Structural and Functional Characterization of a Novel T Cell Receptor Co-regulatory Protein Complex, CD97-CD55*†§

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CD97, the archetypical member of the EGF-TM7 protein family, is constitutively expressed on granulocytes and monocytes and rapidly up-regulated on T and B cells following activation. The key isoform of CD97 expressed on leukocytes binds the complement regulatory protein CD55 (also termed decay-accelerating factor). CD97 has been shown recently to mediate co-stimulation of T cells via CD55. Here, we demonstrate that blocking the interaction between CD55 on monocytes and CD97 on T cells leads to inhibition of proliferation and interferon-γ secretion. This implies that bidirectional interactions between CD97 and CD55 are involved in T cell regulation. Structural studies presented here reveal the molecular basis for this activity. We have solved the structure of EMR2, a very close homolog of CD97, using x-ray crystallography. NMR-based chemical shift mapping of the EMR2-CD55 interaction has allowed us to generate a model for the CD97-CD55 complex. The structure of the complex reveals that the T cell and complement regulatory activities of CD55 occur on opposite faces of the molecule. This suggests that CD55 might simultaneously regulate both the innate and adaptive immune responses, and we have shown that CD55 can still regulate complement when bound to CD97.

T cell activation depends on signaling by the T cell receptor upon binding of peptide-major histocompatibility complex (MHC) complexes displayed by antigen-presenting cells (APCs). However, this appears to be insufficient for the full development of T cell responses, and further stimulation is also required (1). Although many studies have implied that ligation of CD28 on T cells by B7 molecules (CD80/CD86) on APCs is responsible for co-stimulation of naïve T cells, it is now apparent that co-stimulation can also play a part in the regulation of effector T cells. Some molecules co-stimulate the T cell response, whereas others transduce a negative regulatory signal. It appears that the level of T cell activation can be finely tuned by a large number of T cell regulatory molecules with distinct and overlapping functions. Here, we report the identification and structural characterization of a novel receptor-ligand pair (CD97-CD55) that is important in T cell stimulation.

CD55 consists of four N-terminal short consensus repeat (SCR) domains that are expressed on many mammalian cell membrane surfaces via a heavily O-glycosylated spacer that is linked to a C-terminal glycosylphosphatidylinositol anchor. It is a member of the regulators of complement activation protein family (2), and its primary function is to inactivate the C3 convertases (3). The crystal structure of the four SCR domains reveals an extended rod-like arrangement (4).

CD97 is a member of the EGF-TM7 family of proteins, abundantly expressed in smooth muscle cells, macrophages, granulocytes, and dendritic cells and rapidly up-regulated in activated T and B cells (5, 6). It consists of between three to five consecutive epidermal growth factor (EGF) modules linked, via a mucin-like spacer domain, to a seven-span transmembrane (TM7) class B G-protein-coupled receptor (7, 8). EGF domains 2–5 are of the calcium-binding variety. The function of CD97 is not entirely understood, although it is required for neutrophil migration (9). The presence of a class B G-protein-coupled receptor suggests a signaling role. However, G-protein-mediated signaling by EGF-TM7 proteins still awaits functional demonstration. The identification of CD55 as a biological ligand for the three-EGF isoform of CD97 (EGF domains 1, 2, and 5) (10) demonstrates that CD97 can act as a cell adhesion molecule. The largest isoform, with five EGF domains, also binds chondroitin sulfate, which facilitates the interaction of CD97-expressing leukocytes with B cells (11). CD97 expression...
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is higher in carcinomas of different origin compared with corresponding normal cell types (12, 13). A close homolog of CD97 is EMR2, a chondroitin sulfate-binding protein (14) that is expressed on monocytes/macrophages and granulocytes (15). EMR2 is thought to play a role in the migration and adhesion of myeloid cells during cell differentiation, maturation, and activation (16). Of the 143 amino acid residues in the three-EGF isoform only two residues in domain 1 and one residue in domain 2 differ between EMR2 and CD97.

