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The Mechanism by Which Proteolysis Enhances the Ligand-Binding Activity of Guinea Pig Type II Fc Receptor for IgG (FcγRIIB)[1]

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We have previously shown that the ligand-binding activity of type II Fc receptor for IgG (FcγRIIB) on guinea pig peripheral blood polymorphonuclear leukocytes is very low and dramatically increases after treatment of the cells with proteolytic enzymes. In the present study, we analyzed the mechanism of this augmentation. We found that the protease treatment failed to enhance the binding of monomeric IgG to FcγRIIB, increased the binding of small immune complexes (IC) prepared under antigen-excess conditions only modestly, but markedly enhanced the binding of large IC prepared under antibody-excess conditions. These results suggest that proteolysis increases the ligand-binding avidity but not the intrinsic affinity of FcγRIIB. Confocal laser scanning microscopy revealed that the mobility of FcγRIIB on the cell surface was increased after protease treatment. In addition, transfection experiments indicated that the effect of proteolysis on IC binding to CHO cells expressing guinea pig FcγRIIB was strongly dependent on the receptor density. Finally, we demonstrated that the transmembrane and cytoplasmic domains of FcγRIIB were not involved in the proteolysis-induced augmentation of IC binding. Together our results suggest that the mobility of FcγRIIB, which may be restricted due to the association of the ectodomain of the receptor with unknown membrane proteins, is enhanced by proteolysis, allowing the receptors to bind multivalent ligands more readily and hence with higher avidity.

Key words: binding avidity, Fc receptor, guinea pig, polymorphonuclear leukocytes, proteolysis.

Aggregation of the receptors for the Fc portion of IgG (FcγR) on phagocytic cells elicits a variety of responses such as the respiratory burst, phagocytosis, and lysosomal enzyme release. FcγR are heterogeneous and three classes have been defined on leukocytes: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). FcγRI is a 72-kDa glycoprotein that binds monomeric IgG with high affinity and is found on monocytes and macrophages. FcγRII is a receptor with low affinity for monomeric IgG and is broadly distributed on hematopoietic lineage cells, including monocytes/macrophages, polymorphonuclear leukocytes (PMN), mast cells, platelets, and some T and B lymphocytes. In humans, three different genes have been identified, FcγRIIA, B, and C. FcγRIII is also a low affinity FcγR and is expressed on NK cells, macrophages, PMN, and mast cells. Human PMN normally express FcγRII and FcγRIII and both types of FcγR are able to transduce signals to the cells (*1, 2*).

As reported previously, guinea pig PMN express two distinct types of FcγR in terms of IgG subclass binding. One binds both IgG1 and IgG2 (Fcγ1/γ2R) and the other binds only IgG2 (Fcγ2R) (*3, 4*). We recently cloned the cDNA encoding the Fcγ1/γ2R and showed that it was homologous to the human FcγRIIB (*5, 6*). Three isoforms of guinea pig FcγRIIB are present, which may be generated by alternative splicing of sequences coding for part of the cytoplasmic domain (*6*). It has recently been reported that murine FcγRIIB also consists of three isoforms (*7*). On the other hand, the Fcγ2R expressed on PMN is a 120 kDa protein and is structurally different from the Fcγ2R on macrophages that is homologous to the human FcγRIIIA (*4, 8*). Although the structure of the PMN Fcγ2R remains to be determined, the receptor is able to trigger a respiratory burst and the arachidonic acid metabolic cascade upon receptor aggregation, as in the case of the Fcγ2R on macrophages (*9, 10*).

The PMN in the blood stream migrate to extravascular sites in response to chemotactic stimuli, which not only attract the cells, but also activate them. We recently reported a comparative study on guinea pig FcγRIIB expressed on blood PMN and casein-elicited peritoneal PMN (*11*). The level of expression of FcγRIIB on blood

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Abbreviations: Ab, antibody; Ag, antigen; FcγR, Fc receptor for IgG; Fcγ1/γ2R, Fc receptor for IgG1 and IgG2; Fcγ2R, Fc receptor for IgG2; IC, immune complexes; mAb, monoclonal antibody; OVA, hen ovalbumin; PMN, polymorphonuclear leukocytes.

PMN was similar to that on exudate PMN. However, the ligand binding activity of FcγRIIB on blood PMN was extremely low as compared with that on exudate PMN. We also demonstrated that the binding was dramatically augmented by protease treatment of the cells without affecting the number of FcγRIIB on the cell surface (*11*). Other groups have also shown that the ligand binding of FcγRII on human monocytes and neutrophils is modulated by proteolytic enzymes (*12-17*), but the mechanism by which proteases alter the FcγR function has not been thoroughly studied. One possibility is that the effect of protease is direct and induces a conformational change in FcγRII. However, no major structural change in FcγRII after proteolysis has been reported (*14*). It is also possible that the effect of protease is indirect. Proteolysis may affect the membrane environment and promote the clustering of FcγRII which is important for multivalent ligand binding.

To clarify the mechanism, we analyzed the binding of soluble immune complexes (IC) to guinea pig PMN and CHO cells transfected with guinea pig FcγRIIB before and after proteolysis. We demonstrate that the mobility of FcγRIIB, which may be reduced due to the association of the extracellular domain of the receptor with unidentified membrane proteins, is enhanced by proteolysis, and that clustering of FcγRIIB is facilitated when the cells are exposed to multivalent IC. Thus, proteolysis allows the multivalent ligands to bind to FcγRIIB with higher avidity.

MATERIALS AND METHODS

Animals and Cells—Male outbred Hartley guinea pigs obtained from Funabashi Farm (Chiba) were used at 2-4 months of age. Guinea pig peripheral blood PMN were purified as previously described (*11*). Chinese hamster ovary (CHO) cells were maintained in MEM α medium (Life Technology, Tokyo) containing 10% FCS and 50 μM 2-ME.

