Supplementary Material, EMBO-2005-52595

Supplementary Results

Activated CXCR4 is sorted into multivesicular endosomes

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., 2003). Because 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; Raiborg et al., 2003), we investigated whether endocytosed CXCR4 can be found within such structures. Cells for immunoelectron microscopy were transfected with HA-tagged receptor for 24 hours, incubated with CXCL12 (100 nM) for 30 min followed by a 2-hour chase in the presence of 0.3 mM leupeptin. Cells were fixed in 4% formaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at room temperature for 1 hour. Further preparation was essentially as described earlier (Peters et al., 1991). Ultrathin sections were cut on a Leica Ultracut equipped with a Leica EM FCS, labelled with monoclonal HA-antibody, followed by rabbit-anti-mouse secondary antibody and 15 nm protein A gold. Sections were then embedded in 2% methyl cellulose/ 0.3 uranyl acetate, and observed in a Philips CM10 at 80 kV. As shown in Figure S1, gold particles, representing HA-CXCR4, were frequently observed inside MVEs (arrowheads). The receptor could also be observed in smaller endocytic structures (arrows). This demonstrates that endocytosed CXCR4 is targeted into the MVE pathway.

Phosphorylation of CISK by PDK1 in vitro is dependent on hydrophobic motif phosphorylation.

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. Recombinant GST fusion proteins of the kinase domains of CISK S486D and CISK S486A were expressed and purified in E. coli and dialyzed into kinase assay buffer (20 mM Hepes, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, and 0.1% β-mercaptoethanol). In the phosphorylation assay the recombinant CISK proteins were added to a kinase buffer containing ATP (Sigma Aldrich), [γ-³²P]ATP (Amersham Biosciences) and thereafter incubated with PDK1 active (Upstate,
Waltham, MA) as suggested by the manufacture. As shown in Figure S2, we found that CISK S486D was phosphorylated by PDK1 \textit{in vitro}. In contrast, CISK S486A was not phosphorylated, which confirms that prior phosphorylation of the hydrophobic motif of CISK is necessary for PDK1 interaction and phosphorylation.

**EGFR receptor degradation is not regulated by CISK**

AIP4 has multiple cellular functions in addition to regulating the endosomal sorting of CXCR4 (d'Azzo et al., 2005; Fang et al., 2002; Gao et al., 2004; Marchese et al., 2003). Even though some studies have suggested that AIP4 may be involved in EGFR trafficking (Angers et al., 2004; Courbard et al., 2002), our previous studies have shown that depletion of AIP4 have no effect on the lysosomal degradation of EGFR (Marchese et al., 2003). To address if the effect we observe with CISK is cargo-specific or affects the lysosomal sorting of membrane-bound receptors more in general, we sought to investigate the degradation of the EGFR in the presence of CISK. We therefore co-transfected HEK293 cells with empty vector, CISK WT, or CISK S486D and assessed the amount of degraded EGFR by immunoblot analysis (see Materials and Methods for further details). Similar to what we observed with CXCR4, EGFR underwent significant degradation following a 1-hour treatment with EGF (Figure S3A and B). However in stark 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.

**Supplementary Materials and Methods**

**Plasmid constructs**

The plasmid for pEGFPC1-CISK WT has been described in (Nilsen et al., 2004). This plasmid was used as a template for generating pEGFPC1-CISK S486D and S486A using a C-terminal primer encoding an Asp or Alanine instead of a Ser at amino acid 486 during PCR amplification. In addition, these CISK proteins were cloned behind the myc-epitope of pcDNA3-myc (Raiborg et al., 2001). pEGFPC1-CISK S486D PPFA and pcDNA3-myc-CISK S486A PPFA were cloned by quick-change mutagenesis using a primer encoding Ala instead
of a Tyr at amino acid 362 during PCR amplification. pcDNA3-myc-CISK S486D PPFA was cloned by swapping the equivalent CISK S486D PPFA fragment from the pEGFP-vector. The kinase domain (aa 96-496) of CISK WT and CISK S486D were also cloned into pcDNA3-myc for generating \textit{in vitro} translated proteins. The DNAs encoding HA-tagged CXCR4 in pcDNA3, 3×FLAG-ubiquitin in pCMV-10, 3×FLAG-AIP4, and a N-terminal myc-tagged AIP4 have been previously described (Marchese and Benovic, 2001; Marchese et al., 2003). For use in two-hybrid interaction studies, the kinase domain of CISK WT and CISK S486D were cloned into pLexA/pBTM116 (Vojtek et al., 1993) as bait. As prey the WW-domain region of AIP4 (aa 260-486) was cloned into pGAD-GH2 (BD Biosciences Clontech, Mountain View, CA). For expression as glutathione S-transferase (GST) fusion proteins in \textit{Escherichia coli}, the kinase domain of CISK WT, CISK S486D, CISK S486A, and the WW-domain of AIP4 were cloned into pGEX-6P-1 (Amersham Biosciences, Pollards Wood, UK).

