Phosphorylation and Recruitment of Syk by Immunoreceptor Tyrosine-based Activation Motif-based Phosphorylation of Tamalin*

Masayuki Hirose†, Jun Kitano‡, Yoshiaki Nakajima†, Koki Moriyoshi§, Shigeru Yanagis¶, Hirohei Yamamura†, Takanori Muto†, Hisato Jingamis§, and Shigetada Nakanishis‡

From the †Department of Biological Sciences, Faculty of Medicine, and the Department of Molecular and System Biology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, the ‡Department of Genome Sciences, Kobe University Graduate School of Medicine, Chuo-ku, Kobe 650-0017, and the §Department of Molecular Biology, Biomolecular Engineering Research Institute, Suita, Osaka 565-0874, Japan

Tamalin is a scaffold protein that forms a multiple protein assembly including metabotropic glutamate receptors (mGluRs) and several postsynaptic and protein-trafficking scaffold proteins in distinct mode of protein-protein association. In the present investigation, we report that tamalin possesses a typical immunoreceptor tyrosine-based activation motif (ITAM), which enables Syk kinase to be recruited and phosphorylated by the Src family kinases. Immunoprecipitation analysis of rat brain membrane fractions showed that tamalin is present in a multimolecular protein assembly comprising not only mGluR1 but also c-Src, Fyn, and a protein phosphatase, SHP-2. The protein association of both tamalin and c-Src, as determined by truncation analysis of mGluR1 in COS-7 cells, occurred at the carboxy-terminal tail of mGluR1. Mutation analysis of tyrosine with phenylalanine in COS-7 cells revealed that paired tyrosines at the ITAM sequence of tamalin are phosphorylated preferentially by c-Src and Fyn, and this phosphorylation can recruit Syk kinase and enables it to be phosphorylated by the Src family kinases. The phosphorylated tyrosines at the ITAM sequence of tamalin were highly susceptible to dephosphorylation by protein-tyrosine phosphatases in COS-7 cells. Importantly, tamalin was endogenously phosphorylated and associated with Syk in retinoic acid-treated P19 embryonal carcinoma cells that undergo neuron-like differentiation. The present investigation demonstrates that tamalin is a novel signaling molecule that possesses a PDZ domain and a PDZ binding motif and mediates Syk signaling in an ITAM-based fashion.

The multimolecular protein assembly of neurotransmitter receptors is important as a fundamental mechanism for receptor localization, clustering, and signal transduction in neuronal cells (1–3). Neurotransmitter glutamate triggers excitation of neuronal cells and plays a key role in neural plasticity, neural cell development, and neurotoxicity (4–6). Glutamate receptors are categorized into two classes: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs), which belong to the family of G protein-coupled receptors (7, 8). Recently, a number of scaffold proteins that interact with iGluRs or mGluRs have been identified, and their roles in the functional organization and intracellular trafficking of glutamate receptors have been studied extensively (1–3).

Tamalin (also termed GRP1-associated scaffold protein) is a scaffold protein that comprises multiple protein-interacting domains (9, 10). The PDZ domain and the leucine-zipper region of tamalin bind to the carboxyl-terminal tail of group 1/group 2 mGluRs and the coiled-coil region of guanine nucleotide exchange factor cytohesins, respectively (9, 10). Tamalin promotes intracellular trafficking and cell surface expression of group 1 mGluRs in COS-7 cells and cultured hippocampal neurons through the interaction with cytohesins (9). Tamalin also physically associates with several important scaffold proteins involved in postsynaptic organization and protein trafficking in neuronal cells (11). However, its linkage to intracellular signaling cascades remained to be investigated.

In immune cells, paired tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of antigen receptors and their accessory proteins is essential for activation of phosphotyrosine-based, intracellular signaling cascades of antigen receptors (12–14). The ITAM sequence is defined as two tyrosines spaced 9–11 residues apart with leucine or isoleucine positioned 3 residues carboxyl-terminal to each tyrosine (D/E)XX[Y/F]X(L/I/X)3–6[Y/F]; one-letter code for amino acids with X representing any amino acid) (15). Antigen receptor signaling is initiated by paired phosphorylation of tyrosines within the ITAM sequence by a member of the Src protein-tyrosine kinase (PTK) family. The doubly phosphorylated ITAM provides a specific docking site for Syk/ZAP-70 PTKs, and a set of specific protein substrates becomes tyrosine-phosphorylated, resulting in activation of downstream signaling of antigen receptors (16, 17). In this investigation, the amino acid sequence analysis of tamalin revealed the presence of the ITAM sequence at the amino-terminal region of tamalin. Here, we report that tamalin is doubly phosphorylated at tyrosines of the ITAM sequence by the Src family kinases and serves as a scaffold protein that recruits and activates Syk kinase.
**ITAM-based Signaling of Tamalin**

