Targeting of cytoskeletal proteins to the flagellum of *Trypanosoma brucei*

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SUMMARY

The eukaryotic flagellum represents one of the most complex macromolecular structures found in any organism and contains more than 250 proteins. Due to the relative ease of genetic manipulation the flagellum of *Trypanosoma brucei* has emerged as an accessible model system to study the morphogenesis and dynamics of this organelle. We have recently started to characterise the mechanisms by which components of the cytoskeletal fraction of the flagellum, such as the axoneme, the paraflagellar rod and the flagellar attachment zone, are targeted by proteins synthesised in the cytoplasm and assembled. Here, we present the identification of a novel actin-related protein as a component of the axoneme. We show that this protein shares the tripeptid motif histidine-leucine-alanine (HLA) with one of the major proteins of the paraflagellar rod, PFRA. Building on previous work from this lab which showed that a deletion comprising this motif abolished targeting of PFRA to the flagellum we demonstrate in this study that the deletion of the tripeptid motif is sufficient to achieve mistargeting both of the PFRA and the actin-related protein. We propose that this motif represents an essential part of a flagellar targeting machinery in trypanosomes and possibly in other flagellated organisms.

Key words: Trypanosome, Flagellum, Axoneme, Cytoskeleton, Paraflagellar rod, Actin, Targeting

INTRODUCTION

The eukaryotic flagellum can be regarded as a distinct organelle and cellular compartment. It performs specific functions, is morphologically distinct and contains a large number of unique proteins which, due to the absence of ribosomes, have to be transported from the cytoplasm and targeted to the flagellum (Dutcher, 1995). The dominant and highly conserved morphological feature of a eukaryotic flagellum is the axoneme with the classical 9+2 pattern of microtubules. In addition to the axoneme, the flagellum of trypanosomatids and euglenoids contains a paraflagellar rod (PFR), a highly ordered paracrystalline protein structure which is positioned alongside the axoneme (Bastin et al., 1996b). In *Trypanosoma brucei* the PFR consists of two major proteins, PFRA and PFRC, and an unknown number of minor proteins (Bastin et al., 1996b; Maga and LeBowitz, 1999). Ablation of PFRA expression by RNA interference leads to a disruption of the PFR and a paralysed phenotype. Other proteins are still targeted to the flagellum and can be transported to the flagellar tip (Bastin et al., 2000a; Bastin et al., 1998). In trypanosomes the flagellum is not only essential for providing motility but also for certain stages during the differentiation process of the parasite. Epimastigote forms of the parasite are attached to epithelial surfaces of the salivary glands of the tsetse fly via the formation of desmosomal-like structures which connect the flagellar membrane to the host surface (Vickerman, 1966). Furthermore, the flagellum of trypanosomes is attached to the pellicular membrane of the cell body via the flagellar attachment zone (FAZ) (reviewed by Kohl et al., 1999).

Despite the functional and compositional analysis of flagella from various organisms little is know about the pathways by which proteins synthesised in the cytoplasm are delivered to the flagellum. Two dimensional gel electrophoretic analysis has shown that isolated flagella of *Chlamydomonas reinhardtii* consist of over 250 different proteins (Luck, 1984). In trypanosomes, due to additional structural elements such as the PFR and the FAZ, this number is likely to be even higher. Only recently attempts have been made to identify targeting domains in proteins located in or on the flagellum, the flagellar membrane and the flagellar pocket of trypanosomatids. It has become clear that multiple pathways and targeting signals seem to exist by which individual proteins can be targeted to their flagellar destination (reviewed by Bastin et al., 2000b).

In this paper we present the identification of a novel actin-related protein as a component of the flagellar cytoskeleton and provide evidence that flagellar targeting of proteins associated with the cytoskeletal fraction of the flagellum involves a distinct localisation signal contained in flagellar proteins. We demonstrate that an identical tripeptid motif present in two distinct flagellar proteins, the novel actin-related protein and PFRA, is necessary to target these proteins to the flagellum.

MATERIALS AND METHODS

Cell culture

Procyclic *Trypanosoma brucei* 427 were grown in SDM 79 medium containing 10% calf serum at 27°C (Brun and Schönenburger, 1979).

