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Ligand-induced nuclear translocation of $S1P_1$ Cyr61 and CTGF transcription in endothelial cells

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Abstract

Sphingosine-1-phosphate (S1P) receptor subtype 1 ($S1P_1$), a G-protein coupled receptor (GPCR), regulates many biological activities of endothelial cells (ECs). In this report, we show that $S1P_1$ receptors are present in the nuclei of ECs by using various biochemical and microscopic techniques such as cellular fractionation, immunogold labeling, and confocal microscopic analysis. Live cell imaging showed that plasma membrane $S1P_1$ receptors are rapidly internalized and subsequently translocated to nuclear compartment upon S1P stimulation. Utilizing membrane biotinylation technique further supports the notion that nuclear $S1P_1$ receptors were internalized from plasma

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membrane $S1P_1$ after ligand treatment. Moreover, nuclear $S1P_1$ is able to regulate the transcription of Cyr61 and CTGF, two growth factors functionally important in the regulation of vasculature. Collectively, these data suggest a novel S1P–$S1P_1$ signaling axis present in the nuclear compartment of endothelial cells, which may regulate biological responses of endothelium.

Keywords

S1P; $S1P_1$; Endothelial cells

Introduction

Sphingosine-1-phosphate (S1P), a serum-borne bioactive lipid mediator secreted by activated platelets (Yatomi et al. 1995), regulates a variety of cellular responses including proliferation, survival, cytoskeletal remodeling, adhesion, and chemotaxis (Wu et al. 1995 1999, 2008; Spiegel et al. 1998; Lee et al. 1999, 2001, [2006];). In endothelial cells (ECs), most of these responses are mediated by the S1P receptor subtype 1 ($S1P_1$, old nomenclature EDG-1), a prototype of the S1P family of G-protein coupled receptors (GPCRs) (Lee et al. 1999, 2001, 2006; Paik et al. 2001). The $S1P_1$ was cloned from cultured human umbilical vein endothelial cells (HUVECs) stimulated with the tumor promoter phorbol 12-myristate 13-acetate (PMA) (Hla and Maciag 1990). Subsequently, $S1P_1$ was shown to activate mitogen-activated protein kinase (MAPK or ERK1/2) and phospholipase A_2 (PLA_2) and inhibit adenylate cyclase via G_i heterotrimeric G proteins (Lee et al. 1996). In addition, recent studies suggest that S1P/$S1P_1$ signaling plays an important role in regulating blood vessel development and maturation (Lee et al. 1999; Liu et al. 2000). For example, in cultured ECs, S1P/$S1P_1$ signaling regulates endothelial survival, migration, cytoskeletal reorganization, and adherens junction formation, all of which result in endothelial morphogenesis to form capillary-like structures in vitro (Lee et al. 1999, 2001, 2006). Furthermore, deletion of $S1P_1$ results in embryonic lethality in transgenic mice due to a defect in the vasculature (Liu et al. 2000). These results together suggest that S1P/$S1P_1$ signaling plays a critical role in neo-vessel formation and vasculature maintenance.

Ligand-induced internalization and intracellular trafficking of GPCRs is thought to be important in the regulation of signal transduction and biological functions mediated by GPCRs. The spatiotemporal relationship of S1P-induced intracellular traffi cking of the $S1P_1$ receptor was previously examined in HEK293 cells heterologously expressing green fluorescent protein (GFP) tagged $S1P_1$ receptor (HEK293*$S1P_1$GFP*) (Liu et al. 1999). In that study, it was shown that $S1P_1$ receptors were internalized into endosomal compartments and subsequently recycled back to the plasma membrane after S1P stimulation (Liu et al. 1999). However, the relevance of this plasma membrane–endosome–plasma membrane trafficking route of $S1P_1$ receptors has not yet been characterized in a truly physiological setting. Moreover, the functional significance of $S1P_1$ internalization/trafficking in response to S1P stimulation is presently unknown.

There is increasing evidence suggesting nuclear localization of GPCRs (Marrache et al. 2005; Cook et al. 2006; Gobeil et al. 2002, 2003a, b; Lee et al. 2004; Pickard et al. 2006; Liao et al. 2007). However, the underlying mechanisms and functional significance of nuclear-localized GPCRs are poorly understood. In this report, we show that $S1P_1$ is present in the nuclear compartment of ECs. Also, we demonstrate that S1P treatment results in nuclear translocation of $S1P_1$ receptors. Furthermore, we demonstrate that the nuclear S1P–$S1P_1$ signaling axis is able to activate transcriptional activities in endothelial cells. This study presents evidence for the first time showing the functional significance of nuclear-localized

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$S1P_1$ in ECs. These data together suggest that nuclear $S1P_1$ may play a novel role in mediating S1P-regulated endothelial functions.

Materials and methods

Reagents

Sphingosine-1-phosphate (Biomol) was dissolved in fatty acid-free bovine serum albumin (BSA, Sigma, 0.4%). Mouse-derived $S1P_1$ monoclonal antibody (E1-49) was generated by conventional hybridoma technology using bacteria-expressed $S1P_1$ as an immunogen (Lee et al. 2006). E1-49 was shown to specifically react with the human $S1P_1$ receptor by immunostaining, immunoblotting, and immunoprecipitation (Lee et al. 2006). Polyclonal rabbit anti-GFP antibody was purchased from Molecular Probes. Monoclonal mouse anti-calnexin was purchased from BD Transduction Laboratories. Mouse anti-human Na^+/K^+ ATPase (α6F) came from the Development Studies Hybridoma bank. Rabbit anti-human Lamin A/C was purchased from Cell Signaling. Rabbit anti-human Rab4 was purchased from Chemicon. Other reagents, unless otherwise specified, were from Sigma.

Cell culture

HUVECs were purchased from Cambrex Bioproducts. HUVECs and HEK293 stably expressing GFP-tagged $S1P_1$ ($S1P_1$GFP) or GFP polypeptides were cultured as previously described (Lee et al. 1999, 2001, 2006; Liu et al. 1999).

Cell fractionation and nuclei isolation

HUVECs were collected between passages 6–13 at ~80% confluency unless otherwise stated. Isolation of nuclei was achieved by using the hypotonic/Nonidet P-40 lysis method (Kaufmann et al. 1983; Gobeil et al. 2002; Lee et al. 2004; Liao et al. 2007). Briefly, cultures were rinsed twice with ice-cold PBS and collected with cell scrapers. Cells (5×10^6) were resuspended in 0.5 ml of hypotonic/Nonidet P-40 buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40) and incubated on ice for 5 min. After centrifuging at 500*g* for 5 min, nuclear pellets were washed twice with 0.5 ml of hypotonic/Nonidet P-40 buffer. The morphological integrity of isolated nuclei (> 90%) was assessed by light microscopy after trypan blue staining. The purity of subcellular fractions was routinely verified by immunoblotting with antibodies specific for markers of different subcellular organelles, e.g., CD61 and Na^+/K^+ ATPase for the plasma membrane, calnexin for the endoplasmic reticulum, Rab4 for the endosome, and lamin A/C for the nucleus.