Antibodies and Antigens—Guinea pig IgG2 anti-hen ovalbumin (OVA) antibodies were separated and affinity-purified as described (*18, 19*). Some of the purified antibodies were conjugated with biotin, as described (*20*). OVA was labeled with FITC as described (*21*). The molar ratio of FITC to OVA was 7-8. FITC-conjugated goat anti-mouse IgG was purchased from The Jackson Laboratory (West Grove, PA). Rabbit $F(ab')_2$ fragments of anti-mouse IgG and anti-guinea pig IgG were purified as described (*19, 22*) and conjugated with FITC. The molar ratio of FITC to $F(ab')_2$ was 4-5. The $F(ab')_2$ and Fab′ fragments of monoclonal antibody (mAb) to guinea pig FcγRIIB and FcγRIIIA were prepared and purified as described (*23, 24*). The Fab fragments of MP-2 IgG1, a specific mAb to Fcγ2R on PMN, were also prepared as described (*4*). The purity of these fragments was assessed by SDS-PAGE.

Construction of Plasmids and Transfection—The cDNAs encoding guinea pig FcγRII-B1, -B2, and -B3 (*6*) were cloned into pCDM8 mammalian expression vector (Invitrogen, San Diego, CA) and used for transfection. FcγRIIB lacking the cytoplasmic domain was obtained by introducing a stop codon after the sixth amino acid of the cytoplasmic domain. A fragment was amplified with the oligonucleotide 5′-CCAAGGTCACATTCTACCAC-3′, which corresponds to the 570-589 nucleotide sequence in the extracellular domain, as a forward primer and 5′-ATTTCATGGAGGCTGCTTTTTCTT-3′, containing a stop codon (underlined), as a reverse primer and FcγRII-B1-pCDM8 was used as a template. The resultant PCR product was subcloned into pCRII using a TA cloning kit (Invitrogen). The *Xmn*I/*Not*I fragment was reintroduced into the *Xmn*I- and *Not*I-digested FcγRII-B1-pME18S. A chimeric molecule consisting of the extracellular domain of FcγRIIB and the transmembrane and cytoplasmic domains of CD28 was constructed by PCR. The cDNA coding for mouse CD28 (*25*) was isolated by RT-PCR. Briefly, total cellular RNA was extracted from mouse splenic cells and cDNA was synthesized using random primers. CD28 sequences were amplified using the oligonucleotides 5′-CCCGGGATGACACTCAGGCTG-3′ and 5′-GTCGACTCAGGGGCGGTACGC-3′. The PCR product was blunted and subcloned into the pCDM8 vector, which was then used as a template for an additional PCR amplification. Two oligonucleotides were used: 5′-AAGGGCCCAAGTCAAGCGACCTGTTTTGGGCACTGGTC-3′, which contained the 3′ end of the extracellular domain of FcγRIIB (underlined) in which the *Apa*I site was present (in bold) and the 5′ end of the transmembrane domain of CD28, and the oligonucleotide 5′-TAAGGTTCCTTCACAAAG-3′, which is complementary to the 3′ end of the multicloning site of pCDM8. The PCR product was subcloned into pCRII. The *Apa*I/*Not*I fragment was isolated and reintroduced into the *Apa*I- and *Not*I-digested FcγRII-B1-pME18S. FcγRII-positive CHO cells were produced according to protocols provided by the supplier of the LIPOFECT AMINE reagents (Life Technology). A mixture of 1 μg of plasmid constructs and 0.1 μg of pAdD26SVA#3 carrying a dihydrofolate reductase (DHFR) gene was introduced into CHO-DHFR$^-$ cells. After selection in α-MEM without nucleic acids, resistant colonies were screened for FcγRIIB expression by flow cytometry, and cloned by limiting dilution.

Treatment of Cells with Proteases—Cells were suspended in Krebs-Ringer phosphate buffer containing 0.2% BSA at a cell concentration of 5×10^6/ml. The cells were preincubated with 10 μg/ml DNase I (Boehringer Mannheim, Tokyo) at 37°C for 5 min to avoid cell clumping due to sticky DNA released from damaged cells. The cells were then treated with 0.5 mg/ml pronase (from *Streptomyces griseus*; Boehringer Mannheim) at 37°C for 30 min (*11, 26*). The cells were then washed three times and kept on ice until use.

Analysis for Expression of FcγR—The cells (5×10^5) suspended in Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS containing 0.1% BSA and 0.1% NaN_3 (staining buffer) were incubated with saturating amounts of anti-FcγRIIB $F(ab')_2$ (10 μg/ml) for 30 min at 4°C. After incubation, the cells were washed twice with the staining buffer and further incubated with 50 μl of FITC-conjugated rabbit $F(ab')_2$ fragments of anti-mouse IgG (25 μg/ml). Negative controls consisted of cells stained with the secondary Ab alone. The cells thus stained were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Flow Cytometric IC-Binding Assays—Affinity-purified and biotinylated IgG2 anti-OVA antibody and OVA were mixed at a variable molar ratio and incubated for 30 min at 37°C. The IgG2-IC thus formed were kept at 4°C until use. The amounts of soluble IC were expressed in terms of those of antibodies used. The absorbance at 280 nm was used for calculation of the protein concentration (*19*). The cells (5×

10^5) were preincubated with saturating amounts of anti-Fcγ2R Fab for 30 min at 4°C to block the IgG binding through Fcγ2R. Without washing, the cells were then incubated with 0.5 ml of biotinylated IC in the staining buffer for 2 h at 4°C. After incubation, the cells were washed twice with the same buffer and then incubated with 50 μl of FITC-conjugated streptavidin (ZYMED Laboratory, San Francisco, CA) for 20 min at 4°C. Negative control fluorescence was obtained by staining the cells with FITC-streptavidin alone. When unlabeled IgG2-IC were used for binding assays, the cells were incubated with 50 μl of FITC-conjugated rabbit $F(ab')_2$ fragments of anti-guinea pig IgG for 30 min at 4°C.

