Trypanosoma cruzi activates the kinin pathway through the activity of its major cysteine proteinase, cruzipain. Because kininogen molecules may be displayed on cell surfaces by binding to glycosaminoglycans, we examined whether the ability of cruzipain to release kinins from high molecular weight kininogen (HK) is modulated by heparan sulfate (HS). Kinetic assays show that HS reduces the cysteine proteinase inhibitory activity ($K_{\text{inact}}$) of HK about 10-fold. Conversely, the catalytic efficiency of cruzipain on kinin-related synthetic fluorogenic substrates is enhanced up to 6-fold in the presence of HS. Analysis of the HK breakdown products generated by cruzipain indicated that HS changes the pattern of HK cleavage products. Direct measurements of bradykinin demonstrated an up to 35-fold increase in cruzipain-mediated kinin liberation in the presence of HS. Similarly, kinin release by living trypanomastigotes increased up to 10-fold in the presence of HS. These studies suggest that the efficiency of $T$. cruzi to initiate kinin release is potently enhanced by the mutual interactions between cruzipain, HK, and heparan sulfate proteoglycans.

The plasma kallikrein-kinin system is a paradigm of a tightly controlled pro-inflammatory proteolytic cascade activated by vascular injury. Vasoactive peptides structurally related to bradykinin (generally termed as “kinins”) are derived from enzymatic excision from an internal segment (D4 domain) of kinogenins. These peptides are implicated in a broad range of pathophysiological responses, e.g. edema formation, vasodilatation, and pain. Although the nonapeptide bradykinin is released by the action of plasma kallikrein on high molecular weight kininogen (HK), lysyl-bradykinin is liberated from extravascular low molecular weight kininogen (LK) or HK by the activity of tissue kallikreins (2). In inflammatory conditions, oxidized forms of kininogens may be cleaved by the concerted action of neutrophil elastase and mast cell tryptase, liberating Met-Lys-bradykinin (3). Once liberated, kinins activate local endothelial or smooth muscle cells through the constitutively expressed B$_2$ kinin receptor (4) or alternatively through the B$_1$ kinin receptor that is up-regulated during inflammation (5). The effect of kinin stimulation on its receptor(s) is tightly regulated by the action of kinin-degrading peptidases (kininas), such as the angiotensin-converting enzyme and neutral endopeptidase (1).

HK comprises six major domains, and the C-terminal domains (D$_5$H and D$_6$H) mediate plasma contact phase activation; they are not present in LK (6). The other domains, D$_1$–D$_4$, are shared with LK. Domains 1–3 are structures homologous to the cysteine-proteinase inhibitors, cystatins (7), and the bradykinin-containing segment is domain 4. Recent efforts to define the structural basis of HK interaction with endothelial cells have focused on two binding sites. One site is represented by 27 amino acids located in the D3 domain (8), hence overlapping with one of the cystatin-like domains. The second binding site, located in the D$_5$H domain of HK, is a highly basic region formed by clusters of histidine, lysine, and glycine (9). HK binds to a multi-protein receptor complex consisting of gC1q receptor, urokinase plasminogen activator receptor, and cytokeratin 1 (10). Other studies demonstrated that heparan and chondroitin sulfate-type of proteoglycans are high affinity docking sites for HK accumulation on endothelial cells (11, 12). The assembly of HK molecules on human umbilical vein endothelial cells is required to prekallikrein activation, which modulates subsequent factors XI and XII activation (13–15).

