8. ARTÍCULOS

«There is no form of prose more difficult to understand and more tedious to read than the average scientific paper.»

Francis H.C.Crick (1916-2004)

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Interaction between HERC1 and M2-type pyruvate kinase

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Abstract HERC proteins are characterized by having one or more RCC1-like domains as well as a C-terminal HECT domain in their amino acid sequences. This has led researchers to suggest that they may act as both guanine nucleotide exchange factors and E3 ubiquitin ligases. Here we describe a physical interaction between the HECT domain of HERC1, a giant protein involved in intracellular membrane traffic, and the M2 isoform of glycolytic enzyme pyruvate kinase (M2-PK). Partial colocalization of endogenous proteins was observed by immunofluorescence studies. This interaction neither induced M2-PK ubiquitination nor affected its enzymatic activity. The putative significance of the association is discussed.

Key words: HERC1; M2-type pyruvate kinase; HECT domain; RCC1-like domain; E3 ubiquitin ligase; Guanine nucleotide exchange factor

1. Introduction

The human HERC protein family consists of four proteins sharing a HECT domain in their carboxyl-termini and one or more RCC1-like domains (RLDs) elsewhere in their amino acid sequences. The HECT domain (homologous to E6-AP carboxyl-terminus) was first characterized as the domain involved in the E6-AP-mediated ubiquitination of p53 in the presence of papillomaviral E6 oncoprotein [1]. More generally, HECT domains are assumed to confer E3 ubiquitin-protein ligase activity to those proteins containing them through a mechanism involving formation of a thioester bond between ubiquitin's carboxyl-terminus and a conserved active site cysteine residue in the HECT [2,3]. The RCC1-like domain, on the other hand, was initially identified in the regulator of chromosome condensation-1 (RCC1) protein, where it has been shown to stimulate GDP/GTP exchange upon Ran, a monomeric G-protein belonging to the Ras superfamily of GTPases, and thus to regulate important cellular processes such as nucleocytoplasmic transport and mitotic spindle formation [4,5]. For this reason, it is thought that RLDs may act as guanine nucleotide exchange factors (GEFs) for small GTPases [6].

The HERC family can in turn be divided into two subfamilies: the large HERCs (HERC1 and HERC2) are giant proteins almost 5000 amino acid residues long, encoded in chromosome 15 [7] and possessing at least two RLDs and other known sequence motifs in addition to the HECT, whereas the small HERCs (HERC3 and HERC4) are less than one fourth the size of their larger counterparts, are encoded in chromosome 4 [8] and their primary structures display little more than one RLD and the HECT. Very little is known about the cellular functions of these proteins. Mouse HERC2, encoded in the *rjs/jdf2/herc2* locus, has been linked to a genetic syndrome whose major symptoms include dwarfism, a jerky gait and sterility. Interestingly, all these phenotypes appear to be due to the loss of function of HERC2's HECT domain [9,10]. HERC3 is a cytosolic and inner membrane-associated protein which has recently been shown to bind non-covalently to ubiquitin as well as to undergo ubiquitin-mediated proteasomal degradation [11]. HERC4 (also called Ceb-1) was isolated in a yeast two-hybrid screening using both cyclin E and p21 as baits. Unlike all other HERCs whose expression is rather ubiquitous, HERC4 is selectively expressed in reproductive tissues and undergoes upregulation when the functions of both p53 and pRB tumor suppressors are compromised [12]. HERC1, the largest family member (532 kDa) and the first to be described, contains a number of conserved sequence features which are supposed to play different roles in the overall function(s) of the protein. HERC1 possesses two RLDs (RLD1 and RLD2) which have been implicated in different cellular tasks: while RLD1 stimulates guanine nucleotide dissociation on several ARF and Rab family GTPases [6], RLD2 forms a ternary complex with clathrin heavy chain and the chaperone Hsp70 [13]. These data, together with HERC1's localization in inner cell membranes such as the Golgi apparatus and the cytosol [6], have led to the suggestion that this protein has an important function in intracellular membrane traffic. In addition to its RLDs, the most relevant conserved regions in HERC1 include a SPRY (repeats in splA and RyR) domain, a WD-40 domain, both thought to mediate protein–protein interactions [14,15], an F-box motif that might account for HERC1 being a constituent of a so far unidentified

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Abbreviations: aa, amino acid residues; PBS, phosphate-buffered saline; GST, glutathione-S-transferase

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SCF E3 ubiquitin ligase complex [15,16], three putative SH3 binding sites and, finally, the HECT domain, which has already been shown to bind ubiquitin in a dithiothreitol-sensitive manner, thus confirming its ability to act as an E3 ubiquitin-protein ligase [17].

All the above-stated conserved regions present in HERC1 have already been involved in the formation of heteromolecular complexes through protein–protein interactions. In order to identify proteins that interact with HERC1, we have used several HERC1 domains as baits in the yeast two-hybrid system. In this study, we report the identification of M2-type pyruvate kinase as a protein that interacts with the HECT domain of HERC1.

2. Materials and methods

2.1. Plasmids

The 5′-untranslated region of HERC1 cDNA was removed from previously described plasmid pJLR75 [6] by first amplifying a polymerase chain reaction (PCR) fragment in the 5′-coding region with oligos SEQJL135 (5′-CCATCGATGAATTCAACATGGCAACTATGATTCCA-3′) and SEQJL057 (5′-CTTCTCCGAAAGAGGCCATA-3′) and then ligating the *Cla*I–*Nde*I-digested PCR fragment with the larger fragment resulting from digestion of pJLR75 with the same restriction enzymes. This new plasmid was called pFG1 and was used to generate pFG3 by subcloning its *Eco*RI insert into pcDNA3.1/HisC (Invitrogen). pJLR82 was created by cloning into pGBT9 (Clontech) a *Bam*HI–*Sal*I-digested DNA fragment obtained by PCR amplification of pJLR75 with oligos SEQJL118 (5′-CGGGATCCCGAGACAAGTAGTTAAGCTG-3′) and SEQJL119 (5′-ACGCGTCGACGGTCAGTAGTCAGTGTCG-3′). *Eco*RI–*Sal*I insert was extracted from pJLR82 and subcloned into both *Eco*RI–*Sal*I-digested pFastBacHTa (Invitrogen) and *Eco*RI–*Xho*I-cut pcDNA3.1/HisC to form plasmids pFG26 and pCC44, respectively. pFastBacHTa digested with *Rsr*II and *Eco*RI was ligated with a DNA fragment produced by PCR-amplifying vector pGEX4T1 (Amersham Pharmacia Biotech) with oligos RsrIIGST (5′-CTCGGTCCGAAACCATGTCCCCTATACTAGGT-3′) and GSTThr (5′-GGGAATTCCGGGGATCCACGCGGAACCAG-3′) followed by digestion with the same restriction endonucleases. The resulting plasmid, pFastBacGSTa, was subsequently used to create another plasmid, pPM7, by introducing into the former the *Eco*RI insert from pFG1. pJDD7 was similarly created through ligation of the 4 kb *Bam*HI–*Eco*RI fragment of pFG1 with *Bam*HI–*Eco*RI-restricted pFastBacGSTa. pJDD8 and pJDD9 were generated by digesting pPM7 with either *Spe*I (pJDD8) or *Xho*I (pJDD9) followed by religation of the vector-containing fragments. pFG32 was obtained by transferring the *Sma*I–*Xho*I insert from pClone25 (see Section 3) into pGEX4T1. pT7-7-His-UbcH5a was provided by Dr. Kazuhiro Iwai.

2.2. Yeast two-hybrid experiments

Yeast experiments were carried out according to Matchmaker Gal4 Yeast Two-Hybrid System-3 (Clontech). Briefly, *Saccharomyces cerevisiae* AH-109 cells were cotransformed with pJLR82 (encoding HERC1's last 366 amino acid residues) and a HeLa cell cDNA library cloned into pGAD-GH vector (Clontech). Transformants were seeded on appropriate selective media in order to isolate positive clones and library plasmids were isolated from these by complementation of the leuB6 mutation of *Escherichia coli* MH4 strain. For filter β-galactosidase assays see [13].

2.3. Antibodies

Mouse monoclonal anti-M2-PK (clone DF4) was from ScheBo Biotech (Giessen, Germany). Mouse monoclonal anti-His (clone His-1) and anti-glutathione-S-transferase (GST) were from Sigma, as was rabbit anti-ubiquitin antiserum. Affinity purified rabbit polyclonal antibodies against HERC1 (410 and 417) have already been described elsewhere [6].

2.4. Baculoviruses, protein purification and pull-down experiments

2.4.1. Baculoviruses. Recombinant baculoviruses expressing His-HECT, GST, GST-HERC1 (amino acid residues (aa) 1–1413) GST-HERC1 (aa 1–3716), GST-HERC1 (aa 1–4861, full length) and GST-HERC1 (aa 3684–4861) were generated from plasmids pFG26, pFastBacGSTa, pJDD9, pJDD8, pPM7 and pJDD7, respectively. All these baculoviruses were produced according to the Bac-to-Bac system's instructions manual (Invitrogen). M2-PK baculovirus was provided by Dr. Tamio Noguchi.

2.4.2. Protein purification. Nickel-NTA agarose beads (Qiagen) were used to purify both His-UbcH5a from *E. coli* BL21 cells transformed with plasmid pT7-7-His-UbcH5a and His-HECT from baculovirus-infected Sf9 cells. Likewise, GST-M2-PK (aa 406–531) expressed by pFG32-transformed *E. coli* XL1 blue cells, GST and GST-HERC1 (full length) from Sf9 cells were isolated using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Bacterial purifications were carried out according to standard procedures. Insect cell purifications were done basically as described in [6].

2.4.3. Pull-down experiments. In vitro pull-down experiments were performed by mixing in phosphate-buffered saline (PBS) 0.5 μg of either GST or GST-M2-PK (aa 406–531), both bound to glutathione-Sepharose beads, and 1.2 μg of soluble His-HECT. After 3 h of rocking at 4°C, beads were washed three times in PBS and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/immunoblot analysis. In vivo pull-down experiments were carried out 72 h after Sf9 cells had been infected with appropriate baculoviruses. Cells were lysed in ∼1 ml/5×10^6 cells of buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 5 mM β-mercaptoethanol, 5 mM imidazole, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml pepstatin A, 5 μg/ml aprotinin and 5 μg/ml leupeptin and centrifuged 20 min at $15\,000\times g$. 50 μl of either glutathione-Sepharose or nickel-NTA-agarose beads per ml supernatant were then added and the resulting mixture was rocked for 2 h. Beads were washed three times with lysis buffer containing 300 mM NaCl and once with lysis buffer (no protease inhibitors in washes). Pulled-down proteins were studied by SDS–PAGE/immunoblot. The pull-down in Fig. 1E was performed by mixing 100 ng of bead-bound, purified GST or GST-HERC1 and 5 ml of an M2-PK-expressing Sf9 cell lysate (lysis as above). The mixture was rocked for 2 h and beads washed four times with lysis buffer before being analyzed by SDS–PAGE/immunoblot.

2.5. Cell culture and transfection

HEK-293T and HeLa cells were maintained as described in [11]. Transfection of HEK-293T was achieved using the calcium phosphate method when cells were at a confluence of 40–70%. Experiments were performed 40 h after cells were transfected. Sf9 cells were cultured in Petri dishes at 27°C with insect cell medium (BioWhittaker) to which 10% fetal bovine serum was added. Baculovirus infections were carried out using standard procedures.

2.6. Confocal immunofluorescence microscopy

HeLa and HEK-293T cells were grown on glass coverslips and processed as described in [11]. Dilutions used were 1/25 and 1/200 for anti-M2-PK and anti-HERC1 (410) antibodies, respectively. Secondary antibodies were fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit IgGs and Texas red-isothiocyanate-conjugated goat anti-mouse IgGs (both from Amersham Pharmacia Biotech). Samples were observed under a Leica TCS-NT confocal microscope.

2.7. Ubiquitination experiments and enzyme activity measurements

LLnL experiments in HEK-293T cells were performed as previously described [11]. In order to measure pyruvate kinase activity in HEK-293T cells, these were first placed on ice, washed once with ice-cold PBS and extracted in a buffer containing 10 mM Tris–HCl, pH 7.4, 1 mM NaF, 1 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM β-mercaptoethanol. Cells were then lysed with a cell homogenizer and total cell extracts centrifuged 20 min at $15\,000\times g$. Supernatants were then taken and used both to measure total protein levels (BCA kit, Pierce) and to carry out activity assays. These were done in a buffer containing 50 mM Tris–HCl, pH 7.6, 100 mM KCl, 5 mM $MgSO_4$, 2 mM ADP, 0.2 or 2 mM phosphoenolpyruvate (PEP), 0.25 mM NADH and 0.15 mg/ml lactate dehydrogenase (Roche Molecular Biochemicals). Activity was calculated by monitoring the absorbance fall at 340 nm. The effect of UbcH5 on M2-PK activity (Fig. 3C) was determined by adding 2 ng of His-UbcH5a per μg total protein to the HEK-293T extracts followed by a 30-min incubation at 4°C prior to activity measurements.

3. Results

3.1. HERC1–M2-PK interaction in the yeast two-hybrid system

A yeast two-hybrid screening was performed using the last 366 amino acid residues of HERC1 (aa 4496–4861), encompassing its HECT domain, as bait. Plasmid pJLR82 encoding the aforementioned HERC1 fragment fused to Gal4 DNA binding domain was transformed into *S. cerevisiae* AH-109 cells together with a library constructed by inserting HeLa cell cDNAs into *Eco*RI–*Xho*I targets of vector pGAD-GH, which encodes Gal4 transcriptional activation domain. Positive clones were selected by their ability to grow in the absence of histidine, adenine, leucine and tryptophan. Most positives obtained in this manner turned out to possess a library insert corresponding to the C-terminal region (aa 406–531) of glycolytic isoenzyme pyruvate kinase type M2. This interaction was confirmed by a filter β-galactosidase assay (Fig. 1A). The fact that M2-PK failed to interact with HERC3, a homologue of HERC1, appears to indicate that this interaction is specific for HERC1 and does not take place with other HERC family members. On the other hand, neither pJLR82 nor pClone25 (pGAD-GH with the M2-PK insert) gave rise to β-gal$^+$ cells when transformed together with empty pGAD-GH or pGBT9 vectors, respectively.

3.2. In vitro pull-down experiments

In order to confirm the yeast two-hybrid interaction as well as to check whether this was direct or else might be mediated by a bridging protein, histidine-tagged HERC1 (aa 4496–4861, henceforth also referred to as His-HECT) and GST-M2-PK (aa 406–531) fusion protein were purified and pull-downs were carried out with glutathione-Sepharose beads (Fig. 1B, see also Section 2). Results show clearly that His-HECT binds to GST-M2-PK (406–531) and does not to GST, from which it can be concluded that the interaction exists and is likely to be direct between the two proteins.

3.3. In vivo pull-down experiments in insect cells

Next, HERC1–M2-PK interaction was studied in vivo in baculovirus-infected Sf9 insect cells. First, cells were infected with baculoviruses encoding full-length M2-PK and His-HECT and lysates pulled-down with nickel beads (Fig. 1C). When this was done, a fraction of M2-PK was found in the beads, whereas none could be found when pull-downs were carried out in control cells expressing only M2-PK and not His-HECT, indicating that M2-PK bound specifically to His-HECT. Second, full-length M2-PK was heterologously expressed either alone or together with GST or three GST fusion proteins encompassing the entire length of HERC1 and cell extracts were pulled-down with glutathione beads. As expected, M2-PK could only be detected in the beads when it was coexpressed with the fusion protein containing HERC1's carboxyl-terminal region (GST-HERC1 (3684–4861)) and not in all other conditions (Fig. 1D). Finally, we attempted to find the interaction between both full-length proteins. In order to achieve this goal, we incubated purified, bead-bound GST or GST-HERC1 (aa 1–4861, full-length protein) with an M2-PK baculovirus-infected Sf9 cell lysate. Immunoblot analysis showed that a small fraction of M2-PK bound to GST-HERC1 beads, whereas none could be found in control GST beads (Fig. 1E). Taken together, these results demonstrate that M2-PK and HERC1, through its HECT domain, have the ability to interact with each other.

3.4. Immunofluorescence analysis of HERC1–M2-PK colocalization

Subcellular localization of endogenous HERC1 and M2-PK proteins was studied by indirect immunofluorescence confocal microscopy in HeLa and HEK-293T cell lines (Fig. 2). It is noteworthy that both proteins display a similar, mostly perinuclear, punctate staining, which probably means that these proteins somehow interact with membranous intracellular structures in these cell lines [18]. Furthermore, colocalization analysis shows a partial overlapping in the subcellular distributions of both proteins, as seen by the appearance of yellow dots when stainings are superimposed.

3.5. Analysis of M2-PK ubiquitination

In view of the fact that HERC1 is very likely to be an E3 ubiquitin-protein ligase (it possesses a HECT domain and an F-box motif, both generally recognized as hallmarks of such enzymes), we checked whether M2-PK might be a ubiquitination substrate of HERC1. Nevertheless, since ubiquitination of M2-PK has not been described, we first set about determining whether M2-PK undergoes ubiquitination under physiological conditions. For this purpose, we analyzed the effect of proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (LLnL) on M2-PK protein levels in HEK-293T cells (Fig. 3A). While LLnL induced the accumulation of ubiquitinated forms of other proteins, this was not observed for M2-PK nor were its levels affected, as would have been expected of a proteasomally degraded protein. These results notwithstanding, the possibility remained that M2-PK underwent non-proteasome-coupled ubiquitination. To test this hypothesis, histidine-tagged ubiquitin was transfected into HEK-293T cells and cell lysates were incubated with nickel beads to pull-down His-ubiquitinated proteins. Immunoblot analysis showed that M2-PK was not among them (data not shown). Moreover, transcription and translation of radiolabeled M2-PK in rabbit reticulocyte lysates followed by addition of GST-ubiquitin did not result in formation of GST-ubiquitin–M2-PK adducts (data not shown). All results, therefore, point to the direction that M2-PK does not undergo ubiquitination and thus cannot be a ubiquitination substrate of HERC1.

