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HSP90 is a target protein for ubiquitination in *Giardia intestinalis*^a

HSP90 es una proteína blanco de ubiquitinación en Giardia intestinalis

HSP90 é uma proteína alvo de ubiquitinação em Giardia intestinalis

Jenny J Chaparro-Gutiérrez^{1,2}, MV MS; Moisés Wasserman^{1*}, Quim PhD

¹Laboratorio de Investigaciones Básicas en Bioquímica, Facultad de Ciencias,
Universidad Nacional de Colombia, Bogotá, Colombia.

²Facultad de Ciencias Agrarias, Escuela de Medicina Veterinaria, Universidad de Antioquia, Medellín, Colombia

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Summary

Previous studies have demonstrated the existence and expression of genes essential to the process of protein ubiquitination in *Giardia intestinalis*, indicating that the ubiquitin-proteasome system may be involved in the degradation of proteins during its life cycle of the parasite. In this study, purification of ubiquitin was conducted from protein extracts of *G. intestinalis* trophozoites. Then, an anti-ubiquitin specific antibody was obtained to standardize an assay for the detection and evaluation of ubiquitination patterns. Finally, HSP90 was identified as an ubiquitinated protein in this protozoan. This post-translational modification could have regulatory effects associated with the functionality of the protein or its turnover to regulate key molecular events during the parasite's life cycle.

Key Words: protozoan, thermic shock protein, ubiquitin.

Resumen

Estudios previos han demostrado la existencia y expresión de genes esenciales para el proceso de ubiquitinación de proteínas en *Giardia intestinalis*, indicando que el sistema ubiquitina-proteosoma puede estar involucrado en el proceso de degradación de proteínas de este parásito durante su ciclo de vida. En el presente trabajo se realizó la purificación de ubiquitina a partir de extractos proteicos de trofozoitos de *G. intestinalis*, se produjo un anticuerpo anti-ubiquitina específico que permitió la estandarización de un ensayo para la detección y evaluación de los patrones de ubiquitinación, y se identificó la HSP90 como una proteína ubiquitinada en este protozoario. Esta modificación post-transduccional puede tener

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* Corresponding author: Moisés Wasserman. Carrera 45 No 26-85. Unidad Camilo Torres, Bloque 10, Nivel 4, Bogotá D.C., Colombia. E-mail: mwassermannl@unal.edu.co.

efectos regulatorios asociados con la funcionalidad de la proteína o con el recambio para regular eventos moleculares claves durante el ciclo de vida del parásito.

Palabras clave: protozoario, proteína de choque térmico, ubiquitina.

Resumo

Estudos anteriores demonstraram a existência e expressão de genes essenciais para o processo de ubiquitinação de proteínas em Giardia intestinalis, indicando que o sistema ubiquitina-proteassoma podem estar envolvidos na degradação de proteínas do parasita durante seu ciclo de vida. Neste trabalho, foi realizada a purificação da proteína ubiquitina de extratos de trofozoítos de G. intestinalis, foi produzido um anticorpo anti-ubiquitina específico que permitiu a padronização de um ensaio para a detecção e avaliação de padrões de ubiquitinação e foi identificado a HSP90 como uma proteína ubiquitinada no protozoário. Esta modificação pós-transducional pode ter efeitos regulamentares associados com a funcionalidade das proteínas ou com o a substituição para regular eventos moleculares importantes durante o ciclo de vida do parasita.

Palavras-chave: protozoários, proteínas de choque térmico, ubiquitina.

Introduction

The protozoan parasite *Giardia intestinalis* (syn. *G. lamblia*, *G. duodenalis*) is the principal non-viral causing agent of human diarrheic disease throughout the world (Adam, 2001). In addition to its importance as a pathogen, this eukaryote is considered an important model for the study of basic cellular processes (Morrison *et al.*, 2007). During its life cycle, *G. intestinalis* presents two differentiation processes, encystation and excystation. Thus, the parasite alternates between an asexually replicating vegetative form (trophozoite) and an infectious form that is able to survive in the external environment (cyst) (Lujan *et al.*, 1997). Both of these processes involve intracellular modifications, transcription of new genes; synthesis, and probably specific degradation of proteins (Adam, 2001).

