CD147 Inhibits the Nuclear Factor of Activated T-cells by Impairing Vav1 and Rac1 Downstream Signaling*

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CD147 is a transmembrane protein that plays crucial roles in the development and function of the reproductive, visual, and nervous systems. CD147 also exerts positive and negative actions in T-cells by still obscure mechanisms. In this study, we have analyzed the expression, localization, and function of CD147 during T-cell receptor signaling responses. We show here that CD147 is an integral component of the T-cell immune synapse and that its overexpression leads to the inhibition of NF-AT (nuclear factor of activated T-cells) activity induced by Vav1, a Rac1 exchange factor. This inhibitory activity is mediated by the CD147 intracellular tail and is totally independent of its extracellular or transmembrane regions. The molecular dissection of the influence of CD147 on the Vav1 pathway indicates that its inhibitory action takes place downstream of Vav1 and Rac1 but upstream of the serine/threonine kinases JNK and Pak1. The interference of CD147 with these pathways is highly specific because the overexpression of CD147 does not affect the activity of other GDP/GTP exchange factors or the stimulation of the ERK cascade. Finally, we show that the CD147 knockdown in Jurkat cells promotes higher levels of NF-AT stimulation and Pak1 phosphorylation upon T-cell receptor cross-linking. Instead, the lack of CD147 does not affect other signaling cascades that participate in the same cellular response. Taken together, these results indicate that CD147, via the selective inhibition of specific downstream elements of the Vav1/Rac1 route, contributes to the negative regulation of T-cell responses.

The interaction of cells with the extracellular environment requires the use of surface molecules that sense nutritional, hormonal, mitogenic, cell-derived, and substrate-originated signals to induce appropriate cellular responses. Because of this, the elucidation of these human proteome components is crucial for understanding the signaling basis of cell responses and for elucidating and treating human pathologies. CD147, also frequently referred to as Basigin and Emmprin (extracellular matrix metalloprotease inducer), is one of the transmembrane proteins that has been subjected to intense scrutiny during recent times. CD147 is a glycosylated type I transmembrane protein that belongs to the Ig superfamily. Its structure contains an extracellular domain with one or two Ig-like domains depending on the CD147 isoform, a transmembrane region, and a 39-amino acid-long cytoplasmic tail (1–5).

CD147 has become the focus of extensive analysis because of its linkage to diverse pathological states in humans (1–4). Thus, it has been shown that CD147 promotes invasion and metastasis in different tumor types via the induction of MMPs4 and the urokinase-type plasminogen activator system by peritumoral stromal cells. CD147 also favors angiogenesis, anoikis resistance, lactate efflux, multidrug resistance, and cell proliferation in cancer cells (1–4). Furthermore, based on its high expression levels, it has been proposed that its expression status can be used as a prognostic factor in different tumors (1–4). CD147 overexpression and/or function has been associated with other pathological processes such as inflammatory responses (6, 7), pulmonary fibrosis (8), rheumatoid arthritis (9–12), systemic lupus erythematosus (13), heart failure (14), Alzheimer disease (15), and the infectivity cycle of the human immunodeficiency virus and coronaviruses in lymphocytes (16, 17).

Recent genetic studies have also highlighted the important role of this protein in developmental and physiological processes. Thus, disruption of the CD147 gene in mouse leads to blindness (18–20), female and male infertility (21–23), problems in embryo implantation in the uterus (23, 24), nervous system-linked defects in behavior, learning, memory, and sensory activities (25, 26), and immune dysfunctions (25). This phenotype can be aggravated in some mouse genetic backgrounds, leading to a lethal phenotype during the embryonic period (24). In Drosophila, CD147 has been shown to be impor-

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4 The abbreviations used are: MMP, matrix metalloprotease; ATF2, activating transcription factor 2; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HA, hemagglutinin; IKR, in vitro kinase reaction; JNK, c-Jun N-terminal kinase; NF-AT, nuclear factor of activated T-cells; Pak, p21-activated kinase; PBS, phosphate-buffered saline solution; RasGRF2, Ras GDP releasing factor 2; RasGRP1, Ras GTP releasing protein 1; RT, reverse transcription; shRNAmir, microRNA adapted short hairpin RNA; TCR, T-cell receptor; MOPS, 4-morpholinepropanesulfonic acid; F, forward; R, reverse; CMAC, 7-amino-4-chloromethylcoumarin.
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Antibodies—Antibodies to CD147 (8D6), GST (clone Z-5), c-Myc (clone 9E10), and phosphotyrosine residues were all obtained from Santa Cruz Biotechnology. The goat antibody to human CD147 was obtained from R & D Systems. Antibodies to HA, FLAG, and EGFP were obtained from Covance. The rabbit antibodies to the phosphorylated Tyr \(^{174}\) residue of Vav1 and to the Vav1 DH domain (catalog number 301-5) were generated in our laboratory (52). An anti-Rac1 antibody (clone 23A8) was purchased from Upstate Biotechnology, Inc. Anti-tubulin antibodies were from Oncogene. The antibody specific for the phosphorylated Thr\(^{190}\) and Tyr\(^{204}\) residues of ERK1/ERK2, the phosphorylated Thr\(^{183}\) and Tyr\(^{185}\) residues of JNK1/JNK2, the phosphorylated Thr\(^{202}\) and Thr\(^{223}\) residues of Pak1/Pak2, as well as the pan-specific antibodies to ERK, JNK, and Pak family members were obtained from Cell Signaling. Antibodies to CD3 were purchased from Dako. Horseradish peroxidase-conjugated secondary antibodies to rabbit and mouse IgGs were obtained from GE Healthcare. The Cy3- or Cy5-labeled secondary antibodies to either rabbit or mouse IgGs were purchased from Jackson ImmunoResearch.

