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Short communication

**Molecular characterization of the dimer formation of
Fc α / μ receptor (CD351)**

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Abstract

Fc α / μ R (CD351) is an Fc receptor for both IgA and IgM and forms an atypical dimer that is resistant to reduction by 2-mercaptoethanol or boiling. We previously demonstrated that the cytoplasmic portion of Fc α / μ R is required for dimer formation and for its efficient cell-surface expression. However, the biochemical nature of these phenomena has not been determined. By using a BW5147 mouse cell line expressing deletion mutants of the cytoplasmic region of Fc α / μ R, we found that the region spanning amino acids 504 to 523 was required for efficient cell-surface expression, whereas the region spanning amino acids 481 to 490 was required for dimer formation. Immunoblotting analyses of transfectants simultaneously expressing Flag-tagged Fc α / μ R and hemagglutinin-tagged Fc α / μ R suggested that Fc α / μ R does not form homodimers. Instead, our data suggest that Fc α / μ R forms heterodimers with an as-yet-unknown molecule with a molecular weight of 60–70 kDa.

Keywords:

Fc receptor, IgA, IgM, dimer formation

1. Introduction

Fc receptors recognize the Fc portion of immunoglobulins and mediate a variety of immune responses upon binding to immune complexes of immunoglobulins and antigens (Daeron, 1997). Several Fc receptors for IgG and IgE have been shown to play pivotal roles in IgG- or IgE-mediated immune responses, such as antibody-dependent cellular cytotoxicity, mast cell degranulation, phagocytosis, cell proliferation, antibody secretion, and antigen presentation (Daeron, 1997; Lin et al., 1994; Ravetch, 1997). In contrast, the functional characteristics of the Fc receptors for IgM are not as well understood. We previously identified Fc α / μ R (CD351), an Fc receptor for IgA and IgM (Sakamoto et al., 2001; Shibuya et al., 2000). Because the Fc α / μ R gene is located near the polymeric IgR on chromosome 1 (1F in mice and 1q32.3 in humans), these receptors seem to be closely related (Kaetzel, 2005; Shibuya et al., 2000; Shimizu et al., 2001). Fc α / μ R is expressed not only on hematopoietic cells such as B cells and macrophages but also on follicular dendritic cells (FDCs) within follicles of lymphoid organs (Honda et al., 2009). Fc α / μ R mediates endocytosis of the ligands IgA and IgM, for which the cytoplasmic portion of Fc α / μ R is responsible

(Shibuya et al., 2000; Yang et al., 2009). By using *Fcα/μR*-deficient mice, we reported that *Fcα/μR* negatively regulates T-independent antigens retention by FDCs, leading to suppression of humoral immune responses against T-independent antigens (Honda et al., 2009).

We have also reported that the cytoplasmic portion of *Fcα/μR* is important for the formation of dimers with a molecular weight of ~130 kDa. These dimers are resistant to boiling in SDS and to reduction by 2-mercaptoethanol (2-ME). The cytoplasmic portion is also important for the efficient cell-surface expression of *Fcα/μR* (Cho et al., 2010). In this study, we further examined this cytoplasmic region by using BW5147 transfectants expressing deletion mutants of the cytoplasmic portion of *Fcα/μR*. We also examined the formation of *Fcα/μR* dimers and show that *Fcα/μR* does not form homodimers, as we expected, but instead forms heterodimers with a molecule that has a molecular weight of 60–70 kDa.

2. Materials and Methods

2.1. Cells and transfectants

BW5147 transfectants stably expressing wild-type Fc α / μ R or mutant Fc α / μ R lacking the cytoplasmic portion from amino acids 481 to 535 (Δ Cyt.Fc α / μ R), 491 to 535 (Δ 490.Fc α / μ R), 504 to 535 (Δ 503.Fc α / μ R) or 524 to 535 (Δ 523.Fc α / μ R) and tagged with Flag or hemagglutinin (HA) at the N-terminus were established as described previously (Cho et al., 2010; Shibuya et al., 2000). To generate site-directed mutations of the Fc α / μ R di-Leucine motif at residues Leu519 and Leu520 and of the Gln482 and Gln486 residues within the cytoplasmic region spanning amino acids 481 to 490, PCR primers were designed that contained a codon for Ala instead of these residues. The PCR products were subcloned into a pMX retrovirus vector with IRES-GFP or GFP sequences as described previously (Cho et al., 2010; Shibuya et al., 2000).