Immunocytochemical Localization of FcγRIIB on Cell Membranes—The cells to be tested (5×10^5) were incubated with saturating amounts of FITC-conjugated anti-FcγRIIB Fab′ for 30 min at 4°C. After incubation, the cells were washed twice with the staining buffer and further incubated with 50 μl of FITC-conjugated goat $F(ab')_2$ fragments of anti-mouse IgG (25 μg/ml). After staining, the cells were washed three times with the staining buffer and then resuspended in PBS containing 0.1% *p*-phenylenediamine to minimize quenching during observation (*27*). The localization of FcγRIIB was analyzed by confocal laser scanning fluorescence microscopy (Bio-Rad MRC-1024, Nippon Bio-Rad Laboratories, Tokyo).

RESULTS

The Enhancing Effect of Proteolysis on the Binding Activity of FcγRIIB Is Dependent on the Nature of the IC Employed—We previously showed that the IgG2-IC-binding to FcγRIIB on guinea pig blood PMN upon pronase treatment was dramatically increased, although the treatment did not affect the expression of FcγRIIB on the cell surface (*11*). We first analyzed whether the ligand affinity of FcγRIIB was augmented by protease treatment. For this purpose, the cells were treated with pronase and then incubated with various amounts of monomeric IgG2 or OVA-anti-OVA IgG2 IC that had been prepared under Ab-excess conditions (Ag/Ab = 0.1). The cell-bound IgG2 was detected by flow cytometry using FITC-conjugated anti-guinea pig IgG. Although IgG2-IC binding to FcγRIIB was dramatically increased by protease treatment (Fig. 1A), the binding of monomeric IgG2 to FcγRIIB was not affected (Fig. 1B). It should be noted that although the level of cell-bound monomeric IgG2 was very low, this binding was substantial and was completely inhibited by anti-FcγRIIB mAb (data not shown). These results suggest that the protease treatment of the cells fails to increase the intrinsic affinity of FcγRIIB. We further analyzed the binding of IC prepared at various Ag/Ab molar ratios. IC were prepared by incubating a constant amount of anti-OVA IgG2 with various amounts of OVA. Under the conditions we employed, no precipitating complex was observed after incubation. The cell-bound IgG2 were quantitated as described above. The difference in the Ag/Ab molar ratios of the IgG2-IC correlated with a difference in proteolysis-induced enhancement of ligand binding (Fig. 2A). At the Ag/Ab ratio of 0.1, where several IgG2 molecules are expected to bind to a single Ag, the marked enhancement of IC binding to FcγRII was again detected after protease treatment. In contrast, at an Ag/Ab ratio of 10, where only one or two IgG2 molecules are expected to bind to a single Ag, the IC binding to FcγRIIB was less efficiently enhanced after protease treatment. Since in these experiments cell-bound IgG2 were detected with FITC-conjugated anti-guinea pig IgG, there was a possibility that we overestimated the binding activity of FcγRIIB by detecting IgG2 molecules that were present in IC but that had not bound to FcγRIIB. However, similar results were obtained when using FITC-conjugated Ag (Fig. 2B). Thus, the enhancing effect of proteolysis on FcγRIIB activity is strongly dependent on the Ag/Ab ratio of IC employed. These results suggest that proteolysis affects the mobility of FcγRIIB on the cell surface and facilitates clustering of receptors, allowing the multivalent IC to bind with high avidity.

Distribution of FcγRIIB before and after Proteolysis—The localization of FcγRIIB on guinea pig blood PMN before and after proteolysis was determined by immunofluorescence study with a confocal laser scanning microscope. FcγRIIB on pronase-treated or untreated PMN were labeled by incubation with FITC-conjugated Fab′ fragments of mouse anti-FcγRIIB mAb. The cells were then incubated with FITC-conjugated goat $F(ab')_2$ fragment of anti-mouse IgG. As shown in Fig. 3A, FcγRIIB on the untreated cells remained diffusely distributed on the plasma membrane even after incubation with the secondary Ab. In contrast, FcγRIIB on pronase-treated cells redistributed into patches and caps when the cells were reacted with bivalent anti-IgG Ab (Fig. 3B). It should be

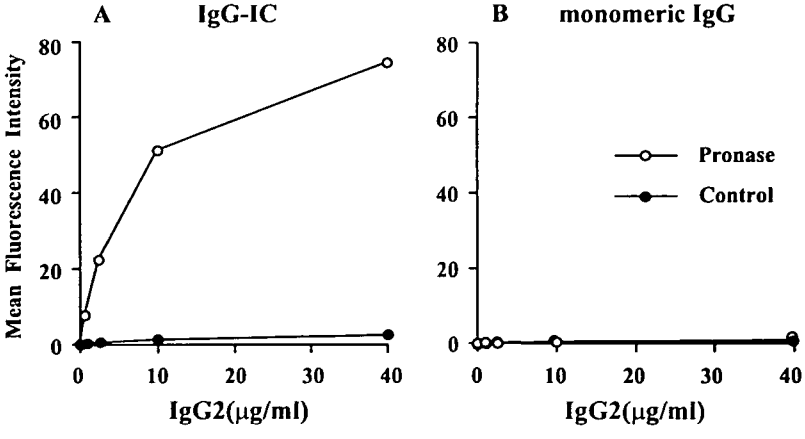

Fig. 1. **The effect of protease treatment on the ligand-binding activity of FcγRIIB on guinea pig peripheral blood PMN.** Pronase-treated (open circles) or untreated PMN (closed circles) were preincubated with anti-Fcγ2R mAb to block the IgG binding through Fcγ2R, then incubated with various amounts of OVA-anti-OVA IgG2 immune complexes (IC) prepared under Ab-excess conditions (Ag/Ab = 0.1) (A) or monomeric anti-OVA IgG2 (B) at 4°C for 2 h. Cells were then washed extensively and the amounts of IC bound were determined by incubating cells with FITC-conjugated rabbit $F(ab')_2$ anti-guinea pig IgG, followed by flow cytometry. The values show the mean fluorescence intensity (linear arbitrary unit) from a single experiment, and are representative of three similar experiments.

