Cloning, molecular analysis and differential cell localisation of the p36 RACK analogue antigen from the parasite protozoon *Crithidia fasciculata*

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Abstract The family of the RACK molecules (receptors for activated C kinases) are present in all the species studied so far. In the genus *Leishmania*, these molecules also induce a strong immune reaction against the infection. We have cloned and characterised the gene that encodes the RACK analogue from the parasite trypanosomatid *Crithidia fasciculata* (CACK). The molecule seems to be encoded by two genes. The sequence analysis of the cloned open reading frame indicates the existence of a high degree of conservation not only with other members of the Trypanosomatidae but also with mammalians. The study of the protein kinase C phosphorylation sites shows the presence of three of them, shared with the mammalian species, additional to those present in the other protozoa suggesting a certain phylogenetic distance between the protozoon *Crithidia fasciculata* and the rest of the Trypanosomatidae. The CACK-encoded polypeptide shows an additional sequence of four amino acids at the carboxy-terminal end, which produces a different folding of the fragment with the presence of an α-helix instead of the β-sheet usual in all the other species studied. A similar result is elicited at the amino-terminal end by the change of three amino acid residues. The immunolocalisation experiments show that the CACK displays a pattern with a distribution mainly at the plasma membrane, different from that of the related *Leishmania* species used as control, that displays a distribution close to the nucleus. Altogether, the data suggest that the existence of the structural differences found may have functional consequences.

Key words: p36 RACK analogue; Protein kinase C activation; *Crithidia fasciculata*

1. Introduction

Crithidia fasciculata is a parasite protozoon that belongs to the family of Trypanosomatidae. The members of this family are mostly parasite protozoa that cause different diseases in a wide range of organisms from plants to humans [1,2]. The family of the RACK proteins (receptors for activated C kinases) are present in many species and are not substrates for protein kinase C (PKC) but increase the phosphorylation of the substrates by stabilising the PKC active form [3]. These proteins are analogues of the β subunits of the G proteins and belong to the family of the WD 40 repeat proteins involved in regulatory functions [4] which have been isolated from different species [5,6].

Interestingly enough, in addition to its physiological role these molecules are able to elicit a strong immune response against the infection produced by the parasites of the related genus *Leishmania* in the animal model [5]. This protective activity, which is dependent on MHC class II molecules, seems to have a relationship to the ability of the RACK molecules to modify the balance between the two Th1/Th2 of $CD4^+$ lymphocyte subpopulations by inducing a down-regulation of the Th2 population [7] and producing the subsequent proliferation of the Th1 subset which leads to the production of the cytokine interleukin-12. These facts are concomitant with the eliciting of a strong immune response against the parasite infection [8–10]. Previous studies in the species *Leishmania infantum* have shown that the RACK analogue LACK (*Leishmania* homologue of receptors for activated C kinase) may be involved in the DNA metabolism and in the early steps of the mechanism of the immune response through its ability to bind DNA replication and RNA synthesis proteins as well as to proteins involved in the mechanisms of antigen presentation [11]. These results suggest the role as regulatory proteins for the members of the RACK family in the Trypanosomatidae.

In this paper we report the cloning of the encoding gene of the RACK analogue from the parasite protozoon *Crithidia fasciculata*. We found that the CACK (*Crithidia* homologue of receptors for activated C kinase) protein is a polypeptide of 35 kDa encoded by an open reading frame of 948 bp with the probable existence of another non-identical gene. The structure of genes arranged in tandem is frequent for these proteins in the related genus *Leishmania* [11]. This protein is very conserved with respect to other species, not only Trypanosomatidae but also mammalians. However, it displays interesting structural differences which may be responsible for a distinct functional behaviour such as their binding ability to different protein kinase C isoforms which have been described in other species [3]. The existence of similarities with mammalian molecules in the PKC phosphorylation sites may indicate a certain phylogenetic distance with other genus of the Trypanosomatidae. The possible relationship between the structural differences found and the display of distinct functional features in related parasite protozoan species is discussed.

