SYNCRIP, a Cytoplasmic Counterpart of Heterogeneous Nuclear Ribonucleoprotein R, Interacts with Ubiquitous Synaptotagmin Isoforms

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Synaptotagmins (Syts) are a large family of membrane proteins consisted of at least 12 isoforms. They are categorized in neuron-specific isoforms (I-V, X, and XI) and ubiquitous isoforms (VI–IX) based on their expression patterns. Syt-I, a neuron-specific and abundant isoform, has been well characterized and postulated to be the exocytotic Ca2+ sensor. However, the functions of other isoforms remain obscure. Here, we report that ubiquitous isoforms of synaptotagmins, Syt-VII, Syt-VIII, and Syt-IX, interacted with a cytoplasmic RNA-binding protein, SYNCRIP (Synaptotagmin-binding, cytoplasmic RNA-interacting protein), through their C2B domains. SYNCRIP was originally found in the Syt-II C2AB domain bound fraction from the mouse brain lysate. cDNA cloning of SYNCRIP cDNA revealed that the protein was highly homologous to heterogeneous nuclear ribonucleoprotein R (hnRNP R) recently identified. SYNCRIP protein was ubiquitously and constantly expressed in various tissues of mice parallel to hnRNP R. SYNCRIP indeed bound RNA with preference to poly(A) RNA; however, in contrast to the nuclear localization of hnRNP R, SYNCRIP was distributed predominantly in the cytoplasm as judged by both biochemical fractionation and immunohistochemical studies. In vitro binding experiments showed the potential interaction of SYNCRIP with C2B domains of Syts except for those of Syt-V, -VI, and -X. Furthermore, the interaction between SYNCRIP and Syt-VII, -VIII, or -IX was revealed by co-immunoprecipitation experiments using COS cells transiently expressing each Syt isoform. These findings suggested that SYNCRIP was a target of ubiquitous type of Syts and implied the involvement of ubiquitous Syts in the regulation of dynamics of the cytoplasmic mRNA.

The restricted distribution of various molecules to proper compartments is an essential process of eucaryotic cells. Vesicles-based transport, which is budding of the vesicles carrying some particular molecules from a donor compartment and fusion of these vesicles with an acceptor membrane, is a fundamental mechanism for this process. Soluble N-ethylmaleimide-sensitive fusion protein (NSF)1 attachment protein (SNAP) receptors (SNAREs) are revealed to be core molecules underlying the mechanism by recent genetic and biochemical studies (1, 2), and the specific pairing of membrane proteins on the vesicle (v-SNAREs) and the target membrane (t-SNAREs) is thought to determine the specificity of docking of the vesicle to the target membrane, and the interaction may generate the force for membrane fusions (3–5).

In neurotransmitter release of the mammalian presynaptic terminus, where electrophysiological studies have been utilized to detect the fusion of synaptic vesicles with axon-terminal plasma membranes and the great advancement in identifying the core molecules has been accomplished, the synaptic vesicle protein, vesicle-associated membrane protein 1 or 2, and the two presynaptic membrane proteins, syntaxin 1 and SNAP-25, serve as the v-SNARE and the t-SNAREs, respectively (2, 6). Although these three SNAREs are essential for neurotransmitter release (6–8), and the formation of a complex among these proteins is the minimal molecular process for synaptic vesicle fusion (9), these molecules are not sufficient for the achievement of rapid synaptic vesicle fusion triggered (within 200 μs) by calcium. Syt-I, an integral membrane protein characterized by a short N terminus, a single transmembrane region, and two C2 domains (the C2A and C2B domains) homologous to the C2 regulatory region of protein kinase C (10, 11), is a most plausible candidate for mediating the calcium signal to the SNARE complex, presented by genetic analysis in various systems (12–14).

Subsequent studies on the SNARE proteins have revealed that each of these molecules constitutes a large gene family and their distinct tissue or intracellular distributions, and suggested their general roles in membrane trafficking and the isoform-specific involvement in the vesicle trafficking at distinct intracellular organelles (15–18). In line with these, Syts have also been found to be a large family consisting of at least 12 isoforms in rats or mice (11, 19). Although the role of Syt-I

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1 The abbreviations used are: NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; v-SNARE, vesicle SNARE; t-SNARE, target membrane SNARE; SNAP-25, synaptosome-associated protein of 25-kDa; Syt, synaptotagmin; GST, glutathione S-transferase; IHPS, inositol high polyphosphates series; IP3, 1,3,4,5-tetakisphosphate; IP4, inositol high polyphosphates series; IP5, 1,3,4,5,6-pentakisphosphate; IP6, inositol hexakisphosphate; PMSE, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; RBD, RNA binding domain; hnRNP R, heterogeneous nuclear ribonucleoprotein R; bp, base pair; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; aa, amino acids.
and -II as a Ca$^{2+}$ sensor in synaptic vesicle fusion has been established and the regulatory roles of other Syts in the SNARE-based vesicle trafficking were examined, detailed characterizations on the intracellular localization of other Syt isoforms remain immature.

