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<tr>
<td>ページ数</td>
<td>23-27</td>
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<td>年度</td>
<td>2013-11</td>
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<td>Molecular immunology</td>
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<tr>
<td>品名コード</td>
<td>molimm</td>
</tr>
<tr>
<td>DOI</td>
<td>10.1016/j.molimm.2013.04.003</td>
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Molecular Immunology

Volume 56
Issue 1-2
Pages 23-27
Year 2013-11

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Short communication

Molecular characterization of the dimer formation of

Fcα/μ receptor (CD351)

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Abstract

Fc\(\alpha/\mu\)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\(\alpha/\mu\)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\(\alpha/\mu\)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\(\alpha/\mu\)R and hemagglutinin-tagged Fc\(\alpha/\mu\)R suggested that Fc\(\alpha/\mu\)R does not form homodimers. Instead, our data suggest that Fc\(\alpha/\mu\)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 Fcα/μR (CD351), an Fc receptor for IgA and IgM (Sakamoto et al., 2001; Shibuya et al., 2000). Because the Fcα/μR 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). Fcα/μR 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). Fcα/μR mediates endocytosis of the ligands IgA and IgM, for which the cytoplasmic portion of Fcα/μR 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 Fcα/μR or mutant Fcα/μR lacking the cytoplasmic portion from amino acids 481 to 535 (ΔCyt.Fcα/μR), 491 to 535 (Δ490.Fcα/μR), 504 to 535 (Δ503.Fcα/μR) or 524 to 535 (Δ523.Fcα/μR) 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 Fcα/μR 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-Fcα/μR 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-Fcα/μR antibody (TX61), followed by allophycocyanin (APC)-conjugated streptavidin. The expression of Fcα/μR on the cell
surface relative to the total amount of Fcα/μR, 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 Fcα/μR

In previous reports, we showed that the cytoplasmic portion of Fcα/μR 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 Fcα/μR, in which the cytoplasmic portion of Fcα/μR was deleted after the 503rd amino acid (Δ503.Fcα/μR) or the 523rd amino acid (Δ523.Fcα/μR) (Fig. 1). Consistent with our previous report (Cho et al., 2010), flow cytometry analyses showed that much less ΔCyt.Fcα/μR than WT.Fcα/μR was expressed on the cell surface (Fig. 2A). Although the cell-surface expression of Δ503.Fcα/μR was even lower than that of ΔCyt.Fcα/μR, Δ523.Fcα/μR was expressed on the cell surface at a level comparable to that of WT.Fcα/μR (Fig. 2A). Thus, the region spanning amino acids 504 to 523 of the cytoplasmic portion is necessary for the efficient cell-surface expression of Fcα/μR. The cytoplasmic region spanning amino acids 504-523 comprises at position 519 and 520 a di-Leucine motif that might be
internalization of Fcα/μR (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 Fcα/μR, we established a BM5147 transfectant stably expressing mutated Fcα/μR 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.Fcα/μR (Fig. 2B). Thus, the di-Leucine motif is not responsible for the efficient cell-surface expression of Fcα/μR.

3.2. The region spanning amino acids 481 to 490 of the cytoplasmic portion is necessary for the formation of Fcα/μR dimers

To determine the region responsible for the formation of Fcα/μR dimers, we carried out an immunoblotting analysis of transfectants stably expressing WT or mutated Fcα/μR. Fcα/μR lacking the cytoplasmic portion (ΔCyt.Fcα/μR) 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, Δ490.Fcα/μR, Δ503.Fcα/μR, and
Δ523.Fcα/μR, as well as WT.Fcα/μR, 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 Fcα/μR 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 Fcα/μR dimers, we established BW5147 transfectants stably expressing Fcα/μR with substitutions of Ala for Gln482 and Gln486. An immunoblotting analysis showed that Fcα/μR mutants with substitutions of each Gln residue still formed dimers (Fig. 3B), indicating that transglutaminase might not be involved in the formation of Fcα/μR dimers. Collectively, these results indicate that dimer formation and efficient cell-surface expression of Fcα/μR are independent phenomena regulated by different regions of the cytoplasmic portion of Fcα/μR.
3.3. *Fcα/μR forms heterodimers, not homodimers*

