Sphingosine 1-phosphate (SIP) generated by cells of innate immunity and the type 1 SIP G protein-coupled receptor (SIP1) on mobile T cells constitute a major system for control of lymphoid organ traffic and tissue migration of T cells. Now we show that T cell activation mediated by the T cell antigen receptor translocates plasma membrane SIP1 to nuclear envelope membranes for association there with G\textsubscript{i\_G} Erk 1/2, and other proteins that plasma membrane SIP1 uses to signal T cell proliferation. However, nuclear SIP1 and plasma membrane SIP1 transduce opposite effects of SIP on T cell proliferation and relevant signaling as exemplified by respective decreases and increases in T cell nuclear concentrations of both phospho-Erk and active (phosphorylated) c-Jun. T cell antigen receptor-mediated activation of T cells therefore both eliminates migration responses to SIP by down-regulation of plasma membrane SIP1 and translocates the SIP-SIP1 axis into the nuclear domain where signals are directed to transcriptional control of immune functions other than migration.

Most studies of the intracellular traffic of G protein-coupled receptors (GPCRs) have focused on their response to occupancy by ligand, which initiates rapid movement from the cell surface to intracellular compartments, including vesiculotubular structures of the perinuclear domain, by diverse pathways and then reinsertion into the plasma membrane (1–3). Recent analyses of cellular traffic of the type 1 GPCR for lysophosphatic acid (LPA\_1) have shown that ligand elicits signal transduction, cell surface down-regulation, and prolonged localization of LPA\_1 in the nuclear domain (4, 5). When localized in the nuclear envelope, LPA\_1 signals transcriptional events by mechanisms not used when LPA\_1 is in the plasma membrane. In endothelial cells and hepatocytes, the LPA-nuclear LPA\_1 axis stimulates prominent transcription of proinflammatory proteins, including type 2 cyclooxygenase (COX-2) and inducible nitric-oxide synthase. However, constituents of the LPA\_1 nuclear signaling complex other than G proteins have not been identified, and it is unclear what elements regulate transcription initiated by LPA\_1 nuclear signals. Most importantly, it is not known how the pathways LPA synthesized principally extracellularly reaches nuclear envelope LPA\_1. In contrast, it is well established that the related lysosphingolipid sphingosine 1-phosphate (SIP) is synthesized intracellularly, taken up readily by non-synthesizing cells, and capable of functioning as an intracellular messenger.

SIP produced by mast cells and mononuclear phagocytes, but not T cells, is secreted into T cell corridors where it elicits and regulates T cell movements in lymphoid organs and chemotaxis in non-immune tissues as well as some migration-independent T cell immune functions (6, 7). The introduction of SIP into suspensions of T cells preincubated without SIP down-regulates plasma membrane type 1 GPCR for SIP (SIP\_1) for a few hours, without changes in mRNA encoding SIP\_1, after which SIP\_1 is re-expressed with full function (8). In contrast, T cell antigen receptor (TCR)-dependent stimulation of T cells evokes greater and more prolonged down-regulation of plasma membrane SIP\_1 as well as highly significant suppression of the level of mRNA encoding SIP\_1 (9). The intracellular fate of T cell plasma membrane SIP\_1 down-regulated by TCR-dependent stimulation has not been examined previously. Our most recent findings indicate that TCR-mediated activation of T cells completely alters their pattern of responsiveness to SIP by both down-regulating plasma membrane SIP\_1 to limit effects of SIP on migration, and translocating most SIP\_1 to the nucleus where it couples principally transcriptionally to non-migration functions of T cells. Now we describe mechanisms of TCR-mediated sustained nuclearization of SIP\_1, the constitution of distinctive SIP\_1 signaling complexes in nuclear envelope membranes, attainment of functionally relevant concentrations of exogenous SIP inside T cells, and novel effects of the SIP-nuclear SIP\_1 axis on proliferation of T cells.