The ubiquitin/proteasome system (UPS) serves as the most important protein degradation pathway in eukaryotes. Ubiquitin is a small globular protein that is covalently attached to target proteins, most commonly at lysine residues. The covalent attachment of ubiquitin to target proteins is catalysed in a three-step enzymatic cascade involving ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes and ubiquitin ligases (E3) (Glickman and Ciechanover 2002). The removal of ubiquitin occurs in one step, catalysed

by deubiquitinases (Reyes-Turcu *et al.*, 2009). Historically, the post-translational modification of proteins by ubiquitin has been associated with protein degradation (DeMartino and Gillette 2007). More recently, non-proteolytic functions of ubiquitination have been revealed in many cellular pathways including membrane trafficking, DNA repair and replication and gene transcription (Welchman *et al.*, 2005; Chen and Sun 2009).

Previous studies have demonstrated the existence and expression of essential genes for the ubiquitination process of proteins in *G. intestinalis* (Gallego *et al.*, 2007; Catic *et al.*, 2007). A ubiquitinated substrate, glucosamine-6-phosphate isomerase has also been identified during the parasite's encystation process (López *et al.*, 2002). This information suggests that the selective intracellular protein degradation system or ubiquitin-proteasome system could be involved in the protein degradation process in *G. intestinalis* during its life cycle. With this study was initiated the characterization of the modification caused by the ubiquitin conjugation in *G. intestinalis* proteins. First, purifying ubiquitin from trophozoites' protein extracts to produce a specific antibody against conjugates ubiquitin-protein. Then, evaluating the ubiquitination patterns on the trophozoite stage and during the encystation process using an immunoblotting assay and finally, identifying an ubiquitinated protein. This post-transductional

modification could have regulatory effects associated with protein's functionality or turnover to regulate key molecular events driving the parasite life cycle.

Materials and methods

Parasite culture

G. intestinalis trophozoites (WB, Clon C6) were cultured in Diamond TYI-S-33 pH 7.0 medium, supplemented with 10% of bovine serum (Gibco, Invitrogen Corp. NY) and 0.5 mg/mL bovine bile (Sigma, St Louis, MO) at 37 °C in borosilicate tubes (BD Falcon, Biosciences, CA). The tubes were filled with medium in order to get an anaerobic atmosphere and they were kept at an inclination of 45 degrees (Keister, 1983). For the *in Vitro* encystation, the trophozoites were cultured in a TYI-S-33 pH 7.8 medium supplemented with 10 mg/mL of bovine bile (Kane *et al.*, 1991). 1.000 IU/mL of penicillin (Sigma, St Louis, MO) and 1.0 µg/mL streptomycin (Sigma, St Louis, MO) were also added; the pH was adjusted with a NaOH 5 N solution before the sterilization by filtration with 0.45 µm pore-size nitrocellulose membranes. Samples were collected during different stages of the encystation process: 0 h (before the stimulus), 1 h, 3 h, 6 h, 12 h, 24 h and 48 h after the stimulus. The number of cysts and the efficiency of the encystation were calculated by cell count in a hemocytometer.

Obtention of G. intestinalis protein extract

The extract was obtained from large scale (5 L) trophozoite cultures treated as was previously described. The 3-day culture cells were put on ice for 15 min in order to detach the parasites that were adhered to the culture tube's surface. Subsequently, they were centrifuged at 900 g for 15 min. In order to separate the culture medium, the cells were washed three times with PBS [137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ (Dibasic) 14.7 mM KH₂PO₄ (Monobasic) pH 7.4] cold, sterile and centrifuged at 900 g for 15 min. After the third wash, the cells were resuspended in 1 mL of PBS and the cellular count was made by diluting 1:1.000 in PBS. The parasites were counted two times in a hemocytometer and were stored at -80 °C until their processing. The number of cells was calculated as:

$N=C*f*10^4$, being N the number of cells in 1 mL; C the average number of cells and f the dilution factor.

