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Phosphorylation of human enhancer of filamentation (HEF1) on serine 369 induces its proteasomal degradation

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ABSTRACT

Human enhancer of filamentation 1 (HEF1) is a multi-domain docking protein of the p130 Cas family. HEF1 is present at focal adhesions and is involved in integrin signalling mediating cytoskeleton reorganization associated with cell migration, adhesion or apoptosis. HEF1 functions are regulated in part by phosphorylation on tyrosine residues. HEF1 is also phosphorylated on serines/threonines leading to two isoforms refered to as p105 and p115. In most cases, the serine/threonine kinase(s) responsible for HEF1 phosphorylation have not been identified. In the present study, we have investigated HEF1 ser/thr phosphorylation. In the HCT-116 cell line transiently overexpressing Flag-HEF1 we showed that Hesperadin, a synthetic indolinone displaying antiproliferative effect and described as an inhibitor of various kinases including Aurora-B, prevented HEF1 phosphorylation induced by the ser/thr phosphatase PP2A inhibitor: okadaic acid (OA). In addition we showed that conversion of endogenous HEF1 p105 to p115 in HaCaT cells was prevented upon treatment with Hesperadin, resulting in accumulation of p105HEF1. We also identified serine 369 as the target site of phosphorylation by this Hesperadin-inhibited kinase in HCT-116. Finally, we provide evidence that phosphorylation on serine 369 but not phosphorylation on serine 296, triggers HEF1 degradation by the proteasomal machinery. These data suggest that conversion of p105 to p115 results from a ser-369-dependent phosphorylation mediated by an Hesperadin-sensitive kinase and regulates the half-life of HEF1.

1. Introduction

Human enhancer of filamentation (HEF1), also known as Cas-L or NEDD9, belongs to the p130 Cas multi-domain docking protein family containing a N-terminal SH3 domain, numerous SH2 binding sites in a "substrate domain", a serine rich domain and a HLH motif in the C terminus [1]. HEF1 was first identified by complementation assay for its ability to induce pseudohyphal growth in *Saccharomyces cerevisiae* [2]. Later on, its role in intracellular signalling and regulation of cytoskeleton has been further characterized. Several reports demonstrated that HEF1 is mainly expressed in lymphoid and epithelial cells [2,3]. It has been implicated in signalling pathways initiated by ligation of integrins or antigen on BCR or TCR [2–5]. Moreover HEF1 is localized to focal adhesion sites where its interaction with various partners is regulated by phosphorylation on tyrosine residues by kinases of the FAK and Src families [6,7]. Phospho-tyrosine residues become docking sites for SH2 domain containing proteins such as CrkL, mediating the activation of small G proteins including Cdc42, Rap1 and RhoA [8–12]. These effectors link HEF1 to regulation of cell morphology, cytoskeleton reorganization and regulation of adhesion [1,13]. HEF1 invalidation in mouse leads to B cells migration defects [14]. Furthermore, the increase in HEF1 expression in melanoma metastasis compared to primary melanomas supports a contribution of this protein to motility and invasiveness [15].

HEF1 is also regulated by proteasomal degradation. In the TGFβ signalling pathway, HEF1 interacts with Smad3 and is consequently targeted to the proteasome through an APC/CDH1 dependent mechanism [16,17]. Alternatively, the Atrophin-1 Interacting Protein-4 (AIP-4) has been proposed as an ubiquitin E3 ligase in Smad3-HEF1 degradation induced by TGFβ [18]. Different studies have reported that proteasomal degradation of HEF1 affects preferentially the serine/threonine phosphorylated form of HEF1 refered to as p115 [16,19].

Phosphorylation of HEF1 on serine/threonine residues and appearance of the p115 isoform have been described as a result of cell adhesion [20,21]. However, the serine/threonine kinase(s) responsible for this HEF1 phosphorylation have not been identified in most cases. A recent study has described the phosphorylation of serine 296 of HEF1 by the cell-cycle-dependent kinase Aurora-A

Abbreviations: HEF1, human enhancer of filamentation 1; FAK, focal adhesion kinase; CrkL, Crk-like; PP, protein phosphatase; Cas, Crk associated substrate; SH, Src homology; APC, anaphase promoting complex.

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[22]. Several reports pointed out a tyrosine dephosphorylation of the components of the focal adhesion complex including FAK, paxillin and p130 Cas associated with ser/thr phosphorylation of these proteins and the dissociation of the complex during the M phase of the cell cycle. This suggests that focal adhesion protein interaction is linked to ser/thr phosphorylation status [23,24].

Aurora-A is a member of Aurora kinase family that includes three members, Aurora-A, -B and -C. All of them are regulators of mitosis and Aurora-A and -B are overexpressed in a variety of tumor cell lines suggesting a role in tumorigenesis [25]. Aurora-A is implicated in mitotic entry, centrosome maturation and spindle assembly. In contrast, Aurora-B ensures correct chromosome alignment and subsequent segregation at the level of kinetochore and progression of cytokinesis [26–28]. Aurora-A activity is down-regulated by dephosphorylation of the threonine 288 by the ser/thr phosphatase PP1 [29]. HEF1 interaction with Aurora-A leads to activation of the kinase while phosphorylated HEF1 fate is still unclear [22]. Aurora-B binds to and is negatively regulated by both PP1 and PP2A and is therefore activated by okadaic acid (OA), an inhibitor of these phosphatases [30]. Hesperadin, described as an inhibitor of Aurora-B kinase is a synthetic indolinone which displays antiproliferative properties and has entered phase I clinical trials as anti-cancer drug. In human cells, Hesperadin treatment prevents phosphorylation of Aurora-B substrates and induces a phenotype similar to Aurora-B siRNA transfection [31]. Hesperadin activity on Aurora-A has not been formally tested.

In the present study, we have further investigated HEF1 phosphorylation. We demonstrate that HEF1 is phosphorylated by a kinase sensitive to Hesperadin and identify serine 369 as the target site of phosphorylation. In addition, we provide evidence that phosphorylation on this residue regulates HEF1 degradation by the proteasomal machinery.

2. Materials and methods

2.1. Cell culture

All cell lines were obtained from ATCC unless specified. SW480 cells are derived from a primary colorectal tumor, they were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (50 μg/ml penicillin, 50 μg/ml streptomycin), with 1 mM sodium pyruvate and 10% fetal calf serum (FCS) at 37 °C with 5% CO_2. The human keratinocyte cell line HaCaT was obtained from N. Fusenig and maintained in the same medium. The colon cancer cell line HT-29 and the lymphopoietic Kit225 cell line were cultured in RPMI 1640 medium with antibiotics as above, with 1 mM sodium pyruvate and 10% FCS. The colon cancer cell line HCT-116 was cultured in McCoy's 5A medium with antibiotics as above and 10% FCS.

