

Identification of SAP as a CTLA-4 Binding Molecule: a Role of SAP in CTLA-4 Signaling Proposed

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ABSTRACT

Background: The precise mechanism by which CTLA-4 regulates T cell immune responses is still not fully understood. Previously we proposed that CTLA-4 could down-regulate T cell function by modulating a signaling cascade initiated from the T cell receptor complex. The evidence for this notion comes from our findings that CTLA-4 associated with the T cell receptor zeta (TCR zeta) chain, and hence regulated TCR zeta phosphorylation by co-associated SHP-2 tyrosine phosphatase (1). In this report, we investigated whether any other signaling molecules could be involved in the CTLA-4 signaling pathway. **Methods:** We have taken biochemical approaches, such as immunoprecipitation followed by autoradiography or immunoblotting, to identify the molecules associated with CTLA-4. To perform these assays, we used activated primary T cells and ectopically transfected 293 cells. Various truncation mutants of CTLA-4 were used to map the interaction site on CTLA-4. **Results:** We found that in addition to TCR zeta and SHP-2, a recently cloned small adaptor molecule, SAP (SLAM-associated protein), was also able to associate with CTLA-4. We identified the domain of SAP association in CTLA-4 being a motif involving GVVVKM. This motif has been previously found to bind SHP-2 through its phosphorylated tyrosine interaction with SH-2 domain of SHP-2. Indeed, co-expression of SAP and SHP-2 reduced their binding to CTLA-4 significantly, suggesting that SAP and SHP-2 compete for the common binding site, GVVVKM. Thus, by blocking SHP-2 recruitment SAP could function as a negative regulator of CTLA-4. **Conclusion:** Taken together, our data suggest the existence of a complicated signaling cascade in regulating CTLA-4 function, and further provide evidence that SAP can act either as a positive or negative regulator depending on the nature of the associating receptors. (*Immune Network* 2002;2(2):72-78)

Key Words: CTLA-4, SAP, SHP-2, tyrosine kinase, transfection, T cells

Introduction

T cell activation depends on the transduction of at least two signals: one via the TCR/CD3 complex through recognition of an MHC/peptide structure, and a second via a co-stimulatory molecule expressed on the surface of professional APCs. In the absence of co-stimulation, T cells become incapable of secreting IL-2 and fail to proliferate, which then leads to the programmed cell death (2,3). CD28 is one of the most potent co-stimulatory receptor that is constitutively expressed on all mouse T cells (4). Upon engagement with its ligands B7-1 and B7-2, CD28 provides co-stimulatory signals that enhance and sustain IL-2 secretion as well as proliferation. In addition, CD28 also mediates strong upregulation of

T cell cytokines, IL-4, IL-5, IL-13, γ -interferon, tumor necrosis factor α , and GM-CSF (5-7). In contrast, CTLA-4, a CD28 homologue that shares its ligands, functions as a negative regulator that restricts IL-2 production, and cell cycle progression (8,9). The most compelling evidence for a regulatory function of CTLA-4 has come from CTLA-4 knockout mice that develop fatal lymphoproliferative disease at 3~4 weeks of age (10,11). This phenotype results from polyclonal activation of peripheral T cells that then infiltrate and cause multi-organ destruction. These studies suggest that CTLA-4 also plays a role in autoimmunity by regulating peripheral homeostasis.

The mechanism by which CTLA-4 regulates T cell immune responses has been under intensive investigation, and the common ground for the current model is that CTLA-4 antagonizes TCR signals to set a higher threshold so that the unwanted T cell activation that might have resulted from weak agonist engagement can be prevented (12,13). Consistent with

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this model, we have shown recently that CTLA-4 forms multimeric complexes with T cell receptor (TCR) ζ chain and a tyrosine phosphatase, SHP-2, in activated T cells (1,14). Using a series of *in vitro* transfection studies, we were able to demonstrate that co-associated SHP-2 can regulate CTLA-4-associated TCR ζ phosphorylation. Indeed, CTLA-4 expressed in activated T cells was shown to be associated with lower phosphorylated species of TCR ζ (mainly p16), but not with the highly phosphorylated p23 TCR ζ . Since TCR ζ phosphorylation serves as a critical proximal signal transducer upon TCR stimulation, we hypothesized that by lowering the extent of TCR ζ phosphorylation CTLA-4 may ultimately antagonize TCR signal transduction, and result in T cell inhibition.

