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The Adaptor Protein Tom1L1 Is a Negative Regulator of Src Mitogenic Signaling Induced by Growth Factors

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The Src family of protein-tyrosine kinases (SFK) play important roles in mitogenesis and morphological changes induced by growth factors. The involved substrates are, however, ill defined. Using an antiphosphotyrosine antibody to screen tyrosine-phosphorylated cDNA expression library, we have identified Tom1L1, an adaptor protein of the Tom1 family and a novel substrate and activator of the SFK. Surprisingly, we found that Tom1L1 does not promote DNA synthesis induced by Src. Furthermore, we report that Tom1L1 negatively regulates SFK mitogenic signaling induced by platelet-derived growth factor (PDGF) through modulation of SFK-receptor association: (i) Tom1L1 inhibits DNA synthesis induced by PDGF; (ii) inhibition is overcome by c-*myc* expression or p53 inactivation, two regulators of SFK mitogenic function; (iii) Src or Fyn coexpression overrides Tom1L1 mitogenic activity; (iv) overexpression of the adaptor reduces Src association with the receptor; and (v) protein inactivation potentiates receptor complex formation, allowing increased SFK activation and DNA synthesis. However, Tom1L1 affects neither DNA synthesis induced by the constitutively active allele SrcY527F nor SFK-regulated actin assembly induced by PDGF. Finally, overexpressed Tom1 and Tom1L2 also associate with Src and affected mitogenic signaling in agreement with some redundancy among members of the Tom1 family. We concluded that Tom1L1 defines a novel mechanism for regulation of SFK mitogenic signaling induced by growth factors.

Src family kinases (SFK) belong to the subfamily of cytoplasmic tyrosine kinases and comprises eight members, three of which (Src, Fyn, and Yes) are widely expressed. SFK share common modular structure, including a myristoylation site at the N terminus for membrane targeting, a unique sequence followed by an SH3, an SH2 and a kinase domain. In addition, they all contain an autophosphorylation site (Tyr416 for chicken Src) in the activation loop of the catalytic domain for kinase activation and a Tyr residue in the short C terminus (Tyr527 for chicken Src) that, when phosphorylated by Csk, inhibits enzymatic activity (51).

SFK play important roles during embryogenesis in mice with significant redundant function for Src, Fyn, and Yes (51). Besides, they have been implicated in growth factor receptor signaling leading to DNA synthesis, receptor endocytosis, and actin assembly (11). In the case of platelet-derived growth factor (PDGF), part of the SFK present in the cell associates with the receptor by interaction of their SH2 domain with the pTyr579 of the receptor, allowing catalytic activation for substrate phosphorylation and mitogenic signal transduction (33). A large body of evidence indicates that, in the context of PDGF, this signaling cascade is largely independent of the Ras/mitogen-activated protein (MAPK) pathway and culminates in the expression of c-*myc* for cell cycle progression (11). Intriguingly, requirement of SFK is dependent upon a functional p53 (12, 20). Although the involved mechanism is completely unknown, one may surmise that p53 inactivation deregulates a downstream element of the Src pathway, thus bypassing SFK requirement for mitogenesis. While Src substrates involved in this pathway remain elusive, several candidates have been recently identified, including the adaptor Shc (5, 21), the transcription factor Stat3 (9), the guanine exchange factor Vav2 (15), and the cytoplasmic tyrosine kinase Abl (20). The latter allowed us to propose the existence of tyrosine kinases cascade (PDGFR/SFK/Abl) that operates on Rac/JNK and Rac/Nox pathways for c-*myc* induction and DNA synthesis (8). Abl mitogenic substrates are however unknown. Whether the other identified substrates mediate c-*myc* expression need to be confirmed (43).

Over the past, Src substrates have been identified by various methods, including direct analysis of candidate proteins, purification of tyrosine phosphorylated proteins, and the analysis of Src-associated proteins (17). However, most of these molecules did not regulate Src mitogenic function. Recently, a genetic approach has been described to identify novel Src substrates (27). This relies on the screening of a Src tyrosine-phosphorylated cDNA expression library using an antiphosphotyrosine antibody. Since SFK play important roles during embryogenesis (51), we surmise that they may phosphorylate substrates required for cell proliferation. Using this approach with an expression library from mouse embryo, we have isolated Tom1L1 (46, 47). Here we report the characterization of this adaptor protein in Src mitogenic signaling. Specifically, we show that while it is a substrate and activator of Src in vitro, Tom1L1 negatively regulates Src mitogenic signal transduction induced by growth factors via regulation of SFK receptor complex formation.

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MATERIALS AND METHODS

Cloning of Tom1L1 cDNA. Search of Src substrates was performed as described in reference 27 by screening a λ Triplex library containing mouse 11-day-old embryo 5′-STRETCH PLUS cDNA (Clontech; ML5015t), with purified Src and the anti phosphotyrosine 4G10 antibody. Full-length Tom1L1 cDNA was deduced by homology with EST sequence found in database (AW012246). To obtain Tom1L1 cDNA, we designed the primers GGAATTCGAGCTCCATGGCGTTTGGG (Tom1L1 5′) GCTCTAGACTGATGCAGTGGCCTACTCAGG (Tom1L1 3′) and performed a PCR using PLATINUM *Taq* DNA polymerase High Fidelity (Life Technologies) on mouse cDNA 12.5-day-old embryo (accession number AJ505007). Tom1L1 was cloned into pcDNA3 and pBabe.

Reagents. Constructs encoding Tom1L1YFPP (Tom1L1 R419D/P421A/P424A/Y457F), Tom1L1ΔL, and Tom1L1ΔLYFPP were obtained by PCR using the QuikChange Site-Directed Mutagenesis System (Stratagene, La Jolla, CA) and/or direct ligation of oligonucleotides onto pcDNA3 and retroviral pBabe vectors. Tom1L1ΔL construct includes deletion of amino acids 292 to 386 of the Tom1L1 sequence. Constructs encoding GST-Tom1L1, GST-Tom1L1/YFPP, GST-VHS (amino acids 1 to 161), GST-GAT (amino acids 155 to 291), GST-Linker (amino acids 284 to 387), GST-C terminus (amino acids 388 to 479), and GST-FynSH1 (amino acids 272 to 522 of human Fyn) were generated by PCR amplification and were cloned into pGEX-4T vectors. Constructs expressing Src, Fyn, SrcY527F, SrcK$^-$, Abl, Myc, and Fos (3, 20), mT (10), Myc-tagged Shc (37), Stam2 (35), and RacV12 and DA-MEK1 (MEK1S218E/S222D) (8) have been described elsewhere. Nef construct was from S. Bénichou, and the constructs encoding hemagglutinin (HA)-tagged human Tom1 members were from W. Hong (45). Polyclonal Tom1L1.1-3 antibodies were raised against glutathione *S*-transferase (GST) fusion proteins containing the full length (αTom1L1.1), amino acids 291 to 474 (αTom1L1.2), or amino acids 1 to 291 (αTom1L1.3) of the murine Tom1L1 and purified as described previously (41). Fusions proteins were generated and purified as described previously (41). Antibodies specific to Src, Fyn and Yes (cst1), PDGFRβ (PR4), and mT (762) have been described elsewhere (39, 40). Antibodies specific to pY_{416}Src were from Biosource International, antibodies specific to pY_{705}Stat3 were from Cell signaling, antibodies specific to Abl (Ab-3) and SU6656 were from Calbiochem, antibodies specific to Stat3 and EC10 were from UBI, pMAPKs from New England Biolabs, and antibromodeoxyuridine (anti-BrdU) antibody was from Becton Dickinson. 9E10 (anti-myc), Y-11 (anti-HA), and anti-MAPK (SC-94) were from Santa Cruz Biotechnology. 4G10 antiphosphotyrosine antibody was from P. Mangeat, anti-Stam2 was from O. Lohi (29), and α-tubulin was from N. Morin. Antibodies coupled to fluorescein isothiocyanate and Texas Red isothiocyanate and poly-(Glu-Tyr) (4:1) were from Sigma, rhodamine phalloidin was from Molecular Probes, BrdU was from Boehringer, PDGF-BB was from Abcys, SU6656 was from Calbiochem, and [γ-^{32}P]ATP was from Amersham. Purified human Src and Fyn that were expressed in Sf9 cells were a generous gift from F. Cruzalegui (Servier Laboratories). Phosphorylated peptide FAKp3/2 derived from residues 367 to 402 of chicken FAK was a gift of S. Arold (1). pTom1L1 proteins were generated as follows: fusions deleted from their GST sequence were phosphorylated in vitro by using GST-FynSH1 bound to glutathione beads with 0.1 mM ATP for 30 min at 30°C. ATP was next removed from the supernatant by using G-50 minicolumns (Amersham).

Cells culture, transfection, microinjection, immunofluorescence, and DNA synthesis. NIH 3T3, p53$^{+/+}$MEF, p53$^{-/-}$MEF, HEK 293, 3T3, and Sf9 cells were described and cultured as described previously (20, 38). Src/Fyn/Yes$^{-/-}$ fibroblasts expressing simian virus 40 (SV40) large T antigen (SYF cells) were a gift from P. Dubreuil. BALB 3T3 (3T3) and BALB 3T3 expressing vSrc (vSrc-3T3) were as described previously (16). Transient transfection was performed with Lipofectamine Plus reagent (Gibco) according to the manufacturer's instructions. NIH 3T3 cells were also microinjected as described previously (37). In the case of biochemical analysis, cells were transfected 40 h before lysis. For DNA synthesis experiments, transfected cells seeded onto glass coverslips were incubated in Dulbecco modified Eagle medium containing 0.5% serum for 30 h and stimulated or not stimulated with the indicated mitogens. DNA synthesis was monitored by adding 0.1 mM BrdU to the medium for 18 h. Cells were fixed for immunostaining as described earlier (20). αTom1L1.2 and αTom1L1.3 were used for Tom1L1 protein expression, and αcst1 was used for Src expression. In the case of Src-promoting DNA synthesis, quiescent cells were further incubated for 18 h in the presence of BrdU before fixation. For small interfering RNA (siRNA) experiments, cells were transfected 48 h before serum starvation. For SU6656 treatment, cells were incubated with the SFK inhibitor (2 μM) 1 h before stimulation. For microinjection experiments, purified antibodies (1 mg/ml) preblocked or not with their purified antigen (1:1) for 30 min on ice, were microinjected prior to PDGF stimulation as described previously (37). For actin assembly, quiescent cells were stimulated with PDGF for 10 min before fixation. Actin was visualized with Texas Red-conjugated phalloidin (20). Fixed cells were observed with a DMRB oil immersion microscope with a PL APO ×40 lens (Leica Microsystems, Deerfield, IL). Images were captured with Micromax camera (ROPER Scientific, Inc., Trenton, NJ) driven by Metamorph (V6.2r5; Universal imaging Corp, Downingtown, PA). The percentage of injected (or transfected) cells that incorporated BrdU for each coverslip was calculated by using the following formula: percent BrdU-positive cells = (number of BrdU-positive injected [or transfected] cells/number of injected [or transfected] cells]) × 100. The percentage of transfected cells that have formed dorsal ruffles was calculated by the following formula: percentage of cells with dorsal ruffle = (number of ruffle-positive transfected cells/number of transfected cells) × 100. For each coverslip, about 150 to 200 cells were analyzed.

siRNA design and transfection. Tom1L1 siRNA (AATGCCGTCCTTGGATATGAT) and control siRNA (AATTCTCCGAACGTGTCACGT) were designed with the QIAGEN design tool and obtained from QIAGEN. Selected sequences were subjected to BLAST searches against the murine genome sequence to ensure that only the desired mRNA was targeted for Tom1L1 siRNA, and no murine mRNA sequence was targeted for control siRNA. Transfection of siRNAs (100 nM) was performed by using Lipofectamine Plus Reagent (Invitrogen) 48 h before cell starvation.

