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CISK attenuates degradation of the chemokine receptor CXCR4 via the ubiquitin ligase AIP4

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HER2 overexpression in cancers causes hyperactivation of the PI 3-kinase pathway and elevated levels of the chemokine receptor CXCR4, which is strongly associated with increased metastatic potential. Here, we provide evidence that the cytokine-independent survival kinase CISK is activated downstream of the PI 3-kinase-dependent kinase PDK1 on endosomes and negatively regulates the lysosomal degradation of CXCR4. We demonstrate that CISK prevents CXCR4 degradation by inhibiting sorting of the receptor from early endosomes to lysosomes. In contrast, CISK does not interfere with ligand-induced degradation of epidermal growth factor receptors. CISK strongly interacts and colocalizes with the E3 ubiquitin ligase AIP4, which is important for the ubiquitin-dependent lysosomal degradation of CXCR4. Moreover, the observed inhibition is both dependent on the interaction between CISK and AIP4 and on the activation status of CISK. Consistent with this, an activated form of CISK but not of the related kinase SGK1 phosphorylates specific sites of AIP4 *in vitro*. Taken together, these results reveal a critical function of CISK in specifically attenuating ubiquitin-dependent degradation of CXCR4, and provide a mechanistic link between the PI 3-kinase pathway and CXCR4 stability.

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Introduction

Chemokines are a small group of low-molecular weight proteins that signal through 7-transmembrane G protein-coupled receptors (GPCRs) to mediate a multitude of cellular functions related to development, leukocyte trafficking, angiogenesis, and immune responses (Bleul *et al*, 1996; Melchers *et al*, 1999). The binding of the chemokine CXCL12/SDF-1α to its receptor CXCR4 induces the activation of multiple signalling cascades, and has been shown to play a crucial role in embryonic development, lymphocyte maturation, and cell migration (Zou *et al*, 1998; Moser and Loetscher, 2001). In addition, CXCR4 has been implicated in several diseases such as asthma, HIV infection, and cancers, suggesting that proper control of the level of activated receptor is essential for accurate activation of downstream signalling events and physiological output (Scarlatti *et al*, 1997; Muller *et al*, 2001; Marchese *et al*, 2003a).

To avoid prolonged activation of the receptors, GPCR complexes are endocytosed and either recycled back to the plasma membrane or sorted into the degradative pathway (Sorkin and Von Zastrow, 2002; Marchese *et al*, 2003a). A ubiquitin-associated system is crucial in regulating these processes and involves the conjugation of ubiquitin onto target proteins destined for degradation, mediated by a family of proteins called E3 ubiquitin ligases (Haglund and Dikic, 2005). A well-characterized example is the agonist-dependent degradation of CXCR4, in which ubiquitination mediated by the E3 ubiquitin ligase AIP4 has been shown to be required at multiple steps in the sorting process (Marchese *et al*, 2003a, b).

Recent studies have revealed overexpression of CXCR4 in colorectal, breast, and non-small cell lung cancers, and elevated CXCR4 levels are strongly associated with increased metastatic potential (Balkwill, 2004; Li *et al*, 2004). The overexpression of CXCR4 in cancer has been found to be mediated via overexpression of HER2, a member of the epidermal growth factor receptor family of RTKs (Benovic and Marchese, 2004; Li *et al*, 2004). HER2 activates the phosphoinositide (PI) 3-kinase pathway, causing enhanced translation of CXCR4 mRNA via activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt/PKB, and their downstream targets. In addition, HER2 inhibits ubiquitin-dependent lysosomal degradation of CXCR4 by a mechanism that remains to be characterized in detail (Benovic and Marchese, 2004; Li *et al*, 2004).

Together with protein kinase C, serum and glucorticoid regulated kinase (SGK), and cytokine independent survival kinase (CISK/SGKL), Akt/PKB belongs to the 'AGC' subfamily of kinases that are activated downstream of PI 3-kinase and play crucial roles in regulating physiological processes relevant to metabolism, growth, proliferation, and survival (Vanhaesebroeck and Alessi, 2000; Mora *et al*, 2004). CISK is the most recently described member of this family and was first identified as an antiapoptotic factor in a screen for IL-3 dependent survival factors (Liu *et al*, 2000). Consistent with the observation that the kinase domain of CISK is very similar to those of Akt and SGK1, CISK becomes activated upon growth factor stimulation and thus becomes able to phosphorylate the same downstream targets such as Bad and FKHRL-1 (Liu *et al*, 2000; Xu *et al*, 2001). The functional

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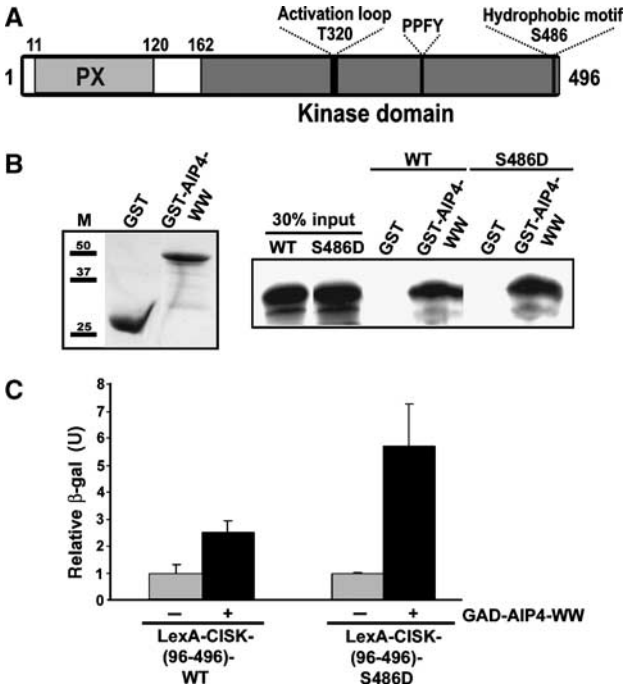

Figure 1 CISK binds the WW domain of the ubiquitin ligase AIP4. (**A**) Domain structure of CISK. The lipid-binding PX-domain and the kinase domain of CISK are shown and the conserved T320 and S486 residues in the activation- and hydrophobic loops, respectively, are indicated. In addition, the PPFY-motif identified in the kinase domain of CISK is shown. (**B**) The kinase domain of CISK interacts with the WW domain of AIP4 *in vitro*. GST-pulldown assay was performed. The amount of input of each protein is indicated. (**C**) CISK interacts with AIP4 in the yeast two-hybrid system. The values indicate β-galactosidase activities presented as fold reporter activation.

importance of this overlapping substrate specificity has, however, been questioned, given that CISK, in contrast to Akt and SGK1, is localized on endosomes via its PX-domain (Liu *et al*, 2000; Virbasius *et al*, 2001; Nilsen *et al*, 2004).

In search of a possible mechanistic link between PI 3-kinase signalling and inhibition of CXCR4 degradation, we hypothesized that CISK could play a role in regulating the latter process. In this paper, we show evidence that CISK controls the endosomal sorting of CXCR4 by regulating the function of AIP4.

Results

CISK interacts with the WW-domains of the E3 ubiquitin ligase AIP4

Previous studies have shown that ligand binding induces endocytosis and lysosomal degradation of CXCR4 (Marchese and Benovic, 2001). Sorting of the receptor from early endosomes to lysosomes requires AIP4-mediated ubiquitination of the receptor, and the ubiquitin-binding endosomal protein Hrs (Marchese *et al*, 2003a, b). Hrs is thought to mediate sorting of ubiquitinated membrane proteins into the intraluminal vesicles of multivesicular endosomes (MVEs) (Lloyd *et al*, 2002; Raiborg *et al*, 2002). Consistent with the idea that endocytosed CXCR4 is targeted into the MVE pathway, we have found by using electron microscopy that CXCR4 is located inside MVEs upon CXCL12 stimulation (see Supplementary Results and Figure S1). Because AIP4 mediates degradation of CXCR4, we considered this E3 ubiquitin ligase as a possible candidate for regulation of CXCR4 levels.

