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Targeting essential *Eimeria ninakohlyakimovae* sporozoite ligands for caprine host endothelial cell invasion with a phage display peptide library

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Abstract

Eimeria ninakohlyakimovae is an important coccidian parasite of goats which causes severe diarrhoea in young animals. Specific molecules that mediate *E. ninakohlyakimovae* host interactions and molecular mechanisms involved in the pathogenesis are still unknown. Although strong circumstantial evidence indicates that *E. ninakohlyakimovae* sporozoite interactions with caprine endothelial host cells (ECs) are specific, hardly any information is available about the interacting molecules that confer host cell specificity. In this study, we describe a novel method to identify surface proteins of caprine umbilical vein endothelial cells (CUVEC) using a phage display library. After several panning rounds, we identified a number of peptides that specifically bind to the surface of CUVEC. Importantly, caprine endothelial cell peptide 2 (PCEC2) and PCEC5 selectively reduced the infection rate by *E. ninakohlyakimovae* sporozoites. These preliminary data give new insight for the molecular identification of ligands involved in the interaction between *E. ninakohlyakimovae* sporozoites and host ECs. Further studies using this phage approach might be useful to identify new potential target molecules for the development of anti-coccidial drugs or even new vaccine strategies.

Keywords

Eimeria ninakohlyakimovae; Goats; Endothelial cells; Phage display; Coccidiosis

Introduction

Coccidiosis is one of the most important parasitic diseases of ruminants and constitutes one of the main real threats for profitability of goat production systems worldwide (Ruiz et al. 2006). *Eimeria ninakohlyakimovae* has been reported as one of the most pathogenic *Eimeria* species, able to produce a massive destruction of infected epithelial host cells resulting in either mild non-haemorrhagic infections (Dai et al. 2006) or severe haemorrhagic typhlocolitis even with fatal outcome (Ruiz et al. 2013). Goat rearing is increasing all over the world, but particularly in emerging countries (FAOSTAT 2013), so there is an urgent need for efficient control strategies to ensure that the goat market remains a solid source of meat, milk and dairy products (Ruiz et al. 2012). Importantly, no new anti-coccidial drugs have been released into the market for many decades. Therefore, basic research focused on the development of new anti-coccidial compounds is a very high priority. For the design of both vaccines candidates and new drugs, a detailed knowledge of the complex host-parasite interactions is crucial.

All species of the genus *Eimeria*, as other members of the phylum Apicomplexa, are obligate intracellular parasites, and therefore, the invasion of host cells is an obligatory step for their lifecycle (for reviews, see Giovannini et al. 2011; Andenmatten et al. 2013). Irrespective of the invasion mechanisms involved, specific molecular interactions between surface host cell receptors and sporozoite ligands seem to be essential for invasion and further development (Singh and Chitnis 2012). Interestingly, *E. ninakohlyakimovae* invades a wide variety of cells in vitro (Ruiz et al. 2010), suggesting that, if host cell receptors are involved in cell invasion, they are conserved in nature. This circumstance would enable sporozoites of *E. ninakohlyakimovae*, which primarily infect endothelial cells (ECs) of the lymph capillaries in the small intestine villi (Vieira et al. 1997), to traverse the epithelial layer and connective tissue until reaching their final host ECs.

The identification of ligands and receptors involved in the interactions between sporozoites and host cells is of significant scientific interest as it may lead to the development of new anticoccidial drugs or even vaccines. Different reports have shown that phage display library can be used for the identification of peptides that specifically bind to the surface of Apicomplexan parasitized cells (Ghosh et al. 2001; da Silva et al. 2002; Ghosh et al. 2011; Vega-Rodríguez et al. 2014). Interestingly, novel peptides isolated via phage display have been demonstrated to specifically and efficiently target gene delivery to human umbilical vein endothelial cells (HUVEC; Nicklin et al. 2000). The present study aimed to investigate the interactions of *E. ninakohlyakimovae* sporozoite ligands with surface peptides of primary caprine umbilical vein endothelial cells (CUVEC) at the molecular level using a phage display library. The phage library consists of random dodecapeptides fused to the N terminus of the major filamentous f88.4 phage coat protein VIII (Bonnycastle et al. 1996).

Material and methods

CUVEC were isolated from goat umbilical cords according to previously described methods (Jaffe et al. 1973; Ruiz et al. 2010). To avoid *Mycoplasma* spp. contamination, CUVEC

monolayers were treated with Plasmocin™ (Invitrogen) at a 0.2 µl/ml final concentration for the duration of the in vitro experiments.