2.2. Reagents and antibodies

Mg-132, Okadaic acid and PP2 were from Calbiochem and were added to the culture medium at final concentration of 10 μM, 125 nM and 10 μM respectively. U0126 was from Promega and used at final concentration of 10 μM. Hesperadin and ZM447439, two inhibitors of Aurora-B [31,32] were kindly provided by N. Kraut (Boehringer Ingelheim, Austria) and N. Keen (Astra Zeneca, Cheshire, UK) and used at final concentration of 1 μM and 10 μM respectively. Cycloheximide and Nocodazole were from Sigma and used at final concentration of 70 μM and 300 nM respectively. Time-course established 8 h as optimal time for okadaic acid treatment, Mg-132 or Hesperadin were added at the same time, in each case. Anti-HEF1 (IQ297) monoclonal antibody was from Immuquest (1/6000). Anti-Flag M2 monoclonal antibody was from Sigma (1/1000). Anti-BubR1 monoclonal antibody was from BD Biosciences (1/1000). Anti-Hsp90 monoclonal antibody was from Stressgen (1/1000). Anti-Aurora-A monoclonal antibody was from Sigma (1/10000). Anti-Aurora-B polyclonal antibody was from Abcam (1/1000).

To generate HEF1 Ser-369 phospho-specific antibody (anti-PhosphoSer-369HEF1), polyclonal rabbit antisera were collected after immunization with the phosphorylated peptide CSRDLVDGINRLpSFSST and subjected to affinity purification (procedure implemented by Covalab, Lyon, France).

2.3. Plasmids and mutations

The construct encoding Flag-HEF1 was generated as follows. HEF1 cDNA was amplified by RT-PCR with a forward primer: 5′-TCCCCCGGGACCGCTGCCGAAATGAAGTAT-3′ and a reverse primer 5′-CCGCTCGAGGAACGTTGCCATCTCCAGCA-3′. PCR product was then digested with SmaI and XhoI and inserted to the BglII/filled in and XhoI sites of the pCMV-Tag1 vector. pCMV-Flag-HEF1 S296A, pCMV-Flag-HEF1 S369A and pCMV-Flag-HEF1 S296/369A were created by using oligonucleotide-directed PCR mutagenesis to create a Ser → Ala change at amino-acid 296, or 369, or both, of full-length HEF1; these constructs are otherwise identical to pCMV-Flag-HEF1wt (wild-type), assessed by DNA sequencing.

2.4. Transfection experiments

For transient transfection of HEF1 or its mutants, cells were seeded in 6-well dishes 24 h before transfection. The different plasmids were adjusted to 2.5 μg of DNA and 7.5 μl of Jet-PEI™ solution (Polyplus transfection, Illkirch, France). After 24 h incubation, cells were supplemented with fresh culture medium and left untreated or incubated with the various reagents, as indicated.

2.5. Preparation of cell lysates and Western-blot analysis

Cells were washed twice with ice-cold PBS buffer and solubilized with lysis buffer (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, pH 7.4, 1% Triton-X100, 2 mM sodium pyrophosphate, 10% glycerol, 25 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin). After incubation on ice for 20 min, cell extracts were centrifuged at 15,000 × *g* for 20 min at 4 °C. The protein concentration of cell extracts was determined using a BCA protein assay reagent (Sigma). Aliquots of cell lysates containing equal amount of protein were subjected to SDS-PAGE under reducing conditions, transferred to a Hybond-C nitrocellulose membrane (Amersham Bioscience) and probed with appropriate antibodies. Immunocomplexes were detected using enhanced chemiluminescence (Amersham, GE Healthcare).

2.6. Small interfering RNA and transfection

The Aurora-A and -B small interfering RNA (siRNA) sequences used have been described previously and are as follows: 5′-AUGCCCUGUCUUACUGUCA-3′ (Aurora-A) and 5′-GGUGAUGGA-GAAUAGCAGU-3′ (Aurora-B) [31,33]. Briefly, 10 or 20 pmol of siRNA and 20 μl of Interferin (Ozyme) were added to 200 μl of Opti-MEM (Invitrogen), mixed gently, and then incubated for 10 min at room temperature. After incubation, the mixture was added to the plate (9 cm^2) in 800 μl of DMEM plus 10% FCS and left for 48 h before lysis for protein analysis.

2.7. Immunoprecipitation

Cell lysates (500 μg-aliquots) prepared as above, were incubated with 4 μg of antibody for 2 h at 4 °C. Next, 40 μl of

protein G-sepharose beads (GE Healthcare) were added, and reaction mixtures were incubated 45 min at 4 °C. The immunoprecipitates were washed three times with lysis buffer.

2.8. In vitro dephosphorylation assay

Lysates were immunoprecipitated as described above. The immunoprecipitates were then washed three times with calf intestinal alcaline phosphatase (CIAP) buffer (50 mM Tris–HCl, pH 8.5, 0.1 mM EDTA) and incubated with 10U of CIAP (Invitrogen) for 1 h at 37 °C.

3. Results

3.1. HEF1 is differentially expressed in various cells

HEF1 mRNA expression is cell type specific, the transcript being mainly present in epithelial and lymphoid cells. We first evaluated HEF1 protein expression in different cell lines including Kit225, HaCaT, SW480, HCT-116 and HT-29. Depending on the cell type tested, HEF1 was either weakly or not expressed, present as a single band (105 kDa) or detected as a doublet (105 and 115 kDa) (Fig. 1). This two bands profile (p105 and p115) is a product of two different ser/thr phosphorylation states of HEF1, previously described in the literature [20,21]. We then decided to examine the significance of this difference.

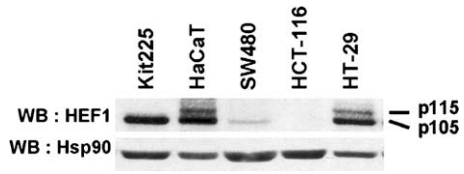

Fig. 1. HEF1 is differentially expressed in cells. Cell lysates of different cell lines, containing an equal amount of proteins, were analyzed by Western-blotting with a monoclonal anti-HEF1 antibody. Membrane was stripped and reprobed with anti-Hsp90 antibody as a loading control.