Biochemistry. Cell lysates, pull-down, immunoprecipitation, Western blotting, kinase assays, and reimmunoprecipitation were performed as described previously (30, 38). Briefly, cells were rinsed twice in ice-cold TBS (20 mM Tris [pH 7.5], 150 mM NaCl, 0.1 mM sodium orthovanadate) and then lysed with LB (20 mM Tris [pH 8], 150 mM NaCl, 1% Nonidet P-40, 1% aprotinin, 20 μM leupeptin, 10 mM NaF, 0.1 mM sodium orthovanadate). Octyl glucoside (60 mM) was added into LB to increase the solubilization of membrane proteins. For drug treatment, cells were treated for 15 min with SU6656 (2 μM) before PDGF-BB (20 ng/ml) stimulation. Purified SFK was incubated for 15 min on ice with GST fusion proteins (0.25 μM), and in vitro kinase assays were performed as described elsewhere (40). In vitro poly(Glu-Tyr) (1:4) phosphorylation assays were performed in the presence of 100 μM ATP and 2 mCi [γ-^{32}P]ATP as described by Koegl et al. (23) using purified SFK that was preincubated for 30 min on ice with phosphorylated FAKp3/2 (50 μM) or Tom1L1 proteins (0.25 μM) for complex formation. The in vitro neutralizing activity of antibodies was analyzed as follows: purified antibody was incubated for 15 min on ice with GST-Tom1L1 that was bound to glutathione beads; in vitro kinase assay was next performed with purified Fyn for 40 min at 30°C in the presence of 100 μM ATP. Beads were then extensively washed, and bound proteins were analyzed by Western blotting as described above. In vitro competition assays were performed as follows: immunoprecipitated human PDGFR that was expressed in Sf9 cells (38) was incubated on ice for 30 min with purified Fyn in the presence of indicated fusion proteins. Fyn associated to the receptor was next analyzed by Western blotting. Fyn bound to Tom1L1 proteins was analyzed after precipitation of the fusion protein complex present in the supernatant with glutathione beads.

RESULTS

Characterization of Tom1L1. We phosphorylated a cDNA expression library derived from mouse embryo (E10.5 to E11.5) with purified Src and then detected phosphorylated proteins with an antiphosphotyrosine antibody. With this approach, we isolated the tyrosine kinase Fak and the adapter p130 Cas (O. Furstoss and S. Roche, unpublished data), two Src substrates with important functions during embryogenesis (51). In addition, we obtained a novel adaptor protein that we originally called Jerry because of this homology with Tom1 (target of myb1) (14) and which turned out to be the likely mouse ortholog of human Tom1L1 (46). In the course of our study, Jerry was also published by Stein et al. as a Src activating and signaling molecule (Srcasm) (47). For clarity, Srcasm/Jerry/Tom1L1 is referred to as Tom1L1 here. This adaptor belongs to the Tom1 family, which comprises three members: Tom1, Tom1L1, and Tom1L2 (46, 47). They all include a VHS (Vps27, Hrs, and STAM) and a GAT (GGA and Tom1) homology domain involved in vesicular trafficking (7, 28), followed by unique sequences. The C terminus of Tom1L1 (amino acids 388 to 479) includes a VY_{457}EEI sequence that

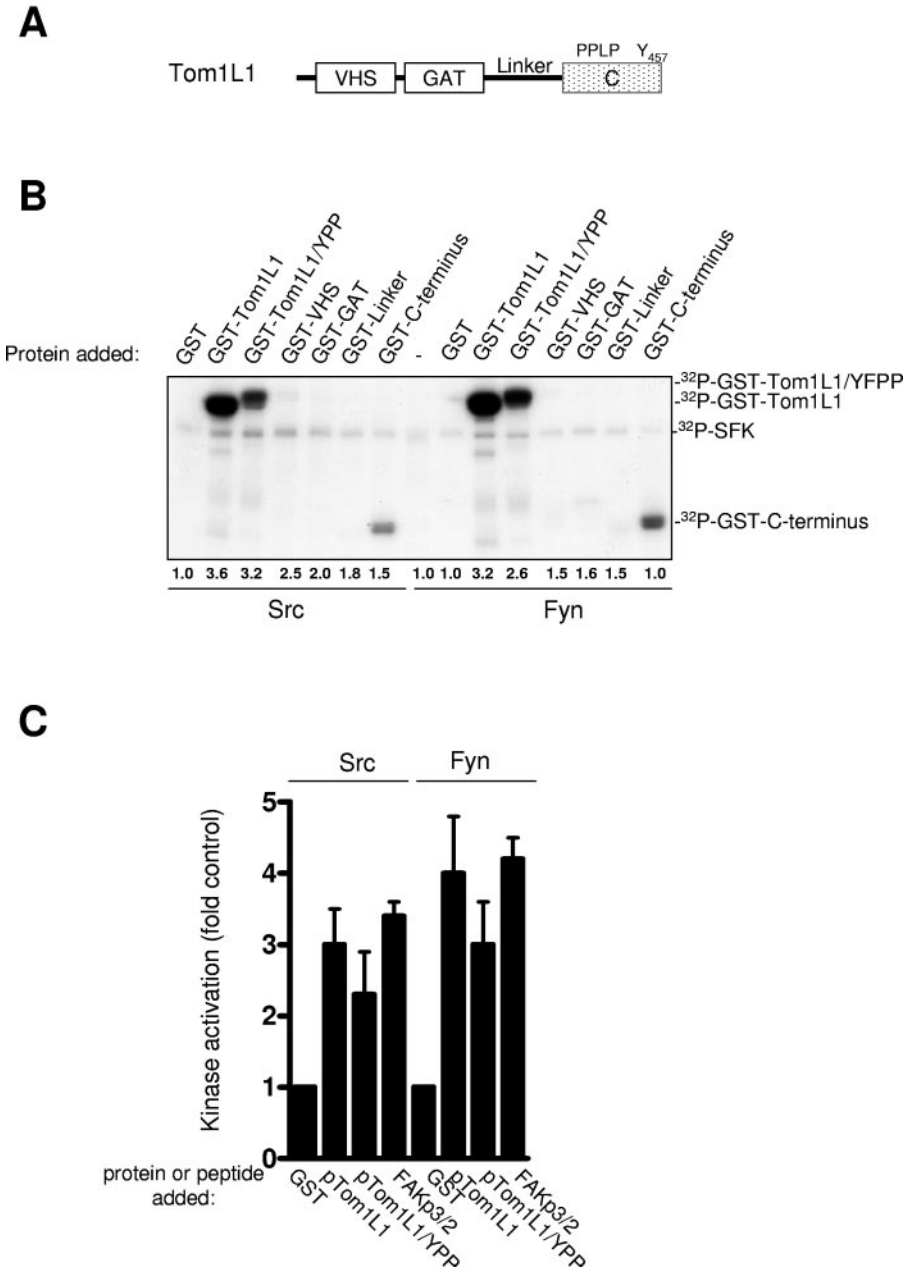

FIG. 1. Tom1L1 activates SFK in vitro, but it does not promote Src-induced DNA synthesis. (A) Schematic representation of Tom1L1. The VHS and GAT homology domains, the Linker region, and the C terminus ("C") are indicated. Potential Src binding sites $PP_{421}LP$ and Tyr457 are also indicated. (B) Tom1L1 stimulates SFK autophosphorylation in vitro. An in vitro kinase assay was performed with purified Src and Fyn as shown and in the presence of the indicated GST fusion proteins. The position of radiolabeled GST fusion proteins (^{32}P-GST fusion) and autophosphorylated SFK (^{32}P-SFK) is shown. The ratio between autophosphorylation obtained with GST fusion protein relative to GST alone is also indicated. (C) Tom1L1 stimulates SFK kinase activity in vitro. Indicated SFK was preincubated with the indicated proteins or peptides, and the kinase activity was analyzed by recording phosphorylation of poly(Glu-Tyr) (1:4). The ratio between the kinase activity obtained in the presence of indicated protein and that obtained in the presence of GST (control) is shown (kinase activation). The mean and the standard deviation are shown and were calculated from four independent experiments. (D) Tom1L1 does not promote Src-induced DNA synthesis. Quiescent NIH 3T3 cells seeded onto coverslips and transfected or not with the indicated constructs were incubated with BrdU for 18 h. Cells were then fixed and processed for immunofluorescence as described in Materials and Method. The percentage of transfected cells that incorporated BrdU was calculated as described in Materials and Methods. The mean and the standard deviation are shown from three to five independent experiments. (E) Tom1L1 does not promote phosphorylation of Src mitogenic substrates. HEK 293 cells were transfected with Src and the mitogenic substrate Abl or Myc-Shc constructs as shown together with indicated construct for 40 h. (Top panel) The level of tyrosine phosphorylation of the immunoprecipitated substrate (pY-Abl and pY-Shc), as well as the immunoprecipitated substrate, is shown. (Bottom panel) The levels of immunoprecipitated SFK and the associated Src interactors Tom1L1 and mT are shown. (F) Tom1L1 does not activate Src in vivo. HEK 293 cells were transfected with shown chicken Src alleles together with the indicated constructs for 40 h. (Top panel) The levels of immunoprecipitated chicken Src (Src), active chicken Src (pY_{416}Src), and associated Tom1L1 are shown. (Bottom panel) Tyrosine phosphorylation content and mT expression were assessed by Western blotting from 10% of the total cell lysate (10% Tot). Arrows indicate tyrosine phosphorylation of expressed mT and Tom1L1. Antibodies used for immunoprecipitation (ip) and Western blotting (wb) are shown.

