Amoebapore is an important virulence factor of *Entamoeba histolytica*

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We have previously demonstrated that inhibition of expression of amoebapore A (AP-A) by antisense RNA caused a marked decrease in the virulence of the parasite. A four-fold over-expression of AP-A was obtained with plasmid (pA7) which has the *ap-a* gene under the control of gene *EhgLE-3-RP-L21*. The virulence of the transfected trophozoites, however, was also decreased. Excess of AP-A protein was found in the cytosol and a significant amount was released into the surrounding media. Transfection of the parasite with a plasmid (psAP-1) in which the *ap-a* gene was introduced with its own regulatory sequences, caused a total suppression of the transcription and translation of both the genomic and episomal *ap-a* genes. The silenced transfectant was not virulent at all. These results demonstrate that important factors need to be expressed at the correct cellular location and that the parasite has additional internal control mechanisms such as transcriptional gene silencing which can prevent excess amounts of gene expression.

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1. Introduction

The major pathogenic function and the most prominent property of *Entamoeba histolytica* is its remarkable cytolytic capacity. Twenty years ago a protein, termed amoebapore which is capable of forming ion channels or pores in lipid membranes and of depolarizing target cells, was discovered in *E. histolytica* (Lynch *et al* 1982; Rosenberg *et al* 1989; Young *et al* 1982). This protein was then extensively investigated and three isoforms of the amoebapore peptides (A, B, C in ratios of 21 : 9 : 1) were isolated and biochemically characterized (Leippe 1991, 1997; Leippe *et al* 1991, 1992, 1994a,b; Nickel *et al* 1999). The structure of the 77-residue peptides was resolved by sequencing and confirmed by molecular cloning of the genes (Leippe *et al* 1992, 1994a,b). More recently, amoebapores were reported to have an interesting structural and functional similarity to other effector cell polypeptides such as NK-lysin and granulysin that are present in natural killer (NK) cells and cytotoxic T cells of pigs (Andersson *et al* 1995; Leippe 1995a, 1997; Pena *et al* 1997; Stenger *et al* 1998). The amoebapores belong to a large superfamily of similar proteins (Zhai and Saier 2000; Bruhn and Leippe 2001).

In *E. histolytica* amoebapores exist as mature and potentially active peptides inside cytoplasmic granules of the trophozoite (Berninghausen and Leippe 1997; Leippe *et al* 1994a,b). The hypothesis on the mode of action of the amoebapore in viable *E. histolytica* trophozoites was that following the lectin-mediated recognition and intimate adherence between the amoeba and its target cell, the amoebapore molecules are inserted into the membrane without depending on the interaction with a specific membrane receptor. One of the problems with this hypothesis was that there was no possibility to prove it in intact trophozoites. Antibodies raised against amoebapore did not inhibit the killing of cells by trophozoites because the amoebapores are not exposed on the amoeba surface. Thus to investigate the role of amoebapore *in vivo*, methods to modulate the internal concentration of amoebapore A (AP-A) in the cell were developed.

Keywords. Amoebapore; *Entamoeba histolytica*; virulence factor

2. Inhibition of amoebapore gene expression by antisense RNA

The stable transfection of eukaryotic cells with plasmid constructs encoding antisense RNA has been extensively used as an efficient tool for down regulation or functional inhibition of expression of genes of interest (Scherczinger *et al* 1992). This same approach was taken to down regulate the expression of AP-A in *E. histolytica*. Although transfections and exogenous gene expression in *Entamoeba* were introduced already several years ago (Hamann *et al* 1995; Vines *et al* 1995), no success was initially obtained with antisense RNA. It turned out that in order to achieve accumulation of high levels of antisense RNA, the *ap-a* gene in its reverse orientation has to be placed in the plasmid under the controlling elements of the g34 copy of the ribosomal protein L21 gene (*EhRP-L21g34*) (Bracha *et al* 2000). A unique property had been found for this g34 gene copy; in contrast to the almost identical second gene copy gLE3, g34 was transcribed but not translated (Moshitch-Moshkovitch *et al* 1997; Petter *et al* 1994). In addition, in order to cause a significant inhibition of gene expression, the sequence of the *ap-a* gene in the antisense pAP-R2 plasmid (figure 1A) has to include also its 3′ UTR sequences. The transfectant produced with plasmid pAP-R2 had high levels of steady state antisense *ap-a* transcript, lower amounts of sense *ap-a* transcript (figure 1B) as well as significantly lower amounts of AP-A protein than the non transfected wild type (figure 2A) (Branch *et al* 1999). The level of AP-A synthesis inhibition was dependent on the concentration of the selective drug (neomycin derivative G418) which the cultures were subjected to. At 100 μg/ml G418, AP-A synthesis was inhibited approximately 60% but at 50 μg/ml it only reached about 48%. The reduction of AP-A content of the transfectants had a direct effect on amoebic virulence. Virulence of the highly AP-A-inhibited transfected trophozoites was found to be significantly diminished as determined both *in vitro* and *in vivo* (figure 2B,C, table 1). The less inhibited transfectant was more virulent (not shown) suggesting that there is a threshold level of AP-A content below which virulence is impaired. As expected lysates of pAP-R2 transfected trophozoites were found to be significantly less active in pore formation in the artificial membrane bilayer system (Bracha *et al* 1999; Leippe 1991; Leippe *et al* 1992). In addition

