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THE DETECTION AND IDENTIFICATION OF THE CR1-LIKE MEMBRANE BINDING PROTEIN OF PORCINE ERYTHROCYTES

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ABSTRACT

Erythrocyte complement receptor I-like (ECR1-like) is a natural immunoreactive molecule on the surface of porcine erythrocyte membrane. The aim of this study was to establish an effective method for the detection and characterization of porcine erythrocyte CR1-like membrane-bound proteins, and to explore their expression characteristics and biological significance in porcine erythrocyte membranes. The CR1-like protein ligands were observed by laser confocal microscopy using fluorescence immunocytochemistry with two types of PDZ-binding domain monoclonal antibodies, FAP-1 (Fas-associated phosphatase-1) and ZO2 (Tight Junction Protein ZO-2); meanwhile, immunoprecipitation and Western blot techniques were used to detect the membrane proteins of porcine blood cells. The immunofluorescence cytochemical staining showed that the specific fluorescence sites of CR1-like and FAP-1 molecules in the porcine erythrocyte membrane skeleton were identical; the sum of the difference squares of the site distances of 253 typical positive erythrocytes was 0.2224, indicating that the difference between the site distances of CR1-like and FAP-1 in each group was approximately 0. The results showed that the distribution of CR1-like and FAP-1 was consistent with a co-local relationship, and the FAP-1 molecule was clearly observed in the examined gel by immunoprecipitation. The results indicate that CR1-like does not bind directly to the erythrocyte membrane skeleton protein, but is distributed on the surface of the porcine erythrocyte membrane through the riveted structure of the FAP-1 protein molecule.

Keywords: CR1-like; FAP-1; Immunoadhesion; Porcine erythrocytes

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INTRODUCTION

Erythrocytes are important intrinsic immune cells in the blood circulation and are widely involved in specific and non-specific immunity, regulation of body immunity and have a complete self-regulatory system in the body. Among them, immune adhesion is the main immune function of human red blood cells. ECR1 (Erythrocyte complement receptor 1) is the most important material basis for the immune adhesion function of erythrocytes. It is a single-chain transmembrane glycoprotein with a short cytoplasmic tail peptide (Kramer *et al*.,2018), genetically polymorphic (Khera *et al*., 2009, Rachid *et al*., 2018), and belongs to a family of complement-activated regulatory proteins with complement C3b binding activity.The most important function of this receptor is to mediate immune adhesion to red blood cells and to deliver IC (Immune complexes). When exogenous or endogenous CR1 (Complement receptor 1), the natural receptor for C3b, specifically binds C3b and adheres CIC to the surface of erythrocytes (Yin *et al*., 2015). Erythrocytes bound with CIC (Circulating immune complexes) travel through the bloodstream to reach tissues such as the liver and spleen, where they deliver CIC to macrophages and promote phagocytosis. During this process, the amount of CR1 on the surface of erythrocytes decreases (Yin *et al*., 2019). When congenital or acquired factors lead to a reduction in the number or viability of CR1, the function of erythrocytes to clear circulating immune complexes is subsequently inhibited (Yu *et al*., 2007). CR1 was first observed to be distributed in clusters of 2-15 or 30-75 particles per cluster on erythrocyte membranes by cryo-etching electron microscopy (Paccaud *et al*., 1988, Chevalier and Kazatchkine, 1989). It is also suggested that this clustered distribution state is the structural basis for the efficient adhesion of CR1 to ICs.To verify CR1 immune function, the fluorescence sites and fluorescence intensities of natural state and paraformaldehyde-fixed erythrocytes were analysed using indirect immunofluorescence techniques. It was found that CR1 in its natural state is not clustered but scattered on the surface of the

