Biochem. J. (1996) 313, 455–466 (Printed in Great Britain)

Antigenicity and conformational analysis of the Zn^{2+}-binding sites of two Zn^{2+}-metalloproteases: *Leishmania* gp63 and mammalian endopeptidase-24.11

Ketty P. SOTERIADOU*§, Athina K. TZINIA*, Evgenia PANOU-PAMONIS†, Vassilias TSIKARIS†, Maria SAKARELLOS-DAITSIOTIS†, Constantinos SAKARELLOS†, Youli PAPAPOULOU‡ and Rebecca MATSAS‡

Laboratories of *Molecular and Biochemical Parasitology and ‡Molecular and Cellular Neurobiology, Department of Biochemistry, Hellenic Pasteur Institute, 127 Vassilissis Sophias, 115 21 Athens, Greece and †Department of Chemistry, University of Ioannina, P.O. Box 1186, 45 110 Ioannina, Greece

The antigenic properties of the Zn^{2+}-binding region of two Zn^{2+}-metalloproteases, *Leishmania* surface protease gp63 and mammalian endopeptidase-24.11 (E-24.11), possessing in their active site the characteristic amino acid sequence **HEXXH**, were investigated by using oligoclonal antibodies raised against two synthetic peptides, V[1]VT**HE**MA**H**ALG[11] (pepgp63) and V[1]IG**HE**IT**H**GFD[11] (pepE-24.11), containing the respective Zn^{2+}-binding sites of the cognate protein. The affinity-purified antibodies, tested on synthetic peptides modelled from the active sites of ten different Zn^{2+}-metalloproteases, showed high selectivity for their respective peptides. However, cross-reactivity was revealed when the antibodies were tested against the gp63 and E-24.11 molecules. A panel of synthetic peptide analogues and peptides of various size was synthesized and used for the fine antigenic characterization of pepgp63 and pepE-24.11. The shortest peptides capable of significant antibody binding were the pentapeptides V[1]VTHE[5] and E[5]ITHG[9] for pepgp63 and pepE-24.11 respectively. His[4] and Glu[5] were found to be indispensable for anti-pepgp63 binding to pepgp63, whereas in the case of pepE-24.11, Glu[5] and His[8] were found to be critical. The conformational characteristics of the two peptides correlate well with the observed differences in their antigenicity. ^{1}H-NMR studies showed that pepgp63 adopts a folded structure whereas pepE-24.11 takes up a rather flexible conformation. Moreover, the antigenically critical His[4] of pepgp63 contributes to the structural stabilization of the peptide. Similarly, the antigenically critical His[8] of pepE-24.11 is involved in partial structural stabilization of its C-terminal region. The generated antibodies may be useful tools for identifying and classifying proteins possessing similar Zn^{2+}-binding motifs and/or environments.

INTRODUCTION

Leishmania surface metalloprotease, gp63, is the major antigenic protein of most *Leishmania* promastigotes [1]. It is also expressed, but at lower levels, by *Leishmania* amastigotes, the intracellular form of the parasite in host macrophages [2–4]. gp63 plays a key role in the attachment of parasites to the macrophage membrane [5,6] and probably contributes to their survival within the macrophage phagolysosomes through its non-specific proteinase activity [7]. Many investigators consider gp63 to be a good candidate for a vaccine against leishmaniosis, a major infectious disease of considerable public-health and economic importance. Mice either immunized with purified gp63 or orally treated with a *Salmonella* mutant (Aro^-) carrying the *Leishmania major* gp63 gene developed significant resistance against *L. major* challenge infection [8]. In addition, synthetic peptides modelled from different regions of the amino acid sequence of gp63 were found to be highly immunogenic and induced protective immunity in mice against challenge infection. A peptide spanning the Zn^{2+}-binding region of gp63, containing residues 258–267 of the propeptide or 158–167 of the protein [9], was found to confer optimal immunogenicity [10]. gp63 has been characterized as a Zn^{2+}-metalloprotease [7,11], the active site of which shares the common pattern HEXXH (where X stands for any amino acid) which constitutes the unique signature of the large superfamily of Zn^{2+}-dependent metalloproteases [12], recently termed zincins [13].

All three conserved residues found in the active site of gp63, i.e. the two histidines and the glutamic acid, have also been shown to be essential for the activity of the mammalian enzyme endopeptidase-24.11 (EC 3.4.24.11; neprilysin; E-24.11) [14,15]. E-24.11 is a well-characterized cell-surface Zn^{2+}-metalloprotease expressed by many different cell types in multiple tissues [16] and is identical with the common acute lymphoblastic leukaemia antigen CD10 [17,18]. The enzyme has its active site exposed at the cell surface and is believed to regulate peptide-induced responses at different tissues. For example, in the central nervous system the function of E-24.11 has been associated with inactivation of the enkephalins [19] and substance P [20] and possibly many other neuropeptides [21], whereas, in the peripheral nervous system, recent studies have revealed a potential, previously unrecognized, role for the enzyme in nerve development and regeneration after injury [22,23]. At other locations, such as the kidney and the vascular endothelium, E-24.11 may regulate atrial natriuretic peptide levels [24,25]. E-24.11 has also been shown to be involved in peptide-mediated inflammatory responses [26,27] and in T-cell activation and regulation of interleukin-2 production [28]. On the basis of these properties, several potent inhibitors of E-24.11 have been synthesized with the aim of them being used as drugs for a number of pathological

Abbreviations used: E-24.11, endopeptidase-24.11; pepgp63, V[1]VTHEMAHALG[11]; pepE-24.11, V[1]IGHEITHGFD[11]; TBS, Tris-buffered saline; RSA, rabbit serum albumin; mAb, monoclonal antibody; Fmoc, fluoren-9-ylmethoxycarbonyl; HOHAHA, two-dimensional homonuclear Hartmann–Hahn spectroscopy; NOE, nuclear Overhauser effect.

§To whom correspondence should be addressed.

conditions (for a review see ref. [29]). However, the design of such highly efficient and orally active inhibitors requires detailed and precise information on the active site of E-24.11.

On the basis of the above considerations, in the present paper we undertook a comparative detailed analysis of the antigenicity of the Zn^{2+}-binding region of *Leishmania* gp63 and mammalian E-24.11. To this end, we produced synthetic peptides and peptide analogues spanning the Zn^{2+}-binding region of the two metalloproteases and generated oligoclonal antibodies against two of these synthetic peptides, V^1VT**HE**MA**H**ALG^{11} (pepgp63) and V^1IG**HE**IT**H**GFD^{11} (pepE-24.11) which correspond to the respective sequences 261–271 and 580–590 of the cognate protein molecules and contain their respective Zn^{2+}-binding sites. Of interest is our finding that, although pepgp63 and pepE-24.11 are antigenically and structurally different, their respective antibodies, i.e. anti-pepgp63 and anti-pepE-24.11, recognize and cross-react with the native protein molecules, thereby representing useful probes that can be used for the study of the active sites of these and other Zn^{2+}-metalloproteases.

EXPERIMENTAL

Materials

Purified pig kidney E-24.11 [30] was kindly provided by Dr. A. J. Kenny, University of Leeds, Leeds, Yorks., U.K.; it was found to be apparently homogeneous by SDS/PAGE. Mouse monoclonal antibody (mAb) 23B11 raised against rabbit E-24.11 was a gift from Dr. P. Crine, University of Montreal, Montreal, Que., Canada. This antibody which was produced and characterized as described by Aubry et al. [31] has been shown to recognize an epitope located at the cytosolic side of E-24.11. Its specificity for pig and rat E-24.11 has been established previously [22,32]. Mouse mAb LD33 raised against purified *Leishmania infantum* gp63 was characterized and used as in previous studies [33,34].

BSA, rabbit serum albumin (RSA), SDS, ELISA substrates, 2-mercaptoethanol and all reagents for peptide synthesis were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. Piperidine and Tween 20 were from Fluka, Buchs, Switzerland and anti-rabbit immunoglobulins from Amersham International, Amersham, Bucks., U.K. Affi-Gel 10 was from Bio-Rad Laboratories, Munich, Germany. All other reagents were of the highest purity available and were purchased from sources reported previously [6,22,23,32].

Peptide synthesis

Two different methodologies were used. (1) Immobilized peptides were synthesized on the tips of small polyethylene rods on which polymers of acrylic acid had been formed by radiation grafting [35]. Rods with attached fluoren-9-ylmethoxycarbonyl (Fmoc)-protected β-alanine, all Fmoc-amino acids and hydroxybenzotriazole were obtained from Cambridge Research Biochemicals, Cambridge, U.K. Peptide synthesis was performed as described by Geysen et al. [35] and in the manufacturer's instructions in 96-well microtitre plates. In order to exclude the possibility of cross-contamination during synthesis, only 24 rods/plate were used.

