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Conservation of a platelet activating domain of Aggrus/podoplanin as a platelet aggregation-inducing factor

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Abstract

Human Aggrus/podoplanin is an identified platelet aggregation-inducing factor of cancer cells, which is also known as a specific marker of lymphatic endothelium. Human Aggrus was known to be expressed in seminoma, squamous cell carcinoma, malignant mesothelioma, sarcomas and several brain tumors. In our previous studies, the sialylated *O*-glycan of human and mouse Aggrus were shown to be critical for its platelet aggregation-inducing activity in the experiments using the glycosylation-deficient Chinese hamster ovary (CHO) cell lines. We newly cloned Aggrus homologues from rat, hamster, dog and bovine cDNAs, in addition to the human and mouse cDNAs, and confirmed there are three tandem repeats of the platelet aggregation-stimulating (PLAG) domain in Aggrus, which were conserved in all homologues. We found that bovine Aggrus has a sporadic deletion mutation in the first PLAG domain, and lacks platelet aggregation-inducing activity. We introduced point mutation in the PLAG domain of Aggrus and showed that either the first or last PLAG domain is critical for activity, but not the middle domain. In addition, we studied the molecular evolutionary process of the PLAG domain of Aggrus. The PLAG domain and its activity appeared after the divergence of avians and mammals. In conclusion, we provide evidence that Aggrus homologues conserved the segment of EDxxVTPG in their extracellular domain which are critical for their platelet aggregation-inducing activities.

Keywords: Platelet aggregation; PLAG domain; Homologous gene

1. Introduction

Platelet aggregation is known to be related to cancer metastasis. Our previous studies have clarified that membranous 44-kDa and 36-kDa sialoglycoproteins, respectively of cancer cells of mice and humans, aggregated platelets with no relation to plasma components (Kato et al., 2003; Kaneko et al., 2004). Ectopic expression of these molecules, designated as Aggrus, was observed in various cancer cells, including testicular tumors, squamous cell carcinomas, mesothelioma, and brain tumors, in which up-regulated expression was evident (Kato et al., 2003, 2004, 2005; Kimura and Kimura, 2005; Martin-Villar et al., 2005; Mishima et al., 2006a,b; Wicki et al., 2006). Unique characteristics of Chinese hamster ovary (CHO) mutant cell lines Lec1, Lec2 and Lec8 revealed that sialylated *O*-glycan is critical for platelet aggregation-inducing activity (Kaneko et al., 2004).

Homologous molecules to Aggrus have been identified independently in several mammals: T1α (Dobbs et al., 1988; Rishi et al., 1995), podoplanin (Breiteneder-Geleff et al., 1997) and PA2.26 (Gandarillas et al., 1997) are isolated from rat alveolar type-I cells in lung, rat glomerular epithelial cells (podocytes) or mouse keratinocytes. Other homologues include the canine receptor for the influenza C virus gp40 (Gandarillas et al., 1997) and its human homologue gp36 (Zimmer et al., 1999) and RANDAM-2 (Kotani et al., 2003), the membrane glycoprotein expressed in neuronal

Abbreviations: CHO, Chinese hamster ovary; PLAG, platelet aggregation-stimulating; PCR, polymerase chain reaction; RT, reverse transcriptase; ORF, open reading frame; PRP, platelet-rich plasma; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; ECL, enhanced chemiluminescence kit; ERM, ezrin, radixin, moesin; WT, wild type; EST, expressed sequence tag; HMM, Hidden Markov Model; PAGE, polyacrylamide gel electrophoresis.

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cells during mouse neurogenesis. Podoplanin is used as a specific marker for lymphatic endothelium in histopathology (Breiteneder-Geleff et al., 1999). Ramirez et al. showed that T1α null mice, generated by a targeted dysfunction of the gene, died at birth of lethal respiratory failure accompanied by immature lymphatic vessel formation (Ramirez et al., 2003; Schacht et al., 2003). Furthermore, T1α/podoplanin conceivably plays an important role in regulating peripheral lung cell proliferation and lymphatic vascular development.

In the present study, we cloned *aggrus* homologues from rat, hamster, dog and bovine cDNAs, in addition to human and mouse *aggrus*, and examined platelet aggregation-inducing activity. All Aggrus homologues, except for the bovine homologue, showed platelet aggregation-inducing activity. We also conducted evolutionary analysis of the *aggrus* genes.