In light of the recent observation that SGK1 regulates the activity of ubiquitin ligases at the plasma membrane (Debonneville *et al*, 2001; Snyder *et al*, 2002), we asked whether the related endosomal kinase CISK might similarly regulate AIP4 on endosomes (Marchese *et al*, 2003a, b; Angers *et al*, 2004). Especially important for the activity of the AGC family of kinases is the C-terminal hydrophobic motif (HM) required for PDK1 recruitment, and the activation loop that is phosphorylated by PDK1 upon binding (Figure 1A) (Frodin *et al*, 2002; Sarbassov *et al*, 2005). Although the kinase(s) that is responsible for phosphorylating the HM is not yet fully characterized, recent studies have shown that this event is PI 3-kinase dependent (Dong and Liu, 2005). Based on sequence analyses and previous studies of other AGC kinases, T320 (in the activation loop) and S486 (in the HM) have been suggested to be required for CISK activation (Liu *et al*, 2000; Virbasius *et al*, 2001; Nilsen *et al*, 2004). In contrast to other AGC family members such as Akt and SGK, CISK harbours an N-terminal PX-domain that has been shown to be required for its endosomal localization (Figure 1A). We noted that CISK contains a PPFY motif in the kinase domain, a motif typically recognized by WW-domains (Figure 1A) that are found in all members of the HECT-ubiquitin ligase family such as Nedd4 and AIP4 (Sudol *et al*, 1995; Ingham *et al*, 2004). We therefore performed protein–protein interaction studies to test if CISK could bind AIP4. To this end we made a GST fusion protein of the AIP4 WW-domains and incubated the immobilized protein with the *in vitro* translated ^{35}S-labelled kinase domain of wild-type (WT) CISK or S486D, a mutant that mimics S486 phosphorylation. As shown in Figure 1B, we found that both CISK WT and S486D strongly interacted with GST-AIP4 WW compared to background binding to GST. These findings were confirmed in the yeast two-hybrid interaction assay (Figure 1C), in which the presence of both CISK and AIP4-WW were required for strong activation of the β-galactosidase reporter. In these interaction studies, we could detect a somewhat stronger binding of CISK S486D compared to CISK WT. This suggests that the binding between CISK and the WW-domains of AIP4 may be enhanced by phosphorylation.

CISK associates with AIP4 on early endosomes

To address whether CISK and AIP4 are located on the same cellular structures, we expressed green fluorescent protein (GFP)-tagged CISK S486D and AIP4 in HeLa cells and stained the cells with antibodies against the early-endosomal marker EEA1. As previously described, we found that CISK mainly was present on EEA1 positive structures (Figure 2A, C, and D) (Liu *et al*, 2000; Virbasius *et al*, 2001; Nilsen *et al*, 2004). A similar pattern was observed for AIP4, although AIP4 to a larger extent than CISK was also detected in other compartments (Figure 2B, C, and E). More importantly, we observed extensive colocalization between CISK and AIP4 on the early endosomes (Figure 2F).

In light of the observed interaction and co-localization of CISK and AIP4, we performed immunoprecipitation studies to check if these proteins also could associate *in vivo*. To this end we transfected HeLa cells with GFP-CISK WT and

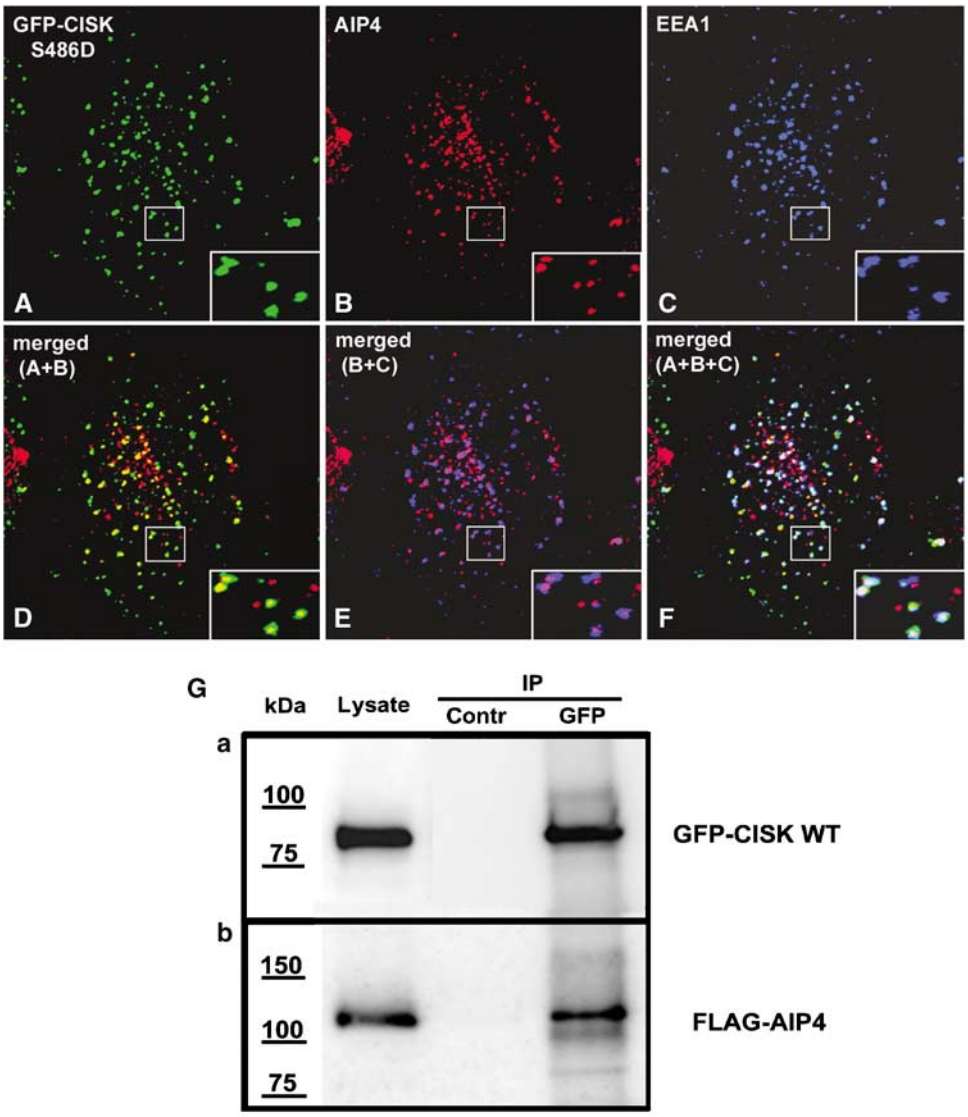

Figure 2 CISK associates with AIP4 on early endosomes. HeLa cells expressing GFP-CISK S486D (**A**) were labelled with anti-AIP4 (**B**) and anti-EEA1 (**C**). Colocalization between CISK and AIP4 is shown in yellow (**D**), between EEA1 and AIP4 in turquoise (**E**), and between all three molecules in white (**F**). A representative region of the cell containing positive structures for all three proteins is enlarged and shown for each picture. (**G**) CISK immunoprecipitates AIP4 from cell lysates. HeLa cell lysate was incubated with beads alone or beads coated with GFP over night and precipitated protein was detected by immunostaining. Upper panel: Immunoprecipitation control of GFP-CISK WT with GFP antibody. Control is uncoated beads in same lysate. Lower panel: Co-precipitated FLAG-AIP4 detected by FLAG-antibody.