(2) V^1VT**HE**MA**H**ALG^{11} (pepgp63) and V^1IG**HE**IT**H**GFD^{11} (pepE-24.11) were synthesized manually by a stepwise solid-phase procedure [36] using the N^{α}-Boc-Gly-OCH_2-Pam and N^{α}-Boc-L-Asp(Bzl)-OCH_2-Pam resins respectively [37] (Pam is phenylacetamidomethyl). N^{α}-Boc/Bzl side-chain protection was carried out by standard methods. Histidine was introduced as N^{α}-Boc-L-His (Tos), and methionine was used without side-chain protection. All protected amino acids were coupled using a ratio in mmol of amino acid/1-hydroxybenzotriazole/$N'N'$-dicyclohexylcarbodi-imide/resin of 3:3:3:1. Completion of the coupling reactions was ensured by the use of the ninhydrin test [38]. After introduction of Zn^{2+}-Boc-L-Met into pepgp63, dimethyl sulphide was added during the removal of Boc groups to avoid oxidation of the methionine side chain. The low/high HF method [39] was used to cleave pepgp63 from the resin support in the presence of dimethyl sulphide, *p*-cresol and *p*-thiocresol, and for the cleavage of pepE-24.11, anisole and phenol were used as scavengers. Crude pepgp63 was subjected to chromatographic purification using Sephadex G-25 equilibrated with aq. 2M acetic acid. The endecapeptide was eluted using a homogeneous butan-1-ol/pyridine/acetic acid/water mixture of (5:5:1:4, by vol.). Highest purity was achieved when the eluent volume, passed through the column before loading the peptide, corresponded to the half-bed column volume. The eluate was subsequently lyophilized and maximum purity was gained by preparative HPLC on a C_{18} column. Isocratic elution (8 ml/min) was performed with the following solvents: A, 84 % water (0.1 % trifluoroacetic acid); B, 16 % acetonitrile (0.1 % trifluoroacetic acid). Purification of pepE-24.11 was achieved by partition chromatography on Sephadex G-25 in butan-1-ol/pyridine/0.1 % acetic acid in water (5:3:11, by vol.).

Oligoclonal antibodies

Oligoclonal anti-pepgp63 and anti-pepE-24.11 antibodies were prepared by immunizing New Zealand White rabbits with synthetic peptides conjugated to RSA by means of 0.1 % glutaraldehyde, using a 20-fold molar excess of peptide to carrier [6]. The animals received repeated subcutaneous injections of 1 mg of peptide in Freund's incomplete adjuvant. The animals were boosted in the absence of adjuvant and blood was collected from the ear 8 days after the final innoculation.

Affinity purification of the oligoclonal antibodies

Peptides were coupled to Affi-Gel 10 according to the manufacturer's instructions. $(NH_4)_2SO_4$-precipitated oligoclonal antibodies in PBS were mixed with the Affi-Gel 10 (200 μl of affinity resin/ml of soluble fraction). The tubes containing the above mixture were rotated end-over-end overnight at 4 °C. The beads were subsequently centrifuged, washed with PBS and the bound antibodies eluted from the affinity resin with 500 mM NaCl/200 mM glycine, pH 2.3, neutralized and stored at −20 °C. The purity of the eluted antibodies was assessed by SDS/PAGE [40].

Parasites

Promastigotes of *L. major* LEM513 were grown in Medium 199 containing 5 % heat-inactivated fetal calf serum as previously described [41].

Purification of gp63

gp63 was purified from stationary-phase promastigotes of *L. major* LEM513 (kindly provided by C. Bordier, Biokema SA, Crissier-sur-Lausanne, Switzerland) [5×10^{11} promastigotes in 100 ml of Tris-buffered saline (TBS) containing a cocktail of enzyme inhibitors and chelators] as previously described [33]. After phase separation with Triton X-114, gp63 was recovered in the detergent-enriched phase. Triton X-114 was replaced by 2.2 mM $N'N'$-dimethyldodecylamine N-oxide on a Fractogel TSK DEAE-650 column, and fractions containing gp63 were identified using non-fat powdered milk as substrate. The proteolytic activity of the fractions was detected as a decrease in the

turbidity of the milk solution [33]. Subsequently the fractions were pooled, applied to a Mono Q column and eluted with a linear gradient of 0–500 mM NaCl in 10 mM Tris/HCl, pH 7.5, as described by Bouvier et al. [42].

Preparation of membrane fractions

Kidneys were removed from adult Wistar rats (bred in the Hellenic Pasteur Institute Animal House) and a membrane fraction was prepared as previously described [22]. *Leishmania* membrane preparations obtained by the method of Dwyer [43] were suspended in TBS containing a mixture of chelators and enzyme inhibitors and stored at −70 °C as previously described [44].

ELISA

Peptides (5 μg/ml), purified gp63 and E-24.11 (500 ng/ml) or membrane preparations (50 μg/ml) in Na_2CO_3/$NaHCO_3$ buffer, pH 9.6, were plated in poly(vinyl chloride) flat-bottomed microtitration plates (100 μl/well). Binding of the purified anti-pepgp63 and anti-pepE-24.11 antibodies to the different antigens was assessed using horseradish peroxidase-conjugated anti-rabbit immunoglobulins as previously described [41].

Binding of the antibodies to peptide-carrying rods was carried out as previously described [6]. Briefly, each peptide-carrying rod was preincubated with 200 μl of PBS containing 0.1 % Tween 20 and 3 % BSA for 1 h at room temperature and then overnight with the test antibody (1 μg/ml) in the same solution. After incubation, the rods were washed (3 × 10 min) with PBS containing 0.05 % Tween 20, followed by incubation with peroxidase-labelled anti-rabbit immunoglobulins (1:1000 dilution) for 1 h. The rods were then washed three times and the bound antibody was detected by reaction for 10 min with a substrate solution (0.05 % azino-di-3-ethylbenzthiazodinsulphonate and 0.03 % H_2O_2 in 0.1 M Na_2HPO_4/0.08 M citric acid buffer, pH 4). The rods were then removed and absorbance was measured at 405 nm. Subsequently, the bound antibodies were released by sonicating the rods in a water bath for 20 min in 0.1 M Na_2HPO_4/1 % SDS/0.1 % 2-mercaptoethanol, at 60 °C. Finally the rods were washed twice with water, once with methanol and air-dried. Peptides retained their antibody-binding capacity for more than 20 assays.

For each specific sequence, about four to ten copies were synthesized, i.e. two to four rods with the same residue sequence, in each of two to three independent synthesis cycles. Standard deviation for antibody-binding to rods with the same peptide sequence, among different experiments, was in almost all cases less than 10 % (usually less than 5 %).

Gel electrophoresis and immunoblotting

SDS/PAGE was performed on a 7–17 % linear polyacrylamide gradient [32]. The separated proteins were then transferred to nitrocellulose, and Western-blot analysis was carried out essentially as previously described [22,32]. After incubation with horseradish peroxidase-conjugated secondary antibodies, filters were developed in diaminobenzidine with nickel enhancement [0.03 % (w/v) diaminobenzidine, 0.03 % (w/v) $NiCl_2$ in TBS].

Immunofluorescence staining

Rat kidney epithelial cells (NRK-52E, purchased from the American Type Culture Collection) were grown on poly-L-lysine-treated coverslips in 48-well Costar culture dishes in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum. Immunofluorescence labelling was performed as previously described [22] on paraformaldehyde-fixed cells. For demonstration of E-24.11 immunoreactivity with mAb 23B11, cells were treated with 0.1 % Triton X-100 for 3 min before staining by the indirect immunofluorescence method [22]. Antibodies were diluted in PBS containing 0.1 M lysine and 10 % fetal bovine serum. Primary antibody was then applied overnight at 4 °C and fluorescence-conjugated secondary antibody for 3 h at room temperature. After immunostaining, coverslips were mounted on glass slides in 90 % glycerol in PBS and viewed with a Zeiss Axiophot photomicroscope.

L. major LEM513 promastigotes were fixed on glass microscope slides [45] and immunostained following the same procedure as for kidney epithelial cells.

^{1}H-NMR

The NMR samples were prepared by dissolving the solid materials in water and adjusting the pH to the desired value with NaOH or HCl. The aqueous solutions obtained were lyophilized, and then weighed amounts of the peptides were dissolved in [^{2}H]DMSO at concentrations of about 6 mmol/dm^3. DMSO was chosen as the solvent because it provides an amphiphilic environment, which mimics physiological conditions and is therefore appropriate for investigating biological structures such as proteins and membranes [46–48].

NMR spectra were recorded on a Bruker AM400 spectrometer at 305 K using standard COSY [49], two-dimensional homonuclear Hartmann-Hahn spectroscopy (HOHAHA) [50] and NOESY [51] microprograms. Spectral width in F_1 and F_2 was 5000 Hz; 256 experiments in 1K data points in the F_2 dimension were performed for COSY, and 512 experiments in 2K data points in the F_2 dimension were applied for HOHAHA and NOESY. Two mixing times (180 and 300 ms) were used for NOESY and one mixing time (100 ms) for HOHAHA.