CD97-CD55 binding is mediated exclusively by protein-protein interactions of the EGF and SCR modules located at the N terminus of each protein (17). The interaction is Ca$^{2+}$-dependent, and the CD97 splice variant with the highest affinity (86 μM) comprises three EGF domains: 1, 2, and 5 (18). EMR2 also binds CD55, but with a $K_D$ at least an order of magnitude weaker than that of CD97 (17). The role of the CD97-CD55 interaction was unknown until now, although it was believed to be involved in adhesion and signaling within the inflammatory immune responses (19). The interaction has been implicated in the pathogenesis of multiple sclerosis (20) and rheumatoid arthritis (21), and we have recently demonstrated that CD97 interaction with CD55 on T cells can mediate co-stimulation in conjunction with CD3 engagement (22). Here, we present data demonstrating that CD97-CD55 acts as a receptor-ligand pair that is important in the regulation of T cell effector function, together with a structural model for the CD97-CD55 ligand pair.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents for Cellular Assays**—The following anti-CD55 antibodies were used: BRIC 216 (binds SCR domain 3), BRIC 110 (binds SCR domain 2), and BRIC 220 (binds SCR domain 1) (Blood Group Reference Laboratory, Bristol, UK) and 791T/36 (binds SCR domains 1 and 2). The anti-CD97 antibodies used were CLB 97/1 (EGF domain 1 of CD97) and CLB 97/3 (stalk region of CD97). T cell medium (TCM) comprised batch-tested RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 20 mM HEPES, 2 mM sodium pyruvate, 1:100 nonessential amino acids (Sigma), and 5% heat-inactivated human male AB serum (5%; First Link UK Ltd.).

**Fluorescence-activated Cell Sorter (FACS) Analysis**—Cells were harvested and immunolabeled with 1 μg of antibody/1 × 10$^6$ cells for 1 h at 4 °C. Cells were then washed, and fluorescein isothiocyanate-conjugated goat anti-mouse polyclonal serum (BD Biosciences) was used as the detecting antibody for 30 min at 4 °C prior to analysis using a FACScan (BD Biosciences). The adherent monocytes were immunolabeled with antibodies to CD55, CD14, MHC classes I and II, and CD3 (as an indicator of T cell contamination). T cell clones were immunolabeled with antibodies to CD3, CD4, CD8, CD16, CD45 RA/RO, and CD56 (DAKO Ltd., Cambridgeshire, UK) and CD97 (gift from Jörg Hamann).

**Generation of T Cell Clones**—Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood, separated by centrifugation over Histopaque 1077 (Sigma), washed, and resuspended in TCM. T cell clones were generated by seeding PBMCs at 2 × 10$^6$ cells/well on a 24-well plate for 7 days with 20 μg/ml peptide. The responding T cells were cloned by limiting dilution at 5, 1, and 0.5 T cells/well in 20-μl Terasaki wells using 1 × 10$^5$/ml irradiated autologous PBMCs as APCs, 50 units/ml recombinant IL (interleukin)-2, and 20 μg/ml peptide. Clones were expanded at 21-day intervals by re-stimulation with 5 × 10$^5$/ml irradiated allogeneic PBMCs, 50 units/ml IL-2, and 2 μg/ml phytohemagglutinin. Antigen-specific proliferation of clones was examined at least 10 days after re-stimulation with phytohemagglutinin. The specificity of clones was assessed by co-culture of 2–4 × 10$^4$ T cells with peptide and either 5 × 10$^5$ irradiated autologous PBMCs or 1 × 10$^5$ PBMCs adhered for 2 h before removal of non-adherent cells.

PBMCs (100 μl) were seeded at 1–2 × 10$^6$/cells/ml in a flat-bottomed 96-well plate (Nunc). The cells were incubated at 37 °C and 5% CO$_2$ for 1 h. Wells were washed three times in phosphate-buffered saline to remove any non-adherent cells, and the phosphate-buffered saline was replaced with 100 μl of TCM. T cells were washed twice in TCM, and 100 μl was added to the relevant wells at 5 × 10$^5$ cells/ml with peptide. Cultures were incubated at 37 °C for 3 days. $[^3]$HThymidine (0.5 μCi/well) was added 6 h prior to harvesting and counting on a Top-Count scintillation counter (BD Biosciences). Blocking assays were set up as described for the proliferation assays with the following modifications. Blocking antibodies were added to the relevant cells at 10–20 μg/ml for 1 h at room temperature. Cells were then washed twice in TCM prior to addition to the cultures.

**Cytokine Measurement**—Proliferation assays were set up as described above. Following 3 days of incubation and prior to thymidine pulsing, 100 μl of culture medium was removed and assayed for the presence of interferon-γ (IFN-γ) using a cytometric bead array (BD Biosciences) according to the manufacturer’s protocol. Briefly, cytokine capture beads were added to the samples or cytokine standards in flow cytometry tubes. These were vortexed, and antibody for fluorescence detection was added to each tube. The samples were incubated at room temperature for 3 h. Beads were pelleted by centrifugation, washed twice, and resuspended prior to reading with a FACScan. Standard curves were plotted from 10-point dilutions of cytokines, and data for samples were converted to picograms/ml cytokine.

