The intracellular localization and oligomerization of chicken invariant chain with major histocompatibility complex class II subunits

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ABSTRACT Invariant chain (Ii) binds to MHC class II (MHCII) to assemble a nonamer in the endoplasmic reticulum. Major histocompatibility complex class II-associated Ii peptide (CLIP) that occupies the peptide binding groove of MHCII prevents MHCII molecules from loading with endogenous antigens. We used the green or red fluorescent protein-fused Ii or MHCII subunits to detect the intracellular localization and oligomerization of chicken Ii with single chicken MHCII subunits. Our results indicated that chicken Ii associates with single MHCII subunits and formed oligomers with MHCII subunits. The Ii mutant with a deleted CLIP sequence blocks the association with single MHCII subunits, but exchanging CLIP with the Newcastle disease virus F_{343} epitope restores this association. Thus, MHCII polymer assembly is not blocked as long as the basic steric molecular structure of Ii is maintained, and different binding models exist in different species or MHCII isotypes.

Key words: chicken invariant chain, major histocompatibility complex class II subunit, colocalization, oligomerization

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INTRODUCTION

In the endoplasmic reticulum (**ER**), the final assembled MHC class II (**MHCII**)-invariant chain (**Ii**) complex is a nonamer, consisting of 1 Ii trimer associated with 3 MHCII heterodimers (Roche et al., 1991; Anderson and Miller, 1992; Lamb and Cresswell, 1992). The association of Ii with MHCII molecules involves the direct occupancy of the peptide-binding groove with a region of Ii known as the MHCII-associated Ii peptide (**CLIP**) domain. Occupancy of the MHCII peptide binding site with CLIP prevents MHCII molecules from loading endogenetic antigenic peptides in the ER (Roche and Cresswell, 1990; Teyton et al., 1990; Odorizzi et al., 1994). Most studies on MHCII and Ii association were done in mammals, particularly humans. The CLIP region (especially amino acids 91 to 99: MRMATPLLM) is completely conserved in human, mouse, and rat (Figure 1A), but this striking conservation does not extend to other species (Dijkstra et al., 2003), including poultry (Zhong et al., 2004, 2006). We found that leucine-based sorting motifs direct the localization of the cytoplasmic tail of chicken Ii (Xu et al., 2008). However, it is unknown if chicken Ii can associate with a single MHCII α or β chain and whether its CLIP plays a key role in the assembly of MHCII-Ii complex, or if the CLIP-substituted Ii can associate with MHCII subunits. We used the green fluorescent protein (**GFP**)- or red fluorescent protein (**RFP**)-fused Ii or MHCII subunits to detect the intracellular localization and oligomerization of chicken Ii with single chicken MHCII subunits and confirmed that chicken CLIP (amino acids 81 to 107) helps maintain oligomerization. We also tested whether Ii mutants with CLIP exchanged for Newcastle disease virus (**NDV**) F_{343} epitopes (amino acids 327 to 359) could still interact with MHCII α or β chains.

MATERIALS AND METHODS

Expression Vectors

The expression vectors pEGFP-C1 and pEGFP-N1 (both Clontech, Mountain View, CA) encode a red-shifted variant or wild-type GFP (Prasher et al., 1992; Chalfie et al., 1994; Inouye and Tsuji, 1994; Plautz et al., 1996), and pDsRed2-N1 vector (Clontech) encodes DsRed2 derived from the *Discosoma* sp. RFP (Fradkov et al., 2000; Yanushevicha et al., 2002). Green fluorescent protein or RFP has been optimized for brighter fluorescence and greater expression in mammalian cells (Prasher et al., 1992; Chalfie et al., 1994; Inouye and

