Memo is a cofilin-interacting protein that influences PLCγ1 and cofilin activities, and is essential for maintaining directionality during ErbB2-induced tumor-cell migration

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Summary
Heregulin (HRG) activates ErbB2-ErbB3 heterodimers thereby stimulating many cellular responses, including motility. Memo and PLCγ1 interact with ErbB2 autophosphorylation sites and are essential for HRG-induced chemotaxis. By tracing HRG-stimulated cell migration in Dunn chambers, we found that Memo- or PLCγ1 knockdown (KD) strongly impairs cell directionality. Memo has no obvious enzymatic activity and was discovered via its ability to complex with ErbB2. Using the yeast two-hybrid approach to gain insight into Memo function, an interaction between Memo and cofilin, a regulator of actin dynamics, was uncovered. The interaction was confirmed in vitro using recombinant proteins and in vivo in co-immunoprecipitation experiments where Memo was detected in complexes with cofilin, ErbB2 and PLCγ1. Interestingly, in Memo KD cells, HRG-induced PLCγ1 phosphorylation was decreased, suggesting that Memo regulates PLCγ1 activation. Furthermore, HRG-induced recruitment of GFP-cofilin to lamellipodia is impaired in Memo and in PLCγ1 KD cells, suggesting that both proteins lie upstream of cofilin in models of ErbB2-driven tumor-cell migration. Finally, in vitro F-actin binding and depolymerization assays showed that Memo enhances cofilin depolymerizing and severing activity. In summary, these data indicate that Memo also regulates actin dynamics by interacting with cofilin and enhancing its function.

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Key words: Heregulin, Cofilin, Breast cancer cells, Dunn chamber assay, Transwell assay, F-actin binding and Depolymerization assays

Introduction
Cell migration is important in normal physiology and in disease. Acquisition of migratory ability by cancer cells is a characteristic that contributes to spread of metastatic tumor cells to distant organs (Gupta and Massague, 2006). Proteins with essential roles in metastasis are under intense scrutiny in order to provide additional targets for cancer therapy. The ErbB2 receptor tyrosine kinase plays an important role in many human tumors (Hynes and Lane, 2005). In breast cancer, ErbB2 gene amplification and overexpression is essential for tumor cell migration. ErbB2 is a member of the epidermal growth factor (EGF; also known as ErbB) family, which also includes EGFR, ErbB3 and ErbB4. ErbB receptor signaling is initiated by ligand binding to the extracellular region, causing formation of receptor homo- and heterodimeric complexes, resulting in autophosphorylation at multiple tyrosine residues in the cytoplasmic domain (Schlessinger, 2000; Yarden and Sliwkowski, 2001). ErbB2 has a central role in the family and is activated by heterodimerization with each of the other ErbB receptors (Graus-Porta et al., 1997). Functional inactivation of ErbB2 in T47D human breast cancer cells (Graus-Porta et al., 1995) impairs the ability of the EGF family ligands, EGF, betacellulin and heregulin (HRG), to stimulate cell migration in Transwell assays (Spencer et al., 2000). Furthermore, introduction of Neu (rat ErbB2 homolog) mutants into the T47D cells showed that two Neu tyrosine add-back mutants, at Tyr1201/1196 and Tyr1227/1222 (human/rat) are able to restore the ability of EGF and HRG to stimulate cell migration (Marone et al., 2004). In a screen for ErbB2 effector proteins with roles in migration, we identified PLCγ1 and Memo. These proteins are recruited to the ErbB2 autophosphorylation sites Tyr1201 and Tyr1227, respectively, and both have essential roles in cell motility (Marone et al., 2004).

By contrast to the well-characterized PLCγ1, Memo is a novel protein. The 2.1 Å crystal structure of Memo revealed that it is structurally homologous to a class of non-heme iron dioxygenases that are mainly found in bacteria (Andujar and Santero, 2003); however, we were unable to detect metal binding or enzymatic activity (Qiu et al., 2008). To gain more insight into Memo function, we used the yeast two-hybrid (YTH) approach to identify Memo-interacting proteins. We report here that Memo interacts with cofilin, a member of the conserved, ubiquitously expressed actin-depolymerizing factor (ADF)/cofilin family, proteins that control
ADF/cofilin proteins control actin dynamics, a process that is essential for cell migration. In fact, depletion of cofilin impairs cell motility (Hotulainen et al., 2005). ADF/cofilin proteins bind actin and are involved in the actin-filament assembly and disassembly processes (dos Remedios et al., 2003). In vitro, it has been shown that cofilin stimulates F-actin disassembly by accelerating the rate of depolymerization at the pointed end, and by severing actin filaments (Carlier et al., 1997; DesMarais et al., 2005; Lappalainen and Drubin, 1997; Paavilainen et al., 2004). Although in vivo the predominant cofilin activity appears to be dependent on cell type, cofilin has an essential role in promoting cytoskeletal dynamics by generating the pool of actin monomers needed for lamellipodium extension at the leading edge (Kiuchi et al., 2007). Cofilin is inactivated by LIM-kinase-mediated phosphorylation at Ser3 (Arber et al., 1998; Yang et al., 1998), and is reactivated by Slingshot-1L (SSH-1L)-induced dephosphorylation (Niwa et al., 2002). PLCγ1 is another regulator of cofilin; indeed, PLCγ1-mediated phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)2] hydrolysis releases PtdIns(4,5)2-bound cofilin, thereby increasing the pool of active cofilin available for interaction with F-actin (Ono, 2007; van Rhenen et al., 2007). Local activation of cofilin by PLCγ1 at the leading edge of migrating cells has been shown to be required for directed protrusion (Mouneimne et al., 2006; Mouneimne et al., 2004).

