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Stimulation of *Trypanosoma cruzi* adenylyl cyclase by an α^D-globin fragment from *Triatoma* hindgut: Effect on differentiation of epimastigote to trypomastigote forms

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ABSTRACT **A peptide from hindguts of the *Triatoma* hematophagous Chagas insect vector activates adenylyl cyclase activity in *Trypanosoma cruzi* epimastigote membranes and stimulates the *in vitro* differentiation of epimastigotes to metacyclic trypomastigotes. Hindguts were obtained from insects fed 2 days earlier with chicken blood. Purification was performed by gel filtration and HPLC on C_{18} and C_4 columns. SDS/PAGE of the purified peptide showed a single band of about 10 kDa. The following sequence was determined for the 20 amino-terminal residues of this peptide: H_2N-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His. This sequence is identical to the amino terminus of chicken α^D-globin. On a Western blot, the peptide immunoreacted with a polyclonal antibody against chicken globin D. A synthetic peptide corresponding to residues 1–40 of the α^D-globin amino terminus also stimulated adenylyl cyclase activity and promoted differentiation. This ^{125}I-labeled synthetic peptide bound specifically to *T. cruzi* epimastigote cells. Activation of epimastigote adenylyl cyclase by the hemoglobin-derived peptide may play an important role in *T. cruzi* differentiation and consequently in the transmission of Chagas disease.**

A major endemia in Latin America is Chagas disease. Its etiological agent is *Trypanosoma cruzi*, a flagellate protozoan that undergoes complex morphological changes throughout its life cycle in both the insect vector and the vertebrate host (1, 2). The hematophagous *Triatoma* vector ingests circulating trypomastigote forms while feeding on the blood of an infected vertebrate host. In the insect digestive tract, ingested trypomastigotes initially differentiate to epimastigotes; in a second stage, which occurs within the vector hindgut, epimastigotes that are proliferative but not infectious convert to metacyclic trypomastigotes. The latter are infectious and nonproliferative parasite forms (3–5). This differentiation process, known as metacyclogenesis, can be induced in epimastigote liquid cultures by mammalian serum components (6), the use of medium mimicking insect urine (7), metabolic stress (8, 9), *Triatoma infestans* hindgut extracts (10, 11), or cyclic AMP (12).

In addition, metacyclic trypomastigotes exhibit higher intracellular cyclic AMP levels than epimastigotes (13), and evidence from this laboratory has shown that *T. cruzi* membranes possess an adenylyl cyclase associated with the α subunit of the stimulatory guanine nucleotide-binding regulatory protein (G protein) (14). $G\alpha_i$ and $G\beta$ polypeptides were also found in these membranes (15).

A basic question arising from these findings is whether a molecule present in *Triatoma* hindgut might be capable of activating epimastigote adenylyl cyclase and thereby causing parasite differentiation.

Here we present evidence showing that a hemoglobin-derived peptide purified from hindgut extracts is responsible for *T. cruzi* epimastigote adenylyl cyclase activation and metacyclogenesis.

MATERIALS AND METHODS

Parasite and Insect Strains. *T. cruzi* epimastigotes of the Tulahuen or RA strain were maintained and harvested as indicated (10, 11).

Noninfected *T. infestans* in the fifth or adult stage were reared at 28°C and 60% relative humidity and were given chickens or mice weekly from which they sucked blood. Hindguts were processed 2 days after feeding.

Membrane Preparation. *T. cruzi* epimastigote membranes were prepared according to Torruella *et al.* (16).

Factor Purification from Hindgut Homogenates. Hindguts were removed and homogenized in phosphate-buffered saline (5 ml/g of tissue). After centrifugation for 15 min at 10,000 × *g*, 10-ml aliquots of the supernate ("crude extract") were passed through a Sephadex G-25 (medium) column (3.5 × 50 cm) and equilibrated with 50 mM Tris·HCl (pH 7.5). Fractions that activated adenylyl cyclase in epimastigote membranes, designated "Sephadex preparation," were pooled and passed through Sep-Pak C_{18} cartridges (Waters; 10 ml per cartridge) equilibrated with water. Each cartridge was washed with 5 ml of water followed by 5 ml of 15% (vol/vol) acetonitrile in water; subsequently, the factor was eluted with 10 ml of 50% acetonitrile in water. "Sep-Pak C_{18} eluates" were pooled, and solvent was removed under reduced pressure. The material corresponding to 400 hindguts was resuspended in 0.5 ml of water and further purified by reverse-phase C_{18} HPLC (Vydac column; 10.0 × 250 mm; 300 Å pore size). Elution was performed with an acetonitrile gradient from 12% to 56% in water at a flow rate of 2.0 ml/min (see Fig. 1*A*). The peak fraction eluting at ≈47% acetonitrile was lyophilized, resuspended in 1 ml of water ("C_{18} HPLC fraction"), and further purified by reverse-phase C_4 HPLC (Vydac column; 4.6 × 250 mm; 300 Å pore size). Elution was performed with a gradient of 0% to 40% acetonitrile in water

