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IgA in the horse: cloning of equine polymeric Ig receptor and J chain and characterization of recombinant forms of equine IgA

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As in other mammals, immunoglobulin A (IgA) in the horse has a key role in immune defense. To better dissect equine IgA function, we isolated complementary DNA (cDNA) clones for equine J chain and polymeric Ig receptor (pIgR). When coexpressed with equine IgA, equine J chain promoted efficient IgA polymerization. A truncated version of equine pIgR, equivalent to secretory component, bound with nanomolar affinity to recombinant equine and human dimeric IgA but not with monomeric IgA from either species. Searches of the equine genome localized equine J chain and pIgR to chromosomes 3 and 5, respectively, with J chain and pIgR coding sequence distributed across 4 and 11 exons, respectively. Comparisons of transcriptional regulatory sequences suggest that horse and human pIgR expression is controlled through common regulatory mechanisms that are less conserved in rodents. These studies pave the way for full dissection of equine IgA function and open up possibilities for immune-based treatment of equine diseases.

INTRODUCTION

After the discovery of antibodies over a century ago, early studies in the horse made important contributions to the understanding of the mammalian adaptive immune system. However, large gaps still remain in our knowledge of the equine immunoglobulin (Ig) system and this is hampering development of specific vaccines and immune-based therapies for many major infectious diseases of the horse. Given the economic importance of the horse globally, it is vital to build a more detailed understanding of equine Ig function, as a key first step toward more effective options for treatment and prevention of equine diseases. A better understanding of the equine IgA (eIgA) system would seem especially important given the numerous equine infections that are manifest in, or gain a foothold at, the mucosal surface.¹ In addition, a wider knowledge of IgA systems in different mammals will provide invaluable insights into both the variety of functions mediated by this Ab class, and the evolution of the IgA system. Moreover, because there are limitations with mouse models of the IgA system (e.g., the mouse lacks the main Fc receptor (Fc α RI) responsible for IgA effector function), it is worthwhile developing a wider knowledge of the IgA systems of other mammals so that relevant animal models may

be identified. For these reasons, we sought to establish systems to facilitate molecular characterization of eIgA.

IgA is present in both the serum and mucosal secretions of the horse, and it is the principal Ig in milk, tears, and secretions of the upper respiratory tract.² In common with most other mammalian species, the horse has a single IgA heavy chain constant region gene (*IGHA*).³ In contrast, humans, along with chimpanzees, gorillas, and gibbons, express two subclasses of IgA (IgA1 and IgA2) encoded by distinct heavy chain constant region genes.

In mucosal secretions, IgA exists primarily as secretory IgA (SIgA). Transepithelial transport of IgA onto the mucosal surfaces is mediated by the polymeric Ig receptor (pIgR), a type I transmembrane glycoprotein. The pIgR, which is expressed on the basolateral surface of epithelial cells, binds to IgA, which has been produced by plasma cells in mucosal effector sites. This IgA is polymeric (pIgA), comprising two or more IgA monomers joined together by an additional 17 kDa polypeptide, the J chain. On binding, both receptor and ligand are internalized and transcytosed across a series of vesicular compartments to the apical plasma membrane. Here, the extracellular portion of the pIgR is cleaved to form secretory component (SC), which

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remains bound to pIgA as an integral part of the SIgA molecule. SC provides SIgA with increased resistance to bacterial proteases, and through its *N*-glycans mediates anchoring to the mucosal surface, which enhances the protective role of SIgA.⁴

SIgA acts as the “first line of immunological defense” against mucosal infection mediating defense through (i) immune exclusion at the mucosal surface, (ii) pIgA-mediated neutralization of pathogens during the process of transcytosis, and (iii) pIgR-mediated excretion of IgA-containing immune complexes formed within the submucosal tissues.⁵ In addition, SIgA participates in antigen presentation and sampling within mucosal tissues, regulation of commensal bacteria, and maintenance of mucosal integrity and homeostasis.⁶ In the horse, mucosal IgA responses seem to have an important role in protection from upper respiratory tract pathogens such as equine herpesvirus-1,⁷ equine influenza virus,⁸ and *Streptococcus equi*.⁹

The presence of J chain in pIgA is essential for interaction with pIgR and, in turn, delivery of pIgA onto mucosal surfaces is dependent on pIgR-mediated transepithelial transport. Hence, J chain and pIgR are critical components of the mucosal IgA system. Proteins consistent with the properties of an equine J chain and SC have been described,^{10,11} but neither these components nor their interaction with equine IgA has been well characterized. In this study, we describe the cloning of equine J chain and pIgR, the generation of recombinant forms of eIgA and SC, and analysis of their interaction. Furthermore, through genomic sequence analysis we provide an insight into both the chromosomal location and gene arrangement of J chain and pIgR, and the potential factors involved in regulation of pIgR expression.

RESULTS

Equine J chain and pIgR complementary DNA (cDNA) clones

The equine J chain cDNA isolated has an open reading frame of 474 bp (Genbank accession no. GQ981317) (Figure 1). The first 22 amino acids are predicted to encode a leader sequence,

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1/1                               31/11
ATGAAGAACCATTGTTTTCTGGGGAGTCCTGGCCATTTTGTTCAGGCTGTTCTTGTG
M K N H L F F W G V L A I F V Q A V L V
61/21 ↓                             91/31
ACAGCCGGAGATGAGGGTGAAGGATTGTTCTGGCTGACAACAAATGTAATGTGTCGGG
T A G D E G E R I V L A D N K C K C V R
121/41                             151/51
GTTACTTCCAGGATCATCCCTCTCTGAAAATCCTAATGAAGACATTCTGGAGAGACAC
V T S R I I P S P E N P N E D I L E R H
181/61                             211/71
ATCAGAATTATTCTGTGAACAGCAGAGAGAATATCTCTGATCCCACTCACCAGTG
I R I I I P V N S R E N I S D P T S P V
241/81                             271/91
AGAACCAAAATTTGTGTACCATTATCCGACCTCTGTAAAAATGTGATCCTGTAGAAGTG
R T K F V Y H L S D L C K K C D P V E V
301/101                             331/111
GAGCTGGATAATCAAGTAGTTACTGCCTCCAGCAAAATATCTGTGATGAAGAAAGTGAG
E L D N Q V V T A S Q S N I C D E E S E
361/121                             391/131
ACCTGTACCGTTATGACAGGAACAAGTGCTACACAAATAGGGTCCCACTTACCTATGGT
T C Y A Y D R N K C Y T N R V P L T Y G
421/141                             451/151
GGTCAGACCAAAATAGTGGAAACAGCCTTGACCCAGATTCTGCTATCTCTGACTAA
G Q T K I V E T A L T P D S C Y P D *

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Figure 1 Nucleotide sequence and amino acid translation of equine J chain. Start of the mature protein at Gly23 is highlighted and indicated by an arrow. The single conserved *N*-glycosylation acceptor site is underlined.

suggesting that the mature protein begins at Gly23 and comprises 136 amino acids. The equine J chain amino acid sequence showed a high degree of identity to other mammalian J chains, with 79, 80, and 74% identity to human, cow, and mouse proteins, respectively (Figure 2a). It shows features typical of

