Transcriptional Gene Silencing Reveals Two Distinct Groups of Entamoeba histolytica Gal/GalNAc-Lectin Light Subunits

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The Entamoeba histolytica cell surface Gal/GalNAc-inhibitable lectin is a heterodimer between a heavy (170 kDa) subunit linked via a disulfide bond to a light (31 to 35 kDa) subunit. Five light subunit genes with high homology have been identified (Eh lgl1 to -5). We have previously shown that silencing of the expression of Eh lgl1, in the G3 trophozoites which had already been silenced in the amoebapore gene (Eh ap-a), also suppressed the transcription of Eh lgl2 and -3 (strain RBV). The total absence of the lgl1 to -3 subunits in the RBV trophozoites affected their ability to cap the surface Gal-lectin molecules to the uroid region. We have now found that in the RBV trophozoites, the lgl4 and -5 subunits (31 kDa) are overexpressed and appear to compensate for the missing lgl1 to -3 in the heterodimer complex. Transcriptional silencing of Eh lgl5 was achieved by transfection of G3 trophozoites with a plasmid containing the open reading frame of Eh lgl5 ligated to the 5′ promoter region of the Eh ap-a gene. The transfected trophozoites (strain L5) were silenced in Eh lgl5 and the closely related Eh lgl4, while the expression of the larger lgl1 to -3 subunits was upregulated. L5 trophozoites retained their ability to cap the Gal-lectin molecules. Attempts to simultaneously silence all of the Eh lgl genes have failed so far, possibly due to their crucial importance to the Gal-lectin functions. Our ability to silence part of the genes belonging to the same family can serve as a tool to study the relationships and functions of the members of other gene families.

The adherence of Entamoeba histolytica trophozoites to mucosal cells and red blood cells (RBC) as well as to some bacteria and colonic mucus is mostly mediated by the cell surface Gal/GalNAc-inhibitable lectin (19). Inhibition of lectin activity with millimolar concentrations of Gal or GalNAc prevents most of the contact-dependent cytotoxicity for which the organism is named (22). The cell surface Gal/GalNAc-lectin molecule is composed of a 260-kDa heterodimer of a disulfide-linked heavy (hgl) (170 kDa) subunit and a light (lgl) (35/31 kDa) subunit which is noncovalently associated with an intermediate subunit (igl) of 150 kDa (10). The structure, role, and functions of the 170-kDa subunit were largely investigated. It mediate subunit (igl) of 150 kDa (10). The structure, role, and functions of the members of other gene families.

E. histolytica culture conditions. Trophozoites of E. histolytica strain HM-1:IMSS and of the plasmidless gene-silenced clone G3 (5) were grown at 37°C in TYI-S-33 medium (11). Transfection of G3 trophozoites was performed as previously described (5), and transfecteds were selected by growing them in the presence of the neomycin derivative G418.

Plasmid constructs. The pEhActNeo shuttle vector, which served as the basic construct, contains the Neo gene, which confers resistance to G418, flanked by the 5′ and 3′ regulatory sequences of the amoeba actin 1 gene Ehea and an E. histolytica autonomous replication sequence, both cloned in pBluescript II SK (−) (2). For the construction of plasmid pL5, the 473-bp fragment of the 5′ upstream region of Eh ap-a was amplified by PCR using primer TCCCCGGGG CTTGTCGACCCCTTTG as the sense primer with a SacII restriction site and CATGCAATGCTATGATTGTGTTAGATGAT as the antisense primer with a Neol restriction site. Since we have previously shown that there was no need to include all of the open reading frame (ORF) of a second gene in order to silence it (7), we amplified a fragment of 311 bp from the 5′ end of the ORF activity to mammalian or bacterial cells but strongly inhibited cytopathic activity and cytotoxic activity as well as the ability to induce liver lesions in hamsters (3).