Plasmids—The pBSG-HA and pBSG-FLAG plasmids containing the pcDNA3 backbone and encoding epitope-tagged versions of human CD147 were kindly provided by Dr. K. Kadomatsu (Nagoya University School of Medicine, Nagoya, Japan). The mammalian expression vector encoding the CD3\(\varepsilon\)-EGFP chimera has been provided by Dr. B. Alarcón (Centro de Biología Molecular, Consejo Superior de Investigaciones Científicas, Madrid, Spain). Plasmids encoding wild type human Vav1 (pJC11), Vav\(^{353}X\)F (pKES26), AU5-tagged Rac1\(^{261}\) (pCEF-UL5-Rac1\(^{261}\)), and EGFP-tagged Rac1\(^{261}\) (pNM42) have been described previously (52–54). The mammalian expression vector pMEX has been referenced before (53). pCEFL-HA-H-Ras (a mammalian expression vector encoding an HA-tagged version of wild type H-Ras), pCEFL-HA-ERK1 (a mammalian expression vector encoding an HA-tagged version of ERK1), pCEFL-GST (a mammalian expression vector encoding the GST protein), pCEFL-Myr (a mammalian expression vector containing a myristoylation signal downstream of a Kozak’s sequence and an ATG codon), pGEX-ATF2 (a bacterial expression vector encoding the ATF2 protein), and pGEX-RafRBD (a bacterial expression vector encoding the GST protein fused to the Ras binding domain of c-Raf1) were obtained from Dr. P. Crespo (University of Cantabria-CSIC, Santander, Spain). The pNF-ATLuc reporter plasmid containing the Photinus pyralis luciferase gene under the regulation of NF-AT sites.
was obtained from Dr. G. Crabtree (Stanford University Medical School, Stanford, CA). pcDNA3-FLAG-JNK (a mammalian expression vector encoding a FLAG-tagged version of JNK1) was provided by Dr. J. Galán (Yale University School of Medicine, New Haven, CT). pcMV6M-Pak1-Myc (a mammalian expression vector encoding wild type Pak1 with a Myc epitope at its C terminus) was provided by Dr. J. Field (University of Pennsylvania School, Philadelphia). pcMV5M-Pak1-(165–544) (a mammalian expression vector encoding a constitutively active version of Pak1) was obtained from Dr. M. H. Cobb (University of Texas Southwestern Medical Center, Dallas). pSGV-H-RasG12V (a mammalian expression vector encoding the constitutively active version of H-Ras) was provided by Dr. J. Downward (Cancer Research UK London Research Institute, London, UK). pcDNA3-FLAG-RasGRF2 (a mammalian expression vector encoding a FLAG-tagged version of mouse RasGRF2) was obtained from Dr. M. Moran (University of Toronto, Toronto, Canada). pFC-MEKK1-(380–672) (a mammalian expression plasmid encoding a constitutively active form of MEKK1) and pRL-SV40 (a mammalian expression vector containing the Renilla reniformis luciferase gene under the regulation of the SV40 promoter) were obtained from Stratagene and Promega, respectively. pMD-G and pNGVL-MLV-gag-pol were provided by Drs. R. C. Mulligan (Children’s Hospital, Boston) and A. Bernard (CNIC, Madrid, Spain), respectively. For the generation of the EGFP-CD147-expressing plasmid (pACC5), the full-length human CD147 cDNA was amplified by PCR from pBSG-FLAG, digested with BamHI, and ligated into the BamHI-linearized pEGFP-N3 plasmid (Clontech). 

For the generation of the vector (pSRM28) expressing the GST-CD147Ct protein (see Fig. 4A), we used the oligonucleotides 5′-CCACGGCACTCTGACTTGGCGACCAGC-3′ (forward) and 5′-GGTGCCGTTGGCCCGGTACTGGCCGGGG-3′ (reverse) to mutate the cysteine residue located at position 123. This mutant plasmid was used in the next mutagenesis step to generate the second mutation in the cysteine residue located at position 183. In this case, we used the oligonucleotides 5′-CCCCGGCCAGTACCGGG-3′ (forward) and 5′-GGGCCTCCTGTTGCGCTTC-3′ (reverse). For the generation of the vector (pSRM26) expressing the GST-CD147Ct protein (see Fig. 4A), the appropriate CD147 cDNA fragment was amplified by PCR from pBSG-HA, digested with BglII and EcoRI, and ligated into BamHI-linearized pEGFP-N3 plasmid (Clontech). To create the mammalian expression vector encoding the CD147Ct-Myc protein (see Fig. 4A), we used the oligonucleotides 5′-CCACGGCACTCTGACTTGGCGACCAGC-3′ (forward) and 5′-GGTGCCGTTGGCCCGGTACTGGCCGGGG-3′ (reverse).
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We used the shRNAmir technology with retroviral vectors to carry out the CD147 mRNA knockdown. To generate the retroviral vectors encoding the different CD147-specific shRNAmir, we designed several pairs of oligonucleotides with microRNA structure. Those included shRNAmir-CD147-1F (5'-TCGAGAAGGTATTTGCTGTTGACCGAGCGCCGGTCAGAGCTACACATTTACATTTGCACTACTTCC-3'), reverse; shRNAmir-CD147-1R (5'-TGGTGCTGCCCGTGCCTACTGCCTCGG-3'), forward; and shRNAmir-CD147-2F (5'-AATTTCCGAGGGCAGTAGGCCACGGGACCCAGAATGCAAAATAG-TGAAGCCACAGATGTATTTGTCATTC-3'), reverse; and shRNAmir-CD147-2R (5'-GGCTCCTGCACTGGAGCGCCCAATATCACATCTGGGTCGCCGCTCTGCCCTCCTGCTCACTGCAACAGCTACATCTCC-3'), reverse. For the generation of the scrambled shRNAmir control, we used the oligonucleotides shRNAmir-CD147-scf (5'-TCGAGAAGGTATTGCTGTTGACCGAGCCGGTCAGAGCTACACATTTACATTTGCACTACTTCC-3'), reverse.

