FEBS Letters 488 (2001) 74–80 FEBS 24462

HERC3 binding to and regulation by ubiquitin

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Received 23 November 2000; accepted 29 November 2000

First published online 20 December 2000

Edited by Felix Wieland

Abstract Members of the HERC (domain homologous to E6 associated protein carboxy-terminus and RCC1 domain protein) family may function both as guanine nucleotide exchange factors and E3 ubiquitin ligases. Here we identify an unstudied member, HERC3. This protein was recognized by specific antibodies in different cell types. HERC3 was located in the cytosol and in vesicular-like structures containing β-COP, ARF and Rab5 proteins. Involvement of HERC3 in the ubiquitin system was suggested by its ability to interact with ubiquitin. The conserved cysteine in HECT proteins was not essential for this non-covalent binding. Moreover, HERC3 was a substrate of ubiquitination being degraded by the proteasome. These observations indicate a fine regulation of HERC3 and suggest a role in vesicular traffic and ubiquitin-dependent processes.

Key words: HERC; RCC1; HECT; E6 associated protein; Intracellular transport; Guanine nucleotide exchange

1. Introduction

Proteins that have a domain homologous to E6 associated protein (E6-AP) carboxy-terminus (HECT domain) and one or more regions with homology to the regulator of chromosome condensation (RCC1) have recently received the HGNC-approved designation of HERC for HECT and RCC1 domains [1]. RCC1 is the only guanine nucleotide exchange factor (GEF) found for the small GTPase Ran [2], which is involved in nucleocytoplasmic transport and the formation of the mitotic spindle [3]. Ran, like the other small GTPases of the Ras superfamily, switches between two states: an inactive GDP-bound form, and an active GTP-bound form. Through this mechanism small GTPases regulate cell proliferation, cytoskeletal morphology and intracellular transport [4–6]. GEFs act as positive modulators by promoting the exchange of GDP for GTP. Proteins containing HECT domains constitute a subclass of E3 ubiquitin ligases [7]. These ligases participate in the ubiquitin pathway which is responsible for the attachment of ubiquitin moieties to substrate proteins, a modification that serves as a signal both for proteasome-mediated proteolysis and for the internalization of membrane proteins. The transference of ubiquitin to the substrate involves a thioester bond between ubiquitin and a specific cysteine residue within E3 molecules, while the attachment of ubiquitin to target proteins occurs through isopeptide bonds between ubiquitin and lysine positions in the substrates [8,9]. The presence of HECT and RCC1-like domains (RLDs) in HERC proteins suggests that this protein family may function both as E3 ubiquitin ligases and as GEFs.

The human HERC family has four members. HERC1/p532 gene was isolated during the course of a gene transfer assay to identify novel oncogenes from human tumor DNA. Biochemical data on HERC1 suggest that it is involved in intracellular trafficking [10,11]. HERC2 was identified as the gene responsible for the *rjs* (runty, jerky and sterile) or *jdf-2* (juvenile development and fertility-2) phenotype in mice [1,12]. HERC2 protein is closely related to HERC1 and HERC2 mutants present secretory defects, which also suggests a function in vesicular trafficking [1,12]. HERC4 has recently been isolated from a two-hybrid screening using cyclin E and p21 as bait [13]. This protein has received the name of Ceb-1, for cyclin E binding protein-1, and it also interacts with cyclin A, B1 and D1. Expression of HERC4 is limited to reproductive tissues where it might have a role in the cell cycle as suggested by its association with cyclins. Finally, HERC3/D25215 gene was identified during a random search for human cDNAs larger than 2 kb [14]. While HERC1 and HERC2 are highly related proteins, the putative HERC3 resembles HERC4 and emerges as the HERC4 homologue in non-reproductive tissues [13,15]. HERC3 protein has not been characterized but the predicted protein contains a single amino-terminal RLD and a carboxy-terminal HECT domain. Here we report the identification of the HERC3 protein. HERC3 shows a subcellular distribution between cytosol and membranes similar to that found for other proteins involved in vesicular traffic. We also show that although HERC3 does not function as a Ran-GEF, it interacts with ubiquitin and is targeted for ubiquitination. Taken together, these results suggest a role for HERC3 in ubiquitin-dependent intracellular processes.

2. Materials and methods

2.1. Plasmids and baculovirus

2.1.1. Mammalian expression plasmids. Human D25215/HERC3 cDNA was kindly provided by Dr. N. Nomura [14]. An *Eco*RI-*Bgl*II polymerase chain reaction (PCR) fragment corresponding to nucleotides 167–1263 of D25215/HERC3 cDNA was fused to nucleotides 1264–3370 (*Bgl*II-*Stu*I) digested from D25215 cDNA and subcloned in frame into *Eco*RI-*Eco*RV sites of pcDNA3.1 HisC (Invitrogen) to generate 6xHis-HERC3 (pCC36). C1018A 6xHis-HERC3 mutant (pCC43) was obtained by site-directed mutagenesis (Stratagene) using

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Abbreviations: HERC, HECT domain and RCC1 domain protein; HECT, homologous to E6-AP carboxy-terminus; E6-AP, E6 associated protein; RLD, RCC1-like domain

0014-5793/01/$20.00
PII: S0014-5793(00)02371-1

oligonucleotides 5′-CGGTGGCCCACACAGCTTACAACCTTCT-TG-3′ and 5′-CAAGAAGGTTGTAAGCTGTGTGGGCCACCG-3′, which contain silent mutations for a diagnostic *Alu*I site in addition to the cysteine to alanine mutation. Myc-tagged HERC4 RLD (residues 1–323) cDNA cloned into pCS2^{+}MT was kindly provided by Dr. M. Ohtsubo [13].

