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Modulation of T Cell Activation by Stomatin-Like Protein 2

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T cell activation through the Ag receptor (TCR) requires sustained signaling from signalosomes within lipid raft microdomains in the plasma membrane. In a proteomic analysis of lipid rafts from human T cells, we identified stomatin-like protein (SLP)-2 as a candidate molecule involved in T cell activation through the Ag receptor. In this study, we show that SLP-2 expression in human primary lymphocytes is up-regulated following in vivo and ex vivo activation. In activated T cells, SLP-2 interacts with components of TCR signalosomes and with polymerized actin. More importantly, up-regulation of SLP-2 expression in human T cell lines and primary peripheral blood T cells increases effector responses, whereas down-regulation of SLP-2 expression correlates with loss of sustained TCR signaling and decreased T cell activation. Our data suggest that SLP-2 is an important player in T cell activation by ensuring sustained TCR signaling, which is required for full effector T cell differentiation, and point to SLP-2 as a potential target for immunomodulation. The Journal of Immunology, 2008, 181: 1927–1936.

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5 Abbreviations used in this paper: IS, immunological synapse; LAT, linker for activation of T cells; PLC, phospholipase C; SEE, staphylococcal enterotoxin E receptor; siRNA, small interfering RNA; SLP, stomatin-like protein; SPFH, stomatin/prohibitin/flotillins/HflK-HflC.

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Materials and Methods

Cells

Human PBMC were isolated from heparinized whole blood of normal donors using Ficol-Hypaque gradients (Amersham Biosciences). Cells were washed in supplemented RPMI 1640 and resuspended at 1 × 10⁶ cells/ml. PBMC blasts were generated by culturing PBMC with PMA (1 ng/ml) and ionomycin (100 ng/ml) for 72 h at 37°C, 5% CO₂. T cell blasts were rested 48 h before use. Primary T cells were isolated from PBMC using a Pan T Cell Isolation Kit (Miltenyi Biotec). Jurkat T cells (E6.1) were obtained from American Type Culture Collection and cultured in supplemented RPMI 1640 medium. The B lymphoblastoid cell line LG2, used as APC in some of these experiments, was provided by E. Long (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD) and cultured in standard supplemented RPMI 1640 medium.

Plasmids, small interfering RNA (siRNA), and T cell transfectants

Human SLP-2 cDNA was subcloned into the pEGFP-N1 expression vector (BD Clontech) to create an in-frame translational fusion of SLP-2 and GFP at the 3' end. Subsequently, the SLP-2 gfp was placed into the doxycycline-inducible pBIG2i vector (38). Stable transfectants were generated by electroporating linearized plasmid into Jurkat E6.1 T cells and screened for stable expression. Doxycycline (Sigma-Aldrich) was added in culture overnight at 1 µg/ml to induce SLP-2-gfp expression. Expression of SLP-2 gfp was confirmed by flow cytometry (BD Biosciences). RNA interference targeting SLP-2 was performed using siRNAs obtained from Ambion (catalogue no. 20643 and 20467) and nucleofected into Jurkat T cells, PBMC, or PBMC blasts using the Nucleofector kit for either cell lines or human primary T cells (Amaxa). As controls for SLP-2 siRNA, we used the scrambled siRNAs provided as a control by the commercial supplier (Ambion). As per manufacturer’s tests, these control siRNAs have no significant similarity to any known gene sequences from mouse, rat, or human, and no toxicity to cells; have been shown to lack any significant effect in cell proliferation and apoptosis assays; and do not modulate the mRNA levels of housekeeping genes (18S rRNA, GAPDH, and cyclophilin) up to 48 h after transfection. siRNAs that target different exons of SLP-2 were mixed to establish whether better knockdown of SLP-2 could be achieved. Through this process, it was established that combining two siRNAs (catalogue no. 20643 and 20467) produced the highest level of knockdown. GFP cDNA in the targeting vector was used as a control for nucleofection efficiency. With the protocols supplied by Amaxa, efficiency of nucleofection for Jurkat T cells was between 40 and 65%. After nucleofection, T cells were rested for 24 h until optimum down-regulation of SLP-2 was observed before proceeding with any functional assays.

