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Phosphorylation of ACAP1 by Akt Regulates the Stimulation-Dependent Recycling of Integrin β1 to Control Cell Migration

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Summary

Components of intracellular signaling that mediate the stimulation-dependent recycling of integrins are being identified, but key transport effectors that are the ultimate downstream targets remain unknown. ACAP1 has been shown recently to function as a transport effector in the cargo sorting of transferrin receptor (TfR) that undergoes constitutive recycling. We now show that ACAP1 also participates in the regulated recycling of integrin β1 to control cell migration. However, in contrast to TfR recycling, the role of ACAP1 in β1 recycling requires its phosphorylation by Akt, which is, in turn, regulated by a canonical signaling pathway. Disrupting the activities of either ACAP1 or Akt, or their assembly with endosomal β1, inhibits β1 recycling and cell migration. These findings advance an understanding of how integrin recycling is achieved during cell migration, and also address a basic issue of how intracellular signaling can interface with transport to achieve regulated recycling.

Introduction

Regulated endocytic recycling involves extracellular stimuli transmitted through intracellular signaling cascades to stimulate internal transport at endosomal compartments. An example of this process is the stimulation-dependent recycling of integrin β1 (Ivaska et al., 2002; Powelka et al., 2004). Integrin recycling is thought to play an important role in cell migration by redistributing integrins from the retracting edges to the migrating front (Bretscher, 1992; Lawson and Maxfield, 1995). Key components of intracellular signaling that participate in integrin recycling are being identified (Ivaska et al., 2002; Ng et al., 1999; Roberts et al., 2004; Woods et al., 2004). However, the identity of key transport effectors that represent the ultimate downstream targets in this process remains unknown.

The ADP-Ribosylation Factor (ARF) family of small GTPases initiates intracellular transport by regulating the recruitment of coat proteins and other cargo-sorting adaptors from the cytosol to membrane (Nie et al., 2003). The GTPase-activating proteins (GAPs) for these small GTPases in the better-characterized transport pathways have been shown to function not only as negative upstream regulators of ARFs, but also as their effectors, by being components of coat complexes (Kuehn et al., 1998; Yang et al., 2002). Taking advantage of this insight, we have shown recently that ACAP1, a previously identified GAP for ARF6 (Jackson et al., 2000), also functions as an effector in cargo sorting at the recycling endosome for the recycling of transferrin receptor (TfR) from this compartment (Dai et al., 2004). However, TfR is an example of a constitutively recycled cargo protein, and, thus, whether ACAP1 also participates in the cargo sorting of regulated recycling proteins remains unknown.

We now identify ACAP1 as a key transport effector in the stimulation-dependent recycling of integrin β1 and also elucidate how ACAP1 functions in this example of regulated recycling.

Results

ACAP1 Colocalizes with Endosomal β1 and Is Critical for β1 Recycling

Using an antibody binding technique, we previously showed that endocytic β1 accumulated at the recycling endosome under the starvation condition, and then recycled upon acute stimulation (Powelka et al., 2004). An alternate biotinylation-based technique showed a similar result (Powelka et al., 2004), indicating that the bound antibody did not influence the endocytic behavior of β1. Thus, using the antibody-based technique, we first determined whether β1 arrested at the recycling endosome in starved cells colocalized with ACAP1. As done previously to reveal the membrane distribution of ACAP1, ACAP1-transfected cells were permeabilized to remove the cytosol (Dai et al., 2004). Upon examination by confocal microscopy, we found that the membrane pool of ACAP1 colocalized extensively with β1 at the recycling endosome (Figure 1A).

To test whether this endosomal ACAP1 played a role in β1 recycling, we first examined whether β1 recycling was affected upon knocking down ACAP1. Using a plasmid that was shown previously to express a small interfering RNA (siRNA) against ACAP1 (Dai et al., 2004), we confirmed its efficient knockdown of endogenous ACAP1 (Figure S1A; see the Supplemental Data available with this article online). In this setting, β1 recycling was also inhibited (Figure 1B). To test whether this effect of the siRNA was specific against endogenous ACAP1, we performed a rescue experiment. In the stretch of the ACAP1 sequence targeted by the siRNA, four bases at the degenerate codon positions were mutated so that the amino acid sequence remained unchanged. When HeLa cells that stably expressed this form of ACAP1 were again treated with the siRNA, we found that total detectable ACAP1 was no longer significantly reduced (Figure 1C), and inhibition of β1 recycling that had been induced by the siRNA was also reversed (Figure 1D).

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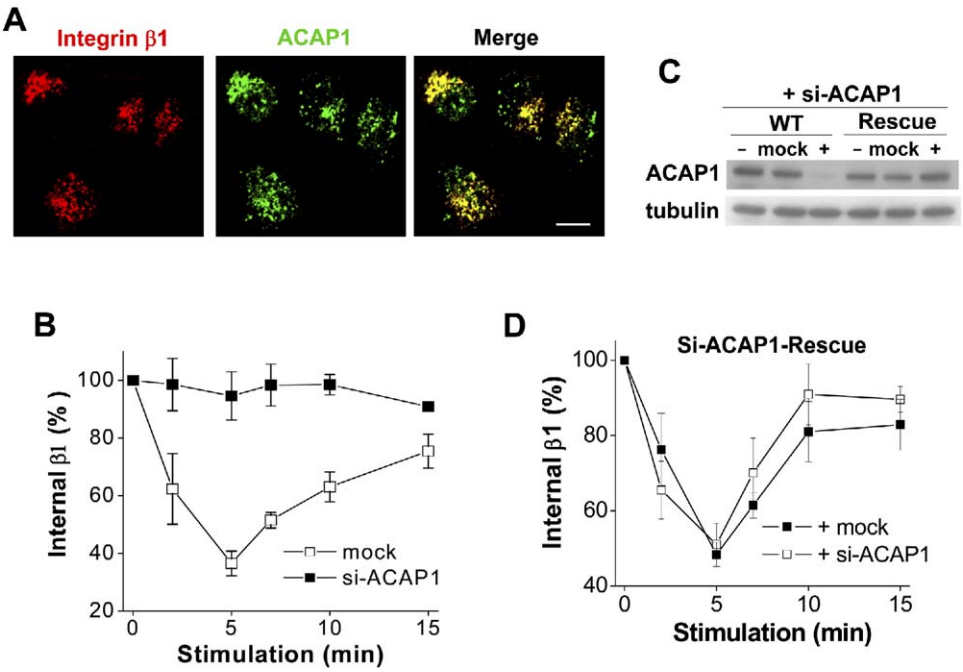

Figure 1. ACAP1 Colocalizes with Endosomal β1 and Is Critical for Its Recycling

(A) Membrane bound ACAP1 colocalizes extensively with endosomal β1. HeLa cells transiently transfected with ACAP1 were starved, and then antibody-bound surface β1 was allowed to accumulate at the recycling endosome. Cells were permeabilized and then fixed for confocal microscopy; bar, 10 μm.

(B) Knocking down of ACAP1 inhibits β1 recycling. HeLa cells, either transfected with siRNA against ACAP1 or mock transfected, were subjected to the recycling assay. The level of internal β1 at the times indicated was quantified, and then calculated as a percentage of that at the initial time point. The mean of this calculated value from three independent experiments is shown with standard error.