CTLA-4 possesses 36 amino acids in its cytoplasmic tail. Our studies using various truncation mutants of CTLA-4 has shown that a membrane proximal sequence of CTLA-4 bearing only 11 intracytoplasmic amino acids (C-11) is responsible for TCR ζ association while a distal domain of CTLA-4 involving GVVVKM motif was engaged in binding to SHP-2¹. These data demonstrate that SHP-2 and TCR ζ bind to distinct motif in CTLA-4 cytoplasmic domain.

Recently, Sayos et al have cloned SAP as a SLAM associating molecule using a yeast two-hybrid approach. In this report, SAP was found to play a critical role in signal transduction initiated by SLAM, and the mutation of SAP, which prevented its binding to SLAM, was attributed to defective function of CD4 T cells and hence resulted in X-linked lymphoproliferative disease (XLP) (15,16). Subsequent studies demonstrated that 2B4, an NK cell activating receptor, also associated with SAP, and was shown to function as a positive regulator of 2B4 signaling in human NK cell lines (17,18). Our initial attempt to understand the CTLA-4 signal transduction pathway was to identify the CTLA-4 associating proteins, and one of the proteins associated with CTLA-4 migrated approximately 15 kDa, whose molecular weight is similar to that of SAP. Indeed, we show in this report that this 15 kDa protein associated with CTLA-4 was found to be SAP. We also found that SAP binds to the GVVVKM motif of CTLA-4, and act as a negative regulator of SHP-2 binding. Thus, SAP plays an important role in CTLA-4 signaling by modulating the level of SHP-2 association.

Materials and Methods

Mice and cell culture. Female BALB/c mice of 6~8 weeks of age were purchased from Fredrick Cancer Research Facility (Frederick, MD) and used for isolating T cells. 293 human embryonic kidney epithelial

cells were obtained from ATCC. Cells were cultured at 37°C, 10% CO₂, in DMEM (Life Technologies, Grand Island, NY) supplemented with 5% or 10% FCS (Hyclone), 25 mM HEPES (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO), 100µg/ml streptomycin (Sigma-Aldrich), 2 mM nonessential amino acids (Life Technologies), and 5µM 2-ME (Sigma-Aldrich). 293 cells were lifted from plastic adherence by incubation with a 0.02% solution of Na₂EDTA (Sigma-Aldrich) in PBS. All tissue cultureware was purchased from Becton Dickinson (Franklin Lakes, NJ).

Antibodies. Anti-CTLA-4 monoclonal Abs (UC10- 4F10; (19)) anti-CD3 (145-2C11; (20)), anti-CD28 (PV-1; (21)) were previously described and kind gifts from Dr. Jeffrey Bluestone (UCSF, San Francisco). Monoclonal anti-hemagglutinin (anti-HA, 12CA5) was purchased from Boehringer Mannheim. Mouse monoclonal anti-phosphotyrosine mAb (4G10) and polyclonal anti-SHP-2 Abs were purchased from Upstate Biotechnology Inc. Goat anti-murine CTLA-4 antiserum (Q20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids. The generation of cDNAs encoding full length and tailless murine CTLA-4 in the mammalian expression vector pCDNA 3.1 (+) (Invitrogen Corp., Carlsbad, CA) have been described previously (1,14). Four truncation mutants of murine CTLA-4 were generated from the full length cDNA by polymerase chain reaction (PCR) using the following primers: Sense (for all mutants): 5'-GAGCTCAAGCCTTATGGCTTGTCTTGGACTCCGG-3'; Antisense (C-27): 5'-GTTTCGCTCTAGATCATTTGCTTTTTCACATTCTGGCTC-3'; Antisense (C-23): 5'-GTTTCGCTCTAGATCATTTCTGGCTCTGTGGGGGC-3'; Antisense (C-17): 5'-GTTTCGCTCTAGATCACATTTTTCACATAGACCCCTG-3'; Antisense (C-11): 5'-GTTTCGCTCTAGATCATTTGTGTAAGAGGACTTCTTTTC-3'. Similarly, HA-tagged SAP was cloned by PCR using oligo dT-primed first strand cDNAs isolated from human PBMC as a template, and inserted into pCDNA 3.1(+) using HindIII/XbaI cloning sites. The forward primer consisted of GAGCTCAAGCCTTATGGCTTATCCTTATGACGTGCCTGACTATGCCGGCGACGCAGTGGCTGTGTATCATGGC and the reverse primer consisted of GTTTCGCTCTAGATCATGGGGCTTTCAGGCAGACATCAGG. Primers were composed of a redundant sequence (italics), restriction sites appropriate for ligation into the pCDNA 3.1(+) expression vector (bold), a stop codon (underlined antisense primers only), and sequence-specific residues (plain). The amino acid sequences of the intracellular region of each of the murine CTLA-4 constructs is illustrated in Fig. 4A.

