with the point mutants or pGFP.M_{654} truncation, these constructs produced different phenotypes for rounding (Fig. 8). A construct with the N terminus of 28-kDa HEF1 deleted promoted rounding comparable to that observed with the pGPF.28 construct, with scored areas of 633 ± 17 (pGFP.28ΔNH_2) and 607 ± 24 (pGFP.28), respectively. However, the two constructs containing deletions of the C-terminal region of 28-kDa HEF1 did not cause rounding, with scored areas of 926 ±50 (pGFP.28ΔCT) and 925 ± 29 (pGFP.M_{654}ΔCT), but had areas equivalent to those observed with cells transfected with vector alone, 1,033 ± 40 (pGFP). Together, these results suggest that the region encoded by pGFP.28ΔNH_2 but not the dHLH within this region is required for the induction of rounding. Characterization of the levels of GFP expression demonstrated that there is a range of fusion protein expression between transfectants. However, the range of expression is similar for all constructs, and further, the GFP expression levels show no correlation with different cellular phenotypes (Fig. 4). Therefore, the observed phenotypes resulting from mutant and truncated peptide overexpression are unlikely to be due to differing levels of fusion protein expression.

FAT determinants in the C-terminal peptide of HEF1. While the experiments with pGFP.M_{654} were being carried out, it was noted that the encoded protein localized to the limited number of focal adhesions residual in the transfected cells (Fig. 9A), as indicated by costaining with paxillin (Fig. 9A′). This raised the possibility that binding of this truncated form of HEF1 to focal adhesions might be displacing part of the population of endogenous Cas family members, thus contributing to focal adhesion destabilization. Alternatively, since it has previously been demonstrated that the C terminus of HEF1 can mediate homodimerization (34), it also seemed possible that pGFP.M_{654} might be localizing to the focal adhesions via dimerization with either HEF1 or p130Cas. While prior studies have reported the SH3 domain of Cas family proteins to be essential for their localization at focal adhesions, some data indicate that additional localizing determinants reside in the C terminus of p130Cas (19, 43), making these points of interest to investigate. We found that while the C-terminal GFP-fused HEF1 derivatives with point mutations in the dHLH pGFP.H1 and pGFP.H2 induced rounding (Fig. 6A), neither of these proteins localized to focal adhesions following transfection into MCF7 cells (Fig. 9B, B′, C, and C′). Similarly, the pGFP.ΔNH2 construct induces rounding (Fig. 8) but does not localize to focal adhesions (data not shown). We conclude, based on these data, that HEF1 truncations do not require localization to focal adhesions to induce cell rounding. Additionally, the pGFP.28ΔCT and pGFP.M_{654}ΔCT HEF1 derivatives did not localize to focal adhesions (results not shown). These data support the idea that integrity of the dHLH, in conjunction with additional sequences encoded between aa 654 and 695, contribute to focal adhesion retention.

In complementary experiments, to determine whether the HEF1 C terminus is necessary for localization of HEF1 to focal adhesions, we prepared a construct with the C terminus deleted in the context of the full-length molecule, pGFP.HEF1ΔCT. When expressed in MCF7 cells, this truncated form of HEF1 does not localize to focal adhesions (Fig. 9E and E′), in contrast to cells transfected with pGFP.HEF1 (Fig. 9D and D′) or pGFP.M_{654} (Fig. 9A and A′). Together with earlier findings, these results indicated that an intact C-terminal domain is necessary and sufficient for effective localization of HEF1 to focal adhesions.

Finally, we examined the effect of deleting the HEF1 C terminus on the rounding and apoptosis of MCF7 cells. As expected, cells transfected with pGFP.HEF1ΔCT did not display the rounding phenotype that is observed in cells transfected with pGFP.28 (compare Fig. 9E and 5A). Similarly,

