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The intracellular localization and association of porcine Ia-associated invariant chain with the MHC class I-related porcine neonatal Fc receptor for IgG

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ABSTRACT

The neonatal Fc receptor (FcRn) transports IgG from mother to young and is involved in antigen presentation. FcRn is structurally similar to MHC class I, but its intracellular trafficking pathway is much more analogous to that of MHC class II. Ia-associated invariant chain (Ii) molecules play an additional role in directing MHC class II trafficking within the endocytic compartments by physical association with MHC class II. This study addresses the question of whether pig Ii chain plays this important role in FcRn trafficking to the endoplasmic reticulum. Red or green fluorescent protein-fused Ii or FcRn was constructed, and the intracellular localization of pig Ii with FcRn was detected using confocal microscopy. Immunoprecipitation and western blotting were used to test for their association. The results indicate that pig Ii chain specifically interacts with both FcRn H chain alone and FcRn–β2m complex, and the CLIP in Ii was required for FcRn–Ii association. A truncated FcRn deletion in the cytoplasmic tail changed the intracellular localization of FcRn. However, the truncated FcRn can still combine Ii. This indicated that the cytoplasmic tail of FcRn fails to affect FcRn association with Ii. These results suggest that association of FcRn with Ii chain is relevant, and appreciation of this process is important to the understanding of how IgG is transported.

1. Introduction

The neonatal Fc receptor (FcRn) was identified in the 1970s as the protein that mediates transfer of maternal IgGs across the neonatal intestine (Jones and Waldmann, 1972). FcRn is composed of an H chain noncovalently associated with an L chain β2m (Simister and Mostov, 1989; Burmeister et al., 1994). Similar to MHC class I, FcRn consists of three extracellular alpha (α) domains, a single-pass transmembrane domain, and a short cytoplasmic tail (Ye et al., 2008a, 2008b). FcRn may play a major role in the passive acquisition of immunity in fetuses of some species and in newborns of most mammals, allowing newborns to obtain humoral immunity against Ags encountered by the mother before they develop their own immune system (Firan et al., 2001; Ghetie and Ward, 2000; Simister, 2000). The latest research showed that FcRn plays an important role in the process of antigen presentation (Liu et al., 2011).

It is clear that FcRn is present in numerous types of polarized epithelia, including those from the intestines, lungs, breasts, and kidneys, as well as other parenchymal cells, including hepatocytes, endothelial cells, and hematopoietic cells (Blumberg et al., 1995; Israel et al., 1997; Kacskovics et al., 2000). All forms of FcRn bind IgG at acidic pH (6.0–6.5) and release IgG at neutral or higher pH, due to conserved basic histidine residues within the CH2–CH3 domain interface of the Fc domain of IgG that interact with corollary acidic residues within the α2 and α3 domains of FcRn (Raghavan et al., 1995). It is well known that FcRn is involved in antigen presentation if the antigen is composed of the antigen-specific IgG immune complex (Wu and Simister, 2001; Zhao et al., 2003).

Invariant chain (Ii), also named CD74, is a type II integral membrane glycoprotein. It acts as a chaperone for the correct folding and the functional stability of MHC class II (Strubin et al., 1986). In the endoplasmic reticulum (ER), the assembled MHC class II–Ii complex is a nonamer, consisting of one Ii trimer associated with three MHC class II heterodimers (Anderson and Miller, 1992; Lamb and Cresswell, 1992; Roche et al., 1991). In epithelial cells, the complexes are highly dependent on the Ii chain's two acidic dileucine-based endosomal targeting motifs, which direct MHC class II to the endocytic pathway (Dugast et al., 2005). On entry of the nonameric complex to endosomes, the C-terminal (lumenal) and N-terminal (cytoplasmic) residues of the

Abbreviations: FcRn, the neonatal Fc receptor; Ii, Ia-associated invariant chain; CLIP, the class II-associated Ii peptide; GFP, green fluorescent protein; RFP, red fluorescent protein.

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Ii chain are proteolytically degraded in stepwise fashion, leaving progressively smaller fragments of Ii associated with the MHC class II (McCormick et al., 2005). The release of the final degradation product, the class II-associated Ii peptide (CLIP) fragment, facilitates the binding of antigenic peptides derived from internalized Ags for Ag presentation (Castellino and Germain, 1995). Thus, the Ii chain plays a key role in the MHC class II antigen presentation pathway.

FcRn is structurally similar to MHC class I, and its intracellular trafficking pathway is somewhat analogous to that of MHC class I. Ye et al. reported that Ii was capable of associating with FcRn and regulating FcRn trafficking (Ye et al., 2008a, 2008b). Basha et al. reported that Ii associated with MHC class I in the ER of dendritic cells (DCs) and mediated the trafficking of MHC class I to endolysosomal compartments for loading with exogenous peptides (Basha et al., 2012). Here we ask if pig Ii can associate with FcRn, and whether the CLIP of pig Ii plays a key role in the assembly of the FcRn–Ii complex, using confocal microscopy to examine the intracellular localization of pig Ii and FcRn, and immunoprecipitation and western blotting to analyze their association.

2. Materials and methods

2.1. Cell lines, Abs

COS-7 African green monkey kidney cells were purchased from the Gibco Company (Grand Island, NY). P815 is a mouse mastocytoma cell line, obtained from Biology Science College, University of Science and Technology of China, which was cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Mouse monoclonal anti-GFP and anti-FLAG were purchased from Sigma-Aldrich (St. Louis, MO). HRP-conjugated goat anti-mouse Ab was purchased from Pierce. Mouse monoclonal anti-Ii and anti-FcRn were prepared by our laboratory (data not shown).