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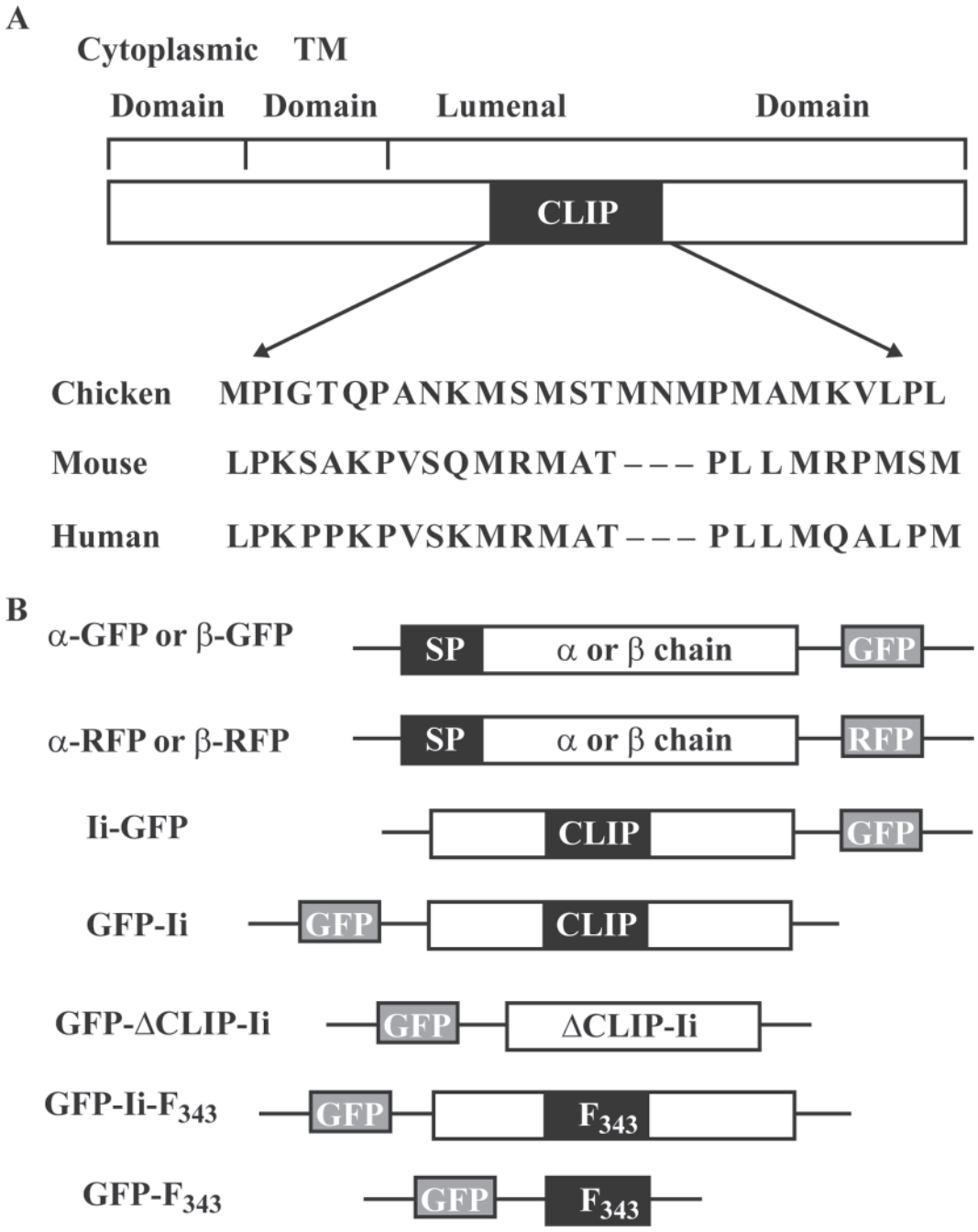

Figure 1. Schematic illustrations of the MHC class II (MHCII)-associated Ii peptide (CLIP) sequence and the tagged constructs used in this study. (A) A schematic illustration of full-length invariant chain (Ii). The CLIP domain (black) from several species is shown. (B) Chicken MHCII α, β chain, Ii, and its mutant tagged green fluorescent protein (GFP) or red fluorescent protein (RFP) constructs are depicted schematically. Chicken MHCII α or β chain was attached at the N-terminus of enhanced green (pEGFP-N1-α, pEGFP-N1-β) or red fluorescent protein (pDsRed2-N1-α, pDsRed2-N1-β); Ii-GFP (pEGFP-N1-Ii) indicated that chicken Ii was fused at the N-terminus of enhanced GFP. GFP-Ii (pEGFP-C1-Ii) indicated that chicken Ii was fused at the C-terminus of enhanced GFP. GFP-ΔCLIP-Ii (pEGFP-C1-ΔCLIP-Ii), GFP-Ii-F_{343} (pEGFP-C1-Ii-F_{343}), and GFP-F_{343} (pEGFP-C1-F_{343}) indicated that chicken Ii mutants and F_{343} epitope fragments were fused at the C-terminus of enhanced GFP. Signal peptide (SP), CLIP, and F_{343} are indicated by a black box, and GFP or RFP tag is indicated by a gray box. TM = transmembrane.

