Molecular Analysis of the Epidermal Growth Factor-like Short Consensus Repeat Domain-mediated Protein-Protein Interactions

DISSECTION OF THE CD97-CD55 COMPLEX

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Epidermal growth factor-like (EGF) and short consensus repeat (SCR) domains are commonly found in cell surface and soluble proteins that mediate specific protein-protein recognition events. Unlike the immunoglobulin (Ig) superfamily, very little is known about the general properties of intermolecular interactions encoded by these common modules, and in particular, how specificity of binding is achieved. We have dissected the binding of CD97 (a member of the EGF-TM7 family) to the complement regulator CD55, two cell surface modular proteins that contain EGF and SCR domains, respectively. We demonstrate that the interaction is mediated solely by these domains and is characterized by a low affinity (86 μM) and rapid off-rate (at least 0.6 s⁻¹). The interaction is Ca²⁺-dependent but is unaffected by glycosylation of the EGF domains. Using biotinylated multimerized peptides in cell binding assays and surface plasmon resonance, we show that a CD97-related EGF-TM7 molecule (termed EMR2), differing by only three amino acids within the EGF domains, binds CD55 with a Kₐ at least an order of magnitude weaker than that of CD97. These results suggest that low affinity cell-cell interactions may be a general feature of highly expressed cell surface proteins and that specificity of SCR-EGF binding can be finely tuned by a small number of amino acid changes on the EGF module surface.

Most cell surface proteins are highly modular in organization and are constructed from different combinations of a limited set of structural domains. In recent years high resolution structures of many of the most common domains have become available, and analysis of the kinetic characteristics of the interactions they mediate is increasing our understanding of processes that underlie the most basic cellular interactions. In particular, cell adhesion interactions mediated by proteins of the Ig superfamily are characterized by multiple weak binding events, each with a low affinity, but with the multivalent nature of the cell surface proteins resulting in a cell-cell interaction of high avidity (1). Two of the most common structural modules used in cell surface proteins are the epidermal growth factor-like (EGF)¹ and short consensus repeat (SCR) domains (Fig. 1). Recent analysis of the human genome (as of March 1, 2001) shows that the EGF family is the fifth most common protein family with 3% of all potential proteins containing EGF domains whereas SCR domains are found in 0.3% of all proteins.

Both of these domain types are commonly used to mediate protein-protein interactions. The EGF module, which often occurs as multiple tandem repeats, is widely distributed among extracellular proteins involved in adhesion, receptor-ligand interactions, extracellular matrix structure, determination of cell fate, and blood coagulation (see Fig. 1). A subset of EGF domains contains a consensus sequence associated with calcium binding (cb): (D/N)(X)₂₋₄(D/N)(X)₄₋₈(X)₆₋₈(Y/F), where m and n are variable and the asterisk indicates possible β-hydroxylation (2–4). Calcium is thought to perform a key role in the orientation of cbEGF pairs by restricting conformational flexibility of interdomain linkages (5, 6) resulting in tandem EGF repeats that are highly resistant to proteolysis (5, 7, 8). SCR domains are frequently found among proteins of the complement system with many of the complement regulatory proteins (e.g. CD55 and CD46, see below) and complement receptors (e.g. CD21 and CD35) consisting entirely of repeated SCR domains. Structural studies (5, 9–10) reveal that both of these small modules (<60 amino acids) fold to form all β-strand domains strengthened by disulfide bonds (two in the case of SCRs and three for EGFs). An understanding of why these domains are so well suited for protein interactions has been hindered by the fact that the disulfide-rich multidomain constructs required for structural analyses are difficult to produce in large yields in the native form. We therefore have very few structures of complexes involving these common modules and

¹The abbreviations used are: EGF, epidermal growth factor; SCR, short consensus repeat; Ig, immunoglobulin; EGF-TM7, epidermal growth factor module-containing seven transmembrane receptor; RCA, regulators of complement activation; cbEGF, calcium-binding EGF motif; EMR2, epidermal growth factor module-containing mucin-like receptor 2; SPR, surface plasmon resonance; PACS, fluorescence-activated cell sorting; RU, response unit; mAb, monoclonal antibody; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.
no structures of EGF-SCR complexes or kinetic characterization of the interaction.