3.6. Effect of HERC1 upon M2-PK enzyme activity

In order to analyze whether the HECT domain of HERC1 might modulate M2-PK enzyme activity, HEK-293T cells were transfected with either control plasmid (pcDNA3.1/His) or plasmid encoding His-HECT (pCC44). His-HECT expression was confirmed by immunoblot (data not shown). Activity was measured in cell lysates at two different phosphoenolpyruvate (PEP) concentrations: 0.2 mM (corresponding roughly to the concentration at which M2-PK has half-maximal activity ($S_{0.5}^{PEP} = 0.25$ mM) [19]) and 2 mM, a saturating PEP concentration. In this manner, we intended to distinguish between effects upon M2-PK's affinity for PEP and effects upon its V_{max}. However, as it can easily be grasped from Fig. 3B, none of those effects were observed. It might be, though, that the HECT domain alone is not sufficient to induce any alterations in M2-PK activity and that full-length HERC1 is needed for them to occur. To test this, we measured M2-PK activity in extracts from control and HERC1-

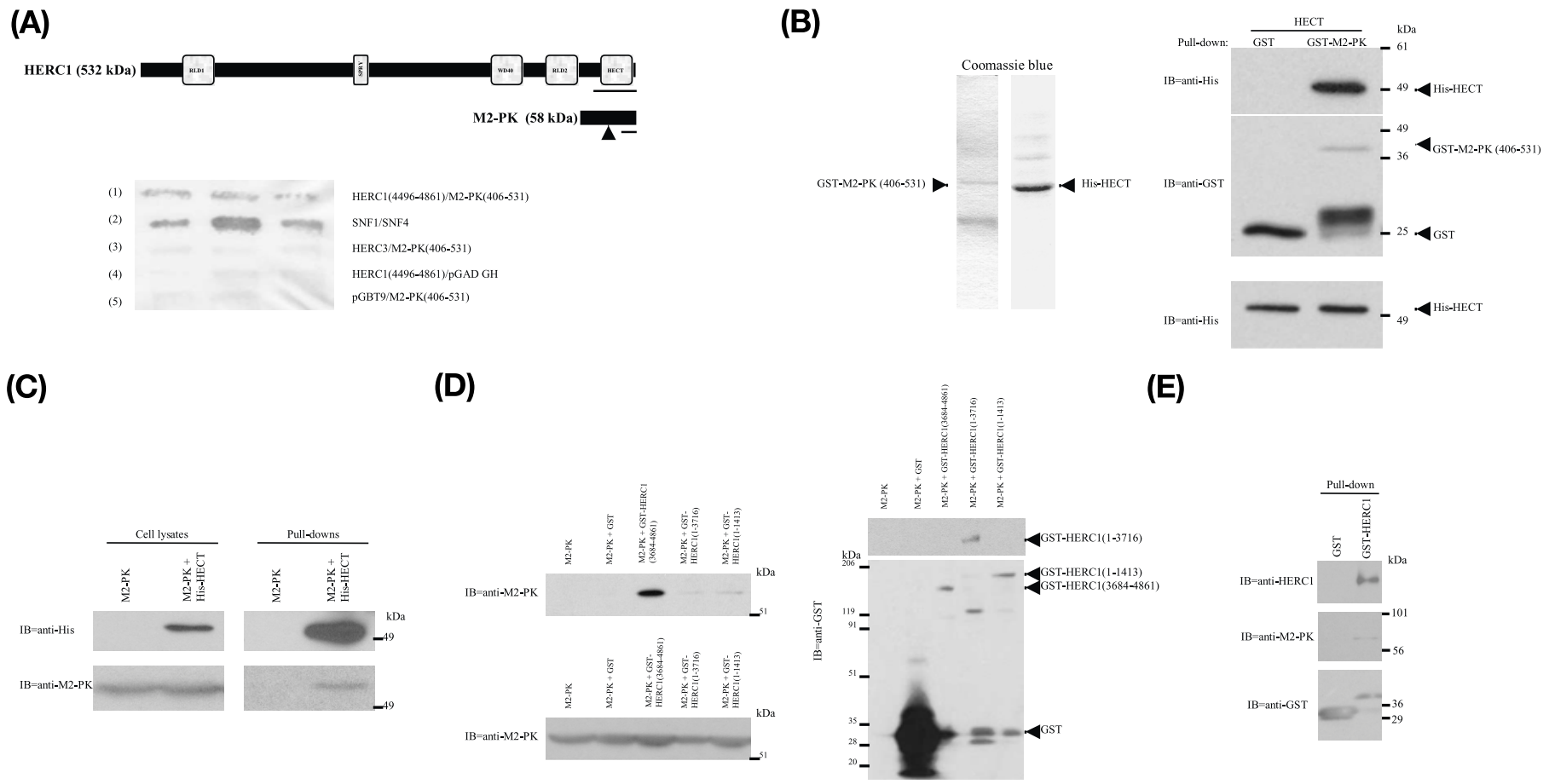

Fig. 1. Interaction between HERC1 and M2-PK. A: The last 366 amino acid residues of HERC1 were used as bait in the yeast two-hybrid system. Among all positive clones analyzed, most corresponded to the last 126 amino acid residues of pyruvate kinase type M2. Top: Schematic representations of HERC1 and M2-PK proteins have been drawn to scale. Major HERC1 domains have also been depicted. M2-PK's active site is marked with an arrowhead. Interacting regions in both proteins have been underlined. Bottom: Filter β-galactosidase assay. Three independent clones are shown for each condition. (1) Interaction between HERC1 (aa 4496–4861) and M2-PK (aa 406–531). (2) SNF1–SNF4 interaction as positive control. (3) Interaction is specific for HERC1, since it doesn't take place with HERC3. (4–5) Negative controls. B: Left: Coomassie blue staining showing purifications of GST-M2-PK (406–531) and His-HECT proteins. The lower band in the former is a degradation product. Right: In vitro pull-downs confirmed the yeast interaction. Pulled-down GST and GST-M2-PK (406–531) and the His-HECT associated to them were resolved by SDS–PAGE and analyzed by immunoblot. Bottom right: 4% of the His-HECT used in pull-downs was loaded into another gel and immunoblotted as well. C: Immunoblots of Sf9 cells infected with baculoviruses encoding full-length M2-PK and His-HECT as indicated. When His-HECT was pulled-down with nickel beads, M2-PK was also found associated. D: Recombinant baculoviruses expressing M2-PK, GST and three GST fusion proteins encompassing the whole HERC1 protein were used to infect Sf9 cells as indicated. Cell extracts were pulled-down with glutathione beads and associated proteins analyzed by SDS–PAGE/immunoblot. M2-PK only interacted with the fusion protein containing HERC1's HECT domain. Top left: M2-PK bound to pulled-down GST fusion proteins. Bottom left: M2-PK levels in cell extracts. Right: Pulled-down GST fusion proteins. E: Glutathione-Sepharose beads bound to either GST or GST-HERC1 (aa 1–4861, full length) were incubated with a lysate from M2-PK baculovirus-infected Sf9 cells and beads were analyzed by SDS–PAGE/immunoblot. A small amount of M2-PK was specifically pulled-down by full-length HERC1.

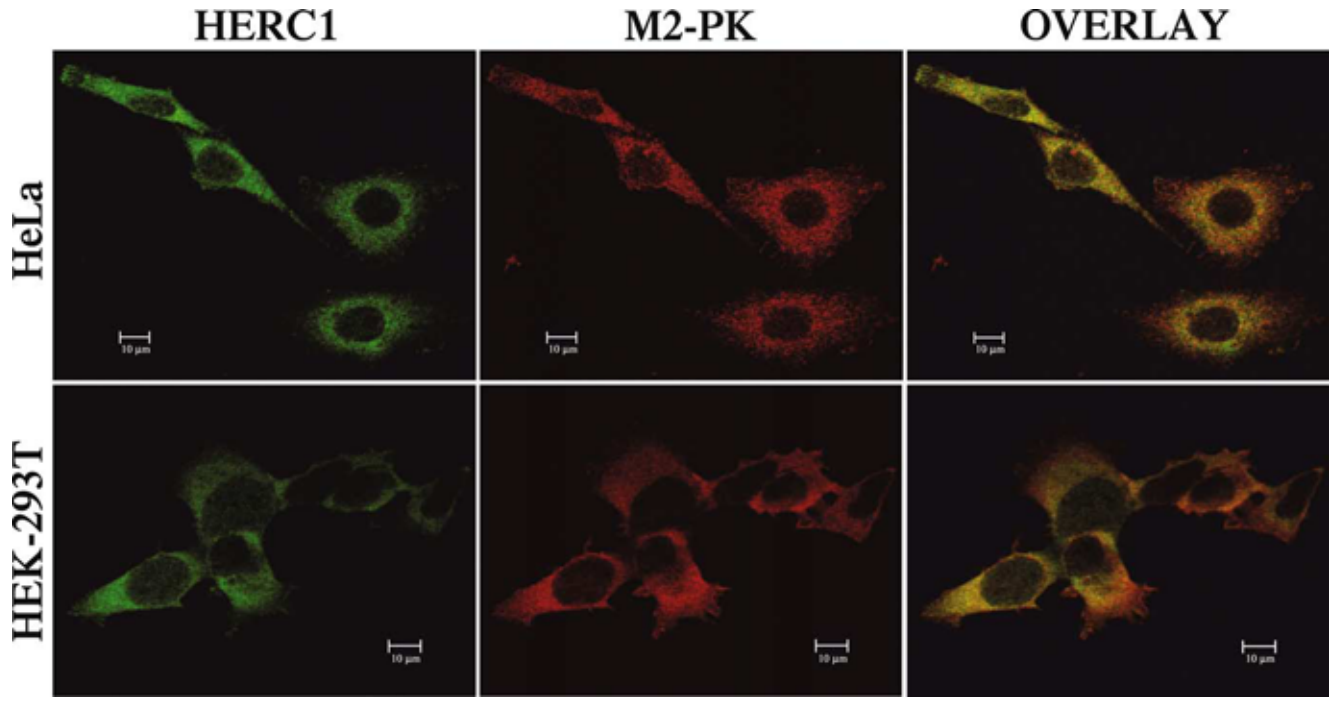

Fig. 2. Localization of endogenous HERC1 and M2-PK proteins was analyzed by confocal immunofluorescence microscopy in HeLa and HEK-293T cell lines. Both proteins display a perinuclear, punctate staining that presumably indicates their association with intracellular membranous structures. HERC1 and M2-PK show a partial colocalization in both cell lines when images are overlaid. All images correspond to one single cellular plane.

overexpressing HEK-293T cells (immunoblot analysis showed a 2–3-fold overexpression, data not shown). As shown in Fig. 3C, no significant changes were detected. Moreover, since HERC1 is known to form a thioester bond with ubiquitin in the presence of UbcH5 [17], we also checked whether the latter protein was necessary for an effect to take place. Nevertheless, addition of purified His-UbcH5a to the former extracts had no effect whatsoever on M2-PK enzyme activity (Fig. 3C). We also wondered whether HERC1 might have an effect upon M2-PK's dimer-to-tetramer ratio, as do other proteins such as papillomaviral protein E7 [19]. To address this issue, we loaded control, His-HECT-expressing and HERC1-overexpressing HEK-293T lysates into a gel filtration chromatography column in order to separate dimeric and tetrameric M2-PK. Elution profiles did not show any significant differences between those samples (data not shown). In summary, all these results appear to indicate that HERC1 does not affect M2-PK enzyme activity.

4. Discussion

In the present study, we have shown a physical interaction between HERC1, a protein involved in intracellular membrane traffic, and glycolytic isoenzyme M2-PK. This interaction was first found in the yeast two-hybrid system and it was shown to take place between the last 366 amino acid residues of HERC1, which encompass its HECT domain, and the last 126 residues of M2-PK including critical residues involved in fructose-1,6-bisphosphate binding and intersubunit contact (see below). The interaction was subsequently demonstrated to be direct by in vitro pull-down assays carried out with purified proteins and to occur in vivo in Sf9 insect cells when both full-length M2-PK and the last 366 or 1178 amino acid residues of HERC1 were expressed using recombinant baculoviruses. An interaction between both full-length proteins, albeit weak, could also be demonstrated in pull-down experiments in Sf9 cells. Moreover, human endogenous HERC1 and M2-PK proteins were shown to display similar, partially overlapping, perinuclear, punctate stainings when their subcellular localization was analyzed by immunofluorescence microscopy. This presumably indicates that both proteins are associated with intracellular membranous compartments [18] and is in agreement with previous data reporting M2-PK activation by phosphatidylserine-containing liposomes [20] and HERC1 localization in inner cell membranes [6]. However, in spite of all this evidence, we have failed to pinpoint the HERC1–M2-PK interaction in mammalian cells. Several reasons might explain this. One of them is the lack of good commercial antibodies to immunoprecipitate endogenous M2-PK or HERC1 proteins. Another possible reason may have to do with the low levels of expression achieved when full-length tagged HERC1 (His-HERC1 or Myc-HERC1) was transfected into mammalian cell lines (HeLa, HEK-293T, COS-1), which in turn is likely to be due to HERC1's giant size (4861 aa). Interestingly, we also could not find the interaction by immunoprecipitation of Myc-HERC1 (aa 3684–4861) or by pull-down of His-HERC1 (aa 4496–4861) in mammalian cells. In our opinion, these data probably indicate that M2-PK binding to HERC1 is tightly regulated in mammals and only happens under very specific conditions.

M2-PK undergoes a very complex regulation. Since it catalyzes the last step in glycolysis, the regulation of its activity is essential for cells to control the amount of carbon channeled into energy production (anaerobic glycolysis or the TCA cycle) and into biosynthetic pathways (M2-PK inhibition in-

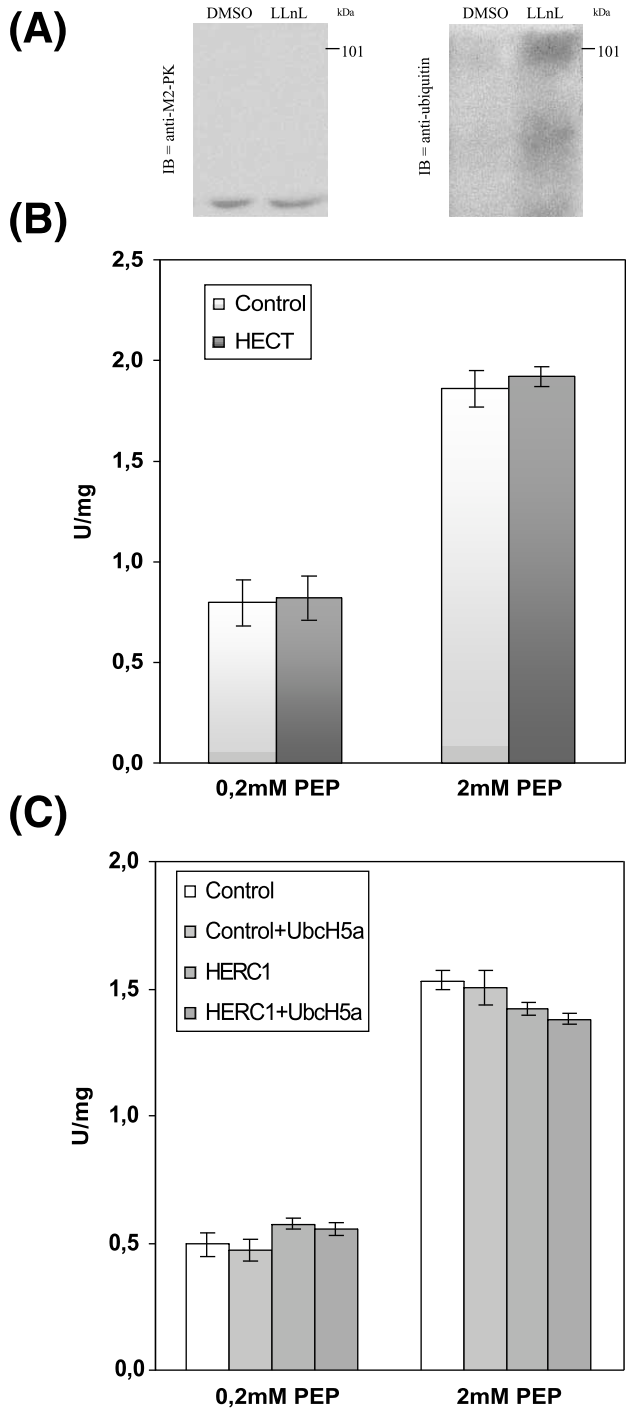

Fig. 3. A: Proteasome inhibitor LLnL does not affect M2-PK levels in HEK-293T cells. Immunoblot analysis showed that while other proteins became mono- or polyubiquitinated as a result of LLnL treatment, M2-PK did not undergo this modification nor were its protein levels significantly affected. B and C: M2-PK activity in HEK-293T cells is not affected by HERC1. B: Activity was analyzed in cells transfected with either control plasmid or a plasmid expressing His-tagged HERC1's HECT domain. C: Activity was analyzed in cells transfected with either control plasmid or a plasmid expressing full-length His-HERC1 (pFG3) in the absence or presence of UbcH5a. Activity was measured at two different phosphoenolpyruvate concentrations and has been expressed as units per mg of protein in the extracts (mean ± S.E.M. of six independent experiments).

duces an accumulation of glycolytic phosphometabolites that may act as biosynthetic precursors [21]). As a matter of fact, M2-PK is only one of four pyruvate kinase isoenzymes in mammals, the other three being L-PK, R-PK and M1-PK. The first two are expressed in liver and red blood cells, respectively, and arise from a single gene (the PKL gene) by differential promoter use. M1-PK, on the other hand, is mainly expressed in muscle and brain and is characterized by its hyperbolic Michaelis–Menten kinetics, in opposition to all other isoforms, which display sigmoidal kinetics. M1- and M2-PK are also synthesized from a single common gene (the PKM gene), which by differential splicing gives rise to the two proteins (these differ only in a short stretch of 56 amino acid residues corresponding to the alternatively spliced exon) [22,23]. M2-PK, like L-PK and R-PK, is allosterically regulated via feed-forward activation by fructose-1,6-bisphosphate (FBP) and via feedback inhibition by adenosine triphosphate (ATP) [21]. Furthermore, M2-PK can be found in cells in two major forms, namely, a highly active tetramer and a less active dimer. The equilibrium between these two forms can be shifted towards one or the other side by several factors: thus, while FBP induces tetramer formation, tyrosine phosphorylation or the E7 oncoprotein of carcinogenic human papillomaviruses inactivate the enzyme by converting it into the dimeric form [21,24]. All these regulatory properties, especially its ability to fine-tune the relative levels of energy production and biosynthesis, appear to make M2-PK the most suitable PK isoform for proliferating cells, which have high energetic and biosynthetic requirements at the same time. This would explain why M2-PK is expressed in all tissues during development and also why it is re-expressed when cells de-differentiate to become malignant or, still, why it is targeted by viral oncoproteins [19,23,24]. In fact, dimeric M2-PK, also known as tumor M2-PK, accumulates in malignant cells and has even turned out to be a good diagnostic marker for a number of cancers [25].

Any of these processes affecting M2-PK function could be regulating its interaction with HERC1. In this regard, we checked whether binding could be dependent on M2-PK being phosphorylated in tyrosine residues, which is known to inactivate the enzyme and could also shed some light on a possible role of HERC1 in tumorigenesis suggested by the fact that this protein is overexpressed in tumor cell lines [6]. Nonetheless, neither expression in HEK-293T cells of constitutively active Src (v-Src is known to be involved in tyrosine phosphorylation of M2-PK in chicken embryos [24]) nor epidermal growth factor treatment of A431 cells induced the association of both proteins (data not shown). It might also be that the interaction is somehow controlled by FBP levels, but this hypothesis, which is all the more reasonable in view of the fact that the HERC1 binding region in M2-PK includes many critical residues for FBP binding and intersubunit contact [26–28], still remains to be explored, as is the case for a possible role of the association of M2-PK to intracellular membranes.

