Vol. 196, No. 3, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 15, 1993 Pages 1155-1162

CYTOPLASMIC-NUCLEAR TRANSLOCATION OF THE HSP70 PROTEIN DURING ENVIROMENTAL STRESS IN TRYPANOSOMA CRUZI

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Received September 14, 1993

The present study provides immunological evidence of the constitutive presence of the Hsp70 protein in the cytoplasm of logarithmically growing T. cruzi parasites cultured at the normal temperature of 28°C and of the translocation of the protein to the nucleus upon a heat shock treatment (2 hours at 37°C). The nuclear translocation of the protein must depend on other factors beside the temperature *per se* since at 28°C, in stationary phase growing parasites, the Hsp70 protein was present in both the cytoplasm and the nucleus. During recovery at 28°C the protein leaves the nuclei but the nuclear-cytoplasmic translocation of the protein is a much more gradual process than its initial transport to the nucleus. Since the isoform of the nuclear Hsp70 is different from that found in the cytoplasm it is likely that before translocation to the nucleus the cytoplasmic Hsp70 nuclear precursor must undergo a specific modification. © 1993 Academic Press, Inc.

When eukaryotic cells are exposed to temperature 5-10°C above the normal temperature of growth they respond by synthesizing a group of proteins known as the heat shock proteins or Hsps. It is known, however, that the Hsps are activated by an enormous varieties of stress conditions. Among the Hsps proteins the 70kd protein (Hsp70) is the most highly conserved with regard to its aminoacid sequence. This protein is encoded in a family of genes and several Hsp70 isoforms have been described (1). Although the physiological significance of hsp70 during heat shock induction has been widely studied in the last few years (2-4) its role is still far from being clear. Most studies point towards the association of the protein with chaperon functions such as folding, assembly, disassembly and degradation of certain proteins and polypeptides (5-8) and with thermotolerance to temperature and anoxia (9). In recent

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Abbreviations: FBS, Faetal bovine serum (inactivated); PBS, Phosphate buffer saline; TBS, Tris buffer saline.

0006-291X/93 $4.00
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years the role of the protein has also been associated with immune surveillance since anti-hsp70s are found in patients having infectious diseases. The fact that the Hsps are induced in stress conditions and the protein has a high concentration in nuclei (10) observed in specific sites of the chromosomes (11) supports the hypothesis that its presence both in the cytoplasm and the nucleus is of an emergency nature. However, its constitutive presence in particular organisms reveals that it has a certain function variability.

Since the T. cruzi parasite, responsible for Chagas' disease and other trypanosomatidae, is subjected to various physiological thermic situations throughout its life-cycle within the insect vector (25°C) and in the mammalian host (37°C), we were interested in the determination of the intracellular localization of the Hsp70 in this parasite at both temperatures. This analysis may be relevant because in contrast with most eukaryotic organisms the Hsp70 gene expression is constitutive at 28°C but a clear increase in the steady state levels of the protein was observed at 37°C. In parasites, moreover, the increase in Hsp70 and its localization may have an important physiological role since in leishmania, schistosoma and naegleria the accumulation of the protein in heat shock conditions has been associated with differentiation and infectivity (12, 13). The present study shows evidence of the translocation to the nucleus of the cytoplasmic Hsp70 and that the transport is heat shock dependent but that it also occurs at 28°C in parasites during the stationary phase of growth. The report shows, moreover, that a specific Hsp70 isoform is the most abundant nuclear form of the protein at 37°C.

MATERIAL AND METHODS

Cell culture.

The Tulahuen strain of T. cruzi, recently obtained from a Colombia isolate, was used in this study. The parasites were cultured at 28°C in LIT medium supplemented with 10% FBS. The initial culture density was 10^6 parasites per ml. The culture was considered to be in the logarithmic phase of growth when the density was 2.0x 10^7 parasites/ml and in the stationary phase when a plateau of 5x 10^8 parasites/ml was reached.

Separation of the nuclear and cytoplasmic fractions.