The light subunit was shown to have a role in enabling the clustering of Gal/GalNAc-lectin complexes, which is required for virulence. This was shown following the dominant-negative expression of an N-truncated lgl1 subunit in the virulent strain (13). Furthermore, the transcriptional silencing of the three (Eh lgl1 to -3) genes in the RBV trophozoites also demonstrated the inability to cluster the Gal-lectin complexes (7). In the present report, we demonstrate that, in the RBV silenced trophozoites the two other lgl genes (Eh lgl4 and -5) are expressed. Moreover, Eh lgl4 and -5 appear to be up-regulated and continue to form heterodimers with the hgl subunits. It thus became interesting to investigate what would be the consequence of silencing the expression of Eh lgl4 and -5 and if it would be possible to simultaneously silence the expression of all five Eh lgl genes.

MATERIALS AND METHODS

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of the Eh\textit{lgl5} gene. The Eh\textit{lgl5} gene fragment was amplified with sense primer CATGCCATGGTTACGTTGTTTTTATTG starting from the first ATG and with a NcoI restriction site and primer TCGAGCTCCATATCTAGTAGTT CCTTTTAC as the antisense primer from /H11001 311 bp of the ORF and with a SacI restriction site. The two fragments were digested with NcoI and ligated, and the cassette was then inserted into the above-mentioned pEhActNeo shuttle vector.

A plasmid (pRB9 [see Fig. 9]) was prepared for the transfection and simultaneous silencing of two genes at once (Eh\textit{lgl1} and Eh\textit{CP-5}) in the G3 clone which already has the Eh\textit{ap-a} gene silenced. Two DNA fragments were prepared by PCR amplification. The first, for silencing the Eh\textit{lgl1} gene, was done using a sense primer from the 5\textendash/H11032 sequence upstream of the Eh\textit{ap-a} gene (TCCCCGCG GCTTGCTGCACCCTTTG) with a SacII restriction site and a specific antisense primer from a region within the ORF of the Eh\textit{lgl1} gene (bp 421; GCGGATC CGAAGTTCATTTCCTTGTTTCAATG) with a BamHI restriction site using pTL plasmid DNA (7) as the template. The second fragment was prepared by using the same 5\textendash/H11032 sequence upstream of the Eh\textit{ap-a} gene primer, as described above, and an antisense primer from a region within the ORF of the Eh\textit{CP-5} gene (bp 396; AGCGGATCCTTTGATCCAGCAACCAAC) with a BamHI restriction site, using the pAP-CP5 plasmid (7) as the DNA template. The two fragments were BamHI digested and ligated to each other, tail to tail, and the resulting cassette was then cloned into the pEhActNeo shuttle vector.

Northern blots and RT-PCR. Total RNA was prepared using the RNA isolation kit TRI Reagent according to the manufacturer's protocol (Sigma, St. Louis, MO). RNA was size fractionated on a 4\% polyacrylamide denaturing gel containing 8 M urea and subsequently blotted to a nylon membrane. Hybridization under stringent conditions was carried out with different probes randomly labeled using the Redi-Prime II kit (Amersham Life Science, Buckinghamshire, United Kingdom) and washed with a mixture of 0.1% sodium dodecyl sulfate (SDS) and 0.1\% SSC (1\% SSC is 0.15 M NaCl plus 0.015 M sodium citrate). For reverse transcription-PCR (RT-PCR), RNA samples were treated with RNase-free DNase (Promega, Madison, WI) and after phenolation and sedimentation, 2\% g of RNA was reacted with avian myeloblastosis virus reverse transcriptase (Promega) according to the manufacturer's protocol using an oligo(dT) adaptor as a primer (Table 1). The cDNA product was diluted five times and used for the subsequent PCRs. In all of the RT-PCR experiments, a control in which the reverse transcriptase was omitted was performed. The results in all cases gave no PCR product, verifying that no DNA was present in the RT samples.