2. Materials and methods

2.1. cDNA cloning of aggrus genes and induction of point mutations

The cDNA encoding the full-length open reading frame (ORF) of rat and dog *aggrus* was obtained by PCR using rat lung cDNA (OriGene Technology Inc., Rockville, MD) and cDNA derived from the Madin-Darby Canine Kidney cell line (American Type Culture Collection) as templates (GenBank Accession Nos. U96449 and Z81018). We designed degenerate primers with reference to alignment of those *aggrus* genes (human, mouse, rat and dog) to obtain other mammalian *aggrus* genes. The primer set for *aggrus* was as follows: *Hin*dIII-AGR-dF1, 5′-ccaagcttATGTGGAVSGYGYCAGYBYTGYTCT-3′ and *Eco*RI-AGR-dR487 5′-gggaattcGGGCGAGWACCTTCCHGAMATYTT-3′. Total RNA was extracted from a hamster: testis; a bovine: lung, colon and muscle. The RNA was isolated from each organ using a QIAGEN RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The accession numbers of hamster and bovine *aggrus* sequences are AB205159 and AB205160, respectively. To assess the platelet aggregation-inducing activity, the full-length cDNAs of human, mouse, hamster and bovine *aggrus*, incorporated with FLAG-tag sequence, were subcloned into pcDNA3 vector (Invitrogen Corp., Carlsbad, CA), or rat and dog *aggrus* were subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen). Substitution of the appropriate amino acid codons to alanine codons in *aggrus* cDNAs was accomplished using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

2.2. Recombinant aggrus expression on CHO cell lines

CHO cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Biocell, Carson, CA), 2 mM L-glutamine (Invitrogen), 100 U/ml of penicillin (Banyu pharmaceutical, Tokyo), and 100 μg/ml of streptomycin (Meiji-Seika, Tokyo). Constructed vectors were transfected into CHO cells by the lipofectamine method (Invitrogen) according to the manufacturer's instruction. For rat, hamster, dog and bovine *aggrus*, stable transfectants were obtained using a selective culture medium containing geneticin (G418; Sigma-Aldrich, St. Louis, MO) at a concentration of 1.0 mg/ml for a couple of weeks. Human, mouse and mock transfectants were transiently obtained.

2.3. Platelet aggregation

Mouse platelet-rich plasma (PRP) was prepared from fresh, heparinized blood extracted from BALB/c mice. An aliquot of 200 μl of PRP was incubated in a cuvette at 37 °C under continuous stirring. After 5 min, 5 μl of phosphate buffered saline (PBS)-washed cells were added. Then light transmittance was monitored at 660 nm for 15 min. This assay was performed with NKK HEMA Tracer I (Niko Bioscientific Co., Tokyo, Japan).

2.4. Western blot analysis

Cultured cell pellets were lysed with a cell lysis buffer (25 mM Tris, 50 mM sodium chloride, 0.2% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2% nonidet P-40, 50 μg/ml aprotinin, and 1 mM phenyl methylsulfonyl fluoride; it was pH 7.4) for 30 min on ice. Western blotting was performed as described previously (Kaneko et al., 2004). For detection of rat and dog Aggrus combined with His tag were performed with anti-His tag antibody (Medical Biological Laboratories Co., Ltd., Nagoya, Japan), whereas hamster and bovine Aggrus with FLAG-tag were done by an anti-FLAG M2 antibody (Sigma-Aldrich). The blotting results were developed using an enhanced chemiluminescence kit (ECL, Amersham biosciences corp., Piscataway, NJ).

2.5. Molecular evolutionary analysis

Multiple alignment was constructed by the CLUSTAL W program (Thompson et al., 1994), and illustrated with MacBoxshade, which was also used to calculate sequence identity and similarity. All Aggrus sequences were analyzed using the NetOGlyc neural network for the identification of *O*-linked glycosylation sites (http://www.cbs.dtu.dk/netOglyc/) (Hansen et al., 1998; Julenius et al., 2005). For constructing a phylogenetic tree, we assumed three topologies as follows: topology 1; ((((mouse, rat), hamster), bovine), human, dog), topology 2; ((((mouse, rat), hamster), human), dog, bovine), and topology 3; ((((mouse, rat), hamster), dog), human, bovine), where rodents form a cluster for all three topologies. The maximum likelihood method with JTT model (Jones et al., 1992) was used to compare these topologies. The CODEML program for amino acid sequence data in the PAML package (Yang, 1997) was used for the estimation. Although the topology 1 showed the highest maximum likelihood value (−1675.628), this is not statistically significant from topologies 2 (−1678.189±3.564) and 3 (−1676.649±4.114). Thus, we used topology 2 as a plausible tree, because human and rodents (both belong to Euarchontoglires) form a cluster.