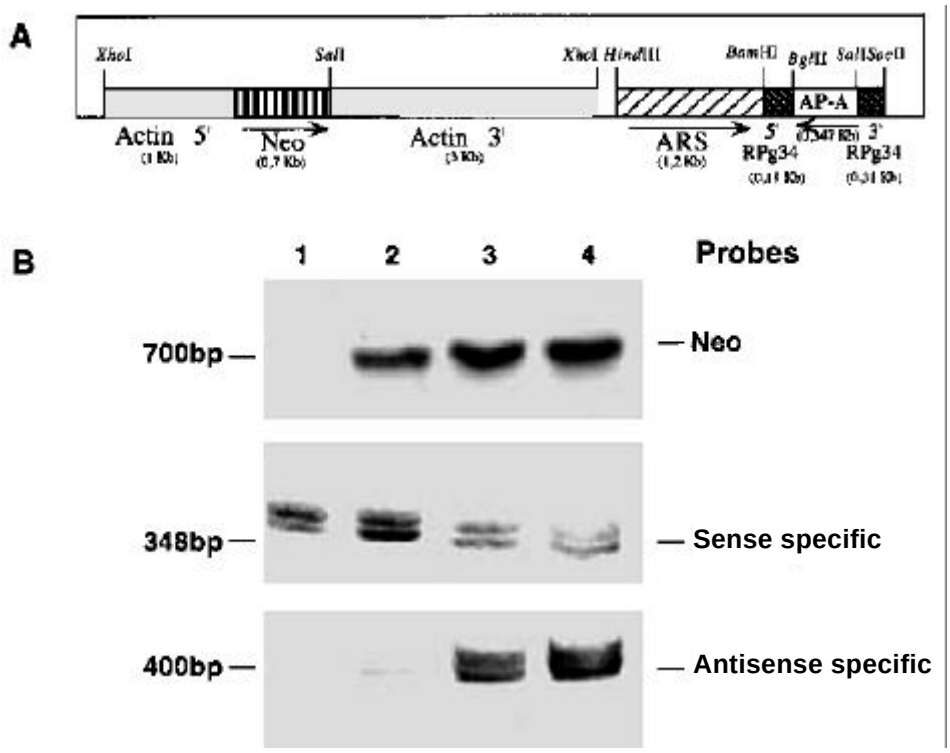

Figure 1. (**A**) Hybrid plasmid pAP-R2, used for antisense inhibition of amoebapore A gene expression. (**B**) Northern blots. Lane 1, non transfected trophozoites; lanes 2–4, transfected with pAP-R2 grown with 6 μg, 50 μg and 100 μg/ml G418 respectively. Hybridizations were done with sense and antisense specific probes for the *ap-a* gene (Bracha *et al* 1999).