The molecular weights of the Fcα/μR monomer and dimer were 65–70 kDa and ~130 kDa, respectively, suggesting that Fcα/μR forms homodimers. To test this, we established BW5147 transfecants simultaneously expressing Flag-tagged Fcα/μR and HA-tagged Fcα/μR (Fig. 4A) and performed an immunoblotting analysis. If Fcα/μR forms homodimers, Flag-tagged Fcα/μR would associate with HA-tagged Fcα/μR as well as with Flag-tagged Fcα/μR. However, an anti-Flag antibody immunoprecipitated Flag-tagged Fcα/μR alone, but not HA-tagged Fcα/μR (Fig. 4B). Conversely, an anti-HA antibody immunoprecipitated HA-tagged Fcα/μR alone, but not Flag-tagged Fcα/μR (Fig. 4B). These results suggest that Fcα/μR forms heterodimers not homodimers. To confirm this, we performed an immunoblotting analysis by using BW5147 transfectants stably expressing Fcα/μR fused to GFP (Fcα/μR–FL). Because the molecular weight of GFP is ~30 kDa, monomeric Fcα/μR–FL should be a protein of ~95 kDa (Fig. 3C). Although the homodimeric form of Fcα/μR–FL was expected to be about ~190 kDa, the dimeric form of Fcα/μR–FL that we observed had a molecular
weight of ~160 kDa (Fig. 3C). These results suggest that Fcα/μR forms heterodimers with an undetermined molecule whose molecular weight is 60–70 kDa.

We previously reported that ΔCyt.Fcα/μR, 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 Fcα/μR. Although Fcα/μR negatively regulates T-independent antigens retention by FDCs (Honda et al., 2009), it remains unclear how Fcα/μR is involved in antigens retention. Identification of the molecule that associates with Fcα/μR in the heterodimer is required for understanding the molecular mechanism of Fcα/μR-mediated immune regulation.
Acknowledgments

We thank S. Mitsuishi for secretarial assistance. This research was supported in part by grants provided by the Ministry of Education, Science and Culture of Japan and the Program for the Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation (NIBIO).
References


Figure Legends

Fig. 1. Schematic presentation of Fcα/μR mutations in the cytoplasmic region

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

Fig. 2. Cell-surface expression of wild-type and mutated Fcα/μR

Flow cytometry was used to analyze GFP expression and the cell-surface expression of Fcα/μR in BW5147 transfectants expressing wild-type (WT) Fcα/μR (A, B), mutants lacking cytoplasmic segments (A), and mutants with Ala substitutions at the di-Leucine residues (B). Fcα/μR on the cell surface was compared with the total amount of Fcα/μR, 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 Fcα/μR 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 Fcα/μR, respectively. Data are representative of more than three independent experiments.

**Fig. 4.** Heterodimer formation by Fcα/μR

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

(B) BW5147 transfectants expressing both Flag- and HA-tagged Fcα/μR 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 Fcα/μR, respectively. (C) BW5147 transfectants expressing wild-type (WT) or GFP-fused Fcα/μR (Fcα/μR-FL) were lysed, immunoprecipitated with anti-Fcα/μR (TX61), and immunoblotted with anti-Fcα/μR (TX7). X indicates an undetermined molecule.
physically associating with Fcα/μR. Data are representative of more than three independent experiments.
Fig. 1

Cytoplasmic region

WT

Δ523

Δ503

Δ490

ΔCyt

WT

Δ523

Δ503

Δ490

ΔCyt

WT

Δ523

Δ503

Δ490

ΔCyt

WT

Δ523

Δ503

Δ490

ΔCyt

519/520LA

482QA

486QA

482/486QA
Fig. 2

A

WT  ΔCyt  Δ523  Δ503

GFP

B

WT  519/520LA  ΔCyt

GFP