RESULTS AND DISCUSSION

Binding of the affinity-purified anti-pepgp63 and anti-pepE-24.11 oligoclonal antibodies to synthetic peptides

Affinity-purified (on peptide–Affi-Gel 10 columns) anti-pepgp63 and anti-pepE-24.11 antibodies were tested for binding to synthetic endecapeptides, the synthesis of which was modelled from the active site of ten known Zn^{2+}-metalloproteases possessing in their active site the characteristic amino acid sequence **HEXXH** (Table 1). Their antibody-binding patterns

Table 1 Amino acid sequence of the active site of the ten putative Zn^{2+}-dependent peptidases [12], given in standard one-letter amino acid symbolism

The ten endecapeptides synthesized by the Pepscan system [6] were modelled according to the sequences shown.

Peptidase	Sequence
E-24.11 (human, rat, rabbit)	VIG**HE**IT**H**GFD
Fibroblast collagenase (human)	VAA**HE**LG**H**SLG
Stromelysin (human)	VAA**HE**IG**H**SLG
Gelatinase (human)	VAA**HE**FG**H**AMG
Aminopeptidase N (human)	VIA**HE**LA**H**QWF
Surface protease gp63 (*Leishmania* sp.)	VVT**HE**MA**H**ALG
Neutral protease (*Bacillus subtillis*)	VTA**HE**MT**H**GVT
Neutral protease (*Serratia* sp.)	TFT**HE**IG**H**ALG
Peptidase N (*Escherichia coli*)	VIG**HE**YF**H**NWT
Thermolysin (*Bacillus stearothermophilus*)	VVG**HE**LT**H**AVT

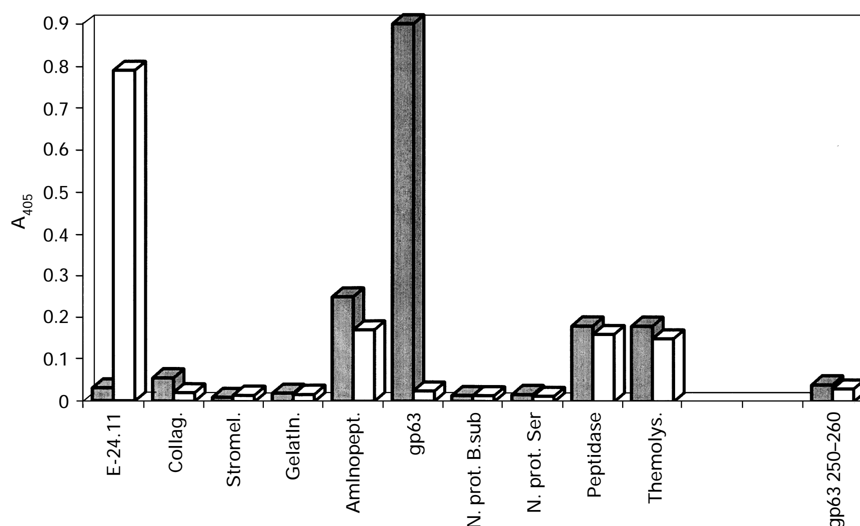

Figure 1 Binding of the affinity-purified antibodies to synthetic peptides modelled from the active site of ten known Zn^{2+}-metalloproteases possessing in their active site the characteristic amino acid sequence HEXXH (Table 1)

Names just below the bars represent abbreviations of the metalloprotease from which the synthetic peptide was modelled. Rods were incubated with approx. 5 μg/ml purified anti-pepgp63 or anti-pepE-24.11 antibodies (first and second bars respectively) and tested by ELISA using peroxidase-labelled anti-rabbit γ-globulin antibody. The 11-residue peptide (gp63 250–260) corresponds to the gp63 adhesion site (residues 250–260 of gp63) and was used as a negative control. This set of results is representative of three independent experiments performed in triplicate. Abbreviations: Collag., human fibroplast collagenase; Stromel., human stromelysin; Gelatin., human gelatinase; Aminopept., human aminopeptidase N; N. prot. B. sub, *B. subtilis* neutral protease; N. prot. Ser, neutral protease from *Serratia* sp.; Peptidase, *E. coli* peptidase N; Thermolys., *B. stearothermophilus* thermolysin B.

are presented in Figure 1. Anti-gp63 and anti-E-24.11 showed by far the most significant selectivity for the peptide against which they were raised, i.e. pepgp63 and pepE-24.11 respectively. Both antibodies exhibited weak binding to the endecapeptides modelled from the active sites of aminopeptidase N (human), peptidase N (*E. coli*) and thermolysin (*B. stearothermophilus*). No binding was observed to the endecapeptide gp63 250–260 which was modelled from the gp63 adhesion site [6] and was used in this study as a negative control.

Binding of anti-pepgp63 and anti-pepE-24.11 to the endecapeptides pepgp63 and pepE-24.11, synthesized by the solid-phase method and used in conventional ELISA, also revealed strong selectivity (see Table 4). Binding of both antibodies to the irrelevant endecapeptide gp63 250–260, to RSA and to rat liver membranes, used as negative controls, yielded A_{492} values of less than 100.

Determination of the antigenic role of each of the three conserved residues constituting the Zn^{2+}-binding site of the active sites of gp63 and E-24.11

The observed selectivity of the generated oligoclonal antibodies for their respective immunogen peptides indicated that the existence of the three common residues in the **HEXXH** motif present in all ten synthetic endecapeptides tested is not sufficient by itself and does not form the basis for antigenic cross-reactivity. In order to elucidate the above finding further and determine the antigenic role of each of the two histidines and the glutamic acid residue present in pepgp63 and pepE-24.11, a series of peptide analogues was synthesized in which each of the three conserved residues of the **HEXXH** motif was replaced with alanine. The peptide analogues of gp63 and E-24.11 were subsequently tested for binding to their respective anti-pepgp63 and anti-E-24.11 oligoclonal antibodies (Figure 2). The antibody-binding pattern of anti-pepgp63 to the pepgp63 analogues (Figure 2a) revealed that replacement of His^4 by Ala decreased antibody binding by 56 %, indicating that this residue significantly contributes to the antigenicity of the epitiope. An even more dramatic loss of antibody-binding activity, amounting to 85 %, was observed when Glu^5 was replaced by Ala. In contrast, substitution of Ala for His^8 had the least effect, decreasing antibody binding by only 15 %. Thus only two of the three conserved residues, namely His^4 and Glu^5 (corresponding to His^{264} and Glu^{265} of the cognate gp63 protein molecule) were found to contribute significantly to the antigenicity of pepgp63. On the other hand, the antibody-binding pattern of anti-pepE-24.11 to pepE-24.11 analogues (Figure 2b) revealed that the second histidine residue, His^8, and Glu^5 were indispensable for antibody binding whereas His^4 did not contribute significantly to the antigenicity of the peptide. In particular, replacement of His^8 or Glu^5 (corresponding to His^{587} and Glu^{584} of the cognate E-24.11 protein molecule) by Ala almost completely inactivated the binding site of pepE-24.11 as a loss of 75–80 % of the binding activity of the antibody was observed whereas a decrease of only 15 % was observed when His^4 (corresponding to His^{583} of the cognate E-24.11 protein molecule) was replaced by Ala.

The above results show that the glutamate residue (Glu^5) in both peptides appears to be indispensable for antibody binding. Moreover, in the case of pepgp63 the first histidine residue in the HEXXH motif contributes significantly to the antigenicity of the epitope whereas in the case of pepE-24.11 it is the second histidine residue that appears to be indispensable for antibody binding.

Determination of the minimum antibody-binding segment within pepgp63 and pepE-24.11

In order to localize further the anti-pepgp63 and anti-pep-E24.11 binding sites, overlapping peptides of various size were synthesized. Binding of anti-pepgp63 and anti-pepE-24.11 anti-

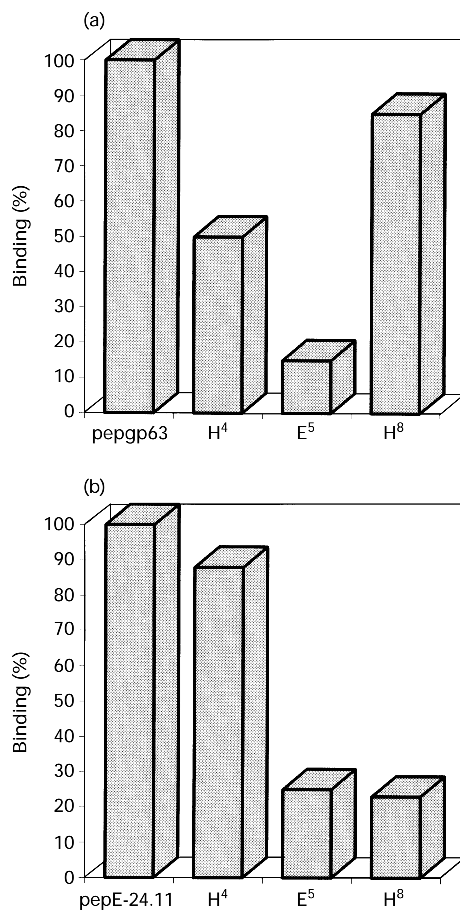

Figure 2 Binding of anti-pepgp63 and anti-E-24.11 antibodies to single-residue analogues of gp63 and E-24.11 endecapeptides as compared with the original peptide (taken as 100%)

The first bar in each graph represents antibody binding to the original unsubstituted peptide. Letters and numbers below the bars represent the substituted amino acid and its corresponding position. (**a**) Analogues of the pepgp63 endecapeptide probed with anti-pepgp63 antibodies; (**b**) analogues of the pepE-24.11 endecapeptide probed with the homologous antibodies. These results are representative of three independent experiments carried out in triplicate.