2.1.2. Glutathione-S-transferase (GST)-fusion proteins. The first half of RLD (residues 1–199), the full RLD (residues 1–392) and the HECT domain (residues 848–1050) were amplified by PCR from D25215/HERC3 cDNA with specific oligonucleotides and subcloned into *Eco*RI and *Xho*I sites of pGEX-4T-1 (Pharmacia) to obtain pCC1, pCC2 and pCC3 plasmids, respectively. GST-HERC3 (pCC38) was obtained by subcloning an *Eco*RI-*Xho*I insert from pCC36 into pGEX-4T-1. Ubiquitin was amplified by PCR with specific oligonucleotides from HeLa cDNA library (Stratagene) and subcloned into pGEX-2TK (Pharmacia) to generate GST-ubiquitin (pJLR86). Human RCC1 cDNA from pJLR8 [10] was subcloned into *Bam*HI/*Not*I pGEX-5X-1 (Pharmacia) to generate GST-RCC1 (pJLR135).

2.1.3. Baculovirus. ARF6-6xHis was obtained by PCR amplification with specific oligonucleotides from human ARF6 cDNA (kindly provided by Dr. J. Donaldson) and subcloned into pFastBac1 (pJLR134). ARF6 baculovirus was generated with the Bac-to-Bac system following the manufacturer's instructions (Gibco BRL). RCC1, HERC1 RLD1, HERC1 RLD2, Ran, Rab3A, Rab5, Rab8 and ARF1 baculoviruses are described elsewhere [10]. All DNA amplified by PCR was sequenced to confirm that no errors were introduced by the polymerase.

2.2. Protein purification and guanine nucleotide exchange assays

2.2.1. Protein purification. RCC1, HERC1 RLD1, HERC1 RLD2, Ran, Ras, Rab3A, Rab5, Rab8, ARF1 and ARF6 were expressed in Sf9 cells and purified as described [10]. GST-Rac and GST-RhoA were kindly provided by Dr. X.R. Bustelo. Proteins encoded by GST-RLD (residues 1–199; pCC1) and GST-HECT (pCC3) formed inclusion bodies and were used to generate antibodies. Soluble GST-RCC1 (pJLR135), GST-HERC3 (pCC38) and GST-RLD (residues 1–392; pCC2) fusion proteins were expressed in BL21 bacteria, purified according to standard procedures with glutathione-Sepharose (Amersham Pharmacia Biotech AB) and used for nucleotide exchange assays. GST and GST-ubiquitin were also purified by standard glutathione-Sepharose affinity.

2.2.2. Guanine nucleotide exchange assays. These experiments were performed essentially as described [10].

2.3. Antibodies

Purified proteins GST-RLD (residues 1–199) or GST-HECT (residues 848–1050) were subcutaneously injected into rabbits to raise anti-HERC3 rabbit polyclonal antibodies CC1, CC2 and CC3, CC4, respectively. For Western blot analysis, either whole antisera or immunoglobulins separated from whole antisera by standard methods [16] were used at 1/1000 dilution. For immunofluorescent staining, antibodies were immunoaffinity purified and used at 1/50 dilution. Mouse monoclonal antibodies (mAbs) against ARF proteins (1D9), anti-Rab5, anti-β-COP (M3A5), and anti-transferrin receptor were kindly provided by R. Kahn, J. Blasi, T.E. Kreis, and M. Camps, respectively. Anti-Golgi protein GMP$_{t}$1 (18B11) and anti-LIMPII (38C7) were a kind gift from I.V. Sandoval. Monoclonal antibodies anti-Hsp70 and anti-c-myc were from Oncogene Science. Anti-α-tubulin (Ab-1) and anti-M2-pyruvate kinase (clone DF4) were from Calbiochem and ScheBo.Tech, respectively. Swine anti-rabbit antibodies conjugated to Texas Red and sheep anti-mouse antibodies labeled with fluorescein were from DAKO.

2.4. Cell culture and transfection

CCD45-SK, MCF-7, HeLa, NRK and COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Peripheral blood lymphocytes (PBLs) were isolated from blood from healthy human donors by centrifugation on a Ficoll gradient. For comparison of HERC3 expression in different cell types, exponentially growing cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in buffer 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 100 μg/ml benzamidine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM NaF, 100 μM orthovanadate; supernatants were recovered after 10 min centrifugation at 13 000×*g*. Transfection of COS-1 cells was performed using the DEAE-dextran method with 6–8 μg DNA per 100 mm plate. After two washes in cold phosphate-buffered saline (PBS) cell extracts were prepared with 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% Triton X-100 supplemented with 1 mM EDTA, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 5 μg/ml leupeptin, 100 μg/ml benzamidine, 0.5 mM PMSF, 1 mM NaF, 100 μM orthovanadate. If treatment with *N*-acetyl-leucinyl-leucinyl-*N*-norleucinal (LLnL) was required, the day after transfection cells were split into two 60 mm plates. After allowing 8–10 h for cell adhesion, cells were treated overnight with 50 μM LLnL (Sigma) or with the same volume of dimethyl sulfoxide (DMSO) in fresh medium. Cell extracts were prepared after washing cells twice in cold PBS by boiling cells for 15 min with 25 mM Tris–HCl, pH 7.5, 1.5% sodium dodecyl sulphate (SDS). Proteins were determined by the BCA Protein Assay (Pierce).

2.5. Cell fractionation

Exponentially growing COS-1 cells were rinsed twice with cold PBS and scraped in buffer containing 10 mM Tris–HCl pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 250 mM sucrose, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 100 μg/ml benzamidine, 0.5 mM PMSF, 1 mM NaF, 100 μM orthovanadate. Cells were disrupted by successive passage through a 27G needle. Cell breakage and nucleus integrity were monitored by trypan blue staining. Nuclei were harvested by 10 min centrifugation at 1000×*g* followed by resuspension and recentrifugation to eliminate traces of cytosol. The postnuclear fraction was subsequently centrifugated at 100 000×*g* for 1 h. After recovering the supernatant (cytosol), the pellet (membranes) was rinsed and recentrifugated. All steps were done in the cold. Final fractions were adjusted to the same final volume before preparing samples for Western blot with sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol (DTT)).

