Identification of CfNek, a novel member of the NIMA family of cell cycle regulators, as a polypeptide copurifying with tubulin polyglutamylation activity in *Crithidia*

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Summary

Post-translational glutamylation of tubulin plays an important role in regulating the interaction between microtubules and associated proteins, but so far the enzymes involved in this process have not been cloned from any cellular source. Using a modified purification scheme that employs a hydroxyapaptite chromatography as the final step we identified a 54 kDa band as the major polypeptide copurifying with tubulin polyglutamylation activity from the trypanosomatid *Crithidia fasciculata*. Based on peptide sequence information we have cloned the corresponding cDNA and identify *Crithidia* p54 as a novel member (termed CfNek) of the NIMA family of putative cell cycle regulators. CfNek is a protein of 479 amino acids that contains an unusual protein kinase domain that lacks the glycine-rich loop in subdomain I. The protein also harbours a PEST sequence and a pleckstrin homology domain. The tubulin polyglutamylase preparation displays the β-casein phosphorylation activity typical for NIMA related kinases. Recombinant His-tagged CfNek expressed in *Crithidia* localises to the flagellar attachment zone/basal body of the parasite. After purification on a Ni^{2+}-column the recombinant enzyme preparation displays ATP-dependent tubulin polyglutamylation activity as well as casein-phosphorylation activity.

Key words: *Crithidia*, NIMA kinases, Polyglutamylation, Tubulin, Post-translational modification, Cell cycle

Introduction

Microtubules are ubiquitous cytoskeletal elements that perform essential functions during cell division, movement, intracellular transport and generation of cell shape. The great diversity of tubulins within a cell is due to the expression of different αβ tubulin isotypes and a large number of different post-translational modifications [PTMs (for a review, see MacRae, 1997)]. Exept for the acetylation/deacetylation of Lys40 on α-tubulins (L'Hernault and Rosenbaum, 1985; Hubbert et al., 2002) these modifications affect the acidic C-terminal region of αβ tubulin, which is not visible in the atomic model of the dimer (Nogales et al., 1998) but is thought to be located at the outer surface of the microtubule (Nogales et al., 1999). These PTMs include removal and readdition of the C-terminal tyrosine of α-tubulins (Argarana et al., 1980; Ersfeld et al., 1993), generation of Δ2 α-tubulin by loss of the penultimate glutamate residue (Paturle et al., 1989) and phosphorylation of β-tubulin (Eipper, 1972).

Polyglutamylation (Eddé et al., 1990) and polyglycylation (Redeker et al., 1994) are two unusual protein modifications consisting of the stepwise addition of glutamate or glycine residues linked via isopeptide bonds to specific glutamate residues in the carboxy termini of α and β tubulin. Recently, Regnard et al. showed that the nucleosome assembly proteins NAP-1 and -2 are also modified by polyglutamylation (Regnard et al., 2000). Both PTMs are evolutionary conserved and already found in tubulins from primitive protists such as *Giardia lamblia* (Weber et al., 1997), but they are absent from yeast. While glutamylation is a general tubulin modification, the occurrence of glycylation seems to be restricted to cells that have either cilia or flagella. The functional importance of both polymodifications of tubulin has recently been highlighted by a number of experiments (reviewed by Rosenbaum, 2000). Xia et al., using in vivo mutagenesis of the modified glutamate residues, showed that polyglycylation of β-tubulin is essential in *Tetrahymena* (Xia et al., 2000). The lethality of the polyglycylation deficiency was due to a failure in assembling functional axonemes and a defect in cytokinesis caused by an incomplete severing of microtubules (Thazhath et al., 2002). Interestingly, trypanosomes have normal axonemes although they completely lack polyglycylation and are instead highly modified by tubulin polyglutamylation (Schneider et al., 1997).

In non-neuronal cells polyglutamylation is largely restricted to the microtubules of the centrioles, the mitotic spindle and the midbody (Bobinnec et al., 1998a). Introduction of the glutamylation specific antibody GT335 into HeLa cells caused a disassembly of centrioles and a transient disappearance of the centrosome as a defined organelle (Bobinnec et al., 1998b). Tubulin polyglutamylase activity as well as the overall level of glutamylated tubulin were shown to be under cell-cycle control

(Regnard et al., 1999) and a specific increase in β-tubulin glutamylation was observed during mitosis (Bobinnec et al., 1998a). The GT335 antibody also interfered with the motility of reactivated sperm axonemes, suggesting a function for tubulin polyglutamylation in flagellar motility (Gagnon et al., 1996). In vitro assays with blot overlays indicate that the length of the polyglutamyl side chain can differentially regulate the binding of microtubule associated proteins (Bonnet et al., 2001; Boucher et al., 1994). Moreover, the processiviy of conventional and single-headed kinesins were shown to be regulated by the interaction between conserved basic residues of the motor proteins with the acidic C-terminus of tubulin (Thorn et al., 2000; Okada and Hirokawa, 2000).

Of the various enzymes involved in tubulin PTMs, so far only the tubulin tyrosine ligase TTL (Ersfeld et al., 1993) and the mirotubule associated deacetylase HDAC6 (Hubbert et al., 2002) have been cloned. While HDAC6 is a member of the histone deacetylase family, TTL shares a fold with the glutathione synthetase ADP-forming family (Dideberg and Bertrand, 1998). We have turned to trypanosomes as a starting material for the purification of a tubulin polyglutamylase. The subpellicular and flagellar microtubules of trypanosomatids are extensively glutamylated (Schneider et al., 1997) and isolated cytoskeletons, obtained by detergent extraction, retain an enzymatic activity that incorporates glutamic acid into tubulin in an ATP-dependent manner (Westermann et al., 1999a). A tubulin polyglutamylase preparation from *Crithidia* accepts mammalian brain tubulin as a substrate and is also able to modify synthetic peptides representing the C-terminal residues of α and β tubulin (Westermann et al., 1999b).

Here we describe the cloning of the major component of a highly purified tubulin polyglutamylase preparation. We identify a 54 kDa polypeptide copurifying with tubulin glutamylation activity from *Crithidia* as a novel member of the NIMA family of putative cell cycle regulators. This finding is especially intriguing since NIMA related kinases from different organisms have been implicated in various aspects of microtubule organisation yet their molecular mechanism of action has largely remained elusive.