**EXPERIMENTAL PROCEDURES**

**Materials—** Reagents and antibodies were purchased from the following sources. Rabbit polyclonal anti-c-Src, anti-Fyn, anti-Syk, and anti-SHP-2 were from Santa Cruz. Mouse monoclonal anti-Fyn (clone 15), anti-Syk (clone 1C6), and anti-SHIP-2 (clone B-1), goat polyclonal anti-SHPI-2, agarose beads conjugated with rabbit polyclonal anti-Myc or mouse monoclonal anti-Myc (clone 9E10), horseradish peroxidase-conjugated anti-Myc and protein A/G PLUS-agarose were from Sigma. Mouse monoclonal anti-Syk (clone SYK-01) was from abcam. Mouse monoclonal anti-phosphotyrosine (anti-P-Tyr) (clone 4G10) and anti-c-Src (clone GD11) and horseradish peroxidase-conjugated anti-P-Tyr were from Upstate Biotechnology, Inc. Rabbit polyclonal antibodies against Syk (P-Tyr352), Syk(P-Tyr356), and Syk(P-Tyr325/326) were from Cell Signaling Technology. Mouse monoclonal anti-FLAG (clone M2), agarose beads conjugated with anti-FLAG, horseradish peroxidase-conjugated anti-FLAG, and Na$_2$VO$_4$ were from Sigma. Mouse monoclonal antibody specific for a large splice variant of mGluR1, mGluR1a, was from Pharmingen. Mouse monoclonal anti-ZAP-70 (clone 29) was from BD Transduction Laboratories. Potassium bisperoxy(1,10-phenanthroline)oxovanadate(V) (bpV(phen)) was from Calbiochem. Rabbit tamalin antiserum was raised against an amino-terminal half of tamalin (residues 1–189) as described previously (11).

**DNA Constructs—** Tamalin was tagged with Myc epitope at its amino-terminal end by inserting tamalin cDNA into the pCMV-Tag3 mammalian expression vector (Stratagene). Tamalin mutants in which phenylalanine replaced tyrosine were generated by site-directed mutagenesis using the QuikChange multi-site-directed mutagenesis kit (Stratagene). A mammalian expression vector for human Syk (pAprohSyk) was described previously (18), and those for human c-Src (pcDNA3-hSrc), constitutively active c-Src (pcDNA3-hSrcY527F), kinase-dead c-Src (pcDNA3-hSrcK298M), and wild-type Fyn (pME-B-Fyn) were kindly provided by T. Yamamoto (19). Kinase-dead Syk was generated by substituting arginine for lysine at position 402 by site-directed mutagenesis. mGluR1a was tagged with FLAG epitope at its amino-terminal end just following the signal sequence. Truncation mutants of FLAG-mGluR1a were generated by introducing stop codons into the carboxyl-terminal tail of mGluR1a by site-directed mutagenesis. Proper mutations were verified for all mutated DNAs by DNA sequencing.

**Cell Culture—** COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37°C in 5% CO$_2$. DNA transfection was performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions; in control experiments, an equivalent amount of the vector DNA was transfected into COS-7 cells. Transfected cells were grown on 6- or 12-well plates in the culture medium for 36–48 h before analysis. In some experiments, 100 μg bpV(phen) or 1 μm Na$_2$VO$_4$ was added to the culture medium 20 min before transfection. All mouse embryo carcinoma cell lines were maintained in α-minimal essential medium supplemented with 10% fetal calf serum at 37°C in 5% CO$_2$. Monolayer cells were cultured in collagen-coated dishes in α-minimal essential medium supplemented with 10% fetal calf serum and 1 μM retinoic acid for 24 and 48 h. bpV(phen) (final concentration, 100 μM) was added 30 min before cell lysis with the radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na$_2$VO$_4$, and 1 mM NaF) supplemented with protease inhibitor mixture Complete (Roche Diagnostics).

