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Research brief

Entamoeba histolytica: expression and DNA binding of CCAAT/enhancer-binding proteins are regulated through the cell cycle

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Index Descriptors and Abbreviations: Entamoeba histolytica; cell cycle; CCAAT/enhancer-binding protein sites. CE, cytoplasmic extracts; C/EBP, CCAAT/enhancer-binding protein; DNA, deoxyribonucleic acid; EMSA, electrophoretic mobility shift assays; NE, nuclear extracts; PAGE, polyacrylamide gel electrophoresis.

Entamoeba histolytica is the protozoan responsible for 50 million cases of invasive amoebiasis and 40,000–100,000 deaths per year worldwide (WHO, 1997). This parasite has two phases in its life cycle, the cyst and the trophozoite. Cysts are involved in the infection and spread of the disease, whereas trophozoites invade tissues and are responsible for pathogenicity. The study of factors regulating gene expression of proteins controlling the pass of cyst to trophozoite and vice-versa, as well as cell cycle and growth in dividing trophozoite, is essential to understand the molecular bases of the infection. However, in general, little is known about gene expression regulation in this pathogen. Several *cis*-regulatory elements involved in transcription have been identified in *E. histolytica* genes promoters (Purdy et al., 1996; Singh and Rogers, 1998). Our group found and cloned the TATA-box-binding protein (EhTBP) (Luna-Arias et al., 1999). Additionally, the EhEBP1 and EhEBP2 transcription factors (Schaenman et al., 2001), and the URE3-binding protein (Gilchrist et al., 2001), which bind to the URE4 and URE3 elements of the *hgl5* gene promoter, respectively, have been cloned and characterized. Recently, we have reported that proteins homologous to the C/EBP transcription factor activate the *EhPgp1* gene promoter (Marchat et al., 2002). The gene encoding a protein showing similarity to one of the members of the C/EBP family was cloned from a genomic library of *E. histolytica*. Its molecular and functional characterization is currently in progress.

C/EBP isoforms belong to the family of leucines zipper DNA-binding proteins, which regulate proliferation, differentiation, and apoptosis in a cell-specific manner in eukaryotic cells (McKnight, 1992). Recent reports indicated that C/EBPs play prominent roles in cell cycle regulation in Mammals. In rat hepatocytes, C/EBPα expression and DNA-binding activity drop after G_1 phase (Rana et al., 1995). Moreover, the steroid-induced expression of C/EBPα is necessary to mediate the glucocorticoid G_1 cell cycle arrest in rat hepatoma cells (Ramos et al., 1996). C/EBPα increases the stability of p21, a cyclin-dependent kinase inhibitor (Timehenko et al., 1996), while C/EBPβ activates the expression of cyclin-E and cyclin-B, which form a complex with cyclin-dependent kinase and promote the cell cycle progress, during liver regeneration (Greenbaum et al., 1998). In serum-deprived mouse mammary epithelial cells, C/EBPδ participates in the induction of G_0 growth arrest and apoptosis (Gigliotti and DeWille, 1998; O'Rourke et al., 1997, 1999a,b).

As in other systems, different proteins are expressed during cell cycle in *E. histolytica*. A protein antigenically related to the oncogene *c-myc* is mainly expressed in G_1 and S phases (Leyva-Leyva et al., 1993). Expression of the cell cycle regulating *Eh cdc2* gene peaks at G_2 phase, whereas the signal transduction *Eh rho* gene shows a

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high level of expression between G_1 and S phases (Gangopadhyay et al., 1997). Recently, our group detected a higher expression of Ehp53, a p53-like transcription factor involved in cell cycle regulation, in G_1 and G_2 phases (our unpublished results). To gain insight into the factors participating in growth regulation in *E. histolytica*, here we investigated the expression of the C/EBP-like protein through the cell cycle using colchicine-synchronized and serum-starved trophozoites.

For the experiments presented here, we used trophozoites of the phagocytosis-deficient mutant L-6 clone (strain HM1: IMSS), which can be easily synchronized by colchicine (Orozco et al., 1983) and whose cell cycle phases lasting is well characterized (Orozco et al., 1988). Near-confluent trophozoites were axenically cultured in TYI-S-33 medium containing 10% fetal bovine serum (Diamond et al., 1978) and 200 μg/ml colchicine were added for 24 h. After washing out the drug, cells were resuspended in fresh complete medium and harvested at 0, 3, 8, and 14 h corresponding to M, G_1, S, and G_2 phases, respectively (Orozco et al., 1988). In some experiments, the medium was supplemented with 5 μCi/ml [^{3}H]thymidine; at 4 h intervals, cells were counted and the amount of radioactivity incorporated in DNA (cpm) was determined as previously reported (Orozco et al., 1988). Our results showed that the trophozoites of L-6 clone were synchronized by colchicine, exhibiting at least two cell cycles, which duration was approximately 12 h. The drops in cpm registered at 20 and 32 h, corresponded to a doubling in the cell population, indicating that cells had passed through mitosis (Fig. 1). Serum-deprived trophozoites (SD), which have been described to be in G_0 phase, were obtained by switching near-confluent cultures from complete medium to serum-free medium for 16 h at 37 °C (Vohra et al., 1988). All experiments presented in this paper were carried out by triplicate. Results shown here were highly reproducible.