DNA techniques

A filter containing a P1 plasmid library of genomic DNA from

Trypanosoma brucei strain 927 (average insert size 60 kb, kindly provided by S. Melville, University of Cambridge) was screened with a digoxigenin-labelled cDNA fragment using the digoxigenin filter hybridisation system (Roche Diagnostics). The cDNA probe represented a sequence unrelated to this project. Purified P1 plasmid DNA from a positive clone was digested with *Eco*RI, separated by agarose gel electrophoreses and probed with a digoxigenin-labelled cDNA as above. A single positive fragment of 5 kb was isolated and subcloned into pBluescript plasmid (Stratagene) and sequenced using the BigDye cycle sequencing protocol (Perkin-Elmer). The Ty epitope-tag, comprising the amino acid residues EVHTNQDPLD (Bastin et al., 1996a) was added to the C terminus between amino acid residue 433 and the stop codon of the full length TrypARP sequence by PCR using the forward primer 5′-CCCAAGCTTATGGTTT-GCAGCACGGAGCGA and the reverse primer 5′-GGATCCCTA-GTCAAGTGGATCTTGGTTAGTATGGACCTCCTGGCACTTGCA-GTGCACAA (tag is underlined). The tagged construct was cloned into the trypanosomal expression vector pHD430 where transcription of the inserted gene is driven by the RNA polymerase I procyclin promoter (Wirtz and Clayton, 1995). The tagged PFRA construct in the same vector was provided by P. Bastin and has been described previously (Bastin et al., 1999a). Deletion of the HLA-motif in both plasmid constructs was done using the GeneEditor mutagenesis system (Promega) employing oligonucleotides with 20 nucleotides of homologous flanking sequences on either side of the HLA-motif. Successful mutagenesis was confirmed by DNA sequencing. Procyclic *Trypanosoma brucei* were transformed by electroporation (Beverley and Clayton, 1993) with the linearised expression vector to achieve homologous integration into the rDNA spacer locus of the genome (Wirtz and Clayton, 1995) and selected in medium containing 2.5 μg/ml phleomycin. Transformations were screened by immunofluorescence as described below. Transformed trypanosomes were cloned by limiting dilution to obtain homogeneous populations.

Immunofluorescence

Procyclic *Trypanosoma brucei* 427 were settled on organosilane-treated slides to enhance adhesion of cells and fixed for 10 minutes with 3.7% formaldehyde in PBS. Cells were then permeabilised with cold 0.5% Nonidet P-40 in PBS for two minutes. Cytoskeletons were prepared by extracting the cells prior to fixation in situ for 2 minutes with cold 0.5% NP-40 in 100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$. The tagged proteins were detected with the mouse monoclonal IgG anti-Ty antibody BB2 (Bastin et al., 1996a) and an FITC-conjugated secondary antibody (Dako, UK). The paraflagellar rod was visualised using mAbs L8C4 (IgG, recognises PFRA) (Kohl et al., 1999) and ROD-1 (IgM, recognises an unknown PFR protein) (Woods et al., 1989b). ROD-1 was detected using a rhodamine-conjugated anti-IgM secondary antibody (Sigma). Basal bodies were visualised with mAb BBA4 (IgM) (Woods et al., 1989b). Images were digitally recorded on a Leica DMR epifluorescence microscope using a cooled CCD-camera (Roper Scientific, UK) analysed with IP-Lab software (Scanalytics, USA) and pseudocoloured in Adobe Photoshop.

Protein analysis

Cells were fractionated into a detergent-soluble and –insoluble (cytoskeleton) fraction by extraction with 0.5% NP-40 exactly as described (Woods et al., 1989a). SDS-PAGE and western blotting was done using standard techniques. Chemiluminescence was used for blot development (Enhanced Luminol, NEN).

RESULTS

Flagellar proteins in trypanosomes

In a previous study (1999a) we have shown that deleting the C-terminal 36 amino acid residues (residues 564-600) of PFRA led to a severe reduction in the ability of the protein to localise to the flagellum. A shorter deletion of only the last 30 amino acid residues had no visible effect. This result indicated that the deletion might have removed a hitherto unknown flagellar targeting signal contained in the C-terminal sequence of the protein. We also showed that in some flagellar proteins of other organisms, such as in the heavy chain of axonemal β-dynein of *Chlamydomonas reinhardtii*, a short sequence of amino acids is found which resembles the putative targeting region of PFRA.

In the course of an unrelated project we have identified a gene encoding for a novel putative actin-related protein in *Trypanosoma brucei* (Fig. 1). The newly identified gene (EMBL accession no. AJ132925) encodes a protein of 433 amino acid residues (M_r 47.5×10^3) whereas the trypanosomal actin contains only 376 amino acid residues (M_r 41.9×10^3) (Ben Amar et al., 1988). The overall identity between the two proteins is 34% and the similarity 69%. The increase in molecular mass is due to the presence of three peptide insertions relative to the actin protein. The moderate degree of identity to conventional actin and the presence of distinct

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ACT_TB M__SDEEQTAIVCDNGSGMVKSGFSGDDAPRHVFPSIVGRPKNEQAMMGSANKKLFVGDE
       |  | |    ** | ||  ***|***|*|||  *|**||*|*|    **** *   *||
ARP_TB MVCSTERAPVVILDGGSHHLRAGYASDGAPRLDIPALVGHPRNRGVAVAAGMNEYEIGDV