The kinin activation pathway was implicated in the spread of infection by several pathogens (16–19). Studies on Trypanosoma cruzi, the etiological agent of Chagas’ heart disease, indicate that activation of bradykinin receptors by infective forms (trypomastigotes) potentiates cellular invasion (20). Bradykinin receptors were activated by bradykinin liberated from kinogenin by the major cysteine protease of $T$. cruzi (21), a papain-like enzyme conventionally designated as cruzipain (also known as, cruzain) (22–24). Because glycosaminoglycans

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*This work was supported in part by a grant from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro, Ministério de Ciência e Tecnologia (Pronex), Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant 97/13133-4), funds from the Deutsche Forschungsgemeinschaft, a grant from Fondes der Chemischen Industrie (to W. M. E.), and by National Institutes of Health Grant 3252779 (to A. H. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡‡ The abbreviations used are: HK, high molecular weight kininogen; LK, low molecular weight kininogen; GAG, glycosaminoglycan; HS, heparan sulfate; n-cruzipain, natural cruzipain; r-cruzipain, recombinant cruzain; DTT, dithiothreitol; CBZ, carbobenzoxy; AMC, arginyl-7-amido-4-methylcoumarin; Abz, O-aminobenzoyl; EDDnp, ethylene-diamine 2,4-dinitrophenyl; E-64, L-aminobenzoyl; EDDnp, ethylene-diamine 2,4-dinitrophenyl; E-64, L-
(GAGs) modulate the catalytic activity of some papain-like enzymes (25–27), the effects of HS on the enzymatic activity of natural and/or recombinant cruzipain isoforms were investigated. These studies indicate that the kinin releasing efficiency of living trypomastigotes is dramatically enhanced by heparan sulfate through its interactions with HK and cruzipain.

**EXPERIMENTAL PROCEDURES**

**Purified Proteases, Kininogen, and GAG—** Natural cruzipain (n-cruzipain) was isolated from crude aqueous extracts of Dm28c epimastigotes as described (28). Recombinant cruzain (kindly supplied by Dr. J. H. McKeen from the University of California, San Francisco, California) designated as r-cruzipain 1, was expressed in Escherichia coli (24); r-cruzipain 2 (80% sequence similarity with r-cruzipain 1) was recombinantly expressed in *Saccharomyces cerevisiae* and purified as described elsewhere (29). These recombinant proteases differ from their natural enzymes by: (i) having a truncated C terminus where residues 216–346 are deleted; (ii) glycosylation in yeast (r-cruzipain 2); and (iii) lack of glycosylation in *E. coli* (r-cruzipain 1). Purified HK was obtained from human plasma as described previously (9). HS from bovine lung (16,000 Da) was a generous gift from Dr. P. Bianchini (Osporic Research Laboratories, Modena, Italy). The characterization of anti-HK monoclonal antibodies was reported in (6). Briefly, MBK3 (IgG) is directed against and HK1 (IgG) against the D1 domain of HK. Antiserum to the light chain of HK was raised in goats and adsorbed with total kininogen-deficient plasma and purified LR (30).

**Cruzipain Proteolysis of HK—** HK (160 nM) was incubated with different concentrations of n-cruzipain (8, 16, 32, and 64 nM) in 50 mM sodium phosphate buffer, pH 6.5, 5 mM EDTA, 200 mM NaCl, 2.5 mM DTT for 1 h at 37°C. In some experiments, the reactions were performed in the presence of 30 μM of HS, which was included in the HK stock. The HK solution was preincubated for 5 min prior to the addition of cruzipain. The reactions were stopped by the addition of SDS-PAGE sample buffer containing 200 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.025% bromphenol blue (1:1 v/v) followed by boiling for 5 min. The samples were subjected to 9% SDS-PAGE, transferred to nitrocellulose, and visualized by Amersham ECL western blot detection kit (Data not shown). The initial rates of hydrolysis at various substrate concentrations were determined by measuring the initial rate of hydrolysis at various substrate concentrations in the presence or absence of different concentrations of sulfated GAGs. The data were analyzed by nonlinear regression using the program GraFit 3.01 (Erithacus Software Ltd.) as described previously (26). The kinetic model depicted in Equation 1 describes the effect of HS on the hydrolysis of Abz-LGMSLMRKPFQ-EDDnp by these cysteine proteinases, where *S* is Abz-LGMSLMRKPFQ-EDDnp, *K*ₚ is the substrate dissociation constant; *K*ₚ is the apparent HS dissociation constant; *α* is the parameter of *K*ₚ perturbation; and *β* is the parameter of *K*ₚ perturbation.