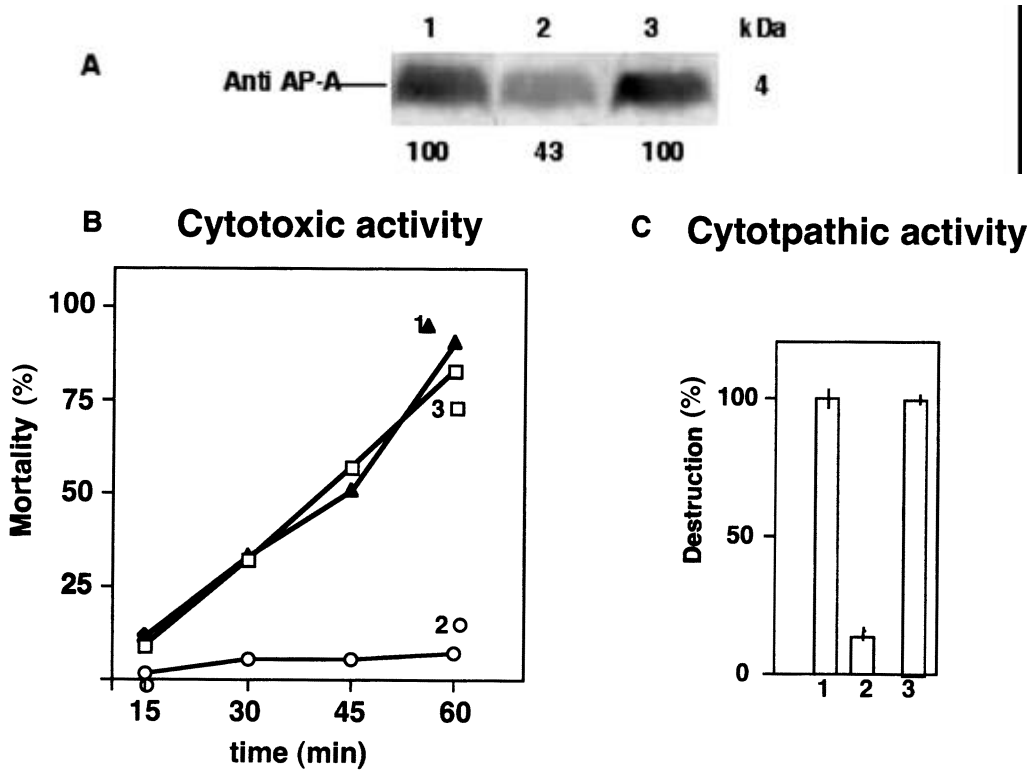

Figure 2. Inhibition of virulence in antisense transfectant pAP-R2 as a function of amoebapore A content. (**A**) Western blots of amoebapore A in gels containing the same amounts of trophozoite protein. Lane 1, non transfected trophozoites of strain HM-1 : IMSS; lane 2, transfectant pAP-R2 grown at 100 μg/ml G418; and lane 3, non related transfectant control grown at 100 μg/ml G418. (**B**) Cytotoxic activity as determined by incubating trophozoites together with suspended BHK cultured cells. Mortality was determined by counting trypan blue positive cell samples at different times of incubation at 37°C. Amoeba samples (1), (2), (3) as above. Cytopathic activity was determined as the destruction of tissue cultured monolayers of BHK cells after incubation with 2×10^5 trophozoites/well for 1 h.

Table 1. Hamster liver abscess formation by different transfectants.

Type of trophozoites	Trophozoites injected/liver ($\times 10^5$)	Animals with abscess	Abscess size (mm)
Non-transfected HM-1 : IMSS	2·5 5·0	(4/4) (4/4)	20–30 30–35
Sham transfected 100 μg/ml G418	5	(4/4)	30–35
Transfected pAP-R2 100 μg/ml G418	2·5 5·0	(0/4) 2/2	None 5–10
Transfected pA-7 6 μg/ml G418	2·5 5·0	(2/2) (4/4)	25–35 35
Transfected pA-7 100 μg/ml G418	5·0	(0/4)	None
Transfected psAP-1 6 μg/ml G418	5·0 10·0	(0/4) (0/2)	None None

to its role in killing mammalian target cells, the amoebapore has been shown to possess bacteriolytic activity (Leippe *et al* 1994a). Lysates of the AP-A-inhibited amoebae had a much poorer capacity to inhibit the growth of *Escherichia coli* bacteria in comparison to the non transfected lysates (Bracha *et al* 1999).

Our results with the pAP-R2 trophozoites, which were inhibited in their amoebapore A expression by antisense RNA clearly demonstrated for the first time the important role of the amoebapore in the parasite's pathogenesis.

3. Overexpression of amoebapore A in transfectants of *E. histolytica*

The above mentioned observation that down regulation of AP-A synthesis correlated with reduced virulence raised the question of what would be the effect of up regulation of the AP-A content in a trophozoite. Overexpression was achieved by transfecting a plasmid in which the

amoebapore gene was placed under the regulatory elements of the *EhRP-L21 gLE3* gene copy (figure 3I-A). The regulatory elements of this gene have been previously shown to drive the expression of exogenous genes in *E. histolytica* (Moshitch-Moshkovitch *et al* 1997). Trophozoites transfected with this plasmid had higher steady state levels of *ap-a* transcripts than the non transfected controls, and the increase of the transcript correlated well with the concentration of G418, used in the cultures (figure 3I-B). The high levels of *ap-a* transcript originate from two sources: from the chromosomal gene and from the plasmid *ap-a* gene where it is flanked by the regulatory sequences of the *EhRP-L21gLE3* gene copy. Differentiation of these two transcripts by RT-PCR revealed that upon increasing the concentration of G418, the amount of transcript originating from the chromosomal *ap-a* gene remained constant, whereas the transcript originating from the plasmid increased as expected (figure 4). In the non transfected trophozoites the only *ap-a* transcript is from the chromosomal gene. Expression of AP-A protein also correlated with G418 concentration of the cultures. At low G418 concentrations of 6 μg/ml, amoebapore expression was about 200% and at 100 μg/ml it reached around 400% (figure 3I-C). This overexpression could be nicely seen also by *in situ* fluorescent immunostaining of AP-A (figure 5). Confocal laser microscope observations revealed some differences in the intracellular localization of AP-A. As previously reported in *E. histolytica* trophozoites of strain HM-1 : IMSS (Leippe *et al* 1994a), and as also shown in

