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The retinal specific CD147 Ig0 domain: from molecular structure to biological activity

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Abstract

CD147 is a type I transmembrane protein that is involved in inflammatory diseases, cancer progression, and multiple human pathogens utilize CD147 for efficient infection. In several cancers, CD147 expression is so high that it is now used as a prognostic marker. The two primary isoforms of CD147 that are related to cancer progression have been identified, differing in their number of immunoglobulin (Ig)-like domains. These include CD147 Ig1-Ig2 that is ubiquitously expressed in most tissues and CD147 Ig0-Ig1-Ig2 that is retinal specific and implicated in retinoblastoma. However, little is known in regard to the retinal specific CD147 Ig0 domain despite its potential role in retinoblastoma. We present the first crystal structure of the human CD147 Ig0 domain and show that the CD147 Ig0 domain is a crystallographic dimer with an I-type domain structure, which is maintained in solution. Furthermore, we have utilized our structural data together with mutagenesis to probe the biological activity of CD147-containing proteins both with and without the CD147 Ig0 domain within several model cell lines. Our findings reveal that the CD147 Ig0 domain is a potent stimulator of interleukin-6 and suggest that the CD147 Ig0 domain has its own receptor distinct from that of the other CD147 Ig-like domains, CD147 Ig1-Ig2. Finally, we show that the CD147 Ig0 dimer is the functional unit required for activity and can be disrupted by a single point mutation.

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Accession Numbers

Coordinates and structure factors for the CD147 Ig0 C67M have been deposited in the Protein Data Bank with accession number 3QQN.

Coordinates and structure factors for the CD147 Ig0 wild-type have been deposited in the Protein Data Bank with accession number 3QR2.

The backbone resonances have been deposited in the Biological Magnetic Resonance Data Bank with accession number 17474.

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Keywords

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Introduction

CD147 (also known as Extracellular Matrix Metalloproteinase Inducer or EMMPRIN) is a type I transmembrane protein that is expressed in nearly all cells including platelets and fibroblasts [1]. Although transmembrane CD147 has been proposed to act as a receptor for several extracellular proteins such as the cyclophilin class of enzymes [2], CD147 also exhibits ligand activity. This ligand activity leads to CD147-mediated stimulation of multiple protein families and is thought to underlie the progression of many diseases such as rheumatoid arthritis and most cancers including retinoblastoma [3,4]. Additionally, CD147 upregulates its own expression creating a positive feedback loop which culminates in upregulated levels of MMPs, cytokines and CD147 itself. Thus, understanding the biology of CD147 is critical for understanding its role in the progression of multiple diseases, potentially revealing targets for therapeutic intervention such as CD147 itself.

Two isoforms of CD147 are expressed in the human body; a ubiquitously expressed form and a retinal specific form, which differ in the number of immunoglobulin-like (Ig-like) domains comprising the extracellular region of the proteins (Fig. 1). Both of these forms comprise an extracellular region, a transmembrane domain and an intracellular cytoplasmic tail. The predominant, ubiquitous form, called CD147 Ig1-Ig2, has two Ig-like domains in the extracellular region (Fig. 1a). The retinal specific CD147 Ig0-Ig1-Ig2 contains an additional Ig-like domain that is distal to the membrane (Fig. 1b) and has remained poorly characterized. Both isoforms are involved in normal development. For example, CD147 Ig1-Ig2 is involved in spermatogenesis [3] while CD147 Ig0-Ig1-Ig2 is involved in normal retinal development [5]. Others and we have shown that the CD147 Ig1-Ig2 isoform stimulates expression of matrix metalloproteinases (MMPs) and pro-inflammatory cytokines [6,7]. Likewise, upregulated expression levels of the CD147 Ig0-Ig1-Ig2 are correlated to increased MMP secretion [8]. Thus, while the two CD147 isoforms differ in their number of Ig-like domains, the overall functions of both isoforms may overlap in that they both appear to act as ligands by directly stimulating the secretion of multiple proteins.

Although CD147 regulation of MMPs and cytokines is critical for normal development, deregulation of both CD147 isoforms is thought to drive cancer progression through subsequent deregulation of downstream proteins. While such downstream effects have been well characterized for the CD147 Ig1-Ig2 isoform, less is known in regard to direct effects of the retinal specific CD147 Ig0-Ig1-Ig2 isoform that may drive retinoblastoma [8]. Since retinoblastoma is associated with both a poor prognosis for adults and pediatric retinoblastoma comprises over 10% of pediatric cancers that develop within the first year of life, novel targets such as CD147 may provide therapeutic targets for the amelioration of disease progression. Thus, the focus of this report concerns the retinal specific CD147 Ig0-Ig1-Ig2 isoform and especially the retinal specific CD147 Ig0 domain itself.

Several novel features distinguish the CD147 Ig0 domain from the other CD147 Ig-like domains. For example, the Ig0 domain does not contain any N-linked glycosylation sites as is often observed for the CD147 Ig1 and Ig2 domains [9,10]. In fact, the only biophysical study thus far has been on the murine CD147 Ig0 homologue that had indicated no post-translational modifications [9]. This report also indicated that the murine CD147 Ig0 domain is a dimer unlike that of the other domains, however, this has yet to be confirmed for the human homologue studied here. Furthermore, the CD147 Ig0 domain has three cysteines

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while the other two CD147 Ig-like domains each comprise two cysteines that form a single disulfide bond within each domain [7,11]. Therefore, a disulfide bond could be formed between two of the cysteine residues within the CD147 Ig0 domain leaving the third cysteine free or poised to form an intermolecular disulfide. A free cysteine is a relatively rare feature for an extracellularly exposed protein and may point to an important biological role for this cysteine [12]. However, thus far there have been no reported studies addressing such activity and a comprehensive analysis of the structure and function of the CD147 Ig0 domain has not been reported.

In this study, we have expressed and purified the human CD147 Ig0 domain both alone and in the context of the entire CD147 Ig0-Ig1-Ig2 ectodomain in order to characterize these proteins from their molecular details to their biological activity. We have solved the first crystal structure of the human CD147 Ig0 domain revealing both a dimeric interaction that persists in solution as well as a freely exposed cysteine residue. Biologically, we have discovered that the CD147 Ig0 domain is a potent stimulator of interleukin-6 (IL-6) secretion in multiple cell lines. By combining our biophysical data with biological assays, we have identified the CD147 Ig0 dimer as the functional unit required for IL-6 secretion and determined that the observed free cysteine is a major contributor to such biological activity. Since CD147 and particularly the CD147 Ig0 domain are implicated in retinoblastoma, our data indicate that CD147 may contribute to cancer progression by stimulating the secretion of proteins such as IL-6.

Results

The crystal structure of the CD147 Ig0 domain

We have solved the crystal structures of both the wild-type CD147 Ig0 domain to 2.4 Å (Fig. 2), and a mutant protein to 2.3 Å resolution (Fig. S1). The CD147 Ig0 domain is a crystallographic dimer in both structures. Based on sequence alignment to other known Ig-like domains, a single disulfide within CD147 Ig0 was predicted to include Cys44-Cys108 with Cys67 left as a potentially free cysteine. Thus, to avoid potential intermolecular cross-linking during the purification process the structure of the mutant CD147 Ig0 Cys67→Met (i.e. CD147 Ig0 C67M) was first elucidated (Fig. S1) and later found to be identical to that of the wild-type protein (Fig. 2). The CD147 Ig0 C67M mutant was initially Selenomethionine labeled in order to solve its structure using the three wavelength MAD method (Fig. S1, Table S1). Using this structure, the crystal structure of the wild-type protein was then solved using molecular replacement (Fig. 2, Table 1). Cys67 is positioned in a loop region distal to the dimer interface and thus does not interfere with the structural integrity of CD147 Ig0 (Fig. 2b). This is further shown by the fact that the CD147 Ig0 wild-type and the mutant CD147 Ig0 C67M are identical with an overall RMSD of all Cα atoms of 0.2 Å (Fig. S1).

