The function and dynamics of the apical scaffolding protein E3KARP are regulated by cell-cycle phosphorylation

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ABSTRACT We examine the dynamics and function of the apical scaffolding protein E3KARP/NHERF2, which consists of two PDZ domains and a tail containing an ezrin-binding domain. The exchange rate of E3KARP is greatly enhanced during mitosis due to phosphorylation at Ser-303 in its tail region. Whereas E3KARP can substitute for the function of the closely related scaffolding protein EBP50/NHERF1 in the formation of interphase microvilli, E3KARP S303D cannot. Moreover, the S303D mutation enhances the in vivo dynamics of the E3KARP tail alone, whereas in vitro the interaction of E3KARP with active ezrin is unaffected by S303D, implicating another factor regulating dynamics in vivo. A-Raf is found to be required for S303 phosphorylation in mitotic cells. Regulation of the dynamics of EBP50 is known to be dependent on its tail region but modulated by PDZ domain occupancy, which is not the case for E3KARP. Of interest, in both cases, the mechanisms regulating dynamics involve the tails, which are the most diverged region of the paralogues and probably evolved independently after a gene duplication event that occurred early in vertebrate evolution.

INTRODUCTION
Polarized cells establish and maintain compositionally and morphologically distinct plasma membrane domains, the classic example being an epithelial cell, with its distinct apical and basolateral domains. The apical domain of epithelial cells is decorated by microvilli that contain a core of actin filaments linked to the plasma membrane in part by activated ezrin, a member of the ezrin/radixin/moesin (ERM) family. ERM proteins can bind directly to plasma membrane proteins and also associate with scaffolding proteins ezrin-binding phosphoprotein of 50 kDa (EBP50)/Na\(^{+}\)-H\(^{+}\) exchanger-3 regulatory factor 1 (NHERF1) or its parologue, exchanger 3 kinase A regulatory protein (E3KARP)/Na\(^{+}\)-H\(^{+}\) exchanger-3 regulatory factor 2 (NHERF2; Fehon et al., 2010). Very little is known about the regulation of scaffolding proteins despite their importance in the functional organization of plasma membrane domains. Recent work has shown that EBP50’s function and dynamics are regulated by its ability to bind ligands (Garbett and Bretscher, 2014). In this study, we explore the biochemical properties of E3KARP and examine whether it is similarly regulated. We find that E3KARP’s function and dynamics are regulated during the cell cycle in a manner mechanistically different from those of EBP50.

EBP50 was identified as a binding partner of activated ezrin (Reczek et al., 1997) and as a factor necessary to confer cAMP regulation on NHE3 (Weinman et al., 1993), hence the alternate names. EBP50 has two postsynaptic density 95/discs large/zona occludens-1 (PDZ) domains, which mediate interactions with multiple PDZ ligands, including transporters and receptors in the plasma membrane (Weinman et al., 1995; Short et al., 1998; Cao et al., 1999; Hernando et al., 2002) and the cytoplasmic protein EBP50 PDZ interactor of 64 kDa (EPI64; Reczek et al., 2001). EBP50 binds ezrin through its C-terminal domain, and this interaction, as well as a functional PDZ1 domain, is required for microvillar assembly and maintenance (Garbett et al., 2010). EBP50 in microvilli is unexpectedly dynamic, exchanging in 5–10 s, and this dynamic behavior is

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Abbreviations used: E3KARP, NHE3 kinase A regulatory protein; EBP50, ERM-binding protein of 50 kDa; EPI64, EBP50 PDZ interactor of 64 kDa; ERM, ezrin/radixin/moesin; FERM, 4.1 ERM; FRAP, fluorescence recovery after photobleaching; NHERF, Na\(^{+}\)-H\(^{+}\) exchanger-3 regulatory factor; PDZ, postsynaptic density 95/disks large/zona occludens-1.

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strongly suppressed when ligand binding to its PDZ domains is inhibited (Garbett and Bretscher, 2012). E3KARP was identified by its ability to bind the cytoplasmic tail of NHE3, recognized as being closely related to EB50 (Yun et al., 1998), and subsequently renamed NHERF2 (Kurashima, 1999). E3KARP and EB50 share 55% sequence identity, and both have two PDZ domains and an ezrin-binding domain (Reczek and Bretscher, 1998). The PDZ domains of E3KARP interact with transporters (PMCA 2b [DeMarco et al., 2002] and SLC26A6 [Lohi et al., 2003]), receptors (β-aradrenergic receptor [Hall et al., 1998] and parathyroid hormone receptor [Mahon et al., 2002]), channels (cyscolysis transmembrane conductance regulator [Sun et al., 2000] and ROMK [Yun, 2002]), and a protein contributing to the glycolalyx (podocalyxin; Li et al., 2002), as well as with cytoplasmic proteins (EB50 [He et al., 2001] and EPI64 [Reczek and Bretscher, 2001]). Of interest, E3KARP exhibits some clear differences from EB50. For example, the expression of EB50 is almost exclusively restricted to epithelial cells, whereas that of E3KARP is not (Ingraffea et al., 2002). Whereas both EB50 and E3KARP can confer CAMP inhibition on NHE3, E3KARP is required for cGMP inhibition of NHE3, a function for which EB50 cannot substitute (Cha et al., 2005; Yun et al., 1998; Weinman et al., 2003). In addition, E3KARP exhibits a much slower exchange rate than EB50 in vivo in microvilli of interphase cells (Garbett and Bretscher, 2012). The region specifying the difference in dynamics between EB50 and E3KARP is located in the region downstream of the PDZ domains (Garbett et al., 2013; Yang et al., 2013).