erythrocyte membrane (Java *et al*., 2015, Lapin *et al*., 2012, Lan *et al*., 2015). When CR1 immunoadheres to sensitised antigens, CR1 clusters to ensure efficient antigen binding by CR1. This clustered CR1 plays an important role in the transport and transfer of IC to macrophages and in the solidification of antigens. The presence of a class of ligand protein, FAP-1, on the surface of human erythrocytes.FAP-1 binds to the CR1 transmembrane region peptide through the PDZ domain, while FAP-1 binds to the erythrocyte membrane skeletal protein through the FERM domain. It is hypothesized that erythrocyte membrane kinesin undergoes conformational changes in response to FAP-1 phosphorylation, causing clustered aggregation of CR1 on the membrane surface (Ghiran *et al*., 2008). It was found that the clustering of CR1 on the erythrocyte membrane surface was accompanied by a transient inward flow of Ca^{2+}, which activated phosphorylation pathways such as PKA (Protein kinase A) and CK2 (Protein kinase CK2) and sent cellular signals, triggering the phosphorylation of FAP-1 protein and membrane skeletal proteins, increasing the consumption of erythrocyte ATP and enhancing the fluidity and variability of cell membrane lipids, thus promoting the change of CR1 from a scattered distribution to a clustered distribution, increasing the affinity of human erythrocyte CR1 to adhere to CIC and ensuring that CR1 can firmly adhere to allergenic antigens even during blood transport (Glodek *et al*., 2010, Melhorn *et al*., 2013). Veterinary studies have shown that the erythrocytes of non-primate mammals also have immune adhesion functions (Deng *et al*., 2018, Wessel *et al*., 2019, Yeo *et al*., 2019, Adams and Jensen., 2022). Changes in erythrocyte immune adhesion function in many animal disease processes, Avian influenza (Zheng *et al*., 2019), infectious bursal disease(Wang *et al*., 2011), malleolar disease (Sohrabian *et al*., 2018) and many other infectious diseases are associated with decreased immune adhesion of red blood cells. In-depth study of the structure of CR1 anchored to the surface of the erythrocyte membrane has become a key scientific question, and the relationship between the distribution state of the erythrocyte CR1 membrane and its immune function has become one of the hot topics of research in this field (Opi *et al*., 2016, Krishna and Nadler, 2016, Yin *et al*., 2016). This study investigates the molecular basis of the interaction between CR1-like and porcine erythrocyte membranes, providing theoretical data to further elucidate the molecular mechanism by which CR1-like mediates the immune function of porcine erythrocytes.

MATERIALS AND METHODS

Ethical statement: The study protocol was formally approved by the Animal Use and Care Ethics Committee of Shanxi Agricultural University (No.SXAU-EAW-2019P.XM.00307006).

Test animals, reagents and consumables: Three healthy adult Landrace pigs were used for sampling. These the pigs were purchased from Zhichao Farm, Beiwang Village, Taigu County, Shanxi province, China. The test animals in this study were used for blood collection operations. Afterwards, erythrocytes were separated and extracted for subsequent tests

Red fluorescently labeled FAP-1 monoclonal antibody (FAP-1 McAb-568), red fluorescently labeled ZO monoclonal antibody (ZO McAb-568), red fluorescently labeled mouse anti-pig IgG (IgG-568) and Protein A-Sepharose were purchased from Invitrogen, (USA); Mouse-derived IgG1 isotype antibodies were presented by UW Proteome Research Center, (Beijing, China); Phenylmethylsulfonyl fluoride (PMSF) and BCA concentration determination kits were purchased from Biyuntian Biological Reagent Company, (Beijing, China).Carbon diimide (EDAC) purchased from Sigma-Aldrich, (Shanghai, China）, The lymphocyte isolate was purchased from Tianjin Hao Yang Biological Products Technology Co, (Tianjin, China). Mouse Anti-Pig CR1-like Monoclonal Antibody (CR1-like Mc Ab) is stored in the laboratory.

Preparation of porcine red blood cell suspensions: Blood was collected from the anterior vena cava of the pig and placed in a sodium citrate anticoagulation tube.1 mL of anticoagulation solution was taken and an equal amount of Hank's buffer was mixed with it. Aspirate 4 mL of lymphocyte isolate, add the diluted blood sample to the isolate and centrifuge at 1,500 rpm for 15 min. aspirate 200 μL of the lowermost erythrocytes, resuspend with 1 mL of Hank's buffer, centrifuge at 1,500 rpm for 5 min and wash twice. Finally, resuspend with Hank's buffer to prepare an erythrocyte suspension and reserve.

Preparation of Protein A-Sepharose suspension: Referring to the instructions, take 1 mL of porcine red blood cell lysate, add rabbit anti-pig IgG, 50 μL of Protein A-Sepharose and incubate at 4°C for 2 h. Centrifuge at 3,500 rpm for 5 min and use the supernatant for subsequent immunoprecipitation.

