Signal Regulatory Proteins (SIRPS) Are Secreted Presynaptic Organizing Molecules*

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Formation of chemical synapses requires exchange of organizing signals between the synaptic partners. Using synaptic vesicle aggregation in cultured neurons as a marker of presynaptic differentiation, we purified candidate presynaptic organizers from mouse brain. A major bioactive species was the extracellular domain of signal regulatory protein α (SIRP-α), a transmembrane immunoglobulin superfamily member concentrated at synapses. The extracellular domain of SIRP-α is cleaved and shed in a developmentally regulated manner. The presynaptic organizing activity of SIRP-α is mediated in part by CD47. SIRP-α homologues, SIRP-β and -γ also have synaptic vesicle clustering activity. The effects of SIRP-α are distinct from those of another presynaptic organizer, FGF22: the two proteins induced vesicle clusters of different sizes, differed in their ability to promote neurite branching, and acted through different receptors and signaling pathways. SIRP family proteins may act together with other organizing molecules to pattern synapses.

To identify novel synaptic organizers, we devised an assay for presynaptic differentiation in isolated, cultured neurons, and used it to guide biochemical purification of active molecules from brain extracts. In initial studies, we showed that fibroblast growth factor (FGF)22 and its close relatives, FGFs 7 and 10, promote differentiation of neuromuscular and cerebellar synapses (7, 10). Some neuronal populations were unresponsive to these FGFs, however, and FGFs accounted for less than half of the active material in brain extracts. We therefore sought additional active species. Here, we show that SIRP-α is a presynaptic organizer.

SIRP-α (signal regulatory protein α; also known as PTPNS1, SHPS1, CD172a, BIT, MFR, and p84) is a transmembrane protein with three immunoglobulin domains (11–14). SIRP-α is highly expressed in neurons and myeloid cells, and is concentrated at synapses in the brain (11–18). Its intracellular domain has tyrosine residues that, when phosphorylated, can interact with phosphatases to send negative signals downstream. SIRP-α is involved in hematopoietic cell functions such as regulation of host cell phagocytosis and clearance, inflammatory mediator production, and control of cell migration (11–13, 19–22). Little is known about roles of SIRP-α in the nervous system, but it is expressed by hippocampal neurons, promotes neurite outgrowth in culture, and enhances the effect of brain-derived neurotrophic factor (23–26). Most of these studies, however, focused on intracellular signals transduced by SIRP-α when it is engaged by its ligand, CD47/integrin-associated protein (16). Here, in contrast, we show that the extracellular domain of SIRP-α is cleaved and acts as a secreted presynaptic organizer. We also show that two little-studied close relatives of SIRP-α, SIRP-β and SIRP-γ, also have presynaptic organizing activity. Finally, we show that SIRPs act in part through CD47 and in part through other receptors, and that effects of SIRPs on axons are distinct from those of FGF22.

EXPERIMENTAL PROCEDURES

Cell Culture—Motoneurons were prepared and cultured as described (7, 10). Briefly, cells were dissociated from E5 chick spinal cord with trypsin. Motoneurons were purified by Metrizamide density gradient centrifugation and plated at 2,000 cells/well in 8-well Lab-Tek Permanox chamber slides coated with poly-D,L-ornithine and laminin (Invitrogen). Motoneu-

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‡ The abbreviations used are: FGF, fibroblast growth factor; SIRP, signal regulatory protein; NMJ, neuromuscular junction; PTX, pertussis toxin; GFP, green fluorescent protein; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; MS/MS, tandem mass spectrometry; MAPK, mitogen-activated protein kinase; RT, reverse transcription.
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rons were cultured in L-15 medium with insulin, putrescine, conalbumin, progesterone, sodium selenite, and 2% horse serum for 48 h, then analyzed as described below. Proteins, blocking antibodies, and inhibitors were added at the time of plating except where indicated.

In some experiments, motoneurons were co-cultured with COS cells, obtained from ATCC. The COS cells were transfected using Lipofectamine 2000 (Invitrogen) and plated on 8-well Lab-Tek Permanox chamber slides. Two days later, motoneurons were prepared as described above and added to the COS cells at 2,000 cells per well. They were co-cultured for 2 days in motoneuron culture media, and the cells were processed for immunohistochemistry.

C2C12 myoblasts (ATCC) were maintained in Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum. Differentiation into myotubes was induced by changing the culture media to Dulbecco’s modified Eagle’s medium containing 2% horse serum.

Histology—Cultures were fixed with 4% paraformaldehyde for 15 min, then incubated successively in 2% bovine serum albumin and 0.1% Triton X-100 for 1 h, with primary antibodies for 2 h at room temperature or overnight at 4 °C, and with secondary antibodies for 1 h at room temperature. Slides were mounted with p-phenylenediamine. Images were captured with a CCD camera (Photometrics) on an inverted microscope.