FLAG-tagged AIP4, harvested after 2 days before CISK was precipitated from the lysate using GFP-coated beads. Then immunoblot analysis was performed to detect co-precipitated AIP4 using the FLAG antibody. As shown in Figure 2G, CISK was specifically precipitated from the lysate by GFP (upper panel). Furthermore, we observed that AIP4 was only pulled down in the presence of CISK, and FLAG-AIP4 protein was not observed when the lysate was incubated with beads alone (lower panel). Together with the interaction data, these results further support the idea that CISK and AIP4 may form a complex on endosomal membranes.

Activation of the hydrophobic loop of CISK is required for translocation of PDK1 to endosomes

Previous studies have shown that phosphoinositide-dependent protein kinase 1 (PDK1), which is activated downstream of PI 3-kinase, is required for the activation of most members of the AGC family of kinases through phosphorylation of the activation loop (Mora *et al*, 2004). In light of the endosomal localization of CISK, we asked whether PDK1, which is typically recruited to the plasma membrane upon PI 3-kinase activation (Mora *et al*, 2004), can be recruited to endosomes by CISK. We therefore co-expressed GFP-tagged CISK and PDK1 in HeLa cells and used confocal microscopy to study the intracellular localization of the proteins. Interestingly, we observed a very high colocalization of CISK and phosphorylated PDK1 on EEA1-positive structures when we expressed the active S486D mutant of CISK (Figure 3). In contrast, when PDK1 was expressed alone or together with the phosphorylation-incompetent mutant CISK S486A, we could not detect PDK1 in the endosomal compartment (data not shown). To verify that PDK1 recruitment by CISK is dependent on prior phosphorylation of the HM in the C-terminus, we performed *in vitro* phosphorylation experiments. We found

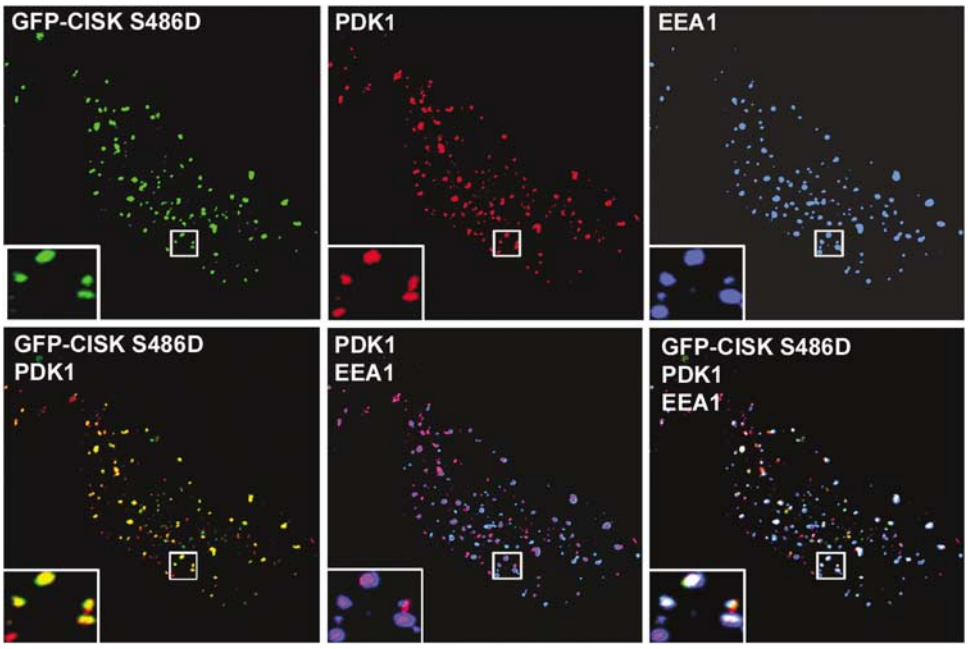

Figure 3 CISK recruits PDK1 to endosomal membranes upon PI 3-kinase activation. HeLa cells were labelled with anti-phospho-PDK1 and anti-EEA1. Colocalization between CISK and phospho-PDK1 is shown in yellow, between phospho-PDK1 and EEA1 in turquoise, and between all three molecules in white. A representative region of the cell containing positive structures for all three proteins is enlarged and shown for each picture.

that CISK S486D was phosphorylated by PDK1 *in vitro* (see Supplementary Results and Figure S2). In contrast, CISK S486A was not phosphorylated, which confirmed that prior phosphorylation of the HM of CISK is necessary for PDK1 interaction and phosphorylation. Together these results suggest that CISK facilitates the recruitment of PDK1 to endosomes only when CISK is preactivated at S486 in the HM through PI 3-kinase signalling.

CISK inhibits ligand-induced degradation of the CXCR4 receptor

AIP4 has been suggested to be involved in controlling the degradation of membrane-bound receptors by promoting their sorting to lysosomes. Recently, it was found that the degradation of CXCR4 was dependent on the ubiquitin ligase activity of AIP4 (Marchese *et al*, 2003a, b). Based on the ability of CISK to interact with AIP4, we therefore asked if CISK could interfere with the agonist-dependent degradation of CXCR4. To address this, we co-transfected HEK293 cells with HA-tagged CXCR4 and empty vector, CISK WT, or CISK phosphorylation mutants and assessed the amount of degraded receptor by immunoblot analysis. As shown in Figure 4A and B, CXCR4 underwent significant degradation in the presence of empty vector following a 2-h CXCL12 treatment. Coexpression of CISK S486D, however, severely reduced the amount of degraded CXCR4, whereas CISK WT abrogated the degradation to some extent. In contrast, the phosphorylation-inactive mutant CISK S486A was not able to inhibit CXCR4 degradation. These results indicate that CISK inhibits the ligand-induced degradation of CXCR4, and that this inhibition requires activation of CISK by phosphorylation of S486.

The degradation of CXCR4 is dependent on multiple complexes on sorting endosomes that recognize the ubiquitinated receptor and facilitate its transfer to the lysosomes (Marchese *et al*, 2003a, b). To investigate if CISK prevents transport of the receptor from early endosomes to lysosomes, we tested if CISK influenced the subcellular localization of CXCR4 during agonist-dependent degradation. We first transfected HeLa cells with only HA-tagged CXCR4 and used confocal microscopy to study the normal distribution of CXCR4 on structures containing EEA1 or LAMP2, well-known markers for early and late endosomes/lysosomes, respectively. As expected, we detected CXCR4 mostly on LAMP2 positive structures upon CXCL12 stimulation (Figure 5A and D), which suggests that CXCR4 undergoes a rapid and extensive agonist-dependent sorting to the lysosomes. A small fraction of CXCR4 was also found in the EEA1-positive compartment, indicating that not all receptors were efficiently targeted for lysosomal degradation. CXCL12 stimulation was, however, required for a punctuated distribution of CXCR4 in the cell, since CXCR4 was mainly localized at the plasma membrane in the absence of agonist treatment (data not shown).

To examine the effect of CISK on CXCR4 localization, we coexpressed HA-tagged CXCR4 and GFP-CISK S486D in CXCL12-stimulated cells and examined whether the distribution of CXCR4 was changed. Indeed, we detected extensive colocalization of CISK and CXCR4 on structures positive for EEA1 (Figure 5B and D). In addition, we found that CISK/CXCR4/EEA1-positive endosomes were enlarged, a characteristic feature also observed previously when the function of AIP4 or Hrs were inhibited (Marchese *et al*, 2003a, b). Consistent with the observation that CXCR4 accumulated in the early-endosomal compartment, we also detected less colocalization of CXCR4 and LAMP2 when CISK S486D was coexpressed (Figure 5C and D). These results suggest that activated CISK interferes with the lysosomal targeting of CXCR4 by inhibiting the activity of proteins involved in the sorting pathway. To investigate whether the inhibition we observed was dependent on the activation status of CISK, we performed the same experiments using the inactive

mutant CISK S486A that has the same endosomal localization as S486D (data not shown). Importantly, we found that coexpressing CISK S486A with HA-CXCR4 did not change the distribution of CXCR4 (Figure 5D), indicating that CISK needed to be activated in order to inhibit the agonist-dependent degradation of CXCR4. Together, these results show that activated CISK inhibits the ligand-induced degradation of CXCR4 by preventing sorting of the endocytosed receptor from early endosomes to lysosomes.