2. Materials and methods

2.1. Parasite strains and culture media

C. fasciculata and *L. infantum* (MHOM/FR/80/LEM 75) parasites

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Abbreviations: CACK, *Crithidia fasciculata* analogue of the receptor for activated protein kinase C (RACK); DAPI, 4,6-diamidine-2-phenylindole dihydrochloride; SSC, sodium citrate/sodium acetate buffer; PMSF, phenylmethylsulphonyl fluoride; ECL, enhanced chemiluminescence

Nucleotide sequence data reported in this paper have been submitted to the GenBank data base with the accession number AFO63022.

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were kindly provided by Drs A. Toraño and J. Alvar respectively (Instituto de Salud Carlos III, Majadahonda, Madrid, Spain). Parasite protozoa were cultured in vitro at 28°C in RPMI 1640 modified medium (Gibco, Middlesex, UK) supplemented with 10% heat inactivated foetal calf serum (Flow Laboratories, UK). For most experiments, parasite cultures were initiated at 1×10^6 parasites/ml and harvested in logarithmic or stationary phase of growth, corresponding to 2–6 days of culture respectively, as defined by morphology and cell concentration [12]. *L. infantum* promastigotes were cultured as previously described [13].

2.2. PCR amplification of the p36 CACK protein

Two oligonucleotides designed according to the LACK protein from *L. infantum* were used to amplify by PCR the complete coding region of the p36/CACK protein from *C. fasciculata*. The forward primer was 5′-GGAATTCACCATGAACTACG-3′ and the reverse was 5′-GGGAAGCTTTTACTCGGCGTCGGAGAT-3′ [11]. The first primer contains the ATG initiation codon whereas the second contains the TAA stop codon. PCR conditions: 1 cycle of 95°C for 5 min, 41°C for 2 min, 72°C for 3 min; 30 cycles of 95°C for 1 min, 41°C for 2 min, 72°C for 3 min and 1 cycle of 95°C for 1 min, 41°C for 2 min and 72°C for 15 min. A 948 bp fragment that contained the whole sequence of the encoding gene was amplified from genomic *C. fasciculata* DNA, gel purified and sequenced. The DNA fragment was labelled with [α-^{32}P]dCTP by the random primer method according to the manufacturer's instructions (Boehringer). DNA sequencing was carried out using the dideoxy chain method on an automated DNA sequencer (ABI PRISM Dye Terminator cycle sequencing with AmpliTaq DNA polymerase, Perkin Elmer).

2.3. Nucleic acid hybridisation

DNA samples were digested with restriction endonucleases, subjected to electrophoresis in agarose gels and transferred to nylon membranes following standard procedures [14]. Blots were hybridised at 65°C for 16 h in: 6×SSC, 5×Denhardt's mix, 0.5% SDS, 100 μg/ml salmon sperm DNA. Filters were washed twice at room temperature for 10 min followed by two washes of 15 min at 65°C in 0.1×SSC, 0.1% SDS. Blots were analysed by autoradiography and the relative intensity of the bands quantified by scanning densitometry (Molecular Dynamics, Computing Densitometer, Image Quant Software Version 3.3).

Total RNA was isolated from logarithmic and stationary growth phases using RNeasy Total RNA kit from Qiagen according to the manufacturer's procedure. For Northern blot analysis, 5 μg of total RNA were fractionated in formaldehyde agarose gels, transferred onto nitrocellulose paper [14] and hybridised with the described PCR probe for the p36/CACK coding region, as in the Southern blot analysis.

2.4. Electrophoresis and Immunoblotting

Total cell lysates were obtained by cell disruption with loading electrophoresis buffer (125 mM Tris pH 6.8 containing 2% SDS, 20% glycerol and 5% 2-mercaptoethanol) as previously described [15]. Cell free fraction was obtained as follows: cells were centrifuged at 990×*g* for 10 min, washed three times with PBS resuspended in lysis buffer (20 mM phosphate pH 7.5 containing 150 mM NaCl, 1 mM iodoacetamide, 1 mM PMSF and 1 mM trypsin inhibitor) and disrupted by sonication, three times for 5 min at 4°C. The sonicated cell suspension was centrifuged at 10 000×*g* for 30 min to remove cell debris and the supernatant was recovered. Fractions were separated by SDS-PAGE on 8% slab gels, according to Laemmli [16]. In all cases, unless indicated, 10 μg of protein was loaded per gel. After electrophoresis, gels were blotted onto nitrocellulose sheets (Hybond-ECL) and blocked in 5% BSA. Immunoblots were incubated with 1:500 polyclonal mouse antiserum raised against p36/LACK from *L. infantum* and developed using the ECL detection system, according to the manufacturer's protocol (Amersham).