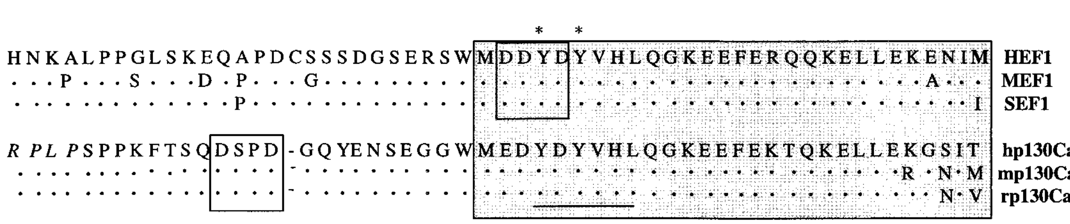


FIG. 11. Caspase cleavage sites are positionally conserved among orthologs of HEF1 and p130Cas. Amino acid alignments of partial sequences from the human (HEF1, aa 599 to 654), mouse (MEF1), and swine (SEF1) orthologs of enhancer of filamentation and the human (hp130Cas, aa 635 to 689), rat (rp130Cas), and mouse (mp130Cas) orthologs of p130Cas are shown. The swine sequence was obtained from an expressed sequence tag in the GenBank database (accession number, BE233552). Positionally conserved DXXD caspase consensus motifs are shown in the two small boxes (one of which is within the shaded area), the reported site for FAK phosphorylation (YDYDVHL) is underlined, and tyrosine residues are marked with an asterisk. The RPLP polyproline motif of p130Cas is in italics, and M_{654} of HEF1 is in boldface. Identical residues are indicated with a dot. The region of greatest similarity between the HEF1 and p130Cas sequences is indicated by the large, shaded box. Dashes (-) indicate where the sequence has been adjusted to facilitate alignment.

this construct did not induce apoptosis. Only 5.8% ± 1.0% apoptotic nuclei were observed in cells transfected with pGFP.HEF1ΔCT, versus 7.5% ± 1.3% (pGFP transfectants) and 22.6% ± 1.1% (pGFP.28) (Fig. 5C). Other work from our laboratory has shown that overexpression of HEF1 in MCF7 cell lines constructed to express inducible HEF1 results in increased cell area when compared with vector control transfectants (S. J. Fashena, M. B. Einarson, G. M. O'Neill, C. Patriotis, and E. A. Golemis, unpublished data). Consequently, we compared the area of cells transfected with pGFP.HEF1ΔCT and pGFP.HEF1 and found that cells transfected with the deletion construct have an area that is significantly smaller (*t* test; $P < 0.05$) than that of cells transfected with pGFP.HEF1 (Fig. 10; compare Fig. 9D and E) but comparable to that of cells transfected with GFP only. Considering that we have defined the C terminus of HEF1 as containing FAT determinants, it seems likely that full-length HEF1 must be able to interact at focal adhesions to induce cell spreading and that the HEF1 SH3 domain is insufficient to confer adequate association for this purpose.

DISCUSSION

Regulation of focal adhesion dynamics is vital to the control of cellular functions determined by cell-ECM interaction. The results presented in this study provide the first evidence that proteolytic cleavage of HEF1 is a product of and may promote focal adhesion disassembly and that functions of HEF1 in cellular detachment can be separated from previously described roles of HEF1 in apoptosis (33). Data in support of this model are as follows: first, loss of hyperphosphorylated full-length HEF1 followed by cleavage of HEF1 correlates with focal adhesion loss. Second, HEF1 cleavage can be prevented by specific integrin receptor-ligand binding and receptor cross-linking in the absence of additional growth factors. Third, overexpression of the 28-kDa HEF1 cleavage product promotes cell rounding under conditions that prohibit apoptosis. Fourth, structure-function analyses indicate that discrete regions of 28-kDa HEF1 contribute to induction of apoptosis versus cell rounding. Fifth, discrete C-terminally encoded residues are required for localization of HEF1 C-terminal peptides to focal adhesions. Further, some C-terminal HEF1-derived peptides which are unable to localize to (residual) focal adhesions still induce cell rounding but show reduced activity in promoting apoptosis. Finally, an overexpressed form of HEF1 lacking C-terminal p28-equivalent sequences fails to localize to focal adhesions and does not induce the spreading observed following overexpression of full-length HEF1. In sum, these data indicate that activity contained in the highly conserved carboxy-terminal region of HEF1 is critical in mediating discrete roles of HEF1 in control of attachment and cell death.

Many focal adhesion molecules are now known to be caspase (5, 16, 28, 35, 55, 56) or calpain substrates (6, 11, 30). Just as altered phosphorylation in response to integrin receptor ligation is a commonly observed mechanism in integrin signaling (17), integrin receptor control of protein cleavage may also prove to be generally applicable in this process. The observation that HEF1 is cleaved by caspases during mitosis (35) and apoptosis (33) has led us to propose that HEF1 integrity may generally reflect the adhesive state of the cell. In this context, it is intriguing to consider recent findings that misexpression of FAK C-terminally derived sequences also induces cell rounding (see above), as this suggests that cleavage of other focal adhesion components may contribute to focal adhesion disassembly. The data presented in this paper imply that HEF1 cleavage is also downstream of focal adhesion loss, based on its dependence on loss of integrin engagement and receptor cross-linking, and that the generation of HEF1-derived peptides in turn contributes to further focal adhesion destabilization. We propose that the observed effects on apoptosis and cell rounding following the overexpression of HEF1-derived peptides simulate the phenotypic consequences of detachment-induced focal adhesion disassembly and the resultant caspase-produced peptides. We note that gelsolin cleavage in apoptosis is reportedly upstream of cellular rounding in apoptosis (29), and we have found that HEF1 cleavage occurs in advance of gelsolin cleavage in apoptosis (33), suggesting that production of HEF1 fragments may constitute an upstream component of the cell-rounding process.