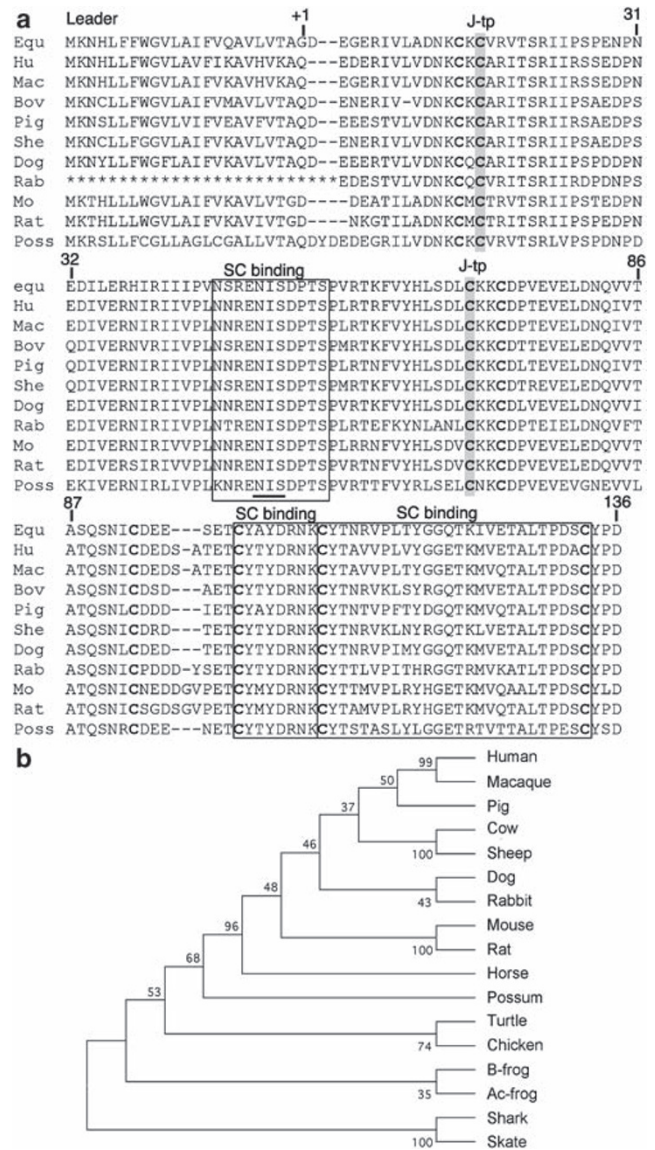


Figure 2 J chain sequence analysis. (a) Alignment of the amino acid sequences of mammalian J chains. Accession numbers and species abbreviations for the sequences used are as follows: human (Hu), NM_144646; rhesus macaque (Mac), CO646198; cow (Bov), NM_175773; pig (Pig), CJ021649; sheep (She), CN823995; dog (Dog), AY081058; rabbit (Rab), P23108; mouse (Mo), NM_152839; rat (Rat), XM_341195; and silver brushtail possum (Poss), AF091138. The rabbit sequence derives from amino acid sequencing of the protein and hence lacks leader sequence. The eight conserved Cys residues are shown in bold and those Cys residues involved in disulfide bonds with the IgA tailpiece are highlighted and marked J-tp. Regions implicated in binding to the secretory component (SC) are boxed. The conserved *N*-glycosylation acceptor site is underlined. (b) Phylogenetic analysis of the amino acid sequences of mammalian J chains (as above) and those from chicken (AB076374), red-eared slider turtle (AB085611), bullfrog (B-frog; AF157508), African clawed frog (Ac-frog; AF036547), nurse shark (AF516711), and clear-nosed skate (AF520475).

J chain such as a preponderance of acidic residues, a single conserved *N*-glycosylation site, and eight Cys residues, involved in intra- and interchain disulfide bond formation, conserved in all known mammalian J chain sequences. In common with a J chain protein isolated from reduced and alkylated equine IgM,¹¹ the predicted amino acid sequence for mature equine J chain lacks methionine. Phylogenetic analysis (**Figure 2b**) showed equine J chain to segregate with other mammalian sequences but to form a separate branch, reflecting the fact that the family equidae is a member of the order perissodactyla, rather than the order artiodactyla (even-toed ungulates), which includes cattle, sheep, and pigs.

EqpIgR cDNA comprised an open reading frame of 2,292 bp (Genbank accession no. GQ981318) (**Figure 3a**). The first 18 amino acids are predicted to encode an N-terminal leader sequence, indicating that the mature protein begins at Lys19 and comprises 746 amino acids. A comparison of eqpIgR with those of other mammalian species (**Figure 4**) reveals a high similarity in amino acid sequence and structural organization. Similar to other pIgRs, the eqpIgR comprises an N-terminal extracellular region of five Ig-like domains (D1–5) and one non-Ig-like domain (D6), a short membrane spanning region, and a long C-terminal cytoplasmic tail. Furthermore, the Ig-like domains of eqpIgR show the conserved Cys residues involved in intradomain disulfide bonds observed in pIgRs from other species.¹² The number of potential *N*-glycosylation sites differs between species and none of the sites are conserved in all species. There are four putative *N*-glycosylation sites in the eqpIgR compared with seven in human, eight in mouse, four in rat, three in bovine, and two in rabbit pIgR. The overall amino acid identity of the eqpIgR with human, bovine, and mouse proteins is 65, 63, and 57%, respectively. However, regions with functional significance, such as those involved in interaction with IgA, and regions of the cytoplasmic domain that govern receptor endocytosis and trafficking¹² are more highly conserved (see **Figure 4**). Phylogenetic analysis (**Figure 3b**) showed the eqpIgR to cluster with the cow, pig, and dog pIgR, but to form a separate branch, again illustrating phylogenetic distance from the even-toed ungulate family.

Purification and analysis of recombinant eqIgA (reqIgA)

An expression vector to drive expression of an α heavy chain (HC) comprising a mouse V_H domain followed by the C α 1, hinge, C α 2, and C α 3 domains of eqIgA was constructed. Transfection of mouse λ light chain (LC)-expressing CHO-K1 cells with this HC vector resulted in the expression of eqIgA, which bound its cognate antigen 3-nitro-4-hydroxy-5-iodophenylacetate (NIP) and was recognized by anti-mouse λ LC and anti-horse IgA antibodies. Analysis of affinity-purified reqIgA by size exclusion chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; **Figure 5a–c**) was consistent with covalently stabilized monomers (H₂L₂). Despite the absence of a Cys residue within the CH1 domain with which to form a HC–LC disulfide bond, LC and HC dimers were not observed. However, unlike human IgA, three Cys residues are present within the hinge region of eqIgA, the most N-terminal of which

may form a covalent bond with the LC. EqIgA purified from serum, which was included for comparison, ran predominantly as dimer with some monomer and higher polymer. This observation is consistent with previous reports that equine serum IgA is predominantly dimeric.^{10,13} Human IgA1 and IgA2 have two common *N*-glycosylation sites, one within the CH2 domain and the other within the C-terminal 18 amino acid tailpiece. These conserved *N*-glycosylation sites are present within eqIgA and treatment of recombinant monomeric eqIgA (reqmIgA) with *N*-glycanase to remove *N*-linked sugars (**Figure 5c**) resulted in a decrease in the size of the IgA HC, suggesting that these sites are occupied in eqIgA.