2.5. p36/CACK treatment with endoglycosidase F

C. fasciculata supernatant containing p36/CACK (see above) was subjected to *N*-deglycosylation as follows: fractions of 400 μg of protein, containing p36/CACK, SDS denatured, were incubated in sodium acetate buffer (50 mM sodium acetate, 50 mM EDTA, 2% NP 40, 1% 2-mercaptoethanol) with 1 U of endoglycosidase F (Boehringer Mannheim). Samples were incubated overnight at 37°C in a total volume of 200 μl and subsequently electrophoresed and blotted (see above).

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1   ATGAACTACGGAGGTTCTTTGAAGGCCACGGCGGGTGAAGTCACGTCCCTGGCGTGCCCGCAGCAG
      M  N  Y  G  G  S  L  K  A  T  A  G  E  V  T  S  L  A  C  P  Q  Q
67  GCCGGCTCCTACATCAAGGTTGTGTCGACGTCCCGCGACGGCACGGCCATCTCGTGGAAGGCCAAC
      A  G  S  Y  I  K  V  V  S  T  S  R  D  G  T  A  I  S  W  K  A  N
133 CCCGACCGCCACAGCGCGGAGGACAACTACGGCATTCCGGACCACCGCATGGAGGGCCACTCCGGC
      P  D  R  H  S  A  E  D  N  Y  G  I  P  D  H  R  M  E  G  H  S  G
199 TTCGTGTCGTGCGTGTCGCTGGCCCACGCCACCGACTACGCGCTGACCGCCTCCTGGGACCACGCG
      F  V  S  C  V  S  L  A  H  A  T  D  Y  A  L  T  A  S  W  D  H  A
265 ATCCGCATGTGGGACCTCCGCACCGGCCAGAGCCAGCGCAAGTTCCTCAAGCACACGAAGGACGTG
      I  R  M  W  D  L  R  T  G  Q  S  Q  R  K  F  L  K  H  T  K  D  V
331 CTCGCCGTGGCGTTCTCGCCGGACGACCGCCTGATCGTGTCGGCTGGCCGGGACAACGTGATCCGT
      L  A  V  A  F  S  P  D  E  R  L  I  V  S  A  G  R  D  N  V  I  R
397 GTGTGGAACGTTGCTGGCGAGTGCATGCACGAGTTCCTGCGCGACGGCCACGAGGACTGGGTGAGC
      V  W  N  V  A  G  E  C  M  H  E  F  L  R  D  G  H  E  D  W  V  S
463 AGCATCTGCTTCTCCCCCTCGCTGGACCTGCCGATCGTGGTGTCGGGCAGCTGGGACAACACCATC
      S  Y  C  F  S  P  S  L  D  L  P  I  V  V  S  G  S  W  D  N  T  I
529 AAGGTGTGGAACGTGAACGAGGGCAAGTGCGTGCACACGCTGCGCGGCCACAAGAACTACGTGTCC
      K  V  W  N  V  N  E  G  K  C  V  H  T  L  R  G  H  K  N  Y  V  S
595 ACGGTGACCGTGTCGCCCGATGGCTCCCTGTGCGCCTCCGGCGGCAAGGACGGCTCCGCGCTCCTG
      T  V  T  V  S  P  D  G  S  L  C  A  S  G  G  K  D  G  S  A  L  L
661 TGGGACCTGAGCAACGGCGAGCAGCAGTTCTCCATCCCGGTGGAGTCGCCGATCAACCAGATCGCC
      W  D  L  S  N  G  E  Q  Q  F  S  I  P  V  E  S  P  I  N  Q  I  A
727 TTTTCGCCCAACCGCTTCTGGATGTGCGTGGCGACGGAGAAGTCGCTGTCGGTGTACGACCTGGAG
      F  S  P  N  R  F  W  M  C  V  A  T  E  K  S  L  S  V  Y  D  L  E
793 ACCAAGGAGGTGATCGCGGACCTGTCCGTGGGCAGCAGCAAGCCGTCCGAGTGCATCTCCGTTGCC
      T  K  E  V  I  A  D  L  S  V  G  S  S  K  P  S  E  C  I  S  V  A
859 TGGTCCGCCGACGGCAACACCCTGTACTCCGGCCACAAGGATAACCAGTTCCGGGCGTGGGACTTC
      W  S  A  D  G  N  T  L  Y  S  G  H  K  D  N  Q  F  R  A  W  D  F
925 AAGAACCTCATCTCCGACGCCGAGTAA
      K  N  L  I  S  D  A  E
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Fig. 1. Nucleotide and deduced amino acid sequences of the *C. fasciculata* CACK gene. The nucleotide sequence is displayed in the 5′ to 3′ direction with the first base of the ATG initiation codon at position 1 and the third base of the TAA stop codon at position 948. The deduced amino acid sequence is in the single amino acid code. The PKC phosphorylation sites are indicated by open boxes. The tryptophan-histidine repeats are indicated by shadowed squares.