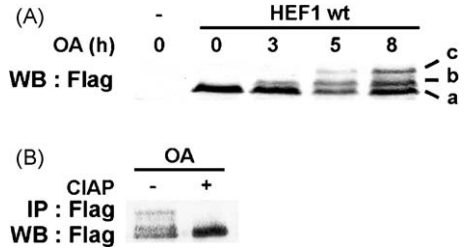

Fig. 2. Okadaic acid induces serine/threonine phosphorylation of HEF1. (A) HCT-116 cells were transiently transfected with pCMV-Flag-HEF1wt (HEF1wt) or empty pCMV-Flag (−). Time-course with okadaic acid (OA) at final concentration of 125 nM was performed. Proteins were separated by SDS-PAGE (acrylamide/bisacrylamide ratio of 30:0.2) and analyzed by immunoblotting with anti-Flag antibody. a–c refered to different HEF1 isoforms (see description in the text). (B) HCT-116 cells transiently transfected with wild type HEF1 were stimulated for 8 h with OA (125 nM). Cells lysates containing an equal amount of proteins were immunoprecipitated with anti-Flag antibody. Immunoprecipitates were treated or not with CIAP (calf intestinal alkaline phosphatase) and immunoblotted with anti-Flag antibody.

3.2. Okadaic acid induces serine/threonine phosphorylation of HEF1

As we did not observe any expression of HEF1 in HCT-116 cell line, we decided to further investigate HEF1 phosphorylation in these cells by transfecting them with a Flag-tagged HEF1. As shown in Fig. 2A, in HCT-116 cells, ectopic Flag-HEF1 is expressed as a single band. PP2A has been described as a phosphatase regulating HEF1 phosphorylation in fibroblast cells [19]. We performed a time-course treatment with okadaic acid (OA), an inhibitor of the serine/threonine phosphatases PP2A and PP1, on these cells and examined HEF1 migration profile after separation on SDS-PAGE using an acrylamide/bisacrylamide ratio of 30:0.2 which improves the separation of ser/thr phosphorylation-induced gel shifts. While in the control cells, HEF1 is expressed as a single band (Fig. 2A, band a), inhibition of PP2A reveals a slower migrating band after 3 h (band b), followed by a third band after 5 h (band c).

We hypothesized that this migration profile resulted from HEF1 ser/thr phosphorylation. We then performed an *in vitro* dephosphorylation assay of ectopic HEF1 immunoprecipitated from cells treated with OA for 8 h. As shown in Fig. 2B, the phosphatase treatment totally abolished slower migrating forms. This data indicates that the two slower migrating forms are the products of at least two phosphorylated states of HEF1 on serine or threonine residues.

3.3. HEF1 is phosphorylated in vivo by an Hesperadin-sensitive kinase

It has been reported that HEF1 phosphorylation is cell cycle-regulated and that HEF1 is a substrate for Aurora-A kinase [22]. The above experiment showed that OA treatment revealed at least two different states of phosphorylation of HEF1. Moreover, OA has also been described as an activator of Aurora kinases A and B [29,30]. To examine the involvement of the Aurora kinases in HEF1 phosphorylation, we transiently transfected HCT-116 cells with Flag-HEF1 and incubated them with OA alone or in the presence of Hesperadin, an inhibitor of Aurora-B kinase. As shown in Fig. 3, co-incubation with OA and Hes resulted in the disappearance of the slower migrating band from the 3 bands pattern of migration of HEF1 visualized upon OA treatment alone. This result strongly suggests that the presence of this particular band (band c) depends on the activity of a kinase that is sensitive to Hesperadin. In contrast, treatment with Hesperadin did not affect the level of band b. This data indicates that other kinase(s) not sensitive to Hesperadin is (are) also involved in OA-induced HEF1 phosphorylation. In parallel, other inhibitors of cell cycle-regulated kinases such as olomoucin or scytonemin failed to prevent OA-induced HEF1 phosphorylation shift (data not shown). We checked the inhibition properties of Hes in our cells by looking for the phosphorylation of a specific Aurora-B substrate: the checkpoint protein kinase BubR1 [32]. Upon 8 h incubation with OA, BubR1 is phosphorylated (visualized by the presence of slower migrating forms) (Fig. 3, lower panel). When OA and Hes were added concomitantly, BubR1 shifts in migration profile were not detected anymore. This indicates that the OA-induced BubR1 migration shift is sensitive to Hesperadin. Thus in these cells, OA effectively increases the phosphorylation of Aurora-B substrates. Taken together, these results indicate that Aurora-B could be the kinase able to phosphorylate HEF1 in HCT-116 cells.

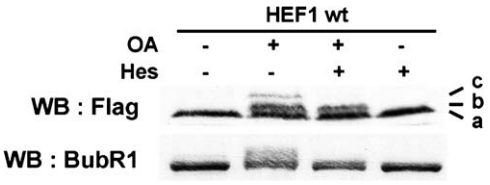

Fig. 3. HEF1 is phosphorylated *in vivo* by a Hesperadin-sensitive kinase. HCT-116 cells were transiently transfected with pCMV-Flag-HEF1wt (HEF1wt). Cells were incubated for 8 h with OA (125 nM) or Hesperadin (Hes) (1 μM) or both. HEF1 migration pattern was revealed by immunoblotting with anti-Flag antibody. Membrane was stripped and reprobed with anti-BubR1 antibody to visualize the activity of Aurora-B.

3.4. An Hesperadin-sensitive kinase phosphorylates HEF1 on serine 369

Serine 296 of HEF1 has been described as a target amino acid for Aurora-A phosphorylation [22]. In addition, the examination of the primary sequence of the protein revealed the existence of an R-X-S located at serine 369 matching the consensus phosphorylation site for Aurora-B but also for other kinases which may be inhibited by Hesperadin [31]. We generated various Flag-tagged HEF1 mutants lacking one or both of these two potential phosphorylation sites. The mutants were overexpressed in HCT-116 cells, and cells were treated or not with OA. The Flag-tagged HEF1 mutant S369A (serine 369 replaced by alanine) does not migrate similarly to wild-type HEF1 after OA treatment (Fig. 4A, lane 1), but exhibits only two bands (lane 2). This two-bands pattern seems to be similar to the one observed previously with wild-type HEF1 upon OA/Hes co-treatment (bands a and b, Fig. 3) suggesting that S369A point mutation abolished the OA induced phosphorylation inhibited by Hes. Upon OA treatment, the Flag-tagged HEF1 mutant S296A (serine 296 replaced by alanine) migrated differently, displaying the basal form (band a) and a shifted protein migrating between the two slower migrating bands observed with the wild-type HEF1 (asterisk indicates the position of band d in lane 3). The Flag-tagged HEF1 double-mutant S296/369A is no longer shifted after OA treatment. Therefore, band d seems to be the signature of a single phosphorylation on serine 369. Thus band a corresponds to the basal isoform of HEF1, bands b, d and c correspond to the shifts induced by HEF1 phosphorylation on ser-296, ser-369 and ser-296 and 369 respectively.