Materials and Methods

Cell culture

Crithidia fasiculata were cultured at 26°C in brain heart infusion medium (BHI, Difco, Detroit, USA) containing 4 μg/ml hemine and 200 μg/ml streptomycin sulfate. Following transfection of parasites, resistant cell lines were grown in BHI-medium containing 200 μg/ml hygromycin. For large scale preparations parasites were grown in 5 l Erlenmeyer flasks with gentle shaking.

Purification of tubulin polyglutamylase

Isolation of *Crithidia* cytoskeletons, solubilisation of tubulin glutamylation activity by salt extraction and subsequent purification steps were as described by Westermann et al. (Westermann et al., 1999a) with certain modifications. The purification was scaled up so that 9 l of *Crithidia* culture were typically used to generate the crude enzyme fraction. For ATP-affinity chromatography on a 4 ml Sepharose-sebacic acid ATP-column, the crude enzyme fractions obtained from three preparations (27 l of *Crithtidia* culture) were pooled. As the final purification step, glycerol gradient fractions containing glutamylation acitivity were pooled (~5 ml) and dialyzed against 10 mM sodium phosphate pH 6.8, 1 mM $MgCl_2$, 1 mM DTT for 1 hour. The partially purified glutamylase was applied on a 1 ml CHT-2 hydroxyapatite column (Bio-Rad, Munich, Germany) at a flow rate of 0.6 ml/minute. The column was developed with a 16 ml linear gradient from 10 mM to 400 mM sodium phosphate, 0.5 ml fractions were collected and 10 μl aliquots were tested for glutamylation activity. Fractions containing glutamylation activity were supplemented with 10% glycerol and 0.1 mg/ml soybean trypsin inhibitor (Sigma, Deisenhofen, Germany), quick frozen in liquid nitrogen and stored at –80°C.

Cloning of p54

Multiple preparations of tubulin polyglutamylase were used to excise the 54 kD band from SDS-polyacrylamide gels. The peptides resulting from an in situ digest with endoproteinase LysC were separated by reversed phase HPLC and sequenced by automated Edman degradation.

Total RNA was isolated from logarithmically growing *Crithidia* cells using the TRIzol reagent (Gibco-BRL, Karlsruhe, Germany). To obtain the corresponding cDNA, RT-PCR was performed using Superscript reverse transcriptase (Gibco-BRL) and an oligo-(dT) primer.

The sequence information from the p54 peptides was used to synthesise degenerate antisense primers that were used in different combinations in PCRs against a spliced leader primer (SL: 5′-CGCTATAAATAAGTATCAGTTTCTGTACTTTATTG-3′) that is directed against a common sequence at the 5′ end of all *Crithidia* mRNAs (Muhich et al., 1987). The following combination gave a correct PCR product: In the first PCR round the primer SW 46 (5′-ACYTCIGGIACIGGRAANACRTC-3′ with R=A/G, Y=T/C, N=A/G/T/C, based on peptide DVFPVPEV) was used against SL and the product was reamplified using the nested Primer SW 42 (5′-TCYTTIGGIGGIATNGTYTCNGA-3′ based on peptide SETIPPKD). The resulting product was gel-purified, cloned into vector pCR2.1 (Invitrogen, Groningen, The Netherlands) and custom-sequenced (MWG-Biotech, Ebersberg, Germany). Based on the established 5′ sequence, two gene-specific primers, SW 47 (5′-AGCATGAAGGCGCTGCTGGACCCGC-3′) and SW 48 (5′-CGACGCAGCAGCTGCTCCAGACAGAG-3′) were synthesized and used in a 3′-RACE-PCR (Gibco-BRL) to obtain the 3′ end of the p54 cDNA.

Expression of recombinant CfNek in *Crithidia*

The full length p54 cDNA was amplified using primers pNus1 (5′-CAGGGAAACATATGCCGACCAACAAGGATGACAAG-3′) and pNus2 (5′-CCTCGGTACCT-CACATGCCACAGGCGCGGT-GAAAC-3′, restricition sites for *Nde*I and *Kpn*I underlined) and inserted into the respective site of the pNUS-HnH expression vector (Tetaud et al., 2002). The vector contains an N-terminal His-tag sequence and a hygromycin resistance gene for the selection of transfected parasites.

Electroporation of *Crithidia* cells was performed as described (Tetaud et al., 2002): Parasites were washed twice in cold BHI-Medium and resuspended at a density of 2×10^7 cells/ml in a 0.4 cm electroporation cuvette containing 30 μg plasmid-DNA. The gene pulser apparatus (Bio-Rad, Munich, Germany) was set to 450 V and 500 μF capacitance. The mixture was subjected to a single pulse, and after 10 minutes on ice the cells were transferred to 5 ml BHI-medium containing 10% FCS and incubated 5 hours at 26°C to allow for recovery. Hygromycin B (Gibco-BRL, Karlsruhe, Germany) was added at a final concentration of 50 μg/ml and after one week the cells were subcultured into BHI-medium containing 200 μg/ml hygromycin.

Purification of His-tagged CfNek

Hygromycin resistant cells were used to prepare a crude enzyme

fraction as described previously (Westermann et al., 1999a). The 0.25 M salt extract was diluted 1:1 with 20 mM Tris pH 8.5, 500 mM KCl, 20 mM imidazole, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol and applied at a flow rate of 0.5 ml/minute on to a 0.5 ml Ni-NTA (Qiagen, Hilden, Germany) column equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl pH 8.5, 1 M KCl, 5 mM 2-mercaptoethanol, 10% glycerol. Finally, the His-tagged protein was cleaved from the column by incubating the resin overnight at 4°C with 20 U thrombin (Amersham Pharmacia, Freiburg, Germany) in 20 mM Tris-HCl pH 8.5, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10% glycerol.

Tubulin polyglutamylase and kinase assays

Tubulin polyglutamylase activity was measured as described (Westermann et al., 1999a) using the filter disc method and brain tubulin as a substrate.

Kinase reactions (25 μl) were carried out in a standard kinase buffer containing 20 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$ and 5 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Hartmann Analytic, Braunschweig, Germany). As an artificial substrate β-casein (Sigma, Deisenhofen, Germany) was used at a final concentration of 1 mg/ml. The kinase reactions were incubated at 30°C for 30 minutes, stopped by the addition of SDS sample buffer and loaded on a 10% SDS polyacrylamide gel. The gel was dried and exposed overnight for autoradiograpy.