Immunoprecipitation and immunoblotting

Cellular or nuclear extracts were prepared with TBST/OG buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 60 mM *n*-octyl β-D-glucopyrano-side) (Lee et al. 1999, 2001, 2006). Extracts were then incubated with anti-$S1P_1$ at 4°C overnight. Subsequently, the immunocomplexes were precipitated by protein-A beads at 4°C for 1 h. After washing, precipitated polypeptides were released by boiling in double-strength Laemmli SDS-sampling buffer (4.6% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (w/v) glycerol, 95.2 mM Tris-HCl, pH 6.8, 0.01% (w/v) bromphenol blue) at 95°C for 5 min and resolved with SDS-PAGE. The electrophoretically separated polypeptides were transferred to nitrocellulose membrane, blocked in 5% non-fat dry milk (LabScientific), and incubated with the indicated primary antibodies on a rotary shaker at 4°C overnight. After washing, the blots were incubated with HRP-conjugated second antibodies and detected with Enhanced Chemiluminescence Reagent (ECL) (Amersham).

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Immunostaining and confocal microscope analysis

Cells were cultured in 35-mm glass bottom dishes (Mat-Tek) and serum-starved in M199 medium for 2 h (for HUVECs) or in DMEM supplemented with 0.05% FBS for 24 h (for HEK293). Cells were then stimulated with S1P (200 nM) for various times, fixed with 4% paraformaldehyde, and permeabilized with 0.25% Triton X-100. $S1P_1$ receptors were stained with the mouse E1-49 antibody (1:1,000 dilutions), followed by staining with Alexa488-conjugated goat anti-mouse antibody (1:1,000 dilutions). GFP-tagged $S1P_1$ polypeptides (Lee et al. 1998; Liu et al. 1999) were directly visualized with an Axiovert 200 M epifluorescence microscope (Carl Zeiss). Confocal microscopic analysis was carried out as previously described (Lee et al. 1999; Liu et al. 1999; Wang et al. 2008).

Quantitative nuclear run-off transcription assay

Nuclear run-off transcriptional assays were performed essentially as described (Rolfe and Sewell 1997; Li and Chaikof 2002; Green and Bender 2000). Nuclei (2×10^6) were collected from HUVECs stimulated with or without S1P (1 μM, 1 h) and suspended in 200 μl of DEPC-treated water. The nuclear fraction (150 μg proteins) was mixed with an equal volume of 2 × reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 1 mM each of ATP, CTP, GTP, and UTP) containing RNase inhibitor (6 U/sample, Promega). Transcription was carried out at 30°C for 2 h in the presence or absence of S1P (1 μM) with gentle shaking. The transcription reaction was then terminated by the addition of DNase I (24 μg/sample) at 30°C for 10 min. Subsequently, 200 μl of SDS-containing buffer (5% SDS, 0.5 M Tris-HCl, pH 7.4, 0.125 M EDTA) was added and proteins were digested by the addition of proteinase K (200 μg/sample). RNAs were then isolated using STAT-60 reagent (Tel-Test Inc.) according to the manufacturer's instructions. One microgram of RNAs was reverse-transcribed with the oligo-dT_{15} primer (Roche Applied Science) by using M-MLV reverse transcriptase (Promega) for first strand cDNA synthesis. PCR detection of gene expression was performed with 1 μl (or 20 ng) of reverse-transcribed cDNAs in a total volume of 20 μl of reaction mixture containing 200 nM of each primer, 0.2 mM of each dNTP and 1 unit of Taq Polymerase (Promega). Reactions were performed using the Gene Amp 9700 PCR System (Applied Biosystems). The PCR conditions were 30 cycles of denaturation at 95°C (30 s), annealing at 58°C (30 s), and extension at 72°C (30 s). Amplified PCR products were analyzed by electrophoresis on a 1.5% agarose gel. PCR primer pairs used were: human Cyr61, (sense) cctcggctgg tcaaagttac, (antisense) aggctccattccaaaaacag; human CTGF: (sense) aaggtgtggctttaggagca, (antisense) tcttgatggctggaga atgc; human actin: (sense) aaactggaacggtgaaggtg, (antisense) tcaagttgggggacaaaaag.

Results

$S1P_1$ receptors are present in the nuclear compartment

To examine whether $S1P_1$ receptors were located in the nuclear compartment, nuclei were isolated from normally growing HUVECs by the well-established nuclear isolation method utilizing NP-40 lysis buffer. The purity of isolated nuclei was confirmed by the absence of markers for the plasma membrane, endosome, and endoplasmic reticulum (Fig. 1a). For example, CD61, Na/K ATPase, Rab4, and calnexin were present in the supernatant fraction after 500*g* centrifugation, which collectively represents the non-nuclear fraction. In contrast, nuclear lamin A/C polypeptides were only detected in the purified endothelial nuclear fraction. Importantly, $S1P_1$ receptors were clearly detected in the isolated endothelial nuclei (top panel, Fig. 1a). In addition, it was previously shown that the GFP-tagged $S1P_1$ ($S1P_1$GFP) exhibits the same characteristics of S1P binding and signal transduction as the wild-type $S1P_1$ (Liu et al. 1999). Similar to ECs, a significant amount of $S1P_1$GFP polypeptides was detected in the nuclei of HEK293*$S1P_1$GFP* cells (Fig. 1b). In contrast, GFP polypeptides were only observed in the non-nuclear fraction of control HEK293*GFP* cells (Fig. 1b). The lack of contaminated

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polypeptides from other cellular compartments (e.g., Na/K ATPase and calnexin; middle two panels, Fig. 1c) suggests that the presence of $S1P_1$ receptors in the nuclear compartment is not an artifact of isolation.

The nuclear presence of $S1P_1$ in ECs was verified by immunogold labeling followed by electron microscope analysis (Fig. 2). Quantitative analysis shows that there are 1.28 ± 0.12 (n = 11) and 0.94 ± 0.10 (n = 14) gold particles/μm^2 in the nuclei and plasma membrane of anti-$S1P_1$ stained ECs. The immunogold labeling is specific, because there are 0.16 ± 0.03 (n = 10) and 0.23 ± 0.09 (n = 11) gold particles/μm^2 in the nuclei and plasma membrane of ECs stained with control isotypical IgG_2b (Fig. 2; $P < 0.001$, t test).

In addition, confocal microscope analysis of the isolated nuclei of HEK293*S1P$_1$GFP* cells shows a punctate distribution pattern of $S1P_1$GFP polypeptides in the isolated nuclei (panels a–e, Fig. 3). In the experiments shown with normal growing HEK293*S1P$_1$GFP* cells at 80% confluency, 29 ± 11% (n = 6) of the isolated nuclei were positive for $S1P_1$GFP polypeptides. Collectively, these data suggest that the nuclear presence of $S1P_1$ receptors is a truly physiological phenomenon.