Here we explore the evolutionary divergence of EB50 and E3KARP and then document E3KARP's biochemical properties to show that, like EB50, it exists as a monomer in solution. We then report that E3KARP is phosphorylated during mitosis on a conserved serine residue, not present in EB50, and that this regulates its function and dynamics. We also identify A-Raf as a kinase necessary for E3KARP phosphorylation at the G2/M stage of the cell cycle. This study reveals that the function and dynamics of E3KARP are regulated but in a manner mechanistically different from those of EB50, suggesting that after the gene duplication event that gave rise to these paralogues, different modes of regulation evolved.

RESULTS
E3KARP and EB50 arose by gene duplication and evolution of their C-terminal regions
Mammalian E3KARP and EB50 are closely related (Donowitz et al., 2005), so we conducted a phylogenetic analysis across metazoan genomes of these two proteins. E3KARP and EB50 are derived from a unique root in early vertebrate evolution, whereas invertebrates have only one form (Figure 1A). Next we examined sequence homology between E3KARP and EB50 in the vertebrate clad. Alignment of 18 E3KARP and EB50 vertebrate species shows a high degree of conservation over the region of their PDZ domains (unpublished data), as well as over the C-terminal residues involved in binding ezrin (Supplemental Figure S1, A and B). Aligning these proteins on the basis of the PDZ regions alone clearly separates the EB50 and E3KARP groups, suggesting a divergence soon after the gene duplication event that gave rise to them. The divergence between the consensus sequences of the full-length proteins reveals the presence of ~20 amino acids upstream of the ezrin-binding domain that is present in all EB50, but not in the E3KARP, homologues (Supplemental Figure S1C). To determine whether the ~20 amino acids are a consequence of an insertion in EB50 or a deletion in E3KARP, we compared their sequences with the species immediately outside the vertebrate clade. The tunicate Ciona species do not have these 20 amino acids (Figure 1, A and B). These data suggest that present-day E3KARP and EB50 arose from a gene duplication event during vertebrate evolution, and soon thereafter EB50 acquired a 20–amino acid insertion, followed by evolutionary divergence of the region between the PDZ domains and ezrin-binding site. This divergent region is partly responsible for the difference in dynamics between EB50 and E3KARP (Garbett et al., 2013).

E3KARP is a slightly elongated monomer
Biochemical and biophysical studies on EB50 and E3KARP have yielded contradictory results on the capacity of EB50 and E3KARP to oligomerize through their PDZ domains (Lau and Hall, 2001). To interpret subsequent results, it was necessary to first document the properties of E3KARP in vitro. Pure EB50 has been shown to exist as an elongated monomer with no tendency to oligomerize (Li et al., 2007; Garbett et al., 2010). To examine the properties of E3KARP, recombinant E3KARP tagged with hexahistidine (6xHis-SUMO) was expressed in bacteria and purified using Ni2+ resin, and the 6xHis-SUMO tag was cleaved to generate free E3KARP (Supplemental Figure S2A). E3KARP migrated as a single species on a Superdex 200 gel filtration column and on a sucrose gradient. By gel filtration, E3KARP has a Stokes radius of 31 Å, corresponding to a molecular weight of 40 kDa for a globular protein (Figure 2, A and C). E3KARP sedimented at 2.99 S (Figure 2, B and C, and Supplemental Figure S2B). These two physical parameters can be combined to predict a native molecular weight of ~39 kDa (Siegel and Monty, 1966), close to the predicted monomeric mass, indicating that E3KARP exists as a monomer in solution with no tendency to oligomerize. Using a protein partial specific volume of 0.74 cm3/g, we calculate a fractional ratio (f/f0) of 1.4, indicating that E3KARP is a slightly elongated monomer in solution. When compared with the physical constants for EB50 (Garbett et al., 2010), this shows that EB50 is more elongated than E3KARP in solution (Figure 2C). This difference is presumably due to the divergence of the tail region between the two proteins.

E3KARP can complement EB50's function in microvilli biogenesis
To explore whether E3KARP has similar functions to EB50, we used the human choriocarcinoma JEG-3 cell line, as these cells normally display abundant microvilli. JEG-3 cells express EB50 but have no detectable E3KARP. Depletion of EB50 by small interfering RNA (siRNA) causes a significant loss of microvilli (Garbett et al., 2010; Figure 3A). When cells are depleted of EB50 by siRNA and transfected to express green fluorescent protein (GFP) targeted to the nucleus (GFP-Nuc), only 44% of the cells show normal microvilli. Expression of GFP-E3KARP also restores normal microvilli in the cells to the same extent as EB50 (Figure 3, A and B). These data suggest that E3KARP shares a similar function to EB50 that is necessary to assemble microvilli.