All amplifications were carried out using the DNA polymerase mixture eLONGase (Life Technologies, Grand Island, NY) and a Geneamp 9600 thermal cycler (Perkin Elmer, Norwalk, CT). PCR products of the predicted length were digested with appropriate restriction endonucleases, purified from low melting point gels, and ligated into the pCDNA 3.1(+) expression vector. Appropriate expression of each construct was initially tested by transient transfection of 293 cells as previously described (21,22). *Metabolic labeling of activated T cells.* BALB/c whole LN and spleen cells were harvested and activated with anti-CD3 (1 μ g/ml) and anti-CD28 (1 μ g/ml) for 48 hours. Activated T cells (20×10^6 cells) were labeled with 35 S-methionine and 35 S-cysteine (Amersham, Arlington Heights, IL) for 4 hours in Methione and Cystein free media. Cells were washed with PBS containing 5% BSA, and subsequently lysed and processed as described in the below.

Transient transfection of 293 cells. Cells were transiently transfected with one or more plasmid constructs by calcium phosphate precipitation (23). The constructs and amounts of DNA used in individual experiments are indicated in the relevant Fig. legends. Transfectants were harvested for biochemical analysis 48h after transfection.

Immunoprecipitation and immunoblotting. Activated T cells or transiently transfected 293 cells were disrupted in lysis in buffer in LB [1% Nonidet P-40, 50 mM tris-HCl (pH 7.4), 150 mM NaCl, 20 mM EDTA (pH 8.0), 1 mM sodium vanadate, leupeptin (10 μ g/ml), 10 μ M aprotinin, 1 mM phenylsulfonylsulfoxide]. Immunoprecipitations were performed with lysates prepared. Lysates were precleared twice with protein A beads before precipitating antibodies were added. Immunoprecipitation was performed overnight at 4°C. Immune complexes were washed five times with LB and subjected to SDS-PAGE. Immunoprecipitates separated on a reducing SDS-12% polyacrylamide gel were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and subsequently immunoblotted (IB) with antibodies listed in the figures. Bound proteins were detected by chemiluminescence (Pierce, Rockford, IL) and quantitated using a Molecular Dynamics ImageQuant[®] densitometer.

Results

CTLA-4 coprecipitation of metabolically labeled proteins. To gain insight into the mechanism of which CTLA-4 transduces its signal, we tried to identify the interacting partners of CTLA-4. Since CTLA-4 expresses only upon activation, we first activated T cells by incubating with maximal dose of anti-CD3 (1 μ g/ml) and anti-CD28 Ab (1 μ g/ml) for 48 hours, and then

added S35-methionine and cystein to the cells for additional 4 hours to radiolabel all the metabolically active proteins. Subsequently, CTLA-4 was immunoprecipitated from cell lysates using mAb UC10-4F10. Upon subjection of CTLA-4 immune complexes to SDS-PAGE and subsequent autoradiography, we found that a number of proteins migrating from 15~26 kDa were co-precipitated with CTLA-4. Previous studies have revealed that a band running around 16 kDa was un- or low phosphorylated TCR ζ (1). More recent data suggested that a band migrating around 25~26 kDa was likely to be CD3 ϵ chain as antibodies against CD3 ϵ chain detected the same band (Fig. 1, arrow a). The association of TCR ζ and CD3 ϵ with CTLA-4 was specific as the p16 and p25~26 bands were absent in immunoprecipitates prepared using control IgGs or anti-CTLA-4 IgGs in the presence of saturating dose (100 μ g) of blocking CTLA-4 Ig (1). These data suggest and confirm the previous notion that CTLA-4 is closely associated with TCR complex, and may regulate TCR signaling.