2.6. Immunofluorescent staining

NRK cells were seeded on coverslips. The following day, cells were fixed in freshly prepared solution (3% paraformaldehyde, 60 mM sucrose, 0.1 M sodium phosphate pH 7.2) for 30 min at room temperature. After three washes in PBS for 5 min, cells were washed with PBS 20 mM glycine for 5 min, permeabilized in PBS 20 mM glycine 0.1% saponin for 10 min at room temperature, washed with PBS 20 mM glycine for 5 min and blocked with PBS 20 mM glycine 1% bovine serum albumin (BSA) for 10 min at room temperature. Incubation with primary antibodies was done in blocking solution for 1 h at 37°C. After washing in PBS glycine for 5 min cells were incubated with secondary antibodies in blocking solution for 45 min at 37°C. Finally, cells were washed for 10 min in PBS and mounted with mowiol (Calbiochem) and analyzed by confocal microscopy.

2.7. Ubiquitin binding assays

2.7.1. Experiments in the rabbit reticulocyte system. Radiolabeled wild-type (pCC36) and mutant (pCC43) HERC3 proteins were produced using T7 Quick coupled TNT kit (Promega) as indicated by the manufacturer. After protein synthesis, aliquots (20 μl) were removed and incubated for 15–30 min with or without 6.5 μg GST-ubiquitin at room temperature. Reactions were stopped at 100°C for 10 min with sample buffer, and proteins were analyzed by SDS–PAGE. After electrophoresis gels were fixed, dried and autoradiographed. For pull-down assays, after the transcription/translation reaction identical aliquots were incubated with 5–10 μg of either GST beads or GST-ubiquitin beads for 15–20 min at room temperature. After binding, the reaction was stopped at 4°C and washed six times with 1 ml of cold buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% Triton X-100). Beads were recovered in sample buffer and proteins analyzed as above. After electrophoresis gels were stained with Coomassie blue, dried and autoradiographed. For competition assays, 5 μg of GST, 5 μg of GST-ubiquitin or 5 μg of GST-ubiquitin plus 10 μg of free ubiquitin were used.

2.7.2. Experiments in bacteria. BL21 cells harboring a control plasmid or GST-HERC3 encoding plasmid (pCC38) were grown to same absorbance ($\lambda = 600$ nm) and induced with IPTG for 2 h. Briefly, cells were sonicated in buffer 10 mM Tris–HCl pH 7.5, 0.2 mM DTT and then Triton X-100 was added at a final concentration of 0.2%.

After centrifugation the cleared lysates were incubated with either GST matrix or GST-ubiquitin matrix for 100 min at 4°C. After washing four times with cold buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.2% Triton X-100) equal aliquots of the affinity matrices were removed, eluted with sample buffer and analyzed by Western blot. Control GST and GST-ubiquitin matrices were obtained by cross-linking 1.5 mg of proteins to a mixture of 0.5 ml Affigel 10 and 0.5 ml Affigel 15 (Bio-Rad) following manufacturer's instructions and were extensively washed before use.

3. Results

3.1. Identification of HERC3 protein

We generated two different classes of sera: CC1 and CC2 against HERC3 RLD, and CC3 and CC4 against the HECT domain (Fig. 1A). Initially these antibodies were tested by Western blot against the purified antigens to compare their sensitivity. As expected, the four antisera detected the purified antigens, as shown in Fig. 1A for CC1 and CC3 antibodies. Similar results were obtained with CC2 and CC4 antisera (data not shown). We found the highest sensitivity for CC1 antiserum. A 1:1000 dilution of CC1 serum detected 0.1 ng of GST-RLD fusion protein (Fig. 1A). Next, we tested the ability of these antibodies to detect endogenous HERC3 protein. Extracts from COS-1 cells were analyzed by SDS–PAGE and Western blot using CC1 and CC3 antibodies. A major band with a molecular mass of approximately 117 kDa, the size expected for the protein encoded by the human HERC3/D25215 cDNA, was observed (Fig. 1B, vector lane of upper panels). To confirm this band as the HERC3 protein, COS-1 cells were transfected with the full-length HERC3 cDNA. Overexpression of HERC3 caused an increase in immunoreactivity of the 117 kDa band (Fig. 1B, HERC3 lane of upper panels). Immunodetection of α-tubulin was used as control for loading. To rule out that CC1 antibodies recognized HERC4/Ceb-1 protein which has a similar size [13], COS-1 cells were also transfected with the myc-tagged RLD of HERC4. CC1 antibodies did not show cross-reaction with the HERC4 RLD (Fig. 1B, HERC4 RLD lane of upper left