(C) A rescue form of ACAP1 is resistant to siRNA against endogenous ACAP1. HeLa cells that stably expressed the wild-type or rescue form of ACAP1 were treated with siRNA directed against the wild-type sequence of ACAP1.

(D) Expression of a rescue ACAP1 overcomes the inhibition of β1 recycling induced by siRNA against endogenous ACAP1. HeLa cells that stably expressed a rescue form of ACAP1 were treated with siRNA against endogenous ACAP1, and β1 recycling was examined in these cells as described in (B).

Stimulation-Dependent Association of ACAP1 with Endosomal β1

As ACAP1 had been shown recently to be critical for TfR recycling through a novel function in cargo sorting that entailed its association with TfR (Dai et al., 2004), we next examined whether ACAP1 also associated with β1 at the recycling endosome. Immunoprecipitation of endosomal β1 (gathered at the recycling endosome in starved cells) revealed a stimulation-dependent association with ACAP1 in both transiently transfected (Figure 2A) and untransfected cells (Figure 2B), indicating that ectopically expressed and endogenous ACAP1 behaved similarly in their stimulation-dependent association with endosomal β1. As control, endosomal β1 did not associate with other surface proteins that underwent recycling (Figure 2C), such as TfR and the MHC class I molecule.

Because TfR is representative of a constitutively recycling cargo protein that has been shown previously to interact with ACAP1 at the recycling endosome (Dai et al., 2004), we also examined this interaction in response to stimulation. As before (Dai et al., 2004), biotin-labeled transferrin was bound to surface TfR and then accumulated at the recycling endosome. Upon isolation with streptavidin beads, we found that the association of endosomal TfR with ACAP1 was not changed significantly by stimulation (Figure 2D).

Residues in ACAP1 Critical for Its Association with β1

As the results thus far suggested the possibility that the stimulation-dependent association of endosomal β1 with ACAP1 could explain why ACAP1 was critical for β1 recycling, we next sought to test this possibility by elucidating how stimulation regulated this association. As different kinase activities have been identified to participate in integrin recycling (Ivaska et al., 2002; Roberts et al., 2004; Woods et al., 2004), we first examined whether β1 and/or ACAP1 were phosphorylated upon acute stimulation. After orthophosphate radiolabeling, phosphorylation of endosomal β1 was not detectable, in either the starved or stimulated condition (Figure 3A). In contrast, phosphorylation of the EGF receptor (EGFR) as control (Figure 3A), and that of ACAP1 (Figure 3B), was readily seen.

However, we also noted that the total phosphorylation of ACAP1 did not change significantly upon stimulation (Figure 3B). In considering how this observation could be reconciled with a potential role for ACAP1 in β1 recycling, we considered the possibility that phos-

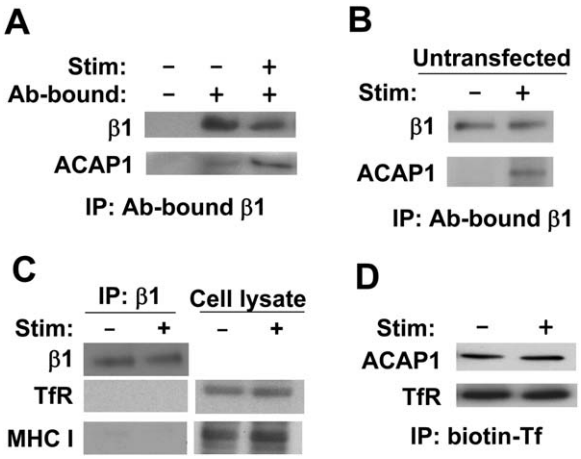

Figure 2. Stimulation-Dependent Association of ACAP1 with Endosomal β1

(A) Stimulation enhances the association of transfected ACAP1 with endosomal β1. HeLa cells transiently transfected with ACAP1 were either starved or stimulated, followed by immunoprecipitation for endosomal β1 and then immunoblotting for ACAP1. As control, the same procedure was also performed on cells whose surface β1 was not bound with antibody.

(B) Stimulation enhances the association of endogenous ACAP1 with endosomal β1. The same coprecipitation study as described in (A) was performed on untransfected HeLa cells.

(C) Endosomal β1 does not associate with other recycling proteins. The same coprecipitation study as described in (A) was performed on untransfected HeLa cells, and then immunoblotted for proteins as indicated.

(D) Stimulation does not enhance the interaction between endosomal TfR and ACAP1. HeLa cells transiently transfected with ACAP1 were starved, and then surface bound transferrin (Tf) that was biotinylated was allowed to accumulate at the recycling endosome. Cells were then continued in starvation or acutely stimulated with 20% serum. After cell lysis, streptavidin bound beads were used to bind biotinylated Tf, followed by immunoblotting for proteins as indicated.

phorylation at selective residues may provide the explanation. Using an algorithm that predicts potential phosphorylation sites based on consensus sites for the known kinases (Yaffe et al., 2001), we identified 12 candidate sites in ACAP1 (Figure 3C). To determine which of these sites may be relevant for β1 recycling, we mutated each serine/threonine residue individually to alanine and tyrosine to phenylalanine, and we then examined whether the point mutation affected the ability of ACAP1 to interact with endosomal β1. Two point mutations, serine at position 554 (S554) or serine at position 724 (S724), were found to reduce the stimulation-dependent association of ACAP1 with β1 (Figure 3D). We also examined the effect of mutating the serine residues to aspartate, which could mimic the effect of phosphorylation. These mutations resulted in enhanced interaction between ACAP1 and β1, so that it was no longer stimulation dependent (Figure 3E).

To elucidate further how mutations at key residues in ACAP1 regulated its interaction with endosomal β1, we considered two mechanistic possibilities. As ACAP1 needs to be recruited from the cytosol onto endosomal membrane to interact with the membrane bound β1, one possibility was that the mutations affected this recruitment step. Alternatively, the mutations could affect the ability of ACAP1 to interact directly with β1, after ACAP1 has been recruited onto the membrane. To test for the first possibility, we generated HeLa cells that stably expressed the different ACAP1 mutants. Cells that stably expressed the wild-type form were also generated as a control to rule out the possibility that the transfection protocol itself contributed to any potential changes noted. To distinguish the transfected ACAP1 from its endogenous pool, the transfected forms were epitope tagged, as this modification had been shown previously not to affect the different functions of ACAP1 (Dai et al., 2004). After cell permeabilization to remove the cytosol, we found that the levels of the different ACAP1 mutants on membrane were similar regardless of stimulation (Figure 4A). Thus, we concluded that the mutations did not have a significant effect on the recruitment of ACAP1 from the cytosol to membrane.

To test for the alternate possibility that these mutations on ACAP1 affected its direct interaction with β1, we used a pull-down approach, as done previously to examine the direct interaction of ACAP1 with other recycling cargo proteins (Dai et al., 2004). When the cytoplasmic domain of β1 was fused to glutathione-s-transferase (GST) and then incubated with the different mutants as recombinant proteins, we found that the aspartate mutants supported an enhanced interaction as compared to that of the alanine mutants (Figure 4B). Strikingly, no significant difference was noted when these different point mutants were incubated with beads that contained the cytoplasmic domain of TfR fused to GST (Figure 4C). Thus, we concluded that mutations at the two key residues in ACAP1 regulated its direct interaction with β1.