\[
 V = \frac{V_{\text{max}}[S]}{1 + \frac{[HS]}{K_\text{d}} + \frac{[HS]}{\alpha K_\text{d}}} \\
 K_{\text{cat}} = \frac{V_{\text{max}}}{[S]} \\
 K_{\text{m}} = \frac{1}{\beta} \frac{[HS]}{K_\text{d}} + \frac{1}{\beta} \frac{[HS]}{\alpha K_\text{d}}
\]  

**Equation 1**

**Sequence Determination of Human Kininogen Fragments—** Human kininogen (40 μg) was incubated with 32 nm of cruzipain in 50 mM sodium phosphate buffer, pH 6.5, 5 mM EDTA, 200 mM NaCl, 2.5 mM DTT for 1 h at 37°C. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiled for 5 min under reducing conditions. The fragments were separated by 9% SDS-PAGE, transferred to a polyvinylidene difluoride microporous membrane (Immobilon, Millipore), and visualized by Amersham ECL western blot detection kit.

**RESULTS**

**Heparan Sulfate Modulates the Endopeptidase Activity of Cruzipain—** Because the catalytic efficiency of some papain-like proteases is modulated by GAGs (25–27), HS may alter the kinetic properties of cruzipain, a member of the C1 peptidase family (32), affecting its ability to function as a kininogenase (21). The effects of HS on the kinetic properties of cruzipain purified from epimastigote extracts (n-cruzipain) were compared with those from two genetically engineered isoforms, r-cruzipain 1 (cruzain) and r-cruzipain 2 (24, 29). Using a short dipeptidyl synthetic substrate, e.g. Z-Phe-Arg-MCA, HS induced *k*ₕₐₜ values of n-cruzipain with the largest inducing differences in the *K*ₚ (data not shown). The kinetic parameters of hydrolysis for each protease were then determined using a longer kinin-like fluorescent substrate, Abz-LGMSLMRKPFQ-EDDnp, which spans the N-terminal flanking site of bradykinin. In the presence of HS, there is a

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were determined (Table I). These studies showed that HS 1 using nonlinear regression, and the values for the constants also caused a marked increase in the enzyme affinity for this significantly as a function of the HS concentration (Fig. 1). The hydrolysis of Abz-LGMISLMKRPQ-EDDnp increased signific-
antly, and the values as a function of HS concentration.

The effect of HS on the activity of n-cruzipain is described by a hyperbolic mixed type inhibition (Equation 1 under Experimental Procedures). These data were fitted to Equation 1 using nonlinear regression, and the values for the constants were determined (Table I). These studies showed that HS

**Fig. 1.** Effect of heparan sulfate on Abz-LGMISLMKRPQ-EDDnp hydrolysis by n-cruzipain. The $k_{cat}$ and $K_m$ values for the hydrolysis of Abz-LGMISLMKRPQ-EDDnp by n-cruzipain were determined in 50 mM sodium phosphate, pH 6.4, 200 mM NaCl, 1 mM EDTA, 2 mM DTT at 37 °C. The kinetics were performed in the presence of increasing concentrations of HS, and the individual parameters were calculated as described under “Experimental Procedures.” A, n-cruzipain $k_{cat}$ values as a function of HS concentration. B, n-cruzipain $K_m$ values as a function of HS concentration.

significant alteration in the kinetic parameters of n-cruzipain for the hydrolysis of the kinin-like substrate (Fig. 1). These data are consistent with that reported for papain (26) and for mammalian cathepsin B (27). The $k_{cat}$ value of n-cruzipain for the hydrolysis of Abz-LGMISLMKRPQ-EDDnp increased significantly as a function of the HS concentration (Fig. 1A). HS also caused a marked increase in the enzyme affinity for this substrate, evidenced by the decrease in the $K_m$ value (Fig. 1B). The effect of HS on the activity of n-cruzipain is described by a hyperbolic mixed type inhibition (Equation 1 under “Experimental Procedures”). The efficiency of hydrolysis of the synthetic substrate was estimated by changing either $K_m$ (parameter $a$) or $k_{cat}$ (parameter $b$). These data were fitted to Equation 1 using nonlinear regression, and the values for the constants were determined (Table I). These studies showed that HS