Recently, SAP was cloned as a SLAM associating molecule, that plays a critical role in SLAM signaling through its ability to bind to tyrosine (Y281) of SLAM and prevent SHP-2 recruitment (16,18). Since CTLA-4 can bind SHP-2 and co-precipitate a protein running at 15 kDa, we tested if this 15 kDa protein might be SAP. As the specific antibodies against murine SAP was not available at the time of study, we decided to check their interaction using a transient transfection approach using 293 cells. Thus, we cloned human SAP by PCR using oligo-dT primed first strands cDNA prepared from human PBMC and subsequently inserted into mammalian expression

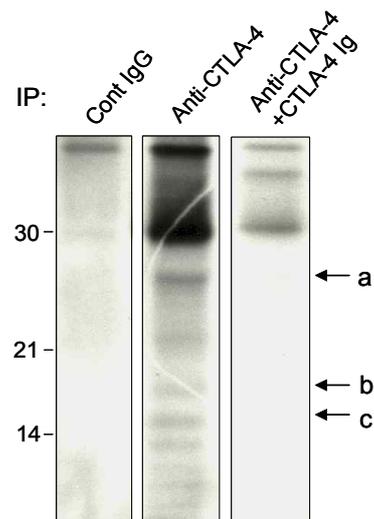


Figure 1.

vector pCDNA 3.1 (+). We added HA sequence it

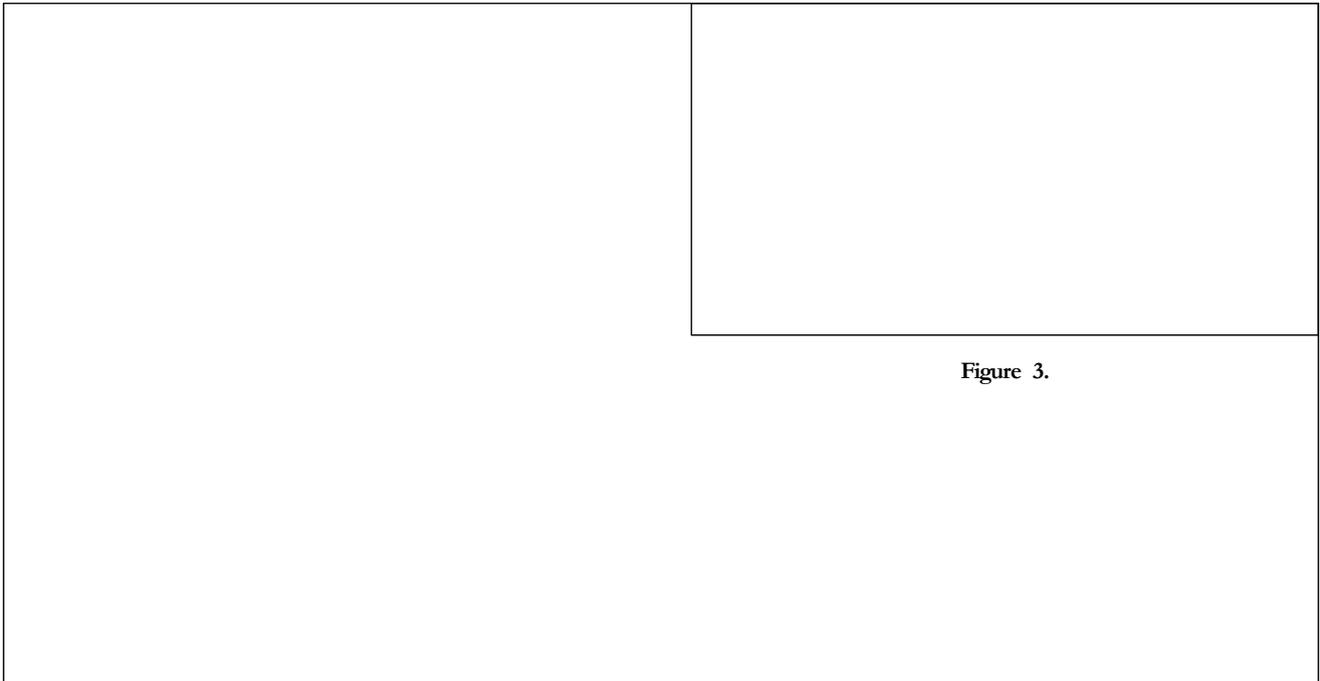


Figure 2.

