Differential trafficking of Src, Lyn, Yes and Fyn is specified by the state of palmitoylation in the SH4 domain

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Summary
Src-family tyrosine kinases (SFKs), which participate in a variety of signal transduction events, are known to localize to the cytoplasmic face of the plasma membrane through lipid modification. Recently, we showed that Lyn, an SFK member, is exocytosed to the plasma membrane via the Golgi region along the secretory pathway. We show here that SFK trafficking is specified by the palmitoylation state. Yes is also a monopalmitoylated SFK and is biosynthetically transported from the Golgi pool of caveolin to the plasma membrane. This pathway can be inhibited in the trans-Golgi network (TGN)-to-cell surface delivery by temperature block at 19°C or dominant-negative Rab11 GTPase. A large fraction of Fyn, a dually palmitoylated SFK, is directly targeted to the plasma membrane irrespective of temperature block of TGN exit. Fyn(C6S), which lacks the second palmitoylation site, is able to traffic in the same way as Lyn and Yes. Moreover, construction of Yes(S6C) and chimeric Lyn or Yes with the Fyn N-terminus further substantiates the importance of the dual palmitoylation site for plasma membrane targeting. Taken together with our recent finding that Src, a nonpalmitoylated SFK, is rapidly exchanged between the plasma membrane and late endosomes/lysosomes, these results suggest that SFK trafficking is specified by the palmitoylation state in the SH4 domain.

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Key words: Src-family tyrosine kinases, trafficking, palmitoylation, Lyn, Fyn, Yes

Introduction
Src-family tyrosine kinases (SFKs), which are non-receptor-type tyrosine kinases, consist of proto-oncogene products and structurally related proteins and include at least eight highly homologous proteins: Src, Lyn, Fyn, Yes, Fgr, Hck, Lck and Blk (Brown and Cooper, 1996; Thomas and Brugge, 1997). SFKs are activated by various stimuli, including growth factors and adhesion proteins, and are involved in a wide range of signaling events at the plasma membrane, resulting in cell proliferation, differentiation, migration, and cell-shape changes. Src, Yes, Lyn and Fyn are widely expressed in a variety of cell types, whereas Blk, Fgr, Hck and Lck are found primarily in hematopoietic cells (Bolen and Brugge, 1997; Thomas and Brugge, 1997).

SFKs are composed of (1) an N-terminal Src homology (SH) 4 domain that contains lipid modification sites; (2) a poorly conserved ‘unique’ domain; (3) an SH3 domain that can bind to specific proline-rich sequences; (4) an SH2 domain that can bind to specific sites of tyrosine phosphorylation; (5) an SH1 tyrosine kinase catalytic domain; and (6) a negative regulatory tail for autoinhibition of kinase activity (Brown and Cooper, 1996; Thomas and Brugge, 1997). All members of the Src family are cotranslationally myristoylated at Gly2 and, with the exception of Src and Blk, are also post-translationally palmitoylated at Cys3, Cys5 or Cys6 (Paige et al., 1993; Alland et al., 1994; Koegl et al., 1994; Resh, 1994; Shenoy-Scaria et al., 1994; Kasahara et al., 2007a). Fatty acylation of SFKs has been shown to influence their interactions with cell membranes (McCabe and Berthiaume, 1999; Resh, 1999) and as a consequence their intracellular distribution.

It is generally thought that SFKs are predominantly located at the cytoplasmic face of the plasma membrane through post-translational myristoylation, usually with subsequent palmitoylation, but in fact, appreciable fractions are found at in variety of intracellular locations, such as endosomes, secretory granules or phagosomes and the Golgi complex (Kaplan et al., 1992; Mohn et al., 1995; Brown and Cooper, 1996; Thomas and Brugge, 1997; Kasahara et al., 2004). Although distinctive localizations of SFK members have been implicated in their specific functions, the mechanism that underlies the targeting of SFKs to their specific locations remains to be elucidated.

We recently showed that Lyn, a palmitoylated SFK, is exocytosed to the plasma membrane via the Golgi region along the secretory pathway (Kasahara et al., 2004). More recently, we demonstrated that Src, a non-palmitoylated SFK, rapidly moves between the plasma membrane and late endosomes or lysosomes, and that mutation of Cys3 in Lyn allows Lyn to traffic in a similar manner to Src (Kasahara et al., 2007a), indicating the importance of palmitoylation for distinct trafficking between Lyn and Src.

In this study, we investigate the localization and trafficking of other ubiquitously expressed SFKs, such as Yes and Fyn. We demonstrate that Lyn and Yes, which are monopalmitoylated SFKs,
are transported to the plasma membrane via the Golgi region along the secretory pathway, whereas a large fraction of Fyn, a dually palmitoylated SFK, is directly targeted to the plasma membrane. Our findings suggest that the state of mono- or dual-palmitoylation makes a difference in trafficking among palmitoylated SFKs.