Using a knockdown (KD) strategy in this study, we observed that depletion of Memo or of PLCγ1 resulted in very similar phenotypes, with a strong impairment of HRG-induced cytoskeletal organization and directional migration of breast tumor cells. By contrast, cofilin KD, or simultaneous KD of Memo and PLCγ1, completely blocked cell movement. Furthermore, co-immunoprecipitation experiments revealed that complexes of active ErbB2, PLCγ1, Memo and cofilin are detected in HRG-treated breast tumor cells. We also provide evidence that Memo positively regulates PLCγ1 phosphorylation and show that Memo and PLCγ1 are both involved in HRG-induced cofilin recruitment to the lamellipodia. Finally, by performing in vitro assays with F-actin, we demonstrate that Memo promotes cofilin depolymerizing and severing activity. Considering these results, we propose that Memo and PLCγ1 lie upstream of cofilin in models of ErbB2-driven breast cancer cell migration.

Results

PLCγ1 and Memo are required for ErbB2-mediated cell motility

PLCγ1 and Memo respectively bind to phosphorylated Tyr1201/1196 and phosphorylated Tyr1227/1222 (in human/rat), two ErbB2 phosphorylation sites that we have shown to be important for cell migration (Marone et al., 2004). In the current study, we performed detailed studies on the role of Memo and PLCγ1 in HRG-induced tumor cell migration. As models, we used T47D and SKBr3 breast cancer cells that are representatives of luminal, estrogen receptor-positive (ER+) cancer cells (Badache and Hynes, 2001; Marone et al., 2004) and ErbB2-overexpressing tumor cells (Lane et al., 2000), respectively. We have previously shown via siRNA-mediated Memo knockdown and general PLC inhibition that, in T47D cells, both proteins have important roles in ErbB2-induced cell motility (Marone et al., 2004). In the following experiment, we decreased the expression of Memo and PLCγ1 in SKBr3 cells using specific siRNAs (Fig. 1A, right panel) and examined their motility. The directional (chemotactic) and random (chemokinetic) migration of control and KD cells was examined in Transwell assay chambers. HRG was added to the lower chamber to measure chemotaxis and to both chambers to measure chemokinetics. KD of Memo or PLCγ1 did not generally affect signaling in response to HRG (Marone et al., 2004) (e.g. ERK-P; supplementary material Fig. S1, left panel). However, individual KD of Memo or PLCγ1 or simultaneous KD of both proteins strongly reduced chemotaxis compared with control lacZ cells (Fig. 1A, left panel; supplementary material Fig. S1, right panel). Interestingly, the chemokinetic response of Memo or PLCγ1 KD cells was similar to that of control cells, whereas double KD of PLCγ1 and Memo resulted in a dramatic blockade of random cell motility (Fig. 1A, left panel), suggesting that Memo and PLCγ1 are cooperating during the migratory process.

![Fig. 1. PLCγ1 and Memo are required for ErbB2-mediated cell motility. (A) (Left panel) SKBr3 cells were transfected with lacZ, Memo and/or PLCγ1 siRNAs. Control responses in the absence of ligand, as well as chemotactic responses towards 1nM HRG and chemokinetic responses in the presence of 1 nM HRG, were determined in Transwell assays. (B) (Left panel) Immunofluorescence microscopy of HRG-stimulated T47D cells treated with lacZ, Memo or/and PLCγ1 siRNAs and labeled with Alexa-Fluor-488-phalloidin (green) or an anti-α-tubulin (red) antibody. Yellow arrows indicate the dense array of actin stress fibers. (A and B, right panels) Extracts were prepared and analyzed for Memo and/or PLCγ1 KD; α-tubulin was used as a control. The data are representative of several independent experiments. Scale bar, 10 μm.](https://example.com/figure1.png)
Signal transduction pathways downstream of ErbB2 temporally and spatially regulate cytoskeleton remodeling during cell migration (Feldner and Brandt, 2002). Therefore, we examined the effect of Memo and PLCγ1 KD, or of simultaneous KD of both proteins on the organization of HRG-dependent microtubules and actin. T47D cells were used for this experiment because following their exposure to HRG, they form large actin-rich lamellipodial protrusions with abundant microtubule extensions (lacZ control, Fig. 1B); however, results with SKBr3 cells are essentially the same (Marone et al., 2004) (our unpublished results). Both Memo KD and PLCγ1 KD cells, as well as double KD cells, displayed similar actin-rich protrusions; however, the F-actin stress fibers appeared thicker and more predominant in the KD cells, suggesting a role for Memo and PLCγ1 in actin organization (yellow arrows, Fig. 1B). Furthermore, compared with control cells, the single and double KD cells showed a strong reduction in microtubule extension to the cell periphery, a phenomenon previously reported for Memo KD cells (Marone et al., 2004). Thus, in response to ErbB2 activation, decreased expression of Memo and of PLCγ1 have very similar consequences on the actin and microtubule network, raising the possibility that Memo and PLCγ1 act in concert during cytoskeleton organization.