S1P treatment results in nuclear translocation of $S1P_1$ receptors

We utilized the immunofluorescent microscopic analysis to examine whether S1P stimulation induces nuclear translocation of the $S1P_1$ receptor (Fig. 4). HEK293*S1P$_1$GFP* cells were synchronized at the quiescent state by serum-starvation in DMEM containing 0.05% FBS for 24 h. $S1P_1$GFP polypeptides were primarily located at the plasma membrane of the serum-starved cultures (upper left panel, Fig. 4a). Addition of S1P induced rapid internalization of $S1P_1$GFP which formed an intracellular dot-like distribution pattern (lower left panel, Fig. 4a). At 30 min after S1P stimulation, $S1P_1$GFP polypeptides were mainly redistributed to the nuclear regions (upper right panel, Fig. 4a). Two hours after S1P addition, $S1P_1$GFP had completely returned to the plasma membrane (lower right panel, Fig. 4a). In contrast, GFP polypeptides were evenly distributed in the cytoplasm of control HEK293*GFP* cells and exhibited no apparent redistribution after S1P addition (data not shown). Furthermore, three-dimensional Z-section analysis of confocal images shows that $S1P_1$GFP polypeptides are mainly relocated to the nuclear envelop region after S1P stimulation (Fig. 4b).

Similarly, S1P stimulation induces nuclear localization of $S1P_1$ receptors in ECs (Fig. 5a). HUVECs undergo apoptosis by overnight serum starvation. Thus, cultured HUVECs were subjected to partial synchronization by plating (1×10^5 cells per 35-mm dish) for 3 days followed by serum-starvation for 2 h at 37°C with serum-free M199 medium. After stimulation with or without S1P, cells were fixed and immunostained with monoclonal $S1P_1$ antibody. The $S1P_1$ receptors were observed at plasma membrane and cytoplasm in these partially synchronized ECs in the absence of S1P stimulation (small arrows, upper right panel, Fig. 5a). In sharp contrast, $S1P_1$ receptors were found in the nuclear regions in more than 80% of ECs after S1P stimulation (large arrows, middle right panel, Fig. 5a). The anti-$S1P_1$ immunostaining was shown to be specific, because the fluorescent signal was barely detected in the absence of anti-$S1P_1$ (lower right panel, Fig. 5a). In addition, anti-$S1P_1$ immunogold labeling showed that $S1P_1$ receptors were significantly relocated to nuclear envelop region after S1P stimulation (arrows, lower panel, Fig. 5b). There were 5.58 ± 2.18 (n = 17) and 1.49 ± 1.22 (n = 16) gold particles/μm^2 in the nuclear envelop regions of ECs treated with or without S1P, respectively ($P < 0.001$, t test). These results together suggest that ligand stimulation is capable of inducing the relocation of $S1P_1$ receptors to the nuclear compartment.

We next utilized the live cell imaging technique to examine the ligand-induced intracellular trafficking and nuclear translocation of $S1P_1$ receptors in HEK293*S1P$_1$GFP* cells (Fig. 6). $S1P_1$ receptors were mainly located at plasma membrane of serum starved

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HEK293*S1P$_1$GFP* cells (arrows at 0 time, Fig. 6) and immediately internalized into cytoplasmic compartments after S1P addition (arrows at 0.5 and 1.0 min, Fig. 6). At 10 min after S1P treatment, $S1P_1$ receptors were clearly detected in the nuclear compartment (arrows at 10 min, Fig. 6), and maintained in the nuclei up to 60 min after S1P stimulation. Subsequently, $S1P_1$ receptors were returned to the plasma membrane. The ligand-induced intracellular trafficking of $S1P_1$ receptors is shown in a real-time movie in Supplemental data 1. In a control, $S1P_1$ receptors remained at the plasma membrane and showed no detectable translocation for the 2-h imaging period in the absence of S1P treatment (data not shown).

In addition, we employed membrane biotinylation technique to confirm that nuclear $S1P_1$ receptors were translocated from plasma membrane. HEK*293S1P$_1$GFP* cells were labeled with cell impermeable Sulfo-NHS-SS-Biotin reagent (Pierce) at 4°C for 20 min. Cells were then separated into nuclear and non-nuclear fractions. As shown in Fig. 7a, only the plasma membrane $S1P_1$ receptors were biotin-labeled, whereas nuclear $S1P_1$ receptors were not (compare top and third panels, Fig. 7a). In contrast, neither nuclear nor non-nuclear Ran polypeptides (Ran locate at cytoplasm and nuclei) were biotinylated (compare second and fourth panels, Fig. 7a). This result indicates that the biotinylation condition exclusively labels the extracellular domains of plasma membrane polypeptides.

Subsequently, biotin-labeled cultures were washed and incubated at 37°C in the presence or absence of S1P for various times. Cell nuclei were prepared, solubilized, and incubated with avidin beads to isolate biotinylated polypeptides. As shown in Fig. 7b, there are no biotin-labeled $S1P_1$ receptors observed in the isolated nuclei in the absence of S1P treatment (top panel, Fig. 7b). In sharp contrast, the biotinylated $S1P_1$ receptors were clearly detected in the isolated nuclei at 10 min after S1P stimulation (top panel, Fig. 7b). In a parallel control immunoblot, nuclear $S1P_1$ polypeptides were shown to be quantitatively increased after S1P stimulation (second panel, Fig. 7b). Together, these data suggest that the nuclear $S1P_1$ was translocated from plasma membrane after ligand stimulation.

Nuclear S1P–$S1P_1$ signaling stimulates Cyr61 and CTGF transcription in ECs

We next investigated the function of nuclear $S1P_1$ receptor in ECs. It was shown that S1P treatment results in transcriptional activation of the Cyr61, CTGF, and COX-2 genes (Han et al. 2003; Muehlich et al. 2004; Kim et al. 2003). Because endothelial S1P/$S1P_1$ signaling has been shown to play a critical role in the regulation of the angiogenic response (Lee et al. 1999; Liu et al. 2000), we initially focused on examining whether nuclear $S1P_1$ regulates the transcriptional activation of Cyr61 and CTGF, two growth factors implicated in angiogenesis (Leu et al. 2002; Gao and Brigstock 2004). In agreement with previous studies, S1P treatment induced Cyr61 and CTGF transcription in a time-dependent manner in cultured ECs (Fig. 8a). In this study, we show that $S1P_1$ was redistributed to nuclear compartment in S1P-treated ECs (Fig. 5). Accordingly, nuclei isolated from S1P-pre-treated ECs were able to induce Cyr61 and CTGF expression following S1P stimulation in an in vitro nuclear run-off transcription assay (Fig. 8b, c). In contrast, $S1P_1$ receptors were mainly located at the plasma membrane of serum-starved ECs (Fig. 5). Consequently, nuclei isolated from serum-starved ECs failed to induce Cyr61 and CTGF transcription. These data indicate that the nuclear S1P–$S1P_1$ signaling axis at least in part regulates S1P-mediated transcription activation of Cyr61 and CTGF in ECs. Also, plasma membrane $S1P_1$ receptor transduced signaling requires the activity of Giα heterotrimeric G protein (Lee et al. 1996, 1998, 1999, 2001). Pretreatment of cells or isolated nuclei with pertussis toxin, an inhibitor of Giα polypeptides, completely abrogated the S1P-induced transcription of Cyr61 and CTGF (Fig. 8d). It suggests that nuclear $S1P_1$ receptors-mediated Cyr61 and CTGF transcription is also regulated by a Giα-dependent pathway.

