Japanese Journal of Veterinary Research 60(1): 23–25, 2012

INFORMATION

Hokkaido University conferred the degree of Doctor of Philosophy on December 26, 2011 to 2 recipients.
The titles of theses and other information are as follows:

Clathrin-mediated endocytosis of mammalian erythroid AE1 anion exchanger regulated by the tyrosine-based YXXΦ motif in the N-Terminal stretch

Chen-Chi Wang

Laboratory of Molecular Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

AE1 is the most abundant integral membrane protein in red cells and is essential for maintaining red cell mechanical stability. However, the mechanism for the assembly of AE1 into the membrane skeletal network remains unknown. The purpose of the present study was to explore the intramolecular signal(s) which participates in vesicular transport and endocytic recycling of AE1. To address this question, several mutants of murine AE1 tagged with an N-terminal EGFP and an extracellular FLAG epitope inserted adjacent to the *N*-glycosylation site (EGFP-mAE1Flag) were prepared, and their expression was analyzed in several different cell lines by confocal laser microscopy, biotinylation, and deglycosylation.

The present study demonstrated that EGFP-mAE1Flag was rapidly internalized, in association with the endocytosis of transferrin and the endogenous AE1 chaperone-like protein GPA in K562 cells. The internalized EGFP-mAE1Flag exhibited localization in the endocytic recycling compartment and the Golgi apparatus. Disruption of the conserved Y72VEL sequence in the N-terminal stretch sequence markedly reduced the internalization and increased the relative abundance of cell-surface AE1, suggesting that the conserved N-terminal YXXΦ-type sequence motif conferred clathrin-mediated endocytic recycling on AE1 as a fundamental characteristic. However, substitution of the N-terminal region from bovine AE1 that lacks the relevant motif for the corresponding region had less of an effect on internalization. Interestingly, deletion or substitution mutations of the Y7EDQL sequence in the bovine N-terminal stretch resulted in the decreased internalization of the AE1 proteins in transfected HEK293 cells.

Cell surface biotinylation and deglycosylation studies showed that approximately 30% of the cell-surface EGFP-mAE1Flag and several other mutants was sorted to the plasma membrane without *N*-glycan maturation in the Golgi apparatus in K562 and HEK293 cells. These findings demonstrate that the processing of *N*-glycan is not a prerequisite to cell surface expression of AE1 and suggest that the endocytic recycling to the Golgi of EGFP-mAE1Flag contributes in further processing of *N*-glycan on the protein after the initial sorting to the plasma membrane.

These findings demonstrate that the conserved YXXΦ sequence or a noncanonical YXXXΦ sequence in the N-terminal stretch facilitates the endocytic recycling of erythroid

AE1 through a clathrin-mediated pathway. Taken together with the previous findings on changes in the cell surface expression and the oligomer formation of AE1 and its interaction with other membrane proteins, the present study suggests that the regulation of YXXΦ motif-dependent endocytosis by interacting proteins including ankyrin is involved in the mechanisms for stable incorporation of AE1 into the plasma membrane during erythroid cell maturation.

The original papers of this thesis appeared in *Jpn. J. Vet. Res.* **59**: 157–164 (2011) and *J. Vet. Med. Sci.* **74**: 17–25 (2012).

Molecular genetic studies on *Ehrlichia ruminantium* for the development of effective prevention strategies against heartwater

Ryo Nakao

Division of Collaboration and Education, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

Ehrlichia ruminantium is a tick-borne intracellular rickettsia that causes heartwater in ruminants. The disease is responsible for significant economic losses in endemic areas such as sub-Saharan Africa and several Caribbean islands, but there is no effective control method available. This thesis describes three molecular genetic approaches for the development of effective prevention strategies against heartwater. First, determinants of bacterial virulence were investigated by comparing virulent and attenuated strains on a genome-wide scale. Second, genetic characterisation of strains from geographically diverse origins and field samples collected in heartwater endemic areas in Uganda was conducted using two different methods, namely multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA). Third, loop-mediated isothermal amplification (LAMP) assays were developed for rapid, simple genetic detection of *E. ruminantium*.