CD55 (or Decay Accelerating Factor; DAF) is a member of the regulators of complement activation (RCA) family. It protects host cells from complement system attack by binding to C3b and C4b preventing formation of the membrane attack complex. It has a widespread tissue distribution and is expressed at high levels on many different cell types. The protein consists of an N-terminal extracellular portion of four SCR domains linked via a heavily O-glycosylated spacer to a C-terminal glycosylphosphatidylinositol anchor (Fig. 1). SCR domains 2–4 are involved in regulation of complement and also in binding to a variety of viral and bacterial pathogens. The most N-terminal SCR (domain 1) also provides the site of interaction for some viruses but until recently the native role of domain 1 was unknown. However, identification of CD97 as a cellular ligand for the N-terminal domains of CD55 (12, 13) has now demonstrated a novel natural function associated with this portion of the molecule. CD97 is a member of the EGF-TM7 family, characterized by the unique chimeric structure in which tandem EGF repeats are coupled to a G protein-coupled receptor moiety via a mucin-like stalk region (14, 15). CD97 is constitutively expressed on granulocytes and monocytes and is rapidly up-regulated on activated T and B cells. It is known to exist in a variety of splice forms containing different numbers of the EGF domains (16, 17) each of which binds CD55 with different affinities. The CD55 binding splice variant comprises three EGF domains (domains 1, 2, and 5), two of which (domains 2 and 5) are predicted to bind calcium (Fig. 1). Although the precise role of the CD55-CD97 interaction is still unknown the unique hybrid structure, the leukocyte-restricted expression pattern of CD97, and the presence of both CD97 and CD55 in arthritic joints (18) suggest possible roles for the CD97-CD55 interaction in adhesion and signaling within the inflammatory and immune responses.

Recently a novel EGF-TM7 molecule, EMR2, sharing highly homologous EGF domains with CD97 but failing to show an interaction with CD55 in biological assays was identified (19). In this study we probe the biophysical, cellular, and molecular properties of the CD55 and CD97 interaction and investigate the sequence-specific requirements for CD55 binding. We show that the interaction is mediated solely by the EGF domains of CD97 and characterized by a low affinity and rapid off-rate. Ca$^{2+}$ is essential for the formation of the CD55 binding face on CD97, but glycosylation of EGF domains from CD97 is not required. The three amino acid differences within the EGF domains of EMR2 that distinguish it from CD97 decrease the affinity for CD55 by at least an order of magnitude. The implications of these data for general properties of cell surface interactions and specificity of EGF domain interactions are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals and reagents were obtained from Sigma unless otherwise specified. Cell culture media and supplements were purchased from Life Technologies, Inc. CD97 monoclonal Abs, BL-Ac/F2, CLB-CD97/1, CLB-CD97/2, and CLB-CD97/3 were kindly provided by Dr. Jörg Hamann (Dept. of Immunobiology, CLB, University of Amsterdam, The Netherlands). cDNA for CD97 was a kind gift of Dr. Celestine O’Shaugnessy (Dept. of Neuropharmacology, GlaxoWellcome, Stevenage, UK).

**Bacterial Expression of CD97 and EMR2**—The three extracellular EGF domains (EGF-1, 2, 5) of the CD55 binding splice variant of CD97 (and analog) of EMR2 were expressed in Escherichia coli using a His tag-based expression system (Qiagen). Primers used for the PCR amplification of EGF domains 1, 2, and 5 from CD97 and EMR2 cDNAs were 5’-TAGTAGGAGATCCATAGAAGGACGATCAGCAGACTCCAGGGCCTGTGCC (forward) and 5’-TAGTAGAAGCTTCTATTATTCACAGACAGTGTCCTTTTG (reverse). Restriction sites used for subsequent cloning into the expression vector pQE30 are underlined. The forward primer also contains a FxI cleavage site and a two-amino acid (SA) spacer sequence prior to the authentic sequence of CD97 and EMR2. Following Ni$^{2+}$ affinity purification under denaturing conditions, peptides were reduced, purified, and refolded according to a well established in vitro refolding protocol (2). The data indicating that the multidomain
constructs adopted the native fold was indirect but substantial. Both peptides showed the characteristic change in HPLC elution profile previously observed for all other cbEGF constructs on refolding (e.g., the profile for ER2 is shown in Fig. 2). Because calcium binding is a property of the native fold of cbEGF domains, correct refolding of these domains in CD97 and EMR2 was indicated by the observed Ca

dependence of CD55-CD97 interaction (see "Results"). After purification, CD97 and EMR2 were lyophilized and reconstituted into appropriate buffers at the desired concentration. The concentration of protein solutions was confirmed using the calculated extinction coefficient at 280 nm (21,000 M