Antibodies

An antisemirum against human SLP-2, generated by immunization of rabbits with a synthetic peptide (aa 343–356, ProSci), was used for immunoprecipitation of SLP-2. A preimmunization serum was used as control for rabbit antiserum immunoprecipitations (ProSci). Commercially available Abs against SLP-2 were purchased from Protein Tech Group. Antiserum against Lck, linker for activation of T cells (LAT), and phospho-LAT were obtained from Upstate Biotechnology. Anti-phospholipase C (PLC)-γ1 and anti-ZAP70 antisera were obtained from Santa Cruz Biotechnology. Immunoblotting for ERK-1/2 was done using a rabbit polyclonal antibody to ERK-1/2 (StressGen Biotechnologies). Anti-phosphopilase C (PLC)-γ1, anti-phospho-PLC-γ1, anti-active ERK-1 and ERK-2 E10, and anti-caspase 3 antisera were obtained from Cell Signaling Technology. CD45 was immunoblotted using a mouse mAb (clone 6H; BD Biosciences). Actin was immunoblotted using an affinity-purified goat polyclonal antiserum (Santa Cruz Biotechnology), and GAPDH was immunoblotted with a mouse mAb from Chemicon International. To ensure that the Western blot signals were within the linear range of detection, we performed signal titration over multiple exposures over a range of time periods from 30 s to 30 min. Signal saturation was detected by image acquisition software (Alpha Innotech). Densitometry and m.w. calibration were done with the Phoretix 1D Database package.

Lipid raft isolation

Jurkat T cells or blast PBMC were stimulated with phytohocolcoccal enterotoxin E superantigen (SEE) and LG-2 cells as APC (at a 5:1 ratio; 60 × 10⁶ T cells with 12 × 10⁵ APC). Whole cell lysates were prepared with 0.5% Triton X-100, and lipid rafts were isolated by sucrose gradient ultracentrifugation, as described (39). Lipid rafts from the same cell equiv-
FIGURE 1. Expression of SLP-2 in human lymphoid tissues. A, Whole cell lysates from the indicated organs were sequentially immunoblotted for SLP-2 and GAPDH. Equal loading of protein per lane was further confirmed by spectroscopy. Results are representative of four independent experiments. B, Lymph node samples from human nonspecific B cell adenitis (top row) or T cell adenitis (middle row), and normal human thymus (bottom row) were stained for SLP-2 using a C terminus-specific rabbit anti-human SLP-2 antiserum or with appropriate controls (CD20bright for B cells, TdT for developing thymocytes), and biotinylated horse anti-rabbit secondary Ab and avidin (Vector Laboratories). High levels of SLP-2 expression were detected in the germinal centers (B cell area) of lymph nodes of nonspecific B cell adenitis, in the paracortical (T cell) area of nonspecific T cell adenitis, and in the cortex of the thymus. Much lower expression of SLP-2 was seen in cells outside these areas. The profile of intracellular localization of SLP-2 was predominantly associated to the periphery of the cell and some aggregates in the central area of the cytoplasm. C, Peripheral blood cells from a normal volunteer in whom expression of SLP-2 could be detected in resting PBMC were used to fractionate these cells into T cells (T), B cells (B), and monocytes (M). Whole cell lysates from these subsets were prepared and sequentially immunoblotted for SLP-2 and GAPDH. Densitometric readings for SLP-2 normalized for GAPDH are shown.
In lymph nodes, SLP-2 was mostly detected in the germinal centers (B cell area) and in the paracortical (T cell) area (Fig. 1B), with a staining pattern of intracellular punctae and peripheral layer consistent with its targeting to mitochondria and plasma membrane association. In the thymus, SLP-2 was detected both in the cortex and in the medulla, although the expression seemed to be higher in the cortex (Fig. 1B). In those individuals in which SLP-2 was detectable in PBMC, it was expressed by monocytes, and to less extent, by T and B lymphocytes (Fig. 1C).