We have found that inositol 1,3,4,5-tetrakisphosphate (IP$_4$) bound the C2B domains of Syts-I, -II, -IV, -VI to IX, and -XI (20, 21) and demonstrated the potential role of inositol high polyphosphate series (IHPS: IP$_6$, inositol 1,3,4,5,6-pentakisphosphate (IP$_{6p}$), and inositol hexakisphosphate (IP$_{6k}$)) in the regulation of Syt's-vesicular trafficking (22). For example, binding of an inositol high polyphosphate to the C2B domain of the neuron-specific and abundant isoform of Syt caused the inhibition of the fusion step of synaptic vesicle with the presynaptic plasma membrane (23–25). During the course of studies to clarify the molecular base of this inhibition, we found that several proteins including the clathrin adaptor complex, AP-2, 66-, 43-, and 33-kDa proteins, interacted with the C2AB domain of Syt-II immobilized on beads matrix and were competitively released from the complex by IHPS (26). Here we report that a 66-kDa protein in the C2AB domain-bound fraction was a RNA-binding protein with preference to poly(A) RNA and directly interacted with the C2B domain of Syts through its C-terminal region in vitro. Thus, we named the protein SYNCRIP (Synaptotagmin-binding, cytoplasmic RNA-interacting protein). Furthermore, in vivo, SYNCRIP specifically interacted with ubiquitous isoforms of Syts, Syt-VII, Syt-VIII, and Syt-IX, in accordance with the ubiquitous tissue distribution of SYNCRIP. On the basis of these results, we discuss the role of ubiquitous Syt isoforms in the dynamics of cytoplasmic mRNA, especially the involvement in vesicle-based transport of mRNA.

**Experimental Procedures**

**Materials**

Lysyl endopeptidase from *Achromobacter lyticus* was purchased from Wako. [$\alpha$-$^32$P]dCTP, L-[35S]methionine, poly(A) RNA, poly(C) RNA, poly(U) RNA, poly(A) DNA, and poly(A)-Sepharose were purchased from Amersham Pharmacia Biotech. Poly(U)-Sephadex and LipofectAMINE reagent were from Life Technologies, Inc. BcaBEST dideoxy sequencing kit and Pyrobest DNA polymerase were from Takara. TNT-coupled reticulocyte lysate systems was purchased from Promega. All other reagents were analytical grade or the highest grade available.

**Preparation of GST Fusion Proteins**

Recombinant glutathione S-transferase (GST) fusion proteins containing various fragments of Syts were expressed and purified on glutathione-Sepharose (Amersham Pharmacia Biotech) as described previously (21).

**Isolation and Partial Amino Acids Sequencing of SYNCRIP from Mouse Brain**

Partial purification of SYNCRIP was performed as described previously with minor modification (26). Fifty grams (wet weight) of mice whole brains were homogenized in 250 ml of 10 mM Hepes-NaOH (pH 7.4), 140 mM NaCl, 2 mM EDTA, 1 mM $\beta$-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM leupeptin, and 10 mM pepstatin A with glass-Teflon homogenizer (930 rpm, 10 strokes). After removing the debris and crude nuclear fraction by low speed centrifugation (1,000 $\times$ g, 10 min), Triton X-100 was added to give the final concentration of 1% (v/v) to the S1 fraction followed by incubation on ice (pH 7.4), 140 mM NaCl, 2 mM EDTA, 1 mM $\beta$-mercaptoethanol, the bound proteins were eluted with 100 mM $\mu$G in wash buffer. The eluted proteins were further separated on MonoQ column chromatography. A 66-kDa protein was eluted at around 0.2 mM NaCl, pH 8.0. The fractions enriched with the 66-kDa protein were separated on 10% SDS-PAGE, followed by staining with Coomassie Brilliant Blue R. The 66-kDa protein band was excised from the gel and digested with lysyl endopeptidase essentially according to the method as described (27). The resultant polypeptides were separated by C-18 reverse-phase column (mRPC C2/C18 SC 2.1/10, Amersharm Pharmacia Biotech) connected on a SMART system (Amersham Pharmacia Biotech). Peptide sequence of each peptide was determined by PE Biosystems 492 protein sequence.