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Abbreviation: G protein, guanine nucleotide-binding regulatory protein.

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at a flow rate of 1.0 ml/min (see Fig. 1*B*). The peak fraction eluting at about 28% acetonitrile was lyophilized, resuspended in 1 ml of water ("C_4 HPLC fraction"), and subjected to further studies. The amount of active material in the different fractions was expressed as its absorbance at 280 nm (A_{280}).

Peptide Sequencing. The hindgut peptide (250 pmol) was subjected to amino-terminal amino acid sequencing by automated Edman degradation using a model 477A protein microsequencer (Applied Biosystems) according to Hewick *et al.* (17).

Peptide Synthesis. Solid-phase peptide synthesis was performed on an Applied Biosystems automated synthesizer (model 431A, version 1.12) using Fmoc chemistry (18). Before use, peptide solutions were purified by passage through Sep-Pak C_{18} cartridges.

Differentiation Assay. Metacyclogenesis was evaluated as described by Isola *et al.* (10, 11). Briefly, cultures in modified Grace's medium (19) supplemented with fractions purified from hindgut homogenates and inoculated with 4×10^6 epimastigotes per ml were incubated at 28°C, and the percentage of flagellates that differentiated to the metacyclic stage was determined on at least 200 forms in wet preparations and in slides stained with May–Grünwald/Giemsa. Cultures were examined every day, and observations were performed in quintuplicate.

Adenylyl Cyclase Assay. The standard incubation mixture contained 50 mM Tris·HCl (pH 7.4), 0.2 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic AMP, 1.5 mM $MgCl_2$, 0.8 mM [α-^{32}P]ATP (specific activity, 200 dpm/pmol), 20 μM GTP, 2 mM phosphocreatine, 0.2 mg of creatine kinase, and about 200 μg of protein from *T. cruzi* epimastigote membranes plus the indicated additions. The final volume was 0.1 ml. Incubations were performed at 37°C for 10 min on triplicate samples. Reactions were stopped, and cyclic AMP was purified and counted for radioactivity as described by Solomon *et al.* (20). Under these conditions, the amount of product formed was proportional to the amount of *T. cruzi* membranes and the incubation time.

^{125}I Labeling of the Peptide and Binding to Epimastigote Cells. The 40-mer synthetic peptide was labeled with chloramine T following the procedure described by Greenwood (21) with the modifications indicated by Molinolo *et al.* (22).

The incubation mixture for the binding assay contained 50 mM phosphate buffer (pH 7.4), 0.2% albumin, 0.4 pmol of labeled peptide (specific activity 200 μCi/μg; 1 Ci = 37 GBq), various concentrations of unlabeled peptide, and about 2×10^6 epimastigote cells. The total volume was 0.3 ml. Incubations were performed for 90 min at room temperature. The bound peptide was separated by filtration through glass fiber filters (Whatman GF-C). Nonspecific binding was determined in the presence of 0.5 μg of unlabeled peptide. The binding constant was calculated according to Cuatrecasas and Hollenberg (23).

Preparation of Globin D and Antibody. Globin D was prepared from chicken adult red cell lysate by chromatography on CM-Sephadex and precipitation with acetone/hydrochloric acid as described by Brown and Ingram (24). Rabbit antiserum was also prepared according to ref. 24.

Other Analytical Methods. The gut peptide was electrophoresed in 22% polyacrylamide gels in the presence of SDS and stained with silver nitrate (25). Western blots were blocked with nonfat milk and incubated with a 1:500 dilution of rabbit antiserum. The immune complexes were detected with the Vectastain ABC-AP kit (Vector Laboratories).

