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ARTICLES

Presence of an Unusually High Concentration of an Ubiquitinated Histone-Like Protein in *Trypanosoma cruzi*

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Abstract The conjugation of ubiquitin to histones H2A and H2B has been established in higher eukaryotes and has been related to changes in chromatin organization. In *Trypanosoma cruzi,* no condensation of chromatin occurs during mitosis. In order to determine the presence of histone ubiquitination in *T. cruzi* epimastigotes, histones were extracted from chromatin and analyzed by three electrophoretic systems: acid-urea, triton-acid-urea and sodium-dodecyl-sulphate polyacrylamide gel. The immunochemical detection of ubiquitin-histone conjugates by Western blotting showed a strong reaction with a slow migrating band of M_r 19 kDa. The high percentage of ubiquitin-histone conjugates present in *T. cruzi* chromatin may be related to the inability of this parasite to condense chromatin into a 30 nm fiber. J. Cell. Biochem. 66:433–440, 1997. © 1997 Wiley-Liss, Inc.

Key words: ubiquitin; *Trypanosoma cruzi;* histone proteins

Trypanosoma cruzi is the etiological agent of Chagas disease [De Souza, 1984]. This intracellular parasite undergoes complex morphological, as well as biochemical changes, as it differentiates and adapts to different environments during its life cycle. These changes should be the result of adaptive modifications in gene expression which are accompanied by modifications of chromatin structure [Wolffe, 1994].

In higher eukaryotic cells, the basic repeating structural unit of chromatin, the nucleosome, consists of approximately 200 pb of DNA wrapped around a histone octamer containing two of each of the core histones (H2A, H2B, H3 and H4). Another histone, histone H1, although not part of the nucleosome core, has an important role in the packaging of nucleosomes into a higher level of chromatin organization [Kornberg, 1974].

Histones are highly conserved throughout evolution. However, several variations in the primary structure of the core histones of higher and lower eukaryotes have been reported [Rizzo, 1985]. In *Trypanosoma cruzi*, as in other lower eukaryotes, chromatin is also organized in nucleosomes of about 200 pb; however, in contrast with higher eukaryotes, its chromatin is physically and enzymatically fragile and no condensation of it into chromosomes occurs during mitosis [Astolfi-Filho et al., 1980; Hecker and Gander, 1985; Hecker et al., 1994].

Histone-like proteins in *T. cruzi* have been extensively characterized by Toro and coworkers [Toro and Galanti, 1988, 1990; Toro et al., 1992, 1993a, 1993b] and differ in sequence, charge and/or size from those of higher eukaryotes. These differences in the primary structure of *T. cruzi* histone-like proteins have been related to the unusual behavior of chromatin condensation observed in this parasite. Another source of variability to be considered is the presence of several chemical modifications that markedly change the nature of the amino acid and modulate histone-DNA interactions in chromatin, such as phosphorylation, acetylation and ubiquitination of these proteins [Bradbury, 1992]. Among these post-translational modifications of histones, the covalent binding of the peptide ubiquitin to H2A and to a lesser extent to H2B has been extensively reported [Busch and Goldknopf, 1981].

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The role of ubiquitination in the degradation of cytosolic proteins by an ATP-dependent proteinase has been well established [for reviews, see Ciechanover and Schwartz, 1994]. In contrast, the function of histone conjugation to ubiquitin remains controversial, and has been related to changes in chromatin organization and function [Bradbury, 1992]. Therefore we have investigated the pattern of ubiquitin conjugation to histone proteins in the non-infective epimastigote forms of *T. cruzi*.

MATERIALS AND METHODS

Materials

All reagents were analytical grade from Sigma Chemical Co. (St. Louis, MO). Anti-rabbit-IgG antibodies coupled to alkaline phosphatase, p-Nitro blue tetrazolium chloride (NBT) and 5-bromo-4 chloro 3-indolyl phosphate-toloidine salt (BCIP) were from Promega. The enhanced chemiluminescence (ECL) Western blotting were from Amersham.