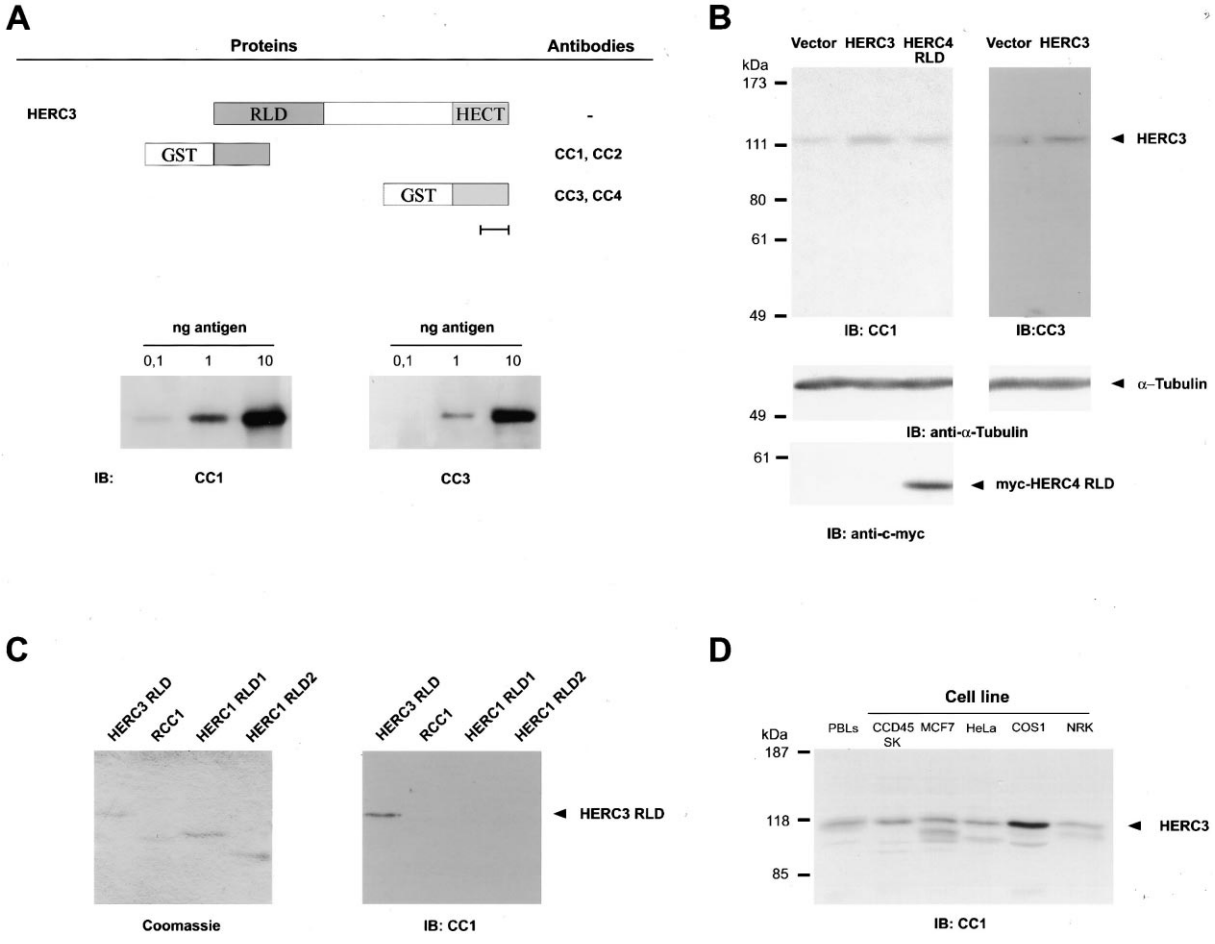

Fig. 1. Anti-HERC3 antibodies and HERC3 protein. A, upper panel: Schematic representation of HERC3 protein indicating the position of RLD and HECT domains. GST-fusion proteins used to generate anti-HERC3 antibodies are also depicted. Scale bar indicates 100 amino acidic residues. Lower panel: Testing anti-HERC3 antibodies. Antisera CC1 (left panel) and CC3 (right panel) at 1:1000 dilution were tested against increasing amounts of their respective antigens. B: Immunoblot of cellular lysates. Protein extracts (70 μg) for COS-1 cells transfected with 6 μg of pcDNA3.1HisC (vector), expression plasmid pCC36 (HERC3) or expression plasmid encoding myc-tagged HERC4 RLD (residues 1–323) were resolved by 8% PAGE/SDS and immunoblotted with anti-HERC3 antibodies CC1 and CC3 (top panel), anti-α-tubulin antibody (middle panel) or anti-c-myc antibody (bottom panel). C: CC1 antibody specificity toward RLD-containing proteins. ≈150 ng of purified HERC3 RLD, RCC1, HERC1 RLD1 and HERC1 RLD2 were resolved by 10% PAGE/SDS and either stained with Coomassie (left panel) or immunoblotted with CC1 antibodies (right panel). D: HERC3 expression in different cell types. Protein extracts (100 μg) from human (PBL, CCD45-SK, MCF-7 and HeLa), monkey (COS-1) and rat (NRK) cells were resolved by 6% PAGE/SDS and immunoblotted with CC1 antibody. Molecular mass markers are indicated in kDa. Arrowheads show the positions of indicated proteins. IB, immunoblot.

panel). We have also analyzed the specificity of CC1 antibodies against RCC1 and the RCC1-like domains of HERC1 (RLD1 and RLD2). Purified RCC1, RLD1, RLD2 and the GST-HERC3 RLD (residues 1–392) (see Coomassie staining, Fig. 1C) were analyzed by Western blot. As shown in Fig. 1C, CC1 antibodies specifically recognized the HERC3 RLD. To analyze the expression of endogenous HERC3 protein in other cell types, we performed Western blot analysis using lysates from human cells (PBLs, peripheral blood lymphocytes; CCD45-SK, skin fibroblasts; MCF-7 and HeLa, epithelial carcinoma cells), monkey cells (COS-1, kidney fibroblasts) and rat cells (NRK, kidney fibroblasts). A major band of approximately 117 kDa was immunodetected in all the cell types using CC1 antibodies (Fig. 1D). Less intense lower immunoreactive bands were also detected in some cases. Whether they represent degradation products or isoforms remains to be determined. Similar results were obtained using CC3 antibodies (data not shown). Altogether these results show that HERC3 antisera are sensitive and specific to detect HERC3 protein and that HERC3 is present in different cell types and species.

3.2. Subcellular location of HERC3 and GEF activity

To localize HERC3 protein, we analyzed its subcellular distribution in COS-1 cells. Cells were fractionated into three fractions: nuclei, membranes and cytosol. Transferrin receptor was used as a marker for membranes; α-tubulin for cytosol; Hsp70 as a protein ubiquitously distributed. Western blot analysis with anti-HERC3 antibodies revealed the presence of HERC3 mainly in the cytosol (Fig. 2A); a small amount was also detected in the membrane fraction after overexposition (data not shown). Similar results were obtained in NRK cells and MCF-7 cells (data not shown). The localization of HERC3 was also studied by confocal microscopy using immunopurified CC1 antibodies. A punctate staining of endogenous HERC3 was observed throughout the cytosol of NRK