**Results**

Yes and Lyn localize to the Golgi region

Src, Lyn, Yes and Fyn are widely expressed in various cell types (Thomas and Brugge, 1997; Kuga et al., 2007). We recently showed that Lyn is biosynthetically transported to the plasma membrane via the Golgi pool of caveolin along the secretory pathway (Kasahara et al., 2004). To examine the localization of other ubiquitously expressed SFKs, we transfected COS-1 cells with Lyn, Yes or Fyn and compared their localization. In the earlier phase of expression, localization of Lyn was predominantly seen in the perinuclear region and gradually changed to the plasma membrane in the later phase (Fig. 1A,B, top), consistent with our report (Kasahara et al., 2004). Similarly to Lyn, Yes was predominantly found at the perinuclear region during the earlier phase of expression and at the plasma membrane in the later phase (Fig. 1A,B, middle). By contrast, the majority of Fyn was found at the plasma membrane from the early phase (Fig. 1A,B, bottom), which is consistent with previous studies (van’t Hof and Resh, 1997). These results suggest that Yes, as well as Lyn, accumulates initially at the perinuclear region and is then transported to the plasma membrane.

To produce a synchronous wave of protein synthesis and trafficking, we used a HeLa cell clone (3-2) (Kasahara et al., 2007b) stably expressing the tetracycline repressor for an inducible expression system. At 12 hours after transfection with Lyn, Yes or Fyn, doxycycline (Dox) was added to medium to induce protein expression. In the course of induction, most Lyn and Yes proteins were initially observed in the perinuclear region and were subsequently detected at the plasma membrane, whereas Fyn was predominantly present on the plasma membrane from the early phase of induction (supplementary material Fig. S1A,B).

**Fig. 1.** Localization of Lyn, Yes and Fyn. (A,B) COS-1 cells transfected with Lyn, Yes or Fyn were cultured for the indicated periods and stained with anti-Lyn, anti-Yes or anti-Fyn antibody. (A) Representative cells exhibiting predominant perinuclear (PeriN) and plasma membrane (PM) localization. Arrows indicate the perinuclear localization. (B) The graph shows the percentage of cells with predominant perinuclear staining. Results (%) are means ± s.d. (n=3-6). The results for Lyn at 18 and 24 hours after transfection were obtained from a representative experiment. (C) COS-1 cells transfected with Lyn or Yes cultured for 18 hours and double stained with anti-Lyn (green) or anti-Yes (green) antibody together with anti-galactosyltransferase (anti-GalT, red) antibody. Boxed areas are magnified in the insets. (D) COS-1 cells transfected with Lyn, Yes or Fyn were cultured for 15 hours in the absence or presence of 5 μg/ml brefeldin A (BFA) during the last 1 hour, and the distribution of expressed proteins were examined with anti-Lyn, anti-Yes or anti-Fyn antibody. (E) Endogenous Lyn (p56/p53) and Fyn (p59) expressed in THP-1 cells immunoblotted with anti-Lyn and anti-Fyn antibodies. (F) THP-1 cells cultured in the absence or presence of 5 μg/ml BFA for 2 hours. Endogenous Lyn and Fyn were visualized with anti-Lyn or anti-Fyn antibody. Arrows indicate the perinuclear region. N, nucleus. Scale bars: 20 μm.
results further support the possibility that Yes is transported from the perinuclear region to the plasma membrane.

Next, we characterized the perinuclear region using Golgi markers, such as β-1,4-galactosyltransferase (GalT), a trans-Golgi protein and GM130, a cis-Golgi protein. A large fraction of perinuclear Yes was also partially colocalized with GalT (Fig. 1C), as also observed with inducible Yes and Lyn (supplementary material Fig. S1C). In addition, Yes was precisely colocalized with caveolin in the perinuclear region (supplementary material Fig. S2), indicating that perinuclear Yes is localized to the Golgi pool of caveolin similarly to the localization of Lyn in the perinuclear region. Perinuclear Yes and Lyn were dispersed by brefeldin A (BFA) (Fig. 1D), further confirming their Golgi localization.

To examine whether localization of endogenous SFKs was similar to those of overexpressed proteins, we used the human monocytic cell line THP-1 in which endogenous Lyn and Fyn were detectable by immunoblotting and immunostaining (Fig. 1E,F). Although endogenous Yes was hardly visualized owing to its low expression (data not shown), endogenous Lyn was localized to the perinuclear region and the plasma membrane. BFA treatment caused dispersal of endogenous Lyn from the perinuclear region (Fig. 1F). Endogenous Fyn was predominantly seen at the plasma membrane and showed no apparent redistribution in the presence of BFA. These results suggest that localization of endogenous SFKs mirror those of the overexpressed proteins.

Newly synthesized Yes initially accumulates in the Golgi region
We next determined whether the presence of Yes in the Golgi region reflected newly synthesized protein in transit to the plasma membrane. COS-1 cells expressing Lyn, Yes or Fyn were treated with cycloheximide (CHX), a protein synthesis inhibitor. CHX treatment markedly reduced perinuclear Lyn within 60 minutes (Fig. 2A,B, top), which is consistent with an earlier report (Kasahara et al., 2004). Reduction of perinuclear Yes was also found within 60 minutes of CHX treatment (middle), indicating that CHX treatment results in a time-dependent chase of newly synthesized Yes from the Golgi region to the plasma membrane. By contrast, CHX treatment did not affect Fyn localization to the plasma membrane (bottom). These results suggest that newly synthesized Yes but not Fyn is initially accumulated at the Golgi region and transported to the plasma membrane in a manner similar to Lyn trafficking.