For styril FM dye imaging (7), cultures were incubated for 90 s with 10 μM FM1-43 in a modified Tyrode solution containing 107 mM NaCl, 47 mM KCl, 2 mM MgCl2, 10 mM glucose, 10 mM HEPES-NaOH (pH 7.4), and 2 mM CaCl2. After washing for 10 min, cultures were treated with 90 mM KCl, Tyrode (KCl plus NaCl held constant). Images were captured with a CCD camera (Photometrics) on an inverted microscope.

Purification and Identification of Synaptic Organizing Molecules—Extracts were prepared from the forebrains of ~100 P7 mouse pups (C57BL6 strain) as described (7) by homogenizing in a Tris buffer (pH 7.5) containing 400 mM NaCl. The extract was centrifuged at 20,000 × g for 30 min and the supernatant was dialyzed against 10 mM Tris (pH 8.0), applied to a DEAE-Sepharose FF column, and eluted with a 0–500 mM linear NaCl gradient. Active fractions were dialyzed against 20 mM sodium acetate buffer (pH 5.0), applied to a Mono S HR 5/5 column, and eluted with a 0–500 mM linear NaCl linear gradient. Active fractions were dialyzed against 25 mM triethanolamine buffer (pH 8.3), applied to a Mono P HR 5/5 column, and eluted with Polybuffer 96 (GE Healthcare; diluted 1:10, pH 6.0). Columns were run using an AKTAprime FPLC System (GE Healthcare). The final active fractions were separated by a 4–15% gradient SDS-PAGE and stained with Colloidal Blue (Novex). Bands were excised, trypsin digested, and subjected to matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) and tandem mass spectrometry (MS/MS) at the Keck Biotechnology Resource Laboratory, Yale University. Sequences were analyzed using the data base searching program ProFound.

Plasmid Constructs and Recombinant Proteins—cDNAs encoding mouse SIRP-α (IMAGE: 5368250), human SIRP-α (IMAGE: 3916836), SIRP-β1 (IMAGE: 5215629), and SIRP-γ (IMAGE: 5588038) were obtained from ATCC. Sequences encoding the entire protein or just the extracellular domain were cloned into the APtag5 expression vector (GenHunter), replacing the alkaline phosphatase coding sequence. A FLAG epitope tag was placed between the signal peptide and the SIRP sequence. Soluble SIRP proteins were produced by transiently transfecting extracellular domain plasmids into COS cells, and purifying secreted SIRP proteins from culture media by affinity chromatography on anti-FLAG-agarose (Sigma). Recombinant FGF22 was prepared as described previously (7).

Western Blotting—Cells and tissues were lysed in lysis buffer (1% Nonidet P-40, 50 mM Tris buffer, pH 7.5) with a freshly added Protein inhibitor mixture tablet (Roche). Western blotting assays were performed as described previously (27). Equal amounts of protein for each group, as ensured by protein assays (BCA, Pierce), were applied to the gel. Equal loading was confirmed by testing the level of α-tubulin by Western blotting. Concentrations of antibodies used for blotting were: anti-SIRP-α extracellular domain, 2.5 μg/ml; anti-SIRP-α intracellular domain, 4 μg/ml; anti-FLAG, 10 μg/ml; anti-α-tubulin, 0.4 μg/ml.

Immunoprecipitation—C2C12 culture media (from myoblasts and myotubes) were collected, precleared with 100 μl of Immobilized Protein-L (Pierce), and incubated with 2.5 μg of anti-SIRP-α extracellular domain antibody at 4 °C overnight. The immune complexes were precipitated with 30 μl of Immobilized Protein-L, washed with phosphate-buffered saline, and subjected to Western blotting to test the amount of secreted SIRP-α.

Reverse Transcription-PCR—RT-PCR was performed using Superscript First-Strand Synthesis System for RT-PCR kit (Invitrogen). Primers used were: 5’-ATA CGC AGA CCT GAA TGT GCC CAA-3’ and 5’-TGG CCA CTC CAT GTA GGA CAA GAA-3’ for SIRP-α, and 5’-AGA GCT GAA GGT GAT CCA ACC TGT-3’ and 3’-ACC CGA ATC AGC AGG AGT GAC ATT-3’ for SIRP-β1.

RESULTS

Assay and Purification of Presynaptic Organizing Molecules—A critical step in presynaptic differentiation is the clustering of synaptic vesicles near neurotransmitter release sites in nerve terminals (28). We therefore used synaptic vesicle clustering as an assay to monitor purification of presynaptic organizing molecules from brain. Embryonic motoneurons were purified, cultured, and
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Presynaptic organizing activity was monitored by the vesicle clustering assay shown in Fig. 1A. Active fractions from each step were pooled and subjected to sequential chromatography. Presynaptic organizing activity was purified 3,313-fold; because multiple active species were present in the starting material (7), purification of SIRP-α was ∼10,000-fold.

