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The molecular interaction of CAR and JAML recruits the central cell signal transducer PI3K

Petra Verdino¹, Deborah A. Witherden², Wendy L. Havran², and Ian A. Wilson^{1,3,*}

¹Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA

²Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA

³Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA

Abstract

Coxsackie and adenovirus receptor (CAR) is the primary cellular receptor for group B coxsackieviruses and most adenovirus serotypes and plays a crucial role in adenoviral gene therapy. Recent discovery of the interaction between Junctional Adhesion Molecule-Like Protein (JAML) and CAR uncovered important functional roles in immunity, inflammation, and tissue homeostasis. Crystal structures of JAML ectodomain (2.2Å) and its complex with CAR (2.8Å) reveal an unusual Ig-domain assembly for JAML and a charged interface that confers high specificity. Biochemical and mutagenesis studies illustrate how CAR-mediated clustering of JAML recruits phosphoinositide-3-kinase (PI3K) to a JAML intracellular sequence motif as delineated for the $\alpha\beta$ T cell costimulatory receptor CD28. Thus, CAR and JAML are cell signaling receptors of the immune system with implications for asthma, cancer, and chronic non-healing wounds.

The coxsackievirus and adenovirus receptor, CAR (1,2), plays a crucial role in both virus-related pathology and adenoviral gene therapy (3–5). The tissue distribution of CAR is broader than the observed virus tropism (6), and significant efforts have been devoted to characterizing and modifying CAR-virus interactions to improve efficacy and safety of adenoviral gene delivery (7). The high conservation of CAR among vertebrates and its low genetic diversity in humans have resulted in its frequent occurrence as a target in the evolution of virus-host interactions (6). Such conservation indicates that CAR is physiologically important. However, although roles in embryonic development, cell-adhesion, tumor cell growth, inflammation, and tissue regeneration have been reported, the underlying mechanisms remain elusive (8,9). The Junctional Adhesion Molecule-Like protein, JAML (10), present on a variety of effector cells of both adaptive and innate immunity, including neutrophils, monocytes, memory T cells, and epithelial $\gamma\delta$ T cells, was recently identified as an endogenous ligand of epithelial and endothelial CAR (11–14). CAR and JAML are both type I transmembrane glycoproteins composed of a globular ectodomain (two Ig domains), a stalk (CAR: 6 residues, JAML: 26 residues), a single transmembrane

* to whom correspondence should be addressed: wilson@scripps.edu.

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helix, and a cytoplasmic domain. The CAR-JAML interaction is important for leukocyte migration and for activating $\gamma\delta$ T cell responses (11–14) with implications for epithelial challenges, such as wound repair or inflammation (15). CAR binding to JAML on epithelial $\gamma\delta$ T cells induces cytokine and growth factor production, MAP kinase pathway activation and, ultimately, cell proliferation (14), indicating that JAML and CAR constitute an important cell signaling complex of the immune system. To elucidate the mechanisms underlying CAR and JAML physiology, we determined the crystal structure of this receptor-ligand pair.

We expressed the mouse JAML ectodomain (residues 1–260) in SC2 cells, purified and crystallized the protein, and collected a 2.2 Å native X-ray dataset (Table S1,S2). The structure was determined initially by MAD phasing at 3.4 Å using a heavy atom derivative and then extended to 2.2 Å by molecular replacement (MR) of the experimental model into a higher resolution native data set. In JAML, two V-set Immunoglobulin (Ig) domains (D1 and D2) associate into a compact assembly (Fig. 1A), where their relative orientation is constrained by an interdomain, parallel β -sheet interaction of the D1 B-strand and the D2 B'-strand, numerous H-bonds, a salt bridge, hydrophobic packing, and a short interdomain linker (Fig. 1B–D, S1). The interface between D1 and D2 buries a total molecular surface area of 1,050 Å² (D1: 540 Å², D2: 510 Å²). The extent of the interface, combined with identical relative orientations of D1 and D2 in four different crystal forms (16), indicates that the highly compact JAML globular domain acts as a single rigid unit in contrast with typical, elongated structures of tandem Ig-domain receptors, such as CAR, CD2, or CD58 (Fig. S2) (17), where flexibly-linked Ig domains facilitate protein recognition.