Immunofluorescence of *Crithidia* cells

Crithidia cells were pelleted, washed twice with PBS and spotted (25 μl, 5×10^5 cells) on poly-L-lysine coated cover slips. Cells were allowed to adhere for 10 minutes and then fixed in methanol at –20°C for 1 hour. Slides were rehydrated in PBS and incubated with primary antibody (Penta-His Antibody, Qiagen, Hilden, Germany, diluted 1:33 in 0.5 mg/ml BSA in PBS) for 1 hour at 37°C. Slides were washed three times with PBS and then incubated with secondary antibody (goat anti mouse rhodamine-conjugated, DAKO, diluted 1:80) for 45 minutes. Slides were given three 5 minute washes in PBS and DNA was stained with Hoechst 33342. Finally, the slides were mounted in Mowiol and examined with a Zeiss Axiophot microscope.

Results

A modified purification scheme for tubulin polyglutamylase

We have previously shown that isolated trypanosomal cytoskeletons retain a tubulin polyglutamylase which is solubilised by 0.25 M salt. The enzyme can be highly enriched using ATP-affinity chromatography, glycerol gradient centrifugation and anion exchange chromatography. Our efforts to identify polypeptide(s) involved in tubulin polyglutamylation were frustrated by the presence of a contaminating 40 kD band in the most purified fractions (Westermann et al., 1999a). This protein was subsequently identified as a trypanosomal homolog of the La RNA binding protein which does not confer any glutamylation activity (Westermann and Weber, 2000). Consequently, we upscaled the purification scheme and replaced the final monoQ step by a hydroxyapatite chromatography on a CHT-2 column. A flow chart of the novel purification scheme is given in Fig. 1A. Prior to hydroxyapatite chromatography the enzyme preparation was dialyzed only briefly (1 hour) to avoid greater losses in

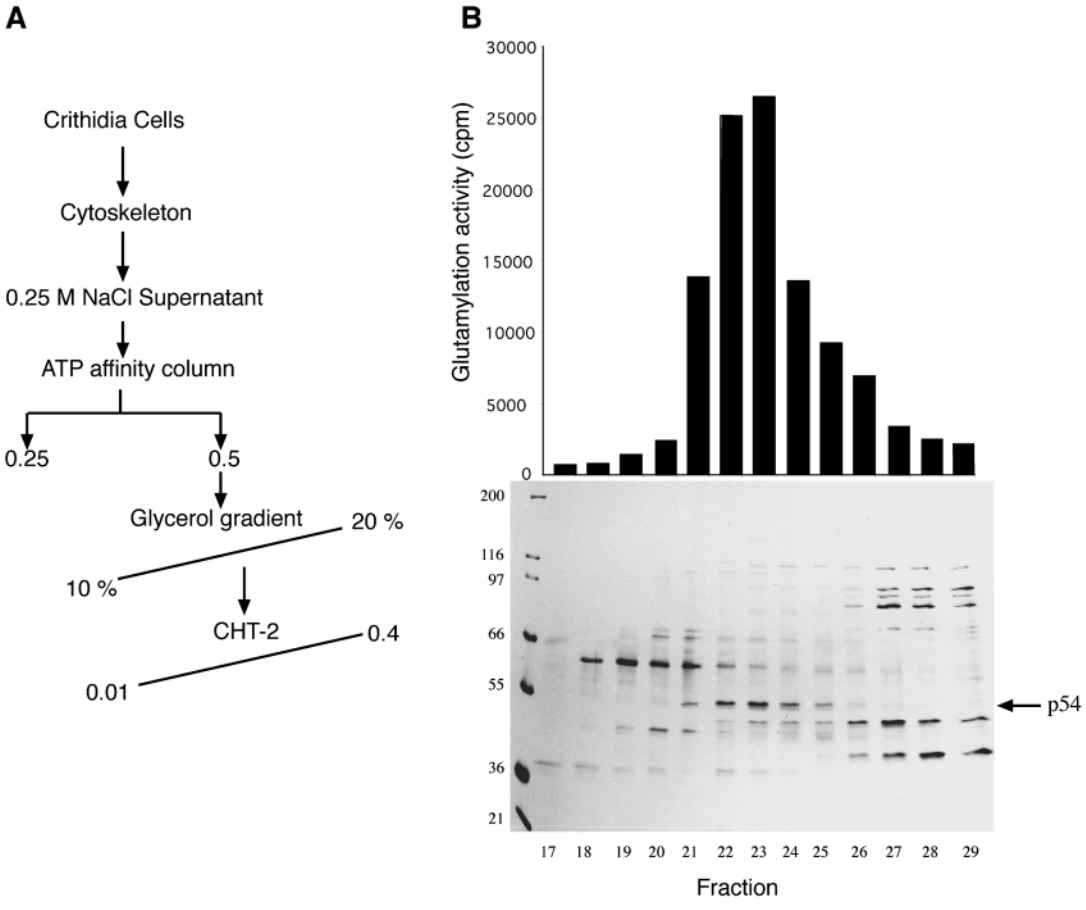

Fig. 1. A modified purification scheme for tubulin polyglutamylase identifies *Crithidia* p54. (A) Flow chart of the purification scheme using hydroxyapatite chromatography on a CHT-2 column as the final step. (B) Aliquots (30 μl) of successive fractions (500 μl) eluting between 100 and 200 mM sodium phosphate from the CHT-2 column were subjected to SDS-PAGE and proteins were visualised by silver staining. The first lane shows marker proteins with molecular masses in kDa. A 10 μl aliquot of each fraction was also tested for tubulin glutamylation activity using the standard assay (upper panel). Note that a 54 kDa polypeptide copurifies with the glutamylation activity (arrow).

enzymatic activity. The glutamylation activity bound quantitatively to the CHT-2 column and consistently eluted as a sharp peak at 160 mM sodium phosphate. 10 μl aliquots of the column fractions were assayed for glutamylation activity while 20 μl aliquots were analyzed by SDS-PAGE. Fig. 1B displays a silver stained gel of the column fractions together with the corresponding tubulin glutamylation activity profile. A 54 kD band was the only visible polypeptide which mirrored the activity profile of the CHT-2 fractions. Attempts to further purify the hydroxyapatite fraction (e.g. by gel filtration on a TSK 3000SW column) were unsuccessful, presumably due to the low protein concentration of the sample. The highly purified CHT-2 fraction could be stored at –80°C after addition of 10% glycerol and retained activity for several months. Using the monoglutamylation assay described by Westermann et al. (Westermann et al., 1999b) which employs unglutamylated yeast tubulin and the GT335 antibody, we also found that the CHT-2 fraction was able to catalyse the initiation of a novel side chain (data not shown).