The Association of ACAP1 with Endosomal β1 Is Responsible for β1 Recycling

The noted effects of the ACAP1 mutants also allowed us to test more definitively whether the association of ACAP1 with endosomal β1 was responsible for β1 recycling. First, with respect to the alanine point mutants, we noted that their distribution appeared to be similar to that of the wild-type (compare Figure S2A and Figure 1A). This observation, together with the above-described finding that they could not interact efficiently with β1, suggested that they would act in a dominant-negative manner to inhibit β1 recycling by preventing endogenous ACAP1 from properly engaging β1 at the recycling endosome. Indeed, whereas β1 in cells that stably expressed the wild-type ACAP1 recycled in a stimulation-dependent manner, as seen in untransfected cells, this recycling in cells that stably expressed the alanine mutants was inhibited (Figure 4D).

Conversely, the prediction for the aspartate mutations of ACAP1, which induced the enhanced association with β1 even under the starvation condition, was that the expression of these point mutants should render β1 recycling constitutive, being no longer dependent on cell stimulation. However, because this situation would prevent the accumulation of endosomal β1, which was needed as the starting point for the biochemical assay to measure β1 recycling quantitatively, we used pri-

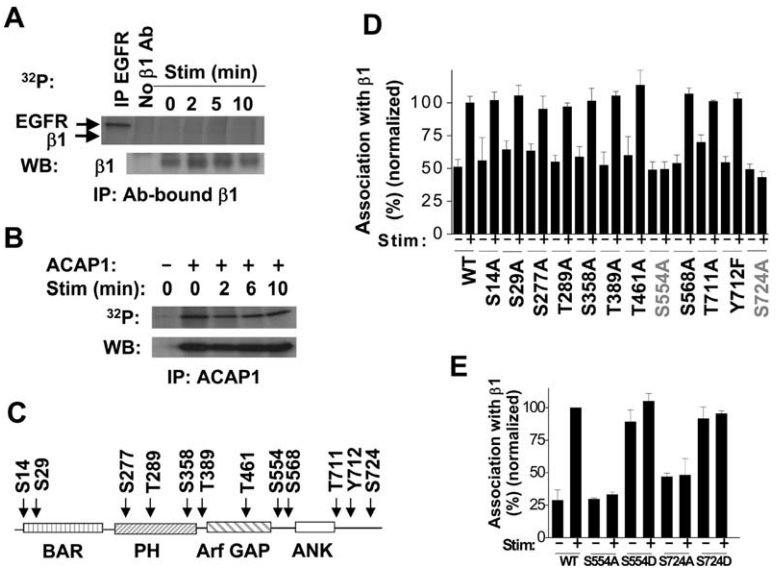

Figure 3. Phosphorylation at Critical Residues in ACAP1 Regulates Its Association with β1 to Modulate β1 Recycling

(A) Integrin β1 is not phosphorylated in either the starved or stimulated condition. Starved HeLa cells were labeled with ^{32}P-orthophosphate and then stimulated with 20% serum for the times indicated. Cells were then lysed and immunoprecipitated for proteins as indicated, followed by autoradiography (upper panel); note that the arrow for β1 indicates where it should migrate in the gel. Immunoblotting (lower panel) shows similar levels of β1 analyzed.

(B) ACAP1 is phosphorylated in both the starved and stimulated condition. Starved HeLa cells were transiently transfected with ACAP1 and then subjected to the same orthophosphate labeling as indicated in (A). Cells were then lysed and immunoprecipitated for ACAP1, followed by autoradiography (upper panel). Immunoblotting (lower panel) showed similar levels of ACAP1 analyzed.

(C) Potential sites of phosphorylation in ACAP1 based on an algorithm that detects consensus sites of known kinases. The different serine, threonine, and tyrosine sites that are potentially phosphorylated by known kinases by using a prediction algorithm are indicated, with the different functional domains of ACAP1 also shown.

(D) Point mutation to alanine at S554 or S724 in ACAP1 prevents its stimulation-enhanced association with endosomal β1. HeLa cells were transiently transfected with different point mutants of ACAP1. Endosomal β1 was then immunoprecipitated from starved or stimulated cells. The level of ACAP1 detected on beads was normalized to the level of β1, and this normalized value was then calculated as a percentage of that derived from the stimulated condition by using the wild-type ACAP1. The mean of this calculated value from three independent experiments is shown with standard error.

(E) Point mutation to aspartate at S554 or S724 in ACAP1 renders its association with endosomal β1 constitutively enhanced. The same experiment was performed as described in (D), with cells transfected with different point mutants of ACAP1 as indicated.

maquine to provide a reversible block in recycling, as done previously (Blagoveshchenskaya et al., 2002). Cells that stably expressed the different aspartate point mutants of ACAP1 were starved and then treated with primaquine to allow the accumulation of antibody bound surface β1 at the recycling endosome. We confirmed that primaquine did not reroute β1 to a different endosomal compartment, because it still colocalized with ACAP1, as assessed by confocal microscopy (Figure S2B). The recycling assay was then performed, by using the point at which primaquine was washed away as the initial time point. As predicted, β1 recycled in cells that expressed either aspartate mutations of ACAP1 even under the starvation condition (Figure 4E). In contrast, β1 did not recycle in cells that expressed wild-type ACAP1, a finding that also confirmed that treatment with primaquine did not itself alter the stimulation dependency of β1 recycling. Thus, we concluded that the association of ACAP1 with endosomal β1 was responsible for the regulated recycling of β1.

Akt Phosphorylates S554 in ACAP1

We next sought to determine more definitively whether the effects of mutations at the two key residues in ACAP1 were due to perturbations in their phosphorylation. For this goal, we used liquid chromatography tandem mass spectrometry (LC-MS/MS) (Stemmann et al., 2001) to target specifically on the phosphorylated and unphosphorylated peptides harboring S554 and S724. To analyze potential phosphorylation events in a physiologically relevant manner, we immunoprecipitated ACAP1 from acutely stimulated HeLa cells. LC-MS/MS analysis of this ACAP1 detected both phosphorylated and unphosphorylated tryptic peptides that contained the S554 residue (Figure 5A). Specifically, diagnostic ions that distinguished between phosphorylation at S554 and S547 in these peptides included a low-intensity b_{12} singly charged ion (m/z = 1254.7; Figure 5B, inset) and a low-intensity b_{14} doubly charged ion (m/z = 754.4; not shown). In addition, MS^3 fragmentation spectra of the doubly charged y_{17} ion (m/z = 966.0 ± 1.5) generated during MS^2 fragmentation of the phosphopeptide precursor measured a significant peak at m/z = 1322.6. This value correlated with the predicted mass (m/z = 1322.8) of a singly charged b_{12} ion (compare to the singly charged b_{14} ion in the MS^2 scan). Thus, these observations unambiguously identified S554 as a phosphorylation site on ACAP1.

