Thy-1 Is a Component Common to Multiple Populations of Synaptic Vesicles

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Abstract. Thy-1, a glycosylphosphatidylinositol-linked integral membrane protein of the immunoglobulin superfamily, is a component of both large dense-core and small clear vesicles in PC12 cells. A majority of this protein, formerly recognized only on the plasma membrane of neurons, is localized to regulated secretory vesicles. Thy-1 is also present in synaptic vesicles in rat central nervous system. Experiments on permeabilized PC12 cells demonstrate that antibodies against Thy-1 inhibit the regulated release of neurotransmitter; this inhibition appears to be independent of any effect on the Ca\(^{2+}\) channel. These findings suggest Thy-1 is an integral component of many types of regulated secretory vesicles, and plays an important role in the regulated vesicular release of neurotransmitter at the synapse.

O ur understanding of the molecular mechanisms involved in secretory vesicle dynamics has advanced substantially in the past 10 yr with the identification of many of the molecular components of secretory vesicles. Results from reconstitution of Golgi transport (Rothman, 1987), yeast secretion mutants (Kaiser and Schekman, 1990), and biochemical characterization of synaptic vesicles (Südhof et al., 1993; Baj jalieh and Scheller, 1995) all point to the existence of common molecular components that carry out membrane fusion. For example, a generalized version of the SNARE\(^1\) hypothesis (Söllner et al., 1993) suggests that a precisely choreographed interplay between synaptic vesicle proteins, plasma membrane proteins, and cytoplasmic proteins results in Ca\(^{2+}\)-stimulated membrane fusion. However, the specific details of many of the processes and molecular components involved in vesicle dynamics remain poorly defined.

Neurons and endocrine cells are highly specialized to carry out regulated secretion from membrane-bounded storage vesicles when an appropriate stimulus is applied. At least two distinct types of regulated secretory vesicles coexist within neurons and endocrine cells; these organelles are typically referred to as small synaptic, or synaptic-like vesicles (SSVs) and large, dense-core vesicles (LDCVs). SSVs and LDCVs differ in a variety of physical and functional properties, including size, density, contents, membrane components, location within cells, biogenesis, and kinetics of release. Despite the differences, these two types of vesicles share many common properties, including transport to the vicinity of specialized release sites, close apposition to specialized sites on the plasma membrane, and the ability to fuse with the plasma membrane in a highly regulated manner, typically in response to an elevation in intracellular free Ca\(^{2+}\) concentration.

A first step in the biochemical approach to understanding regulated secretion involves the identification of the components of the secretory vesicles. A considerable number of such proteins have now been identified. These include the synaptotagmins (Matthew et al., 1981), SV2 (Buckley and Kelly, 1985), synaptophysin/P38 (Jahn et al., 1985; Wiedenmann and Franke, 1985), the synapsins (De Camilli and Greengard, 1986), synaptobrevin, (Trimble et al., 1988; Baumert et al., 1989), rab 3A (Fisher von Mollard et al., 1990), the cysteine string protein (Zinsmaier et al., 1990;
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et al., 1984; Mahanthappa and Patterson, 1992).
outgrowth in both primary neurons and PC12 cells (Leifer
1989; Garnett et al., 1993). Either cross-linking or removal
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plasma membrane (Green and Kelly, 1992). However, a
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with immunoglobulins (Campbell et al., 1981; Cohen et al.,
1983; Morris, 1985). Thy-1 shares structural homologies
of lymphocytes, as it is expressed in nervous sys-
tems, that play a fundamental role in regulated secretion
ions of regulated secretory vesicles in PC12 cells. We re-
port here our unexpected finding that one such protein is
Thy-1, a glycosyl-phosphatidylinositol (GPI)–linked in-
tegral membrane protein of the immunoglobulin super-
family.