10X10⁹ trophozoites of *G. intestinalis* were collected. This cellular pellet of ~ 20 mL was treated with 80 mL of extraction buffer (30 mM Tris-HCl pH 7.5, 20 mM NaCl, 10% glycerol, 0.5% NP40, 10 mM β-mercaptoethanol, 3 mM MgCl₂, 1 mM EGTA). This suspension was maintained in agitation at 4 °C for 1 h, and subsequently was centrifuged at 35.000 g for 15 min. The supernatant was recovered and 500 µL of a protease inhibitor mixture were added (Sigma, St Louis, MO), the final inhibitor concentration was: 2 mM AEBSF (4-(-2-aminoethyl) benzenesulphonyl fluoride, 2.8 µM E64, 3 µM pepstatin A, 8 µM bestatin, 0.6 µM leupeptin, 4 µM aprotinin). The protein quantification of the extract was made by the Bradford method (1976), obtaining a protein concentration of 9.38 g/µL.

Tris-tricine SDS-PAGE gels electrophoresis and western blots

In the discontinuous protein electrophoresis with tricine-SDS-PAGE gels (tricine-sodium dodecyl sulfate polyacrylamide electrophoresis) an anode buffer (20 mM Tris-HCl, pH 8.9) and a cathode buffer (10 mM tris-HCl, 10 mM tricine and 0.1% SDS) were used. In the sample buffer 2X (0.1 M tris-HCl, 24% (v/v) glycerol, 8% (w/v) SDS, 0.2 M DTT, 0.02% (w/v) Coomassie blue G-250), Coomassie blue is used as indicator stain instead of bromophenol blue, because it moves in front of the small peptides. The runs were made at 30 V for 30 min followed by 116 V for approximately 2 h. The proteins that were separated by the previous method were transferred to PVDF (polyvinylidene fluoride) membranes at 25 V overnight in transfer buffer (tris base 25 mM, glycine 192 mM, methanol 10% (v/v), pH 8.3).

For the immunodetection using western blot, the protocol was followed as it will be described a) blocking of the membrane for 1 h with TBST-M (Tris-Buffered Saline-Tween-Milk: 20 mM Tris-HCl, pH 7.5; 150mM NaCl; 0.1% tween 20; 5% (w/v, low fat milk) buffer; b) incubation for 1 h with the primary antibody anti-ubiquitin produced

in rabbit in TBST-M (1:2.000); c) incubation for 1 h in secondary antirabbit IgG biotinylated antibody (Invitrogen) in TBST-M (1:3.000); d) incubation for 30 min with streptavidine-phosphatase alkaline (Promega) in TBSM (1:3.000). After each incubation, 3 successive 10 min washes were made with the respective incubation buffer. Detection was done with the chromogenic substrates BCIP (5-bromo-4-chloro-3-indolyphosphate) and NBT (nitroblue tetrazolium) (Promega, Madison, WI) for the alkaline phosphatase in the buffer substrate (tris-HCl 100 mM pH 9.0, NaCl 150 mM, MgCl₂ 1 mM).

Ubiquitin purification

For the purification process was used a modification of the method previously reported by Sparkman and collaborators (1991). Briefly, 100 mL of trophozoite' protein extract was heated at 85 °C for 20 min in constant agitation; the supernatant was recovered by centrifugation at 30.000 g for 1 h at 4 °C. This solution was precipitated with perchloric acid (final concentration of 5%) in constant agitation for 1 h at 4 °C; the supernatant was recovered as it had been previously done, and this solution was concentrated by precipitation with trichloroacetic acid (final concentration 10%). The pellet was centrifuged at 30.000 g for 5 min at 4 °C; the supernatant was discarded, and the pellet was washed twice with acetone, dried for 30 min at 37 °C and resuspended in 2 mL of deionized sterile water. Lastly, and in order to obtain pure ubiquitin, a preparative extraction from polyacrylamide gels was done following a methodology previously described (Scheer and Ryan, 2001).