PLCγ1 and Memo have important roles in directed tumor-cell migration

Decreased expression of Memo or PLCγ1 reduces HRG-induced motility of tumor cells in Transwell assays (Fig. 1A; supplementary material Fig. S1) (Marone et al., 2004). We investigated these results in more detail using Dunn chambers, a system that allows real-time visualization of migratory cells (Wells and Ridley, 2005; Zicha et al., 1991). The data generated in Dunn chambers provide additional information on the behavior of migratory cells compared with data obtained from Transwell assays, in which only the final distribution of cells can be monitored. For these assays, T47D cells were transiently transfected for 72 hours with lacZ, Memo or PLCγ1 siRNA (Fig. 2A, right panel). Tracks of cells migrating on the bridge of Dunn chambers in a chemo-attractant gradient of HRG were traced using time-lapse video microscopy (Fig. 2A, left panels; supplementary material Movies 1-5). Quantitative analyses of the tracks made by cells with Memo KD or PLCγ1 KD revealed that there was a 44% decrease in the net translocation distance (straight distance from the start to the end point) and a 30-35% decrease in their migration speed, compared with control cells (Fig. 2B, left and middle panels). Thus, as observed in the Transwell assays (Fig. 1A), the cells are still able to migrate although less potently.

Fig. 2. Effect of Memo and PLCγ1 KD on HRG-induced chemotaxis in Dunn chambers. T47D cells were transiently transfected with lacZ, Memo or PLCγ1 siRNAs and 3 days later were analyzed for their ability to migrate in a gradient of HRG. (A) Track displacement was followed during a period of 12 hours. The gradient follows the path from the bottom left to the top right. Gray arrows indicate the direction of individual cells. Cell displacement over the time course is indicated with the colored line (zero, intermediate and final time points are in blue, red and yellow, respectively). KD of Memo or PLCγ1 was confirmed by western blot analysis, α-tubulin was loaded as a control. (B) (Left panel) The net translocation distance is shown as the mean ± s.e.m. of the displacement of 61 cells. (Middle panel) The migration speed is shown as the mean ± s.e.m. of the paths of 61 cells. (Right panel) The directional persistency index of control lacZ, Memo or PLCγ1 KD cells is shown. (C) Decagonal histograms indicating the distribution of cells at their final location. The source of HRG is at the top. The data are representative of several independent experiments.
than control cells. Importantly, there was a dramatic difference in their directional persistence. KD of Memo or PLCγ1 in T47D cells led to a 67% and 72% decrease, respectively, in the directional persistence index (Fig. 2B, right panel). The overall directionality of cell migration in control and KD cells is depicted in Fig. 2C. Considering the final location of migrating cells positioned in the 180° arc facing the HRG source, 86.6% of control cells were located there, whereas only 37.5% and 37.4% of Memo KD and PLCγ1 KD cells, respectively, were in this location. In summary, these results show that KD of either Memo or PLCγ1 does not totally impair cell migration; however, the cells move in a more random manner than control cells that move up the HRG gradient. Thus, both Memo and PLCγ1 have important roles in orchestrating directional cell migration.

Complexes of ErbB2, PLCγ1 and Memo form upon HRG treatment of breast tumor cells

Considering the similar role that Memo and PLCγ1 play in HRG-induced cell migration, we explored the Memo-PLCγ1 connection in more detail. In Memo immunoprecipitations (IPs) from T47D cells (Fig. 3A, left panel), and from HEK293 cells (supplementary material Fig. S2, right panel), complexed PLCγ1 could be detected. In the reverse immunoprecipitation, IPs of PLCγ1 revealed complexed Memo (Fig. 3A, right panel; supplementary material Fig. S2, right panel). In each case, there is an increase in the co-immunoprecipitating protein when lysates from HRG-treated cells are used, probably reflecting stabilized complexes interacting with active ErbB2 (supplementary material Fig. S2, middle panel). Indeed, Memo and PLCγ1 were both detected in ErbB2 IPs from SKBr3 cells that have constitutive receptor activation (supplementary material Fig. S2, left panel).

Memo has a role in HRG-induced PLCγ1 activation

Phosphorylation of PLCγ1 on Tyr783 has been linked to its activation (Rhee, 2001). Upon HRG treatment of T47D breast tumor cells, there is a rapid increase in the Tyr-P content of immunoprecipitated PLCγ1 (Fig. 3B, left panel), showing that HRG activates PLCγ1. Interestingly, in Memo KD T47D cells, there was a strong decrease in the Tyr-P content of the immunoprecipitated PLCγ1 (Fig. 3B, middle panel), whereas the level of PLCγ1 remained the same (Fig. 3B, right panel). These results suggest that Memo has an important role in PLCγ1 phosphorylation in response to ErbB2 activation. Loss of Memo might directly or indirectly affect PLCγ1 activity, perhaps as a result of the alterations in the actin cytoskeleton following Memo KD.