Finally, we attempted to find a possible physiological significance for the interaction. In view of all available information about these proteins, it is reasonable to think that HERC1 could function as an E3 ubiquitin-protein ligase for M2-PK and/or modulate M2-PK enzyme activity. We tested both possibilities. First, since we were not able to find any ubiquitinated forms of M2-PK nor an increase in its expression levels in response to a proteasome inhibitor, we con-

cluded that M2-PK could not be a ubiquitination substrate of HERC1. On the other hand, overexpression of HERC1 or its HECT domain did not affect PK activity in HEK-293T cell lysates, nor did it alter its dimer-to-tetramer ratio. This activity is due only or almost only to the M2-PK isoform, since HEK-293T is an immortalized cell line [23]. Further insight into the functions of both HERC1 and M2-PK will still have to be gained before the physiological relevance of their interaction can be elucidated.

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The giant protein HERC1 is recruited to aluminum fluoride-induced actin-rich surface protrusions in HeLa cells

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Abstract HERC1 is a very large protein involved in membrane traffic through both its ability to bind clathrin and its guanine nucleotide exchange factor (GEF) activity over ARF and Rab family GTPases. Herein, we show that HERC1 is recruited onto actin-rich surface protrusions in ARF6-transfected HeLa cells upon aluminum fluoride (AlF_4^-) treatment. Moreover, the fact that HERC1 overexpression does not stimulate protrusion formation in the absence of AlF_4^-, in conditions where ARNO does, indicates that HERC1 is not acting as an ARF6-GEF in this system, but that instead its recruitment takes place downstream of ARF6 activation. Finally, we suggest a phosphoinositide-binding mechanism whereby HERC1 may translocate to these protrusions.

Key words: HERC1; Aluminum fluoride; ARF6; Phosphoinositide; Actin protrusion; Guanine nucleotide exchange factor

1. Introduction

The human proteome contains four HERC proteins [1–8], which are characterized by possessing both HECT and RCC1-like (RLD) domains. While the former are widely assumed to confer E3 ubiquitin ligase activity to proteins containing them [9,10], the latter have been suggested to act as guanine nucleotide exchange factors (GEFs) for small GTP-binding proteins [1,11]. HERC1, the largest family member (532 kDa), was the first to be identified and so far it has been the most extensively studied [1–4]. Its long amino acid sequence (4861 amino acid residues) contains a number of conserved regions. Among them, the most remarkable are the C-terminal HECT domain, the two RLDs (RLD1 and RLD2), three putative SH3-binding sites, a SPRY domain, a WD-40 domain and an F-box motif [3]. The protein seems to be ubiquitously expressed, with higher levels in brain and testis, and it is over-expressed in tumor cell lines compared to normal ones [1]. Concerning its subcellular distribution, HERC1 is located in both the cytosol and inner cell membranes, the Golgi apparatus among them [1]. Although it has not yet been proven in vivo, the in vitro observations that HERC1 can bind to (through its RLD2) and stimulate (via its RLD1) guanine nucleotide dissociation from ARF1, a small GTPase controlling vesicle coat recruitment in the Golgi, may indicate that HERC1 has an important function in the regulation of membrane traffic in this organelle [1]. Likewise, the ability of HERC1's RLD1 domain to dissociate guanine nucleotides from Rab3a and Rab5 active sites might argue for a role of HERC1 in exo- and endocytosis, respectively [1]. On the other hand, HERC1 has also been shown to form in vivo a cytosolic ternary complex with clathrin heavy chain (CHC) and the chaperone Hsp70, the latter dissociating from it when ATP is present [2]. The interaction between HERC1 and CHC takes place between HERC1's RLD2 and a region in CHC (amino acids 1315–1557) which encompasses the clathrin light chain-binding site [2], thus prompting the suggestion that HERC1 might somehow control clathrin coat assembly on the surface of vesicles. Finally, recent findings show an interaction between HERC1, through its HECT domain, and glycolytic isoenzyme M2-type pyruvate kinase. Nevertheless, the physiological significance of this interaction could not be pinpointed [3]. Taken together, these data generate a rough picture of HERC1 as a regulator of membrane traffic potentially through three different mechanisms: GEF activity over ARF and Rab family GTPases, binding to CHC and ubiquitination of target proteins.

Cortical actin cytoskeleton rearrangements have been implicated in several important cellular functions such as phagocytosis and cell motility [12,13]. Although these actin rearrangements are in most cases orchestrated by members of the Rho family of GTPases [14], ARF6, a member of the ADP-ribosylation factor family, has also been shown to play an important role in their regulation. In particular, experiments carried out in HeLa cells have demonstrated that an increase in the level of ARF6 activation leads to enhanced actin polymerization at the cell surface and to formation of actin-based membrane protrusions which are different from the actin structures formed upon activation of Rho family members [15,16]. A number of empirical approaches have been developed in order to elevate the activity of cellular ARF6. These include transfection of constitutively active or fast cycling mutants of ARF6 [17,18], overexpression of ARF6-GEFs such as ARNO

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Abbreviations: GEF, guanine nucleotide exchange factor; FITC, fluorescein isothiocyanate; TRITC, Texas red isothiocyanate; GFP, green fluorescent protein

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or EFA6 [19,20] and use of the heterotrimeric G protein activator aluminum fluoride (AlF_4^-), which needs to be combined with wild type ARF6 overexpression in order to cause its effects [15]. This drug appears to act in HeLa cells by targeting the α subunit of heterotrimeric G_q. In agreement with these data, a constitutively active mutant of $G\alpha_q$ has been reported to induce protrusions in the absence of AlF_4^- [21]. In any case, it is well established that AlF_4^- exerts its effects upon the cortical actin cytoskeleton specifically through ARF6 activation. This is most obvious from the observation that treatments causing ARF6 to undergo inactivation (transfection of dominant-negative or non-myristoylatable mutants of ARF6 [15] or overexpression of ARF6-GTPase activating proteins such as PAG3 or ACAP1 [22,23]) totally block protrusion formation in response to AlF_4^-. The same outcome can be achieved by transfection of an effector domain mutant of ARF6 likewise incapable of sustaining protrusion formation [21]. Aside from the essential involvement of ARF6, relatively little is known about which physiological stimuli activate ARF6 [15,24] and which are the ARF6 effectors. Concerning this last aspect, however, it has recently been shown that one of the most important mechanisms whereby active ARF6 may give rise to protrusions is by its ability to stimulate phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) synthesis [25–29]. Indeed, this phospholipid is highly enriched in protrusive membranes [29] and it is likely to act as an anchor for other proteins involved in protrusion dynamics.

In this study, we show that HERC1 is recruited onto actin-rich surface protrusions formed in ARF6-transfected HeLa cells upon AlF_4^- treatment. We also show that HERC1 recruitment to these structures occurs downstream of ARF6 activation. Moreover, we demonstrate an interaction between HERC1's RLD1 domain and phosphoinositides, which we suggest may be the underlying mechanism whereby HERC1 translocates to protrusive membranes. Finally, we speculate on the function HERC1 may fulfill in such structures.

2. Materials and methods

2.1. Reagents and antibodies

AlF_4^- was prepared from $AlCl_3$ (Sigma) and NaF (Merck). Azolectin (soybean phosphatidylcholine type II-S) and $PI(4,5)P_2$ were both from Sigma. PIP strips were purchased from Echelon Biosciences (Salt Lake City, UT, USA). Phalloidin-fluorescein isothiocyanate (FITC) and phalloidin-Texas red isothiocyanate (TRITC) (Sigma) were used at 0.1 μg/ml. Goat anti-mouse or anti-rabbit F(ab′)2 fragments conjugated to either Alexa Fluor 488 or Alexa Fluor 568 were purchased from Molecular Probes and used at 0.5 μg/ml. Mouse monoclonal antibodies against hemagglutinin (clone HA-7), Flag (clone M2) and Myc (clone 9E10) epitopes were all from Sigma. Affinity-purified rabbit polyclonal antibodies against HERC1 (410) have already been described [1].

2.2. Plasmids

pJLR155 was obtained by introducing the 15 kb *Eco*RI insert from plasmid pFG3 [3] into vector pEGFP-C2 (BD Biosciences). pJLR130 was constructed by ligating into pET21c (Novagen) the 1.4 kb *Bam*HI-*Not*I insert from pJLR16 (pVL1393-His-RLD2) [1]. Analogously, pJLR131 was created by inserting into pET21c the 1.4 kb fragment resulting from pJLR73 (pBlueBac-His-RLD1) [1] digestion with *Bam*HI and *Eco*RI. pARNO-Myc, pPH-phospholipase Cδ1 (PLCδ1)-green fluorescent protein (GFP) and pMyc-PI(4)P-5Kα were supplied by Dr. James E. Casanova [19], Dr. Tamas Balla [30] and Dr. Michael A. Frohman [25], respectively, while pARF1-HA, pARF6-HA, pARF6-T27N-HA and pARF6-Q67L-HA were a gift from Dr. Julie G. Donaldson [15].

2.3. Protein purification, pull-downs and lipid–protein overlay assays

RLD1- and RLD2-Flag were purified by affinity chromatography from *Escherichia coli* BL21 cells transformed with plasmids pJLR131 or pJLR130, respectively. These purifications were carried out basically as described in [1]. Liposome–protein complex formation assays were basically carried out as reported in [31]. Briefly, 0.5 μg of RLD1-Flag or RLD2-Flag were added to 100 μl of lipid vesicles (1 mg/ml) prepared from either azolectin alone or the same amount of azolectin plus 100 μM $PI(4,5)P_2$ incorporated through co-sonication. The mixture was then incubated for 5 min before ultracentrifugation at $100\,000\times g$ for 30 min. The pelleted vesicles were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and anti-Flag immunoblot analysis. For binding of recombinant proteins to lipids on PIP strips (lipid–protein overlays), strips were blocked 90 min before adding the purified recombinant proteins at 0.5 μg/ml in blocking solution (10 mM Tris pH 7.5+150 mM NaCl+0.1% Tween-20+3% fatty acid-free bovine serum albumin). Strips were then incubated overnight at 4°C, washed and Flag-tagged proteins visualized by immunoblot with anti-Flag antibodies.

2.4. Cell culture, transfection and AlF_4^- treatment

HeLa cells were maintained as described [7] and transfected using either lipofectin (Invitrogen) or the calcium phosphate method. For AlF_4^- treatment, ARF6-transfected cells were incubated for 30 min with complete medium plus 30 mM HEPES pH 7.4 and next for another 30 min with the same solution to which 30 mM NaF and 50μM $AlCl_3$ had been added.

2.5. Confocal microscopy

HeLa cells were processed for immunofluorescence analysis as previously described [7]. Samples were observed under a Leica TCS-NT confocal microscope. The different fluorophores were excited and images captured sequentially so as to avoid channel crosstalk. All images displayed are optical sections.

3. Results

3.1. HERC1 is recruited to aluminum fluoride-induced actin protrusions in HeLa cells

In order to analyze whether HERC1 might be recruited to ARF6-dependent actin protrusions, HeLa cells were transfected with plasmid pARF6-HA, encoding wild type ARF6 fused to a carboxyl-terminal hemagglutinin (HA) epitope, and treated about 24–40 h later with the heterotrimeric G protein activator AlF_4^- for 30 min. Cells were then fixed and processed for immunofluorescence microscopy. As previously described [15], AlF_4^- treatment of ARF6-transfected HeLa cells induced the assembly of surface protrusions to which both F-actin and ARF6-HA (Fig. 1A) translocated. In much the same manner, a subset of endogenous HERC1 also clearly moved from its normal perinuclear localization to these protrusive structures (Fig. 1A). These HERC1-containing structures did not form when HeLa cells were either not transfected (data not shown) or transfected with ARF1 (Fig. 1B). What is more, a dominant-negative mutant of ARF6 (ARF6-T27N) also prevented HERC1 translocation upon AlF_4^- treatment, thus proving the need for ARF6 activation in this process (Fig. 1C, top). Finally, a constitutively active mutant of ARF6 (ARF6-Q67L), which has previously been shown to give rise to actin-containing protrusive structures at the plasma membrane resembling those induced by AlF_4^- [15], did indeed colocalize with HERC1 in these structures (Fig. 1C, bottom).

3.2. HERC1 overexpression does not induce actin protrusions in the absence of aluminum fluoride

Since HERC1 has been shown to catalyze guanine nucleotide dissociation upon ARF and Rab family members [1], we

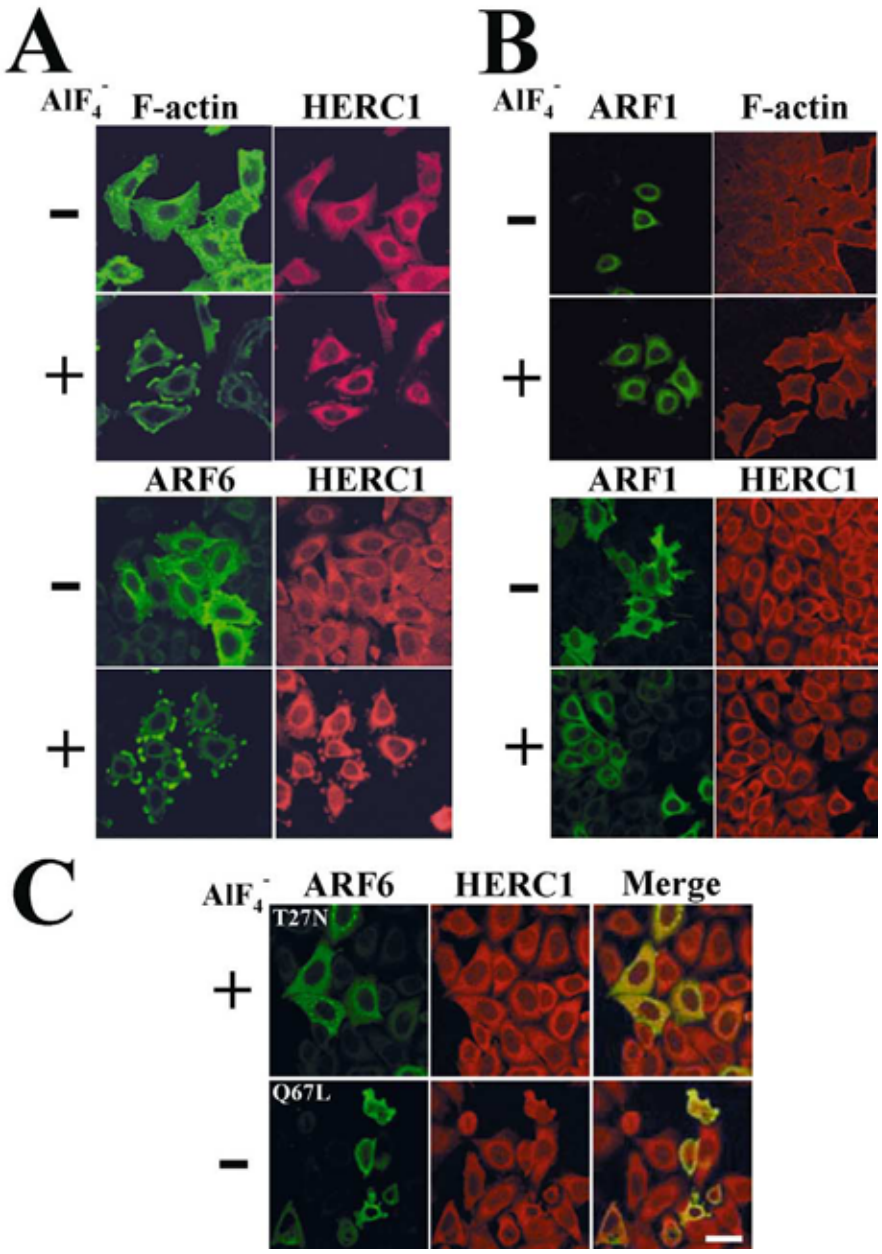

Fig. 1. HERC1 recruitment onto actin protrusions. HeLa cells were transfected with a plasmid encoding ARF6-HA, treated with AlF_4^-, fixed and processed for immunocytochemistry. A: AlF_4^- treatment resulted in the formation of membrane protrusions in which F-actin, detected using phalloidin-FITC, exogenous ARF6, detected using anti-HA antibodies, and endogenous HERC1, detected with specific antibodies, are present. B: Protrusions do not form in response to AlF_4^- when HeLa cells are transfected with a plasmid encoding ARF1-HA instead of ARF6-HA. C: Transfection of dominant-negative ARF6-T27N blocks AlF_4^--induced protrusion formation, while constitutively active ARF6-Q67L induces HERC1-containing protrusions with no need of drug treatment. Scale bar 20 μm.

thought that HERC1 might be involved in ARF6 activation in this system. If this were true, HERC1 overexpression in the absence of AlF_4^- should suffice to evoke actin protrusion formation, as it has already been seen for other ARF6-GEFs such as ARNO and EFA6 [19,20]. In order to test this, we cotransfected HeLa cells with both pARF6-HA and pJLR155, a plasmid encoding a fusion protein between GFP and full-length HERC1. Expression of GFP-HERC1 neither induced protrusion assembly in the absence of AlF_4^- nor prevented their appearance when this compound was added (Fig. 2, bottom panels). At the same time, in a positive control where both pARF6-HA and pARNO-Myc (C-terminal Myc epitope-tagged ARNO) had been cotransfected, protrusions containing both actin and HERC1 formed without any need for AlF_4^- (Fig. 2, top panels). These results most probably indicate that HERC1 is not acting as an ARF6-GEF in this system but that its recruitment to protrusions takes place downstream of ARF6 activation.