To confirm the involvement of the Hesperadin-sensitive kinase in the phosphorylation of ser-369 but not of ser-296, HCT-116 cells were transfected with the two single mutants of HEF1 and incubated with OA alone or in combination with Hes. As expected, Hes has no effect on OA-induced ser-296 phosphorylation shift (Fig. 4B, upper panel), but totally abolished OA-induced ser-369 phosphorylation shift (Fig. 4B, lower panel). Of note, in this experiment band c is not detected as no double phosphorylation occurred.

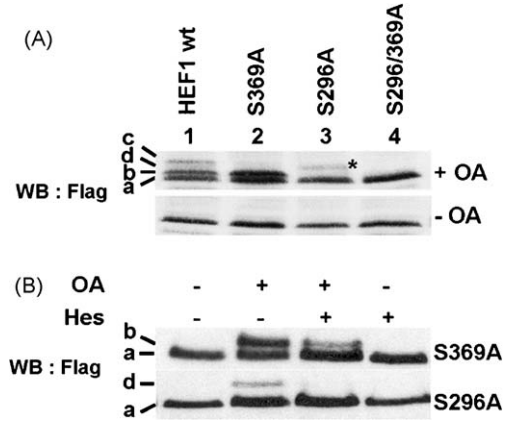

Fig. 4. Hesperadin-sensitive kinase phosphorylates HEF1 on serine 369. (A) HCT-116 cells were transiently transfected with pCMV-Flag-HEF1wt or mutated plasmids, then stimulated or not with OA (125 nM) for 8 h. Lysates were analyzed by Western-blotting with anti-Flag antibody. Lane 1 corresponds to cells transfected with pCMV-Flag-HEF1wt (HEF1wt), lane 2 with pCMV-Flag-HEF1 S369A (S369A), lane 3 with pCMV-Flag-HEF1 S296A (S296A) and lane 4 with pCMV-Flag-HEF1 S296/369A (S296/369A). a–d refered to different HEF1 isoforms. Asterisk in lane 3 indicates the position of band d. (B) HCT-116 cells were transiently transfected with pCMV-Flag-HEF1 S369A (S369A) or pCMV-Flag-HEF1 S296A (S296A), then stimulated by OA (125 nM) or Hes (1 μM) or both. Immunoblotting was performed with anti-Flag antibody. a, b and d refered to different HEF1 isoforms.

In order to ascertain that mutation of serine 369 does not prevent phosphorylation of a different residue in HEF1 but is the actual amino acid phosphorylated, we raised an antibody against an HEF1 peptide encompassing phosphorylated Ser-369. The antibody obtained gave good immuno-reactivity on Western-blot. As shown on Fig. 6C (lanes 1 and 2), all the bands (a–d) described earlier are detected with the M2 antibody. However, only band c from wild type HEF1 and band d from S296A HEF1 are detected with the anti-PhosphoSer-369HEF1 antibody (Fig. 6C, lane 5 and 6). This result confirms that Serine 369 is the amino acid phosphorylated upon OA treatment.

Overall, these results indicate that in cells, ectopically expressed HEF1 is phosphorylated on both serines 296 and 369 following OA stimulation and that serine 369 phosphorylation is dependent on a kinase inhibited by Hesperadin. These two phosphorylations seem to occur independently (Fig. 4A) and with different kinetics, phosphorylation of ser-369 being delayed compared to ser-296 phosphorylation (Fig. 2A).

3.5. HEF1 stability is modulated through phosphorylation of serine 369

Several reports show that HEF1 expression is regulated at the post-transcriptional level [16]. In order to assess the stability of exogenous HEF1 in HCT-116 cells, we performed a time-course treatment with cycloheximide (CHX), an inhibitor of protein synthesis. As shown in Fig. 5A (top left panel), in HCT-116 cells, in the presence of CHX a decrease in HEF1 protein level occurred,

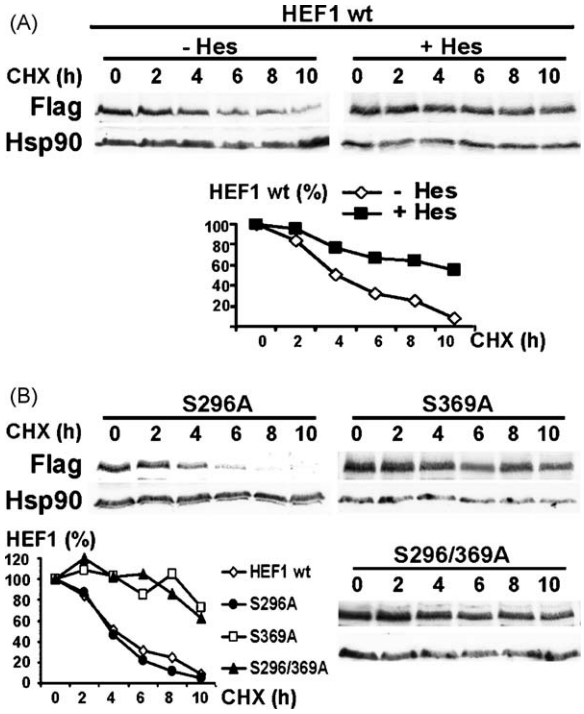

Fig. 5. HEF1 stability is modulated through phosphorylation of serine 369. (A) HCT-116 cells were transiently transfected with pCMV-Flag-HEF1wt (HEF1wt). Time-course was performed with cycloheximide (CHX) at 70 μM alone or with Hes (1 μM). Western-blot analysis of cells extracts were performed using anti-Flag antibody. Membranes were probed with anti-Hsp90 antibody to check for equal protein loading. Amounts of proteins were quantified by densitometric analysis: the HEF1 signal was normalized to the Hsp90 signal and, to facilitate comparison, results were expressed as % of the initial amount (set at 100%) of HEF1wt in the presence or not of Hes. (B) HCT-116 cells were transiently transfected with either pCMV-Flag-HEF1 S296A (S296A), pCMV-Flag-HEF1 S369A (S369A) or pCMV-Flag-HEF1 S296/369A (S296/369A). Time-course with CHX (70 μM) was performed and lysates were resolved by immunoblotting with anti-Flag antibody.

