Original Article

Detection and Localization of Small Nuclear Ribonucleoproteins (snRNPs) in Trypanosomatids Using Anti-m\textsubscript{3}G Antibodies

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SUMMARY: This work reports for the first time the identification and immunolocalization, by confocal and conventional indirect immunofluorescence, of m\textsubscript{3}G epitopes present in ribonucleoproteins of the following trypanosomatids: Trypanosoma cruzi epimastigotes of three different strains, Blastocritidia spp., and Leishmania major promastigotes. The identity of these epitopes and hence the specificity of the anti-m\textsubscript{3}G monoclonal antibody were ascertained through competition reaction with 7-methylguanosine that blocks the Ig binding sites, abolishing the fluorescence in all the parasites tested and showing a specific perinuclear localization of the snRNPs, which suggests their nuclear reimport in the parasites. Using an immunoprecipitation technique, it was also possible to confirm the presence of the trimethylguanosine epitopes in trypanosomatids.

INTRODUCTION

Among the important factors for eukaryotic cell function, there are complexes composed of small ribonucleic acids (RNAs) and proteins. These complexes in turn form small ribonucleoproteins (RNPs) particles with short RNAs (approximately 300 nucleotides), and tightly complexed proteins may appear in different sizes and shapes. RNAs are abundant (approximately 10\textsuperscript{7} copies per mammalian cell, similar to ribosomes) and highly conserved from yeast to humans; describing their cellular roles has remained an important challenge for molecular biologists. In mammalian cells, small nuclear ribonucleoproteins (snRNPs) play an essential role in pre-mRNA processing, primarily in splicing (1-3).

In trypanosomes, Leishmania, and related species, all RNAs are processed via an unusual mechanism known as trans-splicing. Two independent transcript regions, and not a single pre-mRNA, are joined to consistently form a mature mRNA with the same initial sequence (approximately 40 nucleotides), designated as the spliced leader (SL) (4-9).

The trans-splicing reaction in Ascaris lumbricoides has revealed an important functional base pairing between SL RNA intron sequences and U6 RNA. This matching occurs at the Sm binding site region and adjacent nucleotides in SL RNA and other sequences close to the Sm binding site in U6 RNA that are complementary in U2 snRNA (10,11); an equivalent complementarity has not been identified in the cis-splicing process. The presence of this extensive complementarity most likely represents a specific trans-splicing modification that allows exclusion of the U1 and U5 snRNA interaction at the 5’ exon, since the splicing process uses a single leader-intron RNA sequence for all reactions. In cis-splicing, many 5’ exon-intron sequences must be processed by the spliceosome.

Although nematodes, trematodes, and euglenoids all carry out both cis- and trans-splicing (12), the notion has developed over time that trypanosomes lack intervening sequences and consequently the machinery to carry out cis-splicing (13). This assumption was based on observations that all trypanosomatid genes characterized thus far have no introns, and trypanosomes appear to lack a homologue of the U1 snRNA, which plays a crucial role in the initial recognition of the 5’ splice site (14).

Recently, intervening sequences were described in the PAP gene of Trypanosoma brucei and Trypanosoma cruzi, and a candidate T. brucei U1 small nuclear RNA (snRNA) gene was characterized by Dijkstra et al. (15), demonstrating that both cis- and trans-splicing can occur in these organisms, with a prevalence of trans-splicing (13,15,16). Hannon and co-workers (10) also demonstrated that the U1 snRNP is not essential for trans-splicing, but only for cis-splicing, as in nematodes. It has been clearly established that cis-splicing is also present in trypanosomatid protozoa, albeit at low levels.

In most eukaryotic organisms, all snRNA genes are transcribed by RNA polymerase II to produce primary transcripts with a 7-methylguanosine (m\textsubscript{3}G) cap at their 5’ end and short extensions at the 3’ end. The precursors are exported from the nucleus to the cytoplasm, where they assemble into a stable core RNP particle by binding to common proteins, and, as an essential maturation step, the m\textsubscript{3}G cap is hypermethylated to 2,2,7-trimethylguanosine (m\textsubscript{3}G or TMG). This m\textsubscript{3}G cap and the common protein ligations compose the signal to nuclear reimportation of uridine-rich snRNPs (UsnRNPs) (17).