However, in the same sample, we identified the unphosphorylated peptide containing S724 (Figure S3), but we did not detect the phosphorylated form of this peptide. We ruled out technical reasons, such as phosphorylation rendering the peptide excessively polar to prevent its detection. Thus, as we had analyzed an ACAP1 sample isolated from an in vivo condition relevant for stimulation-dependent β1 recycling, we concluded that phosphorylation explained the effects that we had observed (described above) in mutating S554, but not in mutating S724.

As corroborating evidence, we also asked which kinases that may act at either S554 or S724 of ACAP1 based on the prediction algorithm (Yaffe et al., 2001) could be detected to associate with ACAP1 in vivo. Candidate kinases for S554 in ACAP1 included Akt and

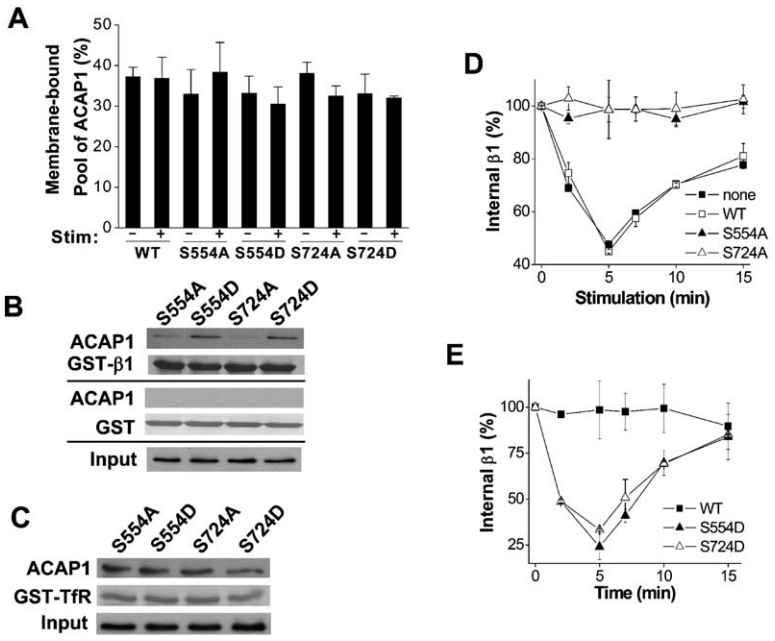

Figure 4. Direct Association of ACAP1 with β1 Responsible for the Role of ACAP1 in β1 Recycling

(A) Mutations at S554 or S724 in ACAP1 do not affect its relative distribution between membrane and cytosol. Cell lines that stably express different tagged forms of ACAP1 were either starved or stimulated and then permeabilized to derive total membranes and cytosol. Both fractions were immunoblotted for the tagged forms of ACAP1, and then the membrane fraction was calculated as a percentage of the total. The mean of this calculated value from three independent experiments is shown with standard error.

(B) Mutations at S554 or S724 in ACAP1 affect the protein interaction between ACAP1 and β1. The different forms of ACAP1 as recombinant proteins were incubated with beads that contained GST-β1, followed by immunoblotting for ACAP1 and detection of GST-β1 by Coomassie staining. As control, beads that contained only GST were subjected to a similar incubation. Input shows that the levels of recombinant ACAP1 added in different incubations were similar.

(C) Mutations at S554 or S724 in ACAP1 do not affect the protein interaction between ACAP1 and TfR. The same pull-down experiment as described in (B) was performed, except GST-TfR was used.

(D) Stable expression of either S554A or S724A inhibits stimulation-dependent recycling of β1. HeLa cells that stably expressed different forms of ACAP1 as indicated, or untransfected, were subjected to the recycling assay. The level of internal β1 at the times indicated was quantified and then calculated as a percentage of that at the initial time point. The mean of this calculated value from three independent experiments is shown with standard error.

(E) Stable expression of either S554D or S724D induces β1 recycling even in the absence of stimulation. HeLa cells that stably expressed different forms of ACAP1 were subjected to the recycling assay as described in (D). In this case, starved cells were also treated with primaquine (0.3 mM) to accumulate β1 at the recycling endosome and were then assessed for β1 recycling under continual starvation when primaquine was washed away at the times indicated.

protein kinase C (PKC) isoforms α, β, γ, and ζ, while S724 was predicted to be targeted only by casein kinase 2 (CK2). In an effort to enhance the detection of any potential interaction of the kinases with the complex of ACAP1 and endosomal β1 in acutely stimulated cells, we deliberately overexpressed each of the candidate kinases by transient transfection. Whereas Akt that was predicted to act on S554 was detected in association with endosomal β1, we did not observe significant association by the other candidate kinases (Figure 5B). Thus, we focused on characterizing how Akt acted on ACAP1.

An antibody has been developed that recognizes a consensus sequence of (K/R)X(K/R)XX(pS/pT) on substrates phosphorylated by Akt, where either S or T must be phosphorylated (Manning et al., 2002). Using this antibody, previously designated as Akt-pSub antibody (Manning et al., 2002), we found that it recognized recombinant wild-type ACAP1 only after incubation with purified active Akt in an in vitro kinase assay (Figure 5C). As ACAP1 contained two residues (T461 and S554) that are potential consensus sites for Akt, based on the prediction algorithm, we also examined the effect of the S554A mutation. Because the Akt-pSub antibody did not recognize this mutant after the incubation (Figure 5C), we concluded that Akt only phosphorylated S554 in ACAP1.

Consistent with Akt acting on ACAP1, we detected endosomal β1 to assemble with ACAP1 and Akt upon acute stimulation in vivo, and we also found that ACAP1 in this complex was recognized by the Akt-pSub antibody (Figure 5D). However, a potential caveat of the results thus far using the Akt-pSub antibody was that its recognition of ACAP1 might not reflect phosphorylation specifically by Akt. Thus, to provide further evidence that S554 of ACAP1 was targeted by Akt rather than another kinase that might recognize a similar consensus sequence, we examined the effect of knocking down Akt. Using a siRNA previously shown to target sequences common to both Akt1 and Akt2 (Katome et al., 2003), we first confirmed efficient reduction of endogenous Akt in HeLa cells by this siRNA (Figure S1B). In these cells, ACAP1 was no longer recognized by the Akt-pSub antibody upon acute stimulation (Figure 6A). Moreover, immunoprecipitation of endosomal β1 from these treated cells revealed that it no longer formed a complex with ACAP1 and Akt upon stimulation (Figure 6B), and β1 recycling in these cells were also inhibited (Figure 6C). As Akt contributes to cell survival, we also ruled out that the observed effects in siRNA-treated cells were due to apoptosis (Figure S4). Taken together, these results provided further evidence that the recognition of ACAP1 by the Akt-pSub antibody represented phosphorylation by Akt rather than by another kinase.