RESULTS

Peptide Characterization and Purification. Preparations from hindguts of *T. infestans*, fed 2 days before with chicken blood, activated adenylyl cyclase in *T. cruzi* epimastigote membranes. The effect was blocked by guanosine 5′-[β-thio]diphosphate, in either the presence or absence of GTP or 5′-guanylyl imidotriphosphate. These "crude preparations" also stimulated the differentiation of epimastigotes to metacyclic trypomastigotes. Adenylyl cyclase activation and differentiation were not observed in Sep-Pak C_{18} eluates from a Sephadex preparation that had been subjected to protease treatment (Table 1). On the other hand, activation and differentiation were maximal after 2 days of blood feeding and decreased in subsequent days (results not shown). Table 1 also shows that a preparation from hindguts of insect vectors fed with mouse blood also activated adenylyl cyclase and promoted differentiation.

Purification of the putative peptidic factor was further performed by reverse-phase chromatography on Sep-Pak C_{18} cartridges and HPLC on C_{18} (Fig. 1*A*) and C_4 columns (Fig. 1*B*). After purification, the compound that activated adenylyl cyclase and stimulated differentiation of the parasite ran as a single band of about 10 kDa on SDS/PAGE (Fig. 2, lane B).

The following sequence for 20 residues of the amino terminus of the peptide was determined: NH_2-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His. Computer analysis of this sequence unequivocally showed that it was identical to the amino terminus of chicken α^D-globin (26), the minor component of adult chicken α-globin chains (Fig. 3).

Further support for the authenticity of the globin-derived peptide was obtained by characterization with a rabbit anti-chicken globin D antibody. On a Western blot, the hindgut peptide immunoreacted with this antibody (Fig. 2, lane C).

For practical purposes the peptide was designated GDF (for globin-derived factor).

Table 1. Effects of *Triatoma* hindgut preparations on *T. cruzi* adenylyl cyclase and differentiation

Addition(s)	A_{280}*	Adenylyl cyclase, pmol per min per mg of protein	% differentiation
None		26 ± 2	4 ± 2
20 μM GTP		24 ± 2	ND
20 μM Gpp[NH]p		24 ± 2	ND
Crude extract	5.0	40 ± 3	75 ± 10
Sephadex prep	2.0	74 ± 4	71 ± 9
Sephadex prep + 20 μM GTP	2.0	78 ± 4	ND
Sephadex prep + 20 μM Gpp[NH]p	2.0	75 ± 4	ND
Sephadex prep + 20 μM GTP + 100 μM GDP[β-S]	2.0	26 ± 4	ND
Sep-Pak C_{18} eluate	0.1	78 ± 5	75 ± 2
Sep-Pak C_{18} eluate†	0.1	26 ± 2	4 ± 2
C_{18} HPLC fraction	0.02	50 ± 3	55 ± 7
C_4 HPLC fraction	0.01	53 ± 3	60 ± 8
10^{-8} M chicken hemoglobin		28 ± 3	5 ± 3
Crude extract from mouse blood-fed insects	5.0	55 ± 3	70 ± 8

Except for the experiment in the last row, all the results correspond to preparations from insects fed chicken blood. ND, not done; Gpp[NH]p, 5′-guanylyl imidotriphosphate; GDP[β-S], guanosine 5′-[β-thio]diphosphate. Ten-microliter aliquots of each fraction were used in all assays.

*Of the fraction added.

†The crude preparation was treated with proteinase K (0.15 mg/ml) for 30 min at 30°C.