ACT_TB AQAKRGVLALKYPIEHGIVTNWDDMEKVWHHTFYNELRVNPESHNVLLTEAPMNPKQNRE
       | ||||*|**  ||| | | *|**|||*| |  |*|||| ||||  ** **   |  **|
ARP_TB ALAKRGMLTVSSPIESGRVVSWENMEKLWGHVMYSELRVRPESHCFIVPQSVNTPASQKE

ACT_TB KMTQIMFETFGVPAMYVGIQAVLSLYSSGRTTGIVLDAGDGVTHTVPIYEGYSLPHAIRR
       |  **| ||| | ****|   |||||| | |||*|*|*| * | *||* |||*| * *
ARP_TB KTLELMMETFHVHSLFLGASQVLSLYSYGLTTGLVIDSGKDRTMAVPVHEGYALGRHVAE

ACT_TB VDMAGRDLTEYLMKILMHTGMTFTTSAEKEIVRNIKEQLCYVALDFDEEMTNSA------
        |*||  ||||*  *|   | *| |* | *** | ||**|||   *      ||
ARP_TB SDVAGEKLTEYFASLLRLEGNSFGTPMEMQVLNNAKEDICYVKPPIFNMTGPSAFFSPSE

ACT_TB ------------------------KSVSEEPFELPDGNVMQVGNQRFRCPEALFKPALI
                               |  |  | ||||| * ** *|    ||||  ***
ARP_TB FPGECDYDLSLEGAPGEGFEDGREDHSSDERVFYLPDGNAIPISTHRSLTTEALFDFGIL

ACT_TB GLDEAPGFHEMT-----------------FQSINKCDIDVRRDLYGNIVLSGGTTMFKN
       | * *|  * ||                | *||*|*  *| *||**||||||   |
ARP_TB GSQYVPKSRYMTELGEIFQPSFPMGVSWLAFAAINNCQPVIRAQLYASIVLSGGNVSFPG

ACT_TB LPERLAKEISNLPPSSIKPKVVAP------PERKYSVWIGGSILSSLTTFQSMWITKSEY
         ||*  |***|   *  *  |*|      | | || |*|||*|** * |  * *** ||
ARP_TB TRERIETEVTQLYRETHTSEAVTPIAVNDIPCRVYSAWVGGSMLAGTSMFPHLAVSRQEY
PFRA                                                  VEYRAHLAKQEEVK

ACT_TB DESGPSIVHSKCF      376aa
       *| |  *|| ||
ARP_TB EEQGHRVVHCKCQ      433aa
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Fig. 1. Alignment of the *Trypanosoma brucei* actin protein sequence (ACT_TB, Swiss Prot accession no. P12432) with the trypanosomal actin-related protein (ARP_TB, EMBL accession no. AJ132925). Actin sequences between organisms are highly conserved and the trypanosomal actin is representative of actin sequences in general. Identical residues are connected by bars, similar residues by star symbols. The flagellar targeting signal in the TrypARP sequence is boxed. Note the three insertions of additional sequence in the TrypARP protein relative to actin. The partial PFRA sequence containing the HLA motif is listed below the relevant part of the ARP sequence.

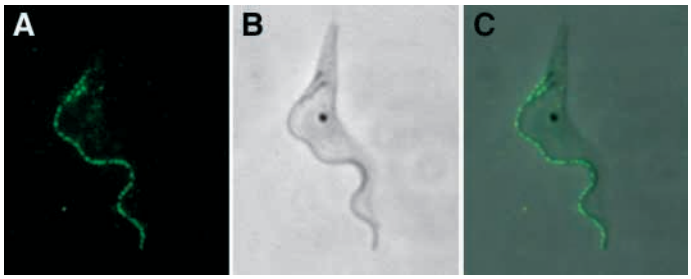

Fig. 2. (A) Immunofluorescence detection of the tagged TrypARP with the monoclonal antibody BB2. The TrypARP-pHD430 construct was stably integrated into the trypanosomal genome and constitutively expressed. (B) Phase contrast image of the same cell. (C) Merged images. The immunofluorescence signal of the TrypARP extended to the distal tip of the flagellum indicating the flagellar localisation of the protein (in contrast to a possible flagellar attachment zone localisation). The cell has just duplicated its basal bodies and has started to assemble a new flagellum. The fluorescence signal is localised on both the mature and emerging daughter flagellum. It emanates in close proximity to the basal bodies. The dark dot in the centre of the cell is the nucleolus. The length of the cell is approximately 20 μm.