Tsuji, 1994; Plautz et al., 1996; Fradkov et al., 2000; Yanushevicha et al., 2002). Genes cloned into the multiple cloning site in pEGFP-C1 vector will be expressed as fusions to the C-terminus of the enhanced GFP (**EGFP**), but genes cloned into the multiple cloning site in pEGFP-N1 vector or pDsRed2-N1 vector will be expressed as fusions to the N-terminus of EGFP or RFP. The fluorescent protein (GFP or RFP) fusion protein expressed from pEGFP-C1, pEGFP-N1, or pDsRed2-N1 can be used to monitor gene expression and protein localization for the gene of interest. Fusions to GFP or RFP retain the fluorescence properties of the native protein, allowing the fusion protein to be localized in vivo. The recombinant EGFP or RFP vector can be transfected into mammalian cells by using any standard transfection method.

DNA Constructs

The cDNA fragment encoding chicken MHCII α (GenBank accession no. AY357254) and β (GenBank accession no. S66480) made in our laboratory were subcloned into the expression vectors pDsRed2-N1 and pEGFP-N1. The cDNA fragment encoding chicken Ii made in our laboratory (Zhong et al., 2004) was subcloned into the pEGFP-N1 and pEGFP-C1 vector.

The primer sequences are as follows: pDsRed2-N1-α and pEGFP-N1-α, forward primer 5′-GAAGATCT-CATGGCGGTGCTGAGCGGAG-3′, reverse primer 5′-GCGTCGACTGGAGCAGCCCCGGTTGG-3′; pDsRed2-N1-β and pEGFP-N1-β, forward primer 5′-GAAGATCTCATGGGGAGCGGCGCGTC-3′, reverse primer 5′-GCGTCGACTGATTCAGCATCCCT-GGAGCGG-3′. Polymerase chain reaction conditions (30 cycles) were as follows: denature for 1 min at 94°C, annealing for 1 min at 62°C, and extension for 2 min at 72°C.

The primer sequences of pEGFP-C1-Ii and pEGFP-N1-Ii are listed: pEGFP-C1-Ii, uni 5′-CCCGAATTC-TATGGCTGAGGAGCAGCGGGAC-3′,pEGFP-N1-Ii, uni 5′-CCCGAATTCTGATGGCTGAGGAGCAGCG-GGAC-3′, pEGFP-C1-Ii and pEGFP-N1-Ii, rev 5′-GCGTCGACTGCTTGGCTTTCACCATGTCC-3′. Polymerase chain reaction conditions (30 cycles) were as follows: denature for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C.

A chicken Ii mutant with a deleted CLIP region (ΔCLIP-Ii) was constructed by overlap extension PCR. The forward round of PCR was performed with the primers: pEGFP-C1-Ii, uni 5′-CCCGAATTCTATG-GCTGAGGAGCAGCGGGAC-3′ and the mutagenic anti-sense primer rev 5′-GTCACCAACAGAGGGAG-CAAGCTTTCTCTGCAGCGACTCC-3′; the back-ward round of PCR was performed with the primers: the mutagenic sense primer uni 5′-GGAGTCGCTG-CAGAGGAAGCTTGCTCCCTCTGTTGGTGAC-3′ and pEGFP-C1-Ii, rev 5′-GCGTCGACTGCTTG-GCTTTCACCATGTCC-3′. Polymerase chain reaction conditions (30 cycles) were as follows: denature for 1 min at 94°C, annealing for 1 min at 62°C, and extension for 2 min at 72°C. Two rounds of PCR products of about 250 bp were amplified. The third round PCR used the products from the forward round and the backward round of PCR above as template and used the same primers and PCR conditions as pEGFP-C1-Ii. Thus, pEGFP-C1-ΔCLIP-Ii was constructed. The removal of CLIP produced a *Hin*dIII restriction site at that position. Newcastle disease virus F_{343} epitope genes (99 bp) were amplified by conventional PCR with primers: uni 5′-CCCAAGCTTGTCGGGTCTGTGA-TAGAG-3′, rev 5′-CCCAAGCTTATAAATACCAG-GAGACAT-3′, whereas the *Hin*dIII restriction site was introduced into each end of the cDNA simultaneously. Polymerase chain reaction conditions (30 cycles) were as follows: denature for 1 min at 94°C, annealing for 1 min at 52°C, and extension for 1 min at 72°C. The amplified F_{343} epitopes and pEGFP-C1-ΔCLIP-Ii were both digested by *Hin*dIII (Takara, Dalian, China), and then pEGFP-C1-ΔCLIP-Ii was phosphorylated and subsequently linked with F_{343} epitope genes to produce the pEGFP-C1-Ii-F_{343} vector. The primers for pEGFP-C1-F_{343} were: uni 5′-GGAAGATCTGTCGGGTCTGT-GATAGAG-3′, rev 5′-GCGTCGACATAAATACCAG-GAGACAT-3′. Polymerase chain reaction conditions (30 cycles) were as follows: denature for 1 min at 94°C, annealing for 1 min at 52°C, and extension for 1 min at 72°C. The PCR reactions were performed by using Py-robest DNA polymerase (Takara) for primer extension with an FTC-312 thermocycler (Barloworld Scientific, Stone, Staffordshire, UK). After all PCR products were cloned into the appropriate eukaryotic expression vectors, they were sequence-verified (Invitrogen, Shanghai, China).