**EXPERIMENTAL PROCEDURES**

**Preparation and Stimulation of CD4 T Cells and HTC4 Cell Transfectants**

C57BL/6 mouse splenic CD4 T cells were isolated at a purity of at least 96% by two cycles of magnetic bead immunoaffinity chromatography as reported previously (10). Replicate 0.6-ml aliquots of purified CD4 T cells at 4–6 $\times$ 10\(^6\)/ml of complete...
RPNI 1640 medium with 10% charcoal/dextran-absorbed fetal bovine serum, 100 units/ml penicillin G, and 50 μg/ml streptomycin in 24-well plates were stimulated with either 10⁻³–10⁻⁶ M S1P for 1 h or 3 μg each of adhesive anti-mouse CD3 + anti-mouse CD28 antibodies (BD Biosciences) for 24 h as described previously (9). S1P₁-null HTC4 rat hepatoma cells were stably transfected with human S1P₁ containing an amino-terminal hemagglutinin (HA) peptide epitope tag using the Amaxa Nucleofection system (Amaxa, Inc., Gaithersburg, MD), designated HTC4-S1P₁(HA) cells, and cultured according to standard procedures (11).

Isolation, Characterization, and Stimulation of Nuclei

Replicate suspensions of 0.5–1 × 10⁷ HTC4-S1P₁(HA) cells and CD4+ T cells, without and after stimulation, were washed twice in phosphate-buffered saline with 0.5 g/100 ml fatty acid-free bovine serum albumin and 2 mM EDTA, and resuspended in 0.1–0.2 ml of lysis buffer composed of 10 mM NaCl with 3 mM MgCl₂, 10 mM Tris-HCl (pH 7.4), 1/100 HALT protease inhibitor mixture (Pierce), and 10 units/ml DNase I (Qiagen, Inc., Valencia, CA) in a 1.5-ml Eppendorf tube. Each suspension was homogenized with a Teflon pestle at 4 °C once using 20 strokes (T cells) or twice separated by 10 min at 4 °C using 40 strokes (HTC4-S1P₁(HA) cells). Free nuclei were pelleted at 700 × g for 10 min at 4 °C, washed three times in lysis buffer with 0.1% Nonidet P-40 as well as HALT and DNase I, and further purified by centrifugation at 4 °C for 15 min at 1200 × g through 0.5 ml of 30% sucrose with 10 mM NaCl and 2.5 mM Tris-HCl (pH 7.4). Some preparations of isolated nuclei were resuspended in 100 μl of lysis buffer with 1% Nonidet P-40 as well as HALT for direct extraction of nuclear proteins that contain immunoreactivity of the nuclear membrane markers nucleoporin p62 and type 1 lamin-associated protein (LAP-1) by Western blot analyses. Assays of the enzymatic markers acid phosphatase for lysosomes (Diagnostic Chemicals, Ltd., Oxford, CT) and 5’-nucleotidase (Diazyme Laboratories, San Diego, CA) for plasma membranes showed that routinely <5% of their total initial activities in intact cells were detected in the nuclei. This level of nuclear purity was confirmed by demonstrating the absence of the plasma membrane marker annexin II (12) in Western blots of nuclear extracts, whereas annexin II was prominent in Western blots of the 700 × g supernatants of homogenates containing membrane and cytoplasmic constituents.

Replicate suspensions of purified nuclei from 0.5 to 1 × 10⁷ T cells and, in some studies, 0.25–0.5 × 10⁷ intact T cells then were resuspended in 200 μl of phosphate-buffered saline with 10 units/ml DNase I, 0.1 g/100 ml fatty acid-free bovine serum albumin, and 1 μM activated sodium orthovanadate phosphatase inhibitor; preincubated for 20 min at 37 °C; and incubated at 37 °C for 1 h without and with 10⁻³–10⁻⁵ M S1P followed by addition of 1/100 HALT protease inhibitor and cooling to 4 °C. Nuclear transcription factors in stimulated nuclei and cells were extracted with the Nu-PER kit for nuclear proteins (Pierce). Phospho-Erk 1/2 (P-Erk 1/2) and total Erk 1/2 in nuclear protein extracts were quantified directly using enzyme-linked immunosorbent assay kits (Calbiochem-EMD Biosciences). Active (phosphorylated) c-Jun (P-c-Jun) was quantified by nucleic acid sequence-based Trans-AM AP-1 enzyme-linked immunosorbent assay kit (Active Motif, Carlsbad, CA). Time course studies compared effects of S1P on transcription factors and other signaling proteins in intact cells and isolated nuclei after 1, 4, and 24 h at 37 °C.

