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DIPLOMARBEIT

MORN-repeat proteins and TbMORN1 targeting in *Trypanosoma brucei*

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Kurzfassung

Im Protist Trypanosoma brucei liegen die meisten seiner kanonischen Organellen- das Flagellum, das Mitochondrium, der Golgi-Apparat- als einfache Kopie vor und daher wird er gerne zur Beantwortung von elementaren zellbiologischen Fragen herangezogen. Bei Studien über die Replikation des Golgi-Apparats wurde eine neue Organelle des Zytoskeletts, genannt Bilobe, entdeckt. Kürzlich wurden zwei strukturelle Komponenten der Bilobe TbMORN1 und TbLRRP1 identifiziert. TbMORN1 besteht aus einer höchst repetitiven Primärstruktur von 15 MORN-repeats, daher muss sich die Zielinformation zur Bilobe innerhalb dieser Wiederholungen befinden. Im Genom von T. brucei gibt es 17 weitere Leserahmen, welche MORN-repeat-Proteine vorhersagen. Keines dieser Proteine ist experimentell charakterisiert. Unser primärer Ansatz war es daher, diese Proteine in prozyklischen Trypanosomen zu exprimieren und auf eine Lokalisierung auf der Bilobe hin zu untersuchen. Sieben Proteine lokalisierten zu dem Axoneme, eines zu dem PFR, zwei wurden am Basal body gefunden, drei waren im Zytosol vorhanden und eines möglicherweise auf dem Mitochondrium. 10 von 14 Proteinen wurden auf Strukturen des Zytoskeletts gefunden, was auf eine strukturelle Rolle der MORN-repeat-Proteine hinweist. Jedoch wurde keines auf der Bilobe entdeckt. Da die Zielinformation in der TbMORN1-Sequenz vorhanden sein muss, führte ich parallel dazu Verkürzungsexperimente durch. Interessanterweise wurden die meisten verkürzten Proteine zusätzlich zur Bilobe am basal body gefunden. Des Weiteren beobachtete ich bei C-terminal verkürzten Proteinen TbMORN1(1-350) – TbMORN1(1-260), ein Muster von Lokalisierung und Misslokalisierung zur Bilobe. Meine Vermutung ist, dass diese ungewöhnliche Beobachtung durch hydrophobische Schlaufen, welche das Protein eventuell an die Plasmamembran binden können, zu erklären ist. Nach Auswertung aller Daten ergaben sich fünf wichtige Domänen. Die Aminosäuren 1-72 und 350-353 sind für eine Lokalisierung ausschließlich zur Bilobe notwendig, um eine starke, stabile Bindung herzustellen. Für die Bildung von sognannten „Spears“, parakistallinen Artefakten, wurden die Aminosäuren 343-350 als wichtig gefunden. Für das Bilobe-targeting sind die Aminosäuren 215-312 notwendig, wobei gleichzeitig ein targeting zum basal body stattfand. Für die Lokalisierung zum basal body war das letzte MORN-repeat ausreichend. Es ist möglich, dass das targeting durch eine Sequenz, welche nicht Teil der MORN-repeats ist, bewerkstelligt wurde. Aus meinen Daten ziehe ich den Schluss, dass das 3D-Motiv der entscheidende Faktor für das targeting von TbMORN1 an die Bilobe ist.

Abstract

The protist Trypanosoma brucei posses most canonical eukaryotic organelles - flagellum, mitochondrion, Golgi - in single copy and is therefore widely used to address basic cell biological questions. Previously a novel cytoskeletal organelle called the bilobe was serendipitously discovered during studies on Golgi replication. Recently two of its structural components TbMORN1 and TbLRRP1 were identified. TbMORN1 consists of a highly repetitive primary structure of 15 MORN - repeats. Therefore the targeting information to the bilobe must be somewhere in these repeats. In the T.brucei genome there are 17 other open reading frames predicted to encode MORN-repeat proteins which are all experimentally uncharacterized. The candidate approach was to express these proteins in procyclic Trypanosomes and screen them for localization redolent of the bilobe. 7 proteins localized to the axoneme, 1 to the PFR, 2 were found at the basal body, 3 were present in the cytosol and one might be mitochondrial. 10/14 proteins where found on cytoskeletal structures which hints at a structural role of MORN-repeat proteins. However none were found at the bilobe. Since TbMORN1 must have the targeting information somewhere in its sequence I in parallel generated truncation constructs to gain information on which MORN-repeats are essential and which are dispensable for bilobe localization. Interestingly most of the truncated proteins were additionally found at the basal body in intact cells which suggests that tagged proteins might traffic to the bilobe through the basal body. I further observed a strange pattern of localization and mislocalization to the bilobe in C-terminal truncations, TbMORN1(1-350)-TbMORN1(1-260). I suggest that this unusual observation could be explained by hydrophobic loops which might bind the protein to the plasma membrane. Combining data from all truncations, I found that 5 domains are important. Amino acids 1-72 and 350-353 are required for targeting exclusively to the bilobe and forming a strong, stable association. The domain 343-350 was found important for the formation of spears, artefactual paracrystaline structures. Further, amino acids 215-312 are necessary for bilobe targeting with impaired targeting to the basal body. For targeting to the basal body the last MORN-repeat was sufficient. It is possible that the targeting is achieved through a sequence that is not part of the MORN-repeat consensus sequence. Concluding from our data I rather suggest that a 3D motif is the crucial factor for targeting TbMORN1.

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1 Introduction

1.1 Trypanosoma brucei

The protist Trypanosoma brucei is a simple eukaryote. As an obligate parasite it is the causative agent of sleeping sickness in humans and nagana in animals. These diseases severely affect social and economic development in sub-Saharan Africa. There are two subspecies Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense which are infective to human (WHO Media Centre, http://www.who.int/mediacentre/factsheets/fs259/en/). The incidence may approach 300 000 to 500 000 cases per year and the disease is invariably fatal if untreated (Berriman et al., 2005). Vaccines or adequate drugs for effective treatment of this disease are currently not available (Simarro et al., 2008). During its complex life cycle Trypanosomes migrate between a mammalian host and an insect vector, the tsetse fly. The main developmental form found in the insect is the gut stage procyclic form; the main type in the human host is the bloodstream form. The cells of both forms are covered in a glycoprotein surface coat. The procyclic form surface coat consists of acidic repeat proteins (procyclins) whereas the bloodstream form is mainly covered by a single isoform of variant surface glycoprotein (VSG) (McConville and Ferguson, 1993; Ferguson, 1999). Trypanosomes evade the host adaptive immune response by changing the composition of this VSG coat. This glycoprotein is encoded in a polycistronic transcribed telomeric expression site and there are also many silent copies found (Horn et al., 2010). Trypanosoma brucei is not only a devastating pathogen but has also become an interesting model organism for basic science contributing to better understanding of common cellular mechanisms. Due to the fact that Trypanosomes are simpler than mammalian cells but posses the canonical eukaryotic organelles they are increasingly used as model systems for eukaryotic cell biology. RNA editing and glycosylphosphatidylinositol anchoring were first discovered in Trypanosomes (Benne et al., 1986; Ferguson et al., 1985). Furthermore the complete Trypanosome genome has been sequenced and the availability of RNA interference make them suitable for application of reverse genetics (Berriman et al., 2005).

1.2 Trypanosome cell structure

T. brucei cells contain a single nucleus, a single flagellum, a basal body and its immature form (the pro-basal body), a single Golgi, and a single elongated mitochondrion containing the mitochondrial genome (Hammarton et al, 2007a) (Fig.1.2.1).

The mitochondrial genome is also unique as it is condensed into a disc-like structure termed the kinetoplast (Hammarton et al, 2007a). The kinetoplast DNA network is composed of several thousand mini-circles that are interlocked. Also intertwined are a few dozen maxi-circles which code for gene products such as rRNA and subunits of the respiratory complexes. Mini-circles encode for guide RNAs that edit maxi-circle transcripts (Klingbeil et al., 2004). RNA editing describes the process in which precursor mRNA sequences are often exchanged by the insertion or less frequently by the deletion of uridine nucleotides (Stuart et al., 2005).

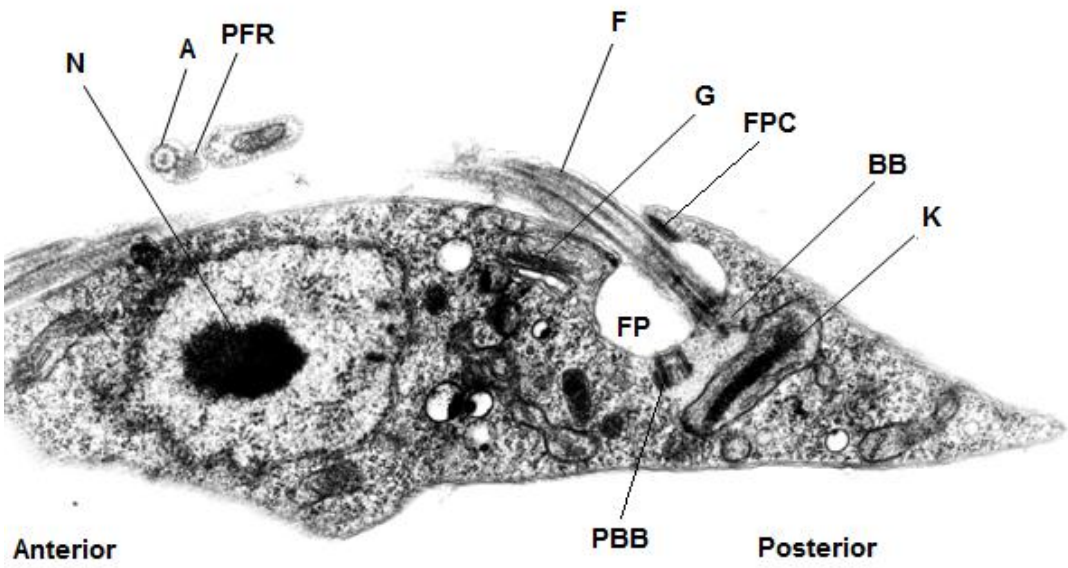

Fig. 1.2.1 Thin-section electron micrograph through a procyclic Trypanosome.

N: nucleus; A: axoneme; PFR: paraflagellar rod; F: flagellum; G: Golgi; FP: flagellar pocket; FPC: flagellar pocket collar; BB: basal body; PBB: pro-basal body; K: kinetoplast. The posterior and anterior ends of the cell are indicated. Taken from Yelinek et al., 2009

The typical vermiform cell morphology of Trypanosomes is generated by an internal cytoskeleton consisting of a cage-like arrangement of more than 100 subpellicular microtubules (MTs) (Sherwin et al., 1989). A precise inter-microtubular distance of 18-22nm is maintained by cross-bridges that link the MTs to each other and to the

inner face of the plasma membrane. The plus ends of the subpellicular MTs terminate at the posterior end of the cell (Fig. 1.2.1).

The direction of cell movement is determined by the flagellum beating which occurs from tip to base (Hammarton et al., 2007a). The flagellum is a highly complex organelle composed of several different structures. Its main component, the axoneme, is formed by a '9+2' arrangement of MTs which is a highly conserved structure among all flagella and cilia in eukaryotes (Ginger et al., 2008). Physically attached to the axoneme is a second large cytoskeletal structure, the paraflagellar rod (PFR). Two main protein components PFR1 and PFR2 (previously PFRC and PFRA) have been identified and are encoded by repeated gene arrays in the T. brucei genome (Gadelha et al., 2004).

The flagellum is nucleated at the basal body, a structure which abuts an invagination of the plasma membrane termed the flagellar pocket (FP). The FP is a plasma membrane sub-domain which is the sole site of endo- and exocytosis (Absalon et al., 2008). The FP is thus the site of trafficking and recycling of glycosylphosphatidylinositol - anchored surface proteins and the VSG antigen, playing an important function in the Trypanosome's defence against the host immune system (Allen et al., 2003). The Trypanosome flagellum is attached to the cell body through the flagellum attachment zone (FAZ). The FAZ is composed of a microtubular quartet with associated membrane and a specific FAZ filament structure (Lacombe et al., 2008). During the cell cycle an additional structure, the flagellar connector (FC), is observed in procyclic Trypanosomes. The FC ensures that the new elongating flagellum migrates along the old flagellum and is placed in a parallel orientation. On top of its structural and mobility functions the flagellum is essential for cell division and further possesses a highly active intra-flagellar transport system (IFT) (Hammarton et al, 2007a). IFT is the bi-directional transport of flagellum components by two IFT complexes A and B. The anterograde transport of IFT particles to the flagellar tip is performed by a heterotrimeric kinesin-2 motor protein. The transport back to the cell body, called retrograde transport, is carried out by dynein-1b motors (Pederson and Rosenbaum, 2008; Pigino et al., 2009).

The main microtubular organizing center (MTOC) of T. brucei is the basal body subtending the flagellar axoneme (Lacombe et al., 2008). The microtubular quartet is nucleated between the basal body and the pro-basal body (Tayler and Godfrey, 1969). This microtubular quartet has the opposite polarity to the corset microtubules. The structure extends along and around the FP membrane in a left-handed helical manner inserting into the subpellicular array of microtubules (Lacombe et al., 2008). Two

boundaries define the flagellar pocket, a structure termed the collar and the neck region (Henley et al., 1978; Sherwin and Gull, 1989). The flagellar pocket collar is visible as an electron-dense structure by electron microscopy (Fig.1.2.1) Recently the first component from the flagellar pocket collar was identified, a cytoskeletal protein termed BILBO1 (Bonhivers et al., 2008). The neck region is a cylindrical region of the plasma membrane and defines the transition between the internal membrane and the external plasma membrane of the cell surface (Lacombe et al., 2008).

The pro-basal body forms a new flagellum in the proximate cell cycle. Both the morphology and function of basal bodies are analogous to the centrioles of mammalian cells (Vaughan et al., 2008).

1.3 Cell division of procyclic Trypanosomes

At the start of the Trypanosome cell cycle the cell contains a single kinetoplast and a single nucleus, this stage is also referred to a 1K1N cell. Cell division in Trypanosomes is unusual as two distinct S-phases need to be co-coordinated - one for the single mitochondrial DNA and one for nuclear DNA. This process starts with the S-phase of the mitochondrial DNA (Vaughan et al., 2008). At the G1-S transition the pro-basal body matures, elongates and invades the FP. Then the new flagellum starts to grow. The new basal body performs a rotation in anti-clockwise direction around the old flagellum while its new axoneme is associated with the pre-existing FP. This rotation results in division of the FP (Lacomble et al., 2010). The new flagellum stays connected to the old flagellum through the FC which migrates simultaneously with the out-growing flagellum (Fig. 1.3.1 B). In T. brucei growth of the new flagellum is crucial for cell division where it ensures transmission of cell polarity. Interestingly the FC stops at a precise length along the old flagellum and this stop coincides with basal body and kinetoplast segregation (Vaughan et al., 2008). Golgi duplication occurs via the formation of a new Golgi next to a new endoplasmic reticulum export site (ERES) (He et al., 2004). Recently it was shown that the entire ERES : Golgi complex is associated with the Trypanosome cytoskeleton, likely via the FAZ (Sevova et al., 2009). Furthermore, in bloodstream form cells it was proposed that single ER : Golgi pairs are present near the nucleus while in cells with two pairs the second is placed near the FP suggesting that it forms the new Golgi (Sevova et al., 2009). Following Golgi duplication the new FAZ is assembled. This 1K1N cell duplicates and segregates its kinetoplast forming a 2K1N cell (de Graffenried et al., 2008).

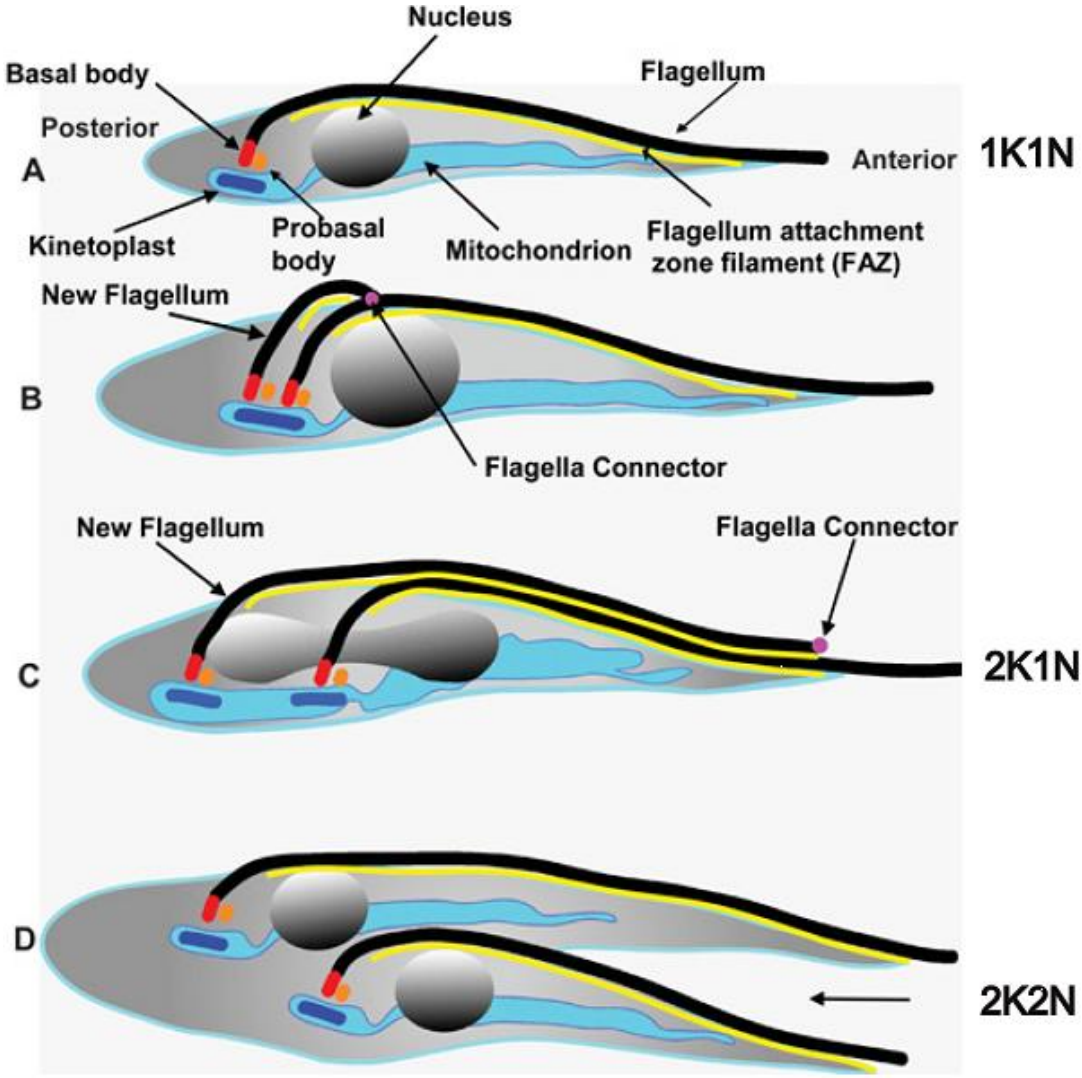

Fig. 1.3.1 Illustration of the cell cycle of procyclic T. brucei.

(A) A 1K1N cell contains one kinetoplast, one nucleus and a single attached flagellum. (B) The pro-basal body matures and a new flagellum extends which is connected to the old flagellum by the flagella connector. (C) The flagellar connector stops at a precise distance. This coincides with basal body and kinetoplast segregation. Then mitosis occurs forming a 2K2N cell. (D) Cytokinesis proceeds from the anterior end to the posterior by flagellar beating. Taken from Vaughan et al., 2008.

Nuclear replication takes considerably longer than mitochondrial S-phase. Mitosis leads to one of the two nuclei positioned between the two kinetoplasts ensuring that cytokinesis will yield two daughter cells (Fig. 1.3.1 C). This stage of cell division is referred to as a 2K2N cell. Interestingly, the mitosis occurs without chromosome condensation or nuclear breakdown (Hammarton et al., 2007b). Cleavage furrow ingression is performed along the cell body from the anterior to posterior end be-

tween the old and the new flagellum (Fig. 1.3.1 D) (Vaughan et al., 2008). Finally the two daughter cells remain attached to each other for some time before final abscission is achieved through flagellar beating.

1.4 Discovery of the bilobe

The work of the Warren group concerns the eukaryotic cell cycle, and specifically the mechanism of Golgi replication. In contrast to mammalian cells Trypanosomes contain a single Golgi and were used as a model system. In mammalian cells the Golgi is juxtaposed the main MTOC, the centrosome. In Trypanosomes however the main cytoplasmic MTOC is the basal body which is found at the wrong side of the FP relative to the Golgi (Fig.1.4.1). In addition to the basal body there needs to be an MTOC component inside the nucleus since Trypanosomes perform a closed mitosis. The question now is if there is an analogous structure to the centrosome which ensures Golgi segregation.

Ca^{2+} binding proteins, called centrins, are conserved components of MTOCs. The monoclonal antibody 20H5, originally raised against centrin from Chlamydomona reinhardtii, labels centrins in a wide range of organisms (Salisbury et al., 1988). In T. brucei it was serendipitously found that this pan-centrin antibody stains a bilobe structure in addition to the basal body (Fig. 1.4.1 A) (He et al., 2005). As a totally novel structure, the group has since then been trying to determine its form, function, and biogenesis. Key to answering these questions is the identification of bilobe – resident proteins.

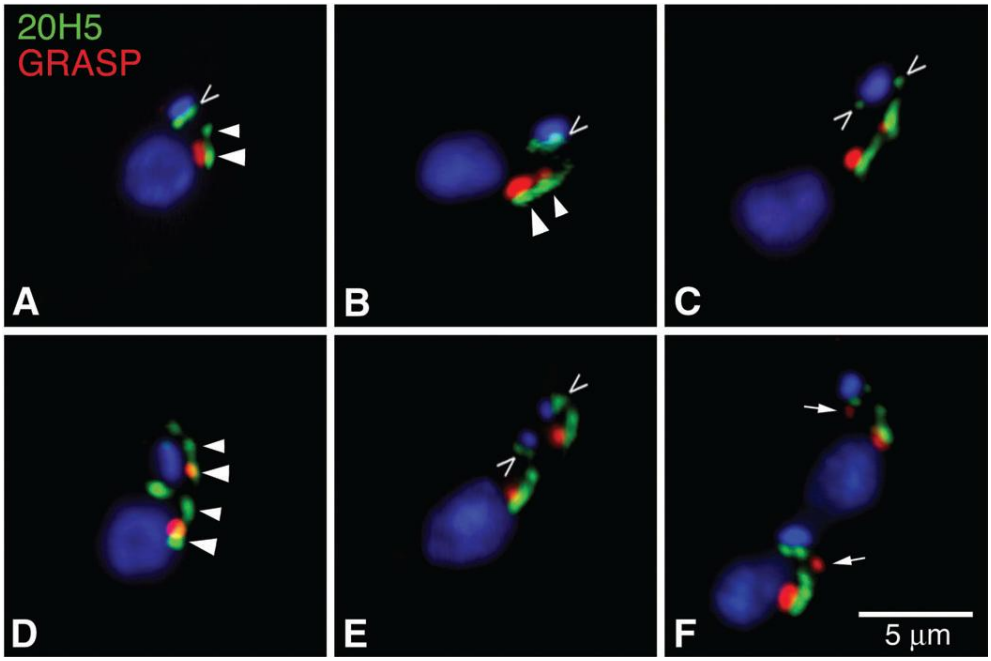

Fig. 1.4.1 Gallery of images through the cell cycle of procyclic Trypanosomes.

GRASP: Golgi reassembly stacking protein; 20H5: pan-centrin antibody; open arrowheads: basal bodies; solid arrowheads: bilobed structure; arrows: Golgi. (A and B) Pan-centrin antibodies label the basal body and additional bilobed structure next to the Golgi. (C and D) The basal body duplicates followed by kinetoplast duplication and segregation (E). (F) 2K2N cell ready for cytokinesis. Taken from He et al., 2005.

1.5 Proteins at the bilobe

1.5.1 TbCentrin2

In T.brucei there are 5 known centrin isoforms. TbCentrin2, along with TbCentrin1 is one of two isoforms detected by 20H5 antibody (He et al., 2005). TbCentrin2 localizes to the flagellum, the basal body and the bilobe. Depletion of TbCentrin2 by RNA interference (RNAi) causes an inhibition of basal body division. Since TbCentrin2 is also found at the basal body this restricts its usefulness as a marker for the bilobe. This also makes it impossible to determine bilobe function because depletion experiments will cause loss from the basal body as well as the bilobe. Therefore, any observed effects could be caused by basal body malfunction. Better understanding of the form and function of the bilobe needs identification of more specific components.

1.5.2 TbCentrin4

TbCentrin4 (previously named TbCen1) localizes to the basal body and to the bilobe structure. This was confirmed by fluorescent protein and epitope tags (Selvapandiyan et al., 2007). Due to the lack of a ten amino acid long epitope TbCen4 is not recognized by 20H5 antibodies. Upon depletion of TbCentrin4 by RNAi (>48 hours) there was disruption of cell cycle progression and an accumulation of multinucleated cells and zoids (cells without a nucleus), was observed. This phenotype was similar to that found in TbCentrin2-depleted cells except for the abundance of zoids (He et al., 2005). The observation of zoids and 1K2N cells suggests that these cells were produced as siblings by incorrect placement of the cleavage furrow and unequal abscission. However all other organelles examined - the basal bodies, kinetoplast, Golgi, lysosomes, flagella and the FAZ - appeared to replicate properly and were found in both cell types. Thus, cell division seems to occur before full segregation of the duplicated nuclei in TbCentrin4 RNAi cells (Shi et al., 2008). Given that both TbCentrin2 and TbCentrin4 have multiple localizations this makes it different to determine exact function of the bilobe.

1.5.3 TbPLK

Polo-like kinases (PLK) are a family of eukaryotic mitotic kinases. T.brucei contains a single PLK homologue that has been linked to cytokinesis (Kumar and Wang, 2006). In Immunofluorescence microscopy TbPLK partially colocalized with the bilobed structure. Interestingly, at early times in the cell cycle TbPLK was concentrated at the basal bodies and the posterior lobe of the bilobe. After basal body replication the signal from TbPLK increased on the duplicating bilobe. Once bilobe duplication was completed the TbPLK signal from the bilobe was lost and the signal appeared at the growing tip of the FAZ. Upon TbPLK depletion by RNAi, cells began to accumulate in the 2K2N state. After 20 hours cells increased in size and had multiple detached flagella. 24hours post induction a large number of multinucleated cells and zoids were observed. Furthermore a clear defect in bilobe morphology and number was seen. The observation that fewer 1K1N cells had two bilobes suggested a delay in bilobe biogenesis. At the same time the number of cells with malformed bilobes and five or more Golgi increased. Thus TbPLK localization and its depletion effects suggest that the kinase plays an important role in bilobe duplication (de Graffenried et al., 2008).

1.5.4 TbLRRP1

The bilobe protein TbLRRP1 was recently identified through comparative proteomic analysis (Zhou et al., 2010). This protein contains several leucine rich repeats in its N-terminal region and a coiled coil motif at the C-terminus. In immunofluorescence microscopy images TbLRRP1 was present on the bilobe together with TbCentrin4 through the entire cell cycle. The TbLRRP1 labelled bilobe was adjacent to the flagellar pocket collar on the posterior side and the FAZ on the anterior side. Upon depletion of TbLRRP1 by RNAi, cell duplication first slowed down and then stopped 72 hours after induction. Accumulation of 1K2N cells was observed which might be due to a lack of kinetoplast segregation. Clustering of duplicated basal bodies and flagella also suggested a failure in segregation of these organelles. Additionally motility defects were observed. New flagella appeared to be shorter than those in control cells although no structural defects in the axoneme or PFR could be observed by electron microscopy. Duplication of the Golgi, flagellar pocket collar and FAZ were all inhibited. In more than 70% of 1K2N cells a single bilobe as well as a single FAZ was observed. Furthermore the new FAZ was often shorter than the old, more anterior one. The lack of the new FAZ might also be an explanation for the inhibited segregation of the basal body and the kinetoplast. Together these data suggest a role of the bilobe in FAZ formation (Zhou et al., 2010).

1.6 Identification of TbMORN1

Prior to discovery of TbLRRP1, the only protein known to localize exclusively to the bilobe was TbMORN1, a MORN-repeat protein (Morriswood et al., 2009). MORN-repeats are 23 amino acid long protein motifs with a general consensus sequence of YXGX(W/F)X6GXGX6GX2 (Im et al., 2007). This bilobe constituent protein was identified by using a comparative proteomic strategy. Using detergent and high salt the Trypanosome flagella also co-purifies with the basal body and the bilobe structure which suggests a tight association with the flagellar cytoskeleton. The flagellum proteome must therefore contain bilobe proteins in addition to axoneme, PFR, and basal body ones. From this flagellum proteome the proteomes of Chlamydomonas reinhardtii and Tetrahymena thermophila flagella/cilia were subtracted (Broadhead et al., 2006). Subtraction resulted a shortlist of 180 putative bilobe/basal body proteins from which 12 proteins were picked randomly and screened for localization by immunofluorescence using a yellow fluorescent protein (YFP) tag. One protein, Tb927.6.4670, had a promising distribution redolent of the bilobe structure. Further

experiments confirmed the localization of Tb927.6.4670 at the bilobe but not the basal body or flagellum (Fig. 1.6.1). Tb927.6.4670, later termed TbMORN1, is a 358 amino acid, approximately 40 kDa protein with 15 tandem MORN-repeats (Morriswood et al., 2009). The YFP-tagged protein, as well as an epitope tagged version and antibodies against the endogenous protein, showed a range of hooked and pronged morphologies in immunofluorescence microscopy analysis. It is not clear if these morphologies represent the bilobe viewed from different angles or show its replication cycle. Besides its different morphologies the overlap of TbMORN1 with 20H5 labelling and TbCentrin4 is extensive but not complete. TbCentrin4 was present in the same cell volume but doesn't colocalize with TbMORN1 and is also present at the basal bodies. It is unclear if the bilobe is composed of several sub domains and if TbCentrin4 and TbMORN1 localize preferentially to different parts of these sub domains. Furthermore in the YFP-TbMORN1 expressing cells intensely bright "spear" structures were additionally observed in 35% of cells. By transmission electron microscopy these structures were found to be paracrystalline artefacts which nonetheless hints at a high capacity for self-organization (Morriswood et al., 2009).

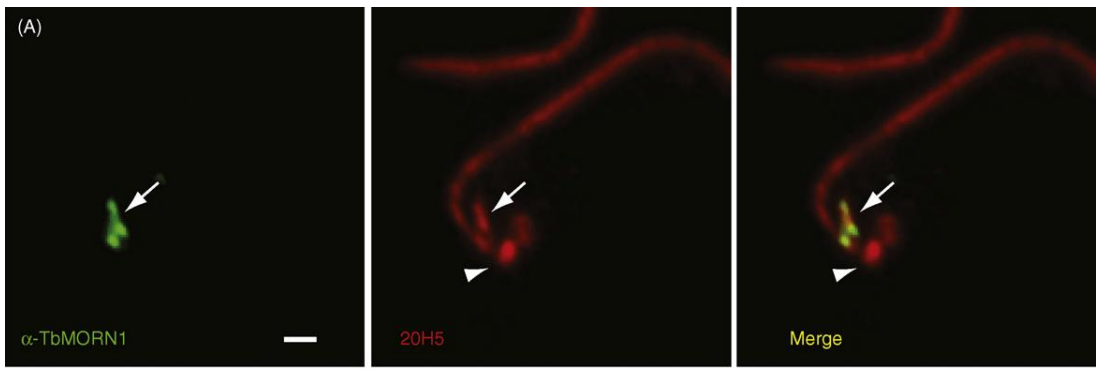

Fig. 1.6.1 Detergent-extracted cytoskeletons of procyclic cells fixed with paraformaldehyde (PFA) and labelled with anti-TbMORN1 and the pan-centrin antibody 20H5.

Anti-TbMORN1 and 20H5 overlap at the bilobe. Arrows: bilobed structure; arrowheads: basal body. Single section of 0.1µm. Scale bar 1µm. Taken from Morriswood et al., 2009.

Depletion of TbMORN1 by RNAi experiments produced a mild growth defect in procyclic Trypanosomes. Surprisingly no alteration of the bilobe labelling with 20H5 could be observed. All 2K2N cells displayed proper duplicated bilobes. One possible explanation could be that because 20% of wild-type TbMORN1 was still present upon induction in RNAi cells this is sufficient for bilobe formation. In bloodstream Trypanosomes in contrast, the depletion of TbMORN1 was lethal 24 hours post-

induction. This implies that TbMORN1 is an essential gene in the bloodstream form (Morriswood et al., 2009).

In other eukaryotes the MORN1 protein is highly conserved among apicomplexan parasites, one of these homologues is TgMORN1 in Toxoplasma gondii (Ferguson et al., 2008). TgMORN1 is composed of 15 MORN-repeats and a short linker sequence of 5 amino acids between MORN repeat 6 and 7 (Gubbels et al., 2006). In Toxoplasma gondii MORN1 localized to the centrocone, a structure within the nuclear envelope, and to the basal and apical ends of the parasitic cell. TgMORN1 is the major component of the basal complex, a cytoskeletal structure at the posterior end of the cell (Hu et al., 2006; Lorestani et al., 2010). Toxoplasma divides through a highly specific mechanism called endodyogeny where two parasites are formed within the mother parasite. TgMORN1 is further involved in the Toxoplasma gondii replication cycle where it forms a ring structure near the duplicated centrioles and moves along the daughter cell and has contraction capacity. Interestingly, TgMORN1 null mutants show a bizarre phenotype where double-headed parasites accumulate. These Janus-headed parasites form the apical complexes but fail to assemble the basal complex. A lack of TgMORN1 leads to incomplete cytokinesis, and more precisely to incomplete abscission. Budding still takes place which can lead to up to 16-headed parasites. Together these data suggests that TgMORN1 is essential for functional basal complex formation. Furthermore the mutants fail to constrict and divide the apicoplast, an organelle found in most apicomplexa (Lorestani et al., 2010).

1.7 MORN-repeat proteins

MORN-repeat proteins were first observed in transmembrane junctional complexes of mammalian skeletal muscle cells where they link the sarcoplasmic reticulum to the plasma membrane (Takeshima et al., 2000). MORN-repeats are thought to be employed as plasma membrane localization devices and are found in conjunction with various enzymatic and protein-protein interaction domains, the best characterized being a phosphatidylinositol phosphate kinase (PIPK) in plants (Ma et al., 2006).

This plant PIPK contains a short N-terminal sequence followed by a MORN-repeat domain with 8 repeats and a C-terminal catalytic domain separated by a short linker sequence. Recent findings proposed that the MORN domain regulated plasma membrane localization and phosphatic acid-inducible activation. However deletion of the

N-terminal region reduced PIPK1 activity 40% compared to the full-length enzyme. Surprisingly the loss of the MORN repeats had no significant effect. The mutant lacking the 8 MORN-repeats showed no difference in activity (Mikami et al., 2010). These findings raise the question of the function of MORN-repeats. In T.brucei there are additional to TbMORN1 17 other open reading frames (ORFs) predicted to encode MORN-repeat proteins which are all experimentally uncharacterized.

1.8 Project outline & Aims

Previously, a novel cytoskeletal structure called the bilobe was identified in T.brucei (He et al., 2005) The function of this organelle remains unclear. So far only two proteins are known to localize exclusively to the bilobe, TbMORN1 (Morriswood et al., 2009) and TbLRRP1 (Zhou et al., 2010). TbCentrin4 doesn't completely overlap with TbMORN1 and is also present at the basal bodies (Shi et al., 2008). TbPLK was transiently present at the bilobe during cell division (de Graffenried et al., 2008).

TbMORN1 consists of a highly repetitive primary structure of 15 MORN-repeats. Therefore the targeting information to the bilobe must be somewhere in these MORN-repeats. The aim of this project was to identify the targeting sequence of TbMORN1 and define which of these MORN-repeats are essential and which are dispensable for proper localization. This would thereby provide more information about MORN-repeats and their possible functions.

Given that TbMORN1 targets to the bilobe the targeting must be mediated by its repeats. Since all other 17 predicted MORN-repeats containing proteins are uncharacterized the candidate approach was to express these proteins in procyclic Trypanosomes and screen them for possible bilobe localization.

2 Materials and Methods

Applied methods are grouped into five categories: (2.1) Gene cloning, (2.2) Cell culture and Transfection, (2.3) Immunofluorescence, (2.4) Protein methods and (2.5) Bioinformatics. Gene cloning covers all steps from the purification of Trypanosome genomic DNA to generation of expression constructs. Cell culture and Transfection describes methods as transient transfection and generation of stable cell lines. The section Immunofluorescence contains protocols for cell fixation and immunofluorescence microscopy. Protein methods include commonly used applications such as whole cell lysate preparation, biochemical fractionation and Western blot analysis. Bioinformatic applications are included in the last section of this chapter.