Preparative electrophoresis were done in denaturing conditions in tricine-SDS-PAGE gels, running 250 µL samples in buffer 2X (0.1 M Tris-HCl, 24% (v/v) glycerol, 8% (w/v) SDS, 0.2 M DTT, 0.02% (w/v) Coomassie blue G-250). In order to visualize the band and the position of the protein, the extreme of the gel containing the ubiquitin standard and part of the partially purified sample was cut. Then it was stained with Coomassie blue [0.025% (w/v) Coomassie brilliant blue G-250, 10% acetic acid]. According to the electrophoretic patterns the corresponding band was cut; it was disrupted with a 2 cc syringe, and the protein

was extracted with the least possible quantity of deionized water maintaining the sample in agitation at 37 °C for 12 h. The process was monitored by vertical electrophoresis in tricine-SDS-PAGE gel and western blot as previously described. The purity of the ubiquitin was verified by tricine-SDS-PAGE. The protein was visualized using silver staining with the Silver Stain Plus (BioRad, Hercules, CA) kit, and was quantified using the Bradford method (1976). This same procedure was done three times.

Anti-ubiquitin antibody production

A specific anti-ubiquitin antibody was produced with the pure ubiquitin following a method that has been previously described (Hershko *et al.*, 1982). Ubiquitin is a small protein (8.5 kDa), it is necessary to cross-link it to a bigger immunogen to generate an immune response; then ubiquitin was coupled to bovine γ -globuline using 3% glutaraldehyde. Bovine γ -globuline (8 mg) and pure *G. intestinalis* ubiquitin (5 mg) were dissolved in 600 µL of buffer potassium phosphate (0.1 M, pH 7.2). The conjugation of ubiquitin to bovine γ -globuline was made by adding 80 µL of glutaraldehyde to the 3% (v/v) in 20 µL aliquots with 10 minutes intervals in constant agitation. The reaction took place at room temperature for an additional period of 90 min after the last addition of glutaraldehyde. The sample was dialyzed over night against 2 L of PBS pH 7.4 at 4 °C, through 3500 Da cut-off membranes (Spectrum Laboratories, Inc USA). *G. intestinalis* ubiquitin was denaturated in its final purification stage. The antigen's denaturation is essential for obtaining an anti-ubiquitin antibody capable of recognizing ubiquitin-protein conjugates (Hershko *et al.*, 1982).

The polyclonal antibody was generated in a two month old New Zealand rabbit. The inoculation chart proposed by Hershko y collaborators was followed (1982). Briefly, the sample of pure ubiquitin was mixed with an equal volume of Freund's complete adjuvant (Sigma, St Louis, MO) for the first inoculation and with Freund's incomplete adjuvant (Sigma, St Louis, MO) (next inoculations- 4 total). The samples containing 0.1 mg of ubiquitin were injected every 2 weeks into rabbits. Antibody titers were determined by western

blot assays as described previously. The antibody obtained against *G. intestinalis* ubiquitin was called Anti-UbGi.

Western blot assays to evaluate ubiquitination patterns

Adequate conditions for a western blot immunodetection assay were standardized with the antiserum (Anti-UbGi). This allowed the analysis of the presence of free ubiquitin and the evaluation of the ubiquitination patterns in trophozoites and during the encystation process of *G. intestinalis*. For these experiments, protein extracts were prepared as previously described Alvarado and Wasserman (2010) and whole parasites (50.000 cells/ μ L) were also used, the adequate conditions for the western blot immunodetection assay were also standardized. Some of these conditions are the optimal titer of the produced polyclonal antibodies, and the adequate concentration of protein extract or parasite quantity for the specific detection of free ubiquitin or ubiquitinated proteins.

The optimal dilution of the antibodies was established with the detection limit that was evident with pure ubiquitin and the parasite's protein extracts. For the *in vitro* encystation, the trophozoites were cultured and collected as it was previously described. The experiments were done by triplicate and with at least two different samples in each experiment. The methodology for electrophoretic and immunodetection assays was the same one that was previously described.

Identification of a ubiquitinated target protein

24 h-trophozoite cultures were treated overnight with 50 μ M of the proteasome inhibitor carbobenzoxy-leucinylleucinylleucinal-H (MG132) (Calbiochem, La Jolla, CA) in DMSO to enrich the ubiquitin-protein conjugates (Vasilescu *et al.*, 2005). Subsequently, the parasites were harvested as previously described containing a cocktail of protease inhibitors (Sigma, St Louis, MO). The suspension was adjusted to 50.000 cells/ μ L in sample buffer 1X (15 mM Tris-HCl, pH 6.8, 0.05% (w/v) SDS, 0.002% (w/v) bromophenol % (v/v) glycerol, 89 mM β -mercaptoethanol), heated at 85 °C for 5 min and was evaluated by SDS-PAGE

(gels of 8%T, 2.6%C, to resolve the region with high molecular weight proteins) and western blot was done as described.