**Protein Expression and Crystallization**—The three extracellular EGF domains (EGF domains 1, 2, and 5) of the CD55-binding splice variants of human CD97 (15,352 Da) and EMR2 (15,579 Da) were expressed in Escherichia coli using a His$_6$ tag-based inducible expression system as described previously (17). After in vitro refolding and purification, the proteins were lyophilized, reconstituted into water, and concentrated to 70.0 and 68.6 mg/ml for CD97 and EMR2, respectively (calculated extinction coefficient at 280 nm; 21,000 M$^{-1}$ cm$^{-1}$ computed from the amino acid composition on ExPASy server (us.expasy.org/tools/protparam.html)). Crystallization was performed as reported previously (23). CD97 failed to crystallize; however, EMR2 co-crystals were successfully grown in the presence of 0.1–0.175 M calcium acetate, 0.1–0.125 M barium chloride, and 0.1 M strontium chloride.

**X-ray Structure Determination and Refinement**—The native EMR2 crystals contain Ca$^{2+}$ and belong to space group P2$_1$,
with two molecules/asymmetric unit. The structure of EMR2 in complex with \( \text{Ba}^{2+} \) was solved by experimental phasing using the anomalous \( \text{Ba}^{2+} \) signal in data collected with radiation of wavelength 1.378 Å on beamline BM14 at the European Synchrotron Radiation Facility. The data were indexed and processed in space group \( \text{P2}_1,2 \), with the assumption of two molecules/asymmetric unit, as the unit cell of the \( \text{Ba}^{2+} \) complexed EMR2 was solved by experimental phasing using \( \text{Ba}^{2+} \) atoms in data collected with radiation of wavelength 0.934 Å on beamline ID14-1. The average SHARP figure of merit for acentric data was 0.34 in the resolution interval 50-2.6 Å, and the average anomalous phasing power of the long wavelength barium data set was 1.8 across the same resolution range. The residual phase ambiguity was resolved by solvent flattening using SOLOMON (25). The main chain was auto-built with ARP-WARP (26), and the side chains were docked manually in Xfit (27). The resulting model was refined by alternating refinement in BUSTER-TNT (28) with manual rebuilding in Xfit. After model building and initial refinement in \( \text{P2}_1,2 \), the detection of a clear pattern of systematic extinctions along the long axis suggested an increase in symmetry in the presence of \( \text{Ba}^{2+} \); the data were re-indexed and reprocessed; and the structures were refined in space group \( \text{P}2_1,2_1,2_1 \), with one molecule/asymmetric unit. The native EMR2 structure (in complex with \( \text{Ca}^{2+} \)) and the structure of the \( \text{Sr}^{2+} \) complex were then determined by molecular replacement using MOLREP, with \( \text{Ba}^{2+} \)-complexed EMR2 as the search model.

Refractive index statistics for all three models are presented in Table 1.

**NMR Data Collection and Assignment**—NMR samples were prepared from uniformly \( ^{15}\text{N} \)- or \( ^{13}\text{C},^{15}\text{N} \)-labeled lyophilized EMR2 in 100 mM deuterated Tris, 150 mM sodium chloride, and 5 mM calcium acetate (adjusted to pH 7.5). All NMR experiments were recorded at 25 °C on homebuilt Omega/GE spectrometers at proton frequencies of 500, 600, and 750 MHz. Backbone assignment was based on the procedure described by Grzesiek and Bax (29) using a double-labeled sample with a concentration of \( \sim 1 \text{ mm} \). Experiments used during the assignment were CBCA(CO)NH, HN(CO)CA, HNCA, \( ^{15}\text{N} \) HSQC-NOESY, \( ^{13}\text{C} \) HSQC-NOESY, and \( ^{13}\text{C},^{15}\text{N} \) HSQC. The collected NMR data were processed using Felix 2.3 (Biosym Inc.) and analyzed with SPARKY 3. Binding of CD55 to EMR2 was observed by titrating lyophilized unlabeled CD55 against \( \sim 2 \text{ mm} \) uniformly \( ^{15}\text{N} \)-labeled EMR2 in increments of 20% up to an equimolar ratio. Using lyophilized CD55 protein ensured that the buffer conditions remained constant throughout the titration.