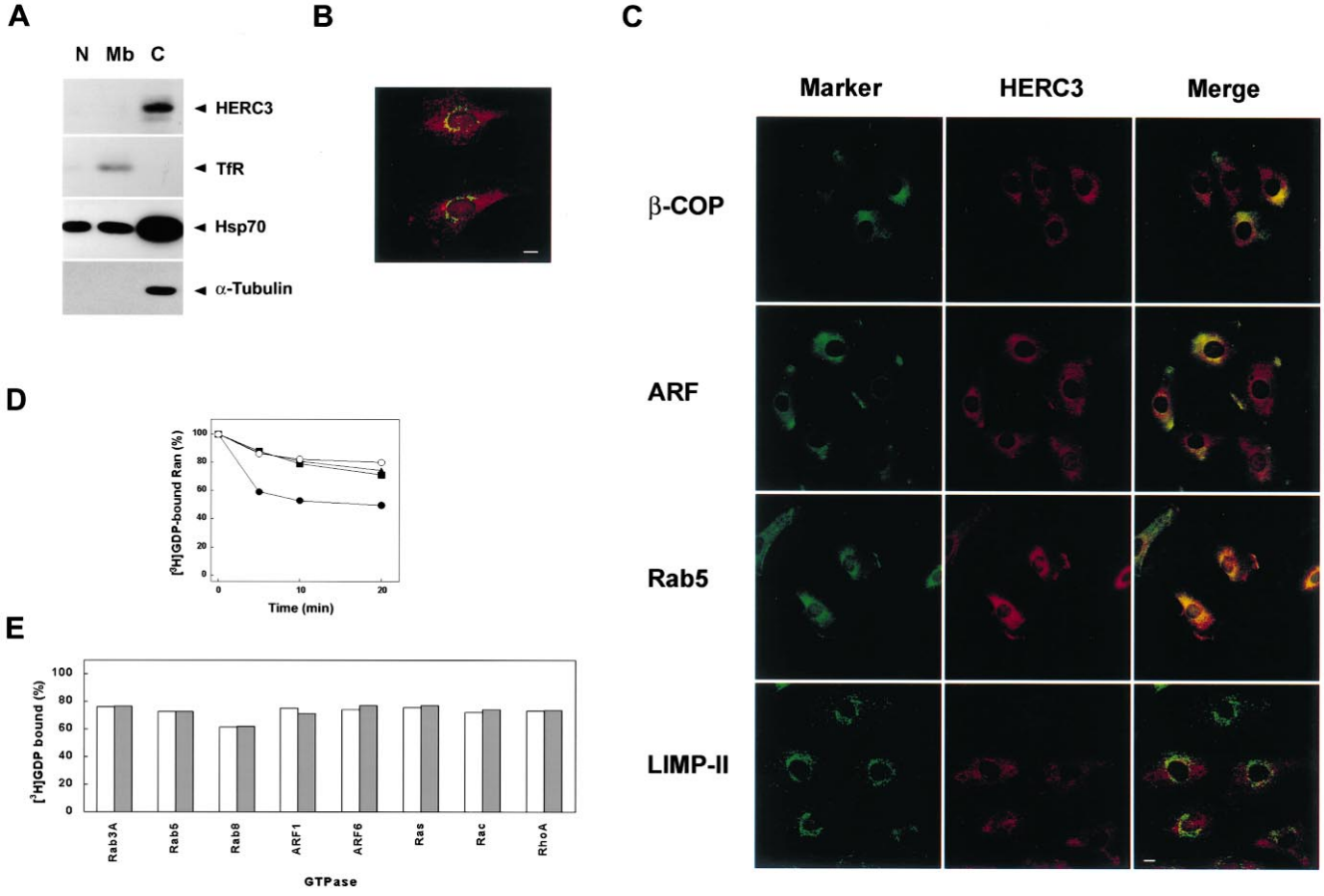

Fig. 2. HERC3 intracellular location and GEF activity. A: Cellular fractionation. COS-1 cells were disrupted and separated into three fractions: nuclei (N), cellular membranes (Mb) and cytosol (C). Protein equivalents of these fractions were resolved by 6% PAGE/SDS and immunoblotted against HERC3, transferrin receptor (TfR), Hsp70 and α-tubulin. The positions of these proteins are indicated by arrowheads. B: Double immunofluorescence analysis with anti-HERC3 and anti-Golgi antibodies. NRK cells were grown on coverslips, fixed, stained with immunopurified rabbit antibodies against HERC3 (red) and mAb antibody against Golgi protein GMP_t1 (green) and analyzed by confocal microscopy. Colocalization is shown in yellow. Scale bar represents 10 μM. C: Immunofluorescence analysis with anti-HERC3 antibodies and lysosomal and vesicular markers. NRK cells processed as in B were stained with anti-HERC3 immunopurified antibodies (middle panel, red) and mAbs against either β-COP, ARF, Rab5 or LIMPII protein (left panel, green). Colocalization analysis was performed by confocal microscopy and colocalization (right panel, merge) is indicated in yellow. Scale bar represents 10 μm. D: Exchange activity on Ran. Ran (1.5 μg) was loaded with [^{3}H]GDP and incubated for the indicated periods of time with buffer (open circles) or the following fusion proteins: GST-RCC1 (solid circles), GST-HERC3 (solid triangles) or GST-HERC3 RLD (residues 1–392) (solid squares). At each time point, aliquots from each condition were taken in duplicate and the amount of [^{3}H]GDP-bound Ran quantified by filter immobilization assay. E: Exchange activity on Rab3A, Rab5, Rab8, ARF1, ARF6, Ras, Rac and RhoA. GTPases were loaded with [^{3}H]GDP and incubated with albumin (open bars) or GST-HERC3 protein (dashed bars). After 10 min, [^{3}H]GDP-bound GTPases were quantified as in A. Results are expressed as percentage of [^{3}H]GDP bound to the GTPases just before the addition of the factors. Each value is the mean of two determinations.

cells (Fig. 2B). The same result was observed in COS-1 and CCD45-SK cells (data not shown). Although HERC3 is mainly a cytosolic protein (Fig. 2A) a minor fraction of HERC3 could be associated with intracellular membranes. To define this distribution, we have analyzed its colocalization with different membrane markers. Double staining with antibodies against β-COP and HERC3 revealed a degree of colocalization, as confirmed when the images were overlaid (Fig. 2C). Similar results were obtained when NRK cells were costained with antibodies against ARF or Rab5 proteins, GTPases involved in vesicular traffic, and HERC3 (Fig. 2C). The greatest colocalization was found with Rab5. In contrast, HERC3 did not colocalize with the trans-Golgi membrane protein GMP_t1 or the lysosomal protein LIMP-II (Fig. 2B and C). Similar results were obtained using immunopurified CC3 antibodies (data not shown).