The CD147 Ig0 domain fold is an intermediate type Ig-like domain fold as defined by Harpaz and Chothia [13]. Each domain consists of anti-parallel β-strands forming two β-sheets as shown in (Fig. 2a). Strands A, B, E and D form one β-sheet while strands A', G, F, C and C' form the second β-sheet. The C' strand is short and there are 64 residues between the two Cys residues that form the disulfide bond (Fig. 2c). A single molecule of CD147 Ig0 is comprised of 9 β-strands (color coded in Fig. 2a), and 2 α-helices (white in Fig. 2a) with the N- and C-termini positioned at the dimer interface. Interestingly, one of the closest structurally related Ig-like domains to the CD147 Ig0 domain is that of the CD147 Ig2 domain and is further described in "Discussion". The single predicted disulfide bond of Cys44-Cys108 was also confirmed in our X-ray crystal structures (Fig. 2c). Since the structures of CD147 Ig0 C67M and CD147 Ig0 wild-type are virtually identical (i.e. only 0.2 Å), Cys67 is not engaged in an intermolecular disulfide interaction and is therefore a free

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cysteine. The closest distance between the two surface exposed Cys67 residues within the CD147 Ig0 dimer is ~15 Å (Fig. 2b), suggesting that only large conformational changes could result in disulfide bond formation. However, no CD147 Ig0 wild-type dimer is observed on a non-reducing SDS PAGE denaturing gel even over the course of weeks (data not shown). Thus, Cys67 is free and surface exposed, leading us to probe its importance in biological activity (see later section below).

The crystallographic dimer is retained in solution and a single mutation can disrupt dimer formation

Unlike the Ig1 and Ig2 domains that we have previously shown to be monomeric even at high millimolar concentrations [7], here we show that the CD147 Ig0 domain is a dimer in solution. Analytical size exclusion chromatography was used to assess whether the CD147 Ig0 dimer is retained in solution. Using a Superdex-12 analytical column, both the CD147 Ig0 wild-type and the CD147 Ig0 C67M constructs migrate as dimers with calculated molecular weights of approximately 30 kDa (Fig. 3a). Our analytical size exclusion chromatography data is consistent with previous reports indicating that the murine CD147 Ig0-Ig1-Ig2 is detected as a dimer [9]. Negligible differences in the elution times were observed for a broad concentration range of 400 μM to as low as 20 μM, suggesting that the self-association affinity is tighter than the lowest concentration used (Fig. 3a, insets). In other words, the dissociation constant is tighter than 20 μM. Thus, the observed CD147 Ig0 dimer in the asymmetric unit within each X-ray crystal structure also forms a stable dimer in solution.

Residues potentially important for dimer formation were first identified from our X-ray crystal structures and then assessed in solution by analytical size exclusion. Two potential intermolecular electrostatic interactions with bond distances of <3.5Å are observed and include Arg130-Gln132 (Fig. 3b) and Glu59-Lys126 (Fig. 3d). Based on the similarity of the CD147 Ig0 domain to that of the CD147 Ig2 domain, Gln132 and Lys126 were mutated to similar residues corresponding to those of the CD147 Ig2 domain that is a monomer. Thus, we purified CD147 Ig0 Gln132→Leu (CD147 Ig0 Q132L) and CD147 Ig0 Lys126→Ala (CD147 Ig0 K126A) and probed the integrity of the dimer by analytical size exclusion. CD147 Ig0 K126A migrated only slightly slower than the intact wild-type dimer (Fig. 3e), while a shift to a significantly lower molecular weight to that expected of the monomer was observed for CD147 Ig0 Q132L (Fig. 3c). This suggests that the K126A mutation only slightly weakens the CD147 Ig0 dimer interaction while the Q132L mutation significantly abrogates dimer formation. Specifically, a total of four intermolecular hydrogen bonds are formed between Arg130 and Gln132, two per each residue at the CD147 Ig0 dimer interaction surface. The Arg130 backbone NH and Gln132 side chain CO form one of the hydrogen bonds while Arg130 backbone CO forms an additional hydrogen bond with the Gln132 backbone NH (Fig. 3b). Thus, the stability of the CD147 Ig0 dimer is only partially stabilized by the intermolecular interaction of Glu59-Lys126 while our data suggest that Arg130-Gln132 contributes significantly to the stability of the dimer. The fact that the CD147 Ig0 dimer can be abrogated by a single point mutation of CD147 Ig0 Q132L allows us to compare the activity of this monomeric mutant to that of the intact dimer.

Solution characterization of CD147 Ig0 at atomic resolution

We used Nuclear Magnetic Resonance (NMR) methods to further characterize the solution behavior of the CD147 Ig0 domain and compare the atomic resolution details to that found within the crystal. The dispersive nature of the resonances within the 2D ^{15}N- Heteronuclear Single Quantum Coherence (HSQC) spectrum is indicative of a well-folded structure in solution (Fig. 4a). Only two stretches could not be unambiguously assigned, which include residues 80–81 and 120–124. Interestingly, the latter stretch of residues comprises a central

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proline (Pro123) that may undergo isomerization and thereby lead to the loss of signals due to intermediate chemical exchange (Fig. 4b, arrow in left dimer).

Since side chain chemical shifts are highly sensitive to secondary structure, we used such information to calculate the predicted secondary structure of the CD147 Ig0 C67M domain in solution (Fig. 4b). Using the program Chemical Shift Index (CSI), residues were assigned to a α–helix, β–sheet or a random coil. β–strands predicted by CSI are largely consistent with the crystal structure of CD147 Ig0 (Fig. 4b). However, CSI also predicts that several loop regions such as residues 66–71 exhibit β-strand propensity suggesting that all 6 of these residues sample more of an extended conformation in solution than that observed in the crystal where only 2 residues are part of the β-strand (Fig. 4b, arrow in right dimer). While the two short helices observed in the crystal structure, which include 54–57 and 80–83, are not predicted by CSI, a comparison of the raw Cα chemical shifts to that of a random coil do indicate that both regions exhibit α–helical propensity in solution. For the crystal, these secondary structure elements may be stabilized. For example, for residues 54–57 the side chain of Asp57 is in close proximity to the amide of Tyr65 in the neighboring asymmetric unit, suggesting that an intermolecular hydrogen bond may be formed only due to crystal packing. Thus, the NMR-derived chemical shift propensities complement our crystal structure data in that most secondary structural elements overlap well between the two methods with only subtle variations.