**Docking**—Docking of the EMR2-CD55 complex was performed using HADDOCK 1.3 (31) with constraints based on the chemical shift perturbation data observed for EMR2 upon complex formation. The starting structures for the docking were chain A of EMR2 (in complex with \( \text{Ca}^{2+} \)) and SCR domains 1 and 2 of CD55 chain A (Protein Data Bank code 1OJV) (4). EMR2 residues that show a significant chemical shift perturbation upon complex formation and that have a high solubility were chain A of EMR2 (in complex with \( \text{Ca}^{2+} \)) and SCR domains 1 and 2 of CD55 chain A (Protein Data Bank code 1OJV) (4). EMR2 residues that show a significant chemical shift perturbation upon complex formation and that have a high solubility were chain A of EMR2 (in complex with \( \text{Ca}^{2+} \)) and SCR domains 1 and 2 of CD55 chain A (Protein Data Bank code 1OJV) (4).
vent accessibility in the free form of the protein (>50% relative accessibility as calculated with NACCESS (32)) were defined as "active." In the absence of any mapping data for CD55, all residues with a high solvent accessibility (>50%) were selected as "passive" residues. The resultant 15 active residues of EMR2 and 80 passive residues of CD55 were used to define ambiguous interaction restraints (AIRs) with a 3-Å distance. To maintain the position of the EMR2 Ca\(^{2+}\) ions, we also defined a set of unambiguous distance restraints, specifying the distance between each Ca\(^{2+}\) ion and its coordinating residues. Residues 1–4, 48–54, 77–83, 124–127, and 130–137 of EMR2 and residues 5–12, 40–45, 56–61, 67–74, 78–91, 98–109, and 114–125 of CD55 were defined as flexible. During the rigid body energy minimization, 2000 structures were calculated. At this stage, the main driving force comes from the AIRs and from van der Waals and electrostatic energy terms once the structures are within the non-bonded cutoff (8.5 Å). The 400 best solutions according to the intermolecular energy were used for the semiflexible annealing in torsion angle space. This was followed by a final refinement of the best 200 solutions with explicit modeling of hydration water molecules. Finally, the resultant structures were clustered using a 2-Å root mean square deviation (r.m.s.d.) based on the pairwise r.m.s.d. matrix after superposition on the backbone of EMR2. An identical HADDOCK procedure was then carried out with a model structure for CD97 (generated by substituting Asp\(^{12}\) with Asn, Met\(^{38}\) with Thr, and Leu\(^{40}\) with Pro) replacing the structure for EMR2. We also carried out an alternative docking procedure with EMR2 and CD55 using the program 3D-DOCK (33). We performed a global scan of translational and rotational space that generated 10,000 structures. We used the same NMR-derived restraints used with HADDOCK as distance filters (EMR2 active residues must lie within 10 Å of any part of CD55). The remaining structures were then compared with those generated by HADDOCK.

**Complement Assay**—The complement inhibition assays were carried out as described previously (40). Briefly, sheep erythrocytes were washed in diluent (0.1 \text{mM} \text{HEPES}, 0.15 \text{mM} \text{NaCl}, and 0.1% gelatin (pH 7.4)), resuspended and sensitized with rabbit hemolytic serum (Serotec) for 3 min, and lysis was determined by measuring the absorbance at 410 nm. Percent hemolysis was calculated from sensitized erythrocytes incubated with human serum, and the negative control used was sensitized erythrocytes in the absence of serum.

**RESULTS**

T Cell Clones Express High Levels of CD97—T cell clones were generated and characterized for expression of T cell markers, CD97, and cytokine profiles. Two clones are described here (Table 2), one characterized as CD4\(^{+}\) and the other CD8\(^{+}\), both of which showed high levels of expression of CD97 using two antibodies, one to the EGF-like domains (CLB 97/1) and one to the stalk region (CLB 97/3). Both clones were shown to produce predominantly a Th1 profile of cytokines in response to peptide presented on autologous APCs, with IFN-\(\gamma\) being the predominant cytokine (Table 2). The response of the clones presented here is representative of multiple CD4 and CD8 cell lines/clones.

As T cells are primarily regulated by APCs, including monocytes and dendritic cells (DCs), we examined the expression of CD55 on monocytes and DCs differentiated from monocytes with granulocyte/macrophage colony-stimulating factor and IL-4. The primary role of DCs is the regulation of naïve T cell responses, whereas monocytes are thought to be more important in the regulation of peripheral T cell activity. The absence of CD97 in naïve T cells and its early up-regulation on T cells following activation might indicate that CD97 has a role in

| **TABLE 2** Characterization of the T cell clones |
|-----------------------------------------------|
| **Marker** | **Mean linear fluorescence** |
| **CM clone** | **JR clone** |
| **Cytokine** | **Amount of cytokine** | **pg/ml** |
| CD3 | 450 | 400 |
| CD4 | 900 | 4 |
| CD8 | 7 | 1007 |
| CD56 | 8 | 76 |
| CD45 RO | 85 | 90 |
| CD16 | 6 | 5 |
| CD97(1) | 980 | 725 |
| CD97(3) | 850 | 745 |
| **Unlabeled** | **Resting monocytes** | **Activated monocytes** | **Activated DCs** |
| **Marker** | **Mean linear fluorescence** | **Mean linear fluorescence** | **Mean linear fluorescence** |
| CD55 | Unlabeled: 10 | 10 | 10 |
| 54 | 155 | 8 |
| CD1a | 54 | 480 | 1370 |
| CD14 | 1050 | 800 | 190 |
| CD80 | 28 | 410 | 380 |
| CD83 | 59 | ND | 120 |
| CD86 | 63 | 435 | ND |
| MHC I | 200 | 900 | 3000 |
| MHC II | 150 | 600 | 200 |