Pharmacological Antagonism of Signal Transduction Pathways

Some aliquots of isolated nuclei or intact cells were preincubated with the MEK inhibitors PD98059 (2’-amin-3’-methoxyflavone, IC₅₀ = 2 μM) or SL327 (α-[amino(4-aminophenyl)thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile, IC₅₀ = 0.2 μM) for 60 min, the protein kinase C inhibitor calphostin C (IC₅₀ = 50 nM) for 60 min (all from BIOMOL International), or pertussis toxin (50 ng/ml) (List Biologicals, Carlsbad, CA) for 16 h prior to addition of S1P.

Microscopy

Control and stimulated nuclei were allowed to adhere to glass slides, which had been pretreated for 30 min at 37 °C with 0.2 ml of 10 μg/ml poly-L-lysine (Sigma) and washed twice with phosphate-buffered saline, prior to fixation in 4% paraformaldehyde and immunostaining with antibodies to nuclear envelope markers, S1P₁, and Gᵢ/o proteins. Sources of purchased antibodies were as follows: mouse IgG1κ anti-phospho-Erk 1/2 (clone 12D4) mAb, Upstate Cell Signaling Solutions-Chemicon, Lake Placid, NY; rabbit anti-Erk 1 and anti-Erk 2, Santa Cruz Biotechnology, Santa Cruz, CA; rabbit anti-Gi₁Gα₂, mouse IgG2b anti-Gi₁, and mouse IgG2b anti-Gi₂α (used as a mixture), Chemicon International, Inc., Temecula, CA; mouse IgG2b anti-nucleoporin p62, Pharmingen-BD Biosciences; mouse IgG1 anti-LAP-1 and mouse IgG2a anti-phosphatidylinositol 3-kinase p110γ, Abcam, Inc., Cambridge, MA; and mouse IgG1 (clone 5) anti-annexin II, BD Transduction Laboratories.

Immunoprecipitation

Fifty-microliter aliquots of extracts of nuclear proteins from 10⁷ T cells in a total of 100 μl of 1% Nonidet P-40 buffer were diluted 10-fold in 0.05 M Tris, 0.1 M NaCl with HALT (pH 7.6) and incubated with purified IgG of rabbit anti-S1P₁ polyclonal antibody or monoclonal antibody to Gᵢ and/or Gₒ proteins in two different precipitation protocols for 1 h at room temperature and overnight at 4 °C. The first protocol was intended to saturate all complementary protein antigen in the sample with antibody and thus assure complete precipitation of that antigen, which involved 25 μg of purified rabbit anti-S1P₁ polyclonal IgG antibody or 10 μg of purified mouse anti-Gᵢ/O monoclonal antibody. The second protocol was designed for equilibrium binding of antibodies to a small percentage of S1P₁ or Gᵢ/O in the sample that would evaluate relative levels of association of other proteins in the receptor complex. This involved 0.5 μg of purified rabbit anti-S1P₁ polyclonal IgG antibody or 0.2 μg of purified mouse anti-Gᵢ/O monoclonal antibody. Then 120 and 25 μl, respectively, for the first and second protocols, of a 50% suspension of protein A-Sepharose (FastFlow, Sigma) was added to each suspension of receptor-antibody complexes followed by incubation at room temperature for 1 h and 4 °C for 4–6 h. After 400 ×
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G centrifugation at 4°C to pellet protein A-Sepharose adsorbent, the supernatant was removed, and the protein A-Sepharose was washed three times with 0.05 M Tris, 0.1 M NaCl with HALT and 0.1% Nonidet P-40 (pH 7.6).

**Western Blots**

Membrane proteins extracted from 5 x 10<sup>6</sup> intact cells or their constituent isolated nuclei or removed from protein A-Sepharose by boiling in Laemmli SDS buffer were separated in a 5–15% gradient polyacrylamide gel (Bio-Rad), blotted onto a polyvinylidene fluoride membrane, and developed with polyclonal rabbit anti-S1P<sub>1</sub> antibody, a monoclonal antibody to a G protein or other signaling complex protein, or one of the antibodies specific for a nuclear membrane protein as described previously (10). A horseradish peroxidase-labeled anti-rabbit IgG light chain antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) was used to develop blots of immunoprecipitates incubated with rabbit primary antibody to avoid obtrusive bands from the rabbit heavy chain.