Cell Culture and Transient Transfection of COS-7 Cells

The COS-7 cell lines were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum (Gibco) and 1% pen-icillin-streptomycin (Sigma, Steinheim, Germany) at 37°C in 5% CO_2.

The transfection procedure has been described previously by Huylebroeck et al. (1988). Briefly, 75% confluent COS-7 cells were split into 24 wells (with coverslips for immunofluorescence microscopy) or 6 wells (subjected to immunoprecipitation) 1 d before transfection. The cells were seeded in 24-well plates with 3×10^4 cells/well or in 6-well plates with 1×10^6 cells/well in Dulbecco's modified Eagle's medium (Gibco) without serum. Amount of DNA was 0.8 μg/well (24-well plates) or 4 μg/well (6-well plates); amount of Lipofectamine 2000 (Invitrogen) was 2.0 μL/well (24-well plates) or 10.0 μL/well (6-well plates). Transfection was performed by using Lipofectamine 2000 for transient transfections, according to the instructions of the manufacturer.

Fluorescence Microscopy

Expression of GFP or RFP was used as a marker of positively transfected cells. At 24 to 48 h after transfection, viable cells grown on coverslips were fixed with 4% paraformaldehyde. The cells were visualized with an Olympus fluorescence microscope (Olympus, Tokyo, Japan).

Immunoprecipitation and Western Blotting

The cells were lysed as described previously by Shachar et al. (1995). The cell lysate was precleared with protein G agarose (Amersham Pharmacia, Upp-sala, Sweden) to reduce background caused by nonspe-cific adsorption of irrelevant cellular proteins to pro-

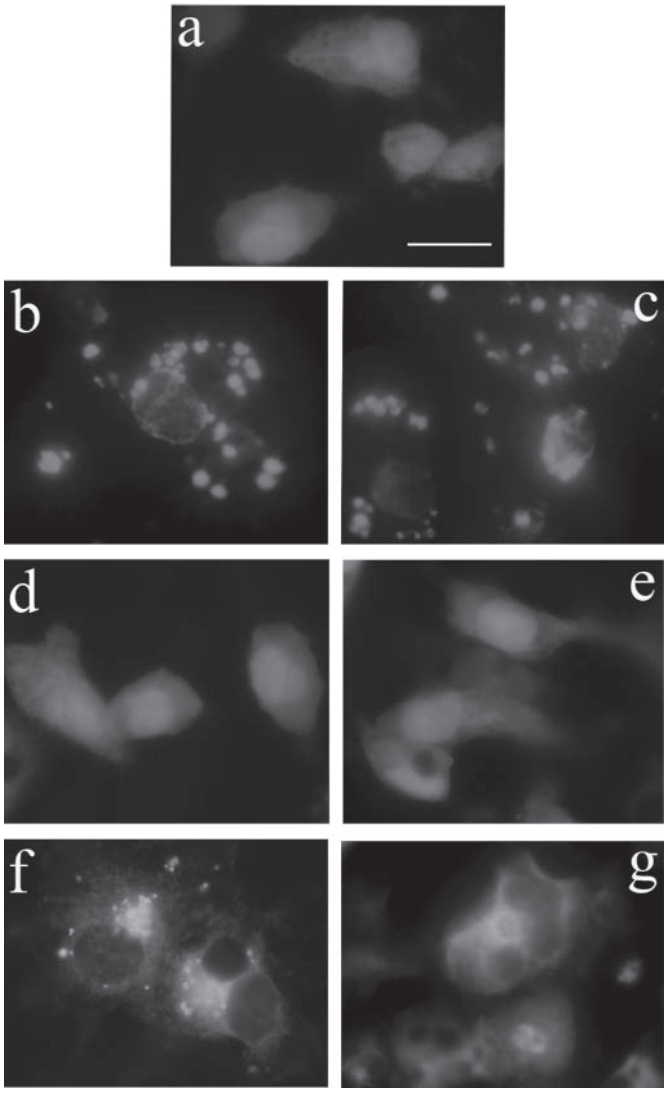

Figure 2. The expression and localization of chicken MHC class II α, β, and invariant chain (Ii) in COS-7 cells. COS-7 cells transiently transfected with pDsRed2-N1 (a), pDsRed2-N1-α (b), pDsRed2-N1-β (c), pEGFP-N1 (d), pEGFP-C1 (e), pEGFP-C1-Ii (f), and pEGFP-N1-Ii (g) were visualized by fluorescence microscopy. Bar, 10 μm.