\textbf{Antibodies}

For the CXCR4 degradation and electron microscopy experiments, anti-myc and anti-HA monoclonal antibodies and anti-HA polyclonal antibodies were purchased from Covance (Berkely, CA). The anti-β-tubulin antibody was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). For EGFR degradation experiments, EGFR antibody was purchased from Stressgen (British Columbia, Canada). For immunofluorescence labelling, anti-HA monoclonal and anti-AIP4 polyclonal antibodies were obtained from Sigma Aldrich and Boehringer (Ingelberg, Germany), respectively. Human anti-EEA1 serum (Mu et al., 1995) and anti-LAMP2 antibodies were gifts from Ban-Hock Toh and Gillian Griffiths, respectively. Phospho-PDK1 antibody was purchased from Cell Signalling, and rhodamine- and Cy5-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Unconjugated rabbit-anti-mouse was from Dako (Denmark) and protein A gold was purchased from George Posthuma, CMC (Utrecht, The Netherlands). Rabbit GFP antibody (Abcam, Cambridge, UK) and FLAG-epitope M2 from mouse (Sigma-Aldrich) was used to detect AIP4 in the immunoprecipitation assay.

\textbf{Two-hybrid interaction study}

The yeast reporter strain L40 (Vojtek et al., 1993) was cotransformed (Schiestl and Gietz, 1989) with the indicated pLexA (bait) and pGAD (prey) plasmids, and β-galactosidase activities of transformants were determined as described previously (Guarente, 1983).
Expression of GST proteins in E.Coli and in vivo transcription/translation

GST fusion proteins were produced in *E. coli* BL21 (DE3, Novagen, San Diego, USA) or C41 (Avidis, France) cells transformed with the respective pGEX constructs. After induction with IPTG (Sigma Aldrich), the cells were spun down, resuspended in Tris-buffer (50 mM Tris pH 7.5, 200 mM NaCl, and 1 mM DTT), and subsequently sonicated before the GST-fusion proteins were purified on glutathione-Sepharose beads according to the instructions from the manufacturers (Amersham Biosciences, Uppsala, Sweden).

pcDNA3-myc-CISK full-length and kinase-domain constructs were translated in the presence of [\(^{35}\)S]methionine (Amersham Biosciences) using the TNT® T7 coupled reticulocyte lysate system (Promega, Southampton, UK) according to the protocol from the manufacturers.

Cell culture, transfection and Confocal fluorescence microscopy

HeLa and HEK293 (Microbix, Toronto, Canada) cell cultures were maintained as recommended by American Type Culture Collection (Manassas, VA) or Microbix (Toronto, Canada), respectively. For expression in mammalian cells, we used the FuGENE (Roche Diagnostics, Indianapolis, IN) or GeneJuice (Novagen, Madison, WI) transfection reagents according to the manufacturer's instructions. Cells were analyzed 24-48 h after transfection. Transfected HeLa cells grown on coverslips were permeabilized with 0.05% saponin, fixed with 3% paraformaldehyde, and stained for fluorescence microscopy as described previously (Simonsen et al., 1998). Coverslips were examined using an LSM 510 META microscope (Carl Zeiss, Jena, Germany) equipped with a Neo-Fluar 100x/1.45 oil immersion objective. Image processing was done with Adobe Photoshop, version 7.0.

GST-pulldown assay

See Supplementary Information.

Lysates from transformed E. coli BL21 or C41 cells containing GST or GST fusion protein were bound in Tris buffer (50 mM Tris, 200 mM NaCl, pH 7.5) to 20-µl aliquots of glutathione-Sepharose (Amersham Biosciences) at RT for 60 min. The beads were then washed with assay buffer, and *in vitro* translated CISK full-length or kinase-domain proteins were added with assay buffer (20 mM Hepes, 140 mM NaCl, 2 mM CaCl₂, pH 7) containing 0.25 % IGEPAL. After rotation at 4°C for 60 min, the beads were washed three times with assay buffer and then analyzed by SDS-PAGE and fluorography using a PhosphorImager (Amersham Biosciences). Images were processed with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).
**Immunoprecipitation experiments**

HeLa cells were transfected for two days with pEGFP-CISK WT and FLAG-AIP4, stimulated with 100 ng/µl EGF for 30 min, and thereafter harvested and added lysis buffer. The lysates were first pre-cleared with Protein-A sepharose beads (Amersham Biosciences/GE, Uppsala, Sweden) for 2 hours before the lysates were incubated with beads alone or beads coated with GFP antibody at 4°C over night. Next day the beads were washed 3 times in lysis buffer and then analyzed by SDS-PAGE. The gels were thereafter blotted onto an Immobilon-P transfer membrane (Millipore, Billerica, MA), stained with GFP or FLAG primary antibodies, and bands detected using HRP-conjugated secondary antibodies and Chemi Genius Image Acquisition System (Syngene, Cambridge, UK).