Cloning of the p54-cDNA

We obtained amino acid sequences of internal p54 peptides and used them to design degenerate antisense oligonucleotide primers. To clone the 5′ end of the p54-cDNA these primers were used in combination with a spliced leader primer in a RT-PCR with total RNA as a template (Fig. 2A). The established nucleotide sequence was then used to design specific sense primers for a conventional 3′RACE-PCR to obtain the full-length cDNA. The p54 cDNA structure is shown in Fig. 2A: the spliced leader sequence is followed by a 423 bp long 5′ UTR which contains 3 stop codons in the same reading frame as the starting methionine. The open reading frame spans 1437 nucleotides and is followed by a 1.7 kb long 3′ UTR and the polyA-tail. Conceptual translation of the open reading frame predicts a protein of 479 amino acid residues with a calculated molecular weight of 54.9 kDa which is in good agreement with the observed size of the protein in SDS-PAGE. The isoelectric point of the protein is pI=6.9 (Fig. 2B) and the amino acid sequence contains all 10 peptide sequences originally obtained from p54. The cDNA sequence and the amino acid sequence of p54 are deposited in GenBank under Acc.No. AJ494838.

p54 is a NIMA related kinase

A Blast search of the *Crithidia* p54 amino acid sequence revealed a high homology to a predicted protein sequence obtained during the genome project of the related trypanosomatid *Leishmania major* (acc. no. QKT3). This sequence, annotated as *Leishmania*-NIMA related-Kinase 1 (LNK-1), showed 88.6% identity to *Crithidia* p54 over the entire polypeptide. Both proteins contain a serine/threonine kinase domain (residues 70 to 325 of p54) related to the catalytic domain of the NIMA protein, which was originally identified as a mitotic regulator necessary for G2/M transition in *Aspergillus nidulans* (Osmani et al., 1988). The domain structure of *Crithidia* p54 (which we named CfNek for *Crithidia fasciculata* NIMA-related kinase) and *Aspergillus* NIMA is shown in Fig. 2C. The similarity between both proteins is largely restricted to the catalytic domain (36% identity), but both proteins contain PEST-sequences commonly found in proteins targeted for rapid degradation (Rechsteiner and Rogers, 1996). The PEST sequence of CfNek is located within the aminoterminal domain of the protein (residues 54 to 65) while the two PEST sequences of NIMA reside in the carboxyterminal extension. Both proteins share basic C-terminal domains (residues 325-479 of CfNek have a pI of 9.6) which are a structural feature of all NIMA-related kinases. The C-terminal domain of CfNek also harbours a

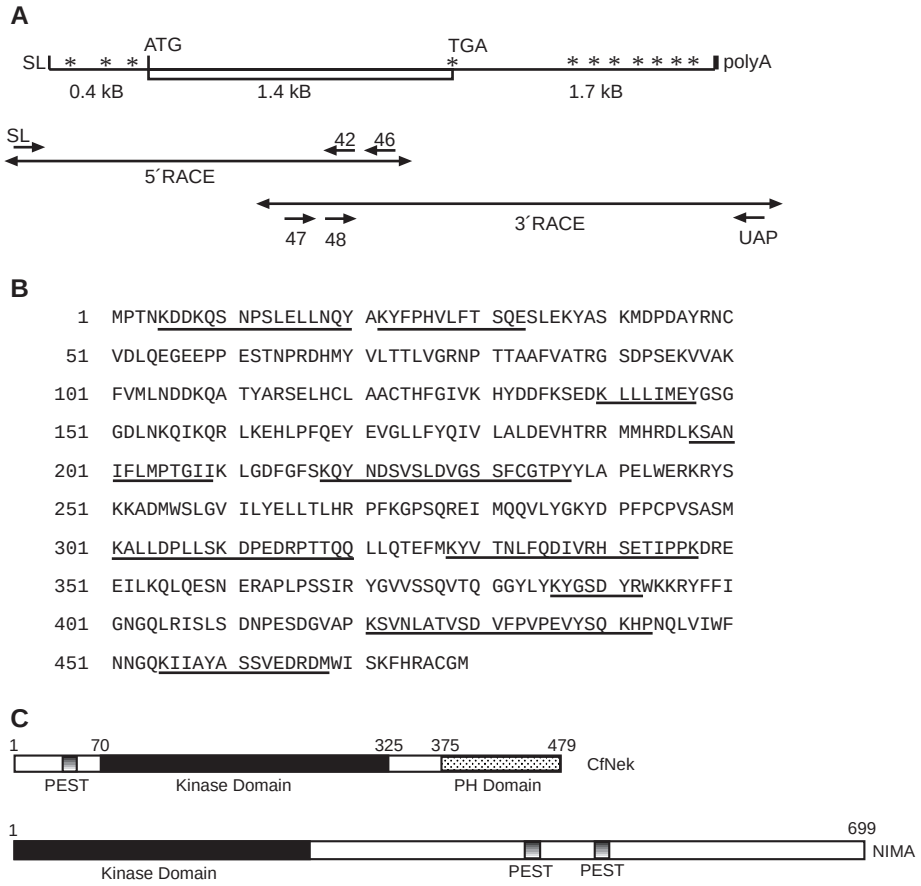

Fig. 2. Cloning the p54 cDNA. (A) Schematic representation of the p54 cDNA and the cloning strategy by 5′ and 3′ RACE (see Materials and Methods for details). SL denotes the spliced leader sequence, UAP is a universal amplification primer. In frame stop codons are depicted by asterisks. (B) Deduced amino acid sequence of *Crithidia* p54. Amino acids identified from direct peptide sequencing are underlined. The cDNA and amino acid sequences have been deposited in GenBank under accession no. AJ494838. (C) Schematic representation of the *Crithidia* p54 (CfNek) domain structure in comparison to the original NIMA protein from *Aspergillus nidulans*.

pleckstrin homology (PH) domain which is found in a number of proteins implicated in intracellular signalling.