Cell Culture

T. cruzi epimastigotes, originally isolated from a Venezuelan patient (EP), were grown at 27°C in a liver infusion tryptose medium with the following composition: 150 mM NaCl, 5.36 mM KCl, 50 mM Na_2HPO_4, 20 mM glucose, 0.03 mM hemin, 1.5% Bacto-tryptose, 0.5% yeast extract, 0.5% liver broth, 10% heat inactivated fetal calf serum. Cells were routinely harvested, at day 6 of culture, by centrifugation at 2,190*g* in a Sorvall RT6000 (H1000B rotor) at 4°C, washed three times in phosphate buffered saline (PBS) pH 7.0 and stored at −70°C until used.

Preparation of Chromatin

Chromatin was obtained from *T. cruzi* epimastigotes following the procedure of Stein et al. [1975] with some modifications [Toro and Galanti, 1990]. Basically, the cells were homogenized in 250 mM sucrose, 1 mM EDTA, 3 mM $CaCl_2$, 10 mM Tris HCl, pH 7.4 and 0.5% saponine, using an Eberbach homogeneizer, at 1,200 rpm for 3 min. After centrifugation at 3,000*g* for 10 min at 4°C, nuclei were washed in PBS and homogenized further in 1% Triton X-100, 150 mM NaCl, 25 mM EDTA, 10 mM Tris HCl, pH 8. This suspension was centrifuged at 12,000*g* in a Sorvall RC-5B (SS-34 rotor) for 20 min at 4°C and the pellet was washed three times with 10 mM Tris HCl, pH 8. The following inhibitors of proteases were used: 2 mM phenyl-methyl-sulphonyl-fluoride (PMSF), 0.5 mM N-α-tosyl-L-lysine chloromethyl ketone (TLCK) and 1 mM N-ethylmaleimide, the latter was used to inhibit isopeptidase activity.

Extraction of Histones

Histones were extracted from chromatin by a modification of the procedure of Panyin and Chalkley [1969], as described by Toro and Galanti [1990]. Briefly, chromatin was extracted overnight with 0.4 N H_2SO_4. After centrifugation at 16,000 rpm in a Sorvall RC-5B (SS-34) for 20 min at 4°C, histones were recovered from the supernatant, dialyzed against distilled water followed by lyophilization. Protein concentration was determined by the method of Lowry et al. [1951].

Polyacrylamide Gel Electrophoresis

The following electrophoretic systems were used.

1) Triton-acid-urea gel electrophoresis (TAU-PAGE). Lyophilized proteins were dissolved in sample buffer containing 4 M urea, 5% acetic acid and 4% β-mercaptoethanol. The gels contained 6 M urea, 0.9 N acetic acid and 0.38% (v/v) Triton DF-16 in 17% polyacrylamide. After a first pre-run (10 mA, up to constant voltage), a second pre-run was performed with 1 M cysteamine in 0.9 N acetic acid per lane at 25 mA for 2 h. The samples (50 µg/lane) were separated according to the method of Alfageme et al. [1974].

2) Acid-urea gel electrophoresis (AU-PAGE). Lyophilized proteins were dissolved in sample buffer containing 4 M urea and 0.9 N acetic acid. Gels containing 0.9 N acetic acid and 2.5 M urea in 17% polyacrylamide were pre-run in 0.9 N acetic acid at 20 mA for 2 h. The samples (50 µg/lane) were separated at 25 mA according to Panyin and Chalkley [1969].

3) SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Lyophilized proteins (30 µg/lane) were dissolved in sample buffer for SDS-PAGE and separated by 10–20% SDS polyacrylamide gel, with a 4% stacking gel as described by Laemmli [1970].

In the three electrophoretic systems described above, gels were stained with 0.1% Coomassie blue R-250 in methanol:acetic acid: water (5:1:5).

Two-Dimensional Gel Electrophoresis

Protein bands obtained by TAU-PAGE and AU-PAGE were cut, equilibrated in 0.25 M Tris-HCl (pH 6.8) containing 0.10% SDS, 10% glycerol, 0.30% β-mercaptoethanol and 0.001% bromophenol blue for 1 h, and applied to separate lanes of a standard SDS-PAGE [Cleveland, 1983].