The distinctive advantage of NMR over other techniques is that dynamics information can be obtained at atomic resolution and thus, both R1 (longitudinal) and R2 (transverse) relaxation rate data were collected to assess the dynamics of CD147 Ig0 C67M (Fig. 4c). Increased R1 relaxation rates are indicative of localized motion within the pico-nanosecond (ps-ns) timescale while R2 relaxation rates have additional contributions from the micro-millisecond timescale (μs-ms). However, we note that the standard pulse sequence utilized here comprises an approximately 500 Hz refocusing pulse during the R2 relaxation period, masking any motions slower than this (i.e. slower than 2 ms). R1 and R2 relaxation rates for 95 and 97 residues, respectively, of CD147 Ig0 C67M were accurately measured with an average R1 of 0.65±0.08 Hz and R2 of 25.2±2.4 Hz (Fig. 4c, d, e). For R1 relaxation rates, only several regions of the protein exhibit rates greater than one standard deviation above the average suggesting that most of the loops are relatively ordered in solution (Fig. 4d). For example, loop residues 66–71 that are predicted in solution to exhibit β-strand propensity from CSI calculations above are also well ordered as shown by their R1 relaxation rates that are similar to the average R1 relaxation rate. This suggests that residues within 66–71 stretch form a much more ordered secondary structure in solution than would be suggested by the X-ray crystal structure. For R2 relaxation rates, multiple residues on different structural elements exhibit rates greater than one standard deviation above the average (Fig. 4e). Interestingly, despite being on different structural elements these residues are adjacent to each other and appear to form a dynamic network within the μs-ms timescale. Since flexible regions of proteins are often involved in function, our initial data here may provide future candidate residues to evaluate through combined mutagenic/function studies.

All three independently folded Ig-like domains do not interact

While we have previously shown that each Ig-like domain within the CD147 Ig1-Ig2 isoform neither self-associate nor interact with each other [7], here we show that all three Ig-like domains within the CD147 Ig0-Ig1-Ig2 isoform do not interact with each other. NMR solution experiments can be used as particularly sensitive markers for protein interactions and offer an obvious means for monitoring interactions that exhibit affinities on the order of the concentrations utilized (micromolar to millimolar concentrations). Thus, we used NMR to probe for inter-domain interactions within CD147 via two different methods.

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The two approaches utilized here to probe inter-domain interactions both employed comparative analyses of ^{15}N-HSQC spectra. Our first experimental approach was to probe potential conformational changes between the entire ectodomain of CD147 Ig0-Ig1-Ig2 to either CD147 Ig0 or CD147 Ig1-Ig2. To this end, we engineered the ectodomain of CD147 Ig0-Ig1-Ig2 described in Materials and Methods to compare with both the CD147 Ig0 domain studied here as well as our previously characterized ectodomain of CD147 Ig1-Ig2 [7]. The majority of resonances from the individual constructs of CD147 Ig1-Ig2 and CD147 Ig0 overlap well with their counterparts in CD147 Ig0-Ig1-Ig2 indicating that there are no stable inter-domain interactions formed (Fig. 5a,b). Many resonances belonging to the CD147 Ig0 domain in the context of the full CD147 Ig0-Ig1-Ig2 construct are weak likely due to the slower tumbling time of the Ig0 dimer relative to the Ig1 and Ig2 monomers. However, most of these resonances can be observed at lower contours (Fig. 5b, right). The second approach employed a straightforward titration of individual constructs where the resonances of one labeled protein were monitored during the addition of another (i.e. chemical shift perturbation mapping). Specifically, using 15N-labeled CD147 Ig0, negligible chemical shift changes were observed upon the addition of up to 250 μM unlabeled CD147 Ig1-Ig2, Fig. S2. Since amide chemical shifts are especially sensitive probes to even subtle changes in the chemical environment that would be expected to occur if these domains remotely interact [14,15], the data presented here suggest that the individual Ig-like domains do not interact with one another. However, it is possible that there are unknown mediators of such interactions that could provide scaffolds for indirect interactions.

CD147 Ig0 is a potent stimulator of interleukin-6 secretion

Enzyme-Linked Immunosorbent Assay (ELISA) was used to assess the biological activity of CD147 constructs and to begin to elucidate the specific functions of the CD147 Ig0 domain as well as its functional unit (i.e. monomer versus dimer). Since the predominant CD147 Ig1-Ig2 isoform stimulates the secretion of many cytokines [7,16], we reasoned that the CD147 Ig0 domain may exhibit a similar activity. Specifically, we hypothesized that the CD147 Ig0 domain directly simulates interleukin-6 (IL-6), since IL-6 drives intraocular tumors [17] and the CD147 Ig0-Ig1-Ig2 isoform is upregulated in retinoblastoma [8]. We assessed the level of IL-6 secretion upon CD147 stimulation in two model cell lines, which included HEK293 and THP-1 cells. THP-1 monocytes are a particularly relevant model cell line to use since monocytes interact with the retinal epithelial cells [18]. Our biophysical and biochemical characterization of CD147 Ig0 and several mutants along with the full CD147 Ig0-Ig1-Ig2 ectodomain allows us to begin exploring the functions of CD147 Ig0 with these cell lines.

We first monitored the response of HEK293 cells with stimulation of our recombinantly expressed CD147 proteins (Fig. 6a). The CD147 Ig0 domain alone results in secretion of IL-6 in a dose dependent manner (Fig. 6a, purple). Since the activity of the CD147 Ig0 domain is comparable to that of the CD147 Ig0-Ig1-Ig2 domain, there is no significant contribution from the Ig1-Ig2 domains (Fig. 6a, purple, red, and cyan). Our data therefore suggests that the CD147 Ig0 domain has a specific receptor. In fact, CD147 Ig1-Ig2 activity is only detectable at 25 μM, which is 5-fold higher than that of CD147 Ig0 containing constructs, suggesting that CD147 Ig1-Ig2 has a lower affinity receptor distinct from the CD147 Ig0 domain. Next, we assessed the difference in the biological activity of the dimer versus the monomer form of the CD147 Ig0 protein. We used the CD147 Ig0 Q132L mutant to probe for the activity of the monomer form of the protein having shown that a mutation at this site results in the abrogation of the dimer. Clearly, abrogation of the CD147 Ig0 dimer results in a complete loss of activity even at the highest CD147 Ig0 Q132L concentration (Fig 6a, green versus purple), indicating that the functional unit of CD147 Ig0 is the dimeric form. Finally, we evaluated the role of the free cysteine, Cys67, in CD147 Ig0 activity, since the presence of such a non disulfide-bonded cysteine residue is relatively rare in the

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extracellular environment. To this end, we used the CD147 Ig0 C67M mutant, which resulted in a marked reduction in IL-6 secretion (Fig. 6a, yellow). Thus, our results indicate that the free cysteine in CD147 Ig0 is important for the activity of the protein. In summary, our data for HEK293 cells indicate that the CD147 Ig0 domain has its own receptor distinct from the other Ig-like domains and that both the dimer and the free cysteine are important for function.

A similar trend was observed in THP-1 monocytes as described above for HEK293 cells for most of the CD147 constructs tested (Fig. 6b). The major distinction between HEK293 cells and the THP-1 monocytes is that there appears to be a synergistic effect of the CD147 Ig1-Ig2 ectodomain to that of the CD147 Ig0 domain. Specifically, the activity of the CD147 Ig0-Ig1-Ig2 ectodomain is higher than that of the CD147 Ig0 domain alone at the equivalent concentrations used (Fig. 6b, red versus purple). The activity is synergistic because stimulation by the individual CD147 Ig0 and CD147 Ig1-Ig2 constructs does not add up to that of the full CD147 Ig0-Ig1-Ig2 ectodomain (Fig. 6b, purple and cyan versus red). Thus, the CD147 Ig0 domain is a potent stimulator of IL-6 secretion in multiple cell lines that include monocytes.