The parasites were collected by centrifugation and washed three times with PBS buffer. Afterwards they were resuspended at 4°C in a lysis solution containing: 820mg of NaCl, 30mg $MgCl_2$, 1 ml 1M Tris-HCl pH 8.4, 1M NP-40, made up to 100 ml with H_2O. The parasites were placed on ice for 15 minutes and centrifuged at 4°C for 15 minutes at 500g. The cytosolic fraction remains in the supernatant and the nuclei in the precipitate. The nuclear pellet was washed twice for 5 min at 4°C in the lysis solution to eliminate cytoplasmic protein contaminants. SDS and PMFS were subsequently added to a 0.2% and 0.2 mM final concentration respectively. Non soluble products were eliminated by centrifugation at 12,000 rpm for 30 minutes at 4°C. A sintase citrate test (14) was used to monitor mitochondrial contamination.

Western blot analysis.

Similar amounts of either cytoplasmic or nuclear protein samples were separated electrophoretically on 10% polyacrylamide and transferred to nitrocellulose paper following standard procedures. Afterwards the filters were incubated for 12 hours in TBS-Tween 20 supplemented with anti $(GMPG)_6$ antibody (C-terminal repeat of hsp70 of T. cruzi (15) at a 1/200 dilution following the procedure previously outlined (16). The filters were, then, incubated for 1 hour with goat anti-rabbit IgG, conjugated with alkaline phosphatase at a dilution of 1/1000. The immunocomplexes were visualized using NTB/IBCP as substrate.

Immunoprecipitation of proteins labeled with ^{35}S methionine.

Logarithmically growing parasites in LIT medium were pelleted, washed twice in PBS and once in RPMI 1640 medium without methionine for 5 min each. Afterwards they were resuspended in the same medium supplemented with 10% dialysed FBS and left for 2 hours before adding ^{35}S methionine to a final concentration of 10uCi/ml. The parasites were incubated in the presence of ^{35}S methionine for 12 hours at 28°C, washed in culture medium and further incubated in that medium with 1000 fold cold methionine for 2 hours before a heat shock at 37°C as outlined in the legend to Fig 4, The isolation of the cytoplasmic and nuclear fractions was performed as described above. For the selective immunoprecipitation of the Hsp70 protein both fractions were incubated for 1 hour with anti $(GMPG)_6$ followed by incubation for another hour with agarose-protein A (Boehringer Manheim)). For the control of the specificity of the reaction both protein fractions were also incubated with preimmune serum. The samples were centrifuged for 15 sec at 12000g. After washing the precipitates were resuspended in 50 µl of buffer (17), heated at 95°C for 3 minutes and subjected to a 5-12% polyacrylamide electrophoresis linear gradient. The immunoprecipitated proteins were visualised by exposing the gel to a Kodax X-Omat autorradiographic film.

RESULTS AND DISCUSSION

Fig 1 shows the Western blot analysis of the Hsp70 protein from the nuclear and cytoplasmic fractions of logarithmicaly growing T. cruzi parasites using an specific antibody raised against the C-terminal $(GMPG)_6$ of the protein (15) in conditions of growth at 28°C

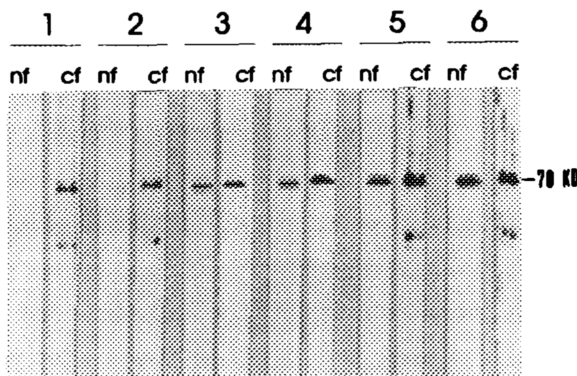

Figure 1. Localization of the Hsp70 in the nuclear (nf) and cytoplasmic (cf) fractions of T. cruzi in the logarithmic phase of growth. The protein fractions were subjected to 10% polyacrylamide gel electrophoresis and visualized by Western blot using an anti $(GMPG)_6$ antibody. Lane 1: Hsp70 from parasites incubated at 28°C; lanes 2, 3, 4, 5, 6: Hsp70 from parasites incubated at 37°C for 2, 6, 12, 24 and 48 hours, respectively. In all of the nuclear fractions the sintase citrate test was negative.