To further examine whether the localization of endogenous Lyn and Fyn was affected by CHX treatment, THP-1 cells were treated with CHX for 3 hours and stained for Lyn and Fyn. Perinuclear accumulation of endogenous Lyn diminished upon CHX treatment (Fig. 2C, top), whereas plasma membrane localization of endogenous Fyn was not affected (Fig. 2C, bottom). These results suggest that the trafficking of endogenous Lyn is different from that of endogenous Fyn, in agreement with the results shown in Fig. 2A,B.

Yes and Lyn traffic to the plasma membrane through the exocytic pathway
Since Yes is localized to the Golgi region and chased to the plasma membrane by CHX treatment, we tested whether Yes, like Lyn, was transported to the plasma membrane through the exocytic pathway. We took advantage of the property of a reduced temperature (19°C) to specifically block TGN membrane vesicle exit (Griffiths et al., 1989; Watson et al., 2003). COS-1 cells expressing Lyn or Fyn were incubated at 19°C for 3 hours in the presence of CHX to block protein synthesis but allow accumulation of the synthesized proteins in the Golgi region. Incubation with CHX at 19°C caused accumulation of Yes as well as Lyn in the perinuclear region (Fig. 3A, middle), whereas perinuclear accumulation of Lyn and Yes was markedly reduced upon incubation with CHX for 3 hours at 37°C (Fig. 3A, left). When the cells incubated at 19°C were warmed to 37°C, the perinuclear accumulation of Lyn and Yes was rapidly dissipated (Fig. 3A, right). In sharp contrast, incubation at 19°C did not allow Fyn to accumulate in the perinuclear region (Fig. 3A, bottom). These results suggest that biosynthetic exocytic traffic is involved in the recruitment of Yes to the plasma membrane in a manner similar to Lyn trafficking.

Overexpression of the dominant-negative Rab11S25N mutant (Rab11DN) causes inhibition of the TGN-to-cell surface delivery (Chen et al., 1998). To ascertain whether the plasma membrane delivery of a conventional exocytic cargo was mediated by Rab11, we used green fluorescent protein (GFP)-tagged vesicular stomatitis-virus-encoded glycoprotein (VSVG-GFP), whose export from the
ER occurs in a temperature-dependent manner (Presley et al., 1997). COS-1 cells transfected with VSVG-GFP or VSVG-GFP plus HA-Rab11DN were incubated at 40°C (nonpermissive temperature) overnight and then shifted to 32°C (permissive temperature) for 30 minutes or for 6 hours, including CHX for the last 3 hours. In cells transfected with VSVG-GFP alone, VSVG-GFP was predominantly found in the Golgi region at 30 minutes after temperature shift, and most VSVG-GFP was transported to the plasma membrane after incubation for 6 hours (Fig. 3Ba, left), consistent with previous reports (Hirschberg et al., 1998; Hirose et al., 2004). Upon overexpression of Rab11DN, VSVG-GFP was largely retained in the Golgi region at 6 hours after temperature shift (Fig. 3Ba, right), in agreement with previous studies (Chen et al., 1998). We then examined whether overexpression of Rab11DN inhibited the transport of Lyn and Yes to the plasma membrane. COS-1 cells transfected with Lyn alone, Lyn plus Rab11DN, Yes or Yes plus Rab11DN, cultured for 24 hours. Cells were doubly stained with anti-Lyn (green) or anti-Yes (green) antibody plus anti-HA antibody (red) (b). Insets show fluorescence images of Rab11DN (red). Cells exhibiting the perinuclear localization of Lyn or Yes were quantified. Results (%) are means ± s.d. (n=3-6). ***P<0.001, Student’s t-test. N, nucleus. Scale bars: 20 μm.

To examine whether the transport of endogenous Yes was also inhibited by temperature block, we used the human megakaryocytic cell line Dami, in which endogenous Yes as well as Lyn and Fyn was easily detected by immunoblotting and immunostaining (Fig. 3C-E). Despite low levels of perinuclear accumulation, endogenous Lyn and Yes were found in the plasma membrane (Fig. 3D, left), and incubation at 19°C for 3 hours increased levels of perinuclear Lyn and Yes in most cells (Fig. 3D, right; Fig. 3E). These results suggest that endogenous Yes as well as Lyn traffics to the plasma membrane through the exocytic pathway. By contrast, Fyn was primarily found at the plasma membrane in cells incubated at 37°C and no apparent redistribution was seen after incubation at 19°C (Fig. 3D, bottom), further confirming the finding that newly synthesized Fyn is directly targeted to the plasma membrane (van’t Hof and Resh, 1997).

In addition, Dami cells were incubated at 15°C to examine the involvement of ER membranes in Lyn and Yes transport. Incubation
at 15°C, which impairs transport from the ER to the cis-Golgi (Presley et al., 1997), resulted in accumulation of Lyn and Yes in the Golgi region but not the ER (supplementary material Fig. S3), suggesting that the trafficking pathways for Lyn, Yes and Fyn do not involve ER membranes.