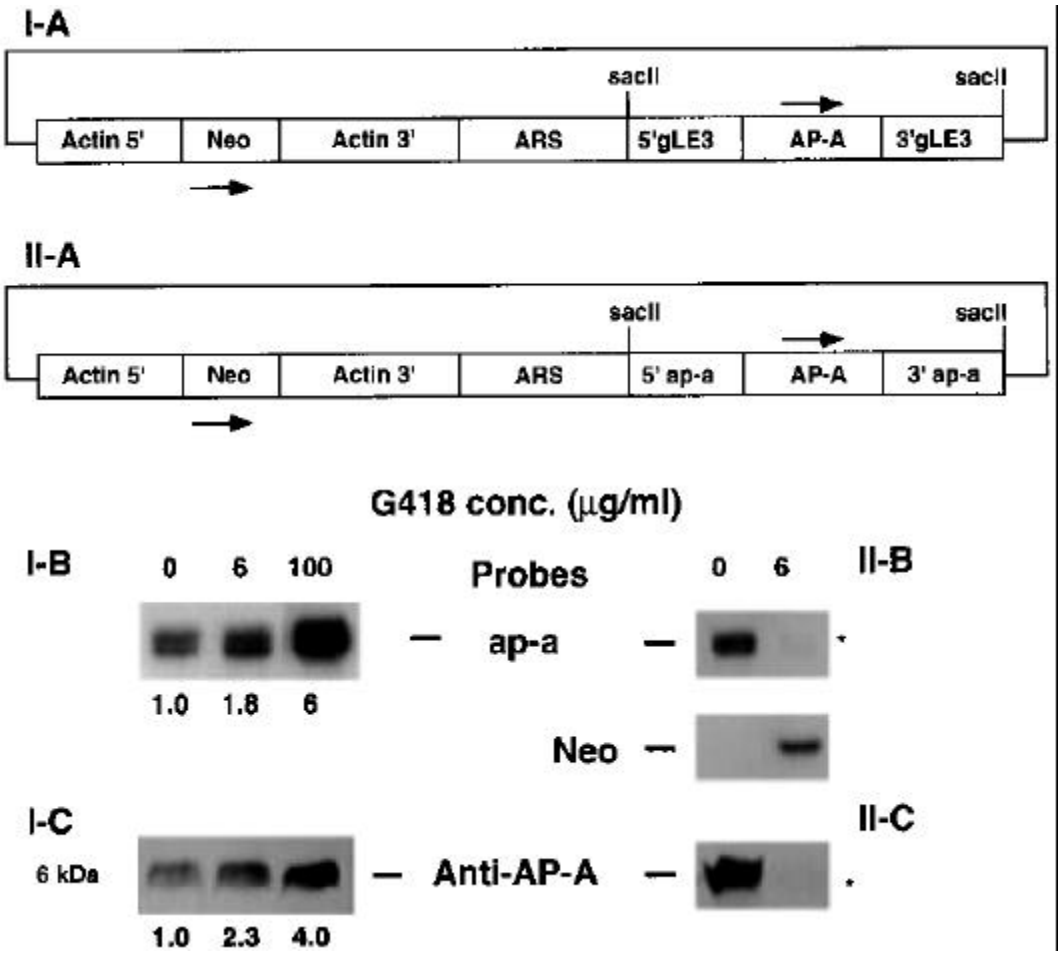

Figure 3. (**I-A**) Hybrid plasmid pA7, used for overexpression of *ap-a* gene. (**I-B**) Northern blot of parent non transfected trophozoites, pA7 transfected and grown in 6 μg/ml G418, and 100 μg/ml respectively. (**I-C**) Western blot of the above mentioned cultures reacted with anti AP-A antibodies. (**II-A**) Hybrid plasmid psAP-1 which causes silencing of *ap-a* gene. (**II-B**) Northern blot of non transfected and psAP-1 transfected trophozoites grown with 6 μg/ml G418. (**II-C**) Western blot of the above cultures. *, points out the absence of *ap-a* transcript and AP-A protein in the psAP-1 transfectant. The presence of plasmid in psAP-1 transfectants is shown by the transcript of *Neo*.

this study for the antisense transfectant, pAP-R2, all the AP-A protein is concentrated in well-defined granules whereas in the high AP-A overproducer, some of the protein was found to overflow into the cytosol. Moreover, we also found that trophozoites of the high amoebapore producer pA7-100, in contrast to those of the parent strain HM-1 : IMSS or pA7-6 trophozoites, released considerable amounts of amoebapore molecules into the surrounding culture medium (figure 6). The mechanism of this release or leakage is not yet understood, but it was certainly not due to lysis of trophozoites since it was not accompanied with any significant decrease in the number of viable trophozoites or a release of other cytoplasmic enzymes such as $NADP^+$-dependent alcohol dehydrogenase (Leippe 1995b). Virulence of the transfectants was determined by *in vitro* and *in vivo* assays. Transfectants grown in the presence of 6 μg/ ml G418 had slightly lower cytopathic and cytotoxic activity than those of the control non-transfected parent strain (figure 7A,B). On the other hand, trophozoites grown in the presence of 100 μg/ml G418 had very poor or almost no virulence in these tests. In addition, trophozoites of transfectant pA7-6 as well as non transfected controls induced the formation of large liver abscesses already at $2{\cdot}5 \times 10^5$ trophozoites/liver whereas, pA7-100 transfected trophozoites did not induce the formation of liver abscesses even at inoculations of 5×10^5 trophozoites/liver (table 1).