Next to the *Leishmania* kinase, p54 is most closely related to NrkA, a NIMA related kinase from *Trypanosoma brucei* (Gale and Parsons, 1993). The two proteins display 46% sequence identity over the catalytic domain, both contain the PH domain at the C-terminal end, but NrkA lacks a PEST-sequence. Fig. 3A shows an alignment of the catalytic domain of CfNek with NIMA related kinases from different species. Strikingly, the *Crithidia* and the *Leishmania* proteins lack one of the characteristic features of protein kinases, namely the glycine rich loop GXGXXG in kinase subdomain I. This sequence is involved in the binding and orientation of ATP (Hanks and Hunter, 1995). The catalytic loop RDXXXXN in subdomain VI, on the other hand, is completely conserved in all Neks. While Neks normally have their catalytic domain at the extreme N-terminus, the catalytic domains of CfNek and LNK-1 start 70 residues downstream of the N-terminus. A phylogenetic analysis of the kinase domains shows that the three trypanosomatid kinases cluster into a subfamily within the NIMA group (Fig. 3B).

Native glutamylase fractions display phosphorylation activity

The finding that p54 is a NIMA-related kinase prompted us to investigate the kinase activity of our tubulin polyglutamylase preparation. While few physiological targets of Neks are

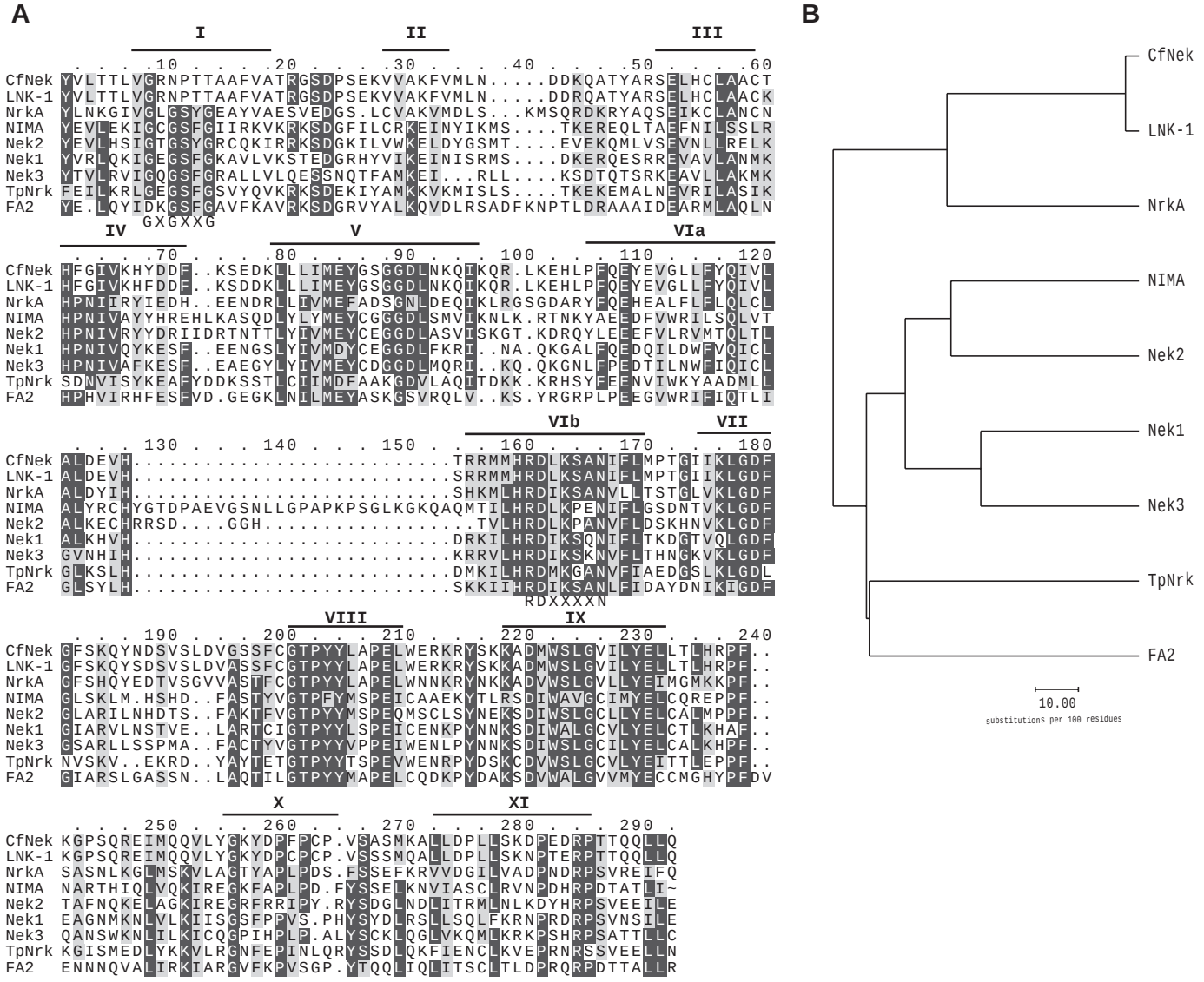

Fig. 3. (A) Amino acid sequence alignment of the kinase domains of CfNek and other NIMA-related kinases. The alignment was generated using PILEUP (GCG-package) and the output was shaded with Macboxshade. Residues conserved in >60% of the proteins are shaded in black, simililar residues are shaded in light grey. The eleven kinase subdomains are denoted above the sequence. Sequences used: *Leishmania major* LNK-1 (Q9NKT3), *Trypanosoma brucei* NrkA (Q08942), *Aspergillus nidulans* NIMA (P11837), *Homo sapiens* Nek2 (P51955), *Mus musculus* Nek1 (P51954) and Nek3 (Q9R0A5), *Tetrahymena pyriformis* TpNrk (O76134), *Chlamydomonas reinhardtii* FA2 (AF479588). The CfNek sequence is from Fig. 2B. (B) Phylogenetic tree derived from the alignment in A. Using the PILEUP output the tree was generated with the GCG-programs DISTANCE and GROWTREE.

known, β-casein is a good artificicial substrate for several NIMA related kinases (Lu et al., 1993). Native glutamylase fractions from the final hydroxyapaptite chromatography were assayed for kinase activity. Fig. 4 shows that these fractions displayed β-casein phosphorylation activity. Moreover, the kinase activity profile exactly mirrored the glutamylation activity profile of the corresponding fractions from the hydroxyapatite column.