its N-terminus as a tag. We then co-expressed SAP with murine CTLA-4 in 293 cells and their association was detected by immunoprecipitation and subsequent immunoblotting. As the cytoplasmic domain of human and murine CTLA-4 is identical, we used murine CTLA-4 as an alternative to human CTLA-4. As shown in Fig. 2A, SAP was present in anti-CTLA-4 immunoprecipitates (lower panel, lane 2), suggesting that the two proteins might be associated. Since lck is one of the very proximal tyrosine kinase activated upon TCR engagement, and has also been shown to phosphorylate CTLA-4 (14), the proximal signaling of TCR and CTLA-4 can be reconstituted more physiologically in the presence of lck. Indeed, coexpression of murine lck augmented SAP association with CTLA-4 (Fig. 2A, lower panel. Lane 3), indicating that either the phosphorylation of CTLA-4 or SAP enhanced the association. Interestingly, phosphotyrosine blot of the same gel showed heavy phosphorylation of CTLA-4, and some minor phosphorylation of SAP co-precipitated with CTLA-4 (Fig. 2B). Since the tyrosines that can be phosphorylated by lck are located within the SH-2 domain of SAP, which are likely to be involved in CTLA-4 binding, the increased SAP binding to CTLA-4 might be due to tyrosine phosphorylation of CTLA-4. Enhanced SAP binding was not due to increased SAP protein level or CTLA-4 level, as the amount of proteins expressed in each condition was comparable (Fig. 2A, upper panel and Fig. 2C). These data suggest that SAP can

interact with CTLA-4 and its binding may be regulated by lck-induced tyrosine phosphorylation. *SAP competes with SHP-2 for CTLA-4 binding.* We next investigated if SAP could act as an inhibitor of SHP-2 binding to CTLA-4, similar to that shown for SLAM binding (16). For this purpose, a plasmid encoding SHP-2 gene was co-transfected with plasmids encoding CTLA-4 and SAP and examined the effect of SHP-2 expression on SAP binding of CTLA-4 (Fig. 3). Since lck phosphorylates CTLA-4 and increases SHP-2 and SAP association, transfection was performed in the presence of lck. In the previous study, we showed that endogenous SHP-2 is present in 293

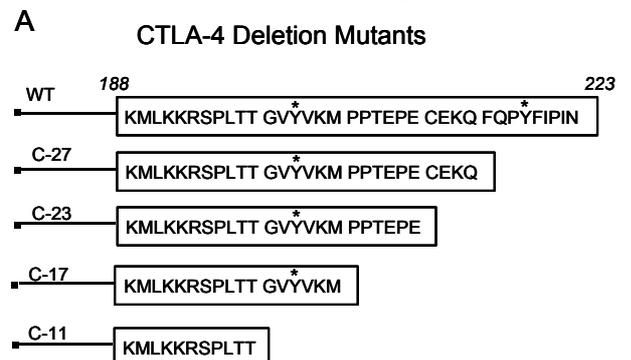


Figure 4.

cells and co-associated with CTLA-4. Indeed, when CTLA-4 was co-expressed with lck, and SAP (lane 1),

we observed the basal level of endogenous SHP-2 binding (upper panel, lane 1). The level of SHP-2 binding to CTLA-4 was significantly increased by SHP-2 overexpression (lane 2, upper panel). However, this augmentation was almost completely abolished in the presence of SAP expression (lane 3, upper panel). Similarly, the level of SAP association with CTLA-4 was significantly decreased when SHP-2 was co-expressed (lane 3 vs lane 1, lower panel). These data demonstrate that SHP-2 and SAP compete for CTLA-4 binding, and SAP could act as an inhibitor of SHP-2 in CTLA-4 function.

SAP binds to GVVVKM motif in CTLA-4. To determine the region of CTLA-4 responsible for SAP binding, we generated a series of cytoplasmic deletion mutants as illustrated in Fig. 4A. These mutants were transiently transfected into 293 cells in the presence of cDNAs encoding SAP and lck, and tested for their ability to bind SAP. All the mutants were expressed at the comparable level as shown by anti-CTLA-4 immunoblot (Fig. 4B, (a)). The CTLA-4 mutants, C-27, C-23, and C-17 became tyrosine phosphorylated when co-transfected with lck (Fig. 4B, (b)), indicating that these mutants could still recruit the tyrosine kinase, lck. In fact, anti-lck immunoblotting showed that all the CTLA-4 mutants were able to co-precipitate lck (data not shown). Mutants, C-27, C-23, C-17, were also able to co-precipitate SAP to the similar level as WT CTLA-4 does, however, C-11 mutant which lost GVVVKM motif failed to co-precipitate SAP (Fig. 4B (c)). The loss of SAP binding to C-11 mutant was not due to loss of SAP expression as SAP expression in each sample was comparable (Fig. 4B (d)). These data demonstrate that SAP binds to GVVVKM motif in CTLA-4. Since this motif is also engaged in SHP-2 binding, SAP can act as a competitive inhibitor of SHP-2 binding to CTLA-4 in activated T cells.