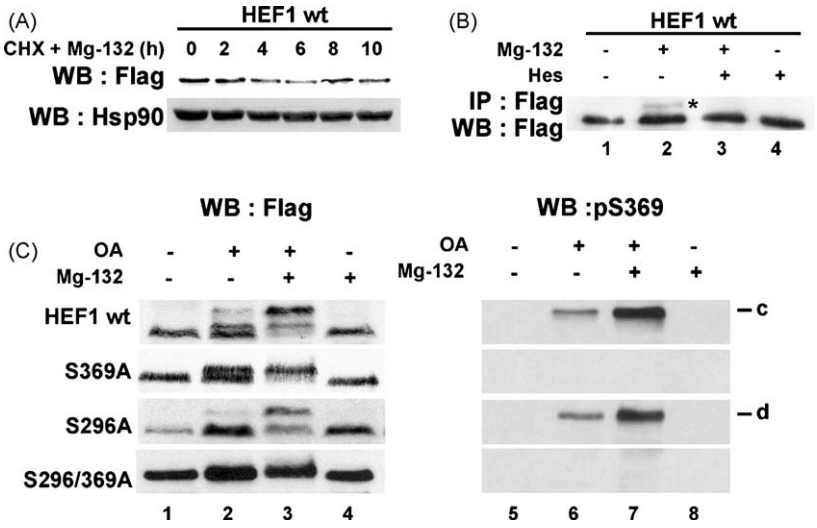

Fig. 6. Phosphorylation of HEF1 on serine 369 induces its proteasomal degradation. (A) HCT-116 cells were transiently transfected with pCMV-Flag-HEF1wt (HEF1wt). Time-course with CHX (70 μM) was performed in presence of Mg-132 (10 μM). Western-blotting was performed using anti-Flag antibody. Membrane was reprobed with anti-Hsp90 antibody as loading control. (B) HCT-116 cells transiently transfected with pCMV-Flag-HEF1wt (HEF1wt) were treated for 8 h with Mg-132 (10 μM) or Hes (1 μM) or both. Cells lysates containing an equal amount of proteins were immunoprecipitated with anti-Flag antibody. The immunoprecipitated protein complexes were then analyzed by Western-blotting with anti-Flag antibody. Asterisk in lane 2 indicates the position of the band similar to band d. (C) HCT-116 cells were transiently transfected with either pCMV-Flag-HEF1wt (HEF1wt), pCMV-Flag-HEF1 S369A (S369A), pCMV-Flag-HEF1 S296A (S296A) or pCMV-Flag-HEF1 S296/369A (S296/369A). Cells were treated with OA (125 nM) alone or in combination with Mg-132 (10 μM) for 8 h. Cell lysates were processed for immunoblotting with the anti-Flag antibody (left panel) and the anti-PhosphoSer-369HEF1 antibody (right panel) sequentially.

pointing out the short half-life of this protein as previously described in other cell lines [21]. We have shown that HEF1 is phosphorylated on ser-369. We then investigated whether in HCT-116 cells, HEF1 degradation is regulated by its phosphorylation on serine 369 by using Hesperadin. As shown on Fig. 5A (top right panel), the decrease in HEF1 is delayed in the presence of Hes, compared to the control time-course. Bands intensities were quantified by densitometric analysis (Fig. 5A bottom panel). Hesperadin strongly increased the stability of HEF1 which indicates that HEF1 instability depends on its phosphorylation by the Hesperadin-sensitive kinase.

To evaluate which serine phosphorylation promotes HEF1 instability, we transiently transfected HCT-116 cells with the HEF1 single and double mutants and CHX was added to the medium for different periods of time (from 2 to 10 h). Compared to wild-type HEF1, virtually no degradation of the S369A mutant of HEF1 was observed even after 10 h of CHX treatment (Fig. 5B, top right panel). This suggests that ser-369 phosphorylation triggers HEF1 degradation. By contrast, the same experiment performed with the HEF1 mutant S296A shows a degradation pattern similar to the one observed with wild-type HEF1, indicating that HEF1 degradation occurs independently of the phosphorylation state of serine 296 (Fig. 5B, top left panel). Finally, the double-mutated HEF1 behaves like HEF1 mutant S369A which agrees with a contribution of ser-369 phosphorylation in instability of HEF1 protein (Fig. 5B, bottom panels).

3.6. Phosphorylation of HEF1 on serine 369 induces its proteasomal degradation

To determine how HEF1 was degraded in our model, we performed the same time-course treatment with CHX on Flag-HEF1 transfected HCT-116 cells as in Fig. 5A, but Mg-132, a proteasome inhibitor was added to the medium concomitantly to CHX (Fig. 6A). We observed very little degradation of ectopic wild-type HEF1 in these conditions, suggesting that its degradation occurs through a proteasomal mechanism. Moreover, when Flag-HEF1 transfected HCT-116 cells were incubated for 8 h with Mg-132 only (without OA) and HEF1 immunoprecipitated from the cell lysates, we were able to detect a slower migrating band (marked by an asterisk, Fig. 6B, compare lanes 1 and 2) which may correspond to a ser/thr phosphorylated form of HEF1 identical to the one observed on Fig. 4A, lane 3 (refered to as band d). Moreover, the additional HEF1 species observed with Mg-132 is no longer detectable when cells were treated concomitantly with Hesperadin (Fig. 6B, compare lanes 2 and 3), which indicates that this additional species might be a product of the Hesperadin inhibited kinase activity. Altogether, these results suggest that HEF1 phosphorylation on ser-369 by the Hesperadin-sensitive kinase triggers its proteasomal degradation.

We next evaluated the effect of Mg-132 on the different mutant forms of HEF1. Wild-type HEF1 protein or mutated forms were overexpressed in HCT-116 cells. Cells were stimulated either with OA or Mg-132 or both. Compared to OA alone, addition of Mg-132 leads to an increase in intensity of both double phosphorylated isoform of wild-type HEF1 and shifted band of S296A HEF1 reflecting a stabilization of these HEF1 isoforms (Fig. 6C, compare lane 3 versus lane 2). This suggests that phosphorylation of serine 369 renders HEF1 more sensitive to proteasomal degradation. By contrast, phosphorylation of serine 296 does not modulate HEF1 susceptibility to proteasomal degradation (S369A panel).