To generate viral particles, 2.5 x 10^7 exponentially growing Jurkat cells were electroporated with 10 μg of either pCMV6M-Pak1-Myc or pCMV5M-Pak1-(165–544) alone or in combination with the indicated expression plasmids. Supernatants were collected, passed through 0.45-μm filters, and 1 x 10^6 Jurkat cells were centrifuged and resuspended in the corresponding retroviral supernatant supplemented with Polybrene (8 μg/ml, Sigma). After this step, Jurkat cells were centrifuged for 45 min at 1500 g and three consecutive cycles of infection were performed as above.
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![Image](image_url)

**FIGURE 1.** CD147 is expressed in the T-cell Jurkat cell line. A, RT-PCR analysis to detect CD147 in two independent cultures of Jurkat cells. As positive control, we included amplification using a human CD147-encoding plasmid as template (lane labeled as Plasmid). As negative control, we performed PCR analysis in the absence of the first step of cDNA synthesis (lane labeled No RT). The molecular weight marker (the δX174 DNA digested with HaeIII) is shown in the right lane. The mobility of the CDNA fragment corresponding to human CD147 is indicated by an arrow on the left. B, Jurkat cells were stained with either no primary antibody (left panel) or with a mouse anti-human CD147 antibody (right panel), counterstained with a secondary antibody to mouse IgGs, and subjected to flow cytometry analysis as indicated under "Experimental Procedures." A minimum of 10,000 live cells were scored in each experiment. Similar results were obtained with a goat anti-human CD147 antiserum (data not shown). C, exponentially growing Jurkat cells were either left untreated (−) or treated (+) with tunicamycin as indicated under "Experimental Procedures." After this period, cellular extracts were obtained and immunoprecipitated with anti-CD147 antibodies. After SDS-PAGE, the immunoprecipitates were analyzed by Western blot using anti-CD147 antibodies. The mobility of the glycosylated and nonglycosylated forms of CD147 are indicated by an arrow and an arrowhead, respectively.

Expression of CD147 in Jurkat Cells—To investigate the role of CD147 in TCR downstream signaling, we used the T-lymphoblastic Jurkat cell line as an experimental model. RT-PCR using Jurkat-derived cDNAs (Fig. 1A), flow cytometry with anti-CD147 antibody-stained Jurkat cell cultures (Fig. 1B), and anti-CD147 immunoblot analysis (Fig. 1C) confirmed the expression of CD147 in this T-lymphoblastoid cell line. As expected (57–60), further experiments confirmed that this protein was glycosylated in Jurkat cells, as determined by its shift in electrophoretic mobility upon incubation of these cells with tunicamycin, a glycosylation inhibitor (Fig. 1C). These results indicated that Jurkat cells express a glycosylated version of CD147 at the cell surface, suggesting that it may play functional roles in T-lymphocytes.

CD147 Translocates to the T-cell/B-cell Immune Synapse—Next, we analyzed whether CD147 could translocate to the immune synapse. These structures, formed in the context of T-cells bound to antigen-presenting cells, cluster MHC-antigen-TCR complexes and in addition engage different intracellular and transmembrane proteins such as integrins, adaptor proteins, protein-tyrosine kinases, and signaling molecules (56). To check this possibility, we first used time-lapse microscopy to follow the subcellular localization of an ectopically expressed CD147 in Jurkat cells during the formation of the immune synapse with superantigen-loaded Raji cells. To facilitate these real time studies, we used a version of CD147 tagged at its C terminus with the EGFP. In nonconjugated Jurkat cells, the CD147-EGFP fusion protein was located in the plasma membrane and, in trace amounts, in the Golgi apparatus (Fig. 2A, upper panel on the left). Upon recognition of a superantigen-carrying B-cell, the CD147-EGFP translocated to the immune synapse (Fig. 2A). The concentration of this protein in the immune synapse was similar to that observed with other immune synapse-localized proteins such as RasGRF2, Vav1, RasGRP1, or the CD3ζ subunit (data not shown, but see Figs. 2B and 5B). As negative control, we could not see the translocation of EGFP or the red fluorescent protein to the immune synapse under the same experimental conditions (data not shown).