**Up-regulation of SLP-2 expression in T and B lymphocytes upon activation**

The high expression of SLP-2 in sites where lymphocyte signaling and activation take place (i.e., Ag activation in lymph nodes and tonsils, positive and negative selection in the thymus) prompted us to examine the effect of activation on SLP-2 expression. Although, in most volunteers tested, peripheral blood T cells express low levels or no SLP-2 under resting conditions, activation of these cells with bacterial superantigens led to a consistent and significant up-regulation of its expression after 36 h (Fig. 2A). Such an up-regulation was already detectable after 2 h of stimulation through the TCR, as observed when cross-linked Abs against CD3 were used to activate T cells (data not shown). Similarly, in B cell preparations from human tonsils fractionated according to their activation status, SLP-2 was mostly detected in the fraction corresponding to activated/memory (CD20<sup>bright</sup>, CD27<sup>-</sup>) B cells (Fig. 2B). Seventy-three percent of cells in this fraction (II) were activated/memory B cells (as indicated by expression of CD27), and only 8% of cells in this fraction were naive B cells. In contrast, 60% of the cells in fraction 4 were naive B cells and only 11% were activated/memory B cells. Fractions 2 and 3 had progressively decreasing numbers of activated/memory B cells (52 and 32%, respectively) and increasing numbers of naive B cells (17 and 45%, respectively). Together, these data led us to conclude that SLP-2 expression is up-regulated by in vivo and ex vivo lymphocyte activation.

**SLP-2 interacts with components of TCR signalosomes and with polymerized actin during T cell activation**

To study the contribution of SLP-2 to T cell activation, and because we had isolated SLP-2 from lipid rafts of activated T cells, we first analyzed the partitioning of SLP-2 within these microdomains following activation. To do this, we used Jurkat T cells that express SLP-2 under resting conditions. As shown in Fig. 3A, SLP-2 was detected in lipid rafts and its partitioning to these microdomains increased soon after activation, subsequently returning to basal levels. Such a profile of redistribution into lipid rafts was consistently documented in multiple experiments (Fig. 3B), and in primary human T cells at discrete time points (Fig. 3C). We failed to see a reciprocal change in the levels of SLP-2 in the soluble fractions in comparison with the increase in SLP-2 levels in the lipid raft fractions, either because the SLP-2 that redistributes into rafts came from the insoluble cytoskeletal pellet or because the amount of SLP-2 that repartitions to the lipid raft fraction from the soluble fraction is small in comparison with the total amount of soluble SLP-2.

Next, we tested whether SLP-2 interacted with the components of TCR signalosomes during activation for up to 60 min, a time window that covers the formation of TCR signaling microclusters and mature IS (Fig. 4A). In complementary coimmunoprecipitation studies, we observed that SLP-2 steadily associated with the CD3<sub>-</sub>-<sub>ε</sub> chain of the TCR complex under resting conditions and during the 60 min of stimulation. Detailed quantitation studies of this association indicated that ~0.09% of the cellular SLP-2 associated with the TCR complex (data not shown). In these studies, we also found that SLP-2 interacted with Lck, ZAP70, LAT, and PLC-γ1 during the 30-min period following stimulation in vitro. Of interest, the magnitude of LAT interacting with SLP-2 was comparable to that of phospho-LAT interacting with SLP-2, suggesting that this interaction involves a single functionally active pool of LAT. In contrast, the interaction between SLP-2 and ZAP70 or PLC-γ1 was steady during the early times of stimulation, whereas tyrosine phosphorylation of these pools only occurred at discrete time points, suggesting the presence of different SLP-2-interacting pools of ZAP and PLC-γ1 in the cell. Such profile of interactions was only observed in specific SLP-2 immunoprecipitations with cross-linked Abs, but not with nonspecific control Abs. Also, these interactions were preserved under stringent lysis conditions, and were selective because no association of SLP-2 with other surface receptors (CD45) or with control intracellular molecules (Ras-GAP, caspase 3) was detected (Fig. 4A). In addition, such interactions were observed using a different detergent (octylglucoside, 2%), and in primary human T cells (data not shown).

