FIP-2, a coiled-coil protein, links Huntingtin to Rab8 and modulates cellular morphogenesis

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Huntington's disease is characterised by the death of cortical and striatal neurons, and is the result of an expanded polyglutamine tract in the Huntingtin protein [1]. Huntingtin is present on both endocytic and secretory membrane organelles but its function is unclear [2,3]. Rab GTPases regulate both of these transport pathways [4]. We have previously shown that Rab8 controls polarised membrane transport by modulating cell morphogenesis [5]. To understand Rab8-mediated processes, we searched for Rab8-interacting proteins by the yeast two-hybrid system. Here, we report that Huntingtin is linked to the Rab8 protein through FIP-2, a tumour necrosis factor-α (TNF-α)-inducible coiled-coil protein related to the NEMO protein [6,7]. The activated form of Rab8 interacted with the amino-terminal region of FIP-2, whereas dominant-negative Rab8 did not. Huntingtin bound to the carboxy-terminal region of FIP-2. Coexpressed FIP-2 and Huntingtin enhanced the recruitment of Huntingtin to Rab8-positive vesicular structures, and FIP-2 promoted cell polarisation in a similar way to Rab8. We propose a model in which Huntingtin, together with FIP-2 and Rab8, are part of a protein network that regulates membrane trafficking and cellular morphogenesis.

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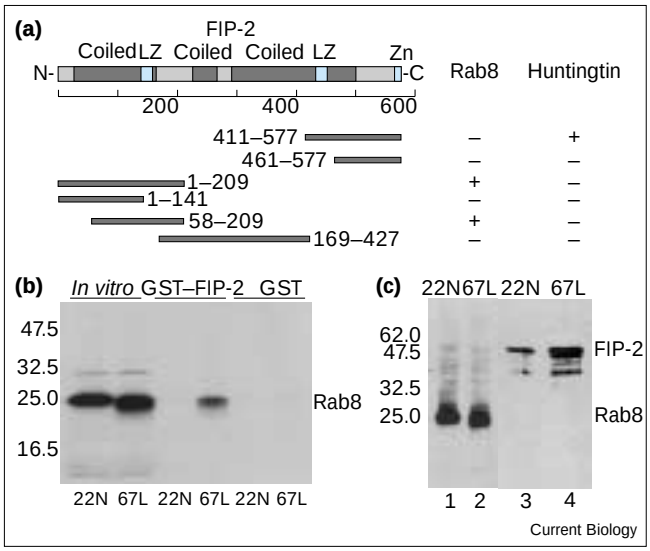
Results and discussion

About 25% of the positive clones isolated in a yeast two-hybrid screen for Rab8-interacting proteins were identical to FIP-2, a protein previously described as a E3-14.7K binder; E3-14.7K is an adenoviral protein involved in protecting a cell against TNF-α-induced apoptosis [6]. We found that FIP-2 was identical to a polypeptide (HYPL) shown to interact with the amino-terminal part of Huntingtin [8]. So we then tested these interactions in the two-hybrid system by pairwise transformations (not shown), and we found that FIP-2 specifically interacts with wild-type Rab8 and the active GTP-bound mutant Rab8-67L, but not with the dominant-negative GDP-bound mutant Rab8-22N. FIP-2 did not interact with Rab3A or Rab2, suggesting that FIP-2 is highly specific for Rab8. However, FIP-2 binds Rab8b, which is closely related to Rab8 [9]. Rab8 and Rab8b differ only in their hypervariable region, making it unlikely that this region is essential

Figure 1



FIP-2 interacts with both Huntingtin and Rab8. (a) Deletion analysis of FIP-2 to determine the regions that interact with Huntingtin and Rab8. The FIP-2 fragments examined in the yeast two-hybrid assay are shown below with the residue number of the first and last amino acids of each truncated FIP-2 protein indicated; +, positive interaction; –, no interaction. Light grey boxes, coiled-coil regions; blue boxes, leucine zipper (LZ) regions; dark grey box, putative zinc finger region. Residues 411–577 were sufficient for interaction with Huntingtin, and residues 55–209 were required for interaction with Rab8. (b) *In vitro* and *in vivo* interactions between FIP-2 and Rab8. The fusion protein GST–FIP-2d was used for *in vitro* binding studies with *in vitro* translated Rab8-22N (22N) and Rab8-67L (67L). Input *in vitro* translated materials are shown in the first two lanes. GST indicates control beads coupled to unfused GST protein. Only the *in vitro* translated Rab8-67L interacted with GST–FIP-2d in this assay. (c) For the *in vivo* binding studies, the construct encoding full-length FIP-2 was cotransfected into fibrosarcoma HT1080 cells with constructs encoding either His–Rab8-22N or His–Rab8–67L. The material cross-linked to His-tagged Rab8 proteins was eluted from metal-chelated beads and analysed by western blotting using anti-FIP-2 and Rab8 antibodies. Lanes 1 and 2 show, respectively, the presence of Rab8-22N or Rab8-67L in the input material. Lanes 3 and 4 show the presence of FIP-2 crosslinked to Rab8-22N and Rab8-67L. The molecular weights in (b,c) are in kDa.