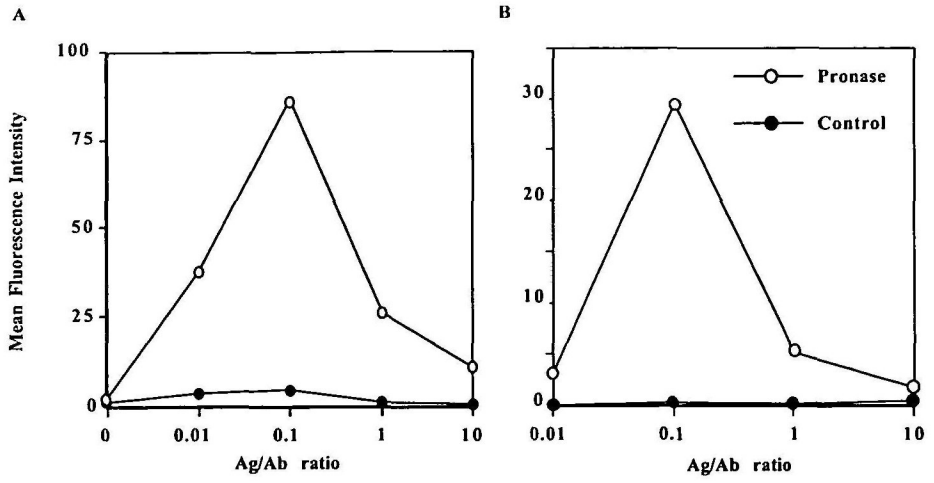

Fig. 2. **The binding of IgG2-IC prepared at various Ag/Ab ratios to PMN.** Pronase-treated (open circles) or untreated PMN (closed circles) were preincubated with anti-Fcγ2R mAb and then incubated with constant amounts of IgG2-IC (40 μg of IgG2/ml) (A) or IgG2-IC prepared by incubating anti-OVA IgG2 with FITC-conjugated OVA (B) at 4°C for 2 h. The amounts of IC bound were determined as described in Fig. 1 (A) or directly by flow cytometric analysis (B). Results from a single experiment are shown and are representative of five similar experiments.

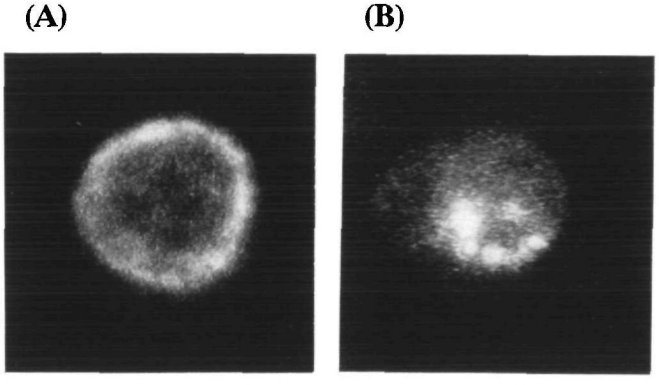

Fig. 3. **Distribution of FcγRIIB on PMN cell surface.** Pronase-untreated (A) or treated PMN (B) were incubated with FITC-conjugated Fab′ fragments of anti-FcγRIIB mAb at 4°C for 30 min. After washing, the cells were incubated with FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse IgG at 4°C for 30 min, and at least 100 cells were analyzed by confocal laser scanning microscopy. Representative cells from a single experiment are shown. Three separate cell preparations were analyzed on different days and the same results were obtained.

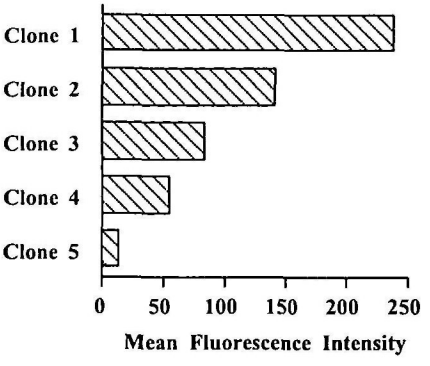

Fig. 4. **Establishment of guinea pig FcγRII-B1 positive CHO cells and their expression level.** Plasmids containing FcγRII-B1 cDNA and DHFR cDNA were co-transfected into CHO cells. After selection, several clones were isolated and stained with anti-FcγRIIB mAb followed by FITC-conjugated goat anti-mouse IgG. The mean fluorescence intensity (linear arbitrary unit) was determined by flow cytometry.

pointed out that clustering of FcγRIIB was not observed before incubation with the anti-IgG Ab (data not shown), suggesting that proteolysis does not induce spontaneous formation of clusters. These results suggest that the mobility of mAb-bound FcγRIIB on PMN is improved by proteolysis, leading to clustering of receptors when the cells are exposed to bivalent anti-IgG antibody. We hypothesized that this increased mobility accounted for the enhanced binding of multivalent IC.