bound free n-cruzipain (E) with a dissociation constant of $K_H = 25 \pm 1 \mu M$, whereas the parameters obtained for the binding to enzyme-substrate complex was $aK_H = 11 \pm 1 \mu M$. The interaction of HS with n-cruzipain resulted in a 2.59-fold increase in the $k_{cat}$ of the enzyme ($b = 2.59 \pm 0.05$) and also increased the affinity of the enzyme for the kinin-like synthetic substrate ($a = 0.446 \pm 0.006$), resulting in an almost 6-fold increase in its catalytic activity ($b/a$). HS also increased the catalytic efficiency of r-cruzipain 1 ($b/a = 3.0$); however, it was at the expense of higher $k_{cat}$ ($b$) values, because it did not alter the affinity of the enzyme for the kinin-like substrate (dissociation constant $a = 1$). There also was a modest increase on the $k_{cat}$ ($b = 1.5 \pm 0.2$) of r-cruzipain-2, but this value was too low to permit a precise estimation of the dissociation constant between this isoenzyme and HS.

**Heparan Sulfate Impairs the Cysteine Proteinase Inhibitory Activity of Kininogen.—**The previous finding that cruzipain can liberate kinins from HK was unexpected because kininogens have two functionally active cystatin-like inhibitory domains (7) that exert potent inhibitory activity toward calpain (33) and papain-like enzymes, such as cruzipain itself (34). Kinetic analysis in the presence of HS revealed that this GAG interfered with the cysteine-protease inhibitory capacity of HK. The apparent inhibition constants of HK over n-cruzipain were determined in the presence or absence of 100 μM of HS. The inhibitory activity of HK ($K_{app} = 0.007 nM$) decreased about 10-fold in the presence of HS ($K_{app} = 0.07 nM$). These results suggested that the HS interaction with HK and/or n-cruzipain significantly reduced the binding affinity of the cystatin-like domains of HK for the parasite proteinase.

**Effect of HS on the Proteolytic Processing of HK by Cruzi-

ipain.—**Because HS impaired the cysteine protease inhibitory activity of HK and enhanced the catalytic activity of n-cruzipain in assays performed with a kinin-like synthetic substrate, we examined whether this GAG changed the proteolysis of HK by n-cruzipain. Assays performed at variable molar ratios of HK/cruzipain ranging from 20:1 to 2:1 were performed in the absence or presence of a molar excess of HS (30 μM), and the HK breakdown products were characterized by immunoblotting and N-terminal sequencing of the cleavage fragments. Heavy chain fragments were defined by using a monoclonal antibody (HKH4) directed to the N-terminal D1 domain of HK. The assays performed with this antibody revealed the presence of two major breakdown products, referred to as H67 and H63 (Fig. 2A, lanes 2–4), in reaction mixtures that did not contain HS. Edman degradation of H67 and H63 did not reveal any sequences, indicating that these heavy chain fragments contain an intact N terminus, which is blocked in the native HK by a pyro-Glu residue (35). Unlike H67, the smaller fragment H63 was not detected by MBK3, an antibody to the bradykinin epoite; consistent with this result, H63 co-migrates with the kinin-free heavy chain fragment (~63 kDa) of HK generated by tissue kallikrein (data not shown). The presence of HS almost completely prevented the formation of H67 in assays performed with relatively low concentrations of n-cruzipain, whereas the H63 form was abundant (Fig. 2A, lanes 6–8). These results indicate that HS redirects cruzipain to more N-terminally located cleavage site(s).