E3KARP is phosphorylated at the G2/M transition of the cell cycle
Phosphorylation of EB50 during both interphase and mitosis can regulate the formation of microvilli (Hall et al., 1999; He et al., 2001; Garbett et al., 2010). To investigate the potential regulation of E3KARP by phosphorylation during the cell cycle, we used epithelial colorectal adenocarcinoma Caco-2 cells, which express endogenous E3KARP. Treatment of Caco-2 cells with nocodazole for 18–20 h arrested the cells in mitosis and resulted in a shift in the mobility of E3KARP, as seen by SDS–PAGE (Figure 4A). Because the shift can be reversed by in vitro phosphatase treatment, this suggests that E3KARP is phosphorylated during mitosis.
riched from the cells grown in heavy medium identified three serine residues whose phosphorylation was enhanced at least threefold during mitosis (indicated in blue in Figure 4C). Serine S43 is localized in the PDZ1 domain, and, of interest, serines S261 and S303 are conserved residues in the C-terminal region of E3KARP and divergent from EBP50 (Figure 1A and Supplemental Figure S1B).

Phosphorylation of Ser-303 regulates the localization, function, and dynamics of E3KARP

To identify the residues phosphorylated during mitosis, we subjected JEG-3 cells stably expressing 3xFLAG-tagged E3KARP to stable isotope labeling of amino acids in cell culture (SILAC) in normal (“light”) or 13C-arginine and 13C-lysine (“heavy”) medium (Figure 4B). 3xFLAG-E3KARP was separately immunoprecipitated from untreated cells grown in light medium or from cells grown in heavy medium, after treatment with nocodazole to arrest them in mitosis. The immunoprecipitates were mixed and subject to trypsin digestion. To enrich for phosphopeptides, we subjected the peptide mixture to immobilized metal affinity chromatography (IMAC) followed by mass spectrometry (Figure 4B). Analysis of tryptic peptides enriched from the cells grown in heavy medium identified three serine residues whose phosphorylation was enhanced at least threefold during mitosis (indicated in blue in Figure 4C). Serine S43 is localized in the PDZ1 domain, and, of interest, serines S261 and S303 are conserved residues in the C-terminal region of E3KARP and divergent from EBP50 (Figure 1A and Supplemental Figure S1B).

FIGURE 1: E3KARP and EBP50 diverged after gene duplication and insertion of 22 amino acids in the EBP50 tail. (A) Phylogenetic tree of EBP50 and E3KARP homologues. The scale bar indicates the number of substitutions. Mammalia, Actinopterygii, Nematoda, Drosophilina, and Nematostella species have each been collapsed into a unique branch. The shaded boxes indicate the E3KARP and EBP50 groups in vertebrate clades. The tree was generated using the EggNOG database and iTOL software. (B) Sequence alignment of C-terminal tails of human EBP50 and E3KARP with two species outside the vertebrate clade, Ciona intestinalis and Ciona savignyi. Conserved residues are highlighted.
S303A, or 3xFLAG-E3KARP S303D, and then FLAG immunoprecipitates were probed for the presence of EPI64 (Supplemental Figure S4C). EPI64 was able to bind E3KARP wild type, as well as the phosphodeficient mutant S303A. The S303D phosphomimetic mutation may slightly reduce the binding of EPI64, but this difference is much more modest than the effect on ezrin coimmunoprecipitation. Thus the major effects of the S303D phosphomimetic mutation are to enhance E3KARP’s dynamics and reduce its in vivo interaction with ezrin.

Finally, we asked whether the phosphorylation on Ser-303 is responsible for the gel shift observed in mitotic cells (Figure 4A). JEG-3 cells stably expressing 3xFLAG-E3KARP, 3xFLAG-E3KARP S303A, or 3xFLAG-E3KARP S303D were treated with nocodazole for 18 h to arrest them in mitosis, lysed in 2× sample buffer, separated by gel electrophoresis, and blotted for FLAG. Of interest, a similar mobility shift is observed for 3xFLAG-E3KARP S303A and 3xFLAG-E3KARP S303D, suggesting that both are phosphorylated at another site during mitosis, most likely at one of the other sites we identified. However, wild-type E3KARP is subjected to a greater gel shift than E3KARP S303A, showing that S303 is indeed responsible for a mitosis-specific gel shift (Figure 5F).

Phosphorylation of S303 regulates the dynamics of the E3KARP tail region

The very different dynamics of full-length EBP50 and E3KARP have been traced to the tail regions, and the tail region of EBP50 alone shows the same dynamics as the full-length protein when the PDZ domains are occupied (Garbett et al., 2013). We therefore examined the dynamics of the tail of E3KARP (FRAP) on interphase JEG-3 cells expressing GFP-E3KARP, GFP-E3KARP S303A, or GFP-E3KARP S303D. Mutation of Ser-303 to alanine (S303A) has no effect on the recovery rate, whereas the phosphomimetic S303D mutant increased the dynamics of E3KARP dramatically to a level similar to that seen for EBP50 (Figure 5C and D). Because E3KARP is not normally present in JEG-3 cells, we repeated our analysis on Caco-2 cells that express endogenous E3KARP and found that each construct behaves the same as in JEG-3 cells (Supplemental Figure S4). We have shown a biochemical correlation between the ability to coimmunoprecipitate EBP50 and ezrin and the in vivo dynamics of EBP50, which is low when EBP50 is highly dynamic and enhanced when it is less dynamic (Garbett and Bretscher, 2012). We used the same assay with cells expressing the 3xFLAG-E3KARP variants. 3xFLAG-E3KARP S303A coprecipitated about as much ezrin as 3xFLAG-E3KARP, whereas 3xFLAG-E3KARP S303D showed very minimal recovery of ezrin (Figure 5E). We also explored whether phosphorylation affected the ability of E3KARP to bind the PDZ1 ligand EPI64 (Reczek and Bretscher, 2001). JEG-3 cells were transfected to express 3xFLAG-E3KARP, 3xFLAG-E3KARP S303A, or 3xFLAG-E3KARP S303D, and then FLAG immunoprecipitates were probed for the presence of EPI64 (Supplemental Figure S4C). EPI64 was able to bind E3KARP wild type, as well as the phosphodeficient mutant S303A. The S303D phosphomimetic mutation may slightly reduce the binding of EPI64, but this difference is much more modest than the effect on ezrin coimmunoprecipitation. Thus the major effects of the S303D phosphomimetic mutation are to enhance E3KARP’s dynamics and reduce its in vivo interaction with ezrin.