### TABLE 1

| Step                | Protein | Total activity | Purification |
|---------------------|---------|----------------|--------------|
| Forebrain (P7, ~100)| 20 μg   | 1,080,000.0    | 1,800,000    |
| S-20                | 61.0    | 68,000         | 609.4        |
| DEAE                | 380.0   | 117,000        | 184.9        |
| Hi-Trap Q           | 6,900.0 | 270,000        | 23.5         |
| Superdex 200        | 8.7     | 48,000         | 3,313.0      |

### FIGURE 1. Assay and identification of presynaptic organizing molecules.

A, assay of presynaptic differentiation. Chick motoneurons were stained with anti-neurofilament antibody to visualize neurites and anti-synapsin antibody to mark synaptic vesicles. The crude brain extract induced neurite elongation, branching, and synaptic vesicle aggregation (middle). The purified fraction (after Mono P; see Table 1) induced vesicle aggregation, but not neurite branching or elongation. Bar is 20 μm for neurofilaments, 10 μm for synapsins. B, the final active fractions were separated by SDS-PAGE and stained with Colloidal Blue. The major band (~70 kDa; arrow) was identified as SIRP-α by mass spectrometry.

treated with brain extract for 2 days, then vesicle distribution was assessed by immunostaining for the vesicle-associated protein, synapsin (28). We used chick motoneuron cultures for the assay because they can be prepared in the large numbers needed to monitor purification, are very pure (>90% purity), and can be cultured at low density to facilitate scoring (7, 10). To purify active factors from the brain, extracts were prepared from the forebrains of 7-day-old mice, because synapse formation is at its peak at this stage (29). Untreated motoneurons extended neurofilament-positive neurites within which synapsin was diffusely distributed (Fig. 1A, top). When crude brain extracts were added to the cultures, synapsin distribution became punctate and varicose. The crude extract also promoted elongation and branching of neurites (Fig. 1A, middle; see below for quantification).

The brain extract was fractionated by anion exchange chromatography (DEAE), and aliquots of individual fractions were added to motoneuron cultures to monitor vesicle-clustering activity. This assay revealed two peaks of activity that induced synapsin aggregation. One peak contains FG22, which we characterized previously (7). The other peak, on which we focus here, was fractionated further on anion exchange (HiTrap-Q), gel filtration (Superdex 200), cation exchange (Mono S), and chromatofocusing (Mono P) columns (Table 1). The most active fractions from the Mono P column retained synapsin aggregation activity but did not detectably affect neurite branching or elongation (Fig. 1A, bottom; number of aggregates were 2.3 ± 0.8/mm for control, 4.98 ± 1.3/mm for crude extract, and 8.0 ± 1.0/mm for purified fraction; neurite lengths were 416.9 ± 12 μm for control, 833.3 ± 15 μm for crude extract, and 424.9 ± 11 μm for purified fraction; branch points per neurite were 1.69 ± 1.1 for control, 4.08 ± 1.5 for crude extract, and 1.50 ± 1.2 for purified fraction; mean ± S.E. for 30 neurites). Vesicle aggregation activity in this fraction was enriched >3,000-fold compared with the original supernatant (Table 1), but because the crude extract contained multiple active components, we estimate that purification of this species was >10,000-fold.

The most active fractions from the Mono P column contained a major protein of 70 kDa (Fig. 1B) along with a minor protein of 40 kDa. The 70-kDa band was subjected to in-gel tryptic digestion. The peptides were processed for MALDI-MS and MS/MS. Data base search with the detected masses identified a protein corresponding to SIRP-α. The 40-kDa band was identified as an N-terminal fragment of rabaptin-5, a 95-kDa intracellular signaling protein (30); it was not studied further.

**Presynaptic Organizing Activity of SIRP-α**—SIRP-α is a transmembrane adhesion molecule of the immunoglobulin superfamily (11–14). As discussed below, the presence of this protein in a soluble extract was unexpected. As a first step in testing whether SIRP-α is capable of promoting synapsin aggregation, we constructed an expression plasmid encoding the full-length protein, and transfected it into COS cells. The motoneurons were co-cultured with COS cells and stained for synapsin. As shown in Fig. 2A, synapsin was diffusely distributed in most neurites contacting COS cells transfected with GFP alone. In contrast, when motoneurons were co-cultured with COS cells expressing SIRP-α in addition to GFP, synapsin was aggregated in ~80% of neurites contacting the transfected COS cells (Fig. 2, A and B). Similar results were obtained in cultures stained for another synaptic vesicle protein, SV2 (Fig. 2C). These results confirm that SIRP-α has presynaptic organizing activity.