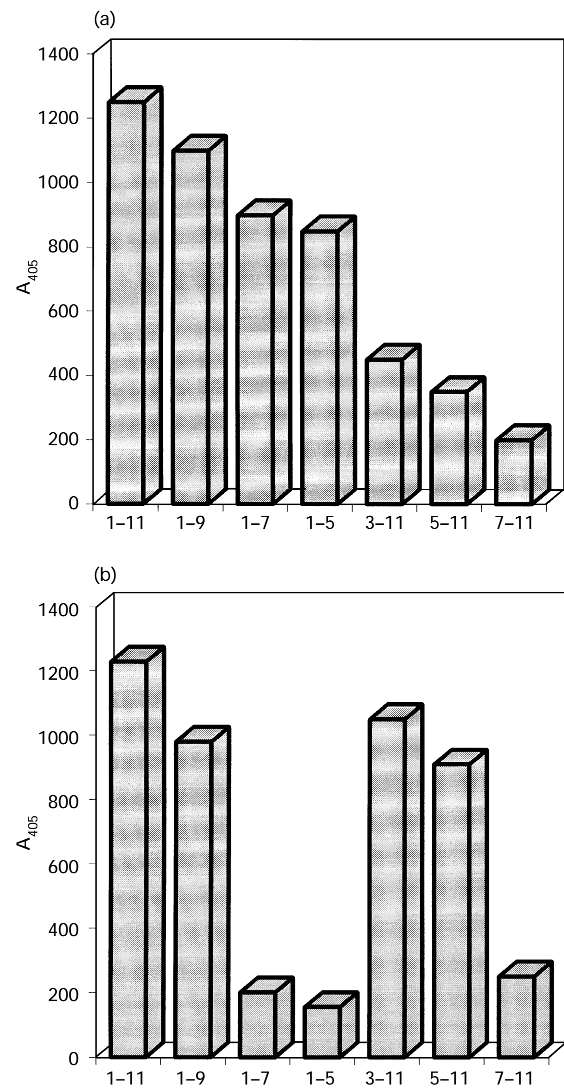

Figure 3 Binding of anti-pepgp63 and anti-pepE-24.11 antibodies to selected gp63 (a) and E-24.11 (b) synthetic peptides of various length

Experimental conditions were as described in the legend of Figure 1. Numbers below the bars represent the position of the amino acid within pepgp63 (V^1VT**HE**MA**H**ALG^{11}) and pepE-24.11 (V^1IG**HE**IT**H**GFD^{11}) (**a**) gp63 endecapeptides, nonapeptides, heptapeptides and pentapeptides probed with purified anti-pepgp63 antibodies; (**b**) E-24.11 endecapeptides, nonapeptides, heptapeptides and pentapeptides probed with purified anti-E-24.11 antibodies. Data shown are means of triplicate measurements. Similar results were obtained in two additional experiments.

bodies to these peptides is presented in Figures 3(a) and 3(b) respectively. Anti-pepgp63 antibodies (Figure 3a) bound with high affinity to the 1–9 and 1–7 gp63 nonapeptide and heptapeptide respectively (exhibiting 88 and 70 % respectively of their binding to the endecapeptide). Binding to the heptapeptide 5–11 and to the pentapeptide 7–11 was negligible. These results indicate that the residues of the segment HALG offer a very moderate contribution to antigenicity. Antibody binding to the pentapeptide 1–5 was almost identical with that to the heptapeptide 1–7, amounting to 70 and 67 % respectively of the binding to the original endecapeptide, suggesting that the sequence V^1VTHE^5 may be the crucial part of the epitope. Moreover, binding of the antibodies to the 3–11 nonapeptide was 50 % of that to the 1–5 pentapeptide indicating that residues V^1V^2 contribute significantly to the antigenicity of the epitope. Anti-pepE-24.11 antibodies (Figure 3b) bound with high affinity to the 1–9 and 3–11 E-24.11 nonapeptides and to the 5–11 heptapeptide (exhibiting 80–90 % of the binding to the original endecapeptide). Thus we conclude that the common segment E^5ITHG^9 contained within all three peptides is crucial for antibody binding. Moreover, binding to the 7–11 pentapeptide is very weak indicating that residues E^5I^6 contribute significantly to the antigenicity of the epitope.

Structural profiles of pepgp63 and pepE-24.11

The complete assignment of all proton resonances (NH, $C^{\alpha}H$ and side-chain aliphatic protons) was based on the combined use of COSY, HOHAHA and NOESY experiments (Tables 2 and 3).

Intense nuclear Overhauser enhancement (NOE) connectivities appeared between amide protons at both ends of pepgp63 (Figure 4), whereas low absolute $\Delta\delta/\Delta T$ values were obtained for H^4NH ($+0.3\times10^{-3}$ p.p.m./K) and G^{11}NH (-1.5×10^{-3} p.p.m./K). This latter indicates that the amide protons of His^4 and Gly^{11} are not entirely exposed to the solvent and they are possibly involved in intramolecular interactions [52,53]. The simultaneous oc-

Table 2 NMR data of ^{261}VTHEMAHALG^{271} (pepgp63) (mmol/dm^3) in [^{2}H]DMSO at 305 K referenced to tetramethylsilane (original aqueous solution at pH 5)

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	Others	$10^3 \times$ Temperature coefficient (p.p.m./K)
V		3.33	2.02	0.89			
				0.83			
V	8.20	4.31	2.02	0.86			−5.8
T	8.09	4.21	4.01	1.02			−7.0
H^{264}	7.81	4.45	2.94			7.54 (C^2H)	+0.3
			2.88			6.84 (C^4H)	
E	8.29	4.18	1.93	2.20			−6.2
			1.76				
M	8.63	4.33	1.96	2.47	2.00		−7.3
			1.82	2.45			
A	8.11	4.19	1.19				−5.3
H^{268}	7.99	4.38	2.91			7.52 (C^2H)	−3.9
						6.84 (C^4H)	
A	8.00	4.19	1.23				−3.4
L	8.49	4.30	1.51	1.61	0.86		−9.3
G	7.74	3.59					−1.5

Table 3 NMR data of V^{580}IGHEITHGFD^{590} (pepE-24.11) (mmol/dm^3) in [^{2}H]DMSO at 305 K referenced to tetramethylsilane (original aqueous solution at pH 5)

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	Others	$10^3 \times$ Temperature coefficient (p.p.m./K)
V		3.48	2.03	0.88			
I	8.45	4.21	1.76	1.41	0.84		
				1.08	0.81		
G	8.43	3.75					−7.6
		3.7					
H^{583}	7.95	4.50	2.87			7.52 (C^2H)	−3.9
						6.77 (C^4H)	
E	8.36	4.21	1.88	2.18			−5.8
			1.76				
I	8.15	4.21	1.76	1.41	0.84		−6.0
				1.08	0.81		
T	7.83	4.24	3.99				−4.4
H^{587}	7.82	4.44	2.97			7.54 (C^2H)	−4.9
			2.87			6.85 (C^4H)	
G	8.10	3.64					−4.5
F	8.29	4.52	3.08			7.23 (C^2H,C^6H)	−5.8
			2.81			7.17 (C^3H,C^4H,C^5H)	
D	7.86	4.18	2.51				−3.7
			2.40				

currence of both consecutive NH_i/NH_{i+1} NOE connectivities and low absolute temperature coefficient values provide evidence for a rather rigid conformation of pepgp63. Thus the presence of the intense $A^9NH/L^{10}NH$ and $L^{10}NH/G^{11}NH$ NOE connectivities in combination with the low absolute $\Delta\delta/\Delta T$ value of $G^{11}NH$ argue in favour of a β_I turn at the C-terminal tetrapeptide ($-H^8$-A-L-G^{11}) stabilized through the formation of a hydrogen bond between $G^{11}NH$ and H^8CO [54].

Strong NOE correlation between the T^3NH/H^4NH successive amide protons was also detected at the N-terminal tetrapeptide (V^1VTH^4-) of pepgp63, whereas V^2NH/T^3NH NOE connectivity was not detected (Figure 4). In addition, the positive temperature coefficient value found for H^4NH ($+0.3\times10^{-3}$ p.p.m./K) may derive from either the shielding effect of the aromatic side chain of histidine or participation of this NH group in a hydrogen-bonding interaction. Although the preceding NMR data argue in favour of a folded structure at the N-terminus of pepgp63, they do not allow us to define with certainty the occurrence of a turn in the V^1VTH^4 sequence.