Next we did N-terminal sequencing of the breakdown prod-

ucts identified by the anti-light chain antibodies (Fig. 2B). L55 and L51, the two major fragments detected polyclonal antibod-

ies directed to the light chain of HK, displayed the sequences NAEVY and APAQ, respectively, at their N terminus (Fig. 2B). This indicates that L55 and L51 are generated by n-cruzipain cleavage of sites that are located at the N-terminal and C-terminal flanking regions of the kinin domain D4 (see Fig. 3 for...
TABLE I

Influence of heparan sulfate on the kinetic parameters of hydrolysis of Abz-LGIMSLMKRPQ-EDDnp by natural and recombinant cruzipain isoforms

| Proteinase | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $k_{cat}$ | $\beta k_{cat}$ | $\beta / \alpha$ |
|------------|-------|----------|-------|----------|---------|---------------|---------------|
| n-cruzipain | 25 ± 1 | 11 ± 1 | 0.12 ± 0.01 | 0.054 ± 0.001 | 1.5 ± 0.1 | 3.9 ± 0.2 | 5.8 |
| r-cruzipain 1 | 15 ± 1 | 15 ± 1 | 0.80 ± 0.09 | 0.82 ± 0.08 | 0.40 ± 0.01 | 1.2 ± 0.2 | 3.0 |
| r-cruzipain 2 | ND | ND | 0.11 ± 0.01 | 0.10 ± 0.01 | 0.50 ± 0.06 | 0.75 ± 0.09 | 1.5 |

**Fig. 2** Degradation of kininogen by n-cruzipain. Increasing concentrations of purified Dm28c cruzipain were incubated with 160 nM of HK in 50 mM Na$_2$HPO$_4$, pH 6.5, 200 mM NaCl, 5 mM EDTA, 2.5 mM, DTT for 1 h at 37 °C, in the absence (left panels) or in the presence of 30 µM of heparan sulfate (right panels). The samples were heat-denatured in SDS-PAGE sample buffer under reducing conditions, separated by SDS-PAGE, blotted onto nitrocellulose, and incubated with the respective anti-HK antibodies. Lanes 1, no enzyme; lanes 2 and 6, 8 nM of cruzipain; lanes 3 and 7, 16 nM of cruzipain; lanes 4 and 8, 32 nM of cruzipain; lanes 5 and 9, 64 nM of cruzipain. A, Western blot using anti-HK heavy chain monoclonal antibody directed to the D1 domain (HKH4). B, Western blot using anti-HK light chain polyclonal antibodies. The N-terminal sequence of each product is indicated at the left.

schematic representation). In the absence of HS, L51 is the major light chain product of HK released by n-cruzipain (Fig. 2B, lanes 2–4), whereas L55 is the principal fragment formed in the presence of the GAG (Fig. 2B, lanes 6 and 7); these data are consistent with the corresponding pattern of the heavy chain fragments, i.e. H67 (−HS) and H63 (+HS). Because HS binds to the histidine-rich region of D5$_{NH}$ (11), the data suggested that this interaction prevented access of the parasite protease to an otherwise susceptible cleavage site on HK, thereby precluding the formation of L51. The finding that H67, the heavy chain fragment complementary to L51, was not generated in appreciable amounts in the presence of HS (Fig. 2A, lanes 6–8) further suggests that the GAG protects the cleavage site in D5$_{NH}$. However, at higher protease concentrations, discrete amounts of H67 are observed, suggesting that cruzipain can overcome the protection of the D5$_{NH}$ domain by HS (Fig. 2A, lane 9). In the presence of excess of cruzipain, the light chain fragments as well as intact HK are completely degraded, unlike H67 and H63 (Fig. 2B, lanes 5 and 9).

Sequence analysis of L55, the major light chain breakdown product released in the presence of HS (Fig. 2B, lanes 6 and 7), provided additional evidence that HS modulates HK processing. The N-terminal sequence of L55, NAEVYVVPW, indicated that the cleavage site is merely 7 residues distal of the cystatin motif VQVAG in the D3 domain. This fact may account for the reduced cysteine protease inhibitory activity of HK in the presence of HS. Trace amounts of proteins that were not recognized by the anti-D1 monoclonal antibodies were also identified (Fig. 2B). The presence of DLEPIL and GLNFRI sequences at their N termini indicated that there was a small fraction of HK molecules that had suffered proteolytic attack in the cystatin-like domain D2 (Fig. 3). Together these data indicate that the proteolytic processing of HK by cruzipain is altered in the presence of HS and that this finding may bear important implications with respect to kinin generation. Therefore, we directly investigated the effects of HS on kinin generation in vitro and in vivo.