**cDNA Cloning of the 66-kDa Protein, SYNCRIP**

Two degener primers corresponding to digested peptide 1 (VTEGLTDVILYHQDPDK) were synthesized as follows: sense primers, 5\'-GTNACNGAAGGCTC7TNA3-3', based on VTEGLT, and anti-sense primers, 5\'(TC7T/AGTC7T/CNGG/TC7T/AGTC7T/AGTFC-3') based on YHQPDDK. 35 cycles of PCR amplification were performed using mouse cerebellum random-primed cDNA library in $\alpha$-32P-labeled primer (Novagen), confirmed by sequencing, and used as the authentic probe. Antibody for re-screening or screening a mouse cerebellum oligo(dT)-primed $\alpha$-32P-labeled cDNA library to obtain full-length SYNCRIP cDNA. Cloned cDNAs were subcloned into pBluescript and sequenced on both directions by dideoxy chain termination method using [$\alpha$-$^32$P]dCTP.

**Preparation of Affinity Purified Anti-SYNCRIP Antibodies**

A His$_6$-tagged recombinant protein containing the N-terminal region of SYNCRIP aa 1–170 was expressed in *E. coli* and partially purified with ProBond resin (Invitrogen), followed by MonoQ column chromatography to thoroughly purify. A Japanese White rabbit was immunized with 300 $\mu$g of the purified protein by subcutaneous injection with the complete Freund’s adjuvant at a 14-day interval. The affinity purified antibody (anti-SYNCRIP-N antibody) was obtained from the antiserum against the affinity column covalently coupled with a GST fusion protein carrying the corresponding region with the antigenic protein. Antibody against a peptide (aa 140–152 of SYNCRIP) was produced in rabbit with the injection of the peptide cross-linked with keyhole limpet hemocyanin via the artificially introduced Cys residue to the N terminus of the peptide. Anti-peptide antibody (anti-SYNCRIP-Pep antibody) was affinity purified with the antigenic peptide-conjugated beads.

**Expression of SYNCRIP in COS-7 Cells and S9 Cells**

For transient expression of SYNCRIP in COS-7 cells, full-length, N-terminally truncated (ΔN, Δaa 1–88) and C-terminally truncated (ΔC, Δaa 401–561) SYNCRIP cDNAs were introduced into *EcoRI* and Xhol sites in 5’ and 3’ ends, respectively, by standard PCR-based methods were subcloned into pCDNA3 (Invitrogen). Each construct was transfected into COS-7 cells using LipofectAMINE reagents according to the manufacturer's instructions. Forty eight hours after transfection, the expression of each construct was examined by Western blotting or indirect immunofluorescence study (see below).

For expression of SYNCRIP in S9 cells, the full-length SYNCRIP cDNA was subcloned into pFASTBAC1 (Life Technologies, Inc.), and recombinant baculovirus carrying SYNCRIP was created and amplified by following the manufacturer's instructions. S9 cells cultured in TNM-FH medium supplemented with 10% fetal calf serum were infected with the amplified recombinant viruses. Cells were harvested 48 h after infection and proceeded for the binding experiments (see below).

**RNA Binding Assay**

$[^{35}S]$-Labeled SYNCRIP was generated by in vitro translation using TNT-coupled reticulocyte lysate systems. Each aliquot of 7S-SYNCRIP T7RIP was incubated with indicated amount of each poly(A) RNA or yeast tRNA in 50 $\mu$l of the binding buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM NaCl) for 30 min at 25 °C. 50 $\mu$l of poly(U)-Sephadex (20% slurry in the binding buffer) was added to each mixture followed by incubation for 45 min at 25 °C. After washing the beads with 500 $\mu$l of the binding buffer 4 times, the bound fractions were loaded on 10% SDS-PAGE and analyzed by Li-Cor imaging system. Direct binding of SYNCRIP to poly(A) RNA was examined by using poly(A)-Sepharose-2B binding buffer containing indicated concentration of NaCl. For comparison of the binding ability of SYNCRIP for poly(A) RNA versus DNA, indicated amount of poly(A) RNA or poly(A) DNA was incubated with $^{35}$S-SYNCRIP, followed by the analysis of poly(A)-Sepharose-bound SYNCRIP at 100 mM NaCl as described above. Inhibitory effects...
of soluble competitors were quantified by FUJIX BAS 2000 system from three independent experiments.

In Vitro Binding Experiments

Full-length SYNCRIP and Truncated SYNCRIPs Expressed in COS-7 Cells Versus GST-Syt-II C2B—The lysate was prepared from COS-7 cells expressing each SYNCRIP protein by homogenization of the cells in 10 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA, 1% Nonidet P-40, 0.2 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin A, followed by centrifugation (12,000 rpm, for 20 min at 4 °C). Each lysate was incubated with equal amount of GST-Syt-II C2B protein for 2 h at 4 °C. GST-Syt-II C2B protein-bound fractions were pulled down with glutathione-Sepharose and examined by Western blotting with anti-SYNCRIP-Pep antibody.