On the basis of its homology to RCC1, we examined whether HERC3 has GEF activity for Ran, GST-HERC3 and GST-HERC3 RLD (residues 1–392) fusion proteins were used in a GEF assay in which we monitored [^{3}H]GDP release from [^{3}H]GDP loaded Ran. As positive control, we used the Ran-GEF RCC1 as a GST-RCC1 fusion protein. As shown in Fig. 2D, neither HERC3 nor the HERC3 RLD promoted [^{3}H]GDP dissociation from Ran in conditions

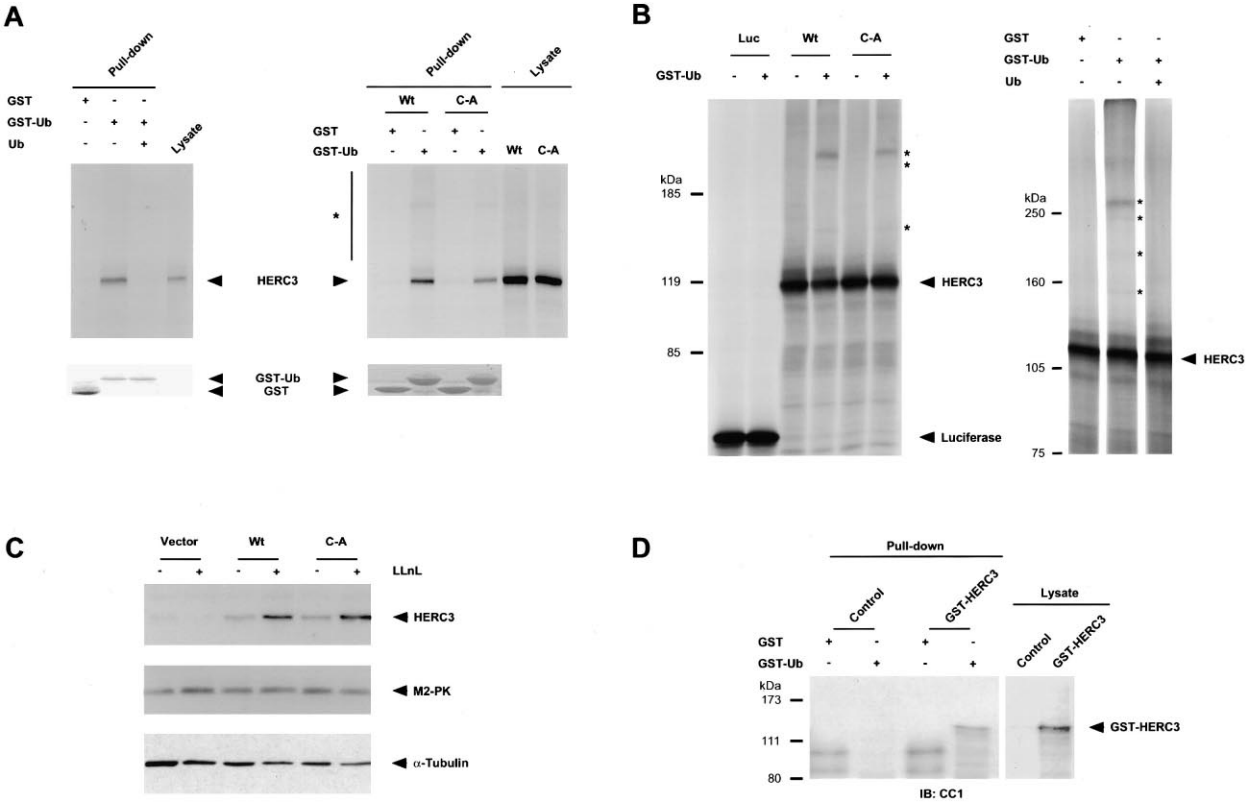

Fig. 3. Ubiquitin binding by HERC3 and HERC3 ubiquitination. A: Pull-down experiments with GST and GST-ubiquitin. Left panel: Competition assay. Wild-type HERC3 protein was synthesized in the rabbit reticulocyte system in the presence of [^{35}S]methionine and incubated with either GST (GST), GST-ubiquitin (GST-Ub), or GST-ubiquitin plus free ubiquitin (Ub). After binding to glutathione-Sepharose and extensive washing, proteins were eluted from the beads with sample buffer containing DTT and resolved by discontinuous 7–12% PAGE/SDS in parallel with rabbit reticulocyte lysate containing the transcribed and translated protein (right lane). After staining with Coomassie blue, gels were dried and autoradiographed. Right panel: Radiolabeled wild-type HERC3 protein (Wt) and C1018A HERC3 protein (C-A) were synthesized as above and incubated with either GST beads (GST) or GST-ubiquitin beads (GST-Ub). After extensive washing, proteins were eluted as above and resolved by discontinuous 7–12% PAGE/SDS in parallel with aliquots of rabbit reticulocyte lysate containing the transcribed and translated protein products. After staining with Coomassie blue, gels were dried and autoradiographed. Comparable amounts of GST and GST-ubiquitin proteins were pulled-down as seen by Coomassie blue staining (bottom panels). Asterisk indicates slower migrating HERC3 bands. B: Ubiquitin adduct formation of HERC3 and mutant HERC3 proteins. Left panel: Wild-type HERC3 protein (Wt), C1018A HERC3 protein (C-A) and control luciferase (Luc) were synthesized as in A and incubated in the presence (+) or absence (−) of GST-ubiquitin (GST-Ub). Reactions were stopped in sample buffer and proteins resolved by 7% PAGE/SDS. After fixation, gels were dried and autoradiographed. Right panel: Competition assay. Radiolabeled wild-type HERC3 protein was incubated in the presence of GST (GST) GST-ubiquitin (GST-Ub) or GST-ubiquitin plus free ubiquitin (Ub). Reactions were stopped as in A and proteins resolved by discontinuous 7–12% PAGE/SDS. After fixation, gels were dried and autoradiographed. Asterisks indicate slower migrating HERC3 bands. C: HERC3 is ubiquitinated and degraded by the proteasome in vivo. 100 mm plates of COS-1 cells were either transfected with 8 μg of pcDNA3.1HisC (vector), expression plasmid pCC36 (wild-type HERC3, Wt) or pCC43 (C1018A HERC3 mutant, C-A). 24 h later, cells from each transfection were split into two 60 mm plates, and treated overnight with DMSO (−) or the proteasome inhibitor LLnL at a concentration of 50 μM (+). Protein extracts (100 μg) were resolved by 7% PAGE/SDS and immunoblotted with CC1 antibody (top panel), anti-M2-pyruvate kinase (M2-PK) antibody (middle panel) and anti-α-tubulin antibody (bottom panel). D: Binding of GST-HERC3 produced in bacteria to a ubiquitin affinity matrix. A control lysate (control) and a GST-HERC3-containing lysate (GST-HERC3) were incubated with either GST or GST-ubiquitin cross-linked to an agarose matrix. After binding and extensive washing, aliquots were removed, eluted with sample buffer, resolved by 7% PAGE/SDS and immunoblotted with CC1 antibody in parallel with aliquots of control and HERC3-containing lysates. Arrowheads show positions of the indicated proteins. Molecular mass markers are expressed in kDa. IB, immunoblot.