During the project I followed two series of experiments- screening of the MORN-repeat family to define subcellular localization, and analyzing truncations of TbMORN1 to determine targeting. Therefore the strategy described in Fig. 2.1 was performed. First the ORF was cloned as described in the section general cloning strategy. The amplified ORF was ligated into the pxS2-YFP expression vector creating N- or C-terminally YFP tagged constructs. Then procyclic T.brucei 29.13 (for screening proteins 1-8) or T.brucei 427 (for all other experiments) cells were transiently transfected and analyzed by immunofluorescence microscopy to determine subcellular localizations. Samples were fixed with either PFA or methanol (MeOH) or detergent-extracted (Xtr) and fixed with PFA. Anti-GFP antibodies were used to enhance the YFP signal. For distinct localization further tests using cytoskeletal and organelle markers were performed. In order to confirm expression of YFP fusion proteins I performed Western blot analysis using antibodies against GFP. These antibodies recognize YFP tags as the primary sequence difference between GFP and YFP is comparatively slight. Additionally I either analyzed whole cell lysates for the MORN-family, or performed biochemical fractionation of the TbMORN1 truncation proteins. To determine hydrophobicity I analyzed truncated TbMORN1 proteins on a Kyte-Doolittle hydrophobicity plot.

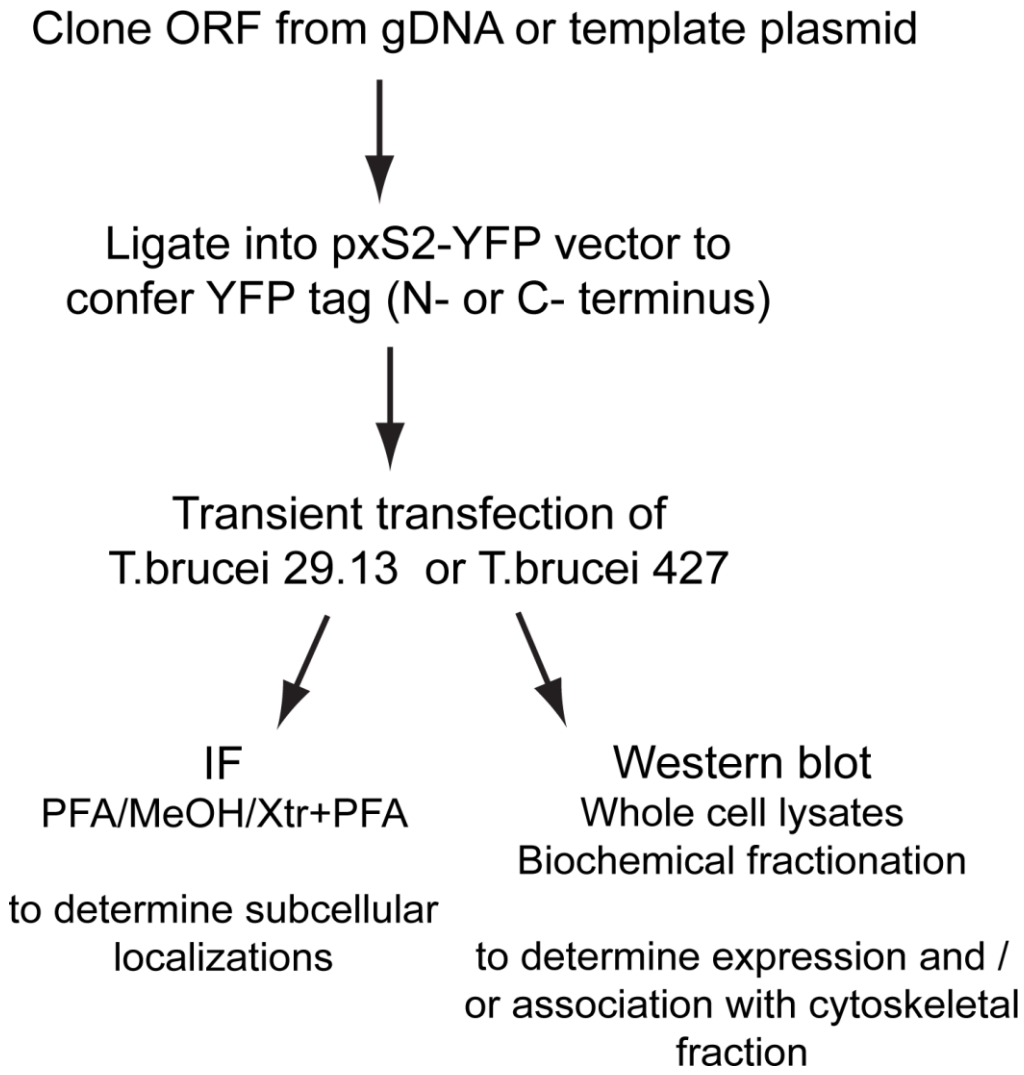

Fig 2.1 Flow chart outlining the performed strategy

2.1 Gene cloning

2.1.1 General strategy

At the beginning of the project the cloning strategy as outlined in Fig. 2.1.1 was performed. For the MORN-family the amplification of genes of interest was achieved by polymerase chain reaction (PCR) using primers annealing to the beginning and end of the selected gene locus on genomic DNA. The TbMORN1 truncation constructs were obtained by subcloning from a parental construct, pGEX-TbMORN1. To generate truncated versions primers binding to indicated positions were used for PCR. These primers additionally introduced sites for restriction enzymes. During the project I used the following enzymes: BamHI, Bgl2, Bcl1, EcoRI, HindIII, NheI either cutting upstream or downstream of the YFP construct (Fig.2.1.2). Blunt-ended PCR

products were separated by gel electrophoresesis and desired product bands were purified by gel extraction. Digestion with the appropriate restriction enzymes enabled ligation into the pxS2-YFP expression vector. The MORN-family constructs were either N- or C-terminally YFP-tagged depending on which restriction sites were available. TbMORN1 truncation constructs were all N-terminally YFP tagged. There is some evidence that a C –terminal tag prevents proper targeting of TbMORN1 (B. Morriswood, unpublished results). The first choice would therefore be to use BamH1 and EcoR1 but unfortunately TbMORN1 has internal restriction sites for both enzymes. These enzymes were therefore used only for short truncation constructs lacking the appropriate sites. For full-length and large constructs we applied Bgl2 which leaves the same overhang as BamH1. For Bgl2 digested PCR fragments, BamH1 and calf intestinal phosphatase (CIP) - treated vector was used. The ligation products were then used to transform bacteria and individual colonies were screened for the presence of the gene of interest by colony PCR. Putative positive colonies were further screened by restriction digestion of purified plasmid minipreps. The isolated minipreps were then sequenced to check if any mutations had been acquired. Due to the fact that Trypanosomes need a high amount of DNA for successful transfection I carried out maxipreps of the expression constructs.

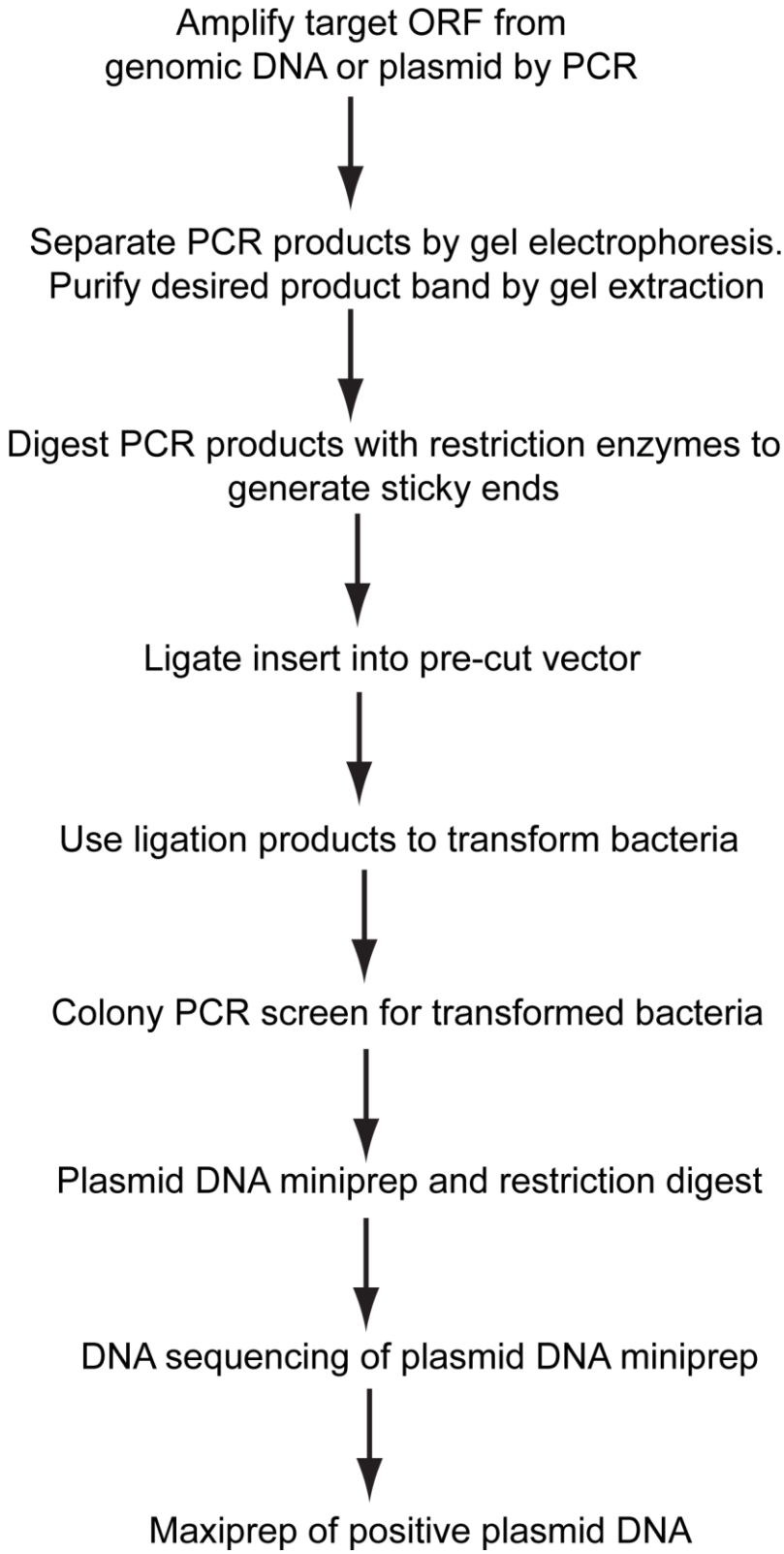

Fig. 2.1.1 Gene cloning.

Flow chart showing performed cloning strategy.

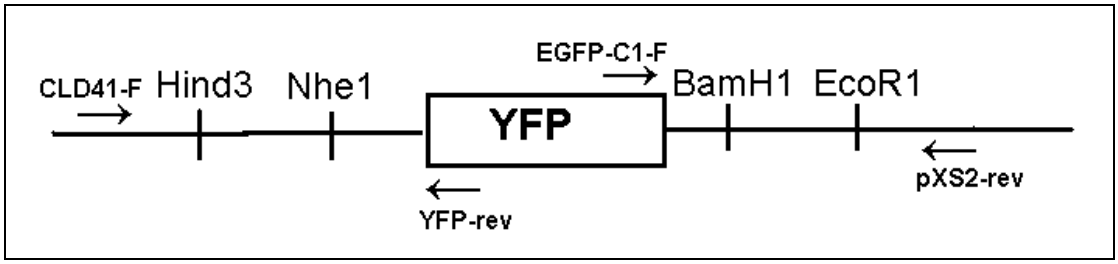

Fig. 2.1.2 Parts of the pxS2-YFP expression vector showing appropriate sites for restriction enzymes.

Arrows indicate primer annealing sites for insert sequencing.

2.1.2 Purification of total DNA

DNA purification was carried out using QIAgen DNeasy Blood & Tissue kit according to the manufacturer's instructions with the following adaptations. In the first step in the protocol, $1x10^7$ cells were utilized and centrifuged for 1 min at 6000rpm. Given that Trypanosomes posses a microtubular corset I used 40µl proteinase K and increased the incubation time to 40 min in order to destroy the tight cytoskeletal structure of these cells. Finally DNA was eluted by applying 100µl of Buffer EB directly onto the membrane and centrifugation for 1 min at 8000rpm after 1 min of incubation at room temperature. This process was performed twice and eluted DNA was aliquoted and stored at -20°C.

2.1.3 Amplification of DNA by PCR

For amplification of DNA by PCR my first choice was to use Vent DNA polymerase which has a high fidelity deriving from an integral 3'- 5' proofreading exonuclease activity. If reactions with Vent polymerase were unsuccessful I turned to high fidelity Taq polymerase, which has an artificially added proofreading function. If this amplification again failed I alternatively used Phusion DNA polymerase or PfuUltra High-Fidelity DNA polymerase. The latter was also used for the site-directed mutagenesis protocol since it provides robust amplification of long, complex targets. Gradient PCR was applied to determine the optimum annealing temperature.

Vent PCR

Vent DNA polymerase (New England Biolabs)

Vent PCR was performed in a 100µl reaction volume containing the following components:

72µl	H_2O
10µl	10x ThermoPol Reaction Buffer
5µl	5mM dNTPs
5µl	10µM Primer 1
5µl	10µM Primer 2
2µl	genomic DNA or plasmid template
1µl	Vent DNA Polymerase (1 unit)
100µl	

In order to obtain appropriate PCR products a program with elongation time and temperature adapted to construct size, using 1 minute per kb of product, was performed.

PCR Program

Temperature	Minutes	Cycles
94°C	5	1
94°C 55°C 72°C	1 2 1min/kb	30
72°C	5	1
10°C	forever	

Taq High Fidelity (HiFi) PCR

Platinum Taq HiFi DNA polymerase (Invitrogen)

PCR with HiFi polymerase was performed in a 50µl reaction volume containing the following components:

36.8µl	H_2O
5µl	10x HiFi Taq Reaction Buffer
2µl	5mM dNTPs
1µl	100mM $MgSO_4$
2µl	10µM Primer 1
2µl	10µM Primer 2
1µl	genomic DNA
0.2µl	HiFi Taq DNA Polymerase (1 unit)
50µl	

Elongation time was adapted to construct sizes using 1 minute per kb.

PCR Program

Temperature	Minutes	Cycles
94°C	2	1
94°C 55°C 68°C	0.5 0.5 1min/kb	30
68°C	5	1
10°C	forever	

PfuUltra High-Fidelity PCR

PfuUltra High-Fidelity DNA polymerase (Stratagene)

PCR with PfuUltra High-Fidelity polymerase was performed in a 50µl reaction volume containing the following components:

39µl	H_2O
5µl	10x PfuUltra Reaction Buffer
1µl	5mM dNTPs
1µl	10µM Primer 1
1µl	10µM Primer 2
2µl	genomic DNA
1µl	PfuUltra DNA Polymerase (2.5 units)
50µl	

Elongation time was adapted to construct sizes using 1 minute per kb.

PCR Program

Temperature	Minutes	Cycles
94°C	5	1
94°C 55°C 72°C	0.30 0.45 1min/kb	30
72°C	10	1
10°C	forever	

Phusion PCR

Phusion DNA polymerase (Finnzyms)

PCR with Phusion polymerase was performed in a 50µl reaction volume containing the following components:

35µl	H_2O
10µl	5x Phusion Reaction Buffer
1.5µl	5mM dNTPs
1µl	10µM Primer 1
1µl	10µM Primer 2
1µl	genomic DNA
0.5µl	Phusion DNA Polymerase (1 unit)
50µl	

Elongation time was adapted to construct sizes using 1 minute per kb.

PCR Program

Temperature	Minutes	Cycles
98°C	2	1
98°C 55°C 72°C	0.15 1 1min/kb	35
72°C	10	1
10°C	forever	

Gradient PCR

Vent DNA polymerase (New England Biolabs)

In order to evaluate optimal annealing temperatures for designed primers gradient PCR with temperatures ranging from 55-65°C (55.1, 55.4, 55.9, 56.9, 58, 59.3, 60.6, 61.9, 63.4, 64.3, 64.9, 65.2°C) was performed. 12 tubes containing following ingredients were prepared. Gradient PCR reactions were carried out using a VWR Quattrocycler.

36.5µl	H_2O
5µl	10x ThermoPol Reaction Buffer
2µl	5mM dNTPs
2.5µl	10µM Primer 1
2.5µl	10µM Primer 2
1µl	genomic DNA
0.5µl	Vent DNA Polymerase (1 unit)
50µl	

PCR Program

Temperature	Minutes	Cycles
95°C	5	1
95°C 55-65°C 72°C	0.5 0.5 1	30
72°C	5	1
10°C	forever	

All PCR reactions, except gradient PCR, were carried out in a Bio-Rad DNAEngine. Finally, PCR products were analyzed by gel electrophoresis.and stored at -20°C.

2.1.4 Colony PCR

Taq DNA polymerase

Colony PCR is a fast method to screen single colonies for the presence of cloned plasmid DNA. In the first step PCR reaction ingredients were mixed together.

13.3µl	H2O
2µl	10x Taq buffer
1µl	5mM dNTPs
1µl	10µM Primer 1
1µl	10µM Primer 2
1.6µl	25mM $MgCl_2$
0.1µl	Taq DNA polymerase
20µl	

Then single colonies were picked using a sterile pipette tip and swirled in PCR reaction and tips transferred to 3ml LB+ampicillin medium.

PCR Program

Temperature	Minutes	Cycles
94°C	5	1
94°C 55°C 72°C	1 2 1min/kb	30
72°C	5	1
10°C	forever	

PCR reactions were analyzed by gel electrophoresis, and LB+ampicillin cultures incubated overnight at 37°C with shaking. Predicted insert-containing cultures were chosen and used for plasmid Mini Preparation.

2.1.5 DNA Gel Electrophoresis

In order to separate and analyze DNA, gel electrophoresis using 1% agarose gels was performed. Electrophoresis grade agarose (Invitrogen) was melted in 1x TAE buffer and cast and run in MGU-202T rigs (C.B.S.Scientific CO.) For DNA visualization SYBR^{R} Safe (Molecular Probes^{TM}) was added according to the manufacturer's instructions. The loading buffer was 40% sucrose solution with bromophenol blue dye. The 1kb DNA Ladder (Invitrogen) served as a size marker. After loading gels were run at 110V for 30 minutes. DNA visualization was carried out in the Safe Imager (Invitrogen).

2.1.6 DNA Gel Extraction

To extract DNA fragments from agarose gels the QIAquick Gel Extraction kit (Qiagen) was used according to the manufacturer's instructions. For DNA elution 30µl Buffer EB were used.

2.1.7 Restriction digestion

In order to generate sticky ends the PCR products and vector (pxS2-YFP) were digested with the appropriate restriction endonucleases. During the project I used six different enzymes; BamHI, Bgl2, Bcl1, EcoRI, HindIII, NheI (all from New England Biolabs). Except Bgl2 all enzymes perform at maximum activity in NE Buffer 2. For Bgl2 I used NE Buffer 3. For digestion following components were mixed together:

30µl	PCR product
5µl	10x NE buffer 2 or NE buffer 3 (New England Biolabs)
5µl	10x bovine serum albumin (BSA)
2µl	Enzyme 1
2µl	Enzyme 2
6µl	H_2O
50µl	

10µl	pxS2-YFP (approx. 10µg)
2µl	10x NE buffer 2 (New England Biolabs)
2µl	10x BSA
1µl	Enzyme 1
1µl	Enzyme 2
4µl	H_2O
20µl	

These ingredients were gently mixed and incubated overnight at 37°C, or at 50°C for digestion with Bcl1.

2.1.8 Test digestion

For analysis of plasmid inserts restriction digests with appropriate enzymes were carried out. A single reaction mix consisted of:

5.4µl	H_2O
2µl	plasmid DNA
1µl	NE Buffer 2
1µl	10x BSA
0.3µl	Enzyme 1
0.3µl	Enzyme 2
10µl	

This reaction was incubated at 37°C for 1½ hour followed by analysis using gel electrophoresis. Plasmids carrying inserts of correct size were sent for sequencing to AGOWA genomics.

2.1.9 PCR Purification

To purify PCR products and vector DNA the QIAquick PCR Purification kit was applied according to the manufacturer's instructions. Eluted DNA was stored at -20°C.

2.1.10 CIP treatment of single cut vector DNA

CIP removes phosphates from the 3' ends of DNA and is therefore useful to inhibit religation of singly-cut vectors. The following components were mixed together and incubated.

25µl	vector DNA (pxS2-YFP)
5µl	10x NE buffer 3
2.5µl	CIP (10U/µl; New England Biolabs)
17.5µl	H_2O
50µl	

These ingredients were gently mixed and incubated for at least 90min at 37°C. To remove residual enzymes a QIAquick PCR purification kit (Qiagen) was applied afterwards.

2.1.11 Ligation reaction

Ligation reactions were usually performed using a 7:1 ratio of insert to vector. Alternatively I measured DNA content by Nanodrop and applied 150ng insert and 50ng cut vector (3:1 ratio). For all reactions additionally a control reaction using water instead of insert, to control for ligation efficiency, was performed. The following components were mixed together and incubated for at least an hour at room temperature or at 16°C overnight.

7µl	cut insert
1µl	cut pxS2-YFP vector
1µl	10x T4 DNA ligase buffer (New England Biolabs)
1µl	T4 DNA ligase (New England Biolabs)
10µl	

2.1.12 Preparation of competent Escherichia coli

For preparation of competent E.coli (DH5α) 3 ml of LB-media were inoculated with DH5α cells and grown overnight at 37°C shaking. The next day this starter culture was used to inoculate 100ml of LB media. E.coli were grown until the OD_{600} reached 0.45-0.55 which corresponds to mid log growth phase. In this stage bacteria are best to transform. The culture was incubated on ice for 15min. Afterwards falcons were centrifuged at 2500rpm for 15min at 4 °C. The supernatant was discarded and falcons thoroughly drained. The pellet was then resuspended in 33ml (1/3 of original volume) RF1 and again incubated on ice for 15min. Then centrifugation at 2500rpm for 15min at 4°C followed. Supernatant and pellet were drained thoroughly and pellet resuspended in 8ml RF2 buffer. Finally aliquots of 250µl were flash frozen in liquid nitrogen und stored at -80°C.

RF1:
100mM KCL
50mM $MnCl_2$-$4H_2O$
30mM Potassium acetate
10mM $CaCl_2$-$2H_2O$
15% w/v glycerol
pH 5.8

RF2:
10mM MOPS
10mM KCl
75mM $CaCl_2$-$2H_2O$
15% w/v glycerol
pH 6.8

2.1.13 Transformation of competent E.coli

Prepared competent E.coli (DH5α) were slowly thawed on ice. 10µl ligation reaction were added to 100µl bacteria and incubated for 5 min on ice. For transformation the DH5α cells were given a heat shock for 45 seconds at 42°C and then again placed on ice for 2 min. After this treatment 500µl LB medium were added to each sample and tubes were shaken at 37°C for an hour in order to give bacteria time for expression of the resistance marker. Recovered E.coli were spread out on prewarmed (1h, 37°C) LB+ampicillin plates and incubated overnight at 37°C.

2.1.14 Bacterial culture

For this project DH5α E.coli were used for all cloning procedures. Bacteria were grown at 37°C with 220rpm agitation in LB media containing antibiotic supplement (100µg/ml ampicillin) if necessary. When single colonies were required E.coli were cultured on LB+ampicillin plates.

2.1.15 Plasmid Miniprep, Maxiprep and DNA sequencing

Plasmid DNA was purified using the QIAgen Plasmid Mini Purification kit.

Since Trypanosomes need high amounts of DNA for successful transfection, sequenced plasmids were further enriched by plasmid maxi prep (QIAgen Plasmid Maxi Purification kit). In the first step transformation was carried out as standard using 1µl of minprep DNA. The next day single colonies were picked and first cultured in 3ml LB+ampicillin at 37°C. After approximately 6 hours this starter culture was used to inoculate 250ml media and bacteria were grown overnight with shaking at 37°C. The next day a maxiprep was carried out according to the manufacturer's instructions and DNA was eluted in 600µl EB buffer.

Sequencing was performed by AGOWA genomics using 4 different primers; CLD41 annealing upstream of the HindIII restriction site and the YFP-rev reverse primer

annealing within the YFP construct, as well as the EGFP-C1-F forward primer and the pxS2-rev (reverse) surrounding the BamHI and EcoRI sites (Fig. 2.1.2).

2.1.16 Site-directed mutagenesis

Site-directed mutagenesis was carried out using the QuickChange Site-Directed Mutagenesis protocol from Stratagene. First mutagenic primers containing the appropriate point mutation were designed according to protocol guidelines. As a template various concentrations of DNA (X=5ng, 10ng, 20ng, 50ng) while keeping the primer concentration constant were used for amplification with PFU Ultra polymerase.

PCR was performed in a 50µl reaction volume containing the following components:

5µl	10x PFU Ultra Reaction Buffer
1µl	5mM dNTPs
1.25µl	10µM Primer 1
1.25µl	10µM Primer 2
1µl	PFU Ultra polymerase
Xµl	template DNA
40.5-Xµl	H_2O
50µl	

PCR Program

Temperature	Minutes	Cycles
95°C	0.5	1
95°C	0.5	12
55°C	1	
68°C	7	
10°C	forever	

PCR products were digested with DpnI at 37°C for 1 hour to digest the non-muatgenic parental strands. This reaction was then directly used to transform bacteria as described before.

2.2 Cell culture and Transfection

Procyclic T.brucei 29.13 or T.brucei 427 cells were transiently transfected and analyzed by immunofluorescence microscopy to determine subcellular localizations. Line 29.13 was generated from T.brucei 427 and expresses T7 RNA polymerase and tetracycline repressor along with linked hygromycin and neomycin resistance genes (Wirtz et al., 1998). For our experiments these circumstances basically did not make any difference. For availability reasons first T.brucei 29.13 cells were used then we switched to T.brucei 427 cells.

2.2.1 Cell culture

The T.brucei 29-13 cell line and T.brucei 427 cell line were maintained in SDM-79 media supplemented with haemin and 15% fetal calf serum, 200µM Glutamine, 10µg/ml Pen/Strep and 10µg/ml Gentamicin at 28°C in the presence of 15µg/ml G418, 50µg/ml hygromycin (T.brucei 29-13) or without hygromycin or G418 (T.brucei 427). Trypanosomes were usually grown in 7ml suspension and split daily.

2.2.2 Cell counting

Cells were counted by using a Z2 Coulter Particle Count and Size Analyser from Beckman Coulter.

2.2.3 Transient transfection

For transient transfection approximately $5x10^7$ cells were used. Cells were centrifuged at 3000rpm for 5 min at 4°C. The pellet was resuspended in 10ml Cytomix and again centrifuged. The final cell pellet was resuspended in 500µl Cytomix and transferred into a 0.4cm electroporation cuvette. 50µg DNA was added and well mixed before electroporation with 2x 10s pulses (1500V, 25µF) using a GenePulser Xcell (Bio-Rad). Transfected cells were transferred to 7ml media and grown overnight at 28°C.

Cytomix:
120mM KCL
0.15mM $CaCl_2$
10mM K_2HPO_4
25mM Hepes (pH 7.6) Molecular Biology Grade
2mM EGTA, pH 8
5mM $MgCl_2$

2.2.4 Generation of a stable cell line

In order to create a stable cell line pxS2-YFP vector containing the target sequence was digested with Nsi1 to linearize it. Linearization is important to enable double homologous recombination at the tubulin locus. The following components were mixed together and incubated at 37°C overnight.

95µl	Maxiprep plasmid DNA (60µg)
15µl	10x NE buffer 3 (New England Biolabs)
15µl	10x BSA
8µl	Nsi1 (New England Biolabs)
17µl	H_2O
150µl	

Since the amount of DNA is too much for usage of the Qiagen kit (column capacity is 10µg) I purified the linearized DNA by mixing the following components together.

150µl	digested plasmid DNA
120µl	H_2O
30µl	3M NaAc (10% total)
750µl	100% Ethanol (EtOH) (2.5 Volume)
1050µl	

This mix was incubated at -20°C for 20 min and then centrifuged at 13000rpm for 15 min at 4°C. After washing with 70% ethanol (EtOH) the tube was again centrifuged at 13000rpm for 1 min at 4°C. The DNA pellet was then air dried for 30 min and resuspended in 60µl water.

For the transfection $5x10^7$cells were transfected with 25µg linearized DNA. For recovery and DNA integration the cells were incubated at 28°C over night. The next day cells were spread in blasticidin containing media (pxS2-YFP carries blasticidin resistance cassette) by serial dilution starting from 1:2, 1:5 and 1:10 diluted cells. The 96 well plates were then incubated at 27°C, 5% CO_2 and checked daily. After 5-14 days a cloudy growth front was visible. At about 20 days after plating the growth

front had proceeded to half of the plate. Clonal populations growing at least 4 wells in front of the growth front were taken to a 24 well plate and 1ml selection media was added on top. The next day 1ml of selection media was added. After overnight incubation the cells were added to 4ml selection media in 25cm^2 flasks. Trypanosomes were then screened for the incorporation of the YFP-construct by immunofluorescence microscopy, PCR from genomic DNA and Western blotting.

2.3 Immunofluorescence

Trypanosomes were transfected with maxiprep DNA by electroporation and grown at 28°C overnight. The next day Trypanosomes transiently expressing the recombinant proteins were harvested and either fixed with MeOH or PFA. Detergent-extracted cytoskeletons were prepared using extraction buffer and fixed with PFA. The cells were then incubated with appropriate antibodies and analyzed by immunofluorescence microscopy.

2.3.1 Fixation and blocking

First 80µl cell suspension per coverslip was harvested by centrifugation at 6000rpm for 1 min at room temperature. The cell pellet containing transfected Trypanosomes was twice washed in 1xPBS and then transferred to a 13mm coverslip placed in a 24-well tissue culture plate. For attachment of cells centrifugation at 3000rpm for 4 min was used.

PFA-Fixation

For PFA fixation Trypanosomes attached to coverslips were incubated in a 4% PFA solution in PBS for 20 min at room temperature. Once washed with 1xPBS, 0.25% Triton X-100 solution in PBS was added and incubated for 5 min. This detergent treatment is necessary to permeabilise the plasma membrane and enable antibody binding. Then coverslips were washed 3 times with 1xPBS and cells visually inspected for attachment and healthy cell morphology. For blocking, the cells were incubated in 3% BSA (in 1xPBS) for 30min on a rocking stage.

MeOH- Fixation

MeOH fixation was performed by incubating coverslips with pre-chilled MeOH for 15min at -20°C. Due to its chemical similarity to water methanol replaces the water

molecules in the cells. When MeOH-treated cell are exposed to air the alcohol quickly evaporates and the cells will dry out. In order to avoid this cells were washed with 1xPBS by continuous infusion and aspiration. Finally coverslips, as PFA fixed cells, were incubated in blocking solution.

Extracted cytoskeletons + PFA-Fixation

To obtain detergent-extracted cytoskeletons, Trypanosomes were attached to coverslips and incubated in extraction buffer ("PEME": 0.5% NP4O, 2mM EGTA, 0.1mM EDTA, 1mM $MgSO_4$, 0.1M PIPES-NaOH, pH 6.9) for 5 min at room temperature. They were then washed 3 times with 1x PBS and fixed with PFA according to the standard protocol.

2.3.2 Antibody binding and Mounting

Primary antibody was added to a humidified chamber at the required dilution (in 1xPBS). Coverslips were transferred onto antibody solution drops and incubated for 1 hour at room temperature. After incubation washing with 1x PBS by 2x rinses and finally 10 min incubation on the rocking stage was performed. Fluorophore-coupled goat anti-rabbit/ anti-mouse secondary antibodies diluted in PBS as appropriate (Molecular Probes) were added to the chamber. Again coverslips were placed on top and incubated for 1 hour at room temperature. To stain DNA coverslips were directly transferred to 4',6-diamidin-2-phenylindol (DAPI) solution, a DNA intercalating substance (1:1000 in PBS). 15 min later DAPI solution was removed by washing 3x with 1xPBS. Finally coverslips were mounted with Fluoromount G (Electron Microscopy Sciences). Later on instead of DAPI staining I used Fluoromount containing DAPI from Southern Biotech. Coverslips were stored at 4°C until used.

2.3.3 Immunofluorescence microscopy

Epifluorescence images were obtained using an inverted microscope (Axio Observer Z1, Carl Zeiss MicroImaging Inc.) equipped with a PCO 1600 camera (PCO) and using the manufacturer's drivers in a custom C++ program.

2.4 Protein methods

In order to confirm expression of YFP fusion proteins I performed Western blot analysis of whole cell lysates from the MORN-family. For the TbMORN1 truncations, fractionation experiments were carried out by extraction with 0.5% NP40 followed by centrifugation at 14000rpm for 5 min. This resulted in three different samples: the input taken before centrifugation, the supernatant containing the soluble cytoplasmic & membrane fraction and the pellet containing the insoluble cytoskeletal fraction. Therefore the supernatant fraction contains cytoplasmic proteins and the pellet fraction represents cytoskeletal-associated proteins. Equal fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. By using α-GFP antibodies expression and tracking of recombinant proteins were examined. To check whether the endogenous MORN1 traffics to the cytoplasmic or cytoskeletal fraction membranes were treated with α-MORN1 antibodies. These blots were used as a positive control for biochemical fractionation experiments.

2.4.1 Whole cell lysate preparation

For Western blot analysis total cell lysates were prepared. Cell concentration was measured and a 5ml sample was centrifuged at 3000rpm for 5min at 4°C. The pellets were taken up in 10ml 1xPBS and again centrifuged. In the next step pellets were resuspended in 1ml 1xPBS, transferred to 1.5ml Eppendorf tubes, and again pelleted (6000rpm, 1min). Finally cells were taken up in sodium dodecyl sulphate (SDS)-loading buffer such that the final concentration was $2x10^5$cells/µl. To denature proteins the samples were boiled for 10 min. Cell lysates were stored at -20°C.

SDS-Loading Buffer
2ml 10% SDS
2ml 0.5M Tris-HCl pH 6.7
3ml 50% Glycerol
1ml Beta-mercaptoethanol
As required Bromophenoblue

2.4.2 Biochemical fractionation

Cells were grown to stationary phase, concentration was measured and a 7ml sample centrifuged at 3000rpm for 5min at 4°C. The pellets were taken up in 1ml PEME+COMPLETE (PEME buffer supplemented with protease inhibitor tablets)

and again centrifuged. In the next step pellets were resuspended in 200µl extraction buffer (0.5%NonidetP-40 in PEME) and incubated for 30 minutes at room temperature on a roller. After incubation 10µl samples, called "Input" (representing 5% of volume) were taken. Extracted cells were then centrifuged for 5 min at 14000rpm at room temperature. Supernatant and pellets were separated and the pellets taken up in 200µl extraction buffer (1%NonidetP-40 in PEME). Of these 2 fractions 10µl samples, referred to P1 and SN1 were obtained. By adding 30µl SDS-LB final volumes of 40µl representing 5% were achieved. To denature proteins the samples were boiled for 10 min. Fractionated cell lysates were stored at -20°C.

2.4.3 SDS-Page

In order to separate proteins according to their sizes, SDS-polyacrylamide gel electrophoresis was performed. Generally a 15% or 12% resolving and 4% stacking gel were cast and run in Mini-PROTEAN Tetra Cells according to the manufacturer's instructions (Bio-Rad).

Resolving gel (1 gel)

Resolving gel	15%	12%	Stacking gel 4%
30% Bis-Acrylamide	5ml	4ml	1.3ml
Tris 1.5M pH8,8	2.5ml	2.5ml	-
Tris 0.5M pH6,8	-	-	2.5ml
H_2O	2.4ml	3.4ml	6.1ml
10% SDS	100µl	100µl	100µl

Shortly before pouring the gel polymerization was started with 100µl 10%APS and 10µl TEMED. SDS gels were usually run at 90mA for 50 min.

2.4.4 Western blot

The transfer of proteins from the SDS-gels to nitrocellulose membranes was performed using Mini Trans-Blot Electrophoretic Transfer cells (Bio-Rad). Therefore the "sandwich", containing in buffer soaked gel, membrane, whatman papers and fiber pads, was assembled. This sandwich was placed into the blotting cassette. Blotting took place for 2 hours at 350mA chilled with ice at 4°C.

In order to check transfer quality, nitrocellulose membranes were stained with Ponceau´s solution (0.2% Ponceau's, 10% acetic acid). After a few minutes this reagent non-specifically stains the membrane but destaining using water reveals transferred proteins from total cell lysates.

To avoid non-specific signals from antibodies bound to nitrocellulose, membranes were blocked with milk solution (10% milk powder, 0.3% TWEEN-20 in 1xPBS) for at least 30 min. Afterwards the membranes were placed into the first antibody solution (diluted in blocking solution) and incubated for 1 hour at room temperature on the rocking stage. Then membranes were washed 3 times in 1xPBST (0.3% TWEEN-20 in PBS) for 5 min to remove any unbound antibodies. The membranes were placed into secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit/anti-mouse) solution and incubated for 1 hour on the rocking stage. In the next step the membranes were again washed 3 times in 1xPBST for 5 min.

For visualization of blots the enhanced chemiluminescence kit (Pierce) was used according to the manufacturer's instructions. X-Ray films (BioMax MS Film, Kodak) were exposed for variable times (a few seconds up to 20 min) and developed.

2.5 Bioinformatics

2.5.1 Immunofluorescence microscopy

Image processing was carried out using ImageJ and Adobe Photoshop CS3 software.