**Quantification of Intracellular S1P**

**Fmoc Derivatization and HPLC Method**—Replicate suspensions of 5–10 x 10<sup>6</sup> purified CD4 T cells in 1 ml of RPMI 1640 medium with 10% charcoal/dextran-absorbed fetal bovine serum, 100 units/ml penicillin G, and 50 µg/ml streptomycin were cultivated for 16 h without and with adherent anti-CD3 + anti-CD28 antibodies, as for stimulation to study nuclear redistribution of S1P, GPCRs, and for 24 h at 37 °C with control absolute amounts. All relevant labeled sphingolipids were well resolved, and the sensitivity limit of detection was 20 fmol.

**LC/MS/MS Method**—Pellets of 5–10 x 10<sup>6</sup> CD4<sup>+</sup> T cells were Dounce-homogenized for 1 min at 4 °C in polypropylene microcentrifuge tubes with 25–50 µl of 80% acetonitrile. These homogenates were Vortex-mixed and centrifuged at 1,000 x g for 5 min at 4 °C; this led to recovery of over 90% of total S1P in the supernatants for quantification by LC/MS/MS (13). The LC/MS/MS system was a Micromass Quattro Ultima equipped with electrospray source, Shimadzu LC-10 AD pumps, and Waters Intelligent Sample Processor 717 Plus. The column was BDS C<sub>18</sub> (4.6 x 50 mm, 5-µm particle size, Keystone), which was eluted isocratically with 80% methanol, 0.1% formic acid, 1 mM ammonium acetate at a flow rate of 1.5 ml/min for optimal sample resolution. The postcolumn flow was split 10:1 for a flow rate of 150 µl/min into the mass spectrometer. The instrument was operated in positive ion mode for monitoring elution of S1P with a multiple reaction monitor scan at 380–264 m/z (14). The cone voltage and collision energy were set at 30 V and 20 eV, respectively.

**RESULTS**

The S1P<sub>1</sub>-S1P<sub>1</sub> Axis May Enhance or Suppress T Cell Proliferation—S1P is a primary growth factor for many types of cells, but we and others have observed that repetitive additions of 10<sup>-8</sup>–10<sup>-6</sup> M S1P suppress the proliferation of T cells evoked by stimulation of the TCR complex with a combination of antibodies specific for its CD3 and CD28 components (10, 15). Proliferative responses of T cells to such TCR-directed stimulation for 48 h were suppressed significantly by introducing 10<sup>-8</sup>–10<sup>-6</sup> M S1P at 12, 24, and 36 h (Fig. 1A). In contrast, the addition of S1P once at the beginning of the incubation had no effect on
T cell suppression of proliferation was observed (Fig. 1B) but did significantly increase proliferation transiently at 24 h to respective mean levels ± S.D. of 122 ± 11% (p < 0.05), 174 ± 16% (p < 0.01), 253 ± 34% (p < 0.01), and 219 ± 24% (p < 0.01) of control for S1P1 levels of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M (not in Fig. 1). When 10^{-9}−10^{-6} M S1P was added only once after 24 h of TCR-mediated stimulation, suppression of proliferation after 48 h was significant at 10^{-8}−10^{-6} M S1P but was not as great as when S1P was added serially (Fig. 1C). At 10^{-6} M, the S1P1-selective synthetic agonist SEW2871, which is \( \frac{1}{50} \) as potent as S1P, had a lower potency against S1P1, whereas transient enhancement of proliferation by S1P added at zero time was thought to be attributable to signals from plasma membrane S1P1. Membrane-enveloped nuclei therefore were isolated from unstimulated and TCR-activated T cells for characterization of nuclear S1P1, by methods applied previously to plasma membrane S1P1, and for successive studies of S1P1-nuclear S1P1, signaling of T cell transcriptional events.