In order to confirm that the proteasome inhibitor stabilized HEF1 isoform phosphorylated on ser-369 we performed a Western-blot with the anti-PhosphoSer-369HEF1 antibody. As shown in Fig. 6C, lane 7, Mg-132 treatment dramatically increased the stability of wild type HEF1 phosphorylated on both ser-296 and ser-369 (band c) and S296A HEF1 phosphorylated on ser-369 (band d) (compare lanes 6 and 7). As a control, no signal was observed with the S369A HEF1 and S269/369A HEF1 mutants treated with OA and Mg-132.

3.7. Endogenous HEF1 is stabilized by Hesperadin treatment

In the following set of experiments, we checked whether the stability of endogenous HEF1 in HaCaT cells depends on its phosphorylation. We performed a time-course treatment with CHX or Hes or Mg-132 on HaCaT cells. Fig. 7A shows that both HEF1 isoforms p105 and p115 disappear upon CHX treatment confirm-

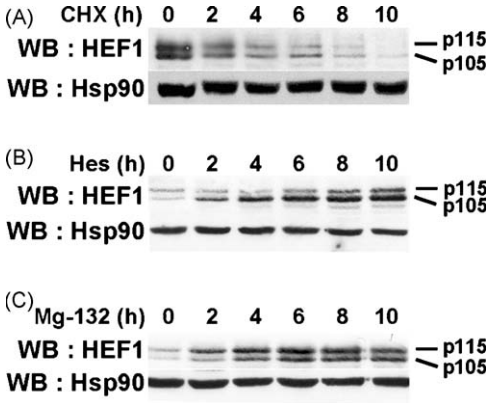

Fig. 7. Endogenous HEF1 is stabilized by Hesperadin treatment. Time-course of 70 μM CHX treatment (A) or 1 μM Hes treatment (B) or 10 μM Mg-132 treatment (C) were performed on HaCaT cells. HEF1 isoforms were resolved using SDS-PAGE migration and subsequent hybridization with monoclonal anti-HEF1 antibody. Membranes were reprobed with anti-Hsp90 antibody to ensure equal protein loading.

ing the instability of endogenous HEF1 in HaCaT cells. Hesperadin addition dramatically increased the intensity of p105HEF1 while we observed a moderate accumulation of p115 (Fig. 7B). This data indicates that, in HaCaT cells, Hes inhibits the conversion of p105 to p115 resulting in an accumulation of p105HEF1. Finally, upon Mg-132 treatment of HaCaT cells, we observed an increase of both p105 and p115 but the intensity is more pronounced for p115HEF1 (Fig. 7C). This result suggests that inhibition of proteasomal machinery decreases the degradation of p115 and to a lesser extent of p105. Taken together, these results indicate that, in HaCaT cells, conversion of p105 to p115 is dependent on phosphorylation and that inhibition of this phosphorylation by Hesperadin stabilizes HEF1 by removing this protein from proteasomal degradation pathways.

3.8. Aurora-B kinase is not involved in HEF1 ser-369 phosphorylation

Finally, we sought to determine whether Aurora-B was the kinase responsible for HEF1 phosphorylation and therefore conversion of p105 to p115. For this, we transfected HaCaT cells with siRNA for Aurora-B, and Aurora-A as a control and analyzed endogenous HEF1 migration profile. As expected transfection of these siRNA leads to depletion of either Aurora-A or -B (Fig. 8A). However inhibition of Aurora-B kinase expression does not correlate with increase of p105 band intensity. This result suggests that the Hesperadin-sensitive kinase responsible for HEF1 phosphorylation may not be Aurora-B. In order to confirm the actual inhibition of Aurora-B kinase activity, we analyzed the phosphorylation status of an Aurora-B substrate (BubR1) following Aurora-B siRNA transfection of HaCaT cells and treatment with Nocodazole, which blocks the cell cycle at M phase when Aurora-B is activated. As shown in Fig. 8B, Aurora-B siRNA transfection dramatically decreases Aurora-B expression; however, in the presence of Nocodazole BubR1 phosphorylation still occurs suggesting that even though it is expressed at a low level, enough protein is present to ensure BubR1 phosphorylation. We then tested another Aurora kinase inhibitor: ZM447439, which has been shown to target both Aurora-A and -B [32]. Surprisingly, compared to Hesperadin, ZM447439 does not inhibit HEF1 phosphorylation shift (Fig. 8C). As a control, cells were treated with Nocodazole to induce phosphorylation of Aurora-B substrate BubR1. Both inhibitors were able to prevent Nocodazole-induced BubR1 phosphorylation shift while in the same lysate, only Hesperadin inhibits HEF1 phosphorylation shift (Fig. 8C). These results indicate that Aurora-B is not the Hesperadin-sensitive kinase which triggers conversion of p105HEF1 to p115HEF1.

However, while Hesperadin is widely used as an Aurora-B inhibitor, several other kinases are also inhibited *in vitro* by Hesperadin, namely, AMP dependent kinase AMPK, the lymphocyte tyrosine kinase Lck, the MAPKK Mek1, the Erk substrate MAPKAPK1/p90Rsk, the checkpoint kinase Chk1 and the phosphorylase kinase PHK [31]. The potential contribution of most of these kinases in HEF1 phosphorylation has been examined. We used pharmacological inhibitors of Src (PP2) and Mek1 (U0126) which inhibits Erk1 substrate MAPKAPK1 activation as well. As shown in Fig. 8D, neither U0126 nor PP2 prevent conversion of p105 to p115, although in the mean time, PP2 seems to decrease HEF1 expression (Fig. 8D). Moreover, we did not observe HEF1 shift inhibition in Chk1 siRNA transfected HaCaT cells and in mouse embryo fibroblasts invalidated for AMPKα1/2 compared to wild type MEF (data not shown).

Altogether, these data suggest that conversion of HEF1 p105 to p115 in HaCaT cells occurs via an Hesperadin-sensitive phosphorylation which is independent of Aurora-B kinase.

4. Discussion

HEF1 is a docking protein present at focal adhesions and phosphorylated on tyrosine residues upon integrin binding, which links focal adhesion to cytoskeleton [1]. However, HEF1 is also phosphorylated on serine and threonine residues; indeed, HEF1 appears as a doublet: p105 and p115 (105 and 115 kDa) which mirrors two different phosphorylation states of the protein [20,21]. In this study we investigated the ser/thr phosphorylation of HEF1 in cells. In HCT-116, over-expressed HEF1 protein is detected as a single band. However, depending on conditions, when HEF1 is immunoprecipitated we were able to detect the p115 isoform with proteasome inhibitor (Fig. 6B, lane 2). So both isoforms seem to be present in these cells but for unknown reason, owing to the ectopic character of the protein (and maybe the fact that it was Flag-tagged), HEF1 is either less subject to phosphorylation or more rapidly degraded in HCT-116 cells.