−1 cm

−1) computed from the amino acid composition on the EXPASY server, Ref. 20).

Fichia Expression of CD55—The expression of the four SCR domains of CD55 as a soluble fragment in the yeast Pichia pastoris has been described in detail elsewhere (21). The protein was dialyzed (10,000 molecular weight cut-off Slide-A-Lyzer, Pierce) against 50 mM Tris/HC1, pH 7.5 to remove imidazole and concentrated to 3000 molecular weight cut-off Centriprep 3 (3500 rpm, 120 min). The protein was dialyzed (10,000 molecular weight cut-off Slide-A-Lyzer, Pierce) against 50 mM Tris/HCl, pH 7.5 to remove imidazole and concentrated to

5000 molecular weight cut-off Centriprep 3 (3500 rpm, 120 min). The concentration of protein was assessed using the calculated extinction coefficient at 280 nm (36,840 M

−1 cm

−1) computed from the amino acid composition on the EXPASY server, Ref. 20).

Surface Plasmon Resonance—Surface plasmon resonance (SPR) experiments were performed on a BLIAcore2000 (BIACORE AB, Steve-

nage, UK). CD55 was covalently immobilized to the carboxylated dextran matrix on the surface of CM5 sensor chips via primary amine coupling using the amine-coupling kit (BIAcore AB) as directed (22) with the following modifications. After the activation step, purified CD55 was injected at 11 μg/ml in 10 mM sodium citrate (pH 4.6) for 5 min (5 μl/min). This repeatedly resulted in the coupling of ~1000 response units (RU) of CD55 to the chip surface. Biotinylated CD97 was coupled to the chip surface via streptavidin according to the protocol provided by Biacore. Interaction data were collected by injecting 30 μl of an appropriate analyte over the coupled chip surface at a flow rate of 20 μl/min at 25 °C, and all traces were corrected for refractive index changes by subtraction of a control trace simultaneously recorded from a mock-immobilized channel. Unless otherwise stated, all experiments were carried out in a buffer (I = 0.15) 5 mM Ca

2+, 135 mM NaCl, 10 mM Tris, pH 7.4.

Mammalian Expression of CD97 and EMR2—All the expression vectors described below were constructed in pcDNA3.1 (Invitrogen). The EMR2 and CD97 expression vectors containing five EGF-like and three EGF-like domains, EMR2 (EGF-1,2,3,4,5), EMR2 (EGF-1,2,5), CD97 (EGF-1,2,3,4,5) and CD97 (EGF-1,2,3,4,5,6) have been described previously (19). The constructs for the EMR2/CD97 domain-swapping chimeras were made by ligating the DNA fragment of the EGF-1,2,5 domain of EMR2 or CD97 to that of the stalk region of CD97 or EMR2, which was ligated in frame with the 7TM region of CD97. Similarly, the EMR2 and CD97 deletion constructs, ΔEMR2 and ΔCD97, were made by ligating the respective EGF-1, 2, 5 domains with the 7TM region of EMR2. The EGF-1,2,5 domains of EMR2 and CD97 were amplified by PCR using primers KE5 (5′-GCTGGATACCAGGGC-