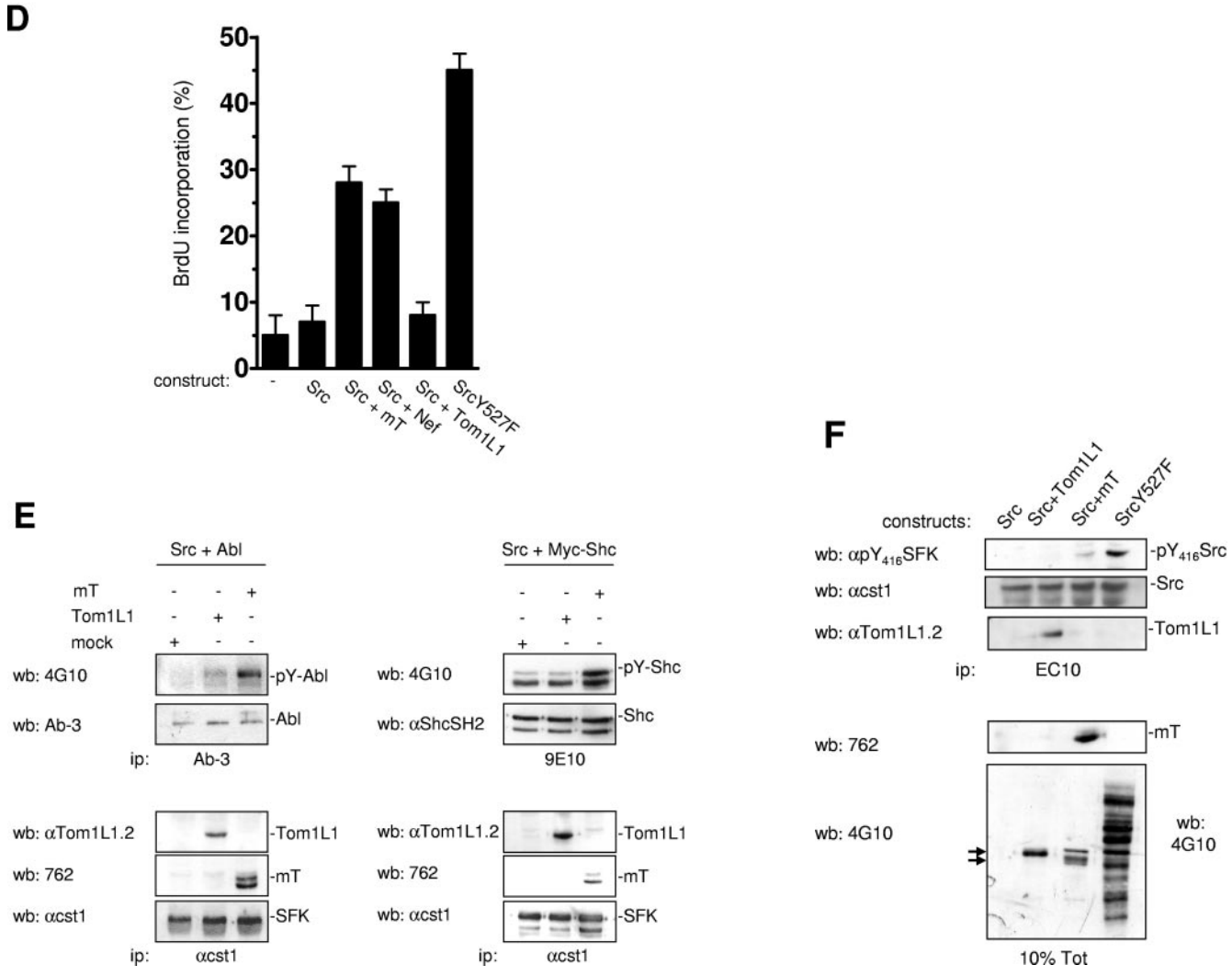

FIG. 1—*Continued.*

creates a perfect phosphorylation site for Src and binding to its SH2 domain (55), plus a $RLP_{421}PLP$ motif with some potential high affinity for SrcSH3 (Fig. 1A). This region also contains several tyrosines that, once phosphorylated, create binding sites for the SH2-containing signaling proteins Grb2 (Tyr441) and the p85 subunit of phosphatidylinositol 3-kinase (Tyr392) (48). However, Tyr457 was identified as the major Src phosphorylation site in vivo (47; Furstoss and Roche, unpublished). We also defined a region between the GAT and the C terminus we called the "Linker" (amino acids 291 to 387) (Fig. 1A) with potential function in Tom1L1 biological activity (see below). Northern blot analysis from mouse adult tissues indicated that this adaptor was ubiquitously expressed at the mRNA level with a single transcript of 2 kb and exhibited the highest expression level in brain and the lowest in lymphocytes (Furstoss and Roche, unpublished).

Activation of SFK by Tom1L1 in vitro. We first investigated the impact of Tom1L1 on SFK activities in vitro. SFK are regulated by intramolecular interactions involving the binding of SH2 to pTyr527 and the SH3 to a sequence present between the SH2 and the small lobe of the kinase (6). Tom1L1 was shown to activate Fyn that was partially purified from bovine brain, through association of pTyr457 with its SH2 and of $RLP_{421}PLP$ with its SH3 domain, allowing stabilization of the kinase in an open and active conformation (47). Using an in vitro kinase assay with purified human Src and Fyn that were expressed in insect cells, we confirmed that Tom1L1 is a strong substrate of SFK (Fig. 1B). It also stimulated catalytic activity as shown by SFK autophosphorylation. However, mutation of Tyr457 into Phe, together with $P_{421}PLP$ into AALA (GST-Tom1L1/YPP), had a reduced effect, suggesting the involvement of additional sequences in catalytic activation. Indeed, various isolated domains of Tom1L1, including VHS, GAT, and the Linker, also increased kinase activity (Fig. 1B). We also looked at the capacity of the Tom1L1-SFK complex to phosphorylate an exogeneous substrate such as poly(Glu-Tyr) (4:1) (Fig. 1C). To this end, we used Tom1L1 that was previously deleted from its GST sequence and phosphorylated by the kinase domain of Fyn (pTom1L1) in order to avoid protein dimerization and substrate competition, respectively. Accordingly, pTom1L1 induced a threefold increase in poly(Glu-Tyr) (1:4) phosphorylation by Src and Fyn, and we observed a min-

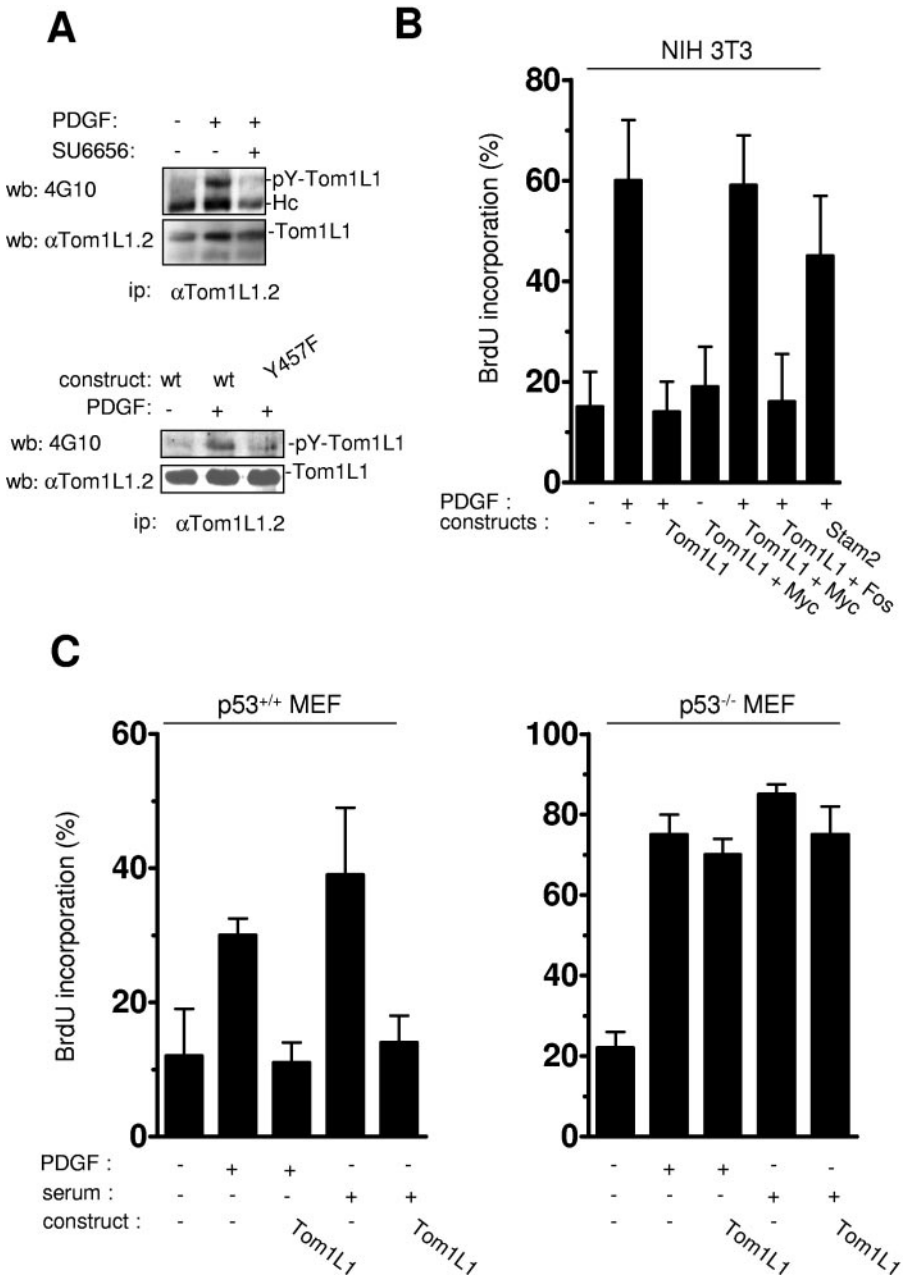

FIG. 2. Tom1L1 overexpression inhibits SFK mitogenic signaling. (A) Tom1L1 is SFK substrate in PDGF-stimulated cells. (Top panel) quiescent NIH 3T3 cells were pretreated or not with SFK specific inhibitor SU6656. (Bottom panel) NIH 3T3 cells were transiently transfected with the indicated construct. Quiescent cells were then stimulated with PDGF (20 ng/ml) for 5 min. The tyrosine phosphorylation of immunoprecipitated Tom1L1 (pY-Tom1L1), as well as the level of the precipitated protein (Tom1L1), is shown. The presence of heavy chain (Hc) is indicated. Antibodies used for immunoprecipitation (ip) and Western blotting (wb) are shown. (B) Tom1L1 overexpression inhibits the mitogenic response induced by PDGF that was overcome by Myc expression. (C) Overexpressed Tom1L1 does not inhibit mitogenesis in fibroblasts with inactive p53. (D) Tom1L1 mitogenic inhibition was alleviated by RacV12, Src, and Fyn expression. (E) Tom1L1 also inhibits the serum response by affecting SFK mitogenic signaling. Indicated cells seeded onto coverslips and transfected or not with indicated constructs were made quiescent by serum starvation of the medium for 30 h, treated or not with SU6656 (2 μM) 1 h before stimulation as shown, and stimulated or not with PDGF (20 ng/ml) or 10% fetal calf serum (serum) as indicated in the presence of BrdU for 18 h. Cells were then fixed and processed for immunofluorescence as described in Materials and Methods. The percentage of transfected cells that incorporated BrdU was calculated as described in Materials and Methods. Note that Src and Fyn constructs were cotransfected in a fourfold excess for significant mitogenic rescue. The results are expressed as the mean ± the standard deviation for three to five independent experiments.

imal contribution to the Tyr457 and the P_{421}PLP on this biochemical effect. Activation was similar to that obtained with a phosphopeptide of the Fak sequence (FAKp3/2) used as a positive control (1). We concluded that SFK regulation by Tom1L1 involves several domains of the adaptor including the Linker region.