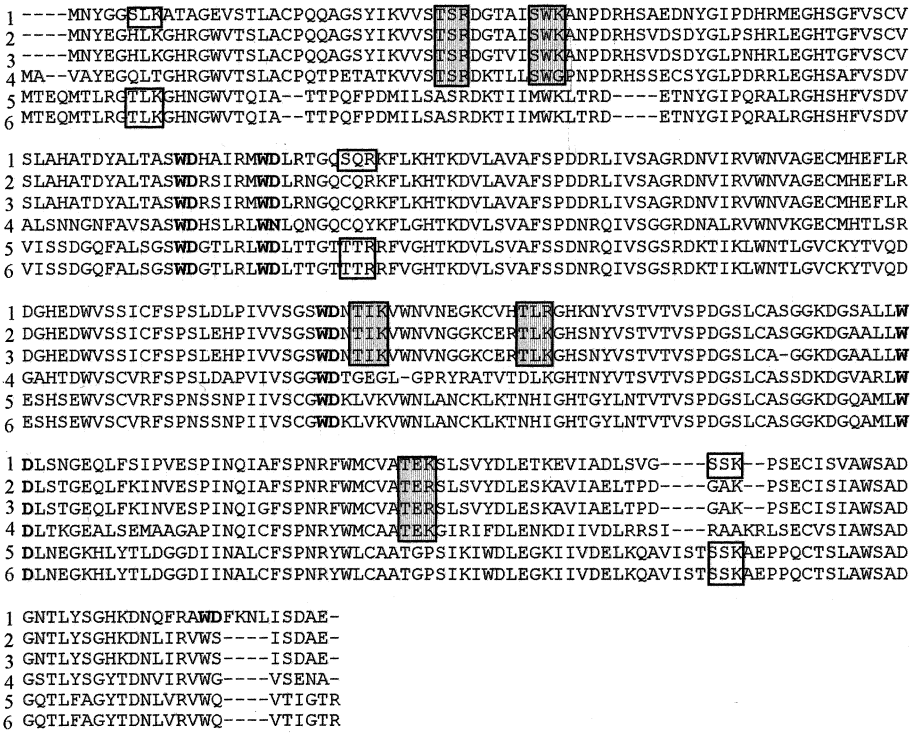

Fig. 2. *C. fasciculata* CACK amino acid sequence and comparison to other RACK molecules from other eukaryotic species. 1, *C. fasciculata* (GenBank AFO63022); 2, *L. infantum* (GenBank U27434); 3, *L. major* (GenBank AFO34804); 4, *T. brucei* (GenBank U72205); 5, mouse (GenBank 2137308); 6, human (GenBank 121027). Missing amino acids are shown as dashes. Open boxes indicate the residues corresponding to PKC phosphorylation sites shared with mammalian species. Shadowed boxes indicate PKC phosphorylation sites shared with Trypanosomatidae. The WD motifs are indicated by bold letters.