Analysis Using CHO Cell Lines Stably Expressing Guinea Pig FcγRIIB—To pursue the above hypothesis and to analyze further the mechanism by which proteolysis enhances the ligand binding of guinea pig FcγRIIB, a series of transfection experiments was performed. Since the binding avidity should in principle be affected both by the valency of IC and by the density of receptors on the cell surface, we attempted to obtain cell lines expressing FcγRIIB at different densities. Stable expression of guinea pig FcγRIIB on CHO cells was obtained by co-transfection of plasmids containing cDNA coding for FcγRIIB and DHFR into DHFR-deficient CHO cells. Individual colonies were screened by flow cytometry using anti-FcγRIIB mAb. Several cloned transfectants expressing different levels of FcγRIIB were obtained, and five clones were used for further experiments (Fig. 4). The density of FcγRIIB on the clone with the lowest level of expression (clone 5) was comparable to that on blood PMN. Clones 3 and 4 expressed 5- to 8-fold larger amounts of FcγRIIB than clone 5. This level of expression was comparable to that on guinea pig peritoneal macrophages. The density of FcγRIIB on the transfectant with the highest level of expression (clone 1) was approximately 20-fold higher than that on clone 5.

Having established a series of transfectants, we next examined the effect of proteolysis on IC binding to these cells. Pronase treatment of the CHO cell lines did not modify their expression of FcγRIIB (Fig. 5, A, C, and E). In addition, the binding of IC (Ag/Ab=0.1) to the CHO cell transfectants was completely inhibited by preincubation of the cells with an excess of anti-FcγRIIB mAb and untransfected CHO cells did not bind the IC (data not shown). The clone with the lowest level of FcγRIIB expression (clone 5) showed almost no ligand-binding activity (Fig. 5B). However, treatment of the cell line with protease markedly

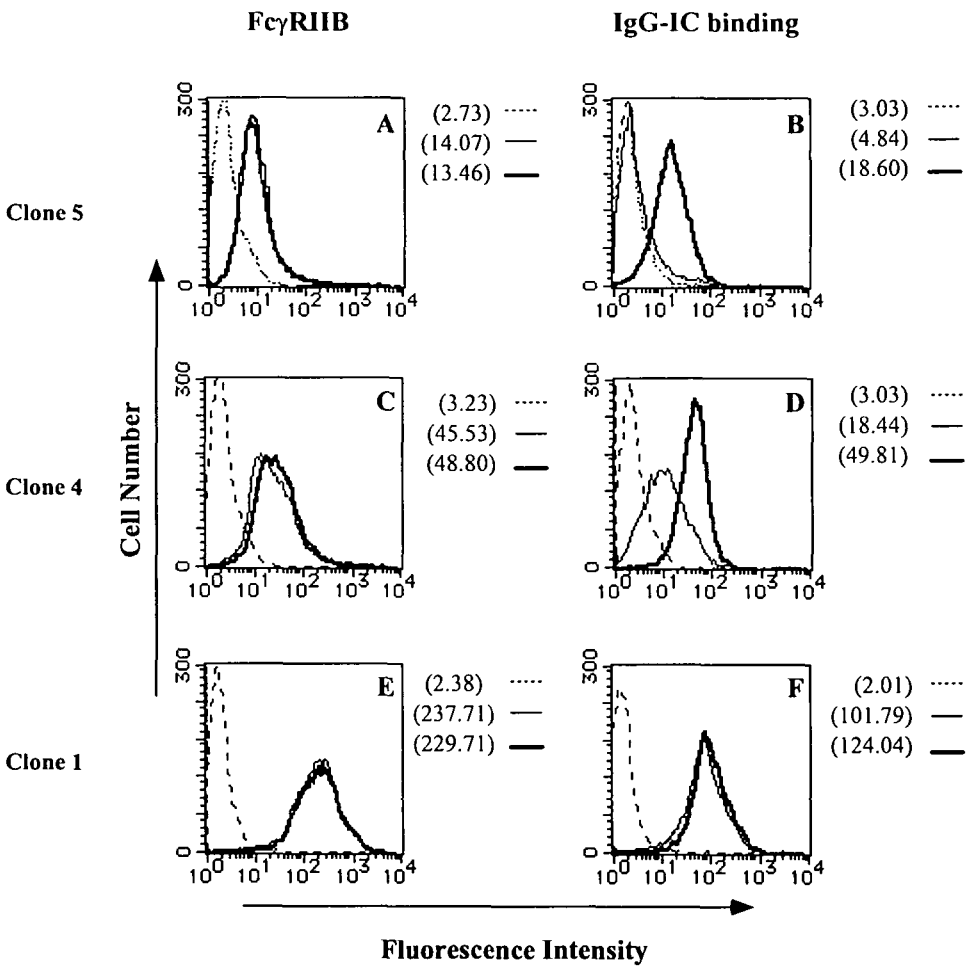

Fig. 5. **Effect of proteolysis on the level of expression and IC binding activity of FcγRIIB on various CHO cell lines.** Pronase-treated (solid bold line) or untreated transfectants (solid line) were stained with anti-FcγRIIB as described in Fig. 4 (A, C, E). Cells were also incubated with IC (Ag/Ab=0.1, 10 μg of Ab/ml) prepared by incubating biotinylated anti-OVA IgG2 with OVA at 4°C for 2 h (B, D, F). The cell-bound IC were detected with FITC-conjugated streptavidin. Negative control cells (dotted line) were obtained by incubating pronase-treated cells with FITC-conjugates alone. The values in parentheses show the mean fluorescence intensity (linear arbitrary unit).

enhanced the binding activity. Thus, this transfectant system was able to reproduce the proteolysis-mediated augmentation of FcγRIIB binding activity seen in blood PMN. Clone 4, which expressed an intermediate level of FcγRIIB, showed moderate IC binding activity before proteolysis (Fig. 5D), and protease treatment significantly enhanced the IC binding activity of the cell line. The clone expressing the highest level of FcγRIIB (clone 1) exhibited a high binding activity before proteolysis (Fig. 5F). In contrast to other transfectants, its binding of IgG-IC to FcγRIIB was not augmented by protease treatment. These findings support the idea that the protease-induced augmentation of IC binding to FcγRIIB was dependent on the density of FcγRIIB as well as on the valency of the IC.