To verify that this translocation step also took place in the case of the endogenous protein, we stained both unconjugated T-cells and T-cell/B-cell conjugates with an anti-CD147 antibody. As a control for immune synapse localization, we used the localization of both total tyrosine-phosphorylated proteins and of a CD3ζ-EGFP chimera. As shown in Fig. 2B (top panel on the left), the endogenous CD147 was localized dispersed through all areas of the plasma membrane in isolated Jurkat cells. Unlike the case of the ectopic CD147-EGFP, no signal was observed in the Golgi apparatus, indicating that the endogenous protein is efficiently and rapidly transported to the plasma membrane upon its biosynthesis (Fig. 2B, top panel on the left). In the case of T-cell/B-cell conjugates, the endogenous CD147 was preferentially observed in the immune synapse, with reduced localization in the areas of the plasma membrane not involved in the formation of that structure (Fig. 2B, 2nd top panel from left). This distribution is highly reminiscent of the distribution of the CD3ζ-EGFP in T-cell/B-cell conjugates (Fig. 2B, 2nd bottom panel from left). The tyrosine-phosphorylated proteins were detected predominantly at the immune synapse with no traces in the rest of the plasma membrane (Fig. 2B, 4th top panel from left). Interestingly, these studies indicated that the anti-CD147 antibodies did not detect the endogenous CD147 protein in the Raji B-cells regardless of whether they were free or bound to T-cells (Fig. 2B, see 1st, 2nd and 5th top panels from left), indicating that the expression of CD147 is not ubiquitous. Taken
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To further characterize the role of CD147 during T-cell responses, we performed luciferase reporter assays to test the possible influence of this transmembrane protein in the modulation of the activity of the NF-AT, a transcriptional factor located downstream of the TCR and Vav1 pathways that is crucial for both T-cell activation and differentiation (43, 44). To this end, we transfected Jurkat cells with the luciferase reporter plasmids and, when appropriate, with a Vav1-encoding vector with or without increasing amounts of a mammalian expression vector encoding the full-length CD147 protein with a C-terminal HA tag. After transfection, cells were left nonstimulated or stimulated with anti-CD3 antibodies, and the levels of NF-AT activity in different conditions were determined as indicated under “Experimental Procedures.” As shown in Fig. 3 (upper panel), CD147-HA induced a dose-dependent inhibition of the Vav1-mediated stimulation of NF-AT. A similar inhibition was observed when a CD147 protein tagged at its C terminus with the FLAG epitope was used (data not shown). The effect of CD147 on NF-AT was not because of improper expression levels of Vav1, as determined by immunoblot experiments (Fig. 3, lower panels).

To identify the structural determinants involved in this inhibitory activity, we generated a collection of CD147 mutants (Fig. 4A). Those included a truncation mutant of CD147 containing only the intracellular tail fused to the C terminus of the GST protein (Fig. 4A, protein 1); a truncated CD147 molecule lacking most of the intracellular domain and with a C-terminal Myc epitope (Fig. 4A, protein 2); the isolated intracellular region of CD147 fused to a myristoylation signal and the EGFP at the N and C terminus, respectively (Fig. 4A, protein 3); and a full-length, HA-tagged version of CD147 containing two missense mutations (C123A/C183A) that disrupt the Ig-like domain present in the extracellular region of the protein (Fig. 4A, protein 4). The expression of these proteins in Jurkat cells indicated that the majority of these proteins could be transported to the plasma membrane as efficiently as the wild type protein (Fig. 4B, 1st and 3rd to 5th panels from left). The only exception was the GST fusion protein containing the CD147 cytoplasmic domain, which displayed an entirely cytoplasmic distribution (Fig. 4B, 2nd panel from left). The CD147C123A/C183A mutant had a predominant localization in the plasma membrane although, in this case, a significant fraction of the protein was seen at the Golgi apparatus (Fig. 4B, 5th panel from left).

When these mutant proteins were co-transfected with Vav1 in NF-AT assays, we observed that the inhibitory action of CD147 was dependent exclusively on the cytoplasmic tail in a subcellular localization-dependent manner. Thus, we found that the myristoylated CD147 cytoplasmic tail abolished Vav1-induced NF-AT activation as effectively as the full-length CD147-HA protein (Fig. 4C, upper panel). However, such inhibitory activity did not occur when the CD147 intracellular tail was expressed cytoplasmically (Fig. 4C, upper panel). No inhibitory activity was detected in the case of CD147 mutants containing the extracellular domain but lacking the tail region (Fig. 4C, upper panel). In agreement with these results, the CD147 mutant protein with a disrupted Ig-like domain still displayed an inhibitory activity in this assay (Fig. 4C, upper panel). Immunoblot experiments confirmed that all proteins were properly expressed in the appropriate samples (Fig. 4C, lower panels), confirming that the NF-AT readouts are a direct consequence of the intrinsic signaling properties of each mutant CD147 protein. These results indicate that CD147 plays inhibitory roles in
both the Vav1 and NF-AT pathway and that such function is mediated by signals engaged by the cytoplasmic tail of this transmembrane protein.

