Brief Definitive Report

The Seven-Span Transmembrane Receptor CD97 Has a Cellular Ligand (CD55, DAF)

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Summary

CD97 is an activation-induced antigen on leukocytes with a seven-span transmembrane (7-TM) region homologous to the secretin receptor superfamily. However, in contrast to this group of peptide hormone receptors, CD97 has an extended extracellular region with three EGF domains at the NH_2 terminus, two of them with a calcium binding site. By demonstrating that lymphocytes and erythrocytes specifically adhere to CD97-transfected COS cells we here show that CD97 in parallel with its molecular evolution has acquired the ability to bind cellular ligands. A mAb selected on its capacity to block the adhesion between CD97 transfectants and red cells was found to be directed to the NH_2-terminal short consensus repeat (SCR) of decay accelerating factor (DAF, CD55), a regulatory protein of the complement cascade. The specificity of the interaction of CD97 with CD55 was established by the observation that erythrocytes that lack CD55, obtained from patients with paroxysmal nocturnal hemoglobinuria (PNH) or the $CD55^-$ phenotype Inab, failed to adhere to CD97 transfectants. This is the first demonstration of a cellular ligand for a 7-TM receptor.

CD97 is an antigen which becomes immediately upregulated on most leukocytes during activation (1). We recently identified CD97 as a 7-TM molecule whose membrane-spanning region is homologous to the secretin receptor superfamily (2). CD97 is different from this group of mammalian and insect peptide hormone receptors (3, 4), in that it has an extended extracellular region with three EGF domains at the NH_2 terminus. The finding of a highly similar architecture in EMR1 (5), which possesses six EGF domains, and its probable murine homologue F4/80 (6) indicates the existence of a new group of 7-TM receptors characterized by several NH_2-terminal EGF domains. We have demonstrated that this new type of 7-TM molecule has recently evolved by exon shuffling to the upstream region of an ancestral gene from the secretin receptor superfamily (7).

All EGF domains in CD97 and EMR1, except the most NH_2-terminal ones, possess a calcium binding site. The Ca^{2+} in this subgroup of EGF domains stabilizes the conformation of the domain and can mediate contact to other proteins (8). The rather recent acquisition of EGF domains raised the possibility that CD97, unlike members from the secretin receptor superfamily, has a cellular ligand (2). In this report evidence is provided that this assumption is valid since we show that CD97 specifically binds CD55 (or decay accelerating factor, DAF), a GPI-linked molecule expressed on most leukocytes.

Materials and Methods

Adhesion Assays. Binding studies were performed with COS cells three days after transient transfection with CD97 cDNA (2) using lipofectamine (GIBCO BRL, Gaithersburg, MD). Typically, 30% of COS cells expressed CD97, as determined by immunoperoxidase staining with CD97 mAbs. At day one, COS cells were replated into six-well culture plates. Mock-transfection was performed by the same procedure, except that no cDNA was added.

To analyze binding, 10×10^6 PBL, obtained from human venous blood by isolation on a Percoll density gradient followed by counterflow centrifugal elutriation, or 100×10^6 erythrocytes were suspended in 1 ml DMEM and overlaid on the COS cells for 30 min at 20°C. Non-adhering cells were removed by gentle washing with PBS before examination by microscopy.

For blocking experiments, erythrocytes were labeled with ^{51}Cr according to manufacturers recommendations (Amersham Co., Buckinghamshire, UK). Binding assays were performed in 12-

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well culture plates in the presence of 5 μg/ml of mAbs. After removing non-adhering cells, the γ emission of well contents lysed with 1% Triton X-100 was determined.

Generation of Monoclonal Antibodies. The mAb CLB-CD97L/1 (IgG1) was produced by fusing mouse myeloma SP2/0 cells with spleen cells from a BALB/c mouse immunized with human erythrocytes. Hybridoma supernatants were screened for the capacity to block the adhesion of erythrocytes to COS cells transfected with CD97 and replated into 96-well culture plates.

Blocking of CLB-CD97L/1 by CD55 mAbs was tested by incubation of PBL with IA10 for 20 min before staining with biotinylated CLB-CD97L/1, followed by PE-streptavidin. Flow cytometric analysis was done on a FACScan® (Becton Dickinson, Mountain View, CA).

CLB-CD97/1 (IgG2a) is a CD97 mAb that was obtained after fusion of spleen cells from a BALB/c mouse that had been immunized with NIH-3T3 cells stably expressing CD97 with SP2/0 cells. CLB-CD97/1 inhibits binding of biotinylated BL-Ac/F2 indicating that both mAbs are directed to the same CD97 epitope (data not shown).