**Regulated Cleavage of the SIRP-α Extracellular Domain**—Although SIRP-α has transmembrane and intracellular domains, all of the peptides obtained by MALDI and MS/MS from the SIRP-α we purified were located in its extracellular domain (Fig. 3A). This result suggested that the presence of this transmembrane protein in a soluble extract resulted from cleavage and release of the extracellular domain. Consistent with this idea, the protein we purified was ~70 kDa, approximately the expected mass of the glycosylated extracellular domain, rather than the ~85 kDa expected for the full-length glycosylated protein (15). The presence of an extracellular fragment in the extract might result from an endogenous, regulated process, or artifactual proteolysis during preparation of the extract. To investigate SIRP-α cleavage, we generated a SIRP-α construct with a FLAG tag at the N terminus and introduced it into COS cells. Media and cells were rapidly separated and treated with a mixture of protease inhibitors. We then probed media and cell lysates with antibodies to the cytoplasmic domain and to the FLAG epitope. As shown in Fig. 3B, a protein of ~85 kDa was detected in lysates but not media by both antibodies; this species presumably corresponds to full-length, glycosylated SIRP-α. In addition, a ~70-kDa band was detected in media but not lysates by the anti-FLAG antibody (arrowhead) but not by
SIRPα from brain (Fig. 1) and full-length SIRPα (Fig. 2), sSIRPα-induced aggregation of synaptic vesicles in neurites of cultured motoneurons (Fig. 4A). We then asked whether the sSIRPα-induced varicosities were capable of recycling vesicles in response to depolarization. sSIRPα-treated cultures took up the fluorescent styryl dye FM1–43 by depolarization with a high K⁺ solution, and released it by an additional round of depolarization (Fig. 4B). Control motoneurons without sSIRPα treatment did not take up the FM1–43 dye (data not shown), indicating that the signal was specific. Thus, sSIRPα promotes formation of varicosities that are not only chemically but also functionally specialized.

To determine the potency of sSIRPα, we added various amounts to motoneurons for 2 days, then assayed vesicle aggregation by immunostaining for synapsin. The synapsin-clustering effect by sSIRPα was dose-dependent with a half-maximal response at ~100 ng/ml or ~1 nM (Fig. 4C), a similar concentration to the effective dose for FGF22 (7).

We next examined the time course of the effects of sSIRPα on vesicle aggregation. In our culture system, motoneurons extend neurites within 24 h of plating, but few vesicle aggregates are seen until after 48 h. We incubated cultures with sSIRPα on only the first day, only the second day, or both first and second days in culture (Fig. 4D). sSIRPα-induced presynaptic differentiation under all three conditions, but the effect was significantly greater when sSIRPα was added on the second day than on the first day. This result suggests that SIRPα does not act by a general effect on neuronal differentiation, but rather by a specific effect on synaptic differentiation and maintenance.

**SIRPα Signals in Part through CD47**—In non-neural cells, CD47 serves as a ligand of SIRPα: binding of CD47 to SIRPα initiates signal transduction in the SIRPα-expressing cells (12, 13). We asked whether SIRPα can also serve as a ligand for CD47, mediating effects of SIRPα on vesicle aggregation. We first used immunostaining to ask whether CD47 was present in cultured motoneurons. CD47 was distributed throughout neurites, but was concentrated near vesicle aggregates as shown by colocalization with synapsin (Fig. 5A; cultured without adding sSIRPα). RT-PCR also detected the CD47 mRNA in motoneurons (data not shown). We then asked whether blocking antibodies to CD47 (34, 35) inhibited vesicle aggregation in the motoneuron-COS cell co-culture system shown in Fig. 2. Blocking antibodies to SIRPα (15) and CD3 were used as positive and negative controls, respectively. Anti-SIRPα (50 μg/ml), a concentration known to completely inhibit SIRPα; Ref. 15) completely blocked the synapsin aggregation in motoneurons induced...
by SIRP-α-expressing COS cells, confirming that the presynaptic differentiation observed is SIRP-α mediated (Fig. 5B). Anti-CD3 (100 µg/ml) had no effect. Anti-CD47 antibodies (combination of 50 µg/ml of each B6H12 and mAP301, concentrations known to completely inhibit human and mouse CD47; Refs. 34–36) inhibited the effect of SIRP-α by ∼50%, indicating that CD47 is involved in the SIRP-α-dependent presynaptic differentiation of motoneurons. Although the partial inhibition may reflect incomplete blockade of chick CD47, the partial inhibition of clustering raises the possibility, discussed further below, that additional receptors are involved in presynaptic effects of SIRP-α.