The phage display library and *Escherichia coli* strain (K81) used in this work (Bonnycastle et al. 1996) had an initial complexity of about 10^9 different peptides. After amplification in *E. coli*, approximately 10^{11} freshly isolated phages (on an average, 100 phages for each sequence) were added to confluent CUVEC monolayers grown on 25-cm² plastic tissue culture flasks (Nunc). CUVEC cultures containing phages were incubated for 1 h at 37 °C in 5 % CO₂ atmosphere and then extensively washed six times with PBS buffer to remove loosely attached phages. Phages bound to CUVEC were amplified in *E. coli* and precipitated from culture supernatants with 16 % PEG (Fisher Scientific). The amplified phages were subjected to a total of four rounds of panning following the same protocol described above. For this purpose, 1×10^8 CFU of phages were subjected to the following panning steps.

After the fourth round, 25 phages were randomly isolated, and colony PCR was performed (Vega-Rodríguez et al. 2014) to determine the nucleotide and the predicted amino acid sequence of the displayed peptide. PCR products were purified using the Cycle-Pure kit (Omega Biotek), and sequencing reactions were performed using the BigDye 3.1 Terminator Cycle Sequencing kit (Applied Biosystems) and analysed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

The strain GC of *E. ninakohlyakimovae* (Ruiz et al. 2013) was used for oocyst and sporozoite production following methods previously described (Fayer and Hammond 1976; Ruiz et al. 2010). For the inhibition assays, six phages showing different nucleotide sequences were employed (PCEC1-PCEC6). Purified sporozoites of *E. ninakohlyakimovae* were mixed with each one of the six selected phages to a final concentration of about 300,000 sporozoites and 5×10^7 phages/ml of ECGM medium (PromoCell) and then added to confluent CUVEC flasks. After 24-h incubation at 37 °C in 5 % CO₂ atmosphere, the infection rate (percentage of infected host cells over a total of 20–30 randomly selected power vision fields) was determined for each phage and compared to those of negative controls (CUVEC without the addition of phages). The inhibition assays for each phage were made in duplicates on different days under the same conditions. Statistical differences were determined by Student's test using the GraphPad Prism® 6 software.

Results and discussion

The present results demonstrated that the peptide cell endothelial clone 5 (PCEC5), and to a lesser extent the PCEC2, inhibited parasite development in caprine endothelial cells by competing with *E. ninakohlyakimovae* sporozoite invasion for an essential CUVEC ligand(s). The phage display library technique employed here was similar to that reported for screening different parasite species, e.g. *Plasmodium* (Ghosh et al. 2001; Narum et al. 2006; Ghosh et al. 2011; Vega-Rodríguez et al. 2014), *Cryptosporidium parvum* (Chen et al. 2005) and *Eimeria* spp. (da Silva et al. 2002). In the case of malaria, this approach made valuable contributions in understanding the biology of ookinete and sporozoite invasion of the mosquito midgut and salivary gland, respectively (Ghosh et al. 2001; Ghosh et al. 2011; Vega-Rodríguez et al. 2014). In general, phage display may provide advantages in

facilitating screening of diverse molecular targets to obtain high-potency (e.g. nanomole affinity) candidates suitable for therapeutic development within a much faster timescale than that achievable for small molecules drugs (Hopkins and Groom 2002).

In the sequencing process of the selected phages, 15 recombinant peptides showed different sequences, which indicate a variability of about 60 %. The PCEC1 represented 28 % of the sequenced clones, while 16 and 8 % of them corresponded to PCEC2 and PCED9, respectively. Phages displaying each of the remaining peptides were recovered once (4 % frequency). Table 1 shows the nucleotide sequences of the 15 sequenced clones and their corresponding predictive peptide sequences. Each displayed peptide is composed of 12-aa fused to the N terminus of protein VIII (XCX8CX), where *X* is any amino acid and *C* stands for cysteine (Bonnycastle et al. 1996).

For the sporozoite-cell invasion inhibition assays, peptides of cell endothelial clones 1, 2, 3, 4, 5 and 6 (PCEC-1-2-3-4-5-6) were used. The selection of these six clones was based on the panning frequency, while some of them were also randomly chosen. The ability of these peptides to inhibit the active *E. ninakohlyakimovae* sporozoite-host cell invasion in CUVEC is depicted in Fig. 1. PCEC5 significantly ($p<0.001$) reduced active sporozoites-host cell invasion resulting in an infection rate of 0.34 % versus non-phage-treated CUVEC (10.9 %). Moreover, PCEC2 caused a moderate non-significant sporozoite-host cell invasion inhibition with 4.25 % (phage treated) versus 1.035 % (non-phage treated) infection rates. Results from PCEC2 should be taken cautiously, as the addition of the phages caused an important detachment of CUVEC monolayer and, besides, the corresponding negative control for this CUVEC isolate had a relatively low infection rate (Fig. 1b) compared to the mean values of infection rate found in the remaining CUVEC isolates and in previous studies published by our group (Ruiz et al. 2010). The detachment of the cell monolayer produced by PCEC2 phage may be explained by the phage ability to produce cellular toxicity, since similar peptides may quickly kill bacteria by physical disruption of cell membranes (Hancock 1997). Finally, the remaining clones (PCEC1, PCEC3, PCEC4 and PCEC6) showed approximately the same infection rate in phage and non-phage-treated cells (Fig. 1).