We separated the nuclei from the rest of the cells to prepare nuclear (NE) and cytoplasmic (CE) extracts (Schreiber et al., 1989) from trophozoites arrested in different cell cycle phases. Through the phase contrast microscope, we checked that the nuclear fraction showed intact nuclei, whereas the cytoplasmic fraction did not present contamination with intact or disrupted nuclei. Proteins (30 μg) were then analyzed by 12% SDS–PAGE (Laemmli, 1976). Densitometrical analyses of Coomassie blue stained gels using Quantity One software (Bio-Rad) showed that similar contents of proteins were loaded in all lanes (Figs. 2a and b). As expected, we observed differences in bands patterns of NE and CE from not synchronized (NS) trophozoites. For example, bands of about 130 and 62 kDa were seen in CE from NS trophozoites, but not in NE (Figs. 2a and b, arrowheads), whereas in NE appeared a doublet of about 26 kDa that was not present in CE (Figs. 2a and b, arrowhead). We also detected differences in NE and CE obtained from trophozoites synchronized in the different cell cycle phases. For example, a band of about 75 kDa was mainly detected in NE from trophozoites arrested in M phase, whereas it was lower in the other phases (Fig. 2a, arrow). In CE, a band of about 70 kDa was present in trophozoites blocked in M, G_1, S, and G_2 phases, but it did not appear in SD trophozoites (Fig. 2b, arrow).

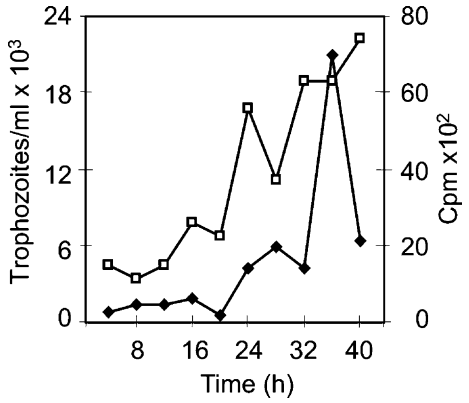

Fig. 1. [^{3}H]Thymidine incorporation in synchronized culture of L-6 clone. Trophozoites were grown in TYI-S-33 medium containing 200 μg/ml colchicine for 24 h at 37 °C. After washing out the drug, cells were transferred to fresh medium. At 4 h intervals, [^{3}H]thymidine incorporation (◆) (cpm) and cells number (□) were determined.

To detect proteins antigenically related to C/EBP, we performed Western blot assays using the anti-human C/EBPβ antibody (Santa Cruz Biotechnology). This antibody recognized two bands of about 65 and 25 kDa in NE and CE obtained from NS trophozoites of clone L-6 (Figs. 2c and d). These results agree with those previously reported for trophozoites of clones A and C2 (Marchat et al., 2002). By Western blot and UV cross linking assays, we demonstrated that these two polypeptides were recognized by anti-C/EBP antibodies and were able to bind to C/EBP DNA sequences. Our results also suggested that the 25 kDa C/EBP-like factor was one of the components of the 65 kDa complex, which disassociation was favored by urea. Here, we could detect the 25 kDa band in the absence of urea, demonstrating that it can also exist as an independent protein in the cell (Marchat et al., 2002). The 25 kDa band was faint in NE and CE from NS trophozoites, but it appeared as a clear band in NE and CE from SD trophozoites and trophozoites arrested in M and G_1 phases. Through the cell cycle phases, both bands presented variations in NE and CE. In NE, the 65 kDa band appeared stronger in M phase, whereas it was hardly detected in S phase. The 25 kDa band expression peaked in G_1 phase, whereas it was very low in the other phases. It also appeared strong in SD trophozoites extracts (Fig. 2c). Interestingly, the 65 kDa band expression did not vary significantly during the cell cycle progression in CE,