Preparation of total membrane protein: Erythrocytes were lysed in NP-40 lysis buffer for 30 min, centrifuged at 15,000 rpm for 30 min at 4°C, washed three times, the precipitate was lysed in 2 % SDS, the protein solution was resuspended in 5 times the volume of TCA/acetone, centrifuged at 15,000 rpm for 1 h at 4°C, washed three times in cold acetone and redissolved in 2 % SDS.

500 μL porcine erythrocyte membrane protein solution mixed with 100 μL tCR1-like McAb, 4°C overnight, 30 μL Protein A-Sepharose after the above pretreatment, incubated for 3 h at 4°C, centrifuged at 3,500 rpm for 5 min, NP-40 lysis buffer washed 3 times,

20.00-50.00 µL 1×Loading Buffer added, boiling water bath for 3 min

Laser confocal detection of CR1-like co-localization with FAP-1 and ZO2: After permeabilisation, the erythrocyte suspension was incubated with tCR1-like McAb-488 at 37°C for 25 min followed by two washes at 1,500 rmp for 2 min.Divided into 3 groups of A, B, C, I, II and III.

Groups A, B and C were erythrocyte suspensions incubated with FAP-1 McAb-568, mouse anti-pig IgG-568 and PBS at 37°C for 35 min respectively; after incubation, smears were centrifuged at 1,500 rmp for 2 min and examined microscopically under a laser confocal microscope. groups I, II and III were erythrocyte suspensions incubated with ZO2 McAb-568, mouse anti-pig IgG -568 incubation and PBS; incubated at 37°C for 35 min and treated as in groups A, B and C. Fluorescence images were recorded and analysed by applying Fluorescence Microscopy Image-Pro Plus 6.0 software to compare the fluorescence overlap effect. A typical cell is selected and the software automatically calculates the cell's near-centre point, measures the distance between the centre point and the positive fluorescent point and evaluates the calculation according to the following formula.

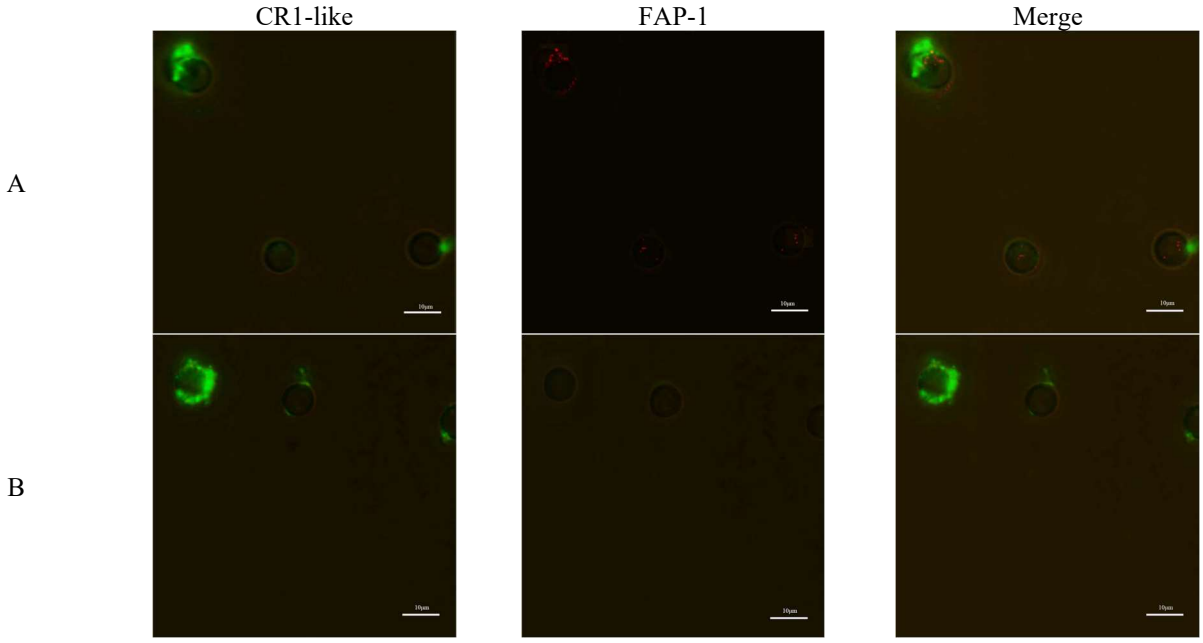
$$Y = \sum (Xi - Xj)^2$$

Where Xi is the average measurement of the length of the CR1-like fluorescent spot from the centre of the cell on selected typical erythrocytes and Xj is the average measurement of the length of the link protein from the centre of the cell.