Regulation of Akt Phosphorylation on ACAP1

We also sought an alternate explanation for how mutations at S724 in ACAP1 affected its interaction with β1, in light of the failure to detect its phosphorylation under

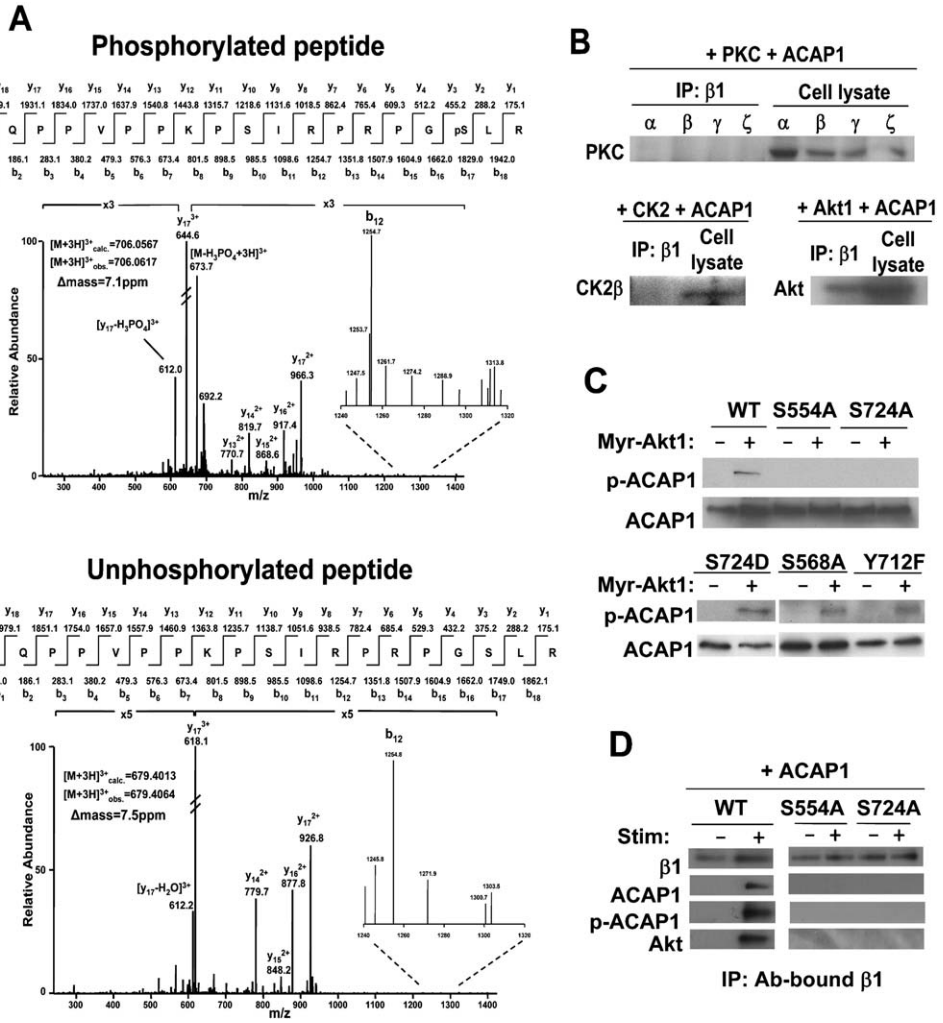

Figure 5. Akt Phosphorylates S554 in ACAP1

(A) LC-MS/MS identifies S554 as an in vivo phosphorylation site on ACAP1. ACAP1 transiently transfected in HeLa cells that had been acutely stimulated was immunoprecipitated and then subjected to trypsin digestion, followed by LC-MS/MS analysis, by using a method to fragment the top ten most intense ions in each precursor scan and also a targeted method to fragment the phosphorylated and unphosphorylated peptides.

(B) Akt associates with endosomal β1 in acutely stimulated cells. HeLa cells were transiently transfected with tagged forms of the different kinases as indicated, and then endosomal β1 was immunoprecipitated, followed by immunoblotting for proteins as indicated. As control, direct immunoblotting of whole-cell lysates (5% of total used in the immunoprecipitation studies) indicates detectable levels of different transfected kinases.

(C) Phosphorylation of S554 in ACAP1 by Akt. Different forms of recombinant soluble ACAP1 as indicated were incubated individually with a constitutively active form of Akt for an in vitro kinase assay. Afterwards, Western blotting was performed by using either an anti-ACAP1 antibody (indicated as ACAP1) or the Akt-pSub antibody (indicated as p-ACAP1).

(D) Acute stimulation induces ACAP1 and Akt to assemble with endosomal β1. HeLa cells were transiently transfected with different forms of ACAP1 as indicated, and then endosomal β1 was immunoprecipitated, followed by immunoblotting for proteins as indicated.

the relevant in vivo condition. In the in vitro kinase assay, incubation of the S724A mutant with Akt did not result in recognition by the Akt-pSub antibody, while a similar incubation with the S724D mutant allowed for this recognition (Figure 5C). To provide evidence that the observed effect of the S724A mutant was specific, we examined two additional alanine mutants, S568A (which is near S554A) and Y712F (which is near S724A), and found that they were also recognized by the Akt-pSub antibody after incubation with Akt (Figure 5C). Thus, we concluded that the S724A mutation in ACAP1 had a specific effect in preventing Akt from acting on the S554 site. Consistent with this explanation, the stimulation-dependent assembly of endosomal β1 with ACAP1 and Akt in stimulated cells was inhibited not only by the S554A mutation but also by the S724A mutation (Figure 5D).

To gain insight into how intracellular signaling might modulate the ability of Akt to act on ACAP1, we also examined the role of a canonical signaling pathway that has been well characterized to regulate Akt. Activation of Akt requires that it binds to specific phosphoinositides

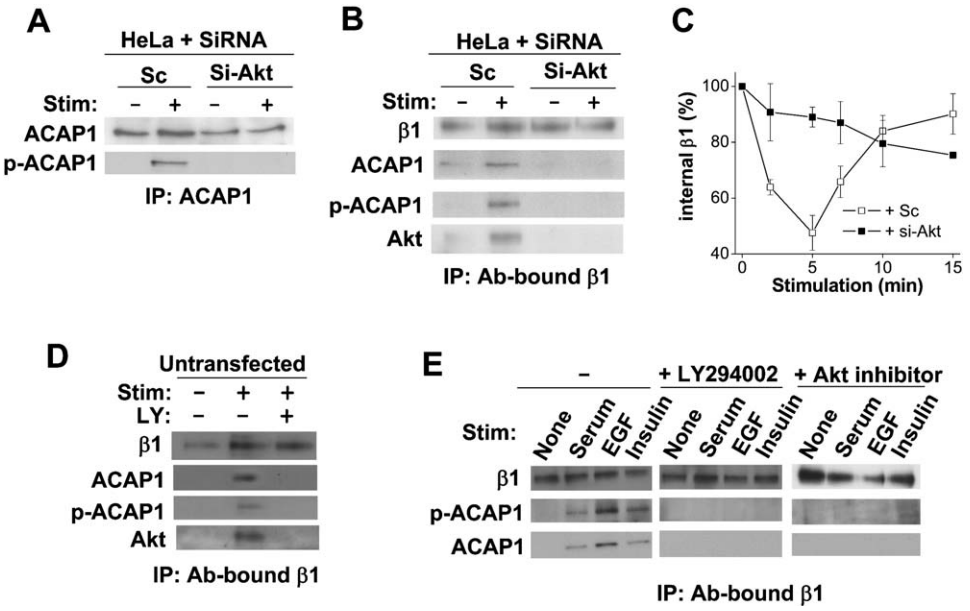

Figure 6. A Critical Role for Phosphorylation of ACAP1 by Akt in the Assembly of an Endosomal β1 Complex that Mediates β1 Recycling

(A) Knocking down Akt abolishes phosphorylation of ACAP1 induced by acute stimulation. HeLa cells were treated with siRNA against Akt, followed by immunoprecipitation for ACAP1 and then immunoblotting with either the anti-ACAP1 antibody or the Akt-pSub antibody.