Thy-1 was initially described on the cell surface of T lym-
phocytes in mouse (Reif and Allen, 1964; Acton et al.,
1974), but in fact this protein is more characteristic of neu-
rons than of lymphocytes, as it is expressed in nervous sys-
tem tissue in virtually all mammalian species (Morris et al.,
1983; Morris, 1985). Thy-1 shares structural homologies
with immunoglobulins (Campbell et al., 1981; Cohen et al.,
1981; Williams and Gagnon, 1982); unlike many other
members of this superfamily, the mature form of Thy-1
does not cross the lipid bilayer, but is anchored to the lu-
menal (extracellular) leaflet by covalent linkage to phos-
phatidylinositol (Low and Kincade, 1985; Tse et al., 1985).
It is known to be expressed by neurons and neuronal cells
in culture (Mahanthappa and Patterson, 1992; Wilkerson
and Touster, 1993), where it has been used as a marker for
plasma membrane (Green and Kelly, 1992). However, a
cue to an intracellular localization of Thy-1 comes from
the observation that Thy-1 accumulates proximal to a
nerve compression, suggesting that there is an intracellular
form that is transported down axons by intraaxonal trans-
port (Morris et al., 1983).

Despite its lack of a transmembrane domain, Thy-1 has
been implicated in signal transduction, through increasing
in intracellular Ca$^{2+}$ (Kroczek et al., 1986; Barboni et al.,
1991) and stimulating tyrosine phosphorylation (Hsi et al.,
1989; Garnett et al., 1993). Either cross-linking or removal
of cell surface Thy-1 triggers differentiation and neurite
outgrowth in both primary neurons and PC12 cells (Leifer
et al., 1984; Mahanthappa and Patterson, 1992).

There have been a few provocative indications that Thy-1
might be involved directly with synaptic transmission. An-
tibodies against Thy-1, when injected into the hypothala-
mus, have been reported to have an effect on drinking be-
havior (Williams et al., 1980). Most intriguing, knockout
mice lacking Thy-1 show a specific deficit in long-term po-
tentiation (LTP) in the dentate gyrus of the hippocampus
(Nosten-Bertrand et al., 1996); this deficit has been con-
firmed by recordings from awake knockout animals (Err-
ington et al., 1997).

We have found that Thy-1 is a component of multiple
types of regulated secretory vesicles, both in cultured cells
and in vivo. We have obtained evidence for the functional
involvement of this protein in the regulated, Ca$^{2+}$-depen-
dent release of norepinephrine (NE), using two indepen-
dent in vitro release systems derived from PC12 cells. The
presence of Thy-1 in multiple populations of secretory ves-
cles in most neuronal cells argues that this protein plays a
fundamental role in regulated secretion at the synapse.

Materials and Methods

Generation of Monoclonal Antibodies

Monoclonal antibodies were prepared using BALB/c mice, according to
standard procedures (Goding, 1986), by fusing spleen cells to SP2/0 my-
eloma cells. Antigen was prepared by homogenizing PC12 cells in Heps-
buffered saline (HBS; 150 mM NaCl, 10 mM Hepes, pH 7.4) in the presence
of 1 mM EGTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin,
1 µg/ml antipain, and 0.2 mM diisopropylfluorophosphate. Secretory ves-
icles were purified by layering homogenates onto 1–27% ficoll gradients in
0.32 M sucrose, 10 mM Hepes, pH 7.4, and centrifuging at 100,000

After selection and cloning of hybridoma cells, antibody-containing
media were screened for their ability to bind to purified PC12
secretory vesicles and for their vesicular pattern of staining by immunoflu-
orescence microscopy.