Expression and localization of His-tagged CfNek in *Crithidia*

The complete CfNek cDNA was expressed in *E. coli* as well as in SF9 cells using a recombinant baculovirus. The recombinant proteins were purified and assayed for glutamylation and phosphorylation activity. In contrast to the native glutamylase fraction from *Crithidia*, neither bacterially nor baculovirus expressed recombinant CfNek displayed casein-phosphorylation or tubulin glutamylation activity (data not shown). We therefore turned to a recently described expression vector for *Crithidia fasciculata* (Tetaud et al., 2002). The pNusHnH vector drives the expression of introduced genes while it is maintained as an extrachromosomal plasmid (episome) which confers resistance to the antibiotic hygromycin.

The CfNek cDNA was cloned into pNusHnH in frame with a N-terminal poly His-sequence. Parasites were transfected by electroporation and selected in liquid culture with 200 μg/ml hygromycine. His_6-CfNek expressing cells, as well as control cells transfected with vector only, were examined by immunofluorescence microscopy using a monoclonal His-tag antibody. Fig. 5 shows that only CfNek expressing parasites displayed a characteristic staining with the anti-His antibody. The cells were labelled strongly at the point where the flagellum is attached to the cell body. In some cells the staining ran along the length of the flagellum but was always stronger at the base than at the tip. An immunblot performed on crude extracts showed that the antibody detected a protein of of the expected size only in the cells transfected with CfNek (Fig. 5C). We conclude that, as judged by immunofluorescence, the recombinant CfNek expressed in *Crithidia* seems to localise to the flagellar attachment zone and presumably to the basal body.

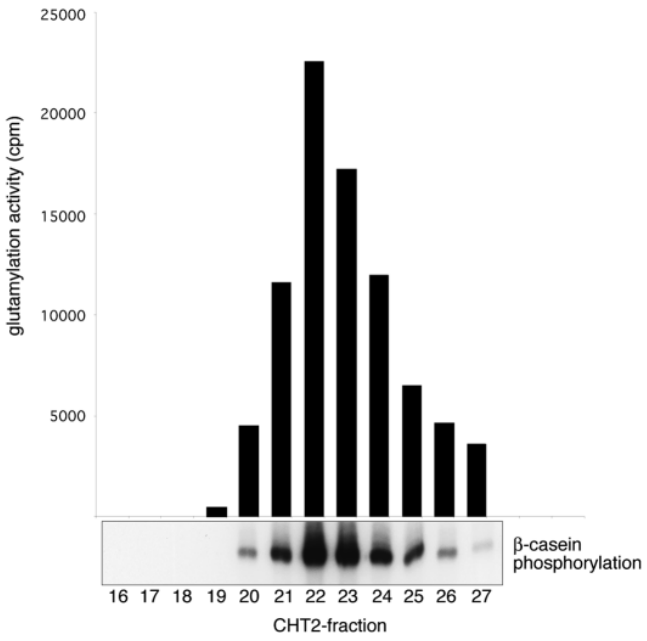

Fig. 4. The tubulin polyglutamylase preparation displays β-casein phosphorylation activity. 10 μl aliquots of successive fractions from the final hydroxyapatite chromatography were assayed for tubulin polyglutamylation activity while 5 μl aliquots were used in kinase assays with β-casein as substrate. The kinase reactions were run on an SDS-gel and analysed by autoradiography. Note that the β-casein phosphorylation activity exactly coelutes from the CHT-2 column with the tubulin glutamylation activity.

Purification of His_6-CfNek from *Crithidia*

We used the introduced His-tag to purify recombinant CfNek from *Crithidia* cells. A crude enzyme fraction obtained from 6 l of CfNek expressing cells was applied on to a Ni^{2+} column. The resin was washed with 1 M NaCl and the recombinant protein was eluted by thrombin cleavage, which leaves the His-tag bound to the column while the remainder of the protein is found in the eluate. Fig. 6A shows a silver stained gel of the thrombin eluate from CfNek expressing and wild type cells. While a contaminating triple band of about 98 kDa was found in both preparations, only the CfNek eluate displayed a protein with the expected size of 55 kDa. Both eluates were assayed for kinase activity and only the CfNek eluate showed significant β-casein phosphorylation activity (Fig. 6B). We also investigated the tubulin polyglutamylation activity of both fractions. Only the CfNek containing eluate catalyzed the incorporation of glutamic acid into TCA-precipitable tubulin (Fig. 6C). To confirm that the observed incorporation of radioactivity was due to tubulin polyglutamylation a series of assays were conducted in which different components of the reaction mixture were omitted. As expected the incorporation of glutamic acid was dependent on the presence of the enzyme fraction, tubulin and ATP (Fig. 6D).

Discussion

We report the first cloning of an enzyme present in a highly purified preparation of tubulin polyglutamylase. We used the 0.25 M NaCl supernatant of isolated cytoskeletons from the trypanosomatid *Crithidia fasciculata* as a starting material and purified the glutamylation activity by ATP-affinity chromatography, glycerol gradient centrifugation and hydroxyapatite chromatography. Due to its low abundance and a pronounced instability it was not possible to obtain a homogenous preparation, but we identified a 54 kDa band as the major polypeptide visible on silver-stained gels which clearly copurified with the glutamylation activity. While tubulin polyglutamylase partially purfied from mouse brain seems to be organised in a multimeric structure of ≥300 kDa (Regnard et al., 1998), we did not find any bands coeluting stoichiometrically with p54 or any other indications for a multi protein complex involved in tubulin polyglutamylation. Moreover, a 54 kDa polypeptide is in reasonable agreement with the sedimentation constant of approximately 3 S which was obtained for *Crithidia* tubulin polyglutamylase by glycerol gradient centrifugation (Westermann et al., 1999a).