Discussion

Using a multidisciplinary approach, we have characterized the CD147 Ig0 domain biophysically, biochemically and biologically. Unlike the widely expressed CD147 Ig1-Ig2 isoform that has been extensively studied [19], the Ig0 domain within the retinal specific CD147 Ig0-Ig1-Ig2 isoform has remained poorly characterized. Considering that CD147 Ig0-Ig1-Ig2 has been implicated in retinoblastoma [8], characterizing the CD147 Ig0 domain may provide a foundation for therapeutically blocking its activity during disease progression. To this end, here we have solved the structure of the CD147 Ig0 domain, characterized the solution behavior and biological activity of the CD147 Ig0 domain alone and in the context of the entire CD147 Ig0-Ig1-Ig2 isoform.

Biophysical studies of CD147 Ig0

Our structural studies of the CD147 Ig0 domain have revealed a classical intermediate Ig-like fold with several fundamental aspects that differentiate it from most other Ig-like domains, including that of both the CD147 Ig1 and Ig2 domain (Fig. 2). First, the CD147 Ig0 domain is a dimer unlike the CD147 Ig1 and Ig2 domains, which are both monomers, and dimerization is unlike that of other Ig-like family members previously characterized. For example, our structural studies suggested that Arg130 and Gln132 are the two most important residues responsible for dimer formation and interact with each other across the dimer interface. Consistent with this structural analysis, disruption of this interaction with the mutant CD147 Ig0 Q132L, abrogates dimer formation as probed by analytical size-exclusion (Fig. 3). Dimerization has also been observed for the murine homologue of CD147 Ig0 [9], which is likely formed by the same two conserved residues (i.e. Arg130 and Gln132). Second, there is a free cysteine residue within the CD147 Ig0 domain (Cys67). This was shown by a comparative structural analysis of both the CD147 Ig0 wild-type protein and the CD147 Ig0 C67M mutant (Fig. S1). Specifically, since the RMSD of the Cα atoms of these two structures is only 0.2 Å, our studies indicate that Cys67 is exposed on the CD147 Ig0 surface and too far away from itself in the neighboring subunit to form a disulfide bond. Finally, NMR relaxation experiments highlight several important dynamic features of the CD147 Ig0 dimer. For example, there is a network of residues that exhibit μs-ms timescale motions (Fig. 4e). Since flexible regions of proteins are often important for function [20], such data may offer insight into future studies aimed at assessing other regions of CD147 Ig0 that are important for activity. In summary, the CD147 Ig0 domain is a dimer and is among a small subset of proteins that exhibit a free cysteine [12].

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NMR studies conducted here have also revealed that none of the CD147 Ig-like domains interact with one-another and therefore dimerization of the entire CD147 Ig0-Ig1-Ig2 isoform is mediated solely by the CD147 Ig0 domain. Titration of unlabelled CD147 Ig1-Ig2 to ^{15}N-labeled CD147 Ig0 did not result in any chemical shift perturbations and was an indication that these domains do not interact (Fig. S2). Consistent with these results, ^{15}N - HSQC spectra superposition of the CD147 Ig0 alone and the CD147 Ig1-Ig2 ectodomain with that of the entire CD147 Ig0-Ig1-Ig2 ectodomain further illustrates a lack of domain-domain interactions. Interestingly, the protein neuroplastin that is closely related to CD147 also exhibits two similar isoforms with either two or three extracellular Ig-like domains that apparently act similarly to CD147 (Fig. 7a). Specifically, the Ig-like domains of neuroplastin are also thought to lack any specific inter-domain interactions and dimerization is only observed in the presence of the first Ig-like domain [21]. Dimerization in the first Ig-like domain of neuroplastin is likely mediated by the same two residues that we observe for Ig0 (Arg130 and Gln132 in CD147 Ig0), since these are conserved. Thus, our biophysical data presented here may have wider implications for how other structurally uncharacterized family members behave.

Biological studies of CD147 Ig0

CD147 is a type I transmembrane protein that serves multiple roles, including the transport of monocarboxylate transporters to the membrane [22], acting as the receptor for extracellular cyclophilins [19], and direct ligand activity studied here. In fact, CD147 is amongst a unique set of proteins integrally involved in cancer that are both expressed on the cell surface and released in microvesicles whereby these proteins can act as ligands to induce signaling events in distal cells [23]. While microvesicle-associated CD147 has been shown to comprise the ubiquitously expressed CD147 Ig1-Ig2 isoform, the CD147 Ig0-Ig1-Ig2 isoform has been shown to be extracellular as well [24]. Thus, both CD147 isoforms may exhibit receptor functions in the context of the cell but also exhibit ligand activity both attached to cells and released from cells. Such ligand activity may be particularly important in the stimulation of proteins involved in disease progression and this may underlie the role of CD147 in tumorgenesis and metastasis. Here, we have shown that CD147 Ig0 either alone or in the context of the full CD147 Ig0-Ig1-Ig2 ectodomain acts as a ligand to stimulate the secretion of IL-6.

Our biological activity data show that CD147 Ig0 is a potent stimulator of IL-6 and we have identified both the dimer as the functional unit and a specific residue involved in activity (Fig. 2, 3 and 6). Specifically, compared to the wild-type protein, the CD147 Ig0 Q132L mutant that is a monomer exhibits no activity even at much higher concentrations. Furthermore, we show that the free Cys67 is important for the activity of CD147 Ig0 as the secretion of IL-6 is significantly reduced with the introduction of the mutation CD147 Ig0 C67M.

We have also been the first to compare the activity of the ectodomain of both CD147 isoforms, CD147 Ig1-Ig2 and CD147 Ig0-Ig1-Ig2, as well as CD147 Ig0 alone, revealing critical insight into the activities of these domains. First, our data suggest that the two isoforms of CD147 may have distinct receptors. The constructs comprising the wild-type CD147 Ig0 domain exhibit much higher activity than the construct lacking this domain. In fact, there is over an order of magnitude increase in IL-6 secretion with the smallest doses of CD147 constructs comprising Ig0 with that of the highest dose of CD147 Ig1-Ig2. Second, in the monocyte cell line there appears to be a synergistic effect of the CD147 Ig0 domain with that of the CD147 Ig1-Ig2 ectodomain. Meaning, CD147 Ig0-Ig1-Ig2 activity is much higher than that of the additive contribution for CD147 Ig0 and CD147 Ig1-Ig2. This may suggest that in monocytes there may be multiple receptors: those that are targeted by either CD147 Ig0, those that are targeted by the ectodomain of CD147 Ig1-Ig2, and those that are

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targeted by the ectodomain of CD147 Ig0-Ig1-Ig2. Identifying the receptors for both isoforms is an important next step in our understanding of CD147 biology.

Comparison of CD147 Ig0 to other Ig-like proteins

CD147 is a member of the immunoglobulin superfamily (IgSF) of proteins and shares many characteristics that are similar to neuroplastin. Like CD147, neuroplastin is expressed as two similar isoforms in the human body called Np65 and Np55 [21] (Fig. 7a). Np65 comprises three extracellular Ig-like domains, Np Ig1-Ig2-Ig3, and Np55 comprises two extracellular Ig-like domains, Np Ig2-Ig3. A second common feature of both proteins is that each comprises a tissue specific isoform, which in both cases are the isoforms containing three Ig-like domains. For neuroplastin, the Np Ig1-Ig2-Ig3 is primarily expressed in the brain [25,26], while CD147 Ig0-Ig1-Ig2 is retinal specific [27]. A third common feature is that both tissue specific isoforms dimerize through the additional membrane distal Ig-like domain. Although the structure of the Np Ig1 domain has yet to be solved, its dimerization may be mediated by the same two conserved residues we have identified in the CD147 Ig0 domain and described above. Finally, both CD147 and neuroplastin exhibit a wide range of functions and both exhibit ligand activity by directly activating signaling pathways [7,21].