Other Antibodies and Reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO),
less otherwise indicated. Reagents for SDS-PAGE were obtained from
Bio-Rad Laboratories (Hercules, CA) and Fisher Scientific Co. (Fairlawn,
NJ). Cell culture medium and reagents were obtained from Gibco BRL
(Gaithersburg, MD). Nitrocellulose membrane filters were from Micron
Separation Inc. (Westboro, MA). *Staphylococcus aureus* pansorbin was
purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Anti-
SV2 antibody was monoclonal 10H3 (Buckley and Kelly, 1985), antisyn-
apotrysin antibody (clone 80.1) was a gift from R. Jahn (Yale University
School of Medicine, New Haven, CT), anti-secretogranin I antibody (clone
219.6) was a gift from P. Rosa (University of Milan, Milan, Italy), and an-
tisidum pump antibody (McK1) was a gift from K. Swadner (Massa-
chusetts General Hospital, Boston, MA). Anti-SNAP-25 antibody was ob-
tained from Sternberger Monoclonals Inc. (Baltimore, MD). Monoclonal
antibody (OXT) against Thy-1 was obtained from PharMingen (San
Diego, CA). Antisynaptophysin antibody, peroxidase-conjugated goat
anti-mouse antibody and alkaline phosphatase-conjugated goat anti-
mouse antibody were obtained from Boehringer Mannheim Biochemicals
(Indianapolis, IN). Biotinylated sheep anti-mouse antibody and Texas
red-streptavidin were from Amersham Corp. (Arlington Heights, IL).
ABC kit was obtained from Vector Labs Inc. (Burlingame, CA).

Cell Culture

Subcloned PC12 cells (Schweitzer and Paddock, 1990), were grown in
DME with 25 mM Hepes plus 5% supplemented calf serum (HyClone,
Logan, UT), 5% horse serum (HyClone), and penicillin plus streptomycin
at 9.5% CO$_2$. Cells to be used for microscopy were pretreated for 3 d
with 10 ng/ml NGF to induce cell differentiation. They were then trypsinized

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and replated onto poly-D-lysine-coated coverslips and grown in the continued presence of NGF for an additional 3 d before fixation and processing. CHO cells were transfected into HAM’s F12 medium containing 5% calf serum (HyClone), penicillin, streptomycin, and fungizone. Monkey kidney (COS) cells were maintained in DME containing 10% calf serum, penicillin, and streptomycin.

**Density Gradient Fractionation**

PC12 cells were labeled overnight with 0.5 μCi/ml of [3H]NE. Cells were harvested with HBS plus 5 mM MgEGTA, and homogenized with a stainless-steel ball bearing homogenizer (EMBL, European Molecular Biology Laboratory), Heidelberg, Germany) at 0°C. The entire cell homogenate was loaded on top of a linear sucrose density gradient (0.6 –1.6 M sucrose in 10 mM HEPES, pH 7.4, 1 mM MgEGTA). The gradient was centrifuged for 18 h at 97,000 g in a Ti rotor (SW41; Beckman Instruments). The contents of the centrifuge tube were recovered by dropwise collection from the bottom of the tube. After collecting the gradients, the material pelleted at the bottom of the tube was resuspended and collected as fraction number one. The fractionated samples were subsequently used for scintillation counting and dot blot immunoassay.

**Dot Blot Immunoassay and Western Blot**

For dot blot immunoassays, nitrocellulose membranes were soaked in TBS (150 mM NaCl, 10 mM Tris, pH 7.4), drained on filter paper, and then mounted on a 9cm well manifold (Bio-Rad laboratories). A 5-μl sample from each fraction of the sucrose density gradient was diluted with 195 μl transfer buffer (192 mM glycine, 20 mM Tris base, 20% methanol; Towbin et al., 1979). Each sample was applied to a separate well of the manifold under low vacuum with a slow filtration rate. The manifold was then disassembled and the filter left to air dry. For Western blots, proteins were dissolved in 2X SDS sample buffer and boiled, with the exception of samples used to quantitate the plasma membrane Na pump, which were allowed to incubate at room temperature for 15 min before loading onto gels. Samples were subjected to SDS-PAGE on 8, 10, or 12% polyacrylamide gels (Laemmli, 1970), in the presence or absence of β-mercaptoethanol, and electrophoresed onto 0.45 μm nitrocellulose filters for 1 h at 100 mA in 25 mM Tris-base, 192 mM glycine, and 20% methanol (Towbin et al., 1979) by a semi-dry transfer apparatus (TE70; Hoefer Scientific Instruments, San Francisco, CA). Molecular weights were estimated by comparison with molecular weight standards purchased from Sigma Chemical Co. or Bio-Rad Laboratories, and stained with Ponceau S dye.