CAGGTTTTCCCGTCTC-3′) and KE3 (5′-GTAATTCACAGACACTTTTCCTG-3′) and KE3 (5′-GTAATTCACAGACACTTTTCCTG-3′). Likewise, the stalk regions of EMR2 and CD97 were generated by using the primer set E65-1 (5′-CTGTGATTCCTAGATGACATTTCTCCACACCTGGACC-3′) with EB3–1 (5′-AGACGAGGATCTCCTCCTTGCACATC-3′) and EB3 with EB3–2 (5′-TCAGATTTGCTCCTTGCACATC-3′), respectively. Specific restriction enzyme sites (underlined) were incorporated in the primers to facilitate the cloning. Site-directed mutants of EMR2 (EMR2-D36N, EMR2-M26T, EMR2-L74P) and CD97 (CD97-N93D, CD97-T59M, CD97-P71L) were made using the EGF-1,2,5 domains of EMR2 and CD97 as template, respectively. Mutagenesis was carried out according to the protocol suggested by the manufacturer (GeneEditor Mutagenesis System, Promega). The resulting mutated EGF-1,2,5 DNA fragments were excised, purified, and ligated with the stalk region of CD97 followed in frame by the 7TM region of EMR2. For the construction of vectors expressing soluble biotinylated proteins, the DNA fragment encoding the consensus peptide sequence, NSGSLH-HILDAQQKMWVHR+, recognized by the E. coli biotin holohemolysin synthetase BirA (23), was generated by PCR using Bio5 (5′-TAGTAGGTTATTACCCACCTTGACC-3′) and Bio5 (5′-TAGTAGGTTATTACCCACCTTGACC-3′) primers and HLA A2 plasmid construct as template (24). Following BamHI and Apal digestion, the DNA fragment was subcloned immediately downstream of the stalk region of EMR2 in pcDNA3.1. Wild-type or site-directed mutant EGF-like domains of EMR2 or CD97 were then inserted into the vector upstream of the EMR2 stalk region. The final constructs therefore contained various EGF-like domains followed by the EMR2 stalk region, a biotinylation signal and a stop codon.

All culture media were supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin, and cells were incubated in a humidified 37 °C, 5% CO

2 incubator. HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium, K562 cells in RPMI 1640 (R10), and CHO-K1 cells in Ham’s F-12 medium. CHO-K1 cells were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s protocol. HEK293T cells were transfected with 40 μg of DNA/175-cm

2 flask using calcium phosphate precipitation. Six hours post-transfection, the medium was replaced with 15 ml of serum-free Dulbecco’s modified Eagle’s medium and incubated for a further 72 h.

Cell Rosette Assays—Cell rosette assays were performed as previously described (19). Briefly, CHO-K1 cells transfected with the appropriate expression vectors were subcultured at day 1 into 12-well dishes and analyzed for their ability to bind human red blood cells 3 days post-transfection. Heparinized human whole blood cells were diluted 1:100 (vol/vol) in R10 and added to transfected cells (1 ml/well) for 30 min at room temperature. Nonbinding red blood cells were removed by gentle washing. The extent of red blood cell adhesion was quantified by measuring the peroxidase activity of hemoglobin (E450) of methanol-

extracted red blood cells. The extent of red blood cell adhesion was determined by FACs analysis using appropriate CD97 mAbs. For cells transfected with EMR2 and CD97 isoforms as well as the domain-swapping and deletion chimera, CLB-CD97/71 and BL-AcF2, which recognize the first EGF-like domain of both EMR2 and CD97, were used. CLB-CD97/2 and CLB-
CD97/3, which specifically recognize the stalk region of CD97, were used for cells transfected with the EMR2 and CD97 site-directed mutants. The red blood cell binding ability of transfected cells was represented by the measurement of peroxidase activities normalized by the median level of cell surface protein expression determined by FACS analysis.

Production of Biotinylated Proteins—Conditioned medium collected from transfected HEK293T cells was concentrated to ~0.5 ml using a 30-kDa cut-off Centriprep tube (Millipore, Bedford, MA), dialyzed with 10 mM Tris- HCl, pH 8 buffer and incubated with 1 μl of BirA enzyme (Avidity, Denver, CO) overnight at room temperature. Excess biotin was subsequently removed by dialysis with 10 mM Tris- HCl, pH 7.3 buffer containing 10 mM CaCl₂ and 150 mM NaCl. The biotinylated proteins were then aliquoted and stored at ~80 °C after quantification by dot-blot analysis using myelin basic protein-biotin (Avidity, Denver, CO) as standard.