Endogenous SYNCRIP in Mouse Brain Versus GST-fused Various Parts of Syt-II and Effect of Ca \(^{2+}\) and Mg \(^{2+}\) on Their Bindings—Mouse whole brain detergent extracts (2.5 mg/ml) were prepared in 10 mM Hepes-NaOH (pH 7.4), 140 mM NaCl, 1% (w/v) Triton X-100, 1 mM β-mercaptoethanol, 0.2 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin A. Each 3 ml of aliquots was added with EDTA (2 mM, the final concentration), CaCl \(_2\) (1 mM), EDTA (2 mM), or MgCl \(_2\) (1 mM) and incubated with 25 μg of each purified GST fusion protein carrying various parts of Syt-II or GST alone for 2 h at 4 °C. After adding 50 μl (50% slurry) of glutathione-Sepharose and another 1-h incubation, the resins were extensively washed with the extract buffer containing each chelator or divalent ion, and bound proteins were examined by Western blotting for SYNCRIP with anti-SYNCRIP-N antibody.

Purified His \(_6\)-tagged recombinant protein containing the C-terminal region of SYNCRIP (aa 401–561) was expressed in E. coli using pRSET plasmid (Invitrogen). The recombinant protein was recovered from inclusion bodies by using denaturing solution (6 M guanidine HCl, 10 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, 1 mM β-mercaptoethanol) and purified with PreBind resin. The denatured recombinant protein was precipitated for refolding by dialysis against 10 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 0.1 mM NaCl, 1 mM EDTA, with gradually reducing the concentration of urea, from 6 to 0.001 M. The dialysate was centrifuged at 12,000 rpm for 20 min at 4 °C to remove malfolded and insoluble parts. After aliquots were incubated with various GST fusion proteins, bound proteins were pulled down and examined by Western blotting with anti-Xpress antibody (Invitrogen).

Co-immunoprecipitation Experiments

Each Syt tagged with T7 epitope in the N terminus (42) was transiently transfected into COS cells. Forty eight hours after transfection, cells were harvested and homogenized into 10 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 0.5 mM β-mercaptoethanol, 1 mM EDTA, 1% Nonidet P-40, 0.2 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin A. The lysates were prepared by centrifugation (12,000 rpm, 20 min) and incubated with affinity purified anti-SYNCRIP-N antibody (3.5 μg/ml) or anti-SYNCRIP-Pep antibody (0.27 μg/ml) or anti-GST-C2B of various Syts. Bound proteins were pulled down with GST fusion protein G-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4 °C. GST-Syt-II C2B protein–bound fractions were pulled down with glutathione-Sepharose and analyzed by Western blotting with anti-SYNCRIP-Pep antibody.

Immunoblotting

Proteins with the amounts indicated in the figure legends were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane by electroblotting. Blots were blocked with 5% skim milk in 0.1% Tween 20, PBS (T-PBS) and then incubated with anti-SYNCRIP-N antibody (0.1 μg/ml) or anti-SYNCRIP-Pep antibody (0.23 μg/ml) for 1 h at room temperature. After extensively washing with T-PBS, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG. Immune-reactive bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech). Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin used as a standard.

RESULTS AND DISCUSSION

Purification and cDNA Cloning of the 66-kDa Protein (SYNCRIP)—We previously reported that several proteins bound the C2AB domain of Syt-II and were competitively released by inositol high polyphosphates series (IHPs) (26). AP2 complex was one of these proteins and seemed to be involved in the IHPs-induced blockade of synaptic transmission (23, 24). However, neither of the subunits of the AP2 complex recombinantly expressed directly interacted with the C2B domain. Thus, we began to seek a molecule that directly interacted with the C2AB domain of Syt-II from the IP \(_3\) eluate. We separated the eluates by MonoQ column chromatography and found that a 66-kDa polypeptide eluted together with the AP2 complex directly interacted with the C2AB domain of Syt-II by far Western analysis using GST-Syt-II C2AB as a probe (Fig. 1A, and data not shown). The partial amino acid sequences of the 66-kDa protein were determined, and based on the sequence, we isolated cDNAs of the 66-kDa protein from mouse cerebellum cdna library. Several overlapping clones were isolated and gave an open reading frame of 1683 nucleotides coding for a protein of 561 amino acids with a calculated mass of 62.5 kDa (Fig. 1B).