Immunoblotting

Immunoblotting was carried out following a modification of the procedure of Towbin et al. [1979] as described by Henriquez et al. [1993]. Briefly, *T. cruzi* histones were separated by gel electrophoresis and transferred electrophoretically to nitrocellulose paper. The filter was blocked with 0.5% Tween 20, 2% gelatin in PBS and immunochemically reacted with an affinity purified antibody raised in rabbit against bovine ubiquitin [Hass and Bright, 1985].

Antigen-antibody complexes were identified with anti-rabbit IgG antibodies coupled to alkaline phosphatase using NBT and BCIP as substrates. Alternatively, detection was performed by enhanced chemiluminescence Western blotting according to the instructions from the manufacturer.

Differential Extraction Procedures

The extraction of high mobility group proteins (HMG) from *T. cruzi* chromatin was carried out according to the procedure described by Busch and Goldknopf [1981]. Briefly, chromatin was resuspended and homogenized in 0.35 M NaCl (twice 3 min at 1,200 rpm) using an Eberbach homogenizer. The homogenate was centrifuged at 1,200*g* for 20 min (Sorvall RC-5B, SS-34 rotor) at 4°C. The supernatant, containing HMG proteins, was stored at 4°C. The pellet was homogenized twice in 5% perchloric acid (PCA) as described above and the homogenate was centrifuged for 20 min at 1,200*g*. The supernatant containing histone H1 was dialyzed at 4°C against distilled water and lyophilized. The pellet was precipitated with 0.4 N H_2SO_4 to obtain nucleosomal histone proteins.

RESULTS

Immunochemical Detection of Ubiquitin-Protein Conjugates in *T. cruzi* Histone Extracts

Figure 1 shows the patterns obtained for histone proteins from *T. cruzi* epimastigotes under three different electrophoretic conditions, TAU-PAGE, AU-PAGE and SDS-PAGE (lanes B), compared with those of standard calf thymus histones (lanes A). The electrophoretic patterns obtained under the different electrophoretic systems confirm the results reported previously by Toro and Galanti [1990]. In TAU-PAGE, which separates proteins according to their hydrophobicity, a cluster of five major protein bands was present in *T. cruzi* (lane B) in the range of migration of mammalian nucleosomal histones (lane A). These protein bands were identified previously by Toro and coworkers as H2A, H3, H2B and H4 [Toro and Galanti, 1988, 1990; Toro et al., 1992, 1993a, 1993b]. The components of the fast migrating, hydrophilic histone complex observed in *T. cruzi* preparations have been extensively characterized and identified as H1 histone variants [Toro and Galanti, 1988; Toro et al., 1993a]. These authors did not identify band X. In AU-PAGE (lane B), six histone protein bands were observed in *T. cruzi* extracts, five of which (bands I, II, III, IV and V) had electrophoretic mobilities similar to those detected in calf thymus. The fast migrating band (band VI) detected in *T. cruzi* has been characterized as an H1 histone [Toro and Galanti, 1990]. Finally, in SDS-PAGE, five protein bands were observed in *T. cruzi* extracts in the region of the core histones of calf thymus, and a slow migrating protein (band 1, M_r 19 KDa) was observed between the latter and the doublets corresponding to H1 in calf thymus. H1 like-histone protein in *T. cruzi* runs in the region of nucleosomal histones; this was supported by its selective extraction with 5% PCA and metachromasia when stained with Coomassie Brilliant Blue (results not shown). To date, no identification for the other protein bands separated by SDS-PAGE is available.

In order to study the presence of ubiquitin-histone conjugates in *T. cruzi*, histone proteins were separated by the three different electrophoretic systems, transferred to nitrocellulose and reacted with an affinity purified anti-ubiquitin antibody. Lanes C of Figure 1 show the results of the immunoblot analysis. In all three systems used, an immunochemical stained band was observed that corresponded to band 1 (SDS-PAGE), band X (TAU-PAGE) and band I (AU-PAGE). The immunoreactive triplet detected in SDS-PAGE was not consistently observed and may represent products of degradation of band 1. In contrast, the large mass of protein present in the other bands did not show any reactivity