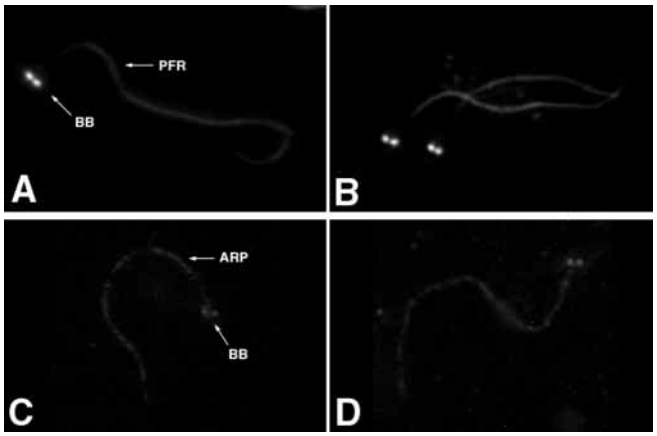

Fig. 3. Simultaneous labelling of the basal bodies and the endogenous PFRA protein (A,B) or the tagged TrypARP protein (C,D). Antibodies against basal bodies (BBA4) and either PFRA (L8C4) or tagged TrypARP (BB2) were mixed and detected with the same secondary FITC-conjugated antibody. In contrast to the PFRA protein which begins further distal from the basal bodies, the TrypARP signal is in very close proximity to the basal bodies. This is indicative that the TrypARP protein is not a component of the paraflagellar rod but of axonemal origin.

insertions is typical of proteins belonging to the family of actin-related proteins (ARPs). The trypanosome actin-related protein (TrypARP) has, however, no similarities with other ARPs as both the composition and length of the ARP-typical insertions are unique. Therefore, the TrypARP represents a novel ARP and the first ARP characterised in trypanosomes. A phylogenetic analysis of ARPs, including data for this TrypARP, has been presented by Schafer and Schroer (Schafer and Schroer, 1999).

To determine the localisation of TrypARP we epitope-tagged the full length coding region and created a stable trypanosome cell line expressing this construct under the control of a strong constitutive promoter. Using western blotting we confirmed that a protein of the expected molecular mass was expressed in transformed trypanosomes (data not shown). Immunofluorescence detection of the tagged protein in cytoskeletal preparations revealed that the protein is localised to the flagellum in detergent-extracted cells (cytoskeletons) (Fig. 2). Superimposition of phase contrast images with the tagged TrypARP immunofluorescence showed that the antibody staining followed the length of the flagellum and terminates at its distal tip (Fig. 2). This result indicated that the TrypARP is not a component of the flagellar attachment zone which does not extend beyond the cell body. The flagellum of trypanosomes contains two prominent internal structures, the axoneme and the paraflagellar rod (PFR). Because the PFR is unique to trypanosomes and the TrypARP has no homologue in the databases it would have been possible that this novel ARP is an additional component of the PFR. To determine its exact intraflagellar localisation we compared the position of the staining pattern of a well characterised PFR protein, PFRA, and the TrypARP relative to the basal body of the cell. The axoneme originates at the basal body whereas the PFR structure is initiated only at the exit of the flagellum from the flagellar pocket of the cell body (Bastin et al., 1996b). Double-labeling immunofluorescence using an anti basal body antibody (BBA4), the anti-tag antibody BB2 or the anti PFRA antibody L8C4 clearly showed that the TrypARP, in contrast to the PFR, originated in very close proximity to the basal body (Fig. 3). Furthermore, we double-labelled cytoskeletons with the anti PFR antibody ROD-1, an IgM, and BB2, an IgG. Merging of images showed that the two structures revealed by the antibodies are distinct from each other (Fig. 4). The ROD-1 staining, representing the PFR, is localised between the cell body and the BB2 staining, which marks the tagged TrypARP localisation. These results suggest that the ARP is an integral component of the cytoskeletal fraction of the flagellum and immunofluorescence colocalisation studies strongly indicate an axonemal localisation.

Targeting of PFRA and TrypARP to the flagellum

The identification of a novel flagellar protein in trypanosomes which is unrelated to PFRA-like proteins enabled us to look for common sequence motifs between these two proteins of the cytoskeletal fraction of the trypanosomal flagellum. Comparing the PFRA sequence (EMBL accession no. X14819) with the TrypARP sequence we noticed no obvious similarities except for a tripeptide sequence comprising the amino acids histidine, leucine and alanine (HLA). In TrypARP, which has 433 amino acids, it is located at residues 412-414, in PFRA (600 amino acids in total) it comprises residues 563-565 and is therefore partially covered by the C-terminal PFRA deletion mutants described in our previous studies (Bastin et al., 1999a). The HLA-motif is not contained within one of the TrypARP insertions relative to actin but part of the actin-similar core sequence, but not present in the actin sequence itself (Fig. 1). Based on the three-dimensional actin structure (Kabsch et al., 1990) we predict that the HLA-motif is positioned on the outside of the TrypARP molecule and therefore potentially accessible for interactions with other proteins. Similarly, the