for FIP-2 binding. Full-length FIP-2 interacted with Huntingtin$^{1–588}$ as expected, whereas no interaction could be detected between Huntingtin$^{1–588}$ and any of the Rab8 constructs (data not shown).

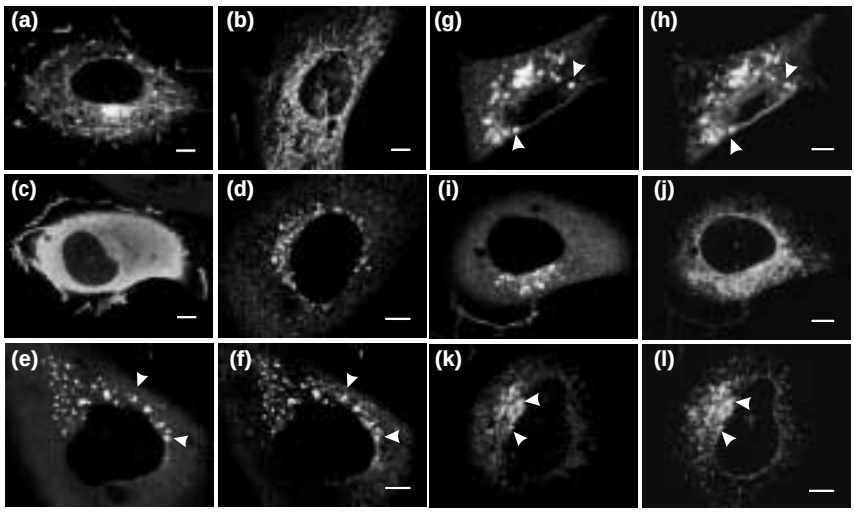
To map the areas in FIP-2 that are involved in the binding of Rab8 and Huntingtin, we generated a number of FIP-2 deletion mutants and tested these in the two-hybrid system (Figure 1a). This analysis demonstrated that the amino-terminal region encompassing amino acids 141–209 of FIP-2 is essential for Rab8 binding. This region contains one of the leucine zipper domains and is located inside the amino-terminal coiled-coil region. We found that a region between amino acids 411–461 of FIP-2 is required for Huntingtin binding. This region also contains a leucine zipper domain and is part of the carboxy-terminal coiled-coil stretch. These results indicate that Rab8 and Huntingtin bind distinct regions of FIP-2.

To analyse the interaction of Rab8 and FIP-2 *in vitro*, we produced recombinant FIP-2 in *Escherichia coli*. Unfortunately, both the full-length Rab8 and FIP-2 were insoluble. However, a glutathione-S-transferase (GST) fusion protein containing amino acids 58–209 of FIP-2 (GST–FIP-2d) was soluble. We incubated *in vitro* translated Rab8-22N and Rab8-67L with beads containing GST–FIP-2d or GST alone. Only Rab8-67L bound GST–FIP-2d, and no binding could be seen to GST (Figure 1b). This result confirms those obtained with the two-hybrid system and shows that only the GTP-bound form of Rab8 interacts with FIP-2.

We also studied the interaction of Rab8 with FIP-2 *in vivo* by using cotransfection of HeLa cells with the T7 vaccinia system. For this purpose, we used constructs expressing His-epitope-tagged Rab8-22N (His–Rab8-22N) and Rab8-67L (His–Rab8-67L) under the control of the T7 promoter. These constructs were mixed separately with a similar plasmid encoding full-length FIP-2. Post-nuclear fractions of these cells were chemically crosslinked and then applied to Talon beads, which bind the His-tagged Rab8 proteins. Western blots showed that equivalent amounts of Rab8-22N and Rab8-67L were expressed in these cells (Figure 1c). FIP-2 was readily detected in the eluate of His–Rab8-67L beads, whereas considerably smaller amounts of FIP-2 was eluted from Rab8-22N, suggesting that the GTP-bound form of Rab8 is the preferred *in vivo* binding partner for FIP-2 (Figure 1c).