3. Results

3.1. Cloning and sequencing of aggrus homologues

We previously subcloned human and mouse *aggrus* genes (Kato et al., 2003). In this study, we newly subcloned and seqenced

rat, hamster, dog and bovine *aggrus*. Fig. 1A showed the multiple alignments of six amino acid sequences of Aggrus. At the amino acid level, identity was determined between human and rodents (means 46%), human and dog (64.5%) or human and bovine (38.6%). Although overall homology was poor, the PLAG domain and transmembrane domain were highly conserved. Fig. 1B shows a schematic representation of the Aggrus protein structure. All Aggrus homologues belong to a type-I transmembrane sialomucin-like glycoprotein that consists of an extracellular domain with three repeats of the PLAG domain, abundant serine and threonine

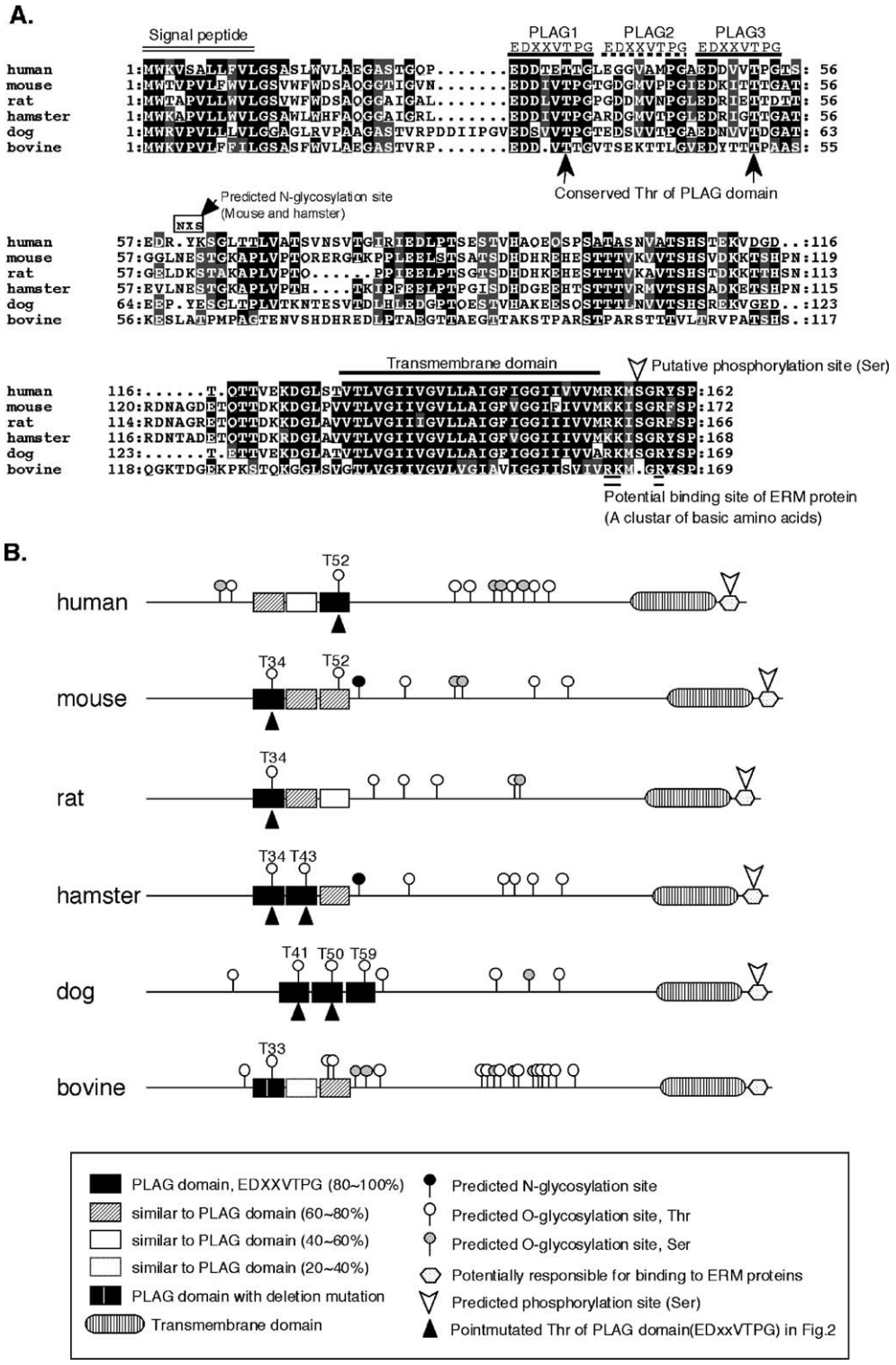

Fig. 1. Comparison of the amino acid sequences of various Aggrus homologues. (A) Multiple alignment of the amino acid sequences. Completely conserved residues are shaded black; conserved substitutions are shaded gray. (B) Schematic representation of the PLAG domain, transmembrane and the predicted glycosylation sites of Aggrus. The PLAG domain is indicated as a box. Box pattern indicated the degree of PLAG domain homology.

residues as potential *O*-glycosylation sites, a single transmembrane portion and a short cytoplasmic tail. The cytoplasmic tail contained potentially binding site of ezrin, radixin, moesin (ERM) protein and potential protein kinase A/protein kinase C-dependent phosphorylation serine residue (Martin-Villar et al., 2005). Bovine Aggrus lacks the potential phosphorylation serine residue. Using NetOGlyc 3.1, we predicted a potential *O*-glycosylation site of these Aggrus sequences. The threonine residues in the PLAG domain were inferred to be *O*-glycosylation sites. Subsequent sequences contained a high proportion of potential *O*-glycosylation