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| Protein      | Theoretical MW (kDa) | Calculated MW (kDa) | Stokes Radius (Å) | Sedimentation Coefficient (S) | Frictional Coefficient (f/f₀) |
|--------------|----------------------|---------------------|-------------------|-----------------------------|-------------------------------|
| EBP50        | 38.8                 | ~47                 | 41.4±1.3          | 2.72±0.02                   | 1.8                           |
| E3KARP       | 37.4                 | ~39                 | 30.9±1.4          | 2.99±0.02                   | 1.4                           |

**FIGURE 2:** E3KARP is a slightly elongated monomer. (A) The Stokes radius of untagged recombinant E3KARP was analyzed by gel filtration on a Superdex 200 10/300GL column and (B) the sedimentation coefficient on a sucrose velocity gradient. The migration of protein standards is shown in blue. (C) Summary of the results for E3KARP compared with those obtained for EBP50 (from Garbett et al., 2010).
the tail for active ezrin. We therefore examined the ability of the E3KARP wild-type tail and the corresponding S303D mutant to bind immobilized ezrin FERM domain in which the E3KARP binding site is fully accessible. Maltose-binding protein (MBP) fusions of both tails bound immobilized FERM beads equivalently over a range of 150–1000 mM NaCl (Figure 6C). We conclude that the S303D mutation has no effect on the ability of the tail to bind active ezrin, and so the different dynamics seen in vivo must be due to some additional factor, most likely one involved in the binding to the S303D tail, thus destabilizing its interaction with ezrin.

The high dynamics of full-length EBP50 is regulated by occupancy of its PDZ domains: the EBP50 tail is intrinsically dynamic, and this is suppressed in the full-length protein by the presence of the PDZ domains when they cannot bind ligand; this suppression is relieved in the wild-type protein upon occupancy of the PDZ domains (Garbett and Bretscher, 2012). To see whether a similar situation exists for E3KARP, we mutated both PDZ domains to inhibit ligand binding in the context of either wild-type E3KARP or the S303D mutant. Surprisingly, mutating both PDZ domains of wild-type E3KARP had no effect on its dynamics, nor did mutating the PDZ domains of the dynamic S303D phosphomimetic mutant (Figure 6, D and E). Thus, in contrast to the situation with EBP50, E3KARP dynamics is not regulated by PDZ domain occupancy but only by phosphorylation.

In cells arrested in mitosis, E3KARP shows a fast exchange rate due to S303 phosphorylation

Our data indicate that E3KARP is phosphorylated on S303 during mitosis and that GFP-E3KARP S303D expressed in interphase cells is much more dynamic than the corresponding wild-type construct. We therefore investigated the localization and dynamics of E3KARP in mitotic cells. JEG-3 cells were transfected to express GFP-E3KARP or the S303A or S303D mutants and then arrested in mitosis by nocodazole treatment. In the rounded mitotic cells, GFP-E3KARP S303A shows a strong cortical localization. However, both GFP-E3KARP and GFP-E3KARP S303D are both largely cytoplasmic in mitotic cells, implying that S303 phosphorylation alters the localization of E3KARP (Figure 7A). FRAP analysis on these constructs shows that GFP-E3KARP S303A has a relatively slow recovery rate, similar to that seen for the wild-type protein in interphase cells, whereas E3KARP S303D and GFP-E3KARP in mitotic cells are both very dynamic with fast recovery rates (Figure 7B). We then
other inhibitors, including ones that affect the c-Jun amino-terminal kinase, mitogen-activated protein kinase (MAPK) kinase (MEK), and MAPK families, had no effect (Supplemental Figure S5A). However, a selective Raf kinase inhibitor reduced E3KARP phosphorylation in a dose-dependent manner (Figure 8A). The mechanism of phosphorylation is conserved among cell types, as Raf inhibitor IV treatment of Caco-2 also inhibited mitotic phosphorylation of endogenous E3KARP (Supplemental Figure S5B).