Tom1L1 does not promote DNA synthesis induced by Src. The role of Tom1L1 was next investigated on Src activity in

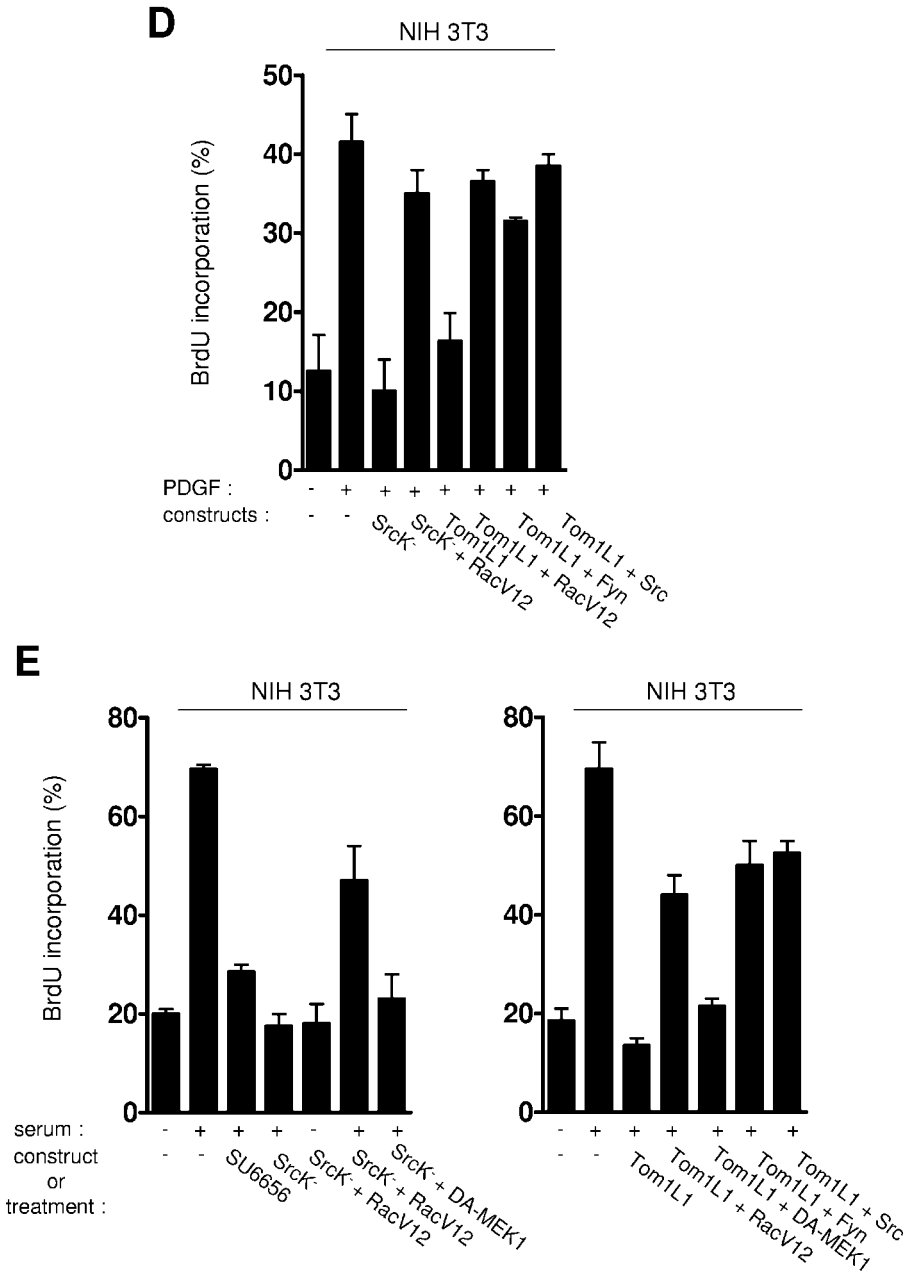

FIG. 2—*Continued.*

vivo. Like mouse middle T antigen of the polyomavirus (mT) (18), Tom1L1 activates Src in vitro, and it is suspected to create binding sites for the signaling proteins involved in the activation of Ras/MAPK and phosphatidylinositol 3-kinase pathways (47), both required for DNA synthesis (18). Therefore, it has been suggested that, like mT, Tom1L1 should activate Src oncogenic activity. To address this issue, we looked at the capacity of these adaptors to promote Src-induced DNA synthesis in the absence of extracellular mitogens. DNA synthesis was monitored by BrdU incorporation assays. As previously reported, overexpression of wild-type Src per se did not induce DNA synthesis, whereas the activated counterpart SrcY527F did it for 50% of the cells (30) (Fig. 1D). Surprisingly, Tom1L1 did not promote Src-induced DNA synthesis in contrast to mT (30%) and the Nef adaptor protein of human immunodeficiency virus (25%), another activator of SFK (31). The activity of Tom1L1 was next confirmed at the molecular level. Unlike mT, Tom1L1 failed to induce phosphorylation of mitogenic substrates required for Src-induced DNA synthesis, such as Shc (18) and the cytoplasmic tyrosine kinase Abl (A. Boureux and S. Roche, unpublished data) (Fig. 1E). Finally, whereas Tom1L1 associated with Src and was tyrosine phosphorylated in vivo, no clear kinase activation was detected when analyzed on the tyrosine phosphorylation content of the cell and on the level of active pY_{416}Src (Fig. 1F).

Tom1L1 inhibits SFK mitogenic signaling induced by PDGF. The effect of Tom1L1 on the Src mitogenic function was next investigated in PDGF-stimulated cells. Phospho-proteomic

analyses have previously implicated members of the Tom1 family in PDGF receptor signaling (24). Here we show that Tom1L1 is an SFK substrate in PDGF-stimulated cells (Fig. 2A): PDGF-induced tyrosine phosphorylation of endogenous Tom1L1, which was inhibited by the SFK specific inhibitor SU6656 (5). Furthermore, transient expression of various Tom1L1 mutants confirmed that Tyr457 is the major phosphorylation site in these cells. The function of Tom1L1 in mitogenesis was then investigated. As shown in Fig. 2B, PDGF induced 60% of S phase entry, and this response was strongly inhibited by Tom1L1. This effect was specific for this adapter since Stam2 (19, 35), another VHS-containing protein did not affect, if any, mitogenesis. Similar results were obtained with primary culture of mouse embryo fibroblasts (Fig. 2C), indicating that this effect was not specific for the cell line used. In agreement to these data, we were unable to generate NIH 3T3 cell lines stably overexpressing Tom1L1, unlike Stam2 (Furstoss and Roche, unpublished). Whether Tom1L1 impinges on the SFK pathway was then addressed. We first observed a link between Tom1L1 and SFK mitogenic targets required for cell cycle progression, including Myc, p53, and Rac (8, 9, 15): constitutive expression of Myc rescued PDGF receptor signaling that was blocked by Tom1L1 (Fig. 2B). This effect was specific to this transcription factor as was not observed with Fos. Second, whereas Tom1L1 inhibited the mitogenic response induced PDGF in wild-type mouse embryo fibroblasts, an inhibitory effect was not observed in cells deficient in p53 (Fig. 2C). Finally, low expression of constitutively active RacV12 was sufficient to alleviate mitogenic inhibition induced by both kinase inactive SrcK$^-$ and Tom1L1 (Fig. 2D). We concluded that Tom1L1 inhibits mitogenesis by affecting SFK signaling. Surprisingly, we also found that Src or Fyn coexpression was sufficient to override the G_1 block induced by Tom1L1, indicating that Tom1L1 inhibits mitogenic signaling at the SFK level.

Previous reports indicated that serum also required SFK for mitogenesis (8, 20, 37). This is exemplified with the mitogenic inhibition obtained with the SFK inhibitor SU6656 or expression of SrcK$^-$ in fibroblasts (Fig. 2E). Mitogenesis is largely independent of PDGF but may require additional mitogens present in fetal calf serum that are still to be identified (20). Whatever the growth factors involved in this response, we found that SFK use common mitogenic targets for signaling including p53 and Rac (Fig. 2C and E) (20). We then examined the role of Tom1L1 in the serum response and found that it also inhibited the induction of DNA synthesis. Interestingly, inhibition was largely dependent upon its capacity to affect SFK signaling (Fig. 2C and E). Most importantly, we also found that Src or Fyn coexpression alleviated the inhibition induced by Tom1L1 (>80%). We concluded that the major mechanism by which Tom1L1 interferes with cell-cycle progression occurs through SFK.

Inactivation of Tom1L1 potentiates mitogenesis. We also evaluated the function of endogenous Tom1L1 by using two independent approaches. Microinjection of neutralizing antibody toward adapter proteins have been successfully used to uncover their function into cells (13, 37); therefore, a similar approach was used for Tom1L1. Three polyclonal antibodies have been raised against various regions of the protein, including the whole Tom1L1 protein (αTom1L1.1), the linker region plus the C terminus (αTom1L1.2), and the VHS plus GAT domains (αTom1L1.3). We found that antibodies 1 and 2 specifically recognized endogenous Tom1L1 expressed in NIH 3T3 cells in Western blotting of a total cell lysate (Fig. 3A). Interaction was specific to the antigen, as Tom1L1 was not detected by the antibody preblocked by its cognate antigen. Note that a protein of 40 kDa was also frequently observed with these antibodies. However, we believe that it is a truncated form of Tom1L1 since it was also detected in mammalian cells expressing the ectopic protein (Fig. 5A). The neutralizing effect of these antibodies was then analyzed in vitro. To this end, bound GST-Tom1L1 was preincubated with antibodies and then incubated with purified Fyn in the presence of ATP for in vitro phosphorylation. After extensive washings, both tyrosine phosphorylation of Tom1L1 and Fyn association were assessed by Western blotting. As shown in Fig. 3B, pY-GST-Tom1L1 and bound Fyn were readily detected in the presence a control antibody. In contrast, both events were strongly reduced by anti-Tom1L1.1 and just abrogated by anti-Tom1L1.2. Similar results were obtained with Src (Furstoss and Roche, unpublished). We concluded that our antibodies inhibit the capacity of SFK to phosphorylate and associate with Tom1L1. Their effect was next assessed in vivo (Fig. 3C). Quiescent cells were microinjected with Tom1L1 antibodies and stimulated with a physiological dose of PDGF in the presence of BrdU for 18 h. We observed that more microinjected cells entered S phase than control cells (twofold). Preincubation of the antibodies with their respective antigens abrogated their potentiating effects, indicating that this was specific to Tom1L1. Specificity was also confirmed by the inability of an antibody specific to Stam proteins (29) to increase mitogenesis. These data were next confirmed by using a siRNA approach to specifically reduce Tom1L1 level into cell (Fig. 3D). From four tested siRNA specific to the murine Tom1L1 sequence, only one induced 70% inhibition of protein level (M. Franco and S. Roche, unpublished data). As shown in Fig. 3D, Tom1L1 downregulation allowed a >2-fold increase in mitogenic response induced by a physiological dose of PDGF. The potentiating effect was dependent upon SFK activity since it was abrogated by SU6656. Biological effect was not restricted to PDGF since a similar effect was obtained with a low dose of fetal calf serum. Again, cellular effects were specific to Tom1L1 as not observed with a control siRNA. Collectively, these data show that Tom1L1 is a negative regulator of DNA synthesis induced by growth factors.