The Cytoplasmic and Transmembrane Domains of FcγRIIB Are Not Involved in the Protease-Induced Augmentation of Ligand Binding—Further studies were conducted to determine which part of FcγRIIB is involved in the proteolysis-mediated enhancement of ligand binding. Besides the FcγRIIB type expressed on PMN and macrophages (FcγRII-B1), two additional isoforms are found on B cells (FcγRII-B2 and -B3) (*6*). They have identical extracellular domains, but their cytoplasmic domains are distinctive. The cytoplasmic domain of FcγRII-B2 is identical to that of FcγRII-B1 except for a 16-amino acid insertion. The cytoplasmic domain of FcγRII-B3 has an insert of 44 amino acid residues, the first 16 of which are identical to the inserted sequence of FcγRII-B2. Individual FcγRIIB isoforms were transfected into CHO cells and the effect of protease on IC binding was examined. As shown in Fig. 6, the established CHO cell lines expressed similar levels of FcγRIIB isotypes. Treatment with pronase did not affect or slightly reduced expression of the receptors. These three cell lines exhibited moderate IC-binding activity before proteolysis and an enhanced binding activity after proteolysis.

We next analyzed whether the cytoplasmic domain and transmembrane domain of FcγRIIB are involved in protease-induced augmentation of ligand binding. For this purpose, we engineered a deletion mutant which lacks the cytoplasmic region of FcγRIIB. We also constructed a FcγRIIB-CD28 chimeric molecule consisting of the extracellular region of FcγRIIB and the transmembrane and cytoplasmic regions of mouse CD28. These cDNA constructs were transfected into CHO cells, and cell lines stably expressing similar levels of FcγRIIB epitope were screened by flow cytometry. As shown in Fig. 7, the binding of IC to the cell lines expressing the mutant FcγRII was markedly augmented by protease treatment, as was the case for the cells expressing wild type FcγRIIB. These data suggest that neither a particular transmembrane nor any cytoplasmic domain of FcγRIIB is required for protease-induced augmentation of FcγRIIB ligand-binding activity.

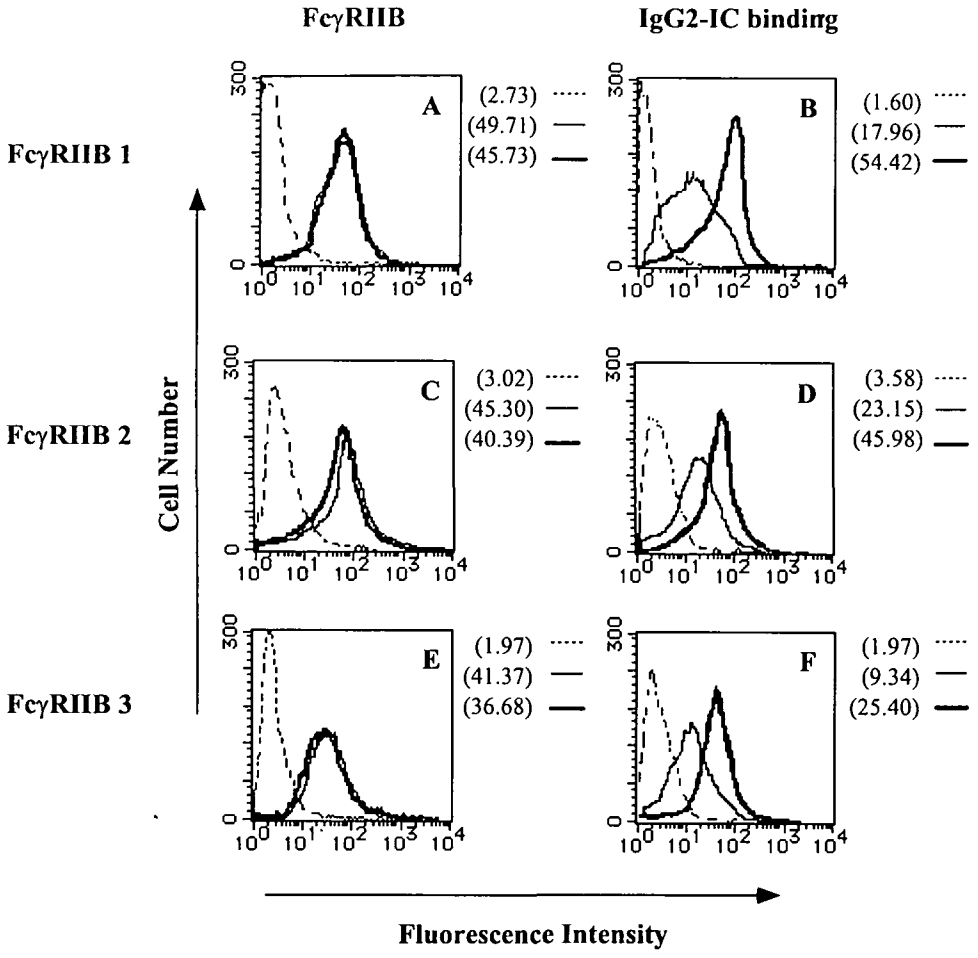

Fig. 6. **Effect of proteolysis on the level of expression and IC binding activity of three different isoforms of FcγRII on CHO cell lines.** The expression of the FcγRIIB epitope and IC binding activity of CHO cells expressing FcγRII-B1 (A, B), -B2 (C, D), or -B3 (E, F) were determined as described in Fig. 5.