We have previously proposed that HEF1 is an apoptotic mediator at focal adhesions (33). As integrin receptor ligation plays a key role in the prevention of apoptosis mediated by focal adhesions (14), it is possible that p28HEF1 may promote apoptosis by stimulating focal adhesion disassembly. Notably, p28HEF1-induced rounding can be separated from apoptosis induction and multiple specific p28HEF1 domains are required for induction of apoptosis. In particular, the sequences between aa 626 and 654 and the dHLH motif found in the C terminus of Cas family proteins contribute to apoptosis induction by 28-kDa HEF1, but mutation or deletion of these sequences has no effect on the induction of rounding. It is suggestive that the identified apoptosis-promoting 28-aa stretch between aa 626 and 654 encompasses at least part of the FAK phosphorylation motif and may therefore compete with full-length HEF1 for phosphorylation by FAK. As the pGFP.M_{654} construct lacks this site, it may not compete for FAK phosphorylation and therefore cannot be proapoptotic. We note that pGFP.28 is not expressed at higher levels than any of the other constructs tested herein, and it is therefore unlikely that the observed differences are due to differences in expression level. Further, treatment with inhibitors of the proteasome (data not shown) demonstrates that the overexpressed 28-kDa peptide undergoes degradation similar to that seen with the native peptide (33; see discussion below), suggesting that the action of this peptide is physiological. Based on the requirement for dimerization-associated sequences, 28-kDa HEF1 may exert its apoptotic effects via dHLH-mediated dimerization with HEF1 or p130Cas and/or physical prevention of HEF1 association with downstream signaling molecules, similar to the manner in which preventing p130Cas interaction with cognate partners is reported to interfere with survival signaling (3). Alternatively, it may act as a dominant negative molecule, titrating other HEF1-interactive partners from functions required to promote cellular viability.

Candidate Cas family C-terminus-interacting-molecules that may transduce Cas signals include the CHAT/NSP3 orthologs that interact with both HEF1 and p130Cas C termini and stimulate the Jun N-terminal kinase signaling pathway (48) and the BCAR3/NSP2/AND-34 proteins that interact with the C

terminus of p130Cas (10, 38). AND-34 acts as a guanine nucleotide exchange factor for Ral GTPase, and coexpression with p130Cas causes a significant reduction in the guanine nucleotide exchange factor activity of AND-34 towards Ral (18). Presently, it is unknown whether 28-kDa HEF1 interacts with additional protein partners capable of inducing apoptosis. It will be interesting to see whether this peptide and the p130Cas C-terminal peptide specifically interact with protein partners unique from the full-length molecule.

In contrast to requirements for apoptosis, a more limited set of sequence elements is required for HEF1 effects on rounding. Of all the C-terminal derivative constructs examined, only those which lack the extreme C-terminal sequences of the protein (pGFP.28ΔCT and pGFP.M_{654}ΔCT) do not induce rounding. Separately, analysis of dHLH mutations indicates that this motif is not required for rounding. The C-terminally encoded FAT sequences that we identify are nonidentical to sequence requirements for rounding: only HEF1- or p28-derived proteins that contain both the aa-626-to-695 sequences and the dHLH sequences localize to focal adhesions; the majority of constructs tested did not localize to focal adhesions. Intriguingly, our observations that some constructs do not localize to the focal adhesions but still cause cell rounding demonstrate that the 28-kDa HEF1 effects on cell rounding do not require localization to focal adhesions. It is also unlikely that 28-kDa HEF1 causes rounding by homodimerizing with HEF1 and p130Cas, thus titrating them away from focal adhesions, as it has been previously shown that the dHLH mutations tested here prevent dimerization (34). As yet, the mechanistic basis of the rounding effect remains unknown. However, studies of the C-terminal region of p130Cas have indicated that deletion of the site of FAK phosphorylation and Src binding prevented localization at focal adhesions (43), while a recent study has shown the sufficiency of the p130Cas C terminus for FAT (19). Deletion of the p130Cas C terminus inhibits Src activation by p130Cas (8), and Src activity has been implicated in cell spreading (27). The cell-rounding phenotype induced by FAK C-terminal caspase peptides is accompanied by decreased tyrosine phosphorylation of focal adhesion components (16), which is likely attributable to the fact that these peptides inhibit FAK interaction with its downstream partners (3), including Cas family members. The observations presented here provide some mechanistic insight into these findings, although the exact sequence of events remains to be determined.