Raf kinases are serine/threonine kinases downstream of Ras and upstream of the MEK/extracellular signal–regulated kinase (ERK) pathway (Cseh et al., 2014). There are three Raf kinases: A-Raf, B-Raf, and Raf-1. The Raf inhibitor IV targets A-Raf and Raf-1 kinases (Shelton et al., 2003; White, 2003). We next examined the localization of expressed GFP–A-Raf and found that it is mostly cytoplasmic but also exhibited a weak enrichment in microvilli (Figure 8B), whereas Raf-1 was cytoplasmic (unpublished data). To explore whether A-Raf might affect E3KARP phosphorylation in mitosis, we knocked down A-Raf using siRNA treatment, arrested the cells in mitosis with nocodazole, and examined the effect on E3KARP phosphorylation. We observed a strong reduction in mitotic phosphorylation of E3KARP in the absence of A-Raf (Figure 8C). Finally, we asked whether knockdown of A-Raf would affect the exchange rate of other inhibitors, including ones that affect the c-Jun amino-terminal kinase, mitogen-activated protein kinase (MAPK) kinase (MEK), and MAPK families, had no effect (Supplemental Figure S5A).

Mitotic phosphorylation of E3KARP depends on A-Raf

The sequence surrounding S303 in E3KARP does not provide a clear indication of the identity of a relevant kinase important for phosphorylation at this residue, although the presence of an adjacent proline suggests that it might be in the CDK1 family. Therefore, cells were arrested in mitosis with nocodazole and the effect of kinase inhibitors on E3KARP phosphorylation assessed. Roscovitine, an inhibitor of CDK1 that phosphorylates EBP50 (Planchais et al., 1997), had no effect on E3KARP phosphorylation under conditions that eliminate EBP50 cell cycle–dependent phosphorylation (Garbett et al., 2010). Similarly, preliminary experiments with used cells expressing 3xFLAG-E3KARP or an empty vector to examine whether the interaction between E3KARP and ezrin is compromised in mitotic cells. Immunoprecipitates of 3xFLAG-E3KARP co-precipitated endogenous ezrin, whereas when the cells were arrested in mitosis, almost no ezrin was recovered (Figure 7C). Thus E3KARP phosphorylated in mitosis behaves very similarly to the expression phosphomimetic E3KARP in interphase cells (Figure 5E).

We conclude that E3KARP is phosphorylated on S303 in mitotic cells and this alters its localization and binding to ezrin and greatly enhances its dynamics.

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FIGURE 5: Phosphorylation of Ser-303 regulates the localization, function, and dynamics of E3KARP. (A) Maximum projection images of the apical surface of JEG-3 cells. Cells were transfected to express GFP-tagged constructs (green) and then stained for ezrin (red) and actin (blue). A vertical cross section through the cells is shown under each maximum projection. The contrast for F-actin was increased for clarity in the panel representing siRNA-treated cells transfected with GFP-E3KARP S303D. Scale bar, 5 μm. (B) Ability of GFP-E3KARP constructs to restore microvilli in cell treated with siRNA against EBP50. Results from scoring individual cells for the presence of microvilli; errors bars indicate mean ± SD; n = 3, **p < 0.01, ***p < 0.001, ****p < 0.0001. (C) FRAP curves of for JEG-3 cells expressing GFP-E3KARP, GFP-E3KARP S303A, and GFP-E3KARP S303D. Error bars show SD. (D) Representative time points from FRAP experiments of GFP-E3KARP, E3KARP S303A, and E3KARP S303D in JEG-3 cells. Scale bars, 2 μm. (E) FLAG-tagged E3KARP, E3KARP S303A, and E3KARP S303D were immunoprecipitated and blotted for FLAG, endogenous ezrin, and E-cadherin. (F) Western blot of total lysates from JEG-3 cells stably expressing FLAG-tagged E3KARP, E3KARP S303A, and E3KARP S303D arrested in mitosis by treatment with nocodazole.
DISCUSSION

Scaffolding proteins are generally believed to bring together two or more proteins to enhance a specific biochemical pathway. Although it is likely that scaffolding proteins are subject to dynamic regulation, little attention has been paid to this aspect of their biology (Garbett and Bretscher, 2014). In this study, we show that the apical scaffolding protein E3KARP is subject to cell-cycle phosphorylation and that this regulates its function and dynamics.

E3KARP in mitosis. First, we treated JEG-3 cells expressing GFP-E3KARP with siRNA against A-Raf for 24 h. We then arrested the cells in mitosis by incubation in nocodazole for 18 h. FRAP analysis showed that A-Raf knockdown partially reduced the exchange rate of GFP-E3KARP in mitotic cells (Figure 8D). Whether the inability to completely reverse the effect on dynamics is due to incomplete knockdown of A-Raf or another kinase that phosphorylated S303 is not clear. Nevertheless, the data suggest that A-Raf kinase contributes to cell-cycle phosphorylation of E3KARP, which enhances its dynamics.

FIGURE 6: S303 phosphorylation of the E3KARP tail regulates its exchange rate, whereas mutation of the PDZ domains in the full-length protein does not. (A) FRAP curves of GFP-tagged E3KARP tail (n = 11), E3KARP tail S303A (n = 14), and E3KARP tail S303D (n = 14) expressed in JEG-3 cells. Errors bars show SD. (B) Representative time points from FRAP experiments of GFP-tagged E3KARP tail, E3KARP tail S303A, and E3KARP tail S303D in JEG-3 cells. Scale bars, 2 μm. (C) MBP-tagged E3KARP tails (WT or S303D) were incubated with ezrin FERM domain beads over a range of 150–1000 mM NaCl. The retained MBP-E3KARP tails were recovered and analyzed by SDS–PAGE and the gel stained with IRDye Blue. The asterisk indicates the MBP tag that was cleaved during MBP-E3KARP tail S303D purification. (D) Maximum projection images of the apical surface of JEG-3 cells expressing the indicated GFP-tagged constructs (green) and then stained for ezrin (red) and actin (blue). Scale bar, 5 μm. (E) FRAP curves of GFP-tagged E3KARP (n = 19), E3KARP PDZmut (n = 15), E3KARP S303D (n = 29), and E3KARP PDZmut S303D (n = 16) expressed in JEG-3 cells. Errors bars show SD.
thought was that it might reduce the affinity of E3KARP for active ezrin.