3.3. The RLD1 domain of HERC1 binds phosphoinositides

It is becoming increasingly clear that many of the events triggered as a result of ARF6 activation are mediated by the

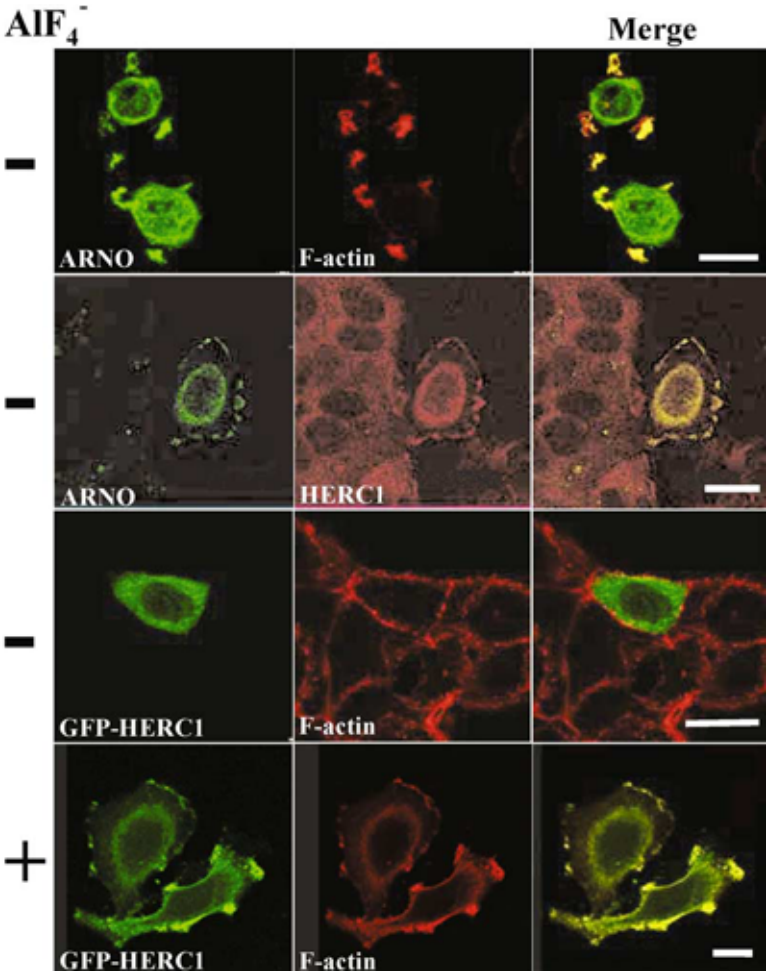

Fig. 2. HERC1 recruitment takes place downstream of ARF6 activation. HeLa cells were cotransfected with wild type ARF6-HA and either ARNO-Myc (positive control, top panels) or GFP-HERC1 (bottom panels). Cells were then treated, when appropriate, with AlF_4^-, fixed and processed for immunocytochemical analysis. ARNO-Myc was detected with anti-Myc antibodies, F-actin with phalloidin-TRITC and HERC1 with specific antibodies. Whereas ARNO induced HERC1-containing protrusions already in the absence of AlF_4^- (top), GFP-HERC1 was not able to do so nor did it block protrusion assembly when AlF_4^- was added (bottom). Scale bars 20 μm.

ability of this GTP-binding protein to elicit $PI(4,5)P_2$ synthesis. Indeed, $PI(4,5)P_2$ has already been shown to be highly enriched in ARF6-dependent actin protrusions [29] and thus $PI(4,5)P_2$-binding proteins are candidates to be recruited onto these structures. In order to find out whether HERC1 is capable of associating to the aforementioned phospholipid, binding assays were performed in which $PI(4,5)P_2$-free or $PI(4,5)P_2$-containing liposomes were incubated together with either the RLD1 or the RLD2 domain of HERC1, both purified from bacteria and possessing Flag epitopes in their carboxy-termini (Fig. 3A). As can be observed in Fig. 3B, RLD1-Flag was pulled down when $PI(4,5)P_2$-containing liposomes were used. A smaller amount of RLD1-Flag was also found with $PI(4,5)P_2$-free ones, which suggests that RLD1-Flag may either have a low, but detectable, affinity for azolectin and/or precipitate due to its intrinsic instability. Even if the latter is true, though, this does not undermine the conclusion that RLD1-Flag specifically binds to $PI(4,5)P_2$. On the other hand, RLD2-Flag was not found associated with any of the liposomes, which allows us to conclude that it is RLD1 and not the Flag epitope that mediates binding to $PI(4,5)P_2$. Next, we wondered whether RLD1 could also bind other phosphoinositides in addition to $PI(4,5)P_2$. In order to answer this question, we performed overlay assays on PIP strips. The strips were blocked, incubated with either RLD1-Flag or RLD2-Flag, washed and the bound RLD domains detected with anti-Flag antibodies. As shown in Fig. 3C, HERC1's RLD1 has affinity for several membrane phospholipids, including all monophosphate phosphoinositides as well as $PI(3,5)P_2$, $PI(4,5)P_2$ and phosphatidic acid. RLD2, on the other hand, does not appear to associate with any of these molecules. Finally, we studied whether HERC1-containing protrusions were also enriched in $PI(4,5)P_2$ (visualized by expressing a fusion protein of the pleckstrin homology domain of PLCδ1 and GFP) and the enzyme involved in its synthesis, namely PI(4)P-5K. As expected, both $PI(4,5)P_2$ and transfected Myc-tagged PI(4)P-5Kα were found in protrusions together with HERC1 (Fig. 3D). Taken together, these results suggest that HERC1 may be pulled to ARF6-dependent membrane protrusions as a result of the capability of its RLD1 domain to interact with phosphoinositides such as $PI(4,5)P_2$.

4. Discussion

In the present study, we have shown that the giant protein HERC1 undergoes recruitment onto actin-based membrane

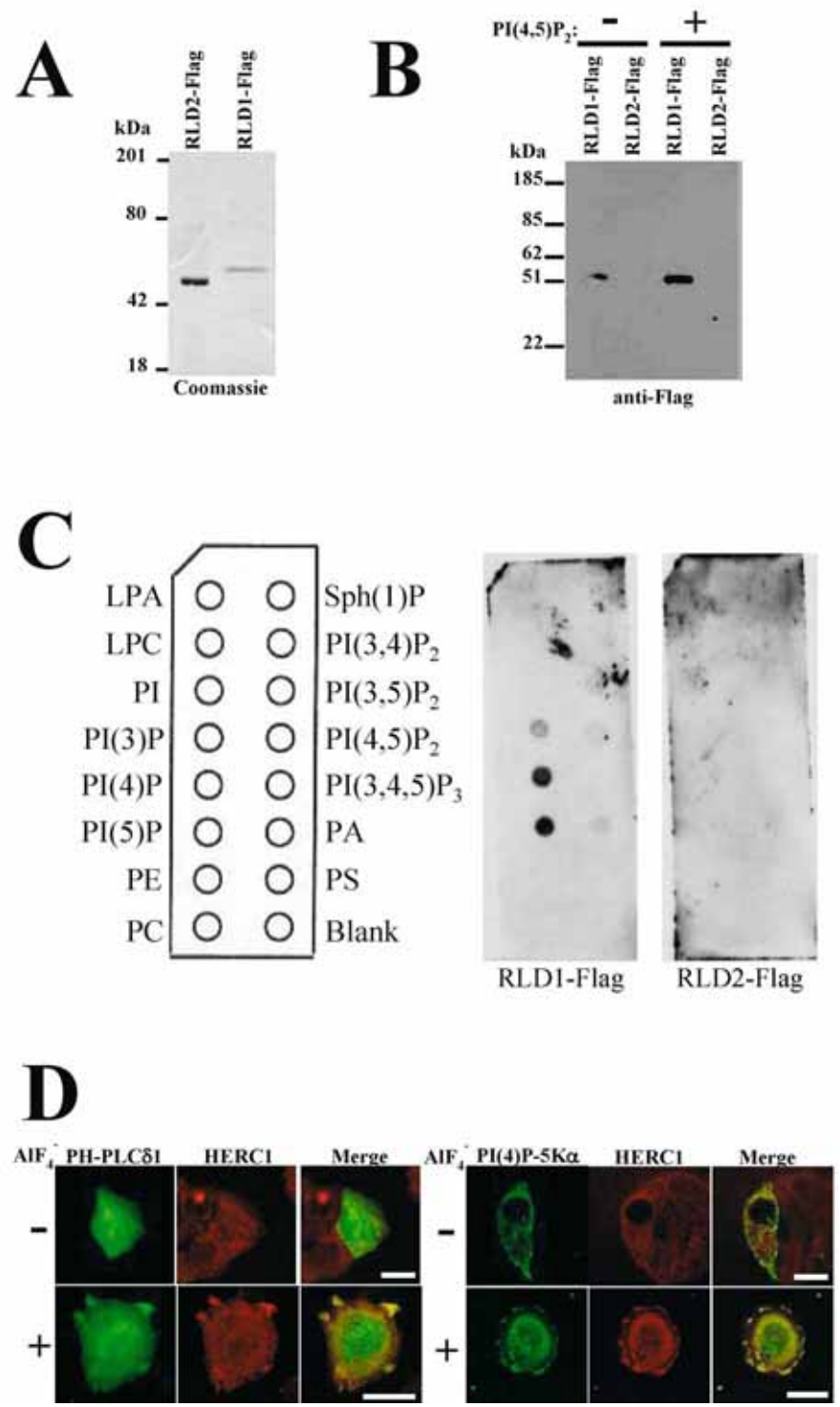

Fig. 3. HERC1's RLD1 domain binds to phosphoinositides. A: RLD1-Flag and RLD2-Flag were heterologously expressed in *E. coli* and purified by affinity chromatography. Purified samples were run on an SDS–PAGE gel which was stained with Coomassie blue dye. B: Liposome–protein complex formation assays were performed in which $PI(4,5)P_2$-free or $PI(4,5)P_2$-containing liposomes were incubated together with either RLD1-Flag or RLD2-Flag. Liposomes were then pulled down by centrifugation and the amount of associated RLDs was analyzed by SDS–PAGE followed by immunoblot with an anti-Flag antibody. C: PIP strips were incubated with recombinant Flag-tagged RLDs (0.5 μg/ml) overnight at 4°C as detailed in Section 2. After washing, bound RLDs were visualized by immunoblotting with anti-Flag antibodies. (Abbreviations: LPA, lysophosphatidic acid; LPC, lysophosphocholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph(1)P, sphingosine-1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine). D: HERC1 colocalizes in AlF_4^--induced protrusions with both $PI(4,5)P_2$ and PI(4)P-5K. HeLa cells were transfected with ARF6 and either PH-PLCδ1-GFP to visualize $PI(4,5)P_2$ or Myc-tagged PI(4)P-5K, which was detected with anti-Myc antibodies. Cells were processed for immunofluorescence microscopy as described in Section 2. Scale bars 20 μm.

protrusions formed in ARF6-overexpressing HeLa cells upon addition of AlF_4^-. We have also shown that these HERC1-enriched protrusions specifically require ARF6 activation in order to form and do not therefore arise as a result of some non-specific action of AlF_4^- drug treatment. In addition, we have demonstrated that HERC1 is not involved in ARF6 activation and thus cannot be acting as an ARF6-GEF in these cells, since otherwise its overexpression would give rise to protrusions in the absence of AlF_4^-, as happens with bona fide ARF6-GEFs such as ARNO and EFA6 [19,20]. The opposite, i.e. that HERC1 may somehow help inactivate ARF6, can likewise be ruled out by the observation that HERC1 overexpression does not prevent protrusions from forming when AlF_4^- is present. HERC1 must therefore be recruited to actin protrusions after ARF6 has already been activated. On the other hand, we have described a previously unknown physical interaction between the RLD1 domain of HERC1 and several membrane phospholipids. Among these, the strongest interaction takes place with PI(4)P and PI(5)P, followed by PI(3)P, PI(4,5)P_2, PI(3,5)P_2 and phosphatidic acid. Since PI(4,5)P_2 has been shown to be highly enriched in actin protrusions [29], we think that HERC1's RLD1 binding to PI(4,5)P_2 may account, at least partly, for the recruitment of HERC1 onto these structures. In this regard, our immunofluorescence studies clearly show that HERC1 colocalizes with both PI(4,5)P_2 and PI(4)P-5K in actin protrusions. Furthermore, preliminary data from our lab show that several HERC1 constructs containing the RLD1 domain go to AlF_4^--induced protrusions. However, the interaction(s) driving HERC1 translocation onto protrusions must still be pinpointed and several alternatives exist apart from the one postulated above. These include among others HERC1 association with other PI(4,5)P_2-binding proteins as well as HERC1's RLD1 binding to phosphatidic acid, the product of phospholipase D, which, like PI(4)P-5K, has also been shown to be activated by ARF6-GTP [32]. On the other hand, binding of HERC1 to PI(4)P and PI(3)P may be of greater significance in the Golgi apparatus and early endosomes, respectively, where these phosphoinositides have recently been shown to perform important functions [33,34] and where HERC1 is also known to be located ([1] and unpublished data).

Regardless of the mechanism whereby HERC1 moves to these protrusions, the important issue concerning HERC1's function in these structures remains unsettled. At first glance, HERC1 does not appear to have a direct role in the enhancement of actin polymerization at the plasma membrane, since its overexpression does not affect protrusion formation (Fig. 2). A more appealing possibility comes from the fact that HERC1's multidomain structure makes it suitable to act as a scaffolding protein by interacting simultaneously with many other proteins, thus bringing them together, in a way similar to the manner in which the protein paxillin works [35]. If HERC1 played such a structural role, it is conceivable that its mere overexpression is not enough to induce great changes in the cell cortex, since the signaling pathways involved in the activation of protrusion formation would not necessarily become activated. Yet another possibility is that HERC1 is involved in macropinocytosis, a process which is strongly stimulated at protrusive sites [17,36]. This would be in better agreement with HERC1's background in membrane traffic as well as with preliminary data from our lab showing HERC1's involvement in fluid-phase pinocytosis (F.R. Garcia-Gonzalo and J.L. Rosa, data not shown). In summary, our findings shed some more light to the issue of HERC1 function and open some new areas of research to be pursued in the future.

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Requirement of phosphatidylinositol-4,5-bisphosphate for HERC1-mediated guanine nucleotide release from ARF proteins

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Abstract HERC1 is a giant multidomain protein involved in membrane trafficking through its interaction with vesicle coat proteins such as clathrin and ARF. Previously, it has been shown that the RCC1-like domain 1 (RLD1) of HERC1 stimulates guanine nucleotide dissociation on ARF1 and Rab proteins. In this study, we have analyzed whether HERC1 may also regulate ARF6 activity. We show that HERC1, through its RLD1, stimulates GDP release from ARF6 but, unexpectedly, it inhibits GDP/GTP exchange on ARF6 under conditions where ARNO stimulates it. Furthermore, we demonstrate that the activity of HERC1 as a guanine nucleotide release factor requires the presence of PI(4,5)P_2 bound to HERC1's RLD1. In agreement with this, we find that purified HERC1 contains PI(4,5)P_2 bound to the RLD1.

Keywords: HERC1; ARF; RCC1-like domain; Phosphoinositide; Guanine nucleotide exchange factor; PI(4,5)P_2

1. Introduction

ADP-ribosylation factors (ARFs) are small Ras-related GTP-binding proteins involved in the regulation of membrane traffic and actin polymerization. Like other GTPases, ARF proteins act as molecular switches that cycle between an inactive GDP-bound state and an active GTP-bound state. Depending on their nucleotide status, ARF proteins interact with several classes of proteins. In their GDP-bound form they bind to guanine nucleotide exchange factors (GEFs) which promote the exchange of GDP by GTP, thus favouring the transition towards the active state. In their GTP-bound conformation they associate with GTPase-activating proteins (GAPs) that catalyze GTP hydrolysis, which leads to the inactivation of the GTPase. The active form also interacts with effectors such as vesicle coat proteins and lipid-modifying enzymes, which are the mediators of ARF's physiological functions. Sequence analysis has identified six ARF family members, of which ARF1 and ARF6 have been the most extensively studied. ARF1 regulates secretory trafficking across Golgi membranes through interaction with vesicle coat proteins, whereas ARF6 regulates both endocytic membrane traffic and the cortical actin cytoskeleton. Both ARF proteins, in their GTP-bound conformation, activate phospholipid-modifying enzymes such as phosphatidylinositol-4-phosphate 5-kinase and phospholipase D, leading to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2) and phosphatidic acid production, respectively [1–7].

HERC family proteins have a domain homologous to E6-associated protein carboxy-terminus (HECT domain) and one or more domains with homology to the regulator of chromosome condensation (RCC1) [8–19]. Proteins containing HECT domains function as a subtype of E3 ubiquitin ligases. These enzymes participate in protein ubiquitination through the transfer of ubiquitin from E2 ubiquitin conjugating enzymes to specific substrate proteins. This covalent modification serves as a signal to regulate different cellular processes such as proteasome-mediated proteolysis or receptor endocytosis [20,21]. On the other hand, RCC1 is an enzyme which catalyzes guanine nucleotide exchange on Ran, a small GTPase of the Ras superfamily. Through this activity, RCC1 is involved in the regulation of important cellular processes such as nucleocytoplasmic transport and mitotic spindle formation [22]. Thus, the presence of HECT and RCC1-like domains (RLDs) in HERC proteins suggests that they may function as both E3 ubiquitin ligases and GEFs. Up to now, six HERC family members have been identified. Sequence analysis makes it possible to classify them into two groups: the large and the small HERCs. The former, including HERC1 and HERC2, are giant proteins containing close to 5000 amino acid residues and more than one RLD in addition to the HECT [8–14], whereas the latter (HERC3-HERC6) have little more than 1000 amino acid residues and only one RLD and the HECT domain [15–19]. Although not much is known about the cellular functions of all these proteins, their molecular characterization so far points to a role in intracellular trafficking. To date, the giant HERC1 protein has been the most studied family member [8–12]. This protein seems to be ubiquitously expressed and it is found overexpressed in tumor cell lines. HERC1 contains multiple domains including two RLDs (RLD1 and RLD2) and a HECT domain at the carboxy terminus. HERC1's RLDs seem to have different molecular functions. Thus, while RLD1 stimulates guanine nucleotide

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Abbreviations: PI(3)P, phosphatidylinositol-3-monophosphate; PI(4)P, phosphatidylinositol-4-monophosphate; PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; GEF, guanine nucleotide exchange factor; RLD, RCC1-like domain; RCC1, regulator of chromosome condensation 1; ARF, ADP-ribosylation factor; His, hexahistidine tag; GRF, guanine nucleotide release factor

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dissociation from ARF1 and members of the Rab family of GTPases, RLD2 interacts with ARF1 and forms a ternary complex with clathrin and the chaperone Hsp70. The HECT domain of HERC1 is known to bind ubiquitin through a thioester linkage. HERC1's ability to act as an E3 ubiquitin ligase, together with its interaction with proteins involved in vesicular transport and its subcellular distribution between cytosol, Golgi and vesicular-like membranes suggest a role for HERC1 in ubiquitin-dependent intracellular membrane traffic.

Recently, we have reported that HERC1 is recruited onto actin-rich surface protrusions formed in HeLa cells upon ARF6 activation by aluminum fluoride treatment [12]. Although HERC1 was shown to colocalize with ARF6, these studies appeared to indicate that HERC1 acts downstream of ARF6 in this context. To study this point in greater detail, we have purified ARF6 and analyzed whether HERC1 and its RLD1 may stimulate guanine nucleotide release from this GTPase similar to what has previously been described for ARF1 [8]. In this paper, we show that HERC1 stimulates guanine nucleotide dissociation from ARF proteins but, unexpectedly, inhibits guanine nucleotide exchange. Furthermore, we demonstrate that this effect requires the presence of $PI(4,5)P_2$ bound to the RLD1 domain of HERC1.