Carbohydrate moieties are present at all potential N-linked glycosylation sites (Asn59, Asn69, and Asn105) of JAML (Fig. 1A). However, these sites are not entirely conserved across species, suggesting that N-glycosylation is not directly correlated with JAML function, but might aid protein stabilization and prevent non-specific interactions on the cell membrane. The stalk, which tethers the extracellular Ig domains of JAML to the cell membrane, and the C-terminal His-tag are disordered in the crystal. This stalk is rich in serine, threonine, proline, and alanine (Fig. S3A) and is likely to be O-glycosylated *in vivo*. The glycosylation would confer some rigidity to the otherwise flexible stalk and would result in a distance of <60 Å between the globular JAML domain and the membrane (Fig. 1A).

JAML can thus be considered as an unconventional Ig-domain receptor where the more compact and rigid assembly of its Ig domains provides the ligand-binding site, while its extended stalk provides sufficient flexibility to facilitate interaction with CAR on the opposing cell surface. To gain insight into this interaction, we determined the crystal structure of the ectodomain complex of JAML and CAR.

The entire mouse CAR ectodomain (residues 1–217) was produced in SF9 cells and crystallized in an equimolar ratio with mouse JAML. The crystal structure was determined at 2.8 Å resolution by MR using unliganded JAML and human CAR D1 (1EAJ (18)) as MR templates. The partially disordered CAR D2 domain was manually built using the NMR structure of human CAR D2 (2NPL (19)) as a model (Table S1). CAR and JAML form a 1:1 complex in the crystallographic asymmetric unit and, as expected for interaction of cell surface receptors from opposing cells, their C-terminal stalks protrude in opposite directions (Fig. 2A). Well-defined electron density was observed for JAML and the V-set CAR D1 Ig domain (3,18) (including the N-linked glycan at Asn87). The C2-set CAR D2 Ig domain (19) was partially disordered indicating flexible linkage of the two CAR Ig domains and classifying CAR as a typical tandem Ig-domain receptor. At the cell surface, CAR D2 is tethered to the membrane by a short stalk and its motion may be additionally restricted by an

N-glycan (Asn182) in a membrane-proximal loop (Fig. S4); thus, the flexibly-linked D1 appears to facilitate interaction with JAML and might also aid in attachment of CAR to coxsackie and adenoviruses (3,4).

The CAR-JAML interface is restricted to the membrane-distal D1 domains of both receptors (Fig. 2A, Table S4) in contrast to previous suggestions (11). Domain deletion experiments demonstrate that JAML D1 is sufficient for CAR binding (14); however, JAML D2 exhibits indirect effects through enhancing both JAML expression levels and CAR-binding affinity to the JAML D1 domain (Fig. S5). The D1 GFCC'C"-sheets of CAR and JAML pack face-to-face comparable to the V_L-V_H arrangement in antibodies or V α -V β in T-cell receptors. Additionally, the CAR A-strand interacts with the JAML CC'-loop. The CAR-JAML interface buries a surface area of 1460 Å² (CAR: 740 Å², JAML: 720 Å²), which is in the typical range for protein-protein complexes. While its shape complementarity (Sc=0.64) is on the low side for protein-protein interactions, it is consistent with the relatively weak CAR-JAML affinity (K_d ~5μM) in solution (Table S3), as in other lymphocyte, cell-cell recognition systems (20). However, avidity and concentration effects for cell membrane-tethered receptors likely result in a greater effective molarity on the cell surface (20–22).

The CAR-JAML interface is unusually hydrophilic (Fig. 2E) and characterized by an array of interdigitated charged residues (Fig. 2C, S6) which are conserved amongst homologues for both proteins (Fig. S3, S4). Two hydrophobic clusters (Fig. 2D) and an antiparallel β-sheet interaction between the CAR C'-strand and the JAML D1 FG-loop (Fig. 2B, S7) provide additional stabilization. A total of 110 protein-protein interactions between CAR and JAML include 13 salt links, 6 H-bonds, and 91 van der Waals' contacts (Table S4). Of the total buried surface area, 36% (CAR: 270 Å², JAML: 260 Å²) is contributed by charged residues (Asp35, Glu37, Asp49, Lys102, Lys104 of CAR; Asp36, Arg37, Asp39, Glu100, Arg102, Lys111 of JAML) (Fig. 2E, S6). The exceptional number of interdigitating salt bridges contributes binding energy but, more importantly, imparts high ligand specificity as demonstrated by mutational analysis (Fig. S8, S9). JAML Asp39, Tyr52, Phe55, Tyr57 and CAR Lys104 compose the functional hot spot of the interaction (Fig. 2D). JAML Tyr52 orients Asp39 such that it binds CAR Lys104 in an extended conformation in which its aliphatic portion packs against JAML Phe55 and Tyr57. The Tyr57 hydroxyl also forms crucial hydrogen bonds to the CAR backbone. The charged interface and functional hot spot of the JAML-CAR complex show significant similarities to the CD2-CD58 interaction (17) (Fig. S10), although low sequence identity (14% and 20% for the D1 domains of JAML/CD2 and CAR/CD58, respectively), and different interface topology, do not suggest any evolutionary relationship between those receptor-ligand pairs.