Using the ubiquitination patterns, the identification of the proteins recognized by the anti-ubiquitin specific antibody was possible. An evident band between 45 kDa and 66 kDa on the parasites that had been treated with the proteasome inhibitor was cut; it was clearly identified by the western blot. The band that was cut had been stained with Coomassie blue and was transferred to low retention Eppendorf tubes. The protein sample was destained by incubation for 2h in 200 μ L of a solution of 50% acetonitrile and 50 mM ammonium bicarbonate.

Then, the gel was dehydrated by treatment with acetonitrile 100% for 5 min. The solvent was removed and the drying process at room temperature was allowed. Subsequently, the gel was incubated in a washing solution (50 mM ammonium bicarbonate) for 10 min and dehydrated once more. After this process the gel was treated with 10 mM DTT for 30 min; then the reductor agent was removed and the gel was washed and incubated with 100 mM of iodocetamide for 30 min. The washing procedure was repeated and the gel was left in digestion for 14 h at 37 °C with 5 μ L (20 ng/ μ L) of Trypsin Gold (Promega) in 50 mM of ammonium bicarbonate. After the enzymatic digestion the peptides were eluted from the gel through incubation for 10 min with 30 μ L ammonium bicarbonate 50 mM. The first elution was recovered and the gel was treated two times with 30 μ L of an acetonitrile 50% and formic acid 5% mixture. These two elutions were mixed with the first one and concentrated. Finally, the sample was analyzed using the mass spectrometry technique MS+MS/MS. The list of the generated masses was analyzed with the MASCOT software (www.matrixscience.com).

Results

Ubiquitin purification

After preparatory electrophoresis SDS-PAGE with tris-tricine buffer, up to 1 mg of pure ubiquitin was obtained from a culture of 10×10^9 trophozoites. In figures 1A and 1B the ubiquitin purification

process is observed. During the entire process a band with molecular weight of ~ 8.5 kDa was evidenced. It migrated at the same height than bovine commercial ubiquitin (Figure 1, lane 6), the identity of the ubiquitin was proved by a western blot assay (Figure 1B), using an anti-ubiquitin polyclonal antibody.

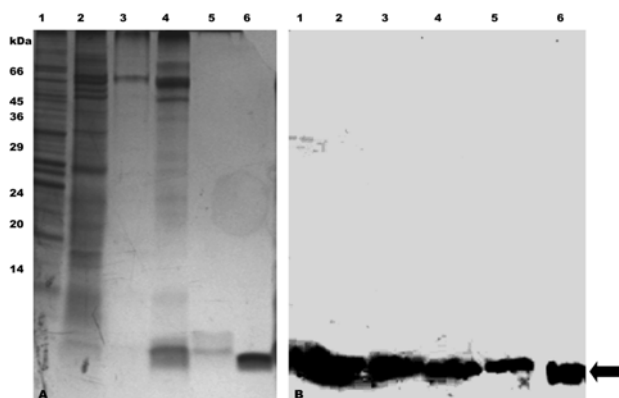


Figure 1. Ubiquitin purification. A. SDS-PAGE T: 18% in tris-tricine gel, stained with silver (Silver Stain Plus. Bio Rad. Hercules, CA). 1. 30 μ g of trophozoite protein extract, 2. 30 μ g of post-heating supernatant, 3. 30 μ g of supernatant after perchloric acid precipitation, 4. 30 μ g of TCA precipitation obtained pellet, 5. 0.05 μ g of pure *Giardia* ubiquitin extracted from the gel after the preparatory electrophoresis. 6. 0.1 μ g of bovine ubiquitin (Sigma, St Louis, Mo). B. Corresponding western blot assay in electrophoresis A gel, Anti-UBGi antibody 1:2000 kDa. was used. Protein low molecular weight mark. The arrow on the right points towards the ubiquitin.