The Gardel strain of *E. ruminantium*, which was originally isolated as a highly virulent strain in Guadeloupe (French West Indies), was attenuated by serial passage in mammalian cell culture for use as a live attenuated vaccine; however, the genetic basis for the reduced virulence of this strain remains unknown. In Chapter I, the genomic sequences of the virulent and attenuated Gardel strains were compared using massively parallel sequencing technology. Comparative genome analysis identified only 16 genomic differences between the virulent and attenuated strains, including one large deletion, five single nucleotide polymorphisms and 10 single bp deletion/insertion polymorphisms. These findings might provide novel insights into the genetic basis of *E. ruminantium* virulence and contribute to the rational design of future vaccines. Transcriptional and translational analysis may be useful to further narrow down the candidate genes in the future.

A better understanding of the population genetics of different *E. ruminantium* strains is needed for the development of novel diagnostic tools, therapeutics and prevention strategies. Specifically, the development of effective

vaccination policies relies on the proper genotyping and characterisation of field isolates. In Chapter II, a recently developed MLST scheme was applied to a panel of reference strains of geographically diverse origins and field samples of *E. ruminantium* obtained from its vector, *Amblyomma variegatum*, in heartwater-endemic areas in Uganda. Only a limited MLST dataset comprising geographically restricted isolates was available from previous reports, and this study expanded the number of allele variants known for each locus. In addition, strong evidence was obtained for the occurrence of recombination events among different strains, suggesting that recombination may play a significant role in maintaining the genetic diversity of *E. ruminantium*. The compilation of MLST data across the African continent will be particularly valuable for understanding the existing genetic diversity of field isolates in African countries where cross-border transport of domestic animals occurs.

To develop a highly discriminatory and cost-effective genotyping method, tandem repeat markers were explored in Chapter III. A multiplex PCR approach combined with capillary electrophoresis separation enabled rapid, precise, and high-throughput typing of each VNTR marker. When tested with *E. ruminantium*-infected *A. variegatum* collected in Uganda, the discriminatory power of MLVA was greater than that of *map1* PCR-restriction fragment length polymorphism analysis. The combination of MLVA with clustering analysis allowed the identification of the genetic structure of *E. ruminantium* populations in the study areas. The high discriminatory power and cost effective performance of MLVA provides the potential for this technique to be used in optimising vaccine trials by identifying local strain diversity, and also raises the possibility of exploring the association between *E. ruminantium* genotypes and phenotypes such as pathological outcome in the ruminant host.

Lastly, Chapter IV describes the development of LAMP assays that allow rapid, sensitive, and specific detection of *E. ruminantium*. Two sets of LAMP primers were designed from the pCS20 and *sodB* genes and successfully amplified DNA from strains of geographically diverse origins. Although LAMP reactions were inhibited in the presence of extracts from blood and ticks, the diagnostic sensitivity of LAMP was higher than that of conventional PCR when tested with field-collected ticks. Since LAMP requires minimal time and equipment, this technique can potentially be used in resource-poor settings where heartwater is endemic. The lack of cross-reactivity with closely related *Ehrlichia* species enhances its utility for active screening in areas threatened with the introduction of the disease.

The work described in this thesis contributes to the development of effective prevention strategies against heartwater, including rational design and appropriate formulation of vaccines. Comprehensive information on the degree of cross-protection between strains and the differences in pathological outcomes in the ruminant host would be useful for further understanding of possible relationships between genotypes and phenotypes, which provides novel insights into the genetic basis of *E. ruminantium* virulence.

The original papers of this thesis appeared in *BMC Microbiol.*, **10**: 296 (2010), *Parasit. Vectors*, **4**: 137 (2011), and *Parasitology*, **5**: 1–14 (2011).