**Immunoprecipitation and Immunoblotting—** For immunoprecipitation of Myc-tamalin and Syk, transfected COS-7 cells were lysed in the TN buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na$_2$VO$_4$, and 1 mM NaF) supplemented with protease inhibitor mixture Complete. For immunoprecipitation of FLAG-mGluR1, cells were lysed with the radioimmunoprecipitation assay buffer supplemented with protease inhibitor mixture Complete. Cell lysates were precleared with normal rabbit or normal mouse IgG-conjugated agarose beads for 60 min. Myc-tamalin and FLAG-mGluR1 were immunoprecipitated with agarose beads conjugated with rabbit polyclonal or mouse monoclonal anti-Myc and anti-FLAG for 3 h at 4°C. Syk was immunoprecipitated with anti-Syk and protein A/G PLUS-agarose. Brain lysates and P2 membrane fractions were prepared from whole rat brains and solubilized with the radioimmunoprecipitation assay buffer as described previously (9). Brain lysates and solubilized membrane fractions were precleared with protein A-Sepharose for 60 min, incubated with antisera or antibodies, and attached to protein A-Sepharose in the radioimmunoprecipitation assay buffer overnight. Immunoprecipitates were eluted with 2× lysis buffer containing 2× SDS-PAGE loading buffer. Cell lysates, solubilized membrane fractions, or immunoprecipitates were separated by 4–12% gradient (Invitrogen) or 8% (Bio-Rad) SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Protran, Schleicher & Schuell), followed by immunoblot analysis with horseradish peroxidase-conjugated or unconjugated antibodies; unconjugated antibodies were reacted with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibody. In immunoblot analysis of anti-tamalin immunoprecipitates, antibody used for immunoprecipitation interfered with detection of both phosphorylated tamalin and Syk. To avoid this problem, anti-tamalin was cross-linked to protein A resin, using an ImmunPure Protein A IgG Plus Orientation kit (Pierce), and immunoprecipitates were blotted with horseradish peroxidase-conjugated antibodies; horseradish peroxidase conjugation was performed using an EZ-Link Plus Activated Peroxidase kit (Pierce). Immunoreactive bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**RESULTS**

**A Protein Assembly of Tamalin with Protein-tyrosine Kinases and Protein-tyrosine Phosphatases—** Tamalin possesses 12 tyrosine residues over its entire sequence (Fig. 1A). The amino acid sequence data analysis disclosed that 2 tyrosines at positions 55 and 67 and their surrounding sequences match the consensus ITAM sequence (Fig. 1A). In addition, tyrosines at positions 214 and 236 are followed by leucine with a 2-amino acid space, and these sequences resemble the immunoreceptor tyrosine-based inhibitory motif (ITIM) involved in recruitment of SHIP and the SHP protein-tyrosine phosphatase (PTP) (Fig. 1A) (20, 21). We first addressed whether tamalin forms a protein complex in vivo with the Src family kinases, c-Src and Fyn, and SHP-2 and SHP-2, all of which are known to be expressed in the brain (22–24). Solubilized rat brain P2 membrane fractions were immunoprecipitated with anti-tamalin and immunoblotted with anti-c-Src, anti-Fyn, anti-SHIP-2, and anti-SHIP-2. Anti-tamalin coimmunoprecipitated c-Src and SHP-2, but not Fyn or SHIP-2 (Fig. 1B).

Our previous study showed that tamalin is enriched at the postsynaptic density fraction and interacts with a large splice variant of mGluR1 in the brain (9). We examined whether mGluR1 has the ability to form a protein complex with PTKs and PTP in the brain. Immunoprecipitation followed by immunoblotting showed that mGluR1 was coimmunoprecipitated from solubilized rat brain P2 membrane fractions with anti-tamalin, anti-c-Src, anti-Fyn and anti-Syk, but not with anti-SHIP-2 (Fig. 1C). The result indicates that mGluR1 associates with not only tamalin but also c-Src, Fyn, and Syk in the brain.

When Myc-tamalin was expressed heterologously together with exogenously transfected c-Src or Fyn or with endogenous SHP-2 in COS-7 cells, none of these proteins was coimmunoprecipitated with Myc-tamalin from cell lysates (data not shown), indicating that tamalin does not bind directly to c-Src, Fyn, or SHP-2. We then investigated an mGluR1-mediated complex formation with PTKs in COS-7 cells. FLAG-mGluR1 was cotransfected with c-Src, Fyn, and Syk in COS-7 cells, and the interaction of these PTKs with mGluR1 was examined by immunoprecipitation of cotransfected cell lysates with anti-FLAG followed by immunoblotting with the respective PTK antibodies. This analysis showed that c-Src, Fyn, and Syk were all coimmunoprecipitated with anti-FLAG (Fig. 2B). SHP-2 was endogenously highly expressed in COS-7 cells, but FLAG-mGluR1 immunoprecipitation failed to coimmunoprecipitate endogenous SHP-2 (Fig. 2B), confirming the inability of SHP-2 to associate with mGluR1.

