Epigenetic silencing of multiple genes in Entamoeba histolytica

Department of
Biological Chemistry

Prof. David Mirelman

Rivka Bracha, Yael Nuchamowitz, Maya Huguenin and Minerva Barrios-Ceballos

☎ 972 8 934 4511
FAX 972 8 934 4112
@ david.mirelman@weizmann.ac.il
www.weizmann.ac.il/Biological_Chemistry/scientist/Mirelman/david_mirelman.html

The human intestinal protozoan parasite *Entamoeba histolytica* possess a number of virulence factors such as a Gal-specific lectin, various cysteine proteinases and a family of small (77 a.a) ampiphatic pore-forming peptides, termed amoebapores. The concerted action of these virulence factors causes intestinal inflammation, kills the mucosal cells and enables the invasion of the parasite. Modulation of the expression of those genes affords a better understanding of their role in the pathogenesis of the amoeba. In earlier work we found that transfection of parasites with a plasmid containing a segment (473 bp) of the 5′ upstream sequences of the amoebapore A gene (*Ehap-a*), surprisingly caused a complete transcriptional silencing of *Ehap-a*. Removal of the plasmid resulted in a plasmid-less clone (termed G3) which retained the silenced phenotype. Sequence analysis of the 473 bp 5′ upstream region of the *Ehap-a* gene revealed the presence of a truncated (140 bp) segment of a neighboring short, interspersed repetitive element (SINE1) which is actively transcribed from the opposite strand, as evidenced by the transcription of a CAT reporter gene. Silencing occurred only if a truncated segment of the SINE element was included in the plasmid. Both, total elimination or the inclusion of the complete SINE sequences prevents the silencing of the *Ehap-a* gene. This prompted us to attempt to silence the expression of additional genes of choice by cloning them under the same *Ehap-a* 5′ upstream region in the G3 amoeba. Genes such as the Gal-lectin (*EhLgl1*), cysteine proteinase 5 (*EhCP-5*) and a Lim-like protein (*EhLim*), were completely silenced resulting in trophozoites that do not express two genes. Moreover, transfection of G3 trophozoites with a plasmid that carries two genes, each under the regulation of the 5′ region of *Ehap-a*, resulted in a triple silenced amoeba in which the expression of three important virulence factors, the AP-A, LGL1 and CP5 was completely suppressed (Fig 1).

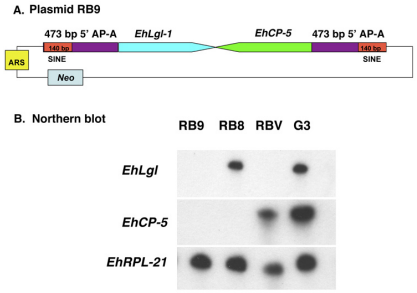

Fig. 1 *A. Plasmid RB9 containing two genes, EhLgl1 and EhCP-5 regulated by the 5′ upstream region (473 bp) of the Ehap-a gene . Transfection was into the plasmid-less G3 trophozoites which were already silenced in the Ehap-a gene. B. Northern blots . RB8 was silenced in the EhCP-5 and RBV was silenced in EhLgl1and RB9 was simultaneously silenced in both genes.*

Understanding the molecular mechanism of this gene silencing phenomenon is of great importance. So far we have established that DNA methylation is not involved in the process both by comparing the bisulfite sequencing of the *Ehap-a* gene and by 5′ Aza Cytidine treatment that failed to reverse the silencing. Nuclear RNA extracts of *Ehap-a* silenced trophozoites were found to contain small amounts of non-productive, single stranded RNA molecules of approximately 140 n with homology to the 5′ end of the SINE1 element, and although neither Dicer nor Drosha genes were identified in the amoeba genome, it is quite possible that these ssRNA strands may play a role in silencing. Some proof for the involvement of heterochromatin in maintaining the silenced state was shown by the digestion of trophozoite nuclei with *Micrococcal* nuclease (MNase). We found that the transcription initiation region of the *Ehap-a* gene in G3 amoeba was more resistant to MNase digestion than that of the parent strain HM-1 (Fig 2).