Immunoprecipitation. Biochemical analyses were performed as described previously (9). Briefly, K562 cells were labeled with ^{125}I (Amersham Co.) by the glucose/lactoperoxidase protocol and lysed in 1% NP-40 buffer, containing 10 mM triethanolamine-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 20 μg/ml ovomucoid trypsin inhibitor, 1 mM Nα-*p*-tosyl-l-lysine chloromethyl ketone, and 20 μg/ml leupeptin. After centrifugation, supernatants were precleared with mouse normal Ig (last preclear is shown in the left lane) and incubated with CLB-CD97L/1 and IA10, a CD55 mAb derived from the Fifth International Leucocyte Typing Workshop (10). Immune complexes were adsorbed onto protein A–Sepharose (Pharmacia, Uppsala, Sweden), eluted under reducing conditions, electrophoretically separated by 5–15% SDS-PAGE, and visualized by autoradiography.

Studies with CD55-deficient Erythrocytes. To deplete CD55-positive cells from erythrocytes of paroxysmal nocturnal hemoglobinuria (PNH) patients, cells were incubated with saturating amounts of CLB-CD97L/1 before addition of anti-mouse IgG magnetic beads (Dynal, Oslo, Norway) and immunomagnetic selection. Expression of CD55 on the erythrocyte populations was determined by flow cytometry with CLB-CD97L/1 or a subclass control mAb. The Inab phenotype erythrocytes examined in this study are from a new, unpublished case of this extremely rare disorder (Dr. G. Daniels, personal communication). Binding of erythrocytes to CD97-transfected COS cells was analyzed as described above.

Results and Discussion

CD97 Can Mediate Intercellular Adhesion. We have previously noticed that the structural features of CD97 suggest an adhesive function for its extracellular region (2). Indeed, as shown in Fig. 1, PBL and erythrocytes adhered to COS cells expressing CD97. This interaction was shown to be specific since pre-incubation of COS cells with CD97 mAbs (either CLB-CD97/1 or BL-Ac/F2 [1]) completely abolished binding.

CD55 Is a Ligand for CD97. Once the binding studies had indicated that a ligand for CD97 is being expressed on erythrocytes, immunization of mice with human erythrocytes combined with standard hybridoma technology was used to generate a ligand-specific mAb. One mAb (CLB-CD97L/1) was identified that blocked the adherence of both erythrocytes and PBL to CD97-transfected COS cells. Biochemical analysis showed that this mAb recognizes a 70-kD protein (Fig. 2 *A*). Among antigens of this size studied during the Fifth International Leucocyte Typing Workshop, a small number was found to be expressed on both erythrocytes and lymphocytes (10). When we tested the capacity of mAbs directed against these antigens to block the binding of erythrocytes to CD97 transfectants, IA10, a mAb recognizing CD55 (11) turned out to be inhibitory. A direct comparison of CLB-CD97L/1 with IA10 revealed that both mAbs immunoprecipitated not only the same major protein of 70 kD which characterizes

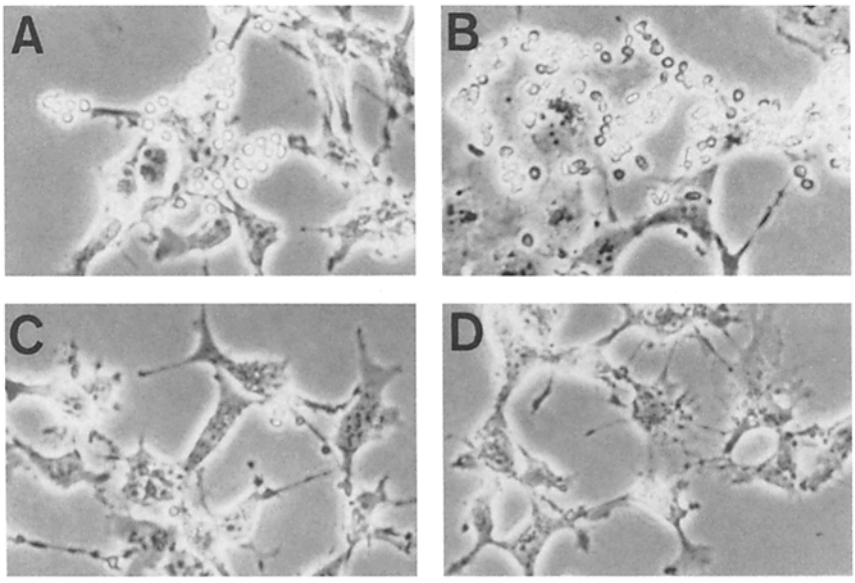

Figure 1. Adherence of human PBL (*A*) and erythrocytes (*B*) to COS cells expressing CD97. Both B and T lymphocytes adhere to CD97-transfected COS cells as revealed from experiments with purified cells (data not shown). No binding is detectable in the presence of 5 μg/ml of CD97 mAbs CLB-CD97/1 (shown) or BL-Ac/F2 (*C*), or when cells are overlaid on mock-transfected COS cells (*D*).