and 37°C. It is known that at 28°C the Hsp70 is constitutively expressed but that at 37°C the abundance of the protein increases several fold (16). It was observed that at 28°C the protein has only a cytoplasmic localization and that when the parasites were subjected to a 37°C heat shock not only a clear increase in cytoplasmic Hsp70 occurred, reaching a plateau after 24 hours of thermic induction, but that the protein gradually concentrated also in large amounts in nuclei. The abundance of the Hsp70 in nuclei seems to be dependent on the length of the treatment at 37°C. An appreciable accumulation of hsp70 in nuclei was only apparent after 2 hours of the heat shock. The densitometric analyses of the protein blot obtained from parasites incubated for 24 hours at 37°C revealed an increase in Hsp70 of 6-fold relative to the constitutive preexisting cytoplasmatic level at 28°C in agreement with previous studies which showed an approximate 4-fold increase in total Hsp70 levels after 2 hours of induction at 37°C (16). Our data suggest, thus, that although the Hsp70 protein is constitutively present in large amounts in T. cruzi parasites cultured at 28°C its function is primarily cytoplasmic at this temperature but that the protein shifts its location to the nucleus when the parasites are submitted to a stress condition at 37°C. In these conditions of growth, however, the protein must have a nuclear as well as a cytoplasmic function since in contrast with what it ocurrs in other organisms (18) a substantial amount of the protein, similar to that detected in nuclei, remained cytoplasmic at 37°C. It is likely that since in T. cruzi parasites the synthesis of proteins is not impered in conditions of Hsp70 induction, in contrast with what ocurrs in most eukaryotic cells, it might well be that the presence of large amounts of Hsp70 in the cytoplasm at 37°C (the temperature of the mammalian host) should be interpreted in the context of protecting the nuclear as well as the cytoplasmic metabolic function of the cell.

Since previous studies from our laboratory indicated that the Hsp70 protein from T. cruzi has a mean half life of about 7 hours we were able to determine the fate of the nuclear Hsp70 during recovery at 28°C following a heat shock treatment at 37°C for 12 hours. Fig 2 (panel B) shows that after 12 hours at 37°C the Hsp70 is present in both the cytoplasm and the nucleus. It was observed (panels C1, C2, C3 and C4) that while the abundance of the nuclear Hsp70 gradually decreases during recovery, the protein remained cytoplasmic after 48 and 72 hours, as expected from its constitutive synthesis, but that the nuclear Hsp70 was practically undetectable after 48 hours. Only a very faint band was visible in some experiments at 48 hours of recovery. The level of the protein in the cytoplasm after 48 hours of recovery at 28°C was similar to that detected in parasites cultured at 28°C without any history of heat shock. The slow release of the protein from the nucleus contrast with data from drosophila (18) in which the shift to the cytoplasmic distribution of the protein is completed in 4-5 hours following the heat shock. We also examined whether the cytoplasmic-

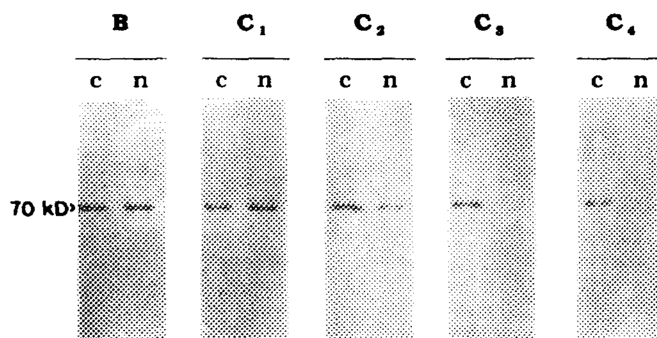

Figure 2. Behavior of the Hsp70 of T. cruzi during recovery at 28°C of epimastigotes cultures in the logarithmic growth phase which were previously subjected to heat shock at 37°C. Lanes n and c: nuclear and cytoplasmic proteins, respectively. Lane B: cultures incubated for 12 hours at 37°C. Lane C_1, C_2, C_3, C_4: cultures recovered at 28°C for 12, 24, 48 and 72 hours, respectively, after a 12 hour induction at 37°C.

nuclear Hsp70 translocation ocurrs also in conditions of Hsp70 induction in the absence of heat shock. Fig. 3 (lane 1), shows that in parasites cultured in the stationary phase of growth, in which induction of the Hsp70 also occurs (16), the Hsp70 protein was observed in the cytoplasmic as well in the nuclear fraction and that the level of the protein did no change even when the parasites were submitted to a heat shock for 12 or 24 hours (panel 2 and 3 respectively) . Thus, as it has been reported for other organisms (10), also in T. cruzi the Hsp70 cytoplasmic-nuclear translocation must be dependent on other factors among which a physiological or nutritional stress could play a fundamental role.