Cell Binding Assay Using Biotinylated Protein-coupled Fluorescent Beads—Cell binding assays using fluorescent beads coupled to biotinylated proteins were performed as previously described (23). In brief, 20 μl of an avidin-coated fluorescent beads (Spherotech, Inc., Libertyville, IL) were washed twice and added to 2 μg of biotinylated protein in a total volume of 50 μl. The bead and protein mixture was sonicated at 20% power for 1 min (Heat systems, Sonicator) and then incubated at 4 °C for 1 h. Nonbinding proteins were removed by washing twice with phosphate-buffered saline/bovine serum albumin, and the beads were resuspended in 50 μl of R10. The bead-protein complex was sonicated again immediately before adding to K562 cells in a 96-well plate (1 × 10⁶ cells/50 μl R10/well). The cell-bead mixture was spun at 1000 g for 1 min (Heat systems, Sonicator) and then incubated at 4 °C for 20 min, incubated for a further 40 min at 4 °C, and finally resuspended in 500 μl of phosphate-buffered saline for FACS analysis. Where necessary, additional reagents (divalent cations, EGTA, and mAbs) were added to the cells 5–10 min before the introduction of the protein-bead complex.

RESULTS

CD55 Binding Is Mediated Exclusively by the EGF Domains of CD97—Previous studies have demonstrated that the EGF domains of CD97 are necessary for CD97-CD55 interaction (13) and that EMR2 is unable to interact with CD55 (19). CD97 and EMR2 share highly homologous EGF domains but are relatively variant (~50% identical) within the supporting stalk region. To investigate the possible contribution of the stalk region to the previously observed CD55 binding activity of CD97, domain-swapping chimera and stalk region deletion mutants were analyzed (Fig. 3) using a quantitative cell rose-tting assay (see “Experimental Procedures”). Proteins containing the EGF-1,2,5 domains of CD97 and the stalk region of EMR2 are CD97-like in their CD55 binding properties, whereas constructs consisting of the EGF domains of EMR2 and the stalk of CD97 are EMR2-like showing no CD55 binding activity in this assay. Deletion of the CD97 stalk retains the ability of CD97 to bind CD55 but reduces the overall binding by ~60%.

We interpret the reduced binding to CD55 observed on deletion of the CD97 stalk as being caused by steric effects; in the absence of the CD97 stalk the EGF domains are less accessible to the cell-bound CD55. Support for this interpretation comes from our surface plasmon resonance studies (see below) that show that constructs of CD97 with or without the stalk have identical affinities for soluble CD55. Controls used in this experiment confirm that different isoforms of CD97 have different CD55 binding abilities with the shortest CD97 isoform, CD97 (EGF-1,2,5), having the highest affinity (100%) whereas the longest isoform, CD97 (EGF-1,2,3,4,5), shows only ~20% of the CD55 binding affinity. All EMR2 isoforms showed no detectable binding to CD55 in this assay (19).