tein G agarose. Fifty microliters of the homogeneous protein G agarose suspension (per 1- to 3-mL sample) was added to the lysate and incubated for 3 h (4°C). Beads were centrifuged at 12,000 × *g* for 20 s at 4°C. The supernatants were either used directly for immunoprecipitation or frozen at −80°C. Protein G Sepharose (Amersham Pharmacia; 25 μL/sample) was conjugated to the mouse polyclonal antibodies anti-Ii (5 μL) for 4 h (4°C) followed by 3 × PBS washes, and the lysates were immunoprecipitated overnight (4°C) with 25 μL of conjugate for each sample. The protein G-bound material was washed 3 times with PBS containing 0.1% Nonidet P40 (Roche, Mannheim, Germany), 0.05% sodium deoxycholate. Subsequently, washed immunoprecipitates were resolved by SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Millipore, Schwalbach, Germany). The blots were blocked with 10% (vol/vol) fetal cattle serum for 1 h and then probed for 1 h with a mouse monoclonal anti-GFP (Clontech), followed by washing and 1 h incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) and peroxidase visualization by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

RESULTS

We measured the intracellular interaction of chicken Ii with individual MHCII polypeptides, especially the CLIP domain and the antigenic peptide, with MHCII α or β chains using the following recombinant eukaryotic expression vectors: pDsRed2-N1-α, pEGFP-N1-α, pDsRed2-N1-β, and pEGFP-N1-β, which contain a gene segment of MHCII α or β chain with an open reading frame of 771 or 798 bp, for a putative 256 or 265 amino acids. It has been found that pEGFP-C1-Ii and pEGFP-N1-Ii contain a gene segment of Ii. In addition, pEGFP-C1-ΔCLIP-Ii contains Ii mutants with a deleted CLIP region, pEGFP-C1-Ii-F_{343} contains Ii mutants with the CLIP region replaced with NDV F_{343} epitope fragments, and pEGFP-C1-F_{343} contains NDV F_{343} epitope fragments. Recombinant constructs were sequence-verified (Figure 1B).

The Expression and Localization of Chicken MHCII α, β, and Ii in COS-7 Cells

First, we observed the expression and the intracellular localization of chicken MHCII α, β, and Ii in COS-7 cells using a fluorescence microscope 48 h after transfection. Control cells transfected with pDsRed2-N1, pEGFP-C1, and pEGFP-N1 showed strong cytoplasmic staining (Figure 2a, d and e), but transfection with pDsRed2-N1-α or pDsRed2-N1-β produced fluorescence in endomembrane vesicles (Figure 2b and c). Similarly, pEGFP-C1-Ii and pEGFP-N1-Ii transfection produced fluorescence in endomembrane vesicles (Figure 2f) or in the cytoplasm (Figure 2g). The pEGFP-N1-α and pEGFP-N1-β fusion proteins were expressed in the cellular endomembrane system as well (data not shown). Thus, fusions to the N-terminus of RFP or GFP (α-RFP, α-GFP, β-RFP, and β-GFP) or to the C-terminus of GFP (GFP-Ii) retain the fluorescence properties of the native protein, allowing the fusion protein to be localized in vivo.

Colocalization of Chicken Ii and MHCII α or MHCII β Chain in COS-7 Cells

To evaluate the colocalization of chicken Ii with individual MHCII α or β polypeptides, we transiently cotransfected pEGFP-C1-Ii and pDsRed2-N1-α or pDsRed2-N1-β into COS-7 cells (Figure 3).The transfection with pDsRed2-N1-α or pDsRed2-N1-β produced red fluorescence in endomembrane vesicles (Figure 3a and c), and pEGFP-C1-Ii transfection produced green fluorescence in endomembrane vesicles (Figure 3b and

d). Three kinds of polypeptides (α-RFP, β-RFP, and GFP-Ii) maintained intracellular localization, with yellow fluorescence showing Ii and MHCII subunit colocalization only in a portion of the green vesicles (Figure 3b, d), indicating oligomerization of the GFP-Ii and α-RFP or β-RFP genes. Normally, these genes are expressed in different parts of the cell, with no fluorescence overlap, but coexpression forms oligomers in the endomembrane system to produce yellow fluorescence.