**TABLE 3** FACS analysis of monocytes and dendritic cells

PBMCs were prepared from Histopaque 1077 treatment of whole blood. Monocytes were obtained following 2 h of culture in tissue culture flasks. Non-adherent cells were removed, and adherent cells were washed. DCs were obtained by treating the fresh adherent monocytes for 5 days with granulocyte/macrophage colony-stimulating factor/IL-4. Both cell types were treated with IFN-\(\gamma\) for 24 h to activate them. The cells were then dislodged using a cell scraper, washed, and counted prior to antibody labeling for FACS analysis. ND, not determined.

| **Marker** | **Mean linear fluorescence** |
| **Resting monocytes** | **Activated monocytes** | **Activated DCs** |
| **CD55** | **CD1a** | **CD14** | **CD80** | **CD83** | **CD86** | **MHC I** | **MHC II** |
| Unlabeled | 10 | 10 | 10 | 8 | 155 | 480 | 1370 |
| CD1a | 54 | 480 | 1370 |
| CD14 | 1050 | 800 | 190 |
| CD80 | 28 | 410 | 380 |
| CD83 | 59 | ND | 120 |
| CD86 | 63 | 435 | ND |
| MHC I | 200 | 900 | 3000 |
| MHC II | 150 | 600 | 200 |
interacting with monocytes, but not DCs. Resting adherent monocytes were analyzed for the expression of a range of markers indicative of the monocyte/DC lineage (Table 3). The monocytes showed a characteristic CD14\textsuperscript{high}/CD1\textalpha\textsubscript{low} phenotype and expression of both MHC classes I and II and the co-stimulatory molecules CD80 and CD86. The monocytes were then subjected to stimulation with lipopolysaccharide/IFN-\gamma, which resulted in a significant increase in the expression of the activation markers CD1\textalpha, CD80, CD86, and MHC classes I and II, an activated phenotype. Similarly, CD55 expression consistently showed a 2–4-fold increase. Differentiation of the monocytes with granulocyte/macrophage colony-stimulating factor/IL-4 resulted in a characteristic change in phenotype indicative of DCs: CD1\textalpha\textsuperscript{high}, CD14\textsuperscript{low}, and MHC class I\textsuperscript{high}. However, there was a significant decrease in CD55 expression on these DCs to a level that was below the background (Fig. 1). This supports the hypothesis that CD97-CD55 interaction occurs primarily between effector T cells and monocytes/macrophages rather than DCs.

**Antibodies to CD55 and CD97 Inhibit T Cell Function**—CD97 was first identified as an early activation marker on T cells. To directly assess whether recognition of CD55 or CD97 has functional consequences, we used antibodies to CD55 and CD97 and measured their effects on T cell function. Pretreatment of monocytes with a range of antibodies recognizing SCR domain 1, 2, or 3 of CD55 resulted in a 50–70% inhibition of proliferation of both the CD4 and CD8 clones. The antibody directed against SCR domain 3 (BRIC 216) showed a 40–50% inhibition of proliferation. Antibodies mapping to SCR domain 1 (BRIC 220) and SCR domains 1 and 2 (791T/36) both inhibited proliferation by >70%. However, the antibody directed against SCR domain 2 (BRIC 110) did not inhibit T cell proliferation of either CD4 or CD8 cells (Fig. 2A). Similarly, isotype-matched control antibodies failed to have any influence on T cell function (data not shown).

Antibodies to CD97 were also used to test this interaction by pretreatment of T cells prior to addition to peptide-pulsed monocytes. These antibodies recognized the first EGF-like domain close to the N terminus of CD97 (CLB 97/1) and the stalk region closest to the membrane (CLB 97/3). Antibody CLB 97/1 was very effective in inhibiting T cell proliferation. However, antibody CLB 97/3, like the control antibody, had no effect on T cell functions (Fig. 2A). The effect was also time-dependent, as the addition of antibodies 2–3 h post-stimulation failed to inhibit proliferation (data not shown).

**Blocking the CD97-CD55 Interaction Inhibits Secretion of IFN-\gamma**—An important component of T cell effector function is secretion of the cytokine IFN-\gamma. This was measured alongside proliferation in the T cell assays. Supernatant from T cell assays

*FIGURE 1.* Adherent monocytes were matured to DCs for 5 days in granulocyte/macrophage colony-stimulating factor/IL-4. Both cell types were analyzed for CD55 expression by indirect staining using anti-CD55 antibody 791T/36 and fluorescein isothiocyanate-conjugated goat anti-mouse polyclonal serum. U represents monocytes treated with the secondary antibody alone. M represents 2-h adherent monocytes treated for 24 h with IFN-\gamma. D represents DCs treated with IFN-\gamma for 24 h.