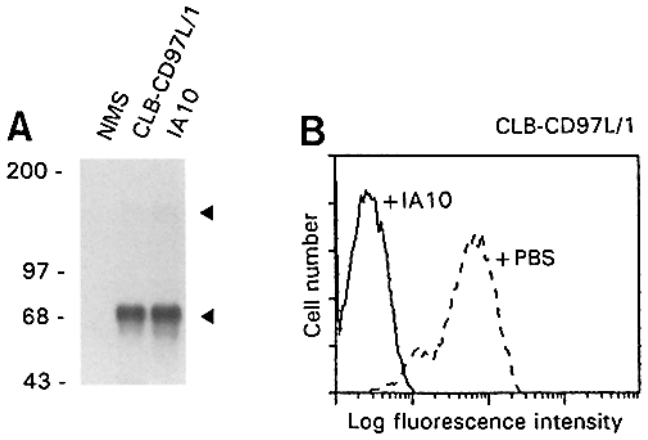

Figure 2. CLB-CD97L/1, a mAb generated to the cellular ligand of CD97 is specific for CD55. (*A*) CLB-CD97L/1 and the CD55 mAb IA10 immunoprecipitate the same major protein of 70 kD from the erythromyeloid cell line K562. Notably, also a smaller band at 140 kD representing dimeric CD55 (13) is detectable in the CLB-CD97L/1 immunoprecipitate. The position of molecular size markers in kD are indicated on the left. (*B*) The binding of biotinylated CLB-CD97L/1 to PBL (*dashed line*) is blocked by the CD55 mAb IA10 (*solid line*).

the CD55 antigen (12), but also a minor band migrating at 140 kD which represents dimeric CD55 (13) (Fig. 2 *A*). Furthermore, IA10 was found to completely block the binding of biotinylated CLB-CD97L/1 to PBL (Fig. 2 *B*), indicating that the mAb generated to the ligand of CD97

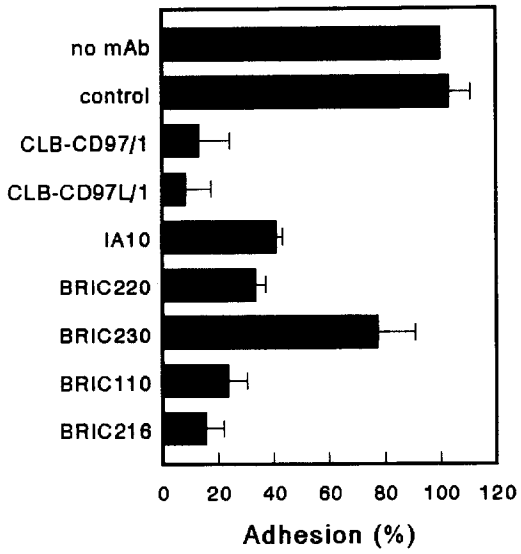

Figure 3. CD55 mAbs inhibit the binding of erythrocytes to CD97-transfected COS cells. Adhesion of ^{51}Cr-labeled erythrocytes to CD97-transfected COS cells was assessed in the presence of 5 μg/ml of mAbs specific for CD97 (CLB-CD97/1), CD55 (CLB-CD97L/1, IA10, BRIC 220, 230, 110, 216) or a mouse IgG1 control mAb. The CD55 mAbs used are directed to the first (IA10, BRIC220, BRIC230), second (BRIC110), or third (BRIC216) SCR domain (14). Results are expressed as the percent of erythrocyte binding, relative to the amount bound in the absence of mAbs. Data shown are mean ± SD of duplicate determinations in three independent experiments.

