Sheep (*Ovis aries*) integrins αvβ1 and αvβ6 related to foot-and-mouth disease virus infection: Molecular cloning, sequence analysis and comparison with homologues

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ABSTRACT

Four members of the αv integrin family of cellular receptors, αvβ1, αvβ3, αvβ6, and αvβ8, have been identified as receptors for foot-and-mouth disease virus (FMDV) in vitro, and integrins are believed to be the receptors used to target epithelial cells in the infected animals. To analyse roles of the αv integrins from a susceptible species as viral receptors, we have cloned sheep αv, β1, and β6 integrin cDNAs and compared them to those of other species. The coding sequences for sheep integrin αv, β1, and β6 were found to be 3147, 2397, and 2364 nuclotides in length, encoding 1048, 798, and 787 amino acids, respectively. The sheep αv, β1, and β6 subunits share many structural features including ligand binding domain and cysteine-rich region with homologues of other species. Phylogenetic trees and similarity analyses showed the close relationship of integrin genes among sheep, pigs, cattle and Bactrian camels that are susceptible to FMDV infection, which were distinct from the order Rodentia, Primates, Perissodactyla, Carnivora, Galliformes. We postulate that host tropism of FMDV may be related to divergence in integrin subunits among different species.

1. Introduction

Integrins are a family of cell adhesion receptors that bind to extracellular matrix ligands, cell-surface ligands, and soluble ligands. They are transmembrane α/β heterodimers and at least 18 α and eight β subunits that interact noncovalently at the cell surface are known in humans, generating 24 heterodimers. Integrins contribute to a variety of processes, including cell proliferation, morphology, migration, and apoptosis [1–3]. Integrin α and β subunits are totally distinct, with no detectable homology between them; sequence identity among α subunits is about 30% and among β subunits 45%, indicating that both the α and the β gene families evolved by gene duplication [4–6]. Each subunit is composed of large extracellular domains, a transmembrane region, and, in most cases, a short cytoplasmic domain. Half of the 18 different α subunits (αD, αE, αL, αM, αX, α1, α2, α10, α11) contain an I domain, also referred to as a von Willebrand factor A domain (A domain), and the head of the

integrin β subunits also appears to contain an A domain-like structure, including a characteristic metal ion-dependent adhesion site (MIDAS). When an A domain is present in an α subunit, it is nearly always the ligand-binding site. When the A domain is absent from the α subunit (α3, α4, α5, α6, α7, α8, α9, αv and α_{IIb}), then the ligand-binding site is typically formed by the β-propeller domain of the α subunit together with an A domain of the β subunit [3,7–9]. Of the 24 known integrins, eight integrins recognize tripeptide arginine-glycine-aspartic acid (RGD) as a binding motif sequence on their natural ligands, including αvβ1, αvβ3, αvβ6, αvβ8, αvβ5, α5β1, α8β1 and $\alpha_{IIb}\beta3$ [10,11].

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animal species including cattle, pigs, sheep, goats, camels, as well as more than 70 wildlife species and is characterized by fever, lameness, and vesicular lesions on the tongue, feet, snout, and teats [12,13]. The disease causes significant economic losses due to high morbidity and loss of production in infected animals. As a result, it is a major hindrance to international trade in animals and animal products [14–16]. The aetiological agent, foot-and-mouth disease virus (FMDV), is classified with the *Aphthovirus* genus as a member of the Picornaviridae family and exists as many subtypes and variants within seven different serotypes (A, O, C,

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Asia1, and South African Territories 1, 2, and 3) [17]. FMDV infects cells by attaching to integrin receptor through a long surface loop, the G–H loop of VP1, which contains a conserved RGD motif that is characteristic of the ligands of several members of the integrin family [18,19]. So far, FMDV has been found to use four members of integrins αvβ1, αvβ3, αvβ6, and αvβ8 as receptors to initiate infection in vitro [20–23]. In addition to integrins, the virus has been shown to utilize other receptors on cultured cells, such as the Fc receptor or heparan sulfate or an artificial single-chain antibody fused to intercellular adhesion molecule 1, these receptors do not require the RGD sequence [24–27]. Field viruses are dependent on integrin receptors to initiate infection in vitro, and integrins are believed to be the receptors used in the infected animals [28,29]. Several other viruses including adenovirus, human parechovirus type 1, coxsackievirus A9, echovirus type 9 have also been reported to utilize RGD-dependent integrins to initiate infection [30–33].