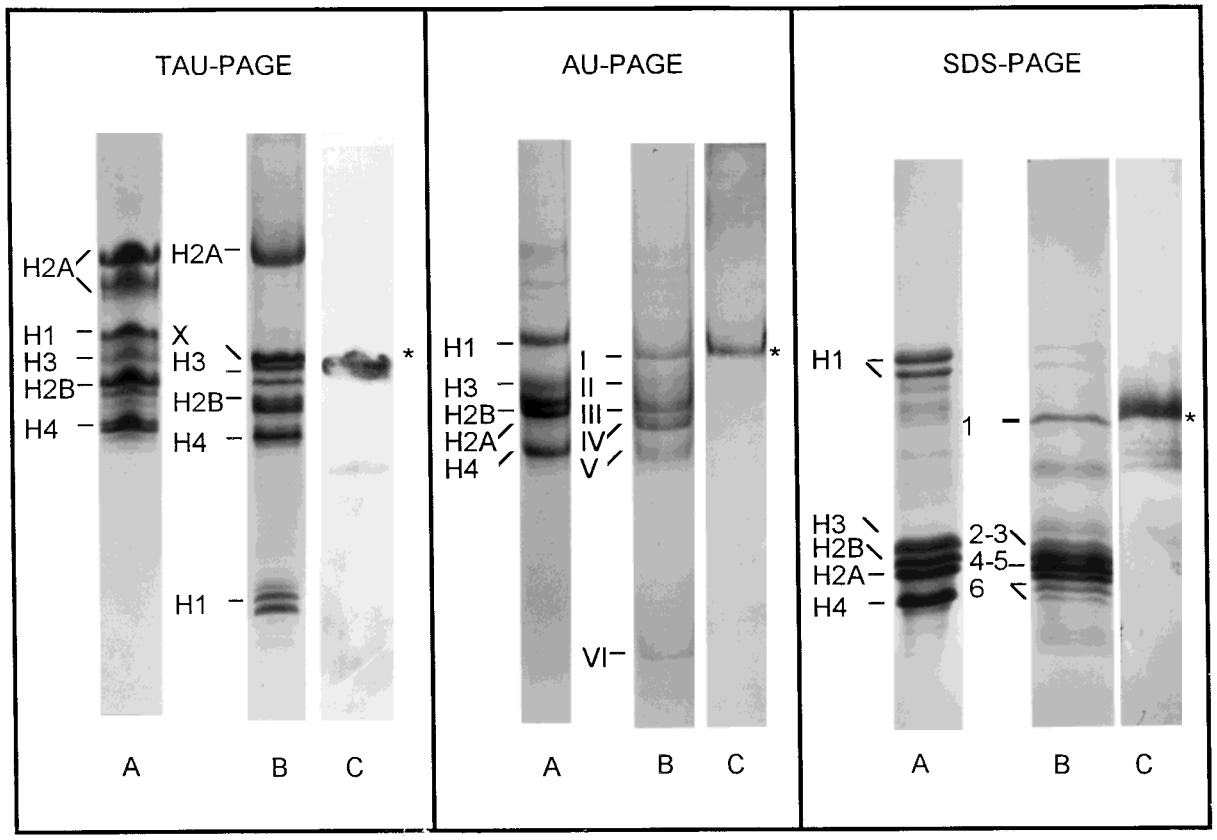

Fig. 1. Immunoblot detection of ubiquitinated histones in *T. cruzi* epimastigotes. Histone proteins isolated from calf thymus and *T. cruzi* epimastigotes were electrophoretically resolved on Triton DF-16/acetic acid/urea, acetic acid/urea or SDS gels. The gels were stained with Coomassie Blue R250 (**lane A,** calf thymus; **lane B,** *T. cruzi*) or in the case of *T. cruzi*, transferred to nitrocellulose filters and stained for ubiquitin conjugates with an anti-ubiquitin antibody (**lane C**). Ubiquitinated proteins are indicated by asterisks.

with the antibody; no reaction was observed when a pre-immune serum was used (data not shown). The strong reaction detected by the anti-ubiquitin antibody in *T. cruzi* histone extracts contrasted with the weak interaction of the antibody with calf thymus histones (results not shown).