Transfection of the reqmIgA-producing CHO clone with the J chain expression vector resulted in the expression of IgA of multiple molecular forms (**Figure 5d–f**), including covalently stabilized monomer (H₂L₂), dimer (H₄L₄J), and larger polymers. This finding is consistent with previous studies of the coexpression of human IgA and J chain in mammalian cells, which have shown that production of IgA polymers is not 100% efficient.^{14,15} Generation of dimeric IgA (dIgA) and pIgA was consistent with J chain incorporation, although the J chain itself could not be directly identified because of lack of a suitable detection antibody. However, recombinant dimeric equine IgA (reqdIgA)-producing CHO clones were selected on the presence of antibody that could bind to human SC (hSC), for which the presence of J chain is an absolute requirement.¹⁶

Analysis of recombinant eqSC (reqSC)

To produce a recombinant form of the pIgR equivalent to SC, the eqpIgR cDNA was truncated by introduction of a stop codon immediately after that encoding Asp590 in D6. A potential cleavage site has previously been identified here (see **Figure 4**) and used for the truncation of recombinant human and mouse pIgR in earlier studies.^{14,17} Transfection of CHO-K1 cells with eqSC cDNA resulted in the secretion of eqSC protein into supernatant. Positive clones were selected on the basis of reqdIgA binding capacity, providing preliminary evidence that the recombinant form of eqSC was able to bind its dIgA ligand. SDS-PAGE analysis revealed that purified reqSC had a molecular mass of approximately 75 kDa (**Figure 6**) and was recognized by a mouse anti-human SC antibody (**Figure 6b**). reqSC migrated slightly faster than the recombinant form of hSC (rhSC), probably because of differences in glycosylation, as mentioned above. The recognition of reqSC by concanavalin A (data not shown) confirmed its glycosylation status. Both reqSC and rhSC were able to bind reqdIgA (**Figure 6c**) and recombinant human dIgA1 (rhIdIgA1; **Figure 6d**), consistent with earlier studies that have described the interspecies binding of SC and dIgA from human and other mammals.^{17,18}

Surface plasmon resonance analysis of dIgA–SC interactions

Injection of 20 nM of reqdIgA or rhIdIgA1 over immobilized NIP-bovine serum albumin gave a response of ~400 RU with a variation between cycles of < 10 RU. Subsequent binding of reqSC to both the equine and human form of dIgA fitted a 1:1 Langmuir binding model (**Figure 7a–c**), in keeping with

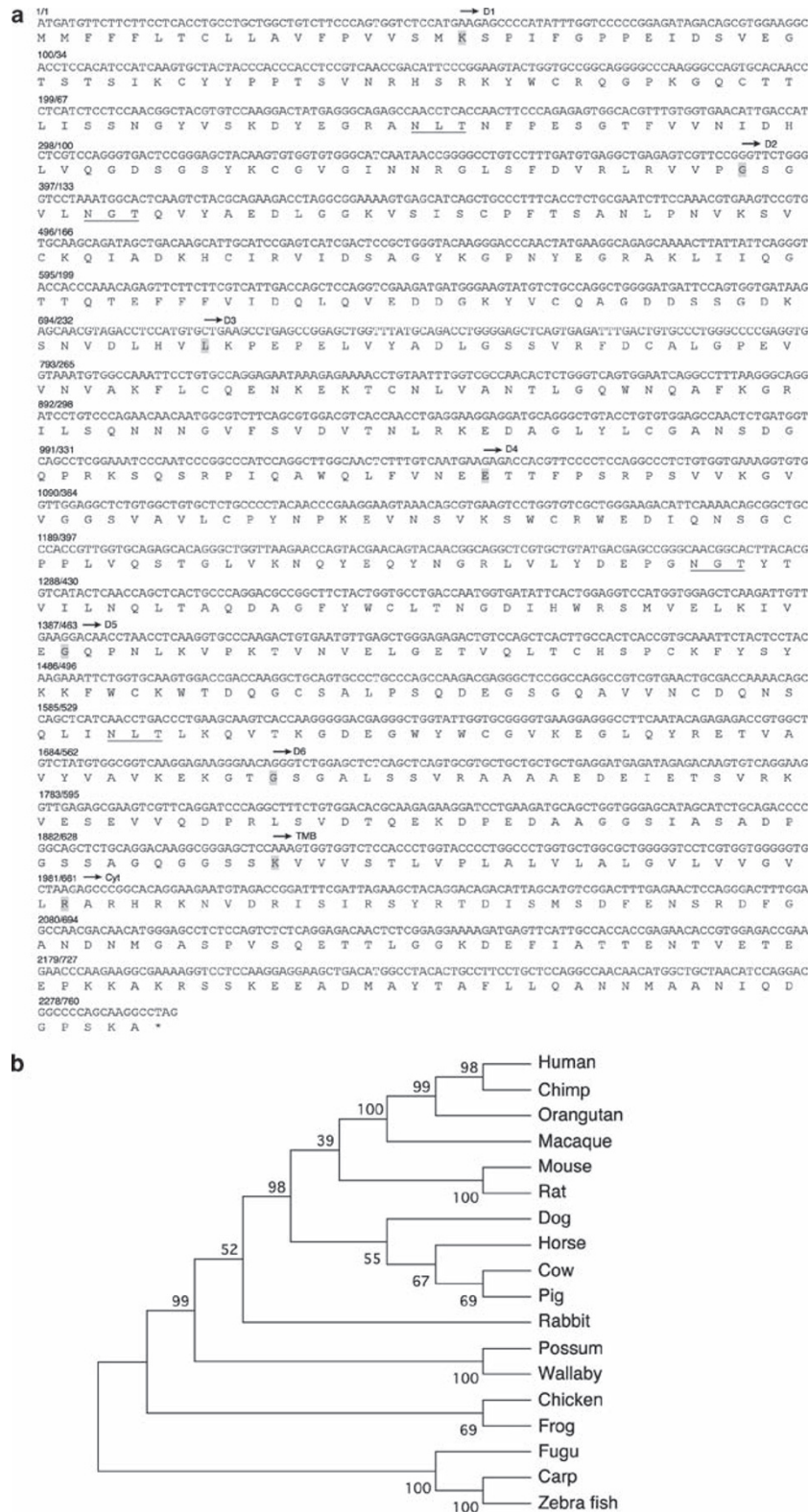


Figure 3 Polymeric Ig receptor (plgR) sequence analysis. **(a)** Nucleotide sequence and amino acid translation of equine plgR. Start of the mature protein at Lys19 is highlighted and indicated by D1. The start of each of the extracellular domains, D1–6 and the transmembrane (TMB) and cytoplasmic (Cyt) domains are highlighted and labeled. Putative *N*-glycosylation acceptor sites are underlined. **(b)** Phylogenetic analysis of the amino acid sequences of various mammalian plgRs. Accession numbers of the sequences used are as follows: human, X73079; cow, X81371; pig, AB032195; dog, AY081057; rabbit, X00412; mouse, NM_011082; rat, NM_012723; silver brushtail possum, AF091137; tammar wallaby, AF317205; chimpanzee, XM_514153; orangutan, CR859163; rhesus macaque, XM_001083307; chicken, XM_417977; African clawed frog, EF079076; Fugu, AB176853; and zebra fish, XM_689741. The sequence for common carp was from Rombout *et al.*⁵³