Memo interacts directly with cofilin – results from yeast two-hybrid and GST-pull-downs

The molecular mechanisms underlying the role of Memo in cell migration are not well understood. To gain more insight into Memo function, a yeast two-hybrid (YTH) screen was performed (Pedrazzi and Stagliar, 2004). By screening a peripheral blood cDNA library, we identified cofilin-1, a ubiquitously expressed protein of ~19 kDa that binds monomeric and filamentous (F)-actin (Ono, 2007), as a potential interacting partner for Memo. Mammalian cells express multiple ADF/cofilin-type proteins. Cofilin-1 is the dominant isoform expressed in many cell lines (Wang et al., 2004; Hotulainen et al., 2005) and we will refer to cofilin-1 as cofilin throughout the text. The specificity of the YTH Memo-cofilin interaction was confirmed using control plasmids (Fig. 4A). A bait-dependency test also demonstrated a specific interaction between Memo and cofilin in the YTH system (supplementary material Fig. S3A).

To confirm the interaction between Memo and cofilin using purified proteins, Memo was expressed as a GST-fusion protein and its interaction with cofilin was tested in pull-down assays. Glutathione-Sepharose-bound GST-Memo was incubated with recombinant cofilin. Cofilin was recovered from GST-Memo beads but not from empty beads (Fig. 4B, lanes 4 vs. 6; supplementary material Fig. S3, panel B, lanes 1 and 3). As a control, cofilin was incubated with the GST-control immobilized beads (supplementary material Fig. S3, panel B, lane 4), where only a small amount of cofilin binding was observed. The interaction of Memo with cofilin is specific, as pre-incubation of cofilin with an excess of soluble Memo decreased the amount of cofilin interacting with GST-Memo beads, whereas pre-incubation of cofilin with an excess of nonspecific BSA protein did not modify the amount of cofilin interacting with GST-Memo beads (Fig. 4B, lanes 7 to 9).

![Fig. 3. Memo complexes with PLCγ1 and ErbB2 and regulates PLCγ1 phosphorylation in tumor cells. (A) (Left panel) T47D cells were stimulated or not with 10 nM HRG for 5 minutes and cell extracts were subjected to immunoprecipitation using a Memo mAb. IPs were probed for PLCγ1 and Memo; a mouse IgG2a mAb was used as a control. (Right panel) T47D cells were stimulated or not with 10 nM HRG for 5 minutes. Cell extracts were subjected to immunoprecipitation using a PLCγ1 antibody and IPs were probed for PLCγ1 and Memo. Whole-cell lysates (W) were loaded as controls. (B) (Left panel) T47D cells were stimulated or not with 10 nM HRG for 5 minutes. Cell extracts were subjected to immunoprecipitation using a PLCγ1 mAb; IPs were probed for Tyr-P and for PLCγ1. (Middle and right panels) T47D cells were transiently transfected with lacZ or Memo siRNA, and stimulated or not for 5 minutes with 10 nM HRG. Cell extracts were subjected to immunoprecipitation using a PLCγ1 mAb (middle panel). PLCγ1 phosphorylation was analyzed by western blotting using a Tyr-P mAb. Blots were reprobed for PLCγ1. (Right panel) Memo and PLCγ1 levels in transfected cells were monitored in whole-cell lysates (W) using the respective antibodies.](image-url)
Memo and tumor-cell migration

Memo is a novel cofilin-interacting protein. Memo and cofilin interact in mammalian cells and complex with active ErbB2. Considering the importance of ADF/cofilin family members in cell migration (Ono, 2007; Hitchcock-Degregori, 2006), we explored the Memo-cofilin interaction in mammalian cells. Memo IPs from lysates of T47D or MDA-MB435 cells, which express high cofilin levels (Wang et al., 2007) (Fig. 4C, upper and middle panel, respectively), were probed with a cofilin antibody (Song et al., 2006) revealing complexed cofilin. ErbB2 was also detected in Memo IPs from lysates of HRG-treated tumor cells, showing that Memo is recruited to the active receptor. The Memo interaction with cofilin and ErbB2 is specific, as neither protein was detected in IPs carried out with an isotype-matched control (Fig. 4C). The cofilin-specific antibody is not suitable for immunoprecipitation, which precluded an examination of cofilin IPs for Memo and ErbB2.

The activity of cofilin is inhibited by LIMK-mediated phosphorylation on Ser3, a modification that prevents the interaction of cofilin with F-actin (Ono, 2007). We examined the ability of Memo to interact with phosphorylated cofilin (cofilin-P) using a specific antiserum. In Memo IPs from T47D cells, cofilin-P was detected (Fig. 4C, lower panel), suggesting that Memo binds both active and inactive cofilin. Interestingly, compared with control cells where low levels of cofilin (Fig. 4C, upper and middle panel) and cofilin-P (Fig. 4C, lower panel) were found in Memo IPs, higher levels of cofilin and cofilin-P were complexed with Memo in lysates from HRG-treated tumor cells (Fig. 4C). Whether or not this result reflects stabilization of the Memo-cofilin interaction at the activated receptor remains to be explored.