Two Dimensional Electrophoresis

The equivalence of the immunoreactive protein bands resolved in the three electrophoretic systems was determined by bidimensional PAGE (Fig. 2). With this purpose histone extracts were first separated in TAU-PAGE according to their hydrophobicity, or in AU-PAGE according to their charge. For AU-PAGE, band I was cut from the gel and applied to a standard SDS-PAGE, and proteins were separated in the second dimension according to their molecular weights (Fig. 2, lane B). In the case of TAU-PAGE, overlapping of bands X and H3 occurs in the first dimension, therefore both protein bands were cut out from the gel and then applied to an SDS-polyacrylamide gel (Fig. 2, lane C). When band I from AU-PAGE was separated by SDS-PAGE in a second dimension it comigrated with protein 1 of SDS-PAGE (lane B). No other proteins become visible when gels were silver stained. When protein bands X and H3 of a TAU gel were separated in a second dimension by SDS (lane C), one band comigrated with band 1 of SDS-PAGE; and H3 comigrated with the cluster of nucleosomal proteins observed in SDS-PAGE. Taken together, these results show that band 1 in SDS-PAGE corresponds to both band X in TAU-PAGE and band I in AU-PAGE. Because different electrophoretic systems were used to separate the immunoreactive band and no further polypeptides were observed with silver staining, we conclude that it corresponds to only one polypeptide.

Characterization of the Immunoreactive Band by Solubility Properties

Densitometric tracing of the protein patterns in SDS-polyacrylamide gels demonstrated that

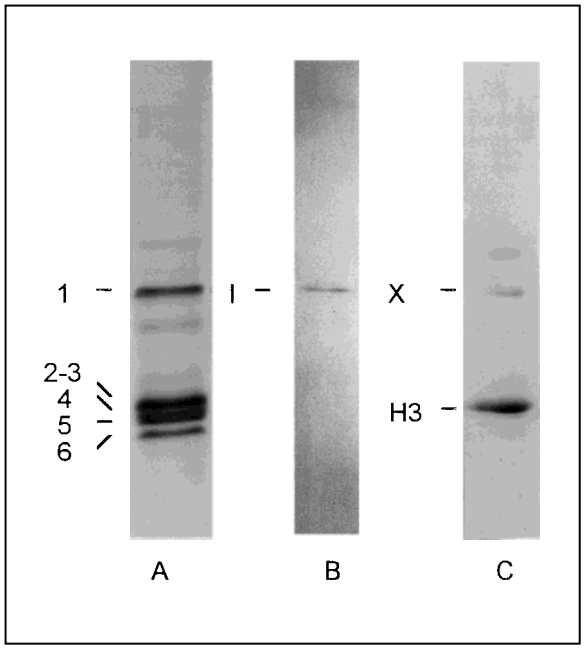

Fig. 2. Two dimensional electrophoresis. Histone proteins extracted from *T. cruzi* chromatin were run in a first dimension in AU-PAGE or TAU-PAGE. Band I (AU-PAGE) and bands X and H3 (TAU-PAGE) were cut and run in a second dimension in SDS-PAGE (**lanes B,** and **C,** respectively). **Lane A** corresponds to the electrophoretic pattern of total histone proteins separated in SDS-PAGE.

the relative amount of this immunoreactive protein varied considerably in different preparations (3%–8% of total histone proteins). Therefore, it was important to discard the possibility that it could correspond to a non-histone chromatin protein contaminating the acid extract, such as the high mobility group (HMG) proteins, present in mammalian chromatin [Busch and Goldknopf, 1981] and identified more recently in *T. cruzi* chromatin by Morales et al. [1992].

With this in mind, *T. cruzi* chromatin was sequentially extracted with 0.35 M NaCl, the salt concentration at which HMG proteins are soluble [Busch and Goldknopf, 1981], and 5% PCA, in order to solubilize H1 histones. The latter was important in order to discard a non-specific cross reaction, with a H1 histone like-protein, that has been previously reported for higher eukaryotic cells [Dawson et al., 1991]. The residual pellet obtained after the sequential treatment with 0.35 M NaCl and 5% PCA was treated with 0.4 N H_2SO_4, for further extraction of nucleosomal histone proteins. Under these conditions, chromatin should be devoid of HMG proteins and H1 histone. Figure 3 shows the electrophoretic pattern in SDS-PAGE of chromatin total proteins (lane A) and proteins extracted from the residual pellet with 0.4 N sulfuric acid (lane B). As can be observed in Figure 3 (lane B), the 19 KDa band was not extracted by 0.35 M NaCl or by 5% PCA. The immunoblot analysis using anti-ubiquitin antibody showed that the 19 KDa protein is the only ubiquitinated protein in the chromatin fraction (lane C) and confirmed its presence in the residual pellet (lane D). The two lower molecular weight immunoreactive bands observed in lane D were not observed in multiple other experiments, and may be a non-specific reaction due to overloading of the gel. These fainter bands were not observed in immunoblots of chromatin extracts (Fig. 1, lane C).