**CD147 Does Not Affect the Phosphorylation/Activation of Vav1 or the Subcellular Localization of Activated Vav1**—Vav1 is a Rac1 GDP/GTP exchange factor whose enzyme activity is dependent on direct tyrosine phosphorylation by upstream, TCR-triggered protein-tyrosine kinases (61, 62). To verify whether the inhibition of NF-AT by CD147 was established at this regulatory level, we investigated whether the overexpression of CD147 could alter the TCR-mediated phosphorylation of Vav1. To this end, we immunoprecipitated Vav1 from total cell extracts derived from anti-CD3-stimulated Jurkat cells expressing the indicated combinations of proteins and monitored its phosphorylation levels by Western blot analysis using antibodies to either total phosphotyrosine residues or to the phosphorylated Tyr174 position of Vav1 (52). Tyr174 is the key regulatory site that mediates the phosphorylation-dependent activation of Vav1 during cell signaling and, therefore, is a good marker to track down the activated fraction of this exchange factor (62–64). As shown in Fig. 5A (upper panels), the levels of total phosphorylation and Tyr174-specific phosphorylation of Vav1 triggered by the TCR were similar regardless of the expression levels of CD147-HA in the samples, indicating that CD147 does not interfere with the upstream activation of Vav1 by tyrosine kinases.

Next, we investigated whether CD147 could affect the subcellular localization of activated, phosphorylated Vav1 proteins. To this end, we monitored the subcellular localization of activated Vav1 in T-cell/B-cell conjugates in the presence or...
absence of overexpressed CD147-HA using an antibody to the phosphorylated Tyr174 residue of Vav1. We found that Tyr(P)174 Vav1 was detected in the immune synapse of T-cells regardless of the CD147 overexpression status (Fig. 5B, 2nd panel from left).

To further confirm that CD147 was not affecting the upstream activation of Vav1, we finally evaluated the effect of overexpressing CD147-HA in the NF-AT stimulation triggered by Vav1Y3xF, a mutant version of Vav1 that shows enhanced GTP/GTP exchange activity because of Tyr to Phe mutations in the inhibitory Tyr142 site and in the adjacent Tyr142 and Tyr160 positions (52). Because of these mutations, this protein is highly active and becomes oncogenic (52, 65). We speculated therefore that if CD147 were acting upstream of Vav1, it should not display inhibitory activity on this Vav1 mutant. Contrary to this hypothesis, we observed that CD147-HA abrogated both the basal and the TCR-stimulated activity of NF-AT triggered by both wild type Vav1 and the Vav1Y3xF mutant (Fig. 5C, left panel). As before, this inhibition was not because of significant effects on the expression of Vav1 proteins, as determined by anti-Vav1 immunoblot analysis (Fig. 5C, upper right panel). As described previously (52), Vav1Y3xF was detected at lower levels than its wild type counterpart because of its preferential distribution in the insoluble, cytoskeletal enriched fractions eliminated during the centrifugation step of the cell lysates. These assays also confirmed the similar expression of CD147-HA in the appropriate samples (Fig. 5C, bottom right panel). Taken together, these observations suggest that CD147 inhibits NF-AT activation by interfering with pathways located either downstream of or in parallel to Vav1.

CD147 Inhibits Selectively the Activation of Both JNK1 and Pak1 Mediated by Vav1 and Rac1Q61L