The PPFY-motif of CISK is required for inhibiting CXCR4 degradation

The WW-domains of Nedd4 and AIP4 have been shown to interact with proteins containing short proline-rich sequences,

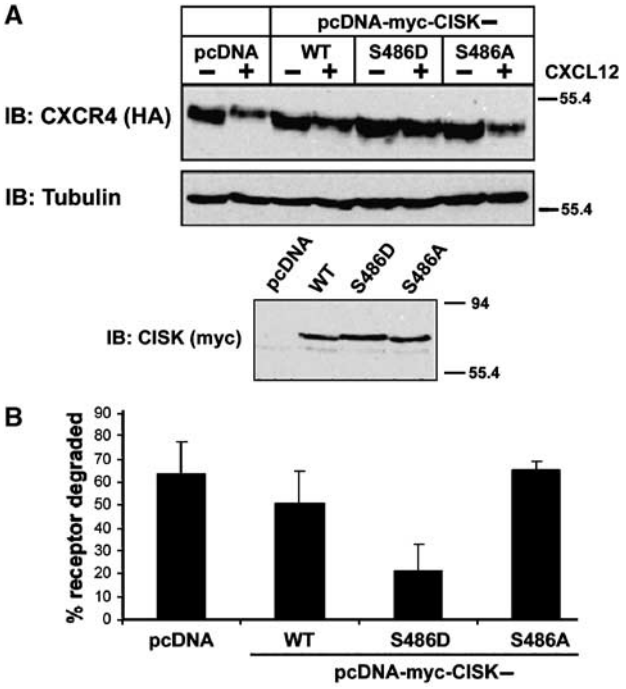

Figure 4 Activated CISK inhibits degradation of the CXCR4 receptor. (**A**) CXCR4 degradation experiments in the presence of WT CISK or the phosphorylation mutants CISK S486D and S486A. The HEK293 lysate were analysed by immunoblotting (IB) using an anti-HA antibody. Additional blots were probed with anti-myc and -tubulin antibodies. Shown are representative blots from three–five independent experiments. (**B**) The amount of degraded CXCR4 receptor was determined using the immunoblots obtained from the analysis described in (A). The bars indicate the amount of CXCR4 receptor degraded in the presence of CXCL12.

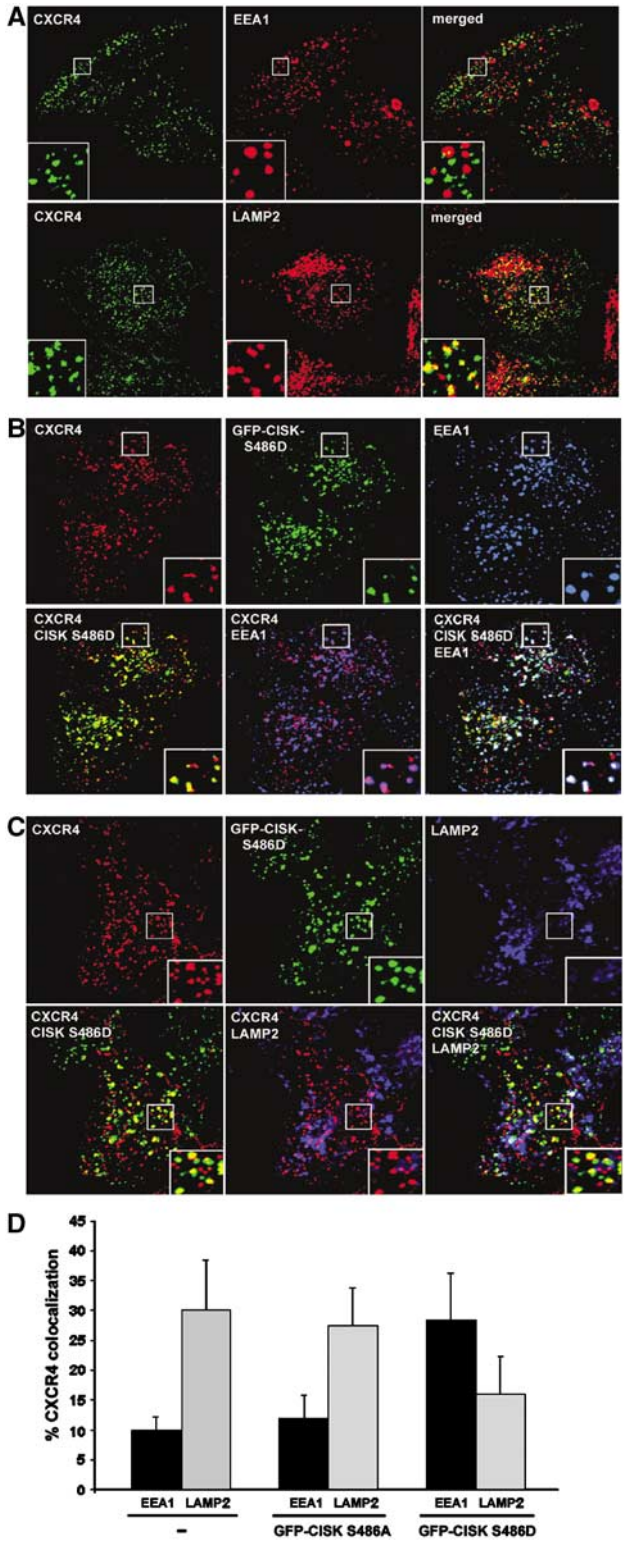

Figure 5 CISK inhibits sorting of CXCR4 from early endosomes to lysosomes. (**A**) CXCR4 is sorted to the lysosomes upon CXCL12 stimulation. HeLa cells were transfected with HA-CXCR4 for 16 h, stimulated with CXCL12 in the presence of cycloheximide and leupeptin, washed, and chased for three more hours before the cells were permeabilized and fixed for immunostaining (see 'Materials and methods' for further details). The cells were labelled with anti-HA (CXCR4), and anti-EEA1 (upper panel) or LAMP-2 (lower panel). Colocalization between CXCR4 and markers is shown in yellow to the right of each panel. (**B**) Same as in (A), but the localization of GFP-CISK S486D is shown in green, HA-CXCR4 in red, and EEA1 in blue. Colocalization between CISK and CXCR4 is shown in yellow, between CXCR4 and EEA1 in turquoise, and between all three molecules in white. A representative region of the cell containing positive structures for all three proteins is enlarged and shown for each picture. (**C**) Same as in (B), but the blue staining represents the LAMP-2 staining in the cell. (**D**) Statistical analysis of the colocalization between CXCR4 and EEA1/LAMP-2 in the absence or presence of GFP-CISK S486A or GFP-CISK S486D coexpression. The bars indicate the average and standard deviation of the colocalization between CXCR4 and EEA1/LAMP-2 compared to total cellular staining of CXCR4 in each of the experiments described in (A–C).

in which the PPXY-motif is the most commonly used interaction surface (Ingham *et al*, 2005). To investigate if the PPFY motif of CISK mediates the binding to AIP4, we substituted the important tyrosine residue with an alanine to abolish the function of this motif. Based on the finding that phosphorylation of the C-terminus also stimulated the binding to AIP4 (see Figure 1B and C), we also wanted to examine the contribution of phosphorylation in the hydrophobic loop of CISK on the AIP4 interaction. For this purpose, we performed GST-pulldown assays in which we incubated a purified GST-fusion protein of the WW-domains of AIP4 with *in vitro* translated ^{35}S-labelled kinase domains of CISK S486A, CISK S486D, CISK S486A/PPFA, or CISK S486D/PPFA. As shown in Figure 6A, we found that both phosphorylation mutants strongly bound to AIP4-WW compared to background GST binding. In line with our previous protein–protein interaction data (see Figure 1B and C), we observed an increased binding to AIP4-WW when CISK had the phosphomimetic S486D mutation in the hydrophobic loop. In contrast, both PPFY-motif mutants of CISK were severely reduced in their ability to interact with the WW-domain of AIP4. These results demonstrate that the PPFY-motif of CISK is required for efficient binding between CISK and AIP4 and suggest that the HM in the extreme C-terminus of CISK is involved in stabilizing this interaction.