Because polarization of signaling molecules and organelles to the IS is cytoskeleton dependent, we examined the association of SLP-2 with actin. We found that SLP-2 interacted with actin under resting conditions and upon TCR stimulation (Fig. 4B). Such an association was not observed using a control preimmune serum, ruling out a nonspecific interaction, and mostly involved T cell actin given the T:APC ratio (5:1) used in these experiments. Furthermore, the association between SLP-2 and β-actin during T cell activation seems to involve mostly the
polymerized form of actin because cytochalasin D, an inhibitor of actin polymerization, completely prevented this interaction (Fig. 4B). We observed a lower level of association between SLP-2 and actin at time 0 in T cells treated with cytochalasin D, most likely reflecting a basal level of interaction between these molecules under steady-state conditions. It is important to note that T cell activation in this experimental system is dependent on actin polymerization, because inhibition of actin polymerization with cytochalasin D led to inhibition of IL-2 production in a dose-dependent manner (Fig. 4C).

**SLP-2 contributes to sustain T cell signaling**

Next, we looked at the functional correlates of SLP-2 expression and its interactions with the components of the TCR signalosome. We reasoned that if SLP-2 played a role in signaling from the TCR signalosomes, then knocking down SLP-2 would decrease TCR signaling. To test this hypothesis, we knocked down SLP-2 expression by RNA interference using two siRNAs against SLP-2. With this technique, we achieved between 65 and 85% reduction of SLP-2 levels. Under these conditions, we found that SLP-2 down-regulation correlated with a significantly shorter timeline of TCR signaling (Fig. 5A). That is, at equal levels of early TCR signaling (as measured by similar levels of initial ERK-1/-2 activation), down-regulation of SLP-2 caused a remarkably shorter duration of ERK activation in response to TCR stimulation, already apparent at 5 min and still significant at 10 min of stimulation. The effect of SLP-2 down-regulation on TCR-dependent signaling was also observed for the kinetic profile of activation of PLC-γ1 (Fig. 5A), and was statistically significant across multiple experiments looking at the profile of ERK activation in response to TCR signaling using Jurkat T cells and primary T cells (Fig. 5B).

**Modulation of IL-2 responses by SLP-2**

Because SLP-2 down-regulation prevented sustained signaling, and because sustained TCR signaling is required for full T cell activation, we predicted that changes in the levels of SLP-2 expression would translate in changes in an effector T cell response such as IL-2 production. We tested this hypothesis in human T cell lines and primary T cells. To determine the effect of SLP-2 overexpression on IL-2 production, we transfected Jurkat T cells with a doxycycline-inducible SLP-2-gfp cDNA. In these cells, SLP-2 expression is increased due to basal leakiness of the vector promoter. Such an expression can be further up-regulated with the addition of doxycycline to the culture, as demonstrated by FACS (Fig. 6A). We found that de novo overexpression of SLP-2 (as corroborated by FACS) significantly increased IL-2 production of these T cells in response to SEE and APC, compared with the parental E6.1 T cell line expressing low levels of SLP-2 (Fig. 6A).
To test whether knocking down the expression of SLP-2 decreased IL-2 responses, we nucleofected Jurkat T cells with two different SLP-2 siRNA constructs. Down-regulation of SLP-2 upon RNA interference was documented by Western blotting, as shown in Fig. 6B. On average, with a reduction of SLP-2 levels between 70 and 80%, we observed that the IL-2 response to APC and SEE significantly decreased compared with the parental SLP-2-expressing Jurkat T cell line (Fig. 6B).