DISCUSSION

The organization of chromatin in *T. cruzi* is similar, although not identical, to that observed in higher eukaryotic cells. Although nucleosomal organization of chromatin is evident in this parasite, nucleosomes are spaced irregularly and no condensation into a 30 nm fiber occurs [Astolfi-Filho et al., 1980; Hecker and Gander, 1985]. The data obtained from *Trypanosoma brucei* [Bender et al., 1992; Burri et al., 1994] and *Crithidia fasciculata* [Duschak and Cazzulo, 1990] and more recently in *Trypanosoma congolense* [Schlimme et al., 1994] confirm these results for other trypanosomatids. The presence of histones in *T. cruzi* was first reported by Rubio et al. [1980] and, in the last 5 years, Galanti and coworkers have undertaken a systematic characterization of *T. cruzi* histones with the aim of understanding the unusual condensation behavior of its chromatin [Toro and Galanti, 1988, 1990; Toro et al., 1992, 1993a, 1993b]. Characterization by these authors of *T. cruzi* histones, according to their banding pattern in different gel systems, solubility properties, amino acid composition as well as sequence analysis, confirmed the presence of a full set of histone proteins in *T. cruzi* chromatin. However, these proteins can be differentiated from higher eukaryotic cells on the basis of their primary sequence as well as by their behavior in different gel systems. Briefly, 1) the strong hydrophilic character and the low molecular weight of H1-like protein [Toro and Galanti, 1988; Toro et al., 1993a; Åslund et al., 1994], 2) divergent amino acid sequence in the amino terminal of H4 [Toro et al., 1992], and, finally, 3) a shorter amino terminal domain in *T. cruzi* histone H2B, where three out of the