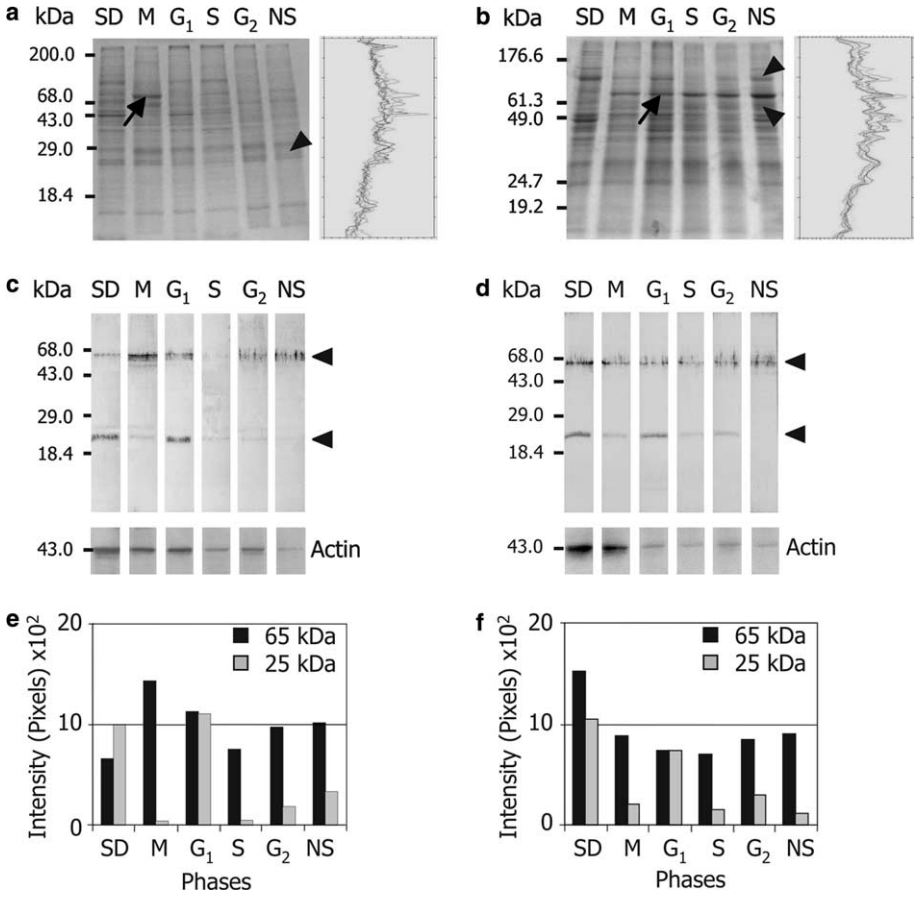

Fig. 2. Expression of nuclear and cytoplasmic proteins recognized by the anti-human C/EBPβ antibody in trophozoites of clone L-6 arrested in the different cell phases. (a) and (b) Coomassie blue stained gels (left). NE (a) or CE (b) (30 μg) were obtained from trophozoites arrested in different cell cycle phases, and separated by 12% SDS–PAGE. SD, serum-deprived trophozoites; NS, not synchronized trophozoites; M, G_1, S, and G_2, cell cycle phases. Densitometrical analyzes (Quantity One Software, Bio-Rad) are shown at the right. Arrows show the 75 and 70 kDa bands; Arrowheads show the 130, 62, and 26 kDa bands. (c) and (d) Western blot assays of gels in (a) and (b). Nitrocellulose membranes were probed with the rabbit polyclonal antibody against the human C/EBPβ (2 μg/ml) (top) or a mouse monoclonal antibody against actin (1:2000 dilution) (bottom). Goat anti-rabbit or anti-mouse peroxydase-conjugated antibodies (1:2000 dilution) were used as secondary antibodies. Blots were developed by 4-chloronaphthol. Molecular weights of standard proteins are at the left. Arrowheads show the polypeptides recognized by the anti-C/EBP antibody. (e) and (f) Densitometry of blots obtained in pixels from Western blot assays shown in (c) and (d). Analyses were done using the Quantity One software (Bio-Rad). All assays were performed in triplicate. Results shown here correspond to a representative experiment.