Previous studies have shown that AIP4 activity is required for efficient sorting of CXCR4 to lysosomes (Marchese *et al*, 2003a, b). Based on our interaction and immuofluorescence data of CISK and AIP4, we sought to investigate if the inhibition we observed with CISK on CXCR4 degradation was mediated through the binding to AIP4. To this end, we compared the effects of WT and PPFA mutant CISK on CXCR4 degradation. To make sure that the difference we observed between these proteins was solely due to a dysfunctional PPFY-motif, we tested the constructs in a S486D background. We thus cotransfected HEK293 cells with HA-tagged CXCR4 and empty vector, CISK S486D or CISK S486D/PPFA and assessed the amount of degraded receptor by immunoblot analysis. As observed previously (Figure 4), we found that co-expression with CISK S486D severely reduced the amount of degraded CXCR4 in comparison with empty vector following a 2-h CXCL12 treatment (Figure 6B and C). In contrast, CISK S486D/PPFA was not able to inhibit the agonist-dependent degradation of CXCR4. This implies that the interaction between CISK and AIP4 is important for the observed ability of CISK to inhibit CXCR4 degradation.

Some studies have suggested that AIP4 may also be involved in EGFR trafficking (Courbard *et al*, 2002; Angers *et al*, 2004). In contrast, our previous studies have shown that depletion of AIP4 has no effect on the lysosomal degradation of this receptor (Marchese *et al*, 2003b). To address if the effect we observed with CISK is cargo-specific or affects the lysosomal sorting of membrane-bound receptors more in general, we also investigated the degradation of the EGFR in the presence of CISK (see Supplementary Results and Figure S3). Interestingly, in contrast to the effect of CISK on CXCR4, neither CISK WT nor S486D had any significant impact on the degradation of EGFR. These results suggest that CISK does not have any general effect on endosomal sorting of membrane bound receptors, but more likely has a role as a specific regulator of receptors/proteins that are regulated by AIP4.

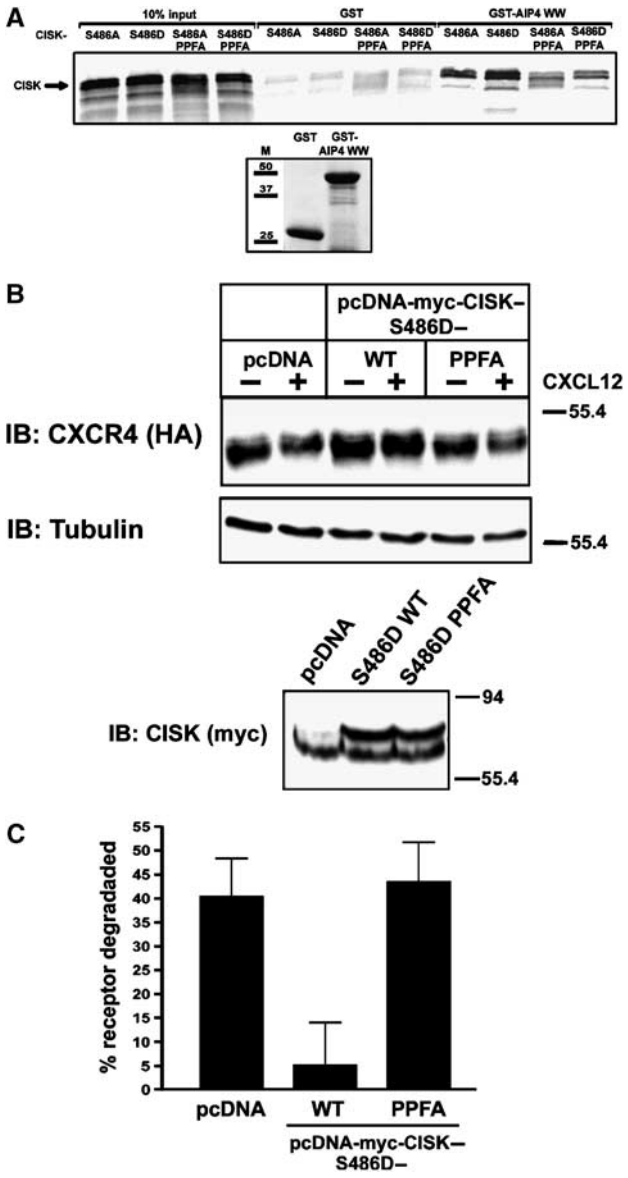

Figure 6 The PPFY-motif of CISK is required for inhibiting CXCR4 degradation. (**A**) GST-pulldown assay of *in vitro* translated CISK and GST-AIP4 WW. The amount of input of each protein is indicated. (**B**) CXCR4 degradation assay (see Figure 4 for more details). Equal amounts of cell lysates from treated and untreated cells were analysed by immunoblotting (IB) using an anti-HA antibody. Control for equal transfection efficiency (myc) and loading control (tubulin) are indicated. (**C**) The amount of degraded CXCR4 receptor was determined using the immunoblots obtained from the analysis described in (B). The bars indicate the amount of CXCR4 receptor degraded in the presence of CXCL12.

CISK phosphorylates AIP4 in the WW-binding domain

AIP4 activity has recently been shown to be regulated through specific phosphorylations of residues close to or inside the WW-binding domain (Gao *et al*, 2004; Yang *et al*, 2006). Based on these reports and the well-characterized regulation of Nedd-4 by SGK1 (Kamynina and Staub, 2002), we addressed the possibility that AIP4 could be a substrate for CISK kinase activity. In light of the suggested overlapping substrate specificities of Akt and CISK, we scanned the AIP4 sequence for predicted Akt phosphorylation sites. Interestingly, we found two predicted Akt phosphorylation

sites in the WW-domain of AIP4 (Figure 7A). Encouraged by this finding we sought to test if the AIP4 WW-domain is a substrate for CISK. To this end we purified GST and GST-AIP4 WW and incubated the recombinant proteins with purified CISK in phosphorylation experiments. Based on our experience that full-length CISK is very difficult to purify *in vitro*, we used a truncated version of CISK that lacks the PX-domain (SGK3), and which was activated *in vitro*. As shown in Figure 7B, we observed that activated CISK/SGK3 induced a phosphorylated band that corresponded to the size of GST-AIP4 WW. In contrast, we could not detect any phosphorylation of GST used as negative control. To further investigate the specificity of the phosphorylation, we tested SGK1 in the same experiments. Interestingly, we found that even though SGK1 and CISK/SGK3 have been reported to have very similar target sequences (Kobayashi *et al*, 1999), SGK1 was