Immunoprecipitation analysis of the role of CR1-like and connexin in porcine erythrocytes: A 20.00 µg protein sample was added to a 10 % separating gel and a 5 % concentrating gel, and at the end of electrophoresis, the protein was transferred to a PVDF membrane, which was then immersed in 5 % skim milk powder blocking solution and blocked at room temperature for 2 h. Mouse anti-pig FAP-1 McAb (1:300 dilution) was added, incubated overnight at 4°C, and the membrane was washed three times for 10 min each by TBST, and horseradish peroxidase-labelled goat anti-mouse IgG (1:10,000 dilution) was added. The luminescent solution was evenly applied to PVDF film, pressed onto film and exposed in the dark room, with the mouse anti-pig IgG incubation gel used as an irrelevant antibody control.

RESULTS

Immunofluorescence analysis: Using immunofluorescence cytochemistry, laser confocal microscopy was performed with two types of PDZ-binding domain monoclonal antibodies, FAP-1 and ZO2, against CR1-like protein ligands. In the FAP-1 McAb incubation group, green fluorescence was clearly seen on the surface of the erythrocytes , and when switched to the red fluorescence exciter, red fluorescence was clearly seen in the same position of the erythrocytes in this field . The images were processed and the distribution of green fluorescence and red fluorescence could be clearly seen in the same position (Figure 1A).



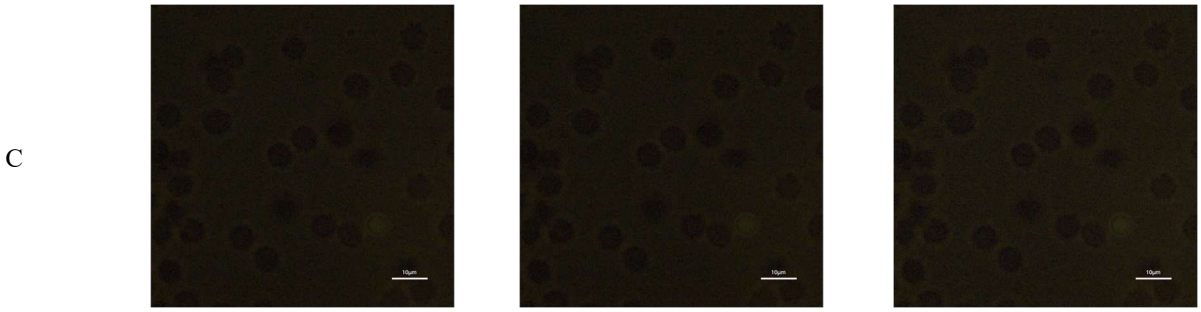


Figure 1. The results of FAP-1 McAb incubation treatment: (A) the green fluorescence exciter detection, CR1-like green fluorescence can be seen on the surface of erythrocytes; the red fluorescence exciter detection, FAP-1 red fluorescence can be seen on the surface of erythrocytes; the overlapping graph of both after Merge;(B) the control group, only CR1-like green fluorescence can be seen.(C) the blank group, Erythrocytes showed no fluorescence under two fluorescent exciters.(bars: 10 μm)

In the ZO2 McAb incubation treatment group, green fluorescence was observed on the surface of porcine erythrocytes when excited with a green fluorescent exciter ; when switched to a red fluorescent exciter, no red fluorescence was observed on the surface of porcine erythrocytes (Figure 2 Ⅰ). Compared with the irrelevant antibody control group and the blank group, there was no significant difference in the fluorescence distribution of porcine erythrocytes(Figure 2 Ⅱ-Ⅲ).