The filters were blocked by soaking in 5% (wt/vol) Carnation nonfat dry milk in TBS for 30 min, or 0.1% Tween-20 in TBS) for 1 h. All the incubation and washing procedures were carried out at room temperature with gentle agitation. After blocking, filters were incubated with tissue culture supernatant or 15,000 dilution of ascites fluid diluted in 1% (wt/vol) nonfat dry milk in TBST for 1 h followed by three washes in TBST.

Bound antibodies were visualized by either enhanced chemiluminescence (ECL) or alkaline phosphatase methods. For ECL, filters were incubated with 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG in TBST containing 1% milk for 1 h, and then washed as above. After draining off excess buffer, membranes were incubated in the detecting reagents (5 mM luminol, 1 mM 4-iodophenol and 0.2 mM H₂O₂ in 150 mM NaCl, 50 mM Tris, pH 9.0) (Thorpe et al., 1985; ECL Western blotting detection system, Amersham, Oakville, Ontario; Dupont REN-ALIANCE Western blot Chemiluminescence system, DuPont, Wilmington, DE) for 1 min. Excess detecting reagents were drained off from the membrane, and it was then wrapped in Saran wrap and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) for 15–60 s. The films were then scanned and analyzed using a scanning densitometer (model GS-700; Bio-Rad Laboratories) with Molecular Analyst software.

For alkaline phosphatase detection, alkaline phosphatase-coupled secondary antibodies were used, and quantitated by incubation with a color substrate (0.01% nitro-blue tetrazolium, 0.005% 5-bromo-4-chloro-3-indolylphosphate and 4 mM MgCl₂ in 45 mM bicarbonate buffer, pH 9.6).

**Immunofluorescence Microscopy**

For standard antibody labeling, PC12 cells treated with NGF were grown on poly-D-lysine-coated eight-well slides. On the third day, cells were rinsed with HBS plus 10 mM CaCl₂ and 10 mM MgCl₂ (CM-HBS) and fixed in modified Bouin’s fixative (Schwetizer and Paddock, 1990) for 10 min. Cells were then washed with HBS, incubated with 50 mM NH₄Cl in HBS for 5 min, permeabilized with 0.1% Triton X-100 in HBS for 2 min, and blocked with 1% nonfat milk (in HBS plus 10 mM MgEGTA) for 10 min. Cells were incubated with primary antibodies for 1 h at 37°C. After washing, cells were further incubated with biotinylated goat anti-mouse antibodies and then Texas red streptavidin. After washing with HBS and distilled water, slides were mounted in 2% triethylene diamine in glycerol, pH 8.6 (Boek et al., 1985). Confocal images were acquired with a Zeiss 410 laser scanning microscope.

To stain nonpermeabilized cells, PC12 cells on coverslips were fixed by placing on ice, and then incubated for 1 h in the presence of and appropriate dilution of primary antibody. After this incubation, the cells were washed three times with ice-cold buffer, and fixed and processed as above. Control experiments with a mouse antitubulin antibody demonstrated that this procedure resulted in no labeling of intracellular structures.