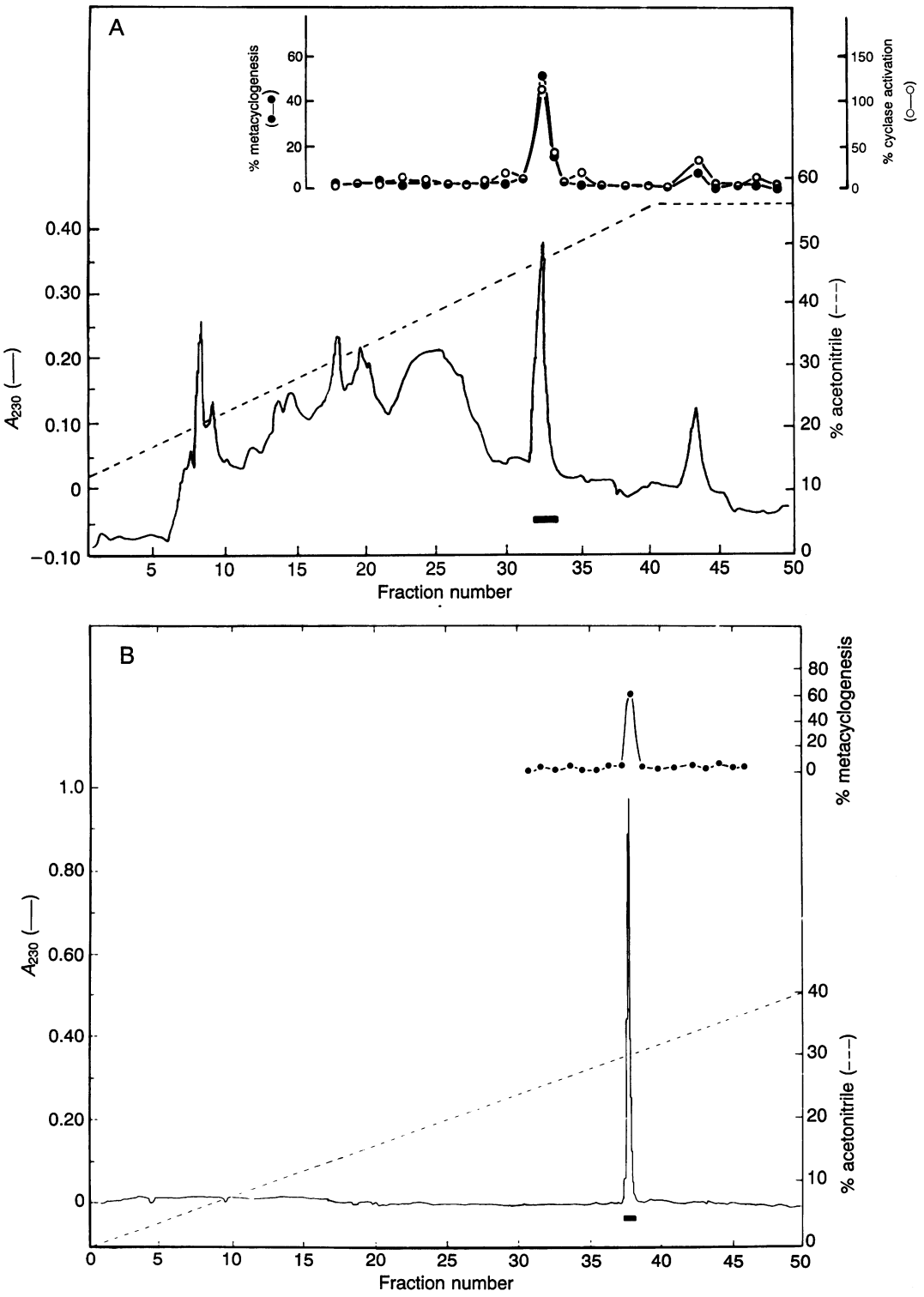

FIG. 1. Purification and characterization of the hindgut peptide. Elution profiles from C_{18} (*A*) and C_4 (*B*) HPLC columns are shown.

Biological Activity of Synthetic Peptides. A peptide corresponding to residues 1–40 of chicken α^D-globin was synthesized [peptide-(1–40)]. This peptide stimulated adenylyl cyclase activity in *T. cruzi* epimastigote membranes and promoted *in vitro* parasite differentiation. These stimulatory effects were observed at peptide concentrations higher than 10^{-10} M. Dose–response curves for both effects were coincident (Fig. 4).

To determine with more precision the primary structure of the active region, other synthetic peptides were tested for their effect on epimastigote adenylyl cyclase. As shown in Fig. 5, peptide-(30–49) was a little less active than peptide-(1–40). Peptide-(35–73) resulted in much less activity, and peptide-(41–73) was inactive. However, the latter peptide enhanced the effect of peptide-(1–40) on adenylyl cyclase.

It is important to point out that chicken hemoglobin was unable to activate epimastigote adenylyl cyclase or to promote differentiation (Table 1).