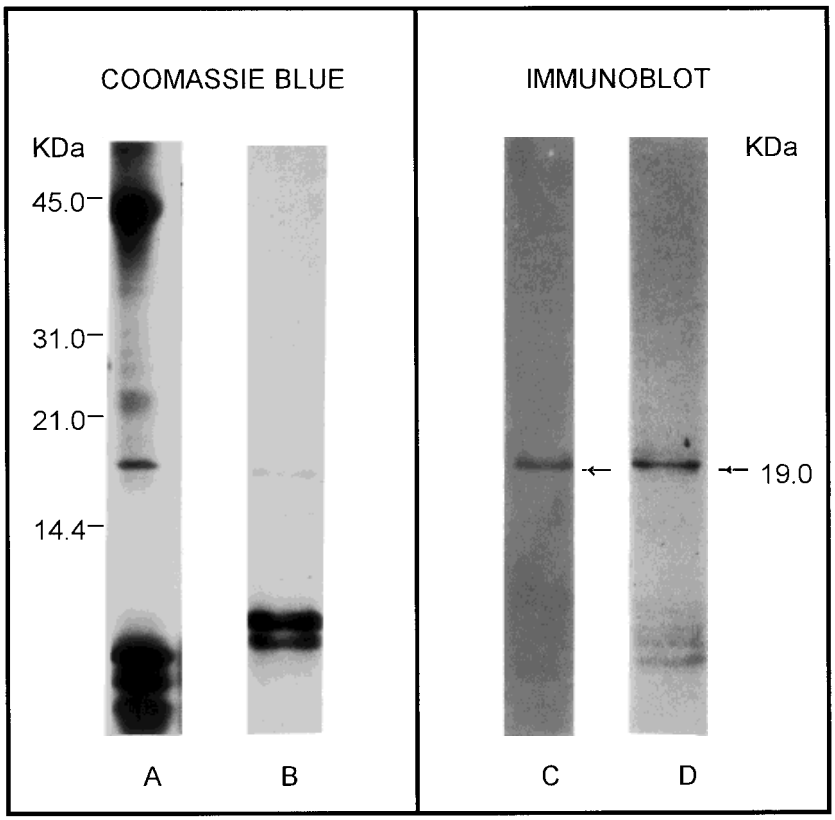

Fig. 3. Application of differential extraction procedures to *T. cruzi* chromatin proteins. Chromatin proteins (**lane A**) and core histone proteins obtained after sequential extraction with 0.35 M NaCl, 5% PCA and 0.4 N H_2SO_4 (**lane B**) were separated by SDS-PAGE and stained with Coomassie Blue R250 or transferred to nitrocellulose for immunoblot analysis using an affinity purified anti-ubiquitin antibody. Ubiquitinated proteins detected in chromatin (**lane C**) and in core histone proteins (**lane D**) are indicated by arrows. Numbers on the left of the gel are the molecular weights of marker proteins.

four possible acetylation sites present in histone H2B of higher eukaryotes are missing [Toro et al., 1993b].

Although the unusual characteristics in the primary structure of *T. cruzi* histones may contribute to the observed abnormal behavior of chromatin condensation, post-translational modifications such as acetylation, methylation, ubiquitination and phosphorylation are important factors to be considered in the modulation of the structure and function of chromatin in this parasite. Among these modifications, the covalent attachment of the C-terminus of ubiquitin to an ϵ-amino group of histones H2A and H2B has been widely reported for several species [Busch and Goldknopf, 1981; West and Bonner, 1980]. However, despite an increasing number of reports in higher eukaryotes, few studies are available on the presence on this modification of histone proteins in lower eukaryotes and none in trypanosomatids.

In the present study, our results have confirmed the results of Toro and Galanti [1990]. When the pattern of *T. cruzi* histones was compared to the pattern obtained for calf thymus histones, differences in electrophoretic mobilities were observed in three different gel systems (Fig. 1). Immunological detection of ubiquitin-histone conjugates using an affinity purified antibody detected the presence, in these three electrophoretic systems, of a high immunoreactive protein band. We propose that this band corresponds to a histone-like protein conjugated to ubiquitin. This is supported by 1) the apparent molecular weight in SDS-PAGE (19 KDa) being in the range of values previously reported for mono-ubiquitinated histones from other organisms [Goldknopf et al., 1975]; and 2) differential extraction procedures with 0.35 M NaCl and 5% PCA indicating that this protein may represent a nucleosomal histone. However, sequence analysis will be necessary to

further identify this ubiquitinated polypeptide as a histone protein.

The densitometric tracing of the protein patterns obtained in SDS-polyacrylamide gels demonstrated that, although the amount of this protein varied among different preparations, it never represented less than 3% of total histone proteins. Two dimensional gel electrophoresis indicated that it corresponds to a unique polypeptide band (Fig. 2). This is unusually high concentration when compared to the levels reported previously for a wide variety of species where these conjugates are present in low concentrations, less than 10% of H2A molecules and 1.5% of H2B molecules are conjugated to ubiquitin [Rechsteiner, 1988].

We speculate that the high concentration of an ubiquitinated histone-like protein in *T. cruzi* may be a factor to be considered, among other features, in understanding the unusual compactation of *T. cruzi* chromatin. It has been proposed in other eukaryotic cells that the addition of bulky globular ubiquitin moieties to core histones can introduce a major structural perturbation in chromatin that may prevent the compactation into a 30 nm supercoil structure [Finley and Chau, 1991]. In accordance with this, the most remarkable changes in ubiquitination of histones have been observed when cells enter mitosis [Mueller et al., 1985] and during erytropoiesis [Pickart and Vella, 1988]. In both cases condensation of chromatin is accompanied by a decrease or total disappearance of ubiquitin-histone conjugates. These results may suggest that ubiquitin has to be removed from chromatin in order to allow close packaging of nucleosomes in both cases.

Further studies will be required to identify this ubiquitin-histone like protein and to determine its role in long-term modulation of chromatin structure and function in this parasite.

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