Structurally, there are several interesting comparisons between CD147 and neuroplastin that can be made to date. Since our determination of the CD147 Ig0 structure completes the structural analysis of all three CD147 Ig-like domains and the crystal structure of the Np Ig2-Ig3 isoform has recently been published [21], the only structural comparison absent is that of the Np Ig1 domain. Thus, no structural comparison can be made between the first Ig-like domains of these two proteins. However, the sequence identity between these tissue specific domains is very high at nearly 50% identity. Both the structure and sequence identity of the remaining domains can be compared (Fig. 7a). For example, in both tissue specific proteins the central Ig-like domains (i.e. CD147 Ig1 and Np Ig2) differ from all the other domains in that they adopt a C-type fold [11,21]. The CD147 Ig0 domain solved here is structurally similar to both its own CD147 Ig2 domain and the Np Ig3 domain, which all adopt an intermediate or I-type fold (Fig. 7b, top) [11,21]. Thus, both proteins share a similar domain composition in their isoforms as well as structurally similar Ig-like folds.

Additional structurally similar Ig-like domains were identified using the Dali search engine and include several contactins (contactin-4 and contactin-2) as well as connectin (also known as titin). Two examples of the close structural homology are shown here (Fig. 7b, bottom). Like both CD147 and neuroplastin, these proteins exhibit a wide range of activities. For example, these proteins act as cellular transmembrane proteins, are important components in muscle fibers, and act as ligands [28,29,30]. However, none of these Ig-like domains dimerize as does the CD147 Ig0 domain characterized here. Thus, our characterization of the CD147 Ig0 dimer highlights the diversity in the oligomeric behavior amongst this Ig-like family of proteins.

In summary, our studies here are the first report on a direct function of the human CD147 Ig0 domain. The distinguishing features of the CD147 Ig0 domain from its structurally similar proteins is that the functional unit is the dimer and there is a surface exposed cysteine that is critical for activity. Like the other CD147 Ig-like domains, at least one direct role for the CD147 Ig0 domain has been identified here in which it acts as a ligand to stimulate the secretion of a well-known cytokine, IL-6. Considering that increased levels of CD147 are correlated to increased levels of several MMPs in retinoblastoma [8] and that IL-6 secretion drives intraocular tumors [17], our studies represent an important contribution towards understanding the molecular details of disease progression. Our studies also pinpoint important structural aspects of the CD147 Ig0 that are critical for the activity of this domain. For example, disruption of the active CD147 Ig0 dimer to the inactive monomer

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may prove to be an important therapeutic strategy for the amelioration of retinoblastoma. Moreover, targeting the region comprising the free cysteine within CD147 Ig0 may also prove effective as a therapeutic strategy, since mutation of this residue was shown to significantly reduce activity. Finally, our data suggests that the two CD147 isoforms have different receptors a yet another important distinction between the two CD147 isoforms. Since CD147 is able to induce signaling pathways via unknown interactions, an important next step in our understanding of CD147 biology and mechanism is to identify these receptors. Current studies in our lab are focused on more widely characterizing the CD147 Ig0-Ig1-Ig2 and identifying the receptors and signaling mechanisms involved.

Materials and Methods

Protein expression and purification

Using the numbering in Swiss-Prot accession code P35613, the CD147 Ig0 wild-type gene encoding residues 22–138 and the entire CD147 Ig0-Ig1-Ig2 ectodomain encoding residues 22–321 were PCR-amplified from a commercially available vector (Open Biosystems, Huntsville, AL). The CD147 Ig1-Ig2 construct has previously been described [7]. All PCR-amplified products were then ligated into the pET15b vector (EMD Biosciences, Inc, San Diego, CA) using NdeI and BamHI restriction sites. Several constructs of the CD147 Ig0 were used in our studies; CD147 Ig0 wild-type protein, CD147 Ig0 C67M, CD147 Ig0 Q132L and CD147 Ig0 K126A. Site-directed mutagenesis was used to introduce single point mutations to obtain all such constructs.

All proteins were expressed in BL21 cells ((DE3) cells, grown at 37 °C, and protein expression was induced using 0.1 mM isopropyl β,D-thiogalactopyranoside at an optical density of approximately 0.6 (600 nm). CD147 constructs expressed in a bacterial expression system are initially unfolded due to improper disulfide bond formation and are thus refolded as described in Schlegel et al. [7]. Slight variations to this previously published method for constructs comprising the CD147 Ig0 domain are described below. The CD147 Ig1-Ig2 ectodomain was purified as described previously [7]. All columns and resins were used on an AKTA FPLC purchased from GE Healthcare.

All proteins were refolded at 4°C as follows. For a typical 2 L growth, cells were lysed via sonication in 35 ml of 100 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM EDTA, centrifuged at 12000g in a Sorvall SS-34 rotor (Sorvall, Asheville, NC), and the insoluble fraction collected. The insoluble fraction was sonicated to homogeneity in 35 ml of denaturation buffer (5 M guanidine-hydrochloride, 100 mM Tris, pH 7.5, 100 mM NaCl, and 2-mercaptoethanol), centrifuged again, and the soluble fraction dialyzed into refolding buffer (1 M arginine, 100 mM Tris, pH 7.5, 100 mM NaCl) for 48 hours. The soluble refolded proteins were then further dialyzed into 50 mM $NaPO_4$, pH 7.5, 0.5 M NaCl, 10 mM imidazole for purification via nickel sepharose equilibrated in the same buffer and eluted in the same buffer supplemented with 0.4 M imidazole. Nickel sepharose fractions were collected and protein samples were further purified with a pre-packaged S-100 size exclusion column equilibrated in 50 mM Tris, pH 7, 150 mM NaCl.

X-ray crystallography sample preparation and data collection

The CD147 Ig0 C67M construct was grown in M9 minimal media supplemented with Selenomethionine and the protein was purified as described above. Crystals were obtained at 1:1 ratio of a 10 mg/mL protein solution to well solution (0.2M NH_4SO_4, 20% w/v 3350PEG) supplemented with 1 μL of 0.1M spermidine tetrahydrochloride additive added to the drop using the sitting drop method at 4 °C. CD147 Ig0 wild-type crystals were obtained at the same concentrations as CD147 Ig0 C67M using a ratio of 1:1 protein to well solution

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(0.2 Na_2SO_4, 20% w/v PEG sulfate). Both crystal structures belong to the hexagonal space group $P6_{522}$, with 2 molecules/asymmetric unit and cell dimensions of a = 80.257 Å, b = 80.257 Å, and c = 160.674Å. The crystals were taken directly from the crystallization drop and flash-cooled in liquid nitrogen for cryo-protection. A three-wavelength MAD experiment was carried out at Advanced Light Source beamline 8.2.1 using an inverse beam strategy. For both proteins, Phenix software was used in structure determination and refinement [31] while Coot software was used for model building [32].