that allowed RCC1 to stimulate this release. The HERC3 colocalization with vesicular proteins, including GTPases ARF and Rab5, raised the possibility that its substrate might be one of these GTPases or other members of these two families of GTPases that control intracellular transport. In order to check this point, we performed GEF assays with Rab proteins (Rab3A, Rab5 and Rab8) and ARF proteins (ARF1 and ARF6). We also tested members of the remaining two families of small GTPases: H-Ras for Ras family and Rac and RhoA for Rho family. As shown in Fig. 2E, HERC3 did not promote guanine nucleotide exchange on any of the small GTPases assayed.

3.3. Ubiquitin binding and polyubiquitination

The presence of a HECT domain in HERC3 suggests that it may bind ubiquitin. To check this possibility, we performed pull-down experiments with GST-ubiquitin bound to glutathione beads incubated with [^{35}S]methionine-radiolabeled HERC3 protein synthesized in the rabbit reticulocyte system. Glutathione beads in the presence of GST-ubiquitin specifically pulled down the wild-type form of HERC3 (Fig. 3A, left panel, arrowhead). This binding probably did not occur through a thioester bond because the same HERC3 mobility was observed by running the samples under non-reducing conditions (data not shown). As it was expected, the addition of free ubiquitin avoided this interaction. A conserved cysteine residue in the HECT domain is essential for the ubiquitin binding and ubiquitinating activity of HECT proteins [7]. The equivalent cysteine in HERC3 is C1018. To analyze the importance of C1018 in HERC3 binding to ubiquitin, we generated a mutant form of HERC3 in which the residue was altered to alanine (C1018A HERC3). Pull-down experiments with ^{35}S-labeled C1018A HERC3 protein showed that it could also bind to GST-ubiquitin, but to a lesser extent (Fig. 3A, right panel, arrowhead).

GST-ubiquitin pull-down experiments also specifically recovered high molecular weight forms of wild-type and C1018A HERC3 proteins (Fig. 3A, asterisk) that might correspond to GST-ubiquitinated forms of these proteins. To investigate whether GST-ubiquitin molecules may be attached to HERC3, [^{35}S]methionine-radiolabeled HERC3 proteins were synthesized in the rabbit reticulocyte system and incubated in the presence or absence of GST-ubiquitin. This incubation resulted in the formation of HERC3 species of higher molecular weight when GST-ubiquitin was added (Fig. 3B, right panel, asterisks). The size increase of these HERC3 species is consistent with the attachment of multiple GST-ubiquitin moieties (35 kDa) to HERC3 protein. These species were DTT-resistant, indicating that this attachment involves isopeptidic bonds rather than thioester bonds. As it was expected, the addition of free ubiquitin avoided the formation of HERC3 species of higher molecular weight. Interestingly, similar results were obtained with the C1018A mutant (Fig. 3B, left panel). The higher molecular weight bands were not observed in ^{35}S-labeled luciferase, which was used as negative control (Fig. 3B, left panel).

The HERC3 ubiquitination might target the protein to proteasome-mediated degradation. To confirm this hypothesis, we tested the effect of the proteasome inhibitor LLnL on HERC3 levels in vivo. As shown in Fig. 3C, the treatment with LLnL of COS-1 cells transfected with HERC3 cDNA resulted in an increase of HERC3 levels (Fig. 3C) and accumulation of ubiquitinated forms of HERC3 after overexposition (data not shown). As expected, similar results were obtained for C1018A mutant (Fig. 3C). Levels of α-tubulin and M2-pyruvate kinase are shown as controls (Fig. 3C). These results show that the HERC3 ubiquitination observed in vitro is also produced in vivo and that it targets HERC3 for proteasome degradation, thereby regulating the levels of HERC3 in the cell.

The HERC3 ubiquitination (Fig. 3B and C) and its interaction with ubiquitin (Fig. 3A) led us to think whether the association of ubiquitin with HERC3 is significant beyond normal substrate recognition by the ubiquitin/proteasome pathway. To answer this question, we have analyzed whether HERC3 can be affinity purified from bacteria using a ubiquitin matrix. To this end, we have cross-linked purified GST-ubiquitin to an agarose matrix. A bacterial lysate of GST-HERC3 fusion protein was incubated with the GST-ubiquitin agarose. Under these conditions, GST-HERC3 protein was specifically purified (Fig. 3D). As negative controls of this experiment, we have used a GST agarose matrix and bacterial lysate not expressing GST-HERC3 (Fig. 3D).