Finally, we examined the kinetics of formation of ErbB2-, PLCγ1-, cofilin- and Memo-containing complexes in response to HRG treatment (Fig. 4D). In lysates made from T47D cells treated for 5 minutes with HRG, there was a strong increase in ErbB2 and cofilin co-immunoprecipitating with Memo, and this complex was maintained throughout the 30-minute time course. PLCγ1 was also...
Cofilin is essential for cell movement in response to HRG in Dunn chambers

Cofilin has been shown to set the direction of tumor cell motility in response to EGF (Ghosh et al., 2004). We used Dunn chambers to examine the effect of cofilin KD on cell motility following ErbB2 activation. Cofilin levels were efficiently decreased in T47D cells (Fig. 5, insert), and tracks of migrating cells in a gradient of HRG were traced. The cofilin KD cells showed a dramatic reduction in their migratory ability. The speed of migration was 16% of that of control cells (Fig. 5, lower panel), resulting in a net translocation distance that was 13% of that achieved by control cells (Fig. 5, upper panel). Double KD of Memo and PLCγ1 in the cells resulted in a stronger block in movement compared with cells with individual KD (Fig. 5 vs Fig. 2). These results are in accordance with those obtained in Transwell assays (Fig. 1A), where double KD of Memo and PLCγ1 had the strongest effect on random migration and chemokinesis. As the cofilin KD cells and the double Memo and PLCγ1 KD cells were essentially stationary, it was not informative to calculate a directional persistence index. These results provide additional evidence that cofilin has an essential role in the motility behavior of cells in response to ErbB2 stimulation. Furthermore, the results suggest that in the initial stages of migration, Memo and PLCγ1 cooperate to stimulate migration and that cofilin is likely mediating these effects.

PLCγ1 and Memo control GFP-cofilin localization in response to HRG

To gain more insight into the effect of ErbB2 on cofilin, we introduced GFP-tagged cofilin into SKBr3 breast tumor cells and examined its cellular distribution in response to HRG. SKBr3 cells were used because T47D cells are not suitable for transient expression of proteins. In the absence of ligand, GFP-cofilin was diffusely distributed in the cytoplasm, whereas following HRG treatment, it was recruited to the actin-rich lamellipodium (Fig. 6A, orange in merged image). These results are similar to those previously reported for cofilin distribution in a variety of cell types following growth factor stimulation (Dawe et al., 2003; Nagata-Ohashi et al., 2004). GFP-tagged Memo also localizes to the lamellipodium in HRG-treated SKBr3 cells (Fig. 6B, orange in merged image).

Next, we monitored the localization of GFP-cofilin in Memo KD and PLCγ1 KD cells. Representative cells are shown in the figure. In contrast to HRG-treated lacZ control cells in which GFP-cofilin localized to the lamellipodia, GFP-cofilin failed to be recruited to the F-actin-rich lamellipodium and remained diffusely distributed in the cytoplasm of ~70% of PLCγ1 KD cells (Fig. 6C, right panel, orange in merged image) and Memo KD cells (Fig. 6C, middle panel, orange in merged image). These results suggest that both PLCγ1 and Memo have a role in cofilin cellular distribution, perhaps reflecting the fact that Memo-depleted or PLCγ1-depleted cells show alterations in the actin cytoskeleton.

We also examined the effect of cofilin KD on the cellular distribution of Memo. Cofilin depletion in SKBr3 cells had a stronger effect on morphology than Memo KD or PLCγ1 KD, with the formation of multipolar lamellipodia. Individual cells (a typical one is shown in Fig. 6D, middle panel) displayed extensions of several protrusions in different directions, a phenotype that has been reported in other models (Nishita et al., 2005; Sidani et al., 2007). Similar results were seen with cofilin KD T47D cells (data not shown). In striking contrast to the effects of Memo or PLCγ1 KD on GFP-cofilin localization, neither cofilin KD nor PLCγ1 KD affected the ability of GFP-Memo to associate with the plasma membrane in response to HRG (Fig. 6D, middle and right panels, orange in merged image). In conclusion, these results suggest that PLCγ1 as well as Memo are upstream of cofilin in response to ErbB2 activation. Cofilin activity is influenced by multiple mechanisms, including PtdIns(4,5)P2 binding (Moon and Drubin, 1995). PLCγ1-mediated hydrolysis of PtdIns(4,5)P2, as reported downstream of EGFR (van Rheenen et al., 2007), contributes to the release of an active pool of cofilin that participates in actin dynamics in stimulated treated cells. Considering that Memo is involved in PLCγ1 activation, the effect of Memo KD on GFP-cofilin localization might be through PLCγ1, something that will be examined in the future.

Furthermore, phosphorylation of cofilin on Ser3 also influences its activity. As a measure of cofilin activity in HRG-treated breast tumor cells, we examined its Ser3-P status with a specific antiserum. The basal level of cofilin-P in T47D and SKBr3 tumor cells is high, and there was no obvious decrease in cofilin-P levels in response to HRG (Fig. 4C, lower panel; supplementary material Fig. S4A). Furthermore, there were no changes in cofilin-P levels in Memo KD or in PLCγ1 KD cells (supplementary material Fig. S4B), suggesting that only a small pool of cofilin might participate in generating the dynamic actin structures observed following ErbB2 activation.