Newly synthesized Yes and Lyn, but not Fyn, traffic to the plasma membrane through the Golgi region

To visualize the trafficking of Lyn, Yes and Fyn just after biosynthesis in living cells, we added a GFP tag to the proteins, which preserves their N-terminal lipid attachment sites (see Materials and Methods) (Kasahara et al., 2004), and performed fluorescence recovery after photobleaching (FRAP) of Lyn-GFP, Yes-GFP and Fyn-GFP in COS-1 cells. When the whole-cell area was photobleached, rapid recovery of Lyn-GFP fluorescence at 37°C was observed in both the perinuclear region and the non-perinuclear region that excludes the perinuclear region from whole cell area. Incubation at 19°C after whole-cell photobleaching inhibited the recovery of Lyn-GFP fluorescence in the non-perinuclear region at 19°C and 37°C were comparable with those in the non-perinuclear region. These results suggest that most of the newly synthesized Fyn traffics directly to the plasma membrane and only a small fraction targets to the Golgi region.

Palmitoylation at Cys6 influences Fyn localization and trafficking

Palmitoylation of SFKs occurs on cysteine residues in the general motif Met-Gly-Cys, where Gly2 is myristoylated (Resh, 1999). Although Lyn and Yes are monopalmitoylated at Cys3 (Koegl et al., 1994; McCabe and Berthiaume, 1999; Kasahara et al., 2007a), Fyn is dually palmitoylated at Cys3 and Cys6 (Alland et al., 1994). Since the trafficking pathway for Lyn and Yes is different to that for Fyn (Figs 3 and 4), we asked whether the lack of the second palmitoylation site in Fyn affected its

Fig. 4. Trafficking of Lyn and Yes just after biosynthesis is different from that of Fyn in living cells. COS-1 cells transfected with Lyn-GFP (A), Yes-GFP (B) or Fyn-GFP (C) cultured for 20 hours. Whole cell area was bleached and monitored at 1-minute intervals at 37°C or 19°C for the next 20 minutes. Times are shown after photobleaching. Mean fluorescence intensities of recovery after photobleaching in the perinuclear area (PeriN) and whole cell area excluding perinuclear region (Non-PeriN) plotted versus time. A representative result (A-C) is shown from at least 2-3 independent experiments. N, nucleus. Scale bars: 20 μm.
trafficking. We generated a Fyn(C6S) mutant in which Cys6 in Fyn was substituted for Ser (Fig. 5A), a substitution previously shown to decrease palmitate incorporation of Fyn (Alland et al., 1994). The levels of protein expression and kinase activity were comparable between wild-type Fyn (Fyn-wt) and Fyn(C6S) (Fig. 5B). Intriguingly, Fyn(C6S) significantly accumulated in the Golgi region 12 hours after transfection compared with Fyn-wt (Fig. 5C,D), although the level of Fyn(C6S) was lower than that for Lyn and Yes (compare with Fig. 1B). The number of cells exhibiting the Golgi localization of Fyn(C6S) was gradually decreased after transfection (Fig. 5D), similar to the results for Lyn and Yes (Fig. 1B). Accumulation of Fyn(C6S) in the Golgi region was dispersed by BFA treatment (Fig. 5E), and accumulation of inducible Fyn(C6S) was also observed in the Golgi region in the early phase of induction (supplementary material Fig. S4 and Fig. S1).

Localization of Fyn(C6S) to the Golgi region in the early phase of expression prompted us to examine whether accumulation of Fyn(C6S) in the Golgi region was diminished by CHX. Upon treatment with CHX, the number of cells exhibiting perinuclear localization of Fyn(C6S) decreased (Fig. 5F), suggesting that following its initial biosynthesis, Fyn(C6S) can enter the secretory membrane-trafficking system. We then examined the involvement of Rab11 in transport of Fyn(C6S) to the plasma membrane. COS-1 cells transfected with Fyn alone, Lyn plus Rab11DN, or Yes plus Rab11DN were incubated for 24 hours. Cells were double stained with anti-Fyn and anti-HA (red) antibodies. Cells exhibiting the perinuclear localization of Fyn(C6S) were quantified. Results (%) are means ± s.d. (n=6-7). **P<0.01, Student’s t-test. Arrows indicate the perinuclear region. N, nucleus. Scale bars: 20 μm.

Fig. 5. Mutation of the second palmitoylation site of Fyn redirects the protein to the Golgi region.
(A) Sequences of the first 16 amino acids of SFKs. The underlined amino acids at position 6 indicate a Cys residue and a Cys→Ser mutation. Myristoylated glycines are highlighted in blue, and palmitoylated cysteines in red. (B) Equal amounts of lysates from COS-1 cells transfected with Fyn or Fyn(C6S) analyzed by western blotting with anti-Fyn, anti-Src[pY418] and anti-actin antibodies.
(C, D) COS-1 cells transfected with Fyn or Fyn(C6S) cultured for the indicated periods and stained with anti-Fyn (green) and anti-GM130 (red) antibodies. (D) Cells exhibiting the perinuclear localization of expressed proteins were quantified. Data represent means ± s.d. (n=3-6). **P<0.01, Student’s t-test.
(E) COS-1 cells transfected with Fyn(C6S) cultured for 15 hours in the absence or presence of 5 μg/ml BFA for the last 1 hour and doubly stained for Fyn (green) and GM130 (red). (F) COS-1 cells transfected with Fyn(C6S) cultured for 12 hours, then treated with 100 μg/ml CHX for the indicated periods, and stained with anti-Fyn antibody. Cells exhibiting the perinuclear localization of Fyn(C6S) were quantified. Results (%) are means ± s.d. (n=3). **P<0.01, Student’s t-test.
Differential trafficking of Src kinases