2.2. Antibodies

Anti-Fc α / μ R antibodies (TX7 and TX61) were generated in our laboratory, as described previously (Cho et al., 2006). Anti-Flag and anti-HA antibodies were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Roche (Indianapolis, IN, USA), respectively.

2.3. Biochemistry

Cells were lysed in 1% NP-40 lysis buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 10 U/mL aprotinin [Sigma–Aldrich]).

Immunoprecipitation was performed by using M280 beads (Sigma–Aldrich) coupled with anti-Flag, anti-HA, or TX61 antibodies. Immunoblotting experiments were performed with anti-Flag, anti-HA, or TX7 antibodies as described previously (Cho et al., 2010).

2.4. Flow cytometry analysis

Cells were stained with a biotinylated anti-Fc α / μ R antibody (TX61), followed by allophycocyanin (APC)-conjugated streptavidin. The expression of Fc α / μ R on the cell

surface relative to the total amount of Fc α / μ R, as determined by GFP expression, was calculated as described previously (Cho et al., 2010).

3. Results and Discussion

3.1. The region spanning amino acids 504 to 523 of the cytoplasmic portion is necessary for the efficient cell-surface expression of Fc α / μ R

In previous reports, we showed that the cytoplasmic portion of Fc α / μ R is required for both dimer formation and efficient cell-surface expression (Cho et al., 2010). To determine the region responsible for these features, we established a BW5147 transfectant expressing mutated Fc α / μ R, in which the cytoplasmic portion of Fc α / μ R was deleted after the 503rd amino acid (Δ 503.Fc α / μ R) or the 523rd amino acid (Δ 523.Fc α / μ R) (Fig. 1). Consistent with our previous report (Cho et al., 2010), flow cytometry analyses showed that much less Δ Cyt.Fc α / μ R than WT.Fc α / μ R was expressed on the cell surface (Fig. 2A). Although the cell-surface expression of Δ 503.Fc α / μ R was even lower than that of Δ Cyt.Fc α / μ R, Δ 523.Fc α / μ R was expressed on the cell surface at a level comparable to that of WT.Fc α / μ R (Fig. 2A). Thus, the region spanning amino acids 504 to 523 of the cytoplasmic portion is necessary for the efficient cell-surface expression of Fc α / μ R. The cytoplasmic region spanning amino acids 504-523 comprises at position 519 and 520 a di-Leucine motif that might be

internalization of Fc α / μ R (Shibuya et al., 2000). Similar motifs are known to be implicated in endosome and lysosome targeting of diverse proteins (Gabilondo et al., 1997). To examine whether the di-Leucine motif is involved in the efficient cell-surface expression of Fc α / μ R, we established a BM5147 transfectant stably expressing mutated Fc α / μ R in which the di-Leucine residues were replaced with Ala residues (Fig. 1). This mutant was expressed on the cell surface at a level comparable to that of WT.Fc α / μ R (Fig. 2B). Thus, the di-Leucine motif is not responsible for the efficient cell-surface expression of Fc α / μ R.