Discussion

In this study, we provided evidence that a recently cloned adaptor molecule, SAP, is associated with CTLA-4 and acts as an inhibitor of SHP-2 binding to CTLA-4. SAP was originally cloned as a SLAM associated protein, and has been shown to be crucial for SLAM function. SLAM is expressed on T cells and B cells, mediates homotypic binding and promotes T cell co-stimulation, proliferation and production of Th1 cytokines (Cocks et al., 1995; Aversa et al., 1997). During EBV infection, SLAM-SLAM interactions at the interface between EBV-infected B cells and T cells may promote the development of EBV specific T helper responses. The cytoplasmic domain of SLAM contains three tyrosine-based motifs; SAP binds to one of these and promotes signaling by preventing the recruitment of the tyrosine phosphatase SHP-2 (16). In XLPD patients, SAP deficiency and subsequent recruitment of SHP-2 may impair SLAM signal transduction, leading to the reduced IFN- γ production by T helper cells and/or reduced T cell help to EBV-specific cytotoxic T cells.

Recent studies have shown that SAP can bind not only to SLAM, but also to 2B4, an activating NK cell receptor mediated in MHC-independent NK cell killing (17). Indeed, 2B4-mediated NK cell activity was impaired in XLPD patients (24,25), suggesting that SAP is also critical in NK cell function. Similarly, our data show that SAP can also bind to CTLA-4 and block CTLA-4 signaling. Thus, the absence of functional SAP can result in crippling of several receptors, all of which can contribute to the lack of T and NK cell function. In the case of CTLA-4, SAP acts as a negative regulator of CTLA-4 and inhibits CTLA-4 function, thus, the net effect will be stimulatory. Therefore, SAP is required for stimulation of T and NK cell responses, and can act as a regulator for both innate and adaptive immune responses.

Recent studies suggest that CTLA-4 act both at the initiation and ongoing stage of T cell activation. CTLA-4 may exert its function by at least two different mechanisms-competing with CD28 and active intracellular signaling (26,27). Both pathways may be operational during the immune response depending on the availability of CD28 and B7s. The close link between CTLA-4 and TCR complex has been demonstrated in several laboratories including ours. CTLA-4 crosslinking resulted in inhibition of LAT (1), ERK and JNK activity (28). Furthermore, CTLA-4 localizes toward the TCR engagement site (29). These results implicate a role of CTLA-4 in the regulation of TCR signaling. In support of this model, we recently demonstrated that CTLA-4 associates with TCR complex by binding to ζ chain of TCR. Our

recent data showed that CTLA-4 associates with TCR ζ within its lysine-rich sequence, KMLKKRS, located in the juxtamembrane domain of CTLA-4¹. Likewise, the membrane proximal domain of TCR ζ involving the ITAM 1 motif seems to be involved in CTLA-4 binding¹, presumably through electrostatic interaction. In contrast, SHP-2 binding of CTLA-4 is mediated through the membrane distal region of CTLA-4 involving GVVVKM motif. Our data in this report showed that SAP was able to compete for SHP-2 binding, presumably by competing for the GVVVKM motif (Fig. 4B). Thus, in activated T cells, the effective CTLA-4 function will favor binding of SHP-2 over SAP. Since the majority of CTLA-4 expressed in 293 cells was localized at the cell surface (data not shown), we suspected that the interaction between CTLA-4 and SAP was taken place at the plasma membrane. However, it is also possible that the interaction can happen at the intracellular organelle, such as early endosome, as CTLA-4 is subject to internalization upon its ligation (30). Taken together, these data suggest that SAP may be a universal regulator of SHP-2 binding in T and NK cells, and control the activation of various receptors that utilize SHP-2 as their signaling partners.

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