**HS Modulates Cruzipain-dependent Kinin Release by T. cruzi Trypomastigotes**—First we asked whether the kininogense activity of n-cruzipain was affected by addition of increasing concentrations of HS in vitro. A bell-shaped curve for liberated bradykinin was observed at different HK-cruzipain stoichiometry of 3.1 and 6.1, with peak values at 30 and 25 µM HS, respectively (Fig. 4A, 4B and B). Time course experiments performed in the presence of fixed concentrations of HS (30 µM) indicated that the amount of released bradykinin increased 15–35-fold over base-line levels within 20 and 120 min, respectively (Fig. 4C). Tests performed with a different isoenzyme, r-cruzipain 2, revealed that HS potentiates kinin liberation by ~4-fold at 60 min (Fig. 4D). Given that HS chains did not significantly increase the catalytic efficiency of r-cruzipain 2 in assays performed with synthetic substrates that span the N-terminal kinin-flanking side (Table I), it is possible that potentiation of the kinin releasing activity of r-cruzipain 2 by this GAG (Fig. 4D) results from facilitated hydrolysis of the more distal C-terminal kinin cleavage site (21).

**T. cruzi** trypomastigotes liberate kinins by the action of secreted forms of cruzipain on cell-bound HK (20). Because HK binds to HS (11), we determined whether living parasites liberated kinins more efficiently when GAG was added to HK (Fig. 5). HS increased the level of bradykinin release 2.5-fold over that liberated in its absence (Fig. 5A). The addition of E-64, an irreversible inhibitor of papain-like cysteine proteinases, completely blocked kinin release by the trypomastigotes (20). Similar to what was observed with purified cruzipain, the increase in kinin liberation changed as a function of the GAG concentration; at 10 µM a 10-fold increase in bradykinin liberation was observed (Fig. 5B). These results suggest that the HK interactions with heparan sulfate may favor the display of the kinin-flanking sites to the kinin-releasing cysteine proteinases of the parasites (20).

**DISCUSSION**

Our interest to investigate the interplay between HK, cruzipain, and heparan sulfate proteoglycans in the context of **T. cruzi** infection was motivated by the recent demonstration that host cell invasion by trypomastigotes is potentiated by activa-
tion of bradykinin receptors (20). HS has been previously recognized as a critical molecule for the initial adhesion of this pathogen to host cell surfaces (36). We reasoned that heparan sulfate covalently bound to proteoglycans might also contribute to kinin signaling because these ubiquitously distributed GAGs serve as platforms for the cell surface accumulation of the kinin precursor molecule, HK (11). Because trypomastigotes depend on secreted forms of cruzipain to liberate kinins, it is conceivable that enzymatic processing involves HK molecules that intimately interact with the structurally heterogeneous chains of heparan sulfate (37).

In the present work, this premise was tested in a model...
system by adding soluble HS and HK to suspensions of living trypomastigotes. A significant increase of kinin output was observed as a function of GAG concentration. Moreover, the irreversible cysteine protease inhibitor E-64 entirely prevented kinin liberation by the trypomastigotes, thus confirming that parasite-mediated excision of the kinin moiety is critically dependent on the catalytic activity of cysteine proteases (21). We have previously demonstrated that the cruzipain-induced kinin liberation could also occur indirectly because of the activation of plasma prekallikrein (21). Analysis of cruzipain-mediated activation of prekallikrein indicated that zymogen processing was not altered in the presence of HS (data not shown).