2. Materials and methods

2.1. Reagents and antibodies

Water soluble L-α-phosphatidylinositol-3-monophosphate, L-α-phosphatidylinositol-4-monophosphate and L-α-phosphatidylinositol-4,5-bisphosphate were from Sigma–Aldrich. Mouse monoclonal antibodies against Flag (clone M2), PI(3)P, PI(4)P and $PI(4,5)P_2$ were from Sigma–Aldrich, Echelon Biosciences and Assay Designs (the last two), respectively. Monoclonal antibodies against ARF (clone 1D9) [23] and $PI(4,5)P_2$ (clone 2C11) were kindly provided by Dr. Richard Kahn and Dr. Gianpietro Schiavo, respectively. Affinity purified rabbit polyclonal antibodies against HERC1 (410) have already been described elsewhere [8]. Goat anti-mouse or anti-rabbit $F(ab')_2$ fragments conjugated to horseradish peroxidase were purchased from Molecular Probes. For immunoblot detection, ECL Plus Western Blotting detection system (Amersham Biosciences) was used. All other chemicals were obtained from Sigma–Aldrich.

2.2. Protein purification from bacteria and insect cells

Plasmid expressing GST-ARNO was kindly supplied by Dr. James E. Casanova [24]. Plasmids encoding RLD1- and RLD2-Flag for bacterial expression have been described previously, as well as the expression and purification of the respective proteins [12]. Sf9 insect cells were maintained as previously described [12]. Recombinant baculoviruses of RLD1-Flag, RLD2-Flag, ARF6-His and HERC1 have already been reported [8,15]. Baculovirus infection, protein expression and purification were performed essentially as described [8]. The purity of these proteins is shown in Figs. 1B and 2A, D.

2.3. Guanine nucleotide binding assays

Guanine nucleotide binding assays were performed essentially as described by Frank et al. [24]. Briefly, for dissociation assays, in general 1 μg of purified ARF6-His was incubated at 30 °C for 45 min in 100 μl of 50 mM HEPES–NaOH (or Tris–HCl) pH 7.5, 1 mM DTT, 2.5 μM [^{3}H]GDP, 5 mM $MgCl_2$ and 1.5 mg/ml azolectin vesicles. After this, 100 μM GTP was added in the presence of 1 μg of RLD1 or RLD2 or of 10 ng of HERC1 or of 2 or 200 μM of water soluble phospholipid. At the indicated times, aliquots of 10 μl were measured for radioactivity using a filter assay [8]. For exchange assays, 1 μg of ARF6-His was incubated at 30 °C for 45 min in 100 μl of 50 mM HEPES–NaOH (or Tris–HCl) pH 7.5, 1 mM DTT, 0.5 μM GDP, 5 mM $MgCl_2$ and 1.5 mg/ml azolectin vesicles. Nucleotide exchange was initiated by adding 1 μM [^{35}S]GTPγS in the presence of 1 μg of GST-ARNO or 10 ng of HERC1. At the indicated times, aliquots of 10 μl were measured for radioactivity using a filter assay [8].

2.4. Slot-blot assays

40 ng/slot of purified RLD1-Flag or RLD2-Flag and 4 ng/slot of HERC1 were loaded in a Bio-Rad slot-blotting unit following manufacturer's instructions. Slots were blocked 60 min in blocking solution (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20 and 3% BSA) at room temperature. They were then incubated overnight at 4 °C with anti-Flag, anti-HERC1 or anti-phosphoinositide antibodies. Immunoblots were carried out as previously described [8].

3. Results

3.1. Purification of ARF6-His and guanine nucleotide binding

In order to analyze whether HERC1's RLDs could stimulate guanine nucleotide dissociation on ARF6 as it was previously reported for ARF1 and Rab proteins, we have used a recombinant baculovirus that expresses ARF6-His in insect Sf9 cells [15]. ARF6-His expression was verified by SDS–PAGE fol-

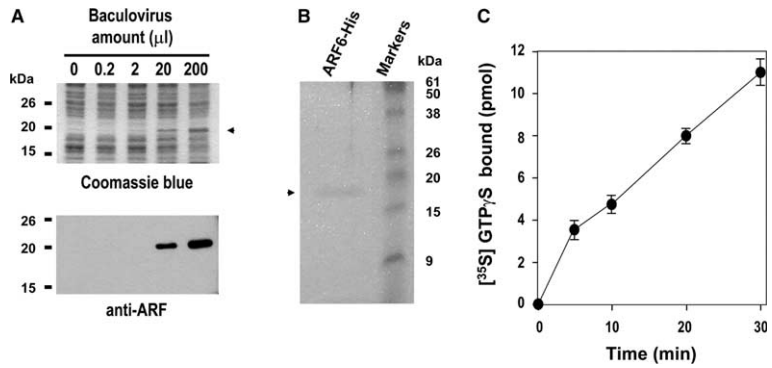

Fig. 1. (A) Expression of ARF6-His with recombinant baculovirus. Different amounts of baculovirus were used to infect Sf9 insect cells. After 60 h of infection, cells were harvested and pellets processed for protein separation by a 12% SDS/PAGE gel. Protein markers are shown on the left. Proteins were stained with Coomassie blue (top). ARF6-His expression is indicated with an arrow. ARF6-His expression was confirmed by immunoblot with anti-ARF antibodies (bottom). (B) Purification of ARF6-His. ARF6-His expressed in insect cells was purified with Nickel-NTA agarose beads as described in Section 2. 0.4 μg of purified protein were analyzed by SDS–PAGE in a 15% gel and proteins were stained with Coomassie blue. Purified protein is indicated with an arrow. Molecular weight markers are also shown (right lane). (C) Time course of [^{35}S]GTPγS binding to ARF6-His. 1 μg of purified ARF6-His was incubated in the presence of 4 μM [^{35}S]GTPγS and, at the indicated times, bound [^{35}S]GTPγS was determined by filter assay as described under Section 2. Data are means ± S.E. from triplicate assays.

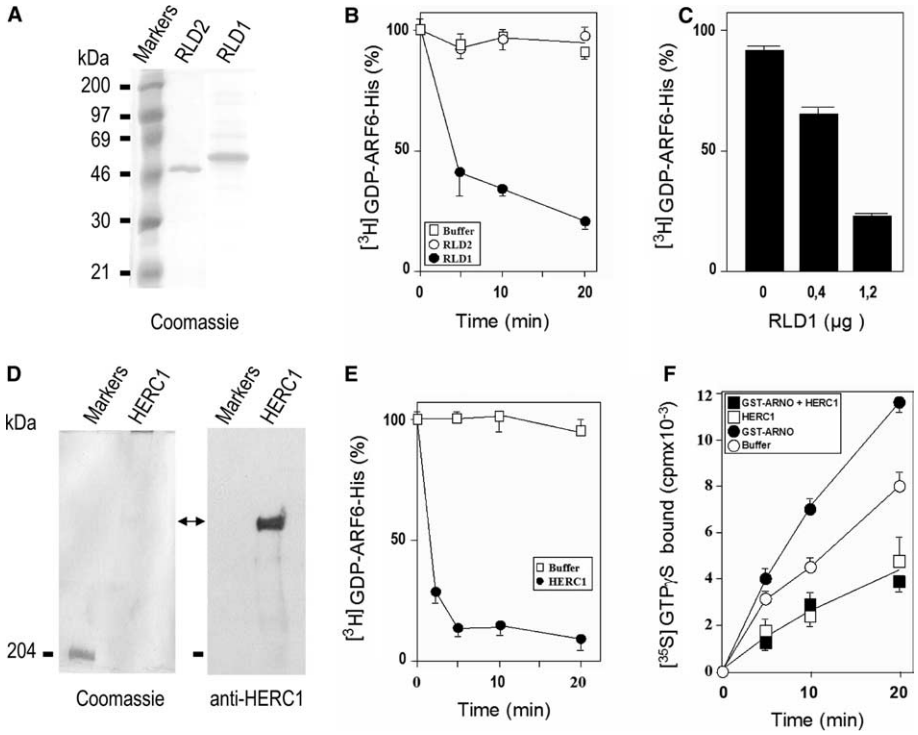

Fig. 2. (A) Purification of RLD1 and RLD2 proteins. His-RLD1-Flag and His-RLD2-Flag proteins were expressed in insect cells and purified with Nickel-NTA agarose beads as described in Section 2. 1 μg of each purification was analyzed by SDS–PAGE in a 10% gel and proteins were visualized by Coomassie blue staining. Molecular weight markers are also shown (left lane). (B) Dissociation of GDP from ARF6-His by RLD1. 1 μg of ARF6-His was loaded with 2.5 μM [^{3}H]GDP for 45 min at 30 °C. [^{3}H]GDP-dissociation was initiated by adding 100 μM GTP in the absence (buffer, white squares) or presence of 1 μg of RLD1 (black circles) or RLD2 (white circles) for the indicated times and analyzed as described under Section 2. (C) GDP dissociation from ARF6-His is dependent on RLD1 concentration. ARF6-His was loaded with [^{3}H]GDP as above. [^{3}H]GDP-dissociation was initiated by adding 100 μM GTP in the presence of different amounts of RLD1. At 20 min, [^{3}H]GDP-bound was determined by filter assay as described in Section 2. (D) Purification of HERC1. His-HERC1 (532 kDa) expressed in insect cells was purified with Nickel-NTA agarose beads as described in Section 2. 0.1 μg of purified protein were analyzed by SDS–PAGE in a 5% gel (acrylamide:bisacrylamide ratio 80:1) stained with Coomassie blue (left panel). Purified protein is indicated with a double-headed arrow. Molecular weight markers are also shown (left lane). An immunoblot with anti-HERC1 antibodies is also shown (right panel). (E) Dissociation of GDP from ARF6-His by HERC1. 1 μg of ARF6-His was loaded with 2.5 μM [^{3}H]GDP for 45 min at 30 °C. [^{3}H]GDP-dissociation was initiated by adding 100 μM GTP in the absence (buffer, white squares) or presence of 10 ng of HERC1 (black circles) for the indicated times and analyzed as described under Section 2. (F) ARNO stimulates guanine nucleotide exchange on ARF6-His but HERC1 does not. 1 μg of ARF6-His was loaded with 0.5 μM GDP for 45 min at 30 °C. Nucleotide exchange was initiated by adding 1 μM [^{35}S]GTPγS in the absence (buffer, white circles) or presence of 1 μg of GST-ARNO (black circles) or 10 ng of HERC1 (white squares) or both (black squares) for the indicated times and analyzed as described under Section 2. Data are means ± S.E. from triplicate assays.

lowed by Coomassie blue staining (Fig 1A, top) and confirmed by immunoblot with monoclonal anti-ARF antibodies (Fig 1A, bottom). ARF6-His was purified using Ni^{2+}-NTA agarose beads and its purity was analyzed by SDS–PAGE and Coomassie staining (Fig. 1B). To assess the functionality of purified ARF6-His, the GTPase was loaded with [^{35}S]GTPγS. In Fig. 1C, a time-dependent [^{35}S]GTPγS incorporation on ARF6-His is shown.

3.2. RLD1 and HERC1 stimulate guanine nucleotide dissociation on ARF6-His

Purified GTPase was loaded with [^{3}H]GDP for 45 min. [^{3}H]GDP-ARF6-His was then incubated with GTP in the absence or presence of RLD1 or RLD2 for the indicated times. As shown in Fig. 2B and C, RLD1, but not RLD2, stimulated the dissociation of [^{3}H]GDP bound to ARF6-His in a time- and dose-dependent manner. These results are similar to those we had found with ARF1 and Rab proteins [8]. To determine if the full-length HERC1 protein may also stimulate this dissociation, purified HERC1 was incubated with [^{3}H]GDP-ARF6-His. A fast release of bound [^{3}H]GDP was observed in the presence of HERC1 (Fig. 2E). Similar results were also found when HERC1 was incubated with ARF6-His loaded with [^{35}S]GTPγS or when purified ARF1 or Rab5 were used as GTPases (data not shown).

3.3. HERC1 inhibits guanine nucleotide exchange on ARF6-His

The above results indicate that HERC1, through its RLD1 domain, stimulates guanine nucleotide dissociation on ARF proteins. In order to analyze whether HERC1 also stimulates guanine nucleotide exchange on these proteins, we loaded ARF6-His with GDP for 45 min and, after this time, [^{35}S]GTPγS was added in the presence or absence of HERC1. As positive control of these experiments, we used purified GST-ARNO, which has previously been shown to catalyze guanine nucleotide exchange on ARF proteins in these assays [24,25]. As expected, the presence of GST-ARNO stimulated the exchange of bound GDP by [^{35}S]GTPγS on ARF6-His (Fig. 2F). Surprisingly, however, the presence of HERC1 not only did not stimulate exchange, but it also caused a clear inhibition of [^{35}S]GTPγS uptake by ARF6-His. This effect was not reversed by the presence of GST-ARNO (Fig. 2F).

3.4. Purified RLD1 and HERC1 contain bound phosphatidylinositol 4,5-bisphosphate

We have recently reported that the RLD1 domain of HERC1 has affinity for phosphoinositides [12]. In this regard, we showed that PI(4,5)P_2-containing liposomes pull down purified RLD1. Moreover, it has also been reported that PI(4,5)P_2 stimulates the dissociation of guanine nucleotide on ARF- and Rho-family proteins [26,27]. With these data in mind, we thought that the stimulation of guanine nucleotide dissociation on ARF proteins by RLD1 and HERC1 might be due to the fact that HERC1, through its RLD1 domain, binds PI(4,5)P_2. To test this hypothesis, we incubated slot blots of these purified proteins with commercial antibodies against PI(3)P, PI(4)P and PI(4,5)P_2. As protein control, we used RLD2 tagged with a Flag epitope. As can be observed in Fig. 3A, anti-PI(4,5)P_2 antibodies displayed immunoreactivity on RLD1 and HERC1 proteins but not on RLD2. These results were totally confirmed with another monoclonal antibody against PI(4,5)P_2 (clone 2C11, data not shown). Anti-PIP antibodies did not detect any phosphatidylinositol monophosphate bound to the purified proteins. This experiment seems to indicate that purified RLD1 and HERC1 contain bound PI(4,5)P_2. To confirm this observation and to exclude any non-specific detection by anti-PI(4,5)P_2 antibodies, we incubated RLD1 in a boiling water-bath during 10 min in order to strip the phospholipid off the protein. After this, the protein was loaded on slots, filtered and washed. Next the slot blots were incubated with anti-Flag or anti-PI(4,5)P_2 antibodies to detect the Flag-tagged protein and its associated PI(4,5)P_2, respectively. As shown in Fig. 3B, this treatment removed the PI(4,5)P_2 bound to RLD1.

3.5. Bound PI(4,5)P_2 is required for the GDP release activity of HERC1 over ARF proteins

Finally, in order to show whether RLD1-associated PI(4,5)P_2 is responsible for the guanine nucleotide dissociation activity on ARF proteins, we have followed several approaches. First, we have purified RLD1 from bacteria. Since these cells cannot synthesize PI(4,5)P_2 [28], purified RLD1 from bacteria does not contain this phospholipid. When the dissociation activity of bacterially purified RLD1 was analyzed on ARF6 and ARF1, we observed no stimulation of guanine nucleotide dissociation (data not shown). Second, PI(3)P, PI(4)P or PI(4,5)P_2 (all at 200 μM) were incubated with ARF6-His loaded with [^{3}H]GDP. As shown in Fig. 3C, PI(4,5)P_2 specifically stimulated [^{3}H]GDP release from ARF6-His. This result is in agreement with previous reports that described a similar effect on ARF1 and Cdc42 proteins [26,27]. Finally, we carried out reconstitution experiments in which inactive RLD1 from bacteria was mixed with tiny

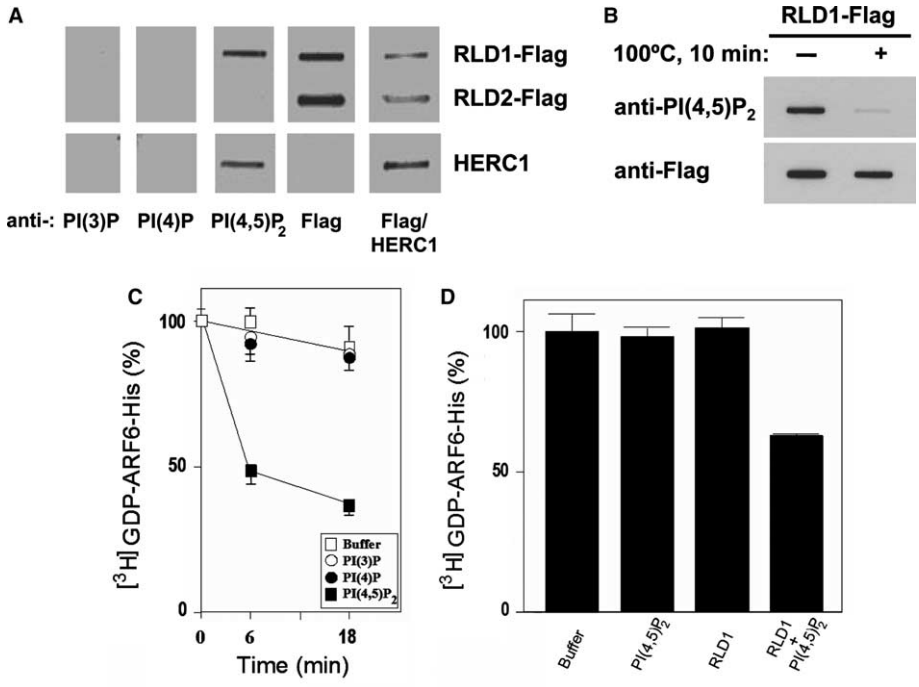

Fig. 3. (A) Purified RLD1 and HERC1 contain PI(4,5)P_2. 40 ng/slot of purified RLD1-Flag or RLD2-Flag proteins and 4 ng/slot of HERC1 protein were loaded in a slot-blot apparatus. Next, slots were incubated with antibodies against PI(3)P, PI(4)P, PI(4,5)P_2, Flag-epitope and HERC1 and detection was as described under Section 2. (B) PI(4,5)P_2 bound to RLD1 is removed by incubation at 100 °C. Purified RLD1-Flag was incubated for 10 min at room temperature or in a boiling water-bath. After this, 40 ng/slot were loaded in a slot-blotting unit and analyzed by immnunoblot with anti-PI(4,5)P_2 or anti-Flag antibodies. (C) 0.5 μg of ARF6-His were loaded with 2.5 μM [^{3}H]GDP for 45 min at 30 °C. [^{3}H]GDP-dissociation was initiated by adding 100 μM GTP in the absence (buffer, white squares) or presence of 200 μM of PI(3)P (white circles), PI(4)P (black circles) or PI(4,5)P_2 (black squares) for the indicated times and analyzed as described in Section 2. (D) [^{3}H]GDP dissociation experiments were carried out as above in the absence (buffer) or presence of 2 μM PI(4,5)P_2, 0.5 μg of bacterially purified RLD1 (which does not contain PI(4,5)P_2) or both (previously incubated for 45 min at 30 °C). After 5 min of incubation, bound [^{3}H]GDP was determined by filter assay. As can be seen, PI(4,5)P_2 addition to inactive RLD1 from bacteria is enough to reconstitute the latter's GRF activity for ARF6-His. Data are means ± S.E. from triplicate assays.

amounts of PI(4,5)P_2 and the resulting mixture was analyzed for [^{3}H]GDP release activity on ARF6-His. As shown in Fig. 3D, while 2 μM PI(4,5)P_2 did not induce an observable [^{3}H]GDP release from ARF6-His after 5 min of incubation, addition of this same amount of PI(4,5)P_2 to the inactive bacterial purification of RLD1 yielded a fully active mixture, with a dissociating activity comparable to that of RLD1 purified from eukaryotic cells. Altogether, these results show that PI(4,5)P_2 bound to RLD1 is required for the guanine nucleotide release activity of HERC1 over ARF6. Even though PI(4,5)P_2 alone already displays some release activity, the synergistical effect seen between this phospholipid and RLD1 suggests that PI(4,5)P_2 is a cofactor for HERC1's activity as a guanine nucleotide release factor (GRF).