Synaptic Organizing Activity of SIRP-β and -γ—SIRPs constitute a small family of closely related proteins encoded by ∼5 genes in mammals (13, 14). We asked whether two of them,
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SIRP-β and -γ, share the synaptic organizing activity of SIRP-α. To this end, we cloned the extracellular domain of human SIRP-α, -β1, and -γ, prepared soluble proteins by purifying them from culture media of transfected COS cells (sSIRP-β1, and sSIRP-γ), and compared their activities to that of sSIRP-α in motoneuron cultures. As shown in Fig. 6, A and C, all three soluble SIRPs had synapsin aggregation activity (p < 0.01 relative to control by analysis of variance). At equal concentrations, sSIRP-α and sSIRP-γ were more active than sSIRP-β1 in this assay.

We then examined the expression of mouse SIRP-α and -β1 in developing muscle and brain by RT-PCR (Fig. 6B; there are multiple SIRP-β but no SIRP-γ in the mouse genome; Refs. 13 and 14). We found that both SIRP-α and -β1 are expressed in these tissues at times of peak synaptogenesis (E16 muscle and P10 brain). Therefore, SIRP proteins comprise a new family of presynaptic organizing molecules.

We next asked whether CD47 is involved in the presynaptic effects mediated by SIRP family members. Under conditions described above (Fig. 5B) anti-CD47 blocking antibodies partially inhibited the presynaptic effect of SIRP-α and SIRP-γ (Fig. 6C). In contrast, the presynaptic effect of SIRP-β1 was not affected by anti-CD47 antibodies (black bars), consistent with reports that SIRP-α and SIRP-γ bind to CD47, but SIRP-β1 does not (13, 16, 37, 38). Interestingly, the CD47-independent activities of the three SIRP family members were similar. These results confirm that CD47 is involved in presynaptic organizing activity of SIRP-γ as well as SIRP-α. The inability of anti-CD47 to inhibit effects of SIRP-β1 provides additional evidence that receptors other than CD47 are involved in mediating effects of SIRPs on neurons.

SIRP-α and FGF22 Promote Presynaptic Differentiation in Distinct Ways—We previously identified FGFs of the 7/10/22 subfamily as presynaptic organizers, and showed that they play roles in synapse formation in the cerebellum (7) and at the neuromuscular junction (NMJ) (10). Here, using similar procedures, we have identified SIRPs as another small family of presynaptic organizers. One possible reason for the presence of multiple organizers is that different factors have distinct roles in presynaptic differentiation (discussed in Ref. 10). As one test of this idea, we asked whether SIRP-α and FGF22 have equivalent or different effects on motoneurons. In fact, effects of these proteins differed from each other in at least two ways. First, vesicle aggregates induced by FGF22 were significantly smaller than those induced by sSIRP-α (Fig. 7, A and C). Second, FGF22 promoted neurite branching but sSIRP-α did not (Fig. 7, A and B). These results indicate that SIRP-α and FGF22 act in different ways.

The differing effects of SIRP-α and FGF22 on motor axons imply that they recruit distinct intracellular signal transduction pathways. As a first step in mapping these pathways, we assayed inhibitors of tyrosine kinases and the G proteins. We used genistein to block tyrosine kinases (39) and pertussis toxin (PTX) to block G protein-receptor interactions (40). The vesicle-aggregating activity of FGF22 was completely blocked by genistein, whereas PTX had no effect on this process (Fig. 7D). Likewise, genistein but not PTX blocked the neurite branching induced by FGF22 (data not shown). Conversely, PTX significantly reduced the number of
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SIRP-α-induced varicosities are capable of depolarization-dependent recycling. Moreover, the effect of SIRP-α on presynaptic differentiation is specific, in that this protein does not affect initial neuronal differentiation, neurite outgrowth, or neurite branching. Finally, we show that two other members of the SIRP family, SIRP-β and -γ, are also presynaptic organizers and that SIRP-α and -γ act in part through CD47. Together, these results introduce a new family of presynaptic organizers that may play important roles in neural development.

The SIRP Family—SIRPs constitute a small family of proteins; the human genome encodes SIRP-α, -β1, -β2, -γ, and -δ and the mouse genome encode SIRP-α, -β1, -β2, and -β3; of these, human SIRP-α, -β1, and -γ and mouse SIRP-α, -β1, and -β2 have been detected at the protein level (13). Among them, SIRP-α has been studied in greatest detail; its cytoplasmic region is phosphorylated upon ligand binding and binds tyrosine phosphatases to serve as a negative regulator of host cells (11–13). In contrast, SIRP-β interacts with an adaptor protein DAP12 to send positive signals and SIRP-γ does not appear to generate intracellular signals (12, 13, 37, 38).