*FIGURE 2.* T cell assays were set up using T cell clones, APCs and 1.0 (A) or 0.1 (B) \mu g/ml specific peptide. Antibodies to CD55 or CD97 (10 \mu g/ml) were used as described under “Experimental Procedures.” Cells were then washed prior to use. A, proliferation was measured after 3 days by 6-h thymidine incorporation and analyzed by scintillation counting. The results were calculated from quadruplicate wells (*, p < 0.01; **, p = 0.25) and are representative of at least three experiments. B, supernatants from 3-day cultures were collected and assayed for IFN-\gamma by a cytometric bead array. Concentrations were calculated from a 10-point standard curve of IFN-\gamma. All samples were assayed in quadruplicate (*, p < 0.002; **, p = 0.2).
was analyzed for the presence of IFN-γ, and the effect of inhibitory antibodies was measured (Fig. 2B). The data show that antibodies to both CD55 and CD97 inhibited not only T cell proliferation, but also IFN-γ secretion. The magnitude of inhibition was similar to that in the proliferation assays and was reproducible at different peptide concentrations and for CD4 and CD8 clones (data not shown). The notable exception was anti-CD97 antibody CLB 97/3 (maps to the stalk region), which had no effect on either T cell proliferation or IFN-γ secretion. This confirms previous observations that antibodies to the stalk region of CD97 do not prevent CD55 binding and that only antibodies to the EGF-like domains are able to block CD55 interactions with CD97.

Structure Determination—The structure of CD55 is known, but to date, no structural information for CD97 has been available to allow a structural understanding of this interaction. We therefore targeted the extracellular portion of the EGF domain 1/2/5 isoform for structure determination. To this end, EGF domains 1, 2, and 5 of CD97 and of EMR2 were expressed in E. coli, purified, and refolded (17). CD97 failed to crystallize, but crystals of EMR2 were produced in complex with Ca²⁺ (native), Ba²⁺, and Sr²⁺ as described previously (23). Data collected from the Ba²⁺-complexed EMR2 crystals on beamlines BM14 and ID14-1 at the European Synchrotron Radiation Facility allowed experimental phasing, and the structure was solved to 2.0 Å. This model was then used, together with data sets collected on beamline ID14-2 at the European Synchrotron Radiation Facility, to solve the structures of EMR2 complexed with Ca²⁺ to 2.4 Å and of EMR2 complexed with Sr²⁺ to 2.4 Å. Data collection and refinement statistics are shown in Table 1.

Structure of the EMR2 Isoform (EGF Domains 1, 2, and 5)—The asymmetric unit of the Ca²⁺ co-crystals consists of two nearly identical chains of EMR2. Ba²⁺ and Sr²⁺ co-crystals both comprise one EMR2 chain/asymmetric unit. Therefore, we have four independent models of the molecule in total. These reveal an extended and gently curving arrangement of three domains, with the metal ions situated in the interdomain junctions (Fig. 3A). The overall dimensions of the molecule are 84 × 35 × 29 Å. Each domain contains a major and minor region of double-stranded antiparallel β-sheet, stabilized by disulfide bonds in a [1–3, 2–4, 5–6] pattern as seen previously in almost all EGF-like structures. The length of the β-sheets varies between the domains, with those in domain 2 being the most extensive. A turn within domain 2 also contains a short α-helix, not seen in domains 1 and 5. The structures of the domains are constant in all crystallographically distinct models (r.m.s.d. < 1 Å). Individual comparisons of the domains with all other EGF domain structures revealed that domain 1 (implicated previously in CD55 recognition) is structurally distinct, there being no structural homologs (r.m.s.d. < 2 Å) detected within SCOP family g.3.11.1, which covers EGF-type modules. The closest homolog to domain 2 is the EGF domain of P-selectin (r.m.s.d. = 1.77 Å) (34), and the closest homolog to EMR2 domain 5 is the first EGF-like domain from human factor IX (r.m.s.d. = 1.95 Å) (35).

Fig. 3B shows an overlay of the four crystallographically different copies of the molecule superimposed by aligning domain 5, demonstrating that there is also very little variation in the structure at the domain interfaces and that EMR2 is a relatively rigid molecule. The divalent ions are probably largely responsible for this rigidity, as they are thought to restrict the conformational flexibility of the interdomain linkages in calcium-binding EGF pairs (36), resulting in resistance to proteolysis (37). Searching for structural homologs at the level of multiple EGF-like domains revealed no similar domain arrangements in the data base, probably due to the presence of longer linkers between the domains in EMR2.