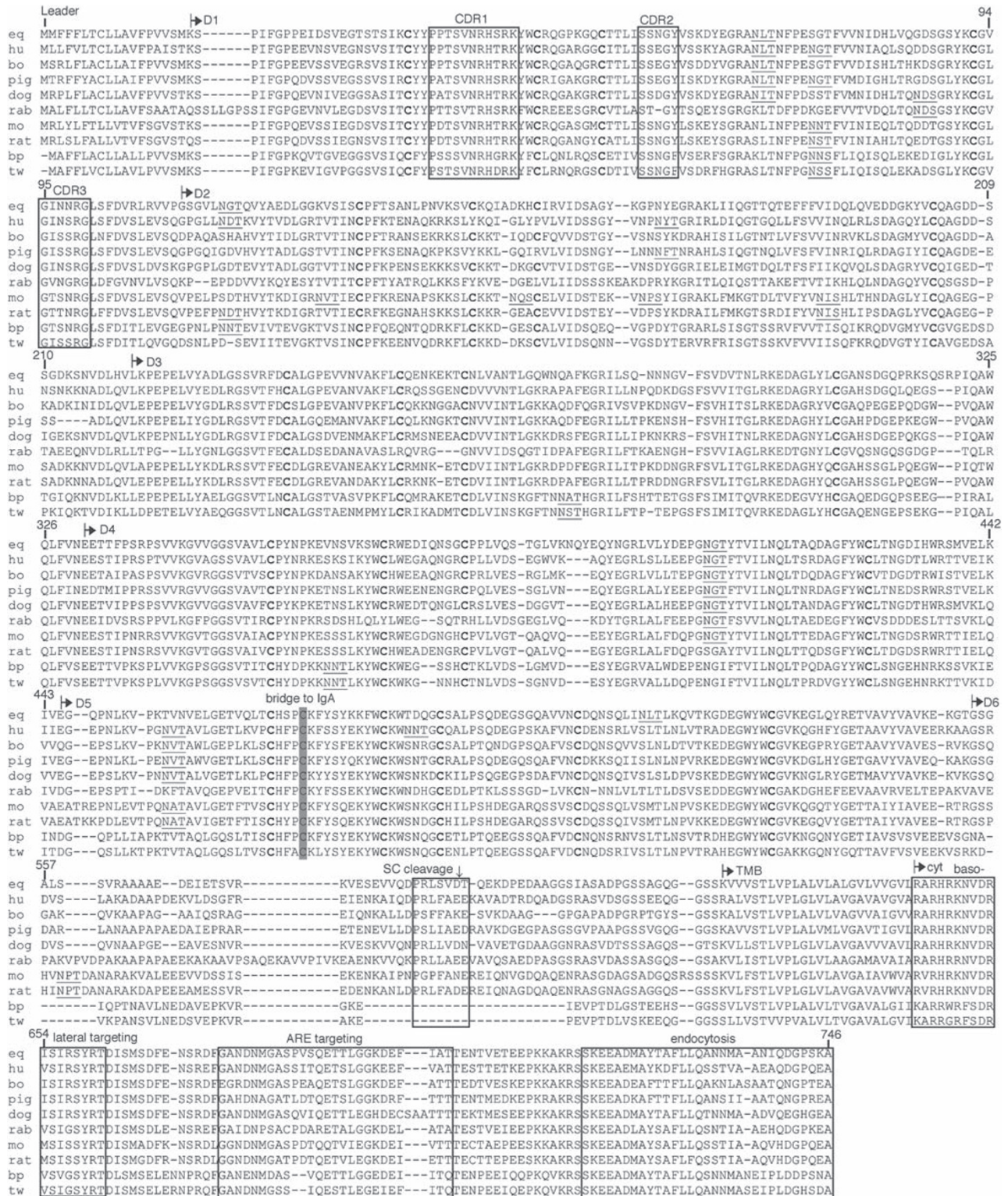


Figure 4 Alignment of the amino acid sequences of mammalian polymeric Ig receptor (pIgR). Accession numbers and species abbreviations for the sequences used are as follows: human (hu), X73079; cow (bo), X81371; pig (pig), AB032195; dog (dog), AY081057; rabbit (rab), X00412; mouse (mo), NM_011082; rat (rat), NM_012723; silver brushtail possum (bp), AF091137; and tammar wallaby (tw), AF317205. Cysteines involved in intradomain disulfide bonds are shown in bold. The Cys residue that forms a disulfide bond with IgA is highlighted. N-glycosylation acceptor sites are underlined. Complementarity determining region (CDR)-like loops in D1, secretory component (SC) cleavage site in D6, and regions involved in basolateral and androgen responsive-element (ARE) targeting and endocytosis are boxed and labeled.

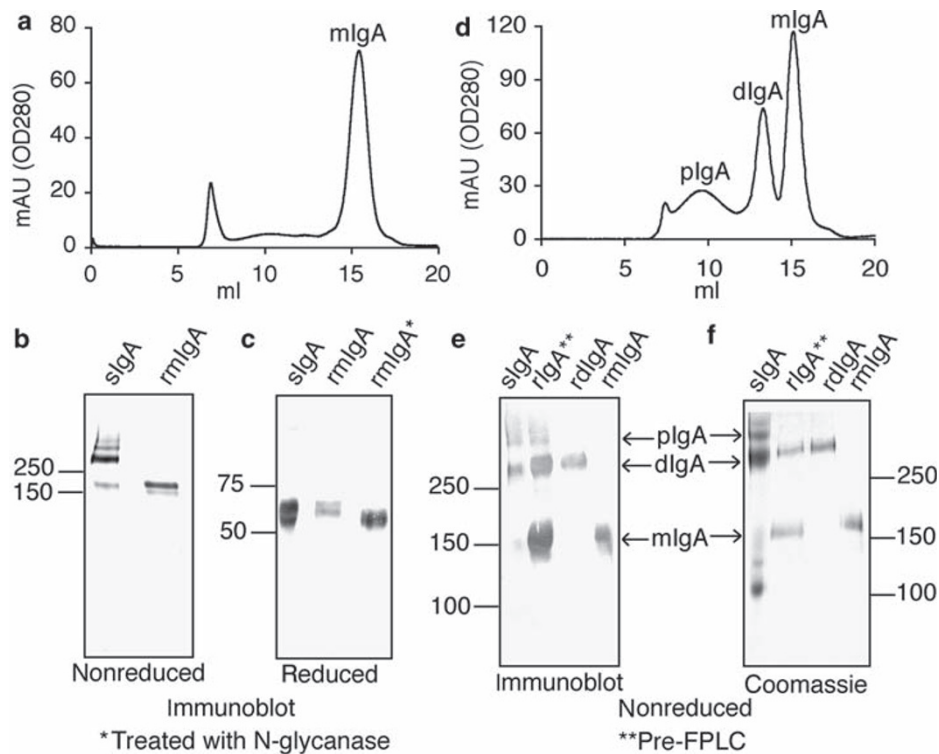


Figure 5 Analysis of recombinant IgAs. (a) Size exclusion chromatography (fast protein liquid chromatography (FPLC)) analysis of recombinant equine IgA affinity purified from supernatant of CHO-K1 cells transfected with heavy chain (HC) and light chain (LC) vectors, showing a single peak corresponding to monomeric IgA. (b, c) Immunoblot analysis of equine serum IgA (slgA) and recombinant monomer IgA (mlgA) probed with goat anti-equine IgA under nonreducing and reducing conditions, respectively. (d) Size exclusion chromatography (FPLC) analysis of recombinant equine IgA affinity purified from supernatant of CHO-K1 cells transfected with LC, HC, and J chain vectors, showing peaks corresponding to mIgA, dimer (dlgA), and larger polymers (plgA). (e, f) Immunoblot probed with goat anti-equine IgA and Coomassie stain of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, respectively, of affinity-purified recombinant equine IgA (rlgA) from cells transfected with LC, HC, and J chain, before size exclusion chromatography (FPLC) showing bands corresponding to mIgA, dlgA, and plgA, and after FPLC separation into mIgA (rmlgA) and dlgA (rdIgA) fractions. slgA is included for comparison. In a and d, minor peaks eluting at ~7 ml correspond to antibody aggregated during the purification process.