Northern blot hybridizations typically include both a prehybridization and a posthybridization wash step. Each step is designed to remove unhybridized probe; the wash step is the more stringent of the two.

qRT-PCR. Quantitative real-time RT-PCR (qRT-PCR) was carried out in a 20-\textmu l reaction mixture containing 1 \texttimes Absolute SYBR green ROX master mix

FIG. 1. Nucleotide sequence comparison of the Eh\textit{lgl} family genes. The different genes that encode the lgl proteins and their accession numbers are as follows: Eh\textit{lgl1}, XM_651053.1; Eh\textit{lgl2}, L20898; Eh\textit{lgl3}, XM_649244.1; Eh\textit{lgl4}, XM_646286.1 (previously annotated as Eh\textit{lgl6}); and Eh\textit{lgl5}, XM_652368.1.
into two tubes, each containing 2×10^6 cells per tube. To each tube, two harvested trophozoites were washed in phosphate-buffered saline and divided surface molecules was performed essentially as previously described (13). Freshly collected fractions of 1 ml each were collected from the top of the gradient. Proteins were precipitated by the addition of trichloroacetic acid. Protein sediments were resuspended in sample buffer and subjected to Ultracentrifuge. Following centrifugation, 12 fractions of 1 ml each were collected at 4°C for 20 h at 39,000 rpm in an SW-41 rotor in a Beckman ultracentrifuge. To each tube, two monoclonal antibodies (3F4 and 7F4; a gift from Richard Vines, TechLab, Buckinghamshire, United Kingdom) were added at a 1:30 dilution. One of the tubes was kept at 4°C and the other at 37°C, to induce capping, for 20 min. Fixation of trophozoites was performed with the addition of paraformaldehyde to a final concentration of 3.7% for 15 min, followed by a wash with NH₄Cl (50 mM) in order to block free aldehydes. Final addition of paraformaldehyde to a final concentration of 3.7% for 15 min, was generated by overlaying this with 30% sucrose and 5% sucrose. Tubes were subjected to SDS-PAGE and immunoblotted as described above.

**SDS-PAGE and Western blots.** For SDS-polyacrylamide gel electrophoresis (PAGE), soluble extracts from trophozoites prepared as previously described (5) were subjected to separation on a 12% polyacrylamide gel under reducing conditions. Gels were blotted onto a nitrocellulose membrane and subjected to immunoreactions with the indicated antibodies. The blots were washed and immunoreactions with the indicated antibodies. The blots were washed and subjected to Western blot analysis using the GeneAmp7300 sequence detection system (PE Applied Biosystems [for further elaboration, see “Sequence detector user bulletin no. 2”]).

**Sucrose gradient flotation.** *E. histolytica* trophozoites were lysed in 1 ml of MES (morpholinethanesulfonic acid)-buffered saline (25 mM MES [pH 6.5], 150 mM NaCl, 1% cold Triton X-100), and the protease inhibitors leupeptin [0.2 mM], iodoacetamide [5 mM], 1,10-phenanthroline monohydrate [5 mM], phenylmethylsulfonyl fluoride [2 mM], sodium orthovanadate [1 mM], and sodium pyrophosphate [1 mM]), and incubated for 30 min on wet ice. Total cell lysates were adjusted to 40% sucrose by adding an equal volume of 80% sucrose and placed at the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was generated by overlaying this with 30% sucrose and 5% sucrose. Tubes were centrifuged at 4°C for 20 h at 39,000 rpm in an SW-41 rotor in a Beckman Ultracentrifuge. Following centrifugation, 12 fractions of 1 ml each were collected from the top of the gradient. Proteins were precipitated by the addition of trichloroacetic acid. Protein sediments were resuspended in sample buffer and subjected to SDS-PAGE and immunoblotted as described above.

**Gal/GalNAc lectin capping assay.** Capping or clustering of the Gal-lectin surface molecules was performed essentially as previously described (13). Freshly harvested trophozoites were washed in phosphate-buffered saline and divided into two tubes, each containing 2×10⁶ cells per tube. To each tube, two monoclonal antibodies (3F4 and 7F4; a gift from Richard Vines, TechLab, Blacksburg, VA) against the heavy (170 kDa) subunit of the Gal-lectin (22) were added at 1:30 dilution. One of the tubes was kept at 4°C and the other at 37°C, to induce capping, for 20 min. Fixation of trophozoites was performed with the addition of paraformaldehyde to a final concentration of 3.7% for 15 min, followed by a wash with NH₄Cl (50 mM) in order to block free aldehydes. Final blocking was done with 2% fetal calf serum. After washing, the fixed trophozoites were incubated with fluorescein isothiocyanate-labeled goat antimouse antibodies (Jackson Immuno Research, West Grove, PA) at a 1:200 dilution. Finally, samples were viewed with a confocal microscope (Fluoview FV500; Olympus, Tokyo, Japan).