2.6. Immunofluorescence

Six day culture cells (10^6), which correspond in the *Leishmania* species with the virulent phase of the protozoon [17], were washed twice with PBS containing 1% foetal calf serum and 0.1% sodium azide and resuspended in the same PBS solution with identical final cell concentration. 25 μl of the cell suspension was spotted onto 12 mm round coverslips. After 1 h at 37°C, the cells were treated with methanol/acetone (1:1) 15 min at −20°C. The fixed cells were incubated at room temperature in DAPI-methanol (0.1 μg/ml). After an additional washing with methanol the cells were treated with PBS containing 0.5% Triton X-100 for 5 min at room temperature and then incubated in an humidity chamber for 1 h at room temperature with mouse polyclonal anti p36/LACK antibody (diluted 1:50). After washing in PBS, cells were incubated for 1 h at room temperature with anti mouse IgG-FITC conjugate (Sigma, diluted 1:64). After washing with PBS, the slides were mounted with Mowiol (Calbiochem) and observed on a Zeiss Axioplan microscope and images were captured by a Hamamatsu CCD camera and processed in Adobe Photoshop on a Macintosh computer.

2.7. Sequence analysis

Sequence comparison analysis of the proteins was carried out using the Altschul algorithm [18]. The sequences of the different species considered have been aligned using the CLUSTALW multiple sequence alignment method using default values [19]. The amino acid sequence based secondary structure prediction was determined according to Chou and Fasman [20] and Frishman and Argos [21].

3. Results

3.1. Cloning of the p36/CACK encoding gene

The 948 bp PCR product obtained with the primers described in Section 2 contained an open reading frame that encodes a polypeptide of 316 amino acids (Fig. 1). The predicted 34.6 kDa protein exhibited homology with the intracellular receptors of protein kinase C (RACKs) that belong to the ancient family of WD 40 regulatory proteins and that contain in their sequence, distributed into organised units and regularly spaced, the motif tryptophan and aspartic amino acids (shadowed letters in Fig. 1) [4,22]. The WD motif appears conserved in all the established units showing a slight difference with respect to the other species considered which lack it in the last one (residues 306–307). There are eight phosphorylation sites for activated C kinases (see open boxes). The deduced p*I* is 5.54, lower than that of the analogue proteins described for *Leishmania* sp.

The CACK from *C. fasciculata* presents a high percentage of homology with the RACKs from other species, not only the related parasites of the genus *Leishmania* but also with higher organisms. Thus, the amino acid homology with other species is 88% with the LACK proteins from *L. infantum* and *L. major*, 74% with *Trypanosoma brucei* and 62% with respect to the mammalian species considered, mouse and human. The analysis of the distinct functional motifs shows several differences. Fig. 2 shows the alignment of CACK with different RACK molecules. As can be seen, the *C. fasciculata* polypeptide (number 1) is four amino acids longer than the *Leishmania* molecules and shorter than the mammalian molecules. This small difference of four additional amino acids (residues 307–310 [FKNL]) in length elicits, according to Chou and Fasman [20], a distinct conformation in the corresponding zone, close to the C-terminal of the protein with an α-helix instead of a β-sheet that appears in the same zone of the molecules of all the other species considered. Furthermore, a variation in the sequence (residues 9–11, GHR→ATA) is concomitant with the same change in the protein folding at

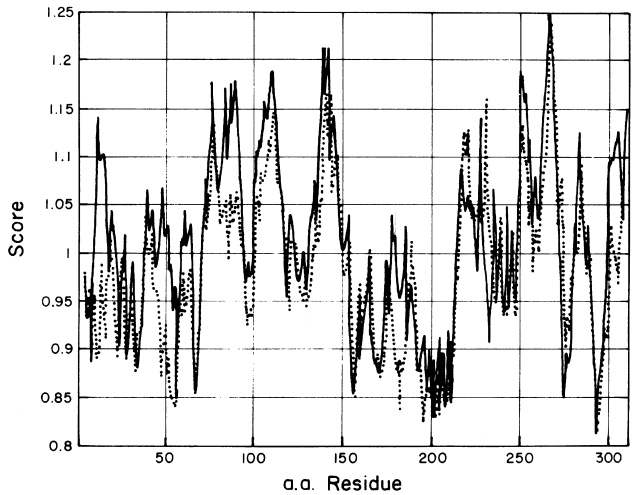

Fig. 3. Alpha-helix profile according to Chou and Fasman [20] of the RACK analogues. Solid line: CACK from *C. fasciculata*. Dotted line: LACK from *L. infantum*.

the amino-terminal end (see Fig. 3). The motif analysis shows several differences in the PKC phosphorylation sites. Thus, the most striking difference is the presence of three phosphorylation sites, two of them identical, at positions 6–8 (SLK) and 276–278 (SSK) and the third changing the tyrosine by serine, 99–101, that are shared with the mammalian species and not with the Trypanosomatidae with which are coincident most of the PKC phosphorylation sites (see shadowed boxes in Fig. 2).