We then investigated the possibility that the HS chains modulate the kinin releasing activity of living trypomastigotes by directly acting on cruzipain. Assays with purified components, i.e. HK, HS, and n-cruzipain, were thus performed to evaluate whether the kinin release reaction was influenced by the interplay of these molecules. In keeping with our concept, the dose-response dependence of HS yielded a bell-shaped activity curve for the liberated kinin peptides, peaking at 30 or 25 μM, depending on the HK-cruzipain stoichiometry. This complex profile is consistent with the formation of ternary molecular complexes between HS, HK, and cruzipain, as previously documented for molecular encounters involving heparin-like sulfated glycosaminoglycans with other physiological ligands (37).

Given that cruzipain is a member of the C1 cysteine-peptidase family (32) and that GAGs modulate the enzymatic activity of other members of this family (25–27), we have examined the effects of HS chains on the catalytic efficiency of natural or genetically engineered cruzipain, using kinin-like synthetic substrates. Our results showed that the presence of HS increased both $k_{cat}$ values ($\beta = 2.59 \pm 0.05$) as well as the affinity of n-cruzipain for the kinin-like substrate ($\alpha = 0.446 \pm 0.006$). Combined, these effects resulted in a 6-fold increase on the catalytic efficiency of the natural protease. Assays performed with r-cruzipain 1 (i.e., cruzain), an enzyme deprived of the C-terminal extension, revealed that HS also enhanced its catalytic efficiency, but not at the expense of the $K_m$ value (not changed). These data suggest that cruzipain may engage the long and highly glycosylated C-terminal extension in secondary, yet productive HS-dependent interactions that alter the active site cleft. Alternatively, the increase in substrate binding affinity that HS promotes on n-cruzipain may stem from differences attributed to post-translational modifications (i.e., N- and O-linked glycosylation) that occur in the natural parasite protease (38) but not in the recombinant enzyme obtained in E. coli. Interestingly, kinetic assays performed with synthetic substrates that span the N-terminal flanking site of bradykinin (Table I) indicated that HS did not significantly affect the catalytic efficiency of r-cruzipain 2, although the same GAG increased about 4-fold the amount of kinins liberated from intact kininogen. Considering that r-cruzipain 2 is poorly inhibited by intact HK (29) and that the C-terminal kinin cleavage site (FR/S) is not efficiently cleaved by this enzyme, our data suggest that GAG interactions with HK and/or r-cruzipain 2 may facilitate the hydrolysis of the C-terminal flanking site by this particular isoenzyme. Thus, these results suggest that the N-terminal and C-terminal kinin-flanking sequences are differentially modulated by HS interactions with HK and/or the closely related cruzipain isoforms (21).

As noted for kinin-like synthetic substrates, the addition of HS enhanced the processing of the natural substrate, HK, by low concentrations of n-cruzipain. Generation of H63, the kinin-free heavy chain breakdown product (Fig. 2A), occurs irrespective of the presence of GAG. This suggests that sequences at the N-terminal flanking site of bradykinin are sensitive to cruzipain cleavage, as predicted from mass spectroscopy analysis of the fragments released from a 20-amino acid-long kininogen D4 mimic (21). N-terminal sequencing and immunoblotting indicated that of the four major cleavage sites identified (Fig. 3), one is placed at the N-terminal flank site (D3) of bradykinin, whereas another is localized further downstream from the kinin domain (D5$\alpha$). In the presence of HS, the cleavage sites juxtaposed to the histidine-rich domain of D5$\alpha$ are not efficiently processed by the parasite protease. Because domain D5$\alpha$ is implicated in HK binding to sulfated glycosaminoglycans (11, 12), these interactions may prevent formation of H67, i.e. the fragment that still displays the kinin moiety, and con-
Heparan Sulfate Modulates Kinin Release by T. cruzi

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Heparan Sulfate Modulates Kinin Release by *Trypanosoma cruzi* through the Activity of Cruzipain

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*J. Biol. Chem. 2002, 277:5875-5881.*

doi: 10.1074/jbc.M108518200 originally published online November 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108518200

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