Emphasis should also be placed on the different behaviour of the histidine residues belonging to the HEMAH Zn^{2+}-binding site of gp63. It appears, therefore that the side chain of His^8 is rather flexible compared with that of His^4. This assumption comes from the chemical-shift-difference values of His^4 and His^8 $C^{\beta}H_2$ protons (Table 2), which indicate that only the His^4 $C^{\beta}H_2$ protons are magnetically non-equivalent ($\Delta\delta His^4$ $C^{\beta}H^2 \sim 0.06$ p.p.m.) because of the rather restricted mobility of this side chain.

From the predicting NMR data, a helical structure for pepgp63 is not validated. Although strong NH_i/NH_{i+1} NOE connectivities are indicative of an α-helix [54–56], the lack of $C^{\alpha}H_i/NH_{i+3}$ and strong $C^{\alpha}H_i/C^{\alpha}NH_{i+3}$ cross-peaks [54] in our spectra demonstrate the absence of a helical structure. This conclusion is also supported by the high absolute temperature coefficient values for

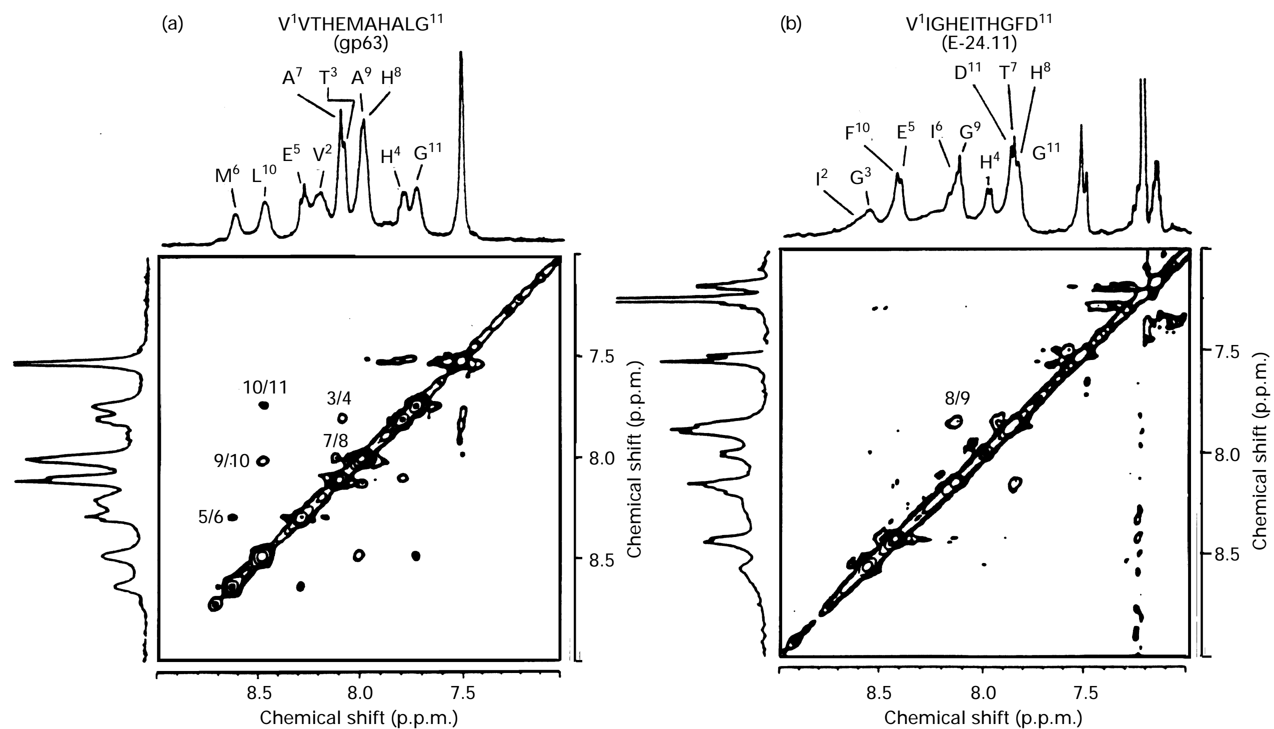

Figure 4 The NH_i/NH_{i+1} region of the 400 MHz NOESY spectra of pepgp63, (V¹VTHEMAHALG¹¹) (a) and pepE-24.11 (V¹IGHEITHGFD¹¹) (b) in [²H]DMSO solution

the amide protons of Leu¹⁰, Ala⁹, His²⁶⁸ (His⁸), Ala⁷, Met⁶ and Glu⁵, which indicate that they are exposed to the solvent. Even though pepgp63 incorporates residues that are not helix breakers [57,58] (e.g. Val, Glu, His), an α-helix cannot be predicted from our NMR study.

The absence of NH_i/NH_{i+1} connectivities (Figure 4) in the peptide backbone of pepE-24.11 (a unique exception is the observed H⁸NH/G⁹NH connectivity at the C-terminus) and the temperature coefficient values (below -3.0×10^{-3} p.p.m./K) determined for all the amide protons (Table 3) indicate that the peptide does not have a hydrogen-bonded structure.

Thus the $\Delta\delta$ values suggest that there are differences in the side-chain rotamer populations. The same conclusion can be drawn from the vicinal coupling constant values ($^3J_{\alpha\beta}$) of the His⁸ side chain [59]. The percentage of the rotamers [$x_1 = 60°$ (g^+), 180(t) and $-60°$ or 300(g^-)] [60,61] (the authors in ref [61] use a nomenclature different from the one used here) estimated on the basis of the coupling constants ($^3J_{\alpha\beta} = 5.3$ Hz, $^3J_{\alpha\beta'} = 5.5$ Hz) indicates that the equilibrium is shifted towards one of them ($g^- = 17\%$, $t = 19\%$ and $g^+ = 64\%$). The g^+ population results from the sum of the coupling constants and does not depend on the stereospecific assignment of the *pro-S* and *pro-R* proton, whereas g^- and t are rather tentatively attributed and could be exchanged. The rotamer populations of the Asp¹¹ side chain were also calculated on the basis of the coupling constant values (Asp¹¹: $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ 5.2 and 3.5 Hz respectively, $g^- = 16\%$, $t = 0\%$ and $g^+ = 84\%$). Shifting of the rotamer equilibrium of His⁸ and Asp¹¹ to the less energetically favourable g^+ (64 and 84% respectively) may derive from conformational restrictions of the His and Asp side chains. In contrast, the main conformer of Phe¹⁰ ($t = 57\%$) is the one energetically favoured (Phe¹⁰: $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ 4.6 and 9.4 Hz respectively, $g^- = 10\%$, $t = 57\%$ and $g^+ = 33\%$).

On the other hand, the absence of amide protons with low absolute temperature coefficient values excludes an interaction between the β-carboxylate of Asp¹¹ and one NH group of the peptide backbone as often proposed in the literature [62,63]. Taking into account the NME results so far discussed, as well as the uniquely observed NOE connectivity between H⁸NH and G⁹NH of the peptide backbone, we can assume that the C-terminal tetrapeptide (–H⁸GFD¹¹) of pepE-24.11 adopts a less flexible conformation, in which the side-chain groups of His⁸ and Asp¹¹ play a key role.

In conclusion, the present NMR study indicates that pepgp63 adopts a folded structure whereas pepE-24.11 takes up a rather flexible conformation. The most prominent feature to emerge is the structural differentiation of the histidine moieties in the HEXXH fingerprint of gp63 and E-24.11. It appears therefore that His⁴ (namely His²⁶⁴) contributes to the stabilization of pepgp63, whereas His⁸ (namely His⁵⁸⁷) is involved in partial stabilization of the C-terminal region of pepE-24.11.

The conformational results correlate well with the antibody specificity of the Zn^{2+}-binding regions of gp63 and E-24.11.

Binding of the anti-pepgp63 and anti-pepE-24.11 antibodies to the cognate gp63 and E-24.11 protein molecules

The binding and specificity of the generated oligoclonal antibodies was further assessed on the gp63 and E-24.11 protein molecules. To this end, anti-pepgp63 and anti-pepE-24.11 antibodies were tested by ELISA for binding to *Leishmania* and rat kidney membrane preparations containing the gp63 and E-24.11 proteins respectively as well as to the purified proteins (Table 4). It was interesting to find that each antibody was capable of recognizing its respective cognate protein molecule, even though the particular sequence against which it was raised should

Table 4 Specificity of the affinity-purified anti-pepgp63 and anti-pepE-24.11 antibodies

Antibody binding was determined by ELISA and was corrected for non-specific binding.