We next determined how FIP-2, Huntingtin and Rab8 affect the localisation of each other in cells. We cotransfected FIP-2, Huntingtin and Rab8 with each other in HT1080 and HeLa cells, and analysed colocalisation by confocal microscopy. When Huntingtin was expressed alone in HT1080 cells, it was mainly found evenly distributed in the cytoplasm (Figure 2c). FIP-2 was also found diffusely distributed in the cytoplasm, but a major fraction of FIP-2 was associated with vesicular structures near the nucleus (Figure 2d). In accordance with previous studies, Rab8-67L was found on perinuclear vesicular structures and in peripheral regions, and the majority of Rab8-22N was confined to reticular structures around the nucleus (Figure 2a,b).

Figure 2



Coexpression of FIP-2, Huntingtin and Rab8 in HT1080 cells. After overnight transfection, cells were fixed with paraformaldehyde, permeabilised with 0.1% triton X-100 and stained with the appropriate antibody (anti-FIP-2, anti-Huntingtin) or localised as a green fluorescent protein (GFP) fusion, and analysed by confocal microscopy. Transfection with only one construct was done as control, as seen for (a) Rab8-67L, (b) Rab8-22N, (c) Huntingtin and (d) FIP-2. Colocalisation of (e,f) FIP-2 and Huntingtin, and (g,h) FIP-2 and GFP–Rab8-67L was detected (h) from the GFP fluorescence, or by using antibodies to (e,g) FIP-2 and (f) Huntingtin. (i,j) FIP-2 and Rab8-22N did not colocalise as detected using antibodies to (i) FIP-2 and (j) Rab8-22N. (k,l) Partial colocalisation of Huntingtin and GFP–Rab8-67L detected from (l) the GFP fluorescence, and (k) using antibodies against Huntingtin. Arrowheads indicate some of the structures colocalising on the merged image (not shown). The scale bars represent 5 μm.

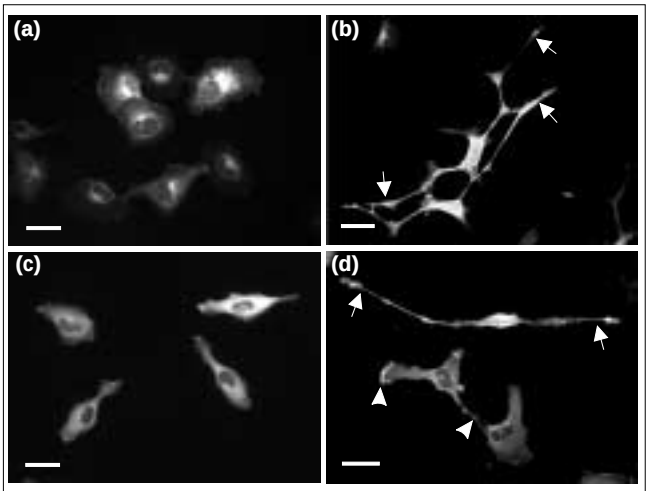
Coexpression of Huntingtin with FIP-2 resulted in redistribution of Huntingtin to FIP-2-positive vesicular structures, suggesting that FIP-2 participates in the targeting of Huntingtin to these structures (Figure 2e,f). Likewise, the expression of Rab8-67L with FIP-2 was also associated with the colocalisation of these proteins (Figure 2g,h). In contrast to Rab8-67L, the Rab8-22N mutant did not colocalise with FIP-2 (Figure 2i,j). Our results do not demonstrate a direct interaction between Rab8 and Huntingtin, but we could nonetheless show partial colocalisation of these proteins in cotransfected cells (Figure 2k,l). Rab8 can presumably recruit Huntingtin to vesicular structures with the help of endogenous FIP-2. Endogenous FIP-2 partially colocalised with the p115 protein, suggesting that at least part of FIP-2 was associated with structures on the Golgi complex (data not shown). We also saw endogenous FIP-2 on vesicles, where it partially colocalised with an enhanced GFP (EGFP)–Rab8 fusion (data not shown).