To assign the interaction sites of mGluR1 with tamalin and c-Src, we constructed a series of truncated mutants at the carboxyl-terminal tail of FLAG-mGluR1 (Fig. 2A) and examined their ability to interact with Myc-tamalin or c-Src coexpressed in COS-7 cells (Fig. 2, C and D). This analysis showed that the interaction of tamalin with mGluR1 was reduced.
greatly by deletion of the carboxyl-terminal 179 amino acids of mGluR1 (mGluR1Δ179) and completely lost in the mGluR1Δ234 mutant (Fig. 2C). The carboxyl-terminal tail of mGluR1 possesses multiple proline-rich sequences including the Src homology 3 binding motif (PXXP) of the Src family kinases (22) (Fig. 2A). Similar to the interaction between mGluR1 and tamalin, the association of c-Src with mGluR1 was reduced greatly by truncation of the last 179 amino acids of mGluR1 (mGluR1Δ179) and abolished completely by removal of multiple proline-rich domains present in the last 234-amino acid sequence of mGluR1 (mGluR1Δ234) (Fig. 2D).

Because the binding sites of tamalin and c-Src are overlapping at the carboxyl-terminal tail of mGluR1, we addressed whether these two proteins can bind concomitantly to mGluR1 or mutually interfere with their binding to mGluR1 (Fig. 2E). FLAG-mGluR1 was cotransfected with either Myc-tamalin or c-Src, or both, and cell lysates were immunoprecipitated with anti-FLAG followed by immunoblotting with anti-Myc and anti-c-Src. Levels of coimmunoprecipitation of both Myc-tamalin mutants were cotransfected with c-Src or Fyn, and their phosphorylation were analyzed by immunoprecipitation followed by anti-P-Tyr immunoblotting (Fig. 3, A-C). These results indicate that the two tyrosines located within the putative ITAM sequence and those at 214 and 236 in the ITIM-related sequences were replaced with phenylalanine (Y55F/Y67F, Y55F/Y61F/Y67F, and Y55F/Y61F/Y214F/Y236F), these mutations almost completely abrogated c-Src-mediated and Fyn-mediated phosphorylation of tamalin. When tyrosines at positions 55 and 67 were doubly replaced with phenyalanine (Y55F/Y67F, Y55F/Y61F/Y67F, and Y55F/Y67F/Y214F/Y236F), these mutations almost completely abrogated c-Src-mediated and Fyn-mediated phosphorylation of tamalin.

Because mGluR1 has the ability to recruit both tamalin and c-Src at its carboxyl-terminal tail, we examined the possibility that coexpression of mGluR1 facilitates phosphorylation of tamalin by c-Src (Fig. 3B). Myc-tamalin and c-Src were coexpressed with and without FLAG-mGluR1, and extents of tamalin phosphorylation were analyzed by immunoprecipitation with anti-Myc followed by immunoblotting with anti-P-Tyr. Coexpression of mGluR1 markedly increased phosphorylation of tamalin by c-Src (Fig. 3D), indicating that mGluR1-mediated recruitment of tamalin and c-Src is capable of facilitating c-Src-mediated phosphorylation of tamalin.

We next tried to identify which tyrosine is phosphorylated by the Src family kinases in COS-7 cells. Tyrosines at positions 55, 61, and 67 within the putative ITAM sequence and those at 214 and 236 in the ITIM-related sequences were replaced with phenyalanine individually or combinatorially. These Myc-tamalin mutants were cotransfected with c-Src or Fyn, and their phosphorylation was analyzed by anti-Myc immunoprecipitation followed by anti-P-Tyr immunoblotting (Fig. 3, C and D). A single mutation at position 55 (Y55F) greatly reduced both c-Src-mediated and Fyn-mediated phosphorylation of tamalin. When tyrosines at positions 55 and 67 were doubly replaced with phenyalanine (Y55F/Y67F, Y55F/Y61F/Y67F, and Y55F/Y67F/Y214F/Y236F), these mutations almost completely abrogated tamalin phosphorylation with both c-Src and Fyn (Fig. 3, C and D). These results indicate that the two tyrosines located within
the consensus ITAM sequence of tamalin serve as preferentially phosphorylated sites with both c-Src and Fyn.