**Degradation assay**

HEK293 cells grown on 10 cm dishes were transfected with DNA encoding empty vector (pcDNA3) or constructs encoding myc-tagged wild-type CISK and mutant versions S486D, S486A, and S486D PPFA. In the degradation experiments, the cells were co-transfected with either HA-CXCR4-pcDNA or epidermal growth factor receptor (EGFR). The following day, cells were passaged onto 24-well plates and allowed to grow for an additional 24 h. For CXCR4 degradation, cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS) plus 5 – 50 µg/ml cycloheximide in the presence or absence of 10 nM stromal derived factor-1α (CXCL12, PeproTech, Rocky Hill, NJ) for 2 h. For EGFR degradation, cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS) plus 50 µg/ml cycloheximide in the presence or absence of 100 ng/ml epidermal growth factor (EGF, PeproTech, Rocky Hill, NJ) for 1 h. Cells were washed once with PBS and lysed by the addition SDS sample buffer. Samples were subject to sonication and equal volumes were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated with anti-HA or anti-EGFR receptor monoclonal antibody followed by chemiluminescence (Pierce, Rockford, IL), as previously described (Marchese and Benovic, 2001). Identical blots were generated and blotted using anti-β-tubulin antibody. Films were subject to densitometric analysis and normalized for total protein using the β-tubulin blots (which typically varied less than 1.2-fold per experiment). Lysates were also examined to detect the expression of ectopically expressed epitope-tagged CISK proteins.
**AIP4 peptides**

For the peptide phosphorylation experiments the following peptides were synthesized (Hybio, Shenzhen, China): AIP4-T344 ‘DHFTRRTTWQRPTLE’, AIP4-T344A ‘DHFTRRTAWQRPTLE’, AIP4-T409 ‘GWEKRTDSNGRVYFVN’, AIP4-T409A ‘GWEKRTDANGRVYFVN’, NEDD4-S325 ‘PQIRRPRSLSSPTVTL’.

**Supplementary Figure Legends.**

**Figure S1**

Immunoelectron microscopic localization of CXCR4. Cells were transfected with HA-tagged CXCR4 and stimulated with 100 nM CXCL12 for 2 hours. The cells were then labelled with monoclonal-anti-HA, followed by rabbit-anti-mouse and 15 nm protein A gold. Receptors were typically found in MVEs (arrowheads) and to a lesser extent in adjacent small vesicular or tubular structures (arrows). Note that receptor labelling is mostly found within the luminal part of the MVE, whereas the limiting membrane does not label.

**Figure S2**

Phosphorylation of CISK by PDK1 *in vitro* is dependent on hydrophobic motif phosphorylation. Recombinant GST fusion proteins of S486A and S486D mutants of the CISK kinase domain (residues 96-496) were incubated in the presence of PDK1. After the samples were run on SDS-PAGE, the gel was fixed and dried. The phosphorylated bands (indicated with arrows) were detected by using a PhosphorImager (Amersham Biosciences). In parallel, the gel was stained in Coomassie Brilliant Blue to detect the amount of CISK protein in each lane. The asterisk indicates the typical degradation band of the GST-CISK fusion proteins.

**Figure S3**

CISK does not inhibit the degradation of the EGF receptor (EGFR). (A) HEK293 cells were transfected with empty vector (pcDNA) or constructs encoding myc-tagged wild-type CISK or the phosphorylation mutant CISK S486D. Cells were incubated in the absence or presence of 100 ng/ml EGF for 1 h at 37°C. Equal amounts of cell lysates from treated and untreated cells were analyzed by immunoblotting (IB) using an anti-EGFR antibody. Additional blots
were probed with anti-myc and β-tubulin antibodies. Shown are representative blots from three independent experiments. (B) The immunoblots obtained from the analysis described in A were subject to densitometric analysis and receptor levels were normalized to tubulin levels. The calculated values from these experiments were analysed using GraphPad Prism 4.0, and the average and standard error of the mean (SEM) were determined. The bars indicate the amount of EGFR degraded in the presence of EGF as compared to vehicle treated cells, and show that CISK does not affect the amount of degraded receptor when compared to empty vector alone.

**Supplementary references**


**A**

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IB: EGFR

**B**

![Bar graph showing % Receptor degraded for pcDNA, WT, and S486D](#)