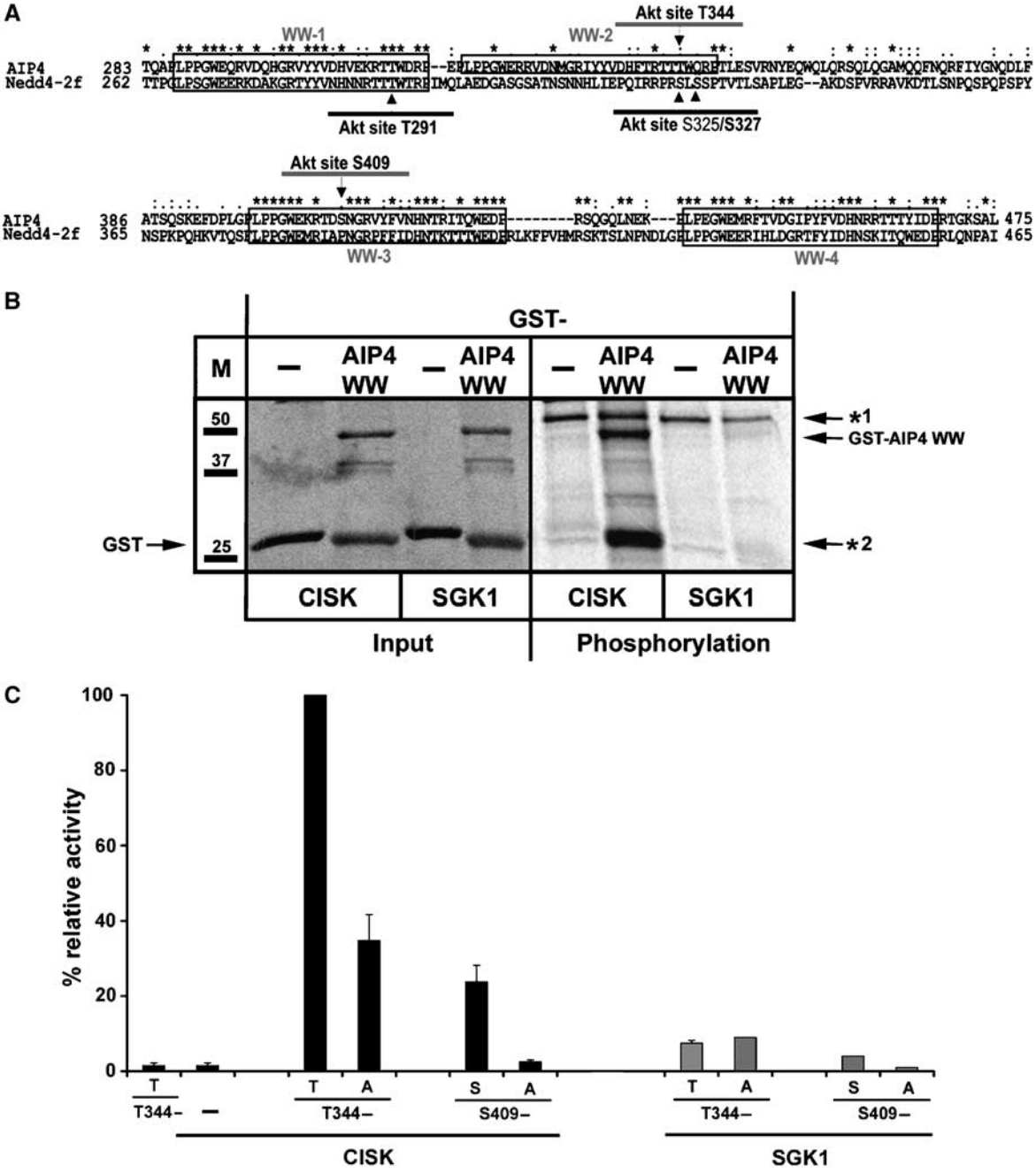

Figure 7 AIP4 is a specific substrate for CISK kinase activity (**A**) Alignment and domain structure of the WW-binding domain of human AIP4 (GI:15079474) and Nedd4-2f (GI:32250387). AIP4 contains four WW-binding modules that are indicated. Two possible Akt phosphorylation sites in the WW-binding domain of AIP4 (T344 and S409) are indicated with red lines. Similarly, the characterized SGK1 phosphorylation sites of Nedd4-2f are shown with black lines. (**B**) CISK phosphorylates the WW-binding domain of AIP4. Phosphorylated bands were detected using PhosphoImager (right panel). The membrane was also stained in Ponceau S solution to determine the size and amount of protein in each reaction (left panel). Phosphorylated AIP4 is indicated with an arrow. In addition, the autophosphorylated kinase (*1) and a putative AIP4 degradation product (*2) are indicated. (**C**) CISK phosphorylates potential phosphorylation motives in the AIP4 WW-domain. CISK/SGK3 or SGK1 was incubated with the respective peptides in kinase buffer as described in (B) and Materials and methods. The average c.p.m. value of CISK/SGK3 with the T344 peptide was put to 100%.

not able to phosphorylate AIP4 WW in these experiments (Figure 7B). AIP4 is thus a specific substrate for CISK/SGK3 phosphorylation.

Based on the finding that there exist at least two potential phosphorylation motifs in the WW-domain of AIP4, we wanted to explore further the possibility that these motifs represent target phosphorylation sites for CISK. We therefore tested whether synthesized peptides covering the two suggested phosphorylation motifs in AIP4 were phosphorylated by activated CISK/SGK3 *in vitro*. As controls we included peptides that were mutated in the putative phosphorylation motifs, T344A and S409A, respectively. As shown in Figure 7C, CISK significantly phosphorylated the peptide that included the T344 motif. In contrast, CISK or the T344 peptide alone did not yield signal over background levels. We also found that the S409 motif is phosphorylated by CISK but to a smaller extent than the T344 motif. Furthermore, we observed a significant reduction in phosphorylation when S409 or T344 was mutated, which suggests that these residues are likely targets for CISK phosphorylation. However, in contrast to S409A, the T344A-containing peptide was still phosphorylated to some extent. This may be explained by the fact that there are at least two more threonine residues in this motif that also may be targets for kinase activity, similar to the corresponding Nedd4-2f region (see Figure 7A). Consistent with our GST-AIP4 WW phosphorylation experiments, we did not detect any significant phosphorylation of any peptide by SGK1, confirming that AIP4 is a specific substrate for CISK kinase activity. In contrast, both CISK and SGK1 were able to phosphorylate a Nedd4-2f peptide that covers the S325/S327 site (Figure 7A, and data not shown). These results confirm that SGK1 is active in our assays and is consistent with earlier reports demonstrating that these kinases may have overlapping target motifs (Tessier and Woodgett, 2006). Taken together, these studies further support our conclusion that CISK kinase activity regulates the activity of AIP4 during receptor degradation.

Discussion

Endocytosis has long been recognized as a means to terminate signalling via degradation of activated receptor complexes after their internalization from the cell surface. Recent reports have, however, shown that several receptors also can signal from endosomes (Sorkin and Von Zastrow, 2002). Proper temporal and spatial control of the receptors in the endosome is therefore essential for accurate signal propagation. In this report we provide evidence that the recently identified survival kinase CISK plays an important role in regulating these events for the chemokine receptor CXCR4. Our findings suggest that CISK attenuates the function of the ubiquitin ligase AIP4 on endosomes, and thereby inhibits the ubiquitin-dependent sorting of CXCR4 to lysosomes.

In light of the recently discovered effect of PI 3-kinase signalling on CXCR4 degradation, we wanted to explore the possibility that CISK might be one of the important mediators linking these processes. Previous studies have shown that CISK is an anti-apoptotic factor that functions downstream of the PI-3 kinase signalling pathway (Liu *et al*, 2000). However, a model in which PDK1 activates CISK on endosomes has been questioned, since CISK and PDK1 are localized to distinct subcellular compartments (Virbasius *et al*, 2001; Gillooly *et al*, 2003). We therefore investigated if PDK1 is the PI 3-kinase mediator that activates CISK on endosomes. By using CISK mutants that can be activated or not with respect to PI-3 kinase signalling, we provide evidence that PDK1 is indeed found on endosomes when CISK is activated through the PI 3-kinase pathway. Consistent with these results, we also demonstrate that activation through the HM of CISK is required for PDK1 binding and phosphorylation.