**RESULTS**

Identification of the EhlgI genes that are expressed in *E. histolytica* G3 and RBV trophozoites. We have previously shown that silencing of the expression of the EhlgI gene in the *E. histolytica* strain G3, which already had the amoebapore genes Ehap-a, Ehap-b, and the saposin-like protein (Suplp1) silenced, caused a simultaneous down-regulation of the closely related Ehlg2 and -3 (7). The plasmidless RBV trophozoites that were selected after the gene silencing were found to be devoid of the characteristic lg1 protein bands which migrate at 35 kDa. RBV trophozoites continued, however, to express the lower-molecular-mass bands of 31 kDa which are seen in Western blots of the reduced PAGE that reacted with the polyclonal antibodies raised against lg1 protein (7) (Fig. 2A).

In order to find out if the 31-kDa protein bands correspond to the other two light subunits of the Gal-lectin, lgl4 and -5, or are due to nonspecific signals, we searched for their transcripts in RNA extracts. RT followed by PCR of the RNA extracted from trophozoites of strains HM-1:IMSS, G3, and RBV revealed that both genes Ehlg1, -2, -3 and -4 were transcribed in the RBV trophozoites that do not express Ehlg1, -2, and -3 (Fig. 2A). The quantitative evaluation of the Ehlg5 transcript level is shown in Fig. 5.

**Distribution of Ehlg4 and -5 in sucrose gradients of the parasite membranes.** It was recently reported that the heavy, intermediate, and light subunits of the Gal/GalNAc-inhibitable lectin were present in raft-like fractions as well as in non-raft membrane fractions (14). A similar sucrose gradient separa-
tion was performed with the membranes of the two strains G3 and RBV. Western blots of the different fractions revealed that in membranes of RBV, only the lower (31 kDa) band was observed with antibodies against the lgl1 subunit in the different fractions, while in the parent G3 trophozoites, the typical profile of the various proteins (31 to 35 kDa) of the light subunit of the Gal-lectin appeared in the fractions (Fig. 3). The sucrose gradient separation profile of actin as well as that of the heavy subunit (hgl) of the Gal/GalNAc-inhibitable lectin appears to be the same in both preparations. These results further demonstrate that the lower band, which appears in

FIG. 3. Distribution of the Gal/GalNac-lectin subunits in sucrose gradients of trophozoite membranes. Total cell lysates prepared with cold Triton X-100 were subjected to sucrose gradient flotation (for details, see Materials and Methods). Twelve fractions were collected from the top of the gradient, and proteins were precipitated with trichloroacetic acid. Following SDS-PAGE and immunoblotting, the distribution of Gal-lectin heavy subunit hgl, Gal-lectin light subunit lgl, and actin in each of the different fractions was analyzed by reacting Western blots with their respective antibodies. Fraction 1 is the top and fraction 12 the bottom of the gradient. T, total lysate.

FIG. 2. (A) Expression of the Ehlg1 proteins in the various transfectants (7). The Western blot of reduced SDS-PAGE was reacted with polyclonal antibodies to lgl1. The different cell lysates are as follows: lane 1, G3; lane 2, pB33, the plasmid that induced the silencing of lgl1 (7); lane 3, pAY, a plasmid that did not silence lgl1; lane 4, RBV trophozoites, which were derived from the pB33-transfected trophozoites after the removal of the plasmid and in which lgl1 remained silenced (7). (B) RT-PCR of RNA samples derived from trophozoites of strain HM-1:IMSS (panel I), G3 (panel II), and RBV (panel III). Each sample underwent a PCR with primer sets for different regions (see Table 1) as follows: lane 1, Ehlg1 (primers 3 and 5); lane 2, Ehlg4 (primers 4 and 5); lane 3 Ehlg1 (primers 9 and 10); and lane 4, ribosomal protein L21 (primers 6 and 2). Notice that Ehlg1 does not appear in the RBV trophozoites, while Ehlg5 and Ehlg4 are transcribed.