Equilibrium Binding Analysis of the Interaction between CD55 and CD97—Surface plasmon resonance (SPR) was used to study the detailed interaction kinetics between domains from CD55 and CD97 implicated in complex formation. Protein constructs consisting of four SCR domains from CD55 and the three EGF domains from CD97 with or without the stalk (Fig. 1) were expressed and purified (see “Experimental Procedures”). SPR measurements were obtained for proteins in both orientations, i.e. either fixed to a static surface or in solution. The CD97 used in the soluble phase consisted of the EGF domains alone and was not glycosylated because it was derived from an E. coli expression system (see “Experimental Procedures”). In contrast, the CD97 bound to the chip containing the full stalk region and was glycosylated because it was obtained using a mammalian cell expression system. Fig. 4a(i) shows an injection of 5 μM CD55 (bar) for 90 s over a chip with 350 RU of CD97 bound whereas Fig. 4b(i) shows an injection 64 μM CD97 (bar) for 90 s over a chip with CD55 bound. A background response is seen in the control trace of each experiment because the BiAcore measures the refractive index near the sensor surface and therefore detects any changes in the bulk refractive index of the injected sample. The response seen when soluble protein is injected over its bound ligand is considerably larger than the response seen in the control trace in each case. Inspection of both sets of sensorgrams reveals that the kinetics of the CD97-CD55 interaction are rapid; binding reaches equilib-
CD55 at 5 mM obtained with 1200 RU of CD55 bound to the chip surface and CD97 in the aqueous phase. CD97 (the same in whichever orientation the experiment was performed irrespective of the order of injections (data not shown). Table I summarizes the values that both the CD55 and CD97 were stable for the duration of injections (low to high concentrations or vice versa), indicating that both the CD55 and CD97 were stable for the duration of injections (fits not shown). Identical results were obtained irrespective of the order of injections (low to high concentrations or vice versa), indicating that both the CD55 and CD97 were stable for the duration of injections (data not shown). Table I summarizes the values of $K_D$ obtained from different experiments (using protein expressed in different preparations) and the quality of the nonlinear fits to the data. The mean of all the experiments yielded a value for $K_D$ of 86 ± 1 μM. The estimates of $K_D$ were the same in whichever orientation the experiment was performed (i.e. CD55 or CD97 immobilized on the chip surface) suggesting that the value of $K_D$ obtained provides a true representation of the in vivo affinity and is not subject to coupling effects. Because $K_D$ is the same for both the soluble and immobilized forms of CD97, glycosylation and the stalk region are not required for CD55 binding. Simultaneous fitting of numerically integrated rate equations derived from the simple Langmuir binding model (A + B → AB) to the sets of sensorgrams (global analysis option BIAevaluation 3.0) shows that the off-rate is at least 0.6 s$^{-1}$ (data not shown).

**Dependence of CD55-CD97 Interaction on the Presence of Ca$^{2+}$**—Previous studies of a cbEGF domain pair from fibrillin-1 have demonstrated that calcium is essential for maintenance of a rod-like interdomain linkage (5). Removal of Ca$^{2+}$ leads to a change in the dynamic properties of this pair, which may be detected by an increase in the susceptibility of the EGF pair to proteolysis (25). Inspection of the sequence of the soluble fragment of CD97 suggests that two of the three EGF domains (domains 2 and 5) are of the Ca$^{2+}$ binding type (Fig. 5). To study the potential role of Ca$^{2+}$ in the CD97/CD55 interaction,
we investigated Ca\textsuperscript{2+} dependence of the interaction. The equivalent concentration of CD97 was injected over immobilized CD55 in Ca\textsuperscript{2+}-containing buffer, in the presence or absence of EGTA (Fig. 6). In Ca\textsuperscript{2+}-containing buffer the presence of EGTA was seen to completely abolish the interaction so that no difference was seen between the response from the control and CD55-coupled chip surfaces. In addition, Mg\textsuperscript{2+} was unable to substitute for Ca\textsuperscript{2+}, because no binding was observed when CD97 was injected over immobilized CD55 in the presence of EGTA and Mg\textsuperscript{2+} (Fig. 6); however, binding was restored by the subsequent addition of Ca\textsuperscript{2+}.

Dissection of the Effect of the Sequence Differences between the EGF Domains of CD97 and EMR2 on CD55 Binding Affinity—EMR2 (19) differs from CD97 by only three amino acid changes within EGF domains 1, 2, and 5, two of which occur in EGF domain 1 and one in EGF domain 2 (98\% sequence identity in the three EGFs contained in this fragment) (Fig. 5). In previous cell-based assays (19) and this study, EMR2 has shown no interaction with CD55 (Fig. 3). Surface plasmon resonance was used to quantitate the effects of the three amino acid differences on CD55 binding. The three EGF domains of EMR2 were expressed and purified using an E. coli expression system (see “Experimental Procedures”) and flowed in the soluble phase over a CD55-coupled chip surface. Only by using high concentrations (~10 mM) of EMR2 could a weak, specific interaction between CD55 and EMR2 be observed, and the binding was sufficiently weak that direct determination of the $K_D$ was not possible with the amounts of protein available. Although it is difficult to accurately compare the absolute values of SPR signals, a direct comparison of the interaction of CD55 with CD97 and EMR2 may be made because these proteins are of the same molecular weight and therefore produce the same refractive index change on binding similar amounts to the sensor surface. Fig. 7 shows the equilibrium response obtained flowing CD97 and EMR2 over the same CD55-coupled flow cell. Data for CD97 are obtained from two independent preparations of CD97 and show that the variation in response between different CD97 preparations is small by comparison to the difference in response observed when comparing CD97 and EMR2. If we assume that the maximal binding capacity of CD55 for EMR2 is the same as its capacity to bind CD97 we can use the initial slope, where the amount of EMR2 or CD97...
bound is approximately proportional to the concentration of protein applied, to provide an estimate of the affinity of EMR2 for CD55 relative to that of CD97. Inspection of the initial slopes of the data presented here suggests that the affinity of EMR2 for CD55 is at least an order of magnitude weaker than that of CD97 for CD55. This is equivalent to a change in the binding energy (using the equation $\Delta G = -RT\ln K_D$) from 5.5 kcal M$^{-1}$ to 4.2 kcal M$^{-1}$.