but it was stronger in SD trophozoites (Fig. 2d). As in NE, the 25 kDa band appeared stronger in SD trophozoites and G_1 phase arrested cells (Fig. 2d). Blots were submitted to densitometrical analysis. Bands intensity, expressed in pixels, confirmed the differences described above (Figs. 2e and f). The anti-actin monoclonal antibody was used as an internal control in the same experiments, to confirm the integrity of protein preparations. It revealed the presence of a doublet of about 43 kDa in NE and CE from NS and synchronized trophozoites, probably corresponding to actin isoforms. Although densitometrical assays demonstrated that the same amount of proteins was loaded in each lane, actin expression also varied during the cell cycle progression in NE and CE, but without correlation with the 25 and 65 kDa bands (Figs. 2c and d), indicating that variation in bands intensity is not related to the amount of proteins used. Moreover, multiple differences and very few similarities were found in blots detected in NE and CE, supporting the absence of cross contamination between both extracts. The differential expression of actin through the cell cycle of *E. histolytica* has been previously detected by us in total extracts (our unpublished data). Moreover, the presence of nuclear actin in this parasite is in agreement with the findings reported by several authors for other organism (Skubatz et al., 2000; Soyer-Gobillar et al., 1996).

To study the ability of the C/EBP-like proteins to bind DNA through the cell cycle, we carried out EMSA using the C/EBPIII oligonucleotide with the sequence of the consensus C/EBP site located at −98 bp in the *EhPgp1* gene promoter (Fig. 3a) (Marchat et al., 2002), and NE and CE from trophozoites arrested in the different cell cycle phases. NE from NS trophozoites of clone L-6 formed a strong specific DNA–protein complex with the C/EBPIII oligonucleotide. The complex

was competed by a 150-fold molar excess of the unlabeled C/EBPIII probe, but not by the unspecific polydIdC competitor (Fig. 3b). These results agree with those previously obtained for NE of clones A and C2 trophozoites (Marchat et al., 2002). The same specific DNA–protein complex was also formed with CE, but its intensity was about 10% of the intensity of the complex formed with NE in the same experimental conditions (Fig. 3c). The binding of proteins to the C/EBPIII oligonucleotide presented variations during the cell cycle progression. Even when we detected a very low amount of proteins antigenically related to C/EBP in NE from trophozoites arrested in S phase, the DNA–protein complex formed with the C/EBPIII oligonucleotide appeared stronger in S phase, than in the other phases. On the other hand, the complex was low in M phase, where a strong 65 kDa band was observed in Western blot assays (Fig. 3d). In general, the binding to C/EBPIII oligonucleotide was lower with CE than with NE. The complex with major intensity was observed in G_2 phase, whereas it was lower in the other phases. Interestingly, no DNA–protein complex was seen when we used CE from SD trophozoites, whereas a high amount of cytoplasmic proteins antigenically related to C/EBP were detected in this phase (Fig. 3e). The densitometrical analysis of the DNA–proteins complexes confirmed these differences (Fig. 3f). In each phase, the specificity of the DNA–protein complex was checked by specific and unspecific competition assays (data not shown) as it is shown in Figs. 3b and 3c for NS trophozoites extracts. Summary of the quantitative data obtained from Western Blot and EMSA experiments is presented in Table 1.

We showed here that, as other transcription factors (Gilchrist et al., 2001), proteins antigenically related to C/EBP are present in nuclear and cytoplasmic fractions of the clone L-6 trophozoites. The expression of proteins homologous to C/EBP varied through the cell cycle of *E. histolytica*. In NE, the bands of 65 and 25 kDa were overexpressed in M and G_1 phases, whereas in CE, both bands were mainly detected in SD trophozoites. Peaks of expression of the 65 and 25 kDa bands did not appear in the same phase of cell cycle in NE and CE. Since the 25 kDa polypeptide is considered as a component of the 65 kDa complex (Marchat et al., 2002), the strong 65 kDa band observed in NE from trophozoites arrested in M phase, could be due to the higher expression of other proteins forming the 65 kDa complex, rather than the amount of the 25 kDa polypeptide itself. In general, as expected for a transcription factor, cytoplasmic C/EBP-like proteins were not able to bind efficiently to the C/EBPIII oligonucleotide, probably because they require modifications to assure an efficient DNA binding. In mammary cells, it has been reported that post-translational control by phosphorylation influences the subcellular localization and DNA-binding activity of C/EBP isoforms (Mahoney et al., 1992; Metz and Ziff, 1991; O'Rourke et al., 1997; Ray and Ray, 1994). Contamination with NE during the preparation of CE can be discarded, since the proteins patterns and the DNA binding obtained with NE and CE differ in all cell cycle phases. Moreover, patterns were highly reproducible in all experiments performed. In NE, the DNA–proteins complex formed with the C/EBPIII oligonucleotide was stronger in S phase. This suggested that C/EBP-like proteins accumulating in the nucleus