**T Cell Activation Translocates S1P1-Gi/o Complexes from Plasma Membrane to Nucleus—Isolated and dilute detergent-washed nuclei from unstimulated and TCR-activated T cells have intact nuclear envelope membranes as shown by the level and native distribution of the membrane pore protein nucleoporin p62 and retention of the LAP-1 of the inner nuclear membrane (Fig. 2, A and B). The absence of plasma membrane and granule proteins from the isolated nuclei was demonstrated by the only marginally detectable levels of 5’-nucleotidase and acid phosphatase and by the absence of annexin II (Fig. 2, C and D). The presence of S1P1 in isolated membrane-enveloped nuclei was shown immunocytochemically (Fig. 3A) where levels were minimally detectable in nuclei from unstimulated T cells and were substantially increased with patchy distribution in nuclei from TCR-activated T cells. The S1P1 complex-associated G protein component, G_{i/o}, also was found in isolated membrane-enveloped nuclei with no apparent difference in staining intensity as a function of prior T cell stimulation. Loss of T cell plasma membrane S1P1 after identical TCR-mediated stimulation has been demonstrated (8). We therefore concluded that T cell activation results in translocation of plasma membrane S1P1 to nuclear membrane. A potentially functional association between S1P1 and G_{i/o} in nuclear membranes was suggested by results of saturating co-immunoprecipitation analyses. The amount of nuclear S1P1-associated G_{i/o}, immunoprecipitated by anti-S1P1 antibody, was increased only slightly by S1P stimulation of the stable transfectants of rat S1P1-null HTC4 hepatocytes but was greatly increased in CD4 T cells by TCR-mediated activation (Fig. 3B, upper blot). Reciprocally nuclear G_{i/o}-associated total S1P1 immunoprecipitated by anti-G_{i/o} antibody, was increased slightly in the stable transfectants and moderately in CD4 T cells by stimulation of intact cells prior to isolation of nuclei (Fig. 3B, lower blot).
To further delineate the signaling proteins associated with nuclear S1P₁, co-immunoprecipitation of only a fraction of total nuclear Gᵢₒ or S1P₁ was performed by equilibrium binding to limiting initial amounts of each precipitating antibody. As in the preceding total co-immunoprecipitation protocols that use antibody saturation, equilibrium binding to a limited amount of each antibody showed that TCR-mediated activation of T cells clearly increased both Gᵢₒ-associated S1P₁ and S1P₁-associated Gᵢₒ with much higher levels of both than in nuclei of S1P₁-stimulated T cells (Fig. 4). Anti-S1P₁ also co-immunoprecipitated nuclear PI 3-kinase (p110) and P-Erk 1/2. Prior stimulation of T cells with S1P or anti-TCR antibodies only increased slightly the levels of nuclear S1P₁-associated PI 3-kinase (p110/γ). However, the increase in nuclear S1P₁-associated P-Erk 1 (p44) was substantial and greater for TCR-mediated stimulation than for exposure to S1P (Fig. 4). As signals transduced by S1P₁-Gᵢₒ through Ras and Raf stimulate generation of P-Erk 1/2, which is coupled to cellular proliferation events, subsequent studies of nuclear S1P₁ signaling mechanisms were directed to P-Erk 1/2 and its downstream factors.

**T Cells Establish High Intracellular Concentrations of Exogenous S1P**—Intracellular concentrations of S1P were quantified in intact T cells without and after exposure to exogenous S1P to ensure that a meaningful range of S1P concentrations was used in studies of the signaling capacity of nuclear membrane S1P₁-Gᵢₒ complexes. The values of pmol/10⁷ T cells (Table 1) were converted to molar intracellular concentrations by assuming that T cells are spheres with a radius of 6 μm and using the
standard geometric formula to calculate T cell volume. Fluorescent labeling of sphingolipids in acidic chloroform-methanol extracts of unstimulated and TCR-activated T cells followed by HPLC resolution and quantification of labeled compounds did not detect any endogenous S1P, whereas a mean of 0.25 pmol/10^7 T cells (midnanomolar range) was found with the LC/MS ion-monitoring method in acetonitrile extracts of TCR-activated T cells (Table 1). After incubation of T cells for 2 h with 10^{-7} M S1P, both methods detected mean intracellular concentrations of 2.1–6.5 pmol/10^7 T cells (high nanomolar to low micromolar range), and incubation with 10^{-6} M S1P led to mean intracellular concentrations of 19–41 pmol/10^7 T cells (midmicromolar range).