3.2. Two genes encode the p36/CACK protein

The Southern blot analysis of the genomic DNA digested with *Hin*dIII, that do not present any target within the protein encoding region, showed two bands. In the case of *Sph*I, *Pvu*I and *Alw*NI, which do present a single target within the CACK encoding region, the pattern showed three bands, two of them being weaker than and accounting approximately for the same intensity as the third one (see Fig. 4A). The Northern blot of *C. fasciculata* (Fig. 4B) shows a single RNA band with a molecular weight of 1.8 kb, similar to the one of higher molecular weight displayed by *L. infantum*, used as a reference control. However, the existence of a faint band close to that main one or the existence of RNA molecules of similar size not resolved under these experimental conditions cannot be ruled out. Altogether, the data would suggest the existence of two non-identical genes encoding the CACK protein.

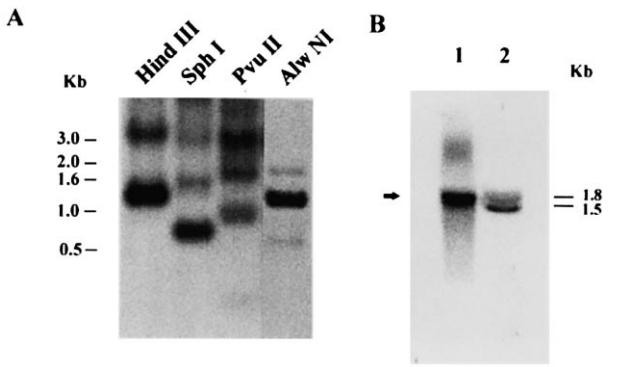

Fig. 4. Southern and Northern blot analysis of the CACK gene. A: Southern blot analysis of *C. fasciculata* genomic DNA. Genomic DNA was subjected to total digestion with the following restriction enzymes: *Hin*dIII, *Sph*I, *Pvu*I and *Alw*NI, molecular weights are indicated on the left. B: Northern blot analysis of the CACK expression. Total RNA (5 μg/lane) was fractionated in 1.5% formaldehyde containing agarose gels and transferred to nitrocellulose membrane. The blot was hybridised with the CACK coding region probe. 1, *C. fasciculata*; 2, *L. infantum*. Similar amounts of RNA were loaded according to the ethidium bromide analysis. For further experimental details see Section 2.

3.3. Expression of the p36/CACK protein

Western blot analysis of the *C. fasciculata* extracts with the antiserum raised against the *Leishmania* LACK (see Fig. 5) showed the presence of a single band with an apparent molecular weight around 36 kDa, slightly higher than that predicted from its amino acid sequence and that of the LACK protein from *L. infantum* used as a control. This higher apparent molecular weight of the polypeptide was not modified by treatment of the extract with endoglycosidase F which indicates that the different molecular weight of CACK with respect to the theoretical one or to the LACK polypeptide is not due to glycosylation. The differences in the protein folding may account for such small differences in the apparent molecular weight in the PAGE.

3.4. Cell localisation of p36/CACK

A polyclonal antibody raised against the LACK protein (see Section 2) was used for immunolocalisation experiments. The results shown in Fig. 6 indicate that CACK mainly localises on the cell plasma membrane (see Fig. 6F) with a minor presence around the nucleus and the kinetoplast. This pattern is different from that displayed in the related species *L. infantum* in which LACK mainly localises in the cell cytoplasm with an increase of the fluorescence intensity close to the kinetoplast and surrounding the cell nucleus, according to its localisation by the DAPI staining (see Fig. 6B,C). This differential localisation pattern may be related to the existence of different PKC isoforms in the cell [23].