Homology analyses of the deduced amino acid sequence of the cloned cDNA revealed that it was highly homologous (81.2% identical) to human heterogeneous nuclear ribonucleoprotein R (hnRNP R); the molecular structure was recently defined by Hassfeld et al. (30). They also reported that hnRNP R was an 82-kDa polypeptide and was localized in the nucleoplasm. During the course of isolating cDNA clones, we found clone 18 coding mouse hnRNP R (99.1% identical to aa298–633 of human hnRNP R) indicating that the 66-kDa protein was a distinct molecule from hnRNP R (Fig. 1, B and C). Like hnRNP R, the 66-kDa protein had a relatively acidic N-terminal region followed by three sets of RNA binding domain (RBD) containing the canonical conserved octameric RNP-1 and hexameric RNP-2 consensus motifs. In the C-terminal region, as a whole, arginine and glycine residues were enriched, some portions of which formed RGG boxes, another type of RNA binding motif. Furthermore, tyrosine residues were also enriched in the N-terminal half of this region (Fig. 1, C and D), and YYGG tetrapeptides were found three times as seen in hnRNP R. The most striking difference was the lack of ~70 amino acids of the C terminus in the 66-kDa protein, and these were found in hnRNP R. More recently, a cellular interactor of NS1, the

\(^2\) A. Mizutani and K. Mikoshiba, unpublished observations.
major nonstructural parvovirus protein, has been cloned from human cDNA library, named NSAP1 (31), whereas its physiological function was not addressed. 99% identical amino acids sequence between the 66-kDa protein and NSAP1 indicated that the 66-kDa protein was a mouse homologue of human NSAP1. Besides these two proteins, the 66-kDa protein showed the high similarity (50–60% identical) to polyadenylate-binding proteins of various species in RBD regions among other RNA-binding proteins.

We produced the two kinds of polyclonal antibodies against the 66-kDa protein as follows: one was against the N-terminal region of the 66-kDa (Fig. 1C, boxed), anti-SYNCRIP-N antibody, and the other was against the peptide whose sequence was completely conserved between the 66-kDa protein and hnRNP R (Fig. 1C, double-underlined); anti-SYNCRIP-Pep antibody. Both of these antibodies recognized the endogenous 66-kDa polypeptide in COS-7 cells lysates (Fig. 2A and Fig. 4B) as well as 82- and 74-kDa polypeptides, hnRNP R itself, and its degradates, respectively (30). When the full-length cDNA was transfected into COS-7 cells, the 66-kDa protein was overexpressed, and the protein was localized in the cytoplasm with both anti-SYNCRIP-N antibody and anti SYNCRIP-Pep antibody (Fig. 2, B and C). The nucleoplasmic staining of non-transfected cells with anti-SYNCRIP-Pep antibody was probably the signal of hnRNP R, since this peptide antibody more preferentially recognized hnRNP R than the 66-kDa protein by Western blotting (Fig. 4B). Moreover, the fluorescence signal of green fluorescence protein-fused 66-kDa protein was also detected only in the cytoplasm (data not shown).

Subcellular fractionation of P7 mice cerebella showed that the 66-kDa protein was enriched in P3 (crude microsome) fraction as well as in P1 (cell debris and crude nucleus) fraction, whereas the 82-kDa hnRNP R was exclusively detected in P1 fraction. These results indicated that the 66-kDa protein, which structure was highly related to hnRNP R, showed the cytoplasmic localization distinct from hnRNP R, suggesting that the 66-kDa protein may be a cytoplasmic counterpart of hnRNP R. Sequence analysis of hnRNP R revealed the bipartite-type nuclear localization signals (32) in the hnRNP R-specific additive region in the C-terminal KRKADGYNPD-SKKR (aa 572–586 of hnRNPR), which may explain the different subcellular distribution of hnRNPR and the 66-kDa protein.