Ubiquitination patterns

The serum obtained during the rabbit immunization, recognized pure *G. intestinalis* ubiquitin and commercial bovine ubiquitin (Sigma, St Louis, MO) using western blot assays with 100 ng ubiquitin (Figure 2). These proteins have an identity of 86.84% using protein-protein blast (<http://ca.expasy.org>). This recognition started 30 days after the first inoculation and the detection increased in time using higher serum dilutions. This evidenced the increase in the antibody titer and in its specificity (data not shown). The pre-immune serum did not detect ubiquitin in any of the studied dilutions. Some variables were determined, one of them was the optimal dilution of the polyclonal antibody when the pure antigen (1:1.000) was used. The protein extract concentration or the right number of parasites for the specific detection of ubiquitin and ubiquitinated proteins was also determined: 100.000 trophozoites and 5 μ g of protein extract were enough to detect the ubiquitin and ubiquitin-protein conjugates in *G. intestinalis*.

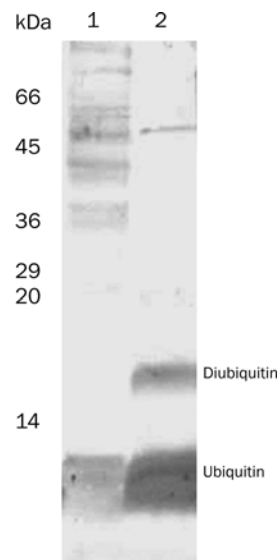


Figure 2. Western blot with Anti-UBGi 1:1000. 1. Trophozoite protein extract (30 μ g) 2. Bovine ubiquitin (Sigma, St Louis, MO), 20 ng.

When the presence of ubiquitin-protein conjugates was being evaluated, the anti-ubiquitin polyclonal antibody (Anti-UBGi) made it possible to identify the ubiquitination patterns in the high molecular weight region during the trophozoite stage (Figure 3, lane T) and during the encystation process (Figure 3, from 0 to 48 h, see parenthesis). The expression of free ubiquitin was constant until the 24 first hours of the encystation process of *G. intestinalis*; at 48 h there was an evident decrease of this type of ubiquitin in the soluble cytoplasm fraction (Figure 3, lane 48 h).

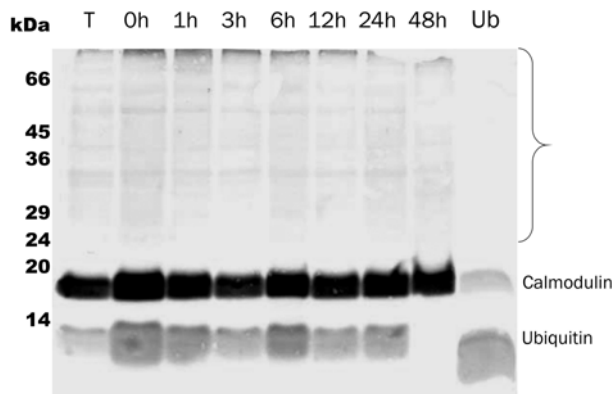


Figure 3. Ubiquitination patterns. 30 μ g of protein extract in each lane. T, Trophozoites; 0, 1, 3, 6, 12, 24, 48 hours of the encystation process, Ub 1000 ng of commercial bovine ubiquitin (Sigma, St. Louis, MO). Calmodulin immunodetection was used as charge control using the Anti-Cam 1:2000 polyclonal antibody. The Anti-UBGi antibody was used in a 1:1000 dilution. The square bracket shows the presence of ubiquitin-protein conjugates.