The C terminus of HEF1 is highly homologous to the C terminus of p130Cas (75% sequence conservation from residues 626 to 834 in HEF1 versus 661 to 870 in human p130Cas). The C-terminal domain of p130Cas is also targeted by caspases during apoptosis (5, 28, 33) (Fig. 11). We note that p130Cas and HEF1 are cleaved at different sites: p130Cas is cleaved at D_{651}SPD (28), while HEF1 is cleaved at D_{627}DYD (33) and lacks a DXXD motif in a position comparable to the DSPD of p130Cas. The predicted HEF1 cleavage site D_{627}DYD comprises part of the highly conserved FAK phosphorylation motif that subsequently binds Src proteins (52). It is not clear which tyrosine residue in the DDYDYVHL sequence of HEF1 is phosphorylated by FAK. Therefore, whether cleavage by caspases after the aspartic acid residue in position 4 of DDYD bisects the FAK phosphorylation motif is not known. Unequivocally, cleavage of p130Cas at D_{651}SPD produces a C-terminal peptide with the YDYVHL FAK phosphorylation motif intact. DDYD is positionally conserved in the human, mouse, and swine homologs of HEF1, while DSPD is positionally conserved between the human, rat and mouse homologs of p130Cas (Fig. 11), suggesting that the different localization of cleavage sites may reflect an evolutionarily conserved and potentially different function for the two peptides.

Correspondingly, overexpression of full-length p130Cas results in increased migration and survival in response to collagen binding to integrin receptors (12). This contrasts with the reported consequences of full-length HEF1 overexpression, which initially stimulates migration (21; Fashena et al., unpublished) but culminates in the induction of apoptosis (33). Relatedly, a p130Cas/FAK interaction is required for survival signaling and FAK C-terminal peptides competent for interaction with p130Cas are sufficient to maintain the survival signal (3). Exogenously expressed p130Cas C-terminal peptides that include the bipartite Src binding motif (encompassing the RPLP polyproline motif and the Src SH2 binding site [Fig. 11]) can activate Src, with this interaction sufficient to stimulate survival pathways (8). The p130Cas peptide studied, however, contains extra sequence N-terminal to the predicted caspase cleavage site of p130Cas and includes both the RPLP polyproline motif of p130Cas upstream of the predicted caspase cleavage site and the Src SH2 binding motif located downstream (44). Given that the polyproline region of p130Cas is absolutely required for interaction with Src (8), it seems likely that the p130Cas caspase-derived C-terminal peptide would be unable to bridge the interaction and maintain the survival signal. To date, the significance of the altered sites of cleavage between HEF1 and p130Cas remains to be determined.

Finally, an interesting implication of this study is that if HEF1 cleavage occurs under conditions in which there is sustained reduction of focal adhesions but no apoptosis, there should nevertheless be caspases active under the same conditions. While it is well established that caspases are active during apoptosis, only recently has it become clear that caspases are also active during proliferation and development (54). There is, as yet, no direct evidence to support a role for increased caspase activity during mitosis. However, expression of the caspase-inhibitory molecule survivin is cell cycle regulated, with peak expression during mitosis (37). This indirectly suggests that there may be a requirement for increased control of caspase activity during mitosis, potentially restricting the extent of cleavage of substrates, such as HEF1. Intriguingly, it has very recently been demonstrated that overexpression of FAK correlates with increased survivin expression (51). In contrast, uninhibited caspase activity during apoptosis could contribute to the permanent disassembly of focal adhesions. We have earlier reported that HEF1 cleavage products appear to be degraded by the proteasome (33). It is noteworthy, therefore, that there is increased proteasome activity in proliferating cells (4) and that the proteasome localizes to the periphery during the G_2/M phase of the cell cycle. Proteasomal activity may further ensure that the loss of focal adhesions during mitosis is not permanent, by destabilizing the proapoptotic 28-kDa peptide.

In conclusion, the results presented in this paper support an important role for HEF1 in focal adhesion disassembly. Exact

definition of the manner in which HEF1 acts in concert with other structural and signaling molecules at focal adhesions to achieve this function will be a fascinating target of future study.

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