We noted that perinuclear accumulation of Fyn-wt was moderately induced by long-term coexpression of Rab11DN with Fyn-wt (~38% for Fyn-wt (Fig. 5G) and ~75% for Lyn or Yes (Fig. 3Bb)). About 15% of cells expressing Fyn-wt showed perinuclear Fyn-wt, which partially colocalized with GM130, a Golgi marker (Fig. 5G; data not shown), and weak perinuclear fluorescence of Fyn-GFP could be detected at 19°C after whole-cell photobleaching (Fig. 4C). Also, a few percentages of Dami cells showed perinuclear staining for endogenous Fyn (supplementary material Fig. S3F). These results suggest that a small fraction of Fyn-wt might traffic from the Golgi region to the plasma membrane along the Rab11-mediated exocytic pathway.

In addition, we transfected COS-1 cells with Fyn-wt, Fyn(C6S) or Lyn, and examined tyrosine phosphorylation at the early phase of expression when Golgi accumulation of Fyn(C6S) and Lyn but not Fyn-wt was visible. Similarly to results observed with Lyn, Fyn(C6S) but not Fyn-wt increased tyrosine phosphorylation of pp40 (supplementary material Fig. S5), supporting the finding that localization of Fyn(C6S) is similar to that of Lyn.

Palmitoylation at Cys6 in Fyn contributes to its plasma membrane targeting

A Fyn-Src chimera exhibits by far the greatest incorporation of the palmitate analog, whereas mutation of Ser3 and Ser6 in viral Src to Cys shows mild incorporation (Alland et al., 1994). To examine whether palmitoylation at Cys 6 affected localization of Lyn and Yes, we first created a Fyn-Lyn chimera (Fyn24-Lyn), in which the SH4 domain of Lyn was substituted for that of Fyn-wt (Fig. 6A). Expression and kinase activity were confirmed in COS-1 cells transfected with each construct (Fig. 6B). Intriguingly, localization of Fyn24-Lyn was different from that of Lyn, despite containing 95% of the Lyn protein sequence, and Fyn24-Lyn was mainly localized at the plasma membrane in the same manner as Fyn (Fig. 6C). To assess whether the palmitoylation site at Cys6 was important for the targeting of Fyn24-Lyn to the plasma membrane, we introduced a mutation of Cys6 to Ser in Fyn24-Lyn and created Fyn24(C6S)-Lyn (Fig. 6A). Although Fyn24-Lyn was mainly localized to the plasma membrane, Fyn24(C6S)-Lyn localized to the perinuclear region and the plasma membrane (Fig. 6D), similarly to Lyn localization (Figs 1-3) (Kasahara et al., 2004). Additionally, perinuclear Fyn24(C6S)-Lyn was partially

Fig. 6. Dually palmitoylated N-terminus of Fyn leads Lyn and Yes to the plasma membrane localization. (A,F) Schematic representations of the constructs used in this study are shown with the Src homology (SH) domains and the kinase domain. Fyn24-Lyn, Fyn24-Yes and Fyn24(C6S)-Lyn contain the first 24 amino acids of Fyn-wt or Fyn(C6S). (B-F) COS-1 cells transiently transfected with the indicated constructs cultured for 15 hours. (B,F) Cell lysates analyzed by western blotting with anti-Lyn, anti-Yes, anti-Fyn, anti-Src[pY418] and anti-actin antibodies. (C-F) Expressed proteins visualized with anti-Lyn, anti-Yes or anti-Fyn antibody. Cells expressing Fyn24-Lyn or Fyn24(C6S)-Lyn doubly stained with anti-Lyn (green) and anti-GalT (red) antibody. N, nucleus. Scale bars: 20 μm. Cells exhibiting the perinuclear localization of expressed proteins were quantitated. Data represent means ± s.d. (n=3-4). **P<0.01, *** P<0.001, Student’s t-test. NS, not significant.
colocalized with GalT [Fig. 6D; see Fig. 5 and supplementary material S4 for Fyn(C6S)], which is consistent with the perinuclear localization of Lyn and Yes (Fig. 1C; Fig. 3E; supplementary material Fig. S1C and Fig. S3B,D). These results suggest that the N-terminus preserving the monopalmitylation site is responsible for Golgi localization of SFKs.

We then created a Fyn-Yes chimera (Fyn24-Yes) and confirmed its expression and kinase activity (Fig. 6B). Similarly to the results of Fyn24-Lyn, Fyn24-Yes was also localized at the plasma membrane (Fig. 6E), suggesting that the Fyn N-terminus, which contains one myristoylation site and two palmitoylation sites, allows Lyn and Yes to traffic directly to the plasma membrane. Furthermore, we generated a Yes(S6C) mutant in which Ser6 in Yes was substituted for Cys (Fig. 6F). The levels of protein expression were comparable between wild-type Yes and Yes(S6C) (Fig. 6F). Intriguingly, Yes(S6C) was mainly localized at the plasma membrane but not at the Golgi region, similarly to Fyn localization (Fig. 6F). Taken together, these results suggest that the presence of the second palmitoylation site in the N-terminus has an important role in the direct targeting of SFKs to the plasma membrane.