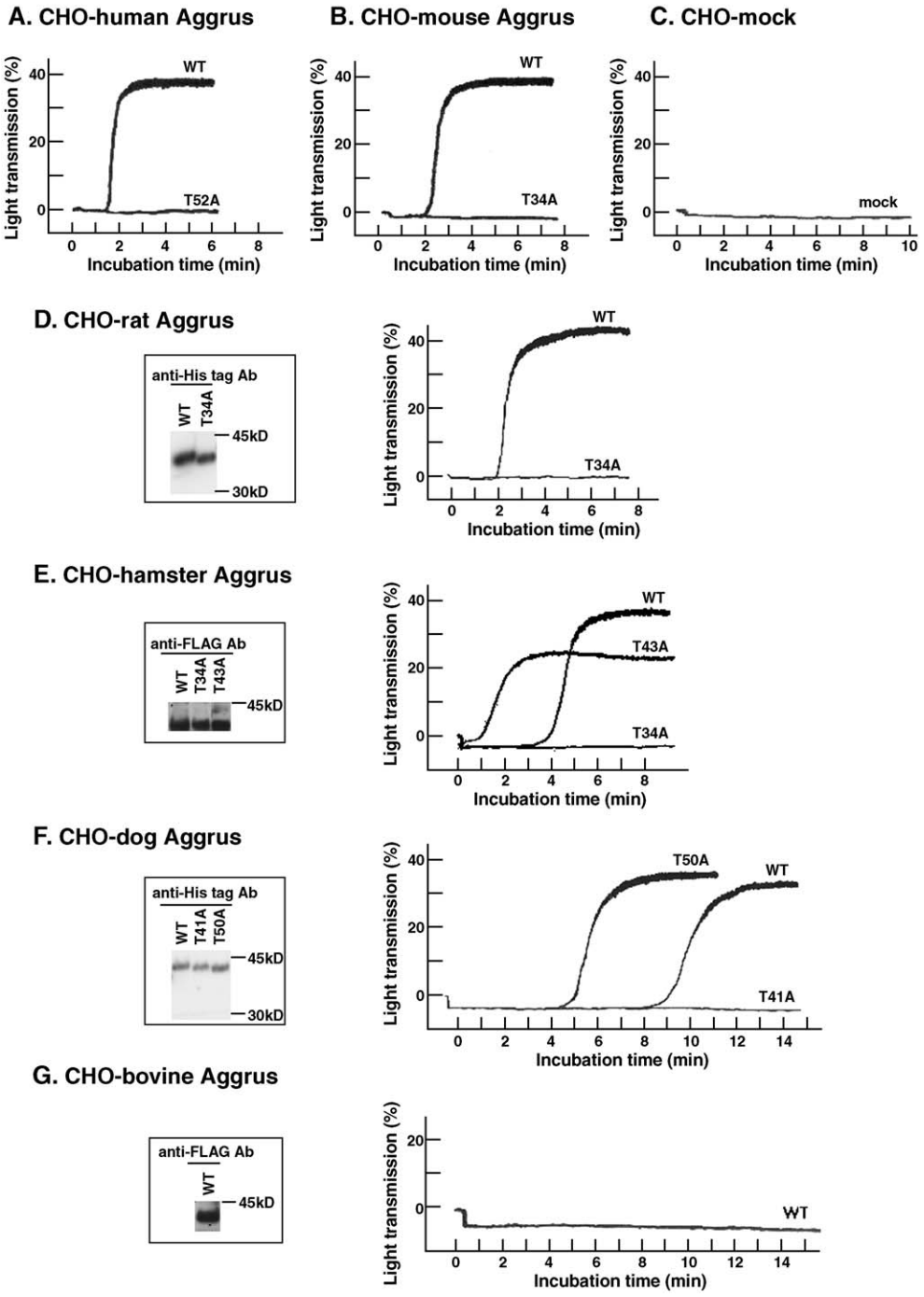

Fig. 2. Platelet aggregation-inducing activity of transfected cells. Mouse platelet-rich plasma (PRP) was prepared from fresh, heparinized blood extracted from BALB/c mice. An aliquot of 200 μl of PRP was incubated in a cuvette at 37 °C under continuous stirring with transiently transfected (A) human Aggrus WT, T52A; (B) mouse Aggrus WT, T34A; (C) mock transfectant, or with stably transfected (D) rat Aggrus WT, T34A; (E) hamster Aggrus WT, T34A, T43A; (F) dog Aggrus WT, T41A, T50A; (G) bovine Aggrus WT. Then light transmittance was monitored at 660 nm for 15 min. This assay was performed with NKK HEMA Tracer I. Transfected cell pellets were lysed with a cell lysis buffer for 30 min on ice. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 10% constant gels. After proteins were transferred onto immobilon polyvinylidene difluoride membrane, the strips were incubated with anti-His tag antibody for rat and dog Aggrus, or with FLAG-tag antibody for hamster and bovine Aggrus. The blotting results were developed using an enhanced chemiluminescence kit.

serine and threonine residues. However, neither the exact number nor the positions of glycosylated residues seem to be conserved among human, rodents, dog and bovine Aggrus.

3.2. Confirmation of platelet aggregation-inducing activity of Aggrus homologues

Expression of recombinant molecules was confirmed by Western blot analysis in Fig. 2. We previously found out the platelet aggregation-inducing activity of human and mouse Aggrus (Fig. 2A and B; Kato et al., 2003). In this study, wild type (WT) of