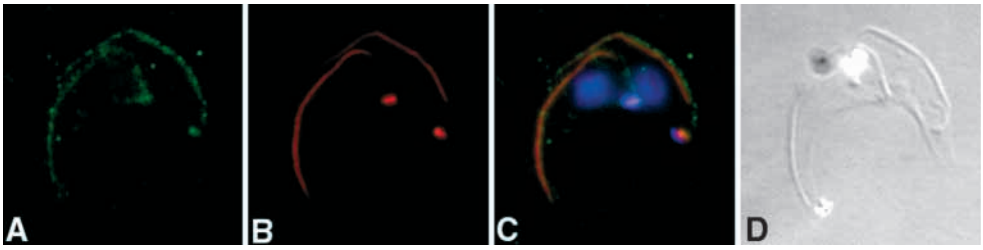

Fig. 4. Along the entire length of the flagellum the localisation of ARP is distinct from PFRA. Labelling of the tagged TrypARP with BB2 (A), the paraflagellar rod and the basal body with a mixture of the anti PFR antibody ROD and the basal body with BBA4 (B). The very bright staining of BBA4 causes a certain amount of bleed-through into the FITC-channel in A. The merged image (C), which also includes the DNA staining pattern using DAPI, shows that the two structures can be resolved into two distinct, non-overlapping patterns. (C) A DIC image of the same cell. This cell is in mitosis and has two flagella. In the right flagellum, which is the new daughter flagellum, the ROD/PFR signal is located between the cell body and the TrypARP signal. The location of the TrypARP signal again suggests an axonemal localisation of the protein.

position of the HLA-motif near the C terminus of PFRA which contains a number of charged residues also makes an exposed localisation likely.

To test whether this motif is part of a flagellar targeting signal in both proteins we deleted the three amino acids by in vitro mutagenesis and localised the tagged, mutagenised protein by immunofluorescence. First, we determined whether the mutagenised and non-mutagenised tagged proteins are expressed in stably transformed trypanosomes. In both cases, a protein product of the expected size was detected by western blotting with no apparent differences in the expression levels of the mutagenised and non-mutagenised versions (data not shown, but see Figs 5, 6 and 7). Although the PFRA protein is one of the most abundant proteins in the cell, the deletion led to an almost complete ablation of the immunofluorescence signal from the PFR of the flagellum in cytoskeletons and a massive accumulation in the cytoplasm. The cytoplasmic protein is soluble because it was not detectable in detergent-extracted cytoskeletons. The measured intensity of the flagellar fluorescent signal compared to that of cells expressing non-mutagenised tagged PFRA indicates a reduction of >95% (Fig. 5).

A very similar result was obtained with the TrypARP-ΔHLA cell line (Fig. 6). Immunofluorescence microscopy revealed the ablation of the mutated TrypARP protein signal in the flagellum of the TrypARP-ΔHLA cell line below the threshold of detectability. The strong diffuse signal in the cytoplasm was, however, maintained. In situ extraction of unfixed cells with the non-ionic detergent NP-40 abolished this cytoplasmic

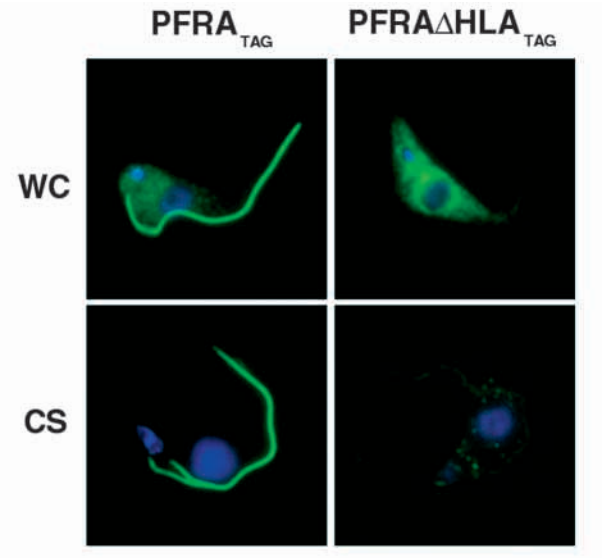

Fig. 5. Localisation of the tagged PFRA protein and the PFRAΔHLA protein in fixed whole cells (WC, whole cells) and cells extracted with 0.5% NP-40 prior to fixation (CS, cytoskeletons). The signal of the non-mutated protein (left panels) in non-extracted cells (WC, upper panels) is localised in the cell body and the flagellum, whereas it is localised only in the cell body in mutated cells (right panels). In detergent-extracted cells (CS, lower panels) only the flagellar staining remains in non-mutated cells. In mutated cells, a faint outline of the flagellum is recognisable and some residual dot-like staining within the cell body. Exposure times were the same within each figure but differ between Figs 5 and 6 and a quantitative comparison is therefore not possible. The DNA of the nucleus and kinetoplast is stained with DAPI (blue).