Association of Chicken Ii with Individual MHCII Polypeptides

To determine whether the Ii-MHCII interaction required the CLIP binding segment in Ii, we tested the interaction of pEGFP-C1-Ii or pEGFP-C1-ΔCLIP-Ii with pEGFP-N1-α or pEGFP-N1-β. We performed immunoprecipitation with mouse antibodies against Ii, separation with SDS-PAGE, and then immunoblotting for individual MHCII polypeptides using monoclonal antibodies against GFP. The full Ii could bind to the monomorphic α or β chain and form GFP-α-Ii or GFP-β-Ii oligomers (Figure 4, lane 3 and 2), whereas CLIP deletion blocked the association with MHCII α or β chain (Figure 4, lane 7 and 6).

Interaction of the NDV F_{343} Epitope Substituted for CLIP and Individual MHCII Polypeptides

The binding of CLIP to the MHCII peptide binding site prevents MHCII molecules from loading endogenetic antigenic peptides and ensures association with exogenous peptides. Deletion of CLIP in Ii abolished the interaction with MHCII peptides. We therefore constructed an Ii chimera gene (pEGFP-C1-Ii-F_{343}), in which the NDV F_{343} epitope fragment was substituted for CLIP. Transfection with pEGFP-C1-F_{343} or pEGFP-C1-Ii-F_{343} produced fluorescence staining in the cytoplasm (Figure 5A, a) or intracellular vesicles (Figure 5A, b), suggesting that the NDV F_{343} peptides could efficiently load MHCII into the endomembrane system. The Ii-F_{343} mutant could also bind to MHCII α or β chain (Figure 5B, lane 2 and 1).

DISCUSSION

Green fluorescent protein or RFP, as a marker for gene expression, can monitor target gene expression and protein localization in live cells (Prasher et al., 1992; Chalfie et al., 1994; Inouye and Tsuji, 1994; Plautz et al., 1996; Fradkov et al., 2000; Yanushevicha et al., 2002), but N- or C-terminal attachment to these proteins can affect intracellular localization of the fusion protein. A SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/) analysis based on amino acid sequence showed that the N-terminal segment of MHCII α or β chains contains the signal peptide, whereas the N-terminal of Ii contains a signal anchor, namely an uncleaved signal peptide. Signaling peptides and anchoring activity mediate arrival at the ER and maintenance of intracellular localization. When a protein biosynthesis is finished, its signal peptide will be cleaved by the signal peptidase I (SPase I) in the side of ER lumen, but the signal anchor will be not cleaved (Zhai et al., 2001). If MHCII α and β containing signal peptide are attached to the C-terminal of GFP or RFP, GFP or RFP will be cleaved along with signal peptide, as a result GFP or RFP, as a reporter gene, cannot monitor target gene expression and protein localization in live cells. Therefore, MHCII α and β was linked to the N-terminal of GFP or RFP. But Ii containing an uncleaved signal peptide can be linked to the C-terminal of GFP. This strategy allowed direct observation of the biological activity of the fusion protein.

As polymorphic molecules, some MHCII isotype and allotype subunits are expressed in an antigen-presenting cell, and it is difficult to examineMHCII assembly. So far, many studies focus on how and where mammalian Ii binds to MHCII α or β, and they finally finish assembly as a complex of a nonamer (Roche et al., 1991; Anderson and Miller, 1992; Lamb and Cresswell, 1992; Neumann and Koch, 2005; Neumann and Koch, 2006). In this study, a series of experiments were carried out to