RBV Western blots and reacted with antibodies against lgl1, represents the 31-kDa light subunits of lgl4 and -5 which prior to the reduction were covalently linked by a disulfide bond to the heavy hgl subunits.

Transcriptional silencing of Ehlg4 and -5. In order to silence the expression of Ehlg5, a plasmid construct similar to the previous construct which silenced Ehlg1 (7) was prepared (Fig. 4A). In brief, the 5′ upstream region of the Ehap-a gene (473 bp) was ligated to a truncated ORF (311 bp) of the Ehlg5 gene. (B) RT-PCR done on RNA extracted from the trophozoites G3 (panel I), RBV (panel II), and L5 (panel III). Each panel contained three pairs of primers. The primers are listed in Table 1 and the legend to Fig. 2B. Lane I, Ehlg5; lane II, Ehlg4, and lane III, EhRP-L21, the ribosomal protein transcript. Note that in L5 the transcripts of both genes Ehlg4 and Ehlg5 are missing. (C) The Western blot of reduced SDS-PAGE was reacted with polyclonal antibodies to lgl1. The different cell lysates are as follows: lane 1, HM-1:IMSS; lane 2, G3; lane 3, RBV; and lane 4, L5. Notice that the 31-kDa bands are missing in L5.

Our results show that it is possible to differentiate between two groups of lgl lectin genes and that these two groups can be
silenced separately. The first group includes genes *Ehlg1* to -3, which are cosuppressed when *Ehlg1* is silenced while the genes *Ehlg4* and -5 are expressed, whereas in the second group, silencing of *Ehlg5* also cosuppressed *Ehlg4* but not *Ehlg1* to -3.

**Changes in the transcription levels of the expressed *Ehlg1* genes.** In order to obtain more accurate data for the levels of expression of *Ehlg5* in RBV trophozoites and the expression of *Ehlg1* in L5, a qRT-PCR was performed. Figure 5 shows the increase in the relative transcription level of *Ehlg5* in RBV trophozoites in the absence of transcription of *Ehlg1*, and an inverted result was seen for the L5 trophozoites, namely a higher level of *Ehlg1* with no *Ehlg5* transcript. The overexpression of the lower 31-kDa protein bands, which corresponds to *Ehlg4* and -5, can also be clearly seen in the Western blots of the RBV trophozoites (see also Fig. 2A). These results indicate that overexpression happened among the members of one of the *Ehlg1* gene groups whenever the expression of the other group was suppressed, enabling the compensation for the missing lgl subunits which form the Gal/GalNAC-lectin heterodimer.

**Silencing of the *Ehlg1* members does not influence the expression of the other gene members of the Gal/GalNAC-lectin complex.** A semiquantitative RT-PCR (shown in two different concentrations in Fig. 6) indicated that silencing of either *Ehlg1* to -3 in RBV trophozoites or of *Ehlg4* and -5 in L5 trophozoites did not significantly alter the transcription of the heavy subunit *Ehhl* or the *Ehhl* intermediate subunits, which are components of the Gal-lectin family (10).

**Comparison of phenotypes between G3, RBV, and L5 trophozoites.** G3 trophozoites and all of the descendants of G3 trophozoites in which we have silenced additional genes are nonvirulent because of the lack of the toxic amoebapore proteins and cannot be tested for regular virulence properties (5). We have previously shown that RBV trophozoites that are devoid of lgl1, -2, and -3 were incapable of clustering or capping of the Gal/GalNAC-specific lectin molecules upon exposure to antibodies against the lectin (7). The inability of RBV trophozoites to induce the capping of the Gal-lectin occurred, although the light subunits lgl4 and -5 were expressed, but their presence did not enable the capping. As can be seen in Fig. 7, capping of the Gal-lectin complex in the L5 trophozoites which don’t have the light subunits lgl4 and -5 (but do have lgl1, -2, and -3) was found to proceed at 37°C, as in the parent strain G3 but differed from RBV trophozoites. This indicates that the lgl4 and -5 subunits are not essential for the surface capping activity and the presence of lgl1 to -3 was sufficient for the capping formation.