Association of Syk with the Phosphorylated ITAM of Tamalin—We examined whether tamalin has the ability to recruit Syk, depending on PTK-mediated phosphorylation at the ITAM sequence of tamalin. Myc-tamalin was cotransfected in different combinations with active and inactive forms of c-Src and Syk in COS-7 cells (Fig. 4A). Myc-tamalin was immunoprecipitated with anti-Myc (C) and anti-c-Src (D). Expression levels of Myc-tamalin and c-Src were quantified by immunoblotting of cell lysates (one-tenth of immunoprecipitation) with the respective antibodies. E, the concomitant association of tamalin and c-Src with mGlur1 was analyzed. FLAG-mGlur1 was coexpressed in the indicated combinations of Myc-tamalin and c-Src in COS-7 cells. Cell lysates were immunoprecipitated with anti-FLAG followed by immunoblotting with anti-Myc and anti-c-Src. Note that endogenous c-Src was detected in both cell lysates and mGlur1 immunoprecipitates of c-Src-untransfected COS-7 cells. Omission of cotransfection of the corresponding cDNA is indicated as a minus sign.

We then examined whether tyrosine phosphorylation in the ITAM sequence of tamalin is necessary for interaction with tamalin and Syk (Fig. 4B). Wild-type and a series of tyrosine-mutated forms of Myc-tamalin were coexpressed with c-Src and Syk in COS-7 cells. The extents of tamalin phosphorylation and the interaction of Syk with various forms of tamalin were analyzed by immunoprecipitation of Myc-tamalin followed by immunoblotting with anti-P-Tyr and anti-Syk, respectively (Fig. 4B). In every case, phosphorylation signals of tamalin were increased prominently by coexpression of c-Src and Syk, but relative extents of c-Src-mediated phosphorylation of various forms of tamalin remained unchanged between the presence and absence of Syk (compare the data of Fig. 3C and 4B). Importantly, paired substitutions at positions 55 and 67 (Y55F/Y67F)
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Fig. 3. Tyrosine phosphorylation of the tamalin ITAM sequence with c-Src and Fyn. A, phosphorylation of tamalin with c-Src was analyzed in COS-7 cells. Cells were transfected with Myc-tamalin with or without wild-type (WT), kinase-dead (KD), and constitutively active (CA) c-Src, and cell lysates were immunoprecipitated (IP) with anti-Myc followed by immunoblotting with anti-P-Tyr; P-tamalin, phosphorylated tamalin. Cell lysates were immunoblotted with anti-c-Src to quantify expression levels of c-Src in cell lysates. B, the effects of mGluR1 coexpression of c-Src-mediated phosphorylation of tamalin were analyzed in COS-7 cells. Cells were transfected with Myc-tamalin and c-Src with or without FLAG-mGluR1. The amounts of Myc-tamalin and c-Src and extents of tamalin phosphorylation were determined as in A. C and D, the effects of tyrosine replacement with phenylalanine on tamalin phosphorylation were analyzed by immunoprecipitation with anti-Myc followed by immunoblotting with anti-P-Tyr. COS-7 cells were transfected with c-Src (C) or Fyn (D) in combination with wild-type and a set of tyrosine-mutated forms of Myc-tamalin; phenylalanine replaced tyrosine at the indicated positions of tamalin. Expression levels of c-Src and Fyn in cell lysates were quantified by immunoblotting with the respective antibodies.

Y67F, Y55F/Y61F/Y67F, Y55F/Y67F/Y214F/Y236F) almost completely abolished the interaction between tamalin and Syk (Fig. 4B). In contrast, single or combinatorial mutations at positions 61, 214, and 236 (Y61F, Y214F, Y236F, Y214F/Y236F, Y61F/Y214F/Y236F) had no appreciable effect on the interaction between tamalin and Syk (Fig. 4B).

The importance of ITAM phosphorylation in the association between tamalin and Syk was analyzed further in Fyn-mediated phosphorylation of tamalin by coexpression of Fyn, Syk, and a series of tyrosine-mutated forms of tamalin in COS-7 cells (Fig. 4C). The association of tamalin with Syk was greatly and moderately reduced in Y55F and Y67F mutants, respectively, and almost completely abrogated by paired substitutions at both positions (Y55F/Y67F, Y55F/Y61F/Y67F). In contrast, Y61F and Y214F/Y236F mutations had no appreciable effect on the association between tamalin and Syk (Fig. 4C). These results demonstrate that paired tyrosine phosphorylation of the tamalin ITAM plays a critical role for binding of Syk to tamalin.