To assess the possibility that CD147 was interfering with Vav1 downstream pathways, we investigated the effect of overexpressing the CD147-HA protein in the activation of JNK1, a well known downstream element of the Vav1 route (66). To that end, we expressed a FLAG-tagged version of JNK1 in Jurkat cells alone or in combination with Vav1Y3xF plus/minus increasing concentrations of CD147-HA. After this step, we immunoprecipitated the FLAG-JNK1 and monitored the enzyme activity of the kinase in each of those experimental conditions using in vitro kinase reactions. Similarly to the results obtained in NF-AT assays, we observed that CD147-HA induced a dose-dependent inhibition of the activity of JNK1 triggered by the Vav1Y3xF mutant (Fig. 6, A and B, upper pan-
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**FIGURE 6.** CD147 inhibits selectively downstream Vav1 and TCR pathways. **A,** CD147 inhibits the JNK activity induced by Vav1Y357F. Anti-FLAG immunoprecipitates (IP) derived from the indicated transfections (top) were subjected to IKRs as indicated under “Experimental Procedures.” Reactions were processed as in A and subjected to autoradiography to develop the radioactive signals (upper panel). Aliquots of the same lysates were analyzed by Western blot (WB) with the indicated antibodies (right) to monitor the expression levels of Vav1Y357F (2nd panel from top), CD147-HA (3rd panel from top), and FLAG-JNK1 (bottom panel). The mobility of the 32P-labeled substrate (ATF2) and ectopic proteins is indicated by arrowheads (left). The decreasing size of + signs in the CD147-HA lane (top) reflect the serial reduction in the amount of the CD147-HA-encoding vector used in the transfections (10, 5, 3, 1, and 0.5 µg). **B,** CD147 inhibits JNK activity induced by constitutively active Rac1. Anti-FLAG immunoprecipitates derived from the indicated transfections (top) were subjected to IKRs as indicated in A. The dried SDS-polyacrylamide gel was finally exposed to autoradiography films to develop the radioactive signals (two top panels, each of them showing different exposure times of the same experiment). Aliquots of the same lysates were analyzed by Western blot with the indicated antibodies (right) to monitor the expression levels of Vav1Y357F (3rd panel from top), AUS-Rac1Q61L (4th panel from top), CD147-HA (5th panel from top), and FLAG-JNK1 (bottom panel). The mobility of the 32P-labeled substrate (ATF2) and ectopic proteins is indicated by arrowheads (left). **C,** CD147 inhibits Pak1 activation by Rac1Q61L. Anti-Myc immunoprecipitates derived from the indicated transfections (top) were subjected to IKRs as indicated under “Experimental Procedures.” Reactions were processed as in A and subjected to autoradiography to develop the radioactive signals obtained in each condition (upper). Aliquots of the same lysates were analyzed by immunoblot analysis with the indicated antibodies (right) to monitor the expression levels of AUS-Rac1Q61L (2nd panel from top), CD147-FLAG (3rd panel from top), and Pak1-Myc (bottom panel). The mobility of the 32P-labeled substrate (myelin basic protein) and ectopic proteins is indicated by arrowheads (left). **D,** CD147 does not affect the activation of JNK1 induced by MEKK1 (600–672) (left panel) or the intrinsic kinase activity of Pak1165–544 (right panel). Anti-JNK (left panel) or anti-Pak1 (right panel) immunoprecipitates obtained from the indicated transfections (top) were subjected to IKRs as indicated in A and C. Panels show the autoradiography obtained in one of the representative IKRs. The mobility of the 32P-labeled ATF2 and myelin basic protein is indicated by arrowheads in the left and right panel, respectively. **E,** CD147 does not disrupt the activation of H-Ras by RasGRF2. Cell lysates derived from the indicated transfections (top) were subjected to GTP-H-Ras pulldown assays as indicated under “Experimental Procedures.” Bound proteins were electrophoresed, transferred onto nitrocellulose filters, and analyzed by anti-HA immunoblot to reveal the levels of GTP-bound H-Ras in each experimental condition (upper panel). Aliquots of the same lysates were analyzed by Western blot with the indicated antibodies (right) to determine the expression levels of RasGRF2 (2nd panel from top), CD147-FLAG (3rd panel from top), and HA-H-Ras (bottom panel). The mobility of the indicated proteins is shown with arrowheads (left). **F,** CD147 does not affect ERK1 activation downstream of the TCR. Anti-HA immunoprecipitates derived from the indicated transfection (top) and stimulation (bottom) conditions were analyzed by serial immunoblot analysis using anti-phospho-ERK (upper panel) and anti-HA (2nd panel from top) antibodies. Selected aliquots of the lysates obtained from nonstimulated cells were immunoblotted with the indicated antibodies (right) to detect the expression of CD147-FLAG (3rd panel from top) and HA-ERK1 (bottom panel). The mobility of the detected proteins is indicated by arrowheads (left).
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To confirm that the inhibitory action of CD147 was not a general action on TCR-dependent signals, we carried out four independent control experiments. First, we demonstrated that CD147 did not affect the stimulation of JNK1 triggered by a constitutively active mutant of MEKK1 (Fig. 6D, left panel), a Rac1 downstream element that participates in JNK activation (68). Second, we observed that CD147 was also ineffective at inhibiting the intrinsic kinase activity of a constitutively active version of Pak1 (Fig. 6D, right panel). Finally, we demonstrated that CD147 did not have any effect on the activation of H-Ras triggered by another GDP/GTP exchange factor (RasGRF2, Fig. 6E) or in the activation of ERK1 induced by TCR cross-linking (Fig. 6F). Because ERK1 kinase activation requires the Vav1/Rac1 pathway in lymphocytes (47–50), these results indirectly suggest that CD147 only affects a subset of the signaling branches that emanate from Vav1 and Rac1. Taken collectively, these results indicate that CD147 affects specific signal transduction pathways of the Vav1/Rac1 route. In addition, the lack of effect of CD147 on activated mutants of MEKK1 and Pak1 suggests that the action of CD147 takes place somewhere between Rac1 and its downstream elements JNK1 and Pak1.

The CD147 mRNA Knockdown Promotes the Hyperphosphorylation of Pak1 upon TCR Engagement—Finally, we used shRNAmir technology to investigate the effect of the CD147 knockdown in the signal transduction pathways of Jurkat cells. To this end, we used a retrovirus-based approach to generate a Jurkat cell pool containing an shRNAmir directed against the human CD147 transcript. In addition, we engineered two control Jurkat cell pools, one derived from a mock infection and a second one generated using a scrambled shRNAmir-containing retrovirus. To further explore the inhibitory effects of CD147 in the signal transduction pathway of Vav1 and Rac1, we next tested the influence of that protein in the stimulation of a second Vav1/Rac1 downstream element, the serine/threonine kinase Pak1 (67, 68). These experiments indicated that CD147-HA inhibited both the basal and the Rac1-induced activation of Pak1 (Fig. 6C, upper panels) without affecting the expression levels of either Rac1 or Pak1 (Fig. 6C, lower panels). These results indicate that CD147 exerts a global inhibitory effect on the downstream elements of the Vav1/Rac1 route.

CD147-HA is acting downstream of Vav1 and Rac1. Immunoblot analyses confirmed that the expression levels of Vav1 Tyr33F, Rac1G61L, and FLAG-JNK1 were not affected by the overexpression of CD147-HA (Fig. 6, A and B, lower panels).

To further explore the inhibitory effects of CD147 in the signal transduction pathway of Vav1 and Rac1, we tested the influence of that protein in the stimulation of a second Vav1/Rac1 downstream element, the serine/threonine kinase Pak1 (67, 68). These experiments indicated that CD147-HA inhibited both the basal and the Rac1-induced activation of Pak1 (Fig. 6C, upper panels) without affecting the expression levels of either Rac1G61L or Pak1 (Fig. 6C, lower panels). These results indicate that CD147 exerts a global inhibitory effect on the downstream elements of the Vav1/Rac1 route.