2.5.2 Databases, Internet resources

T.brucei GeneDB: Data base which provides the latest sequence data and annotation for a range of pathogens including Trypanosomes. www.genedb.org/genedb/tryp

TriTrypDB: TriTryp data base includes a genomic resource for Trypanosomes. http://tritrypdb.org/tritrypdb/

NEB: This sites gives information about products (restriction enzymes, polymerases) and applications (PCR conditions) from New England Biolabs.
http://www.neb.com/nebecomm/default.asp

2.5.3 Tools and Software

JPred is a consensus method for protein secondary structure prediction. http://www.compbio.dundee.ac.uk/www-jpred/

NCBI provides BLAST sequence analysis service for DNA and protein. http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

Hydrophobicity plot: Through this tool plots demonstrating hydrophobic character of a protein sequence can be obtained. These plots display the distribution of polar and apolar residues along a protein sequence. This analysis is commonly used for the prediction of membrane-spanning segments (highly hydrophobic) or regions that are likely exposed on the surface of proteins (hydrophilic domains). To generate data for a plot the sequence is scanned with a moving window of fixed size. At each position the mean hydrophobic index of the amino acids within the window is calculated and plotted as the midpoint of the window. In the Kyte-Doolittle scale hydropobic regions achieve positive values. Negative values have hydrophilic character. The window size refers to the number of amino acids examined at a time to determine appoint of hydrophobic character. So one should choose a window that corresponds to the expected size of the structural motif.

http://www.vivo.colostate.edu/molkit/hydropathy/index.html

Mitoprot II:

Mitoprot II produces an output using a set of parameters that calculate the N-terminal protein region that can support a mitochondrial targeting sequence and a predicted cleavage site. Thus, it searches for the existence of two close glutamic acids or aspartic acids takes in account the isoelectric point, the enrichment of positively charged residues, the paucity of acidic residues and further parameters (Claros and Vincens, 1996). This program has been used before to predict possible mitochondrial targeting sequences in Leishmania major (Li et al., 2007).
http://ihg.gsf.de/ihg/mitoprot.html

CSS-Palm: Prediction of palmitoylation sites:

http://csspalm.biocuckoo.org/

3 Results

3.1 The MORN-repeat protein family

In T.brucei there are 17 other proteins containing MORN-repeats which are all uncharacterized so far (Fig.3.1.1). In this project I cloned the MORN-repeat proteins and screened for possible bilobe localization. Two of these proteins, Protein 1 and 7, were previously detected in the flagellar proteome (Broadhead et al., 2006). Protein 3, 4, 5, 6, 7 and 9 were found in the plasma membrane and cytoskeletal fraction of bloodstream form cells (Bridges et al., 2008). Additionally Proteins 2, 6 and 8 have potentially interesting features- like a VPS9, kinase, or kinesin motor domain respectively. Protein 12 has predicted transmembrane domains and Protein 17 carries a zinc finger motif.

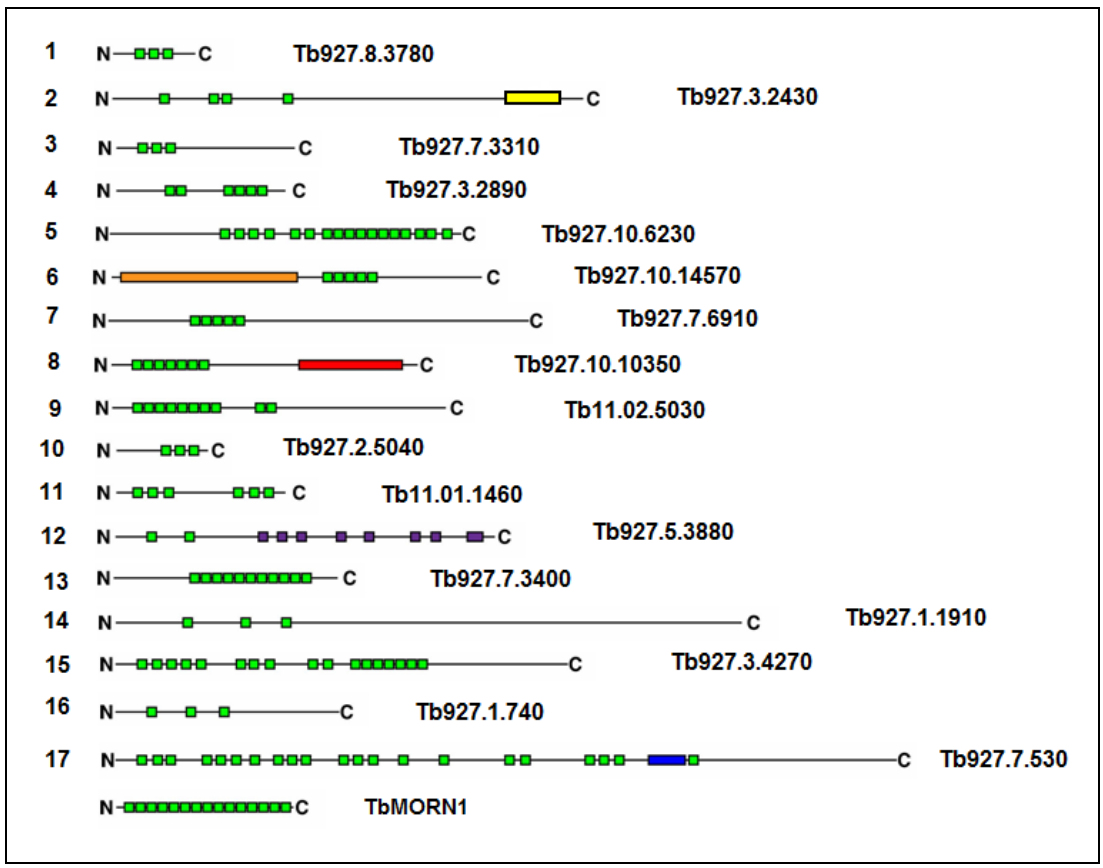

Fig. 3.1.1 The family of MORN-repeat proteins in T.brucei.

Protein maps of MORN-repeat family members showing predicted protein domains. Green box: MORN-repeat; yellow box: VPS9 domain; orange box: kinesin motor domain; red box: kinase domain; purple box: transmembrane domain; blue box: zinc finger motif.

3.2 General experimental strategy

The general experimental strategy was to clone each ORF into the pxS2-YFP expression vector (Fig.2.1.1) using standard molecular biology techniques. During cloning the YFP tag was placed at the 5' or 3' end of the cloned gene depending on the availability of restriction sites (Table 3.2-1).

Name	Gene ID	YFP gene at	Restriction site(s)	Notes
Protein 1	Tb927.8.3780	5'end	BamH1/EcoR1	Q49H, A152S
Protein 2	Tb927.3.2430	5'end	BamH1/BamH1	A59V, A63P, A303M, M364T, E767K, F772L, Q825E
Protein 3	Tb927.7.3310	5'end	BamH1/EcoR1	
Protein 4	Tb927.3.2890	3'end	Hind3/Nhe1	
Protein 5	Tb927.10.6230	3'end	Nhe1/Nhe1	
Protein 6	Tb927.10.14570	3'end	Hind3/Nhe1	
Protein 7	Tb927.7.6910	3'end	Hind3/Nhe1	Q221M, P493A
Protein 8	Tb927.10.10350	3'end	Hind3/Nhe1	V310A, D315N, D466G
Protein 9	Tb11.02.5030	5'end	BamH1/EcoR1	R21H, T264I, P380T
Protein 10	Tb927.2.5040	5'end	BamH1/EcoR1	
Protein 11	Tb11.01.1460	5'end	BamH1/EcoR1	
Protein 12	Tb927.5.3880	3'end	Hind3/Nhe1	S580P
Protein 13	Tb927.7.3400	3'end	Hind3/Nhe1	
Protein 14	Tb927.1.1910	5'end	Bcl1/EcoR1	
Protein 15	Tb927.3.4270	3'end	Hind3/Nhe1	S210L, Y268H, S549G, M669L, V706A, R761K, E823D, N830S
Protein 16	Tb927.1.740	5'end	BamH1/EcoR1	
Protein 17	Tb927.7.530	3'end	Hind3/Nhe1	

Table 3.2-1 Summary of cloning

These constructs were used to transiently transfect procyclic T.brucei cells. Localization of each protein were then investigated by immunofluorescence microscopy. Whole cells and extracted cytoskeletons were fixed with PFA or MeOH. α-GFP antibodies were used to amplify the YFP signal. Protein localizations were confirmed by costaining with antibodies against marker proteins- α-PFRA for PFR, YL1/2 for

basal body, and α-LRRP1 for bilobe. Protein expression was further checked by Western blotting of whole cell lysates using α-GFP antibodies.

3.2.1 Protein 1, Tb927.8.3780

Tb927.8.3780 is a 501 amino acid, approximately 20kDa protein with 3 MORN-repeats (Fig. 3.2.1). The protein was previously detected in the flagellar proteome (Broadhead et al., 2006). Sequencing results on the cloned construct showed 2 point mutations, specifically Q49H and A152S. Since these two aberrations were not found within the MORN-repeats we expected them not to strongly influence protein activity. Furthermore both point mutations were also detected in repeated PCR cloning reactions and therefore we suggest that they might be real. Generally, some natural variations between lab populations and the genome from GeneDB are to be expected.

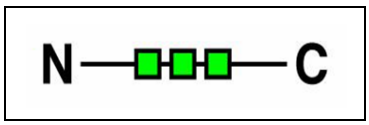

Fig. 3.2.1 Schematic of Protein 1 (Tb927.8.3780).

MORN-repeats are represented by green boxes.

Immunofluorescence studies of Protein 1 using α-GFP antibodies revealed a whole cell labelling in MeOH- and PFA-fixed cells suggesting a pool of YFP-tagged proteins in the cytosol (Fig.3.2.2 A and B). Detergent-extracted cytoskeletons showed that the recombinant protein was present on the flagellum (Fig.3.2.2 C). This is consistent with the annotation of Protein 1 in the flagellar proteome. To better determine the localization of YFP-Protein 1 a second set of experiments using α-PFRA antibodies to label the PFR was performed. The α-GFP signal was found on top of the PFRA staining and furthermore extended beyond the PFR (Fig.3.2.2 D). This suggested that the YFP-tagged protein localized to the flagellar axoneme.

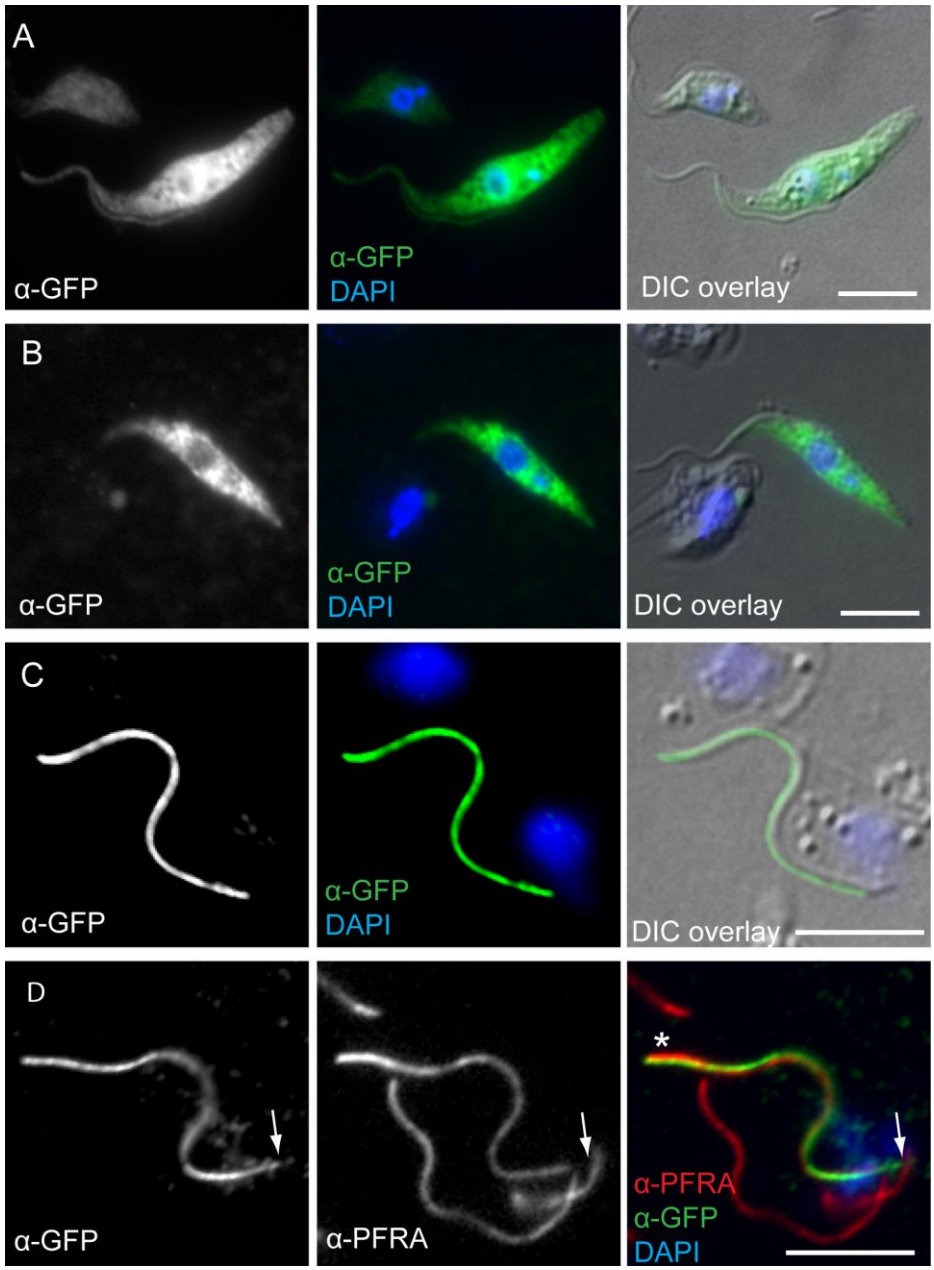

Fig. 3.2.2 Localization of YFP-Protein 1 (Tb927.8.3780) examined by immunofluorescence microscopy.

Transiently transfected procyclic Trypanosomes fixed with either (A) PFA or (B) MeOH showed cytosolic labelling with α-GFP antibodies. (C,D) Detergent-extracted cytoskeletons from YFP-Protein 1 expressing cells were fixed with PFA and labelled with α-GFP and α-PFRA antibodies (D). The recombinant protein appeared to be present on the flagellum on top of the PFR (*) and extended beyond the PFR towards the kinetoplast (arrow). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Bars, 5µm.

3.2.2 Protein 2, Tb927.3.2430

Tb927.3.2430 is a 103kDa protein composed of 4 MORN-repeats, and has a VPS9 domain near the C-terminus (Fig.3.2.3). The latter is present in yeast vacuolar sorting protein 9 and is a Rab5 Guanidine exchange factor (GEF). Amplification of the Protein 2 ORF with Vent polymerase was unsuccessful. Magnesium concentrations were varied from 2-8mM but no product could be obtained. For this reason I used a High Fidelity Taq DNA polymerase which has an artificially added proofreading ability to minimize the misincorporation rate of normal Taq polymerase. Using High Fidelity Taq and a higher annealing temperature of 58°C a 2763bp product was amplified. The ORF was ligated into the expression vector using only a single restriction site and with the YFP tag at the 5'end. Sequencing revealed that the initial clones carried 7 point mutations (A59V, A63P, A303M, M364T, E767K, F772L, Q825E) which are not found within MORN-repeats but the last was found within the VPS9 domain. In order to obtain a mutation-free PCR product I tried two further polymerases, Phusion and PFU Ultra. Except for the first mutation, A59V, all others were found in a repeated PCR and therefore we supposed them to be real in the T.brucei culture.

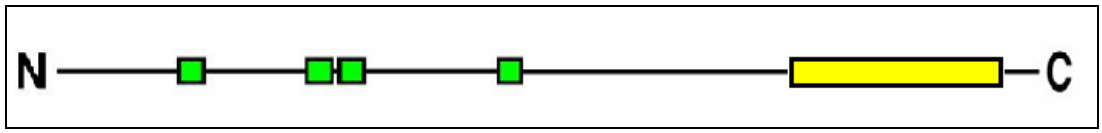

Fig. 3.2.3 Schematic image of Protein 2 (Tb927.3.2430).

The 103kDa protein is composed of 4 MORN-repeats and has a VSP9 domain near the C-terminus.

Procyclic cells were treated for immunofluorescence as standard. In intact cells, a cytoplasmic labelling pattern was observed (Fig.3.2.4 A and B). This suggested that the fusion protein was expressed and present in the cytosol. In detergent extracted cytoskeletons no specific signal was obtained in two independent transfections (data not shown). Therefore I classified YFP-Protein 2 as a cytosolic protein.

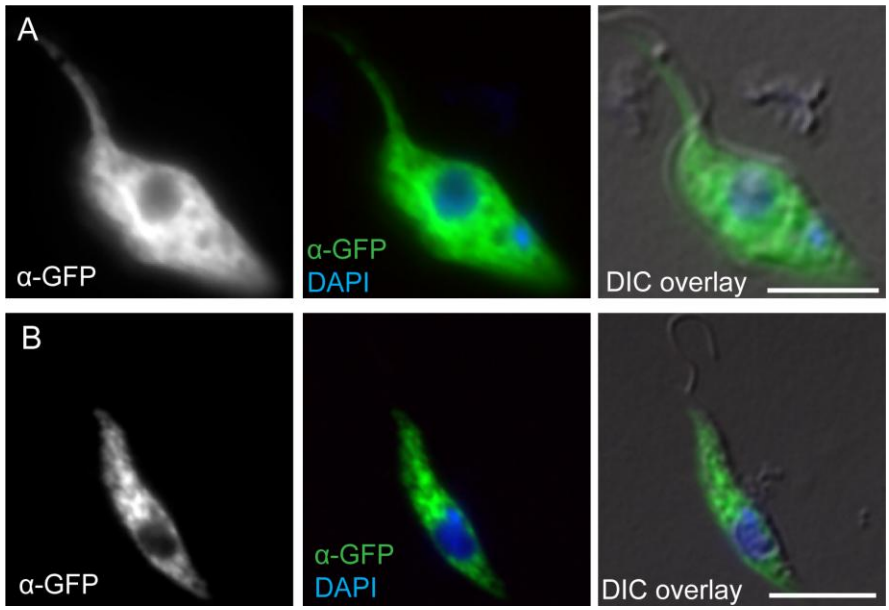

Fig. 3.2.4 Trypanosomes expressing YFP-Portein 2 (Tb927.3.2430).

Cells were fixed with PFA (A) or MeOH (B) 15 hours after transfection and labelled with α-GFP antibodies. Nuclear and kinetoplast DNA were labelled with DAPI. Under both fixation conditions whole cell staining was observed suggesting that a large pool of cytosolic recombinant proteins was present. Bars, 5µm.

3.2.3 Protein 3, Tb927.7.3310

Tb927.7.3310 was detected in the plasma membrane and cytoskeletal fraction of bloodstream form cells (Bridges et al., 2008). The 1071bp long gene coding for 3 MORN-repeats is located on chromosome 7 (Fig.3.2.5).

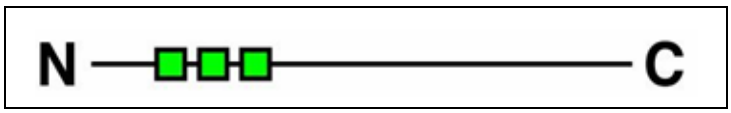

Fig. 3.2.5 Schematic of Protein 3 (Tb927.7.3310).

Sequencing of cloned DNA showed that the amplified inserts contained a deletion at base 63. This region of Tb927.7.3310 locus is composed of a stretch of ten As coding for multiplephenylalanine residues and therefore the polymerase is probably at risk of skipping bases and introducing mutations. This deletion leading to a frameshift would alter the sequence and encode a completely different protein. Therefore a second amplification of Protein 3 by PCR was started but sequencing results again showed the same aberration. Unfortunately trials with Phusion and PFU Ultra polymerase were similarly unsuccessful. Therefore site-directed mutagenesis to introduce the missing adenosine was performed. Sequencing of the mutagenic plasmid revealed

that it contains one mutation, K343E. Since this aberration was also detected in a repeated PCR reaction I considered it to be present in the Trypanosome genome of the culture.

Immunofluorescence microscopy revealed cytosolic labelling in PFA and MeOH-fixed cells (Fig.3.2.6 A and B). This suggested that the recombinant protein was expressed and present in the cytosol. Trypanosomes treated with extraction buffer showed that YFP-Protein 3 was found on the flagellum. The localization is likely to be to the axoneme since the α-GFP staining was observed on top of the PFR and extended beyond it towards the kinetoplast (Fig.3.2.6 C).

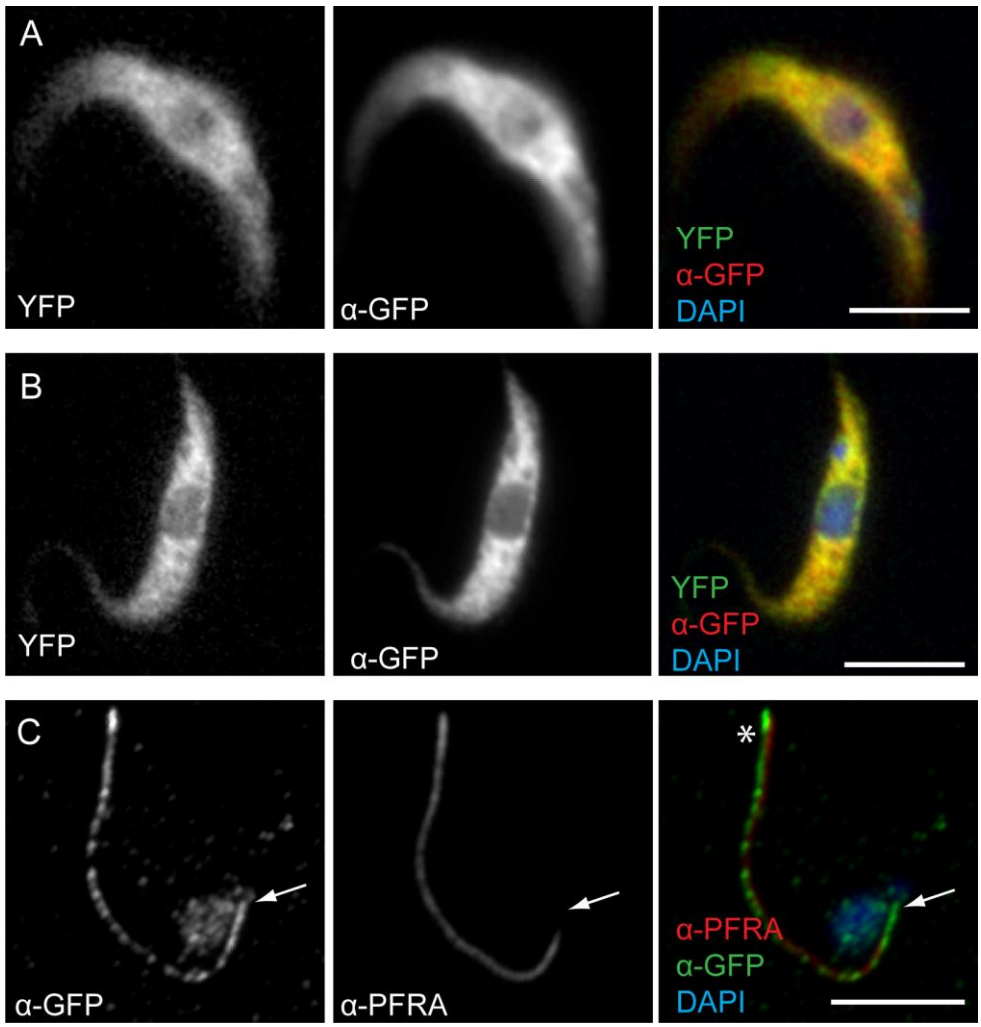

Fig. 3.2.6 YFP-Protein 3 (Tb927.7.3310) expressing cells examined by immunofluorescence microscopy.

Procyclic Trypanosomes were fixed with (A) PFA or (B) MeOH. α-GFP antibodies were used to enhance the YFP signal. Nuclear and kinetoplast DNA were labelled with DAPI. Uniform whole cell labelling was observed suggesting that there was a

pool of recombinant protein in the cytosol. (C) Detergent extracted cytoskeletons reveal that the tagged protein was present on the flagellum on top of the PFR (*) and extended beyond the PFR (arrow). Bars, 5µm.

3.2.4 Protein 4, Tb927.3.2890

Tb927.3.2890 is a 37kDa protein with 6 MORN-repeats (Fig.3.2.7). This 322 amino acid long polypeptide was detected in the plasma membrane and cytoskeletal fraction of bloodstream form cells (Bridges et al., 2008).

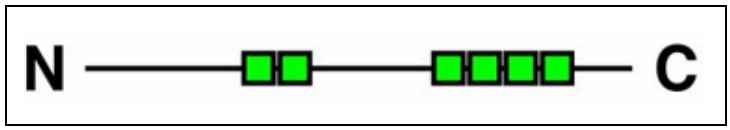

Fig. 3.2.7 Protein 4 (Tb927.3.2890).

Trypanosomes transiently expressing the YFP-tagged recombinant protein fixed with MeOH or PFA showed cytosolic staining after labelling with α-GFP antibodies (Fig.3.2.8 A and B). Detergent-extracted cytoskeletons showed that the fusion protein was present on the flagellum (Fig.3.2.8 C). By costaining with PFRA antibodies it was further shown that the YFP-tagged protein was probably present on the axoneme. The α-GFP signal was found on a structure lying on top of the PFR and extended beyond it (Fig.3.2.8 D).

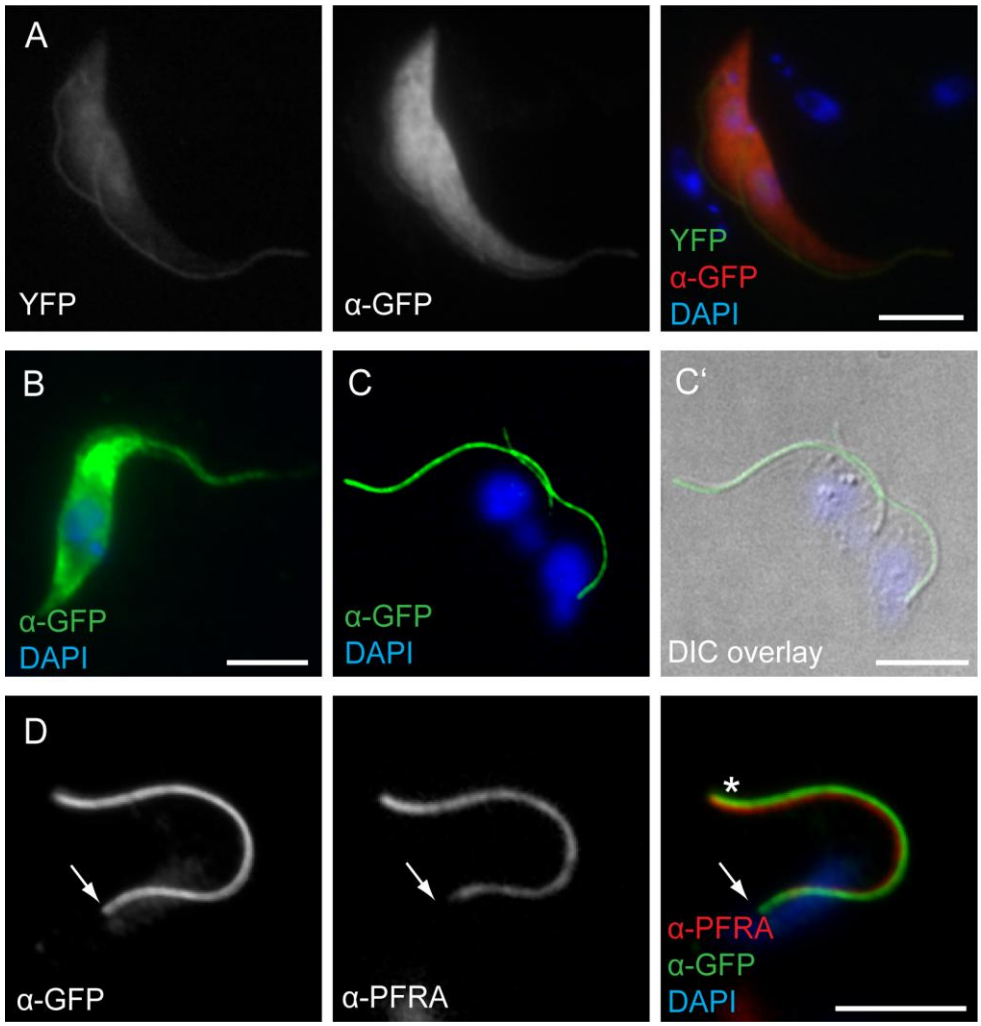

Fig. 3.2.8 Protein 4-YFP (Tb927.3.2890) is a flagellar protein.

(A) PFA fixed Trypanosomes labelled with α-GFP antibodies showed cytosolic staining. (B) Uniform whole cell staining was also observed for MeOH-fixed cells. (C, D) Detergent-extracted Trypanosomes fixed with PFA and labelled with α-GFP displayed a clear flagellum staining. (D) The costaining with PFRA revealed that Protein 4-YFP was probably present on the axoneme as labelling was found on top of the PFR (*) and extended beyond the PFR (arrow). Nuclear and kinetoplast DNA were labelled with DAPI. Bars, 5µm.

3.2.5 Protein 5, Tb927.10.6230

For amplification of the Tb927.10.6230 gene all trials with Vent DNA polymerase, even with magnesium concentrations ranging from 2-8mM, were unsuccessful. Using High Fidelity Taq DNA polymerase finally gave rise to a 2073bp long product. The 77kDa protein (Fig.3.2.9) was previously detected in bloodstream plasma membrane and cytoskeletal fraction (Bridges et al., 2008).

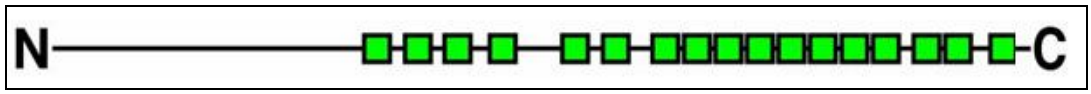

Fig. 3.2.9 Protein 5 (Tb927.10.6230) the primary structure contains 17 MORN-repeats.

Transient expression of Protein 5-YFP was challenging too since no cells expressing the recombinant protein were observed (n=2). For the third transfection I used cells at a concentration of $4x10^6$ cell/ml which means that cells were in mid log phase and therefore should take up DNA easier than in stationary phase. But even so no transfected cells were observed. As a consequence I decided to generate a stable cell line. Unfortunately no clones were obtained in the selection media. A second transfection lead to one clone growing in selection media, but expression of the YFP-tagged protein was not observed by IF. Western blots of cell lysates from the clonal population were also negative. PCR using primers annealing to the 3'end of the ORF of Protein 5 and the 5'end of the YFP gene was unsuccessful. The basticidin gene in contrast was successfully amplified by PCR. This means that the cells acquired the resistance gene but not the YFP cassette. From all these circumstances I considered that the YFP-tagged protein might be toxic for the cells.

3.2.6 Protein 6, Tb927.10.14570

The approximately 80kDa protein contains 5 MORN-repeats and a kinesin motor domain (Fig.3.2.10). Kinesins are microtubule-associated motor proteins that can play a role in organelle transport. Protein 6 was additionally detected in both the plasma membrane and cytoskeletal fractions of bloodstream Trypanosomes (Bridges et al., 2008).

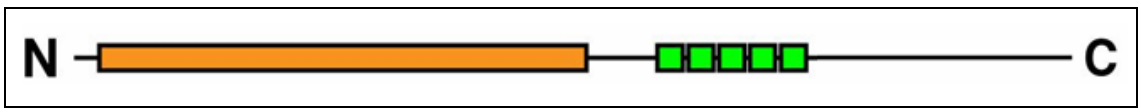

Fig. 3.2.10 Schematic of Protein 6 (Tb927.10.14570).
The kinesin motor domain is shown in orange.

Anti-GFP antibodies binding to the YFP tag labelled a pool of cytosolic recombinant proteins in PFA- and MeOH-fixed cells (Fig.3.2.11 A and B). In detergent-extracted cytoskeletons of Trypanosomes fixed with PFA Protein 6-YFP was found on the flagellar axoneme (Fig.3.2.11 C). This was shown by costaining with α-PFRA antibodies where the α-GFP staining was found on top of the PFR and extended beyond it towards the kinetoplast (Fig.3.2.11 D).

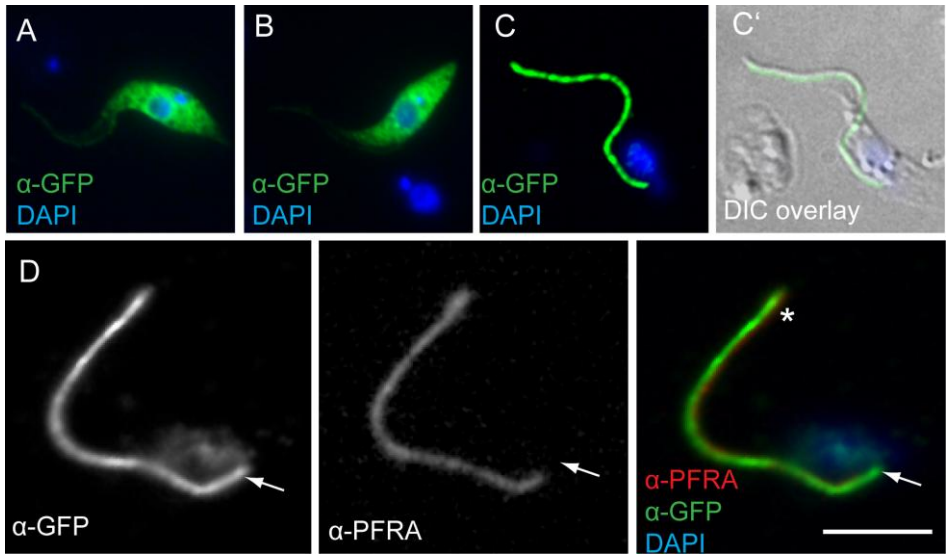

Fig. 3.2.11 Localization of Protein 6-YFP (Tb927.10.14570) examined by immunofluorescence microscopy.

Nuclear and kinetoplast DNA were labelled with DAPI. Transiently transfected Trypanosomes fixed with (A) PFA or (B) MeOH showed cytoplasmic α-GFP labelling. (C) Detergent-extracted cytoskeletons from Protein 6-YFP expressing cells were fixed with PFA. Labelling of α-GFP was observed on the flagellum. (D) Costaining with PFR binding antibodies. Protein 6-YFP was found on a structure which lied on top of the PFR (*) and extended beyond the PFR (arrows). Bars, 5µm.

3.2.7 Protein 7, Tb927.7.6910

Protein 7 was also detected in the flagellum proteome. It was also found in the bloodstream plasma membrane and cytoskeletal fraction (Bridges et al., 2008) and contains 5 MORN-repeats (Fig.3.2.12). For amplification Hifi Taq polymerase was used and 2 point mutations (Q221M, P493A) outside MORN-repeats were detected.

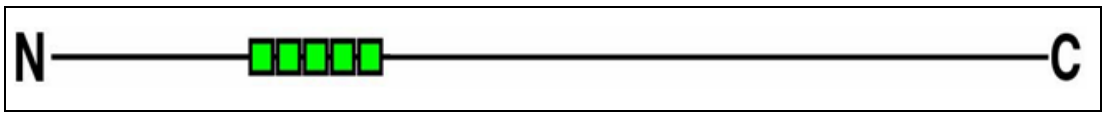

Fig. 3.2.12 Schematic of Protein 7.

The expression patterns in PFA- and MeOH-fixed cells were a whole cell labelling (Fig.3.2.13 A and B). Detergent-extracted cytoskeletons from Protein 7-YFP transfected cells show flagellum labelling with localization on the axoneme. Costaining with α-PFRA antibodies showed that the α-GFP labelling was found on top of the

PFR and extended beyond it towards the kinetoplast (Fig.3.2.13 C). Therefore Protein 7 probably localizes to the axoneme.