4. Discussion

We have cloned an intracellular receptor for activated C kinases: CACK, the analogue of the LACK protective antigen in several Trypanosomatidae. This protein seems to be encoded by two non-identical genes. This type of gene structure would fit with the usual structure shown in the related

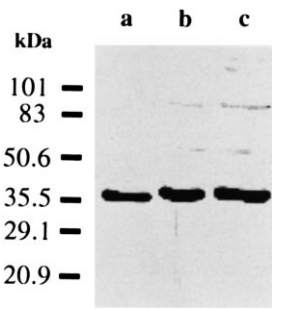

Fig. 5. Western blot of the CACK protein and endoglycosidase treatment. Lane 1, *L. infantum* cell extract; lane 2, *C. fasciculata* cell extract; lane 3, *C. fasciculata* endoglycosidase treated fraction. For further experimental details see Section 2.

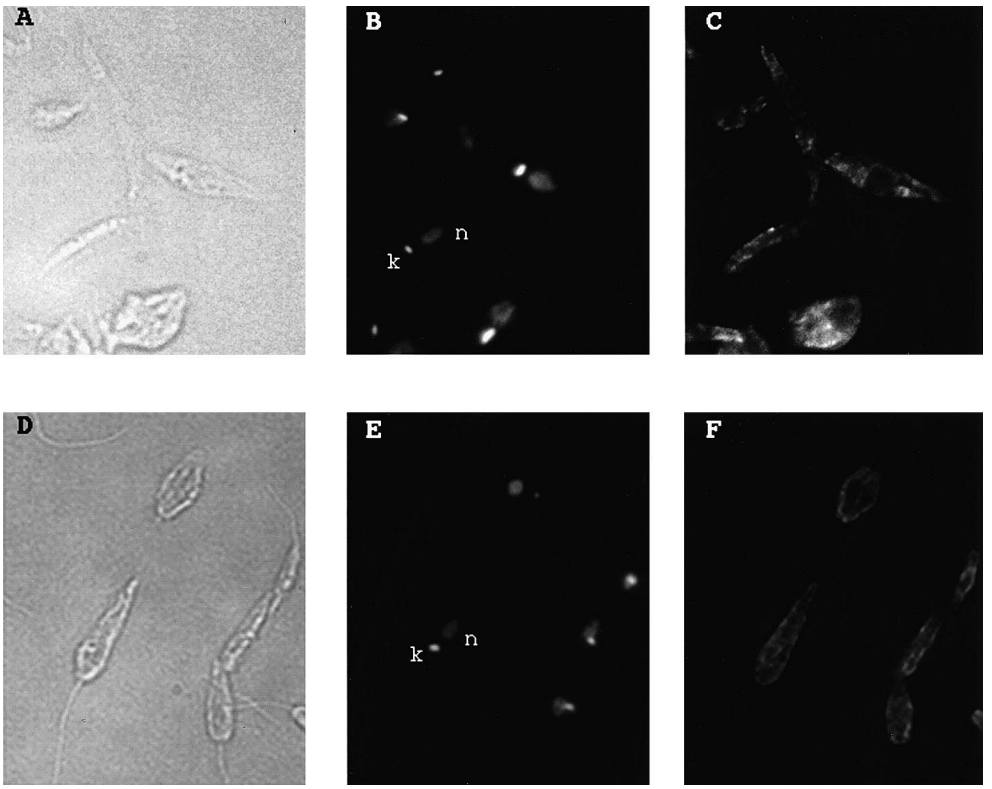

Fig. 6. Immunofluorescence. A, B, and C: *L. infantum* phase contrast, DAPI staining and fluorescence with anti-LACK antibody respectively. C, D and F: *C. fasciculata* phase contrast, DAPI staining and fluorescence with anti-LACK antibody. For further experimental details see Section 2.

parasite *L. infantum*, which displays a gene structure with genes tandemly associated, a common feature for different genes in the genus *Leishmania* [24]. Like the other members of the RACKs, CACK belongs to the WD 40 repeat family of regulatory proteins. However, it presents several structural differences with the considered proteins from other Trypanosomatidae. Thus, the WD motif is present in all the repeating units including the last one at the carboxy-terminal end. The motif is absent in this position in the rest of the studied species. The existence of four additional amino acids at the carboxy-terminal region of the protein and a change in a small sequence at the amino-terminal end apparently induce changes on the protein folding with respect to the structure of the analogue molecules from the other Trypanosomatidae species studied: *L. infantum*, *L. major* and *T. brucei*. These differences also appear in the phosphorylation sites for PKC in which the *C. fasciculata* receptor shows several sites shared with mammalian species, in addition to the common sites present in all the protozoan species studied suggesting that the *C. fasciculata* RACK presents a certain phylogenetic distance between this species and the other components of the family.