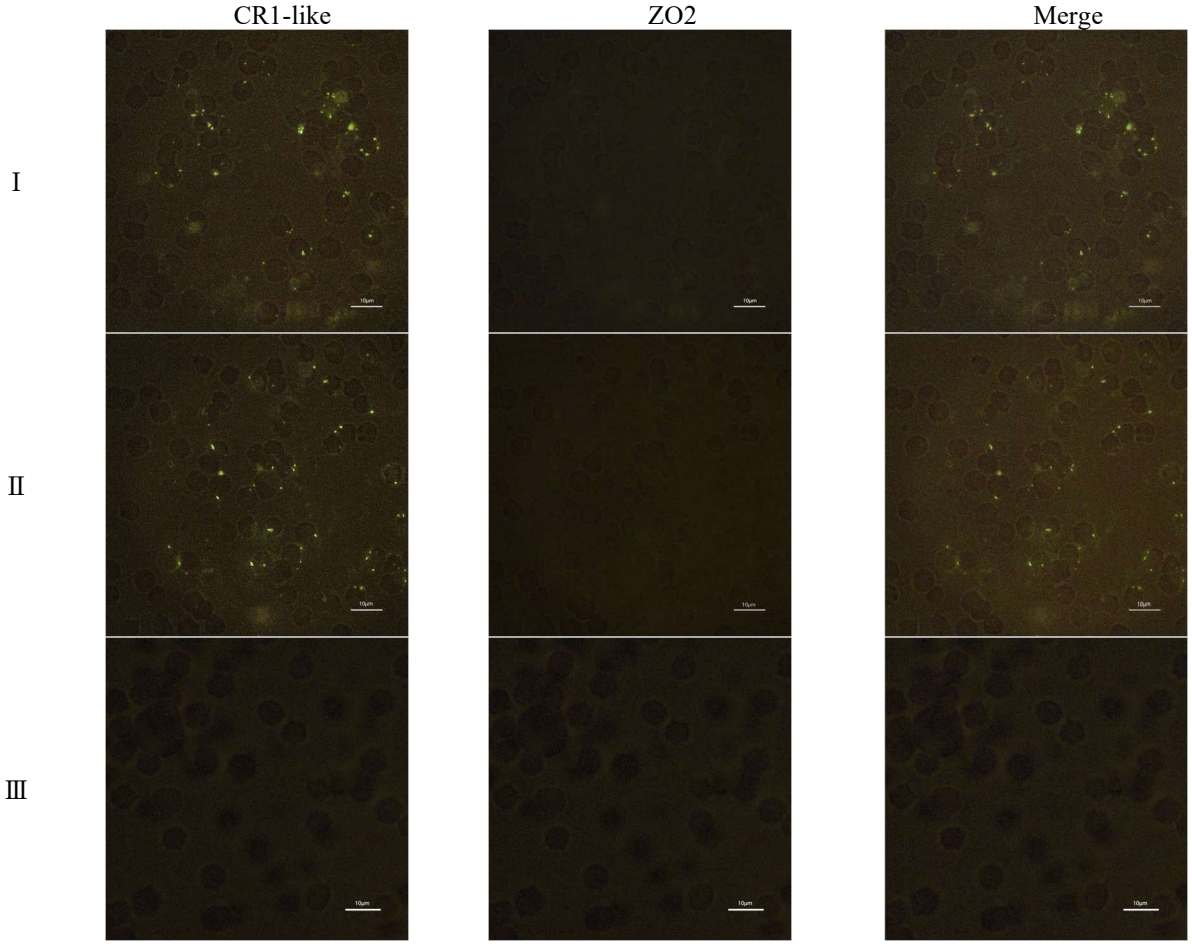


Figure 2. Incubation microscopy results of ZO2 McAb-568. (Ⅰ-Ⅱ) the CR1-like green fluorescence detected by the green laser; no fluorescence detected by the red exciter; Ⅲ is the blank group, Erythrocytes showed no fluorescence under two fluorescent exciters.(bars: 10 μm)

Overlap effect calculation analysis: In this experiment, the distance values of nearly 3000 positive sites on 258 FAP-1 fluorescence-positive erythrocytes were calculated and evaluated with Image-Pro Plus: IPP 6.0 software. brought into equation $Y = \sum (Xi - Xj)^2$ for calculation. The result was 0.2224. It can be assumed that there is a geometric co-location relationship between CR1-like and FAP-1, and the measurement results (Figure 3).