The candidate glutamylase is a NIMA related kinase

Cloning of the p54 cDNA clearly identified the enzyme as a novel trypanosomal member of the NIMA family of mitotic kinases (CfNek). The original NIMA enzyme from *Aspergillus* was identified as a factor critical for G2/M transition (Osmani et al., 1988). Detailed analysis of NIMA function in *Aspergillus* revealed that elevated NIMA activity, dependent on phosphorylation by $p34^{cdc2}$/cyclinB (Ye et al., 1995) is necessary for the correct organization of spindle microtubules and for the integrity of the nuclear envelope (Osmani et al.,

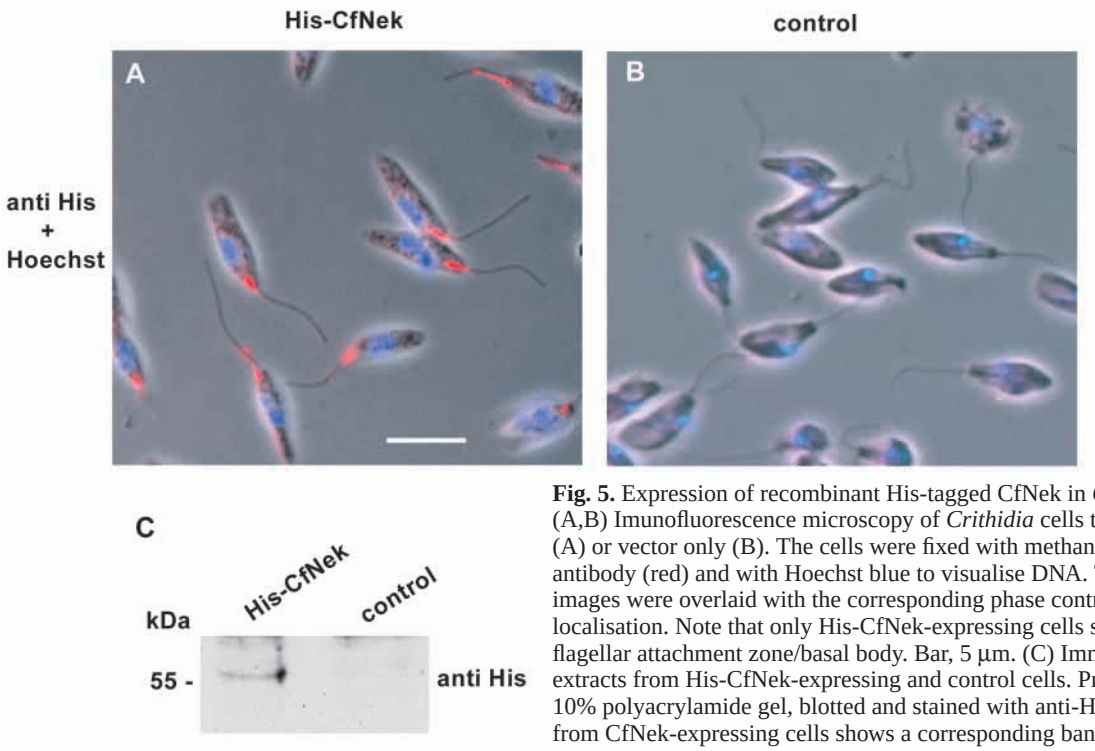

Fig. 5. Expression of recombinant His-tagged CfNek in *Crithidia*. (A,B) Imunofluorescence microscopy of *Crithidia* cells transfected with His-CfNek (A) or vector only (B). The cells were fixed with methanol and stained with a His-antibody (red) and with Hoechst blue to visualise DNA. The immunofluorescence images were overlaid with the corresponding phase contrast images to allow easier localisation. Note that only His-CfNek-expressing cells show a staining of the flagellar attachment zone/basal body. Bar, 5 μm. (C) Immunoblot analysis of total cell extracts from His-CfNek-expressing and control cells. Proteins were separated on a 10% polyacrylamide gel, blotted and stained with anti-His antibody. Only the extract from CfNek-expressing cells shows a corresponding band of 56 kDa.

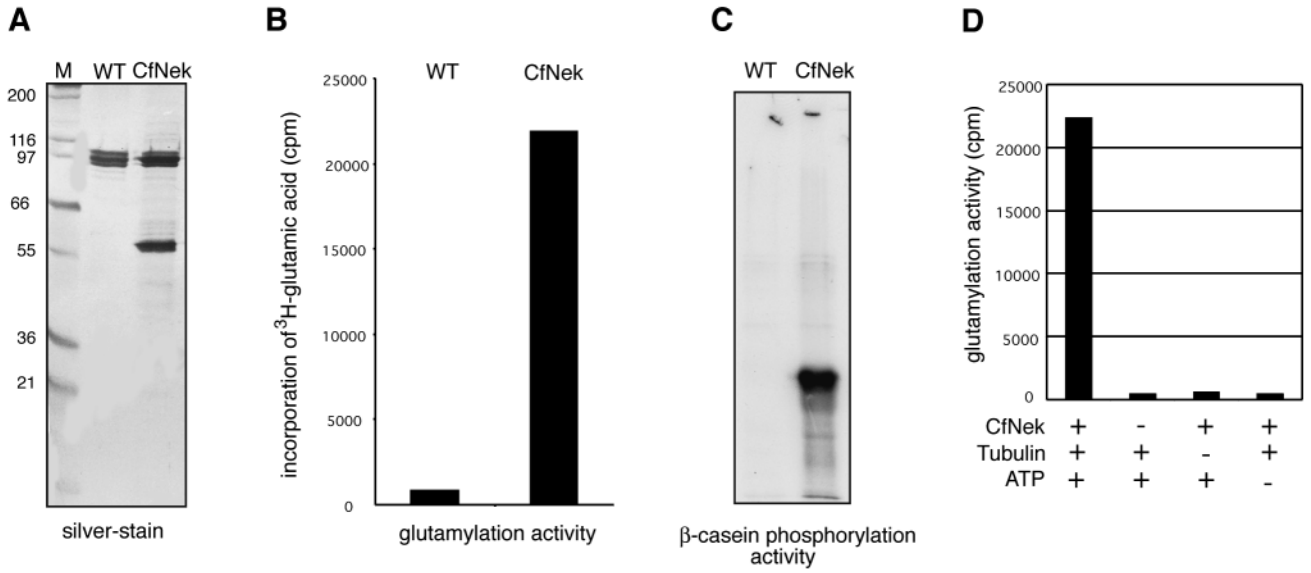

Fig. 6. Purification and analysis of His-tagged CfNek from *Crithidia*. (A) Purification of His-CfNek over a Ni-column. 10% polyacrylamide gel stained with silver of the thrombin eluate from wild-type (WT) or CfNek-expressing cells. Note that a contaminating triple band of about 98 kDa is present in both eluates while only the CfNek eluate contains the expected 56 kDa polypeptide. (B) 5 μl aliquots of the thrombin eluates from A were assayed for tubulin polyglutamylation activity. Only the CfNek eluate displays significant tubulin polyglutamylation activity. (C) 5 μl aliquots of the thrombin eluates from A were used in kinase assays with β-casein as substrate. The kinase reactions were run on an SDS-gel and analyzed by autoradiography. Again, only the CfNek eluate catalyses the incorporation of radioactve phosphate into β-casein. (D) 5 μl aliquots of the CfNek eluate from A were used in different glutamylation assays with the indicated components. Glutamylation activity is only observed with CfNek, tubulin and ATP in the reaction mixture.