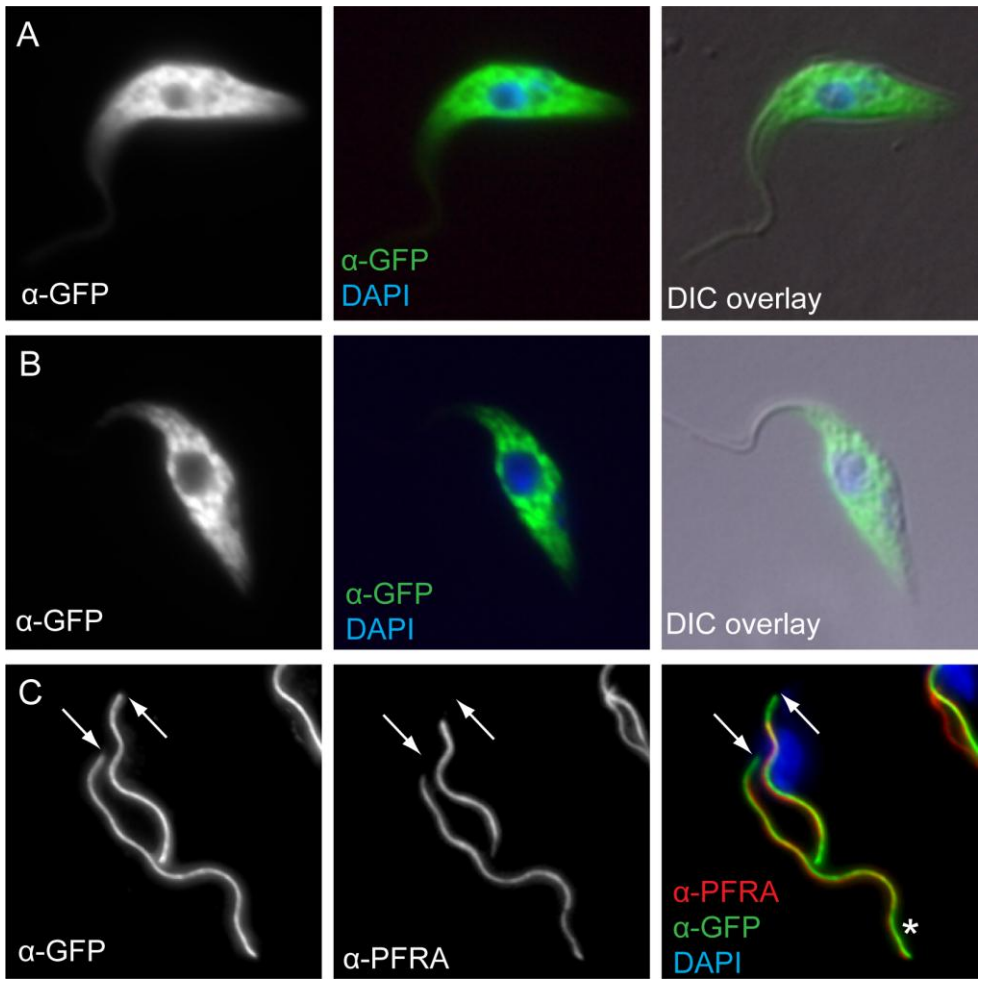

Fig. 3.2.13 Trypanosomes transiently expressing Protein 7-YFP (Tb927.7.6910).

(A) PFA- or (B) MeOH-fixed cells showed cytosolic localization. (C, D) Detergent-extracted cytoskeletons revealed distinct flagellar staining. Costaining with α-PFRA antibodies showed that the YFP-tagged protein was lying on top of the PFR(*) and extended beyond it (arrows) towards the kinetoplast. Bars, 5µm.

3.2.8 Protein 8, Tb927.10.10350

The 66.7kDa protein Tb927.10.10350 is composed of 7 MORN-repeats and a kinase domain which is a predicted serine/threonine kinase (Fig.3.2.14).

Sequencing of the clned gene revealed 3 point mutations: V310A, D315N, D466G. None were found within a MORN-repeat but the last was within the kinase domain.

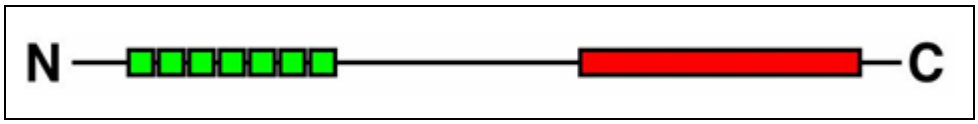

Fig. 3.2.14 Schematic of Protein 8 (Tb927.10.10350).

The kinase domain is shown in red.

A bright green whole cell staining was observed in PFA- and MeOH-fixed cells (Fig.3.2.15 A and B). In detergent-extracted cytoskeletons a punctate structure was recognized by α-GFP antibodies (Fig.3.2.15 C). This pattern was observed in many cells at a position next to the kinetoplast which suggested basal body staining. The fact that 2N2K cells showed 2 distinct punctae further supported this idea (Fig.3.2.15 C').

The α-GFP signal strongly overlapped with the basal bodies as labelled with YL1/2 monoclonal antibody (Fig.3.2.15 D). I concluded that Protein 8-YFP is present at the basal body.

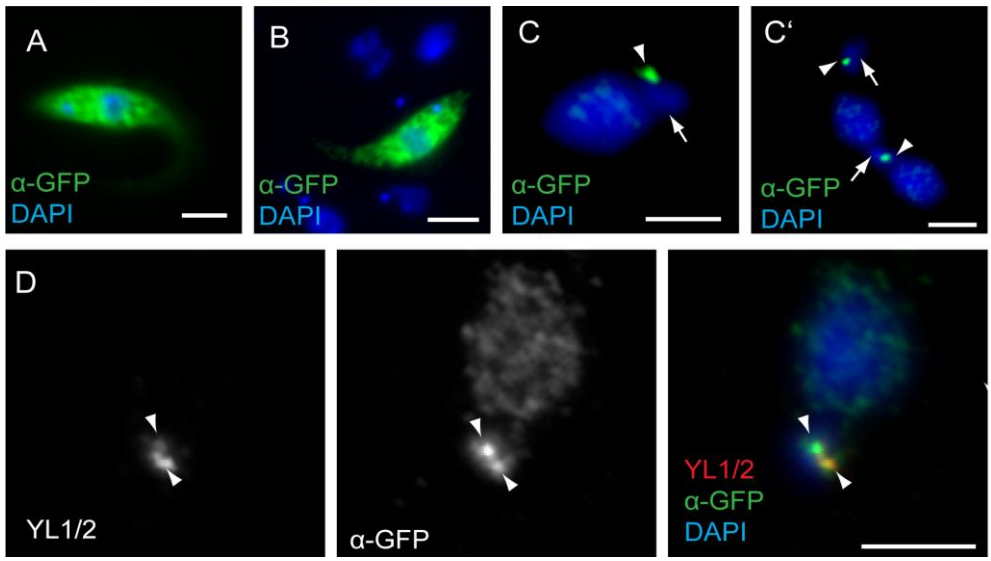

Fig. 3.2.15 Protein 8-YFP (Tb927.10.10350) localized to the basal bodies.

Cells were fixed with (A) PFA or (B) MeOH and labelled with α-GFP antibodies. Nuclear and kinetoplast DNA were labelled with DAPI. Whole cell staining was observed under both conditions. (C) Detergent-extracted cytoskeletons from Protein 8-expressing cells fixed with PFA and labelled with α-GFP antibodies. A punctate structure (arrowheads) was recognized by α-GFP antibodies that due to its consistent position near the kinetoplast (arrow) might be basal body staining. (D) Costaining showed that Protein 8 and YL1/2 significantly overlap (arrowhead). Bars, 5µm.

3.2.9 Protein 9, Tb11.02.5030

Tb11.02.5030 is a 72,56kDa protein which contains 10 MORN-repeats (Fig.3.2.16). This protein was detected in the plasma membrane fraction of blood stream form cells (Bridges et al., 2008). Sequencing revealed that this clone carries three mutations (R21H, T264I, P380T).

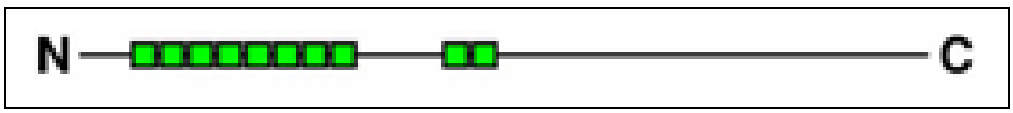

Fig. 3.2.16 Schematic of Protein 9 (Tb11.02.5030).

Immunofluorescence studies of Protein 9 showed whole cell labelling in intact cells which suggested that the protein was present in the cytosol (Fig.3.2.17 A and B). Cells treated with extraction buffer and fixed with PFA displayed flagellum labelling (Fig.3.2.17 C). For specific localization a second set of experiments with PFR binding antibodies was performed. The α-GFP staining was found on a structure which was found on top of the PFR and extended beyond it (Fig.3.2.17 D). Therefore Protein 9 probably localizes to the flagellar axoneme.

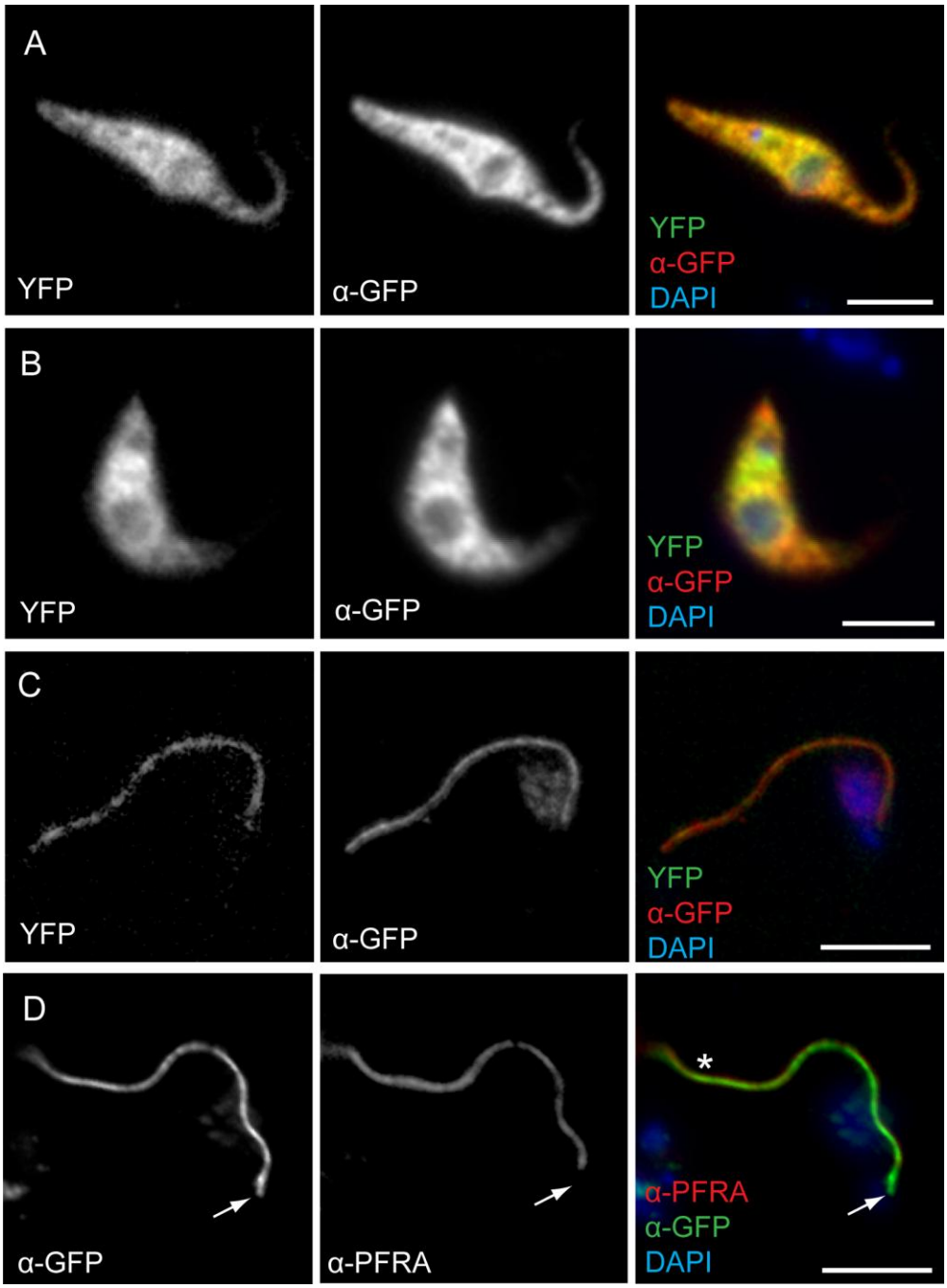

Fig. 3.2.17 Localization of YFP-Protein 9 (Tb11.02.5030)

Transiently transfected cells were fixed with (A) PFA or (B) MeOH and labelled with α-GFP antibodies. Nuclear and kinetoplast DNA were visualized with DAPI. Cells displayed cytosolic staining suggesting that there was a pool of recombinant proteins in the cytosol. (C, D) Trypanosomes treated with extraction buffer and fixed with PFA were labelled with α-GFP and α-PFRA. YFP-Protein 9 appeared to be present on the flagellar axoneme (arrows) since the α-GFP signal was found on top of the PFR (*) and extended beyond it. Bars, 5µm.

3.2.10 Protein 10, Tb927.2.5040

Tb927.2.5040 is a 19,63kDa protein containing 3 MORN repeats near the C-terminus (Fig.3.2.18).

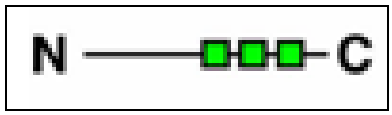

Fig. 3.2.18 Schematic of Protein 10 (Tb927.2.5040).

By immunofluorescence microscopy, a strong signal from the nucleus was observed in PFA-fixed cells (Fig.3.2.19 A). With α-GFP antibodies a whole cell labelling pattern was instead observed. This might be due to the fact that α-GFP antibodies also recognize unfolded proteins present in the cytosol. After treatment with extraction buffer the protein was found on the flagellum (Fig.3.2.19 B). Colabelling with α-PFRA showed that YFP-Protein 10 was found on top and extended beyond the PFR (Fig.3.2.19 C). Therefore Protein 10 probably localizes to the axoneme.

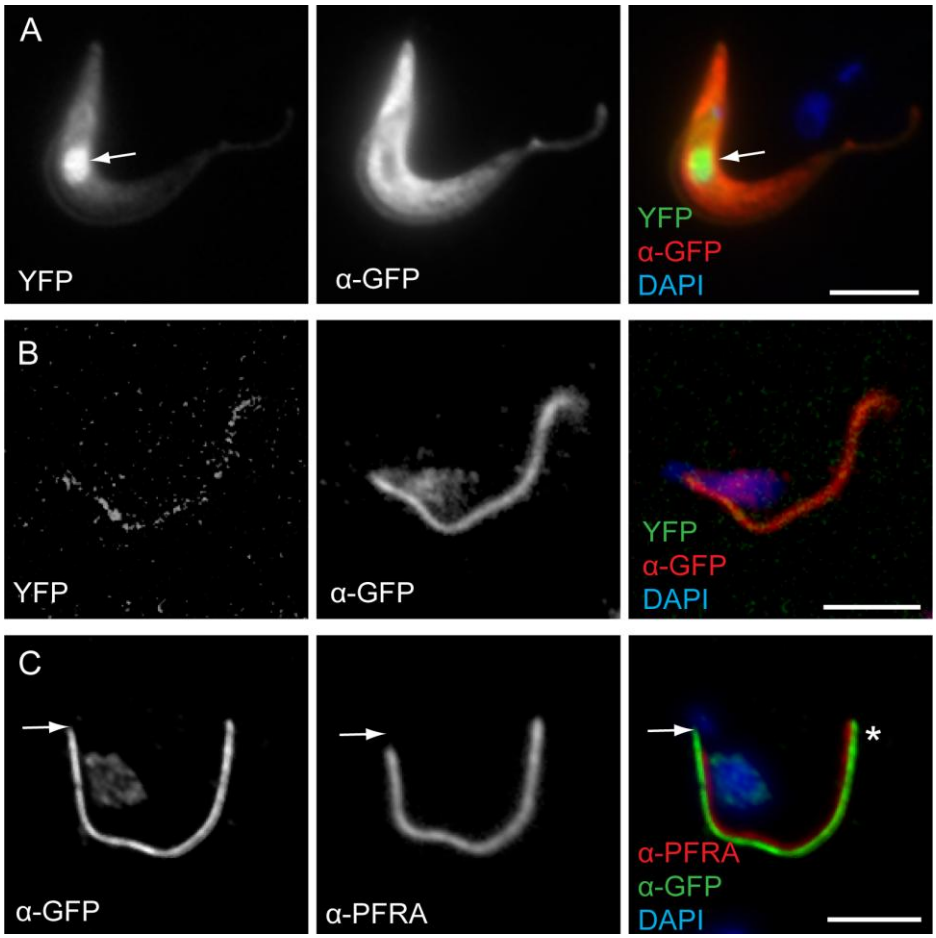

Fig. 3.2.19 Trypanosomes 427 cells transiently expressing YFP-Protein 10 (Tb927.2.5040).

Cells were fixed with (A) PFA or (B,C) pretreated with extraction buffer and fixed with PFA. α-GFP antibodies were used to amplify the YFP signal. Nuclear and kinetoplast DNA were labelled with DAPI. (A) PFA-fixed cells displayed a strong signal from the nucleus (arrow) and some cytosolic labelling with α-GFP. (B, C) In detergent-extracted cytoskeletons the fusion protein was found on top of the PFR (*). The α-GFP signal further extended beyond the PFR (arrow) towards the kinetoplast. Bars, 5µm.

3.2.11 Protein 11, Tb11.01.1460

The 37kDa protein Tb11.01.1460 contains 6 MORN-repeats (Fig.3.2.20).

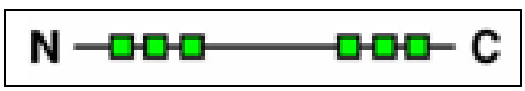

Fig. 3.2.20 Schematic of Protein 11 (Tb11.01.1460).

YFP-Protein 11 appeared in the cytosol and on a structure next to the kinetoplast (Fig.3.2.21A). After extraction the signal was solely found on a punctate structure (Fig.3.2.21 B). Costaining with α-TbCen2, which labels the flagellum and the basal body, showed that the recombinant protein colocalized with the basal bodies (data not shown). The basal body localization was further confirmed by colocalization with YL1/2 (Fig.3.2.21 C).

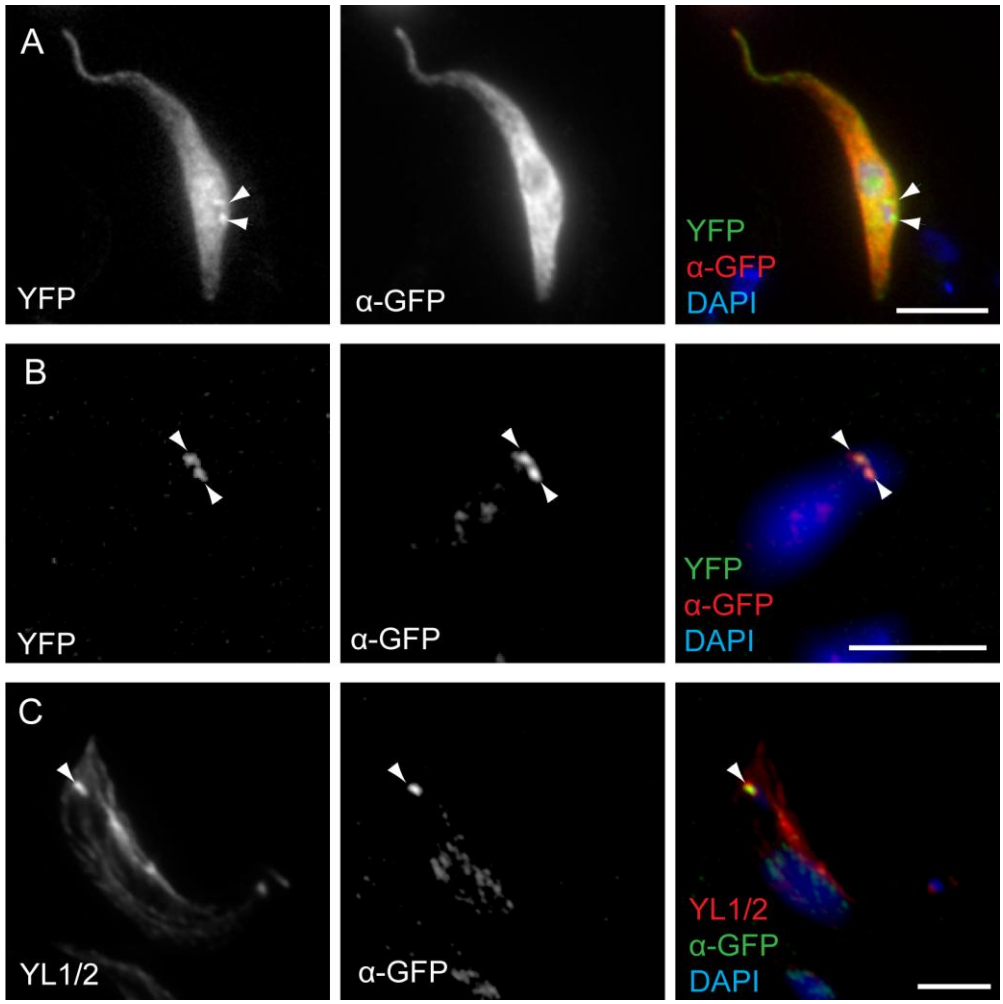

Fig. 3.2.21 Trypanosomes expressing YFP-Protein 11 (Tb11.01.1460).

Cells were fixed with (A) PFA or (B, C) treated with extraction buffer and fixed with PFA. To increase the YFP signal α-GFP antibodies were used. Nuclear and kinetoplast DNA were labelled with DAPI. (A) In PFA-fixed cells the tagged protein appeared in the cytosol and on a structure next the kinetoplast (arrowheads). (B) Cells treated with detergent show punctuate labelling (arrowheads) next the kinetoplast. (C) This punctuate labelling partially overlapped with the YL1/2 signal (arrowhead). Bars, 5µm.

3.2.12 Protein 12, Tb927.5.3880

Amplification of the ORF of Protein12 (Tb927.5.3880) with Vent polymerase did not lead to any product. Adjusting PCR conditions and using HiFi Taq polymerase finally gave rise to a 2223 base pair long product. The Tb927.5.3880 ORF codes for a 83kDa protein which contains 2 MORN-repeats and 8 predicted transmembrane regions (Fig.3.2.22). Since this transmembrane protein carries an N-terminal signal peptide the YFP tag was placed at the C-terminus. The construct carries one mutation, S580P, which was not found in any domain and therefore I consider it unlikely to influence localization.

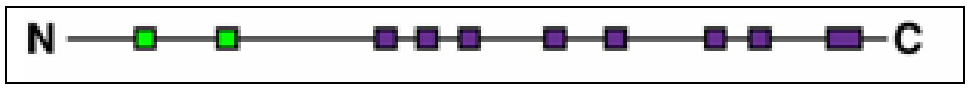

Fig. 3.2.22 Protein 12 (Tb927.5.3880).

Transmembrane regions are represented by purple boxes.

PFA-fixed cells displayed a meshed labelling pattern across the whole cell body (Fig.3.2.23). This might be mitochondrial labelling. In MeOH- fixed or extracted cells no signal was observed (data not shown).

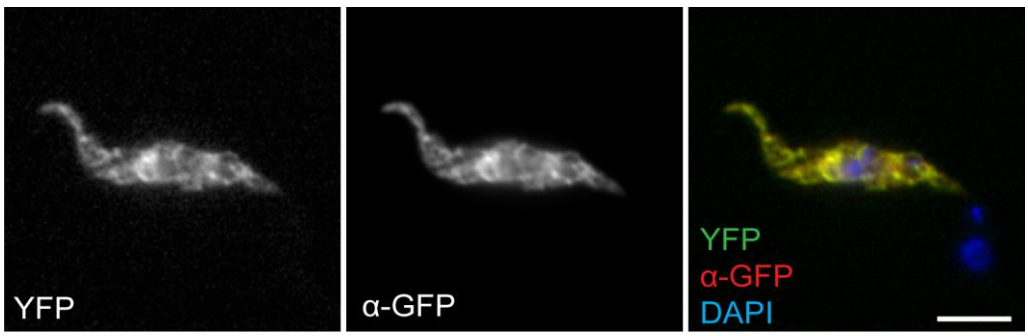

Fig. 3.2.23 Trypanosomes 427 cells expressing Protein 12-YFP (Tb927.5.3880).

Cells were fixed with PFA and α-GFP antibodies were used to amplify the YFP signal. Nuclear and kinetoplast DNA were labelled with DAPI. YFP and α-GFP showed a meshed labelling pattern across the whole cell body. Bars, 5µm.

3.2.13 Protein 13, Tb927.7.3400

Tb927.7.3400 encodes a 49kDa large protein with 11 MORN-repeats (Fig.3.2.24). Protein 13 ORF was also amplified by HiFi Taq since Vent polymerase did not lead to any product. A C-terminal YFP tag was necessary as this protein has a predicted signal peptide at the N-terminus.

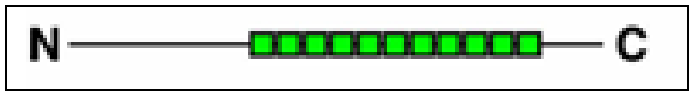

Fig. 3.2.24 Schematic image of Protein 13 (Tb927.7.3400).

Protein 13-YFP was present in the cytosol in intact cells (Fig.3.2.25 A). This suggested that the recombinant protein was found in a pool of cytosolic proteins. Extraction further revealed flagellum localization (Fig.3.2.25 B). PFRA costaining indicated that the PFR and the α-GFP staining colocalized (Fig.3.2.25 C). Thus Tb927.7.3400 seemed to localize to the PFR.

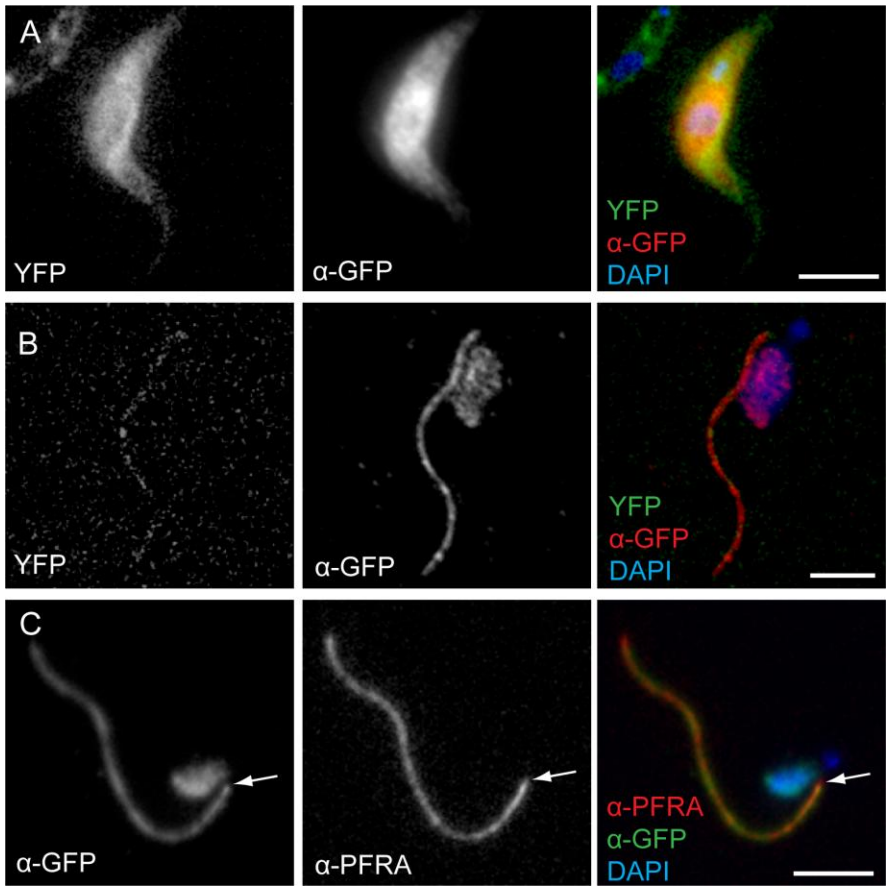

Fig. 3.2.25 Localization of Tb927.7.3400-YFP.

Cells were fixed with (A) PFA or (B,C) extracted and fixed with PFA. Nuclear and kinetoplast DNA were labelled with DAPI. (A) Uniform cytosolic signal was observed. (B) Trypanosomes displayed flagellum staining. (C) α-PFRA labelling and α-GFP staining colocalized (arrow). Bars, 5µm.

3.2.14 Protein 14, Tb927.1.1910

Tb927.1.1910 is a 1258 amino acid long, approximately 136kDa protein with 3 MORN-repeats (Fig.3.2.26). RNAi experiments in bloodstream form Trypanosomes had previously shown abnormal cell cycle progression (Subramaniam et al, 2006).

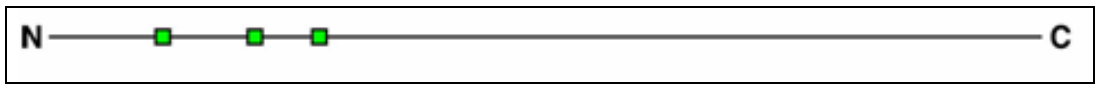

Fig. 3.2.26 Protein 14 (Tb927.1.1910).

Amplification of the ORF of Protein 14 was first performed with Vent polymerase. Despite repeated cloning attempts with two different polymerases (Vent, HiFi Taq) amplification of the gene ORF was unsuccessful.

3.2.15 Protein 15, Tb927.3.4270

The 100kDa protein Tb927.3.4270 contains 17 MORN repeats (Fig.3.2.27). Amplification of protein 15 ORF with Vent polymerase first lead to multiple PCR products. Therefore we used a higher annealing temperature of 60°C which resulted in no product. Finally HiFi Taq polymerase gave rise to a 2751 base pair long product. Sequencing of the selected clone revealed several mutations (S210L, Y268H, S549G, M669L, V706A, R761K, E823D, N830S) but all mutations were found in repeated PCR. Unfortunately the first and third mutation are found within MORN-repeats. Despite big efforts in cloning, and screening of several clones, fewer mutations could not be achieved.

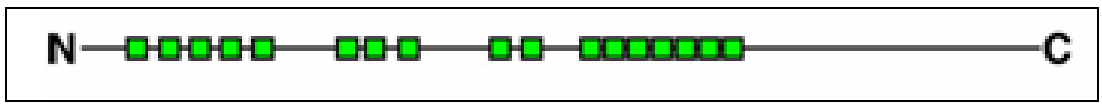

Fig. 3.2.27 Schematic of Protein 15 (Tb927.3.4270).

Transient expression of Protein 15-YFP and PFA fixation of Trypanosomes resulted in cytosolic staining by α-GFP antibodies (Fig.3.2.28 A). This showed that the fluorescent protein was expressed and present in the cytosol. MeOH fixation also lead to whole cell labelling (Fig.3.2.28 B). After detergent extraction this staining was lost and no signal could be detected (data not shown).

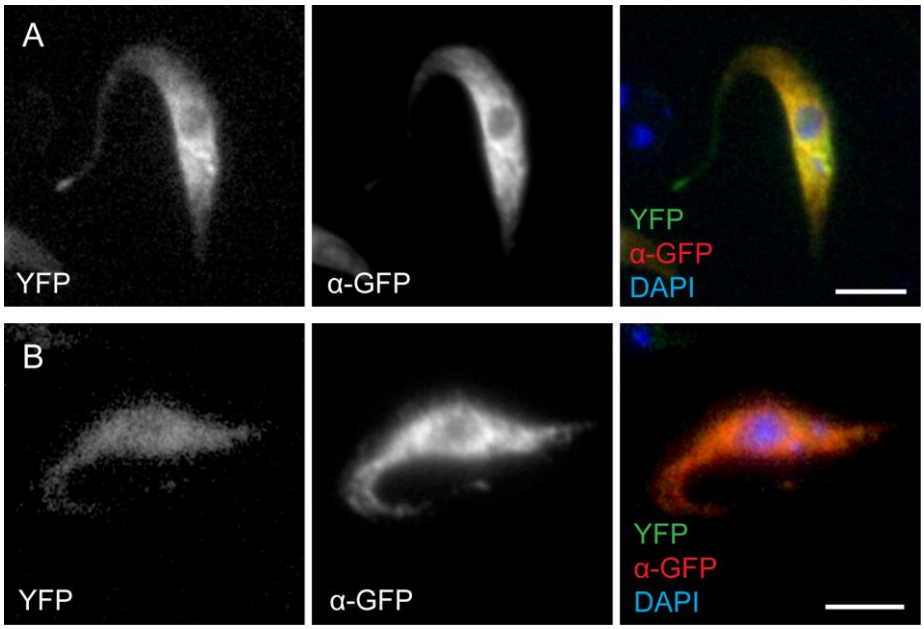

Fig. 3.2.28 Expression of Protein 15-YFP (Tb927.3.4270) examined by immunofluorescence microscopy using α-GFP antibodies.

Nuclear and kinetoplast DNA were labelled with DAPI. Cytosolic signal and labelling were observed in PFA-fixed cells (A). Cells after MeOH treatment also showed cytosolic labelling pattern (B). Bars, 5µm.

3.2.16 Protein 16, Tb927.1.740

Tb927.1.740 ORF codes for a 49kDa protein with 3 MORN-repeats (Fig.3.2.29).

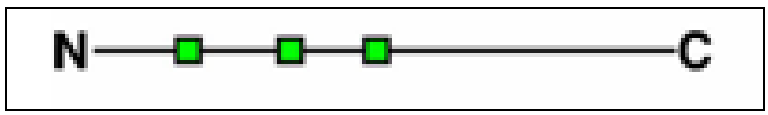

Fig. 3.2.29 Schematic of Protein 16 (Tb927.1.740).

Immunofluorescence microscopy revealed a cytosolic labelling pattern in YFP-Protein16 expressing cells (Fig.3.2.30). PFA-fixed Trypanosomes additionally show signal from a structure next the kinetoplast which according to size and shape might come from the basal bodies. In MeOH-fixed and extracted cells fixed with PFA no signal could be observed, which implied that the signal was lost after extraction (data not shown).

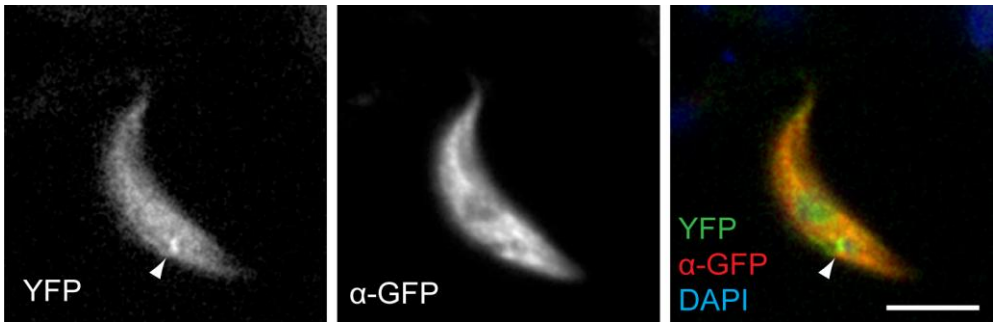

Fig. 3.2.30 YFP-Protein 16 (Tb927.1.740) expressing cells were fixed with PFA and stained with α-GFP antibodies.

Nuclear and kinetoplast DNA were labelled with DAPI. A cytosolic labelling pattern was observed. Additionally a YFP signal from a structure (arrowhead) next to the kinetoplast was seen. Shape and size might indicate basal body localization. Bar, 5µm.

3.2.17 Protein 17, Tb927.7.530

Tb927.7.530 is a protein of 171kDa and contains 21 MORN-repeats and a predicted zinc finger motif (Fig.3.2.31).

Fig. 3.2.31 Protein 17 (Tb927.7.530).

The predicted zinc finger domain is represented by the blue box.

Cloning of the Tb927.7.530 gene was unsuccessful despite the use of 3 different polymerases (Vent, Taq, PFU Ultra) and multiple optimization attempts. The PCR product always displayed several deletions.

3.2.18 Western blot analysis

In order to confirm the expression of the YFP-tagged proteins in transfected Trypanosomes I performed Western blot analysis of whole cell lysates using antibodies against GFP (Fig.3.2.32). For 9 recombinant proteins, namely Protein 1,4,6,7,8,10,11,13 and 15, bands of proper size were detected confirming the expression of YFP-tagged proteins. Despite experimenting with higher transfer (increased current for transfer and longer transfer time) and loading of $1{,}2x10^7$ cells, no signals for Protein 2,3,9,12 and 16 were observed (data not shown). Expression of YFP alone would lead to a band at 27kDa. This was not observed. I rather think that YFP-

tagged proteins were expressed at a lower level and therefore were not detected by Western blot.

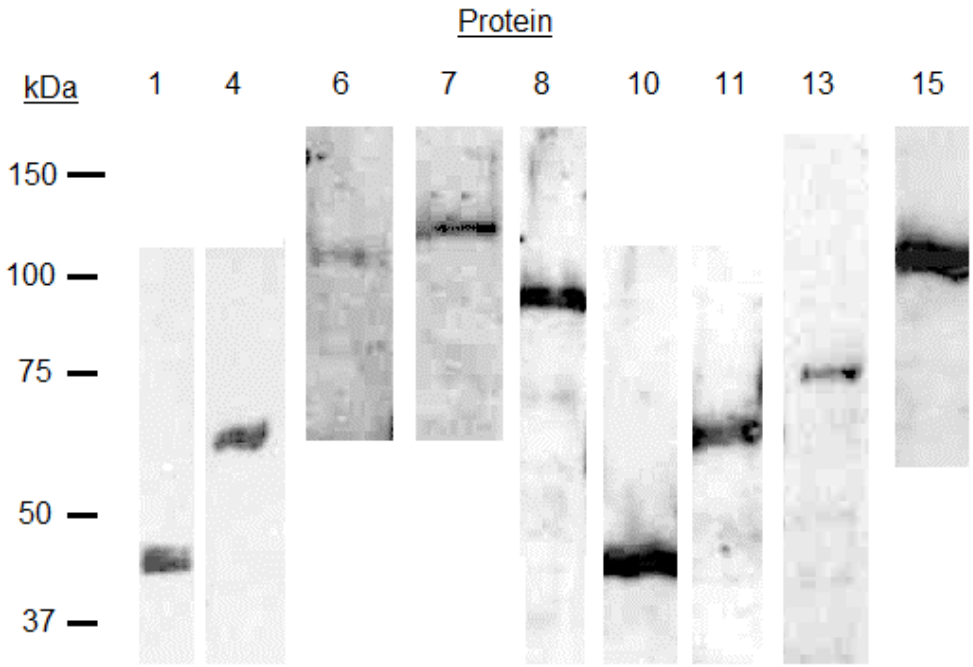

Fig. 3.2.32 Western blot analysis of whole cell lysates.

