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

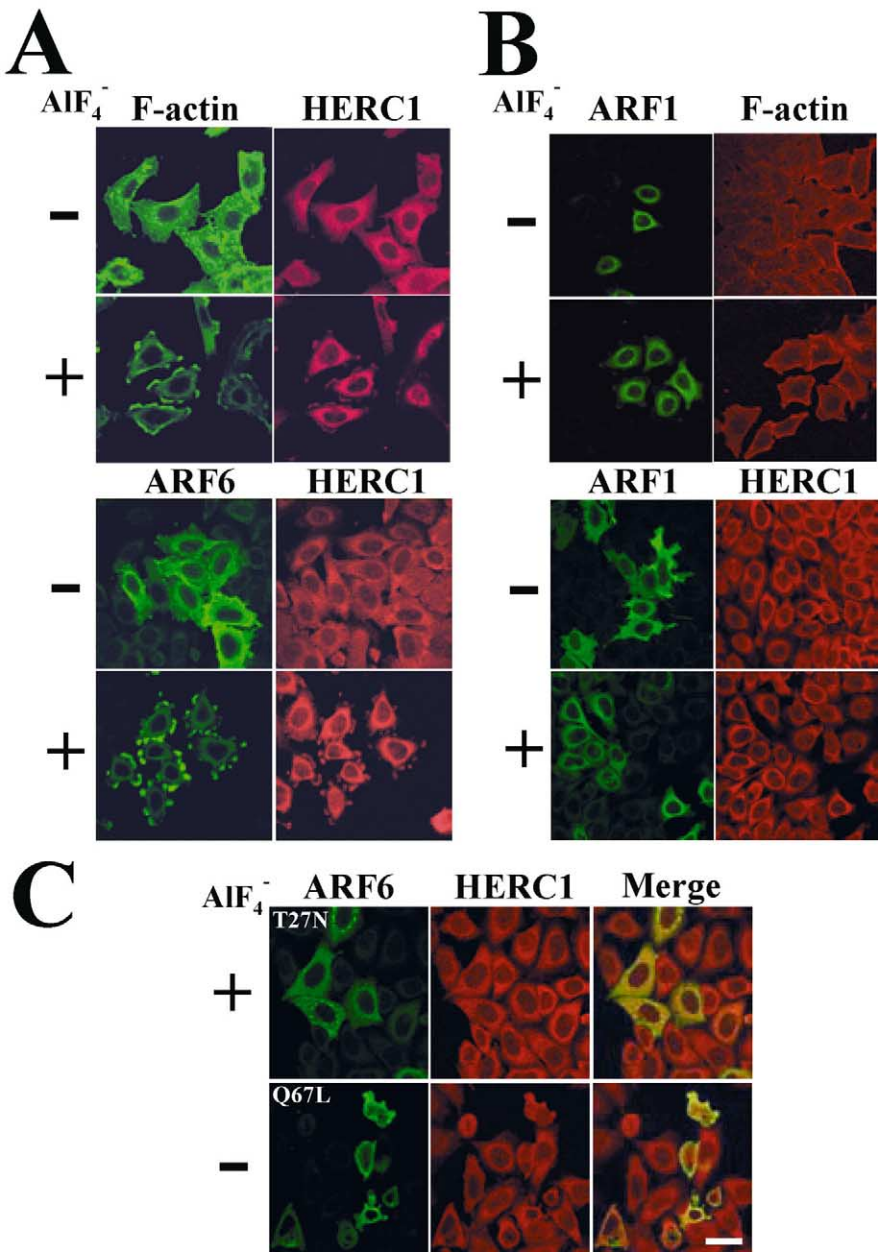

Fig. 1. HERC1 recruitment onto actin protrusions. HeLa cells were transfected with a plasmid encoding ARF6-HA, treated with AlF_4^-, fixed and processed for immunocytochemistry. A: AlF_4^- treatment resulted in the formation of membrane protrusions in which F-actin, detected using phalloidin-FITC, exogenous 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