To assess the individual contribution of the three amino acid residues to CD55 binding, two assays were carried out using site-directed single residue mutants of CD97 and EMR2. Cell rosetting analysis of transfected CHO-K1 cells showed that the CD55-binding activities of the CD97-N33D, CD97-T59M and CD97-P71L mutants were all reduced compared with that of wild-type CD97 (EGF-1,2,5). Conversely, cells transfected with the EMR2-D36N-, EMR2-M62T-, or EMR2-L74P-expressing constructs displayed increased CD55 binding abilities (Fig. 8).

Cell surface protein expression in CHO-K1 cells was monitored by FACS and shown to be comparable for all proteins studied (see “Experimental Procedures”). Consistent with the quantitative data, fewer and smaller rosettes of erythrocytes were observed around the cells expressing mutant proteins (data not shown).

A second ligand binding assay using multimeric forms of soluble extracellular domains of CD97 and EMR2 proteins was also employed to analyze the binding properties of mutant proteins (Fig. 9). Biotinylated extracellular domains of CD97 and EMR2 were coupled to avidin-coated fluorescent microspheres to form the multimeric protein probes for use in a FACS-based assay system (Fig. 9a) (26). K562 human myelogenous leukemia cell line, expressing a homogenous cell surface CD55 expression pattern (data not shown; Ref. 27), was used as a source of CD55 in the assay. As expected, wild-type CD97 protein-microsphere complexes bound to K562 cells and showed a strong shift in fluorescence intensity (Fig. 9b). In contrast, wild-type EMR2 protein-microsphere complexes did not bind K562 cells. As previously demonstrated, the binding of CD97-microsphere complexes to K562 cells was found to be calcium-dependent and mediated by CD55 as the addition of EGTA without Mg$^{2+}$ (data not shown), with Mg$^{2+}$, or a blocking anti-CD55 mAb completely ablated the binding (Fig. 9, c...
In this study we have identified the molecular basis of the CD55-CD97 cell surface interaction using biological and biophysical methods. Binding is mediated exclusively by protein-protein interactions of the SCR and EGF module families located at the N terminus of each protein. Surface plasmon resonance studies of the EGF-SCR interaction show that it is characterized by a low affinity caused by a rapid off-rate. Because CD55 and CD97 are known to be expressed at high levels on the surface of cells (27–29), our data predict an in vivo interaction of high avidity. Prior work by others has demonstrated that cell-cell interactions mediated by proteins of the Ig superfamily are also characterized by multiple, low affinity interactions. Our results are therefore of general interest because they suggest this property may prove to be a widespread characteristic of cell-cell interactions mediated by a wide variety of protein folds; perhaps because fine titration of cell-cell binding affinity may be easily achieved by regulating expression of the interacting proteins on the cell surface. Interestingly the $K_D$ of the CD55-CD97 interaction is at least an order of magnitude weaker than the previously characterized interaction between CD55 and Echovirus 11 (22). The requirement of the virus-CD55 interaction to be of a higher affinity than the CD97-CD55 interaction probably reflects the fact that an icosahedral virus can, at most, present 60 binding sites for its receptor and must therefore have a reasonably high affinity for its receptor to achieve a sufficiently avid interaction. However, an activated leukocyte will have many more than 60 copies of CD97 presented on the cell surface, and a high avidity interaction may therefore be promoted by a protein-protein interaction of a much lower affinity.