Although the identification of the caprine endothelial cell receptor(s) and the protein moiety on the parasite (ligand) which interacts with this receptor requires further experiments, the finding that the use of a phage display library led to the identification of the inhibitory peptides PCEC5 and PCEC2 is of high scientific interest. Additionally, these findings constitute a further contribution to the field of host cell invasion mechanisms of apicomplexan parasites, a process that has proven to be extremely complex (Chen et al. 2005; Taubert et al. 2010; Hermosilla et al. 2012).

In summary, we present first preliminary evidence that PCEC5 and, perhaps PCEC2, can hinder active sporozoite invasion and further *E. ninakohlyakimovae* development in primary caprine endothelial cells, most likely by competing for a CUVEC receptor required for sporozoite invasion. The molecular identification of critical host cell receptor(s) as well as *E. ninakohlyakimovae* sporozoites ligand(s) involved in host cell invasion will provide better understanding of this process.

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Fig. 1.

Inhibition of *E. ninakohlyakimovae* sporozoite invasion of CUVEC by peptides from the phage library. After four panning rounds, a total of six cell endothelial clones (PCEC-1-2-3-4-5-6) were selected and tested in order to evaluate the inhibition of sporozoite invasion of CUVEC. Purified sporozoites of *E. ninakohlyakimovae* were mixed with each of the six selected phages and then added to confluent CUVEC flasks (SPZ + PCEC-1-2-3-4-5-6, cross panels). After 24-h incubation, the infection rate (percentage of infected cells) was determined for each phage and compared to those of negative controls (same CUVEC isolate and passage, infected with sporozoites but not with phages) (SPZ,

black panels). The statistical difference between the two replicates using Student's test is represented: *** $P < 0.001$)

Table 1

Sequence process was used to analyse 25 selected clones after fourth panning

Clone name	Frequency	Nucleotide sequence	Predicted peptide sequence
CEC1	7/25 (28 %)	GCATGCGACTCTTGTTCCCTATGCTAAGCTTTGCCAA	ACDSCSYAKLCQ
CEC2	4/25 (16 %)	CCTTGCAACCTCTTGTTCCCTATGCTAAGCTTGCCAA	PCNLLFLC*ACQ
CEC3	1/25 (4 %)	AAGTGTCTTACGCAGCAGCCGACGACGATGTGTGAT	KCLTQQPTTMCD
CEC4	1/25 (4 %)	GCTTGCCAGGTGTCAGCCTAAGGGTTAGTATGTTAG	ACQVSA*GLVC*
CEC5	1/25 (4 %)	TGCTGTACAGCACGCGTGAGGTACGCATGTGTAAG	CCHSTREVRMCK
CEC6	1/25 (4 %)	AGCTGCGTTGGACTCTCTTCAGGCTAAGCTTGCCAA	SCVGLSSG*ACQ
CEC7	1/25 (4 %)	TTGTGTTGCCGGTTTGCGTCGCGTTCGTCTTGTTGT	LCCRFASRSSCC
CEC8	1/25 (4 %)	CACTGTAGGATTCCGCCTTCGCAGACTGTTGTGAG	HCRIPPSQTVCE
CEC9	2/25 (8 %)	GCGTGTGCGAAGTATTTGCCTACTATTCCTTGTCG	ACRKYLPTIPCP
CEC10	1/25 (4 %)	CCGTGTCAGAATCGGCTTCCTAGGTATATTTGTTCT	PCQNRLPRYICS
CEC11	1/25 (4 %)	CTGTGTGAGCATACTTTGACTATTCTTGCTTGTTAG	LCEHTLTILAC*
CEC12	1/25 (4 %)	CCTTGTTTGATGCTGGCTACTTAGGCGAGTTGTCCT	PCLMLAT*ASCP
CEC13	1/25 (4 %)	TTCTGTCCTAGGCTTGAGGACTAAAGACTTTGTCAT	FCPRLED*RLCH
CEC14	1/25 (4 %)	GCCTGTAGTTATGTACGGTTCGTTAGCCGTGTATG	AC*LCTVR*PCM
CEC15	1/25 (4 %)	CCTTCTGCAGGACAGTCCTTAGCTAAGCTTTGCCAA	PCAGQSLAKLCQ

Fifteen different groups of nucleotide and peptide sequences were found; this represent 60 % of variability of the selected phages encoded. CEC1 and CEC2 clones were the most frequent with 28 and 16 %, respectively; the rest were recovered only once (4 %)