The association of phosphorylated tamalin with ZAP-70 was also examined by cotransfection of c-Src, Myc-tamalin, and ZAP-70 in COS-7 cells. Immunoprecipitation with anti-Myc followed by immunoblotting with anti-ZAP-70 failed to detect the association between phosphorylated tamalin and ZAP-70 in cotransfected cell lysates (data not shown).

Enhanced Phosphorylation of Tamalin-associated Syk—We examined whether recruitment of Syk to phosphorylated tamalin influences Syk phosphorylation by immunoprecipitation of cotransfected cell lysates with anti-Syk followed by immunoblotting with anti-P-Tyr (Fig. 5). c-Src phosphorylated Syk (lane 2), and this phosphorylation of Syk was enhanced considerably by coexpression with tamalin (lane 3). The tyrosine phosphorylation of Syk was dependent on the Src kinase activity (lane 4), but appreciable Syk phosphorylation was observed with kinase-dead Syk (lane 5). The results indicate that the association of Syk with tamalin in an ITAM-based manner increases c-Src-mediated phosphorylation of Syk.

The Src family kinases have been shown to phosphorylate Syk at multiple sites and induce autophosphorylation of Syk (25, 26). The resultant activation of Syk stimulates distinct downstream signalings, depending on different phosphorylation sites of Syk (27). We analyzed tamalin-dependent phosphorylation sites of Syk, using antibodies specific for phosphorylated Tyr\(^{323}\), Tyr\(^{352}\), and Tyr\(^{525}/\text{Yyr}^{526}\) of Syk. Antibodies against P-Tyr\(^{323}\) and P-Tyr\(^{525}/\text{Yyr}^{526}\) failed to reveal immunoreactive signals of phosphorylated Syk in cell lysates cotransfected with c-Src, Syk, and tamalin (data not shown). Antibody against P-Tyr\(^{352}\), in contrast, yielded immunoreactive signals that were dependent on active c-Src (Fig. 5, lanes 1–4) and significantly enhanced by coexpression with tamalin (lane 3). This phosphorylation was slightly enhanced when tamalin was coexpressed with kinase-dead c-Src (compare lane 1 and lane 4), and this enhancement may be attributed to endogenous c-Src in COS-7 cells (lane 1). More importantly, the enhanced phosphorylation at position 352 of Syk was seen explicitly when kinase-dead Syk was expressed in conjunction with coexpression of c-Src and tamalin (lane 5). The results indicate that the interaction of phosphorylated tamalin with Syk specifies c-Src-mediated tyrosine phosphorylation at position 352 of Syk.

Enhancement of Tamalin Phosphorylation and Syk Recruitment by Protein Phosphatase Inhibitors—To assess the susceptibility of phosphorylated tamalin to dephosphorylation with PTPs, we examined the effects of PTP inhibitors on tamalin phosphorylation in COS-7 cells. COS-7 cells were coexpressed...
with Myc-tamalin and c-Src and incubated with a tyrosine phosphatase-selective inhibitor, bpV(phen) (28), for 10 or 20 min before cell lysis. Cell lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-P-Tyr. Expression levels of c-Src and Syk were quantified by immunoblotting of cell lysates with anti-c-Src and anti-Syk, respectively. B and C, the association of Syk with c-Src-phosphorylated (B) or Fyn-phosphorylated (C) tamalin was analyzed by immunoblotting of Myc-tamalin immunoprecipitates with anti-Syk. COS-7 cells were transfected with wild-type and tyrosine-mutated forms of Myc-tamalin in combination either with c-Src and Syk (B) or with Fyn and Syk (C). Cell lysates were immunoprecipitated with anti-Myc followed by immunoblotting with anti-Myc, anti-P-Tyr, and anti-Syk. Expression levels of c-Src, Syk, and Fyn were quantified by immunoblotting of cell lysates with the respective antibodies.