4. Discussion

The structural features of the HERC proteins suggest that they are involved in the regulation of intracellular transport, cell cycle control and ubiquitin-dependent processes [1,10–13]. HERC3 protein is a previously unstudied member of this family. Now, we report the identification of HERC3 protein. This protein was found in all the cell types tested suggesting that it is ubiquitous and confirming the expression previously reported by Northern blot [13,14,17]. The subcellular distribution of HERC3 between the cytosol and vesicular membranes suggests that its association with vesicles might be transient, a common trait of vesicular traffic proteins [6]. Moreover, its colocalization with β-COP, ARF and Rab5 proteins suggests a possible role for HERC3 in intracellular transport. The vesicular-type pattern observed by confocal microscopy resembles that found for HERC1 except that HERC3 was not found in the Golgi [10].

The presence of a conserved RLD indicates that HERC proteins are putative guanine nucleotide exchange factors. This also applies to the RLD-containing proteins that do not belong to HERC family, some of which have been implicated in human diseases: Retinitis Pigmentosa GTPase regulator (RPGR), which is the protein product for the gene whose mutation is the major cause of X-linked Retinitis Pigmentosa [18–20]; DelGEF, for deafness locus associated putative guanine nucleotide exchange factor [21]; a protein associated with myc (Pam) [22]; and CHC1L (chromosome condensation-1 like), a protein with a potential role in tumors and retinal disease [23]. Little is known about which GTPases may be regulated by these putative GEFs which have a potential role in human pathologies. The only information available is that HERC1 RLD1 can stimulate guanine nucleotide dissociation for ARF1, Rab3A and Rab5. The homology between HERC1 and HERC3 and their subcellular location in vesicular structures led us to analyze whether HERC3 can stimulate guanine nucleotide exchange in various GTPases of Ras superfamily (Ran, Ras, Rac, RhoA, Rab3A, Rab5, Rab8, ARF1 and ARF6). HERC3 did not stimulate guanine nucleotide dissociation on any of the GTPases tested. The

colocalization between HERC3 and Rab5 and ARF proteins also led us to check a possible association similar to that described between RCC1 and Ran [24] or HERC1 and ARF1 [10]. Although our attempts to find these protein–protein interactions using insect cells infected with recombinant baculovirus expressing these proteins failed (data not shown), we cannot rule out that HERC3 could act on some other GTPase, yet to be identified.

HERC proteins are predicted to have E3 ligase activity. The functionality of the isolated HECT domains has been reported for HERC1 and HERC3, and their preference for different E2s, UbcH5 and UbcH7, respectively [17]. Now, the experiments with GST-ubiquitin show that full-length HERC3 protein is also able to bind ubiquitin (Fig. 3), which is compatible with a function as an E3 ubiquitin ligase. Although the cysteine 1018 is not essential for this association, the fact that the C1018A mutant binds less to GST-ubiquitin beads (Fig. 3A) seems to indicate that the cysteine could stabilize the ubiquitin binding.

GST-ubiquitin HERC3 adducts were insensitive to DTT suggesting the ubiquitination of HERC3 on lysines. Data obtained in vivo further support HERC3 ubiquitination by showing the increase in the levels of HERC3 and appearance of higher molecular weight forms of HERC3 after inhibition of the proteasome in COS-1 cells (Fig. 3C and data not shown). HECT proteins RSP5 and E6-AP display self-ubiquitination activity. Reported data suggest an intramolecular transfer of ubiquitin for RSP5 and an intermolecular transfer for E6-AP [7,25,26]. HERC3 ubiquitination is unlikely to be self-ubiquitination since mutant HERC3 is also ubiquitinated and to the same extent (Fig. 3B, asterisk). HERC3 ubiquitination seems to indicate a fine regulation of HERC3 levels and supports the hypothesis that ubiquitination is common to HECT proteins [25]. Moreover, the F-box component of SCF ligases, another subclass of E3 ubiquitin protein ligases is also regulated by ubiquitination [27].

Structural comparison between four members of the HECT family groups them in two subfamilies: the large proteins HERC1 and HERC2, which contain other domains in addition to RCC1 and HECT in their primary structure, and the smaller proteins HERC3 and HERC4, which contain only the RCC1 and HECT domains. Chromosomal location also points out this relationship. Human HERC1 and HERC2 map closely in chromosome 15q and human HERC3 and HERC4 in chromosome 4q. These proximal locations suggest that genomic duplication led to each protein subfamily [1,12,13,15,28]. These characteristics probably indicate similar functions for each subclass. HERC4 interacts with several cyclins and is regulated by tumor suppressor proteins p53 and retinoblastoma [13]. Whether HERC3 interacts with cyclins and whether its expression is regulated by tumor suppressor proteins remains to be determined.

In summary, we identify HERC3 protein. Its subcellular localization is compatible with a role in vesicular trafficking and its association with ubiquitin suggests that it may regulate ubiquitin-dependent processes. Identification of HERC3 interacting proteins may help us to better understand the function of HERC3 in cellular processes.

Acknowledgements: We thank Mònica Vallés and Álvaro Jiménez for their technical help in antibody generation and Drs. R.P. Casaroli and S. Vilaró and the members of the Departament de Ciències Fisiològiques II for their many valuable suggestions. We also thank Drs. R.A. Khan, J. Blasi, I.V. Sandoval, T.E. Kreis, M. Camps and M. Schöndorf (ScheBo.Tech) for providing antibodies against ARF, Rab5, $GMP_1$1/LIMPII, β-COP, transferrin receptor and M2-pyruvate kinase, respectively, and N. Nomura, M. Ohtsubo and J. Donaldson for the D25215, HERC4 RLD and ARF6 cDNAs, respectively. This work was supported by grants from Ministerio de Educación y Ciencia (SAF98-0129) and Generalitat de Catalunya (99SGR-73). C.C. has a doctoral fellowship from Generalitat de Catalunya.

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