**Cloning of the 7C8 Antigen**

**Library Screening.** A rat PC12 cell line cDNA library was constructed in the plasmid expression vector CDMS (Seed and Aruffo, 1987) and transfected into CHO cells as previously described (Liu et al., 1992). The stable transformants were plated at low density (~1,500 cells/15-cm plate) and overlaid the following day with a 17-μm pore polyester replica filter (Esko, 1986). After 5 d, filters containing cell colonies from 20–150 plates were removed, fixed in 4% formaldehyde from paraformaldehyde, washed in Ca²⁺- and Mg²⁺-free phosphate-buffered saline, blocked in 3% normal goat serum, 0.01% Triton X-100, and incubated overnight at 4°C in 7C8 ascitest diluted 1:10,000 in 0.1% Tween-20, 0.1% BSA, and 0.01% Triton X-100. The filters were washed six times in 1% goat serum and 0.01% Triton X-100 and incubated in biotin-conjugated goat anti-mouse IgM antiserum, followed by an avidin–biotin complex conjugated to alkaline phosphatase (Vector Labs, Inc.). For secondary screening, a lower density of cells (~100 cells/10-cm plate) was used. Double-stranded DNA sequencing was performed using fluorescent nucleotides in an automated sequencer apparatus.

**Purified Rescue.** Genomic DNA was extracted by homogenization with 5 M guanidinium thiocyanate and precipitation with isopropanol. The integrated plasmid DNA was excised, religated, and transformed into Escherichia coli as described previously (Feany et al., 1992). From each of the three sets of colonies transformed with DNA from independent CHO cell lines, plasmid DNA was prepared from 30 to 60 colonies. DNA from one of the three sets was pooled, and then radiolabeled using a random-primer labeling kit (Stratagene, La Jolla, CA). DNA preps derived from the other two sets of colonies were subjected to a PstI/HindIII digest to excise the cDNA insert from the CMV8 vector, electrophoresed through agarose, and blotted to nitrocellulose filters that were probed with the radiolabeled, independently isolated DNA.

**Cell Transfections.** For transient transfections, COS cells were electroporated with 15 to 30 μg DNA using a Gene Pulser apparatus (Bio-Rad Laboratories) as previously described (Peter et al., 1994).

**Cell Surface Biotinylation**

Biotinylation was performed by a modification of the procedure described by Schmidt et al. (1997). All steps were performed at 4°C. Subconfluent 15-cm dishes of PC12 cells were rinsed three times with 10 ml CM-PBS (136 mM NaCl, 2.5 mM KCl, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 0.5 mM CaCl₂, and 2 mM MgCl₂). After removing the rinse, the biotinylation was started by the addition to each dish of 4 ml CM-PBS containing 4 mg sulfo-NHS-LC-biotin (Pierce, Rockford, IL), followed by incubation at 0°C for 30 min with slow shaking. Biotinylation was terminated by removing the medium and rinsing dishes three times with CM-PBS buffer and three times with HBS.

**Immunoprecipitations**

**Immunosolation of Vesicles.** For immunosolation of SV2-containing vesicles, S. aureus cells (SAC, Calbiochem-Novabiochem, Corp.), pretreated with rabbit anti-mouse antibody, were incubated with anti-SV2 monoclonal antibodies, clone 10H3 (Buckley and Kelly, 1985) or normal mouse serum (NMS) diluted in HBS for 1 h on ice. SV2-coated SAC, or NMS-treated SAC, was washed in HBS and incubated with postnuclear supernatant of PC12 cells for 1 h at 4°C under slow rotation. Samples were spun for 5 min at 2,000 g and washed three times in HBS. The pellet and supernatant were then subjected to SDS-PAGE and immunoblotting.

**Thy-1 Immunoprecipitation.** Ox7-coated SAC was prepared by pretreating SAC with rabbit anti-mouse antibodies and then with OX-7 antibody. A postnuclear supernatant of PC12 cells was solubilized with Det-
HBS (1% Nonidet P40, 0.3% SDS, 0.4% deoxycholate, 66 mM EDTA, 10 mM Hepes, pH 7.4), and then incubated with OK7-coated SAC for 1 h at 4°C with slow rotation. Samples were centrifuged for 5 min at 2,000 g, and washed once with Det-HBS and twice with HBS. The pellets and supernatants were then boiled in SDS sample buffer and subjected to SDS-PAGE and immunoblotting.