Identification of HSP90 as a ubiquitinated protein

After the proteasome inhibition with MG132 the accumulation of several proteins with molecular masses higher than 50 kDa was evident. A protein between the 45 kDa and the 66 kDa marks (Figure 4 and Figure 5) with low parasite concentration could be observed. This band was cut and processed for its identification through mass spectrometry MS+MS/MS technique and it was identified as “heat shock protein HSP90- α (*Giardia lamblia* ATCC 50803)” with a molecular mass of 43.752 kDa (Figure 6). To corroborate the identity of this protein and validate this result, western blot assays were done using a monoclonal antibody Anti-HSP90 (Figure 5, Anti-HSP90). One of the bands recognized by the monoclonal antibody anti-HSP90 was also clearly marked with the anti-ubiquitin antibody both in the control trophozoites and the inhibited trophozoites (Figure 5, Anti-UbGi, lanes 1 and 2, respectively) and it corresponded to a ~52 kDa protein. This result indicates that the protein identified by mass spectrometry is the monoubiquitinated (8.5 kDa) HSP90- α (43.752 kDa), which is located in exactly 52 kDa (Figure 5, Anti-UbGi, lane 2 and Anti-HSP90, lane 2). With the monoclonal antibody anti-HSP90 a bigger band corresponding to the whole protein can be identified (Figure 5, Anti-HSP90, lane 2).

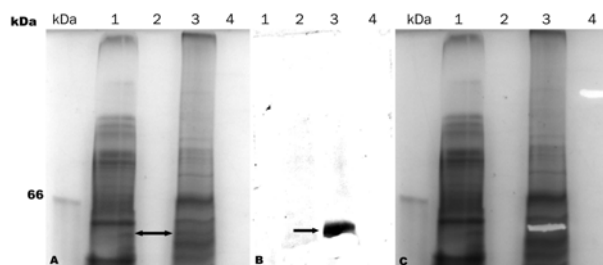


Figure 4. Identification of a ubiquitination target protein in *G. intestinalis*. Through the use of a proteasome inhibitor was possible the accumulation and separation of a ubiquitinated protein in trophozoites. A. SDS-PAGE in tris-glycine gel 8% T stained with Coomassie blue solution. kDa, low molecular weight marker for proteins; 1. 150,000 *G. intestinalis* trophozoites; 2. Electrophoresis sample buffer 1X B. Corresponding A gel western blot with the Anti-UbGi antibody 1:1000 . The specific detection of a ubiquitin-protein conjugate of approximately 52 kDa is evidenced in the MG 132 treated parasites. C. Protein band and a negative band were cut for mass spectrometry analysis. The arrows show the protein that is accumulated post proteasome inhibition and its identification by western blot.

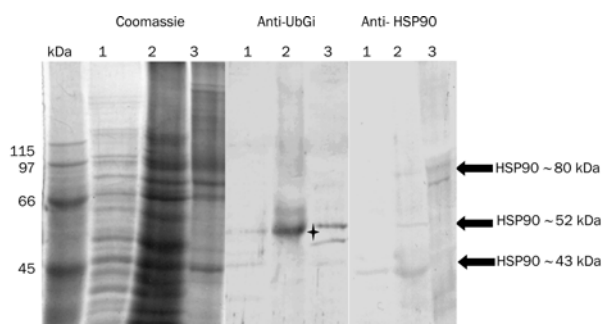


Figure 5. HSP90 ubiquitination in *G. intestinalis*. The parasite concentration was adjusted to 200.000 cells. kDa, high molecular weight protein marker; 1. *G. intestinalis* trophozoites treated for 12 h with DMSO; 2. *G. intestinalis* trophozoites treated for 12 h with 50 μ M of MG132; 3. Control with *Plasmodium falciparum* trophozoites. The arrows show the detection of HSP90 with different molecular mass. The asterisk shows the detection of 52 kDa, monoubiquitinated HSP90.

Table 1. Results Mass Spectrometry: MS+MS/MS. Laboratory: Servicio Central de Apoyo a la Investigación. Universidad de Córdoba (Spain).