The JAML binding site on CAR overlaps with the receptor binding site for adenoviruses (3,4) (Fig. 3). Adenoviruses interact with up to 16 of the 18 CAR residues that mediate JAML binding (Fig. 3B, S11), thus inhibiting JAML-CAR interaction (14) and induction of γδ T cell signaling (Fig. S15). In contrast, only 6 residues of the Coxsackie B virus binding site overlap with the functional CAR interface, but notably includes hot spot residue Lys104. The high degree of conservation and the sensitivity of the key residues of the CAR-JAML interface to mutation support the idea that some viruses ensure long-term entry into host cells by interaction with highly conserved receptor binding sites as any mutations would compromise receptor function (9,18).

The CAR-JAML ectodomain interaction induces cell signaling events at the JAML intracellular domain (ICD) (Fig. 4A, S3A) (14), but the mechanism of signal transduction across the cell membrane has not previously been determined. While no significant structural rearrangements occur in the JAML ectodomain upon CAR ligation, JAML and CAR can apparently exist as both monomers and dimers (12,18). In the crystal, a dimer of

the CAR-JAML complex, with an interface size ($\sim 1,400 \text{ \AA}^2$) and shape complementarity ($Sc=0.73$) within the range of typical protein-protein interactions ($Sc=0.70-0.76$), is assembled on the crystallographic 2-fold axis (Fig. S12, S13). While the biological relevance of this particular assembly remains to be established (23), JAML dimerization/clustering as a mechanism for signal transduction is strongly supported by the finding that binding of dimeric, but not monomeric, CAR or antibody ligands recruits PI3K to the JAML ICD (Fig. 4D,E).

How does the interaction between CAR and JAML activate $\gamma\delta$ T-cell responses during epithelial insults (14)? The JAML ICD is highly conserved, suggesting interaction with intracellular proteins, and contains putative serine, threonine, and tyrosine phosphorylation sites (Fig. S3A). Most strikingly, it includes a YMxMxPxxP signaling motif similar to that in the major costimulatory $\alpha\beta$ T-cell coreceptor CD28 (Fig. S3B). The tyrosine-phosphorylated motif in CD28 binds the universal signal transducer PI3K 1A (24–26) leading to activation of MAP kinase pathways and IL-2 production (26,27).

As resting and activated epithelial $\gamma\delta$ T cells lack the major PI3K-binding $\alpha\beta$ costimulatory receptors, CD28 and ICOS, and ligand-binding to JAML leads to MAPK activation and IL-2 production (14), we tested whether JAML mediates costimulation through interaction with PI3K. Indeed, on epithelial $\gamma\delta$ T cells, PI3K associates with JAML within 1min of CAR ligation (Fig. 4A). Once PI3K has been recruited, disruption of the CAR-JAML interaction leads to rapid decline of PI3K levels (Fig. 4B), tightly linking extracellular protein recognition events to intracellular signaling. PI3K-binding to JAML also strongly correlates with affinity of the CAR-JAML interaction, as demonstrated by decreased kinase recruitment by CAR mutants that have reduced binding to JAML (Fig. 4C,S9). To further dissect the JAML-PI3K interaction, mutants of the JAML intracellular signaling motifs were generated. In JAML-transfected CHO cells, PI3K binds constitutively to wild-type JAML and a Tyr³¹⁴Phe mutant, but not to a Tyr³³⁶Phe mutant, of the YMxM motif (Fig. 4F), identifying this region as the PI3K binding site. In Pro^{340,343,346}Ala JAML mutants, negligible PI3K association occurs and suggests that, as for CD28 (24–26), the polyproline motif serves to recruit a primary kinase to phosphorylate YMxM and that PI3K association to JAML critically depends on the phosphorylation state of the PI3K binding motif. Despite low sequence identity (11% for JAML/CD28, 16% for CAR/B7-1) and the different domain organization of JAML and CD28, these results demonstrate a striking functional similarity between costimulation and cell signaling through CAR-JAML on epithelial $\gamma\delta$ T cells and B7-CD28 on $\alpha\beta$ T cells.