As previously shown, the erythrophagocytosis capability of the G3 amoeba was not impaired by the lack of amoebapore (9). This ability was also not affected in the different lgl-silenced amoeba, RBV or L5 (Fig. 8). A slight difference, however, was observed in the sensitivity of the erythrophagocytosis to inhibition by galactose (0.1 M). RBC ingestion by RBV trophozoites was most sensitive to galactose (89% inhibition); in L5, inhibition was 77%; while in the parent strain, G3 inhibited was only 63% (Fig. 8).

A somewhat similar result was seen when the adherence of the different trophozoites to fixed BHK cells was examined (not shown). This finding suggests that in the absence of lgl1 to -3, as in the RBV trophozoites, the CRD of the hgl (20) is more favorably exposed and the added galactose molecules are...
more effective at competing with the mammalian cell target for the carbohydrate binding domain.

Is it possible to silence all of the members of the light subunit of the Gal/GalNAc-lectin? We have previously reported that we failed in our attempts to silence a third gene family following transfection of either RBV or RB8 trophozoites (7). We recently managed to simultaneously silence two additional genes in the G3 strain that is already silenced for the amoebapore (6). The two genes, Eh\textit{lgl1} and Eh\textit{CP-5}, were each ligated, as before, to the 473 bp of the upstream region of the Eh\textit{ap-a} gene. The two fragments were then ligated tail to tail to each other and cloned into the pEhActNeo vector described before (2). Transfection of the pRB9 plasmid (Fig. 9A) into G3 trophozoites caused the silencing of both the Eh\textit{lgl1} and Eh\textit{CP-5} genes in addition to the amoebapore genes that were already silenced (Fig. 9B). A plasmid built on the same principles described above, carrying the Eh\textit{lgl1} gene and the Eh\textit{ap-a} gene, was prepared and transfected into G3, but in the process of selection with G418, in all three cases that we tried, the transfected trophozoites died. It is possible that silencing of the entire family of Eh\textit{lgl} genes is lethal for the trophozoites, but we cannot exclude, at this stage, experimental difficulties.

**DISCUSSION**

In recent years, a lot of unequivocal evidence has been published indicating that the Gal/GalNAc-inhibitable lectin plays an important role in the physiology of \textit{Entamoeba histolytica} (3, 4, 13, 15, 20, 23). Among the three main protein subunits that compose the Gal/GalNAc-lectin complex, the heavy (hgl [170 kDa]) subunits were investigated the most, probably because they possess the CRD which mediates the trophozoite’s ability to adhere to different targets and because antibodies to the hgl are usually found in human hosts infected with the parasites (1, 12). Much less is known about the function of the other two subunits that belong to the Gal/GalNAc-
lectin family, the two genes of the intermediate subunits (Eh\textit{lgl}) (10), and the five genes found for the light subunits (Eh\textit{lg}l) (3, 17, 24).

In the present investigation, we tried to learn more about the relationship between the different genes of the light subunit group and their function using our ability to silence the expression of only a part of these genes with a given plasmid construct. The members of the light subunit gene family bear high sequence homology in their nucleotide and protein sequences, and the polyclonal antibody raised against the lgl1 protein cross-reacts with all the light subunits (3, 13). The main sequence difference between the first group (lgl1 to -3) and the second group (lgl4 and -5) is a deletion of 17 amino acids, which also accounts for their smaller molecular mass. Moreover, from the distribution of the lgl and hg\textit{l} proteins seen in the sucrose gradient fractions, we conclude that all of the lgl subunits are present on the amoeba surface as heterodimers. Differences were observed in the regulation of the transcription of the two groups of Eh\textit{lgl} genes. As previously shown, silencing of the Eh\textit{lgl}1 gene resulted in the down-regulation of the closely related Eh\textit{lgl}2 and -3 genes (all of which are considered to belong to the 35-kDa proteins) in the RBV strain. In the present work, we show that Eh\textit{lgl}4 and -5 were up-regulated when Eh\textit{lgl}1, -2, and -3 were silenced in the RBV trophozoites. Moreover, when we prepared a plasmid construct containing the Eh\textit{lgl}5 gene using the same principle as for the silencing of Eh\textit{lgl}1 (7), the G3 transfected trophozoites that were obtained (strain L5) were found to be suppressed in the transcription of two genes—Eh\textit{lgl}5, as expected, as well as Eh\textit{lgl}4 (both produce 31-kDa proteins)—while the other genes, Eh\textit{lgl}1 to -3 were transcribed. This indicates that it is possible to distinguish between two subgroups among the Eh\textit{lgl} gene family members and that the expression of these two subgroups may be differently regulated.