Discussion

In the present study, we demonstrate that SFK trafficking is specified by its palmitoylation state. Lyn and Yes, which are both nonpalmitoylated SFKs, are biosynthetically transported to the plasma membrane via the Golgi pool of caveolin along the secretory pathway, whereas Fyn, a dually palmitoylated SFK, is directly targeted to the plasma membrane. We further show that Fyn(C6S), which lacks the second palmitoylation site, behaves in a manner reminiscent of Lyn and Yes by accumulating in the Golgi region early after biosynthesis, and that Yes(S6C), in which a second palmitoylation site is created, becomes localized to the plasma membrane.

Three major pathways for SFK trafficking

Palmitoylation of SFKs has been shown to contribute to their localization (Bijlmakers et al., 1997; Carreno et al., 2000). Although Src is not palmitoylated, Lyn and Yes are monopalmitylated at Cys3 (Koeogl et al., 1994; McCabe and Berthaume, 1999; Kasahara et al., 2007a) and Fyn is dually palmitoylated at Cys3 and Cys6 (Alland et al., 1994). We recently showed that the trafficking of Src (a nonpalmitoylated SFK) is different from that of Lyn (a monopalmitylated SFK) because of the state of palmitoylation (Kasahara et al., 2007a).

Here, we propose a model of three major trafficking pathways for SFKs (Fig. 7). (1) The cycling pathway for myristoylated but not palmitoylated SFKs: Src is rapidly exchanged between late endosomes/lysosomes and the plasma membrane, possibly through its cytosolic release (Kasahara et al., 2007a). (2) The secretory pathway from the Golgi region to the plasma membrane for myristoylated and monopalmitylated SFKs: newly synthesized Lyn and Yes initially enter the Golgi system, where palmitoylation probably occurs, providing entry into the membrane secretory transport pathway en route to the plasma membrane (Fig. 3A,D, Fig. 4) (Kasahara et al., 2004). Rab11 is involved in exocytic transport of Lyn and Yes (Fig. 3B). (3) The direct plasma-membrane-targeting pathway for myristoylated and dually palmitoylated SFKs: newly synthesized Fyn is targeted directly to the plasma membrane (Fig. 2A, Fig. 3D, Fig. 4) (van’t Hof and Resh, 1997).

Importantly, the results for endogenous Lyn, Yes and Fyn in THP-1 and Dami cells (Fig. 1F; Fig. 3D) agree with our proposed model. Although perinuclear staining for endogenous Lyn and Yes in Dami cells is not so strong as that observed in THP-1 cells, build-up of Lyn and Yes in the Golgi region after temperature block in Dami cells (Fig. 3D) allows us to assume that newly synthesized Lyn and Yes can reach the plasma membrane rapidly in Dami cells compared with THP-1 cells. Furthermore, we found that like Lyn, Fyn(C6S), which lacks the second palmitoylation site, accumulates in the Golgi region (Fig. 5), and that the dually palmitoylated N-terminus of Fyn leads Lyn and Yes directly to the plasma membrane whereas the monopalmitylated N-terminus of Fyn redirects Lyn to the Golgi region (Fig. 6). We also found that creation of the second palmitoylation site in Yes enables its transfer directly to the plasma membrane (Fig. 6). These results suggest that the state of palmitoylation (Lyn and Yes) or dual palmitoylation (Fyn) makes a difference in the trafficking pathways between Lyn-Yes and Fyn. Our model is also consistent with previous findings that nonpalmitoylated p61^Hck, like Src, is localized to lysosomes whereas monopalmitylated p59^Hck localizes to the Golgi and the plasma membrane (Carreno et al., 2000). Taken together, these results provide evidence that SFK trafficking is classified into three groups depending on the state of palmitoylation, as depicted in our model.

Notably, a very small fraction of Fyn was found to localize to the Golgi region (Fig. 4C, Fig. 5C, Fig. 5D; supplementary material Fig. S3F) (van’t Hof and Resh, 1997). Given that palmitoylation of Fyn does not occur perfectly (Liang et al., 2004), it is plausible that mono- or non-palmitoylated Fyn can follow a pathway other than the direct targeting pathway to the plasma membrane. Imperfect palmitoylation, if any, might affect the trafficking of the other SFKs including Lyn and Yes.

Cys3 is considered to have a greater role in the membrane localization of Fyn than Cys6 judging from biochemical data, although it would be difficult to detect localization changes among various cell membranes (Shenoy-Scaria et al., 1994; Wolven et al., 1997). In the present study, we reveal that mutation at Cys6
influences intracellular localization of Fyn by accumulating the protein in the Golgi region (Fig. 5C). Taken together with the finding that Yes(S6C) becomes localized at the plasma membrane (Fig. 6), we thus hypothesize that the second palmitoylation site at Cys6 is important for plasma membrane targeting by keeping Fyn away from the Lyn-type trafficking. The presence of the second cysteine residue in the SFK N-terminal domain might influence recognition by proteins that are involved in protein palmitoylation or sorting to the plasma membrane. This hypothesis may be also explained by the results that Lck, which is dually palmitoylated at Cys3 and Cys5 (Koeegl et al., 1994; Yurchak and Sefton, 1995), accumulates in the Golgi region by mutation of the second palmitoylation site at Cys5 in transfected NIH-3T3 cells (Bijlmakers et al., 1997).