Fig. 4. Association of Syk with the ITAM-phosphorylated tamalin. A, COS-7 cells were transfected with wild-type (WT) Myc-tamalin in combination with wild-type and kinase-dead (KD) mutants of c-Src and Syk as indicated. Cell lysates were immunoprecipitated (IP) with anti-Myc followed by immunoblotting with anti-Myc and anti-P-Tyr. Expression levels of c-Src and Syk were quantified by immunoblotting of cell lysates with anti-c-Src and anti-Syk, respectively. B and C, the association of Syk with c-Src-phosphorylated (B) or Fyn-phosphorylated (C) tamalin was analyzed by immunoblotting of Myc-tamalin immunoprecipitates with anti-Syk. COS-7 cells were transfected with wild-type and tyrosine-mutated forms of Myc-tamalin in combination either with c-Src and Syk (B) or with Fyn and Syk (C). Cell lysates were immunoprecipitated with anti-Myc followed by immunoblotting with anti-Myc, anti-P-Tyr, and anti-Syk. Expression levels of c-Src, Syk, and Fyn were quantified by immunoblotting of cell lysates with the respective antibodies.

Fig. 5. Enhancement of tyrosine phosphorylation of the tamalin-associated Syk. The effects of the protein association of tamalin and Syk on Syk phosphorylation (P-Syk) were analyzed. Myc-tamalin was coexpressed in indicated combinations of wild-type and kinase-dead (KD) mutants of c-Src and Syk in COS-7 cells. Cell lysates were immunoprecipitated (IP) with anti-Syk followed by immunoblotting with anti-P-Tyr. Levels of phosphorylated Syk at position 352 were determined by immunoblotting of cell lysates with anti-Syk/P-Tyr(352). Expression levels of Myc-tamalin, c-Src, and Syk were quantified by immunoblotting of cell lysates with the respective antibodies.

Tyramine was added into the presence and absence of the PTP inhibitor and is highly susceptible to dephosphorylation with PTPs. Single and paired tyrosine mutations at the ITIM-related sequences had no effect on bpV(phen)-enhanced tamalin phosphorylation (Fig. 6A, lanes 8–10). In addition, phosphorylated tamalin immunoprecipitated from bpV(phen)-treated cell lysates showed no association with endogenous SHP-2 (data not shown). It is thus unlikely that the ITIM-related sequences of tamalin serve to recruit SHP-2 to tamalin.

Enhanced tamalin phosphorylation and its predominant effect on paired tyrosines at the tamalin ITAM sequence were also observed after treatment with a broad spectrum of phosphatase inhibitor, Na₃VO₄, for 20 min (Fig. 6B). In this experiment, binding of Syk to phosphorylated tamalin was analyzed by immunoprecipitation of cell lysates with anti-Myc followed by immunoblotting with anti-Syk (Fig. 6B). Syk binding commonly increased in a variety of tamalin mutants, probably reflecting a weak interaction of Syk with phosphorylated tyrosines at multiple sites of tamalin. Importantly, this binding was apparently reduced by paired tyrosine mutations at the ITAM sequence of tamalin (Fig. 6B, lanes 5, 7, 11–13), confirming that the interaction of Syk with tamalin occurs in an ITAM-based manner.

Tamalin Phosphorylation and Syk Recruitment in P19 Embryonal Carcinoma Cells—We next addressed whether tamalin is phosphorylated endogenously and can form a protein assembly with Syk in neural cells. This analysis was first conducted using whole rat brain lysates or solubilized P2 membrane fractions by anti-tamalin immunoprecipitation followed by immunoblotting with anti-P-Tyr and anti-Syk. Upon this analysis, neither phosphorylated tamalin nor tamalin-associated Syk was detected in either of the brain preparations (data not shown). This failure could result from possible technical problems such as a rapid turnover of phosphorylation/phosphorylation of tamalin or insufficient PTK activation for tamalin phosphorylation in the normal brain. We thus analyzed P19 embryonal carcinoma cells, which are capable of differentiating into neuron-like cells in culture (10). In this cell line, tamalin has been shown to be highly induced by exposure to retinoic acid (10). Furthermore, Syk has been implicated in neuron-like differentiation of these cells (29). P19 cells in monolayer culture were treated with retinoic acid for 24 or 48 h, and cell lysates were subjected to immunoprecipitation with anti-tamalin followed by immunoblotting with anti-P-Tyr and anti-Syk (Fig. 7). This analysis showed not only a signal of tyrosine phosphorylation of tamalin but also coimmunoprecipitation of Syk with immunoprecipitated tamalin. The result demon-
cating the dependence of tamalin phosphorylation on c-Src and enhanced considerably with constitutively active c-Src, indicating the dependence of tamalin phosphorylation on c-Src kinase activity. Once the ITAM is phosphorylated, it can recruit Syk and enables it to be phosphorylated by the Src family kinases. Phosphorylated tamalin at the ITAM sequence is highly susceptible to dephosphorylation with PTPs. The functional tamalin is thus regulated by phosphorylation and dephosphorylation in an ITAM-based fashion. Importantly, tamalin is endogenously phosphorylated and associated with Syk in P19 cells. Tamalin is thus a novel signaling adaptor that serves not only as a PDZ scaffold protein but also as an ITAM-based signaling molecule.