Bacteria which interact with amoebic trophozoites are known to become susceptible to solubilization by dilute detergent (Triton X-100, 0·2%) (Bracha and Mirelman 1984), whereas unexposed control bacteria are resistant to such treatment. Following the interaction of [^{14}C]-glucose labelled *E. coli* cells with the different trophozoites (Bracha and Mirelman 1984; Bracha *et al* 1982), it was found that the number of bacteria which became associated was very similar. The bacteria that attached to trophozoites of transfectant pA7-100 were, however, much less susceptible to solubilization by detergent

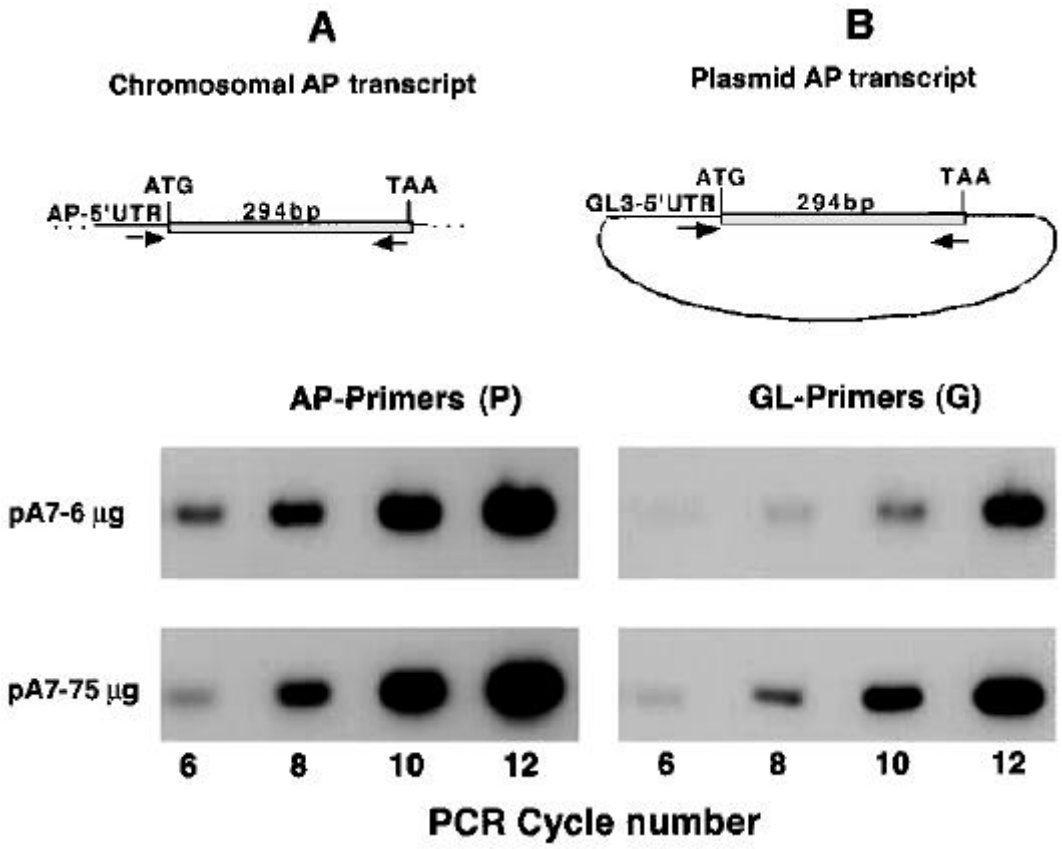

Figure 4. mRNA levels of *ap-a* from the chromosomal and episomal genes of transfectant pA7. The differentiation between the two mRNAs was made by RT-PCR using different 5′ oligonucleotide primers. (**A**) Complementary to the 5′ UTR of *ap-a* (Leippe *et al* 1994b). (**B**) Complementary to the 5′ UTR of the ribosomal protein *L21* gene *gLE3* (Moshitch-Moshkovitch *et al* 1997), together with a common antisense primer from the ORF of the *ap-a* gene for amplification of the cDNA. Samples were removed from the PCR tube after different amplification cycles. Non transfected control did not contain any plasmid coded transcript (not shown).

than those in the non-transfected trophozoites. The bacteria remained intact and viable, almost as the control bacteria which were not interacted with amoebae (figure 7C).

4. Trophozoites with a silenced *AP-A* gene

In view of the reduced virulence of transfectant pA7-100 which occurred in spite of its increased content of AP-A protein, we searched for a more "natural" way to over-express AP-A. Assuming that one of the problems might be the regulating elements in the plasmid, we prepared a new plasmid psAP-1 in which the *ap-a* gene was placed under its original, regulatory elements. Transfections with plasmid psAP-1 (figure 3II-A) surprisingly produced an amoeba in which the transcription and translation of the *ap-a* gene was totally abrogated (figure 3II-B, II-C). The molecular basis for this silencing phenomenon is currently being summarized and will be published elsewhere. We took advantage however, of the functional knockout phenotype of this transfectant, in order to look at the intracellular fate of bacteria or red blood cells