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Discussion

Intracellular trafficking plays an important role in GPCRs signaling and function. Using HEK293 cells expressing $S1P_1$GFP polypeptides, we showed that S1P treatment results in the internalization of $S1P_1$ receptors into the intracellular endosomal compartment (Liu et al. 1999). Also, it was recently demonstrated that $S1P_1$ is relocated to caveolae for regulation of cortical actin formation (Singleton et al. 2005) and to proteasomes, where the receptor was ubiquitinylated and degraded (Oo et al. 2007). These results together suggest that $S1P_1$ may be redistributed to multiple intracellular organelles to execute distinct signaling pathways. Thus, we utilized time-lapse microscope analysis to examine the S1P-stimulated intracellular trafficking of $S1P_1$ receptors in a spatiotemporal manner (Fig. 6; Supplemental data 1). Surprisingly, we constantly observed that $S1P_1$ receptors were rapidly internalized into intracellular compartments upon ligand stimulation, traveled to nuclear regions, and then recycled back to plasma membrane (Fig. 6; Supplemental data 1). This observation suggests that cellular nucleus may be a novel intracellular destination of ligand-induced $S1P_1$ trafficking.

Subsequently, we rigorously examined this speculation by utilizing various biochemical and microscopic techniques. Cellular fractionation studies showed that $S1P_1$ receptors were present in the nuclei of ECs and HEK293 cells stably expressing $S1P_1$GFP polypeptides (Fig. 1). The isolated nuclear fraction was lack of contaminated polypeptides from other cellular compartments (e.g., CD61, Na/K ATPase for the plasma membrane, Rab4 for the endosome, and calnexin for endoplasmic reticulum; Fig. 1a, c). Moreover, lamin A/C polypeptides (a specific nuclear marker) were only detected in the isolated nuclear fraction (Fig. 1a, c). These data suggest that the presence of $S1P_1$ receptors in the nuclei is not an artifact of isolation. Furthermore, anti-$S1P_1$ immunogold labeling followed by high resolution electron microscopic analysis showed that $S1P_1$ receptors are specifically present in both plasma membrane and nuclear envelop of ECs (Fig. 2). In addition, $S1P_1$ polypeptides were clearly present in the nuclei when the isolated nuclei were examined by confocal microscope analysis (Fig. 3). Collectively, these data suggest that nuclear presence of $S1P_1$ receptors is a truly physiological phenomenon.

There are increasing lines of evidence showing the presence of GPCRs in the nuclear compartment (Marrache et al. 2005; Cook et al. 2006; Gobeil et al. 2002, 2003a, b; Lee et al. 2004; Pickard et al. 2006; Liao et al. 2007). The topological aspects of nuclear GPCRs are completely unknown at present, because GPCRs are highly hydrophobic due to their seven trans-membrane segments. Moreover, since the canonical nuclear localization sequences (NLS) have not been identified in GPCRs, the mechanisms underlying nuclear translocation of GPCRs have not been defined. Immunogold labeling showed that $S1P_1$ receptors are mainly located at the nuclear envelop region (arrows, Figs. 2, 5). Thus, it is possible that the structure of nuclear $S1P_1$ might be stabilized in the nuclei by integration into nuclear membrane, which is similar to the topological characteristics of plasma membrane $S1P_1$.

Specific nuclear translocation is generally thought to occur via NLS, which contains either a stretch of basic amino acids or bipartite clusters of basic amino acids (Silver 1991; Dingwall and Laskey 1991). A close examination of the primary sequence of $S1P_1$ polypeptides does not reveal the presence of such a canonical NLS. Interestingly, 11 of 34 amino acids (32.4%) in the third intracellular loop (i_3 domain) of the $S1P_1$ receptors are either Arg or Lys (RIYSLVRTRSRRLTFRKNISKGSRSSEKSLALLK). It suggests that the i_3 domain of $S1P_1$ receptor might function as a non-canonical NLS; and thus, mediate nuclear translocation of $S1P_1$ receptors. However, this notion is highly speculative and awaits empirical determination by site-directed mutagenesis of i_3 domain of $S1P_1$ receptors in the future.

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It should be noted that the size of the $S1P_1$ receptor in the nucleus is the same as that in the plasma membrane as detected by immunoblotting (Fig. 1a), which is consistent with a full-length receptor (Fig. 1a). Also, the nuclear $S1P_1$ receptor could be detected by either anti-GFP for the C-terminal GFP tag (Figs. 1b, c, 7) or an antibody for the N-terminal Flag tag (data not shown). This data indicates that nuclear $S1P_1$ has not been proteolytically processed and is full length during the process of nuclear translocation. However, it is presently unknown whether subtle modifications (e.g., phosphorylation) of $S1P_1$ receptors are required for nuclear translocation.

Confocal microscopic analysis and live cell imaging showed that S1P treatment stimulates the translocation of plasma membrane $S1P_1$ receptors to nuclear compartment (Figs. 4, 6; supplemental data 1). The ligand-induced plasma membrane–nucleus trafficking route was further confirmed by the membrane biotinylation technique (Fig. 7). We used the cell impermeable Sulfo-NHS-SS-Biotin reagent to exclusively label the extracellular domains of plasma membrane polypeptides (Fig. 7a). After ligand stimulation, the biotin-labeled $S1P_1$ receptors appeared in the isolated nuclei in a time-dependent manner (top panel, Fig. 7b). Thus, these data suggest that the nuclear $S1P_1$ was translocated from plasma membrane after ligand stimulation.