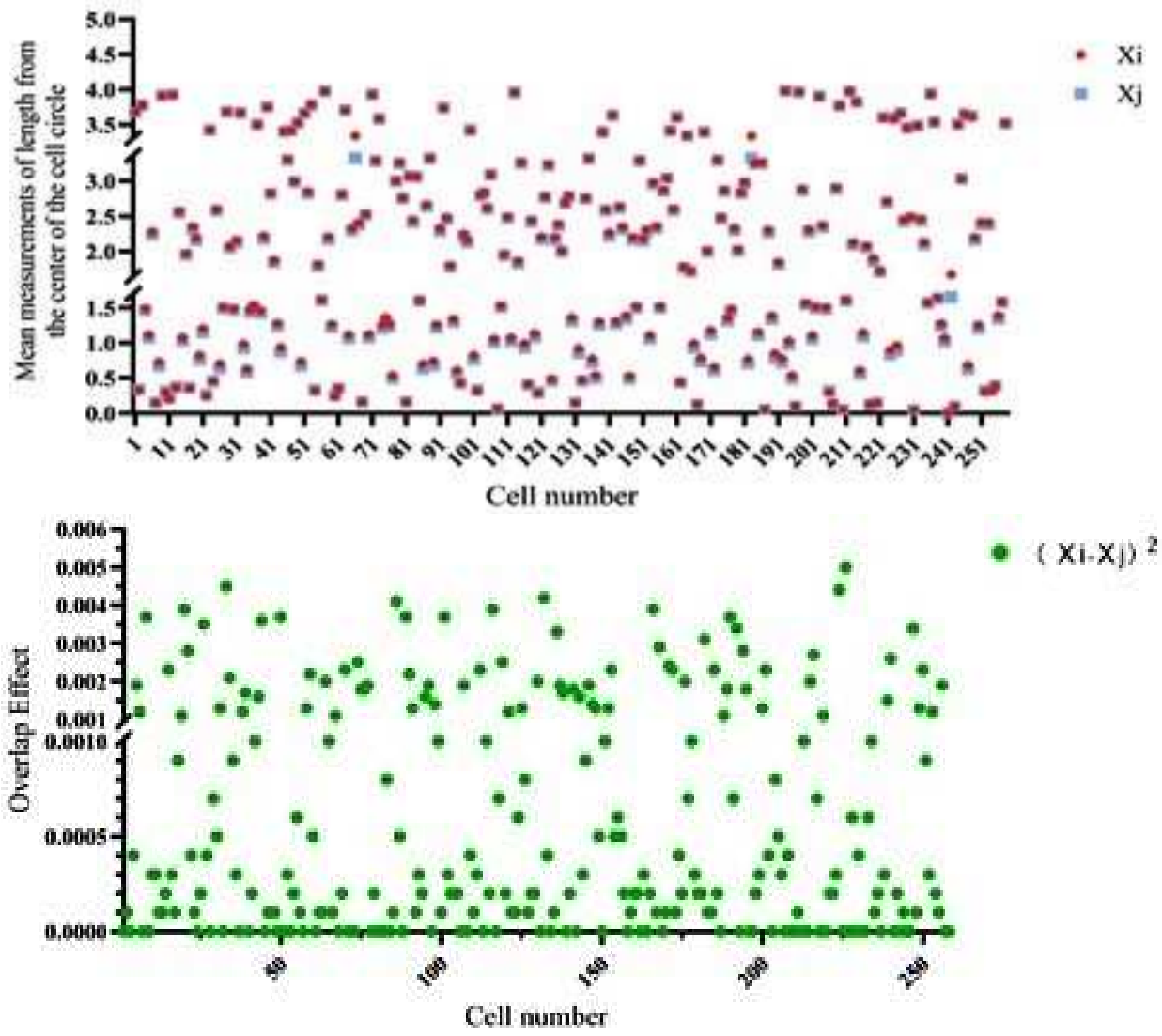


Figure 3. Scatter plot of geometric co-location relationship between CR1-like and FAP-1. (A) the scatter plot of the average measured values of CR1-like and FAP-1 lengths from the center of the cell circle. (B) the scatter plot of overlapping effect between CR1-like and FAP-1.

Western Blot: The membrane proteins of porcine blood cells were extracted according to ReadyPrep™ Protein Extraction kit. The extracted total membrane proteins were analyzed by SDS-PAGE. As seen from the figure, the total membrane protein bands of porcine erythrocytes were clear and neat. The molecular weight of membrane proteins was widely distributed, and the molecular weight of the bands for the test purpose was also included.(Figure 4)

Fig. 5 shows that the gel is incubated with FAP-1 monoclonal antibody and exposed to color analysis, and positive bands can be seen in lane 1; 2 and 3 are parallel samples, which also show the positive band of interest. 4 and 5 are mouse anti-pig IgG incubation groups with no visible bands; 6 and 7 are blank controls, and there are no visible bands.