Clinical signs of FMD may be severe but are generally milder in sheep and goats than in pigs and cattle, it may therefore go unnoticed through out the infection, maintaining infectivity for a long period. Furthermore, sheep and goats may become carriers of FMDV for long periods of time [12,34–36]. The outbreak of FMD in the United kingdom (UK) in 2001 has provided a good example of how difficult it is to make a clinical diagnosis of the disease in sheep, where over 50% of samples from sheep were not confirmed as positive for FMDV, viral antigen or genome [37]. There are many examples of FMD outbreaks involved in sheep and goats. In 1983, FMD spread from Spain into Morocco with infected sheep. In 1989, FMD was introduced to Tunisia by infected sheep. Outbreaks also occurred in Turkey during 1995 and 1996, in which 20% of the total cases were associated with sheep and goats [14,38]. FMD serotype O outbreak in 1996 in Greece was suspected to be caused by illegally imported sheep from Turkey. In the Middle East and North Africa where small ruminants make up the most important livestock species, the FMDV O type is believed to have predilection for sheep [14,37]. These facts strongly suggest that sheep may play an important role in the epidemiology of FMD. However, despite these cumulative evidences, research concerning FMDV pathogenesis in sheep has been very limited in comparison to cattle and pigs. So far, little information is available for FMDV receptor in sheep, though integrins are important molecules in the susceptibility of cloven-hoofed animals to FMDV infection [19,28,29]. In this report, as the first step to understand the pathogenesis of FMDV in sheep, which would help understand the reasons for its mild clinical signs and persistent infection, we molecularly cloned the sheep αv, β1, and β6 subunit cDNAs for the first time and compared them to those of other species including order Artiodactyla, Primates, Perissodactyla, Carnivora, Rodentia, Galliformes.

2. Materials and methods

2.1. RNA isolation

Tongue and lung tissues were collected from four healthy domestic sheep (1–3 years of age) immediately after slaughter. Approximately 500 mg of each sample were kept in liquid nitrogen until use. Tissues were grinded thoroughly with an RNase-free, liquid-nitrogen–cooled mortar and pestle. Total RNA from each tissue was extracted with RNeasy Mini Kit (Qiagen, Germany) as described by the manufacturer. All animal experiments were perfomed according to protocols approved by the institutional committee for use and care of animals.

2.2. Amplification of cDNA

An aliquot of the total RNA (5 μg) was reverse transcribed by the AMV reverse transcriptase (20 U/μl, Takara, Japan) and the oligo-dT$_{18}$ primer (20 pmol/μl) and the random primer (20 pmol/μl) in a total volume of 40 μl reaction according to the manufacturer's instructions. The αv, β1 and β6 cDNAs were amplified from cDNAs of sheep lung and tongue tissues by PCR using primers based on bovine and other animal integrin sequences reported in GenBank (Table 1). Because we could not amplify the complete αv gene as a single fragment by using alphavF1 and alphavR2 primers located at the outside of the open reading frame, we designed two additional primers from the internal region of the αv gene, which will allow to amplify two fragments that share the overlapping sequence of 390 nucleotides between them, but no unique restriction enzyme site that could be used to join these fragments. We have reconstructed the complete sheep av gene that contains the open reading frame using fusion-PCR method. PCR was carried out in the total volume of 100 μl containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.25 mM MgCl$_2$, 0.2 mM dNTPs, 5 U of Taq polymerase (Takara, Japan), 40 pmol each of the primers and 10 μl of the cDNA sample. The procedures for PCR recommended by the manufacturer were used, with the following cycling parameters: 5 min at 95 °C for predenaturation, 35 cycles of 1 min at 95 °C, 30s at annealing temperatures depending on integrins to be amplified (Table 1) and 3 min at 72 °C, followed by the final extension for 10 min at 72 °C. The resulting PCR products were run on 1% agarose gel containing ethidium bromide and the DNA bands were visualized using a UV transilluminator.

2.3. Cloning and sequencing

The amplified products corresponding to integrin cDNAs were gel-purified using the Qiaquick Gel Extraction Kit (Qiagen, Germany). The purified products were ligated into the pGEM-T easy vector (Promega, USA), and the resultant recombinant plasmids

Table 1
Primers sequences used to cloning of integrin cDNAs from sheep.

Primers	Sequence (5′ to 3′)	Target gene	Size of PCR products	Annealing temperature
AlphavF1	5′-TCGGCGATGGCTTTTCCGCCGCG-3′	5′ part of integrin αv[1]	1.7 kb	56 °C
AlphavR1	5′-GTTTGTCTCTAAATTCAGATTCATCCC-3′			
AlphavF2	5′-AATGGATATCCAGACTTAATTGTAGG-3′	3′ part of integrin αv[2]	1.8 kb	55 °C
AlphavR2	5′-CAGTTAAGTTTCTGAGTTTCCTTC-3′			
Beta1F1	5′-CGGGAGAAGATGAATTTACAACTGA-3′	5′ part of integrin β1[3]	1.2 kb	54 °C
Beta1R1	5′-ATTCACCCCATTCTTGCAGTAAGAC -3′			
Beta1F2	5′-CCTTCTATTGCTCACCTTGTCCAG-3′	3′ part of integrin β1[4]	1.5 kb	59 °C
Beta1R2	5′-TCATTTTCCCTCATACTTCGGAT-3′			
Beta6F	5′- CTGAGACCGATGGCGATTGATCT-3′	Integrin β6	2.4 kb	58 °C
Beta6R	5′-CTTAAGGGTACCTTACTATCCATCCGT-3′			

1 and 2: the 5′ part and 3′ part of integrin αv overlap by 390 bp.
3 and 4: the 5′ part and 3′ part of integrin β1 overlap by 310 bp.