It is interesting to mention in this context that when the down-regulation of the Eh\textit{lgl}1 gene was performed by antisense transcripts (3), the major inhibitory effect was on all the 35-kDa proteins of the lgl family but no effect was seen on the 31-kDa proteins (see Fig. 3 in reference 3). Separate regulation of different members of gene families during a gene-silencing event was also observed in the case of the amoebapore genes, which were simultaneously silenced with the Eh\textit{ap-a} gene (Eh\textit{ap-b} and Sap\textit{ip1}). These genes have high sequence homology and are located on the same evolutionary branch (7, 8). Similarly, in the Eh\textit{lg}l group there might be phylogenetic differences between the two groups of Eh\textit{lg}l genes. Eh\textit{lg}l4 and -5, which are the low-molecular-weight species, might have emerged later, whereas the other genes, Eh\textit{lgl}1, -2, and -3, which have a distinct role in the clustering of the surface lectin molecules, probably appeared earlier.

The 260-kDa Gal/GalNAc-lectin complex that is present on the surface membrane of the trophozoite has been reported to consist of one molecule of the heavy (hg\textit{l}) subunit (170 kDa) covalently ligated to one molecule of the light (lg\textit{l}) subunit (35/31 kDa) (21, 22). The relative abundance of each of the light subunits in the lectin complex is, however, unknown. From the distribution of the lg\textit{l} proteins in the RBV trophozoite membranes, we can conclude that lg\textit{l}4 and -5 replace the missing lg\textit{l}1, -2, and -3 subunits in the Gal/GalNAc-lectin heterodimer and their increased amount appears to compensate to some extent for the loss of lg\textit{l}1 to -3. In the L5 trophozoites, the reverse occurred, namely lg\textit{l}1, -2, and -3 compensate for the lack of lg\textit{l}4 and -5. Our results also enable us to distinguish between the biological functions of the two subgroups of lg\textit{l} subunits. lg\textit{l}1, -2, and -3, as already shown (7), play a role in clustering of Gal/GalNAc-lectin molecules, a function that is lost when these lg\textit{l}s are down-regulated. In contrast, silencing of lg\textit{l}4 and -5 did not have a significant effect on the usual clustering and capping of the lectin complex. We also noted that there was some slight difference between RBV and L5 trophozoites in their adherence to RBC or BH\textit{K} cells. The adherence of RBV trophozoites which lack lg\textit{l}1 to -3 as well as strain L5, which lacks lg\textit{l}4 and -5 were more sensitive to inhibition by galactose molecules than the parent G3 strain. This suggests that the CRD of the hg\textit{l} subunit maybe somewhat masked in the presence of lg\textit{l}1 to -3 and somewhat less by lg\textit{l}4 and -5, perhaps due to a slightly different conformation that the shorter lg\textit{l}4 and -5 subunits confer to the 260-kDa lectin complex. It is of major interest to find out if the light subunit proteins of the Gal/GalNAc-inhibitable lectin are essential for parasite survival and adhesion functions. We attempted to silence all of the Eh\textit{lgl} genes using a method that proved successful for the simultaneous silencing of Eh\textit{lgl}1 to -3 and cysteine proteinase 5 (EhCP-5) in the same G3 trophozoites (6). Unfortunately, and despite our repeated efforts, the simultaneous silencing of all Eh\textit{lgl} genes in the same G3 trophozoites was not successful in three separate transfections, and the transfectants died during the selection with G418. It is possible that the simultaneous silencing of expression of all the Eh\textit{lgl} subunits could be lethal for the trophozoites, but more experiments are currently being conducted in order to exclude technical problems.

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