To date, no function of CISK in endosomes has been reported. It has been suggested that CISK operates in parallel with Akt/PKB (Liu *et al*, 2000; Xu *et al*, 2001), but the distinct localizations of CISK and Akt suggest that CISK has a more specialized function on endosomes. The observation that SGK1 regulates ubiquitin ligases at the plasma membrane (Kamynina and Staub, 2002), prompted us to consider if CISK could have a similar function on endosomes. In light of the important role of the ubiquitin ligase AIP4 in regulating the degradation of CXCR4, it was therefore intriguing to find that CISK strongly interacts with AIP4, which has previously been shown to localize to endosomes (Courbard *et al*, 2002; Marchese *et al*, 2003b). In line with the idea that these proteins also exist as a complex, we found that CISK and AIP4 co-immunoprecipitate from cell lysates and co-localize on early endosomes. Furthermore, we found that the PPFY-motif of CISK is crucial in mediating this interaction, which is consistent with the role of such motifs in binding WW-domains of ubiquitin ligases (Ingham *et al*, 2004). We also observed that the phosphomimetic mutation of S486 in the HM stimulated the binding between AIP4 and CISK. This is consistent with the previous observations of the WW-domains of Nedd-4 and Pin1, which both were shown to bind phosphoserine and -threonine modules in addition to proline-rich regions (Lu *et al*, 1999; Ilsley *et al*, 2002). Although the mechanism is not known at present, this implies that phosphorylation of the HM in CISK has a positive effect on the binding to AIP4 by stimulating and/or stabilizing the interaction between the WW-binding domain and the PPFY motif of CISK.

The observation that CISK and AIP4 can exist in the same complex on endosomes encouraged us to address the possibility that CISK may interfere with the AIP4-mediated lysosomal degradation of CXCR4 (Marchese *et al*, 2003b). Upon binding of CXCL12, CXCR4 is phosphorylated and thereafter internalized in a nonvisual-arrestin-dependent manner from the cell surface (Orsini *et al*, 1999; Cheng *et al*, 2000). Although CXCR4 is ubiquitinated at the plasma membrane, the rate-limiting step during degradation appears to be the sorting of CXCR4 in endosomes (Marchese and Benovic, 2001). Interestingly, we found that activated CISK strongly reduced the agonist-dependent degradation of CXCR4, which suggests that PI 3-kinase signalling via CISK interferes with the endosomal sorting process. We also demonstrated that the inhibition we observed was mediated through the interaction of CISK and AIP4, since a PPFY-motif mutant of CISK was not able to interfere with the degradation of CXCR4. Based on the observation that AIP4 is found in complex with the endosomal protein Hrs (Marchese *et al*, 2003b), it is unlikely that CISK is required for AIP4 targeting to the endosomal membrane. Furthermore, we found that the PPFA-motif mutant displayed the same subcellular localization as WT and CISK S486D (data not shown), which suggest that

CISK and AIP4 have to interact at the endosomal membrane in order to inhibit CXCR4 degradation. Further evidence for the involvement of CISK in the sorting pathway of CXCR4 was obtained by investigating the subcellular localization of CXCR4. Like previously reported (Marchese and Benovic, 2001; Marchese *et al*, 2003b), we detected CXCR4 mainly in late endosomes/lysosomes upon CXCL12 stimulation, with a minor fraction remaining on early endosomes. In contrast, the co-expression with CISK strongly increased the localization of CXCR4 to EEA1-positive early endosomes, which is consistent with an inhibitory role of CISK in the sorting of the receptor from early to late endosomes.

The finding that CXCR4 stimulates PI 3-kinase signalling upon CXCL12 activation (Peng *et al*, 2005) raises the possibility that ligand binding may cause two opposing effects on CXCR4 degradation. On the one hand, ligand-induced ubiquitination favours degradation of the receptor (Marchese and Benovic, 2001; Marchese *et al*, 2003a, b), whereas, on the other hand, ligand-induced activation of PI 3-kinase is predicted to activate PDK1 and thereby CISK, thus attenuating degradation. We thus propose that the fraction of CXCR4 that becomes degraded following CXCL12 binding may depend on the balance between AIP4-mediated ubiquitination and PI 3-kinase signalling, and perhaps additional signalling inputs. It is worth noting that overexpression of WT CISK caused only a partial inhibition of CXCR4 degradation in our experiments, whereas the phosphorylation-negative mutant had no effect and the phosphomimetic mutant had a strong effect. This suggests that additional activation of PI 3-kinase by other receptors might be required for a full inhibitory effect. In cancers, HER2 would be a good candidate for such heterologous activation of PI 3-kinase signalling.

Consistent with the idea that AIP4 becomes phosphorylated upon PI 3-kinase signalling, we observed that CISK-mediated inhibition of CXCR4 degradation is dependent on the activation status of CISK. Recent studies have demonstrated that SGK1 stimulates sodium influx by inhibiting the ubiquitin-dependent degradation of the ion channels mediated by Nedd4-2 (Debonneville *et al*, 2001; Snyder *et al*, 2002). SGK1 phosphorylates residues in the WW-domain, and thereby obliterates the binding of Nedd4-2 to the channel components. AIP4 also contains potential SGK1/CISK motifs in the WW-domain, and we here provide evidence that the AIP4 WW-domain is a target of CISK but not SGK1 kinase activity. This finding is further supported by the observation that the two possible peptides representing the potential target sequences in this domain are also phosphorylated by CISK.

Previous studies have suggested that the PX-domain negatively regulates the kinase activity of CISK (Liu *et al*, 2000). On the endosomal membrane, the ability of CISK to phosphorylate its substrates is dependent on their prior interaction in the lipid bilayer, whereas the specificity of the kinase is probably not affected. From our *in vitro* studies it seems that the T344 site in AIP4 is the most likely target site for CISK phosphorylation. This motif is also located in the region of the WW-domain corresponding to the one that is important for the negative regulation of Nedd4-2 by SGK1. Furthermore, alignment studies of the sequence surrounding the T344 site indicated that this motif is very well conserved among AIP4 homologues and the WWP1 and WWP1 family members (data not shown). Based on these findings, we suggest that CISK phosphorylation inhibits the activity of AIP4 on endosomes, possibly by reducing binding to target proteins. Of note, two recent reports have demonstrated the importance of specific phosphorylation events for AIP4 activity (Gao *et al*, 2004; Yang *et al*, 2006). While Jnk1 phosphorylation in the proline-rich region just upstream of the WW-domain results in enhanced activity of AIP4, Fyn phosphorylation in the vicinity of the WW-domain (Y371) significantly reduces the ability of AIP4 to ubiquitinate JunB. Phosphorylation of AIP4 may regulate an auto-inhibitory interaction between the WW-domains and the catalytic HECT-domain (Gallagher *et al*, 2006). CISK-mediated control of AIP4 ubiquitin ligase activity might control CXCR4 sorting in several ways. For instance, while ubiquitination of CXCR4 by AIP4 promotes its degradation (Marchese *et al*, 2003b), AIP4-mediated ubiquitination of the endosomal sorting component Hrs is predicted to have the opposite effect by causing auto-inhibition of Hrs (Hoeller *et al*, 2006).