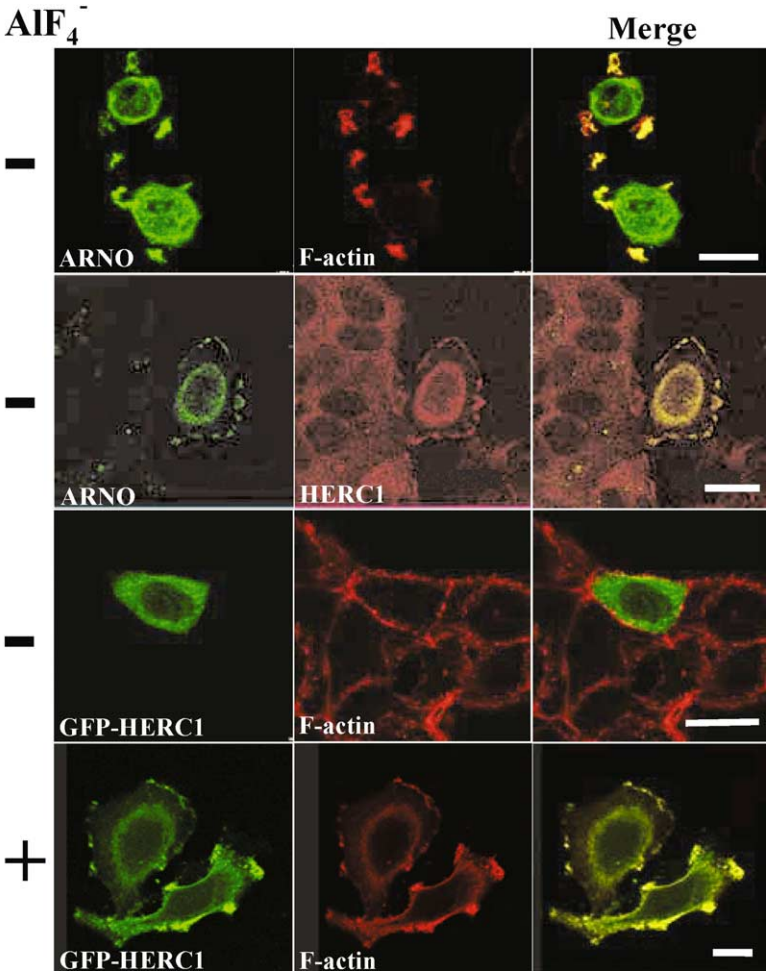

Fig. 2. HERC1 recruitment takes place downstream of ARF6 activation. HeLa cells were cotransfected with wild type ARF6-HA and either ARNO-Myc (positive control, top panels) or GFP-HERC1 (bottom panels). Cells were then treated, when appropriate, with AlF_4^-, fixed and processed for immunocytochemical 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

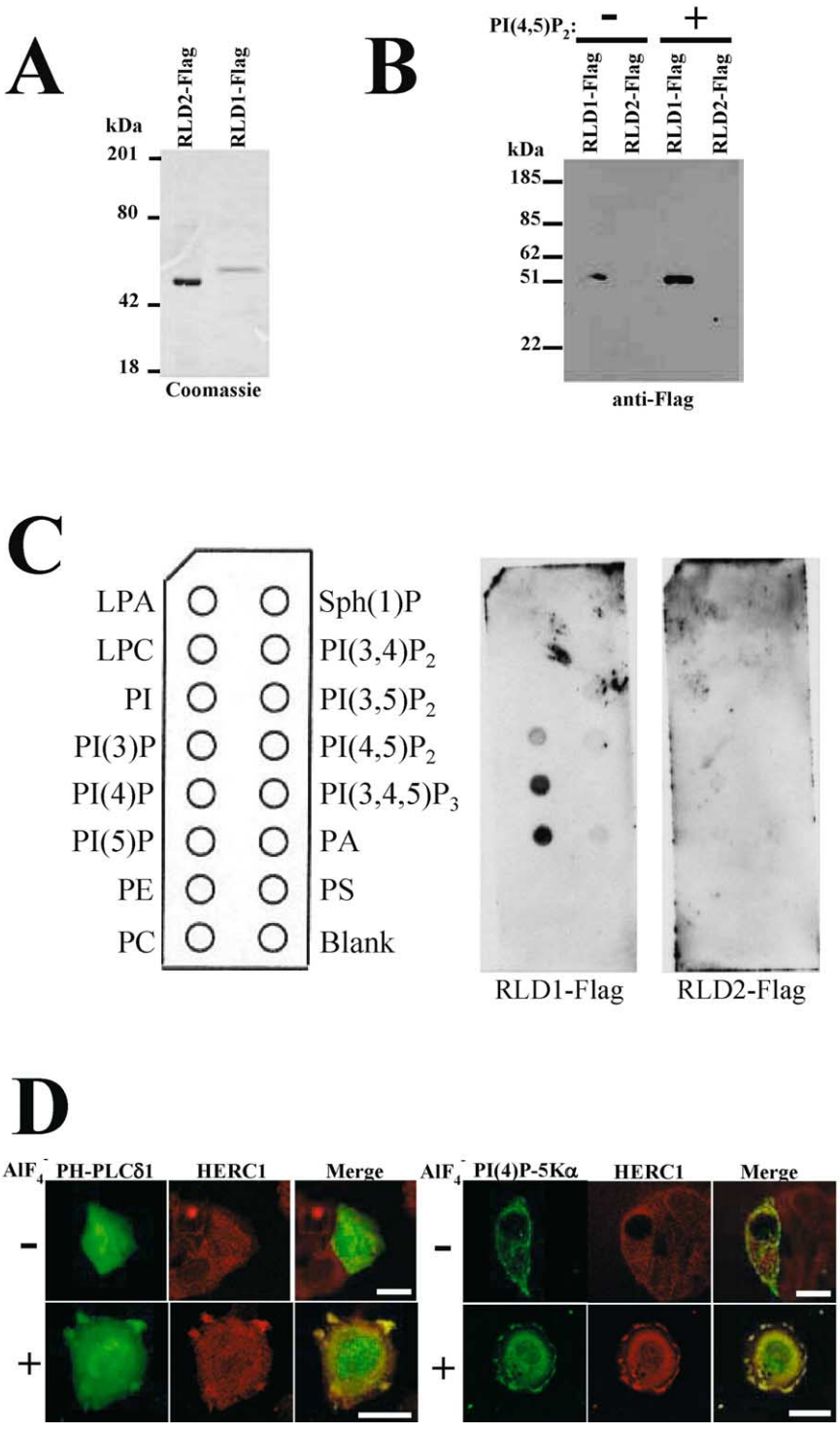

Fig. 3. HERC1's RLD1 domain binds to phosphoinositides. A: RLD1-Flag and RLD2-Flag were heterologously expressed in *E. coli* and purified by affinity chromatography. Purified samples were run on an SDS–PAGE gel which was stained with Coomassie blue dye. B: Liposome–protein complex formation assays were 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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