3.2. The region spanning amino acids 481 to 490 of the cytoplasmic portion is necessary for the formation of Fc α / μ R dimers

To determine the region responsible for the formation of Fc α / μ R dimers, we carried out an immunoblotting analysis of transfectants stably expressing WT or mutated Fc α / μ R. Fc α / μ R lacking the cytoplasmic portion (Δ Cyt.Fc α / μ R) existed as a monomer with a molecular weight of ~65 kDa in BW5147 cells (Fig. 3A), consistent with our previous report (Cho et al., 2010). However, Δ 490.Fc α / μ R, Δ 503.Fc α / μ R, and

$\Delta 523.Fc\alpha/\mu R$, as well as WT.Fc $\alpha/\mu R$, formed dimers (Fig. 3A), suggesting that the region spanning amino acids 481 to 490 in the cytoplasmic portion is required for dimer formation. Because the Fc $\alpha/\mu R$ dimers are remarkably stable and resistant to reduction by 2-ME (Cho et al., 2010), we hypothesized that a posttranslational modification might be involved, such as tissue transglutaminase-mediated cross-linkage between a Gln and a Lys residue to form an ϵ -(γ -glutamyl) lysine isopeptide bond between the cytoplasmic portions (AbdAlla et al., 2004; Griffin et al., 2002). Indeed, there are two Gln residues, Gln482 and Gln486, within the region spanning amino acids 481 to 490. To examine the possible involvement of transglutaminase-mediated cross-linkage in the formation of Fc $\alpha/\mu R$ dimers, we established BW5147 transfectants stably expressing Fc $\alpha/\mu R$ with substitutions of Ala for Gln482 and Gln486. An immunoblotting analysis showed that Fc $\alpha/\mu R$ mutants with substitutions of each Gln residue still formed dimers (Fig. 3B), indicating that transglutaminase might not be involved in the formation of Fc $\alpha/\mu R$ dimers. Collectively, these results indicate that dimer formation and efficient cell-surface expression of Fc $\alpha/\mu R$ are independent phenomena regulated by different regions of the cytoplasmic portion of Fc $\alpha/\mu R$.

3.3. Fc α / μ R forms heterodimers, not homodimers

The molecular weights of the Fc α / μ R monomer and dimer were 65–70 kDa and ~130 kDa, respectively, suggesting that Fc α / μ R forms homodimers. To test this, we established BW5147 transfectants simultaneously expressing Flag-tagged Fc α / μ R and HA-tagged Fc α / μ R (Fig. 4A) and performed an immunoblotting analysis. If Fc α / μ R forms homodimers, Flag-tagged Fc α / μ R would associate with HA-tagged Fc α / μ R as well as with Flag-tagged Fc α / μ R. However, an anti-Flag antibody immunoprecipitated Flag-tagged Fc α / μ R alone, but not HA-tagged Fc α / μ R (Fig. 4B). Conversely, an anti-HA antibody immunoprecipitated HA-tagged Fc α / μ R alone, but not Flag-tagged Fc α / μ R (Fig. 4B). These results suggest that Fc α / μ R forms heterodimers not homodimers. To confirm this, we performed an immunoblotting analysis by using BW5147 transfectants stably expressing Fc α / μ R fused to GFP (Fc α / μ R–FL). Because the molecular weight of GFP is ~30 kDa, monomeric Fc α / μ R–FL should be a protein of ~95 kDa (Fig. 3C). Although the homodimeric form of Fc α / μ R–FL was expected to be about ~190 kDa, the dimeric form of Fc α / μ R–FL that we observed had a molecular

weight of ~160 kDa (Fig. 3C). These results suggest that Fc α / μ R forms heterodimers with an undetermined molecule whose molecular weight is 60–70 kDa.

We previously reported that Δ Cyt.Fc α / μ R, which is expressed as a monomer on the cell surface, can bind both IgA and IgM, indicating that dimer formation is not required for ligand binding (Cho et al., 2010). In the current study, we showed that dimer formation was also not associated with efficient cell-surface expression of Fc α / μ R. Although Fc α / μ R negatively regulates T-independent antigens retention by FDCs (Honda et al., 2009), it remains unclear how Fc α / μ R is involved in antigens retention. Identification of the molecule that associates with Fc α / μ R in the heterodimer is required for understanding the molecular mechanism of Fc α / μ R-mediated immune regulation.

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Figure Legends

Fig. 1. Schematic presentation of Fc α / μ R mutations in the cytoplasmic region

Amino acid sequences in the cytoplasmic region of wild-type (WT) Fc α / μ R, and various deletion (Δ) and site-directed (substitution of Ala for Leu [LA] or Gln [QA]) mutants of Fc α / μ R.