In summary, we show that $S1P_1$ is present in the nuclear compartment of normal growing ECs. Also, we provide evidence supporting that S1P stimulation induces the plasma membrane–nucleus trafficking of $S1P_1$ receptors in ECs. Importantly, we show that the nuclear S1P–$S1P_1$ signaling axis in ECs directly induces expression of Cyr61 and CTGF, two growth factors which are functionally implied in angiogenic responses (Leu et al. 2002; Gao and Brigstock 2004). These data together strongly suggest that nuclear localization of the $S1P_1$ receptor is a truly physiological phenomenon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

S1P	Sphingosine-1-phosphate
$S1P_1$ (old nomenclature EDG-1, endothelial differentiating gene-1)	The high affinity GPCR for S1P
GPCR	G-protein coupled receptor
HUVECs	Human umbilical vein endothelial cells
ECs	Cultured endothelial cells
GFP	Green fluorescent protein
Cyr61	Cysteine-rich angiogenic protein 61
CTGF	Connective tissue growth factor
NLS	Nuclear localization sequences

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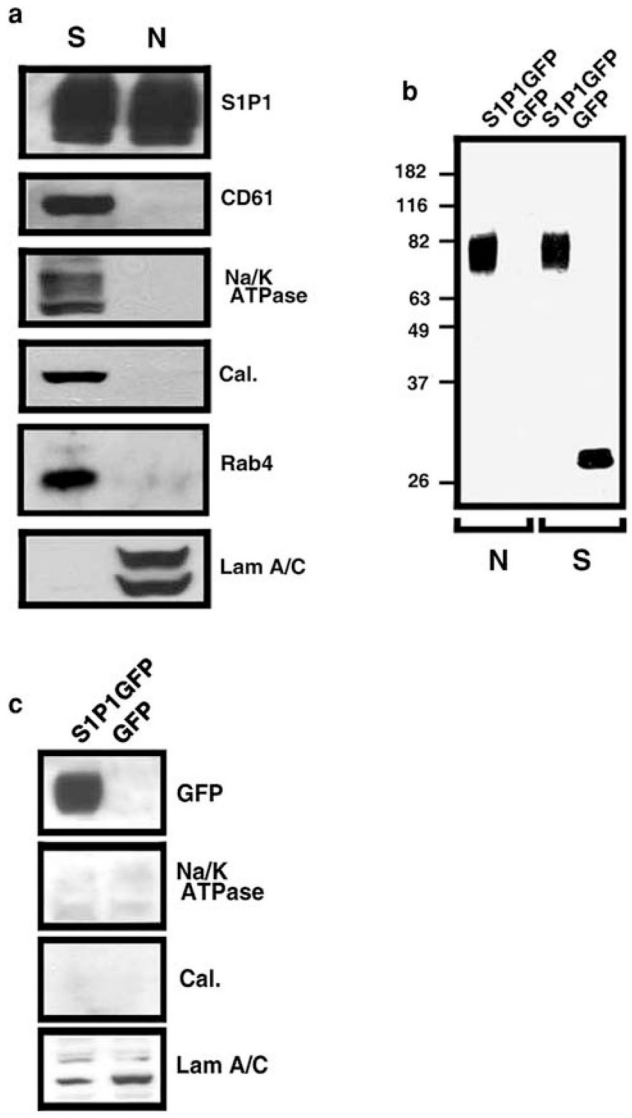

Fig. 1.

Nuclear localization of $S1P_1$ receptor. **a** HUVECs (5×10^6 cells) were used to isolate nuclear (*N*) and non-nuclear (*S*, the 500*g* supernatant after cellular disruption in lysis buffer) fractions as described in Sect. "Materials and methods". Extracts were immunoprecipitated with anti-$S1P_1$ followed by immunoblot with anti-$S1P_1$ (*top panel*). *Second–sixth panels* 50 μg of each fraction were probed with marker antibodies; e.g., CD61 and Na/K ATPase (plasma membrane), calnexin (Cal, endoplasmic reticulum), Rab4 (endosome), and lamin A/C (Lam A/C, nuclei). **b** Nuclear (*N*) and non-nuclear (*S*) fractions were prepared from HEK293*S1P*$_1$*GFP* and HEK293*GFP* cells, followed by immunoblotting with anti-GFP. Note that $S1P_1$GFP polypeptides were present in both nuclear and non-nuclear fractions, whereas GFP were only present in the non-nuclear fraction. **c** Nuclei were isolated from HEK293*S1P*$_1$*GFP* and HEK293*GFP* cells. The nuclear extracts were then precipitated with anti-GFP followed by immunoblot with anti-GFP (*top panel*). *Second–forth panels* 50 μg of extracts were immunoblotted with marker antibodies

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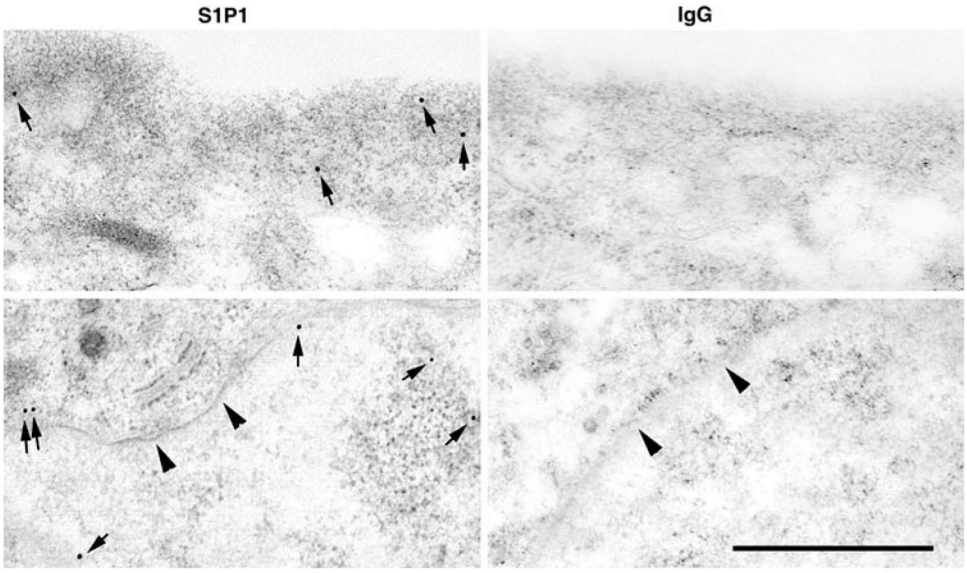

Fig. 2.

Immunogold detection of nuclear $S1P_1$ in ECs. HU-VECs were incubated with anti-$S1P_1$ (*left*) or isotypical mouse IgG_2b (*right*) followed by gold-particle conjugated secondary antibody as described (Mednieks et al. 1987; Wang et al. 1997). Note that immunogold labeling shows that $S1P_1$ are present both in plasma membrane (*top*) and nuclear compartments (*bottom*). *Arrows* gold particles, *arrowheads* nuclear envelop. *Scale bar* = 0.226 μm