DISCUSSION

In this study, we have explored the mechanism by which proteolysis enhances the ligand-binding activity of FcγRIIB. Cosio *et al.* (*12, 13*) found that bacterial pronase enhanced the avidity of FcγR on human phagocytic cells. Recently, Tax and van de Winkel (*15*) reported that the avidity of human FcγRII is increased by proteolytic enzymes. More recently, a similar effect of protease on human neutrophil FcγRII was described (*16*). We also found that the ligand-binding capacity of FcγRIIB on guinea pig PMN is dramatically improved by protease treatment of the cells (*11*). Since the ligand-binding activity of FcγRIIB on guinea pig blood PMN is significantly lower than that on casein-elicited peritoneal PMN, it seems possible that FcγRIIB on blood PMN is activated by protease in areas of inflammation where proteases are abundantly present (*28*). In fact, PMN-derived serine proteases such as elastase were reported to enhance the avidity of FcγRII (*17*). We also found that leukocyte-derived elastase and cathepsin G enhanced the ligand-binding capacity of guinea pig FcγRIIB, whereas trypsin, collagenase, and pancreas-derived elastase failed to do so (data not shown). Thus, locally secreted protease may play an important physiological role by augmenting the ligand-binding capacity of FcγRII on phagocytes.

The mechanism of the induction of enhanced binding activity of FcγRII has been unclear. Based on the fact that proteolysis does not affect the m.w. or the IEF pattern of FcγRII (*14*), it has been postulated that the enhancing effect of protease results from a conformational change of FcγRII (*14, 15*). Others have suggested that protease removes some membrane proteins that affect general membrane properties such as fluidity, leading to the clustering of receptors which is important for multivalent ligand binding (*13*). Unlike FcγRI, the affinity of FcγRII for monomeric IgG is rather low, the affinity constant being less than $10^7\ M^{-1}$ (*29*). Nevertheless, the binding *via* FcγRII is readily demonstrated by soluble IC and insoluble IC such as IgG-sensitized erythrocytes. In this study, we determined whether the intrinsic affinity of guinea pig FcγRIIB is increased after proteolysis by measuring the binding of monomeric IgG. We found that the binding of monomeric IgG was not enhanced by proteolysis. Moreover, we demonstrated that the enhancing effect of proteolysis is closely related to the Ag/Ab ratio of the soluble IC employed. At an Ag/Ab ratio of 0.1, where a single IC contains multiple Fc regions, the highest enhancement of IC binding to FcγRIIB is observed after protease treatment. In contrast, at an Ag/Ab ratio of 10, where individual IC contains principally only one Fc region, the ligand-binding activity is less efficiently enhanced. These results suggest that proteolysis does not enhance the intrinsic affinity of

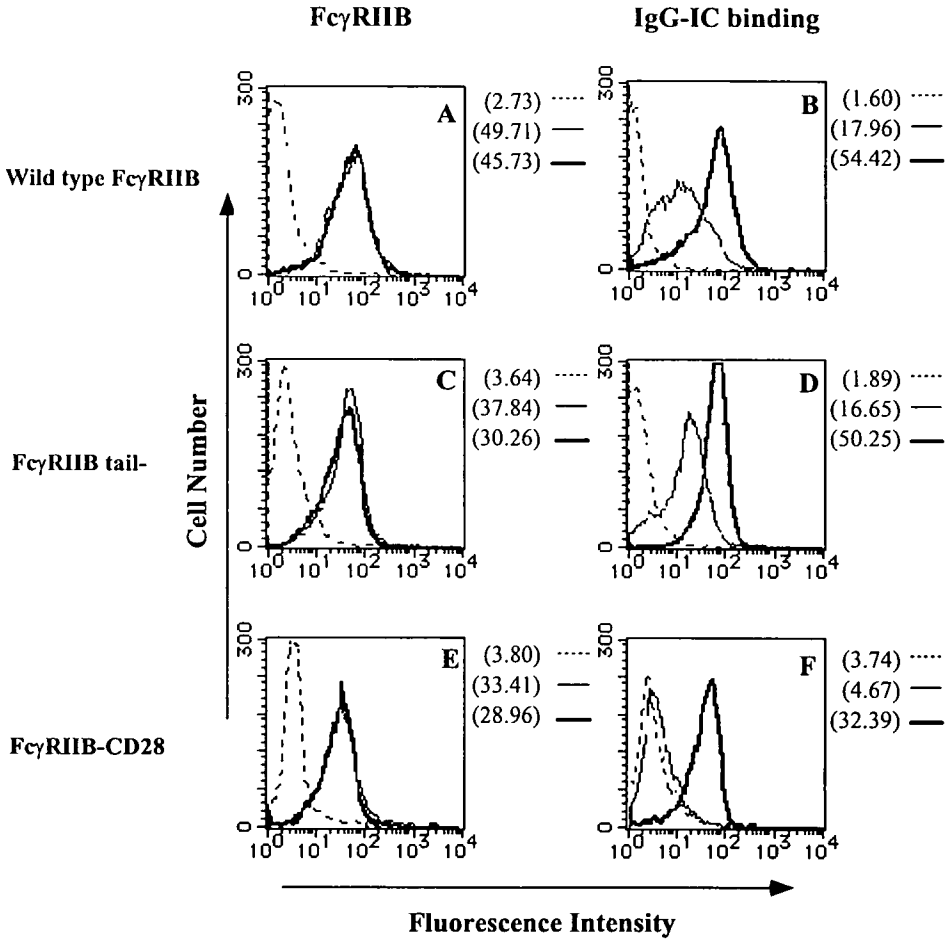

Fig. 7. **Effect of proteolysis on the level of expression and IC binding activity of FcγRIIB on various CHO cell lines expressing the wild type of FcγRIIB (A, B), FcγRIIB-tail-minus mutant (C, D), or chimeric FcγRIIB, which consisted of the extracellular domain of FcγRIIB and the transmembrane and cytoplasmic domains of mouse CD28 (E, F).** Their expression level and IC binding activity were determined as described in Fig. 5.