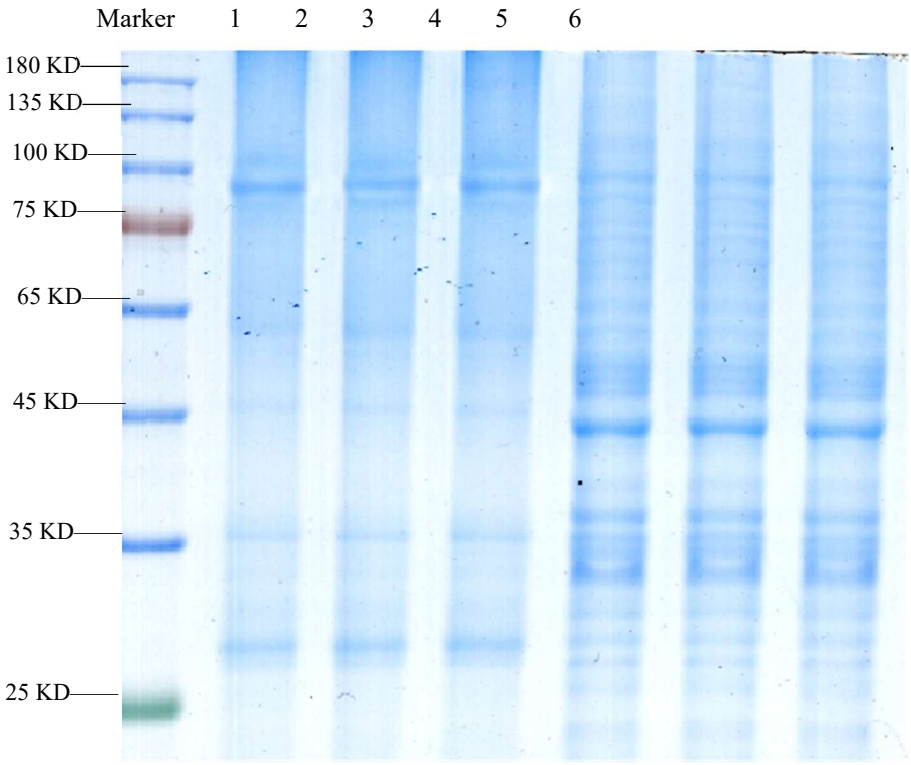


Figure 4. Results of porcine erythrocyte membrane total protein SDS-PAGE

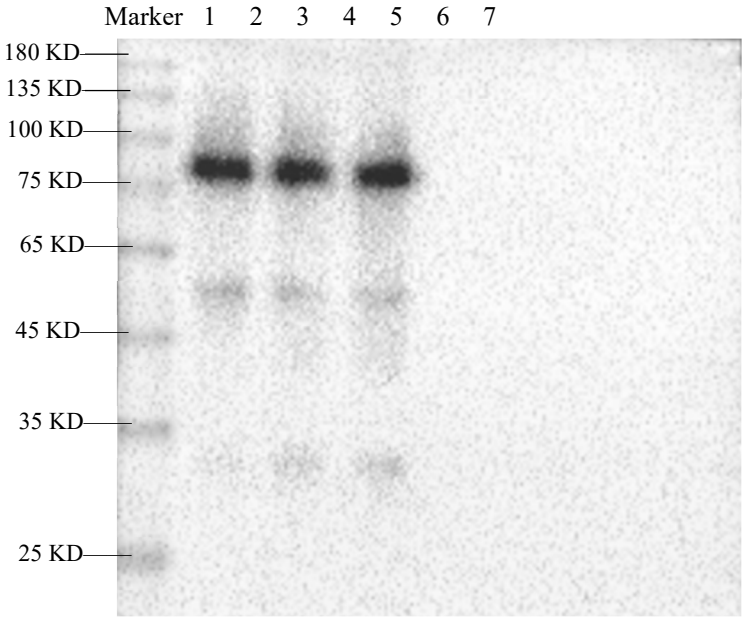


Figure 5. Results of porcine erythrocyte membrane total protein Western Blot. (1-3) test group, primary antibody is FAP-1 McAb;(4, 5) negative control group, mouse anti-pig IgG antibody incubation; (6, 7) blank group.