The extracellular domains of all three of the SIRPs we tested have presynaptic organizing activity (Fig. 6), indicating that SIRP proteins are a new presynaptic organizer family. The role of the SIRP family in the nervous system is largely unknown. SIRP-α might be involved in apoptosis (26, 42) and promotion of neurite and filopodia formation in culture (23–25), but its in vivo function is not known. Functions of other members in the nervous system have not yet been reported, but at least SIRP-1 and -2 have been detected in the brain (43). To examine whether SIRP family members have roles in synapse formation in vivo, and to understand if they have distinct functions from each other, it will be important to assess synaptic development in the absence of SIRP-α, -β, and -γ singly and in combination.

Although SIRPs have been studied most intensively as transmembrane signal-transducing receptors, we isolated SIRP-α as a soluble ligand, and showed that extracellular domains of SIRP-β and -γ are also active. For SIRP-α, we showed that the transmembrane form is also active, but it remains possible that the transmembrane molecules were shed either constitutively or in response to neurite contact. It was reported that a mutant SIRP-α resistant to ectodomain shedding impaired cell migration (31); such mutants can be used to address the role of shedding in presynaptic differentiation. Shedding may not be nec-

FIGURE 7. Different presynaptic organizing effects and mechanisms for SIRP-α and FGF22. A, motoneurons were cultured for 2 days with phosphate-buffered saline (control), sSIRP-α, or FGF22, and stained for synapsin. sSIRP-α induced relatively bigger synapsin-positive aggregates than FGF22, whereas FGF22 induced branching in addition to vesicle clustering. Bar is 15 μm. B, extent of neurite branching induced by FGF22 and sSIRP-α. Bars show mean ± S.E. for 100 neurites per condition. *, differs from control at p < 0.01. C, size of vesicle-rich aggregates induced by FGF22 and sSIRP-α. Bars show mean ± S.E. for 100 aggregates per condition. *, differs from control at p < 0.01. D, effect of genistein (a tyrosine kinase inhibitor; 100 nM), pertussis toxin (PTX, an inhibitor of G protein-receptor interaction; 100 ng/μl), dibutyryl-cAMP (dbcAMP; a cAMP analogue; 100 μM), and U0126 (a MAPK inhibitor; 10 μM) on FGF22- and sSIRP-α-induced vesicle aggregation. FGF22-induced formation of synapsin aggregates is completely blocked by genistein, whereas sSIRP-α-induced varicosity formation is partially inhibited by PTX, dbcAMP, and U0126. None of the inhibitors affected basal levels of aggregation. Bars show mean ± S.E. for at least 50 neurites per condition. *, differs from corresponding controls without inhibitors at p < 0.01.

SYNAPSES ARE A NOVEL SECRETED PRESYNAPTIC ORGANIZER—Using the aggregation of synaptic vesicles in cultured motoneurons as an assay, we purified an active protein ~10,000-fold from brain extracts and identified it as the extracellular domain of a transmembrane protein, SIRP-α. We then characterized the effect of SIRP-α as a presynaptic organizer. We show that the extracellular domain of SIRP-α is cleaved and released; that both the transmembrane form of SIRP-α and its soluble extracellular domain promote formation of synaptic vesicle-rich aggregates; that soluble SIRP-α acts at a concentration of ~1 nm and that synapsin aggregates induced by SIRP-α, whereas genistein had no effect (Fig. 7D). We further analyzed signaling molecules involved in SIRP-α- and FGF22-mediated presynaptic differentiation and found that dibutyryl-cAMP (a cAMP analogue) and U0126 (a MAPK inhibitor) significantly inhibited the effect of SIRP-α but not that of FGF22. None of the agents significantly affected neurite length, neurite branching, or basal levels of synapsin aggregation in control motoneurons (Fig. 7D and data not shown). The partial effect of PTX on SIRP-α-induced clustering provides additional evidence that its effects are mediated in part by CD47, which is known to recruit G proteins (41) and in part by other receptors. Together, these results indicate that SIRP-α and FGF22 use distinct signaling pathways to organize presynaptic differentiation.

DISCUSSION

SIRP-α as a Novel Secreted Presynaptic Organizer—Using the aggregation of synaptic vesicles in cultured motoneurons as an assay, we purified an active protein ~10,000-fold from brain extracts and identified it as the extracellular domain of a transmembrane protein, SIRP-α. We then characterized the effect of SIRP-α as a presynaptic organizer. We show that the extracellular domain of SIRP-α is cleaved and released; that both the transmembrane form of SIRP-α and its soluble extracellular domain promote formation of synaptic vesicle-rich aggregates; that soluble SIRP-α acts at a concentration of ~1 nm and that synapsin aggregates induced by SIRP-α, whereas genistein had no effect (Fig. 7D). We further analyzed signaling molecules involved in SIRP-α- and FGF22-mediated presynaptic differentiation and found that dibutyryl-cAMP (a cAMP analogue) and U0126 (a MAPK inhibitor) significantly inhibited the effect of SIRP-α but not that of FGF22. None of the agents significantly affected neurite length, neurite branching, or basal levels of synapsin aggregation in control motoneurons (Fig. 7D and data not shown). The partial effect of PTX on SIRP-α-induced clustering provides additional evidence that its effects are mediated in part by CD47, which is known to recruit G proteins (41) and in part by other receptors. Together, these results indicate that SIRP-α and FGF22 use distinct signaling pathways to organize presynaptic differentiation.