NMR sample preparation, spectroscopy, and data analysis

All Nuclear Magnetic Resonance (NMR) spectra were collected at 25ºC on a Varian 800- or 900-MHz spectrometer using 0.25 mM protein in 50 mM Tris, pH 7, 150 mM NaCl. Spectra were collected on CD147 Ig0 wild type and CD147 Ig0 C67M mutants with a 6xHis tag. All samples were supplemented with 7% D_2O. All pulse sequences were obtained from standard Varian Biopack libraries, data were processed using nmrPipe software [33] and analyzed using CCPNmr software [34]. Transfer Relaxation Optimized Spectroscopy (i.e. TROSY-based) HSQC experiments were used for spectral comparisons of different constructs but non-TROSY sequences were used for relaxation rate collection. The backbone assignments of the CD147 Ig0 domain were determined using $^{2}H,^{15}N,^{13}C$-labeled protein. Standard multidimensional NMR experiments that included an HNCACB, HNCA, HN(co)CA, HN(ca)CB were used, while a 3D-^{15}N-NOESY was used to confirm amide-amide interaction.

For relaxation experiments, standard R1 and R2 relaxation experiments were applied with recycle delays of 2.5 s at 900 MHz. Relaxation delays for R1 experiments were 0.01, 0.1, 0.3, 0.5, 0.7, 0.9, 1.1 and 1.3 s, and relaxation delays for R2 experiments were 10, 30, 50, 70, and 90 ms. Compensating pulses prior to the recycle delay were utilized for R2 measurements to account for potential sample heating and all relaxation experiments were arrayed within a single experiment to also account for any potential field inhomogeneities during the course of data collection. Individual amide relaxation rates were fit using a combination of both NMRPipe software and Gnuplot software.

Activity Assay

Secretion of IL-6 was measured in two different cell lines using ELISA detection kits (ELISA Tech, Aurora, CO). HEK293 cells were cultured in DMEM media containing high glucose, L-glutamine and sodium pyruvate supplemented with 10% Fetal Bovine Serum (FBS). THP-1 cells were cultured in RPMI 1640 media containing L-glutamine supplemented with 10% FBS. Cells were stored at 37 °C and 5% CO_2 at all times. Stimulation of both cells lines with recombinant CD147 constructs was performed in serum free media.

Supernatant from cell culture experiments was applied to a sandwich ELISA and the assay was performed according to the manufacturer's protocol. For HEK293 cells, 80% confluent cells were stimulated with CD147 constructs for 24 hours at 37 ºC. THP-1 cells were stimulated at a cell concentration of $1x10^6$ cells/mL for 24 hours at 37 ºC. Cells were collected and centrifuged at 16,000gfor 2 minutes to remove unattached cells (HEK293 cells) or to pellet the cells (THP-1 cells). 100 µl of the supernatant was applied to the plate and ELISAs were performed under the manufacturer's protocol (ELISA Tech).

Supplementary Material

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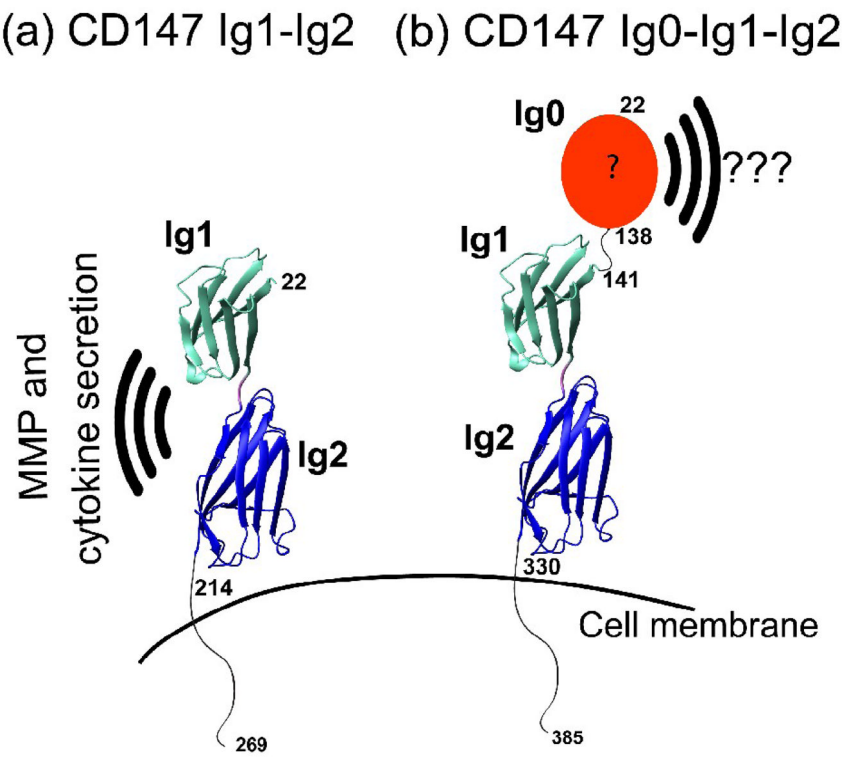

Fig. 1.

Two human CD147 isoforms are expressed that differ in their number of extracellular Ig-like domains. Both CD147 isoforms are comprised of an extracellular region, a transmembrane region and a short cytoplasmic tail. a) CD147 Ig1-Ig2 isoform contains two extracellular Ig-like domains and is the predominantly expressed CD147 isoform that is present in most tissues. The structure of this ectodomain comprising Ig1 (aquamarine) and Ig2 (blue) has recently been solved and characterized in solution [7,11]. b) The CD147 Ig0-Ig1-Ig2 isoform contains an additional Ig-like domain called Ig0 (orange sphere) and is retinal specific. While the CD147 Ig1-Ig2 ectodomain is known to induce MMP and cytokine secretion, little is known in regard to the CD147 Ig0 domain that includes both its structure and function.

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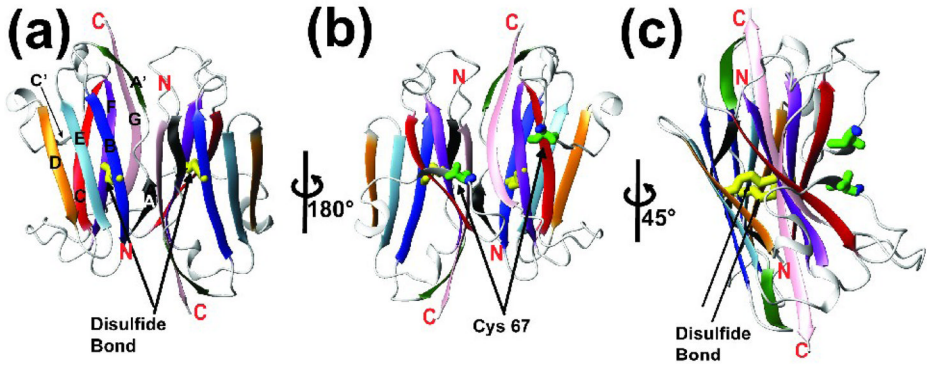

Fig. 2.

The crystallographic dimer of CD147 Ig0. a) X-ray crystal structure of the CD147 Ig0 dimer shows that each domain takes on an intermediate type Ig-like fold. Each monomer of CD147 Ig0 is comprised of 9 β-strands forming two β-sheets (color coded). The β-strand designation, residues comprising each strand and colors are as follows: strand A (24–27, black), strand A' (32–35, green), Strand B (40–47, blue), strand C (53–60, red), stand C' (68–69, grey), stand D (79–84, gold) strand E (89–95, light blue), strand F (104–111, purple) and strand G (128–137, pink). A disulfide bond is formed between Cys44 and Cys 108 (yellow). b) The position of the third, free cysteine residue within the CD147 Ig0 domain is mapped onto the crystal structure (green). c) Side view showing a single disulfide bond (yellow) within each of the monomer molecules of the CD147 Ig0 domain.