Despite the evolutionary conservation of the secondary structure, the SL RNA sequence is not conserved among different organisms capable of processing their mRNAs by trans-splicing. However, within the trypanosomatids, the exon sequence shows a considerable degree of conservation at the primary sequence level. One characteristic feature of the SL RNA is the presence of a hypermodified cap structure, which is referred to as cap 4 because the four nucleotides after the m\textsubscript{3}G are modified (18,19).

The aim of this study was to identify by conventional and
confocal indirect immunofluorescence the m,G epitopes present in the RNPs of trypanosomatids, which include T. cruzi epimastigotes of three different strains, Blastocritidia ssp., and Leishmania major promastigotes, thus showing for the first time the immunolocalization of the snRNPs in these parasites.

MATERIALS AND METHODS

Parasites: T. cruzi epimastigotes forms (Y, NCS, and Bolivia strains) and Blastocritidia ssp. promastigote forms were grown at 28°C (10 days) in LIT medium (20). The L. major promastigote forms were kindly provided by Dra. Regina Dórea (Departamento de Ciências Biológicas, Faculdade de Ciências e Letras, Unesp, Sao Paulo, Brazil).

Indirect immunofluorescence (IIF) reactions: Parasite cultures were centrifuged for 15 min at 1,000 x g, and the pellet was washed 3 times with PBS (pH 7.2); after the last wash, an aliquot was collected for quantitation under a Neubauer chamber (approximately 10^6 cells/ml). Cells suspended in PBS (approximately 10^6 cells/ml) were fixed overnight under agitation with 2% formaldehyde in PBS. Fixed cells were then washed three times in PBS and 10^6/ml cells suspensions were dropped in predrawn circles on microscopy slides and left at 37°C to air dry. Immunofluorescence assays were carried out using rabbit anti-m,G serum (provided by Dra. Montserrat Bach-Elias, CSIC, Barcelona, Spain) (diluted 1:10), normal human or rabbit serum (diluted 1:80) as a negative control, and chagasic human serum (diluted 1:50) as a positive control for the parasites on the slides; all sera were diluted in PBS. T. cruzi-Y strain reactions were also performed with mouse monoclonal anti-m,G antibodies (ICN Biomedicals, Costa Mesa, Calif., USA) diluted 1:5, as suggested by the manufacturer. Anti-human IgG (Salck, São Paulo, Brazil), anti-rabbit IgG (Sigma, St. Louis, Mo., USA), and anti-mouse IgG (Pierce, Rockford, Ill., USA) FITC conjugates were diluted 1:40, 1:100, and 1:100, respectively, in 0.001% Evans Blue/PBS, as suggested. All incubations with sera and secondary antibodies were carried out for 30 min at 37°C. After each incubation, the slides were washed in PBS. All preparations were examined by fluorescence microscopy (Leitz Dm RXE; Leica, Wetzlar, Germany) using the Leica DC100 software. The IIF reactions using monoclonal antibody with T. cruzi (Y strain) epimastigotes were also stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Molecular Probes, Eugene, Oreg., USA) and imaged by confocal microscopy using a Zeiss Axiovert 100 microscope with a 100 × Plan-Apochromatic 1.4 oil immersion objective attached to an MRC-1024UV confocal system (BioRad, Hercules, Calif., USA). Specificity controls were performed using competition reactions in which both rabbit and monoclonal antibody anti-m,G were previously and separately incubated with 7.5 mM 7-methylguanosine (Sigma) in 1M Tris-HCl pH 7.5 at 4°C for 2 h. The treated antibody solutions were then used as described above. Cross-reactivity between T. cruzi and insect trypanosomatids has been previously detected in studies of sera from chagasic patients or from rabbits immunized with different trypanosomatids (21).