We showed that conversion to p115HEF1 is a result of phosphorylation of p105 by a Hesperadin-inhibited kinase and we identified ser-369 of HEF1 as the target residue of this kinase. We confirmed, using an anti-PhosphoSer-369HEF1 antibody that serine 369 is indeed the residue targeted by this kinase. Moreover, our experiments also suggest that phosphorylation of HEF1 on ser-369 does not appear to depend upon either Aurora-A or Aurora-B kinase activity. So far, only the Aurora-A kinase was identified as an HEF1 kinase in MCF-7 cells, targeting ser-296. HEF1 interaction with Aurora-A during mitosis leads to kinase activation and its dissociation from phosphorylated HEF1 [22].

Two studies suggested that adhesion of Y1F cells induced ser/thr phosphorylation of HEF1 (conversion of p105 to p115 isoform) through a mechanism involving PP2A-dependent inhibition [19,21]. Such a regulation of phosphorylation did not occur in HaCaT cells (data not shown). Using the PP2A inhibitor, okadaic acid, we have been able to discriminate two serine phosphorylations on HEF1 (ser-296 and ser-369) occurring independently and with different kinetics (Fig. 2A and 4A). Mutation of these residues clearly prevents HEF1 shifting (Fig. 4A). A role of PP1 and/or PP2A in regulating Aurora kinases has been described previously [29,30]. Autophosphorylation of a threonine residue in the kinase domain of Aurora is necessary to activate the kinases [29,34]. Their inactivation occurs via a PP1 and/or PP2A-dependent dephosphorylation of this threonine. Furthermore, besides Aurora-B, other target kinases of Hesperadin (AMPK, Chk1, Mek1,...) are also substrates of PP2A, although at a higher IC_{50}, which make them candidates for HEF1 phosphorylation [35–37]. Furthermore, PP2A

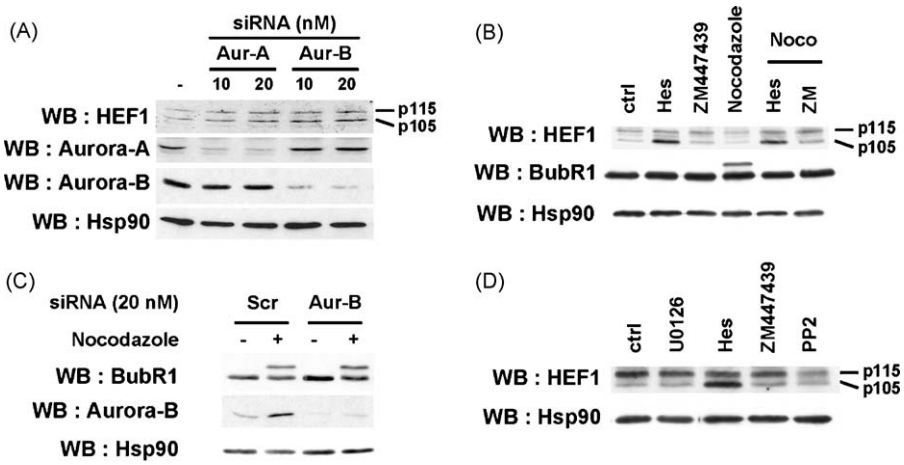

Fig. 8. Aurora-B kinase is not involved in HEF1 ser-369 phosphorylation. (A and B) HaCaT cells were transfected with siRNA targeting Aurora-A or Aurora-B at various concentrations as described. HEF1 isoforms were resolved using SDS-PAGE migration and subsequent hybridization with monoclonal anti-HEF1 antibody. Membranes were reprobed with anti-Hsp90 antibody to ensure equal protein loading and with anti-Aurora-A or anti-Aurora-B antibody to assess siRNA efficiency. (B) HaCaT cells were transfected with siRNA as described in A and treated with Nocodazole (300 nM) for 16 h before harvesting. Membrane was stripped and reprobed with anti-BubR1 antibody to visualize the activity of Aurora-B. (C) Cells were treated with Nocodazole (300 nM) for 16 h and with Hes (1 μM) or ZM (10 μM) for 8 h before harvesting. HEF1 and BubR1 isoforms were resolved using SDS-PAGE migration and subsequent hybridization with monoclonal anti-HEF1 and anti-BubR1 antibodies respectively. Membranes were reprobed with anti-Hsp90 antibody to ensure equal protein loading. (D) HaCaT cells were treated with the indicated drugs as described in Materials and Methods. HEF1 shift of migration was revealed using an anti-HEF1 antibody. Hsp90 was used as a loading control.

dephosphorylates another member of HEF1 docking protein family, p130 Cas [38]. This suggests that PP2A might then target both protein, the Hesperadin sensitive kinase and HEF1. We therefore cannot conclude as to whether increased phosphorylation of HEF1 in OA-treated HCT-116 cells results from inhibition of PP2A-mediated dephosphorylation of HEF1 or from increased kinase activity. Of note, even though we added PP2A inhibitor to observe HEF1 phosphorylation more readily, such a basal phosphorylation of HEF1 by the Hesperadin-inhibited kinase seems to actually occur even in the absence of OA treatment as observed in the presence of proteasome inhibitor without Hesperadin (Fig. 6B).

We also attempted to identify the kinase inhibitable by Hesperadin which is responsible for HEF1 phosphorylation on ser-369 and conversion of endogenous HEF1 p105 to p115. While Aurora-B was a good candidate as ser-369 matches perfectly the phosphorylation consensus site for this kinase, transfection of siRNA for Aurora-B failed to prevent HEF1 phosphorylation. In the mean time, the depletion of Aurora-B by siRNA did not prevent phosphorylation of BubR1, a known Aurora-B substrate, while Hesperadin did; indicating that Aurora-B extinction by siRNA was not complete. However, the use of another inhibitor, ZM447439 inhibiting Nocodazole-induced BubR1 phosphorylation but not HEF1 conversion to p115 allowed us to exclude Auroras as HEF1 kinases in HaCaT cells. Moreover, Aurora-A and -B depletion in HCT-116 cells transfected with HEF1 did not prevent phosphorylation of HEF1 on ser-369 and ser-296 following OA treatment; thus confirming the results obtained on endogenous HEF1 in HaCaT cells (Fig. 8C and data not shown). Specificity of Hesperadin has already been studied. Besides Aurora-B, six other kinases turned out to be inactivated by this drug *in vitro,* although with different IC_{50}. These kinases include AMPK, Chk1, Lck, Mek1, MAPKAPK1/p90Rsk and phosphorylase kinase PHK [31]. Use of a specific inhibitor of Mek1 and siRNA targeting Chk1 excluded the potential contribution of these kinases in HEF1 ser-369 phosphorylation. The fact that p90Rsk is a target of the Mek1/Erk pathway and thus is sensitive to Mek inhibition makes it unlikely to be the HEF1 kinase (Fig. 8D and data not shown). The p56 Lck, as a tyrosine kinase cannot directly phosphorylate HEF1 on serine but could somehow regulate the activity of the HEF1 kinase. However the Src family kinase inhibitor PP2, known to target p56Lck [39], has no effect on the HEF1 shift. Further experiments are needed to precisely identify the kinase.