Recently, several cytoplasmic signaling molecules that possess the ITAM sequence have been identified and characterized (30, 31). Proteins of the ezrin/radixin/moesin family contain the ITAM sequence at their amino-terminal regions and act as adaptor molecules that mediate Syk phosphorylation in leukocyte adhesion receptor signaling cascades (30). STAM is another ITAM-containing adaptor molecule involved in signaling pathways of cytokine receptors, although STAM has been reported not to interact physically with Syk (31, 32). Tamalin is the first example of a signaling molecule that possesses not only the functional ITAM sequence but also the PDZ domain and PDZ binding motif. The antigen receptor complexes comprise multiple copies of the ITAM sequences (13, 33). Tamalin contains a single ITAM motif but has ability to form an oligomer via interaction between the PDZ domain and its own PDZ binding motif (11). The oligomeric tamalin, like the antigen receptor complexes, could contribute to amplifying intracellular signaling cascades.

It has been reported that stimulation of group 1 mGluRs induces an activation of c-Src and Fyn in cultured cortical neurons in a protein kinase C-independent, Ca\textsuperscript{2+}/calmodulin-dependent manner (34). Furthermore, stimulation of group 1 mGluRs has been shown to activate c-Src at the mossy fiber-CA3 synapses in a G protein-independent manner (35). Tamalin is enriched at the postsynaptic density fraction and interacts with both mGluR1 and mGluR5 (9). We therefore examined whether glutamate stimulation of mGluR1 could enhance c-Src-mediated tyrosine phosphorylation of tamalin in heterologously mGluR1-expressing COS-7 cells. However,
these experiments failed to detect any change in tamalin phosphorylation by incubation with 100 μM glutamate for 1–15 min (data not shown). We also examined the possibility that mGluR1-associated tamalin is capable of enhancing recruitment of Syk to mGluR1 (data not shown). The role of tamalin phosphorylation in mGluR1 signaling transduction thus remains elusive in this investigation. Nonetheless, it has been revealed that both tamalin and c-Src were coimmunoprecipitated with mGluR1 from brain extracts, and both interacted with the carboxy-terminal tail of mGluR1 in COS-7 cells. Therefore, it is possible that the ITAM-based interaction of tamalin with Syk regulates intracellular signaling of mGluR1 by some extracellular signals that activate the Src family kinases. Because the tamalin/Syk signaling molecules are now revealed to form a protein assembly in P19 cells, the P19 cell line would provide a nice system to investigate the role of this signaling pathway in neuronal cell function and differentiation.

The activation of Syk by phosphorylation leads to stimulation of several important signaling pathways that culminate in the activation of transcription factors in the nucleus (36, 37). The activated Syk phosphorylates downstream adaptor proteins and PTKs, which in turn activate phospholipase C-γ (25). Phospholipase C-γ cleaves phosphatidylinositol bisphosphate to yield diacylglycerol and inositol trisphosphate (17). Inositol trisphosphate increases intracellular concentrations of Ca²⁺, which activates a phosphatase, calcineurin, and also protein kinase C with a conjunctive action of diacylglycerol (25). Because group 1 mGluRs activate phospholipase C-β through direct coupling to G proteins (38, 39), Syk-mediated activation of phospholipase C-γ may not necessarily participate in group 1 mGluR signal transduction but may be involved in signaling cascades of other tamalin-associated receptors. Another important pathway of the Syk signaling cascade is generated by activation of small G proteins, which is achieved by coordinate actions of adaptor proteins and guanine nucleotide exchange factors (40, 41). Interestingly, the leucine-zipper region of tamalin binds to the coiled-coil region of cytohesins which are guanine nucleotide exchange factor specific for the ADP-ribosylation factor family of small G proteins (9, 10). Molecular assembly of cytohesins and Syk may thus be built on the tamalin scaffold protein and may efficiently link Syk signaling to small G protein cascades.

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Phosphorylation and Recruitment of Syk by Immunoreceptor Tyrosine-based Activation Motif-based Phosphorylation of Tamalin
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