	Antibody binding (A_{492})	
Antigen	Anti-pepgp63	Anti-pepE-24.11
pepgp63 (0.5 μg/well)	975	185
pepE-24.11 (0.5 μg/well)	150	850
Purified gp63 (50 ng/well)	560	420
Purified E-24.11 (50 ng/well)	450	375
Leishmania membranes (5 μg/well)	1100	640
Rat kidney membranes (5 μg/well)	880	1170

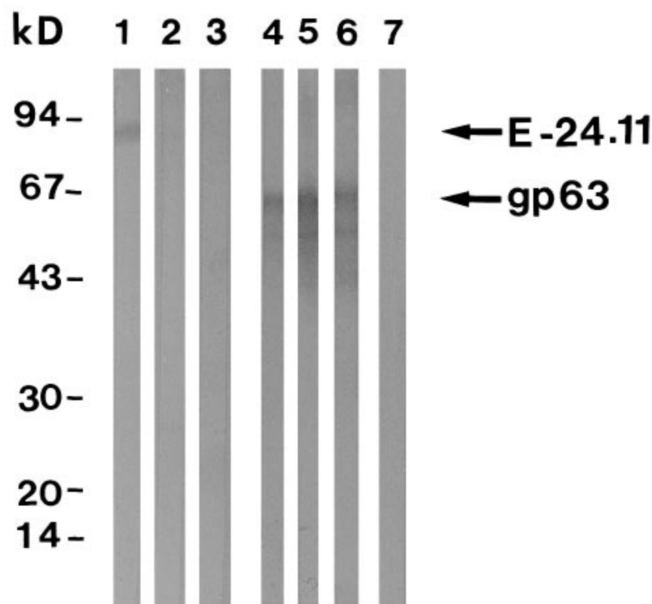

Figure 5 Binding of purified anti-pepgp63 and anti-pepE-24.11 to Western blots of *Leishmania* or kidney membrane preparations

Membrane preparations were analysed by SDS/PAGE under reducing conditions on discontinuous 10% minislab gels. Rat kidney membrane preparations were probed with mAb 23B11 recognizing rat E-24.11 (lane 1), anti-pepE-24.11 (lane 2) or anti-pepgp63 (lane 3). *Leishmania* membrane preparations were probed with mAb LD33 raised against purified gp63 (lane 4), anti-pepgp63 (lane 5) or anti-pepE-24.11 (lane 6). *Leishmania* membrane preparations probed with rabbit preimmune serum (1:200 dilution) was used as a negative control (lane 7). Abbreviation: kD, kDa.

have a folded pocket-like conformation on the native protein [64]. Moreover, it was noteworthy that, although both antibodies showed significant selectivity for their respective immunogen peptides, cross-reactivity was revealed when they were tested on membrane preparations or purified protein molecules, i.e. both antibodies were found to cross-react effectively with either protein molecule (Table 4). Binding of both antibodies to the irrelevant endecapeptide gp63 250–260, to RSA or to rat liver membranes, used as negative controls, yielded in all cases A_{492} values of less than 100.

The specificity of binding of the purified anti-pepgp63 and anti-pepE-24.11 antibodies to their homologous peptides as well as to their homologous and heterologous proteins was also demonstrated by preabsorbing the affinity-purified antibodies to purified gp63 bound to CNBr-activated Sepharose beads. Binding of the preabsorbed antibodies was measured by ELISA on microtitration plates coated with pepgp63 and pepE-24.11 as well as on purified gp63 and E-24.11. It was thus shown that preabsorption of both antibodies to gp63–Sepharose beads resulted in a significant decrease in binding to their homologous peptides ($\sim$ 60–70%) as well as to their homologous and heterologous purified proteins ($>$ 85%) (results not shown).

The observed antibody cross-reactivity was in part confirmed by Western-blot analysis of *Leishmania* and rat kidney membrane preparations (Figure 5) as well as on immunoblots of purified proteins (results not shown). It was thus demonstrated that both antibodies recognize SDS-denatured gp63 but not SDS-denatured E-24.11. This finding suggests that unfolding of gp63, evoked by the action of SDS, does not affect the antigenicity of the active-site region of the protein. On the contrary, binding of the antibodies to E-24.11 appears to be conformation-dependent and is lost on treatment of the protein with SDS.

Finally, the observed cross-reactivity of the antibodies was further supported by immunostaining of gp63 and E-24.11 on *L. major* promastigotes and kidney epithelial cells (Figures 6 and 7). Both antibodies immunostained paraformaldehyde-fixed parasites and NRK cells (Figures 6 and 7 respectively). Their binding pattern was comparable with that of their respective positive controls, namely mAbs LD33 and 23B11 which recognize gp63 and E-24.11 respectively [22,23]. It should be noted that all immunostaining was abolished in the absence of primary antibodies or in control experiments where the purified anti-pepgp63 and anti-pepE-24.11 antibodies were preabsorbed with purified gp63 bound to Sepharose beads (not shown).

Of interest is our finding that, although pepgp63 and pepE-24.11 were found to be antigenically and structurally different, their respective antibodies, i.e. anti-pepgp63 and anti-pepE-24.11, cross-react with and recognize the native molecules.

The different structural profiles of the two peptides as determined by NMR are in good agreement with their different antigenic profiles and may explain the selectivity of the generated oligoclonal antibodies for their respective peptides, indicating that the conformational motif of each of the two peptides is critical for antibody binding. It is also very probable that the structural differentiation of the two histidine residues within each peptide as well as between the two peptides contributes to the different antigenic roles of these conserved residues. On the other hand, the observed cross-reactivity of the antibodies with both peptidases, in their native form, suggests a similar conformation of their active-site regions.

X-ray-diffraction studies of thermolysin and carboxypeptidase A have shown that the Zn^{2+} ion is co-ordinated by three amino acid side chains and a water molecule [65]. Recently, unique signatures within the amino acid sequences of the Zn^{2+}-metalloproteases were identified and a classification of these enzymes into distinct superfamilies and subfamilies on the basis of sequence and structural similarities was proposed. On the basis of the first two Zn^{2+}-co-ordinating ligands, metalloproteases were divided into two major categories, one containing the HEXXH motif and the other containing the HXXEH motif [13].

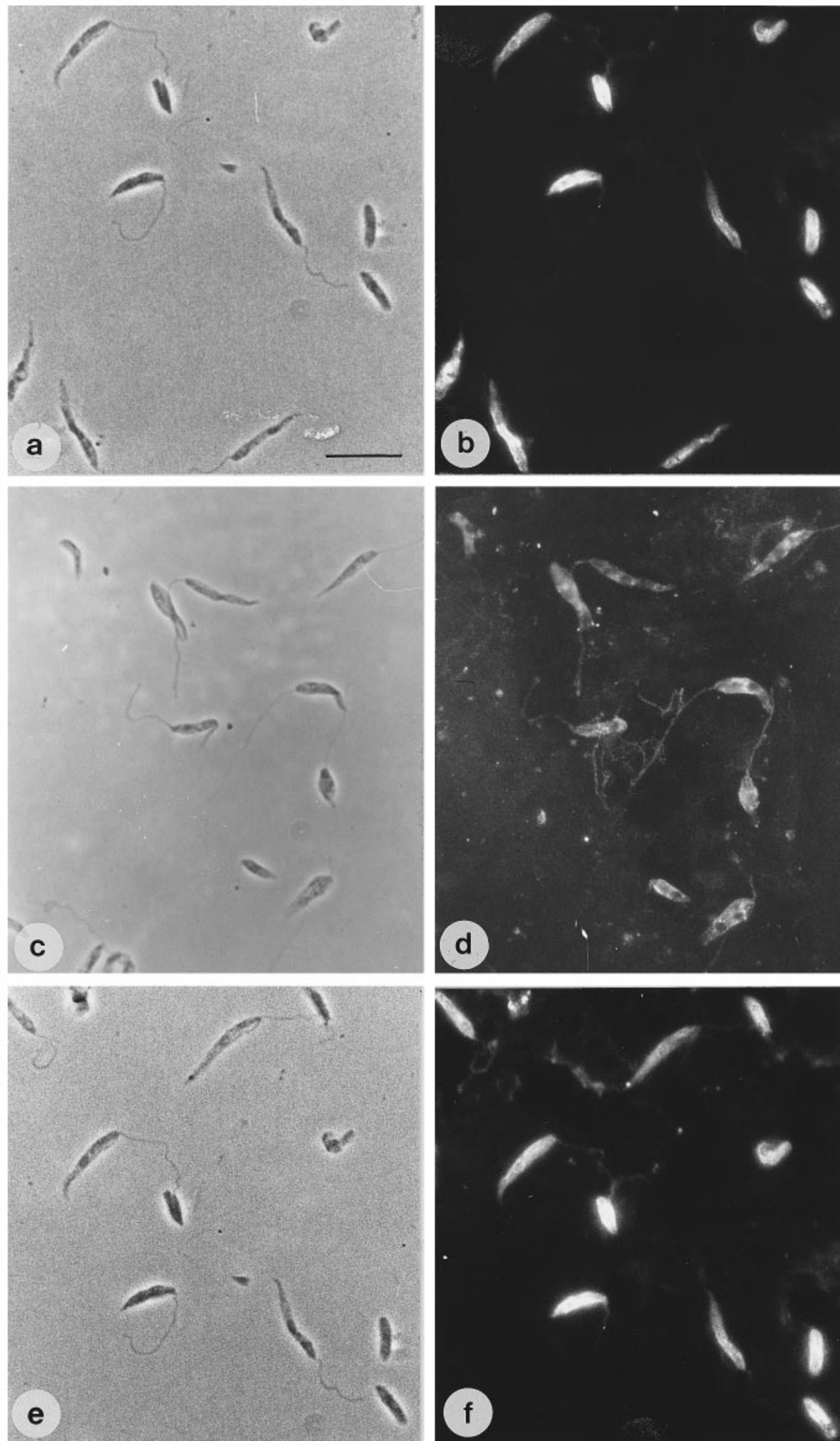

Figure 6 Immunofluorescence labelling of *Leishmania* parasites cells with anti-pepgp63 oligoclonal antibodies