**Subcellular Fractionation and Purification of Synaptic Vesicles from Rat Brains**

Synaptic vesicles were purified under isoosmotic conditions as described previously (Floor et al., 1995). Briefly, rat brains were homogenized in 0.32 M sucrose, 5 mM NaHPO4, at pH 6.6, 1 mM EGTA, and 0.02% NaN3. An equal volume CK buffer (0.16 M KCl, 5 mM NaHPO4 at pH 6.6, 1 mM EGTA, and 0.02% NaN3, and 1 mM dithiothreitol) was added, the suspension was centrifuged at 7,500 g for 25 min, and the resulting supernatant was centrifuged at 60,000 g for 3 h. The resulting pellet was resuspended in CK buffer and loaded on 10–30% (wt/vol) sucrose gradients and centrifuged at 100,000 g for 2 h in a vertical rotor. Fractions containing synaptic vesicles were pooled and further chromatographed on a Sephacryl S-1000 column in CK buffer. Both LDCVs and SVs are enriched ~30-fold in peak fractions after Sephacryl S-1000 chromatography.

**Immunohistochemistry**

Rats were anesthetized and perfused with 4% paraformaldehyde/HBS. Tissue blocks were further fixed by immersion in the same fixative for 3 h at 4°C and then soaked in 20% sucrose/HBS overnight at 4°C for cryostat sectioning. Sections (10 μm in thickness) were rinsed with HBS buffer, permeabilized with 0.3% Triton X-100/HBS for 15 min, and then treated with 0.3% H2O2 for 20 min to destroy endogenous peroxidase. Sections were washed three times in HBS, blocked with 5% BSA/HBS for 30 min, and then incubated with primary antibodies (1:500 dilution SV2 ascites or 7C8 culture supernatant) at 4°C for 1 h. After washing three times in HBS, sections were incubated with 1:200 dilution of biotinylated anti-mouse IgG for 30 min, washed three times in HBS and then further incubated in 1:500 dilution of avidin–biotin complexes for 30 min. Signals were detected by color reaction (0.05% diaminobenzidine tetrahydrochloride in 50 mM Tris, pH 7.2, and 0.03% H2O2). The peroxidase reaction was stopped by transferring to distilled water. Stained specimens were then mounted for viewing in the light microscope.

**NE Release from Intact and Semi-intact PC12 Cells**

To monitor the release of neurotransmitter from semi-intact cells, PC12 cells were labeled with [3H]NE, washed, removed from culture dishes in cold K glutamate buffer (120 mM K glutamate, 20 mM K acetate, 2 mM EGTA, 0.1% bovine serum albumin, and 20 mM Hepes, pH 7.2), and permeabilized by passage through a narrow clearance ball homogenizer (Hay and Martin, 1992). The protein identified by the 7C8 antibody some-what specifically to a single protein band with a relative mobility of gradient-purified vesicles, and showed a punctate staining pattern in NGF-differentiated PC12 cells. Antibodies exhibiting these characteristics were further analyzed by Western blotting. Some of these antibodies recognized previously described antigens (e.g., synaptophysin), and some bound to multiple bands on blots, but several bound specifically to single bands that did not correspond to known vesicle proteins. An example of this last category was the clone designated 7C8.

The initial identification of the 7C8 antigen is shown in Fig. 1. Fig. 1 A illustrates the cofractionation of 7C8 binding with both populations of regulated secretory vesicles in PC12 cells. The cells were loaded overnight with [3H]NE, harvested, and homogenized; cellular components were then fractionated on a sucrose density gradient, and the fractions analyzed for the presence of various vesicle markers. As indicated by the circles, 7C8 binding exhibited a bimodal pattern, with peaks in both the lower (denser) third of the gradient and a second, smaller peak in the upper third of the gradient. The lower peak corresponds to the location of the NE-containing LDCVs (Schweitzer and Kelly, 1985), as shown by the single peak of [3H]NE peak (Fig. 1 A, diamonds). The upper peak corresponds to the location of the cholinergic SSVs (Blumberg and Schweitzer, 1992), as shown by the second peak of SV2 (squares), which is a component of both SSVs and LDCVs. This distribution was not simply the result of all cellular or vesicular proteins sedimenting in this fashion; in contrast to 7C8 and SV2, another of the monoclonal antibodies we isolated, denoted 15C12, showed a single peak of binding corresponding only to the denser of the two peaks of 7C8 binding (triangles). In experiments to be described elsewhere, we have shown that this antigen is an integral membrane protein associated exclusively with the LDCVs (data not shown).