Immunoprecipitation reactions with anti-m,G antibody blocked by m,G: To further characterize the specificity of the antibody, immunoprecipitation assays were performed with the total RNA of T. cruzi (epimastigote form of the Y strain) and rabbit anti-m,G serum. Briefly, the serum was precipitated with an equal volume of 100%-saturated ammonium sulfate solution, and the samples were washed two times with a 50%-saturated solution; the precipitated IgGs were bound to protein A-Sepharose (Gibco BRL, Rockville, Md., USA) during overnight incubation at 4°C. After the complex had been washed, it was incubated with 10 mM m,G diluted in NET-2 buffer (Tris-HCl, NaCl, Nonidet P40, Sodium Azide) for 2 h at 4°C to block the IgG binding site. Twenty-five micrograms of total RNA were added to the reaction and the samples were incubated again for 2 h at 4°C under constant rotation; the samples used for the reaction were washed, treated with proteinase K (20 mg/mL), and 1% SDS for 15 min at 37°C followed by phenol/chloroform extraction. The reaction samples were precipitated with 100% ethanol and submitted to electrophoresis in 10% denaturing urea/polyacrylamide/TBE buffer gel followed by silver staining. A control reaction was performed without the blocked antibody.

RESULTS

IIF reactions: Figures 1, 2, and 3 present the results of confocal and conventional IIF reactions with different species of trypanosomatids: T. cruzi (Y, NCS, and Bolivia strains), Blastocritidia ssp. (insect parasite), and L. major promastigote forms, respectively. Positive controls for the parasites on the microscopy slides were performed with serum from a chagasic patient (Figs. 2 and 3, panel 1), since they have cross-reactivity with chagasic sera. Parasites incubated with normal human serum or normal rabbit serum, as negative controls, showed no detectable fluorescence under the experimental conditions used in this study (data not shown). L. major and Blastocritidia ssp. promastigotes also reacted with chagasic serum, which is similar to findings previously described for other trypanosomatids (22).

Figure 1 shows the TMG epitopes detected in the T. cruzi UsnRNPs analyzed by confocal microscopy. In the competition reaction, abrogation of the perinuclear fluorescence was observed, and only nuclei and kinetoplasts (labeled with DAPI) were detected (Fig. 1B, in red). Panels C and D present phase-contrast pictures of the reactions without and with competition, respectively, to observe the limits of the cellular membrane of the parasites outlined in panels A and B, respectively. Parasites incubated with anti-m,G antibody showed perinuclear fluorescence, indicating that the TMG cap epitopes of the snRNPs were localized predominantly in discrete regions close to the nuclei (arrows in Fig. 1A). A similar fluorescence pattern was observed for the three different parasites after reaction with anti-m,G antibody (Figs. 2 and 3, panel 2). The identity of these epitopes, and hence the specificity of the rabbit anti-m,G antibody, was ascertained by competition reactions with m,G that block the Ig binding-sites, thus abolishing fluorescence in all of the parasites tested (Fig. 1B, Figs. 2 and 3, panel 3).

Immunoprecipitation reactions: This reaction revealed a pattern of bands that represented the immunoprecipitated snRNAs (Fig. 4B). The TMG antibody reaction was then blocked with m,G and no bands were detected (Fig. 4A). These reactions confirmed the specificity of the anti-m,G antibody used in this study, since the TMG antibodies in which binding sites were blocked by m,G did not react with the m,G cap localized in the 5’ ends of the snRNAs. The immunoprecipitated bands shown in Fig. 4B reflect not only antibody specificity, but also refer to the snRNAs of the trypanosomatid snRNPs. These results, taken together, provide support for
DISCUSSION

These data confirm the presence of TMG cap epitopes of UsnRNPs, which were immunolocalized in different trypanosomatids, independent of the reactivity with chagasic serum or pathogenicity. This region is a relevant target in these parasites in particular, and in eukaryotes in general, since they are markers of 5’ end snRNAs. All mRNA precursors transcribed by RNA polymerase II have a 5’ cap added by polymerase-associated enzymes. The 5’ cap protects mRNAs from exoribonucleases and also plays an important role in mRNA translation and processing, and therefore also in the splicing process (23).

In trypanosomatids, the UsnRNAs are transcribed by RNA polymerase III are also capped at the 5’ end (24). The pre-mRNAs receive this structure during trans-splicing when a capped SL sequence of 39 nucleotides is transferred, forming SL RNA at the 5’ end of each mRNA. SL RNA is also transcribed by RNA polymerase II (25).