Thus, this delineation of the molecular details of the CAR-JAML-PI3K regulatory circuit has now uncovered a physiological role of the major virus receptor CAR and its partner JAML in cell signaling. These insights may serve as a basis for modulating cellular responses during tissue homeostasis and immunity, and have implications for treatment of chronic non-healing wounds, asthma, and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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affinity CAR-JAML heterotetramer was intrinsically hampered by experimental constraints (e. g. the limited protein concentrations that could be used in the experiments or that soluble CAR proteins are a poor probe to investigate the heterotypic interactions that naturally occur between the cell-bound JAML and CAR counter-receptors).

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Fig. 1.

Crystal structure of the unliganded mouse JAML ectodomain reveals a novel prototype for tandem Ig-domain receptors. **(A)** Ribbon diagram of JAML D1 (membrane-distal, residues 10–121, light blue) and D2 (membrane-proximal, residues 122–236, salmon) Ig V-set domains comprising 9 β -strands arranged into two antiparallel β -sheets (A'GFCC'C''':DEB) linked by canonical disulfide bridges (Cys25 to Cys99 and Cys138 to Cys216, green). D1 tightly packs its BED β -sheet against the D2 B'C- and FG-loops. The D1 A-strand (residues 1–9) and the C-terminal stalk region (residues 234–260, dashes) which tethers the JAML Ig domains to the cell membrane were disordered. Carbohydrate moieties attached to the three N-linked glycosylation sites (yellow sticks) are labeled. **(B–D)** Several interdomain interactions stabilize the relative orientation of D1 and D2 (see inset boxes). **(B)** A hydrophobic cluster is formed by Leu22, Val74, Leu85 of D1, and Ile140, Val220, and Leu223 of D2. Arg147 forms a salt bridge with Asp81, whose side-chain orientation is restrained by H-bonds to Ser83. **(C)** A short β -strand (B', red) in the BC-loop of D2 with the B-strand of D1 creates a 4-stranded DEBB' interdomain β -sheet. Additional interdomain H-bonds (some water-mediated) are not shown: Arg14 - Gln141, Glu19 - Gln141, Glu19 - Ser139, Glu19 - Glu122, Ser20 - Ser225, Gln87 - Glu224, and Gln87 - Ser225. **(D)** A short, rigid linker (residues 120–123) directly connects the D1 G-strand to the D2 A'-strand, as D2 lacks an A-strand. The linker is stabilized by two prolines, an H-bond network, and a salt bridge between Glu122 and Arg137.

Fig. 2.

CAR-JAML ectodomain complex structure. **(A)** Ribbon diagram of the CAR-JAML complex. JAML (D1, residues 11–121, light blue; D2, residues 122–236, salmon; disordered stalk, dashes), CAR {D1, residues 3–120, light green; D2, residues 121–216, gold; n.b. CAR D2 loops that were disordered in the crystal structure were grafted on from the CAR D2 NMR structure (2NPL, dots)}, and carbohydrates (gray sticks). CAR and JAML interact with their D1 A'GFCC'C'' β -sheets in a face-to-face, hand shake-like fashion and **(B)** An antiparallel β -sheet is formed between the G-strand (Ser107-Lys111) of JAML and the C'-strand (Ile47-Val51) of CAR which moves ~ 6.2 Å as compared to apo CAR (Fig. S7). **(C)** The interface is characterized by an array of interdigitated charged amino acids. Specifically, JAML Arg37 forms salt bridges with CAR Asp35 and Glu37 and JAML Asp36. CAR Glu37 also interacts with JAML Arg102, which forms a salt bridge to JAML Asp36. CAR Lys102 interacts with JAML Glu100 and Asp39, which in turn forms bidentate interactions with CAR Lys104. **(D)** The functional hot spot of the CAR-JAML interaction: JAML Asp39, Tyr52, Phe55, Tyr57 and CAR Lys104. **(E)** Open book representation of the CAR-JAML interface. Atoms which form contacts are colored green and blue on the molecular surfaces of JAML and CAR, respectively. Above, the electrostatic potential was mapped onto the molecular surface and contoured at ± 35 kT/eV (blue/red). CAR residues contacting JAML are shown as green sticks above the JAML surface. JAML residues contacting CAR are shown as blue sticks above the CAR surface. The surfaces of CAR and JAML are highly complementary which ensures high ligand specificity. The strong electrostatic component of this interaction accounts for rapid kinetics and likely a high sensitivity to the environment.