F-actin binding and depolymerization assays

Cofilin is a well-described actin-binding protein that stimulates F-actin turnover to provide a pool of actin monomers needed for cell motility (Bamburg, 1999; Paavilainen et al., 2004). In the final experiments, we examined the effect of Memo on cofilin binding to actin and on in vitro cofilin-induced depolymerization and

Fig. 5. PLCγ1, Memo and cofilin are required for ErbB2-mediated directional migration in Dunn chambers. T47D cells were transiently transfected with either lacZ or cofilin siRNAs, or simultaneously transfected with Memo and PLCγ1 siRNAs. Cell migration was analyzed in a gradient of HRG in Dunn chambers for 4.5 hours. Net translocation distance and migration speed were calculated for 78 cells. Cofilin, Memo and PLCγ1 levels in KD cells were checked by western blotting with the respective antibodies. α-Tubulin levels were monitored as a control. The data are representative of several independent experiments.
severing. Accordingly, actin was polymerized then mixed with Memo or cofilin alone, or in combination. Actin filaments were sedimented by centrifugation and the supernatant and F-actin-containing pellet fractions were recovered. Scanning densitometry of the gels stained with Coomassie brilliant blue (CBB) (Fig. 7A, upper panel) was performed to quantify the amount of protein in each fraction (Fig. 7A, lower panel). Assays with α-actinin and BSA served as positive and negative binding controls, respectively. When incubated with F-actin, most α-actinin was found in the pellet (sample 3), whereas BSA remained in the supernatant (sample 4). As expected, cofilin shifted from 35% in the pellet when alone (sample 5) to 58% in the pellet in the presence of F-actin (sample 6). There was also a shift for Memo from 18% in the pellet when alone (sample 7) to 38% when incubated with F-actin (sample 9), suggesting that Memo might bind F-actin. When Memo and cofilin were both incubated with F-actin (sample 10), there were no obvious differences in cofilin or Memo content in the F-actin pellet (58% and 33%, respectively), showing that Memo does not interfere with cofilin binding.

The ability of a protein to depolymerize and sever F-actin can be assessed by measuring the shift of actin from the pellet to the supernatant fraction. Memo addition to F-actin did not alter the amount of actin in the supernatant (Fig. 7A, sample 9), whereas the addition of cofilin, a known F-actin depolymerizing-severing...
protein resulted in a 1.21-fold increase of actin in the supernatant fraction (Fig. 7A, sample 6). Interestingly, there was a further 1.52-fold increase of actin in the supernatant when cofilin and Memo were added together with F-actin (Fig. 7A, sample 10), suggesting that Memo might influence the activity of cofilin.

Additional experiments were performed to test the effect of Memo on cofilin depolymerizing and severing activity. Increasing amounts of Memo were mixed with fixed amounts of G-actin and cofilin; actin polymerization was initiated, then the pellet and supernatant fractions were analyzed as above. The experiment shown in Fig. 8 was performed three times and the results of all experiments are summarized in the tables. A typical gel is shown in the upper panel (Fig. 8). Addition of cofilin to actin resulted in a 1.65-fold increase of actin in the supernatant (Fig. 8, sample 2), reflecting cofilin-induced depolymerization and severing activity. In samples 5-6, increasing amounts of Memo were included in the assay resulting in a further enhancement of actin in the supernatant (1.32- to 1.69-fold increase of actin compared with sample 2). Furthermore, addition of Memo also resulted in an increased amount of cofilin in the supernatant fraction (1.35- to 1.67-fold increase of cofilin compared with sample 2), reflecting enhanced cofilin-induced actin depolymerization and severing activity. In summary, these results suggest that in the presence of Memo, the ability of cofilin to mediate F-actin depolymerization and severing is promoted.

Discussion

In breast cancer, ErbB2 overexpression correlates with an aggressive metastatic phenotype (Hynes and Lane, 2005). We have been exploring signaling pathways activated by ErbB2 with the aim of identifying proteins recruited by the receptor that are essential for tumor cell migration. Activated ErbB2 is complexed with multiple proteins that couple the receptor to a variety of intracellular signaling pathways (Olayioye et al., 2000). We show here that two proteins, Memo and PLCγ1, are required for HRG-induced tumor cell migration. Depletion of either Memo or PLCγ1 has similar effects on cell motility and on the actin and microtubule cytoskeleton. Furthermore, in Dunn chambers, we observed that in the absence of either Memo or PLCγ1, cells could migrate in response to HRG, albeit more slowly than control cells. However, loss of Memo or PLCγ1 had a dramatic effect on cell directionality. Indeed, both proteins are essential for the persistent directed migration of tumor cells up a gradient of HRG. Furthermore, we show that Memo stimulates cofilin-induced F-actin depolymerization and severing activity in an in vitro assay. Finally, simultaneous KD of Memo and PLCγ1, like cofilin KD, almost completely blocked migration. Taken together, these results suggest that both Memo and PLCγ1 converge on cofilin, in an ErbB2-initiated migratory pathway.