Tom1L1 modulates SFK association with the PDGF receptor. The mechanism by which Tom1L1 inhibits mitogenesis was next analyzed in further details. The Src mitogenic signaling is initiated by the association of SrcSH2 with the PDGF receptor (PDGFR), leading to catalytic activation (11). Since pY457Tom1L1 also associates with SrcSH2, we postulated that the phosphorylated adaptor will compete with PDGFR for SFK association. This hypothesis was first evaluated in vitro (Fig. 4A). To this end, human PDGFR that was expressed in Sf9 cells was immunoprecipitated and incubated with purified Fyn in the presence of various GST-Tom1L1 proteins. After extensive washings, the presence of bound Fyn was detected by Western blotting. We found that neither GST alone nor GST-Tom1L1 affected Fyn/PDGFR complex formation, although some Fyn was associated with Tom1L1. In contrast

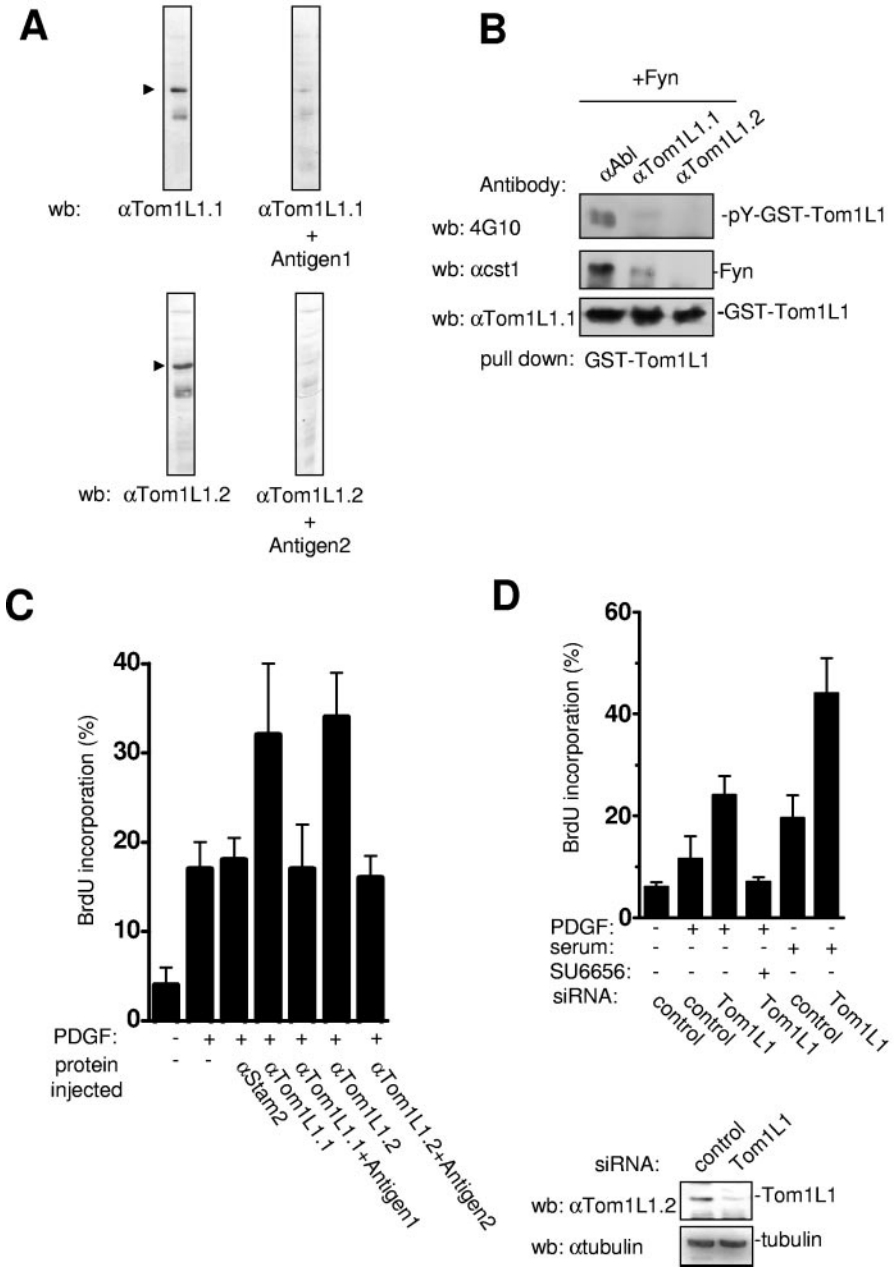

FIG. 3. Tom1L1 regulates mitogenesis. (A) Characterization of Tom1L1 antibodies. Western blotting of a NIH 3T3 total cell lysate with indicated affinity-purified Tom1L1 antibodies that were preblocked or not with their respective antigen. The arrows indicate the presence of Tom1L1. (B) Neutralizing activity of Tom1L1 antibodies in vitro. GST-Tom1L1 that was bound to glutathione beads (pull down) was preincubated with indicated antibody and subjected to an in vitro kinase assay with purified Fyn and subjected to extensive washings. The levels of bound GST-Tom1L1, bound phosphorylated GST-Tom1L1 (pY-GST-Tom1L1), and associated Fyn are shown. Antibodies used for immunoprecipitation (ip) and Western blotting (wb) are shown. (C) Microinjection of αTom1L1 antibodies potentiate the PDGF mitogenic response. Quiescent NIH 3T3 cells seeded onto coverslips were microinjected with the indicated antibody, stimulated or not with PDGF (5 ng/ml) in the presence of BrdU for 18 h. Cells were then fixed and processed for immunofluorescence as described in Materials and Methods. Shown is the percentage of BrdU incorporation present in injected and noninjected cells under the specified conditions and calculated as indicated in Materials and Methods. The results are expressed as the mean ± the standard deviation of six independent experiments. (D) The top panel shows increased mitogenesis in cells with a reduced level of Tom1L1. Quiescent NIH 3T3 cells transfected with the indicated siRNA and treated or not with SU6656 (2 μM) 1 h before stimulation as shown were stimulated or not stimulated with PDGF (5 ng/ml) or 2.5% fetal calf serum (serum) as indicated and in the presence of BrdU for 18 h. The percentage of BrdU incorporation under the specified conditions is shown. The data from three independent experiments have been averaged, and the mean and standard deviation are shown. The bottom panel shows the reduction of Tom1L1 level by siRNA transfection. The level of Tom1L1 and tubulin in cells transfected with indicated siRNA is shown and was assessed by Western blotting from a total cell lysate. Antibodies used for Western blotting (wb) are shown.

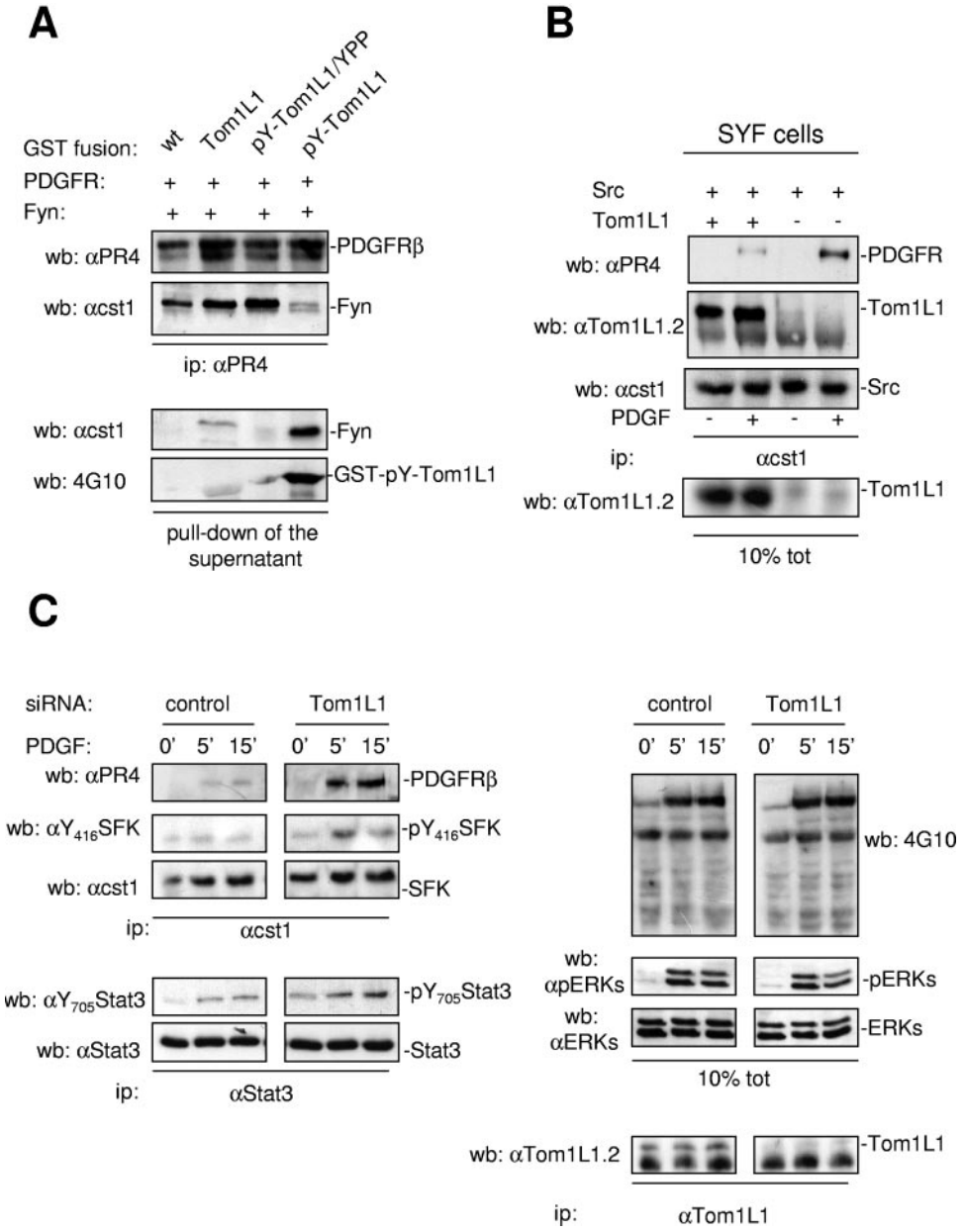

FIG. 4. Tom1L1 regulates SFK/PDGFR complex formation for mitogenic signaling. (A) Tom1L1 competes with PDGFR for Fyn association. Immunoprecipitated PDGFR that was expressed in Sf9 cells was incubated with purified Fyn together with the indicated GST fusion proteins. (Top panel) Levels of precipitated PDGFR and associated Fyn are shown. (Bottom panel) Fyn associated with the GST proteins was precipitated from the supernatant using glutathione beads. The level of Fyn associated to GST fusion proteins, as well as the level of tyrosine phosphorylated Tom1L1 proteins (pY-GST-Tom1L1), is shown. (B) Tom1L1 overexpression competes with PDGFR for Src association in vivo. Quiescent SYF cells that were transiently transfected with indicated constructs were stimulated or not stimulated with PDGF (25 ng/ml) for 10 min. The levels of immunoprecipitated Src, associated Tom1L1 and PDGFRβ are shown. The level of Tom1L1 is also shown and was assessed from 10% of the total cell lysate. (C and D) Increased SFK association with the PDGFRβ in cells with reduced Tom1L1 level. Quiescent NIH 3T3 cells that were transfected with indicated siRNA were stimulated with PDGF (5 ng/ml) for the indicated times. The levels of immunoprecipitated SFK, active SFK ($pY_{416}SFK$) and associated PDGFRβ are shown. The levels of immunoprecipitated Stat3 and phosphorylated Stat3 (pY705Stat3), as well as immunoprecipitated Tom1L1, are shown. Levels of tyrosine phosphorylation content, active MAPKs (pMAPKs), and MAPKs are also shown and were assessed from 10% of total cell lysates (10% tot). (D) Kinase assay of immunoprecipitated SFK from indicated cells. The presence of associated PDGFR was confirmed by reimmunoprecipitation experiment with normal rabbit serum as a control (NRS) or the PR4 antibody. Autophosphorylated SFK (^{32}P-SFK) and labeled PDGFR (^{32}P-PDGFR) are shown. Antibodies used for immunoprecipitation (ip) and Western blotting (wb) are indicated.