S1P Suppresses Levels of P-Erk and P-c-Jun in Nuclei Isolated from Activated but Not Naive T Cells—Isolated membrane enveloped-nuclei were incubated for 1 h with 10^{-9}–10^{-6} M S1P prior to extraction of nuclear proteins for quantitation of P-Erk 1/2, total Erk 1/2, and P-c-Jun. Both P-Erk 1/2 and P-c-Jun were quantified with an enzyme-linked immunosorbent assay method by their binding to adherent nucleic acid recognition sequences specific for the activated factors and then detection with labeled antibodies. S1P suppressed the level of P-Erk 1/2 in nuclei from TCR-activated T cells, which express S1P_1, in an S1P concentration-dependent relationship that attained significance at 10^{-7}–10^{-6} M S1P but not in nuclei from unactivated T cells lacking S1P_1 (Fig. 5, A and B). The levels of total Erk 1/2/Î¼g of nuclear protein were not altered by S1P in these or any other studies of T cell nuclei. As for P-Erk, levels of active P-c-Jun in nuclei from TCR-activated T cells, but not from unactivated T cells, were suppressed significantly by 10^{-7} and 10^{-6} M S1P (Fig. 5, C and D). At 10^{-6} M, the S1P_1-selective synthetic agonist SEW2871 suppressed P-Erk 1/2 in nuclei from TCR-activated T cells by 57\% (p < 0.01) but increased P-Erk 1/2 in nuclei from unactivated T cells by 12\% (not shown in Fig. 5).

S1P-Nuclear S1P_1 Complexes Signal Erk 1/2 through Gi/o Proteins and MEK Kinase—The possibility that S1P suppression of nuclear concentrations of P-Erk is an active inhibitory process, which results from S1P-S1P_1-Gi/o complex signaling through expected downstream members of the known MEK-mitogen-activated protein (Erk) kinase pathway, was investigated by applying two structurally different MEK kinase inhibitors known to reduce signaling by this pathway. At respective concentrations optimally inhibitory of MEK kinase, both PD98059 and SL327 prevented completely S1P

![Figure 5](image-url). Dependence of S1P suppression of the nuclear concentrations of P-Erk 1/2 and activated P-c-Jun on nuclearized S1P_1 in prestimulated T cells. Each column and bar depict the mean ± S.D. of the results of quantification of P-Erk 1/2 (A and B) and P-c-Jun (C and D) in three to six sets of isolated nuclei from T cells. The control values (100%) for P-Erk 1/2 and P-c-Jun in 1-Î¼g samples of nuclear proteins ranged from 49 to 473 and 0.046 to 0.444 pg/ml, respectively. An S1P concentration of 3–7 is 3 \times 10^{-7} M. The p values from paired two-tail t tests were as follows: +, p < 0.02; *, p < 0.001.
suppression of P-Erk 1/2 in nuclei from TCR-activated T cells (Fig. 6). Both pertussis toxin and the protein kinase C inhibitor calphostin C also prevented S1P-S1P1 axis suppression of the levels of P-Erk in nuclei isolated from TCR-activated T cells at concentrations that had blocked elevations of P-Erk in intact T cells stimulated through their TCRs. In isolated nuclei from TCR-activated T cells incubated for 1 h with $10^{-8}$, $10^{-7}$, and $10^{-6}$ M S1P, mean P-Erk levels were not significantly different from those of the controls without S1P (100%) at 89, 112, and 122% when preincubated with pertussis toxin and 94, 88, and 108% when preincubated with calphostin C. Thus S1P active suppressive signaling through nuclearized S1P1-Gi/o complexes attains a decrease in nuclear concentration of P-Erk, which had been elevated by prior TCR-mediated activation. That this decrease contrasts with the direct increase in nuclear concentration of P-Erk and subsequently T cell proliferation mediated by plasma membrane S1P1-Gi/o signals prompted further studies of this difference.

Opposite Signals to Erk from Plasma Membrane and Nuclear S1P1-Gi/o Complexes—The different responses to S1P signaling through nuclear as contrasted with plasma membrane S1P1-Gi/o complexes were studied further by analyzing events in intact unstimulated and TCR-activated intact T cells, from which plasma membrane S1P1-Gi/o complexes had been down-regulated, but did significantly suppress the level of P-Erk 1/2 in their isolated nuclei at $10^{-8}$–$10^{-6}$ M S1P through highly expressed nuclear S1P1-Gi/o complexes.
DISCUSSION