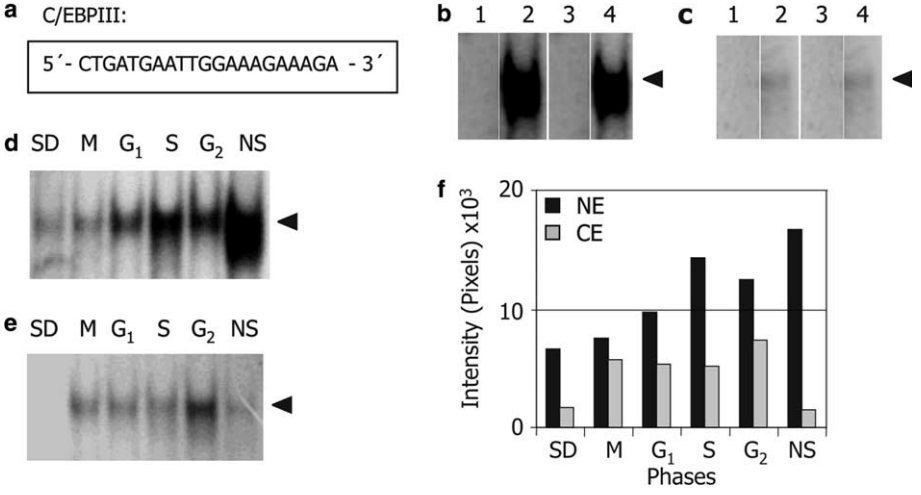

Fig. 3. Protein binding to C/EBP sequence in trophozoites of clone L-6 arrested in the different cell cycle phases. (a) C/EBPIII oligonucleotide sequence. (b) and (c) C/EBP-binding activity. EMSA were performed using 20 μg of NE (b) and CE (c) from NS trophozoites, and 10 000 cpm of the [γ-^{32}P]ATP C/EBPIII probe. Lane 1, free probe; lane 2, no competitor; lane 3, 150-fold molar excess of unlabeled C/EBPIII oligonucleotide; and lane 4, 150-fold molar excess of unlabeled polydIdC. Arrowheads show the specific DNA–proteins complexes. (d) and (e) C/EBP-binding activity through the cell cycle. EMSA were performed as in (b) and (c), using NE (d) and CE (e) from SD and NS trophozoites, and trophozoites arrested in M, G_1, S, and G_2 cell cycle phases. Arrowheads show the specific DNA–proteins complexes. (f) Densitometry of DNA–proteins complexes analyzed using the Quantity One software (Bio-Rad). All assays were performed by triplicate. Results shown here correspond to a representative experiment.

Table 1
Quantitative detection of C/EBP-like proteins and its DNA-binding activity through the cell cycle

	NE					CE				
Phase:	SD	M	G_1	S	G_2	SD	M	G_1	S	G_2
65 kDa (WB)	46	**100**	78	53	68	**100**	58	48	45	55
25 kDa (WB)	90	3	**100**	4	16	**100**	20	70	13	28
DNA-binding (EMSA)	46	52	68	**100**	87	26	92	86	82	**100**

Numbers (expressed in percentage) correspond to the normalization of densitometrical values. 100% values (bold) were arbitrary assigned to major values obtained for each horizontal line in the densitometric analyses. WB, Western blot assays; EMSA, electromobility shift assays.

during M and G_1 phases are unable to bind DNA efficiently. This suggests again that C/EBP-like proteins require some maturation processes, such as phosphorylation, as reported for C/EBPδ during the hepatic acute phase response (Lacorte et al., 1997). Moreover, C/EBP isoforms can bind DNA as homo- or heterodimer, with another C/EBP family member or another leucine zipper transcription factor. Even high levels of nuclear C/EBP may be ineffective to bind C/EBPIII oligonucleotide during M and G1 phases if the putative appropriate dimerization partner is absent or inactive in the synchronized cells. Then, changes in DNA-binding activity through cell cycle could also be due to changes in the abundance of associated proteins. Finally, increased C/EBP-binding activity in S phase could suggest that the C/EBP-like protein could be one of the factors, which regulate the expression of genes involved in replication and DNA synthesis in *E. histolytica*.

In summary, we showed that the trophozoites of *E. histolytica* have nuclear and cytoplasmic proteins antigenically related to the human C/EBPβ. These proteins exhibited differential expression pattern and DNA-binding activity during cell cycle progression (Table 1), which suggests that they could be participating in cell cycle regulation. The study of such factors involved in the control of cell cycle and growth in dividing trophozoites will help us to understand the molecular bases of the differentiation processes in *E. histolytica*.

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