Rank	Protein Score	Protein Score C.I.%	Protein Name	Species	Protein MW	Protein PI	Access No	Pep Count	Total Ion Score	Total ion C.I.%
1	101	99.997	Heat shock protein HSP90 alpha	<i>Giardia lamblia</i> ATCC 50803	43752.3	5.51	gi[159115537	9	46	99.067
PEPTIDES INFORMATION										
Cal. mass	Observ mass	\pm da	\pm ppm	Start seq..	End seq.	Sequence	Ion score	C.I. %	Modification	Rank result type
1156.5269	1156.5576	0.0307	27	164	172	DYFGEELQR				Mascot
1347.7015	1347.7139	0.0124	9	260	270	KEVEVHEVNVK				Mascot
1364.6416	1364.6771	0.0355	26	325	335	RAPFDMWDAQK				Mascot
1380.6365	1380.6736	0.0373	27	325	335	RAPFDMWDAQK			Oxidation m[6]	Mascot
1551.6849	1551.729	0.0441	28	281	292	DVTEDEYKDFYK				Mascot
1555.8955	1555.9001	0.0046	3	45	58	LHFTSLTRPSVLGK				Mascot
1631.8785	1631.8866	0.0081	5	72	86	DNILVIRDTGVGMTK				Mascot
1969.9515	1969.9976	0.0461	23	293	309	QINPSDYEGHLAVSHFR				Mascot
1969.9515	1969.9976	0.0461	23	293	309	QINPSDYEGHLAVSHFR	46	99.06		Mascot

Origen: Servicio Central de Apoyo a la Investigación. Universidad de Córdoba (Spain).

Discussion

G. intestinalis ubiquitin was purified through a three step method that uses this protein's thermostability and solubility in the perchloric acid 5% solution. The pure protein made it possible to obtain a specific polyclonal antibody for *G. intestinalis* ubiquitin (Anti-UbGi) which recognized free ubiquitin and ubiquitin-protein conjugates both in soluble protein extracts and in whole parasites. The detection of ubiquitin by the pre-immune serum could not be seen in any of the studied dilutions, confirming that the detection was because of the immunization process.

The decrease in the ubiquitin levels at 48 h after the encystation stimulus in cytoplasm soluble protein extracts suggests that a ubiquitin conjugation to proteins or a relocalization of this protein from the parasite's cytoplasm to the cyst's walls could be happening. Stefanic and collaborators (2006) reported the association of the 26S proteasome with the encystation specific vesicles and demonstrated the relocalization of this protease towards the parasite's periphery during the early stages of *G. intestinalis*' encystation. It is possible to suggest that the ubiquitination machinery could go through the same relocalization or that the ubiquitin is linked to proteins related with the periphery of cyst.

The protein identified as ubiquitinated is a cytoplasm protein involved in several cellular processes: the protein degradation in cells exposed to stress, in a key regulatory protein conformational development, and in the growth and development regulation of various protozoan (Pallavi *et al.*, 2010). HSP90 is strongly regulated through several control mechanisms that regulating its activity. Among them are: conformational changes; cofactor presence and post-transductional modifications such as acetylation (Scroggins *et al.*, 2007), S-nitrosylation (Martínez-Ruiz *et al.*, 2005), and phosphorylation (Mollapour *et al.*, 2010).

Even though in various eukaryote cell systems HSP90 has been identified as a ubiquitinated

protein (Kirkpatrick *et al.*, 2005; Vasilescu *et al.*, 2005; Morales and Perdew 2007), the physiological role of this modification is not yet known. In our experimental model the detection of HSP90 ubiquitination enhanced in the proteasome inhibited parasites, suggest that its ubiquitination level is too low (Figure 5, Anti-UbGi); an evident level can be reached only after the accumulation of ubiquitin-protein conjugates has been induced (Figure 5, Anti-UbGi, lane 2). It is well documented, in other eukaryote systems, that the cell treatment with proteasome inhibitors causes the induction of a heat shock response which generates the expression of the heat shock proteins (HSP), including HSP90 (Bush *et al.*, 1997; Lee and Goldberg 1998; Mitsiades *et al.*, 2002).

It is important to consider that this type of proteins can be involved in the differentiation process of *G. intestinalis*. Kim and collaborators (2009) found a peak of messenger RNA for HSP90 at 6 h after the induction of the encystation process. This suggests the expression of these proteins in a differential way during *G. intestinalis*' life cycle. On the other hand, it has recently been proved that HSP90 protein in *G. intestinalis* goes through a post-transcriptional modification to produce the entire ~ 80 kDa protein (Nageshan *et al.*, 2011), making it possible the production of the two fragments of the protein. With this research we have been able to demonstrate that the alpha fraction of *G. intestinalis* HSP90 is monoubiquitinated. This post-transductional modification could have regulatory effects associated with this protein's functionality or turnover.

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