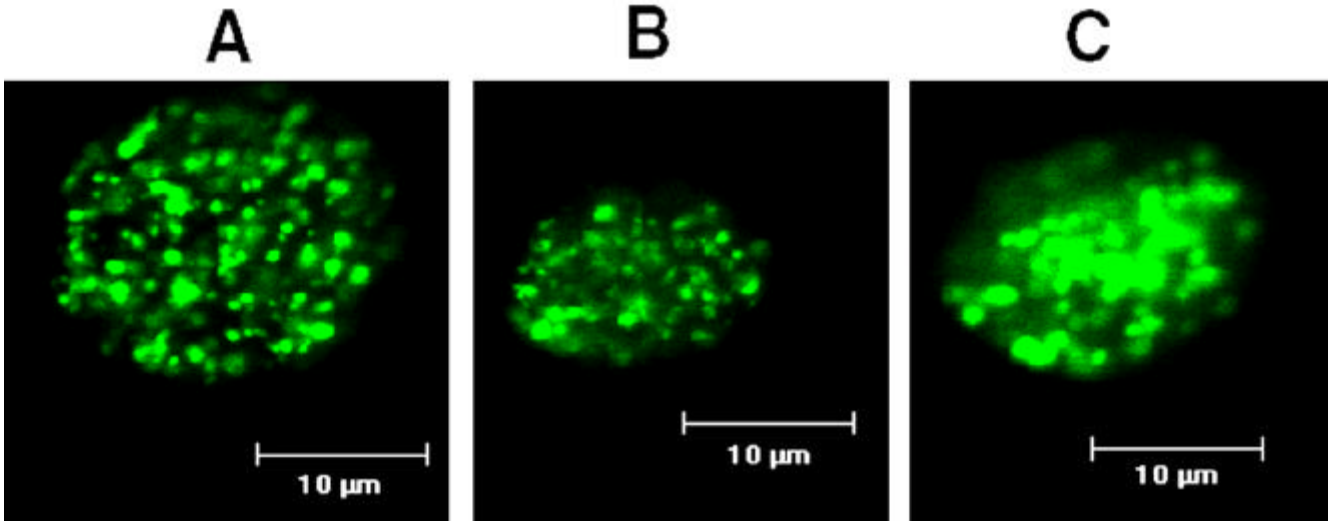

Figure 5. Confocal microscopy of immunostained trophozoites. (**A**) Non transfected control. (**B**) pAP-R2 antisense inhibited transfectant. (**C**) pA7 transfectant overexpressing AP-A. Fixed trophozoites were treated with saponin 0·05% and incubated with anti-amoebapore A antibodies. The second antibody used was an FITC labelled goat-anti rabbit.

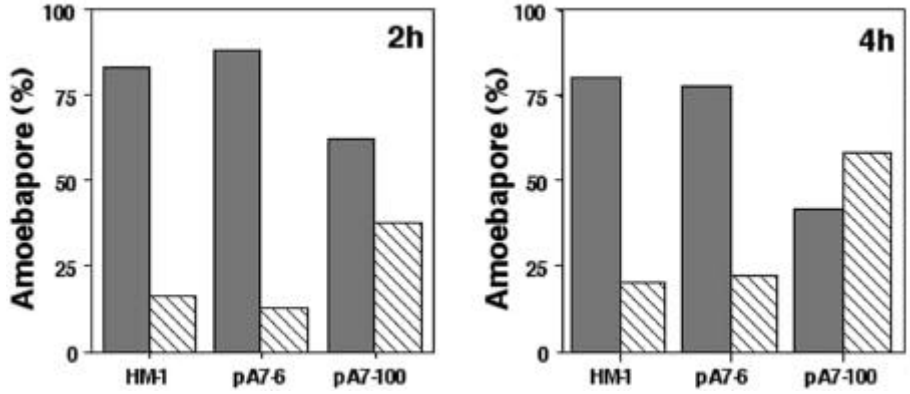

Figure 6. Release of amoebapore A into conditioned medium by non transfected trophozoites as well as by pA-7 transfected trophozoites grown with 6 µg and 100 µg/ml G418 after 2 and 4 h incubation in a serum free TYI-33 medium. Amoebapore A was detected and determined both in the trophozoites (filled columns) as well as in the conditioned medium (striped columns) by Western blot analysis. Less than 10% of trophozoites lysed during the incubations as determined by trypan blue exclusion as well as by determinations of the cytoplasmic enzyme $NADP^+$ dependent alcohol dehydrogenase in the cells and in the conditioned medium.