We found that the E3KARP tail alone, like the full-length protein, is intrinsically not dynamic but becomes dynamic upon S303 phosphorylation, with the PDZ domains playing little or no role in regulating the dynamics. This is in contrast to EBP50, in which the tail is intrinsically dynamic, with the unoccupied PDZ domains suppressing the dynamics, and this suppression is relieved by PDZ ligand binding (Garbett and Bretscher, 2012). Furthermore, in vitro analyses of E3KARP revealed that the interaction with active ezrin is unperturbed even when E3KARP is highly dynamic, indicating that in vivo dynamics is mediated by unknown cytoplasmic proteins. It is important to note that in vitro, pure EBP50 and E3KARP both exist as monomers in solution (Garbett et al., 2010; this study) and do not oligomerize through their PDZ domains as had been suggested (Lau and Hall, 2001), so the regulation of their dynamics conferred by the tail regions is unlikely to be explained by oligomerization status.

Phylogenetic analysis reveals that EBP50 and E3KARP arose by gene duplication followed by sequence divergence in their tails. The regions in the tail responsible for regulation of their dynamics are especially divergent, but in the case of E3KARP, for which only one residue is involved, this residue is highly conserved among vertebrate homologues. Therefore it appears that the regulation of the dynamics of EBP50 and E3KARP evolved independently, which is consistent with the mechanistic differences in regulation. We found that the longer EBP50 tail is a result of an insertion of 20 amino acids and that this insertion is in the region identified as necessary for conferring the higher dynamic of EBP50. Because EBP50 and E3KARP show specific and mutually exclusive tissue distributions (Ingraffea et al., 2002), the modes of regulation are presumably reflective of their different functions.

Interphase cells, in which EBP50 has been depleted, had greatly reduced microvilli, which were restored upon expression of GFP-E3KARP. Thus E3KARP can provide a function normally performed by EBP50, despite the differences in their dynamics. Expression of GFP-E3KARP with the phosphomimetic S303D mutation displayed an impaired ability to restore microvilli in EBP50-depleted cells. It is likely that the S303D mutation is a good mimic for S303 phosphorylation, as its dynamics is indistinguishable in mitotic cells from that of E3KARP phosphorylated in vivo on S303. Thus phosphorylation of S303 enhanced the dynamics of E3KARP and compromised its function in microvilli biogenesis. By contrast, GFP-EBP50 was highly dynamic after expression in EBP50-depleted cells, restoring microvilli, thereby indicating that enhanced dynamics alone does not necessarily impair the microvillar functions of these scaffolding proteins but may reflect the mechanistically different ways the dynamics is regulated.

Using several different approaches, we found that A-Raf contributes to S303 phosphorylation of E3KARP. A-Raf is the least-studied member of the three members of the Raf serine/threonine kinase family, with much more known about B-Raf and Raf-1 (Roskoski, 2010; Cseh et al., 2014). These kinases are upstream of the MEK-ERK cascade and are regulated by phosphorylation and subsequent binding to 14-3-3 proteins (Fischer et al., 2009; Roskoski, 2010). For example, 14-3-3 proteins can bind and activate Raf-1 at the G2/M transition (Hayne et al., 2000). We do not know whether A-Raf is selectively activated during mitosis, which will be an interesting topic for future studies. Our results with MEK-ERK inhibitors suggest that they are not involved in E3KARP phosphorylation; thus E3KARP may be a direct substrate of A-Raf or of an unknown downstream kinase. However, knockdown of A-Raf only partially eliminated the enhanced dynamics of E3KARP seen in mitotic cells. It is therefore
FIGURE 8: A-Raf contributes to the cell cycle–dependent phosphorylation of E3KARP. (A) JEG-3 cells stably expressing 3xFLAG-tagged E3KARP were treated with DMSO, nocodazole (50 ng/ml), and/or Raf inhibitor IV at the indicated concentrations overnight at 37°C. Cells were lysed, E3KARP was detected by Western blotting, and the level of phosphorylation was quantitated using Odyssey software (n ≥ 3). Errors bars show SD. (B) Maximum projection images of the apical surface of JEG-3 cells expressing the indicated constructs. Cells were stained for FLAG (red) and actin (blue). Scale bar, 5 μm. White box indicates area magnified on the right, in which a single z-plane is shown. Scale bar, 2 μm. (C) Cells stably expressing 3xFLAG-tagged E3KARP were treated with 10 nM control (siGL2) or A-Raf (siA-Raf) siRNA for 72 h. At 18 h before lysing of the cells, 50 ng/ml nocodazole was added. Cell extracts separated by SDS–PAGE were Western blotted for FLAG (red) and A-Raf (blue). Scale bar, 55 kDa. Anti-FLAG, anti-A-Raf. (D) FRAP curves of GFP-tagged E3KARP in interphase JEG-3 cells (n = 19), cells arrested by nocodazole treatment (n = 13), and cells treated with siRNA against A-Raf and then arrested by nocodazole treatment (n = 20). Errors bars show SD.
likely that another kinase contributes to S303 phosphorylation in mitosis to regulate E3KARP's dynamics. It will be interesting to understand the implication of E3KARP phosphorylation during the cell cycle. Because the actin cytoskeleton has been involved in membrane tension regulation during the cell cycle to prevent endocytosis (Boulant et al., 2011; Kaur et al., 2014), it will also be interesting to examine how E3KARP is involved in this mechanism.