The immunolocalisation experiments showed a different distribution pattern with respect to the one of *L. infantum*. Thus, whereas the CACK localised mainly at the plasma cell membrane, presumably bound to PKC activated forms, in the *Leishmania* species it appears close to the kinetoplast, the cell single mitochondria, and the nucleus. One possible explanation for this distinct cell localisation would be that the described changes on the CACK molecular structure, although not very remarkable, may have a direct influence on the functional behaviour of the receptor that probably binds a distinct isoform of the activated kinase C. The questions raised by the present data deserve further study to find out whether the structural and functional differences found are representative of an intermediate position of *C. fasciculata* between the other Trypanosomatidae and higher species and more importantly, if they are the result of a usual method of differentiation through small variations in structure in the Trypanosomatidae.

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References

[1] Neva, F. and Sacks, D.L. (1995) Leishmaniasis in Tropical and Geographical Medicine (Warren, K.S. and Mahmoud, A.A., Eds.), pp. 296–308, McGraw-Hill, New York.
[2] Li, C. and Englund, P.T. (1997) J. Biol. Chem. 272, 20787–20792.
[3] Mochly-Rosen, D., Henrich, C.J., Cheever, L., Khaner, H. and Simpson, P.C. (1990) Cell. Regul. 1, 693–706.
[4] Neer, E.J., Schmidt, C.J., Nambudripad, R. and Smith, T.F. (1994) Nature 371, 297–300.
[5] Mougneau, E., Altare, F., Wakil, A.E., Zheng, S., Coppola, T., Wang, Z.E., Waldman, R., Locksley, R.M. and Glaichenhaus, N. (1995) Science 268, 563–566.
[6] Lardans, V., Serra, E., Capron, A. and Dissous, C. (1998) Exp. Parasitol. 88, 194–199.
[7] Soldera, S., McSorley, S.J. and Glaichenhaus, N. (1997) Eur. J. Immunol. 27, 848–854.
[8] Titus, R.G., Theodor, C.M., Kimsey, P.B., Shankar, A., Hall, L., McGurn, M. and Povielli, L. (1992) Subcell. Biochem. 18, 99–129.

[9] Trinchieri, G. and Scott, P. (1994) Immunol. Today 15, 460–463.
[10] Mossman, T.R. and Sad, S. (1996) Immunol. Today 17, 138–146.
[11] Gonzalez-Aseguinolaza, G., Taladriz, S., Marquet, A. and Larraga, V. (1999) Eur. J. Biochem. 259, 1–8.
[12] Zarley, J.H., Britigan, B.E. and Wilson, M.E. (1991) J. Clin. Invest. 88, 1511–1521.
[13] Gonzalez-Aseguinolaza, G., Almazan, F., Rodriguez, F.J., Marquet, A. and Larraga, V. (1997) Biochem. Biophys. Acta 1361, 92–102.
[14] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[15] Roberts, S.C., Swihart, K.G., Agey, M.W., Ramamoorthy, R., Wilson, M.E. and Donelson, J.E. (1993) Mol. Biochem. Parasitol. 62, 157–172.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Da Silva, R. and Sacks, D.L. (1987) Infect. Immun. 61, 2802–2806.
[18] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[19] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acid Res. 22, 4673–4680.
[20] Chou, P.Y. and Fasman, G.D. (1978) Annu. Rev. Biochem. 47, 251–276.
[21] Frishman, D. and Argos, P. (1996) Protein Eng. 9, 133–142.
[22] Ron, D., Chen, C.H., Caldwell, J., Jamieson, L., Orr, E. and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. USA 91, 839–843.
[23] Kiley, S., Schaap, D., Parker, P., Hsieh, L.L. and Jaken, S. (1990) J. Biol. Chem. 265, 15704–15712.
[24] Vanhamme, L. and Pays, E. (1995) Micobiol. Rev. 59, 223–240.