Mapping the Ligand-binding Face—We used NMR chemical shift perturbation experiments to study the interaction between EMR2 and CD55. 1H,15N HSQC spectra were acquired while titrating 15N-labeled EMR2 against lyophilized unlabeled CD55 and revealed that a large number of EMR2 NH and HN nuclei are significantly perturbed upon interaction with CD55 (Fig. 3E). Any peaks that showed a >4-fold reduction in intensity at the 60% titration point were defined as being significantly perturbed. Details of the assignment data and the characteristics of the chemical shifts changes are presented in supplemental Table 1. Most of the peaks that correspond to residues affected by the CD55 titration decreased in intensity upon addition of CD55, with new peaks appearing with different chemical shift position. This is characteristic of an interaction that is in the slow exchange regime on the NMR time scale. A handful of peaks showed intermediate exchange characteristics, broadening at initial titration points and disappearing under saturating ligand conditions. Only a few peaks showed fast exchange characteristics. Given the weak affinity between EMR2 and CD55 (17), the observation of slow and intermediate exchange kinetics in the NMR tube was unexpected.

To identify residues with perturbed resonances, it was necessary to assign EMR2 in its unbound form. A set of triple-resonance experiments allowed assignment of 76% of the backbone N¹, HN, and Cα nuclei in EMR2 (calculated excluding the N-terminal residue and all prolines), which were fairly evenly distributed throughout the three domains. This, in turn, enabled us to assign >80% of the 1H,15N HSQC peaks that were significantly perturbed during the titration experiment. As shown in Fig. 3 (C and D), all the assigned amino acids that experienced significant backbone shifts upon CD55 binding are found within domains 1 and 2. None of the assigned residues in domain 5 were affected by CD55 binding. Residues implicated by the chemical shift perturbation experiment suggest a nearly contiguous surface on domains 1 and 2 (Fig. 3C). The binding surface could be even more contiguous than these data suggest, as two loop regions that are part of this surface could not be assigned and so could also be part of the binding site. On the side of the molecule opposite the implicated binding surface, the protein largely showed no chemical shift changes during the titration (Fig. 3D). On this face, only two significant regions showed changes, and these are sequences close to cysteine residues (for example, sequence Cys⁷⁰-Val⁷¹-Cys⁷² in domain 2) that are likely to experience small conformational changes as a result of being disulfide-linked to residues on the binding surface.

Docking the CD97/EMR2-CD55 Complex—A full understanding of the role of CD97-CD55 in T cell regulation requires a structure for the complex formed by the two proteins. We
FIGURE 3. A, secondary structure of EMR2, colored from blue at the N terminus to red at the C terminus. Disulfide bonds are shown in yellow, and the Ca\(^{2+}\) ions are displayed as purple spheres. B, main chain ribbon representations of the four copies of EMR2 superimposed on EGF domain 5. Ca\(^{2+}\) chain A is shown in dark blue, Ca\(^{2+}\) chain B in light blue, Ba\(^{2+}\) in green, and Sr\(^{2+}\) in red. C and D, surface representations of EMR2. C is rotated 180° around the vertical axis relative to D. Residues implicated by the chemical shift perturbation experiments are colored red. E, \(^1\)H,\(^{15}\)N HSQC spectra showing titration start (blue peaks) and end (red peaks) points acquired while adding unlabeled CD55 to \(^{15}\)N-labeled EMR2. Intermediate titration points were collected, but for clarity, only end points are shown.
generated a model for the complex between CD97 and CD55 using the high ambiguity-driven protein-protein docking approach, HADDOCK (31), which combines NMR chemical shift mapping information with in silico docking of the individual protein structures.

As the structure of CD97 remains unknown, we generated models for both the EMR2-CD55 complex (using the experimentally determined structures) and the CD97-CD55 complex (using a model for CD97 constructed by substituting the altered side chains without modification of the main chain conformation). The complexes generated were the same within the experimental errors associated with the methods and will therefore not be separately discussed. The EMR2 residues shown by NMR mapping to be involved in the interaction with CD55 were used to generate AIRs as described under “Experimental Procedures.” HADDOCK was used for the docking calculations with the structure of EMR2 in complex with Ca\(^{2+}\) (or the CD97 model) and the structure of CD55 SCR domains 1 and 2 as input. In light of previous work demonstrating that the interaction with CD97/EMR2 is mediated by the SCR domains located at the N terminus of CD55 (10, 17, 18) and the data from the NMR titration experiments, the decision to use only domains 1 and 2 of CD55 was made to reduce computational time. The calcium ions were explicitly included in the docking calculations.