pY-Tom1L1 inhibited binding to the receptor by 80%. Accordingly, most Fyn was found associated with the phosphorylated adaptor. Inhibition implicated pTyr457 since it was not observed with the phosphorylated Tom1L1/YFPP mutant. Whether a similar mechanism occurs in mammalian cells was then investigated (Fig. 4B). To this end, Src was transiently expressed together or not with Tom1L1 in SV40 large-T-antigen-transformed fibroblasts deficient in Src, Fyn and Yes (SYF

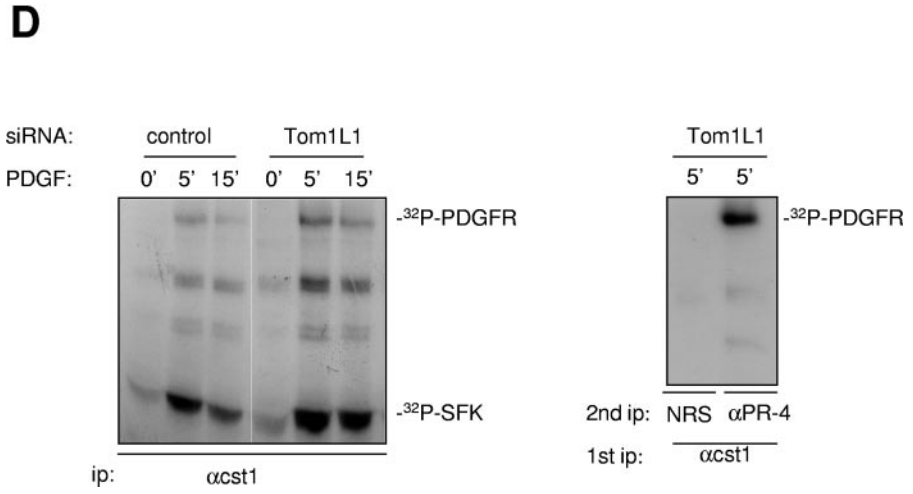

FIG. 4—*Continued.*

cells). Cells were made quiescent and stimulated with PDGF. We found that Tom1L1 overexpression strongly associated with Src, allowing a reduction of the Src/PDGFR complex formation. However, Tyr457 mutation poorly affected Tom1L1 effect (S. Roche, unpublished data), suggesting the existence of additional mechanisms for in vivo inhibition.

The role of endogenous Tom1L1 was also addressed on SFK-PDGFR complex formation in NIH 3T3 cells (Fig. 4C and D). We found that at a physiological dose of PDGF both association and activation of SFK by the receptor was strongly increased in cells with reduced Tom1L1. In vivo activation was further confirmed on phosphorylation of the Src mitogenic substrate Stat3. In contrast, Tom1L1 inactivation neither affected tyrosine phosphorylation content nor early MAPK activation. Nevertheless, a small but significant reduction of MAPK activation was frequently observed at longer times of stimulation (Fig. 4C). Collectively, these data indicate that Tom1L1 regulates Src mitogenic signaling by modulating the association of SFK with PDGFR.

Role for the Linker domain in Tom1L1 activity. Since Tyr457 was not sufficient to mediate Tom1L1 mitogenic inhibition, we reevaluated Src-Tom1L1 complex formation (Fig. 5A). Tom1L1 associated with Src when it was coexpressed in HEK 293 cells. Interaction was reduced by 80% with the Tom1L1 allele deleted from the described SrcSH2 and SrcSH3 binding sites (Tom1L1/YFPP). This allowed us to surmise an additional sequence within Tom1L1 for Src interaction. Although neither VHS nor GAT deletion affected Src/Tom1L1 complex formation (M. Franco, V. Simon, and S. Roche, unpublished data), kinase association was reduced with the Tom1L1ΔL allele deleted from the Linker, implicating this region in protein interaction. Indeed, Src failed to associate with Tom1L1ΔL/YFPP that was deleted from both SrcSH2 and SrcSH3 binding sites and the Linker region. We concluded that both regions are required for Src association in vivo. Src-Tom1L1 interaction was next confirmed in vitro. As shown in Fig. 5B, purified Src specifically interacted with GST-Tom1L1. Src also strongly interacted with GST-C terminus and GST-Linker regions of Tom1L1, confirming in vivo data. Similar results were obtained with purified Fyn (Franco and Roche, unpublished), suggesting a common mechanism for Tom1L1/SFK complex formation. In addition, our in vitro data suggest the existence of a physical interaction between the kinase and these regions of Tom1L1. It should be noticed that a low but significant interaction could be also observed with GST-VHS sequence, but this was not reproduced in vivo (Franco and Roche, unpublished). Finally, the biological incidence of Src association on Tom1L1 mitogenic activity was also evaluated (Fig. 5C). To this end, Tom1L1 alleles were expressed under the control of a retroviral promoter that allows a low ectopic protein expression (Fig. 5C). In these conditions, we found that unlike the wild-type molecule, Tom1L1ΔL/YFPP did not affect the PDGF mitogenic response. We concluded that Tom1L1 requires SFK association for mitogenic inhibition.

Tom1L1 does not affect DNA synthesis induced by oncogenic Src. Whether Tom1L1 also regulates DNA synthesis induced by a constitutive allele of Src was next investigated. First, we found that endogenous Tom1L1 is strongly phosphorylated by vSrc (Fig. 6A), implicating Tom1L1 as a mediator of Src oncogenic activity. Nevertheless, its overexpression did not affect the capacity of the active SrcY527F to induce DNA synthesis per se (Fig. 6C). It should be noted that a small but significant inhibition was obtained with the adaptor Stam2, originally used as a negative control. The meaning of this inhibition is currently unknown. Similarly, SrcY527F did not require endogenous Tom1L1 for the induction of DNA synthesis since reduction of the protein level did not affect this cellular response (Fig. 6D). The absence of Tom1L1 on oncogenic Src biological activity was also confirmed at the molecular level. As shown in Fig. 6B, its overexpression did not affect Src activity, as shown by the tyrosine phosphorylation content and the level of pY416Src. We concluded that Tom1L1 is not involved in DNA synthesis induced by deregulated Src.

Tom1L1 does not regulate SFK-mediated actin assembly induced by PDGF. Whether Tom1L1 regulates additional SFK signaling pathways was also addressed. In addition to mitogenesis, PDGF induces F-actin enriched dorsal ruffles that were linked to cell migration into extracellular matrix (49). We have recently shown that this response also implicates SFK. In ad-

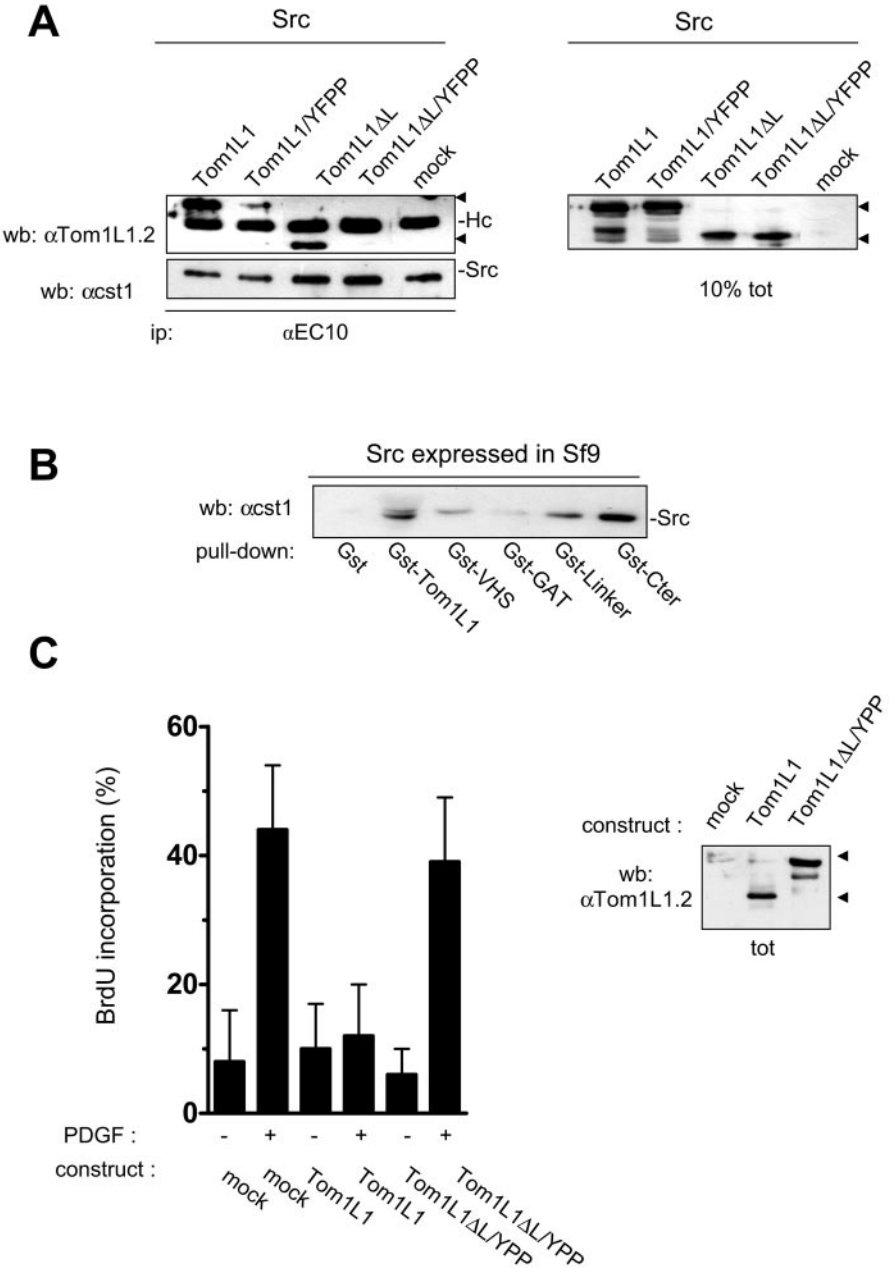

FIG. 5. Tom1L1 requires SFK association for in vivo mitogenic activity. (A) In vivo association of Tom1L1 with Src additionally requires the Linker region. HEK 293 cells were transfected with chicken Src together with empty vector (mock) or indicated Tom1L1 constructs. The level of immunoprecipitated Src together with associated Tom1L1 mutants is shown. The level of Tom1L1 proteins is also shown and was assessed from 10% of the total cell lysate. Antibodies used for immunoprecipitation (ip) and Western blotting (wb) are shown. Arrows indicate the presence of indicated Tom1L1 mutants. (B) In vitro association of Tom1L1 with Src additionally requires the linker region. The indicated GST fusion proteins bound to glutathione beads were incubated with purified Src that was expressed in Sf9 cells. The level of bound Src that was assessed by Western blotting with the indicated antibody is shown. (C) Overexpression of a Tom1L1 mutant that does not associate with SFK does not inhibit the PDGF mitogenic response. The left panel shows results for quiescent NIH 3T3 cells seeded onto coverslips that were transfected with empty vector (mock) or the indicated constructs, that were stimulated or not with PDGF as indicated, in the presence of BrdU for 18 h. Cells were then fixed and processed for immunofluorescence as described in Materials and Methods. The percentage of BrdU incorporation under the specified conditions is shown. The results are expressed as the mean ± the standard deviation for three to five independent experiments. The right panel shows the level of Tom1L1 alleles that were transfected with the indicated constructs and that was assessed by Western blotting (wb) from a total cell lysate (tot) with the indicated antibody.