Our results also clearly showed that in HaCaT cells, endogenous p115HEF1 depends on an Hesperadin-sensitive phosphorylation of p105HEF1 and this isoform is targeted to the proteasome (Fig. 7). We were able to show that in HCT-116 cells, the S369A but not the S296A mutation of HEF1 increases its stability (Fig. 5B). Recent published works agree with the preferential degradation of p115HEF1 isoform [16,18], although no kinase was identified yet. Interestingly, haematopoietic isoform of Cas/HEF1 Associated signal Transducer (CHAT-H), a binding partner of the Cas family proteins, is required to induce ser/thr phosphorylation of HEF1 and N-terminal domain of CHAT-H is necessary for this phosphorylation to occur [11]. Using several inhibitors such as PKC, Mek, PDK, PI3K inhibitors the authors failed to identify the kinase responsible for HEF1 ser/thr phosphorylation. Moreover, in their work, they deleted a short region of the serine-rich domain of HEF1 (from ser-369 to pro-396) that removes ser-369. Such deletion mutant does not undergo ser/thr phosphorylation anymore, which is in agreement with our results. It would be interesting to check which phosphorylated residue is targeted through CHAT-H interaction with HEF1.

Several reports demonstrated that ser/thr phosphorylation of HEF1 mediates its proteasomal degradation; p115HEF1 being the main isoform interacting with Smad3 and triggering the degradation of both proteins via an APC/CDH1 pathway [16,17]. Moreover AIP-4, an E3 ubiquitin ligase for HEF1, interacts more strongly with p115HEF1 than with the p105 isoform and ectopic expression of AIP-4 triggers preferentially p115HEF1 degradation [18]. The fact that ser-369 phosphorylated HEF1 undergoes proteasomal degradation is confirmed by the strong increase of this phosphorylated isoform upon Mg-132 treatment compared to OA alone (Fig. 6C). However, in the HCT-116 cell line, none of these proteins interacts with HEF1, which suggests that HEF1 degradation is not mediated through AIP-4, Smad3 or CDH1 (data not shown). In addition, besides its degradation through the proteasome, Law et al. showed that upon TNFα stimulation in MCF-7 cells, HEF1 can be processed in a caspase-dependent manner [20,40]. Although the ser-369 is very close to the $D_{363}LVD_{367}$ consensus caspase cleavage site, we did not observe a stabilization of this HEF1 isoform with addition of caspase

inhibitor (data not shown), indicating that HEF1 clearance does not occur in a caspase dependent manner.

Finally, we showed that in HaCaT cells, endogenous HEF1, detected as a doublet, undergoes degradation. When expressed in these cells, ectopic Flag-HEF1 is detected as a single band as observed in HCT-116 cells; moreover ectopic expression of HEF1 in HaCaT cells results in the same pattern as in HCT-116 cells, upon OA and Mg-132 treatment (data not shown). Zheng and McKeown-Longo demonstrated that ser/thr phosphorylated p115HEF1 is the major isoform targeted to the proteasome [19]. We showed that Hesperadin treatment leads to a dramatic increase of the p105HEF1 isoform which confirms that p105 is less sensitive to proteasomal degradation than p115. Moreover, we observed a moderate increase of the p115 isoform. This augmentation remains unexplained; although it is possible that the over accumulation of p105HEF1 might result in increased phosphorylation. Alternatively, there are examples of proteins in which a single residue is the target of different kinases [41,42]. So we can not exclude that other kinase(s) phosphorylate HEF1 on ser-369 but also on another residue leading to an HEF1 isoform that would be less sensitive to degradation and would be part of the p115 band. Comparatively, proteasome inhibitor treatment leads to accumulation of p115HEF1, even though p105 also increases. Indeed, this phosphorylation/dephosphorylation of HEF1 can be considered as a dynamic process, the kinase and PP2A activities regulating the switch between the two isoforms. It has also been hypothesized that different pools of HEF1 may be present in different subcellular compartments and have distinct functions [43]. Pugacheva and Golemis showed that HEF1 interacts with and activates Aurora-A during mitosis [22]. One can imagine that phosphorylation of a specific pool of HEF1 may modify the fate of this protein. In fact, Dadke et al. reported that by interacting with the RhoA-GEF ECT2, HEF1 positively regulates RhoA activity during early mitosis. Late in mitosis, the cleavage furrow regression needs RhoA to be inactivated and these authors showed that down-regulation of HEF1 expression is required for the cytokinesis to progress [10]. Phosphorylation of this specific pool of HEF1 at this moment might be a way for the cell to trigger RhoA inactivation. This may also explain why Hesperadin and Mg-132 treatment do not lead to a complete disappearance of p115 or p105 respectively.

We showed that HEF1 phosphorylation increases its degradation. The coupling of HEF1 phosphorylation on ser-369 and proteasomal degradation might not be cell type specific, but rather reflects a more general mechanism. It would be interesting to explore the mechanisms underlying the difference in HEF1 isoforms expression patterns between cell types.

In conclusion, HEF1 is a downstream target of unidentified integrin-regulated ser/thr kinases but also of cell cycle dependent kinases. It is likely that more than one kinase is involved in the phosphorylation of distinct residues mediating different processes. Indeed ser/thr phosphorylation of HEF1 is required for migration [15], adhesion [21], cell cycle [22], but may lead to degradation as well [19]. According to its localization, ser/thr phosphorylated HEF1 seems to have different functions. Here we demonstrated that HEF1 is phosphorylated on ser-369 by a Hesperadin-sensitive kinase. Furthermore, we showed that HEF1 phosphorylation at this site is required for its degradation by the proteasomal machinery.

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