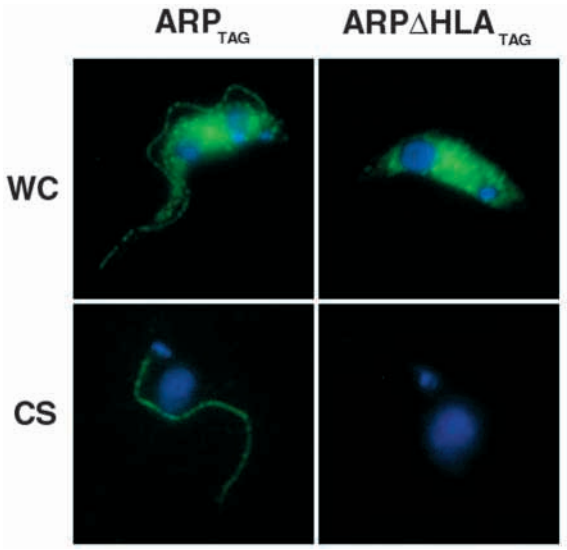

Fig. 6. Localisation of the tagged ARP and the ARPΔHLA mutant in fixed whole cells (WC, whole cells) and cells extracted with 0.5% NP-40 prior to fixation (CS, cytoskeletons). In non-extracted cells (upper panel) the anti-tag antibody BB2 recognises the tagged non-mutated protein in the cell body and in the flagellum. In mutant cells only the cell body is labelled. The cell body staining is completely extractable with detergent (lower panels). Only the flagellar labelling is resistant to detergent extraction indicating that the ARP protein has been integrated into a structural element of the flagellum. The DNA is stained with DAPI (blue).

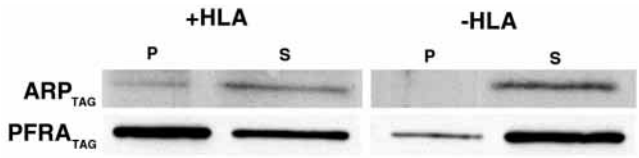

Fig. 7. Western blot analysis of the distribution of mutated and non-mutated tagged ARP and PFRA proteins after fractionation of cells into a detergent-insoluble (P, pellet) and detergent-soluble (S, supernatant) fraction using 0.5% NP-40. In non-mutated cells (+HLA) the tagged ARP and PFRA proteins is detectable in both the soluble and insoluble fractions. In mutated cells (–HLA) the tagged ARP protein is no longer detectable in the insoluble fraction. In the case of tagged mutated PFRA, the bulk of the protein is found in the soluble fraction and only a small amount remained in the insoluble fraction. These data are a semiquantitative reflection of the in situ observations described in Figs 5 and 6.

staining. This indicated that the protein accumulated in a soluble form in the cytoplasm instead of being targeted and incorporated into a flagellar structure, rendering it resistant to detergent extraction.

Western blot analysis of cytoskeletal and soluble extracts of mutated and non-mutated cell lines of both proteins confirmed the microscopical analysis. The detection of the non-mutated TrypARP showed a clear signal in both the cytoskeletal and soluble fraction whereas in TrypARP-ΔHLA extracts the signal had decreased below detection level in the cytoskeletal fraction but remained detectable in the soluble fraction (Fig. 7). The fractionation and western blot analysis of the PFRA mutant led to similar results. In non-mutated cells a strong signal is detected in the cytoskeletal fraction but in cells expressing the PFRAΔHLA mutation, the signal in the cytoskeleton fraction is drastically reduced (Fig. 7).

DISCUSSION

The definition of a flagellar targeting signal

A sequence comparison of the trypanosomal actin-related protein revealed a shared peptide motif with PFRA, an otherwise unrelated flagellar protein in *T. brucei* which is one of the two major proteins of the PFR structure. The fact that a C-terminal deletion mutant of PFRA which comprises this motif displays a severe PFRA targeting phenotype led us to the hypothesis that this common motif might have a shared function in these proteins. We confirmed the importance of this motif for correct flagellar targeting of both proteins by specific deletion mutagenesis in both the PFRA and the ARP proteins. Whereas the targeting of epitope-tagged ARP to the flagellum was completely abolished, the amount of epitope-tagged PFRA in its proper location was reduced by more than 95%. Both sets of cytological data were congruent with cell fractionation experiments.