Reuter et al. (26) studied the localization and distribution of UsnRNAs during mitosis in mammalian cells by immunofluorescence microscopy with snRNA cap-specific anti-m3G antibody. Whereas the snRNAs are strictly nuclear at late prophase, they are distributed throughout the cell plasma at metaphase and anaphase; snRNAs then reenter the newly formed nuclei of the two daughter cells at early telophase, producing speckled nuclear fluorescent patterns typical of interphase cells. Independent immunofluorescence studies

Fig. 1. Confocal fluorescence microscopy of Trypanosoma cruzi epimastigotes forms (Y strain). snRNPs were localized by labeling with monoclonal anti-m3G antibodies. (A) Arrows indicate snRNPs fluorescence (green) and nuclei and kinetoplasts labeled with DAPI (red). (B) Same as A, but reaction of anti-m3G serum was abolished by the competition reaction. (C, D) Corresponding differential interference contrast of the same parasites in A and B. Magnification bar = 10 μm.

Fig. 2. Subcellular localization of snRNPs epitopes in Trypanosoma cruzi Y (A), NCS (B) and Bolivia (C) strains epimastigotes forms by conventional immunofluorescence microscopy. 1) chagasic human serum; 2) rabbit anti-m3G serum diluted 1:10, and 3) rabbit anti-m3G treated with 7-methylguanosine. Arrows indicate reaction with epitopes localized with anti-m3G serum. Magnification ×1,000.
with anti-(U1)RNP auto antibodies, which react specifically with proteins unique to the U1 snRNP species, showed the same distribution of snRNP antigens during mitosis, as was observed with the snRNA-specific anti-m3G antibody.

Previous studies already have used specific antibodies for m3G to detect snRNA-containing snRNPs in interphase cells (27). Immunofluorescence microscopy was also used by Bridge et al. (28) to investigate the effects of adenovirus infection on the nuclear organization of splicing snRNPs in HeLa cells; the results showed that splicing snRNPs were localized at the center of viral DNA synthesis, as determined using specific antibodies against common snRNP antigens and the snRNA-specific m3G 5’ cap structure. Also using immunofluorescence, Potashkin et al. (29) employed specific antibodies for the m3G-cap structure of snRNAs to examine the distribution and localization of snRNPs particles within the yeast cell nucleus of Saccharomyces cerevisiae. It was found that most of the abundant snRNAs were localized to portions of the nucleus previously referred to as the “nucleolus”. These findings were in contrast to the results obtained with mammalian cells, in which the majority of the abundant snRNAs were present in a non-nucleolar network within the non-chromatin-containing regions of the nucleoplasm (29).

In the present study, we confirmed the presence of TMG epitopes in different trypanosomatids; specific localization of these epitopes close to the nuclei was confirmed after competition reactions with m7G were carried out using either polyclonal or monoclonal anti-m3G antibodies. To date, several studies have highlighted the importance of the TMG cap structure in UsnRNAs, particularly with respect to the distribution of these structures inside cells.

Using specific polyclonal antibodies, Palfi and Bindereif (30) described the localization of and characterized several common snRNPs in T. brucei. Based on immunofluorescence microscopy, snRNPs proteins in these trypanosomes appear to be predominantly localized in the nucleus, with a fraction of proteins residing in the cytoplasm. This differential distribution might reflect an ordered assembly pathway for snRNPs that involves both cytoplasmic and nuclear stages, similar to the cytoplasmic-nuclear assembly pathways proposed for the mammalian U2snRNP.

The present results clearly demonstrated for the first time direct evidence of the presence of UsnRNAs in the cytoplasmic region near the nucleus, which most likely suggests the nuclear reimporting of UsnRNPs in these parasites. The predominance of fluorescence in the cytoplasm was shown to be due to the parasite treatment before IIF reaction (e.g., in the absence of Triton X-100 pretreatment). The specificity of the antibody was demonstrated by immunoprecipitation assay, as shown in Fig. 4, since antibody competition with m7G confirmed that the snRNP bands disappeared.

The results demonstrated that the accurate modification of the entire cap structure requires that SL RNA is in the SL RNP complex, and SL RNA capping takes place in the nucleus. Recently, it was suggested that SL RNA might also be found in the cytoplasm. Treatment with the karyopherin-specific inhibitor leptomycin B eliminated the cytoplasmic SL RNA and exerted an influence on modification at the +4 position and 3’ end formation, suggesting that the cytoplasmic stage is necessary for SL RNA biogenesis (18). Our studies are now focused on the characterization of all UsnRNPs in various strains of T. cruzi in order to evaluate the role in trans-splicing reactions (manuscript submitted).
REFERENCES