It is clear from this analysis that the 7C8 antigen copurified with both the catecholamine-containing LDCVs (Fig. 1 A, fractions 6–10) and the lower density SSVs (fractions 13–15). As is typical for such density gradients, the vesicles are spread out over several fractions; this probably reflects the micro-heterogeneity that is observed even in highly purified preparations of synaptic vesicles (Carlson et al., 1978). In addition to this spreading, there is a slight variation in the precise locations of the peaks for the different vesicle markers. This difference suggests that the relative amounts of these markers may vary in vesicles of slightly differing densities.

Fig. 1 B shows that this antibody bound exclusively and specifically to a single protein band with a relative mobility of 27,000. For comparison, Fig. 1 B also shows two known vesicle antigens, synaptophysin (P38; Jahn et al., 1985; Wiedenmann and Franke, 1985) and SV2 (Buckley and Kelly, 1985), both of which are present in PC12 cell vesicles. The protein identified by the 7C8 antibody sometimes appeared on Western blots as a closely spaced doublet rather than a single band (Fig. 1 C and D).

If the 7C8 antigen were a legitimate component of synaptic vesicles, and not a cell culture artifact, it should be present in high amounts in brain tissue. This prediction is confirmed by the Western blots shown in Fig. 1. C and D, demonstrating the presence of a single immunoreactive band in rat brain tissue, having a slightly higher mobility.
than seen with PC12 cells. These experiments also indicated that the 7C8 antigen was different from known vesicle proteins of similar molecular weight, including rab-3A (Fig. 1 C), SNAP-25, (Fig. 1 D), and synaptogyrin (Fig. 1 D). In addition to the differences in mobility, these proteins were distinguished by the differences in distribution: in contrast to the 7C8 antigen, which behaved as an integral membrane protein (Fig. 1 C and data not shown) and consistent with previous reports (Darchen et al., 1990; Johnston et al., 1991), rab3A was found in both the soluble and membrane fractions of a rat brain homogenate (Fig. 1 C). SNAP-25, although exhibiting similar mobility to the 7C8 antigen, did not show the difference in mobility that the latter exhibited between PC12 cell and brain tissue (Fig. 1 D). Synaptogyrin was present in detectable (not shown) but much lower amounts in PC12 cells than rat brain, again in contrast to 7C8 (Fig. 1 D).

We carried out immunofluorescence microscopy to localize the 7C8 antigen within PC12 cells. Cells used for these experiments were differentiated with NGF to facilitate the visualization of vesicles within different regions of the cells. Fig. 2 shows a single confocal optical plane of three NGF-treated PC12 cells after standard fixation and permeabilization, illustrating the intracellular punctate appearance of 7C8 staining. Many brightly stained puncta, with an apparent diameter of ~0.4 μm can be seen scattered throughout the cytoplasm, particularly in the terminal swellings of the neurites. In addition, there are numerous smaller and fainter puncta that are not well resolved, which probably represent vesicles that are smaller than the resolution limit of the light microscope. Both these puncta are absent from the nuclei of the cells. This appearance suggests that the 7C8 antigen is localized to at least two populations of vesicles in the cytoplasm of PC12 cells. This impression was confirmed by morphological analysis of the overall distribution within cells and neurites.