The remaining small amounts of tagged PFRA which entered the flagellum were correctly targeted to the PFR. This indicates that the deletion of the HLA motif is not essential for proper incorporation into the appropriate flagellar structures but that its function affects events upstream of assembly itself. In a recent study on flagellar assembly and dynamics we (Bastin and Gull, 1999) have dissected the steps required for flagellar assembly in trypanosomes into a number of discrete events from protein synthesis to cytoplasmic transfer, entry into the flagellar compartment, intraflagellar transport and assembly. Using *Chlamydomonas* and sea urchin as model organisms to study flagello- and ciliogenesis several routes of intraflagellar transport (IFT) have been described (Rosenbaum et al., 1999). The most intriguing route to transport proteins within the flagellum has been initially described by Kozminski et al. (1993) and involves the formation of raft-like particles which can be observed moving along the axoneme towards the distal tip of the flagellum. This bulk movement of proteins is mediated by kinesins such as FLA-10 in *Chlamydomonas* as mutations in the corresponding gene can abolish this process (Kozminski et al., 1995). Morphologically similar particles have also been observed in the flagellum of trypanosomes by electron microscopy (Sherwin and Gull, 1989) but it is unknown whether they represent the functional equivalent of the ‘rafts’ described in *Chlamydomonas*. Because it is not known whether PFR proteins or ARP are transported via such a mechanism we can only speculate on the possibility that the HLA-motif has a function in associating different proteins with such transport particles. IFT is further complicated by the observation that other routes of anterograde transport may exist beside the ‘raft’-transport (Stephens, 1995). In addition, the flagellum is also characterised by the presence of specific sets of membrane proteins. For example, in the trypanosomatid *Leishmania enriettii* two isotypes of a glucose transporter have been described: one is targeted to the cell body membrane and the other is targeted exclusively to the flagellar membrane (Snapp and Landfear, 1997).

The import of precursor proteins into the flagellar compartment represents a step in the assembly of the flagellum where proteins can potentially be actively selected for the flagellar compartment. Using two-dimensional gel electrophoresis it has been estimated that the flagellum contains more than 250 proteins (Luck, 1984). Although only a small number of these proteins have been characterised it has been shown that many proteins are exclusively found in this organelle, the classical example being flagellar- or cilia-specific dyneins (Dutcher, 1995). Also, many cytoplasmic proteins seem to be unable to enter the flagellar compartment by default, a notable exception being the ubiquitous HSP70 which might assist in assembling flagellar components (Bloch and Johnson, 1995; Stephens and Lemieux, 1999). Morphologically, a distinct feature of the flagella of many organisms including trypanosomes is a necklace-zone which is localised close to the transition zone between the proximal triplet and the distal doublet axonemal structure (Dentler, 1981; Ringo, 1967; Vickerman and Preston, 1976). This zone contains structures which connect the axoneme to the flagellar membrane and it is possible, though unproven, that this structure forms a barrier or selective valve controlling movement of proteins into the flagellar compartment.

The presence of a functional targeting signal in two unrelated proteins, although from the same organism, raised the possibility that identical or similar signals might occur in other flagellar proteins from different organisms. A screen for this motif in flagellar proteins revealed that a number of flagellar proteins contain the HLA-motif, but by no means all flagellar proteins. If the HLA motif is a more general flagellar targeting signal two arguments can explain the absence of the

motif in many flagellar proteins. Firstly, the signal occurs only in proteins which use one of several possible modes of flagellar entry and transport. Possible alternative pathways of protein transport within the flagellum can be flagellar membrane-associated, axoneme-associated or via the flagellar matrix (Stephens, 1994; Stephens, 1995). Godsel and Engman (1999) have identified a 24 kDa calcium binding protein (FCaBP) in *Trypanosoma brucei* which is associated exclusively to the flagellar membrane. They were able to show that the N-terminal 24 amino acids of FCaBP which are myristoylated and palmitoylated in position 2 and 4, respectively, and binds calcium via internal EF-hands, are necessary and sufficient for targeting this protein to the membrane of the flagellum. An analogues example for alternative targeting to the same organelle is the variety of signals and mechanisms by which proteins are imported/exported into and out of the nucleus (Nakielny and Dreyfuss, 1999). Secondly, some flagellar precursor proteins enter the flagellum as complexes preassembled in the cytoplasm. The pathway of cytoplasmic pre-assembly and co-transport into the flagellum is well documented for the dynein arm complexes of the axoneme (Fok et al., 1994; Fowkes and Mitchell, 1998). Interestingly, only very few of the dyneins contain the HLA-motif. It occurs only once in the more than 1200 amino acids of the *Chlamydomonas* heavy chain isoform DHC1b, which is involved in retrograde intraflagellar transport (Rosenbaum et al., 1999). Many other dynein components do not have this motif at all. In trypanosomes it has been shown that the ROD-1 antigen is unable to enter the flagellum in the absence of the PFRA protein, which contains the HLA motif (Bastin et al., 1999b). Complexed proteins would need a targeting signal only on one component to enter the flagellum. Therefore, the occurrence of the HLA-motif is compatible with the concept of preassembly of flagellar protein complexes before entering the organelle. In this context the occurrence of the HLA –motif in tektins, a family of three proteins (tektins A, B, C) which form unique intermediate filament-like protofilaments in the connecting zone of the A and B tubules in the axoneme, is interesting. The current model indicates that tektins A and B form a stable heterodimer which is associated to a tektin C homodimer (Norrander et al., 1996; Pirner and Linck, 1994). Of the three tektins only tektin C contains the HLA-motif, raising the possibility that tektin C might serve as a carrier for tektins A and B. The HLA motif is also found in the recently cloned human and mouse sperm tektin-t (Iguchi et al., 1999).