Fig. 3.

Comparison of functional and viral epitopes of CAR. **(A)** Schematic representations of CAR complex structures: from left to right: mCAR-mJAML {3MJ7, this work}, hCAR-hAd12 {1KAC (3)}, hCAR-hCVB3 {1JEW (4)}, and hCAR homodimer {1EAJ (18)}. **(B)** Molecular surface of the CAR D1 domain in the various CAR complex structures. CAR atoms that are part of the epitope for the respective ligands are colored blue (CAR-JAML), red (CAR-Ad12), brown (CAR-CVB3), and light green (CAR homodimer). The CAR-JAML binding site mostly coincides with both the adenoviral and the homodimerization interface of CAR, while the coxsackievirus B binding site is located at the periphery of the functional CAR interfaces.

Fig. 4.

Ligand-induced PI3K recruitment to the JAML ICD. Anti-PI3K Western blots of JAML immunoprecipitated with anti-JAML HL4E10 IgG from epithelial $\gamma\delta$ T cell lysates after stimulation with ligands (A–E) or from JAML mutant-transfected CHO cell lysates (F). (A) Time course of stimulation with CAR-Fc. After 1 min, PI3K association to JAML is significantly elevated over the basal level. (B) PI3K-recruitment is rapidly switched off once CAR is released from the JAML ectodomain. $\gamma\delta$ T cells were incubated with buffer (DPBS), Adenovirus F5 protein (F5), or stimulated with CAR (CAR-Fc) followed by an excess of F5 (which competes with JAML for CAR-binding). F5 alone does not alter basal PI3K levels (DPBS), while a 1 min addition of F5 (CAR-Fc+1min F5) significantly decreases PI3K

recruitment in CAR-stimulated $\gamma\delta$ T cells (CAR-Fc+1min). **(C)** Functional consequences of mutations in the CAR-JAML interface on downstream signalling by JAML. wt CAR-Fc and a Asp49Ala mutant induce significant PI3K recruitment, consistent with their comparable JAML-binding characteristics (Fig. S9) and location of CAR Asp49 at the periphery of the JAML-binding site (Fig. 2E), where mutations would be expected to cause smaller effects on binding. Mutation of CAR Asp35 induces less, but does not entirely abolish, PI3K-binding to JAML, consistent with its interaction with JAML Arg37, for which mutations are well tolerated. In contrast, CAR mutants Glu³⁷Ala, Lys¹⁰²Ala, and Lys¹⁰⁴Ala, all of which interact with JAML hot spot residues (Fig. 2C), do not induce significant PI3K recruitment. **(D+E)** Dimeric, but not monomeric, ligands induce PI3K-recruitment to JAML. PI3K levels are increased by JAML binding of bivalent HL4E10 IgG, bivalent HL4E10 Fab'₂ fragment, and dimeric CAR (CAR-Fc fusion protein), but not by monovalent HL4E10 Fab or monomeric CAR (CAR-His). Fc- or His-tagged control proteins (CD62L-Fc, CD1d-His) do not induce PI3K recruitment. **(F)** Dissection of the role of conserved JAML intracellular signaling motifs in PI3K binding. PI3K constitutively binds to wild-type and Tyr³¹⁴Phe JAML, while binding is abolished to any Tyr³³⁶Phe mutants in the YMxM PI3K-binding motif. PI3K association is greatly reduced in JAML polyproline motif mutants Pro^{340,343,346}Ala and Tyr³¹⁴Phe/Pro^{340,343,346}Ala even although they still exhibit an intact YMxM motif. Data are representative of at least three independent experiments.