Fig. 2. Cell-surface expression of wild-type and mutated Fc α / μ R

Flow cytometry was used to analyze GFP expression and the cell-surface expression of Fc α / μ R in BW5147 transfectants expressing wild-type (WT) Fc α / μ R (A, B), mutants lacking cytoplasmic segments (A), and mutants with Ala substitutions at the di-Leucine residues (B). Fc α / μ R on the cell surface was compared with the total amount of Fc α / μ R, as determined by GFP expression (bar graphs in lower panels). Data are representative of more than three independent experiments.

Fig. 3. The cytoplasmic region is required for dimer formation

BW5147 transfectants expressing mutated Fc α / μ R were lysed with 1% NP-40 lysis buffer, immunoprecipitated with an anti-Flag antibody, and then immunoblotted with an anti-Flag antibody. The closed and open arrows indicate the monomers and dimers of Fc α / μ R, respectively. Data are representative of more than three independent experiments.

Fig. 4. Heterodimer formation by Fc α / μ R

(A) Flow cytometry analysis of BW5147 transfectants simultaneously expressing Fc α / μ R with a Flag tag at the N-terminus and Fc α / μ R with an HA tag at the N-terminus.

(B) BW5147 transfectants expressing both Flag- and HA-tagged Fc α / μ R were lysed, immunoprecipitated, and immunoblotted with anti-Flag or anti-HA antibodies, as indicated. The closed and open arrows indicate the monomer and dimer of Fc α / μ R, respectively. (C) BW5147 transfectants expressing wild-type (WT) or GFP-fused Fc α / μ R (Fc α / μ R-FL) were lysed, immunoprecipitated with anti-Fc α / μ R (TX61), and immunoblotted with anti-Fc α / μ R (TX7). X indicates an undetermined molecule

physically associating with Fca/ μ R. Data are representative of more than three independent experiments.