Finally, we corroborated the effect of SLP-2 expression on IL-2 responses in primary human T cells from normal volunteers. Human naive T cells express low levels of SLP-2, but the levels are up-regulated significantly upon activation. Thus, to induce high...
expression of SLP-2 in primary human T lymphocytes, we preactivated these cells with a mitogenic combination of PMA and ionomycin for 3 days, followed by a resting period. At this point, these effector T cells and their naive T cell counterparts from the same donor were nucleofected with SLP-2 siRNA or control siRNA and restimulated with SEE and APC for 24 h, and their IL-2 response was examined. As shown in Fig. 7A, knocking down the expression of SLP-2 by more than 60% correlated with a significant decrease in the IL-2 response of the effector T cells, as illustrated by the shift to the right of the dose-response curve to SEE (p < 0.001). Little effect was observed for SLP-2 siRNA in resting T cells in which SLP-2 expression is almost absent (Fig. 7A), suggesting that the SLP-2 siRNAs used for these experiments do not have an off-target (non-SLP-2-dependent) effect on the IL-2 response of T cells. SLP-2 siRNA had no effect on the IL-2 response of human T cells to mitogenic stimulation with PMA and ionomycin, which bypasses early TCR signaling events (Fig. 7B). Together, the findings from Jurkat T cells and from primary T cells demonstrate that T cell activation can be modulated by changing the levels of SLP-2. We documented that, in primed primary human T cells, there is a more sustained profile of TCR-dependent ERK activation compared with naive primary T cells (Fig. 7C), with stronger phospho-ERK signal still detectable by 30 min postactivation. This finding is consistent with our claim given that primed T cells up-regulate SLP-2 expression, and complements the results shown in Fig. 5 of less sustained ERK activation in Jurkat T cells when SLP-2 was down-regulated by siRNA.

Discussion

The results presented here show that SLP-2 expression in T and B lymphocytes is up-regulated by activation in vivo and ex vivo. Upon expression in T cells, SLP-2 interacts with molecules involved in early TCR signaling and with polymerized actin, and contributes to sustain TCR-dependent signaling. Consistent with these findings, one can modulate effector T cell responses such as IL-2 production by changing the levels of SLP-2: overexpression of this protein increases IL-2 responses, whereas down-regulation of its expression correlates with decreased IL-2 responses, both in T cell lines as well as in primary human T cells. To our knowledge,
these results provide the first biological evidence of a function for SLP-2 in an eukaryotic cell type, that of sustaining TCR signaling and enhancing T cell activation. The findings of SLP-2 partitioning in lipid rafts, its interactions with TCR signalosome components and polymerized actin, and its contribution to sustain TCR-dependent signaling are consistent with the emerging role assigned to proteins containing an SPFH domain. This domain has been linked to protein interactions with cell membranes (23–25). In addition, proteins containing such a domain are enriched in lipid rafts, and have been previously implicated in the regulation of cell signaling (42, 43). For example, prohibitin is indispensable for the activation of the Ras-ERK signaling pathway (44), and a similar role has been proposed for the flotillins (45–47). Together this evidence positions SLP-2 in the proper subcellular compartment to participate in TCR-dependent signaling.

The structural basis of the molecular interactions identified for SLP-2 is currently under study. The primary sequence of SLP-2 predicts that there are at least two cellular pools of this protein: one in mitochondria (25) and the other associated with the plasma membrane (16, 48–50). Preliminary data from our laboratory confirm these two pools and suggest that both pools of SLP-2 coalesce in the peripheral area of the IS, a region in which signaling TCR microclusters accumulate (14) (M. Kirchhof, C. Lemke, and J.

**FIGURE 7.** Modulation of human peripheral blood T cell responses by SLP-2. A, PBLs from a normal volunteer were isolated and used as resting cells, or after 3 days of ex vivo activation with PMA and ionomycin and 48 h of resting. Cells were nucleofected with two SLP-2 siRNAs or with control scrambled siRNAs and used 24 h later for stimulation with autologous APC and SEE. IL-2 production after 24 h was assessed by ELISA (mean ± SD). Additional controls included cells nucleofected without any DNA to rule out nonspecific effect of nucleofection. The efficiency of nucleofection was controlled by a GFP cDNA provided by Amaxa, with efficiencies ranging from 40 to 65%. Inlet figure shows Western blot for SLP-2 to confirm knockdown of SLP-2, and β-actin as a loading control in the two groups tested. Similar results were obtained in four separate experiments. *** p < 0.001. B, Down-regulation of SLP-2 does not affect the IL-2 response to mitogenic stimulation with PMA and ionomycin. PBLs from the experiment shown in A were used after 3 days of ex vivo activation and 48 h of resting. Cells were nucleofected with SLP-2 siRNA and used 24 h later for stimulation with PMA and ionomycin. IL-2 production after 24 h was assessed by ELISA (mean ± SD). Cells nucleofected without any DNA or with nonsense siRNA were used as controls. The effect of SLP-2 siRNA on SLP-2 levels is shown in the right inlet panel of A. C, Resting and blast human peripheral blood T cells were stimulated with APC and SEE for the indicated times. Cell lysates were prepared and immunoblotted for dually phosphorylated, active ERK-1/-2, total ERK-1/-2, and SLP-2.
Madrenas, preliminary observations). Such an observation is also consistent with the recent report of mitochondrial polarization to the IS during T cell activation (11). The primary sequence of SLP-2 does not stand out with an enzymatic domain or with a conventional protein-protein interaction domain that could explain its direct interactions with signaling molecules. It is therefore plausible to assume that the interactions of SLP-2 with TCR signalosomes and with polymerized actin involve indirect associations between SLP-2 and other molecules, with SLP-2 potentially acting as a scaffolding/assembling protein.