FIGURE 7. Endogenous CD147 contributes to the down-modulation of Pak1 and NF-AT activities. A, effective CD147 knockdown in Jurkat cells. Stable Jurkat cell pools derived from either a mock infection or from infections with retrovirus containing the CD147 shRNAmir or a control shRNAmir containing a sequence of scrambled nucleotides (top) were generated as indicated under “Experimental Procedures.” Total cell extracts from these pools were subjected to immunoblot analysis with the indicated antibodies (right) to detect the expression levels of endogenous CD147 (upper panel), the ectopic EGFP (middle panel), and the endogenous tubulin (bottom panel). The electrophoretic mobility of the detected proteins is indicated by arrowheads (left). B, CD147 knockdown reduces the surface expression of this protein in Jurkat cells. The indicated Jurkat cell pools (insets) were analyzed by flow cytometry to quantify the expression levels of CD147 in the plasma membrane. A minimum of 10,000 live cells was scored in each condition. The line inside the histogram indicates the area of the fluorescence that was considered as CD147+. The estimated percentage of CD147+ cells in each clone is indicated above those lanes. C, CD147 knockdown favors higher levels of NF-AT activity in Jurkat cells. The indicated Jurkat cell pools (top) were stimulated with anti-CD3 for the indicated periods of time (top) and lysed. Clarified extracts were then subjected to immunoblot analysis with the indicated antibodies (right) to monitor the phosphorylation and total protein levels of JNK, Pak1, and ERK1. As a control, cell lysates were also monitored for the expression of tubulin (bottom panel).

Transcriptional and translational experiments.
CD147 Inhibits NF-AT Activity during T-cell Signaling

These pools were tested in two independent functional assays. First, we performed NF-AT transactivation assays by transfecting the pNF-AT reporter plasmid and measuring the NF-AT-dependent luciferase activity in nonstimulated and anti-CD3 stimulated Jurkat cells. These experiments indicated that the CD147 knockdown pool had approximately a 5-fold higher basal activity than the one containing the scrambled shRNAmir construct. This higher NF-AT activity was maintained, although at lower levels (~1.45-fold change) upon TCR cross-linking (Fig. 7C). Second, we evaluated the consequence of the CD147 knockdown in the TCR-dependent activation of serine/threonine kinase cascades implicated in T-cell functions. To that end, total cellular extracts obtained from Jurkat cell pools stimulated for the indicated periods of time with anti-CD3 antibodies were analyzed by immunoblot using phospho-specific antibodies to phosphorylated JNK, Pak, and ERK family members. As loading controls, aliquots of the same lysates were analyzed in parallel with antibodies to JNK, Pak, ERK, and tubulin. We observed that the CD147 knockdown had no major effects in the kinetics and/or levels of phosphorylation of JNK or ERK family proteins (Fig. 7D, 1st and 5th panels from top, respectively). Instead, we found that Pak1 displayed much higher phosphorylation levels in the CD147 shRNAmir-containing Jurkat pool than in that harboring the scrambled shRNAmir (Fig. 7D, 3rd panel from top). In all cases, we did not observe any consistent change in the total expression levels of JNK, Pak, or ERK proteins (Fig. 7D, 2nd, 4th, and 6th panel from top, respectively). Likewise, comparable amounts of lysates were used, as determined by the similar level of tubulin in all the samples (Fig. 7D, bottom panel). Taken together, these results indicate that CD147 appears to play a major role in controlling the activation kinetics of Pak1 and the stimulation levels of the transcriptional factor NF-AT. The moderate (in the case of NF-AT activity) or lack of effect (in the case of JNK activity) of the CD147 knockdown in TCR-stimulated cells can be explained if we consider that, unlike the Rac1-dependent activation of Pak1, the stimulation of those two signaling pathways by the TCR relies on both Vav1/Rac1-dependent and Vav1/Rac1-independent signals (69–72). Alternatively, it is possible that the effective inhibition of NF-AT and JNK signals may require much stronger signals than those needed for the blockage of Pak1 and therefore that its phosphorylation can normally proceed under the TCR stimulation conditions used in our tissue culture experiments.

DISCUSSION

The efficient, properly tuned, and homeostatic activation of T-lymphocytes during immune responses requires the simultaneous or, in same cases, sequential generation within the activated T-cell of intracellular signals that emanate from the TCR and other ancillary surface molecules. These ancillary proteins provide both positive and negative signals that modulate the intensity, temporal extension, and termination of the T-cell response to an specific antigenic insult. For example, it is known that T-lymphocytes require the co-engagement of the CD28 molecule to bypass the anergy state and progress into the proliferative phase (73). Instead, CTLA-4 engagement is required for shutting down T-cell responses (74). As a consequence, disruption of the expression of this molecule induces a spurious stimulation of T-lymphocytes that induce lymphadenopathy, splenomegaly, elevated levels of plasma IgGs, severe myocarditis, and pancreatitis (74). Other negative T-cell surface molecules include CD95, CD148, killer cell inhibitory receptors, PD1, and B- and T-lymphocyte attenuator (75–78).