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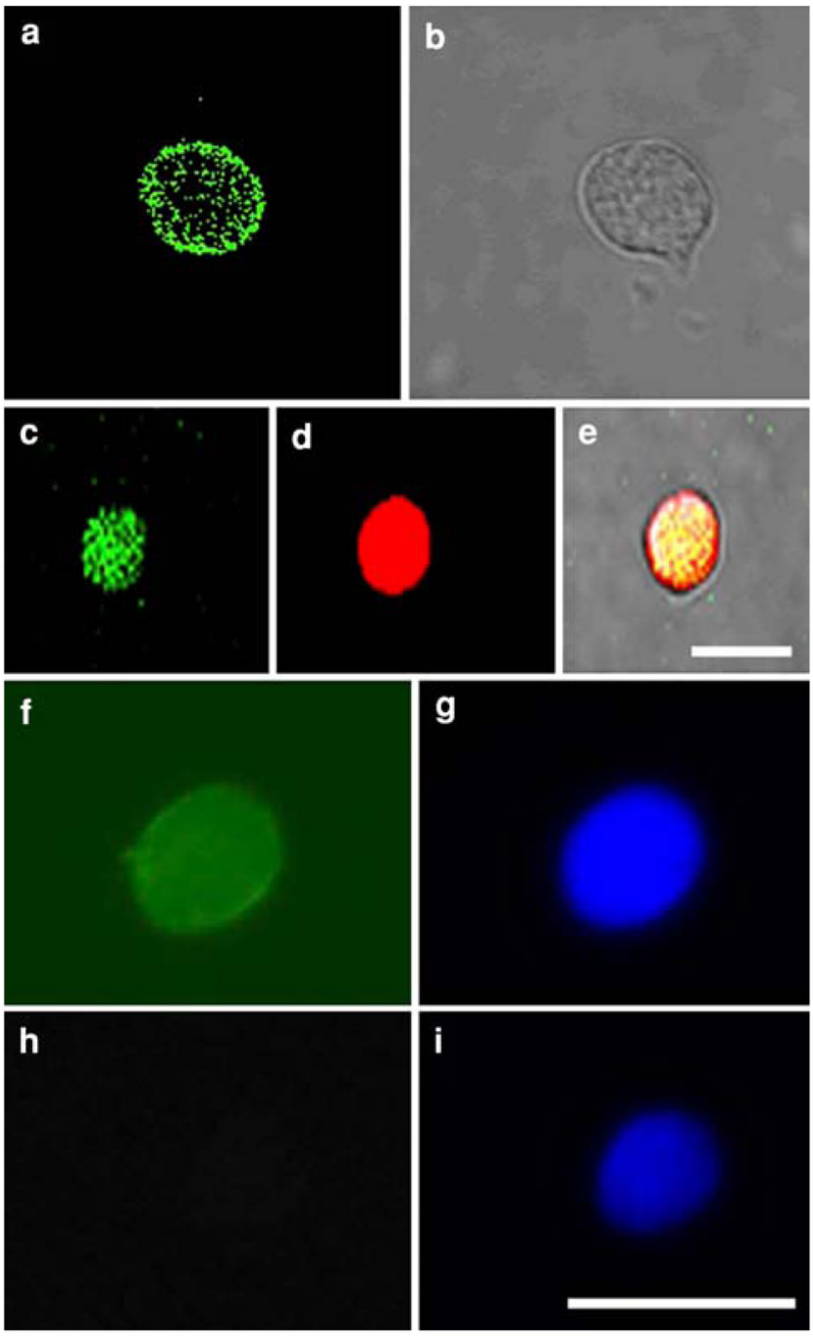

Fig. 3.

Confocal microscopy analysis of $S1P_1$ in isolated nuclei. Nuclei were isolated from HEK293*S1P$_1$GFP* cells (Lee et al. 1998; Liu et al. 1999) and analyzed by a confocal microscope (Lee et al. 1999; Liu et al. 1999; Wang et al. 2008) (*Panels* **a**–**e**) or a fluorescent microscope (Axiovert 200M, Zeiss) (*panels* **f**–**i**). *Panels* **a**, **c** $S1P_1$GFP localization in isolated nuclei; *panel* **b** phase contrast image; *panel* **d** propidium iodine stained nuclei; *panel* **e** merged image of *panels* **c** and **d**. *Panels* **f**, **g** nuclei of HEK293*S1P$_1$GFP* cells; *panels* **h** and **i** nuclei of HEK293*GFP* cells. *Panels* **f**, **h** fluorescent images; *panels* **g**, **i** DAPI-stained nuclei. *Scale bars* in *panels* **i** (for *panels* **a**, **b**, **f**–**i**) and **e** (for *panels* **c**–**e**) = 20 μm. Images are representative of three experiments with identical results

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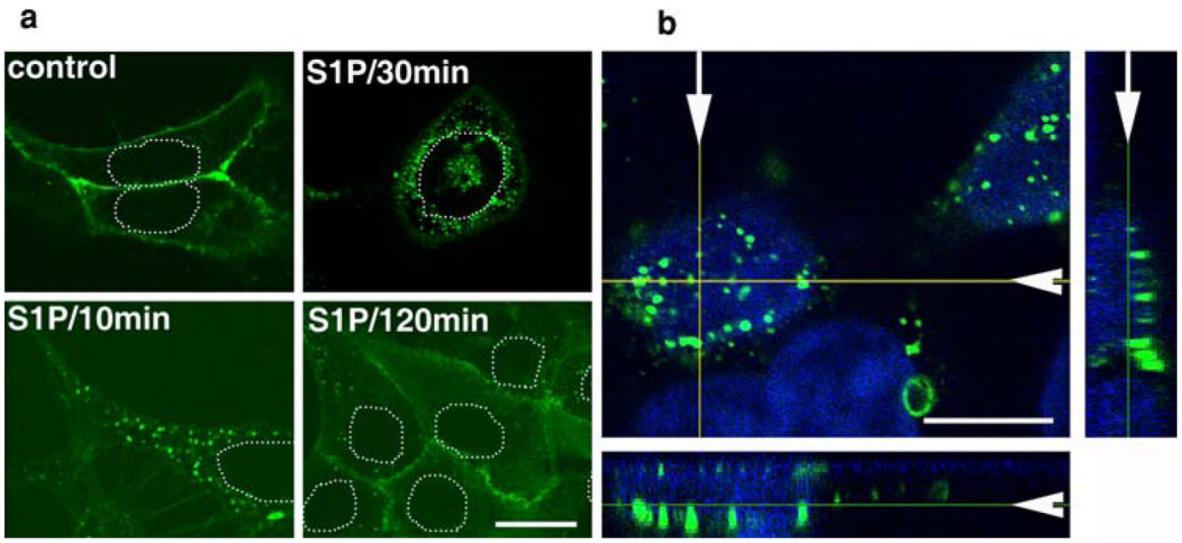

Fig. 4.