How can SLP-2 contribute to sustain TCR-dependent signaling? We propose two, nonmutually exclusive ways by which SLP-2 can do that. One way is that SLP-2 contributes to the assembly of the multimolecular signalosomes on the transmembrane adapter LAT. Such a facilitating role in signalosome assembly would stabilize signalosomes for sufficient time to deliver sustained signaling. This possible mechanism is supported by the observed sequential interactions between SLP-2 and signaling molecules and polymerized actin, and by the prevention of sustained TCR signaling seen with down-regulation of SLP-2 expression. This possibility is also consistent with the formation of TCR signalosomes within lipid rafts (51–53), where SLP-2 is enriched, and with the recent claim that SPFH family members bind cholesterol, and contribute to the formation of signaling-permissive protein-lipid complexes (30, 54).

Another way to explain how SLP-2 contributes to sustain TCR-dependent signaling is by claiming that SLP-2 modulates mitochondrial function, which is in itself linked to TCR signaling, as indicated by the polarization of mitochondria to the IS. The specific role that SLP-2 plays in mitochondrial function is unknown, but may be linked to the assembly of the electron transport supercomplexes in lipid rafts of the inner mitochondrial membrane in a way analogous to what we propose for the SLP-2 pool in the plasma membrane. According to this hypothesis, polymerization of actin in response to TCR signaling would bring TCR signalosomes and mitochondria to the IS. In this way, the energetic requirements of TCR signaling can be met because it warrants close proximity between the TCR signalosomes and the compartmentalized mitochondria. This possibility is in line with the changes in mitochondria positioning during cell responses through fission and fusion, processes that make use of the cytoskeleton and/or microtubules (12, 55). We cannot rule out that SLP-2 may scaffold mitochondria with the cytoskeleton through its interaction with mitochondrial external membrane proteins (56) such as mitofusin-2 (although we have to date failed to document SLP-2-mitofusin-2 association in T cells). This proposed role for SLP-2 in T cell activation points to a novel regulatory mechanism of signal transduction based on cytoskeleton-dependent interaction between signalosomes and cellular organelles (9), a mechanism that may be applicable to other cell surface receptors in addition to the TCR (our preliminary observations).

We have shown that, following T cell activation, the levels of SLP-2 increase, and this translates into enhanced effector responses (e.g., IL-2 production). In contrast, decreasing SLP-2 expression decreases IL-2 responses. Such observations have in our opinion, several important biological implications. One is that it may partially explain the long-standing finding that, at equal requirement for sustained signaling, primed T cells show much stronger signal transduction and functional responsiveness than naive T cells. One may argue that this is, in part, due to primed T cells having higher levels of SLP-2 than naive T cells (57, 58). Another implication is that the increased effector phenotype seen with neoplastic transformation may be due, in some cases, to higher SLP-2 expression (59–62). Finally, the ability to modulate effector T cell responses by regulating SLP-2 expression identifies SLP-2 as a potentially useful target for immunotherapy. This could take the form of down-regulation of SLP-2 expression to decrease T cell reactivity (e.g., in the course of autoimmune disease) or alternatively, enhancement of SLP-2 expression to increase T cell responsiveness (e.g., vaccine development).

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Disclosures
The authors have no financial conflict of interest.

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