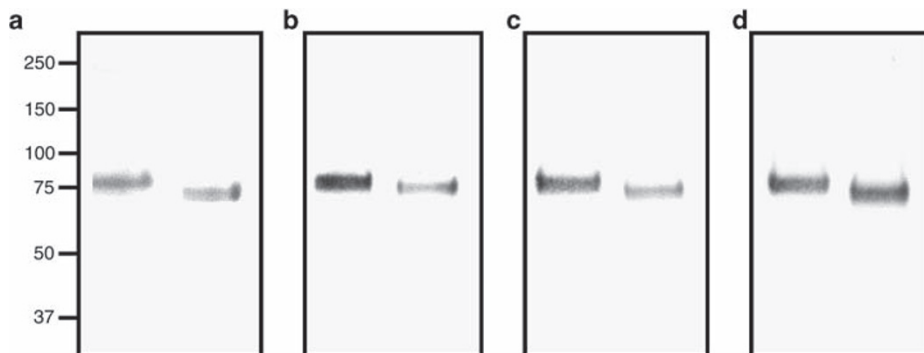


Figure 6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of recombinant equine secretory component (SC). (a) Gel stained with PageBlue. (b–d) Immunoblots probed with (b) mouse anti-human SC, (c) recombinant dimeric equine IgA (reqdIgA), and (d) recombinant human dlG1A (rhdIgA1). For each panel, the recombinant form of human SC (rhSC) is shown in the left lane and recombinant equine SC (reqSC) in the right lane.

the known 1:1 stoichiometry between human pIgA and SC. Consistent with studies in other species, we found that eqSC was unable to bind to mIgA, regardless of whether it was equine or human. This finding supports early reports that a molecule consistent with SC associated only with high-molecular-weight species of IgA (>350 kDa) in equine secretions.¹⁰ Equine SC bound to reqdIgA with a K_D of 5.1×10^{-9} M and to rhdIgA1

with a K_D of 1.4×10^{-9} M, consistent with earlier reports¹⁸ of the binding of human pIgA to SC from various species (human, bovine, and rabbit) with K_D values ranging from 1.3×10^{-9} to 3.2×10^{-9} M. The slightly lower affinity of reqSC for reqdIgA was almost entirely because of a slower rate of association with the equine ($2.5 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$) when compared with the human ($5 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$) ligand.

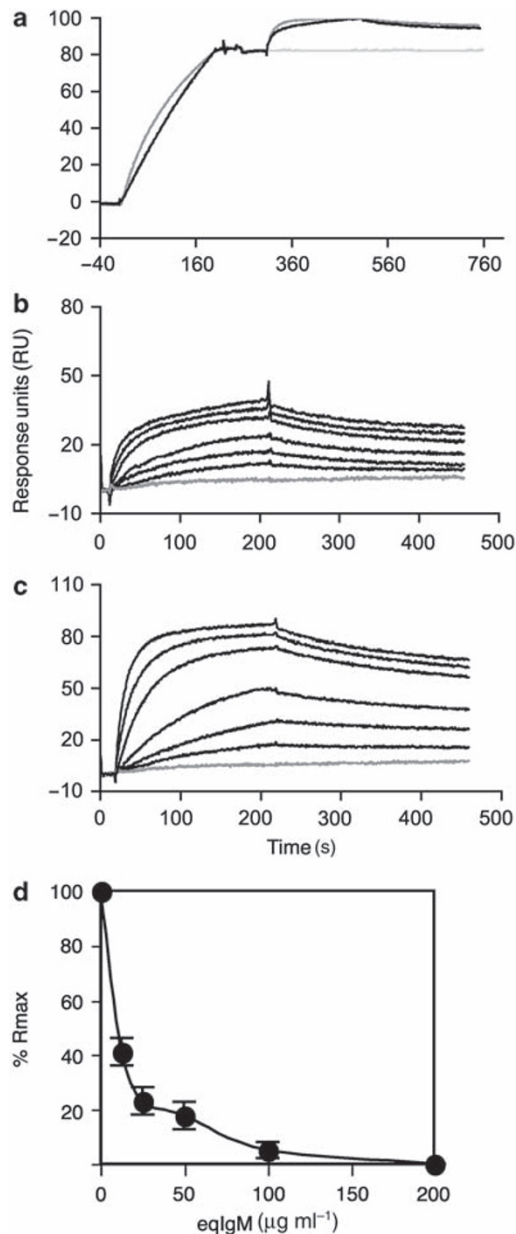


Figure 7 Surface plasmon resonance analysis of the equine secretory component (eqSC)-dimeric IgA (dIgA) interaction. **(a)** Normalized sensorgram showing the binding of recombinant dimeric equine IgA (reqdIgA; black) and recombinant human dIgA1 (rhdlgA1; dark gray), both at 20 nM, to a NIP-derivatized sensor chip and the subsequent application of recombinant eqSC (reqSC) (133 nM) or buffer (light gray line). **(b, c)** Sensorgrams (black lines) for the binding of reqSC (at concentrations of 4.1, 8.3, 16.6, 33, 66, and 133 nM) to prebound **(b)** reqdIgA and **(c)** rhdlgA1. The gray line represents the binding of reqSC to either prebound **(b)** reqdIgA or **(c)** rhmlgA1. **(d)** Inhibition of eqSC binding to immobilized dIgA by preincubation of eqSC with equine serum IgM. Results are shown as the percentage of maximum response observed in the absence of inhibition.

Preincubation of reqSC with polyclonal serum eqIgM was found to inhibit the binding of reqSC to immobilized reqdIgA (**Figure 7d**), indicating that reqSC can also bind to eqIgM with appreciable affinity.

Analysis of the equine J chain and eqpIgR genomic DNA

Searches of the equine genome with equine J chain and eqpIgR cDNA sequences identified sequences on *Equus caballus* chromosome 3 (ECA3) and ECA5, respectively. Human and mouse J chain and *PIGR* genes have been localized to HSA4 (*Homo sapiens* chromosome 4) and MMU5 (*Mus musculus* chromosome 5) and HSA1 and MMU1, respectively.^{19–22} Comparative mapping of the human, mouse, and equine genomes has aligned regions of HSA4 and MMU5 to ECA3 and regions of HSA1 and MMU1 to ECA5^{23,24} providing support for our assignment of the equine genes. Close to the human *PIGR* gene on chromosome 1q31–q42 are the Fc receptors for IgG (FcγR), IgE (FcεRI), and IgA/IgM (Fcα/μR). We located a receptor homologous to human and mouse Fcα/μR (accession no. XM_001489848) downstream of *PIGR*, and a receptor homologous to mammalian FcγRIII as well as the equine FcεRI γ-chain are located on ECA5, suggesting comparable arrangement of genes in the horse.

Exon number, length, and exon–intron boundaries of the equine J chain and *PIGR* genes bear a close resemblance to those of human and mouse,^{22,25–27} with the coding sequence of J chain distributed across 4 exons and that of pIgR across 11 exons (**Figure 8**).

The proximal promoter region and exon 1 of the eqpIgR gene were compared with human (Y08254), rat (AF039920), and mouse (U83426) sequences to identify conserved binding sites for transcription factors.^{28,29} DNA elements previously implicated in regulation of basal transcription of pIgR mRNA were identified (**Figure 9a**), including an E-box motif, activator protein 2 binding site, and an inverted repeat motif in the proximal promoter region. Binding sites for inducible factors included a conserved interferon-sensitive response element in exon 1 and steroid-responsive elements, including an androgen responsive-element in exon 1 and a glucocorticoid DNA-responsive element in the proximal promoter region. Interestingly, two interferon-sensitive response elements approximately 100 bp upstream of the transcription start site are conserved between the horse and human but are lacking in the rodent pIgR genes. As the interferon-sensitive response element motif is a binding site for the interferon regulatory factor family of cytokine-inducible transcription factors, these evolutionary differences of rodent from both human and horse may reflect differences in pIgR regulation by proinflammatory cytokines such as interferon-γ and tumor necrosis factor.