Leishmania parasites were fixed on glass microscope slides and immunostained with anti-pepgp63 (**a**, **b**), anti-pepE24.11 (**c**, **d**) or mAb LD33 recognizing gp63 (**e**, **f**). Phase-contrast (left) and fluorescein optics (right) were used. All three antibodies immunostained the paraformaldehyde-fixed parasites. The bar corresponds to 15 μm.

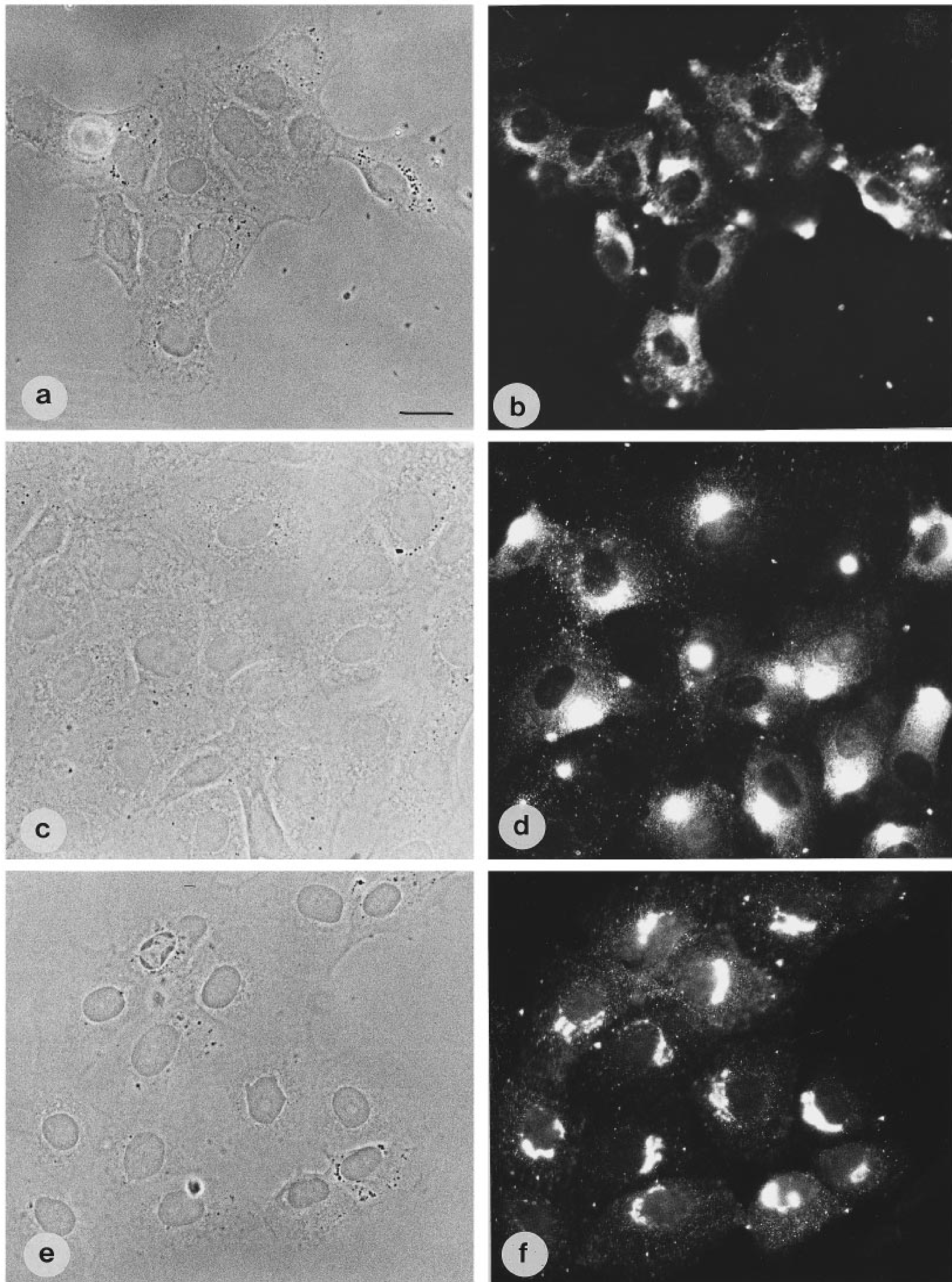

Figure 7 Immunofluorescence labelling of NRK52 cells with anti-pepE-24.11 oligoclonal antibodies

Kidney epithelial cells (NRK cell-line) were grown on glass microscope slides, fixed and immunostained with anti-pepgp63 (**a**, **b**), anti-pepE-24.11 (**c**, **d**) or mAb 23B11 which recognizes rat E-24.11 (**e**, **f**). Phase-contrast (left) and fluorescein optics (right) were used. All three antibodies produced a similar immunofluorescence labelling pattern on the NRK cells. The bar corresponds to 20 μm.

The two histidine residues within these motifs serve as Zn^{2+} ligands, and the glutamic acid residue polarizes a water molecule involved in nucleophilic attack at the scissile peptide bond [66]. The superfamily of HEXXH metalloproteases, termed zincins [13], can be further divided into two groups which contain proteases having either a histidine or a glutamic acid residue as the third distant Zn^{2+}co-ordinating ligand [67]. E-24.11 and gp63 as well as metalloproteases belonging to the thermolysin family are examples of the latter group. Glu^{646} of E-24.11 and Glu^{407} of gp63 are equivalent to Glu^{166} of thermolysin [42,67,68]. It was thus suggested that E-24.11 and thermolysin exhibit, in spite of their low overall sequence similarity, a virtually equivalent active-site Zn^{2+} environment and that their Zn^{2+}-binding sites probably possess a similar conformation [68]. Our data, in particular the cross-reactivity of the antibodies for the two protein molecules despite selectivity for their respective peptides, suggest that the geometrical conformation of the Zn^{2+}-binding sites of E-24.11 and gp63 are very similar.

The antibodies generated here may be useful tools for identifying and classifying proteins possessing similar Zn^{2+}-binding environments. These antibodies, in conjunction with further conformational studies on the Zn^{2+}-binding region of gp63 and E-24.11, may contribute to the design of highly specific and orally active inhibitors for the two enzymes, the determination of their natural substrates at their site(s) of action and the elucidation of their role in pathophysiological conditions. In the case of gp63 in particular, the acquired information may help towards the design of chemotherapeutic agents for leishmaniosis. Inhibition of gp63 and E-24.11 protease activity by the anti-pepgp63 and anti-pepE-24.11 antibodies is worth investigating and is currently being studied in our laboratory.

We thank Dr. A. J. Kenny and Dr. P. Crine for gifts of purified E-24.11 and mAb 23B11 respectively, and Dr. M. Marraud, Director of the Laboratory of Macromolecular Physical–Chemistry, ENSIC-INPL, Nancy, France, for providing the NMR facilities and for helpful discussions. We also thank Dr. S. Tzartos for valuable discussions and for critically reading the manuscript. This work was supported by grants from the EU Human Capital and Mobility Program (CHRXCT930266), Biotechnology Program (BIO2CT930326), Biomedicine and Health Program (BMHCT941378), the Greek General Secretariat of Research and Technology and the United Nations Industrial Development Organization.