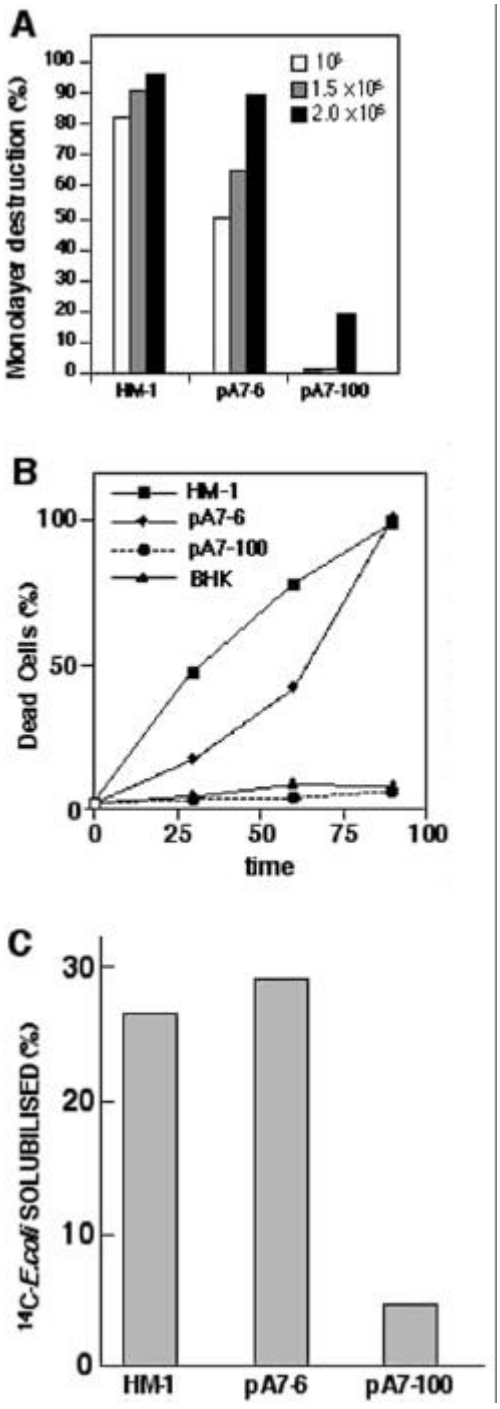

Figure 7. Virulence of pA7 transfectants grown at 6 µg or 100 µg/ml G418. (**A**) Cytopathic activity was determined by the rate of destruction of BHK nonolayers as previously described (Bracha *et al* 1999). (**B**) Cytotoxic activity as determined by the number of trypan blue cells following exposure of BHK cells in suspension with trophozoites at a ratio of 6 : 1. (**C**) Sensitivity of [^{14}C]-labelled *E. coli* cells to detergent (Triton X-100, 0·2%) following their interaction with trophozoites as described (Bracha and Mirelman 1984). The number of *E. coli* cells which became associated with each type of trophozoite was comparable.

which became phagocytized by the amoeba, so as to pinpoint the role of amoebapore in this process. For this purpose we used *E. coli* cells expressing GFP protein. Incubation of bacteria with amoeba results in fast (1 h) phagocytosis of numerous (> 100) bacteria which, as seen by confocal fluorescence microscope (figure 8), are rapidly taken up into vacuoles of the trophozoite. The same picture was observed in the silenced transfectant, psAP-1, in non transfected cells, and in a control unrelated transfectant. The question was what would happen to those bacteria after 24 h of continued culture. As shown in figure 8, a low intensity diffused fluorescence of the GFP protein of the bacteria could be seen in the vacuoles of the Sham transfected cells. A similar observation was seen in non-transfected trophozoites. On the other hand, after 24 h cultivation, the bacteria ingested by psAP-1 transfected trophozoites were still, to a high proportion, intact and their GFP protein remained localized in the bacteria. Similar results were obtained when red blood cells were ingested by the different trophozoite; red blood cells were hardly degraded in the AP-A amoeba. These observations clearly demonstrate the importance of the amoebapore for the disruption of ingested cells.

5. Discussion

Our investigations of the transfectants in which the AP-A content of the trophozoites was modulated clearly indicate that down regulation by antisense transcripts results in lower virulence. A similar lowering of virulence was reported also when other important virulent factors such as cysteine proteinase CP-5 (Ankri *et al* 1998) and the 35 kDa light subunit of the Gal/GalNac lectin (Ankri *et al* 1999) were down regulated by the same technique. In contrast, most of the attempts to increase trophozoite virulence as a result of increasing the amount of a virulent factor have so far failed. In both our amoebapore overexpressing transfectant pA-7, as well as in those overexpressing CP-2 (Hellberg *et al* 2001), there was a substantial increase in transcription and expression of the protein, but virulence didn't increase. This may be the result of a cooperative action among the different virulence factors. Thus, the decrease of a single factor can lower the overall virulence, but an increase in any one of them will probably cause only a slight effect, if any, because the other virulence molecules become the limiting factor. Our results also indicate that not only is the amount of the overexpressed protein important for its function but also its correct localization in the cell. In pA7-100 transfectants, the overexpressed AP-A seems to overflow from its original localization in vesicles into the cytosol and some of it is even released to the outside. This mislocalization seems to influence the trophozoite