Intron 1 of the *eqpIgR* gene was compared with those of the human (accession no. X95880) and mouse (accession no. AB001489) to identify a conserved intronic enhancer (**Figure 9b**). This enhancer region contains at least seven target elements for DNA-binding factors.^{28,29} All seven elements were identified within intron 1 of the eqpIgR, including conserved binding sites for tissue-specific (hepatocyte nuclear factor-1) and cytokine-inducible transcription factors (signal transducer and activator of transcription 6 and nuclear factor-κB).

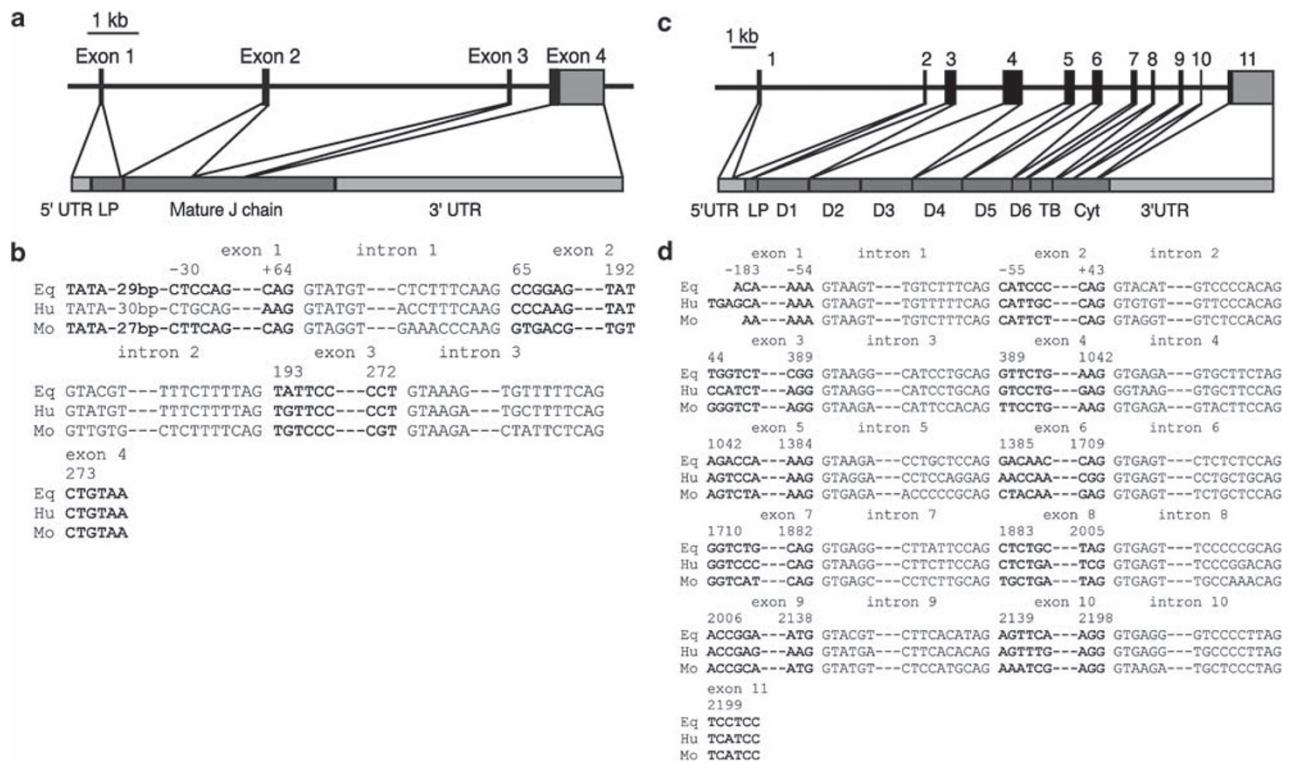


Figure 8 Gene organization. Structure of the (a) equine J chain and (c) plgR genes, and comparison of (b) equine J chain and (d) plgR exon-intron boundaries with those of the human and mouse genes. Equine J chain coding sequence is arranged as follows: exon 1, 97 bp encoding 33 bp untranslated region (UTR) and the first 21 residues of the leader peptide; exon 2, 126 bp encoding the last amino acid of the leader peptide and amino acids +1–41; exon 3, 81 bp encoding amino acids 42–68; and exon 4, 1.1 kb encoding amino acids 69–136 (205 bp) and a long 3' UTR. Equine polymeric Ig receptor (plgR) coding sequence is arranged as follows: exon 1, 128 bp of 5' UTR; exon 2, 97 bp encoding 5' UTR and 14 amino acids of the leader peptide; exon 3, encodes 4 amino acids of the leader peptide and all of D1; exon 4, encodes D2 and D3; exon 5, encodes D4; exon 6, encodes D5; exon 7 encodes D6; exon 8 encodes the last 5 amino acids of D6, the transmembrane (TMB) and the first 8 amino acids of the cytoplasmic domain; exons 9 and 10, encode the cytoplasmic domain; and exon 11 encodes the last 31 amino acids of the cytoplasmic tail and 1,700 bp of 3' UTR. Numbering shown in b and d is relative to the start of the mature protein.

DISCUSSION

SIgA provides a “first line” of immune defense, critical for the protection of mucosal sites that are vulnerable to attack by infectious microorganisms. In this study we report the cloning of equine J chain and plgR, thereby completing the cloning of all three specific components of equine SIgA, namely IgA HC, J chain, and SC. In addition, we document the first expression of these three components in recombinant form, with subsequent functional characterization.

The recombinant version of eqIgA appeared representative of its native counterpart and assembled as anticipated.¹⁰ An early study suggested that not all eqIgA molecules have covalent links between LC and HC,¹³ but this was not observed to be the case with reqIgA. It is possible that these different observations reflect allotype differences between the IgAs analyzed, given the example in mouse IgA, in which different allotypic forms differ in their ability to form H-L disulfide bonds.³⁰ Certainly, allelic variation of IgA has been observed in equine genomic DNA,³¹ although it is presently unclear whether any differences lie within coding regions.

Molecular models of human IgA1 and IgA2^{32,33} have revealed a striking effect of hinge length on antibody structure. The shorter hinge region (6 residues) of human IgA2 enforces a more

compact structure on the IgA2 molecule compared with that of IgA1, which has a more extended hinge region (19 residues). Horse IgA has a relatively long hinge (11 residues) but the presence of three Cys residues suggests that it may be constrained by inter-H-chain disulfide bonds. The extended human IgA1 hinge is thought to provide this subclass with a capacity for more avid binding to antigens spread further apart across the surface of pathogens. Possibly, such a capacity is of less importance in mammals such as the horse, in which both SIgA and serum IgA are found chiefly in dimeric form. The proposed structure of dIgA is of two IgA monomers linked tail to tail via J chain,³⁴ an arrangement clearly compatible with the ability to bind widely spaced antigens through at least one of the Fab arms of each monomer.