The results presented in this study indicate that CD147 probably belongs to this subset of negative regulatory receptors, at least is the context of some defined T-cell responses. Thus, we have observed that the overexpression of CD147 blocks Vav1-dependent pathways such as the activation of the NF-AT transcriptional factor and the stimulation of both JNK and Pak. These effects are rather specific, because other GDP/GTP exchange factors expressed in T-cells (i.e. RasGRF2 (55)) or intracellular responses (i.e. TCR-driven ERK activation) are not affected by the overexpression of this protein. In agreement with these results, our loss-of-function studies have shown that the knockdown of CD147 transcripts leads to enhanced NF-AT stimulation and Pak1 phosphorylation in T-cells upon TCR engagement. Again, this effect is specific, because other intracellular cascades function at normal levels and kinetics in the absence of CD147.

In agreement with the concept that CD147-mediated signals act downstream of Vav1, we have observed that CD147 does not affect the phosphorylation levels of Vav1, a post-translational modification that represents a condition sine qua non for the catalytic activation of this Rac1 GDP/GTP exchange factor (61, 62). CD147 also does not affect the subcellular localization of activated Vav1 proteins at the plasma membrane. Finally, we have demonstrated that CD147 inhibits the signals derived from a Rac1 mutant whose activation is totally Vav1-independent. Instead, we observed that the inhibitory activity of CD147 is lost when co-transfected with constitutively active downstream elements of the Vav1 and Rac1 route such as MEKK1 and Pak1, suggesting that CD147 signals interfere at the level of Rac1/effecter interaction or in immediate steps between Rac1, MEKK1, and Pak1. At this moment, the mechanism involved in this blockage is unknown. Based on previous evidence regarding the effect of CD147 cross-linking in the sequestration of co-receptors such as CD48 and CD58 away from lipid rafts (31), it can be argued that such inhibitory effect could be due to the removal of activated Rac1 from some specific subdomain of the plasma membrane that is not compatible with the binding of some effectors. However, preliminary experiments have shown that the overexpression of CD147 does not seem to alter the normal distribution of Rac1Q61L in sucrose gradient fractionation and immunofluorescence studies.5 Alternatively, it is possible that CD147 serves as an adaptor molecule for an inhibitor of Rac1 downstream signals or of Rac1 itself. In agreement with this possibility, we have shown that the inhibitory activity of CD147 toward the NF-AT response can be recapitulated with the constitutive expression of the CD147 cytosolic tail in the plasma membrane. The tethering of negative regulatory molecules via the intracellular region of other inhibitory co-receptors is not an unprecedented observation. Thus, it has been

5 S. Ruiz, A. Castro-Castro, and X. R. Bustelo, unpublished observations.
shown that CTLA-4/CD152, PD1, and B- and T-lymphocyte attenuator contribute to the down-modulation of TCR signals by recruiting phosphatases such as SHP-2 and/or PP2A (74, 75). CD148, a phosphatase itself, also binds to the intracellular signaling protein syntenin (78). We are currently performing both proteomic and two-hybrid experiments to identifying the proteins involved in the CD147 route. Interestingly, we have also observed that a CD147 protein with an inactive Ig-like domain (CD147C123A/C183A mutant) displays a reproducible higher inhibitory activity than the wild type version or the myristoylated CD147 tail when tested in NF-AT transactivation assays. Whether this is because of the elimination of interactions with other membrane proteins, to a differential localization in specific plasma membrane subdomains, or to lower internalization cycles remains to be determined.

To date, the exact role of CD147 in T-cells has been rather ambivalent. In favor of a negative role during TCR-dependent responses, previous studies have shown that the cross-linking of CD147 with specific antibodies to its extracellular region blocks TCR-mediated lymphocyte proliferation, an effect probably derived from the displacement of specific T-cell co-receptors (CD48, CD59) from lipid raft-enriched membrane fractions (31). Studies conducted in CD147−/− mice have also shown that CD147-deficient T-cells show more robust mitogenic responses during mixed lymphocyte reactions than their wild type counterparts (25). Finally, it has been shown that an antibody-mediated CD147 receptor cross-linking leads to a thymocyte maturation block (33). In contrast to these results, there are observations compatible with a positive action of CD147 in T-cells. For example, it has been demonstrated that CD147 favors T-cell chemotaxis by binding to extracellular cyclophilins (7, 34). CD147 also promotes interactions of lymphoid cancer cells with normal fibroblasts that favor the synthesis and secretion of MMP2 by the latter cells (30). The frequent overexpression of CD147 by different types of T-cell lymphomas is also an indirect observation that favors the concept of a positive role for this transmembrane protein (30), at least in specific aspects of cancer T-cell biology. Although apparently contradictory, this functional duality is not entirely puzzling because it is possible that CD147 plays negative roles in the context of TCR-dependent responses and positive roles in other functional scenarios such as adhesion/migration-dependent pathways or integrin signaling. Indeed, antagonistic functions are not a rare event in the T-cell co-receptor field (74).

It has been shown before that Pak is required for NF-AT activation in T-cells (67), so it is quite possible that the inhibitions of NF-AT and Pak1 by CD147 shown here are intertwined in the same biological response. Notwithstanding these observations, we cannot exclude the possibility that CD147 may also affect the intensity of NF-AT signals by interfering with additional pathways. The NF-AT response involves a complex series of intracellular signaling proteins such as phospholipase C-γ1, the GDP/GTP exchange factors RasGRP1 and RasGRF2, calcineurin, and a complex subset of transcriptional factors such as NF-AT and AP1 family members (43, 44, 51, 55). Therefore, it will be interesting to address in the near future whether CD147 action is circumscribed to the Vav1/Rac1/Pak1 route or, alternatively, whether it can be generalized to other elements that participate in this signaling network.

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