(B) Knocking down Akt prevents the assembly of endosomal β1 with ACAP1. HeLa cells were treated with siRNA against Akt, followed by immunoprecipitation for endosomal β1 and then immunoblotting for proteins as indicated.

(C) Knocking down Akt inhibits β1 recycling. HeLa cells treated with siRNA against Akt or a scrambled siRNA were subjected to the recycling assay. The level of internal β1 at the times indicated was quantified and then calculated as a percentage of that at the initial time point. The mean of this calculated value from three independent experiments is shown with standard error.

(D) The stimulation-dependent assembly of endosomal β1 with endogenous ACAP1 and Akt requires PI3K activity. HeLa cells were subjected to different conditions as indicated, and then endosomal β1 was immunoprecipitated, followed by immunoblotting for proteins as indicated. LY294002 was used at 50 uM.

(E) Addition of serum, EGF, or insulin enhances the interaction of endosomal β1 with ACAP1, which is blocked by inhibitors of PI3K and Akt. HeLa cells were subjected to different conditions as indicated, and then endosomal β1 was immunoprecipitated, followed by immunoblotting for proteins as indicated. The following concentrations were used: serum, 20%; EGF, 0.1 μg/ml; insulin, 1 μg/ml; LY294002, 50 μM; and Akt inhibitor, 20 μM.

that are the product of phosphatidylinositol 3-phosphate kinase (PI3K), and, thus, inhibitors of PI3K activity can be used to block Akt activity (Burgering and Coffer, 1995). When cells were first pretreated with one of these inhibitors, LY294002, we found that acute stimulation could no longer induce the formation of endosomal β1 with ACAP1 and Akt (Figure 6D). Notably, this result was observed in untransfected cells, indicating that the previously observed complex formation for transfected proteins (Figure 5D) also occurred for endogenous proteins (Figure 6D).

We also sought to identify upstream cell surface receptors that are stimulated upon the addition of serum in our stimulation protocol. Specific ligands that bind either the insulin receptor or the EGF receptor have been shown to be upstream of the PI3K/Akt signaling pathway (Burgering and Coffer, 1995). We found that the addition of these ligands induced the formation of endosomal β1 with ACAP1 and Akt, and that this complex formation was inhibited in the presence of either LY294002 or another Akt inhibitor, a phosphatidylinositol analog that binds to the pleckstrin homology domain of Akt to inhibit its activation by phosphorylation (Hu et al., 2000) (Figure 6E). Taken together, these results revealed that a canonical signaling pathway well known to regulate Akt activation was also involved in modulating the role of Akt in regulating ACAP1.

A Critical Role for ACAP1, Akt, and Their Assembly with Endosomal β1 in Cell Migration

Finally, as integrin recycling and Akt have both been implicated in cell migration (Brazil and Hemmings, 2001; Webb et al., 2002), we tested whether perturbing the ability of Akt to act on ACAP1 in modulating β1 recycling would affect cell migration. For this purpose, we examined two assays of cell migration. In one assay, migration was measured across Transwell membrane coated with fibronectin to assess β1-dependent movement, as HeLa cells express mainly β1 integrins to mediate adhesion to this matrix substance (Shaw et al., 1995). Initially, examining the roles of Akt (Figure 7A) and ACAP1 (upper panel of Figure 7B), we found that cells treated with siRNA against either target inhibited migration. Subsequently, to disrupt more specifically the association of endosomal β1 with ACAP1, we examined HeLa cells that stably expressed either the S554A or the S724A point mutants, and we found that these cells also exhibited reduced cell migration (Figure 7C). Confirming these results by a different migration assay that examines wound healing, we found that

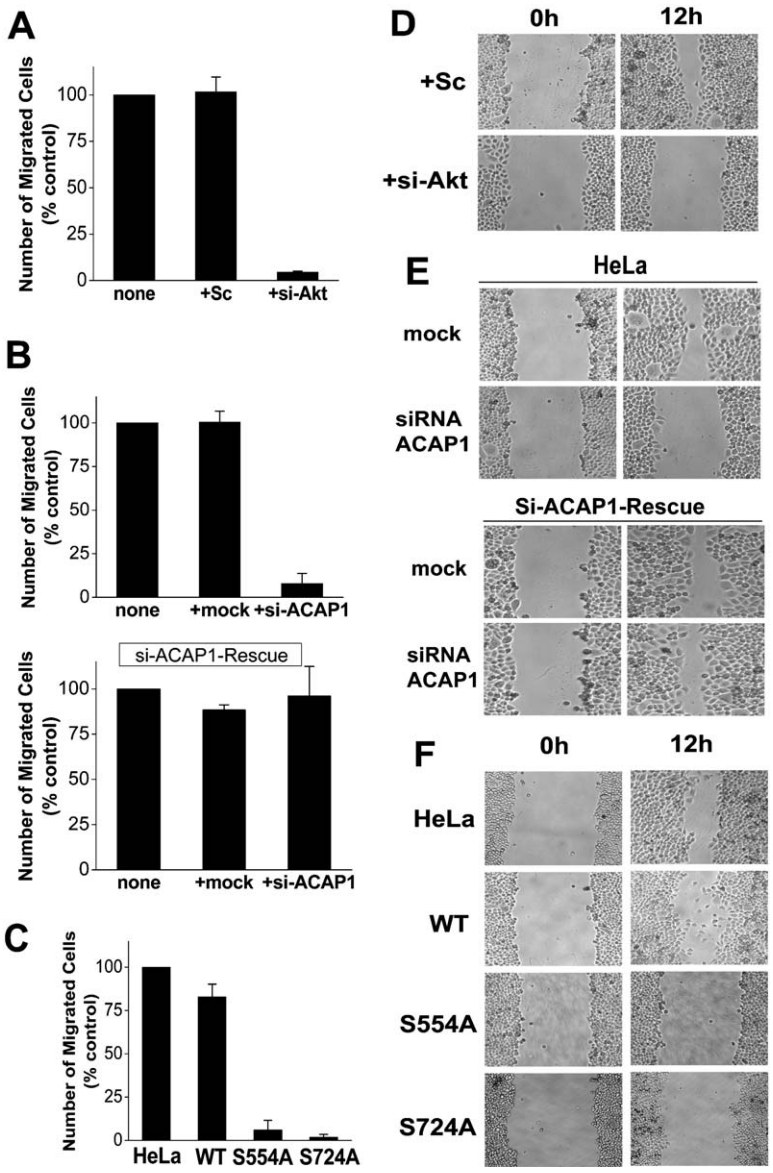

Figure 7. A Critical Role for ACAP1, Akt, and Their Assembly into an Endosomal Complex with β1 in Cell Migration

(A) Knocking down Akt inhibits migration across Transwell membrane coated with fibronectin. HeLa cells treated with siRNA against Akt or a scrambled siRNA were examined in a migration assay across Transwell membrane coated with fibronectin. The number of migrating cells was compared to the condition of no treatment and then expressed as a percentage. The mean with standard error was then derived from three independent experiments.