PLCγ1 has a well-described role in PtdIns(4,5)P2 hydrolysis (Patterson et al., 2005) and is known to mediate chemotaxis.
towards a number of growth-factor-activated receptor tyrosine kinases (RTKs) (Wells, 2000). We show here that transient KD of Memo in T47D cells causes an impairment of PLCγ activation in response to HRG. These results were confirmed using T47D cells with stable shRNA-mediated Memo KD (provided by Gwen MacDonald) (data not shown). Although we cannot rule out a direct effect of Memo on the activity of PLCγ recruited to ErbB2, we consider it more likely that the effects of Memo are indirect. There is accumulating evidence that PLCγ has an important role in integrin-mediated adhesion and migration. PLCγ has been detected in integrin complexes (Choi et al., 2007), where it is activated by Src (Jones et al., 2005). It is possible that alterations in the actin cytoskeleton resulting from Memo depletion impact on the ability of integrin complexes to activate PLCγ.

Considering the novel role of Memo in PLCγ activation, it is possible that the major effect of Memo depletion is via decreased PLCγ activity. Although it cannot be completely ruled out, we consider this unlikely for various reasons. Compared with cells with individual depletion of Memo or PLCγ, simultaneous KD of both proteins has a more dramatic effect on the actin cytoskeleton of the cells (Fig. 1B), and on their migration in Transwell assays (Fig. 1A) and in Dunn chambers (Fig. 5). Interestingly, the double KD cells behave like add-back cells expressing an ErbB2 mutant lacking all autophosphorylation sites (NYPD cells). These cells are impaired in their migratory ability in Transwell assays (Marone et al., 2004) and are totally blocked in the Dunn chamber assay (supplementary material Fig. S5). These results would not be expected if the only cellular role of Memo was to ensure proper PLCγ activation. Furthermore, in vitro F-actin sedimentation assays showed that Memo positively influences cofilin activity, which in the in vitro assay is independent of PLCγ.

We favor the hypothesis that Memo and PLCγ each have several roles in migration. Following acute ErbB2 activation, when both proteins are recruited to the receptor, they act in concert to stimulate the migratory process. PLCγ activation promotes the localized release of PtdIns(4,5)P2-bound cofilin, which stimulates alterations in actin dynamics. Indeed in the MTLn3 rat cancer cell model, rapid PLCγ activation following EGF treatment (van Rienen et al., 2007) has been implicated in an increase in cofilin-mediated severing and an increase in actin barbed ends at the leading edge of the cell (Chan et al., 2000; DesMarais et al., 2005). However, PLCγ is not only activated by RTKs, but has also been detected in integrin complexes (Choi et al., 2007), where it is activated by Src (Jones et al., 2005). Indeed, in some cellular models it has been found that PLCγ has migratory functions that are independent of RTK activation (Jones et al., 2005).

Memo might also have multiple roles in the migratory process. First, results from in-vitro assays suggest that Memo might bind F-actin. Indeed, complexes of Memo and actin were found in lysates of T47D breast tumor cells (supplementary material Fig. S6). Moreover, our results suggest that Memo has an important role in cofilin function. Considering that Memo and cofilin were detected in complexes with activated ErbB2, and that Memo directly binds cofilin, it is possible that one role of Memo is to escort cofilin to the leading edge of the cell. Indeed, both GFP-Memo and GFP-cofilin rapidly associate with lamellipodia following HRG treatment (Fig. 6). In the receptor complexes, Memo might also bind the pool of cofilin released by PLCγ-mediated PtdIns(4,5)P2 hydrolysis. Both of these activities would result in an enrichment of cofilin at the cell periphery where it could participate in regulating actin dynamics. In co-immunoprecipitation assays, Memo was also detected in complexes with inactive cofilin-P. Memo might also have a role in the dephosphorylation and ensuing activation of cofilin. It should be mentioned, however, that in the breast cancer models used in our studies, we did not detect any changes in cofilin-P levels in response to HRG. Importantly, by testing recombinant Memo and cofilin in the F-actin sedimentation assay, we found that Memo stimulates the F-actin depolymerizing and severing activity of cofilin. ADF/cofilin family proteins are regulated by multiple mechanisms, including interactions with other proteins, such as Aip1 and CAP (Paavilainen et al., 2004), that also influence actin dynamics. Future experiments will be aimed at determining the domains of Memo and cofilin that interact and the mechanism underlying the role of Memo in cofilin regulation. Future work will be aimed at a closer analysis of Memo-actin binding.

In summary, the results presented here show that Memo is a novel player in the control of actin cytoskeleton dynamics. The essential role of Memo in cell migration might make it an interesting target for metastatic cancer therapy.