Fig.1

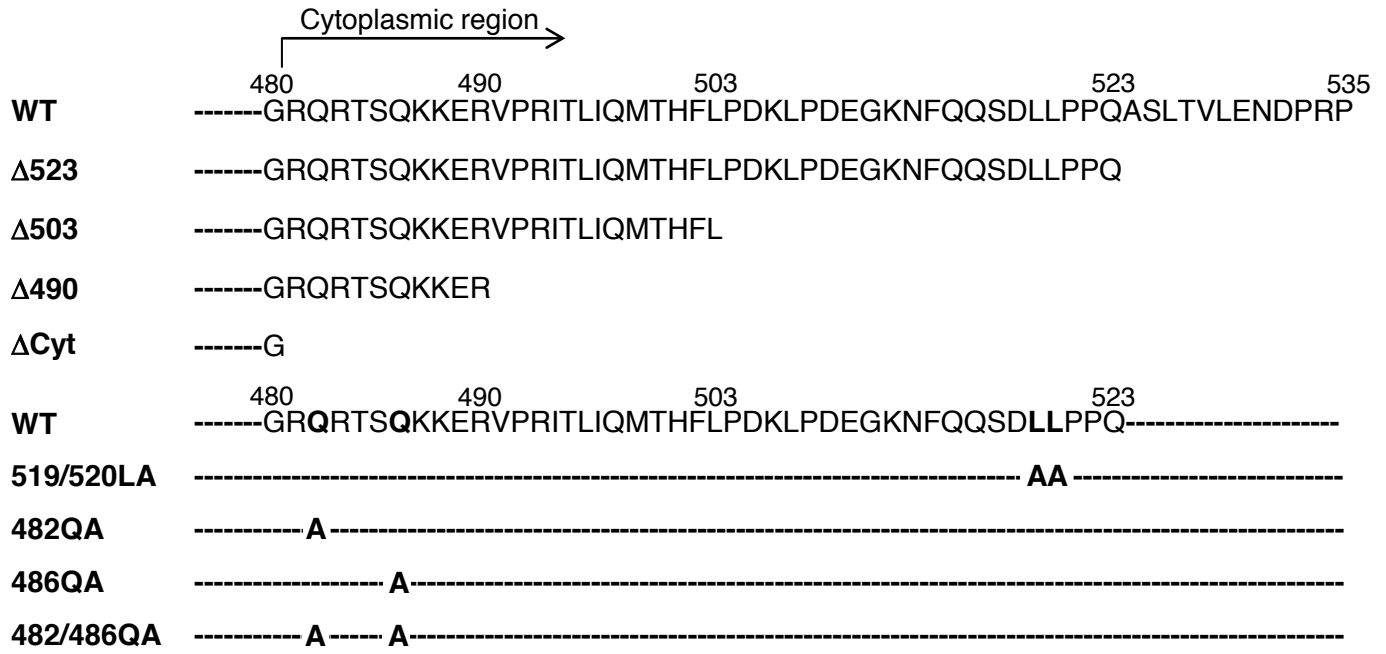


Fig.2

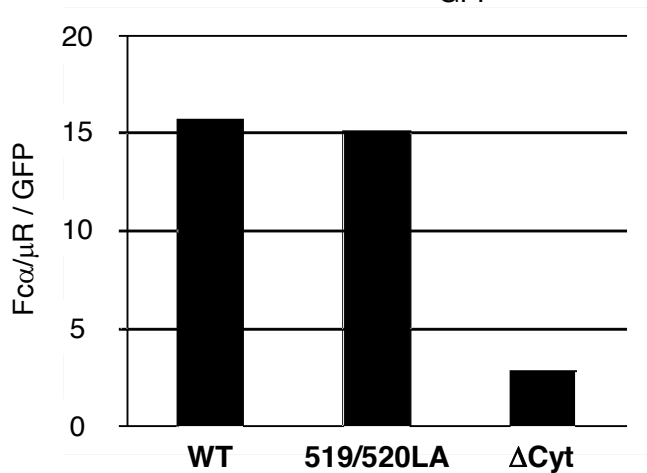
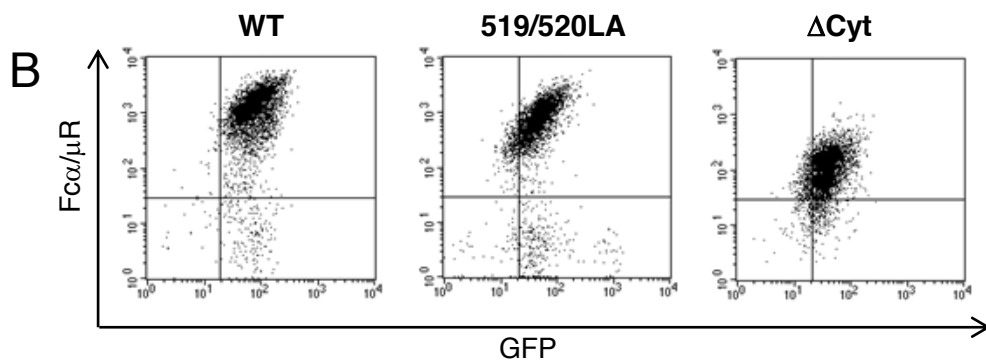
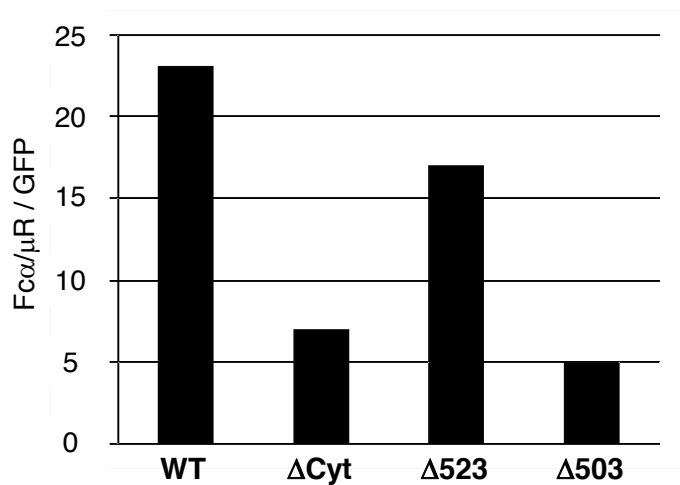