(B) Knocking down ACAP1 inhibits migration across Transwell membrane coated with fibronectin. (Upper panel) HeLa cells transfected with a plasmid that expressed siRNA against ACAP1, or a scrambled siRNA, or not transfected were examined in a migration assay across Transwell membrane coated with fibronectin. The results are expressed as described in (A). (Bottom panel) HeLa cells that stably expressed a rescue form of ACAP1 were examined by the same experiment described in the upper panel.

(C) Expression of either the S554A or the S724A mutant inhibits migration across Transwell membrane coated with fibronectin. HeLa cells that stably expressed the different point mutants of ACAP1 as indicated were examined in a migration assay across Transwell membrane coated with fibronectin. The results were expressed as described in (A).

(D) Knocking down Akt inhibits wound-healing migration. HeLa cells treated with siRNA against Akt or a scrambled siRNA were examined in the wound-healing assay. Results are representative of three independent experiments.

(E) Knocking down ACAP1 inhibits wound-healing migration. (Upper panel) HeLa cells transfected with a plasmid that expressed siRNA against ACAP1, or a scrambled siRNA, or not transfected were examined in the wound-healing assay. (Bottom panel) HeLa cells that stably expressed a rescue form of ACAP1 were examined by the same experiment described in the upper panel. Both results are representative of three independent experiments.

(F) Expression of either the S554A or the S724A mutant inhibits wound-healing migration. HeLa cells that stably expressed the different point mutants of ACAP1 as indicated were examined in the wound-healing assay. Results are representative of three independent experiments.

siRNA against Akt (Figure 7D) or ACAP1 (upper panel of Figure 7E), or the ectopic expression of the S554A or the S724A mutant of ACAP1 (Figure 7F), also inhibited cell migration. Moreover, in both migration assays, we confirmed the specificity of the siRNA against ACAP1, as the stable expression of the rescue construct reversed the effects seen by the treatment of siRNA against endogenous ACAP1 (lower panels of Figures 7B and 7E).

Discussion

We have elucidated how ACAP1 functions in the regulated recycling of integrin β1. Taking advantage of the previous insight that ACAP1 functions not only as a GAP for ARF6, but also as its effector in cargo sorting (Dai et al., 2004), we first show that ACAP1 associates with β1 at the recycling endosome in a stimulation-dependent manner. Investigating how external stimulation regulates this association, we identify two key residues in ACAP1 and then elucidate their roles. For S554, its role is due to phosphorylation by Akt, which is, in turn, regulated by a canonical signaling pathway instigated by surface growth factor receptors. For S724, our findings suggest an alternate mechanism that modulates the ability of ACAP1 to be engaged by Akt at the S554 site (which will be discussed in more detail below). These observations then allowed us to show that the assembly of ACAP1 and Akt with endosomal β1 mediates β1 recycling during cell migration.

Besides having identified a key transport effector that represents the ultimate downstream target in integrin recycling, our findings also contribute to a general

understanding of how a transport effector can mediate both constitutive and regulated transport, as we provide a mechanistic explanation for how ACAP1 is able to participate in the cargo sorting of both constitutively recycling proteins and regulated recycling proteins. In this explanation, we have provided an unexpected insight. Initial analysis of how mutations at two key residues in ACAP1 affect its association with β1 reveals similar effects, leading to the impression that both residues would exert their roles through phosphorylation at these sites. However, using mass spectrometry to identify phosphorylation sites more definitively, we detect phosphorylation only at S554, when ACAP1 is isolated from an in vivo condition relevant for β1 recycling. Thus, while the mutational effects at S554 are explained by phosphorylation at this site, the mutational effects at S724 require an alternate mechanism.

In considering a plausible explanation, we are led by the consideration that mutation from serine to aspartate should render a residue more hydrophilic, while mutation from serine to alanine should have the opposite effect of causing the residue to be more hydrophobic. Thus, an intriguing possibility is that serine at position 724 supports a conformation of ACAP1 that is intermediate in the spectrum of two extremes, with one end being a conformation that would result in a high-affinity interaction between ACAP1 and β1 (represented by the effect of mutating to aspartate) and the other end being a conformation that would result in either no or low-affinity interaction between ACAP1 and β1 (represented by the effect of mutating to alanine). Such a mechanism would also readily explain some key observations that we have made. First, one can readily envision how phosphorylation at S554 in ACAP1 would regulate a change in the conformation of ACAP1 to modulate its interaction with β1. Second, one can envision a conformational change in ACAP1 that is sufficiently global so that it affects not only binding to β1, but also access to its S554 site by Akt. This scenario can, in turn, explain why mutation at S724 affects phosphorylation of S554 by Akt, and also why single mutations at either S554 or S724 would have a dominant effect on the association of ACAP1 with β1.

Finally, we note that an important implication of our cumulative findings on ACAP1 as a cargo-sorting device is that this role will be relevant for a broad range of cellular activities that are known to involve endocytic recycling. Besides cell migration, which itself underlies a wide range of physiologic and pathologic events, other important examples that require endocytic recycling include insulin-stimulated recycling of glucose transporters, cell polarity, cytokinesis, and phagocytosis. Thus, the future investigation of a potential role for ACAP1 in these examples will likely contribute to a better mechanistic understanding of how these events are achieved.

Experimental Procedures

Reagents and Cells

Reagents obtained include: EGF, insulin, biotinylated transferrin, and LY294002 (all from Sigma; St. Louis, MO), and the Akt inhibitor, 1L-6-hydroxymethyl-chiro-inositol [2-(R)-2-*O*-methyl]-3-*O*-octadecylcarbonate (from Calbiochem; La Jolla, CA). HeLa cells were cultured as previously described (Dai et al., 2004).

Antibodies

Antibodies have been described previously (Dai et al., 2004; Jackson et al., 2000; Powelka et al., 2004), and they include: TS2/16 against β1 integrin, W6/32 against MHC I, M3A5 against β-COP, H68.4 against TfR, 9E10 against the Myc epitope, 15E6 against C-terminally-tagged HA epitope, M2 against the FLAG epitope, antiserum against ACAP1 peptide, antiserum against ACAP1 whole protein, antiserum against cellubrevin, and secondary antibodies conjugated to Cy2 or Cy3.

Additional antibodies used include: DM1α against α-tubulin (Sigma; St. Louis, MO), the Akt-pSub antibody (Cell Signaling Technology; Beverly, MA), and antisera against Akt, the CK2-β subunit, and PKC (recognizing all isoforms) (all from Santa Cruz Biotechnology; Santa Cruz, CA).