Since the level of Golgi-accumulated Fyn(C6S) was lower than that of Lyn and Yes (Fig. 1B; Fig. 5D), other factors besides palmitoylation might influence localization of SFK. Given that Fyn24-Lyn and Fyn24-Yes, which contain their SH3, SH2 and kinase domains, tend to stay in the Golgi, unlike Lyn-wt (Fig. 6D-E), we assume that the SH3 and SH2 domains of Lyn and Yes might contribute to retention of Lyn and Yes in the Golgi region by protein-protein interactions with Golgi components.

Palmitoylation is mediated by protein acyltransferases (PATs), which include a large family of integral membrane proteins that contain a DHHC cysteine-rich domain (DHHC PATs) (Linder and Deschenes, 2007). More than 20 DHHC PATs were isolated in mammals, and recent progress has shown that DHHC PATs have exquisite substrate specificity (Fukata et al., 2004; Ohno et al., 2006; Linder and Deschenes, 2007). Most of the DHHC PAT members are localized to the ER and/or Golgi, but some are in fact found at the plasma membrane (Ohno et al., 2006). Although DHHC PATs are likely to account for most palmitoylation events, there might be substrate-specific PATs that are not members of the DHHC family (Linder and Deschenes, 2007). Taken together with the hypothesis that Fyn is targeted directly to the plasma membrane without a requirement for the exocytic machinery (van’t Hof and Resh, 1997; Bijlmakers and Marsh, 2003), it would be reasonable to assume that PATs localizing to the Golgi region and the plasma membrane, although they have not yet been identified, are responsible for palmitoylation of Lyn-Yes and Fyn, respectively.

Lyn and Yes are precisely colocalized with the Golgi pool of caveolin (supplementary material Fig. S2) (Kasahara et al., 2004); they also partially colocalize with GaIT and GM130, which are Golgi-resident proteins (Fig. 1C, Fig. 3E; supplementary material Fig. S1C and Fig. S3B,D). Inhibition of protein synthesis by CHX results in a chase of Lyn and Yes to the plasma membrane, with a concomitant chase of the Golgi pool of caveolin (this study) (Kasahara et al., 2004), but it does not allow GaIT and GM130 to move from the Golgi complex to the plasma membrane (data not shown). Given that the Golgi pool of caveolin accounts for the intracellular pool of the protein in the presence of continual exit of caveolin in exocytic vesicles (Nichols, 2002), we presume that perinuclear Lyn and Yes localize to caveolin-positive exocytic vesicles in the Golgi region.

Implications for roles of the distinct trafficking of SFKs

Recent evidence provides a novel view that endomembranes, such as Golgi and ER membranes, serve as a platform of signaling molecules. For example, Ras that is restricted to endomembranes was shown to couple to the ERK-signaling cascade (Chiu et al., 2002). It is also shown that phosphatidylinositol-3,4,5-

trisphosphate levels are increased to a larger extent at endomembranes than at the plasma membrane, which is triggered by endocytosed receptor tyrosine kinases (Sato et al., 2003). In the present study, we demonstrate that each SFK member localizes to various intracellular organelles because of the distinct trafficking pathway depicted in Fig. 7. Thus, our findings raise the intriguing possibility that the distinct localization brings about specific functions of SFKs by allowing SFKs to interact with differently located substrates.

Indeed, we recently showed that Src is involved in targeting of macropinosomes to lysosomes and accumulation of lysosomes (Kasahara et al., 2007b; Kasahara et al., 2008). It is also reported that Src activation on RhoB-associated late endosomes leads to Src translocation from the perinuclear region toward discrete cell peripheral structures (Sandilands et al., 2004). However, Lyn phosphorylates annexin II on endomembranes including Golgi membranes (Matsuda et al., 2006). Moreover, p61Hck, but not p59fck, triggers the biogenesis of podosomes by exocytosis from lysosomes (Cougoule et al., 2005). These results strengthen our idea that specific functions among SFK members stem from their distinct intracellular localizations. In addition, we found that, like Lyn, Fyn(C6S) but not Lyn-wt increased tyrosine phosphorylation of pp40 (supplementary material Fig. S5), suggesting that an alteration in the subcellular localization of Fyn influences its access to SFK substrates. Blk and Src, both of which are nonpalmitoylated SFKs, phosphorylate coexpressed immunoglobulin-α, whereas palmitoylated SFKs do not (Saouaf et al., 1997). Taken together, the distinct trafficking of SFKs specified by the state of palmitoylation might be important for their specific roles and functional compensation.

Many signaling proteins are palmitoylated (Milligan et al., 1995; Resh, 1999; Smotrys and Linder, 2004). The notion that palmitoylation of SFKs is crucial for proper trafficking and function reinforces the possibility that the trafficking of other palmitoylated proteins, such as Ras GTPases and eNOS, is specified by their palmitoylation state and thus might lead to their specific functions. In fact, monopalmitoylation of H-Ras on Cys184 results primarily in a Golgi localization and impairs its ability to activate Raf, MEK and ERK (Roy et al., 2005). For eNOS, mutation of the palmitoylation sites blocks tight perinuclear targeting and nitric oxide release from cells (Liu et al., 1996). Palmitoylation might be essential for many proteins as well as SFKs, for proper trafficking and localization to fulfill their function. Furthermore, SFKs are abundantly expressed throughout the central nervous system (CNS) and regulate ion channel activity and synaptic transmission in the developed CNS (Wang and Salter, 1994; Thomas and Brugge, 1997). In neurons, palmitoylation has a key role in targeting proteins for transport to nerve terminals and for regulating trafficking at synapses (El-Husseini and Bredt, 2002; Huang and El-Husseini, 2005). It is therefore attractive to speculate that SFK members might be deeply involved in neuronal functions through their palmitoylation-regulated, spatiotemporal trafficking.