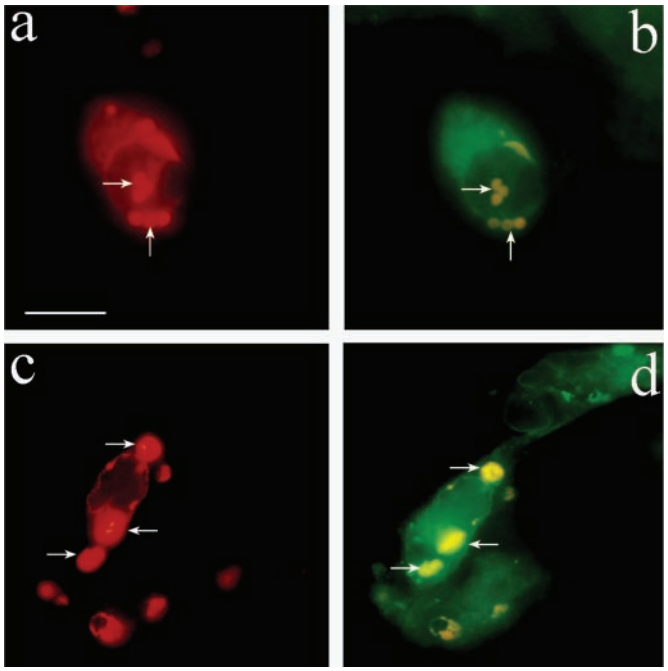

Figure 3. Colocalization of chicken invariant chain (Ii) and MHC class II α or β chains in COS-7 cells. COS-7 cells transiently transfected with pEGFP-C1-Ii and pDsRed2-N1-α were visualized by fluorescence microscopy: a = α-red fluorescent protein (RFP), b = green fluorescent protein (GFP)-Ii in the same cell, yellow indicating colocalization of GFP-Ii and α-RFP. Cells transiently transfected with pEGFP-C1-Ii and pDsRed2-N1-β were visualized by fluorescence microscopy: c = β-RFP, d = GFP-Ii in the same cell, yellow indicating colocalization of GFP-Ii and β-RFP. Bar, 10 μm. The arrows indicate the sites for the colocalization of chicken invariant chain (Ii) and MHC class II α or β chains in COS-7 cells.

investigate the association of chicken Ii and its mutants with single chicken MHCII subunits. In a comparison of the interaction of chicken Ii with MHCII subunits with that of mammal, we found that there were similarities and differences. Human Ii can only associate with 3 MHCII α isotypes, and mouse Ii can bind to I-Aα and I-Eα, but only partially bind to the monomorphic I-Eβ (Neumann and Koch, 2005). Chicken Ii was found to bind to both MHCII α and β chains (Figure 3 and 4), which indicates that different binding models exist among species or MHCII isotypes. Furthermore, it is known that the CLIP fragment binds to the MHCII αβ dimer DR3 (Ghosh et al., 1995), and the MHCII groove contains several anchor positions for CLIP binding (Liang et al., 1996; Serwe et al., 1997). A chicken Ii mutant with a deleted CLIP cannot bind to MHCII α or β chain, which indicates that the CLIP plays a key role on the assembly of Ii with MHCII subunits, and that the chicken CLIP shares functional properties with its mammalian homologs in spite of their sequence differences.

In addition, according to the characteristics of the CLIP occupying the peptide binding groove of class II before the MHCII·Ii complex targeting to acidic endosomal compartments (Bangia and Watts, 1995; Thayer et al., 1999), we constructed the chimera gene encoding chicken Ii chain containing NDV F_{343} epitope fragment (amino acids 327 to 359) replacing CLIP. An interesting phenomenon was found (Figure 5A): the Ii chimera changed the localization of F_{343} antigenic peptides in COS-7 cella. Under the direct guidance of the sorting motifs of the cytoplasmic tail of chicken Ii (Xu et al., 2008), the CLIP-substituted Ii can efficiently load F_{343} antigenic peptides into the vesicles of the endomembrane system in the transfected cells. Moreover, it is more important that a chicken Ii mutant with a deleted CLIP cannot bind to MHCII α or β chain, but a substituted NDV F_{343} sequence can (Figure 4 and 5), indicating that CLIP replacement does not block MHCII polymer assembly as long as Ii maintains its basic steric molecular structure. All of the data above suggest that the CLIP-substituted Ii vector targets the peptide to endosomes as well as to the groove of MHCII. Therefore, in the designing of effective DNA vaccines, chicken Ii could act as a potential carrier of the antigenic peptides based on its function mentioned before.