Is the HLA motif on its own sufficient to target cytoskeletal proteins to the flagellum? A statistical analysis of the complete SWISSPROT database revealed that HLA occurs on average as often in non-flagellar proteins as in proteins of established flagellar localisation (data not shown). Furthermore, GFP fused to the carboxy terminus of PFRA, containing the HLA-motif, and expressed in trypanosomes localises to the flagellum but also to the cell body. Therefore, the HLA sequence is necessary but not sufficient for exclusive targeting of proteins to the flagellum. The lack of sequence similarity between unrelated flagellar proteins such as PFRA and TrypARP beyond this common motif seems to indicate that no other simple linear sequence elements are involved in targeting but that probably the overall structural context of the HLA signal within a protein may be an important factor for its function as a targeting signal. It is possible to superimpose the TrypARP structure onto the known crystal structure of actin. Kellheher et al. have shown that the overall structure of many ARPs is very closely related to that of actin (Kelleher et al., 1995). A comparison of the modelled TrypARP 3-dimensional structure with actin reveals that the HLA motif is most likely contained within a short α-helix and localised on the surface of the protein. Such additional structural requirements might be necessary for the functionality of this targeting motif. We also cannot exclude the presence of more complex, not easily recognisable non-linear protein motifs which contribute to flagellar protein targeting.

The presence of a flagellar localisation signal and the selective targeting of proteins to this organelle implies the existence of receptor or carrier molecules which mediate this process. The definition of a targeting signal will facilitate the identification the components of proteins involved in flagellar targeting.

A novel actin-related protein as a component of the flagellum

Actin-related proteins (ARPs) are members of a growing family of proteins belonging to a superfamily of ATPases which includes actin, heat-shock proteins, hexokinases and other proteins (for a review, see Schafer and Schroer, 1999). ARPs are usually slightly larger than actin due to the presence of short sequence insertions relative to the consensus actin sequence. They share between 30-40% identity to actin. Based on structural predictions in none of the ARPs studied on a primary sequence level these insertions appear to disrupt the core three-dimensional structure described for actin (Kabsch and Vandekerckhove, 1992; Lorenz et al., 1993). Molecular modelling studies indicate that the insertions are positioned as loops on the outside of the molecules and may determine the individual functions of different ARPs by mediating specific protein-protein interactions (Dyche Mullins et al., 1996; Kelleher et al., 1995). The functions of most ARPs are unknown. Only ARP1 (centractin), as part of the dynactin complex, and the role ARP2/3 in regulation of actin dynamics have been extensively characterised. Whereas these ARPs occur in the cytoplasm some ARPs, e.g. ARP7 and 9 in yeast, have been found in the nucleus as part of the SWI/SNF chromatin remodelling complex (Schafer and Schroer, 1999). Analysis of kinetoplastid-specific databases revealed that an ARP3 homologue (accession no. AQ902473) and an as yet unclassified actin-related protein (accession no. AL359776, ID CAB95256) has been identified in *Leishmania major* as a result of the genome sequencing project.

Here we describe the first identification of a typical actin-related protein in the eukaryotic flagellum. Previously, it has been shown that actin itself constitutes one of the light chains of the inner arm dynein complex in the flagella of *Chlamydomonas* (Kagami and Kamiya, 1992; Kato-Minoura et al., 1997; Piperno and Luck, 1979). It has been mapped to the inner projections of the dynein arms I2 and I3 but is absent from I2. An unusual form of actin occurring in the flagellum of the green alga *Chlamydomonas reinhardtii* has been described previously. The sequence of the *Chlamydomonas* NAP protein has a much higher degree of identity (64%) with actin than actin-related proteins and also lacks the ARP-typical insertions and therefore has virtually the same molecular mass as actin (Kato-Minoura et al., 1997; Kato-Minoura et al.,

1998). Interestingly, it has been shown that in actin-minus mutants of *Chlamydomonas* this divergent actin seems to be able to complement most of the deficiencies expected to occur by the loss of actin by upregulating its own expression (Kato-Minoura et al., 1998). Southern blotting analysis (data not shown) indicate that the TrypARP protein is coded by a single copy gene in the diploid genome. Therefore, the trypanosomal ARP represents an interesting and accessible model system to study the role of this novel protein in the structural organisation of the flagellum.

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