Our findings suggest that CISK is important for regulating the amount of CXCR4 to be sorted to lysosomes for degradation (Figure 8). The observation that CISK has no effect on EGFR degradation is consistent with the proposed role of AIP4 in specifically regulating the sorting of CXCR4. By controlling the activity of ubiquitin ligases on endosomes, CISK may fine-tune the intensity of the signalling events mediated by CXCR4 on endosomal membranes. Consistent with this hypothesis, it was recently reported that CISK null mice displayed a defect in hair follicle morphogenesis, probably due to impaired cell migration during follicle formation (McCormick *et al*, 2004; Alonso *et al*, 2005). In light of the important role CXCR4 plays in cell migration (Florin *et al*, 2005; Kucia *et al*, 2005), it is tempting to speculate that the observed defects are due to an absence of PI 3-kinase mediated regulation of CXCR4 levels in the CISK deficient cells. Recent reports have also highlighted the role of CXCR4 signalling in cancer progression and metastasis (Balkwill, 2004; Kucia *et al*, 2005). This is based on the ability of CXCL12-CXCR4 to promote cell invasion, migration, and adhesion during metastasis in breast and prostate cancers. It has been demonstrated that in breast cancer cell lines the defect in CXCR4 degradation is due to overactive PI 3-kinase signalling (Benovic and Marchese, 2004; Li *et al*, 2004). Based on the observation that AIP4 function was inhibited in these cancer cells, our findings suggest a mechanism to explain how PI 3-kinase signalling could control CXCR4 levels during cancer development.

In summary, we present a novel mechanism for attenuation of CXCR4 degradation and provide the first evidence for a function of CISK on endosomes. Through PI 3-kinase signalling, CISK can regulate the sorting of CXCR4 to lysosomes by interfering with the endosomal function of AIP4. Adding to the potential role of CISK as a survival effector during normal development, our results provide new insights into how PI 3-kinase signalling, through activation of PDK1 and CISK, may promote CXCR4-mediated cancer progression.

Materials and methods

Analysis of the subcellular localization of CXCR4

HeLa cells were transfected with HA-CXCR4 in the absence or presence of the GFP-fusion constructs expressing CISK S486D or

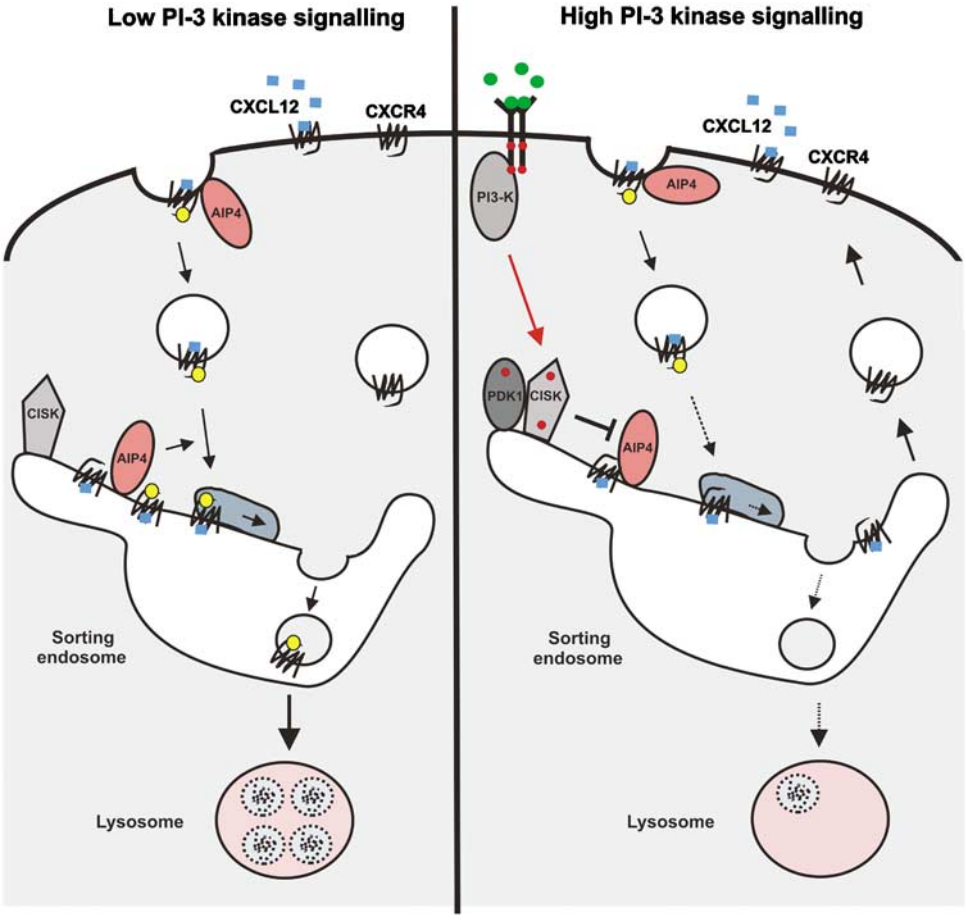

Figure 8 Model of CISK-mediated inhibition of CXCR4 sorting and degradation. Upon CXCL12 activation, CXCR4 is internalized into clathrin-coated pits from the plasma membrane. Consequently, CXCR4 is ubiquitinated and transported into the endocytic pathway for degradation in lysosomes. As well as keeping CXCR4 ubiquitinated at the endosomes, AIP4 may also regulate the sorting machinery itself through ubiquitination. When high PI 3-kinase signalling is turned on by various growth factors during development or cancer progression, the degradation of CXCR4 is strongly inhibited. PI 3-kinase stimulation leads to the activation of PDK1 and CISK on endosomal membranes. We propose that CISK negatively regulates the function of endosomal AIP4 through phosphorylation and thereby prevents CXCR4 from being efficiently sorted to lysosomes (see text for further details).

CISK S486A. The next day the cells were incubated for 1 h with 100 nM CXCL12 in the presence of 10 ng/μl cycloheximide and 0.3 mM leupeptin. After incubation with CXCL12, the cells were washed once with DMEM and chased for 3 more hours in DMEM containing 10% FBS, cycloheximide, and leupeptin. Then the cells were permeabilized, fixed, and stained for immuofluorescence microscopy as previously described (see Materials and methods). To calculate the colocalization between CXCR4 and EEA1 or LAMP-2 using the Adobe Photoshop 7.0 software, the colour of each probe was selected before the amount of pixels over a defined threshold were counted. To determine the percentage of colocalization between CXCR4 and the endosomal markers, the amount of costaining of CXCR4 and either EEA1 or LAMP-2 was compared to the total staining of CXCR4 in the cell. More than 20 cells from two to three independent experiments were calculated, and the values were put into Microsoft XL for further statistical analysis.

In vitro phosphorylation of AIP4 WW

Recombinant GST and GST fusion protein of the WW-domain of AIP4 was expressed and purified in *Escherichia coli*. GST or GST-AIP4 WW were incubated in kinase buffer (8 mM MOPS pH 7, 0.2 mM EDTA, 1 μg/μl BSA, 0.1% Igepal, and 0.1% β-mercaptoethanol) containing ATP (Sigma Aldrich), [γ-^{33}P]ATP (Amersham Biosciences) and thereafter incubated with a preactivated CISK variant that lacks the PX domain (SGK3) or PDK1-activated SGK1 (Upstate, MA, USA). After 20 min incubation at 30°C, SDS-stop solution was added and the samples were subsequently run on SDS–PAGE. The gels were blotted onto an Immobilon-P transfer membrane (Millipore, Billerica, MA) and signals detected using a PhosphorImager (Amersham Biosciences). Images were processed with Adobe Photoshop 7.0. In the peptide phosphorylation assays, 250 μM of each peptide was incubated in MOPS buffer as previously described and added activated CISK/SGK3 or SGK1 for 20 min 30°C. The reactions were stopped by adding 0.3% phosphoric acid and signals detected using P81 phosphocellulose filter squares (Upstate) and scintillation counter (Perkin-Elmer, MA, USA).

Plasmid constructs, antibodies, protein interaction studies, immunoprecipitation studies, expression and purification of recombinant proteins, confocal fluorescence microscopy and degradation assays.

See Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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