DISCUSSION

Phosphorylation is the most abundant post-translational modification (PTM) in proteins. Its importance is reflected in the space allocated for kinases in the genome (Buljan M *et al*., 2020). Phosphorylation occurs most commonly on serine, followed by threonine and tyrosine (Hornbeck PV *et al*., 2015). CR1 cytoplasmic domain peptide (CR1-tail peptide) bound PDZ domains 2, 3, and 5 of Fas-associated phosphatase 1

(FAP-1), a scaffolding protein with tyrosine phosphatase activity (Ghiran *et al*., 2008). Meanwhile, This research group investigated the interaction between porcine CR1-like and C3b using molecular simulation to clarify the molecular mechanism of the immune adhesion of porcine erythrocytes (Hou Z *et al*., 2023). Tyrosine phosphorylation, on the other hand, has a more important function of binding to proteins to provide specific structural genes to facilitate their interaction with other proteins to form multi-protein complexes to perform related functions, in addition to conformation and activation of the protein's activity. It has been found that FAP-1, as an important complex phosphatase molecule, is widely distributed on the surface of T lymphocytes, macrophages and other important immune cells and has an important role in the immune response of the body (Sato *et al*., 1995, Lu *et al*., 2016). However, whether FAP-1 is distributed on the surface of porcine erythrocytes and its function have not been clarified. In our study, we found that under physiological conditions, the distribution of CR1-like in porcine erythrocyte membranes was dispersed. When the CR1-like functioned as an immunoadhesive agent, the CR1-like exhibited multivalent and efficient binding to the sensitized particles, and its distribution changed to a particle-like cluster. It was further found that when the membrane fluidity of porcine erythrocytes was reduced by glutaraldehyde, the immunoadhesive activity of CR1-like was also reduced, which showed that the distribution state and membrane fluidity of CR1-like were the important physiological basis for the immunoadhesive function of CR1-like in porcine erythrocytes, but the related molecular mechanism was not yet clear. In view of this, the present study focused on the binding region of CR1-like to the erythrocyte membrane, and explored the molecular basis of the CR1-like distribution status affecting its immune function by using in situ fluorescence staining, geometric position calculation evaluation and immunoprecipitation assay. First, the immunofluorescence cytochemical staining results showed that the specific fluorescence site distribution of CR1-like and FAP-1 molecules in the porcine erythrocyte membrane skeleton were the same for both; second, the summation of the difference squares of site distances was performed for 253 typical positive cells, and the result was 0.2224, indicating that the difference of site distances of CR1-like and FAP-1 in each group was approximately 0, and the distribution of CR1 -like, FAP-1 distribution is consistent with the characteristics of co-location relationship; Third, the existence of CR1-like, FAP-1 interaction was further analyzed by immunoprecipitation technique, and the total membrane protein samples were treated with CR1-like incubation and then detected by Western Blot with FAP-1 monoclonal antibody, and the FAP-1 molecule was clearly observed in the detected gel. 1 molecule was clearly observed in the examined gels. Taken together, the results showed that porcine erythrocyte CR1-like does not bind directly to the erythrocyte membrane backbone protein, but is distributed on the surface of the porcine erythrocyte membrane through the riveted structure of the FAP-1 protein molecule. It has been confirmed that excessive oxidation of erythrocyte membrane leads to excessive phosphorylation of membrane proteins, which in turn affects erythrocyte membrane fluidity and its physiological functions, and FAP-1, as an important dephosphorylation molecule, has an important regulatory function in maintaining the normal state of membrane fluidity.

Conclusion: The study confirmed that porcine CR1-like molecules were distributed on the surface of porcine erythrocyte membranes through FAP-1 molecules, and preliminarily explored the molecular basis of the distribution state of CR1-like on porcine erythrocyte membranes.

Conflict of interest: None of the authors has any conflict of interest.

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Authors' contribution: Zhang zeting conceived and designed the experiments, performed the experiments, analyzed the data, prepared the graphs and/or tables, wrote the draft paper, and approved the final draft.

Ru Qing, Zhang Zheng, Fan Kuohai, Sun Na, Sun Panpan and Li Hongquan authored or reviewed drafts of the paper, approved the final draft.

Yin Wei conceived and designed the experiments, contributed reagents/materials/analysis tools, approved the final draft.

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