In summary, we have uncovered a new mechanism regulating the apical scaffolding protein E3KARP during the cell cycle. The phosphoregulation of E3KARP brings specific properties to the protein that have not been explored before. Together with our earlier studies on EB5p0, our results highlight how two homologous proteins can provide similar functions despite having unique mechanisms of regulation.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Anti-ERP and affinity-purified antibodies against human ezrin, E3KARP and EPI64 have been described (Bretsch, 1989; Ingrafea et al., 2002; Hokanson and Bretsch, 2012). The mouse anti-FLAG, mouse anti–E-cadherin, and rabbit anti–A-Raf (C20) antibodies were purchased from Sigma-Aldrich (St. Louis, MO), BD Biosciences (Franklin Lakes, NJ), and Santa Cruz Biotechnology (Dallas, TX), respectively. Goat anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and MP Biomedicals (Solon, OH), respectively. Hoechst 33258 DNA stain, Alexa Fluor 568 donkey anti-rabbit antibody, and Alexa Fluor 660–conjugated phalloidin were purchased from Invitrogen (LifeTechnologies, Grand Island, NY). IRDye 680– and 800–conjugated secondary antibodies were from Li-COR Biosciences (Lincoln, NE). Dimethyl sulfoxide (DMSO) and roscovitine were obtained from Sigma-Aldrich. RAF Inhibitor IV was obtained from Santa Cruz Biotechnology. Sorafenib, PD 98058 was obtained from Calbiochem (EMD Millipore, Billerica, MA). The siRNAs targeting human EBP50 (5′-CGCGGAAUACUUCGA-3′) were obtained from Thermo Fisher Scientific; FBS from Invitrogen. JEG-3 cells were transfected with 1–2 μg of DNA at low confluence, allowed to recover for 24 h, and then transfected again with 10 nM of siRNA using Lipofectamine RNAiMAX (LifeTechnologies). Cells were then selected and maintained with 2μg/ml puromycin (Sigma-Aldrich).

**DNA constructs**

All EB5p0 and E3KARP constructs were created in pEGFP-C2 (Takara Bio, Otsu, Japan) and pE-SUMO (LifeSensors, Malvern, PA). The 3xFLAG tagged E3KARP construct was created using PCR and inserted into pQXIP (BD Biosciences; Garbett et al., 2013). All point mutations in pEGFP-E3KARP were generated using the mutagenesis kit (QuikChange; Agilent Technologies, Santa Clara, CA).

**Cell culture and transfection**

JEG-3 cells, Caco-2 cells, and Phoenix-AMPHO (American Type Culture Collection, Manassas, VA) were maintained in a 5% CO2 humidified atmosphere at 37°C in MEM with 10% fetal bovine serum (FBS) or DMEM with 10% FBS (MEM and DMEM from Thermo Fisher Scientific; FBS from Invitrogen). JEG-3 cells, Caco-2 cells, and Phoenix-AMPHO (American Type Culture Collection, Manassas, VA) were maintained in a 5% CO2 humidified atmosphere at 37°C in MEM with 10% fetal bovine serum (FBS) or DMEM with 10% FBS (MEM and DMEM from Thermo Fisher Scientific; FBS from Invitrogen). JEG-3 cells were transfected with polyethyleneimine (PE; Polysciences, Warrington, PA) and DNA as described previously (Hanono et al., 2006). Caco-2 were transfected with Lipofectamine 2000 (LifeTechnologies) according to the manufacturer’s instructions. For microvillus rescue assays, cells were first transfected with 1–2 μg of DNA at low confluence, allowed to recover for 24 h, and then transfected again with 10 nM of siRNA using Lipofectamine RNAiMAX (LifeTechnologies). Cells were then allowed to grow for another 48 h and then processed for immunofluorescence and counting. For mitotic arrest studies, JEG-3 and Caco-2 cells were incubated with 50 ng/ml and 1 μg/ml nocodazole (Sigma-Aldrich), respectively, for 18–20 h before preparation for analysis. For siRNA A-Raf assays, cells were transfected with 10 nM siRNA using Lipofectamine RNAiMAX at low confluence, allowed to recover for 48 h, and then treated with 50 ng/ml nocodazole. At 72 h after siRNA transfection, cells were lysed in 2x sample buffer. To analyze the effect of A-Raf knockdown on the dynamics of E3KARP in mitosis, JEG-3 cells were transfected with PE and 1 μg of DNA to express GFP-E3KARP and with 10 nm siRNA to A-Raf using Lipofectamine RNAiMAX for 24 h. The cells were then arrested in mitosis by 18-h nocodazole treatment and subjected to FRAP analysis.