S1P induces nuclear localization of $S1P_1$ receptors. **a** Serum-starved HEK293 *S1P$_1$GFP* cells were treated with or without S1P (200 nM) for various times. The intracellular trafficking of $S1P_1$GFP polypeptides was visualized by a confocal microscopy. Note that $S1P_1$ receptors were mainly located at the plasma membrane at serum-starved cells, and were rapidly internalized into intracellular compartments (S1P, 10 min). After S1P stimulation for 30 min, $S1P_1$ receptors are translocated to nuclear compartment. Two hours later, $S1P_1$ are returned to the plasma membrane. *Dashed circles* demarcate the cell nuclei. **b** After serum starvation, HEK293*S1P$_1$GFP* cells were stimulated with S1P (100 nM) for 30 min. The nuclear localization of $S1P_1$GFP receptors were analyzed by a confocal microscope (FV1000, Olympus). Note that $S1P_1$GFP polypeptides were translocated to the nuclear regions (DAPI-stained, blue colored) after S1P stimulation. *Right and bottom strips* the three-dimensional Z-stacks of images, sectioned through areas pointed by *arrow* and *arrowhead*, respectively. Note that $S1P_1$GFP polypeptides mainly locate at nuclear envelop. *Scale bars* in *panels* **a** and **b** are 14 and 15.7 μm, respectively

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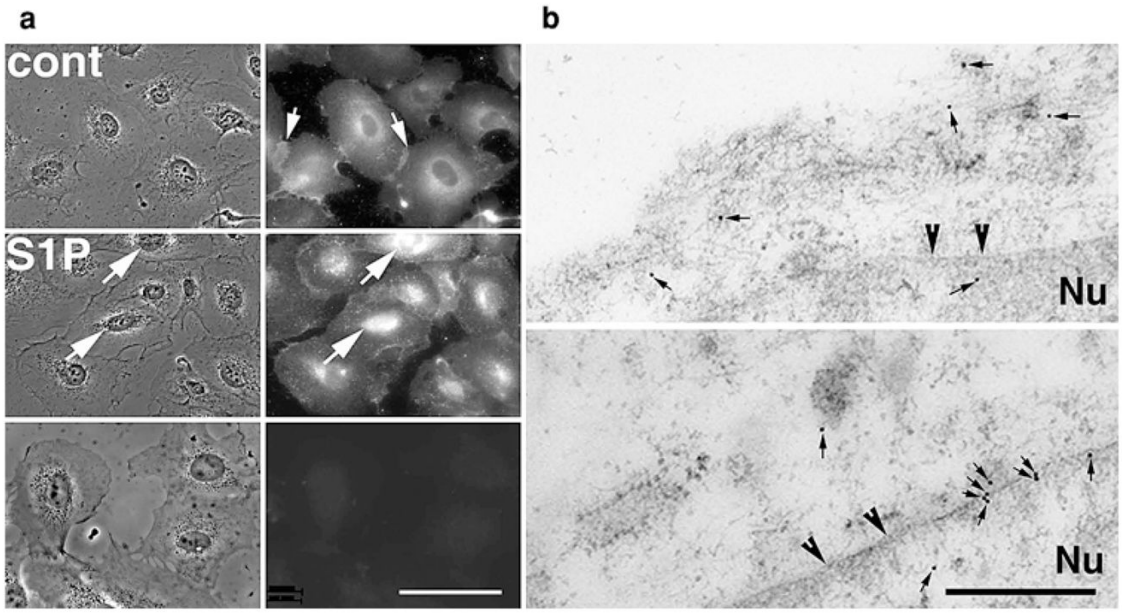

Fig. 5.

S1P induces nuclear translocation of $S1P_1$ in ECs. **a** HUVECs were stimulated in the absence (*upper panels*) or presence (*middle panels*) of S1P (100 nM, 60 min), followed by immunostaining with anti-$S1P_1$ (*right panels*). *Lower panel*, immunostain was performed without anti-$S1P_1$ primary antibody. Note that the $S1P_1$ receptors were detected at cell membrane in the absence of S1P stimulation (*small arrows*). In sharp contrast, $S1P_1$ receptors relocated to nuclear regions in more than 80% of ECs after S1P stimulation (*large arrows*). The anti-$S1P_1$ immunostaining is specific, because the fluorescent signal was barely detected in the absence of anti-$S1P_1$. **b** HUVECs were plated for 3 days followed by serum-starvation for 2 h at 37°C with serum-free M199 medium. After stimulation with (*low panel*) or without (*upper panel*) S1P (100 nM, 30 min), cells were immunogold labeled with $S1P_1$ antibody. Note that S1P treatment significantly induces the translocation of $S1P_1$ receptors to nuclear envelop regions (*arrows*, *lower panel*). There are 5.58 ± 2.18 (n = 17) and 1.49 ± 1.22 (n = 16) gold particles/μm^2 in the nuclear envelop regions of ECs treated with or without S1P, respectively ($P < 0.001$, t test). *Arrows* gold particles, *arrowheads* nuclear envelop, *Nu* nuclei. Scale bars in *panels* **a** and **b** are 34 and 0.142 μm, respectively

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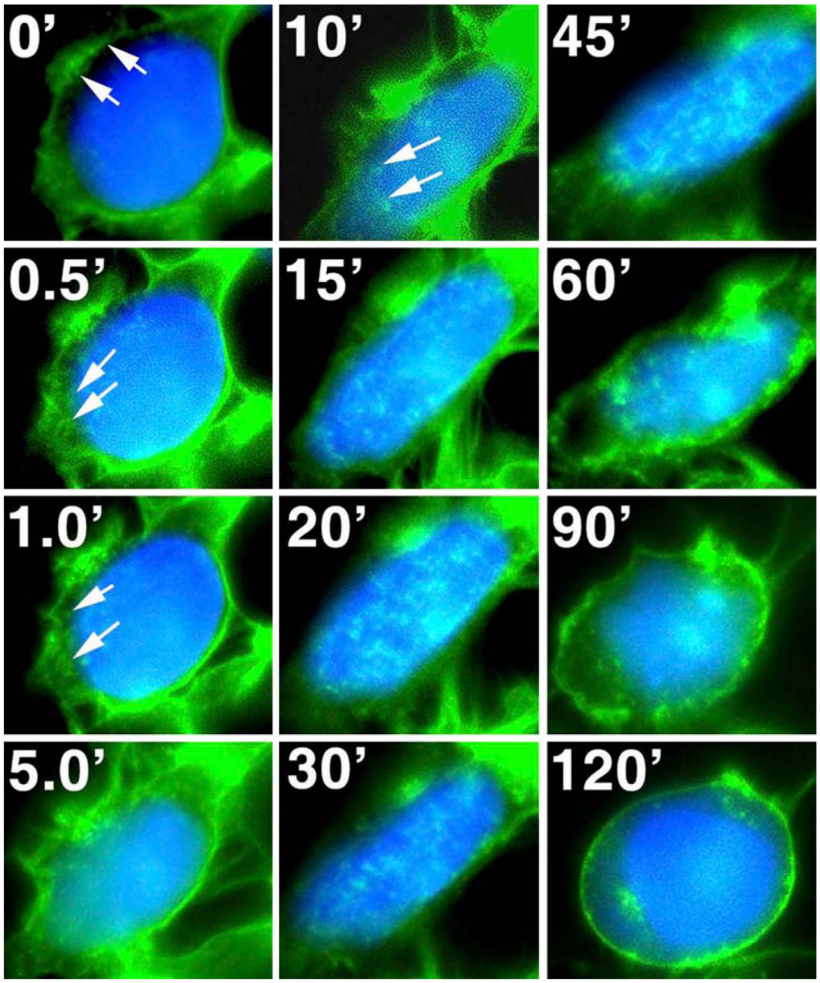

Fig. 6.