The nuclearized S1P-S1P1 axis fulfills many of the cytological and biochemical requisites expected of an intracellular messenger system. Activation of T cells through the TCR mechanism by antigen, which evokes a full program of functional immune responses, results in nuclearization of most plasma membrane S1P1 and assembly of a functional signaling complex in nuclear membranes. Membrane-enveloped nuclei isolated for the first time from unstimulated and TCR-activated T cells, using modifications of a method used by us for their recovery from several other types of cells (4, 5), strongly expressed established nuclear membrane markers and lacked protein markers characteristic of plasma membranes and lysosomal granules (Fig. 2). Membrane-enveloped nuclei from TCR-activated T cells showed S1P1 and G\textsubscript{i/o} immunocytochemically and by Western blots, S1P1 was expressed at higher levels in nuclei from TCR-activated than from unstimulated T cells, and the level of association of S1P1 and G\textsubscript{i/o} also was greater in nuclei from TCR-activated than unstimulated T cells (Figs. 3 and 4). Other signaling proteins, including P-Erk and PI 3-kinase-p110\textgamma, which are considered to associate with some S1P1-G\textsubscript{i/o} complexes, were found in immunoprecipitates of T cell nuclear membrane proteins and were more highly represented in nuclear membranes of TCR-activated than in those from unstimulated T cells (Fig. 4).

That increased levels of nuclear membrane S1P1 do not simply represent increased biosynthesis and expression of S1P1 in the perinuclear endoplasmic reticulum was excluded by the failure of cycloheximide inhibition of protein synthesis to alter the pattern of expression of nuclear S1P1. The appearance of G\textsubscript{i1/2} in nuclear membranes at levels unchanged by T cell activation (Fig. 3A) was not unexpected as G proteins associate readily with many cellular membranes at two or more small areas of contact (16). However, movement of the integral membrane protein S1P1 from plasma membranes to nuclear membranes was unexpected. S1P1 has no conventional nuclear localization sequence and no known mechanism for such translocation. Nonetheless S1P1-G\textsubscript{i/o} signaling complexes were established in nuclear membranes of TCR-activated T cells, but not unstimulated T cells, and transduced significant suppression of nuclear levels of P-Erk and P-c-Jun, which correlate with S1P suppression of T cell proliferative responses to TCR-mediated stimulation (Figs. 1, 5, and 7). In contrast, the plasma membrane S1P1-G\textsubscript{i/o} complex expressed by unstimulated intact T cells transduced increases in nuclear P-Erk and P-c-Jun, which correlate with transient enhancement of TCR-mediated T cell proliferation that is terminated by translocation of S1P1 to nuclear membranes (Figs. 1 and 7 and data in text).

The other requirement for consideration of the S1P-S1P1 axis as an intracellular messenger system is the presence of functionally relevant concentrations of S1P in T cells. Although T cells do not generate sufficient endogenous S1P for optimal signaling, uptake of extracellular S1P that presumably would come from mast cells and mononuclear phagocytes is highly efficient, independent of the state of T cell activation, and capable of establishing intracellular concentrations as high as the midmicromolar range (Table 1). Longstanding interest in nuclear sphingolipids has been heightened recently by findings of association of sphingomyelin with chromatin as well as the nuclear envelope, a capacity of sphingomyelin to bind to RNA in a complex that affords protection from RNases, opposing effects of ceramide and sphingosine on protein kinase C activity, the preferential localization of S1P-generating type 2 sphingosine kinase in nuclei, and potential regulatory roles of ceramide and S1P in cellular proliferation and apoptosis (17–20). The discovery of nuclear membrane expression of S1P1, with clear functional capabilities adds poignancy to prior knowledge of the presence there of biosynthetic precursors and a complete enzymatic cascade necessary to generate S1P. The relative roles of locally produced nuclear S1P and that taken up and transported from extracellular fluid into the nucleus are likely to depend on the type of cell and its functional state. Pharmacological reduction of the endogenous cellular sources by suppression of synthesis will require plasma membrane- and nuclear membrane-permeant inhibitors of type 2 sphingosine kinase or enzymes earlier in the pathway.