Fig. 4A shows a plot of intermolecular energy (sum of intermolecular van der Waals, electrostatic, and AIR energy terms) for the 200 refined complex structures after water refinement as a function of their backbone r.m.s.d. from the lowest energy structure. After analysis, four main clusters were obtained. For each cluster, a representative structure was chosen, the lowest energy structure of those with the fewest AIR violations. For each of the representative structures, we used AREAIMOL (38) to analyze the change in solvent-accessible areas of EMR2 upon formation of the complex. One would expect that, if a primary amide experiences a change in surface exposure upon formation of the complex, it will also experience a change in chemical environment over the course of the NMR titration. Of the EMR2 primary amides with assigned resonances whose degree of surface exposure changes upon formation of the cluster 1 complex, 100% \((n = 3)\) also display an experimental chemical shift during the titration. This compares favorably with the representative structures for clusters 2–4. Of the buried EMR2 primary amides with assigned resonances, 80% \((n = 5)\) in the cluster 2 complex, 50% \((n = 2)\) in the cluster 3 complex, and 60% \((n = 5)\) in the cluster 4 complex are significantly perturbed during the titration. Based on these analyses, the representative structure from cluster 1 is likely to provide the best model of the EMR2-CD55 complex at present. In this complex model, 82% \((n = 22)\) of all buried residues either definitely shift (50%) or are unassigned and might shift (32%). Only 18% of the buried residues definitely do not shift, and all of these are adjacent to a shifting residue. As expected, HADDOCK analyses using the CD97 model also led to four clusters, where the lowest energy cluster contained complexes with essentially the same domain arrangements as the preferred EMR2-CD55 complex (r.m.s.d. = 1.2 Å).

An alternative docking program, 3D-DOCK (33), was also used to generate 10,000 structures of the complex formed between CD97/EMR2 and CD55 based on a global scan of translational and rotational space. These structures were then filtered using the chemical shift data to select complexes that were consistent with the experimental measurements. None of
Structural and Functional Characterization of CD97-CD55

In this study, we have provided the first functional evidence for monocyte-associated CD55 regulating T cell effector function through its interaction with CD97 on T cells. By blocking this interaction with antibodies to either CD55 or CD97, we detected a significant reduction in both proliferative function and secretion of IFN-γ. These findings identify a potential role for CD97-CD55 in T cell regulation. CD55 is expressed at a low level by most epithelial and endothelial cells, and its expression is increased up to 40 times that of normal tissue (42) by inflammatory signals. The modest affinity (86 μM) demonstrated for the CD97-CD55 interaction (17) may provide a mechanism by which CD55 levels on normal cells have little or no effect on T cells. However, when overexpressed, a level may be reached where the avidity effect on the T cells increases to promote changes in effector functions. We have previously defined CD55 as a co-stimulator of effector T cells and demonstrated that these effects can be mediated by CD97 (22). However, no direct effect of antibodies to either CD55 or CD97 has been seen when added to cells alone. Similarly, addition of these antibodies to T cell cultures 2–3 h post-stimulation failed to inhibit proliferative responses (data not shown). This indicates that early interaction of CD55 with CD97 may be required to stabilize T cell receptor-MHC engagement, enhancing T cell function as is seen with interaction of LFA-1 with ICAM-1 (intercellular adhesion molecule 1). However, this does not exclude the possibility of direct signaling via CD97. Further investigations into the role of CD55 and CD97 will address their function as a co-stimulatory receptor pair and may identify new potential therapeutic approaches.

Our structural data reveal that both molecules exist in an extended form (the estimated height of each molecule above the cell membrane being ~300 Å), with the N-terminal (interacting) domains being presented at or near the surface of the mass of molecules presented on the cell surface. This implies a role for this binding event at an early stage of monocyte-T cell interactions. The structural data also suggest that formation of the complex does not involve dramatic rearrangements in CD97, so how is signaling transmitted from the extracellular contact to the cytoplasm? Presumably, the fact that CD55 is concentrated in lipid rafts (43) and those molecules with which it interacts are therefore likely to become clustered is of importance in understanding signaling. Clustering of receptors on the cell surface is a common theme in generation of intracellular signals (44), although the evidence that antibody ligation of CD97 does not have the same functional consequences as CD55 binding implies subtle requirements for particular levels of clustering (or for other stimuli) to generate a productive interaction.
The observation that CD55 can simultaneously act to stimulate T cell effector function and also to promote decay acceleration adds to the complexity of interactions that must be integrated at the cell surface. Although it is obviously desirable that the monocytes promoting proliferation are protected against the innate immune system, the ability of a single protein to regulate both innate and adaptive immunity simultaneously reminds us that any compartmentalization of these two arms of defense does not reflect the level of integration that exists in vivo.

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