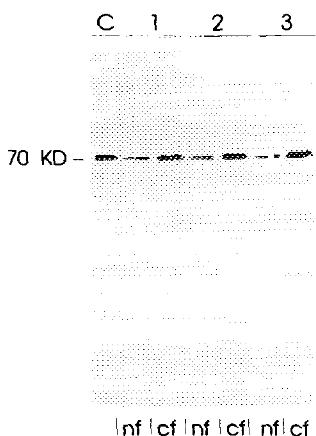

Figure 3. Localization of the Hsp70 protein in stationary phase cultures of T. cruzi. Lines nf and cf: nuclear and cytoplasmatic fraction, respectively. The proteins were subjected to 10% polyacrylamide electrophoresis and analyzed by Western blot using an anti $(GMPG)_6$ antibody. C (control): Hsp70 from parasites in the logarithmic phase of growth incubated at 28°C; Lane 1: Hsp70 from parasites in the stationary phase of growth incubated at 28°C; Lanes 2 and 3: Hsp70 from parasites in the stationary phase of growth incubated at 37°C for 12 and 24 hours, respectively.

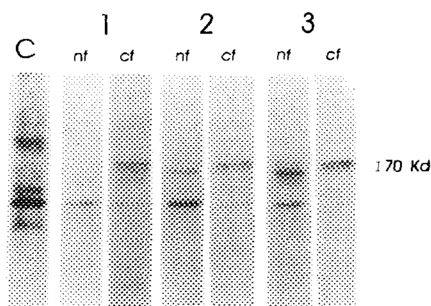

Figure 4. Immunoprecipitation of the Hsp70 protein from epimastigotes of T. cruzi in the logarithmic phase of growth using an anti $(GMPG)_6$ antibody. The proteins were labeled in vivo with ^{35}S methionine as indicated in material and methods. nf: nuclear fraction; cf: cytoplasmic fraction. Lane C: immunoprecipitation of the proteins synthesized at 28°C using a preimmune serum. Lane 1: labeled protein fractions from parasites incubated at 28°C. Lane 2: labeled protein fraction from parasites incubated at 28°C and heat shock treated for 2 hr at 37°C in the absence of label. Lane 3: labeled protein fractions from parasites incubated at 37°C for 12 hours. It was observed that the protein fraction of about 50Kd present in lane C immunoprecipitated with the preimmune serum was mainly detected in the nf fractions.

In order to determine whether the hsp70 protein translocated to the nucleus was that synthesized during the heat shock or that constitutively present in the cytoplasm we labeled the Hsp70 with ^{35}S methionine in parasites grown either at 28°C or 37°C for 12 hours and after been cultured for 1 hour in the absence of the labeling they were submitted to a heat shock at 37°C. For these experiments a 5-12% polyacrylamide gradient gel was used in an attempt to improve the resolution of the Hsp70 isoforms. Fig 4 (lane 1) shows that as expected in parasites cultured at 28°C the Hsp70 protein is present only in the cytoplasm but that after the heat shock treatment a fraction of the labeled protein synthesized at 28°C is translocated to the nuclear fraction (lane 2). Interestingly the most intensively labeled form of the nuclear Hsp70 has an electrophoretic mobility different from the most intensively labeled cytoplasmic form of the protein suggesting that either before its transport to the nucleus the protein has to be modified or that only one of the cytoplasmic isoforms migrates to the nucleus. Although our data can not discriminate between these two possibilities they favor the hyphotesis of a modification of the cytoplasmic Hsp70 before its translocation to the nucleus since the amount of the isoform of low molecular weight present in the nucleus at 37°C is significantly higher than the same form detected in the cytoplasm at 28°C. It should be noticed that the amount of parasites labeled in lane 1 and 2 is the same. This interpretation is reinforced by the fact that the nuclear isoform of low molecular weight in parasites cultured at 37°C was 80% of all the rest of the Hsp70 forms (lane 3) while this form is present in the cytoplasmic fraction in negligible amounts. In the cytoplasm most of the labelling accumulates in the isoform of the highest molecular weight. The higher

abundance of an specific isoform of the Hsp70 in parasites cultured at 37°C relative to the one detected in parasites cultured at 28°C has also been reported (16).