FcγRIIB; rather, it enhances the ability of receptors to cluster upon exposure to a multivalent ligand and therefore to bind the ligand with higher avidity. Indeed, without proteolysis, FcγRIIB on PMN, which had been labeled with Fab′ fragment of anti-FcγRIIB mAb, remained diffusely distributed on the plasma membrane even after incubation with the bivalent anti-IgG Ab, as assessed by immunofluorescence and confocal laser scanning microscopy. In contrast, clustering of FcγRIIB was observed on protease-treated cells when the cells were allowed to react with secondary Ab. Thus, the clustering of FcγRIIB on the surface of PMN appears to be impeded, although the basis for the restricted mobility is unclear. Proteolysis may increase the mobility of FcγRIIB by affecting nonspecifically a membrane protein component or specifically some membrane proteins associated with FcγRIIB. It will be interesting to determine directly whether the mobility of FcγRIIB is modified by methods such as fluorescence recovery after photobleaching (FRAP) and single particle tracking (SPT).

This concept was further supported and extended by transfection experiments in which guinea pig FcγRIIB were expressed on FcγR-negative CHO cells. We have previously reported that the ligand binding affinity of rat FcγRII on transfected CHO cells is significantly augmented by protease treatment (*26*). In this study, the cells expressing guinea pig FcγRIIB at low density (clone 5) showed an enhanced IC binding after protease treatment, as in the case of guinea pig blood PMN. This result suggests that CHO cells also express putative membrane molecules that impede free diffusion of FcγRIIB. The cell line expressing FcγRIIB at intermediate density (clone 4) exhibited a moderate binding activity and protease treatment enhanced the activity. This level of expression is comparable to that observed on guinea pig peritoneal macrophages. In fact, the macrophages could bind larger amounts of IC than PMN, and when treated with pronase, the cells exhibited only about twofold enhanced IC binding *via* FcγRIIB (data not shown). Importantly, the cells expressing FcγRIIB at extremely high density (clone 1) exhibited an elevated IC binding activity before proteolysis, but proteolysis did not further enhance the activity. These results clearly indicate that the enhancing effect of protease is strongly dependent on the cell surface density of FcγRIIB. It is likely that a dense expression of FcγRIIB could minimize the influence of reduced mobility of the receptor and allow the multiple receptors to bind to multivalent IC without proteolysis. It is also possible that under conditions of high receptor density, some FcγRIIB could be free from putative associated molecules due to the limited number of the latter and thus be mobile even without protease treatment.

In contrast to FcγRIIB on PMN and macrophages, Tuijnman *et al.* (*17*) have reported that FcγRII on B cell lines such as Raji and Daudi do not show an enhanced IC binding activity after treatment with protease. We have also found that proteolysis of guinea pig splenic B cells fails

to induce an enhanced ligand-binding activity of FcγRIIB (data not shown). The B cells express approximately the same level of FcγRIIB as PMN. Phagocytes and B cells express different isoforms of FcγRIIB. The phagocyte isoform (human and murine FcγRII-B2, guinea pig FcγRII-B1) is identical to the B cell isoform (human FcγRII-B1, murine FcγRII-B1, -B1′, guinea pig FcγRII-B2, -B3) except for an insertion in the cytoplasmic tail of the B cell form (*6, 7, 30, 31*). It has been reported that the inserted sequence is important for preventing FcγRII-mediated endocytosis and contributes to the formation of caps in response to receptor cross-linking (*7, 32, 33*). Therefore, the findings of Tuijnman *et al.* and ourselves raised the possibility that the inserted sequence may impede mobility by interacting with cytoskeletal components, and that proteolysis may not be able to affect this association. However, transfection experiments indicated that the ligand binding by the B cell isoform of FcγRIIB on CHO cells could also be enhanced by proteolysis. This result is in conflict with the phenomenon observed on B cells. We speculate that FcγRIIB on B cells are expressed in a different way from that on CHO transfectants, especially in terms of association with the cytoskeleton. Alternatively, putative associated molecules found on B cell membranes that tightly regulate the mobility of the receptor are not expressed in CHO cells. For instance, B cell FcγRIIB may be closely associated with the Ag receptors, since co-ligation of receptors aborts the Ag receptor-triggered B cell activation (*34-36*). Transfection of FcγRIIB into an FcγR-negative B cell line would make it possible to analyze this difference further.

We also demonstrated that neither the specific transmembrane domain of FcγRIIB nor any cytoplasmic domain is required for the proteolysis-mediated modulation of ligand-binding activity. As shown in Fig. 6, the FcγRIIB mutant that lacks the entire cytoplasmic domain exhibited an enhancing ligand binding after proteolysis. Moreover, the chimeric FcγRIIB in which the transmembrane and cytoplasmic domains were substituted with those of an unrelated transmembrane protein (mouse CD28) also showed proteolysis-induced enhanced binding. These results suggest that the extracellular domain of FcγRIIB is involved in the enhancing mechanism; protease treatment affects, specifically or nonspecifically, the association of the ectodomain of FcγRIIB with some membrane proteins, leading to the increase in receptor mobility. It is also possible that the receptor interaction with the cytoskeleton is mediated by the external domain (*37, 38*) and disrupted by proteolysis. Besides proteolytic enzymes, the deglycosylating enzyme neuraminidase is capable of enhancing FcγR function (*39*). Therefore, FcγRIIB could interact with some putative membrane molecules *via* their sugar chains. It remains to be explored which region of the extracellular domain is involved. One approach might be transfection of FcγRIIB mutated in its ectodomain.

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