dition, our results suggest that in contrast to Src mitogenic function, SFK signal transduction promoting actin assembly does not require receptor association. Rather, it involves the lipid second messenger sphingosine 1 phosphate that may activate SFK through the seven transmembrane receptor of the EDG family and an associated heterotrimeric Gi protein (52; L. Veracini, M. Franco, A. Boureux, S. Roche, and C. Bénistant, submitted for publication). In NIH 3T3 cells, PDGF

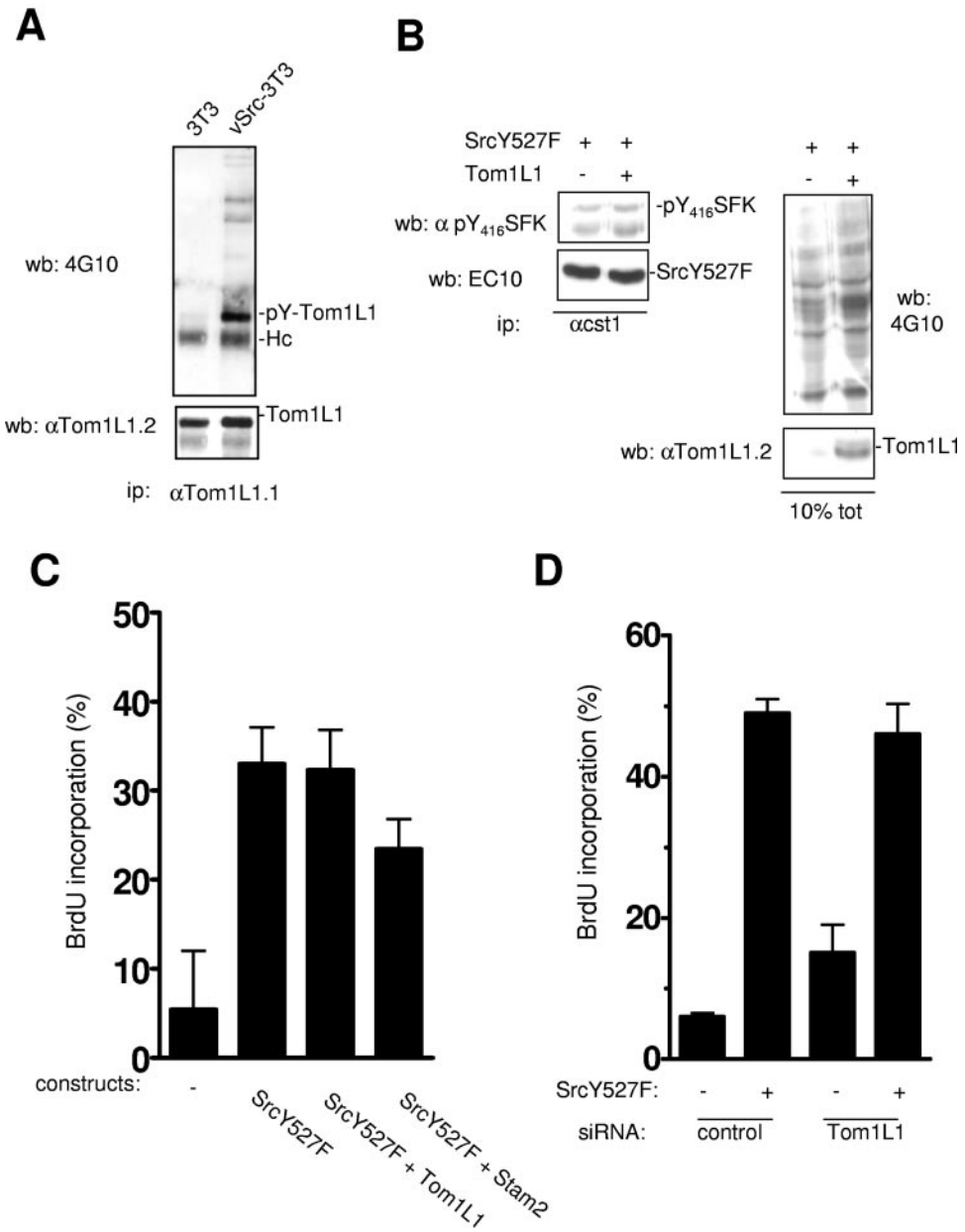

FIG. 6. Tom1L1 does not regulate DNA synthesis induced by active SrcY527F. (A) Tom1L1 is tyrosine phosphorylated in cells transformed by vSrc. Tom1L1 was immunoprecipitated from BALB 3T3 (3T3) and BALB 3T3 expressing by vSrc (vSrc-3T3). The levels of immunoprecipitated Tom1L1, tyrosine-phosphorylated Tom1L1 (pY-Tom1L1), and associated tyrosine-phosphorylated proteins are shown. The presence of heavy chain is indicated (Hc). (B) Tom1L1 does not affect in vivo activity of SrcY527F. HEK 293 cells were transfected with the indicated constructs. The level of immunoprecipitated SFK and active SFK ($pY_{416}SFK$) is shown (left panel). The tyrosine phosphorylation content and the expression of Tom1L1 are also shown (right panel) and were assessed from 10% of the total cell lysate (10% tot). Antibodies used for immunoprecipitation (ip) and Western blotting (wb) are indicated. (C) Tom1L1 overexpression does not affect DNA synthesis induced by SrcY527F. Quiescent NIH 3T3 cells seeded onto coverslips and transfected or not with Src527F, together with indicated constructs were incubated with BrdU for 18 h. (D) SrcY527-induced DNA synthesis does not require Tom1L1. Quiescent NIH 3T3 that were transfected with indicated siRNA together or not with SrcY527F as shown were incubated with BrdU for 18 h. Cells were then fixed and processed for immunofluorescence as described in Materials and Methods. The percentage of BrdU incorporation under the specified conditions is shown. The results are expressed as the mean ± the standard deviation of three independent experiments.

induced dorsal ruffle formation in 50% of the cells (Fig. 7). Accordingly, the kinase dead allele $SrcK^-$ reduced this response by 60%. In contrast, neither Tom1L1 overexpression nor endogenous protein downregulation had an effect on this cytoskeletal rearrangement (Fig. 7). Therefore, Tom1L1 may not regulate all biological responses regulated by SFK.

Tom1 and Tom1L2 overexpression also regulate mitogenesis. Finally, we analyzed whether some functional redundancy

exist between members of Tom1 family. First, we observed that all human Tom1 members associate with Src when coexpressed in HEK 293 cells, with a stronger interaction for Tom1L1 and Tom1L2 (Fig. 8C). As observed with murine Tom1L1, the human ortholog was heavily phosphorylated by Src. Surprisingly, Tom1 was also found to be tyrosine phosphorylated, although the responsible kinase was not formerly identified. The role of Tom1 adaptors was next evaluated on mitogenesis (Fig. 8A). Overexpression of all adaptors affected the induction of DNA synthesis induced by PDGF and serum with the following relative potencies: Tom1L1 (both murine mTom1L1 and human hTom1L1) > Tom1L2 > Tom1. Tom1 had the smallest effect, suggesting that it may not have a major function in this cellular response. Finally, we observed that mitogenic inhibition induced by human Tom1L1 and Tom1L2 was largely reversed by Myc expression, in agreement with a regulatory function in SFK mitogenic signaling (Fig. 8B). We concluded that human and murine Tom1L1 have similar functions during mitogenesis. Furthermore, these data raise the notion that Tom1 members have partial redundant functions in the regulation SFK mitogenic signal transduction.

DISCUSSION

We have characterized here the role of Tom1L1 in SFK mitogenic signal transduction. We have isolated Tom1L1 with the use the genetic screen previously described by Courtneidge for the search of novel Src substrates (17). Such an approach has been successfully used for the identification of Tks5/Fish, a substrate involved in cancer invasion (44). Interestingly, neither Tom1L1 nor Tks5 has not been detected by phosphoproteomic approaches (24, 42), while it is heavily tyrosine phosphorylated in cells expressing oncogenic Src (27; the present study). This confirmed that while promising, phospho-proteomic analyses are not exhaustive, and complementary approaches are still valuable for identification of specific tyrosine kinase substrates.

Tom1L1 has been previously characterized as a Src activator, based on its interaction with SrcSH2 and SrcSH3 (47). Indeed, Tom1L1 stimulates SFK enzymatic activity in vitro. Tom1L1 has been also suggested to exhibit oncogenic activity like mT (47). However, our report strongly suggests that it is neither a good activator nor an oncogenic inducer of SFK in vivo. The reason for such differences it not known, but we believe that it additionally interacts with a SFK regulator that may prevent catalytic activation at least on the promotion of mitogenic signaling. In agreement with this idea, SFK activation can be observed in cells that express very high concentrations of Tom1L1, which may titrate out such an interactor (Franco and Roche, unpublished). Similarly, Tom1L1 may activate SFK in cells with a reduced level of such a binder. This would explain the moderate SFK activation observed in keratinocytes expressing very high level of Tom1L1 (26). The search for such interactors has been undertaken in the lab, and we have recently isolated a potential candidate that might confirm this hypothesis. Characterization of this novel regulator is under investigation (M. Franco, V. Simon, C. Benistant, and S. Roche, unpublished data).