Lysates obtained from cells around 16 hours after transfection with indicated YFP constructs. Membranes were probed with antibodies against GFP. $2x10^6$ cells were loaded per lane ($4x10^6$ for construct 15).

3.3 TbMORN1-truncations

3.3.1 General experimental strategy

The aim of this project was to identify the targeting sequence of TbMORN1 and define which of these MORN-repeats are essential and which are dispensable for proper targeting. To address these questions TbMORN1 N- and C-terminal truncation constructs (Fig.3.3.1 and Table 3.3-1) were N-terminal YFP-tagged and expressed in T.brucei 427 cells. Recombinant proteins were screened for bilobe localization by immunofluorescence microscopy. Cells and extracted cytoskeletons were fixed with PFA. α-GFP antibodies were used to enhance the YFP signal. Costaining with antibodies against marker proteins, as for the MORN-repeat protein family experiments, were performed if necessary. Localization was also investigated by biochemical fractionation of transfected cells. Equal fractions of input, cytoplasmic supernatant and cytoskeletal pellet were analyzed by Western blotting using α-GFP antibodies. For detection of endogenous TbMORN1 α-MORN1 antibodies were used.

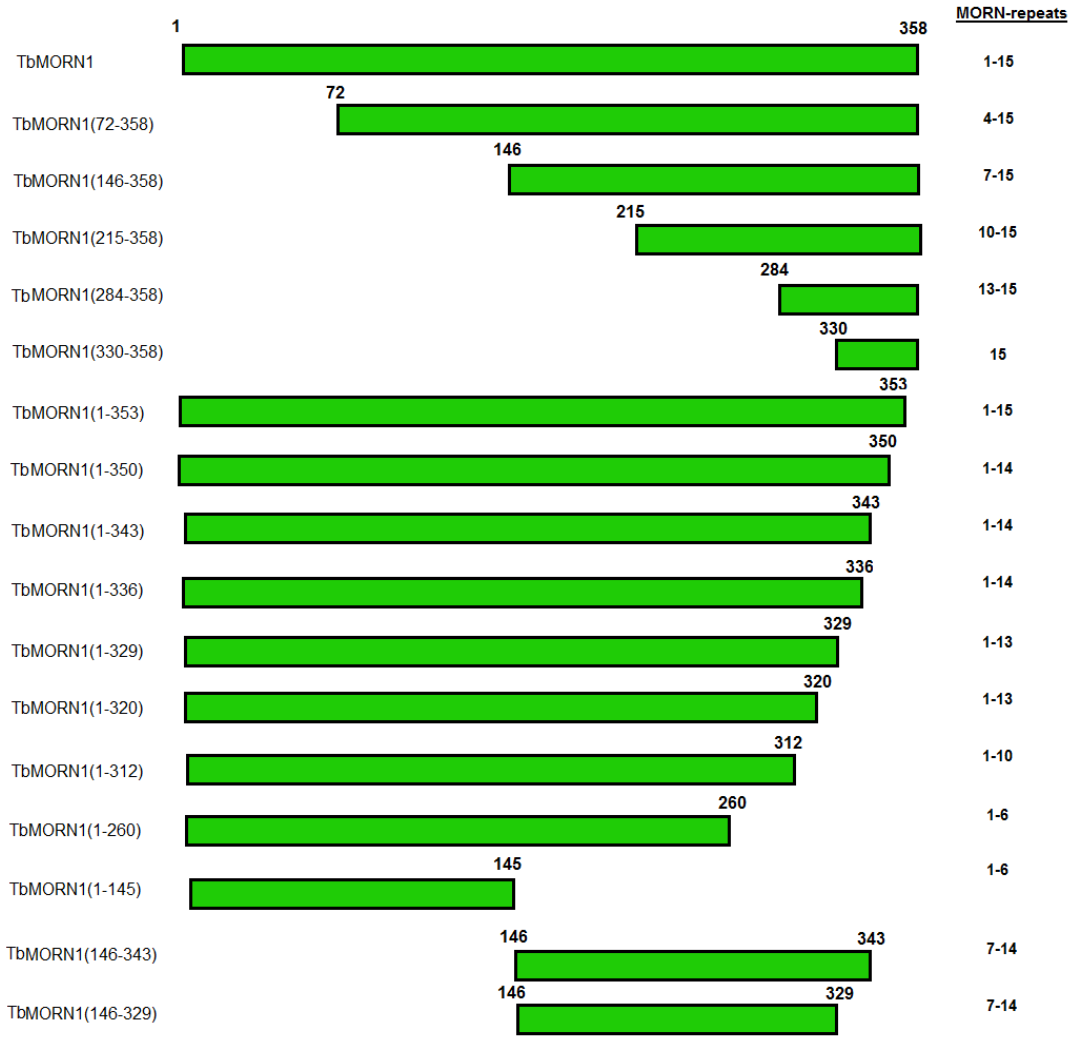

Fig. 3.3.1 Truncation constructs

construct	AA	MORN-repeats
TbMORN1	1-358	1-15
TbMORN1(72-358)	72-358	4-15
TbMORN1(146-358)	146-358	7-15
TbMORN1(215-358)	215-358	10-15
TbMORN1(284-358)	284-358	13-15
TbMORN1(330-358)	330-358	15
TbMORN1(1-353)	1-353	1-15
TbMORN1(1-350)	1-350	1-14
TbMORN1(1-343)	1-343	1-14
TbMORN1(1-336)	1-336	1-14
TbMORN1(1-329)	1-329	1-14

TbMORN1(1-320)	1-320	1-13
TbMORN1(1-312)	1-312	1-13
TbMORN1(1-260)	1-260	1-10
TbMORN1(1-145)	1-145	1-6
TbMORN1(146-343)	146-343	7-14
TbMORN1(146-329)	146-329	7-14

Table 3.3-1 TbMORN1 truncation constructs

3.3.2 TbMORN1

Screening of full length TbMORN1(1-358) by immunofluorescence microscopy showed a hook-shaped structure next the kinetoplast in PFA-fixed cells. For 2K1N cells two of these structures were observed (Fig.3.3.2 A). Additionaly "spear" shaped structures of constant width but varying length were observed (Fig.3.3.2 B). This artefactual paracrystalline structure was previously observed for YFP-tagged TbMORN1 (Morriswood et al., 2009). "Spear" formation was observed in 44% of transfected cells fixed with PFA (data not shown). α-GFP antibodies further recognize a pool of recombinant proteins in the cytosol visible by whole cell labelling (Fig.3.3.2 A). This indicates that the YFP-tagged protein is expressed and present in the cytosol but might also be due to the detection of unfolded tagged protein. Detergent-extracted cytoskeletons showed localization of the YFP fusion proteins at the bilobe (Fig.3.3.2 C) which was confirmed by colocalization with TbLRRP1 in single 0.1µm sections (Fig.3.3.2 D).

Immunoblotting of fractionated cell lysates with α-GFP antibodies detected an approximatly 67kDa protein which confirmed the expression of YFP-TbMORN1(1-358) observed by IF. The YFP-tagged protein was present in all three fractions but a stronger signal from the cytoskeletal fraction than from the cytoplasmic sample was observed (Fig.3.3.3 A). This indicated that YFP-TbMORN1 preferentialy associates with the cytoskeletion. α-MORN1 antibodies detected endogenous TbMORN1 at 41kDa which was targeted to the cytoskeletal fraction (Fig.3.3.3 B). Comparing YFP-TbMORN1 and endogenous TbMORN1, a strong signal for cytoskeletal fractions was observed for both. YFP-TbMORN1 is also found in the supernatant fraction whereas endogenous TbMORN1 is soley in the pellet fraction. This indicates that more of the YFP-tagged version is found in the cytosol compared to the endogenous but overall YFP-tagged proteins are a good model for studing targeting of TbMORN1.

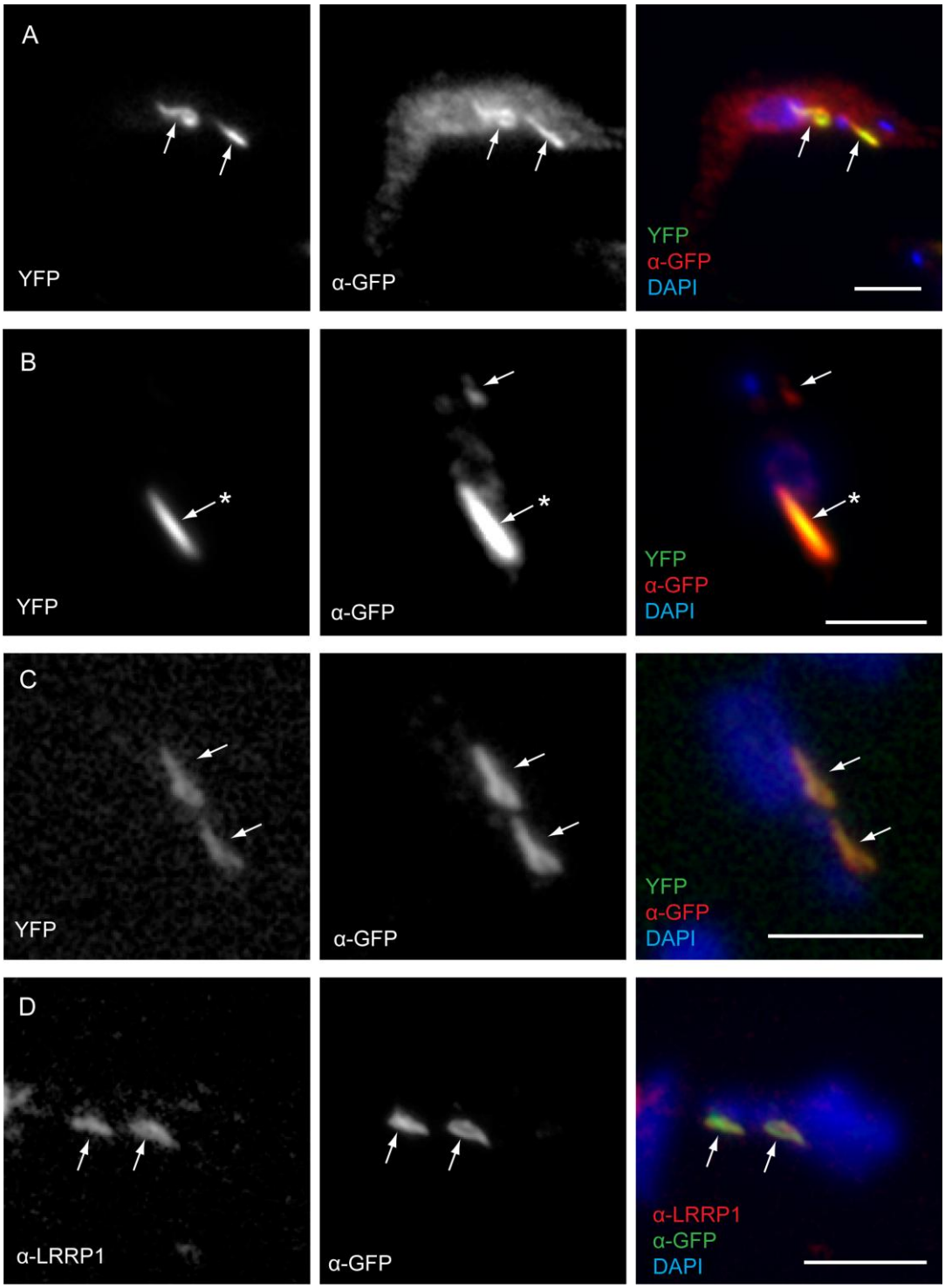

Fig. 3.3.2 Localization of YFP-tagged TbMORN1(1-358).

Transiently transfected procyclic cells were fixed with PFA; α-GFP antibodies were used to enhance the YFP signal (A). PFA-fixed cells showed a hook shaped structure (arrow) next to the kinetoplast and some cytosolic labelling. (B) Additionally artifactual "spear"-shaped structures (*) were observed. (C, D) T. brucei cells were treated with detergent and the extracted cytoskeletons were fixed with PFA (C). Localization

of the YFP fusion protein at a bilobe shaped structure (arrow) was also observed under these conditions. (D) Colocalization with TbLRRP1 in single sections showed localization of YFP-TbMORN1 at the bilobe (arrow). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Bars, 5µm.

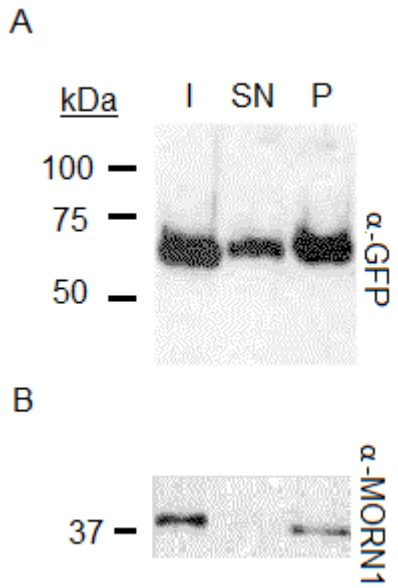

Fig. 3.3.3 Fractionation of YFP-TbMORN1-transfected cells.

Procyclic cells transiently expressing YFP-TbMORN1 were treated with detergent to solubilise cytoplasmic and membrane-associated proteins, and the fractions separated by centrifugation. Equal fractions of Input (I), soluble supernatant (SN) and insoluble pellet (P) were separated by SDS-PAGE and analyzed by western blotting. (A) Samples probed with α-GFP antibodies. The predicted MW of YFP-TbMORN1 is approximately 67 kDa. (B) Samples probed with α-MORN1 antibodies. Endogenous TbMORN1 is 40 kDa. Lanes contain a 1% fraction of each sample.

3.4 N-terminal truncations

3.4.1 TbMORN1(72-358)

This truncated construct was missing the first 213 base pairs coding for 71 amino acids and contains the last 12 MORN repeats (4-15). Sequencing revealed that this construct carries a mutation Y215C which was found in the original template construct and was shown not to affect proper localization at the bilobe (B. Morriswood, personal communication).

T.brucei 427 cells were used to transiently express the truncated construct. In PFA fixed cells the fusion protein was present in the cytosol and on a hook-shaped structure next to the kinetoplast (Fig.3.4.1 A). For MeOH fixation faint cytosolic labelling was observed (data not shown). In detergent extracted cytoskeletons YFP-MORN1(72-358) localized to the bilobe observable by YFP and α-GFP labelling (Fig.3.4.1 B) . Colocalization with TbLRRP1 confirmed that the truncated protein proper targeted to the bilobe (Fig.3.4.1 C).

Western blot analysis revealed that the YFP-tagged protein was found in the input, the supernatant and also the pellet fraction (Fig.3.4.2 A). Compared to the full-length TbMORN1 (Fig.3.3.3 A) less tagged protein was found in the pellet and more in the supernatant. The latter indicates that the truncated version is more present in the cytosol.

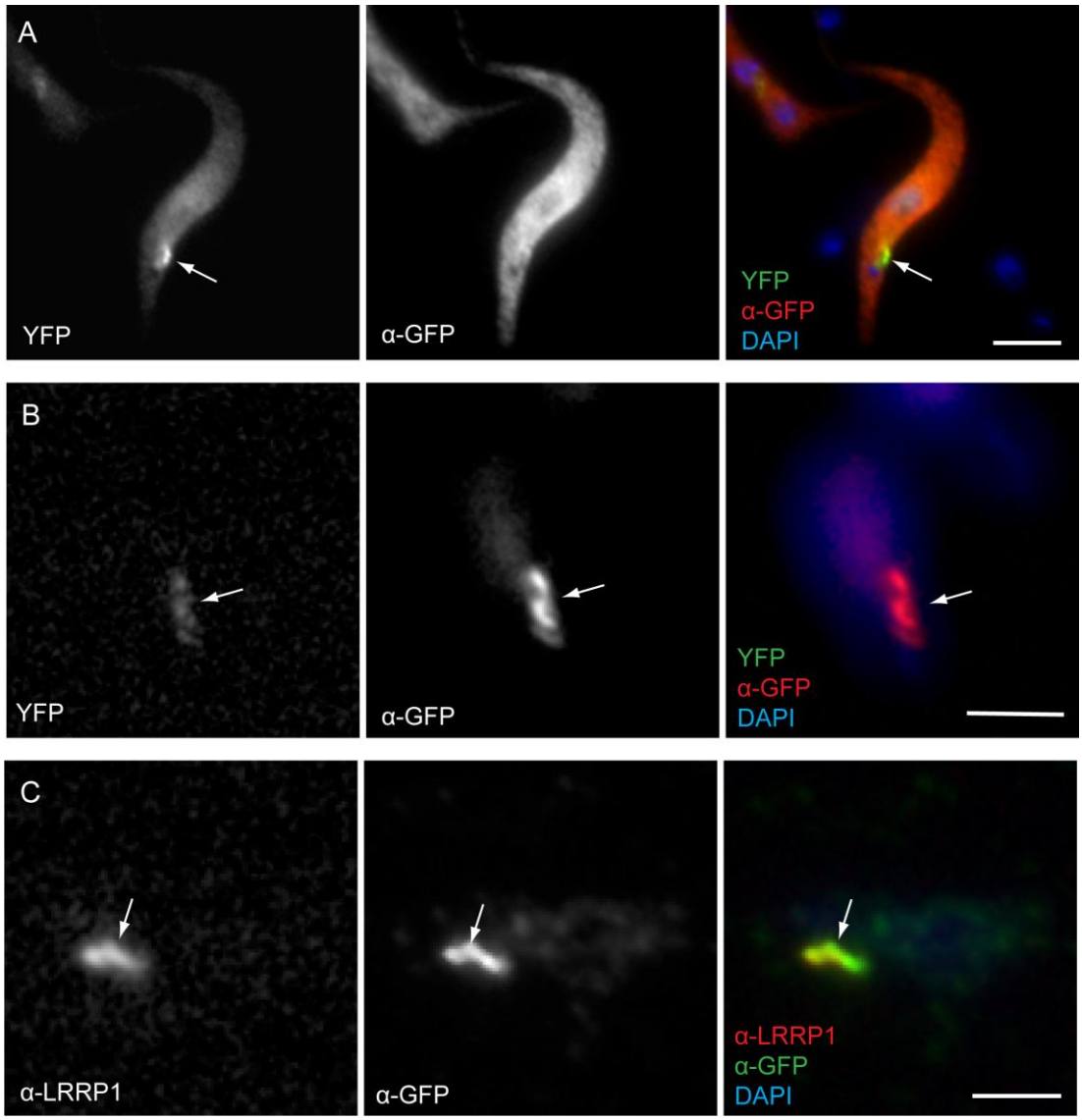

Fig. 3.4.1 Localization of YFP-TbMORN1(72-358).

Procyclic cells were fixed with PFA (A) or extracted with detergent and fixed with PFA (B, C) and analyzed by immunofluorescence microscopy. YFP-TbMORN1(72-358) was present in the cytosol and on a hook-shaped structure (arrow) near the kinetoplast (A). The labelling was preserved in detergent-extracted cytoskeletons (arrow) (B). Colocalization with TbLRRP1 confirmed the presence of YFP-TbMORN1(72-358) at the bilobe (arrow) (C). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Images are representative of 2 independent transfections. Bars, 5µm.

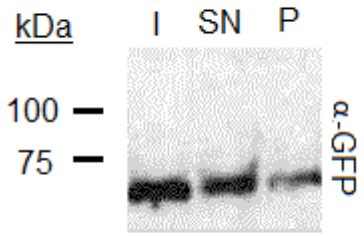

Fig. 3.4.2 Fractionation of cell lysates obtained after transfection of YFP-TbMORN1(72-358).

Procyclic cells transiently expressing YFP-TbMORN1(72-358) were treated with detergent and soluble and insoluble fractions were separated by centrifugation. Equal fractions of input (I), soluble supernant (SN) and insoluble pellet (P) were analyzed by SDS-PAGE and Western blotting. A-GFP antibodies recognize a 59kDa protein. Lanes contain 1% fraction of each sample.

3.4.2 TbMORN1(146-358)

Transient transfection of YFP-TbMORN1(146-358) showed cytosolic labelling and two structures near the kinetoplast in PFA-fixed cells. The shape and size of these structures were reminiscent of the basal bodies and the bilobe (Fig.3.4.3 A). Interestingly the punctated structure colocalized with YL1/2 staining indicating that the truncated protein was present at the basal bodies (Fig.3.4.3 B). In detergent extracted cytoskeletons the signal from the basal bodies was lost and the YFP-tagged protein localized only to the bilobe-shaped structure (Fig.3.4.3 C). Costaining with α-LRRP1 confirmed the presence of YFP-TbMORN1(146-358) at the bilobe (Fig.3.4.3 D).

After biochemical fractionation, the 24kDa protein was found in the soluble supernatant fraction and only very little was present in the insoluble pellet fraction (Fig.3.4.4 A). α-MORN1 antibodies showed that the postive control, the endogenous TbMORN1, was present in the cytoskeletal fraction but not the supernatant (Fig.3.4.4 B).

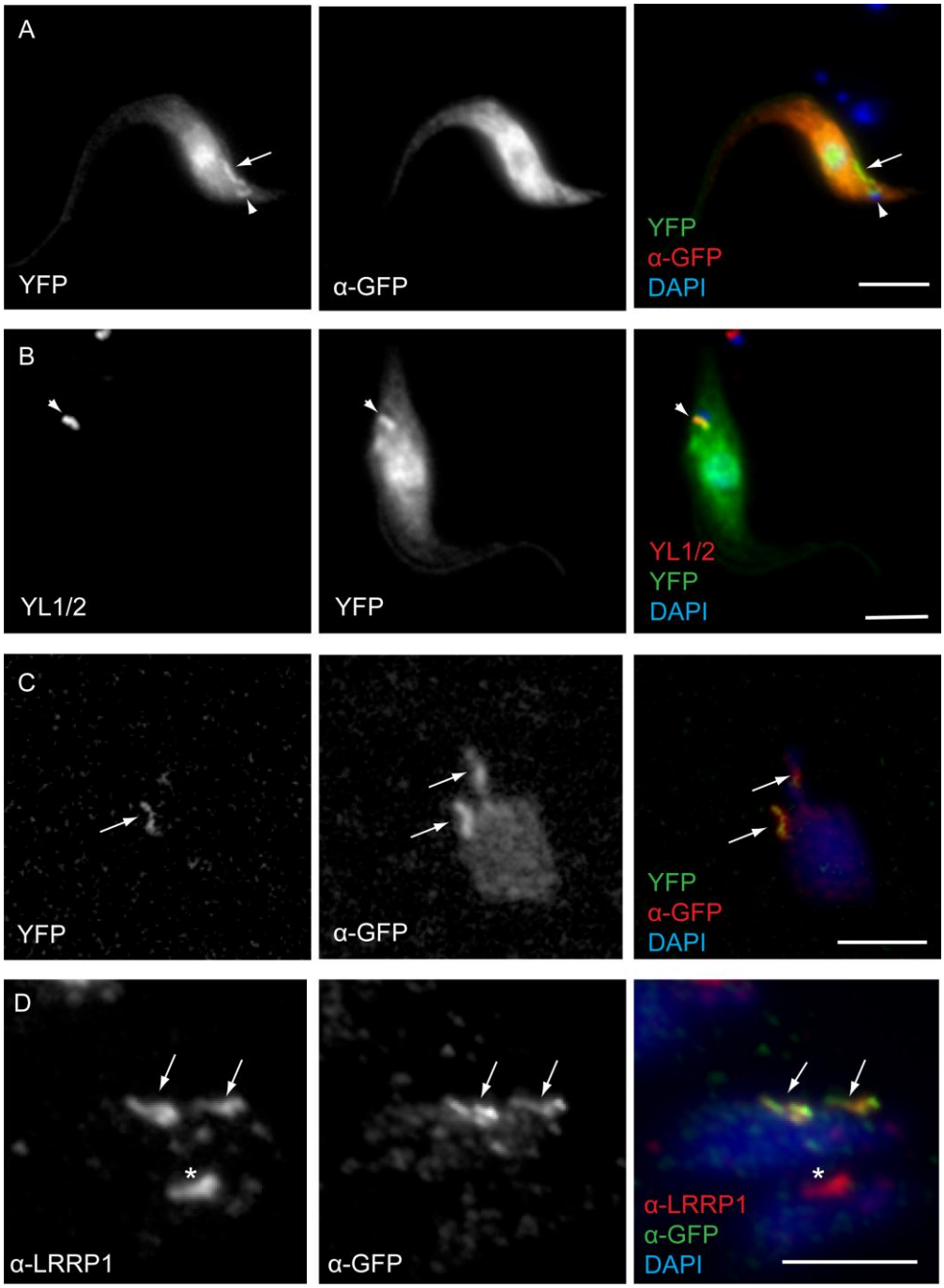

Fig. 3.4.3 Localization of YFP-TbMORN1(146-358).

Procyclic cells transiently expressing YFP-TbMORN1(146-358) were fixed with PFA and analyzed by immunofluorescence microscopy (A). The YFP-tagged protein was present in the cytoplasm as well as localizing to two structures near the kinetoplast. The shape and size of these structures were reminiscent of the basal bodies (arrowhead) and the bilobe (arrow). (B) The presence of the construct at the basal bodies was confirmed by colocalization with YL1/2 labelling (arrowhead). (C) Detergent-extracted cytoskeletons were fixed with PFA and examined by immunofluorescence microscopy. Under these conditions, the signal from the basal bodies was

lost and localization only to the bilobed structure (arrows) was observed. (D) This structure was confirmed to be the bilobe by colocalization with TbLRRP1 (arrows). Bilobe of an untransfected cell (*). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Images are representative of 2 independent transfections. Bars, 5µm.

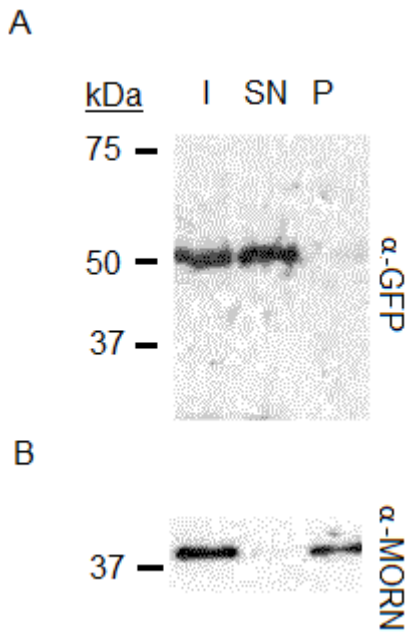

Fig. 3.4.4 YFP-TbMORN1(146-358)-expressing cells were fractionated and examined by Western blot analysis.

Fractions of cell lysates were obtained by detergent extraction and centrifugation. Equal fractions of input (I), soluble supernatant (SN) and insoluble pellet (P) were separated by SDS-PAGE and membranes were treated with α-GFP (A) and α-MORN1 (B) antibodies. (A) YFP-TbMORN1(146-358) has a predicted MW of 51kDa. (B) Samples probed with α-MORN1 recognize endogenous TbMORN1, 40kDa. Lanes contain a 1% fraction of each sample.

3.4.3 TbMORN1(215-358)

Immunofluorescence microscopy of YFP-MORN1(215-358) transfected cells revealed whole cell labelling of a cytosolic pool of fusion protein (Fig.3.4.5 A). The YFP-tagged protein furthermore localized to two structures near the kinetoplast (Fig.3.4.5 A). One was basal body localization as it was shown by overlap with YL1/2 (Fig.3.4.5 B) The second structure was the bilobe, as confirmed by colocalizing with α-LRRP1 antibodies (Fig.3.4.5 C).

Expression of the YFP-tagged protein was confirmed by western blot analysis as the α-GFP antibodies detected an approximately 43kDa protein. This band was present in all three fractions which proves that the truncated protein was targeted to the cytoskeleton (Fig.3.4.6).

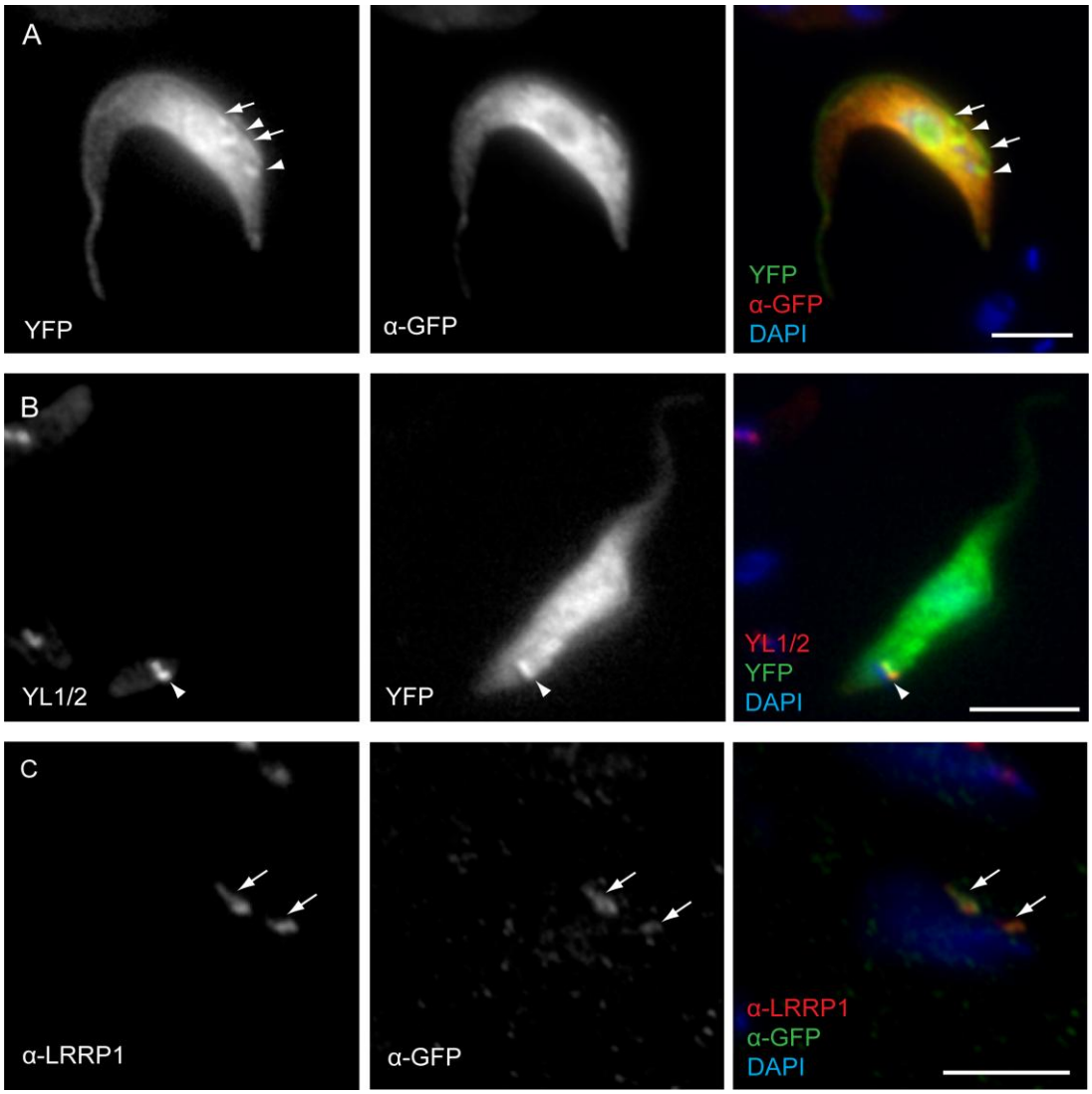

Fig. 3.4.5 Localization of YFP-TbMORN1(215-358).

Procyclic cells expressing YFP-TbMORN1(215-358) showed cytosolic labelling with α-GFP antibodies after PFA fixation. Furthermore labelling of two structures near the kinetoplast reminiscent of the basal body (arrowheads) and bilobe (arrows) was observed in PFA-fixed cells (A). The presence of YFP-TbMORN1(215-358) at the basal body (arrowhead) was confirmed using costaining with YL1/2 antibodies (B). Detergent-extracted cells were fixed with PFA and stained with α-LRRP1 and α-GFP antibodies. YFP-TbMORN1(215-358) colocalized with TbLRRP1 (arrows) (C). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Images are representative of 2 independent transfections. Bars, 5µm.

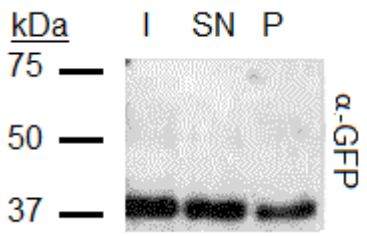

Fig. 3.4.6 Western blot analysis of fractionated YFP-TbMORN1(215-358)-transfected cell lysates.

Procyclic cells were transfected with YFP-TbMORN1(215-358) and lysates extracted with detergent and separated by centrifugation. This resulted in three fractions input (I), soluble supernatant (SN) and insoluble pellet (P). Equal fractions were examined by SDS-PAGE and Western blotting. Samples probed with α-GFP. The truncated protein is 43kDa. Lanes contain a 1% fraction of each sample.

3.4.4 TbMORN1(284-358)

Observation of T.brucei 427 cells transfected with YFP-TbMORN1(284-358) and fixed with PFA showed that the protein was present in the cytosol (Fig.3.4.7 A) and additionally on a structure next the kinetoplast which overlaps with YL1/2 labelling (Fig.3.4.7 B). In contrast to other constructs no signal from the bilobe was observed in PFA or Xtr+PFA fixed cells (data not shown). This suggested that the truncated protein was no longer targeted to the bilobe.

Western blot of fractionated cell lysates showed that YFP-TbMORN1(284-358) was expressed and present in the input, the supernatant and very little in the pellet fraction. This means that the tagged protein mainly targeted to the cytoplasm and that very little was present at the cytoskeleton (Fig.3.4.8 A). Using α-MORN1 antibodies the endogenous TbMORN1 protein, 40kDa, was recognized. As expected this protein was found in the cytoskeletal but not cytoplasmic fraction (Fig.3.4.8 B).

As previously observed the signal from the bilobe got weaker after extraction, especially if few cells were transfected there is a chance to miss the signal. In order to make sure that YFP-TbMORN1(284-358) did not target to the bilobe and that we might not see the signal we tried to create a stable cell line. Unfortunately after two rounds of transfection and plating the only clone obtained was positive for presence

of the blasticidin gene but negative for the YFP construct. This implies that cells must have picked up the selection gene but not the YFP construct.

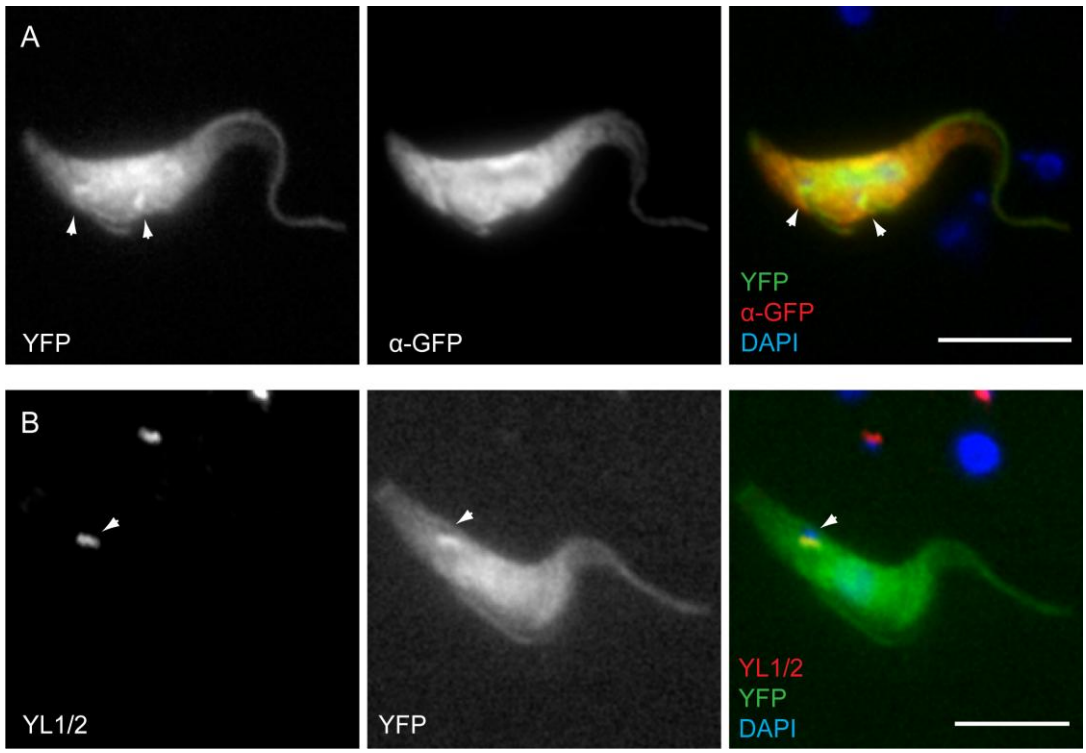

Fig. 3.4.7 Localization of YFP-TbMORN1(284-358).