To facilitate production of polymeric eqIgA, we cloned equine J chain. Its sequence contains the conserved Cys residues (see Figure 2) required for covalent interaction with the IgA tailpiece Cys,^{34,35} and we found that it was able to promote efficient polymerization of eqIgA. The conserved nature of the J chain Cys residues across diverse species (human, mouse, chicken, and bullfrog) seems sufficient to promote dimerization when heterogeneously expressed with human IgA.³⁶ However, the degree of polymerization



Figure 9 Identification of transcription factor binding sites. **(a)** Alignment of the 5' flanking region and exon 1 of the horse, human, rat, and mouse *PIGR* showing binding sites for constitutive and inducible transcription factors. AP2, activator protein 2; ARE, androgen response element; GDRE, glucocorticoid DNA-response element; tissue-specific, sequence that may have a role in tissue-specific regulation; ISRE, interferon-sensitive response element; repeat, inverted repeat motif; TATA, non-consensus TATA-box. Numbering shown is relative to the transcriptional start shown by gray highlight. **(b)** Alignment of the *PIGR* intron 1 enhancer from horse, human, and mouse showing the seven regulatory elements identified in this region, including hepatocyte nuclear factor (HNF-1), signal transducer and activator of transcription 6 (STAT-6), and nuclear factor (NF)-κB binding sites.

is variable, suggesting that subtle amino acid differences between species may influence the efficiency of interaction. Indeed, additional residues within the tailpiece and the Cα3 domain of IgA seem to have a role in polymerization.^{35,37} Through incorporation of equine J chain, rather than J chain from another mammalian species, our expression system offers the advantage of producing authentic versions of polymeric eIgA, suitable for in-depth analysis from which reliable conclusions relevant to the horse may be drawn.

Separate to its role in IgA polymerization, J chain seems to be required for the binding of dIgA to pIgR/SC through direct, noncovalent interactions involving a C-terminal loop comprising Cys109 to Cys134 (human numbering) and two other

regions (boxed in **Figure 2**).³⁶ These elements are observed to be well conserved in equine J chain.

Turning to the role of pIgR, the binding of dIgA to pIgR involves both covalent and noncovalent interactions. Three loops within pIgR D1 that are analogous to the complementarity determining regions of Ig variable domains are critical for noncovalent interaction with dIgA.^{18,38} These complementarity determining region-like loops are conserved in eIgR (**Figure 4**). After noncovalent binding between IgA and pIgR, a covalent bond is formed between the pIgR and one of the IgA monomer subunits. In the human system, the Cys residues involved in this covalent bond are Cys468 in the pIgR D5 and Cys311 within the Cα2 domain of IgA. These Cys residues are conserved in eIgR (**Figure 4**).

Of the HC domains of IgA, the C α 3 domain shows the highest degree of identity across species and seems to be the most important for noncovalent interaction with pIgR.^{14,39} Motifs within the C α 3 domain of IgA required for pIgR binding are centered on three regions. In human IgA these include a loop region comprising residues 402–410, adjacent residues 411–414, Lys377, and residues Pro440–Phe443, the so-called “PLAF” loop.^{14,40} In the horse, 15 of these 18 amino acids are conserved or highly conserved substitutions, suggesting a common mode of binding.

The pIgR from certain species (human and cow) can bind and transport pIgM as well as pIgA, whereas pIgR from rabbits and rodents transports only pIgA.^{12,36} Our preliminary results with a polyclonal preparation of eqIgM suggest that pIgR in the horse also binds pIgM. However, further detailed studies, ideally using recombinant eqIgM, will be required to ascertain the precise affinity of eqSC for eqIgM.

In humans, transcytosis of pIgR occurs in the absence of its IgA ligand, resulting in release of free SC into secretions in which it acts to inhibit the binding of bacteria and bacterial toxins to intestinal cells.⁴¹ In addition, free SC produced by bronchial epithelial cells has a regulatory role by complexing with and sequestering soluble interleukin-8. Thus, SC may form part of a feedback mechanism that downregulates interleukin-8-mediated recruitment of neutrophils to the airway and attenuates the inflammatory response.⁴² The presence of free SC in equine milk¹⁰ suggests that constitutive release of free SC into the secretions also occurs in horses. Given the upregulation of interleukin-8 and its role in promoting airway neutrophilia in horses with recurrent airway obstruction,⁴³ it would be interesting to investigate the existence of this feedback mechanism in horses.

Efficient export of pIgA into the secretions requires coordinated transcriptional regulation of pIgR expression by a number of mediators.^{28,29} We found that many of the binding sites for transcription factors identified within the proximal promoter region, exon 1, and intron 1 of the human *PIGR* gene are conserved in the horse *PIGR* gene. These include the interferon-sensitive response element (exon 1), nuclear factor- κ B (intron 1), and signal transducer and activator of transcription 6 (intron 1) sites that are required for interferon- γ , tumor necrosis factor, and interleukin-4-mediated upregulation of pIgR mRNA. Thus, it seems likely that these cytokines similarly have a role in the horse, in regulation of pIgR expression at sites of infection and inflammation.

The effector functions of equine IgA are not yet well characterized. However, evidence of strong opsonophagocytic activity, for example, against the important pathogen *S. equi*,⁹ suggests that equine IgA is able to mediate killing through FcR on phagocytes or through the complement pathway. Indeed, a receptor for eqIgA homologous to human CD89 (Fc α RI) is readily detected in equine polymorphonuclear neutrophils and is able to bind equine serum IgA and SIgA.⁴⁴ Interestingly, comparisons between human, bovine, and equine CD89 suggest that although their interaction sites for IgA are related, each has distinct features.⁴⁴ Thus, to gain insights into this interaction in a particular species, it is essential to investigate the IgA and receptor in that species, rather than making assumptions based on studies in other species. The availability of reqIgA now

opens up the possibility of both defining the precise interaction site on eqIgA Fc for eqCD89, and further elucidating the killing mechanisms that eqIgA is capable of triggering.

A mucosal IgA response in horses makes a key contribution to immunity against viral (equine influenza virus and equine herpesvirus-1) and bacterial (*S. equi*, *Rhodococcus equi*, *Salmonella enterica*, and *Clostridium botulinum*) infections.^{7–9,45–48} Our studies pave the way for development of pathogen-specific reqIgA suitable for therapeutic intervention in the horse, mirroring developments in the human system in which recombinant hIgAs targeting various bacterial, viral, and parasitic antigens are under analysis. Mucosal administration of reqIgA could prevent or treat equine infectious disease in which effective vaccines are unavailable or provide only partial protection, such as *R. equi* pneumonia in foals.

IgA also acts as an architect of the mucosal immune response by participating in antigen presentation to mucosal dendritic cells and induction of appropriate T-cell responses and immunological memory.⁶ In addition to a role in protection against pathogenic microorganisms, a significant role for IgA in shaping and regulating the population of commensal bacteria within the mucosa has been recognized. The availability of defined recombinant versions of eqIgA should now facilitate studies into these phenomena in the horse.

In conclusion, we have produced and characterized the first reqIgA to reflect the different molecular forms found in nature, namely polymeric and secretory eqIgA. These show both similarities with other mammalian IgA, and important species-specific distinctions. The latter underline the value of detailed investigations within particular species, and caution against simple extrapolation of findings in one species to another. The recombinant eqIgA, J chain, and SC described in this study are valuable sources of pure and homogenous material that can be used as reference proteins for the production and screening of equine-specific monoclonal antibody reagents. Furthermore, production of reqIgA of defined specificity and molecular form will permit delineation of the precise functions of IgA in equine immunity, and open up possibilities for development of pathogen-specific reqIgA suitable for therapeutic intervention in equine infectious disease.