The ^{125}I-labeled synthetic peptide-(1–40) bound specifically to *T. cruzi* epimastigote cells. Displacements of the labeled ligand by unlabeled peptide-(1–40) or peptide-(30–49) were identical, giving an estimated dissociation constant of

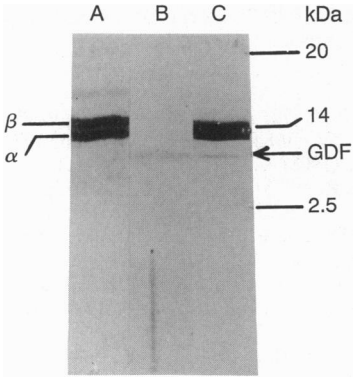

FIG. 2. SDS/PAGE of hindgut fractions. Silver-stained gels of the crude extract (lane A) and C_4 HPLC fraction (lane B) are shown. Lane C shows a Western blot of the crude extract incubated with the anti-globin D antibody. α and β, globin chains.

2×10^{-9} M and about 2000 receptors per cell. Peptide-(35–73) and peptide-(41–73) were less efficient in the displacement of the labeled ligand (Fig. 6).

DISCUSSION

Results reported here indicate that a peptide, designated GDF, which naturally exists in hindguts of *Triatoma* fed with chicken blood, causes activation of *T. cruzi* epimastigote adenylyl cyclase and stimulation of metacyclogenesis. The peptide is a breakdown product of α^D-globin, a minor component of chicken α-globin chains.

A possible model for *T. cruzi* differentiation can be postulated. The globin fragment, upon binding to a specific receptor present in epimastigote membranes, causes adenylyl cyclase activation. A G_S protein is probably involved in the signal transduction pathway responsible for the rise of intracellular cyclic AMP levels. Thereafter, the cyclic nucleotide causes protein kinase A activation, which might determine the phosphorylation of specific protein targets involved in the differentiation of epimastigotes to trypomastigotes. Evidence for such a mechanism is supported by the finding in this laboratory of protein kinase A in *T. cruzi* epimastigotes, which is similar to type II bovine heart cyclic AMP-dependent protein kinase (27).

In addition to this, *T. cruzi* transduction pathways might also involve a protein kinase C whose existence in epimastigotes was also demonstrated in this laboratory (28). These facts raise the possibility that Trypanosomatidae differentiation and proliferation can be controlled by transduction mechanisms resembling those occurring in mammalian cells.

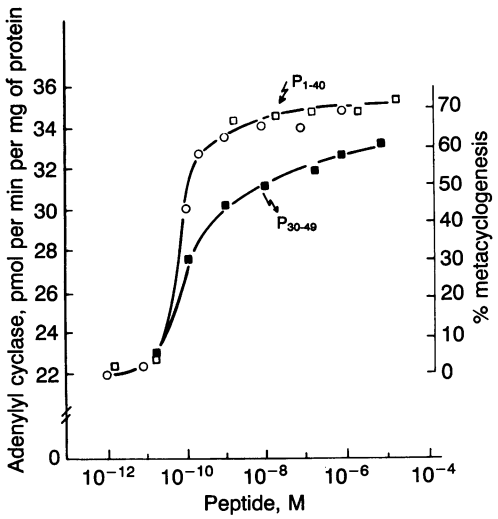

FIG. 4. Stimulation of epimastigote adenylyl cyclase and metacyclogenesis by synthetic peptides (P). □ and ■, Adenylyl cyclase activity as a function of peptide-(1–40) and peptide-(30–49), respectively; ○, metacyclogenesis as a function of peptide-(1–40) concentration.

The model presented here is based on the use of insect vectors fed with chicken blood. However, hindgut extracts from insects fed with mouse blood also stimulated epimastigote adenylyl cyclase and promoted differentiation. This could indicate that other fragments from a variety of globin species might have a similar effect. Thus, *T. cruzi* could have several receptors with different specificity for globin-derived peptides, or alternatively, only one receptor specific for a common domain shared by several α-globin chain species.

As shown in Fig. 3, α-globin chains from at least three vertebrate species, chicken, mouse, and human, have extensive amino acid homologies (26, 29, 30). Considering the results obtained with synthetic peptides (Figs. 5 and 6) and accepting that *T. cruzi* has only one receptor entity, it could be postulated that this receptor is specific for peptides having sequences of the α-globin chain domain between positions 30/31 and 62.