4. Discussion

In this study, we have analyzed whether HERC1 may regulate the activity of ARF6. We show that HERC1, through its RLD1 domain, stimulates guanine nucleotide release from ARF6 (Fig. 2A–E). These data are in agreement with what we had described for ARF1 and Rab proteins [8]. Although in our former report this dissociation activity was taken as evidence that HERC1 was a GEF for these GTPases, our current studies indicate that this is not the case. Indeed, guanine nucleotide exchange experiments have now clearly demonstrated that not only does HERC1 not stimulate GDP/GTP exchange on ARF6 as does ARNO, but it also inhibits this process relative to the uncatalyzed reaction. Moreover, this inhibition is dominant over ARNO's stimulatory effects (Fig. 2F). These results, therefore, indicate that HERC1 rather than being an activator of ARF and Rab proteins as formerly thought is actually an inhibitor of them, at least in vitro. Furthermore, we have proven that HERC1, through its RLD1 domain, is non-covalently bound to PI(4,5)P_2 (Fig. 3A and B) and that this phosphoinositide is required as a cofactor for HERC1's activity as a GRF (Fig. 3C and D).

Since it has been established that Sec7 domain-containing ARF-GEFs such as ARNO act by stimulating GDP dissociation on their targets without directly affecting the rate of subsequent GTP incorporation [29], we wondered how it is possible that a protein that triggers GDP release from ARF6 does not at the same time stimulate its GTP uptake. In this regard, it has been shown that a mammalian protein called Mog1 is a GRF for the small GTPase Ran [30]. Mog1 induces GDP dissociation from Ran but not GDP/GTP exchange and these effects have been explained as the result of the formation of a stable complex between nucleotide-free Ran and Mog1. The fact that Mog1 remains bound to Ran after the dissociation step has been accomplished would occlude Ran's active site and therefore prevent subsequent GTP binding. In the case of HERC1, however, we have not been able to detect any stable interaction between HERC1 and ARF6 (data not shown). For this reason, the most likely explanation for our data is that HERC1 is more efficient in releasing nucleotide from ARF6 active site than are ARF-GEFs such as ARNO. This higher dissociation activity would preclude the accumulation of GTP-bound ARF6 in our assays by very rapidly removing the GTP once it has associated with ARF6. By contrast, ARNO-induced dissociation would be slow enough to allow a faster accumulation of ARF6 · GTP. Furthermore, this explanation would also account for the dominant effect of HERC1 over ARNO.

On the other hand, we have shown that HERC1's GRF activity depends on the presence of bound PI(4,5)P_2 on its RLD1 domain. This is best demonstrated by the fact that RLD1 purified from *Escherichia coli*, which does not contain phosphatidylinositol or its derivatives [28], does not show GRF activity, whereas PI(4,5)P_2-containing RLD1 from insect cells is highly active. What is more, addition of a small amount of PI(4,5)P_2 (2 μM) to bacterially purified inactive RLD1 is enough to reconstitute the latter's GRF activity to values which are comparable to those seen with RLD1 purified from eukaryotic sources (Fig. 3D). As a matter of fact, PI(4,5)P_2 alone already displays some GRF activity on ARF6 (Fig. 3C), in agreement with what has previously been reported for other GTPases, namely ARF1 and Cdc42 [26,27]. Nevertheless, the GRF activity of isolated PI(4,5)P_2 on ARF6 is much lower than that observed for either the RLD1 of HERC1 (Fig. 2B and C) or the full HERC1 protein (Fig. 2E), therefore further indicating that HERC1 and PI(4,5)P_2 act synergistically in order to enhance the reaction rate. These data, in conjunction with similar results found for ARF1 and Rab5, suggest that HERC1 is a PI(4,5)P_2-dependent GRF for these proteins.

The mechanism whereby Sec7 domain-containing ARF-GEFs catalyze the dissociation of GDP from ARF proteins has been elucidated in considerable detail [29]. This involves a glutamic acid residue in the GEF, known as the glutamic finger, which during the reaction course is brought into close proximity to the alpha- and beta-phosphate groups of ARF-bound GDP. As a result of the repulsion forces thus generated, GDP is displaced from ARF's active site. Based on this knowledge, we envisage an analogous mechanism which we hypothesize could account for HERC1-catalyzed GDP release from ARFs. According to this model, the role fulfilled by the glutamic finger of Sec7 domains would in the case of HERC1 be taken up by the RLD1-associated PI(4,5)P_2. Thus, upon binding of ARF to HERC1's RLD1, the former would undergo a rotation which would bring together the phosphate groups of both GDP and PI(4,5)P_2, thereby triggering nucleotide release.

PI(4,5)P_2 has recently emerged as a crucial player in diverse cellular activities such as membrane trafficking and cytoskeletal dynamics [31,32]. The cellular levels of this molecule are controlled by the relative activities of two sets of enzymes, namely phosphoinositide kinases and inositol phosphatases, which are in charge of its synthesis and degradation, respectively. One of the former enzymes, PI(4)P 5-kinase, has been shown to be one of the major downstream targets of active ARFs [2–5]. Therefore, according to current models, ARF activation in specific membrane patches would lead to localized PI(4,5)P_2 production, which would in turn recruit PI(4,5)P_2-binding effector complexes to these membrane locations. One important cellular process known to be regulated in this manner is the formation of clathrin-coated vesicles (CCVs). Here, PI(4,5)P_2 acts in concert with ARF · GTP in order to nucleate clathrin coat assembly at specific membrane sites [33]. If one takes into account the results presented in this paper as well as the previously reported interaction between HERC1, clathrin and Hsp70, a chaperone involved in CCV uncoating [9,34], it is very tempting to speculate about the possibility of HERC1 playing an important role in the dynamics of CCVs. Nonetheless, future studies will be required in order

to ascertain the validity of these hypotheses and understand the function of HERC1 in these processes.

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The HERC proteins: functional and evolutionary insights

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The HERC proteins: functional and evolutionary insights

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Abstract

HERC proteins are defined as containing both HECT and RCC1-like domains in their amino acid sequences. Six HERC genes have turned up in the human genome, which encode two different sorts of polypeptides: while the small HERC proteins possess little more than the two aforementioned domains, the large ones are giant proteins with a plethora of potentially important regions. It has now been almost ten years since the discovery of the first family member and information is starting to accumulate pointing to a general role for these proteins as ubiquitin ligases involved in membrane trafficking events. In this review, the available data on these six members are discussed, together with an account of their evolution.

Keywords: HERC proteins, RCC1-like domain, HECT domain, ubiquitin ligase.

1. Definition

According to the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC), all proteins containing both HECT and RCC1-like domains in their amino acid sequences shall be referred to as HERC proteins and numbered according to the order in which they are approved at HGNC [1].

2. The HECT domain

HECT (Homologous to E6AP COOH Terminus) domains are those protein domains showing a high degree of similarity (usually around 50%) with the carboxyl-terminal region of E6-associated protein (E6AP). The latter was originally identified as the cellular protein mediating the association between the tumor suppressor p53 and the E6 oncoprotein of tumorigenic human papillomaviruses HPV-16 and 18 [2,3]. Shortly afterwards, it was discovered that the E6-E6AP complex functions as a ubiquitin ligase for p53, thereby inducing its proteasomal degradation [4]. This and later studies also showed that E6AP's ubiquitin ligase activity is not restricted to pathological conditions, but that it instead constitutes the normal function of this protein, with the sole difference that under physiological conditions it is not p53 but other proteins which undergo E6AP-mediated ubiquitination [5-7]. In addition, a few years after the initial identification of E6AP a number of proteins were found possessing C-terminal regions very similar to that in E6AP. What is more, several of these proteins were shown to resemble E6AP not only in the sequence of its approximately last 350 amino acid residues, but also in its ability to form thioester bonds with ubiquitin, indicating that they may also be active ubiquitin ligases [8,9]. Thus, the HECT domain, as this C-terminal stretch of conserved amino acids came to be known, became established as a

structural feature that endowed those proteins containing it with the ability to act as ubiquitin ligases. Protein ubiquitination is accomplished through a hierarchical enzymatic cascade, consisting of a single ubiquitin-activating enzyme (E1), a limited number of ubiquitin-conjugating enzymes (E2) and many ubiquitin ligases (E3). The great abundance of E3s enables them to specifically recognize substrates, this being the most important function of ubiquitin ligases. Currently, HECT domain proteins are regarded as one of two major classes of E3s (for a review on the ubiquitin system see [10]). Unlike all other E3s, HECT ubiquitin ligases utilize a covalent mechanism involving the formation of a thioester bond between a highly conserved cysteine residue in the HECT's active site and the C-terminus of ubiquitin [9]. Some insights into how this actually happens can be obtained from the crystal structure of the HECT domain of E6AP bound to the E2 UbcH7 (Figure 1 and [11]). In this structure, the HECT consists of two lobes with the active site cysteine located at the interface between the two, directly facing the active site cysteine in UbcH7, from which ubiquitin must be transferred to the HECT. For this to take place, though, the long distance separating the two cysteines in the crystal (41Å) must first be surmounted, which presumably entails a conformational change induced by ubiquitin upon its binding to UbcH7 [11]. Once the HECT-ubiquitin conjugate has been formed, ubiquitin must be transferred to a substrate's lysine residue to form a stable isopeptide bond. However, since it has been shown that substrates usually bind outside the HECT [12], ubiquitin conjugation to substrates probably requires an intramolecular rearrangement so that the target lysine residue in the substrate can get close enough to the HECT's active site. So, it seems clear that both structure and function of HECT domains have been conserved during evolution. As it will be seen now, the same does not appear to have occurred with RCC1-like domains.

3. The RCC1-like domain (RLD)

The RCC1-like domain or RLD is a structural feature found in many proteins which displays high similarity to the sequence of the RCC1 protein (Regulator of Chromosome Condensation-1). The RLD is characterized by the presence of several (usually seven) repeats of 51-68 aa each, thus making for a domain of up to 400 amino acid residues. The three-dimensional structure of RCC1's RLD reveals a seven-bladed β-propeller fold wherein each blade corresponds to the previously identified sequence repeats (Figure 2 and [13,14]). Up to now, close to 20 proteins harboring RLDs have been described in the literature, even though many more are present in databases [15]. The first to be discovered was RCC1 itself, which is also the best known. RCC1 was identified as the labile component in thermosensitive baby-hamster kidney tsBN2 cells which, when placed at the restrictive temperature, were unable to enter S-phase or, if they had already entered it, went into precocious chromosome condensation and mitosis without completing DNA replication [16,17]. Further studies showed RCC1 to be a nuclear, chromatin-associated protein which interacts with the Ras-related GTP-binding protein Ran and catalyzes guanine nucleotide exchange upon it [18-20]. More recent studies have established that RCC1, through its ability to activate Ran, regulates a panoply of cellular processes including nucleocytoplasmic transport, mitotic spindle assembly and nuclear envelope formation (for a recent review on the functions of Ran refer to [21]). Therefore, the RLD of RCC1 has a double role: while one face of the β-propeller binds to Ran and acts as a guanine nucleotide exchange factor (GEF) for this small GTPase [14], the opposite face associates with histones H2A and H2B and thus tethers RCC1 to chromatin, which is essential for its proper function (Figure 2 and [21,22]). The enzyme activity of RCC1 raises the question of whether other RLD-

containing proteins may also function as GEFs for small GTPases. In fact, a few years after it was demonstrated for RCC1, the HERC1 protein (see below) was also shown to stimulate guanine nucleotide dissociation on ARF and Rab family small GTPases [23]. Although at the time this was taken to mean that HERC1, through one of its two RLDs, was a GEF for these GTPases, a recent report has challenged this view, since the stimulation of GDP dissociation induced by HERC1 is not accompanied by a concomitant increase in GTP uptake by the small G protein [24]. Moreover, nucleotide dissociation activity in HERC1 has been shown to rely on its RLD1 binding to phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), making the case of HERC1 still more different from that of RCC1 [24]. Aside from RCC1 and HERC1, two other RLDs have been purported to behave as GEFs: these are the RLDs of PRAF, a phosphoinositide-binding *Arabidopsis thaliana* protein [25] and of Claret, a protein involved in eye pigment granule biogenesis in *Drosophila* [26]. Both cases share the fact that the RLD substrates belong to the Rab family (plant Rab8 and the fly equivalent of human Rabs 32 and 38, respectively) and that their GEF activity has not been proven conclusively: in the first case it has been taken for granted on the basis of nucleotide dissociation experiments only, whereas in the second only a preferential binding for the GDP-bound form of Rab32/38 has been shown. Even though it is likely that these two proteins are bona fide GEFs, a definitive proof of it should include GTP incorporation experiments, too. Although it is possible that other RLD-based GEFs are awaiting discovery, the actual fact is that no more of them have been reported so far (if we allow for the exception of Alsin, a GEF for Rac1 and Rab5 that uses other conserved domains for these activities and whose RLD has no known function [27]). Instead, RLDs have been shown to fulfil other roles, all of which involve interactions with other proteins. Examples of this include the RLD2 of HERC1, which binds to clathrin [28], and the

RLDs of Nercc1, PAM, RPGR and DelGEF. The case of the Nercc1 kinase is a curious one: even though its RLD binds to the inactive, GDP-bound form of Ran, this most probably does not lead to guanine nucleotide exchange, since Nercc1's RLD lacks key catalytic residues which are needed for the GEF activity of RCC1 [29,30]. Also interesting is the case of PAM, a giant protein involved in synaptogenesis and nociception, whose RLD2 specifically binds to and strongly inhibits adenylate cyclase type V [31]. On the other hand, RPGR, the protein affected in X-linked retinitis pigmentosa (RP3), uses its RLD to interact with two other proteins, namely RPGRIP and the δ subunit of cGMP phosphodiesterase. These interactions, especially the first one, have been shown to be important for retinal function and are disrupted by all known RP3 mutations [32-35]. Finally, DelGEF has been shown to associate through its RLD with the human orthologue of yeast Sec5 as well as with a very small 9 kDa protein called DelGIP1. Both interactions appear to have a role in proteoglycan secretion [36,37]. Despite other RLD proteins having been reported, nothing is known about the functions of their RLDs, so they will not be dealt with in this review. Altogether, then, the available information indicates that the RLD is a structurally conserved, yet functionally very versatile domain, whose functions may include interactions with other proteins or phospholipids and in some cases GEF activity on small G proteins.

4. The HERC proteins

The human genome encodes more than 20 proteins containing a HECT domain in their C-termini [15]. The N-terminal regions of these proteins are diverse, but even so many of these polypeptides can be placed in one of two subfamilies. In particular, the Nedd4 family of HECT ubiquitin ligases includes nine human members, all of which have

similar N-termini containing a single calcium-binding C2 domain and 2 to 4 WW domains [38]. On the other hand, the second subfamily of HECT ligases is made up of the six human HERC proteins, which, as noted above, all possess one or more RLDs upstream of their HECTs. The HERC family members can in turn be subdivided into two subgroups according to their sizes and domain architecture (Figure 3 and Table I). All these members have already been published and characterized to varying extents. Next, the information available on each of them is discussed.