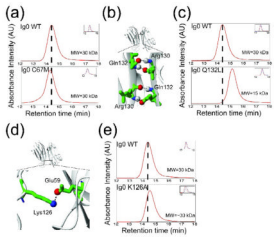

Fig. 3.

A single point mutation in the CD147 Ig0 dimer interface disrupts the integrity of the dimer. a) Both CD147 Ig0 wild-type and the mutant CD147 Ig0 C67M migrate as that expected for a dimer as assessed by analytical size-exclusion. b) From the X-ray crystal structures, two residues at the dimer interface identified as important for dimer formation include Arg130 and Gln132, which form a total of four H-bonds described in the text. c) The mutant CD147 Ig0 Q132L disrupts the stable formation of this dimer as the mutant migrates more closely to that predicted of the monomeric species. Insets show that dimer formation in solution is not concentration dependent as the elution time is the same at 20 μM (blue) and 400 mM (red). d) The interaction of Lys126 and Gln59 is not a critical determinant of dimer formation. The side chain Nζ of Lys126 is within 3.2 Å of the side chain CO of Gln59. e) Size exclusion chromatography data of the CD147 Ig0 K126A construct shows that there is only a slight shift in the elution time however, the calculated molecular weight is still that of the dimer, i.e. 30 kDa.

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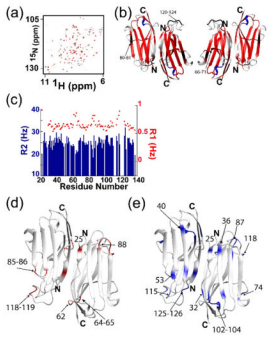

Fig. 4.

The structural integrity and dynamics of the CD147 Ig0 domain are revealed by NMR solution studies. a) The dispersive nature of the ^{15}N-HSQC of 0.25 mM CD147 Ig0 C67M is indicative of a well-folded protein in solution. B) Secondary structure propensity predictions, which include a β-strands (red) and α–helices (blue), are mapped onto the X-ray crystal structure of the CD147 Ig0 dimer using the NMR assigned resonance chemical shifts. CSI as well as the explicit differences of Cα chemical shifts relative to random coil agreed well for all calculated β-strands. However, only the latter predicted α–helical structure suggesting a transient nature for these helices in solution. The two regions that were not unambiguously assigned (black) include residues 80–81 and 120–124. C) Amide N^H R1 (red dots) and R2 (blue bars) relaxation rates were obtained for CD147 Ig0 C67M to identify dynamic regions of the protein. D) Residues with R1 relaxation rates greater than +1.0 standard deviation (SD) above the average are mapped onto the crystal structure of CD147 Ig0 (red). Only a subset of loops exhibit elevated R1 relaxation rates, indicating that most of the loops form stable structures in solution. E) Residues with R2 relaxation rates greater than +1.0 SD from the average are mapped onto the crystal structure of CD147 Ig0. Interestingly, adjacent residues that exhibit elevated R2 relaxation rates are shown (blue) and form a potential network. All data were collected at 25°C and 900 MHz.

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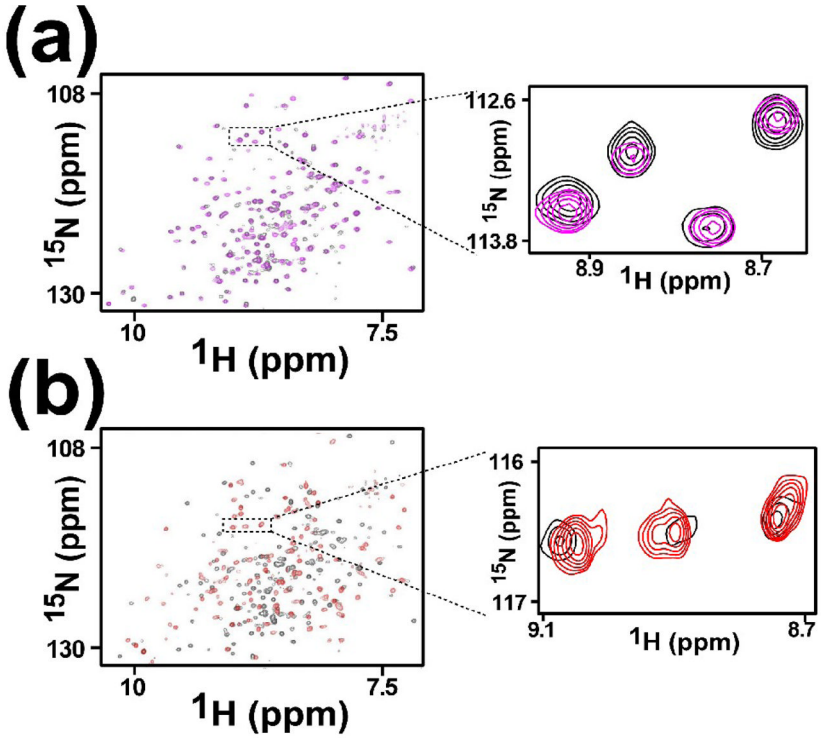

Fig. 5.

NMR solution studies reveal that the independently folded Ig-like domains of CD147 do not interact with one another. A) ^{15}N-trHSQC of 0.5 mM CD147 Ig0-Ig1-Ig2 (black) overlaid with the 0.5 mM CD147 Ig1-Ig2 (purple) domain indicates that the dispersion profiles of the two proteins overlap very well (enlarged region). B) ^{15}N-trHSQC of 0.25 mM CD147 Ig0-Ig1-Ig2 (black) overlaid with 0.25 mM CD147 Ig0 (red) domain also indicates that the two proteins overlap well (enlarged region). The contour levels were lowered for CD147 Ig0-Ig1-Ig2 (enlarged area) to show the observed overlap with the CD147 Ig0 resonances. Such lower signal/noise was likely necessary due to the lower tumbling time of the Ig0 dimer in the context of the entire ~70 kDa ectodomain dimer. The lack of significant chemical shift changes in all spectra indicates that the domains do not form a stable interaction and this is further shown by titration of individual constructs with one another (see Fig. S2).