For generation of stable JEG-3 cell lines expressing 3xFLAG-E3KARP, Phoenix-AMPHO cells were cotransfected with the foregrowing construct in pQXIP in addition to a plasmid encoding VSV-G using PE. The resulting retroviruses were then used to infect JEG-3 cells, which were then selected and maintained with 2μg/ml puromycin (Sigma-Aldrich).

**Immunoprecipitation and Western blotting**

JEG-3 cells transiently expressing 3xFLAG-E3KARP, 3xFLAG-E3KARP S303A, and 3xFLAG-E3KARP S303D constructs were lysed in lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1% IGEPAL-630, 50 mM NaF, 0.1 mM Na3VO4, 10 mM β-glycerol phosphate, and 2.5% glycerol) and incubated with anti-FLAG M2 affinity resin (Sigma-Aldrich) while nutating at 4°C for 2 h. The resin was then washed three times in lysis buffer with 0.1% IGEPAL-630, and the remaining bound material was eluted using 3xFLAG-peptide (Sigma-Aldrich) for 30 min at room temperature. Then protein samples were boiled in reducing sample buffer. Boiled protein samples were then separated by SDS–PAGE, transferred to Immobilon-FL (EMD Millipore) for Western blotting, and then visualized using ECL (GE Healthcare) or an Odyssey infrared imaging system (LI-COR Biosciences).

**SILAC and mass spectrometry**

For SILAC, JEG-3 stable cell lines expressing 3xFLAG-E3KARP were grown for ~3 wk in MEM (Thermo Fisher Scientific) containing diazylated FBS (Invitrogen) and either C12-arginine and lysine or C13-arginine and lysine (Sigma-Aldrich), respectively. FLAG immunoprecipitations were as described, with slight modifications for mass spectrometry processing, as described previously (Smolka et al., 2007; Viswanatha et al., 2012). Briefly, after immunoprecipitation, bound protein was eluted in 50 mM Tris (pH 8.0) and 1% SDS and then precipitated with 50% ethanol, 49.9% acetone, and 0.1% acetic acid. Protein samples were then mixed, trypsin digested (Promega, Madison, WI), and desalted in a C18 column (Waters, Milford, MA). The tryptic peptides were dehydrated in a speed vacum and then resuspended in 1% acetic acid. Phosphopeptides were enriched by IMAC as previously described (Smolka et al., 2007; Albuquerque et al., 2008; Ohouo et al., 2013), reconstituted in 85 μl of solution containing 80% acetonitrile and 1% formic acid, and fractionated by hydrophilic interaction liquid chromatography. The resulting fractions were injected into a mass spectrometer (QExactive LC-MS/MS; Thermo Fisher Scientific) and the data analyzed using Proteome Discoverer (Thermo Fisher Scientific).

**Immunofluorescence**

For immunofluorescence, cells grown on glass coverslips were fixed in 3.7% formaldehyde/phosphate-buffered saline (PBS) for 10 min at room temperature. Cells were permeabilized in 0.2%
and complete protease inhibitor by sonication, centrifuged, and run over amylose resin column (New England Biolabs, Ipswich, MA), washed with PBS, and then eluted in PBS with 10 mM maltose.

**Gel filtration and sucrose gradients**

From 1 to 2 mg of untagged full-length E3KARP was run over a Superdex 200 10/300GL column on an AKTA FPLC (GE Healthcare) in 150 mM NaCl and 10 mM Tris, pH 7.4, with 1 mM DTT. Stokes radii were calculated as described previously (Begg et al., 2001). Full-length E3KARP was run on 5–20% sucrose gradients with size standards bovine serum albumin (4.3S), carbonic anhydrase (3.2S), and myoglobin (2.04S) in a rotor (SW28Ti; Beckman Coulter, Brea, CA) at 50,000 rpm for 26 h at 4°C. Fractions were collected manually, run out by SDS–PAGE, and stained with IRDye blue protein stain (LI-COR Biosciences) for band densitometry and sedimentation coefficient calculation. Frictional ratios were calculated from $f/f_0 = R_S/(4\pi\eta\rho/3)$, where $R_S$ is the experimentally determined Stokes radius, $\rho$ is Avogadro's number, $\eta$ is the partial specific volume (taken to be 0.74 cm$^3$/g for protein), and $M$ is the calculated molecular mass.

**In vitro binding assays**

For binding of MBP-tagged E3KARPtail to ezrin-FERM, 20 μg of MBP-E3KARPtail constructs was incubated with 7 μl of ezrin FERM-CNbr bead slurry (coupled to be 2 μg/μl on the beads) in a range of 150–1000 mM NaCl, 0.1% Triton X-100, 5% glycerol, and 20 mM Tris, pH 7.4, for 1 h at 4°C. After washing, the bound protein was eluted by boiling in SDS sample buffer. Samples were run on SDS–PAGE, stained with IRDye blue protein stain, and scanned using an Odyssey infrared imaging system.

**Phylogenetic analysis**

The phylogenetic tree was generated using the database EggNOG on the metazoan clades of E3KARP (Powell et al., 2012) and the online software iTol (Letunic and Bork, 2011). All alignments were generated using Clustal Omega and processed for publication using Jalview (Waterhouse et al., 2009).

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