METHODS

Cloning equine J chain and pIgR. cDNA synthesized from equine ileal total RNA using the ImProm II Reverse Transcription System (Promega, Southampton, UK) was used as template for PCR amplification of J chain and pIgR using degenerative primers based on the known nucleotide sequences from other mammalian species (detailed in **Table 1**). After sequencing of J chain and pIgR PCR products, 5'/3' RACE was carried out using total ileal RNA and a 5'/3' RACE kit (Roche, Mannheim, Germany). Forward and reverse primers (**Table 1**) were designed according to the 5'/3' RACE sequences and the complete J chain and pIgR coding sequences were amplified from cDNA. J chain and pIgR PCR products were cloned, respectively, into pcDNA3.1 (Invitrogen, Paisley, UK) and pcDNA3.1/Hygro (Invitrogen) and sequenced as before. Amino acid sequence analysis was carried out using ClustalW (<http://www.ebi.ac.uk/clustalW/index.html>). Phylogenetic analysis was performed using the neighbor-joining method with 1,000 bootstrap replications available in MEGA3 molecular evolutionary genetics analysis software.⁴⁹

Table 1 Primers used in the study

| Primer | Sequence |
|------------------------|---|
| <i>J chain primers</i> | |
| J chain forward A | 5'- <u>AAGCTT</u> AGTCAAGATGAAGAA [C/T][C/T][A/G/C]TTTGC-3' |
| J chain reverse A | 5'- <u>GAATTC</u> TTATTAGTCAGG [A/G]TAGCAGG[A/C]ATC-3' |
| J chain forward B | 5'- <u>AAGCTT</u> GTAAGTCAAGATGAAG AAC-3' |
| J chain reverse B | 5'- <u>GAATTC</u> TTATTAGTCAGGATAGCAGG-3' |
| <i>pIgR primers</i> | |
| pIgR forward A | 5'- <u>GCGGCCG</u> CAA[G/A]AG[T/C]CC [C/A]AT[A/C]TT[T/C]GG[T/G/C]CCC-3' |
| pIgR reverse A | 5'- <u>CTCGAG</u> [G/A/T]GC[T/C]TC[T/C] TCCTTGGAT[C/G/A]GA[C/T]C[T/G]-3' |
| pIgR forward B | 5'- <u>AAGCTT</u> GCTGCCGTCTGGCTGACA GGC-3' |
| pIgR reverse B | 5'- <u>CTCGAG</u> GCCTAAGTGGAGTATGAG TGC-3' |
| SC reverse | 5'- <u>CTCGAG</u> TCATCAGTCCACAGAAAGC CTGG-3' |

Abbreviations: pIgR, polymeric Ig receptor; SC, secretory component. Primers denoted A were used for initial PCR amplification and primers denoted B were used for PCR amplification after 5'/3' RACE. Underlining denotes restriction sites incorporated into primers (HindIII for J chain forward A and B and pIgR forward B; EcoRI for J chain reverse A and B; XhoI for pIgR reverse A and B and SC reverse; and NotI for pIgR forward A); bracketed bases denote degenerate nucleotides; and italics denote an introduced stop codon.

Expression of monomeric and dimeric equine IgA in CHO-K1 cells.

Genomic DNA for the horse *IGHA* gene³ was amplified by PCR and subcloned as a *Bam*HI fragment downstream of a mouse V_H gene specific for NIP in the vector pcDNA3.1VNip.⁵⁰ To produce reqmIgA, CHO-K1 cells expressing a mouse λ LC specific for NIP were transfected with the IgA HC vector, using previously described protocols.⁵¹ Supernatant from individual resistant CHO clones was screened for IgA production by antigen-capture enzyme-linked immunosorbent assay as previously described,⁵¹ except that detection antibodies used were either goat anti-mouse λ LC-horseradish peroxidase (HRP) conjugate (0.2 μ g ml⁻¹; Bethyl Laboratories, Montgomery, TX) or goat anti-horse IgA-HRP conjugate (0.1 μ g ml⁻¹; Serotec, Oxford, UK).

To produce reqdIgA, a CHO-K1 cell line stably producing reqmIgA was transfected with the equine J chain expression vector. Supernatant from individual resistant CHO clones was screened for dIgA production using a hSC capture enzyme-linked immunosorbent assay,¹⁴ detecting with goat anti-mouse λ LC-HRP (0.2 μ g ml⁻¹; Bethyl Laboratories).

Purification and analysis of reqIgA. reqIgA was purified using NIP-affinity chromatography and size exclusion chromatography as previously described.^{14,51} Fractions corresponding to intact monomeric (H₂L₂) or dimeric (H₄L₄) eqIgA were pooled for further analysis by SDS-PAGE and western blotting. Equine IgA from serum (Accurate Chemical and Scientific Corporation, Westbury, NY) was included for comparison. Gels were stained with Coomassie brilliant blue and western blots were probed with HRP-conjugated goat anti-horse IgA (0.1 μ g ml⁻¹; Serotec). Using a GlycoPro enzymatic deglycosylation kit (Prozyme, Leandro, CA), N-linked sugars were removed from reqmIgA.

Expression of reqSC in CHO-K1 cells. A truncated form of equine pIgR cDNA, equivalent to the coding sequence for SC, was amplified using the pIgR forward primer B and SC reverse primer (Table 1), inserted into pcDNA3.1/Hygro and sequenced. ReqSC was expressed in CHO-K1

cells, as previously described for rhSC.¹⁴ Supernatant from individual resistant CHO-K1 clones was screened for SC production by dotblot on nitrocellulose, detecting bound reqdIgA with HRP-conjugated goat anti-mouse λ LC (0.2 μ g ml⁻¹ in phosphate-buffered saline with Tween 20).

Purification and analysis of reqSC. reqSC was purified using a human pIgA-Sepharose column as previously described,¹⁴ and analyzed by SDS-PAGE and western blotting. Gels were stained with PageBlue protein staining solution (Fermentas, St Leon-Rot, Germany) and western blots were probed with rhdIgA1 or reqdIgA (both 1 μ g ml⁻¹) followed by goat anti-LC-HRP (0.2 μ g ml⁻¹), or mouse anti-human SC (1 μ g ml⁻¹; Monosan, Uden, The Netherlands) followed by goat anti-mouse Fc-HRP (1/1,000; Sigma-Aldrich, Poole, UK). rhSC¹⁴ was included as a comparison.

Interaction of IgA with reqSC. Surface plasmon resonance experiments were carried out on a BiacoreX instrument (GE Healthcare, Little Chalfont, UK) with NIP-derivatized bovine serum albumin immobilized onto a CM5 chip. Either reqdIgA or hdIgA1 (100 μ l, 20 nM) was applied at a flow rate of 30 μ l min⁻¹. reqSC at concentrations up to 133 nM (100 μ l, flow rate of 30 μ l min⁻¹) was then applied and binding to the prebound IgA analyzed. To determine whether reqSC can bind eqIgM, the effect of preincubating reqSC with polyclonal serum eqIgM (0–200 μ g ml⁻¹)⁵² on its ability to bind to prebound reqdIgA was assessed. BIAevaluation 3.2 software (GE Healthcare) was used for data analysis.

Analysis of equine J chain and pIgR genomic DNA sequences. Equine J chain and pIgR cDNA sequences were used to search the equine genome (available at <http://www.ncbi.nlm.nih.gov/Genbank>) for corresponding genomic DNA sequences. The putative transcriptional start sequences and intron–exon arrangements were identified by comparison of cDNA and genomic sequences. Conserved transcription regulatory elements in the equine pIgR gene were identified by comparison with human, mouse, and rat *PIGR* genes.

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DISCLOSURE

The authors declared no conflict of interest.

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