REFERENCES

1 Bordier, C. (1987) Parasitol. Today **3**, 151–153
2 Medina-Acosta, E., Karess, R. E., Schwartz, H. and Russell, D. G. (1989) Mol. Biochem. Parsitol. **37**, 263–274
3 Frommel, T. O., Button, L. L., Fujikura, Y. and McMaster, W. R. (1990) Mol. Biochem. Parasitol. **38**, 25–32
4 Schneinder, P., Rosat, J. P., Bouvier, J., Louis, J. and Bordier, C. (1992) Exp. Parasitol. **75**, 196–206
5 Russell, D. G. and Wilhelm, H. (1986) J. Immunol. **136**, 2613–2620
6 Soteriadou, K. P., Remoundos, M. S., Katsikas, M. C., Tzinia, A. K., Tsikaris, V., Sakarellos, C. and Tzartos, J. S. (1992) J. Biol. Chem. **267**, 13980–13985
7 Chaudhuri, G., Chaudhuri, M., Pan, A. and Chang, K. P. (1989) J. Biol. Chem. **269**, 7483–7489
8 Yang, D. M., Fairweather, N., Button, L., McMaster, W. R., Kahl, L. P. and Liew, F. Y. (1990) J. Immunol. **145**, 2281–2285
9 Button, L. L. and McMaster, W. R. (1988) J. Exp. Med. **167**, 724–729
10 Yang, D., Rogers, M. V., Brett, S. J. and Liew, F. Y. (1993) Immunology **78**, 582–585
11 Etges, R., Bouvier, J. and Bordier, C. (1986) J. Biol. Chem. **261**, 9098–9101
12 Jongeneel, C. V., Bouvier, J. and Bairoch, A. (1989) FEBS Lett. **242**, 211–214
13 Hooper, N. M. (1994) FEBS Lett. **354**, 1–6
14 Devault, A., Sales, V., Nault, C., Beaumont, A., Roques, B. P., Crine, P. and Boileau, G. (1988) FEBS Lett.**231**, 54–58
15 Devault, A., Nault, C., Zollinger, M., Fournier-Zaluski, M. C., Roques, B. P., Crine, P. adn Boileau, G. (1998) J. Biol. Chem. **263**, 4033–4040
16 Kenny, A. J. (1986) Trends Biochem. Sci. **11**, 40–42
17 Letarte, M., Vera, S., Tran, R., Addis, J. B., Omizuka, J. R., Quackenbush, M. M., Jangenel, C. V. and McInnis, R. R. (1988) J. Exp. Med. **168**, 1247–1253
18 Shipp, M. A., Vijayaraghavan, J., Schmidt, E. V., Masteller, E. L., D'Adamio, L. D., Hersh, L. B. and Reinherz, E. L. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 297–301
19 Malfroy, B., Swertz, J. P., Guyon, A., Roques, B. P. and Schwartz, J. C. (1978) Nature (London) **276**, 523–526
20 Matsas, R., Fulcher, I. S., Kenny, A. J. and Turner, A. J. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3111–3115
21 Erdos, E. G. and Skidgel, R. A. (1989). Fed. Proc. Fed. Am. Soc. Exp. Biol. **3**, 145–151
22 Kioussi, C., Crine, P. and Matsas, R. (1992) Neuroscience **50**, 69–83
23 Kioussi, C., Mamalaki, A., Jessen, K. R., Mirsky, R., Hersh, L. B. and Matsas, R. (1995) Eur. J. Neurosci. **7**, 951–961
24 Stephenson, S. L. and Kenny, A. J. (1987) Biochem. J. **243**, 183–187
25 Soleihac, J. M., Lucas, E., Beaumont, A., Turcaud, S., Michel, J. B., Ficheux, D., Fournier-Zaluski, M. C. and Roques, B. P. (1992) Mol. Pharmacol. **41**, 609–614
26 Connelly, J. C., Skidgel, R. A., Schultz, W. W., Johnson, A. R. and Erdos, E. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8737–8741
27 Shipp, M. A., Stephano, G. B., D'Adamio, L., Switzer, S. N., Howard, F. D., Sinistera, J., Scharrer, B. and Reinherz, E. L. (1990) Nature (London) **347**, 394–396
28 Mari, B., Checler, F., Ponzio, G., Peyron, J. F., Manie, S., Farahifar, D., Rossi, B. and Auberger, P. (1992) EMBO J. **11**, 3875–3885
29 Roques, B. P., Noble, F., Dauge, V., Fournie-Zaluski, M. C. and Beaumont, A. (1993) Pharmacol. Rev. **45**, 87–146
30 Gee, N. S., Matsas, R. and Kenny, A. J. (1983) Biochem. J. **214**, 377–386
31 Aubry, M., Crine, P., Fortin, S. Legrimellec, C., Venien, C. and Zollinger, M. (1988) Biochim. Biophys. Acta **967**, 56–64
32 Kioussi, C. and Matsas, R. (1991) J. Neurochem. **57**, 431–440
33 Tzinia, A. K. and Soteriadou, K. P. (1991) Mol. Biochem. Parasitol. **47**, 83–90
34 Soteriadou, K. P., Tzinia, A. K., Mamalaki, A., Pheluzat, M. A. and Robert-Gero, M. (1994) Eur. J. Biochem. **223**, 61–68
35 Geysen, H. M., Meolen, H. R. and Bacteling, S. J. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3998–4002
36 Merrifield, R. B. (1963) J. Am. Chem. Soc. **85**, 2149–2154
37 Mitchell, A. R., Kent, S. B. H., Engelhard, M. and Merrifield, R. B. (1978) J. Org. Chem. **43**, 2845–2852
38 Kaiser, E., Colescott, R. L., Bossinger, P. D. and Cook, P. F. (1970) Anal. Biochem. **34**, 595–598
39 Tam, J. P., Heath, W. F. and Merrifield, R. B. (1983) J. Am. Chem. Soc. **105**, 6442–6455
40 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
41 Voyiatzaki, C. S. and Soteriadou, K. P. (1990) J. Biol. Chem. **265**, 22380–22385
42 Bouvier, J., Bordier, C., Vogel, H., Reichelt, R. and Etges, R. (1989) Mol. Biochem. Parasitol. **37**, 235–246
43 Dwyer, D. M. (1980) J. Protozool. **27**, 176–182
44 Soteriadou, K. P., Tzinia, A. K., Hatjiantoniou, M. G. and Tzartos, J. S. (1988) Infect. Immun. **56**, 1180–1186
45 Voyiatzaki, C. S. and Soteriadou, K. P. (1992) J. Biol. Chem. **267**, 9112–9117
46 Vaisman, I. I. and Berkowitz, M. L. (1992) J. Am. Chem. Soc. **114**, 7889–7896
47 Anchordoguy, T. J., Cessini, C. A., Crowe, J. N. and Crowe, L. M. (1991) Cryobiology **28**, 467–473
48 Tsikaris, V., Detsikas, E., Sakarellos-Daitsiotis, M., Sakarellos, C., Vatzaki, E., Tzartos, S. J., Marraud, M. and Cung, M. T. (1993) Biopolymers **33**, 1123–1134
49 Aue, W. P., Bartholdi, E. and Ernst, E. R. (1976) J. Chem. Phys. **64**, 2229–2246
50 Bax, A. and Davis, D. G. (1985) J. Magn. Reson. **65**, 355–360
51 Bodenhausen, G., Kogler, H. and Ernst, R. R. (1984) J. Magn. Reson. **58**, 370–388
52 Wuthrich, K. (1976) NMR in Biological Research: Peptides and Proteins, North Holland, Amsterdam
53 Cung, M. T., Tsikaris, V., Demange, P, Papadouli, I., Tzartos, S. J., Sakarellos, C. and Marraud, M. (1992) Pept. Res. **5**, 14–24.
54 Wuthrich, K. (1986) NMR of Proteins and Nucleic Acids, J. Wiley and Sons, New York
55 Billeter, M., Braun, W. and Wuthrich, K. (1982) J. Mol. Biol. **155**, 321–346
56 Marion, D., Zasloff, M. and Bax, A. (1988) FEBS Lett. **227**, 21–26
57 Chou, P. Y. and Fasman, G. D. (1974) Biochemistry **13**, 222–224
58 Schulz, G. E. (1988) Annu. Rev. Biophys. Chem. **17**, 1–21
59 Cung, M. T. and Marraud, M. (1982) Biopolymers **21**, 953–967
60 Benedetti, E., Morelli, G., Nemethy, G. and Scheraga, H. A. (1983) Int. J. Pept. Protein Res. **22**, 1–15
61 McGregor, M. J., Islam, S. A. and Stenberg, M. J. E. (1987) J. Mol. Biol. **198**, 295–310
62 Dyson, H. J. and Wright, P. E. (1991) Annu. Rev. Biophys. Biophys. Chem. **20**, 539–557
63 Ebina, S. and Wuthrich, K. (1984) J. Mol. Biol. **179**, 283–291

64 Vallee, B. L. and Auld, D. S. (1989) FEBS Lett. **257**, 138–140
65 Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N. Jr., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H. and Coppola, J. (1986) Brookhaven Symp. Biol. **21**, 24–90
66 Borde, W., Gomis-Ruth, F. and Stockler, W. (1993) FEBS Lett. **331**, 134–140
67 Jiang, W. and Bond, J. S. (1992) FEBS Lett. **312**, 110–114
68 Le Moual, H., Devault, A., Roques, B. P., Crine, P. and Boileau, G. (1991) J. Biol. Chem. **266**, 15670–15674

Received 22 March 1995/8 September 1995; accepted 13 September 1995