The YFP tagged protein was present in the cytosol (A) as well as on a structure (arrowheads) next to the kinetoplast which colocalized with YL1/2 labelling (arrowhead) (B). α-GFP antibodies showed cytosolic labelling. Nuclear and kinetoplast DNA were labelled with DAPI. Images are representative of 3 independent transfections. Bars, 5µm.

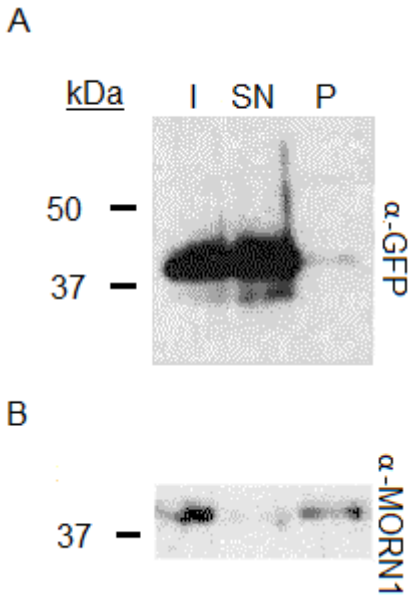

Fig. 3.4.8 Fractionation of YFP-TbMORN1(284-358)-expressing cells.

Procyclic cells transiently expressing YFP-TbMORN1(284-358) were treated with extraction buffer and separated into soluble and insoluble fractions by centrifugation. Equal fractions of input (I), supernatant (SN) and pellet (P) were examined by SDS-PAGE and Western blotting. (A) α-GFP antibodies recognize the 38kDa YFP-tagged protein. (B) Membrane treated with α-MORN1 antibodies showed the 40kDa endogenous TbMORN1. Lanes contain a 1% fraction of each sample.

3.4.5 TbMORN1(330-358)

Immunofluorescence microscopy reveals that the fluorescent protein was present in the cytosol and on a structure next the kinetoplast (Fig.3.4.9). The shape and size of this structure are reminiscent of basal body localization.

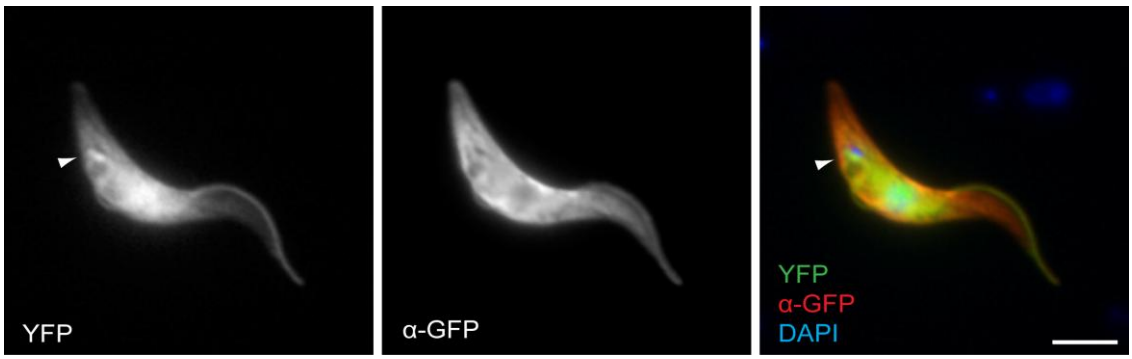

Fig. 3.4.9 Localization of YFP-TbMORN1(330-358).

Procyclic cells were fixed with PFA and labelled with α-GFP antibodies. Expression of the YFP-tagged protein in the cytoplasm as well as its localization to a structure (arrowhead) next the kinetoplast was observed. Nuclear and kinetoplast DNA were labelled with DAPI. Bar, 5µm.

The 30kDa protein was present in the input and the supernatant fraction but absent in the pellet fraction as it was shown by Western blot analysis (Fig.3.4.10 A). Figure 3.4.10 B shows the positive control endogenous TbMORN1.

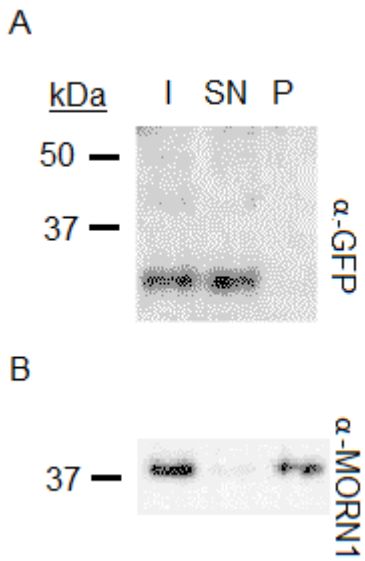

Fig. 3.4.10 Analysis of fractionated cell lysates after expression of YFP-TbMORN1(330-358).

Procyclic cells transiently expressing YFP-TbMORN1(330-358) were treated with detergent and the lysates separated by centrifugation. Input (I), supernatant (SN) and pellet (P) samples were loaded in equal fractions on polyacrylamide gels and separated by SDS-PAGE. Western blotting with α-GFP antibodies showed the 30kDa YFP-tagged protein (A). (B) Endogenous TbMORN1, 40kDa, was recognized by α-TbMORN1 antibodies. Lanes contain a 1% fraction of each sample.

3.5 C-terminal truncations

3.5.1 TbMORN1(1-353)

PFA fixation of YFP-TbMORN1(1-353) transfected cells showed some nuclear labelling and a very faint bilobe-shaped structure (Fig.3.5.1 A). This structure overlaped with α-LRRP1 labelling (Fig.3.5.1 B). Therefore this truncated construct was present at the bilobe although it seemed to be expressed at a lower level than other constructs. This might also be an explanation why spear formation was not observed.

On Western blot no expression of YFP-TbMORN1(1-353) could be detected despite multiple attempts (data not shown).

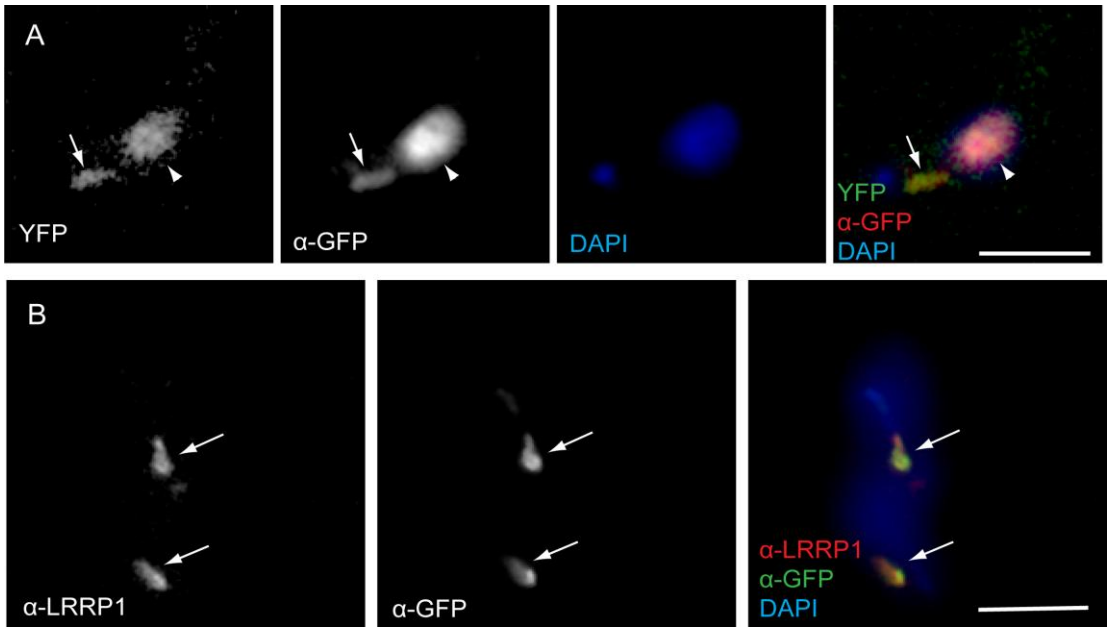

Fig. 3.5.1 Localization of YFP-TbMORN1(1-353).

Immunofluorescence microscopy images of procyclic cells transiently expressing YFP-TbMORN1(1-353). In PFA-fixed Trypanosomes nuclear labelling (arrowhead) and a very faint signal from a bilobe-shaped structure (arrow) were observed (A). In detergent extracted cells fixed with PFA, α-GFP antibodies recognized a hook-shaped structure which colocalized with α-LRRP1 labelling (arrows) (B). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Bars, 5µm.

3.5.2 TbMORN1(1-350)

Trypanosomes transiently expressing YFP-TbMORN1(1-350) were fixed with PFA and MeOH (data not shown). The truncated protein was observed in the cytosol and on the basal body and the bilobe (Fig.3.5.2 A). Additionally the YFP protein was present on a spear shaped structure of fixed width but random length (Fig.3.5.2 A and B). This paracrystalline structure was artefactual and was also observed for the full length TbMORN1. For PFA-fixed cells this spear formation was detected in 53% of cells (data not shown). Upon MeOH fixation or extraction and PFA fixation the recombinant protein was only present at the bilobe (Fig.3.5.2 C). This localization was confirmed by costaining with α-LRRP1 (Fig.3.5.2 D).

The 66kDa protein was recognized by α-GFP antibodies on Western blot analysis. The protein was present in all three fraction (Fig.3.5.3).

YFP-TbMORN1(1-350) localized to the basal bodies and the bilobe as it was shown by fluorescence microscopy. Fractionation backed up this idea since the YFP tagged protein was present in cytoplasmic as well as in cytoskeletal fraction.

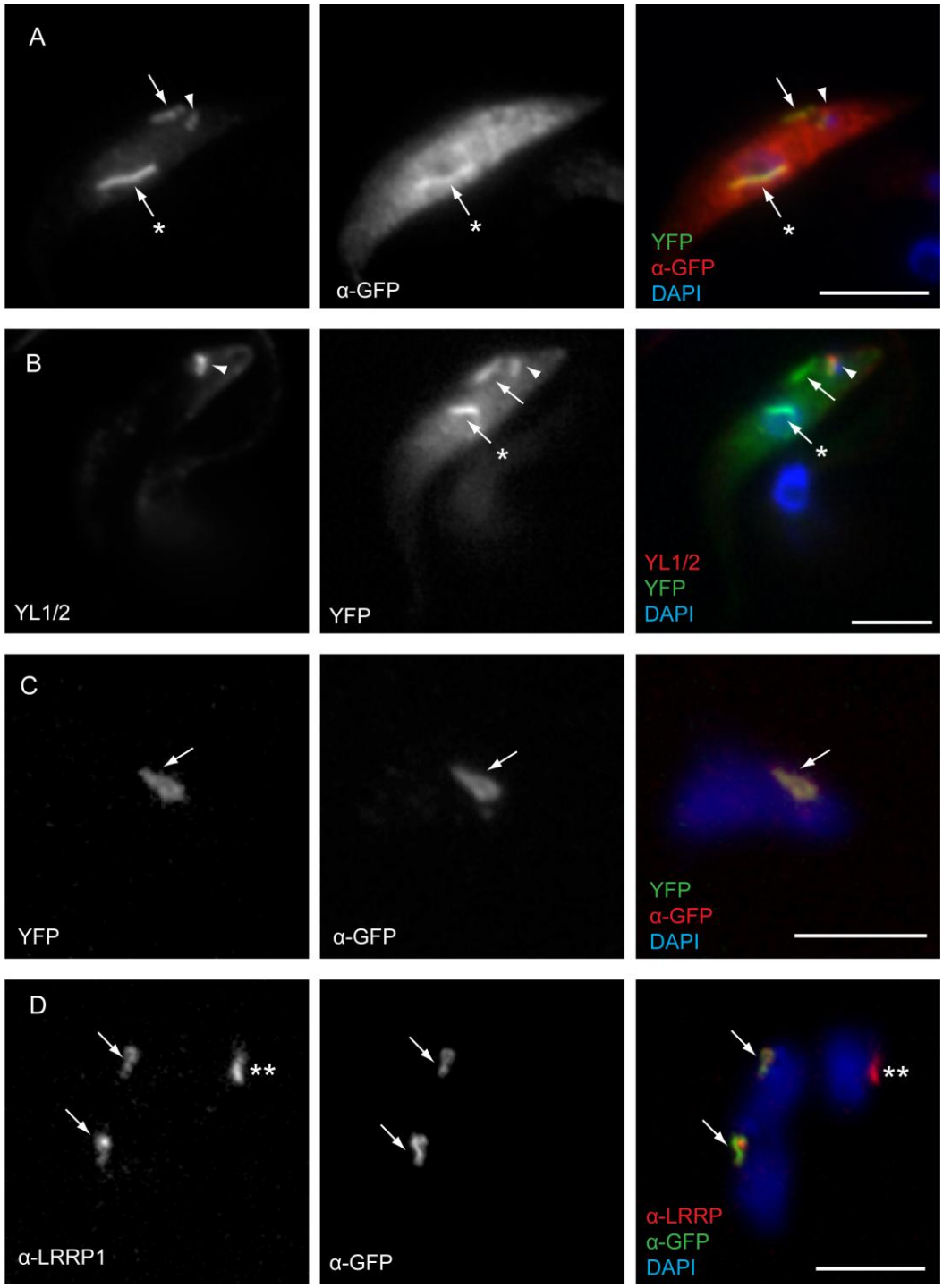

Fig. 3.5.2 Localization of YFP-TbMORN1(1-350).

Procyclic cells fixed with PFA and treated with α-GFP antibodies were examined by immunofluorescence microscopy. Upon PFA fixation YFP signal was observed in

the cytosol and on two structures near the kinetoplast, suggesting basal body (arrowhead) and bilobe (arrow) labelling (A). Colocalization with YL1/2 confirmed that the construct was at the basal bodies (arrowhead) (B). Additionally YFP was present on a "spear" shaped structure (*). Transfected cells were extracted with 0.5% NP40 and fixed with PFA (C,D). A-GFP antibodies recognized a hook shaped structure (arrows) (C) which colocalized with α-LRRP1 labelling (arrows) (D). Bilobe of an untransfected cell (**). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Images are representative of 2 independent transfections. Bars, 5µm.

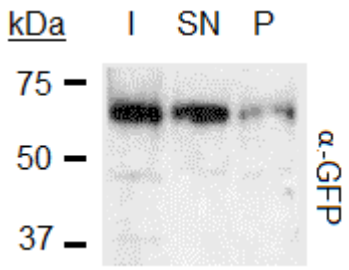

Fig. 3.5.3 Fractionated cell lysates of YFP-TbMORN1(1-350) transfected cells.

Input (I), supernatant (SN) and pellet (P) samples were obtained after detergent extraction and centrifugation of YFP-TbMORN1(1-350) expressing cells. Equal fractions were examined by SDS-PAGE and Western blotting. (A) A-GFP antibodies recognize the 66kDa YFP-tagged protein. Lanes contain a 1% fraction of each sample.

3.5.3 TbMORN1(1-343)

Immunofluorescence microscopy of YFP-TbMORN1(1-343) transfected cells showed cytosolic labelling by α-GFP antibodies. The YFP construct was also found at two structures near the kinetoplast suggesting basal body and bilobe localization (Fig.3.5.4 A). By costaining with YL1/2 antibodies the basal body localization could be confirmed (Fig.3.5.4 B). In detergent extracted cytoskeletons in contrast no signal from the bilobe could be observed (data not shown).

After transfection with YFP-TbMORN1(1-343) fractionated cell lysates were obtained for western blot analysis. α-GFP antibodies recognized an approximately 66kDa protein in the input and cytoplasmic samples (Fig.3.5.5 A). This suggested that the YFP tagged protein stayed in the cytosol and was not transported to the cy-

toskeleton. The endogenous TbMORN1, recognized by α-MORN1 antibodies, in contrast was targeted to the cytoskeletal fraction (Fig.3.5.5 B).

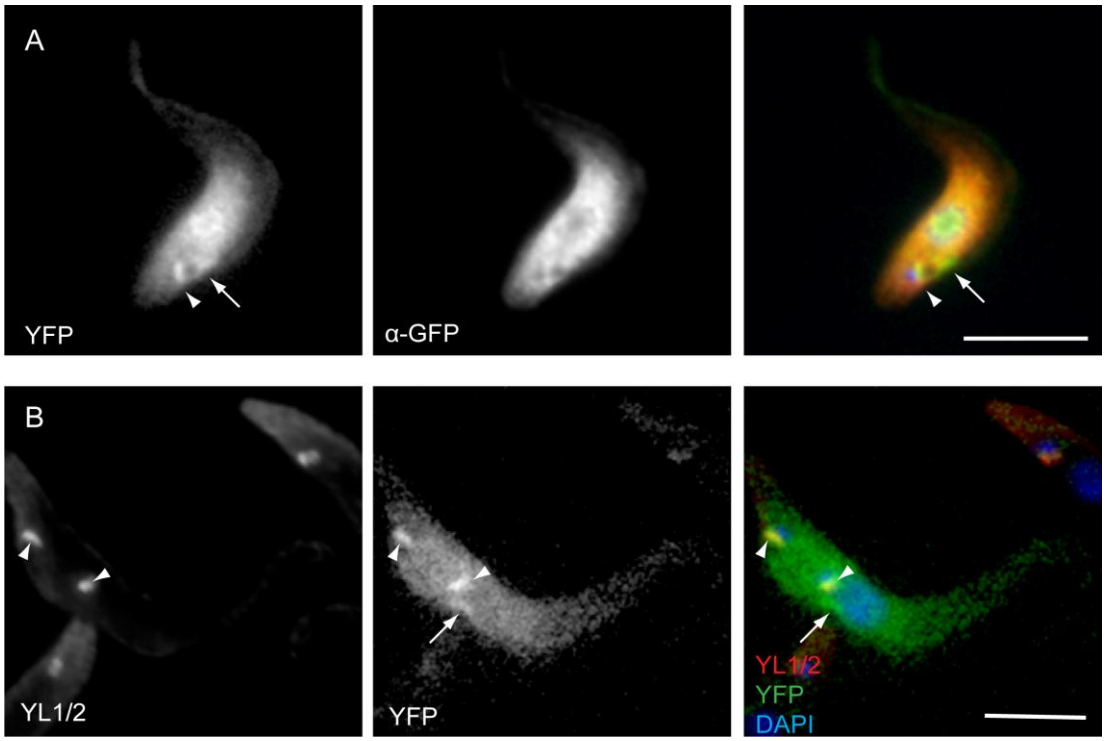

Fig. 3.5.4 Localization of YFP-TbMORN1(1-343).

The YFP-tagged protein was present in the cytosol as well as on 2 structures near the kinetoplast. Size and shape suggested basal bodies (arrowhead) and bilobe (arrow) labelling (A). Colocalization with YL1/2 antibodies confirmed presence of YFP-TbMORN1(1-343) at the basal bodies (arrowhead) (B). Transiently transfected cells were fixed with PFA. Nuclear and kinetoplast DNA were labelled with DAPI. Images are representative for 2 independent transfections. Bars, 5µm.

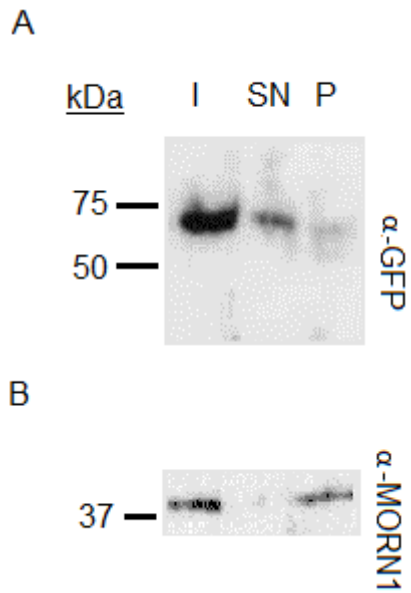

Fig. 3.5.5 Fractionated cell lysates of YFP-TbMORN1(1-343) transfected cells.

In order to solubilise cytoplasmic and membrane-associated proteins YFP-TbMORN1(1-343)-transfected cells were treated with extraction buffer. Centrifugation of lysates then lead to three fractions: input (I), supernatant (SN) containing soluble proteins and pellet (P) containing insoluble proteins. Equal fractions were analyzed by SDS-PAGE and Western blotting. (A) Samples probed with α-GFP. The recombinant protein is 66kDa. (B) The membrane was treated with α-TbMORN1 antibodies. Endogenous TbMORN1 is 40kDa. Lanes contain a 1% fraction of each sample.

3.5.4 TbMORN1(1-336)

Trypanosomes transfected with YFP-TbMORN1(1-336) and fixed with PFA showed a cytosolic labelling and also on a structure next to the kinetoplast (Fig.3.5.6 A). By using YL1/2 antibodies its localization at the basal bodies was confirmed (Fig.3.5.6 B). Detergent extracted cytoskeletons revealed that the truncated protein was present at the bilobe and colocalized with α-LRRP1 antibodies (Fig.3.5.6 C).

The 38kDa protein was present in all three fractions, with less in the cytoskeletal sample (Fig.3.5.7). This showed that the fusion protein mainly stays in the cytosol and that little was targeted to cytoskeletal proteins.

Immunofluorescence microscopy images showed that the YFP-TbMORN1(1-336) construct was present in the cytosol, at the basal bodies and at the bilobe. By Western blotting it was shown that the tagged protein was targeted to both fractions but mainly to the cytoplasmic fraction.

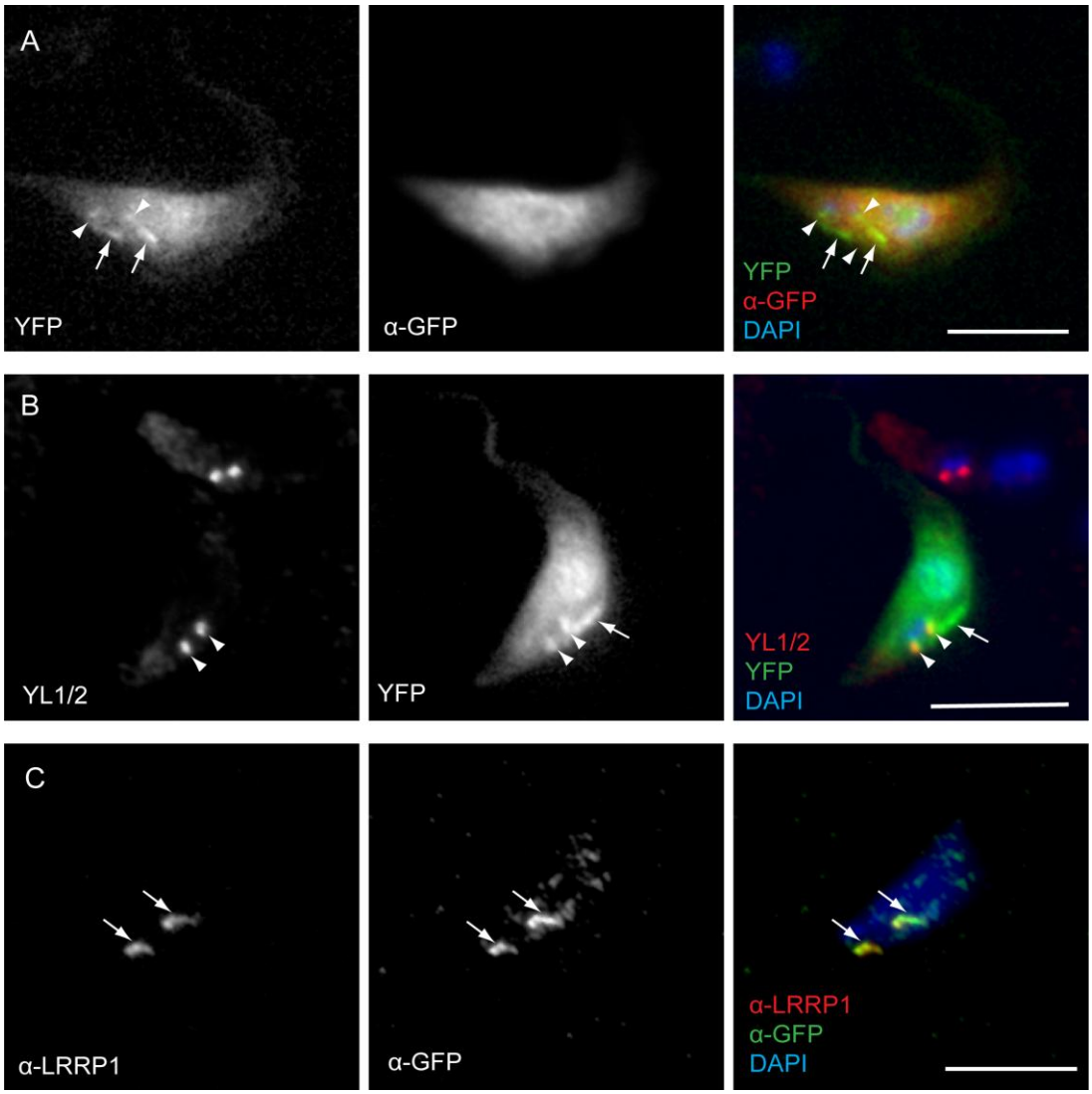

Fig. 3.5.6 Localization of YFP-TbMORN1(1-336).

(A) Procyclic Trypanosomes were transiently transfected, fixed with PFA, and labelled with antibodies against GFP. The YFP signal revealed cytosolic labelling and some additional signals which might come from the basal bodies (arrowheads) and the bilobe (arrows). α-GFP antibodies showed uniform cytosolic staining. (B) Using YL1/2 antibodies colocalization of YFP-tagged protein with the basal bodies (arrowheads) was observed. (C) In detergent extracted cytoskeletons the structure recognized by α-GFP antibodies colocalized with α-LRRP1 labelling (arrows). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Images are representative of 2 independent transfections. Bars, 5µm.

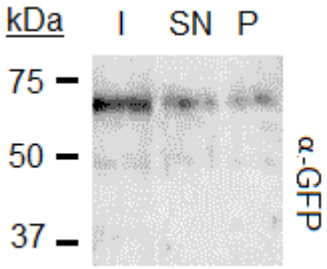

Fig. 3.5.7 YFP-TbMORN1(1-336) fractionated cell lysates.

Trypanosomes transiently expressing YFP-TbMORN1(1-336) were treated with extraction buffer to solubilise cytoplasmic and membrane associated proteins. Samples of input (I), supernatant (SN) representing the cytoplasmic fraction and pellet (P) representing the cytoskeletal proteins were loaded in equal fractions on a gel. After SDS-PAGE proteins were transferred onto a nitrocellulose membrane and analyzed by Western blotting. Membrane treated with antibodies against GFP. The YFP-tagged protein is 65 kDa. Lanes contain a 1% fraction of each sample.

3.5.5 TbMORN1(1-329)

In transient transfection experiments YFP-TbMORN1(1-329) localized to the cytosol, to the basal body, and some structure near the kinetoplast (Fig.3.5.8 A). Basal body localization was confirmed by colocalization with YL1/2 (Fig.3.5.8 B). Surprisingly no signal from any of these structures was observed in detergent extracted cells (data not shown).

Fractionated cell lysates analyzed by Western blot showed that the recombinant protein was expressed and present in all three fractions (Fig.3.5.9). So according to blotting the protein was targeted to the cytoplasm and the cytoskeleton.

Since there was a chance of missing bilobe labelling after extraction and to correct for low transfection efficiency we created a stable cells line with YFP-TbMORN1(1-329). Unfortunately the one clone obtained did not express the YFP-tagged protein as determined by immunofuorescence and western blotting. Again the PCR for the blasticidin resistance gene was positive which implies that cells had acquired that but not the target DNA.

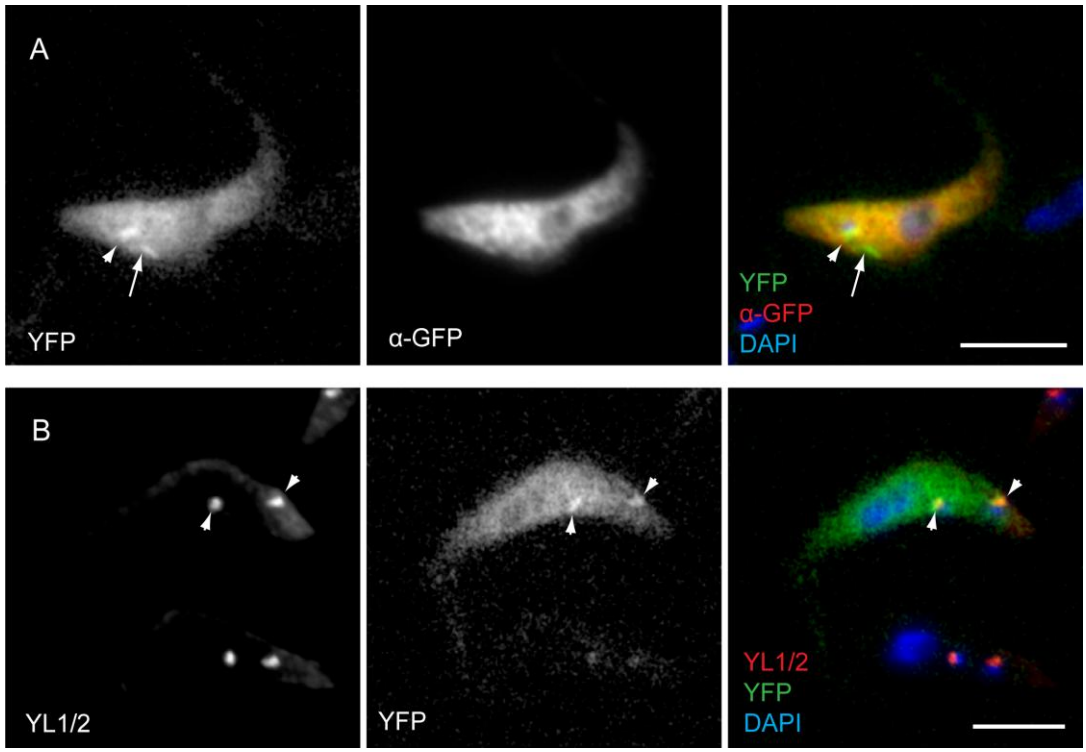

Fig. 3.5.8 Localization of YFP-TbMORN1(1-329).

Procyclic Trypanosomes were fixed with PFA, and α-GFP antibodies were used to enhance the YFP signal. (A) A-GFP antibodies showed cytosolic staining. The YFP-tagged protein localized to the cytoplasm and a structure (arrow) near the kinetoplast and a structure (arrowhead) which overlapped with YL1/2 antibodies (arrowheads) (B). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Immunofluorescence microscopy images are representative of 3 independent transfections. Bars, 5µm.

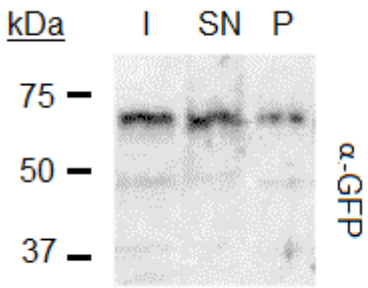

Fig. 3.5.9 Fractionation of YFP-TbMORN1(1-329)-expressing cells.

Procyclic cells were transiently transfected with YFP-TbMORN1(1-329). Cell fractions were obtained by centrifugation after treatment with detergent. Fractions of input (I), soluble proteins present in the supernatant (SN) and insoluble proteins in the pellet (P) were loaded in equal amounts for SDS-PAGE. Immunoblotting was performed with α-GFP antibodies. The truncated protein, 64

kDa, was expressed. Lanes contain a 1% fraction of each sample.

3.5.6 TbMORN1(1-320)

After fixation with PFA the tagged protein was observed in the cytosol and at two structures (Fig.3.5.10 A). YL1/2 treatment showed that the signal next to the kinetoplast came from the basal bodies (Fig.3.5.10 B). The second, fork-shaped structure was the bilobe as shown by costaining with α-LRRP1 antibodies (Fig.3.5.10 C). In MeOH-fixed cells no signal could be detected (data not shown).

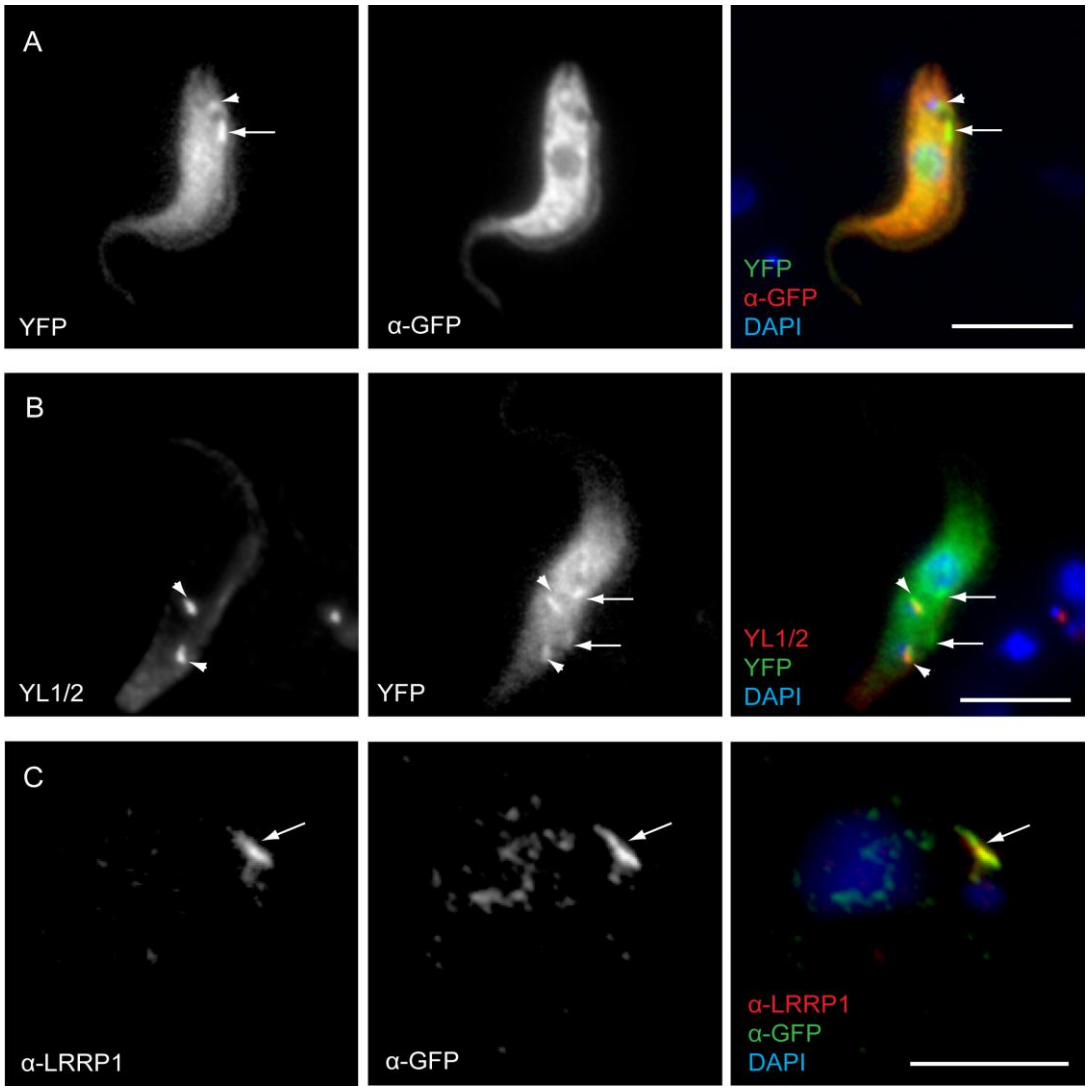

Fig. 3.5.10 Localization of YFP-TbMORN1(1-320).

Procyclic Trypanosomes were stained with α-GFP antibodies (A) or YL1/2 antibodies (B) after fixation with PFA. For the YFP-tagged protein cytosolic labelling and additionally two structures (arrows and arrowheads) near the kinetoplast were observed. Merged pictures with YL1/2 showed that one of these structures represented the basal bodies (arrowheads). (C) Detergent extracted cytoskeletons revealed localization of YFP-TbMORN1(1-320) at the bilobe (arrow) which was confirmed by colocalization with α-LRRP1 labelling. Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Images represent data from two independent transfections. Bars, 5µm.

Western blot analysis revealed that the fusion protein, 63kDa, was expressed and present in the input, the cytoplasmic and very little in the cytoskeletal fraction (Fig.3.5.11 A). The endogenous TbMORN1 in contrast was found in the input and in the pellet fraction (Fig.3.5.11 B).