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essary in the brain, but could be important at synapses like the NMJ, where a basal lamina separates the pre- and postsynaptic membranes from each other (1); at this synapse, nerve terminals may have limited access to the myotube membrane. We found that more SIRP-α is released from myotubes than from myoblasts (Fig. 3F). In that motor axons synapse on myotubes but not myoblasts, developmental regulation of SIRP-α shedding might contribute to appropriate synapse formation. There are no reports of whether SIRP-β or -γ can be shed; it would be interesting to test if shedding is a common regulation for SIRP family proteins.

SIRP Receptors—Use of blocking antibodies showed that SIRP-α and SIRP-γ promote presynaptic differentiation at least in part by interacting with CD47. This result is consistent with reports that SIRP-α and SIRP-γ signal through CD47 to regulate hematopoietic cell function, migration, and apoptosis (43–46) and that SIRP-α promotes filopodia formation of CD47-overexpressing neurons (47, 48).

On the other hand, three lines of evidence suggest that receptors other than CD47 are also involved in mediating the synaptogenic effects of SIRPs. First, anti-CD47 only partially blocked effects of SIRP-α and SIRP-γ at the highest concentration tested. Second, anti-CD47 had no effect on the presynaptic organizing effect of SIRP-β1, consistent with reports that SIRP-β does not bind to CD47 (13, 37). Interestingly, the CD47-resistant activities of SIRP-α and SIRP-γ were similar to that of SIRP-β1. Third, pertussis toxin, which is known to block signaling through CD47 (41), only partially blocked presynaptic organizing activities of SIRP-α. Together, these results suggest that SIRPs act in part through additional receptors. Indeed, there are other ligands that can bind and activate SIRP-α in the lung and myeloid cells (49, 50). In addition, previous experiments in which CD47 was overexpressed in cultured neurons suggested that CD47 is a general modulator of neuronal differentiation (51). Our data, which indicates that SIRP-α is more specifically involved in presynaptic differentiation, supports the idea that CD47 cannot completely account for the effects of SIRP-α. Identification of additional SIRP receptors involved in presynaptic differentiation is underway.