4.1. HERC1

The HERC1 protein can be regarded as the founding member of the family, insofar as it was the first to be discovered. HERC1, originally named p619 and later also p532, was identified during a search for human oncogenic sequences from breast cancer DNA cells using so-called nude mouse tumorigenicity assays [39]. As a result of this search, a 150 kb DNA sequence containing fragments of at least two different human loci was isolated which preserved the ability to induce tumors in nude mice [23]. Subsequently, this DNA fragment, referred to as OncH, was used for the identification of expressed sequences with exon trapping techniques. These yielded a single exon whose sequence encoded part of a protein with similarity to RCC1 (HERC1's RLDs display ~40% similarity to RCC1), and therefore a good candidate for a cell-cycle regulatory protein. Thus, the sequence of this exon was used to retrieve the full-length cDNA sequence of this novel gene from a human fetal brain library. So, after seven rounds of screening, eight overlapping clones were obtained which encompassed a 15 kb cDNA sequence containing a single ORF encoding a predicted protein of 4861 aa and 532 kDa [23]. Analysis of HERC1 sequence reveals the presence of a number of regions which are conserved in other proteins. These include two RLDs (RLD1 and RLD2), the C-

terminal HECT, a WD40 domain homologous to the β subunits of heterotrimeric G proteins, a SPRY domain and other minor motifs such as putative SH3-binding proline-rich sequences, a potential leucine zipper and several regions enriched in polar and acidic sidechains. While the WD40 domain is well-known both structurally, it adopts a seven-bladed β-propeller fold similar to the one found in RLDs [40], and functionally, it usually mediates protein-protein interactions, sometimes even with other WD40 proteins [41], not much is known of the SPRY domain (spl A and RyR), even though it has been suggested that it might fold in a similar way to immunoglobulin-like domains [42] (the latter are found in many proteins of diverse function, including many immune system and cell adhesion molecules, and adopt a β-sandwich fold [43]) and that it may likewise participate in interactions with other proteins or even RNAs [44]. Although the functions of all these domains in HERC1 have not yet been fully elucidated, several studies have contributed interesting insights which will now be summarized. So, it has been shown that the mRNA of HERC1 displays a ubiquitous expression pattern with slightly higher levels in brain and testis [23]. Similarly, HERC1 mRNA [23] and protein (FRG and JLR, unpublished data) have been detected in all tested cell lines, with the potentially relevant observation that mRNA levels are significantly higher in a number of tumor cell lines with respect to two normal cell lines of fibroblast origin [23]. This observation, together with the way in which HERC1 was originally discovered, raise the possibility of HERC1 having oncogenic properties. However, unlike the original OncH fragment (see above), HERC1 cDNA does not induce tumor formation in nude mouse assays. There are at least three potential explanations for this, namely that (1) out of the two human loci in OncH only one contained an oncogene and this was not HERC1, (2) OncH expressed a mutated oncogenic version of HERC1 different from the one eventually cloned and (3) HERC1 overexpression in the nude mouse assays was not

sufficient for it to induce efficient tumor formation [23]. Therefore, the issue of the potential oncogenic capacity of HERC1 remains open to this day. At the subcellular level, the HERC1 protein has an exclusively cytoplasmic localization, where it can be found soluble or associated with vesicular membranes and with the Golgi apparatus, from which HERC1 is dislodged upon treatment with the fungal metabolite brefeldin A [23]. As to its function, the first studies were oriented to determine whether HERC1, like RCC1, could act as a GEF for small GTPases. In accordance with its observed subcellular distribution, HERC1 was found to stimulate guanine nucleotide dissociation from ARF and Rab family GTPases (ARF1, Rab3a and Rab5), both involved in membrane trafficking, while no stimulation was observed for other GTPases such as Ran or R-Ras2/TC21. This activity was found to reside in HERC1's RLD1[23] and, although it originally looked like the RLD1 acted as a GEF for these GTPases, a more recent paper has shown that HERC1's RLD1, rather than as a GEF, acts as a guanine nucleotide release factor (GRF), since it prevents rather than stimulates GDP/GTP exchange on the active site of these GTPases [24]. Moreover, the GRF activity of HERC1 has been shown to require the presence of $PI(4,5)P_2$ bound to the RLD1 of HERC1 [24]. On the other hand, HERC1's RLD2 does not possess either GEF or GRF activity over any of the above small G proteins. Instead, RLD2 has been shown to bind to ARF1 as well as to the heavy chain of clathrin [23,28]. These two interactions support the idea of HERC1 playing an important role in intracellular membrane trafficking both in the Golgi, where ARF1 is a crucial player, and elsewhere in the cytoplasm (clathrin is a major coat component of many sorts of intracellular vesicles). Some clue as to how this role for HERC1 in vesicle traffic is accomplished may lie in several observations related to the clathrin-HERC1 interaction. Thus, only the cytosolic fraction of HERC1, but not the membrane-associated pool, was shown to be bound to

clathrin heavy chain (CHC). Moreover, the chaperone Hsp70, which has been involved in the uncoating of clathrin coated vesicles (CCVs) [45,46], was also found in this complex, from which it dissociates in the presence of ATP [28]. This multiprotein complex also contains clathrin light chain (CLC), thus ruling out the initially considered possibility that the CHC-HERC1 interaction might compete with CHC-CLC binding (the RLD2-binding site in CHC was mapped very close to the CLC-binding site [28]) (FRG and JLR, unpublished data). Aside from the observed functions for HERC1's RLDs, several data are also available concerning the HECT domain. In particular, the HECT of HERC1 has been shown to conjugate ubiquitin through its active site cysteine in a reaction that requires the presence of the E2 UbcH5, but that does not take place with other E2s such as UbcH1, UbcH6 or UbcH7, thus indicating that HERC1 is probably a functional E3 ubiquitin ligase that specifically transfers ubiquitin from UbcH5 on to the substrates [9]. Unfortunately, none of the proteins found to interact with HERC1 have been shown to undergo HERC1-mediated ubiquitination. Apart from the ones already mentioned, HERC1 has been shown to bind to the glycolytic isoenzyme pyruvate kinase M2 [47]. This protein was originally identified by its ability to associate with the HECT of HERC1 in the yeast two-hybrid system and, even though the interaction was confirmed in vitro and in vivo, no effect of HERC1 on either its enzyme activity or its ubiquitination could be found [47]. Finally, HERC1 has also been found to be recruited to sites of active actin polymerization at the plasma membrane induced upon activation of the GTPase ARF6 [48]. Even though HERC1 can act as a GRF for ARF6 in vitro [24], HERC1 does not appear to be regulating ARF6 activity in HeLa cells, since HERC1 overexpression neither activates nor prevents the activation of this GTPase. Instead, HERC1 appears to be recruited to these membrane protrusions as a consequence of ARF6 activation [48]. Given the ability of ARF6 to activate $PI(4,5)P_2$

synthesis at the plasma membrane [49], it has been postulated that HERC1 might be recruited to ARF6-induced protrusions as a result of the ability of its RLD1 to interact with this phosphoinositide. In agreement with this, HERC1, $PI(4,5)P_2$ and the $PI(4,5)P_2$-synthesizing enzyme, PI(4)P-5-kinase, all colocalize in these structures. Although the role of HERC1 in ARF6-induced, actin-rich protrusions has not yet been addressed, it might have to do with the active macropinocytosis occurring at these locations [48].

4.2. HERC2

Although HERC1 was the first HERC protein to be identified, the history of HERC2 can be traced back to much earlier times. Indeed, the discovery of HERC2 was the result of complementation studies carried out with mutant alleles of the pink-eyed dilution (p) locus in mouse chromosome 7C. The first mutants to be reported from this locus displayed varying degrees of coat and eye hypopigmentation but were otherwise fully viable and fertile. However, already in 1960 [50] some mutants were described which, besides the typical hypopigmentation phenotype, presented a much graver set of abnormalities including reduced viability, smaller size, a jerky gait and sterility (a phenotype that would later become known as the *rjs* syndrome: runty, jerky, sterile). Even though initial reports found it difficult to believe that such pleiotropic effects could be due to the dysfunction of a single gene [50], later studies eventually proved this to be the case. In particular, the first clear indication of the monogenic origin of the *rjs* phenotype came from complementation analyses of a number of recessive p-locus alleles in which it was established that all alleles leading to the *rjs* syndrome when in homozygosis fail to complement each other when in heterozygosis while they are fully complemented by those alleles involved in the milder, exclusively pigmentation-related phenotype [51]. However, definitive proof of the existence of a single gene underlying

the *rjs* phenotype still had to wait until a chemical mutagenesis protocol involving the use of N-ethyl-N-nitrosourea allowed Rinchik et al. to generate single base-pair mutants of this locus boasting a full-fledged *rjs* syndrome [52]. Once it had been demonstrated that a single gene accounted for all *rjs*-related symptoms, it was only a matter of time till the *rjs* gene was finally cloned. This was achieved in 1998 by Lehman et al. [53], who showed that three of the previously studied *rjs* mutants contained deletions in a gene encoding a giant 528 kDa protein, later to be renamed as HERC2. Of the three deletions described, two were deletions of large chunks of DNA which gave rise to severely truncated HERC2 mRNAs and proteins whereas the third one was an intragenic deletion yielding a protein lacking 321 internal amino acid residues. While the first two deletions were too large to rule out the possibility of other genes downstream of HERC2 also being deleted, the third deletion was confined within HERC2 and thus strongly suggested that alterations in HERC2 underlie the *rjs* disorder. Nevertheless, the possibility still remained of *rjs* being due to deletion of another gene located inside an intron of the HERC2 gene. Therefore, ultimate proof of HERC2 mutations being the causal agent of the *rjs* syndrome still had to wait one more year until it was shown that some of the previously reported single base-pair mutants leading to *rjs* were in fact splice junction mutations exclusively affecting HERC2 [54]. In this same paper, Ji et al. also cloned the human orthologue of mouse HERC2 and showed that recent (i.e. during the last 20 million years) duplication and translocation events involving fragments of the HERC2 gene have given rise to the transcribed low-copy repeats, also called duplicons, which are found at the deletion breakpoint hotspots in human chromosome bands 15q11 and 15q13 involved in most cases of Prader-Willi syndrome (PWS). The latter is a so-called genomic imprinting disorder caused by lack of expression of a group of genes located within a 2 Mb stretch of DNA in the region

between 15q11 and 15q13. In normal individuals, these genes are expressed only from paternal chromosome 15 since the maternal counterparts become silenced during oogenesis or early embryonic development in a process known as genomic imprinting. In patients with PWS, though, paternal expression of these genes also fails to take place owing, in 70% of cases, to a 4 Mb deletion occurring during spermatogenesis. More precisely, this deletion appears as a result of a mistake in homologous recombination during meiotic prophase I and HERC2 duplicons play a crucial role in increasing the likelihood of this mistake happening. However, in spite of HERC2 having been found altered in some PWS patients and the symptoms of PWS resembling those of murine *rjs* to some extent, it appears that HERC2 mutations do not underlie PWS, since the HERC2 gene does not undergo imprinting and therefore PWS patients express their maternal HERC2 allele, which, given the recessive nature of HERC2 mutations in mice, should be sufficient to avoid complications (for a review on PWS and the role of HERC2 duplicons in its etiopathogenesis see [55]). In any case, HERC2 duplicons, despite being transcribed, should be regarded as pseudogenes given their high mutation rates (equivalent to those of introns), the presence of premature stop codons in all reading frames and the fact that they only constitute parts of the only functionally relevant, ancestral HERC2 gene found in 15q13 (the region equivalent to mouse 7C by conservation of synteny) [56]. Concerning the HERC2 protein, it is, as mentioned above, a giant, highly conserved, 528 kDa protein containing a number of conserved regions including the HECT, three RLDs, a DOC domain, an M-H domain, a cytochrome b5-like region and a ZZ-type zinc finger. The DOC domain was initially described as a roughly 200 aa region homologous to the APC10/DOC1 subunit of the APC ubiquitin ligase complex involved in progression through mitosis [57]. Recently, APC10/DOC1 has been shown to adopt a β-sandwich fold and to significantly enhance

the affinity of the APC complex for its substrates, thereby stimulating their polyubiquitination [58-61]. Since all proteins with DOC domains have also been found to possess either HECT or cullin domains, both involved in protein ubiquitination, it seems reasonable that the function described for APC10/DOC1 could be conserved in the other DOC-domain proteins. If so, it might then be expected that the DOC domain in HERC2 stabilized the association between HERC2 and its substrates. Another domain which might be involved in HERC2 activity as an E3 is the M-H domain (Mib-Herc2), which is also found in a zebrafish RING-finger-containing ubiquitin ligase called Mind Bomb (Mib) [62]. Regarding the cytochrome b5-like structural motif, the absence of the two histidine residues involved in heme coordination in cytochrome b5 makes it extremely unlikely that HERC2 may also bind a heme group. Nonetheless, as has been shown for membrane-associated progesterone receptors (MAPRs), it might well be that the original heme-binding cytochrome b5 domain has turned in HERC2 into a hydrophobic binding pocket for some non-heme ligand [63]. On the other hand, HERC2 also contains a ZZ-type putative zinc finger motif with six conserved cysteines and two outlying histidines that may allow it to bind Zn^{2+} ions. Even though no studies have yet been performed with the HERC2 protein, some interesting data are available from the analysis of its mRNA as well as the effects of its mutations in mice. Murine HERC2 mRNA is expressed ubiquitously but at significantly higher levels in brain and testis [53]. While most studied mutations have been shown to give rise to drastically truncated polypeptides, two of them are of special interest since they yield proteins that lack only a limited number of internal residues. The fact that one of these *rjs* mutants lacks only part of the HECT (aa 4428-4748) clearly attests to this domain's importance in HERC2 function and suggests that the *rjs* phenotype arises as a result of defective ubiquitination of one or more HERC2 substrates. By contrast, the second *rjs* mutant has

an intact HECT but lacks a stretch of 53 aa (3716-3768) located right after the RLD2. Therefore, it seems likely that these 53 aa are required for the binding of HERC2 substrates (curiously, this mutation, in contrast to all other studied, also leads to increased HERC2 mRNA levels, although the significance of this is currently unknown [64]). Finally, the physiological events for which HERC2-mediated ubiquitination might be important can be vaguely outlined on the basis of *rjs* symptoms. Thus, reduced growth and genital hypoplasia in *rjs* mice might be due to problems in hormonal secretion in the pituitary, which in turn might be due to the dysfunction of hypothalamic neurosecretory neurons whose axons have been found degenerated in a HERC2 mutant [65]. Likewise, other neurons might account for the jerky gait and maternal behavior defects also seen in *rjs* mice. On the other hand, it has been shown that defects in spermatogenesis are intrinsic to the germ line [66] and arise as a result of the formation of an abnormal acrosome, a secretory organelle derived from the Golgi complex. Therefore, all data are consistent with a role for HERC2 in the secretory trafficking pathways of mainly neurons and sperm cell precursors.

4.3. HERC3

The cDNA of human HERC3, by then still referred to as D25215, was originally identified in 1994 in a random search for cDNAs larger than 2 kb [67]. However, it was not until seven years later that the protein encoded by this cDNA was described for the first time [68]. HERC3 is a 117 kDa protein that is located in the cytosol and in cytoplasmic vesicular-like structures in all tested cell lines, where it colocalizes with markers of intracellular membrane trafficking pathways such as β-COP, Rab5 and ARF, but not with lysosomal (LIMP-II) or Golgi (GMPt1) markers [68]. Even though it cannot be ruled out that the RLD domain of HERC3 has dissociation activity for some

as yet unidentified small GTPase/s, such activity was not detected for any of nine of these proteins, namely Ran, Rab3a, Rab5, Rab8, ARF1, ARF6, Ras, Rac and RhoA [67]. On the other hand, the HECT domain of HERC3 has been shown to be fully functional, insofar as it can form a thioester bond with ubiquitin, as long as the latter is supplied by an appropriate E2 (in vitro, both UbcH7 and UbcH5 have been shown to act as ubiquitin donors for HERC3, although the former does it more efficiently) [9]. As a matter of fact, HERC3 has been shown to bind ubiquitin, albeit with a reduced affinity, even when its thioester-forming active site cysteine (C1018) has been mutated to alanine [68]. This C1018-independent binding may be due to the non-covalent association of ubiquitin to either the HECT's active site or, alternatively, to a different ubiquitin-binding site elsewhere in the HERC3 molecule. Anyhow, there still is another way in which HERC3 binds to ubiquitin. Indeed, not only does HERC3 appear to be a ubiquitination enzyme for other so far undiscovered proteins, but it also undergoes ubiquitination itself in one or more of its lysine residues, as shown by in vitro ubiquitination assays performed in the rabbit reticulocyte lysate system [68]. In other words, HERC3 appears to be both a ubiquitin ligase and a ubiquitination substrate. This raises the possibility of HERC3 ubiquitinating itself. However, this seems unlikely, since HERC3 ubiquitination is not at all affected by the C1018A mutation. In any case, it has been established that HERC3 undergoes polyubiquitination and that it is degraded in the proteasome [68]. Regarding the expression of HERC3 mRNA, a recent report shows that, in mouse, it is expressed throughout the brain, with especially high levels in the piriform cortex, the hippocampus and the amygdala [69]. HERC3 expression in other tissues has also been found [9,69]. In summary, then, HERC3 is probably a ubiquitin ligase involved in membrane traffic whose own levels seem to be regulated by ubiquitin-dependent proteasomal degradation.

4.4. HERC4

Similar to what has just been said for HERC3, the HERC4 cDNA was originally identified during a search for new human brain cDNAs encoding large proteins [70]. However, the initially reported sequence [70] was later found to be incomplete and publication of the full-length HERC4 cDNA sequence still had to wait until a very recent paper [71]. This last article also reports all currently available knowledge on the HERC4 protein [71]. The mRNA of HERC4 has been found in all examined tissues, with its levels being significantly higher in brain and testis than in placenta and heart. Analogous to what has been seen for other HERCs, the subcellular localization of the overexpressed HERC4 protein appears in immunocytochemical studies as a cytoplasmic, punctuate staining indicating its association with membranous structures. A shocking aspect of the work on HERC4 is undoubtedly the complexity in the processing of its pre-mRNA. So, the HERC4 gene contains 29 potential exons, out of which only 25 or 26 usually end up in the mature mRNA (4.45 kb), which thus gives rise to two proteins of 1049 and 1057 aa which constitute the major forms of HERC4 in the cell. However, albeit with a lower frequency, the pre-mRNA can be spliced in different ways from the ones already mentioned. These alternative splicings may include events such as the exclusion of the first five exons and substitution of them by another one containing an alternative translation initiation codon (which renders a protein lacking part of its RLD), the exclusion of exons 24 and 25 (thus creating a protein with a deletion in its HECT), the inclusion after exons 3 or 11 of an additional exon containing a Stop codon (which generates severely truncated proteins) or the exclusion of exons 5 and 9, which alters the reading frame and gives rise to a small

protein of only 118 aa. The physiological relevance of all these splicings is currently unknown, as is HERC4 protein function.

4.5. HERC5

HERC5, initially described as Ceb1 (Cyclin E-binding protein-1) [72] and later also designated HERC4 [47,48,68], is a 117 kDa protein whose mRNA is highly expressed in testis and fetal brain and is found at much lower levels in other organs such as the ovaries, pancreas, heart, placenta and skeletal muscle. The HERC5 protein was originally identified in a yeast two-hybrid screen using cyclin E as bait [72]. More recently, HERC5 was also found during a search for genes upregulated after pro-inflammatory cytokine treatment of human skin microvascular endothelial cells (HSMECs) [73]. Both papers provide very valuable insights into the function of HERC5, which will now be reviewed in some detail. Aside from the above-stated tissular distribution, HERC5 subcellular localization is analogous to what has already been shown for the other small HERCs, i.e. cytoplasmic with both a soluble and an inner membrane-bound component [72]. Interestingly, HERC5 gene expression has been shown to be the object of a fine regulation. Indeed, even though HERC5 levels are normally very low in most tissues, its expression increases considerably when cells are subject to certain treatments, such as expression of viral oncoproteins that inactivate the tumor suppressors p53 and Rb [72]. Similarly, HERC5 mRNA levels also rise in response to pro-inflammatory stimuli [73]. In particular, a clear increase in the amount of HERC5 mRNA is seen 8 hours after treatment of HSMECs with either LPS, TNF-α or IL-1β. This upregulation fails to occur if either NFκB activation or protein synthesis are blocked, thus probably indicating that HERC5 gene expression depends on some transcription factor whose own expression is in turn regulated by NFκB. Moreover, this

interpretation is in agreement with the observation that the proximal region of HERC5 gene promoter does not contain any consensus NFκB-binding site but it instead harbors sites for other inflammation-related transcription factors [73]. Nevertheless, the changes observed in HERC5 mRNA after pro-inflammatory cytokine treatment of HSMECs are not accompanied by a concomitant increase in HERC5 protein levels. This apparent paradox can be explained on the basis of an independent effect of LPS on HERC5 protein's half-life. This effect is much faster than the one affecting the mRNA and entails a very swift degradation of the protein, whose half-life descends from 9 hours in the absence of LPS to a mere 2 hours in its presence. As a consequence of this dual effect, HERC5 protein levels rapidly fall in response to LPS and are not restored until about 12 hours later, thanks to the rise in the amount of mRNA [73]. From all the above data, a broad picture of HERC5 protein function starts to emerge. First of all, its high expression in testis suggests that HERC5 may be playing a special role at this location. In this regard, HERC5, by virtue of its E3 ubiquitin ligase activity, may participate in the massive protein ubiquitination and destruction taking place during spermatogenesis [74,75]. On the other hand, the fine regulation of HERC5 during inflammation also points to this protein having an important say in this process. As it has been seen, HERC5 disappears during the early phase of inflammation, which might lead to temporal substrate stabilization, only to reappear several hours later, presumably in order to contribute to the end phases of inflammation: this would be in accordance with the proved importance of ubiquitin-mediated protein degradation during the resolution of inflammation [76]. Finally, the regulation of HERC5 by p53 and Rb, together with its interaction with cyclin E and other cyclins [72], makes it appealing to think of a possible role for HERC5 in cell cycle progression. However, nobody has yet shown any changes in the levels or activity of HERC5 during the cell cycle. Therefore, there is

plenty of work ahead before the functions of this interesting protein are eventually unveiled.