Because both mammalian-expressed CD97 (immobilized CD97 bound to chip surface) and E. coli-derived material (when CD97 is present in the soluble phase) give the same value for $K_D$, glycosylation of CD97 is not involved in determining the specificity or affinity for CD55. There has been much debate about the role of glycosylation in determining the specificity and affinity of cell adhesion interactions (30, 31), and recent work has shown that for another family of EGF-containing proteins, the Notch family, control of the glycosylation state of the EGF domains by selective expression of the glycosylating enzyme is used to regulate the interactions of Notch with its ligands (32). However, prior work studying another adhesive interaction has shown that the interaction of CD2 with its ligand is glycosylation-independent (32). Our demonstration of the glycosylation independence of the CD97-CD55 interaction shows that CD2-ligand interactions are not an exception that “proves the rule,” and that glycosylation of extracellular domains is not necessarily required to modulate protein-protein interactions.

Although the cell-based binding assays employed in this study showed no detectable CD55-EMR2 interaction, SPR assays have detected a weak but specific interaction between CD55 and EMR2. It is possible that the much weaker CD55-EMR2 interaction has fallen beyond the detection limit of the cell-based assay systems, which are less sensitive than SPR. It would be reasonable to speculate that, given high enough levels of cell-surface CD55 proteins one would be able to detect the CD55-EMR2 interaction using the cell-based assay systems. Because both CD97 and EMR2 are predominantly expressed by granulocytes, monocytes, and macrophages, this provides a mechanism whereby the CD55 binding ability of these important immune cells could be regulated by the cell surface expression levels of a pair of closely related EGF-TM7 proteins.

The Ca$^{2+}$-dependence of the CD97-CD55 interaction indicates that the Ca$^{2+}$ binding sites within the EGF-2 and 5 domains of CD97 are crucial for intermolecular interactions. Structural data from a fibrillin-1 cbEGF pair have shown that Ca$^{2+}$ binding is required for the maintenance of interdomain rigidity (5, 6). As a consequence, tandem repeats of cbEGF domains or EGF-cbEGF domains with similar conservation of residues are predicted to form extended rod-like structures, which present specific protein surfaces for protein-protein interactions. Inspection of the sequences of EGF-cbEGF and cbEGF-chEGF pairs from CD97 and EMR2 suggest they are of the fibrillin-1 or class I type (5), because they have one residue between the last Cys residue of the N-terminal cbEGF and the first calcium binding residue of the C-terminal cbEGF (Fig. 5). In addition hydrophobic packing residues also implicated in maintaining the rod-like conformation of fibrillin-1 cbEGFs are conserved in CD97 and EMR2. Calcium binding to cbEGF domains is therefore probably critical in maintaining CD97-CD55 interaction by sustaining an overall rod-like structure of the three EGF domains.

The complete abrogation of the CD55/97 interaction in the absence of Ca$^{2+}$ (Figs. 5 and 9d) suggests that the protein surface on CD97 recognized by CD55 extends over the domain 1–2 boundary rather than being localized on a single domain, and this observation is confirmed by the locations of the amino acid residues that were mutated.
The amino acid sequence of the biotinylation site is also shown. a, CD97-fluorescent beads bound to K562 cells. b, CD97-EGF + Mg\(^{2+}\) abolishes the binding of CD97-fluorescent beads to K562 cells, whereas addition of EGTA + Ca\(^{2+}\) restores the binding. c and f, biotinylated site-directed mutants of EMR2 and CD97 show progressively increased and reduced CD55 binding activities, respectively.

The binding site for CD55 is likely to be located on the first two domains of CD97. Mapping of the three amino acid changes to CD97-CD55 interaction is Ca\(^{2+}\)-dependent. Introduction of EGTA or EGTA + Mg\(^{2+}\) abolishes the binding of CD97-fluorescent beads to K562 cells, whereas addition of EGTA + Ca\(^{2+}\) restores the binding. e and f, biotinylated site-directed mutants of EMR2 and CD97 show progressively increased and reduced CD55 binding activities, respectively.

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