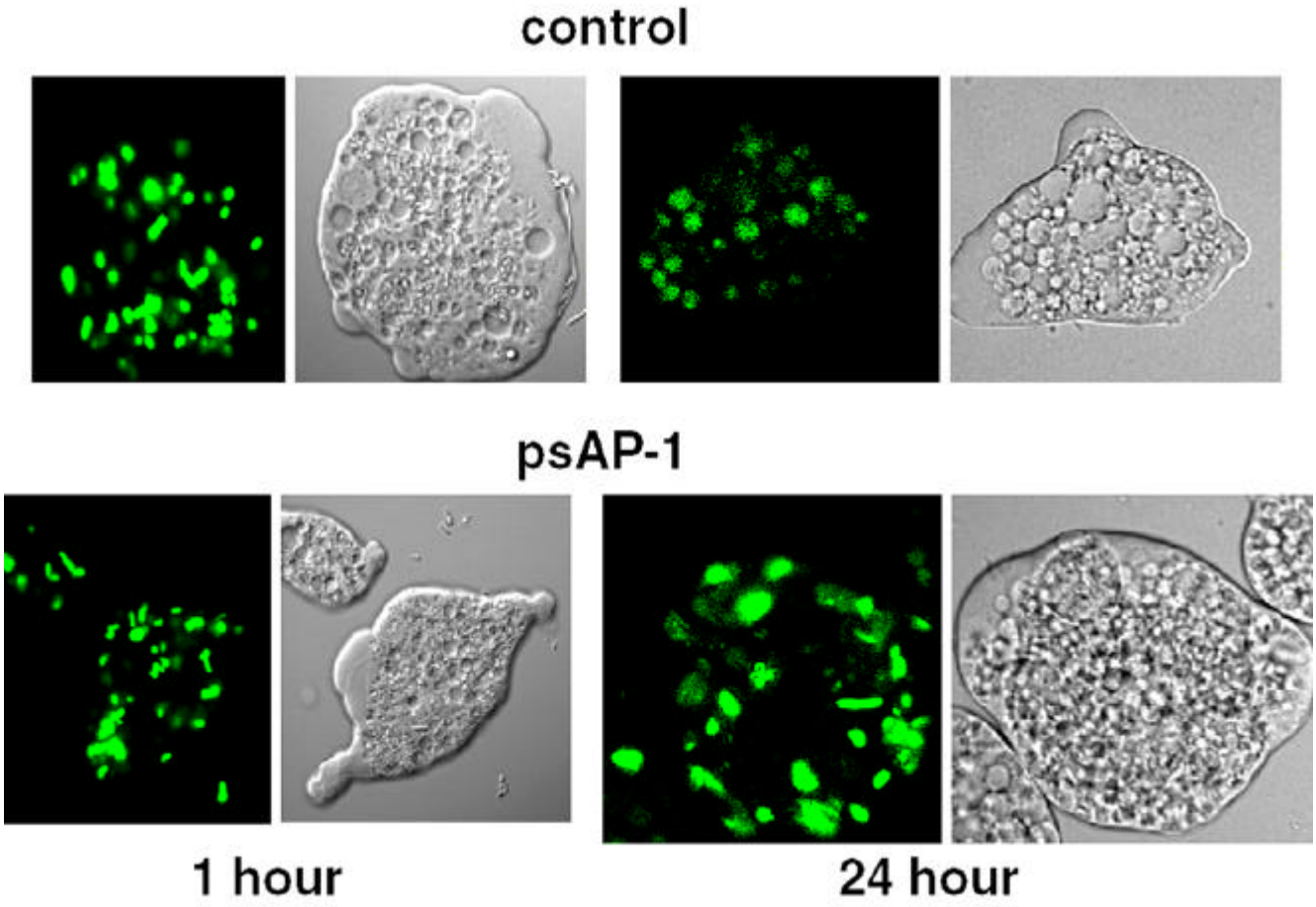

Figure 8. GFP-labelled bacteria following their ingestion by psAP-1 transfected trophozoites which are totally devoid of AP-A expression. For comparison we examined non transfected trophozoites as well as a non related Sham transfectant. Trophozoites were incubated with bacteria at a ratio of 1 : 1000 for 1 h, after that the trophozoites were washed, half of them were immediately fixed in formalin 3·7% and the remaining were recultured for 24 h in TYI-S-33 medium containing Claforan to kill any remaining bacteria after which they were fixed as above and examined in a confocal fluorescent microscope.

itself and might be a cause for the reduction of virulence. A somewhat analogous phenomenon was described in *Aspergillus* where over-expressed RNase was shown to overflow from the secretory apparatus into the cytosol and this in turn had toxic effects on the cells (Nonaka *et al* 2000). Another consequence of overexpression of the AP-A isoform might be a change in the stochiometry with the two other amoebapore isoforms, AP-B and AP-C which the trophozoites produce, and whose relative abundance was reported to be 21 : 9 : 1 respectively (Nickel *et al* 1999). Although each isoform has been shown to have its individual pore-forming activity and is able to lyse bacterial spheroplasts, it is still not clear if the ratio between the different isoforms bears any relevance or is important for the organization of the amoebapore molecules within the cytoplasmic granules and for the exertion of their toxic effect in the membranes of target cells.

The elucidation of the molecular events which cause the silencing of amoebapore gene in transfectant, psAP-1, will hopefully shed more light on another very important phenomenon, the apparent ability of trophozoites to control the synthesis of some of its proteins. Moreover, it will be interesting to see whether the avirulent AP-A minus trophozoites would be effective in immunoprotection of mammalian hosts.

In conclusion, the results obtained from analysis of the transfectants modulated in AP-A, either by downregulation, overexpression or silencing, clearly demonstrate that AP-A is an important virulent factor and any changes in its amounts, cellular localization or organization can cause marked changes in the virulence capacity of the cell.

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