4.6. HERC6

The HERC6 gene, which was identified due to its high similarity to HERC5 (~50% nucleotide sequence identity in the HECT domain region) [71], has been preliminarily characterized, together with HERC4, in a very recent paper [71]. Analogous to what has already been said for HERC4, the most bewildering aspect of HERC6 studies concerns the multiple splicing products of its pre-mRNA. So, the initial HERC6 transcript contains 25 potential exons, of which normally 23, all but numbers 9 and 17, end up in the mature messenger molecule (3.89 kb). This mRNA encodes the most common form of HERC6 protein, containing 1022 aa. Less frequently, though, the primary HERC6 transcript undergoes alternative splicings, leading to at least three other mRNAs, where either exons 9, 16 and 17, exons 9, 10 and 14 or exons 7, 9, 10, 14 and 17 are missing. As a result, the proteins encoded by these mRNAs have internal deletions (36 non-conserved amino acid residues are deleted due to absence of exon 16) or are truncated (connection between exons 8 and 11 causes frameshift, leading to proteins of only 322 and 364 aa). As with HERC4, it is not known whether these minor forms of HERC6 have any functional relevance, although it is tempting to speculate that the truncated proteins containing only the RLD domain may act as dominant negative regulators of HERC6-mediated ubiquitination, since they would be expected to bind to substrates without ubiquitinating them. Finally, expression analyses show that HERC6 mRNA levels are higher in brain and testis than in placenta and heart [71], while subcellular distribution studies with the overexpressed HERC6 protein show that, like other family members, it is located in cytoplasmic, vesicular-like structures [71].

5. Evolution of HERC genes

The impressive development experienced by the genomics field in the course of the last decade is providing researchers with an increasingly profound comprehension of the ways in which the genomes of living creatures have evolved during the history of life on Earth. So, a recent article has used the available genomic data on HERC genes in order to create a broad picture of their evolution [71]. This study has some important implications which will now be discussed. First of all, the elaboration of a phylogenetic tree with the sequences of all published HERC genes from different species firmly establishes the existence of the two aforementioned HERC subfamilies, i.e. the large and the small HERCs [71]. Interestingly, the *C.elegans* HERC4 orthologue emerges directly out of the tree's basal line [71]. This observation, together with the fact that HERC4 is the only HERC protein to be found in the nematode genome, has led to the suggestion that HERC4 might represent the most ancient family member, from which all others are derived [71]. Nevertheless, a more detailed analysis of the available data on animal as well as HERC gene evolution suggests another possibility. In particular, given the fact that both chordates and arthropods possess at least one member of each HERC subfamily (e.g. HERC4 and HERC2 in *Drosophila* and all 6 members in humans), this indicates that the last common ancestor of both phyla (i.e. the so-called Urbilateria [77]) already must have had one representative of each of these two subfamilies. In view of this, the absence of large HERC genes in nematodes (the last common ancestor of nematodes and arthropods was a protostome who lived much later than Urbilateria) should be interpreted as the result of secondary gene loss in the direct ancestors of nematodes, rather than as the small HERC genes having arisen first in evolution (Figure 4 and [78]). If this turns out to be true, then both HERC subfamilies

would have already appeared by the time the first bilateral animals existed. Whether the small HERC genes gave rise to the large ones (or vice versa) or both arose independently of one another cannot be figured out at present. On the other hand, the fact that HERC genes are missing from the genomes of other eukaryotes such as fungi or plants, which, however, do possess RLD and HECT domains in separate proteins, suggests that the first HERC gene may have appeared as a result of a gene fusion event very early in animal evolution. Concerning the more modern family members, the first to appear were HERC1 and HERC3, which may have arisen from HERC2 and HERC4 as a consequence of whole genome duplication events known to have occurred early in vertebrate evolution [71,79]. The next member to appear was HERC6, owing to the duplication of the HERC3 gene at some time during tetrapod evolution [71]. Finally, HERC5 was the last family acquisition, existing in primates but not in rodents. Like HERC6, HERC5 must have appeared after a gene duplication event, probably from HERC6, to which it is most closely related (HERC3, HERC5 and HERC6 are all located within a 330 kb cluster in human chromosome 4) [71]. As the genomes from other taxonomic groups within the animal kingdom are made public, it will be possible to learn more details about the key steps in the evolutionary history of this gene family.

6. Final remarks

Almost ten years have elapsed since the initial description of HERC1 in 1996 and more than forty since the obtention of the first HERC2 mutants in 1960. During these years the cloning and initial characterization of all existing human HERCs has been achieved. In addition to fascinating insights into the evolutionary history of this gene family, these last years have witnessed an accumulation of evidence suggesting that these proteins may be active ubiquitin ligases (HERC1, HERC3 and HERC5 have been shown to form

thioester bonds with ubiquitin [9,73], while a mouse HERC2 mutant lacking only the HECT domain has been shown to display a full-fledged *rjs* syndrome [53]). Nevertheless, since no ubiquitination substrates have yet been reported for any of these proteins, the possibility that they may have other functions, which may or may not be related to ubiquitin-dependent processes, should not be ruled out. On the other hand, evidence has been gathered which suggests that, at least some family members, may have important roles in intracellular membrane trafficking (the evidence for this is especially compelling for HERC1 [23,24,28], although it is also available for HERC2 [53] and HERC3 [68], let alone the fact that all members appear to localize in cytoplasmic vesicle-like structures). Also of potential interest is the observation that most HERC proteins, or at least their mRNAs, appear to be highly expressed in brain and/or testis, where they could have specialized functions (this has been proven for HERC2, which is essential for mouse spermatogenesis [53,54,66], but it may also be the case for other members [23,69,71,72]). In any case, there is still a very long way ahead until the workings of these proteins can be figured out in detail, so many new discoveries on the HERC proteins should be expected in the forthcoming years.

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Figure legends

Table I. The human HERC family. Gene information was obtained from [56,71,80,81] and from Genbank (www.ncbi.nlm.nih.gov/Genbank/) and the Ensembl Genome Browser (www.ensembl.org). Pairwise protein alignments were done using the Align program at www.ebi.ac.uk/emboss/align, whereas protein molecular weights were calculated using the Protein Parameters tool at www.expasy.ch. It should be noted that there is a discrepancy between the HERC3 chromosomal localization reported by different sources. So, according to the Ensembl Genome Browser the human HERC3 gene is located downstream of HERC6 and HERC5 in 4q22.1, whereas reference [81] and Genbank place it at 4q21 (n.a. = not applicable; HERC5 does not exist in mouse).

Figure 1. HECT domain structure. The 2.6Å-resolution structure of the HECT domain of E6-associated protein (E6AP) bound to the E2 ubiquitin-conjugating enzyme UbcH7 is shown [11]. The HECT domain (residues 495-852 of human E6AP, shown in pink) consists of a large, mostly α-helical, N-terminal lobe (N-lobe, residues 495-737), which is connected by a three-residue hinge (residues 738-740) to the smaller C-lobe (residues 741-852, top left), which displays an α/β structure and contains the active site cysteine that forms the thioester bond with ubiquitin (Cys-820). Regarding UbcH7, it also has a mixed α/β structure and binds to the N-lobe of the HECT, in a position that allows its catalytic cysteine residue (Cys-86) to directly face the HECT's Cys-820, which is 41Å away in an open line of sight, as shown in the figure (both catalytic cysteines have been depicted in yellow). All data were obtained from [11]. The figure was generated using

the program Cn3D (www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). The protein data bank (PDB) entry for the E6AP-UbcH7 structure is 1D5F .

Figure 2. Human RCC1 structure. The 1.7Å-resolution structure of the Regulator of Chromosome Condensation-1 (RCC1) is shown [13]. (A) Frontal view of the β-propeller that allows visualization of its seven blades. (B) Lateral view of the β-propeller showing the β-wedge and the N- and C-termini of the protein. The side of RCC1 containing the N- and C-termini is the one interacting with chromatin, while the opposite side interacts and catalyzes nucleotide exchange on Ran. The β-wedge is a crucial player in the nucleotide exchange mechanism due to its ability to wedge itself between residues of the switch II (a loop in Ran that undergoes major conformational changes upon activation of this GTPase) and the P-loop of Ran (i.e. the loop in Ran which binds to the β-phosphate group of GDP·Mg^{2+}). This is thought to lead to Mg^{2+} release from Ran, which in turn decreases Ran's affinity for GDP, thus prompting nucleotide release [14]. After this, exchange is believed to be completed by the spontaneous binding of a GTP molecule (much more abundant in cells than GDP) to Ran's empty active site, which in turn triggers dissociation of RCC1 from Ran·GTP. [82]. The figure was generated using the Cn3D program (see Figure 1). The PDB entry for the human RCC1 structure is 1A12.

Figure 3. The human HERC family of proteins. The HERC proteins can be divided into two subfamilies, the small and the large HERCs. Whereas the former possess little more than the RLD and HECT domains, the latter are giant proteins with more than one RLD, a HECT and several other conserved regions. Protein domains were identified using the InterProScan program at www.ebi.ac.uk/InterProScan/. (RLD: RCC1-like domain [13],

HECT: homologous to E6AP C-terminus [8,11], SPRY: spl A and RyR [44], WD40: G protein β subunit-like repeats [40], Cyt b5: cytochrome b5-like domain [63], M-H: Mind bomb-Herc2 domain [62], ZZ: ZZ-type zinc finger [83], DOC: DOC1/APC10 domain [57]).

Figure 4. HERC family evolution. The fact that both arthropods and chordates possess small and large HERC genes suggests that the last common ancestor of all bilateral animals must already have possessed such genes. In turn, this indicates that the absence of large HERCs in nematodes must be due to a more recent gene loss event (arrow) having taken place during the evolution of nematodes or their immediate ancestors (HERC2 orthologues have been found in at least four arthropod species, namely *Drosophila melanogaster* (GenBank accession number NP_608388), *Drosophila pseudoobscura* (EAL32685), *Anopheles gambiae* (EAA00368) and *Apis mellifera* (XP_395007). Instead, only HERC4 orthologues have been found in nematodes: *Caenorhabditis elegans* (NP_490834) and *Caenorhabditis briggsae* (CAE63916)). The evolutionary tree, which shows only major taxa, has been adapted from [77]. Distances in the tree bear no relation with actual evolutionary time

Family member	*Chromosomal localization*	*Gene size (kb)*	*Exon number*	*Human-Mouse identity (%)*	*Protein size (kDa)*
HERC1	15q22	225	78	96	532
HERC2	15q13	211	93	95	528
HERC3	4q21-22	116	26	92	117
HERC4	10q21	153	29	91	118
HERC5	4q22	49	23	n.a.	117
HERC6	4q22	64	25	66	115

Table I

Garcia-Gonzalo and Rosa (2005)

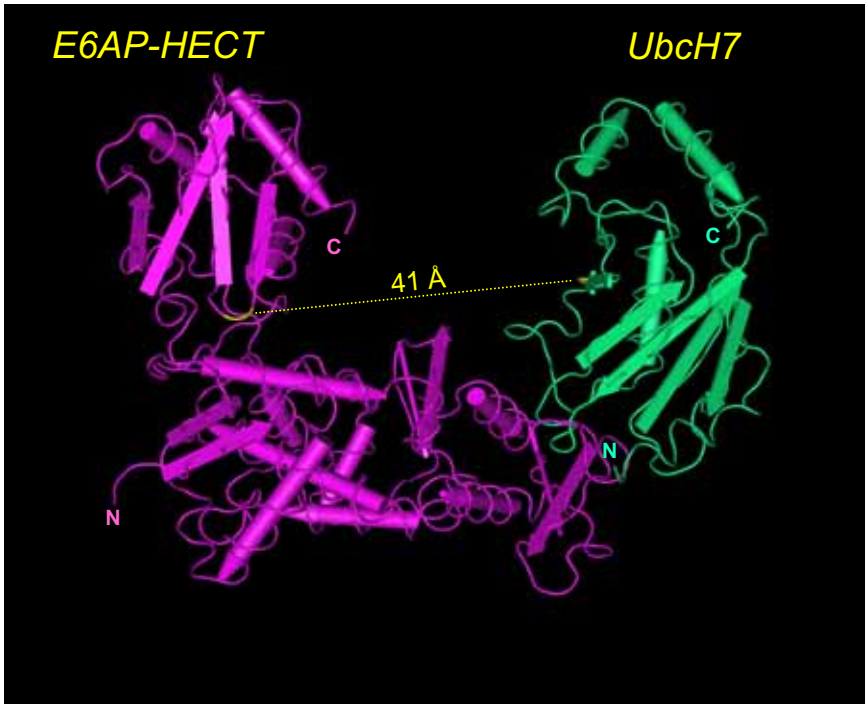

Figure 1

Garcia-Gonzalo and Rosa (2005)

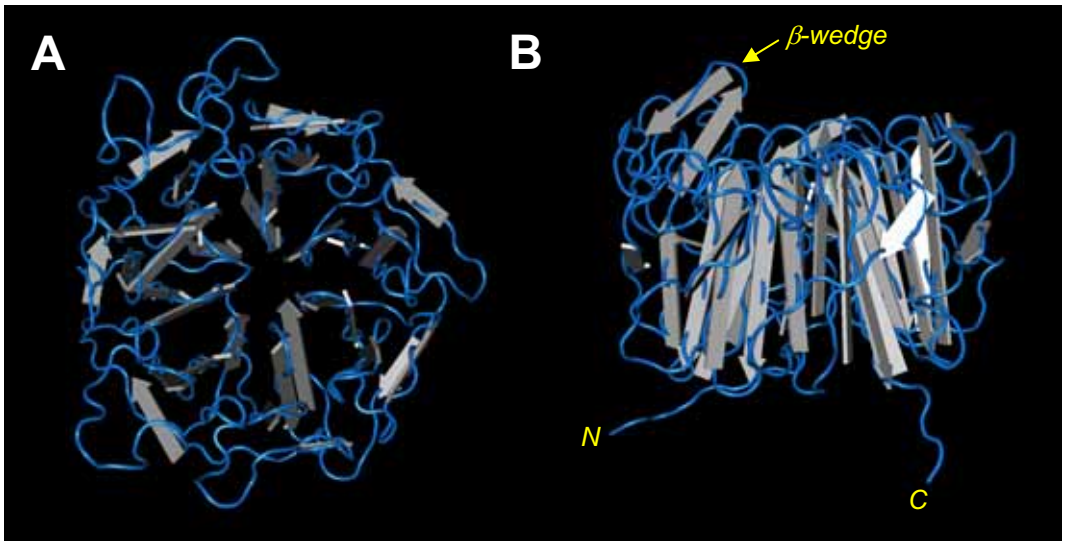

Figure 2

Garcia-Gonzalo and Rosa (2005)

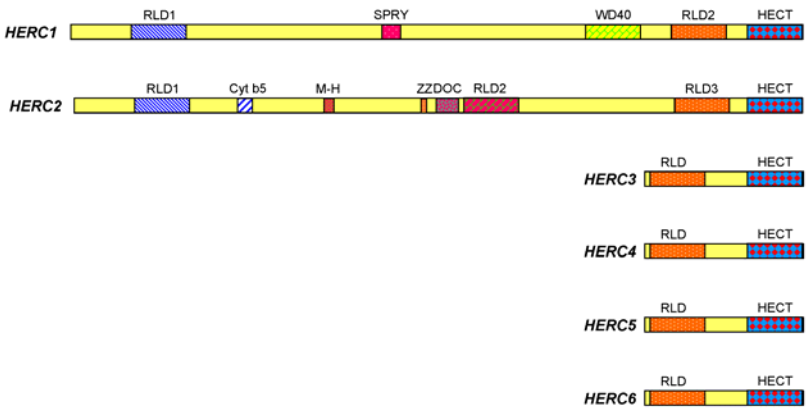

Figure 3

Garcia-Gonzalo and Rosa (2005)

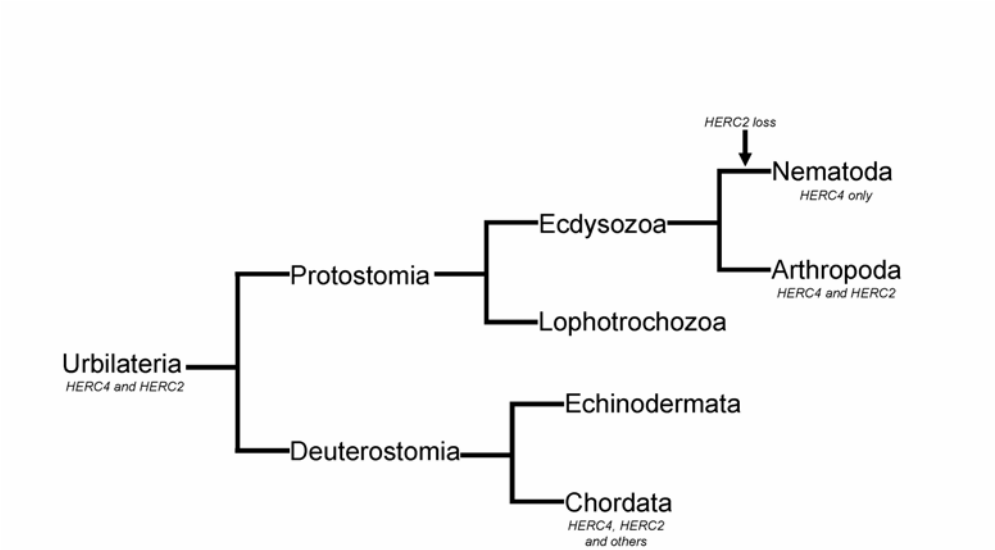

Figure 4

Garcia-Gonzalo and Rosa (2005)