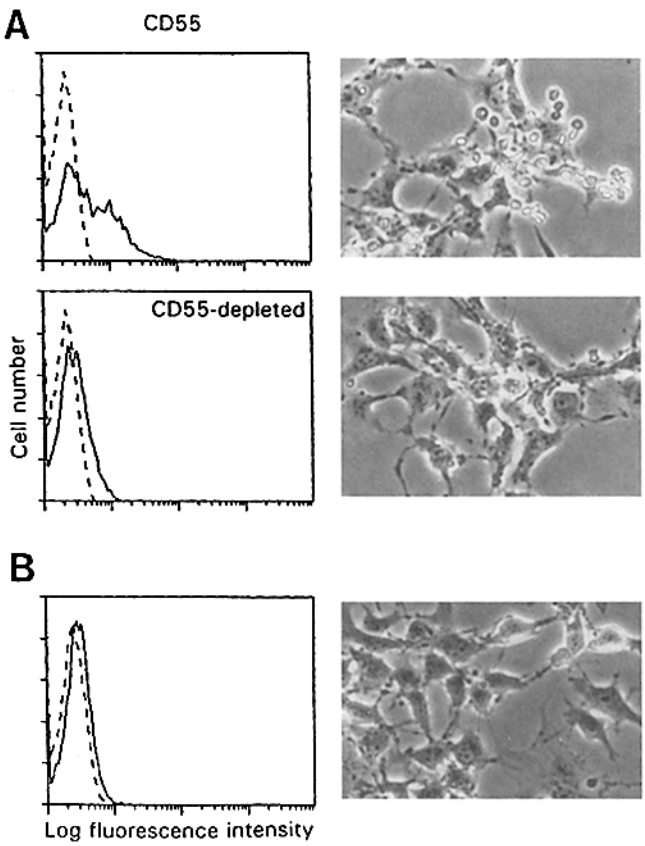

Figure 4. CD55-deficient erythrocytes are not able to adhere to CD97-transfected COS cells. (*A*) Erythrocytes from an PNH patient bind to CD97-transfected COS cells (*upper right panel*) due to the presence of non-afflicted cells in this clonal disease (16, 17) (*upper left panel*). After removing the CD55-positive erythrocytes by immunomagnetic sorting (*lower left panel*), adherence was completely abolished (*lower right panel*). One representative experiment out of four is shown. (*B*) The complete absence of CD55 expression in the Inab phenotype (18, 19) (*left panel*) prevents erythrocytes from binding to CD97-transfected COS cells (*right panel*).

recognizes the same epitope as IA10 that has been previously mapped to the first (of four) SCR of CD55 (14).

CD55 is a GPI-anchored molecule expressed by all blood cells and cells in contact with blood and tissue fluid that by inhibiting C3/C5 convertases protects them from complement-mediated damage (12). To investigate the specificity of the interaction between CD97 and CD55, a larger panel of CD55 mAbs, directed against distinct SCR domains within the molecule, was tested in the above described adherence assay. As shown in Fig. 3, inhibition of erythrocyte adhesion to CD97-transfected COS cells between 23 and 92% was observed. The finding that also mAbs mapping to the second (BRIC110) and third (BRIC216) SCR of CD55 are able to block binding suggests that these domains, in addition to the first SCR, are involved in ligation of CD97. The ability to dissociate and prevent assembly of C3/C5 convertases in both the classical and alternative pathway of the complement cascade has recently been mapped to the SCR domains two, three and four (14, 15). The finding that the first SCR of CD55 is involved in ad-

hesion to CD97 is the first demonstration of a molecular function for this domain.

CD55-deficient Erythrocytes Do Not Bind to CD97 Transfectants. Further evidence that CD97 specifically interacts with CD55 came from observations that erythrocytes lacking CD55 expression fail to adhere to CD97 transfectants. First, PNH is an acquired somatic defect in GPI-anchor synthesis that leads to the absence of GPI-anchored molecules (16, 17). Due to the clonal character of this hematopoietic stem cell disorder, both CD55-positive and CD55-negative red cells circulate in the blood of afflicted patients. CD55-positive erythrocytes from these patients have retained the ability to bind CD97 transfectants (Fig. 4 *A, upper panels*). However, after depletion of the unaffected, CD55-expressing erythrocytes, a complete abrogation of adherence was seen (Fig. 4 *A, lower panels*). Second, the Inab phenotype represents an inherited deficiency in CD55 expression due to truncative mutations in the CD55 gene (18, 19). Erythrocytes with this phenotype completely lacked the ability to bind COS cells expressing CD97 (Fig. 4 *B*).

In conclusion, the interaction between the activation-regulated CD97 antigen and CD55 implies the existence of a novel adhesion pathway (20) primarily used by primed but not quiescent leukocytes. Remarkably, among the hundreds of known 7-TM receptors CD97 is the first molecule for which a cellular ligand has now been demonstrated (21–23). It needs to be investigated if this is a common feature of the new group of 7-TM receptors with NH_2-terminal EGF domains to which CD97 belongs. Although the physiological consequences of the interaction between CD97 and CD55 remain to be determined, our findings indicate that complement regulation is probably not the exclusive function of CD55. Notably, transgenic CD55 is currently being used to downmodulate complement activation by xeno-transplants (24). Our data imply that, although the effect on complement activation might be beneficial for graft survival, attraction of activated leukocytes to the graft might be an unwanted (and so far unanticipated) side effect of this approach.

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