Several mechanisms have been reported for regulation of SFK mitogenic signaling induced by growth factors. This in-

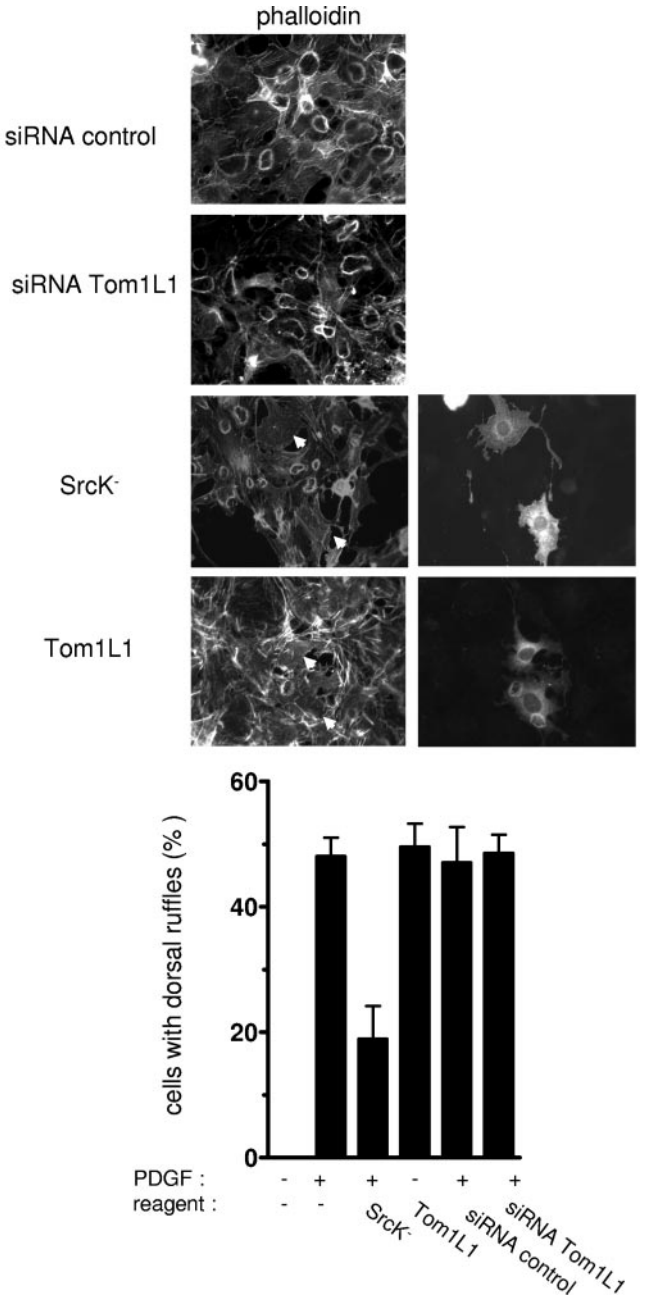

FIG. 7. Tom1L1 does not regulate SFK-mediating actin assembly induced by PDGF. (Top panel) Illustration of a typical effect obtained in cells transfected with indicated siRNA or indicated construct on PDGF-induced dorsal ruffle formation. White arrows indicate cells expressing the transfected construct. (Bottom panel) Statistical analysis on PDGF response (percentage of cells with dorsal ruffle) of cells expressing indicated construct or transfected with indicated siRNA. Quiescent NIH 3T3 cells that were transfected or not with indicated construct or siRNA, were stimulated or not with PDGF (15 ng/ml) for 10 min. Cells were fixed and proceeded for actin staining using rhodamine-phalloidin and ectopic protein expression as described in Materials and Method. The percentage of cells that formed dorsal ruffles was calculated, and statistical analysis was assessed as described in Materials and Methods. The results are expressed as the mean ± the standard deviation of three independent experiments.

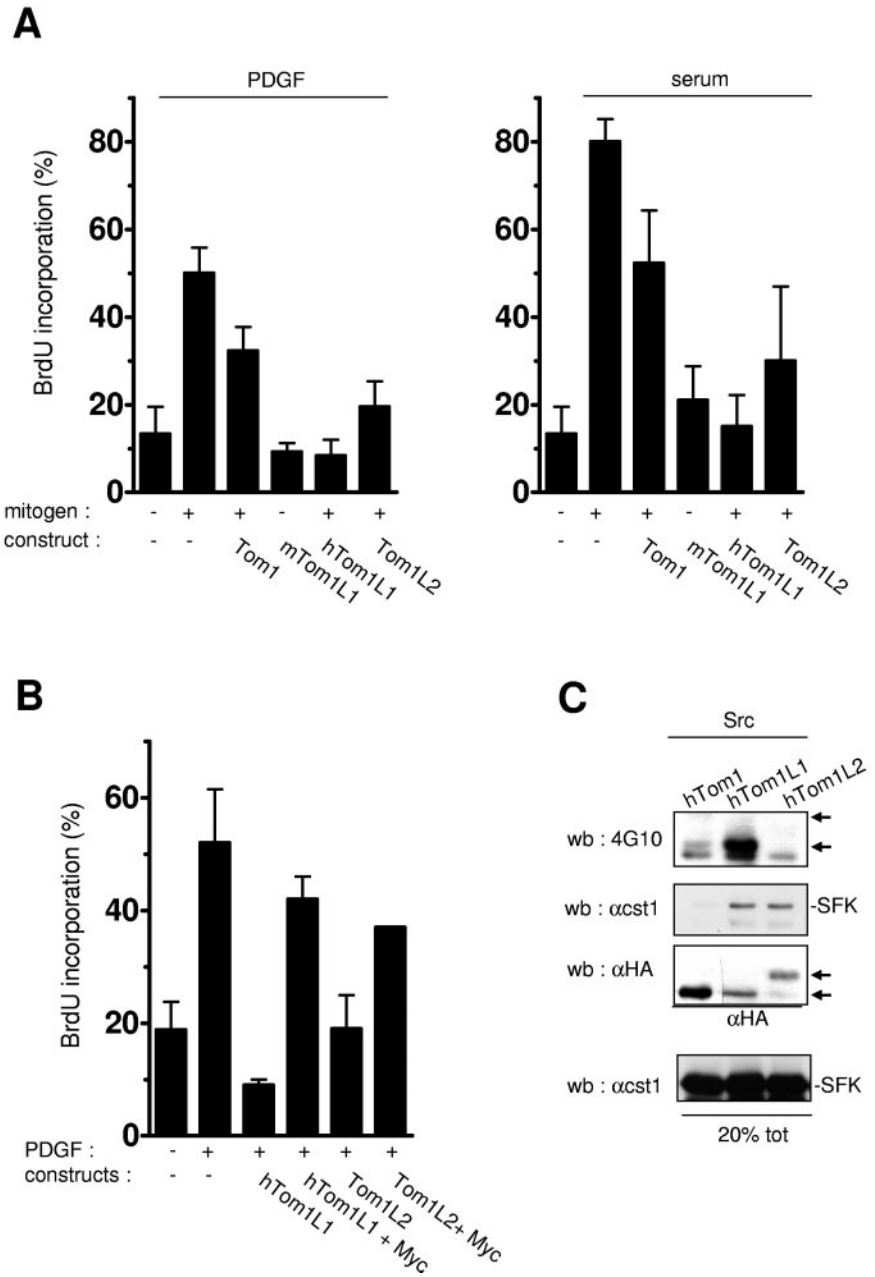

FIG. 8. Effect of other members of Tom1 family on mitogenesis. (A) Overexpression of members of the Tom1 family affected mitogenesis. (B) Inhibitory effects are overcome by Myc expression. Quiescent NIH 3T3 cells seeded onto coverslips that were transfected or not with indicated constructs were stimulated or not with PDGF (20 ng/ml) or 10% fetal calf serum (serum) as indicated and in the presence of BrdU for 18 h. Cells were then fixed and processed for immunofluorescence as described in Materials and Methods. The percentage of BrdU incorporation under the specified conditions is shown. The results are expressed as the mean ± the standard deviation of three independent experiments. (C) Tom1 members associate with SFK. HEK 293 cells were transfected with Src together with the indicated constructs expressing HA-tagged Tom1 members. The level of immunoprecipitated Tom1 proteins, tyrosine phosphorylation, and associated SFK is shown. The level of SFK is also shown and was assessed from 20% of the total cell lysate (20% tot). Antibodies used for immunoprecipitation (ip) and Western blotting (wb) are shown. Arrows indicate the presence of ectopic Tom1 proteins.

cludes the Src-like adaptor protein (Slap), which may act by competition with Src for binding to the receptor (37) and/or its mitogenic substrates (30). Another mechanism involves the transmembrane protein of the TRAP family and Csk binding protein Cbp/PAG (22). When phosphorylated, Cbp/PAG recruits Csk to the membrane, allowing SFK inactivation (22). While indeed this adaptor regulates SFK mitogenic signaling induced by growth factors, the involved mechanism is still not clearly defined (54; L. Veracini, S. Roche, V. Horejsi, and C. Benistant, unpublished data). We report here an additional mechanism with the example of Tom1L1. Our data indicate that it regulates mitogenic signaling by modulating SFK/recep-

tor association. In agreement to this notion, Tom1L1 inhibition is overcome by SFK coexpression. Its overexpression reduced association, whereas protein inactivation increases complex formation. Furthermore, Tom1L1 does not affect proliferative signaling induced by a constitutive active allele of Src. Finally, it does not regulate SFK-mediating actin assembly, a cellular process that does not require receptor association (52; Veracini et al., submitted). How Tom1L1 regulates complex formation is still not completely resolved. A simple competition mechanism between pY579-PDGFR and pY457-Tom1L1 has been observed in vitro, and a similar mechanism may exist in vivo. Accordingly, Tom1L1 overexpression reduced association with the receptor in SYF fibroblasts. However, mutagenesis analysis suggests the existence of a more complicated mechanism that is still under current investigation (M. Franco, C. Benistant, and S. Roche, unpublished data). Nevertheless, our mutagenesis analysis implicated SFK association for Tom1L1 mitogenic function.

Tom1L1 may share common functions with other members of the Tom1 family during mitogenesis. Although not analyzed in details, our data suggest that they may also regulate SFK mitogenic signaling, although inhibitory effect observed with Tom1 was reduced. Interestingly, all of these adaptor proteins include VHS and GAT homology domains involved in vesicular trafficking (7, 28) and are thought to regulate receptor endocytosis and/or recycling (36, 45, 53). Therefore, Tom1L1 may have a role in growth factor receptor trafficking. This hypothesis is further supported by the identification of endocytic regulators, Hrs and Tsg101 as two potential interactors of this adaptor (36). An association in mammalian cells was, however, not provided, leaving questions about their involvement in Tom1L1 activity. Furthermore, most of our knowledge on growth factor receptor trafficking has been reported for the epidermal growth factor receptor (25), and only few data are available on PDGFRβ. Nevertheless, mutagenesis analysis in PDGFRβ suggested an implication of SFK association for receptor internalization (32). More recently, Avrov and Kazlauskas surmised the existence of a SFK substrate required for PDGFRα endocytosis (2), and Tom1L1 could be one of these substrates. Therefore, Tom1L1 may play a role in endocytosis, recycling, and/or degradation of PDGFR. Whether mitogenic and potential trafficking functions of Tom1L1 are directly related is currently unknown. Intriguingly, no major defect in PDGFR mitogenic signaling has been observed in cells with reduced Tom1L1 except for the SFK pathway, suggesting that its mitogenic function is largely dissociated from its role in PDGFR endocytosis and/or recycling. How Tom1L1 contributes to both SFK mitogenic and endocytic functions is an important issue to be addressed.

The function of Tom1L1 in mitogenesis may have important implications in cell physiology. Our results suggest that it is an important Src substrate during embryogenesis. We believe that it may participate in modulation of cell proliferation and/or cell responses induced by growth factors. For example, Tom1L1 regulates mitogenesis without affecting morphological changes induced by PDGF. Therefore, its protein level may influence the cell response induced by growth factors (i.e., proliferation versus migration). The role of Tom1L1 on cell fate has been also illustrated in keratinocyte differentiation induced by EGF (26). Knockout of the corresponding gene in mouse will be very informative in this regard. Finally, Tom1L1 may be also implicated in cancer, based on its negative regulatory function in cell proliferation. In agreement with this hypothesis, the Tom1L1 level was downregulated in melanoma cells, which may impact on SFK oncogenic activity (26). However, the exact role of Tom1L1 may be more complex than originally thought. Indeed, TOM1L1 was recently found coamplified with HER2 in human breast cancer (34). SFK have been largely implicated in this type of cancer (50). Specifically, these kinases cooperate with members of the epidermal growth factor receptor family for tumorigenesis (4). Whether Tom1L1 is a positive or negative regulator of SFK signaling in these cells is an important issue that we are currently investigating.

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