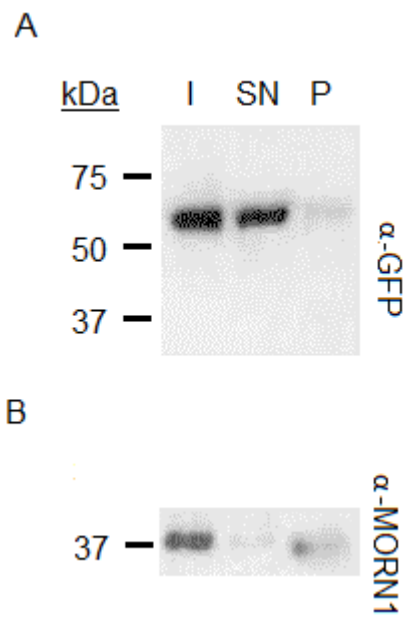

Fig. 3.5.11 Western blot analysis of fractionated cell lysates obtained after transfection with YFP-TbMORN1(1-320).

Fractionated lysates were obtained by centrifugation after extraction with detergent. Equal fractions of input (I), soluble supernatant (SN) and insoluble pellet (P) were analyzed by SDS-PAGE and Western blotting. (A) α-GFP antibodies recognized a 63kDa band which confirmed the expression of the YFP-tagged protein. (B) The endogenous TbMORN1, 40kDa, was recognized by α-TbMORN1 antibodies. Lanes contain a 1% fraction of each sample.

3.5.7 TbMORN1(1-312)

YFP-TbMORN1(1-312) localized to the bilobe. The protein was found in the cytosol, at the basal bodies, and at the bilobe (Fig.3.5.12 A). These localizations were

confirmed by costaining with YL1/2 (Fig.3.5.12 B) and TbLRRP1 (Fig.3.5.12 C). In MeOH fixed cells no signal was detected (data not shown).

The 63kDa protein was found in the input, cytoplasmic and very little in the cytskeletal fraction (Fig.3.5.13 A). Therefore the YFP tagged protein mainly stayed in the cytosol. Additionaly we performed Western blot analysis with α-MORN1 antibodies which revealed that the endogenous MORN1 was present in the input and the pellet fraction (Fig.3.5.13 B).

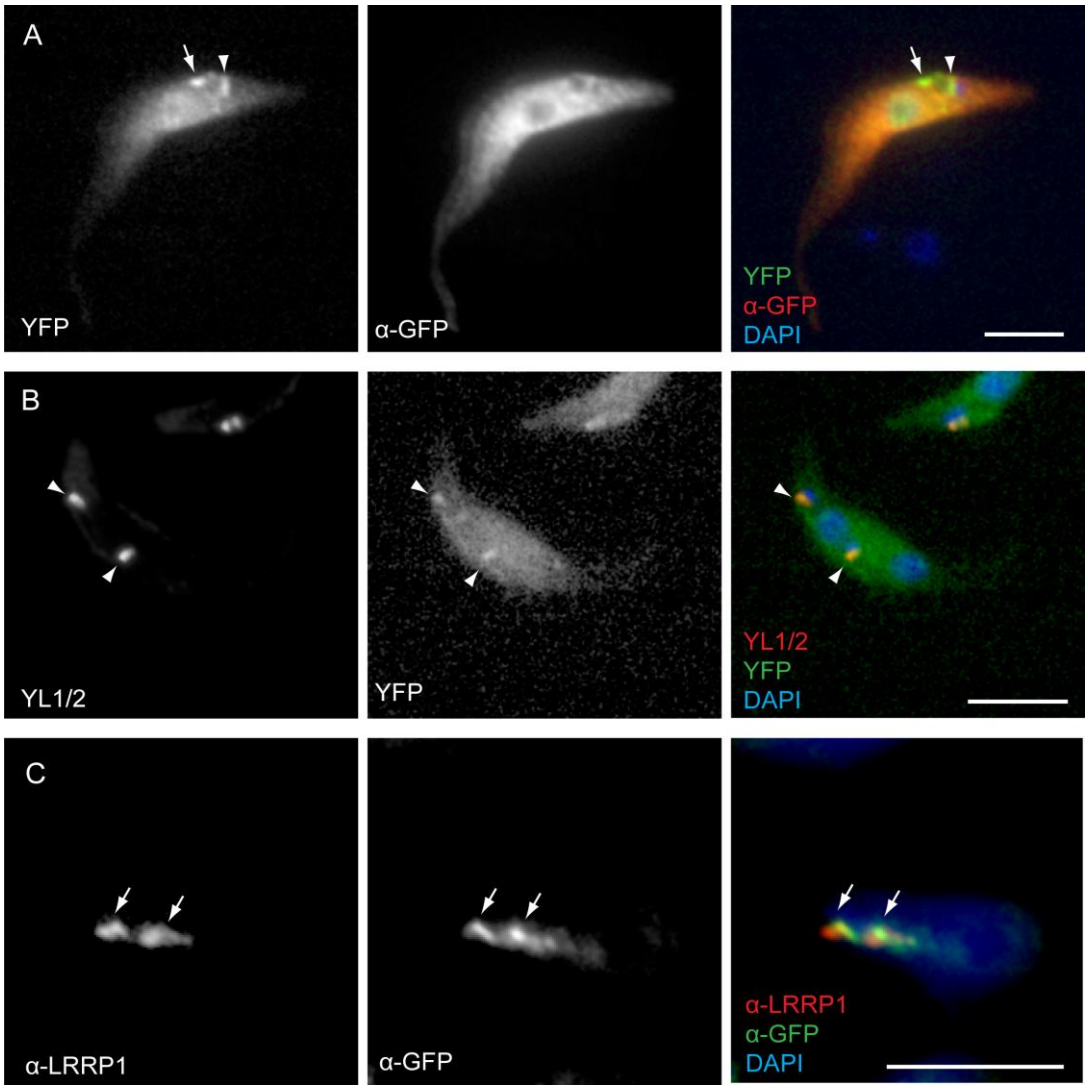

Fig. 3.5.12 Localization of YFP-TbMORN1(1-312).

(A) Procyclic Trypanosomes expressing YFP-TbMORN1(1-312) were fixed with PFA and stained with α-GFP antibodies. The YFP-tagged protein was found in the cytosol and at two structures near the kinetoplast reminiscent of basal body (arrow-

head) and bilobe (arrow) labelling. α-GFP showed cytoplasmic staining. (B) Costaining with YL1/2 revealed that the punctate structure next to the kinetoplast (arrowheads) was the basal body. (C) Cells treated with extraction buffer and fixed with PFA showed colocalization of YFP-MORN1(1-312) with α-LRRP1 labelling (arrows). Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Images are representative of 2 independent transfections. Bars, 5µm.

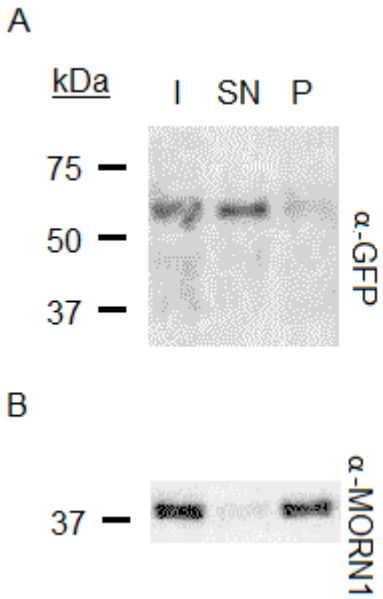

Fig. 3.5.13 Immunoblotting of YFP-TbMORN1(1-312)- transfected cells.
Fractionated cell lysates were obtained by centrifugation after solubilization of cytoplasmic and membrane-associated proteins. Three fractions- input (I), supernatant (SN) containing cytoplasmic proteins, and pellet (P) containing cytoskeletal proteins were loaded in equal fractions for SDS-PAGE. Western blotting with α-GFP (A) and α-MORN1 (B) antibodies was performed. (A) The expected size of the truncated protein is 63kDa. (B) The endogenous MORN1 protein, 40kDa, appears after treatment with α-MORN1 antibodies. Lanes contain a 1% fraction of each sample.

3.5.8 TbMORN1(1-260)

YFP-TbMORN1(1-260) was present in the cytosol and on two faint structures next to the kinetoplast (Fig.3.5.14 A). Costaining with YL1/2 confirmed that one of these structures was the basal body (Fig.3.5.14 B). MeOH-fixed cells showed uniform cytosolic labelling which might be due to the detection of a cytosolic pool of recombinant protein (data not shown). In detergent extracted cytoskeletons the YFP-tagged protein was observed at the bilobe and colocalized with α-LRRP1 labelling (Fig.3.5.14 C).

Expression of the YFP-tagged protein was confirmed by Western blot analysis where α-GFP antibodies detected a 56kDa large protein (Fig.3.5.15 A). Furthermore the fusion protein mainly stays in the cytoplasm and only very little was targeted to the cytoskeleton as was shown by the fractionation experiment. The endogenous MORN1 recognized by α-MORN1 antibodies targeted to the cytoskeleton (Fig.3.5.15 B).

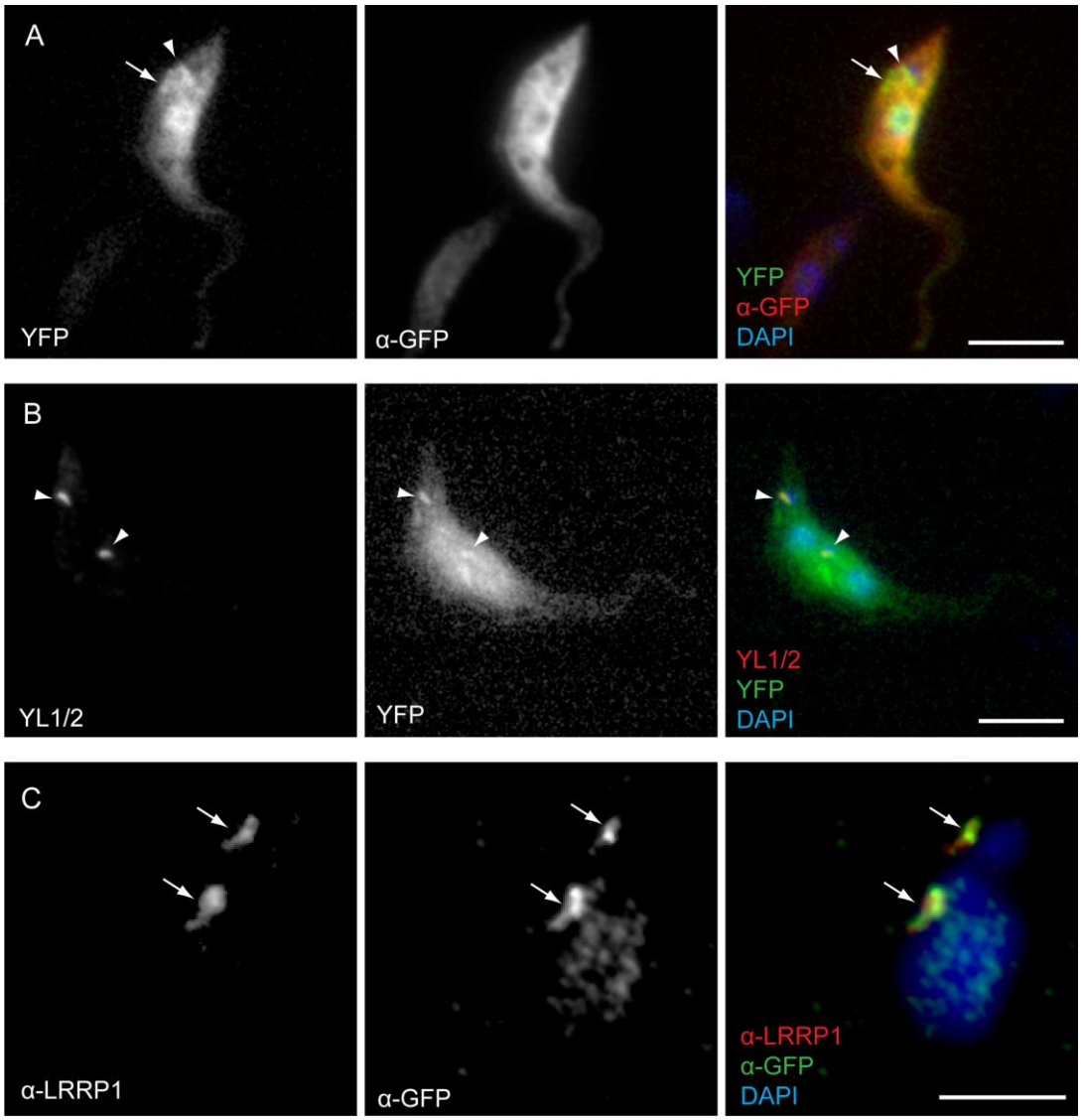

Fig. 3.5.14 Localization of YFP-TbMORN1(1-260).

Procyclic cells were fixed with PFA and treated with α-GFP antibodies to increase the YFP signal. (A, B) Upon PFA fixation the cells showed cytosolic labelling and additionally some faint structures next to the kinetoplast (arrowhead) (A). This structure colocalized with the basal body marker YL1/2 (arrowheads) (B). (C) In detergent extracted cytoskeletons YFP-TbMORN1(1-260) targeted to the bilobe (arrows)

as confirmed by colocalization with TbLRRP1. Nuclear and kinetoplast DNA were in all cases labelled with DAPI. Bars, 5µm.

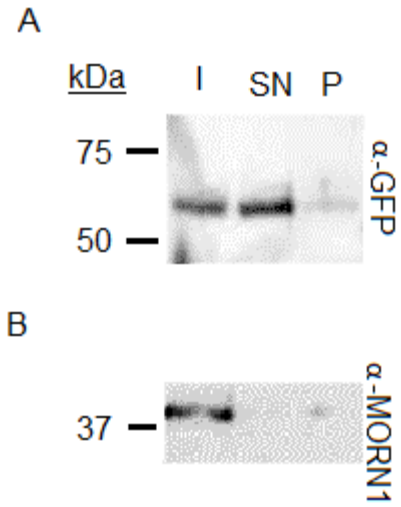

Fig. 3.5.15 Fractionation of YFP-TbMORN1(1-260)-transfected cells. Procyclic cells transiently expressing YFP-TbMORN1(1-260) were treated with extraction buffer and fractions separated by centrifugation. Input (I) soluble supernatant (SN) and insoluble (P) proteins were analyzed by SDS-PAGE and Western blotting. (A) Membranes treated with α-GFP antibodies confirmed the expression of the 56kDa large protein. (B) Samples probed with α-MORN1 antibodies. These recognized the endogenous TbMORN1, 40kDa. Lanes contain a 1% fraction of each sample.

3.5.9 TbMORN1(1-145)

YFP-TbMORN1(1-145) showed cytosolic staining (Fig.3.5.16). After extraction with detergent no signal was monitored (data not shown).

Western blot of fractionated cell lysates confirmed that YFP-TbMORN1(1-145) was expressed and present in the input and the cytoplasmic fraction (Fig.3.5.17 A). As expected there was no signal obtained from the cytoskleletal fraction. α-MORN1 antibodies further revealed that the endogenous TbMORN1 protein targeted to the cytoskeleton (Fig.3.5.17 B).

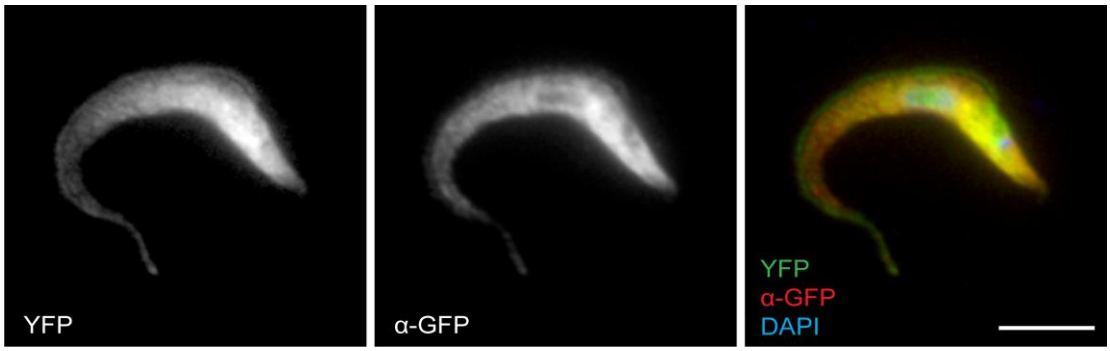

Fig. 3.5.16 Localization of YFP-TbMORN1(1-145).

Procyclic Trypanosomes transiently expressing YFP-TbMORN1(1-145) were fixed with PFA. Immunofluorescence images showed cytosolic staining with α-GFP antibodies. Nuclear and kinetoplast DNA were labelled with DAPI. Data was representative of 2 independent transfections. Bars, 5µm.

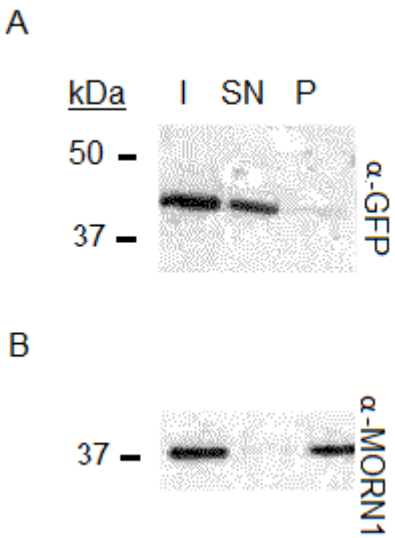

Fig. 3.5.17 Immunoblotting of YFP-TbMORN1(1-145) transfected cell lysates.

Procyclic 427 cells were transfected with YFP-TbMORN1(1-145). Fractionated cell lysates were obtained by extracting with 0.5% NP40 followed by centrifugation. The input (I), supernatant (SN) and pellet (P) fractions were examined by SDS-PAGE and Western blotting. (A) An approximately 45kDa large protein was detected by α-GFP antibodies. (B) Samples probed with α-TbMORN1. The endogenous TbMORN1, 40kDa, was recognized. Lanes contain a 1% fraction of each sample.

3.6 Truncations from both ends

3.6.1 TbMORN1(146-343)

Sequencing revealed that this clone carried two mutations, the Y215C as expected and in addition A279T.

Immunofluorescence microscopy of YFP-TbMORN1(146-343) transfected cells showed cytosolic labelling with α-GFP antibodies (Fig.3.6.1). After detergent extraction no signal from any structure could be observed (data not shown).

α-GFP antibodies recognized a faint signal at approximately 50kDa in all fractions (Fig.3.6.2).

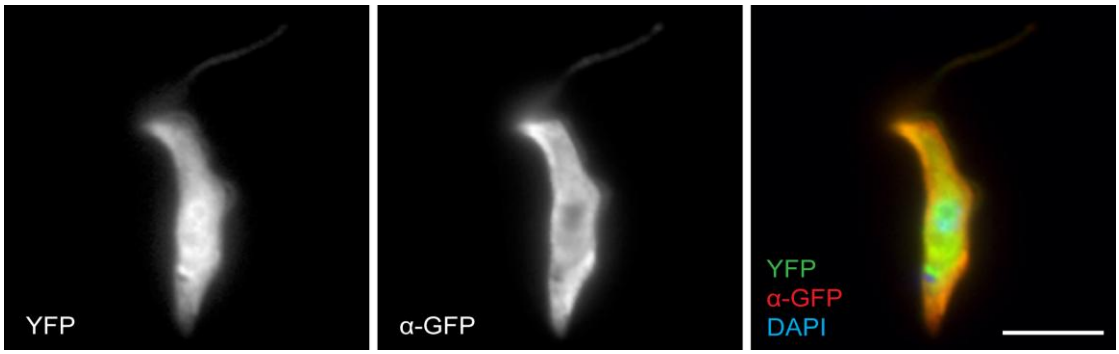

Fig. 3.6.1 Localization of YFP-TbMORN1(146-343).

Transfected cells showed whole cell labelling with α-GFP suggesting that antibodies recognized a pool of recombinant protein in the cytosol. Nuclear and kinetoplast DNA were labelled with DAPI. Bars, 5µm.

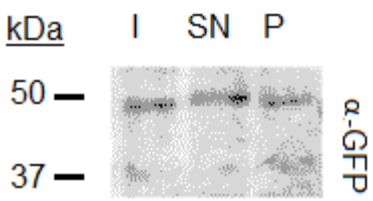

Fig. 3.6.2 Western blot analysis of fractionated cell lysates.

Lysates were obtained from YFP-TbMORN1(146-343) transfected cells. The input (I), the soluble supernatant (SN) and the insoluble pellet (P) were analyzed by SDS-PAGE and Western blotting. Samples probed with α-GFP. The expected protein is approximately 50kDa.

3.6.2 TbMORN1(146-329)

YFP-TbMORN1(146-329) showed cytosolic labelling (Fig.3.6.3). After detergent extraction no signal from any structure could be detected (data not shown).

The truncated protein was found in all three fractions as it was shown by Western blot analysis (Fig.3.6.4).

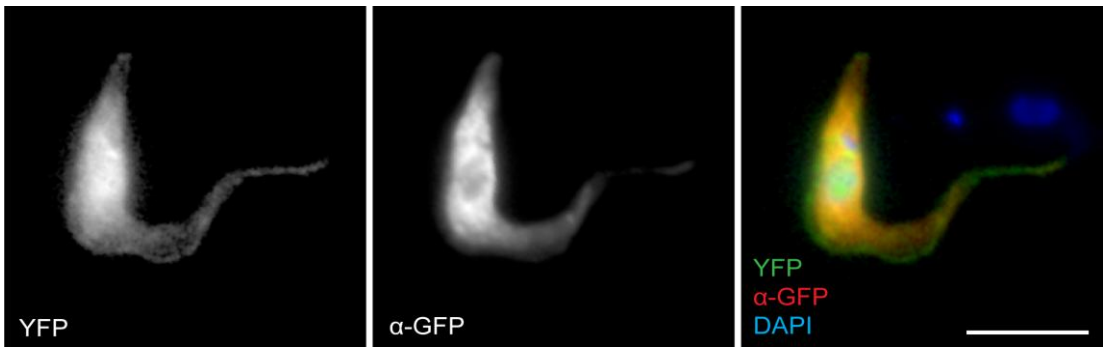

Fig. 3.6.3 Localization of YFP-TbMORN1(146-329).

α-GFP antibodies were used to boost the YFP signal. Both YFP and α-GFP staining showed cytosolic labelling. Nuclear and kinetoplast DNA were labelled with DAPI. Bars, 5µm.

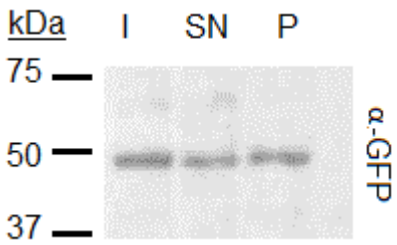

Fig. 3.6.4 Fractionated cell lysates obtained after transfection with YFP-TbMORN1(146-329).

Procyclic Trypanosomes expressing YFP-TbMORN1(146-329) were treated with detergent and cytoplasmic and membrane-associated proteins were separated by centrifugation. Equal fractions of input (I), soluble supernatant (SN) and insoluble pellet (P) were analyzed by SDS-PAGE and Western blotting. Immunoblots were treated with

α-GFP antibodies. The 47kDa truncated protein was observed. Lanes contain a 1% fraction of each sample.

3.7 Basepair exchange

At the beginning of the project I had been using Bgl2 digested fragments which were ligated into BamH1 cut vectors. By this combination the digested fragment can be inserted in both orientations. To avoid insertion in wrong orientation and to increase cloning efficiency I wanted to get rid of the internal EcoR1 restriction site. Thus this construct carried a basepair exchange at base 1035 from TTC>TTT to abolish the EcoR1 site. There is little known about codon usage in Trypanosomes but data from Alonso et al. suggests that this basepair might be okay (Alonso et al., 1992).

By immunofluorescence microscopy it was shown that the construct was expressed and targeted correctly to the bilobe as it was shown by colocaliaztion with TbLRRP1 (Fig.3.7.1 A and B).

Western blotting supports the idea that the base pair exchanged protein targeted to the cytoskeleton as a stronger signal from this fraction was observed (Fig.3.7.2).

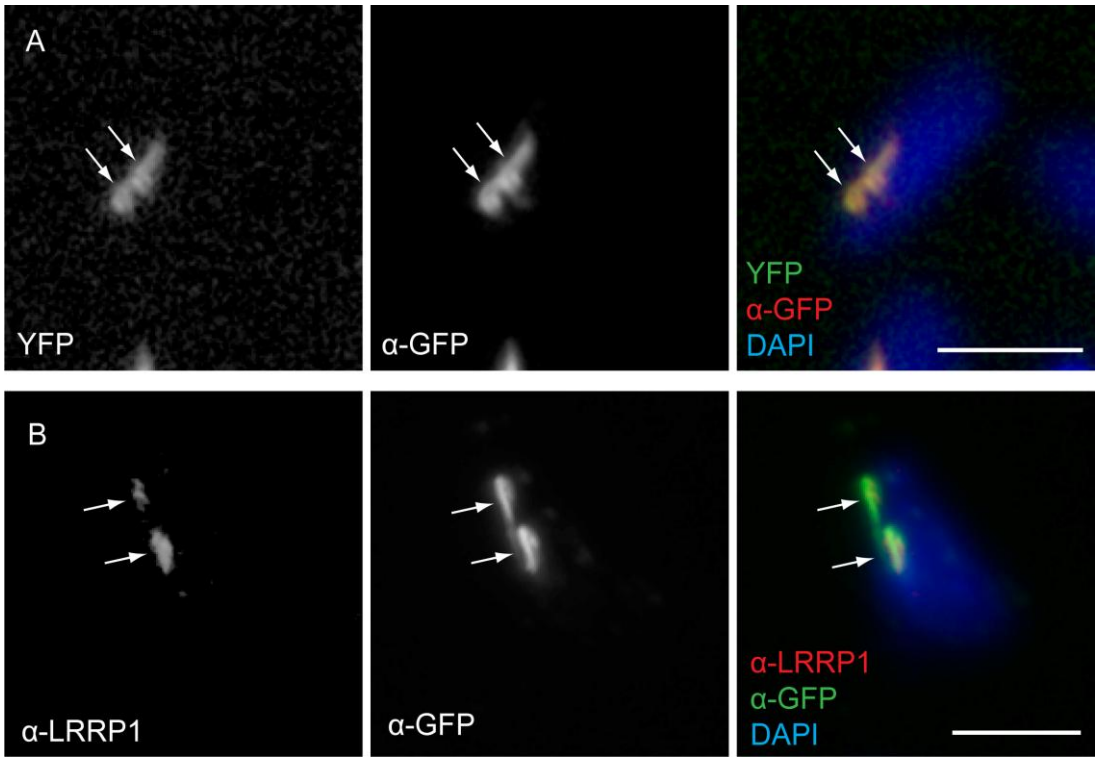

Fig. 3.7.1 Localization of base pair exchanged full length TbMORN1.

(A) Procyclic ells were fixed with PFA (A) or extracted and fixed with PFA (B) and stained with α-GFP antibodies. The YFP - tagged protein was present at the bilobe (arrows) as shown by colocalization with LRRP1. Nuclear and kinetoplast DNA were visualized with DAPI. Bars, 5µm.

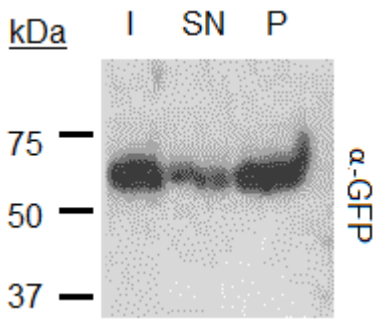

Fig. 3.7.2 Western blot analysis of fractionated cell lysates obtained from base pair exchanged YFP-TbMORN1 transfected cells.

Procyclic cells were treated with extraction buffer and fractions were separated by centrifugation. The Input (I), supernatant (SN) and the pellet (P) samples represent cytoplasmic and cytoskeletal proteins. Analysis by SDS-PAGE and Western blotting showed a 67kDa large protein recognized by α-GFP antibodies. Lanes contain a 1% fraction of each sample.

3.8 Transfection efficiency

During the project I performed transient transfections which means that not all cells will express the tagged protein. Since I was worried that low numbers of transfected cells could have an influence on the strength of localization I evaluated transfection efficiencies (TE) (Table 3.8-1.). Therefore I counted transfected and untransfected cells in 5 separate fields of view. I calculated the efficiency of single transfections and the mean TE. I observed that the TE of a single transfection varied between 3-23% but that low TE did not reflect the paucity of localization. Some constructs with low TE did localize properly and others did not. Interestingly, the highest TE was counted for TbMORN1(1-358) and TbMORN1(1-350) where spears were also observed. Further I was interested if the cell concentration at the time of transfection played a role for TE. I therefore plotted cell concentration and TE values (Fig. 3.8.1). No obvious correlation between TE and concentration was observed. There is in general though a tendency for higher TEs at lower cell concentrations and lower TEs when the cell culture was denser. I think that this reflects that cells in mid log phase, 10^6 cell/ml, were easier to transfect than cells that were already in stationary phase, 10^7 cells/ml, and therefore take up DNA less efficiently.

	Σ transfected	Σ untransfected	TE single Transfection	No. of transfections	Mean
TbMORN1	14	58	0,241	2	0,182
	19	155	0,123		
TbMORN1(72-358)	15	110	0,136	2	0,124
	17	153	0,111		
TbMORN1(146-358)	21	133	0,158	2	0,157
	12	77	0,156		
TbMORN1(215-358)	24	198	0,121	2	0,097
	8	111	0,072		

TbMORN1(284-358)	11	106	0,104	2	0,157
	28	133	0,211		
TbMORN1(330-358)	6	175	0,034	1	
TbMORN1(1-353)	9	136	0,066	1	
TbMORN1(1-350)	30	131	0,229	2	0,213
	23	117	0,197		
TbMORN1(1-343)	5	48	0,104	2	0,088
	11	154	0,071		
TbMORN1(1-336)	8	95	0,084	2	0,077
	12	174	0,069		
TbMORN1(1-329)	8	107	0,075	2	0,106
	15	109	0,138		
TbMORN1(1-320)	10	166	0,060	2	0,075
	9	101	0,089		
TbMORN1(1-312)	17	159	0,107	2	0,124
	17	120	0,142		
TbMORN1(1-260)	17	165	0,103	1	
TbMORN1(1-145)	12	142	0,085	2	0,131
	18	101	0,178		
TbMORN1(146-343)	6	61	0,098	1	
TbMORN1(146-329)	9	89	0,101	1	

Table 3.8-1 Transfection efficiency.

For each transient transfection the cells in 5 fields of view were counted. The transfection efficiency of a single transfection was defined as transfected cells relative to untransfected ones. Mean is the mean transfection efficiency where multiple datasets were available.

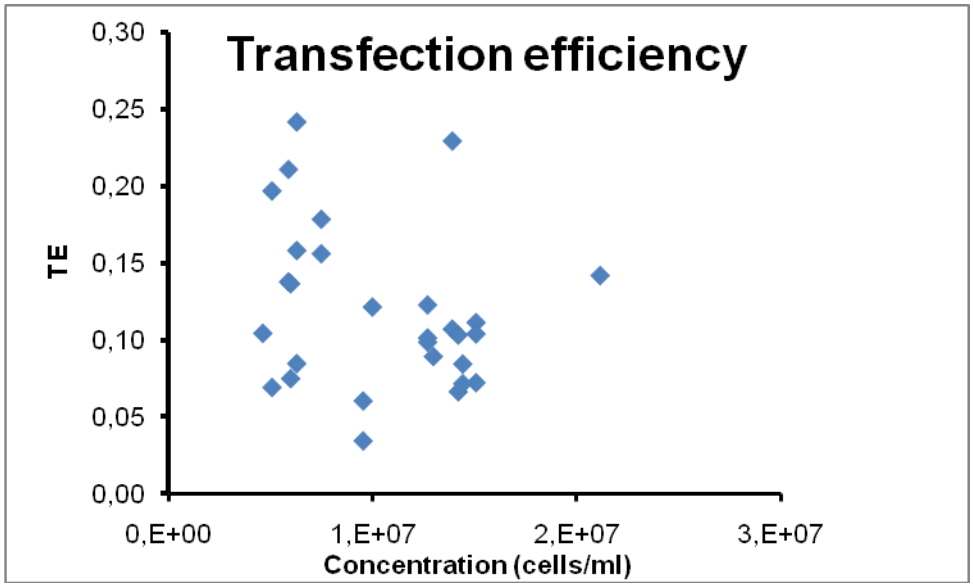

Fig. 3.8.1 Transfection efficiency.

Transfection efficiency was plotted against the culture concentration at the time of transfection.

4 Discussion

My project had two main aims: screening the MORN-repeat proteins for bilobe localization, and determining the targeting sequence of TbMORN1. These two projects will be considered in turn.

4.1 The MORN-repeat protein family

The aim of this project was to screen the MORN-repeat family proteins for possible bilobe localization. Therefore YFP-tagged fusion proteins were transiently expressed in Trypanosomes. All recombinant proteins expressed showed cytoplasmic α-GFP labelling in PFA- and MeOH-fixed cells. This uniform whole cell staining suggests a pool of YFP-tagged proteins present in the cytosol. Interestingly for protein 10 strong nuclear labelling in PFA-fixed cell was observed. This was surprising since protein 10 does not have a nuclear localization signal. More specific localizations of fusion proteins were observed in detergent-extracted cytoskeletons (Table 4.1-1and Fig.4.1.1).

Protein	ID	PFA	MeOH	Xtr+PFA	WB	Localization
1	Tb927.8.3780	cyt.	cyt.	flagellum	yes	axoneme
2	Tb927.3.2430	cyt.	cyt.	-	no	cyt.
3	Tb927.7.3310	cyt.	cyt.	flagellum	no	axoneme
4	Tb927.3.2890	cyt.	cyt.	flagellum	yes	axoneme
5	Tb927.10.6230	-	-	-	-	-
6	Tb927.10.14570	cyt.	cyt.	flagellum	yes	axoneme
7	Tb927.7.6910	cyt.	cyt.	flagellum	yes	axoneme
8	Tb927.10.10350	cyt.	cyt.	BB	yes	BB
9	Tb11.02.5030	cyt.	cyt.	flagellum	no	axoneme
10	Tb927.2.5040	cyt. nuc.	-	flagellum	yes	axoneme
11	Tb11.01.1460	cyt.	-	BB	yes	BB
12	Tb927.5.3880	cyt.	-	-	yes	cyt.

		mito.				mito.
13	Tb927.7.3400	cyt.	-	flagellum	yes	PFR
14	Tb927.1.1910	-	-	-	-	-
15	Tb927.3.4270	cyt.	cyt.	-	yes	cyt.
16	Tb927.1.740	cyt. BB?	-	-	no	cyt. BB?
17	Tb927.7.530	-	-	-	-	-

Table 4.1-1 Summary of MORN-repeat proteins and their localizations

cyt.: cytosolic; nuc.: nuclear; mito.: mitochondrial; BB: basal body

Of the 17 screened proteins a total of 7, namely Proteins 1,3,4,6,7,9 and 10, localized to the axoneme. The flagellum is a highly complex organelle, essential for motility, has a role in cell morphogenesis and is crucial for cell polarity and division. The axoneme and the PFR form the flagellum of T.brucei. The axoneme ring structure of nine outer doublet microtubules and central pair of single microtubules is highly conserved amongst eukaryotes. This part of the flagellum also contains conserved elements such as radial spokes, dynein arms and nexin links (Hammarton et al., 2007a). Furthermore the flagellum is supported by all kinds of molecular motor proteins through a highly active intra-flagellar transport. Due to this component variety it is possible that selected proteins might be interacting with one of these elements. The kinesin motor domain-containing Protein 6 is especially likely to be involved in flagellar transport.

Protein 13 colocalizes with the PFRA staining therefore I suggest that this protein could be part of the PFR. Three distinct zones- proximal, intermediate and distal divide the PFR. The PFR is linked to the axoneme by robust filaments. The two major proteins are PFR1 and PFR2 (Gadelha et al., 2004) but its complete composition is still unknown. Therefore Protein 13 might be a structural component within the PFR.