1991). Recent evidence suggests that NIMA is a mitotic histone H3 kinase, which also localises to spindle microtubules and spindle pole bodies (DeSouza et al., 2000). The fact that overexpression of NIMA in mammlian cells caused premature mitotic events such as chromosome condensation (Lu and Hunter, 1995) suggested the existence of a NIMA like pathway in vertebrates. The relationship between NIMA and several mammlian Neks, however, has remained unclear, as the only protein that could complement the original *nimA*-mutation, was nim-1 from the related fungus *Neurospora crassa* (Pu et al., 1995). Of the various mammalian Neks, Nek2 is most closely related to NIMA (Fig. 3B) and part of its function affects the centrosome cycle as overexpression causes premature splitting of the centrosome while expression of dominant negative Nek2 leads to loss of centrosome integrity (Fry et al., 1998). A centrosome-related function has also been reported for the *Xenopus* Nek2 homolog (Uto and Sagata, 2000; Fry et al., 2000) and another related kinase is involved in the formation of microtubule organizing centres in *Dictyostelium* (Gräf, 2002).

Crithidia p54 has a clear homolog in the predicted *Leishmania major* protein LNK-1 (Fig. 3A), while the similarity to *Trypanosoma brucei* NrkA is significantly reduced, leaving the question open whether these proteins are true homologs. All three trypanosomatid proteins, however, share the pleckstrin homology domain at the C-terminus. The unusual catalytic domain which lacks the glycine-rich loop in subdomain I is restricted to the *Crithidia* and the *Leishmania* protein (Fig. 3A). We do not know whether this unique sequence has consequences for the catalytic activity, but we note that a special ATP-affinity resin with a long spacer had to be used for the purification of the glutamylation activity (Westermann et al., 1999a).

Properties of recombinant CfNek expressed in *Crithidia*

When expressed in *E. coli* or insect cells we did not observe phosphorylation or glutamylation activity of the purified recombinant CfNek. The failure to obtain an active enzyme by expression in a heterologous system could be due to a misfolded protein or to the lack of some activation step. NIMA for example has been shown to depend on the phosphorylation by $p34^{cdc2}$/cyclinB for full enzymatic activity (Ye et al., 1995). As judged by immunofluorescence, only upon expression in *Crithidia* was a specific localisation of the recombinant protein to the basal body/flagellar attachment zone of the parasite observed. Future studies, involving the generation of antibodies and the use of immuno-electronmicroscopy on extracted cytoskeletons, will have to establish the precise localisation of the endogenous CfNek. When the enzyme was expressed in HeLa cells only a cytoplasmic staining was seen in immunofluorescence (our unpublished observations). More importantly, basal bodies are structures homolgous to centrioles and are known to contain highly glutamylated microtubules (Geimer et al., 1997). We also observed CfNek staining along the flagellum which is interesting in light of the fact that a polyglycylation deficient mutant in *Tetrahymena* shows defects in axonemal architecture with large gaps in the flagellar transition zone (Thazhath et al., 2002). As trypanosomes lack glycylated tubulins (Schneider et al., 1997) but have functional axonemes, it seems likely that they compensate for the essential function of tubulin glycylation by generating a high level of tubulin glutamylation.

We did not observe any obvious effects on growth rate or motility of the *Crithidia* cells when expressing the recombinant CfNek. The pNus expression vector lacks promotor sequences and therefore the introduced gene is expressed only at a moderate level (Tetaud et al., 2002). With the future development of novel vectors allowing overexpression of genes in *Crithidia* it will be possible to investigate the consequences of a highly increased CfNek concentration.

Possible relationship between glutamylation and NIMA related kinases

Surprisingly, the amino acid sequence identifies the p54 polypeptide copurifying with glutamylation activity as a phosphotransferase and recombinant CfNek displays casein-phosphorylation activity. As we were unable to obtain an active enzyme preparation by expression in a heterologous system, we cannot rule out the possibility that CfNek does not directly catalyse the glutamylation reaction but that the actual glutamylase is instead associated with CfNek and possibly regulated through phosphorylation. On the other hand, it is tempting to speculate that the glutamylation reaction could directly require the phosphotransferase activity of CfNek as the generation of a peptide bond is likely to proceed via the generation of an intermediary acylphosphate. NIMA related kinases comprise a group of biochemically distinct kinases that can transfer a phosphate group within an acidic environment. We noted previously that upon incubation with partially purified polyglutamylase, synthetic C-terminal tubulin peptides became both glutamylated and serine-phosphorylated (Westermann et al., 1999a; Westermann et al., 1999b). Thus, a definite decision whether CfNek and tubulin polyglutamylase are identical or associated will need further experimentation, for example the knock-out of the homologous *Leishmania* protein. As centrosome stability depends upon tubulin polyglutamylation (Bobinnec et al., 1998b) and Nek2 kinases from different organisms are involved in centrosome maturation and integrity (Fry et al., 1998; Uto and Sagata, 2000) these enzymes appear good candidates to be tested for glutamylation activity in the future.

Conclusions

We identify an unusual protein kinase of the NIMA family as the first enzyme involved in tubulin polyglutamylation. We base this conclusion on four observations: First, CfNek was cloned as the major polypeptide copurifying with glutamylation activity from *Crithidia* (Fig. 2A). Second, a preparation of recombinant His-tagged CfNek purified from *Crithidia* displays casein-phosphorylation and tubulin glutamylation activity (Fig. 4). Third, as judged by immunofluorescence, the recombinant His-tagged enzyme localizes to sites (basal body/flagellar attachment zone, Fig. 5) known to be critically regulated by glutamylation (Bobinnec et al., 1998a; Bobinnec et al., 1998b). Fourth, NIMA related kinases from other organisms have been shown to perform functions related to tubulin polyglutamylation including centrosome maturation and stability, spindle integrity and cell cycle control (Fry et al., 1998; DeSouza et al., 2000). The

cloning of the first candidate glutamylase from *Crithidia* indicates an unsuspected link between a post-translational tubulin modification and a group of mitotic kinases which deserves further investigation.

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