Materials and Methods

Reagents, recombinant proteins, antibodies and plasmids

HRG-B1, referred to as HRG, was purchased from R&D Systems (Minneapolis, MN). Recombinant cofilin from Sigma was used to test the specificity of the anti-cofilin antibody for various cell lines. For actin codedeposition and depolymerization assays, the actin-binding spin-down assay kit (BK001) from Cytoskeleton was used. Purification of Memo has been described previously (Qi et al., 2008). Polyclonal antibodies to a 19-amino acid peptide of Memo were produced in rabbits (aa 25–43 human Memo NM_015955: NQLEGGLSLSQVSTKRPAR); the same peptide was used for Memo mAb production in mice. Isotype-matched IgG2a control serum for Memo and IgG3 control serum for PLCγ were purchased from Sigma. The affinity-purified chicken anti-cofilin antibody AE774 (Song et al., 2006) was provided by John Condeelis (Albert Einstein College of Medicine, New York, NY) and the rabbit anti-cofilin antibody (no. 3312) was from Cell Signaling (Danvers, MA). Phospho-cofilin (rabbit anti-Ser3-P-cofilin no. 3311) and phospho-p44-42 MAPK (Thr202-204) were from Cell Signaling. Rabbit and mouse anti-PLCγ antisera (sc-81 and sc-7290, respectively) were from Santa Cruz (Santa Cruz, CA) and were used at 1:50 and 1:100, respectively. Mouse anti-PLCγ antibodies were a gift from John Condeelis.

Yeast two-hybrid analysis

The analysis was carried out as described previously (Pedrazi and Staglar, 2004). Full-length human Memo cDNA was cloned into pLexA-Kan (Dualsystems Biotech, Schlieren, Switzerland) as a fusion with the LexA DNA binding domain, and was used as bait. The peripheral blood cDNA library was fused to the GAL4-activation domain in the PACT2 vector (Clontech Laboratories, Mountain View, CA). The yeast reporter strain L40 was used. Transformants were selected in dropout plates and positive clones were identified. Growth in the absence of histidine and in the presence of 10-galactosidase indicates an interaction. Plasmid DNA was isolated from the yeast clones and rescued into Esherichia coli. A bait-dependency test was performed whereby each isolated plasmid was retransformed into yeast together with the control bait (pLexA-Kan without Memo), followed by growth selection and quantitative lacZ assay. Bait-dependent positive clones were sequenced and subjected to BLAST analysis.

Cell culture and transfections

T47D, NYPD, SKBr3, MDA-MB435 breast carcinoma cells and HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (GIBCO Invitrogen, Basel, Switzerland). GFP-Memo and GFP-cofilin constructs were transfected into SKBr3 cells using FuGene-6 transfection reagent (Roche Diagnostics, Indianapolis, IN). T47D and SKBr3 cells were transfected with siRNA using HiPerFect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. For dual transfection of SKBr3 cells with siRNA and plasmid constructs, DharmaFECT DUO reagent (Dharmacon, Lafayette, CO) was used. The following siRNAs were used: p53 (Santa Cruz, CA) for p53 silencing, p53A (no. 3311) and psedu-p44-42 MAPK (Thr202-204) were from Cell Signaling. Rabbit and mouse anti-PLCγ antisera (sc-81 and sc-7290, respectively) were from Santa Cruz (Santa Cruz, CA) and were used at 1:50 and 1:100, respectively. Mouse anti-PLCγ antibodies were a gift from John Condeelis.

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maximal after 72 hours; accordingly, cells were plated in Transwell assay chambers 3 days after siRNA transfection and migration assays were performed for 24 hours. For Dunn migration assays, cells were plated on coverslips 3 days after siRNA transfections and migration tracks were visualized over 4 to 12 hours.

**Immunoprecipitations, GST-pull-down and immunoblotting**

Cells were stimulated or not with 1 nM HRG, extracted in NP-40 buffer, and protein lysates were immunoblotted as described previously (Marone et al., 2004). For immunoprecipitation experiments, cells (stimulated or not with 10 nM HRG) were lysed in Triton X-100 buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1.5 mM MgCl₂, 25 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100, 1% glycerol, 10 μg ml⁻¹ leupeptin, 10 μg ml⁻¹ aprotinin, 2 mM sodium orthovanadate and 0.5 mM phenylmethylsulphonylfluoride). Equal amounts of cell lysates were incubated overnight at 4°C with antibodies. During the course of our studies, we have noted that pre-treatment of cells with CoCl₂ (50 μM) enhanced the levels of Memo in the IPs. This was not due to a CoCl₂-induced increase in Memo protein levels but is probably a consequence of increasing epitope availability. Thus, in some experiments, Memo IPs were made with lysates from untreated cells (Fig. 4C); however, cofilin could also be detected in Memo IPs made with lysates from untreated cells (Fig. 4D). Immunocomplexes were collected with protein-A or protein-G-Sepharose beads (Sigma), and centrifuged. The supernatant were subjected to a second round of immunoprecipitation. Proteins were then released by boiling in sample buffer, blotted onto polyvinylidene difluoride membranes (Millipore Corporation) and probed with the appropriate antibodies. Immunoprecipitations were also performed with lysates from CoCl₂-treated cells, in order to represent the directionality of cell migration. The percentage of cells with final migration paths was quantified using the Imaris software and the data were plotted as described above. Molar ratios of cofilin or Memo to actin were calculated using the following equation:

\[
\text{Molar ratio} = \frac{\text{Band intensity for cofilin or Memo}}{\text{Band intensity for actin}}
\]

**Statistical analysis**

Statistical analyses were performed using one-sided paired Student’s t-test for comparisons. A P-value of <0.05 was considered statistically significant.

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