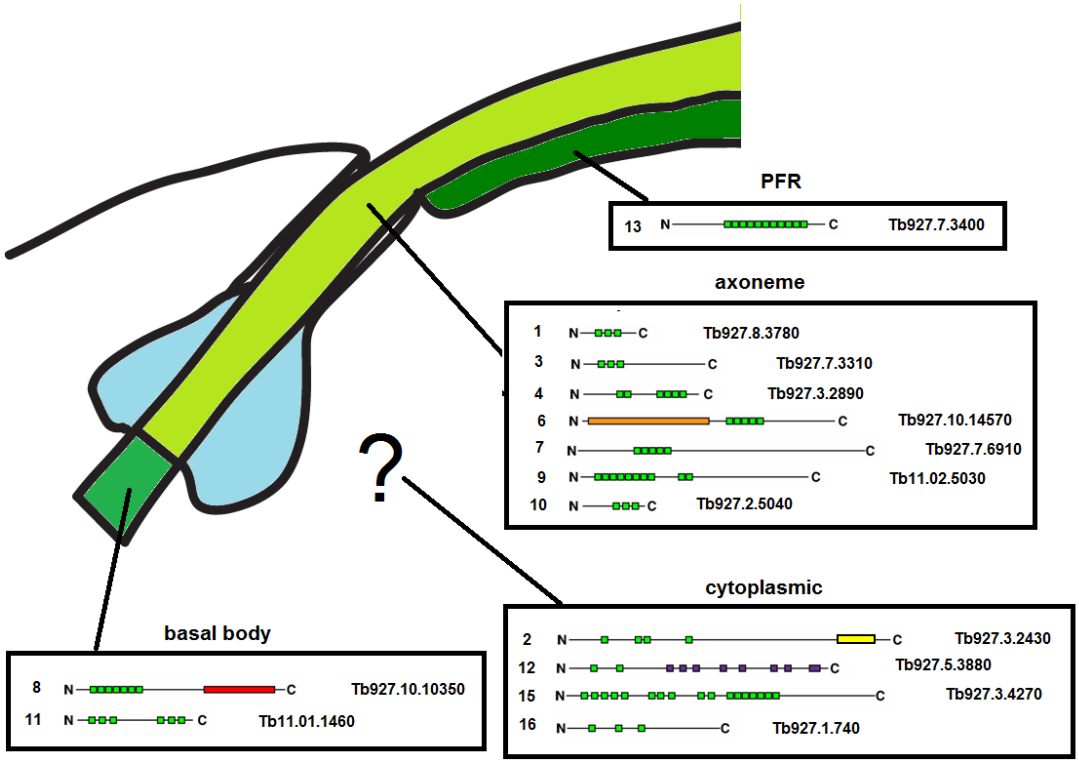

Fig. 4.1.1 Summary of MORN-repeat protein family localization.

7 proteins localized to the axoneme, 1 to the PFR, 2 were present at the basal body and 4 were found in the cytosol.

The main MTOC in Trypanosomes is the basal body which is responsible for duplication and segregation of organelles. Furthermore the basal body is the nucleation site for the new flagellum. Our results show that two Proteins- 8 and 11 localize to both the mature and the pro-basal body. Due to their localization these proteins might be involved in segregation of the kinetoplast or assembly of the new axoneme. Protein 8 further has a predicted kinase domain which might also implement a regulatory function perhaps a role in cell cycle.

Protein 2, 15 and 16 were considered as cytosolic proteins and Protein 16 might additionally be present on the basal body. Protein 2 has a predicted VPS9 domain which is a Rab5 GEF. It is therefore possible that this protein might play an important regulatory function by activating small GTPases.

Interestingly, a meshed labelling pattern across the whole cell body was observed for Protein 12. This might be “mitochondrial” labelling although the analysis by Mitoprot II shows a mitochondrial targeting chance of 0,038 and does not predict a cleavage site. I first also considered endoplasmatic reticulum localization but the typical ring around the nucleus was not observed. In Trypanosomes there are hardly any pro-

teins known which are exported to the mitochondrium it is therefore conceivable that Protein 12 is one of these or interacts with one of these components.

Unfortunately, I encountered trouble cloning two constructs- Protein 14 where no positive clones were obtained and Protein 17 which is a huge protein and showed several deletions after sequencing. For these two constructs I suggest recloning by endogenous replacement. This method means cloning of the 5' untranslated region and the first approximately 500 bp of the ORF into a special vector which allows replacement of the endogenous gene by the YFP- flanked cassette through double crossover of homologous recombination. This method should lead to positive clones and abolish deletions. Owing to time limits this step could not be carried out.

For Protein 5 I could not observe expression after transient transfection. Despite repeated attempts, it was not possible to generate a stably transfected cell line. I therefore consider that this tagged protein might be toxic for the cells. One possible experiment to solve this problem could be to put the YFP tag at the N-terminus instead of the C-terminus or try a different, smaller tag like the Ty1 epitope for example. If over expression is the problem then endogenous replacement might be an option too. This would allow normal expression levels.

Western blot of total cell lysates confirmed the expression of 9/14 proteins (1,4,6,7,8,10,11,13,15). Unfortunately for 5 proteins (2,3,12,16) no signal could be detected. I consider that the expression level might be too low in these cases. This is especially feasible for protein 12 and 16 where only a faint YFP signal was ever observed. For better detection, cell lysates should be prepared at earlier time points after transfection and higher amounts of cells should be loaded on the gel.

In summary 7 proteins localized to the axoneme, 1 to the PFR, 2 where found at the basal body, 3 where present in the cytosol and one might be mitochondrial. To study their possible functions RNAi experiments depleting the proteins would be the method of choice. In all cases the screened proteins were not targeted to the bilobe which implies that MORN-repeat sequences alone are not sufficient for bilobe localization. This further suggests that a 3D motif in TbMORN1 might be the crucial feature. But 10/14 proteins were found on cytoskeletal structures which hints at a structural role of MORN-repeat proteins.

4.2 TbMORN1 truncation

During this project the aim was to find the targeting sequence of TbMORN1 and define which MORN-repeats are essential and which are dispensable for targeting. Therefore N- and C-terminal truncation constructs were transiently expressed in procyclic Trypanosomes and screened by immunofluorescence microscopy for localization redolent of the bilobe (Fig.4.2.1 A).

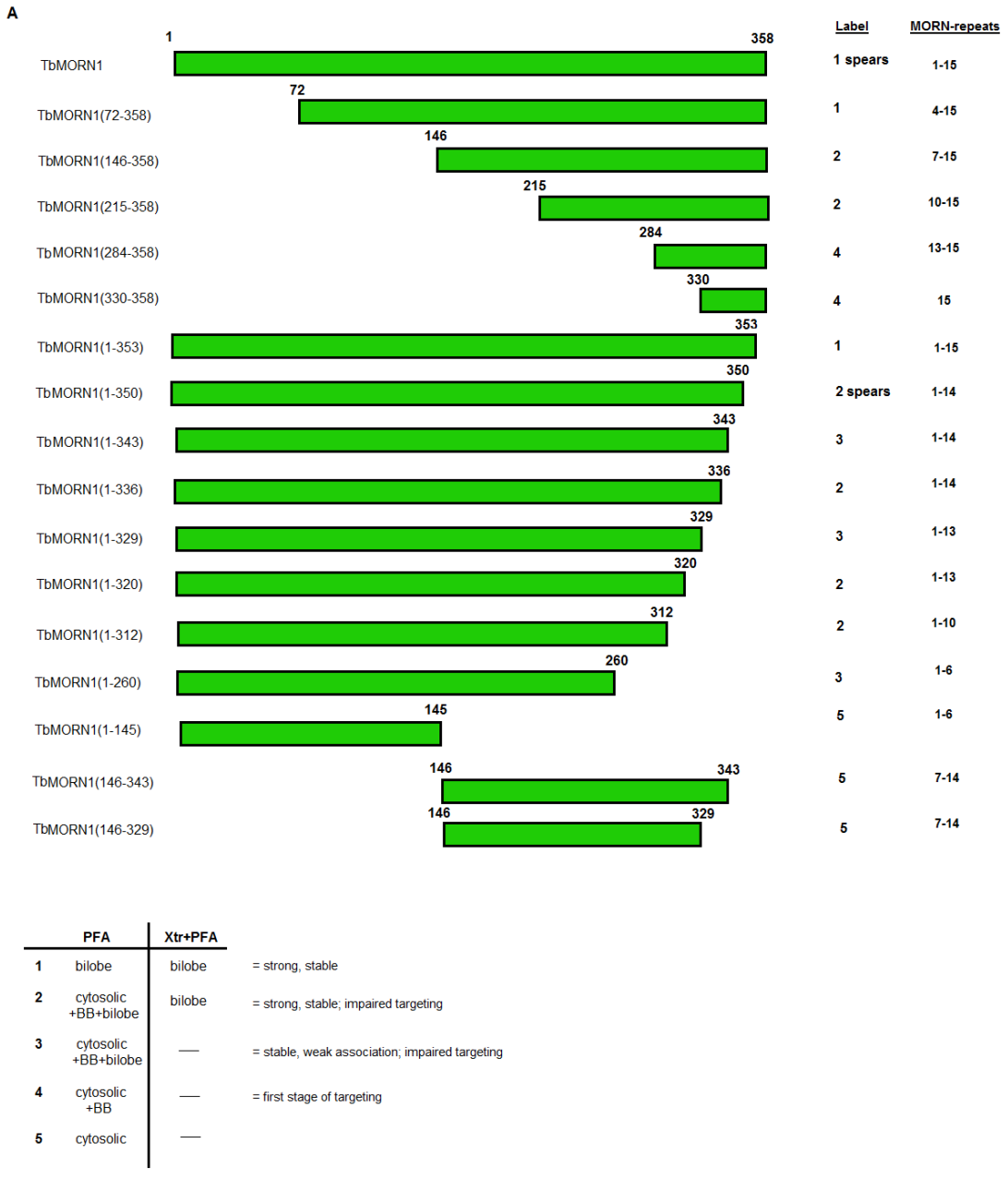

Fig. 4.2.1 Summary of truncated constructs

Transient transfections with full-length YFP- tagged TbMORN1 showed that in intact cells as well as in extracted cytoskeletons the fusion protein was found at the bilobe and colocalized with TbLRRP1 labelling. Additionally YFP-TbMORN1 formed artefactual paracrystalline structures referred to as "spears". Further I performed immunoblotting of biochemically fractionated cell lysates obtained from YFP-TbMORN1 transfected cells. The YFP-tagged protein was present in all three fractions but a strong signal from the pellet fraction was observed. This indicates that YFP-TbMORN1 preferentially associates with the cytoskeleton. α-MORN1 antibodies detected the endogenous TbMORN1 which was targeted exclusively to the cytoskeletal fraction.

The N-terminal truncation, TbMORN1(72-358), had no effect on proper localization to the bilobe. Less protein was observed in the cytoskeletal fraction in western blots implying that more YFP-tagged protein stays in the cytosol. This coincided with the observation of faint cytosolic staining in PFA-fixed cells. A truncation construct missing the first 6 MORN-repeats, TbMORN1(146-358), was still targeted to the bilobe but was additionally found in the cytosol and at the basal bodies. This implies that amino acids 72-146 are necessary for localization exclusively to the bilobe. However on Western blot no signal for the cytoskeletal fraction was observed which remains an unresolved question so far. I suggest that the exposure time was not set long enough to detect fainter bands. The construct TbMORN1(215-358) also localized to the cytosol, the basal body and the bilobe. The Western blot analysis revealed that some protein was targeted to the cytoskeleton. The construct containing amino acids 284-358 was no longer observed at the bilobe in intact cells, but in the cytosol and at the basal bodies. However extraction of cytoskeletons lead to the loss of basal body signal. Thus, amino acids 215-284 are necessary for targeting to the bilobe. This matches with the observation that for the cytoskeletal fraction only a very faint signal was detected. The last MORN-repeat, TbMORN1(330-358), still showed basal body labelling which suggests that amino acids 330-358 are sufficient for basal body localization. In Western blot analysis only signals from the input and the supernatant could be observed. The missing signal from the cytoskeleton could be due to the fact that the basal body signal might be too weak and that little amount of protein might not be detected. Combining the data from the N-terminal truncations there are 3 important domains that are: 1-72 for localization only to the bilobe, 215-284 for bilobe targeting and 330-358 for basal body localization.

The C-terminal truncations complicated this picture. TbMORN1(1-353) localized correctly to the bilobe but also showed nuclear labelling. It is known that TbMORN1

does not target to the nucleus and therefore I suggest that it is due to antibody which is nonspecifically bound. Furthermore no spears could be observed and the signal from the bilobe was less strong than for other constructs which might indicate that this protein was expressed at a lower level. This low level expression might also explain why no signal could be recognized on Western blot. However the construct missing the last 8 amino acids, TbMORN1(1-350), did localize properly but was also present in the cytosol and at the basal body. Interestingly the formation of spears was observed in 53% of transfected cells. Blotting of fractionated cell lysates backed up the idea that the YFP-tagged protein was mainly present in the cytosol and little was targeted to the cytoskeleton. TbMORN1(1-343) was observed in the cytosol, at the basal bodies and a structure next the kinetoplast in intact cells. After detergent extraction however no signal from any structure could be detected. TbMORN1(1-336) showed the same labelling pattern in intact cells but localized to the bilobe even after detergent extraction. Oddly for construct TbMORN1(1-329) no signal could be observed in detergent extracted cytoskeletons. TbMORN1(1-320) in contrast did again localize to the bilobe even after treatment with extraction buffer. On Western blot all these constructs showed a strong band in the supernatant and a faint signal in the pellet fraction. TbMORN1(1-312) showed the common labelling pattern of cytosolic, basal body and bilobe structure in the intact cells. After extraction the protein was still found at the bilobe. Western blots also displayed the pattern that most of the signal was present in the cytoplasmic fraction and little was found in the pellet. The construct containing amino acid 1-260, TbMORN1(1-260) was also found in the cytosol, at the basal body and at the bilobed structure, but the signal from these cytoskeletal structures was lost after extraction. Only MORN1(1-145) showed exclusively cytosolic staining and no basal body signal. Western blots back up this idea since no band for the cytoskeletal fraction was observed. In summary the importance of 3 domains were determined. Amino acids 350-353 seems to the necessary for targeting exclusively to the bilobe all other proteins are additionally found in the cytosol and at the basal bodies. The domain 343-350 seems to be important for spear formation suggesting that the far C-terminus was needed to form paracrystalline artefacts. Furthermore amino acids 145-260 are indispensible for bilobe and basal body targeting.

Further constructs truncated from both ends were created; TbMORN1(146-343) which we would expect to localize to the cytosol, the basal bodies and the bilobe as it was observed for TbMORN1(146-358) and TbMORN1(1-343). Interestingly the YFP-tagged protein was only observed to be cytosolic in PFA-fixed cells. This implies that amino acids 145-260 are not sufficient for bilobe or basal body targeting.

The same behaviour was observed for construct TbMORN1(146-329). This suggests that truncated proteins might be too short and that the localization was achieved through structural features of TbMORN1. This indicates that the structure might play an important role in targeting.

Furthermore I was worried that low numbers of transfected cells could have an influence on the strength of localization. But as evaluation of TEs showed the variation of TEs was little and there was no connection between low TE value and abundant localization observed.

For nearly all truncation constructs basal body labelling in intact cells was observed. Interestingly, the signal from the basal body was lost after extraction which might indicate a weak, reversible association. I think that this labelling might reflect trafficking of TbMORN1 through the basal body and that truncated versions are possibly delayed at the basal body. Something similar was noted in recent experiments on the small G protein Rab-like 5 in Trypanosomes. RABL5 exhibited cytosolic, basal body and flagellum staining. Interestingly, in IFT RNAi cells this protein was only found at the basal body (Adhiambo et al., 2008). This indicates that the basal body might play an important role as a staging post in cytosolic protein trafficking.

During the project I observed that the constructs exhibited different strength of bilobe localization. Some showed a good bilobe signal whereas other displayed faint bilobe signalling. It is possible that truncated constructs alone would not localizae to the bilobe but that truncated proteins can bind to endogenous TbMORN1 and therefore target properly.

Surprisingly, the spear formation was only observed for TbMORN1 and TbMORN1(1-350). All other N- or C-terminal truncations did not show these artefactual structures. This implements that the far C-terminus is needed for spear formation and self- association.

Combining data from all truncations I found that 5 domains are important. Amino acid 1-72 and 350-353 are required for targeting exclusively to the bilobe. The domain 343-350 was found to be important for the formation of spears. Amino acid 215-312 are necessary for bilobe targeting. For targeting to the basal body, the last MORN-repeat, was sufficient.

I also observed a strange pattern of localization and mis-localization to the bilobe in C-terminal truncations, TbMORN1(1-350)- TbMORN1(1-260). I suggest that this unusual observation could be explained by hydrophobicity. In figure 4.2.2 I plotted

TbMORN1 sequence by Kyte & Doolite approximation to delineate the hydrophobic character of the protein (Fig.4.2.2). Regions with values above 0 are hydrophobic in character and negative values represent hydrophilic residues. Interestingly, TbMORN1 shows 3 hydrophobic loops from amino acid 300 to 358. When we introduce the constructs we can see that when we cut after a hydrophobic loop which is true for TbMORN1(1-350), TbMORN1(1-336) and TbMORN1(1-320), the truncated protein did properly localize to the bilobe. In contrast when we cut within one of these loops, TbMORN1(1-343) and TbMORN1(1-329) the targeting to the bilobe is inhibited. I therefore suggest that the hydrophobicity of the far C-terminus has an important role in targeting TbMORN1.

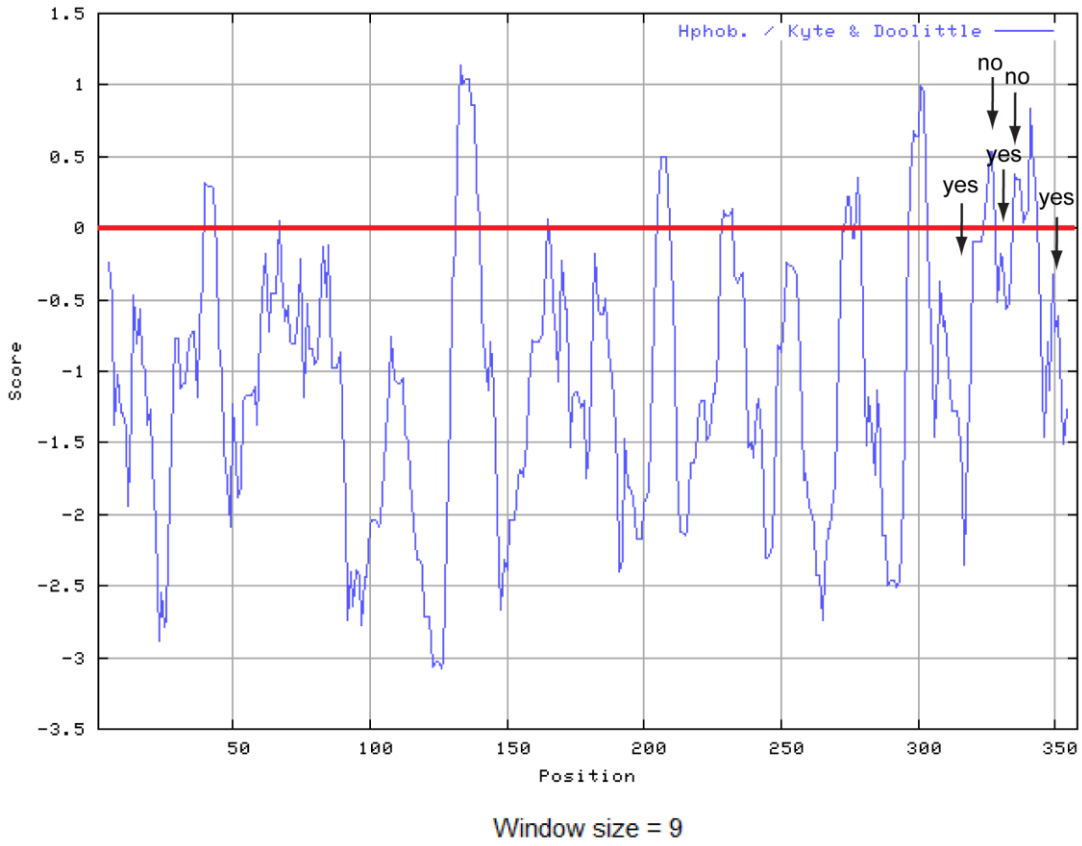

Fig. 4.2.2 Hydrophobicity plot of TbMORN1.

This plot shows the distribution of polar and apolar residues along the protein. Therefore the sequence is scanned with a moving window of fixed size (= 9). At each amino acid the mean hydrophobic index within the window is calculated and plotted. Hydrophobic regions have values above 0. Hydrophilic characters have negative values.

Recently it was shown that the MORN1 protein in T. gondii has a predicted palmitolyation site and that mutation of this site leads to localization to the centrocone

instead of to the basal end (Lorestani et al., 2010). Surprisingly, TbMORN1 has a predicted palmitolyation site too. This site at Cysteine 17 is predicted even under the most stringent conditions. We know from studies on palmitoylation that for many proteins the palmitoylation is essential for stable membrane association (Salaun et al., 2010).

Only TbMORN1(1-358), TbMORN1(72-358) and TbMORN1(1-353) localized exclusively to the bilobe which we consider as a strong, stable association (Fig.4.2.1. B). All others that localized to the bilobe also showed basal body labelling therefore showed impaired targeting. Basal body labelling is only observed in intact cells and not in extracted cytoskeletons. This observation might be explained by a weak association at the basal body. Furthermore we sometimes observed bilobe labelling in PFA-fixed cells but not necessarily after extraction (Fig.4.2.1. B, class 2,3). This stable and weak association might be explained by the hydrophobicity plot. Sometimes we only observed localization to the basal body (Fig.4.2.1. B, class 4) which we consider as the first stage of targeting.

Over all I propose the following model - I think that TbMORN1 traffics to the bilobe via the basal body since I observed basal body labelling for most of the truncated constructs (Fig.4.2.3). This coincides with the observation that by biochemical fractionation more protein comes down in the supernatant. Data from the hydrophobcity plot further suggests that TbMORN1 might be reversibly bound to the plasma membrane by its hydrophobic loops at the C-terminus. The final stable association might then be performed by palmitoylation linkage at Cysteine 17.

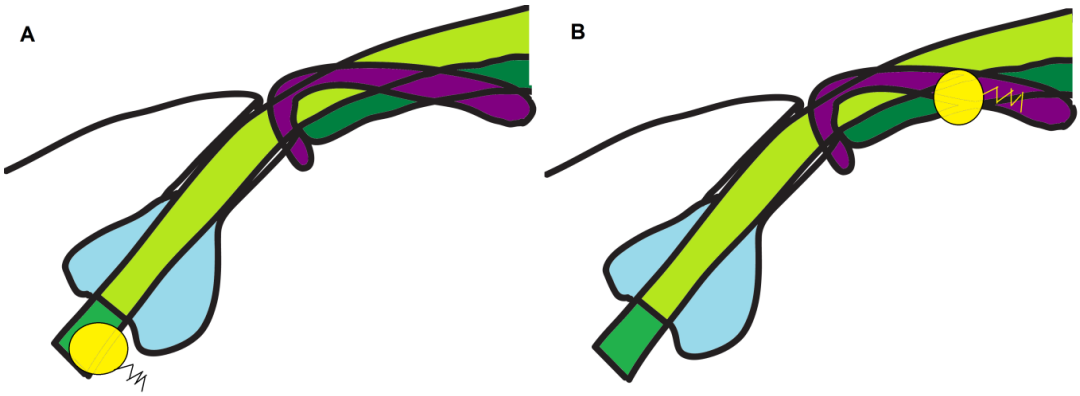

Fig. 4.2.3 Model for TbMORN1 targeting.

(A) New molecules of TbMORN1 dock at basal body. Weak association. (B) Molecules traffic up to the bilobe (maybe on the microtubular quartet). At bilobe they oli-

gomerize forming a strong and stable association. Palmitoylation occcurs to achieve a strong and stable association with the plasma membrane.

To support the conclusions from TbMORN1(145-358) further truncation constructs, shorter than amino acid 145 need to be done. Additionally I suggest preparing stable cell lines for TbMORN1(1-145) and TbMORN1(284-358) to check for the lack of localization to the bilobe. To confirm basal body localization electron microscopy on truncated proteins could be performed. To further support conclusions from the hydrophobicity plot a construct TbMORN1(1-346) could be generated. Finally a palmitoylation assay should be carried out to check if the predicted site shows this post-translational modification.

From the MORN-repeat protein family we can conclude that the MORN-repeat sequence alone does not lead to targeting to the bilobe. 10/14 MORN-repeat proteins were found on cytoskeletal structures which suggests a structural role. However further studies investigating the possible function of MORN-repeats still need to be done. Something in TbMORN1 is targeting the protein to the bilobe. It is possible that the targeting is achieved through a sequence that is not part of the MORN-repeat consensus sequence. From my data I suggest that a 3D motif is the crucial factor for targeting TbMORN1.

5 List of Abbreviations

BSA	bovine serum albumin
CIP	calf intestinal phosphatase
DAPI	4’2-diamidin-2-phenylindol
E.	Escherichia
ERES	endoplasmic reticulum export site
EtOH	ethanol
FAZ	flagellum attachment zone
FP	flagellar pocket
GEF	guanidine exchange factor
GFP	green fluorescent protein
HiFi	high fidelity
I	input
IFT	intra-flagellar transport
MeOH	methanol
min	minute(s)
MT	microtubule
MTOC	microtubular organizing center
MORN	membrane occupation and recognition nexus
ORF	open reading frame
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFR	paraflagellar rod
PLK	polo-like kinase
PIPK	phosphatidylinositolphosphate kinase
RNAi	RNA interference
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SN	supernatant
TE	transfection efficiency
T.	Trypanosoma
VSG	variant surface glycoprotein
Xtr	detergent - extracted
YFP	yellow fluorescent protein

6 List of Figures

7 List of Tables

8 References

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WHO Media Centre: http://www.who.int/mediacentre/factsheets/fs259/en/) letzter Zugriff 19.08.11

9 APPENDIX

List of antibodies

	Name	Dilution	Company/Reference
Primary Antibodies	rabbit-α-GFP	1: 5000	Seedorf et al., 1999
	mouse-α-PFRA	1: 10000	Kohl et al., 1999
	rat YL1/2	1: 1000	Abcam
	mouse-α-BB4A	1:10	Woods et al., 1989
	mouse-α-Cen2	1.25	Warren lab, unpublished to date
	mouse-α-LRRP1	1:5000	Zhou et al., 2010
Secondary Antibodies	goat-α-rabbit 488	1: 3000	Molecular Probes
	goat-α-rabbit 568	1:3000	Molecular Probes
	goat-α-mouse IgG 568	1: 3000	Molecular Probes
	goat-α-mouse IgM 568	1:3000	Molecular Probes
	goat-α-rat 555	1: 3000	Molecular Probes

List of constructs

Name	Protein	PCR primers
1. Tb927.8.3780	Tb927.8.3780	Tb927.8.3780_Bam_F Tb927.8.3780_Eco_R
2. Tb927.3.2430	Tb927.3.2430	Tb927.3.2430_Bam_F Tb927.3.2430_Bam_R
3. Tb927.7.3310	Tb927.7.3310	Tb927.7.3310_Bam_F Tb927.7.3310_Eco_R
4. Tb927.3.2890	Tb927.3.2890	Tb927.3.2890_Hind_F Tb927.3.2890_Nhe_R
5. Tb10.70.0600	Tb927.10.6230	Tb10.70.0600_Nhe_F Tb10.70.0600_Nhe_R
6. Tb10.61.2100	Tb927.10.14570	Tb10.61.2100_Hind_F Tb10.61.2100_Nhe_R
7. Tb927.7.6910	Tb927.7.6910	Tb927.7.6910_Hind_F Tb927.7.6910_Nhe_R
8. Tb10.406.0580	Tb927.10.10350	Tb10.406.0580_Hind_F

		Tb10.406.0580_Nhe_R
9. Tb11.02.5030	Tb11.02.5030	Tb11.02.5030_Bam_F Tb11.02.5030_Eco_R
10. Tb927.2.5040	Tb927.2.5040	Tb927.2.5040_Bam_F Tb927.2.5040_Eco_R
11. Tb11.01.1460	Tb11.01.1460	Tb11.01.1460_Bam_F Tb11.01.1460_Eco_R
12. Tb927.5.3880	Tb927.5.3880	Tb927.5.3880_Hind_F Tb927.5.3880_Nhe_R
13. Tb927.7.3400	Tb927.7.3400	Tb927.7.3400_Hind_F Tb927.7.3400_Nhe_R
14. Tb927.1.1910	Tb927.1.1910	Tb927.1.1910_Bcl_F Tb927.1.1910_Eco_R
15. Tb927.3.4270	Tb927.3.4270	Tb927.3.4270_Hind_F Tb927.3.4270_Nhe_R
16. Tb927.1.740	Tb927.1.740	Tb927.1.740_Bam_F Tb927.1.740_Eco_R
17. Tb927.7.530	Tb927.7.530	Tb927.7.530_Hind_F Tb927.7.530_Nhe_R
TbMORN1(1-358)	TbMORN1(1-358)	MORN1-5' Blg2 MORN1-3'Bgl2
E, TbMORN1(72-358)	TbMORN1(72-358)	MORN1(72-358)_Bgl2_F MORN1-3'Bgl2
2, TbMORN1(146-358)	TbMORN1(146-358)	MORN1-IntBGL2-F MORN1-3'Bgl2
A, TbMORN1(215-358)	TbMORN1(215-358)	6xMORN1-IntBGL2-F MORN1-3'Bgl2
B, TbMORN1(284-358)	TbMORN1(284-358)	3xMORN1-IntBGL2-F MORN1-3'Bgl2
L, TbMORN1(330-358)	TbMORN1(330-358)	MORN(330)_BamH_F MORN1-3'Bgl2
K, TbMORN1(1-353)	TbMORN1(1-353)	MORN1-5' Blg2 MORN(354)_Mut_Eco
I, TbMORN1(1-350)	TbMORN1(1-350)	MORN1-5' Blg2 MORN(350)_Mut_Eco_R
C, TbMORN1(1-343)	TbMORN1(1-343)	MORN1-5' Blg2 cutCterMORN1-IntBgl2-R
H, TbMORN1(1-336)	TbMORN1(1-336)	MORN1-5' Blg2

		MORN1(1-336)_Eco-R
F, TbMORN1(1-329)	TbMORN1(1-329)	MORN1-5' Blg2 MORN1(1-329)_Eco_R
P, TbMORN1(1-320)	TbMORN1(1-320)	MORN1-5' Blg2 MORN(320)_Eco_R
O, TbMORN1(1-312)	TbMORN1(1-312)	MORN1-5' Blg2 MORN(312)_Eco_R
Q, TbMORN1(1-260)	TbMORN1(1-260)	MORN1-5' Blg2 MORN1(260)_Eco-R
1, TbMORN1(1-145)	TbMORN1(1-145)	MORN1-5' Blg2 1.MORN1sequence
D, TbMORN1(146-343)	TbMORN1(146-343)	MORN1-IntBGL2-F cutCterMORN1-IntBgl2-R
G, TbMORN1(146-329)	TbMORN1(146-329)	MORN1-IntBGL2-F MORN1(1-329)_Eco_R

List of Primers

Name	**F / R**	**Site**	**Sequence**
Tb927.8.3780_Bam_F Tb927.8.3780_Eco_R	F R	BamHI EcoRI	cggggatccATGCCACCCAAAAA-GAAGGTG cgggaattcC-TAACCCTCTTTTTCATCCTTC
Tb927.3.2430_Bam_F Tb927.3.2430_Bam_R	F R	BamHI BamHI	cggggatccAT-GAAACGATGCAACAGTGAG cggggatccTTATAAAATA-GATGCAATAAA
Tb927.7.3310_Bam_F Tb927.7.3310_Eco_R	F R	BamHI EcoRI	cggggatccATGTGTA-TATGCATGTATGCC cgggaattcCTACAGAGAATCCTCTA-CAAT
Tb927.3.2890_Hind_F Tb927.3.2890_Nhe_R	F R	HindIII NheI	cggaagcttATGTCGAAGCAAAA-GAATGTG cgggctag-cATCGGTCCACGTCATGCCGTC
Tb10.70.0600_Nhe_F Tb10.70.0600_Nhe_R	F R	NheI NheI	cgggctagcATGTTAACTGTAACGGAG-GAT

			cgggctagcGGCAGCGTAAATACCGTTTTT
Tb10.61.2100_Hind_F Tb10.61.2100_Nhe_R	F R	HindIII NheI	cggaagcttATGGAGAATTCCAGTTCGTT cgggctagcCTCTACATC-CAAGCCCCATCC
Tb927.7.6910_Hind_F Tb927.7.6910_Nhe_R	F R	HindIII NheI	cggaagcttATGTCGGGAAGTGACGCA-GAA cgggctagcTTCCTTGGTCGCCACAG-CAGC
Tb10.406.0580_Hind_F Tb10.406.0580_Nhe_R	F R	HindIII NheI	cggaagcttATGAGTACACAA-GAATCTCAT cgggctagcAAACGTAA-GAAATGGATGTTT
Tb11.02.5030_Bam_F Tb11.02.5030_Eco_R	F R	BamHI EcoRI	gccggatccATGACAACGAAGGAAG-CAGTG ccggaattcTCACACCCCGTCATGAGAGG
Tb927.2.5040_Bam_F Tb927.2.5040_Eco_R	F R	BamHI EcoRI	gccggatccATGCAGAGGGACGCTGACAG ggcgaattcCTATAAAC-TAACCTCCGCAAGC
Tb11.01.1460_Bam_F Tb11.01.1460_Eco_R	F R	BamHI EcoRI	ggcggatccATGCTAGACAAAGTTGTAGG ggcgaattcTCACCGGACAGTAAGAG-GAAAG
Tb927.5.3880_Hind_F Tb927.5.3880_Nhe_R	F R	HindIII NheI	cggaagcttATGGTCGGCAGCGCATCGCGG ggcgctagcTCTCCCTCCCGTATCCGCAA
Tb927.7.3400_Hind_F Tb927.7.3400_Nhe_R	F R	HindIII NheI	cggaagcttATGGCTTTGGGATCAG-GAATC ggcgctagcTGTTGGTGCATCCGCCATAC
Tb927.1.1910_Bcl_F Tb927.1.1910_Eco_R	F R	BclI EcoRI	cggtgatcaATGTTACCATCCTCTCAGGAC ccggaattcTCAAACATCTCCGGTATCAG
Tb927.3.4270_Hind_F Tb927.3.4270_Nhe_R	F R	HindIII NheI	cggaagcttATGGGGCATCCGAG-CGACAGC ggcgctagcCATGAGGCTGGCAA-GATCCTG
Tb927.1.740_Bam_F Tb927.1.740_Eco_R	F R	BamHI EcoRI	gccggatc-cATGTTTGTGCGCCGGTTGGTG ggcgaattcTTATCCCTTTGAGGTAAAGG
Tb927.7.530_Hind_F Tb927.7.530_Nhe_R	F R	HindIII NheI	cggaagcttATGCCCGATAACAATAACAA ggcgctagcACTTTCACTACTAGCTGGC
Internal sequencing primers			

2 Tb927.3.2430 internal primer F	F		GGTTCCATGACACTGGCACGC
2Internal-reverse	R		CTCACTACTAAGGTCCACCAGC
5Int-Fbeginn	F		GGCAACTCCTGTGGCGAGGGCGTCGC TACTCGG
5TbInternal-F	F		GGGAAGAAGGAGGGTCTTGGTGT
6 Tb10.61.2100 internal primer F	F		GGGCGTTGCAGGTGATCAGC
7 Tb927.7.6910 internal primer F	F		CGTCGTCACGGTGTCGGCGTG
8Tb10.406.0580 internal primer F	F		GCGTATGCTCGGAGAGGGTC
12Int-F	F		CACTGCCCCAGTTCTCCTTCTCGCC
Site-directed mutagenesis			
3SDM-Fnew	F		CTGTACATACAAGGAAGGAACAAAA AAAAAAGCATCAGCACAACCTGCAG
3SDM-Rnew	R		CTGCAGGTTGTGCTGATGCTTTTTTTT TTGTTCCTTCCTTGTATGTACAG
Sequencing primers			
EGFP-C1-F	F		GAAGCGCGATCACATGGTC
Primer YFP reverse	R		GCGGTTCACCAGGGTGTCGCCCTCG
MORN1-5' Blg2	F	Bgl2	gaagatctATATACTCTGGTGAGATA-GAAAATGG
MORN1-3'Bgl2	R	Bgl2	gaagatctATAATCCGGATTGCTGAGAT-CACAG
MORN1(72-358)_Bgl2_F	F	Bgl2	cggagatctTATACTGGAGAATGGAG-TATGGGCC
MORN1-IntBGL2-F	F	Bgl2	cggagatctTACGACGG-CGAATGGAACGAAG
6xMORN1-IntBGL2-F	F	Bgl2	cgga-gatctTACGAGGGCTACTGGCACCTCG
3xMORN1-IntBGL2-F	F	Bgl2	cgga-gatctTACGAGGGTGAGTGGGCTGAGG
MORN(330)_BamH_F	F	BamH1	cggggatccGTACATTGGAACCTGGAAG-GATG
MORN(354)_Mut_Eco	R	EcoR1	gccgaattcCTGAGATCACAGTTCTCA-GACAATCGAAATTCACCC
MORN(350)_Mut_Eco_R	R	EcoR1	ggcgaattcGTTCTCAGACAATCGAAATT-CACC
cutCterMORN1-	R	Bgl2	ccgagatctACCCTGACCCACAAT-

IntBgl2-R			GACCCCATCC
MORN1(1-336)_Eco-R	R	EcoR1	ggcgaatt-cATCCTTCCAGGTTCCAATGTAC
MORN1(1-329)_Eco_R	R	EcoR1	ggcgaattcCATGGAGCCGTCCTTGA-GAATTATCTTCCC
MORN(320)_Eco_R	R	EcoR1	ggcgaattcCCCGGGGCCATCCTTCTTTCCATGCG
MORN(312)_Eco_R	R	EcoR1	ggcgaattcCGCAAAT-GATCCCTCATAGCTGG
MORN1(260)_Eco-R	R	EcoR1	ggcgaattcATGTGTCCCCATTGCTATAGC

LEBENSLAUF

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2004-2009	Studium der molekularen Biologie, Universität Wien
2009-2011	Diplomarbeit am Max F. Perutz Laboratory, Group Graham Warren

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