Ligand-induced intracellular trafficking of $S1P_1$ receptor. HEK293*S1P*$_1$*GFP* cells were transfected with nuclear localization sequence (NLS) tagged YFP polypeptides to locate the nuclei (*blue*). Cultures were serum-starved for 24 h followed by stimulating with S1P (200 nM). The intracellular trafficking of $S1P_1$GFP polypeptides (*green*) was recorded by live cell imaging technique as we previously described (Jala et al. 2005). The micrograms are from a representative of seven independent experiments which have similar results

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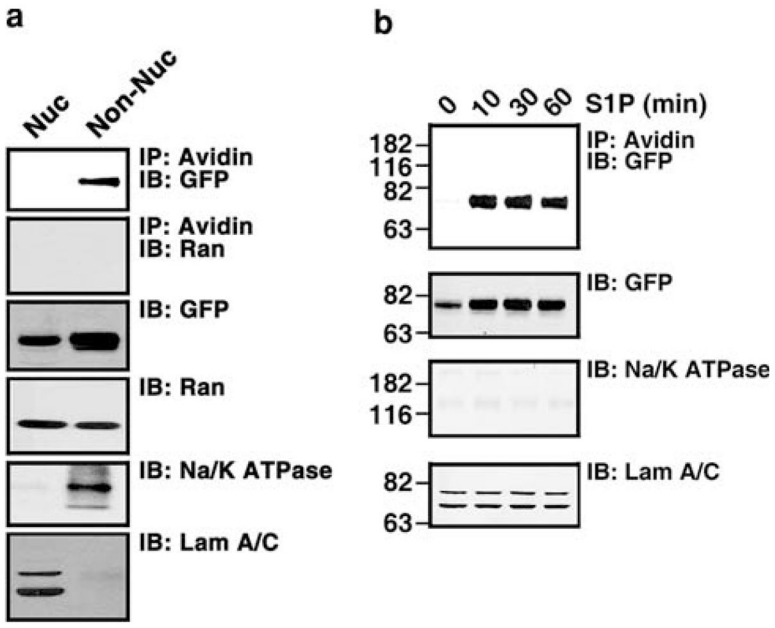

Fig. 7.

S1P induces nuclear localization of $S1P_1$ receptors. **a** HEK293*S1P$_1$GFP* cells were labeled with cell impermeable Sulfo-NHS-SS-Biotin reagent (Pierce) at 4°C for 20 min. Cells were separated into nuclear and non-nuclear fractions as described above. Extracts were incubated with avidin beads to isolate biotin-labeled polypeptides followed by immunoblotting with GFP or Ran antibody (*top two panels*). *3rd–6th panels* 50 μg extracts were directly probed with indicated antibodies. Note that the biotinylation condition only label plasma membrane polypeptides since neither nuclear $S1P_1$ nor Ran (a cytoplasmic/nuclear polypeptide) was labeled. **b** After biotinylation, cells were washed and incubated at 37°C with or without S1P (200 nM) for various times. Cell nuclei were prepared and nuclear extracts were incubated with avidin beads. *Top panel* the avidin beads isolated complexes were blotted with anti-GFP. *2nd–4th panels* 50 μg extracts were directly immunoblotted with indicated antibodies. Note that biotin-labeled $S1P_1$ receptors were not detected in the nuclei in the absence of S1P stimulation (*left lane*, *top panel*). In contrast, biotin-labeled $S1P_1$ appeared in the nuclear compartment after S1P stimulation (*2nd–4th lanes*, *top panel*). Also note that the amount of nuclear $S1P_1$ receptors was significantly increased after S1P treatment (*second panel*). The experiments in **a** and **b** were repeated at least three times with similar results

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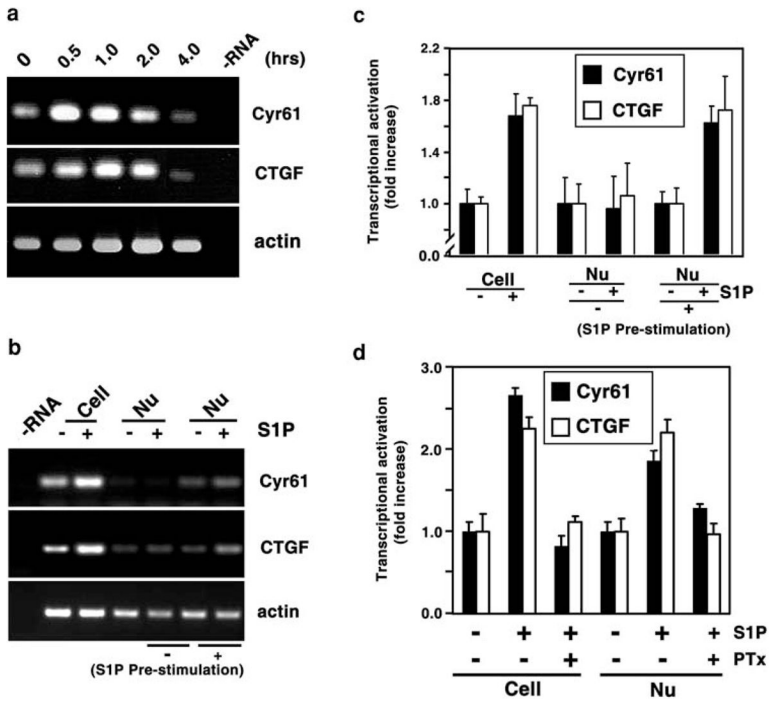

Fig. 8.

Induction of Cyr61 and CTGF gene expression by nuclear S1P-S1P$_1$ signaling. **a** ECs were treated with S1P for various times. Subsequently, the expression of Cyr61 and CTGF transcripts was measured by RT-PCR as described in Sect. “Materials and methods”; RNA, RT-PCR reaction was performed in the absence of RNA transcript. **b** Nuclei (*Nu*) were isolated from ECs pre-treated with or without S1P. Subsequently, the isolated nuclei were stimulated in the presence or absence of S1P during the in vitro nuclear run-off transcription reaction as described in Sect. “Materials and methods”. Cells (*2nd and 3rd lanes*), RT-PCR of total RNAs isolated from intact ECs pretreated with or without S1P for 1 h. **c** Mean ± SE. (n = 3) of in vitro nuclear run-off transcription assay of S1P induced Cyr61 and CTGF gene expression in isolated endothelial nuclei. **d** Cell, ECs were pretreated with or without pertussis toxin (PTx, 200 ng/ml, 2 h) followed by stimulating with S1P for 1 h. *Nu* Nuclei were isolated from S1P-prestimulated cells. The isolated nuclei were stimulated with or without S1P (1 μM) in the presence or absence of PTx (500 ng/ml) during the in vitro nuclear run-off transcription reaction. Data are mean ± SE. of two experiments with duplicate determinants