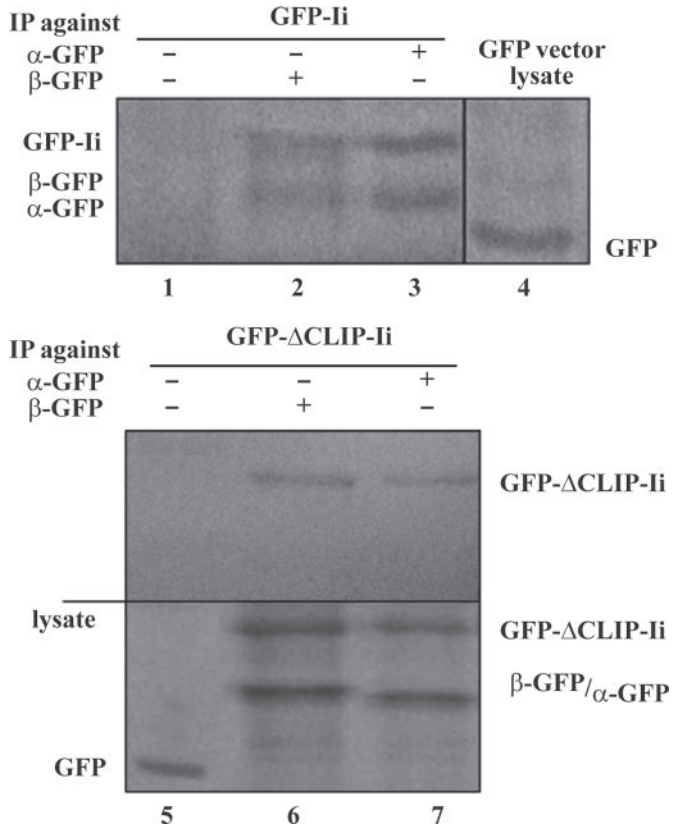

Figure 4. Interaction of chicken invariant chain (Ii) with single MHC class II (MHCII) polypeptides. COS-7 cells were transfected with green fluorescent protein (GFP)-Ii and β-GFP (lane 2) or α-GFP (lane 3), with GFP-ΔCLIP-Ii and β-GFP (lane 6) or α-GFP (lane 7). Chicken Ii or ΔCLIP-Ii was immunoprecipitated (IP) with mouse polyclonal antibodies against chicken Ii; subsequently, MHCII α or β polypeptides tagged with GFP were conducted with monoclonal antibodies against GFP for Western blotting. Lanes 1 and 5 are GFP empty vector control. Lane 4 is GFP empty vector lysate. CLIP = MHCII-associated Ii peptide.

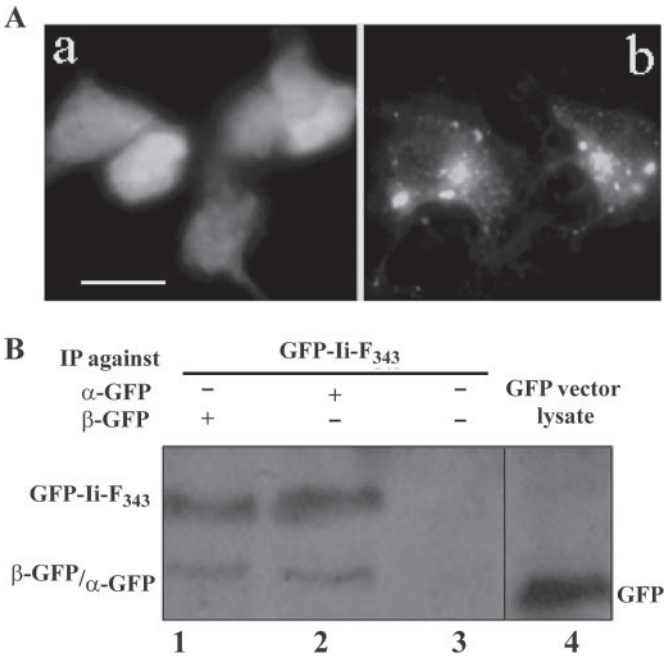

Figure 5. Interaction of a mutant with the Newcastle disease virus (NDV) F_{343} epitope substituted for MHC class II (MHCII)-associated Ii peptide (CLIP) and individual MHCII polypeptides. (A) The CLIP-substituted invariant chain (Ii; pEGFP-C1-Ii-F_{343}) can change the intracellular localization of the NDV F_{343} epitope peptides and efficiently load it onto the endomembrane system. (a) pEGFP-C1-F_{343}; (b) pEGFP-C1-Ii-F_{343}. Bar, 10 μm. (B) The interaction of CLIP-substituted Ii with single MHCII polypeptides. COS-7 cells were transfected with green fluorescent protein (GFP)-Ii-F_{343} and β-GFP (lane 1) or α-GFP (lane 2). GFP-Ii-F_{343} was immunoprecipitated (IP) with polyclonal antibodies against chicken Ii. α-GFP and β-GFP polypeptides tagged with GFP were conducted with monoclonal antibodies against GFP for Western blotting. Lane 3 is empty vector control. Lane 4 is GFP empty vector lysate.

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