SIRPs and FGFs, Multiple Presynaptic Organizers for Synaptic Specificity?—We previously identified FGF22 as a presynaptic organizer through biochemical purification and showed that FGF22 is critical for synapse formation between pontine neurons and cerebellar granule cells (7) as well as at the NMJ (10). However, we also found that there are synapses that are not organized by FGF22 in the brain (7). In addition, FGFs are involved in the induction of the NMJ, but not in the maturation or maintenance (10). Therefore, it appears that different synapses use different presynaptic organizers, and different steps require different molecules to organize functional synapses. Here, we have identified SIRPs as presynaptic organizers. We have compared the effects of SIRP-α and FGF22, and shown that the effects are different from each other in culture (Fig. 7, A–C). Moreover, SIRP-α and FGF22 use distinct signaling pathways for presynaptic differentiation (Fig. 7D). FGF22 use tyrosine kinases for presynaptic differentiation, which is consistent with the fact that the receptor for FGF22, FGFR2 (52, 53), is a tyrosine kinase. Presynaptic effects by the SIRP-α sig-
23. Wang, X. X., and Pfenninger, K. H. (2006) J. Cell Sci. 119, 172–183
24. Ohnishi, H., Kaneko, Y., Okazawa, H., Miyashita, M., Sato, R., Hayashi, A., Tada, K., Nagata, S., Takahashi, M., and Matozaki, T. (2005) J. Neurosci. 25, 2702–2711
25. Mitsuhashi, H., Futai, E., Sasagawa, N., Hayashi, Y., Nishino, I., and Ishiura, S. (2008) J. Neurochem. 105, 101–112
26. Araki, T., Yamada, M., Ohnishi, H., Sano, S. I., and Hatanaka, H. (2000) J. Neurochem. 75, 1502–1510
27. Umemori, H., Sato, S., Yagi, T., Aizawa, S., and Yamamoto, T. (1994) Nature 367, 572–576
28. Murthy, V. N., and De Camilli, P. (2003) Annu. Rev. Neurosci. 26, 701–728
29. Vaughn, J. E. (1989) Synapse 3, 255–285
30. Stenmark, H., Vitale, G., Ullrich, O., and Zerial, M. (1995) Cell 83, 423–432
31. Ohnishi, H., Kobayashi, H., Okazawa, H., Ohe, Y., Tomizawa, K., Sato, R., and Matozaki, T. (2004) J. Biol. Chem. 279, 27878–27887
32. Mitsuhashi, H., Yoshikawa, A., Sasagawa, N., Hayashi, Y., and Ishiura, S. (2005) J. Biochem. (Tokyo) 137, 495–502
33. Kontaridis, M. I., Liu, X., Zhang, L., and Bennett, A. M. (2001) J. Cell Sci. 114, 2187–2198
34. Brown, E., Hooper, L., Ho, T., and Gresham, H. (1990) J. Cell Biol. 111, 2785–2794
35. Lindberg, F. P., Bullard, D. C., Caver, T. E., Gresham, H. D., Beaudet, A. L., and Brown, E. J. (1996) Science 274, 795–798
36. Babic, I., Schallhorn, A., Lindberg, F. P., and Iirim, F. R. (2000) J. Immunol. 164, 3652–3658
37. Seiffert, M., Brossart, P., Cant, C., Cella, M., Colonna, M., Brigger, W., Kanz, L., Ullrich, A., and Bühring, H. J. (2001) Blood 97, 2741–2749
38. Piccio, L., Vermi, W., Boles, K. S., Fuchs, A., Strader, C. A., Facchetti, F., Cella, M., and Colonna, M. (2005) Blood 105, 2421–2427
39. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukushima, Y. (1987) J. Biol. Chem. 262, 5592–5595
40. Rens-Domiano, S., and Hamm, H. E. (1995) FASEB J. 9, 1059–1066
41. Brown, E. J., and Frazier, W. A. (2001) Trends Cell Biol. 11, 130–135
42. Koshimizu, H., Araki, T., Takai, S., Yokomaku, D., Ishikawa, Y., Kubota, M., Sano, S., Hatanka, H., and Yamada, M. (2002) J. Neurochem. 82, 249–257
43. Liu, Y., Bühring, H. I., Zen, K., Burst, S. L., Schnell, F. I., Williams, I. R., and Parkos, C. A. (2002) J. Biol. Chem. 277, 10028–10036
44. de Vries, H. E., Hendriks, I. J., Honing, H., De Lavalette, C. R., van der Pol, S. M., Hooijberg, E., Dijkstra, C. D., and van der Berg, T. K. (2002) J. Immunol. 168, 5832–5839
45. Kato, H., Honda, S., Yoshida, H., Kashiwagi, H., Shiraga, M., Honma, N., Kurata, Y., and Tomiyama, Y. (2005) J. Thromb. Haemostasis 3, 763–774
46. Brooke, G., Holbrook, J. D., Brown, M. H., and Barclay, A. N. (2004) J. Immunol. 173, 2562–2570
47. Murata, T., Ohnishi, H., Okazawa, H., Murata, Y., Kusakari, S., Hayashi, Y., Miyashita, M., Itoh, H., Oldenborg, P. A., Furuya, N., and Matozaki, T. (2006) J. Neurosci. 26, 12397–12407
48. Miyashita, M., Ohnishi, H., Okazawa, H., Tomonaga, H., Hayashi, A., Fujimoto, T. T., Furuya, N., and Matozaki, T. (2004) Mol. Biol. Cell 15, 3950–3963
49. Gardai, S. J., Xiao, Y. Q., Dickinson, M., Nick, J. A., Voelker, D. R., Greene, K. E., and Henson, P. M. (2003) Cell 115, 13–23
50. Johansen, M. L., and Brown, E. J. (2007) J. Biol. Chem. 282, 24219–24230
51. Numakawa, T., Ishimoto, T., Suzuki, S., Numakawa, Y., Adachi, N., Matsunoto, T., Yokomaku, D., Koshimizu, H., Fujimori, K. E., Hashimoto, R., Taguchi, T., and Kunugi, H. (2004) J. Biol. Chem. 279, 43245–43253
52. Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G., and Goldfarb, M. (1996) J. Biol. Chem. 271, 15292–15297
53. Zhang, X., Ibrahimii, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., and Ornitz, D. M. (2006) J. Biol. Chem. 281, 15698–15707
54. Scheiffele, P., Fan, J., Choi, J., Fetter, R., and Serafini, T. (2000) Cell 101, 657–669
55. Biederer, T., Sara, Y., Mozhayeva, M., Atasoy, D., Liu, X., Kavalali, E. T., and Südhof, T. C. (2002) Science 297, 1525–1531
56. Gao, A. G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J., and Frazier, W. A. (1996) J. Biol. Chem. 271, 21–24