Plasmids and Transfections

Flag-tagged ACAP1 in pFlag-CMV2 has been described (Jackson et al., 2000). The following plasmids were obtained: PKCα, PKCβ, Flag-tagged PKCγ, and Myc-tagged PKCζ (from A. Newton, University of California, San Diego, CA), HA-tagged Akt and the constitutively active myristoylated Akt (from M. Birnbaum, University of Pennsylvania, Philadelphia, PA), and CK2 α, α′, and β subunits (from D. Litchfield, University of Western Ontario, London, Ontario, Canada).

Myc-tagged ACAP1 in pcDNA 3.1 was generated by subcloning the coding sequence of ACAP1, which was generated through the polymerase chain reaction. Point mutants and the rescue form (generated by mutating the nucleotides 276–279 in the siRNA-targeted DNA sequence of ACAP1 from CAGC to TTCG) of ACAP1 were generated by using QuickChange XL Site-Directed Mutagenesis (Strategene; La Jolla, CA). To append the cytoplasmic domain of β1 to the carboxy terminus of GST, the cDNA encoding different domains was amplified by PCR, and then subcloned into the BamH1 and EcoRI sites of the pGEX-4T-3 vector (Amersham Pharmacia Biotech; Piscataway, NJ).

Transient transfections were performed by using Fugene 6 (Roche Biochemicals; Indianapolis, IN). HeLa cell lines that stably express the different forms of ACAP1 were generated by transient transfection, followed by selection in 1 mg/ml G418 (Life Technologies, Inc.; Gaithersburg, MD).

siRNA

Expression of siRNA against ACAP1 with the pSUPER plasmid has been described (Dai et al., 2004). For siRNA against Akt, a sequence (TGCCCTTCTACAACCAGGA) that is common to both Akt1 (nucleotides 1040–1058) and Akt2 (nucleotides 1043–1061) (Katome et al., 2003), along with a scrambled sequence as control, were obtained (Ambion; Austin, TX), and then transfected with Oligofactamine (Invitrogen; Carlsbad, CA).

Confocal Microscopy

Colocalization studies were done as previously described (Dai et al., 2004).

β1 Recycling Assay

The endocytic recycling of β1 was assessed by using a previously established biochemical assay for recycling proteins (Blagoveshchenskaya et al., 2002), which was adapted to examine β1 recycling as previously described (Powelka et al., 2004). Unless indicated otherwise, stimulation was performed by adding 20% serum to cells that had been starved overnight.

Immunoprecipitation of Endosomal β1

Immunoprecipitation of endosomal β1 was performed as follows. Surface β1 bound to the anti-β1 antibody TS2/16 was accumulated at the recycling endosome in starved cells as previously described (Powelka et al., 2004). Cells were then either continued in starvation or acutely stimulated for 2 min with 20% serum (or specific agent as stated otherwise), cooled to 4°C, followed by lysis in a Triton buffer (consisting of 1% Triton X-100, 20 mM Tris [pH 7.4], and 300 mM NaCl) and incubation with Sepharose beads coated with protein A (Amersham Pharmacia Biotech; Piscataway, NJ). Beads were then washed three times with the Triton lysis buffer and analyzed by SDS-PAGE under nonreducing conditions.

Pull-Down Assay

Pull-down assays were performed essentially as previously described (Dai et al., 2004), except different forms of recombinant ACAP1 were expressed as myc-tagged proteins by using the TNT T7 coupled reticulocyte lysate system (Promega; Madison, WI), and then purified by binding to anti-myc antibody on beads, followed by elution from beads by adding excess soluble myc peptide (Sigma; St. Louis, MO).

In Vitro Kinase Assay

The in vitro kinase assay was performed essentially as previously described (Zheng et al., 2000). Recombinant forms of purified ACAP1 were first dephosphorylated with calf intestinal phosphatase prior to use in the kinase assay. Because Akt requires multiple phosphorylation for its activation (Brazil and Hemmings, 2001), we purified active Akt by transfecting a construct that encodes an HA-tagged form of a constitutively active Akt (by the addition of a myristoylation signal [Kohn et al., 1996]) in Hela cells, followed by immunoprecipitation with an anti-HA antibody.

Orthophosphate Labeling

HeLa cells were starved overnight in DMEM with 0.01% bovine serum albumin (BSA, Sigma; St. Louis, MO), followed by incubation in 1 ml of the same medium with 2 mCi ^{32}P-labeled ATP added for each 100 mm plate at 37°C for 2 hr. Cells were then stimulated with 20% serum that had been prewarmed to 37°C. At different time points as indicated, cells were lysed in the Triton buffer with phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 mM β-glycerophosphate) for analysis by immunoprecipitation as described above.

LC-MS/MS

The general procedure was performed as previously described (Ballif et al., 2005). Digestion was allowed to proceed for 100 min at 37°C. Longer digests resulted in cleavage C-terminal to Arg at Arg-Pro sites near S554 and made even the unphosphorylated form of the peptide harboring S554 undetectable. Precursor scans were performed in the FTICR cell at 100,000 resolution and an AGC setting of two million. Targeted MS^2 scans (m/z = 706.1 ± 1.5 and m/z = 679.4 ± 1.5) and a targeted MS^3 scan (m/z = 966.0 ± 1.5 generated from fragmentation of the 706.1 precursor) were performed in the ion trap mass spectrometer by using one microscan and a 500 ms fill time. MS^2 spectra were searched against the protein sequence for human ACAP1 by using SEQUEST and a mass tolerance of 1.1 Da with no enzyme specificity.

Apoptosis Assay

Cells were stained with PE-conjugated Annexin V according to the manufacturer's protocol for adherent cells (BD Pharmingen; San Diego, CA).

Migration Assays

Migration across membrane in a Transwell chamber (6.5 mm insert diameter, 8 μm pore size, polycarbonate membrane; Corning Glassworks; Corning, NY) was assessed by first coating both sides of the membrane with 10 μg/ml fibronectin in PBS overnight at 4°C and then blocked with 1% BSA in DMEM for 1 hr at 37°C. Cells starved in DMEM with 0.01% BSA were then stimulated by adding 20% fetal bovine serum, were then plated (1×10^5 cells in 100 μl) to the top chamber, and were allowed to migrate for 4 hr at 37°C. Membranes were fixed in 4% paraformaldehyde for 15 min. Cells remaining on the top side of the membrane were removed by using a cotton swab. The remaining cells on the bottom side were stained with propidium iodide (Sigma; St. Louis, MO) and then counted by using fluorescence microscopy with a 10× objective of a Nikon Optiphot2 microscope (Nikon Instruments; Melville, NY).

For the wound-healing migration assay, confluent cells on coverslips were starved overnight in DMEM with 0.01% BSA. After marring coverslips with a linear scratch by a sterile pipette tip, cells were stimulated with 20% fetal bovine serum at 37°C for the times indicated. Images were taken with the 4× objective of a Nikon TE2000 inverted microscope coupled to a CCD-SPOT RT digital camera (Nikon Instruments; Melville, NY).

Supplemental Data

Supplemental Data contain additional experimental results, including knockdown by siRNA, distribution of ACAP1 mutants by immunofluorescence microscopy, mass spectrometry of the ACAP1 peptide that contains the S724 residue, and assessment for apoptosis upon knocking down Akt. These results are available at http://www.developmentalcell.com/cgi/content/full/9/5/663/DC1/.

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