**RNA Binding Activity of the 66-kDa Protein (SYNCRIP)**—To test whether the 66-kDa protein can bind to RNA molecules, we performed RNA binding analysis in vitro. The 66-kDa protein was in vitro translated with $^{35}$S and incubated with poly(U)-Sepharose with various amount of competitors. As shown in Fig.
3A, 35S-labeled 66-kDa protein indeed bound poly(U)-RNA Sepharose. Soluble poly(A) RNA and, to a lesser extent poly(U) RNA, competed for the 66-kDa protein binding. However, yeast tRNA or poly(C) RNA were ineffective competitors, indicating that the 66-kDa protein binds preferentially to poly(A) RNA. To confirm the direct binding of the 66-kDa protein to poly(A) RNA, we carried out similar experiments using poly(A)-Sepharose and compared the binding to poly(A) RNA and poly(U) RNA at various concentrations of NaCl. The 66-kDa protein showed stronger binding to poly(A) RNA than to poly(U) RNA at 100 mM NaCl, and the binding is obvious at high concentration of NaCl up to 400 mM (Fig. 3B, left panel). We also examined the comparative binding ability of the 66-kDa protein for homopoly(A) RNA and DNA. Soluble poly(A) RNA strongly reduced the 66-kDa protein binding to poly(A)-Sepharose; with 50 µg of soluble poly(A) RNA, the binding was reduced to 17.4 ± 3.7% (n = 3). In contrast, soluble poly(A) DNA showed the weaker inhibition; 50 µg of soluble poly(A) DNA, the binding was reduced to 58.6 ± 4.3% (n = 3) (Fig. 3B, right panel). These evidences showed that the 66-kDa protein directly bound to poly(A) RNA with higher affinity than to poly(A) DNA. Thus, we hereafter refer to this protein as SYNRIP (Synaptotagmin-binding, cytoplasmic RNA-interacting protein). At present, the in vivo target of RNA molecules has not been determined; however, binding preference to poly(A) RNA of SYNRIP and its high similarity to polyadenylate binding proteins suggested that SYNRIP most likely bound RNA molecules containing poly(A) tract, e.g. messenger RNAs in vivo.

The C-terminal Domain of SYNRIP Interacts with C2B Domain of Syts—SYNRIP was isolated through affinity chromatography using Syt-II C2AB domain-immobilized matrix. We examined which C2 domain of Syt was responsible for the binding and the effect of divalent cations against the binding. Lysates were prepared from mouse whole brain as described under "Experimental Procedures" and incubated with various GST fusion proteins in the presence of Ca2+ (1 mM), Mg2+ (1 mM), EGTA (2 mM), or EDTA (2 mM). The binding of SYNRIP
to the fusion proteins was detected by immunoblotting with anti-SYNCRIP-N antibody. As shown in Fig. 4A, SYNCRIP bound to GST-Syt-II C2B and GST-Syt-II-C2AB to same extent but not to GST-Syt-II C2A nor GST alone. Furthermore, the binding was independent of Ca\(^{2+}\) and Mg\(^{2+}\). The obvious binding in the presence of 1 mM MgCl\(_2\) which is near the physiological concentration suggested the possible binding in vivo. The immunoreactive band with slower migrating than the 66-kDa SYNCRIP was visible in the Ca\(^{2+}\) plus fractions, which was probably due to some modifications of SYNCRIP during incubation in the presence of Ca\(^{2+}\), which was not yet defined.

Next, we examined which region of SYNCRIP was important for its binding to Syt. Wild type (W), N-terminally truncated (\(\Delta N\)), and C-terminally truncated (\(\Delta C\)) SYNCRIP (Fig. 1D) were transiently expressed in COS-7 cells. All these constructs expressed with expected sizes of molecular mass were confirmed by immunoblotting with anti-SYNCRIP-Pep antibody (Fig. 4B, lane 1). The lysate was prepared from COS-7 cells expressing each construct and incubated with GST-Syt-II-C2B. The binding of each construct was detected by immunoblotting with anti-SYNCRIP-Pep antibody. Correct expression of each construct was confirmed by immunoblotting with anti-SYNCRIP-Pep antibody (Fig. 4B, lane 1). For control, the same experiment was performed with GST alone. Bound proteins were pulled down with glutathione-Sepharose and examined by Western blotting using anti-SYNCRIP-N antibody. As shown in Fig. 4A, SYNCRIP bound to GST-Syt-II C2B and GST-Syt-II-C2AB to same extent but not to GST-Syt-II C2A nor GST alone. Furthermore, the binding was independent of Ca\(^{2+}\) and Mg\(^{2+}\). The obvious binding in the presence of 1 mM MgCl\(_2\) which is near the physiological concentration suggested the possible binding in vivo. The immunoreactive band with slower migrating than the 66-kDa SYNCRIP was visible in the Ca\(^{2+}\) plus fractions, which was probably due to some modifications of SYNCRIP during incubation in the presence of Ca\(^{2+}\), which was not yet defined.