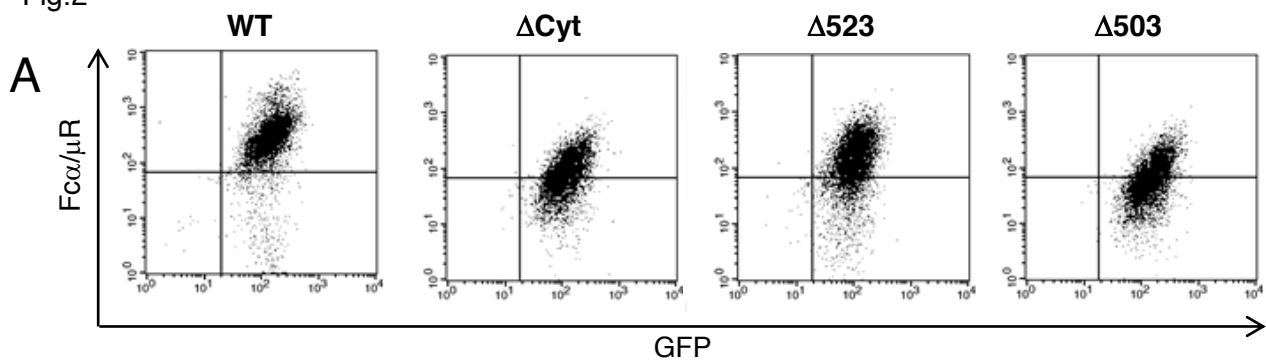
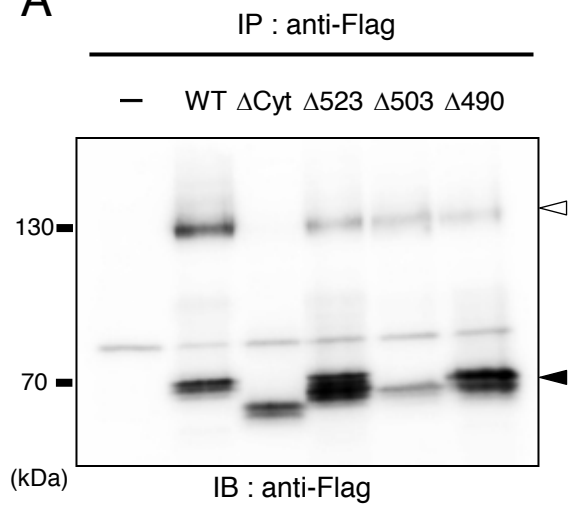


Fig.3

A



B

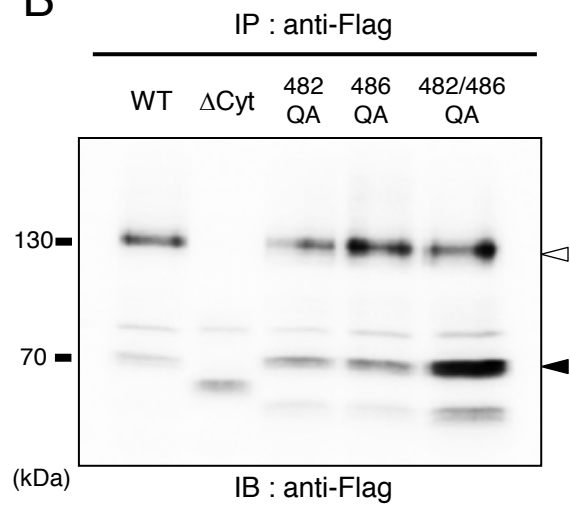


Fig.4