In addition, a loss of methylation at Lysine 4 of Histone H3 was detected by ChIP analysis in the chromatin domain of the *Ehap-a* gene of G3 silenced trophozoites, indicating a transcription inactivated domain. The process of silencing of the *Ehap-a* gene also caused the silencing of other members of the pore-forming family of genes which resides on the same evolutionary branch (*Ehap-b* and SAPLIP1). A similar observation was found with the family of *EhLgl* genes. When *EhLgl1* was silenced, two other members, *EhLgl2* and *EhLgl3* were down-regulated as well, while *ELgl4* and *EhLgl5* were up-regulated. In contrast, when *EhLgl5* was silenced *EhLgl4* was also down-regulated while the first three continued to be expressed. This ability to silence part of the genes belonging to a same family can serve as a tool to study the relationships and functions of the members of other gene families.

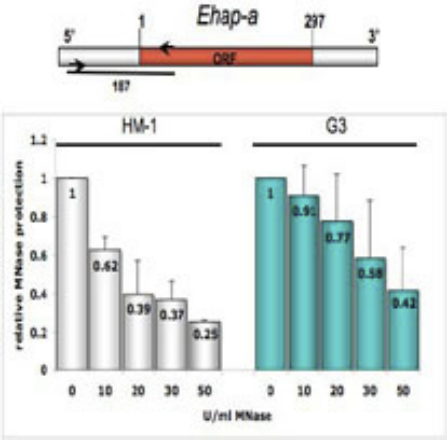

Fig. 2 *The MNase accessibility of the Ehap-a promoter region in the HM-1 and G3 amoeba strains was determined by digesting chromatin with the indicated amount of MNase and quantifying the undigested DNA by qPCR. The relative resistance of the target sequence was determined by normalizing with a control gene that is similarly expressed in both strains. The average of 3 independent experiments are shown with S.D.*

Preliminary experiments comparing the expression profiles of the parent strain HM-1 versus the G3 amoeba on microarray chips revealed that numerous additional genes such as a

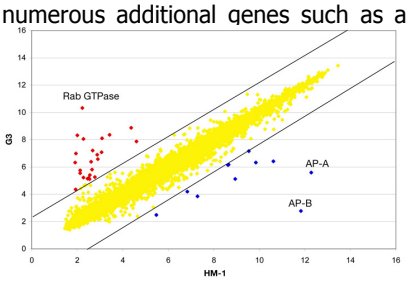

Fig 3. Expression fold comparison between the parent strain HM-1 and the Ehap-a silenced G3 trophozoites performed on the Affimetrix E. histolytica microarrays in collaboration with Dr. C. Gilchrist, U.VA. Only spots with a pValue <0.05 were evaluated and the figure shows only genes which had a > 7 fold difference. Red dots- genes overexpressed in G3.

RabGTPase, are highly over-expressed in G3 trophozoites (Fig 3).

The G3, amoebapore-silenced trophozoites were previously found to be virulence-attenuated. The new double and triple gene silenced trophozoites have additional virulence deficiencies such as the inability of *EhLgl1* silenced amoeba (RBV) to cluster antibodies bound to the cell surface lectin molecules to the uroid region of the amoeba or of *EhCP-5* silenced amoeba (RB8) to digest mucus and sIgA. Our novel method to

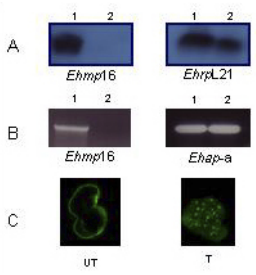

Fig. 4 . The metalloprotease Ehmp16-inverzincin gene of E. histolytica. A. Northern Blot analysis on E. histolytica HM-1 (1) and E. dispar (2). B. RT-PCR analysis on cDNA from E. histolytica HM-1 (1) and E. dispar (2); C. Immunolocalization of the mp16 in detergent treated and untreated trophozoites of E. histolytica.

simultaneously silence additional genes may be useful for the generation of virulence–attenuated trophozoites that could have the potential to serve as a live vaccine.

Another family of virulence genes which we are investigating are the metallo-proteases. One of these genes, coding for an *Ehmp16* (inverzincin), is not expressed in the non-pathogenic *Entamoeba dispar.* Antibodies prepared against the recombinant *Ehmp16* revealed that it is membrane bound (Fig 4). We are trying to suppress its expression so as to determine its role in the pathogenicity of the parasite.

Selected publications (since 2006)

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