Since in drosophila cells following 30 min of the temperature elevation Hsp70 is visible in nuclei and in this period Hsp70 mRNAs have to be made de novo, transported to the cytoplasm and translated into proteins, the nuclear translocation of the protein has been considered as a extremely rapid phenomenon being of an emergency nature. We expected a similar or even faster behavior in parasites since large amounts of the protein already exist in the cytoplasm before the heat shock. Our data indicate that in T. cruzi the cytoplasmic-nuclear translocation of the Hsp70 is slow since the protein was visible only after h of the shock. Thus, either the nuclear presence of the Hsp70 in the nucleus is not of an emergency nature in parasites or the mechanism of translocation in not as efficient as in other higher eukaryotic cells. It should be pointed out that although incubation at 37°C is a truly heat shock for promastigotes cultured at 28°C it does not per se represents an stressful situation for these parasites since 5-10°C elevation in temperature is part of their life cycle, contrary to what it occurs in other eukaryotic cells. It should be noticed that probably in parasites the Hsp70 plays also other specific roles besides those described for the protein as chaperon. In fact, in Schistosoma high levels of Hsp70 expression have been observed coincidental with the transformation process of the larva form to adult (13) and in leishmania a shift in temperature from 26°C to 37°C induces differentiation from promastigotes to amastigotes (19) connected with acquisition of infective capability (20,21). The slow rate of translocation could also be connected with the need for the Hsp70 modification.

ACKNOWLEDGMENT

This research was supported by Plan Nacional I+D grant N° BIO90-0786.

REFERENCES

1. Lindquist, S., and Craig, E. A. (1988) A. Rev. Genet. 22, 631-677.
2. Craig, E. A. (1985) CRC Crit. Rev. Biochem. 18, 239-280.
3. Lindquist, S. (1986) A. Rev. Biochem. 55, 1151-1191.
4. Craig, E. A., and Gross, C. A. (1991) A. Trends Biochem. Sci. 16, 135-140.
5. Gething M-J, and Sambrook J. (1992) Nature 355, 33-44.
6. Beckmann, R. P., Miaaen, L. A., and Welch, W. J. (1990) Science 248, 850-854.
7. Chirico, W. J., Waters, M. G., and Blobel, G. (1988) Nature 322, 805-809.
8. Kang, P. J. et al. (1990) Nature 348, 137-143.
9. Sanchez, Y., and Lindquist, S. (1990) Science 248, 1112-1115.
10. Velazquez, J. M., Didomenico B. J., and Lindquist S. (1980) Cell 20, 679-689.
11. Laran, E., Requena, J. M., Jimenez-Ruiz, A., Lopez, M. C., and Alonso, C. (1990) Chromosoma 99, 315-320.
12. Van Der Ploeg, L.H.T., Giannini, S.H., Cantor, C. R. (1985) Science 228, 1443-1445.

13. Newport, G., Culpepper J., and Agabian N. (1988) Parasitol. Today 4, 306-312.
14. Shepherd, D., and Garland, P.B. (1973) Methods Enzymol. 13, 11-16.
15. Requena J. M., López M. C., Jimenez-Ruiz A., de la Torre J.C., and Alonso C. (1988) Nucleic Acids Res. 16, 1393-1406.
16. Requena J. M., Jimenez-Ruiz A., Soto M., Assiego R., Santarén J. F., López M. C., Patarroyo M. E., and Alonso C. (1992) Mol. Biochem. Parasitol. 53, 201-212.
17. Kessler, S. W. (1975) A. J. Immunol. 115, 1617-1624.
18. Velazquez J.M.,and lindquist, S. (1984) Cell 36, 655-662.
19. Hunter, K. W., Cook, C. L., and Hayunga, E. G. (1984) Biochem. and Biophy. Res. Commun. 125, 755-760.
20. Smejkal, R. M., Wolff, R., and Olenick, J. G. (1988) Exp. Parasitol. 65, 1-9.
21. Alcina, A., Urzainqui, A. and Carrasco, L. (1988) Eur. J. Biochem. 172, 121-127.