rat, hamster and dog Aggrus, which were expressed on CHO cells, respectively, induced platelet aggregation in the same way (Fig. 2D, E and F). However, wild type bovine Aggrus did not induce platelet aggregation (Fig. 2G). The bovine Aggrus has a sporadic deletion mutation in amino acid sequence of the PLAG domain (refer to Fig. 1).

Previously, we generated point mutants of human-T52A and mouse-T34A Aggrus, which did not induce platelet aggregation-inducing activity (Fig. 2A and B; Kato et al., 2003). In this study, we newly generated several point mutants of *aggrus* homologues. Mutant Aggrus (rat-T34A, hamster-T34A and dog-T41A) did not induce platelet aggregation, but mutant hamster (hamster-T43A) and dog Aggrus (dog-T50A) did (Fig. 2D, E, and F). These results indicated that the most conserved PLAG domain, either the first or the last, was critical for activity, whereas the middle one did not affect platelet aggregation-inducing activity.

3.3. Molecular evolutionary analysis

We constructed a phylogenetic tree of six mammalian Aggrus amino acids sequences (Fig. 3). The rodents and the bovine lineages showed longer branches in contrast with the human and the dog lineages. The longer branch of rodents results from higher rates of amino acid substitution in rodents (Gu and Li, 1992). It is likely that some kinds of functional changes are related to higher rates of amino acid substitution in the bovine lineages. In fact, bovine Aggrus did not induce platelet aggregation, as shown in Fig. 2G, probably because of a sporadic deletion mutation in amino acid sequence of the PLAG domain.