2.2. RT-PCR analysis

Duodenum samples were collected from newborn piglet using the Animal Use Protocol approved by the China Institutional Animal Care and Use Committee. For total RNA extraction, cells were pelleted and resuspended at 10^6 cells/ml in Trizol reagent (Invitrogen, Carlsbad, CA). The pig FcRn gene was amplified by primers (FcRn1: 5′-GAAGATCTGG TCTCCCGCTCAGGAT-3′, FcRn2:5′-GCGTCGACGACCAGCAGGCAGTTG CT-3′) with a one-step RT-PCR kit (Qiagen). The mRNA was also amplified with specific primers (β2m1: GAAGATCTCATGGCTCCCCTCG TGGC, β2m2: GCGTCGACTGGTGGTCTCGATCCCAC) to obtain the pig β2m gene. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

2.3. Construction of FcRn and Ii wild-type and mutant expression plasmids

The cDNA fragment encoding pig Ii made in our laboratory was subcloned into the peCherry-C1, pCDNA3, and pEGFP-C1 vectors (Xu et al., 2013a, 2013b). The cDNA fragments encoding pig β2m and FcRn were subcloned into pEGFP-N1 expression plasmids and pcDNA3-FLAG, respectively. The PCR primers FcRnM1 (5′-gaagatctATGCGGGTCCCCCGGCCTCAGCCCTG-3′) and FcRnM2 (5′-GCGTCGAC-CTTCCTCATCCTCCTCCACAGCAGAGC-3′) were used to construct a plasmid encoding a truncated FcRn protein lacking the cytoplasmic tail. The DNA fragment was digested and ligated into the plasmids pEGFP-N1 and pcDNA3-FLAG to generate the plasmids pEGFP-N1-FcRn-M and pcDNA3-FLAG-FcRn-M. The construction of expression plasmid pCDNA3-ΔCLIP-Ii for a pig Ii mutant protein lacking the CLIP region (ΔCLIP-Ii) has been described previously (Xu et al., 2013a, 2013b). All constructs were sequenced to verify the accuracy of the amplification.

2.4. Transient transfection of COS-7 cells

The process of transient transfection has been described previously (Xu et al., 2008; Ye et al., 2009). COS-7 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, USA). Briefly, 70% confluent COS-7 cells were split into six wells (for immunoprecipitation) or 24 wells (with coverslips for immunofluorescence microscopy) 1 day before transfection. COS-7 cells were transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Protein expression was examined 36-48 h after transfection.

2.5. Stable transfection of P815

The stable cells, $P815^{FcRn}$ and $P815^{Ii}$, have been described previously (Ye et al., 2008a, 2008b; Zhu et al., 2002). Briefly, P815 cells were cultured in RPMI-1640 including 25 mM HEPES, 2 mM glutamine, 10% heat-inactivated fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 pg/ml streptomycin. P815 cells were stably transfected with pCDNA3-Ii or pCDNA3-FLAG-FcRn by use of Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, and G418 was used to select the positive clones. After transfection and selection, positive clones were determined for RT-PCR and protein expression by western blotting using anti-Ii or anti-FcRn Ab. Transfectants were maintained in the medium containing G418 (400 μg/ml).

2.6. Confocal fluorescence microscopy

Expression of green fluorescent protein (GFP) or red fluorescent protein (RFP) was used as a marker of positively transfected cells. At 36–48 h after transfection, images of the COS7 cells were acquired with a Zeiss confocal laser-scanning microscope (CLSM) using a 60× oil objective (excitation at 488 nm for RFP and emission at 515 nm for GFP).

2.7. Immunoprecipitation and western blotting

At 36 h post-transfection, the cells were harvested and lysed in 1 ml immunoprecipitation lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and 1 protease inhibitor cocktail tablet (Roche)] at 4 °C for 1 h. The cells were then centrifuged at 12,000 *g* at 4 °C for 1 h, and 20 μl Protein A/G Plus-Agarose beads (GE Healthcare, USA) were added to the supernatants and incubated at 4 °C for 2 h. Immunoprecipitations were done as described previously (Chen et al., 2012) Briefly, immunoprecipitation was performed with an anti-GFP mAb or anti-FLAG mAb and Protein A/G Plus-Agarose beads. The unbound proteins were removed with PBS. Subsequently, washed immunoprecipitates were resolved on a 12% SDS-PAGE gel under reducing conditions and electrotransferred to a nitrocellulose membrane (Millipore, Germany). The membranes were blocked with 10% calf serum, incubated separately with anti-FcRn Ab or anti-Ii Ab for 1 h, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse Ab (Pierce, Rockford, USA). All blocking, incubation, and washing steps were performed in PBST solution (PBS and 0.05% Tween 20).

2.8. IgG binding assay

An IgG binding assay was performed as previously described (Zhu et al., 2005), with the following modifications. Cells were lysed in PBS (pH 6.0 or 7.5) with 0.5% CHAPS (Sigma-Aldrich) and a protease inhibitor mixture on ice for 1 h. Supernatants containing soluble proteins were incubated with IgG Sepharose (Amersham Pharmacia Biotech) at 4 °C overnight. The unbound proteins were removed with PBS (pH 6.0 or 7.5) containing 0.1% CHAPS. The adsorbed proteins were boiled with reducing electrophoresis sample buffer at 95 °C for 5 min. The