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**Spatio-temporal Expression Patterns of SYNCRIP Differ from That of Syt I**—The above binding experiments were all in vitro, so the next question was whether the binding between SYNCRIP and Syts really occur in vivo. To address this issue, we first performed immunohistochemical studies on the postnatal 7th day mouse cerebellum when SYNCRIP expression was highest during the postnatal development of mouse cerebellum (see below) with anti-SYNCRIP-N antibody, which recognized SYNCRIP with more preference than hnRNPR R on both Western blotting and immunofluorescence studies (Fig. 2, A–C, and Fig. 4B). SYNCRIP was expressed in most of neurons, i.e. granule cells in both external germinatal layer and internal granular layer and Purkinje cells. Although the biochemical fractionation study showed that a significant amount of SYNCRIP was observed in the LP2 fraction representing the fraction enriched with synaptosomal vesicles, morphologically anti-SYNCRIP-N antibody-reactive signals were concentrated in perinuclear and somatic regions of these cells and were almost lacking in molecular layers without migrating granule cells therein (Fig. 5A), where dendrites of Purkinje cells and synaptic structures were concentrated, and Syt-I signals were highly positive (Fig. 5B). The parallel experiment with anti-SYNCRIP-Pep antibody, which more preferentially recognized hnRNPR than SYNCRIP (Fig. 4B), showed that the immunoreactive signals were exclusively apparent in nuclei, suggesting that the perinuclear and somatic immunosignals with anti-SYNCRIP-N antibody indeed represented the SYNCRIP localization in neurons and that the SYNCRIP signals detected in the LP2 fraction by the fractionation study (Fig. 2D) might be derived from SYNCRIP on the other membrane structure rather than on the synaptic vesicles. Furthermore, these distinct subcellular distributions between SYNCRIP and Syt I were supported by the finding that SYNCRIP and Syt I were not co-immunoprecipitated from neuronal tissue with neither anti-SYNCRIP-N antibody nor anti-Syt I antibody (data not shown). The cytoplasmic localization of SYNCRIP in native...
tissues was consistent with the findings in transient expression experiments as described above (Fig. 2). Although the possibilities of nuclear localization of small parts of SYNCRIP and shuttling between nucleus and cytoplasm remain, SYNCRIP exists in cytoplasm at least in the steady state of cells and functions differently from hnRNP R.

In developing mouse cerebellum, SYNCRIP protein expression was highest in P7 and declined afterward (Fig. 6A, upper). In contrast, expression of Syt I and II was increased during the development (Fig. 6A, lower, and data not shown). In various tissues of P7 mouse, SYNCRIP protein was expressed not only in central nervous tissues but also in all peripheral tissues examined with almost constant level (Fig. 6B). 82-kDa hnRNP R was expressed in parallel to SYNCRIP in various tissues.

These results clearly indicated the discrepancy in spatio-temporal expression pattern of SYNCRIP and neuronal major Syt isoforms, Syt-I and Syt-II, suggesting that SYNCRIP was not likely involved in the regulation of neurotransmitter release and that Syt-I and Syt-II were not likely to be physiological targets for SYNCRIP.

SYNCRIP Interacts with Syt C2B Domains of Selected Isoforms—The expression patterns of SYNCRIP in developing neuron and the ubiquitous expression in various tissues suggested that physiological targets for SYNCRIP were likely other Syts isoforms rather than major neuronal Syts, Syt-I and Syt-II. Then we tested the binding of SYNCRIP to C2B domains of 11 Syts. To eliminate the effects of co-existing proteins in the lysate from native tissues, which are expected to differ from tissue to tissue, we expressed SYNCRIP in Sf9 cells. The lysate was prepared from Sf9 cells, and each aliquot was incubated with almost equal amounts of GST-Syt-C2Bs. The bound SYNCRIP was detected by immunoblotting with anti-SYNCRIP-N antibody. As shown in Fig. 7, SYNCRIP bound the C2B domains of Syt-III, -IV, -VII, -VIII, -IX, and -XI as well as those of Syt-I and Syt-II. No significant binding to Syt-V, -VI, nor -X was detected even with longer exposure for detecting SYNCRIP signals. Although this experiment was only qualitative, the binding between SYNCRIP and Syt-IX-C2B domain seemed to be relatively weaker than with other Syt C2B domains. Several proteins including AP2, β-SNAP, N- and P/Q-type Ca$^{2+}$ channels were reported to bind C2B domain of Syt (33–35), and only AP2 complex, among them, was examined for binding to various C2B domains of Syts by Li et al. (19). In their reports, AP2 complex interacted with all C2B domains of Syts examined, i.e. Syt I–VIII, except Syt-I. The different binding specificity of SYNCRIP and AP2 for Syts isoforms suggested that SYNCRIP recognized the surface of C2B domains of Syts in a different manner.