These facts are relevant in the pathogenesis of the parasite infection. In an animal host, the risk for a dissemination of infective forms through the whole organism might be parallel to the capacity to generate globin proteolytic fragments having such a domain. In mammals, this function is displayed

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                    10        20        30        40        50
CαD  MLTAEDKKLI|QQAWEKAASH|QEEFGAEALT|RMFTTYPQTK|TYFPHFDLSH|
CαA  MV-SAA--NNV KGIFT-IAG- AE-Y---T-E ---TTY-P-- --------L-P
Hα   MV-SPA--TNV KAAWG-VGA- AG-Y---A-E ---LSF-T-- --------L-H
Mα   MV-SGE--SNI KAAWG-IGG- GA-Y---A-E ---ASF-T-- --------V-H

                    60        70        80        90       100
CαD  GSDQVRGHGK KVLGALGNAV|KNVDNLSQAM|AELSNLHAYN|LRVDPVNFKL|
CαA  --A-IK---- --VA--IE-A NHI-DIAGTL SK--D---HK ----------
Hα   --A-VK---- --AD--TN-V AHV-DMPNAL SA--D---HK ----------
Mα   --A-VK---- --AD--AS-A GHL-DLPGAL SA--D---HK ----------

                   110       120       130       140
CαD  LSQCIQVVLA|VHMGKDYTPE|VHAAFDKFLS|AVSAVLAEKY|R
CαA  -GQ-FL-VVA I-HPAAL--E ---SL----C A-GT--TA-- -
Hα   -SH-LL-TLA A-LPAEF--A ---SL----A S-ST--TS-- -
Mα   -SH-LL-TLA S-HPADF--A ---SL----A S-ST--TS-- -
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FIG. 3. Amino acid sequences of chicken α^D (CαD; 141 aa), chicken α^A (CαA; 142 aa), mouse α (Mα; 142 aa), and human α (Hα; 142 aa) globin chains.

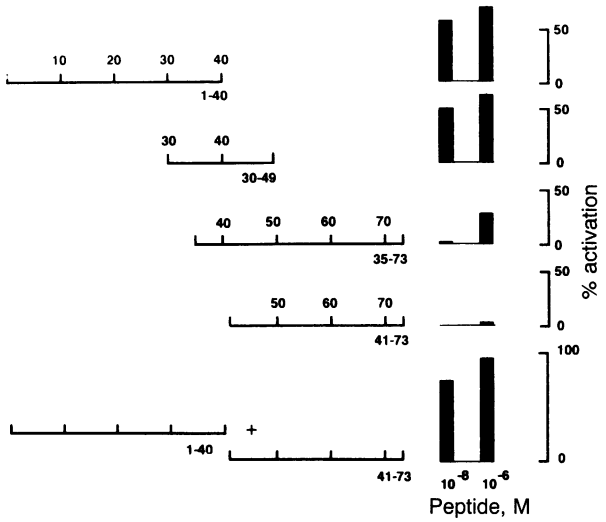

FIG. 5. Effect of synthetic peptides on epimastigote adenylyl cyclase activity. Basal adenylyl cyclase activity was 22 pmol per min per mg of protein.

by macrophages, which constitute the first cellular step for both hemoglobin degradation and *T. cruzi* infection.

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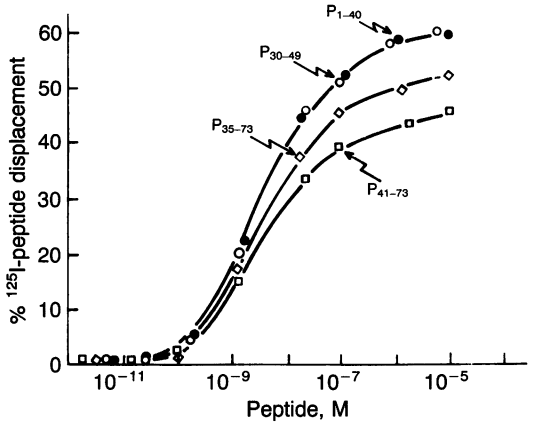

FIG. 6. Competition of ^{125}I-labeled peptide-(1–40) binding to epimastigote cells by unlabeled peptides (P).

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