Distinctive regulatory roles are suggested for nuclear membrane S1P1 in activated effector T cells, where they predominate quantitatively, because they suppress nuclear P-Erk levels and thereby inhibit proliferation of the T cells that is required for most immune functional responses (Fig. 7). As for other components of the mitogen-activated protein kinase pathways, phosphorylated Erks are critical for many aspects of T cell survival, proliferation, differentiation, and immune functional responses. Clearly this is the explanation for decreases in TCR-mediated proliferation induced by S1P that is introduced after T cell activation leads to nuclearization of S1P1 (Fig. 1). Thymic maturation was defective in Erk 1-null mice where Erk 1 is required for complete differentiation of thymocytes to the CD4/8+ stage, but most effector functions of mature Erk 1-null T cells were normal (21, 22). The polarization toward Th1 cell development and activity in Erk 1-null mice was attributable to enhanced generation of Th1-promoting cytokines by dendritic cells and not to an intrinsic alteration in the T cells. Thus any defects in functions other than proliferation of activated T cells receiving suppressive signals from nuclearized S1P1 are most likely due to resultant decreases in activity of related S1P1-associated signaling factors suppressed in parallel, such as PI 3-kinase-p110\textgamma bound to \beta\gamma dimers of S1P1-G protein complexes (Fig. 4), which are required for numerous GPCR-mediated responses including chemotaxis to chemokines (23).

Most circulating naïve and memory T cells express levels of plasma membrane S1P1 sufficient to transduce both chemotactic responses to S1P and suppression of chemotactic responses to chemokines. The levels of functional S1P1 on T cells from secondary lymphoid organs, however, differ substantially as a result of the combined effects of sustained S1P-induced downregulation, loss of post-translational modifications necessary for S1P1 activity, and diminished coupling of S1P1 to signaling pathways (24, 25). The plasma membrane expression of S1P1 also is substantially reduced on activated effector T cells, largely due to down-regulation, to levels that do not support any migration-related responses to S1P until after reversion to a non-activated state (26). The present results suggest that migration responses mediated by plasma membrane S1P1 are
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replaced in activated T cells by responses of proliferation and possibly some effector functions to signals from intracellular S1P through nuclearized S1P1 complexes. In addition to a novel distribution within the T cell, exposure to ambient S1P concentrations in the nuclear domain as contrasted with that in extracellular fluid, and unique coupling to nuclear functions, there may be distinctive aspects of the signaling cascades for nuclear membrane S1P1 that are not seen for plasma membrane S1P1 and will be elucidated by future studies. Completely different roles for nuclear S1P1s may be envisioned based solely on their localization. For example, nuclear S1P1 could facilitate nuclear translocation of a G protein-associated signaling factor such as RhoA, which in turn may mediate activation of nuclear phospholipase D and its multiple roles in nuclear signaling (27). Of greatest immunological interest is the possibility that nuclear membrane S1P1, which does not influence T cell migration in any way, may transduce S1P-suppressive signals not only to proliferation but also to non-migration functions such as cytokine generation.

Two intracellular roles of S1P were identified in studies of the effects of some protein growth factors on non-immune cells. S1P may act as a selective messenger for protein growth factor receptor signaling and as a regulator of protein growth factor receptor stimulation through transactivation by specific S1P GPCRs (28). Pharmacological inhibition of intracellular generation of S1P blocked signaling by platelet-derived growth factor receptors, but not epidermal growth factor receptors, on the same cells, and this was reversed by addition of exogenous S1P (29). S1P transactivates epidermal growth factor receptors and some other protein growth factor receptors by stimulating tyrosine kinase-mediated phosphorylation through intracellular signals from cell type-specific S1P GPCRs (30, 31). Some roles of S1P as intracellular messenger assume the existence of intracellular S1P receptors of either a conventional type or a different design, but these have not been identified definitively. The potential involvement of nuclear S1P GPCRs in activating interactions of S1P with protein growth factors and their receptors will require additional investigations.

The discovery of nuclear S1P GPCRs and high levels of intracellular S1P suggests both new roles for these systems in normal immunology and the need to develop cell-permeant agonists and antagonists that can affect these targets in intact cells. Such agents may potentiously influence T cell trafficking through plasma membrane S1P1, and also non-migration activities of activated effector T cells through nuclear S1P1 GPCRs. It is further postulated that effects on T cell migration through plasma membrane S1P1 are short lived, whereas effects on proliferation and effector functions through nuclear S1P1 are sustained and may require longer administration of pharmacological agents.

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