SYNCRIP Interacts with Syt-VII, -VIII, and -IX in Intact Cells—Although in vitro binding experiments showed that SYNCRIP potentially interacted with C2B domains of Syt-I–IV, -VII–IX, and -XI, these findings did not always represent the interaction under physiological conditions as was the case in SYNCRIP-Syt-I. Moreover, the findings of ubiquitous tissue distribution of SYNCRIP led us to focus on ubiquitous isoforms of Syts as a physiological target for SYNCRIP. Thus, we finally...
examined the interaction of SYNCRIP with ubiquitous types of Syts and Syts-IV and -V in intact cells by immunoprecipitation. At present, we have no specific antibodies that recognize each Syt isoform; T7 epitope-tagged full-length Syt-IV, -V, -VII, -VIII, and -IX were transiently expressed in COS cells. The lysates were prepared from each COS cell and immunoprecipitated with anti-SYNCRIP-N antibody. We examined each lysate by immunoblotting with anti-T7 antibody and confirmed the expression of each T7-tagged Syt with almost the same extent (Fig. 8, upper). The immunoprecipitates were examined by immunoblotting with both anti-SYNCRIP-N antibody (Fig. 8, middle) and anti-T7 antibody (Fig. 8, lower). Endogenous SYNCRIP were constantly immunoprecipitated, and Syt-VII, -VIII, and -IX were specifically co-immunoprecipitated. Among them, Syt-IX was reproducibly co-precipitated to more extent than the other two isoforms, whereas the in vitro binding of SYNCRIP to the C2B domain of Syt-IX appeared relatively weak (see above, Fig. 7), suggesting that much greater populations of Syt-IX were interacting with SYNCRIP in steady state cells than those of Syt-VII and Syt-VIII. Syt-V was not co-immunoprecipitated, which was consistent with the finding that the C2B domain of Syt-V did not bind to SYNCRIP in vitro. Although the C2B domain of Syt-IV bound SYNCRIP in vitro, full-length Syt-IV was not co-immunoprecipitated, which was probably due to the different intracellular compartmentalizations of Syt-IV and SYNCRIP. These results indicate that this immunoprecipitation experiment indeed represented the interaction in intact cells and suggested that ubiquitous isoforms of Syts, Syts-VII, -VIII, and -IX, were likely to be physiological targets for SYNCRIP.

Conclusions—We found SYNCRIP during the course of studies to elucidate molecular events in the IHPS-induced neurotransmission blockade. SYNCRIP bound the C2B domain of Syt via its C-terminal region and also bound RNA with preference to poly(A) RNA. The primary structure was highly homologous to hnRNP R; however, the subcellular localization was distinct from that of hnRNP R, suggesting that SYNCRIP was involved in the mRNA dynamics in cytoplasm such as mRNA transport to proper sites, stabilization, and degradation. SYNCRIP potentially bound C2B domains of Syts except for Syt-V, -VI, and -IX in vitro and indeed interacted with Syt-VII, -VIII, and -IX even in intact cells. These three Syt isoforms were expressed in non-neuronal tissues as well as in neurons (19, 36). Together with the tissue distribution and subcellular localization of Syt, it is plausible that SYNCRIP interacts with these three Syt isoforms in physiological situations. To our knowledge, the subcellular distribution as well as physiological function of these three Syt isoforms were not known, so we must obtain specific probes for these isoform proteins and examine the subcellular localization to confirm our idea. Syt-I, -II, and -IV also interacted with SYNCRIP through their C2B domains in vitro; however, immunohistochemical and co-immunoprecipitation experiments showed no evidence of the interactions in native tissues and intact cells probably due to their distinct subcellular compartmentalization. However, in some special situations, such as in stimulated neurons, their transient interaction may occur.

What is the biological meaning of the interaction between SYNCRIP and Syt-VII, -VIII, and -IX? Considering the regulatory functions of Syts on the SNARE-based vesicle trafficking, one of the attractive hypotheses is that these interactions are involved in the transport of mRNA. Mature mRNAs are exported from nucleus and transported to proper subcellular sites, generating the asymmetrical subcellular distribution of the mRNA and its translated products (37, 38). One mechanism considered is that some mRNAs utilize organelles as vehicles and are transported along the cytoskeleton (39, 40). Recent studies on Syt-III and Syt-VI showed that these isoforms were not concentrated in the synaptic vesicle and Syt-VI was, in particular, distributed in all fractions examined, suggesting the localization of Syt isoforms on various organelles and versatile functions of them (41). We certainly need further analyses on whether non-neuronal Syts are involved in the organelle-based mRNA transport with SYNCRIP; however, our findings will shed light on the function of ubiquitous Syts and augment the idea of diverse functions of Syts.
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SYNCRIP, a Cytoplasmic Counterpart of Heterogeneous Nuclear Ribonucleoprotein R, Interacts with Ubiquitous Synaptotagmin Isoforms
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