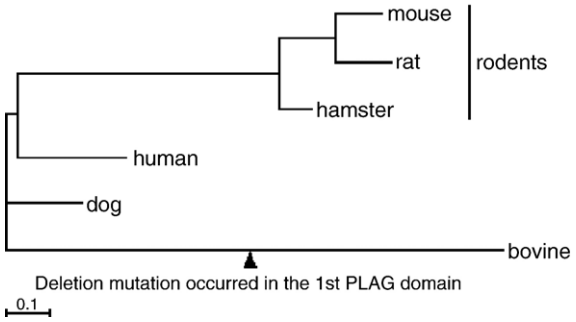

Fig. 3. A phylogenetic tree of *aggrus* genes. Rates of amino acid substitutions were calculated by using JTT model with gamma correction. An estimated gamma shape parameter ($\alpha = 1.6$) was used for the gamma distribution. A root was not defined and showed by trichotomy style of bovine, dog, and human–rodents.

Furthermore, we found a similar gene in chicken expressed sequence tag (EST) database using the TBLASTN algorithm. A putative chicken orthologue had neither a PLAG domain nor *O*-glycosylated sites (data not shown). It suggests that the PLAG domain arose and was triplicated before the appearance of mammals. Speciation of mammals might have occurred after the domain was triplicated. Then, a deletion mutation of the first PLAG domain happened in the bovine lineage (Fig. 3).

4. Discussion

We cloned rat, hamster, dog and bovine *aggrus*, in addition to previously cloned human and mouse *aggrus*. Human, rodents and dog Aggrus exhibit similar structural and biochemical characteristics and likely share an identical function: platelet aggregation-inducing activity. Unexpectedly, bovine Aggrus showed no activity. The introduced point mutations were located in the last PLAG domain for human Aggrus and the first PLAG domains for rodents and dog Aggrus, which were the most conserved part among three PLAG domains. Furthermore, we introduced point mutation in hamster-T43A (the middle domain) and dog-T50A (the middle domain). Human-T52A, mouse-T34A, rat-T34A, hamster-T34A and dog-T41A showed no platelet aggregation-inducing activity, but hamster-T43A and dog-T50A did. These results indicated that the most conserved PLAG domain is critical for activity. However, the middle PLAG domain did not affect their activity, even though the PLAG sequence is highly conserved. It will be interesting to investigate the tertiary structure of the three tandem PLAG domains, which might yield important information for exploitation in a novel anti-coagulant.

Fig. 1B shows that the first (rodents, dog and bovine) or the last (human and dog) PLAG domain was highly conserved. The middle domain seems to be released from evolutional pressure. For that reason, mutations were accumulated in the middle domain. However, in the case of rodents and dog Aggrus, the middle PLAG domain was also conserved: it is speculated to be overwritten with the sequence of first PLAG domain by gene conversion or crossing-over.

Using the TBLASTN algorithm, we found that mouse Homeobox protein Nkx-3.2 has a PLAG sequence — EDxxVTPG. An evolutionary conserved homologue of the Drosophila bagpipe homeobox gene, Nkx-3.2, was expressed in the splanchnic mesoderm and the embryonic skeleton. Furthermore, using profile Hidden Markov Model (profile HMM) method, we found a PLAG domain-containing sequence in Neurocan. The profile HMM method turns a multiple sequence alignment into a position-specific scoring system that uses databases to search for remote homologous sequences (Eddy, 1998). Neurocan is a core protein of a chondroitin sulfate proteoglycan; it is abundant in fetal brain tissue. It is inferred to be involved in modulation of cell adhesion and migration. Both Nkx-3.2 and Neurocan seem to be unrelated to platelet aggregation-inducing activity. We think that the platelet aggregation-inducing activity is not the only function of Aggrus. This newly identified PLAG domain might have a novel biological activity.

To our knowledge, this is the first detailed study of Aggrus PLAG domain. There are three tandem repeats of PLAG domain,

EDxxVTPG, in their extracellular domain of Aggrus/podoplanin homologues. Either the first or last PLAG domain is critical for activity, but not the middle domain. Furthermore, we studied the molecular evolutionary process of PLAG domain of Aggrus. It would lead us to understand the mechanism of the platelet aggregation, and help us to exploit anti-coagulant therapies.

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