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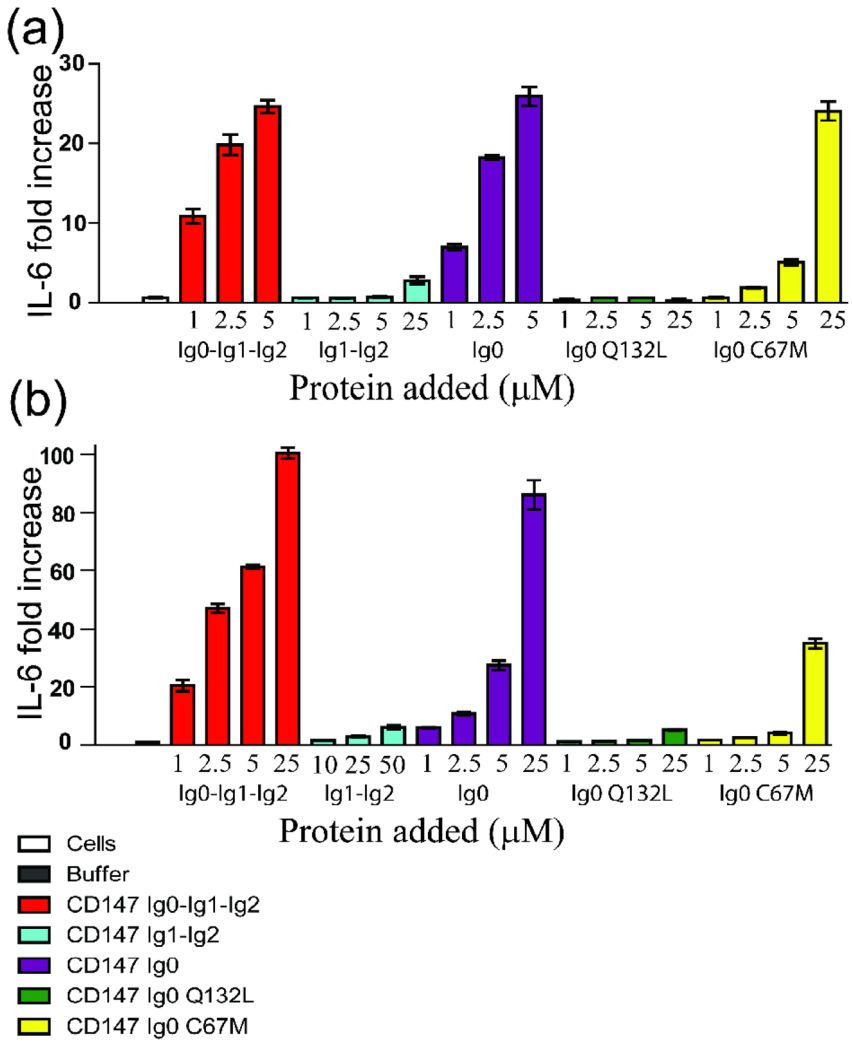

Fig. 6.

Biological activity assays of CD147 Ig-like domains show that CD147 Ig0 is a potent stimulator of interleukin-6 (IL-6) and reveal fundamental insights into CD147 activity. Two cell lines were used that include a) HEK293 cells and b) THP-1 monocytes. The data include unstimulated cells (white), buffer (black), CD147 Ig0-Ig1-Ig2 ectodomain (red), CD147 Ig1-Ig2 ectodomain (cyan), wild-type CD147 Ig0 (purple), CD147 Ig0 Q132L (green), CD147 Ig0 C67M (yellow) at the indicated concentrations used. Similar trends were observed for both cell lines where the CD147 Ig0 dimer is the functional unit required for activity and the free cysteine (i.e. Cys67) is important for activity.

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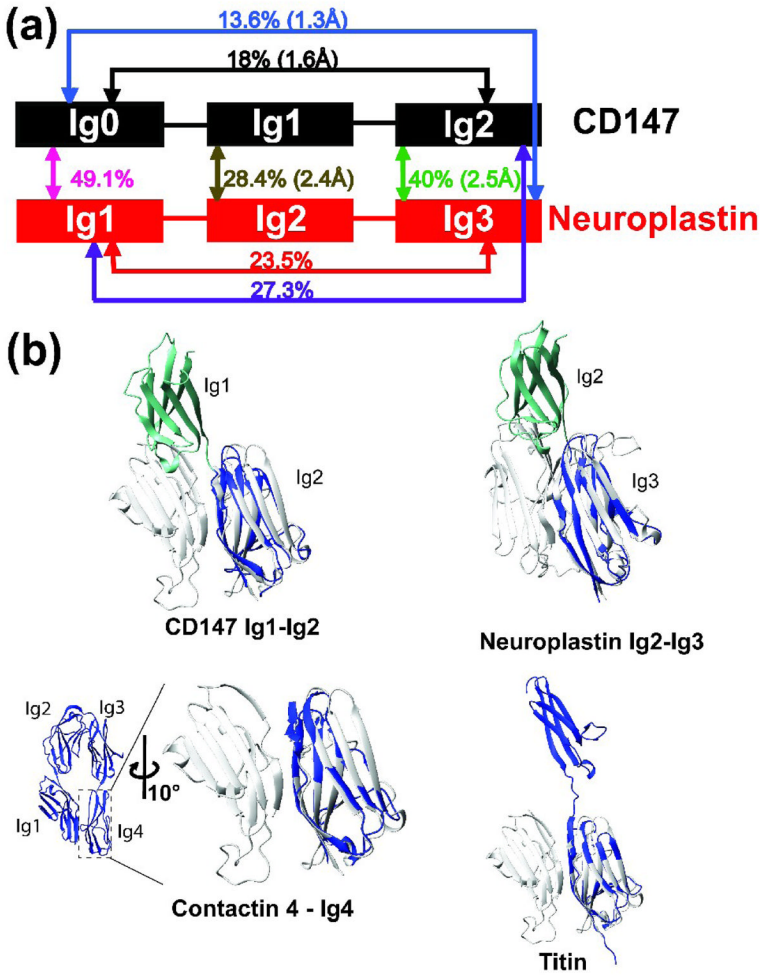

Fig. 7.

Structure and sequence comparisons of CD147 to other Ig-like proteins. a) Comparison between CD147 and neuroplastin Ig-like domains, which both have a tissue specific isoform comprising all three Ig-like domains and a ubiquitously expressed isoform that exclude the N-terminal Ig-like domain. Both the sequence identity and RMSD across all structurally similar Cα atoms are provided (RMSD in parenthesis), excluding that of the neuroplastin Ig1 domain that has yet to be solved. Both the N- and C-terminal Ig-like domains are highly similar both within each protein and between each protein. b) Structural comparisons of the CD147 Ig0 domain in the context of the dimer (white) solved here with other Ig-like domains. Top left: comparison to the CD147 Ig2 domain (blue) that shares 18.0% identity with an RMSD of 1.6 Å (PDB accession 3B5H). Top right: comparison to the neuroplastin Ig3 domain (blue) that shares 13.6% identity with an RMSD of 1.3 Å (PDB accession 2WV3). Bottom left: comparison to the contactin Ig4 domain that shares 21.5% identity with an RMSD of 1.1 Å (PDB accession 3KLD). Bottom left: comparison to the connectin Z2 domain that shares 20.3% identify with an RMSD of 1.3 Å (PDB accession 1YA5).

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Table 1

X-ray data processing and refinement statistics for CD147 Ig0 wild-type

Data Collection	
Space group	$P6_522$
Unit cell dimensions	
a,b,c (Å^2)	80.257, 80.257, 160.674
αβ γ (°)	90, 90, 120
Wavelength	1.2053
Resolution Range (Å)[a]	42.42–2.3 (2.38–2.3)
No. of reflections	
Total	274270
Unique	14231
Completeness (%)	99.6
$I/I\sigma$[a]	10.2 (2.6)
R_{merge}[a,b]	0.186 (0.771)
Mosaicity (deg)	0.8
Refinement	
R_{cryst}[c]	26.4%
R_{free}[c]	30.7%
No. of reflections	14231
No. of atoms	1930
No. of waters	98
RMSD Deviation	
Bond Distances (Å)	0.008
Bond Angles (deg)	1.1
B factor (Å^2)	
Protein	34.4
Water	12.7
Ramachandran Plot	
Most favored (%)	86.1
Additionally allowed (%)	9.5
Generally allowed (%)	3.0
Disallowed (%)	1.5

[a] Values for the highest resolution shell are indicated in the parentheses.

[b] $R_{merge} = \Sigma \mid I_{obs} - I_{ave} \mid / \Sigma I_{obs}$.

[c] $R_{cryst} = \Sigma \parallel F_{obs} \mid - \mid F_{calc} \parallel / \Sigma \mid F_{obs}$