Considering the importance of protein trafficking in their proper localization and function, there is great interest in understanding the mechanisms that regulate palmitoylation-induced protein sorting and ‘cracking’ the palmitoylation codes within proteins to reveal how a palmitoylated peptide sequence relates to the final destination of a protein in the cell. Like the search for palmitoylation consensus sequences, these questions are likely to present a significant challenge.
Materials and Methods

Plasmids
cDNA encoding human Lyn (Yamashita et al., 1987), human Yes (Sugekawa et al., 1987), and human Fyn (Tezuka et al., 1999) were provided by Tadashi Yamamoto (The University of Tokyo, Japan). Lyn and Yes were subcloned into the pcDNA4/TO vector (Invitrogen) as described (Kasahara et al., 2004; Kuga et al., 2007), and Fyn was also subcloned into pcDNA4/TO. Fyn24-Lyn was generated by fusion of the N-terminal sequence of Lyn (1-24; with 1 designating the initiator methionine) with Lyn lacking the N-terminal sequence (25-543), and the linker sequence Gly-Arg-Ser-Thr was inserted between Fyn(1-24) and Lyn(26-512). The Cys→Ser mutation at position 6 in Fyn(CS) and Fyn(24CS) was created by PCR using Fyn or Fyn24-Lyn as a template with the sense primer 5'ACAGGATCCGCCATGCTGAGTACGGAAGTTAGAGGAGA 3' and the respective antisense primer 5'TTGCATATGCGTATGACTCCAC-3' or 5'GGAGCGCCGGCCCTGGTCAAGTCGACTGC-3'. The Ser→Cys mutation at position 6 in Yes(S6C) was created by PCR using Yes as a template with the sense primer 5'ACAGGATCCGCCATGCTGAGTACGGAAGTTAGAGGAGA 3' and the antisense primer 5'ACAGGATCCGCCATGCTGAGTACGGAAGTTAGAGGAGA 3'.

Antibodies
The following mouse monoclonal antibodies were used: Lyn (H6; Santa Cruz Biotechnology), Yes (clone 1; BD Biosciences, and IB7; Wako Pure Chemicals, Osaka, Japan), Fyn (F301; Wako Pure Chemicals, Osaka, Japan), HA epitope (4G10; 4G10), and the linker sequence Gly-Leu-Glu-Arg-Pro-Ser-Asn-Ser-Cys-Ser-Pro-Gly-Asp-Pro-Leu-Val-Leu-Ala-Leu-Pro-Val-Ala-Thr was provided by Jennifer Lippincott-Schwartz (Presley et al., 1997) through Mitsuo Tagaya (Hirose et al., 2004).

Cells and transfection
COS-1 cells were cultured in Iscove's modified DME containing 5% fetal bovine serum at 37°C in a 5% CO2 incubator. Transient transfection was performed using TransIT transfection reagent (Mirus) (Kasahara et al., 2007b; Kasahara et al., 2008) or linear polyethyleneimine (25 kDa; Polysciences, Warrington, PA) (Durocher et al., 2002). Dami cells (Hirao et al., 1998) were grown in suspension in Iscove's modified DME containing 5% fetal bovine serum at 37°C in a 5% CO2 incubator. Transient transfection was performed using TransIT transfection reagent (Mirus) (Kasahara et al., 2007b; Kasahara et al., 2008) or linear polyethyleneimine (25 kDa; Polysciences, Warrington, PA) (Durocher et al., 2002).

Immunofluorescence
Immunofluorescence staining was performed as described (Yamaguchi and Fukuda, 1995; Tada et al., 1999; Yamaguchi et al., 2001; Kasahara et al., 2004; Nakayama and Yamaguchi, 2005; Kuga et al., 2007). In brief, cells were washed in warmed PBS and blocked in PBS containing 0.1% saponin and 3% bovine serum albumin for 30 minutes, and then incubated with a primary and a secondary antibody for 1 hour each. After washing with PBS containing 0.1% saponin, cells were mounted with ProLong anti-fade reagent (Molecular Probes). Confocal images were obtained using an LSM510 (Carl Zeiss) and a Fluoview FV500 (Olympus, Tokyo, Japan) laser-scanning microscope with an ×40 0.75 NA or ×63 1.4NA oil-immersion objective, or a ×<sup>−</sup>0.1 1.00 NA water-immersion objective. 200-400 cells were scored for each assay. For immunofluorescence of THP-1 and Dami cells, cells in suspension were directly fixed with 3% paraformaldehyde and then attached on coverslips by brief cytocentrifugation. Composite figures were prepared using Photoshop 5.0 and Illustrator 9.0 software (Adobe).

References

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Additional notes and references are included in the text as appropriate.
Differential trafficking of Src kinases