Rab8 is an extraordinary Rab protein in that it promotes radical changes in the cell shape by reorganising actin and microtubules [5]. In accordance with this, we observed that the expression of wild-type Rab8 or Rab8-67L polarised HT1080 cells; the polarisation was characterised by the formation of leading and trailing edges. High-level expression of these proteins resulted in the formation of neurite-like extensions (Figure 3b), whereas cells expressing Rab8-22N were more symmetric (Figure 3a). Because FIP-2 and Huntingtin directly and indirectly bind to Rab8, it is conceivable that they might play a role in modulating cellular morphogenesis. Expression of Huntingtin$^{1–588}$ did not notably change the cell shape (Figure 3c), but moderate expression of FIP-2 promoted the formation of extended lamellar and tail structures, and we observed formation of extended protrusions in cells expressing higher levels of FIP-2 (Figure 3d). This FIP-2-mediated change in cell shape was inhibited by Rab8-22N, suggesting that FIP-2 might act upstream of Rab8 (data not shown).

Several studies have proposed that Huntingtin participates in processes mediating vesicle trafficking [2,3]. Our results provide further evidence for this by showing that Huntingtin is indirectly linked to the small GTPase, Rab8. Huntingtin has been shown to localise to the plasma membrane, the *trans*-Golgi network and to vesicles in the cytoplasm [3]. Interestingly, a similar intracellular distribution has also been shown for Rab8 [5,10]. Moreover, we have shown here that coexpression of Huntingtin and Rab8–GTP results in partial colocalisation to a perinuclear region. The FIP-2 protein most likely mediates this colocalisation as, according to our results, FIP-2 binds both Huntingtin and Rab8. Furthermore, FIP-2 promoted relocalisation of cytosolic Huntingtin to vesicular structures.

Rab8 modulates cellular morphogenesis by a currently unknown mechanism. Our finding that a Rab8-binding protein, FIP-2, associates with Huntingtin may offer some clues. It has recently been shown that the expression of the Huntingtin-binding protein HAP-1 in PC12 cells promotes neurite extension [11]. Moreover, coexpression of a mutant Huntingtin protein with HAP-1 inhibits this process. The fact that FIP-2, another Huntingtin-binding protein, also modulates changes in the cell shape strongly support a function for Huntingtin in processes controlling cell morphogenesis. HAP-1 links Huntingtin to the p150^{Glued} subunit of dynactin, suggesting that Huntingtin is present in a protein complex that is required for motility of membrane organelles along microtubules [12]. HAP-1 also binds to a Trio-like polypeptide with a Rac1 guanine nucleotide exchange factor domain that is involved in controlling actin dynamics [13]. Furthermore, Huntingtin interacts with HIP1, a protein that links membrane vesicles to the actin cytoskeleton [14]. Whether the FIP-2-induced cell polarisation is mediated through these Huntingtin-binding proteins or by other Rab8-binding proteins awaits further studies. However, the fact that FIP-2 is induced by TNF-α suggests that it could mediate this signal through Rab8 [6]. This is an intriguing possibility because Rab8–GTP also binds another protein, Rab8ip/germinal centre kinase (GCK), which is activated by TNF-α [15]. Thus, it is likely that Rab8 plays a central role in mediating responses to stress.

Figure 3



The effects of FIP-2, Huntingtin and Rab8 mutants on cellular morphogenesis. HT1080 cells were transiently transfected, with separate constructs encoding (a) Rab8-22N, (b) Rab8-67L, (c) Huntingtin and (d) FIP-2. After 16 h, cells were fixed with 4% paraformaldehyde, permeabilised with triton X-100, stained with the appropriate antibodies and analysed by immunofluorescence microscopy. Arrows indicate the presence of extensive processes, while arrowheads indicate protrusion and tail structures. The scale bars represent 20 μm.

Although the cell biological function of Huntingtin is still unknown, some evidence implicates an important role for Huntingtin in apoptosis [16]. This is also suggested by the fact that overexpression of wild-type Huntingtin protects cells from a variety of apoptotic stimuli [17]. How this anti-apoptotic effect of Huntingtin is achieved is still not clear. FIP-2 interacts with the adenoviral E3-14.7K protein, which protects cells from TNF-α's cytolytic activity [6]. Interestingly, both the E3-14.7K protein and Huntingtin bind the carboxy-terminal region of FIP-2. Thus, the intriguing possibility exists that the binding of Huntingtin to FIP-2 could counteract cytolytic signals mediated through FIP-2. This possibility is in agreement with recent results showing that expression of mutant Huntingtin increases the expression of several inflammatory-related mRNAs [18].

Supplementary material

Supplementary material including additional methodological detail is available at http://current-biology.com/supmat/supmatin.htm.

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