The confocal image shown in Fig. 2 clearly illustrates the staining of individual vesicles; conventional immunofluo-
rescence microscopy, which is not as capable of resolving individual vesicles, provides more complete information about the overall distribution of a protein in various regions of a cell. This distribution is of interest because PC12 cells exhibit a differential localization of LDCVs and SSVs, with the LDCVs being relatively concentrated in the neurite tips, while the SSVs are most prevalent in the perinuclear cell bodies (Schweitzer and Paddock, 1990).

A comparison of the localization of 7C8 staining within NGF-differentiated PC12 cells with that of other vesicle markers is shown in Fig. 3. Fig. 3A shows a phase contrast image of three PC12 cells and their associated neurites, whereas Fig. 3B shows the corresponding immunofluorescence image of these cells after staining with the 7C8 antibody. In this view, both the perinuclear cell bodies (Fig. 3B, arrowheads) and the neurite terminals (arrows) exhibit approximately equal levels of staining with 7C8; this appearance is consistent with the staining pattern illustrated in Fig. 2 above, combined with the fact that a considerably greater volume of cytoplasm is contained in the perinuclear cell body than in the neurites.

In contrast with the localization of 7C8 staining, markers selective for either the SSVs or the LDCVs showed distinctly different staining patterns. Fig. 3D illustrates the staining of cells and processes with an antibody against secretogranin I, a protein that is contained exclusively within the LDCVs in PC12 cells (Rosa et al., 1985). Although there was some level of staining throughout the cell, the tips of the neurites (arrows) stained more intensely with the antibody against secretogranin I than did the cell body, reflecting the relatively larger numbers of LDCVs that accumulate in the neurite tips as compared with the cell body.

Conversely, synaptophysin is an integral membrane protein that is present predominantly in the SSVs in PC12 cells (Jahn et al., 1985). The localization of synaptophysin also differed from that of the 7C8 antigen. The staining pattern for synaptophysin (Fig. 3F) appeared as the complement of that seen for secretogranin I; that is, the perinuclear cell bodies (arrowheads) stained more brightly than the neurite tips, reflecting the relatively greater concentration of SSVs in the cell bodies than in neurite terminals (Schweitzer and Paddock, 1990).

Previous morphological and biochemical studies have shown that the vesicle marker SV2 is present on both SSVs and LDCVs (Wiedenmann and Franke, 1985; Navone et al., 1986; Lowe et al., 1988). We therefore expected that SV2 staining would appear similar to that of 7C8. The staining pattern of SV2 is shown in Fig. 3H; like 7C8, this was of approximately equal intensity in the perinuclear cell bodies and neurite tips, reflecting its presence in both populations of secretory vesicles.

For purposes of illustration, cells with short neurites were chosen for these figures, because it was not feasible...
to combine high magnification images of cells with the large fields necessary to include the terminals of long neurites. However, we have observed the same patterns of staining shown in all the panels in Figs. 2 and 3 in cells with neurites as long as 10 cell body diameters in length.

**Identification of the 7C8 Antigen as Thy-1**

To determine the identity of this novel component of vesicles, we used the 7C8 antibody to screen a PC12 cell cDNA library in the plasmid expression vector CDM8 (Seed and Aruffo, 1987), and introduced these sequences into CHO fibroblasts (Liu et al., 1992); wild-type CHO cells do not express the 7C8 antigen (data not shown). When replica colonies from ~30,000 independent transformants were immunostained with the 7C8 antibody, we identified 37 colonies positive for antibody binding. We selected 10 of these more intensely positive colonies for further analysis. Of these ten, three clones expressed a protein of ~27 kD that comigrated on Western blot analysis with the 7C8 antigen seen in PC12 cells (data not shown).

We used plasmid rescue to isolate the sequences encoding the 7C8 antigen from the immunoreactive CHO cell clone. Genomic DNA was extracted from each clone, and the integrated plasmids were excised, religated, and introduced into transformation-competent *E. coli*. Because the stable CHO transformants contained multiple distinct integrated plasmids, we used Southern analysis to identify cDNAs common to the three independently isolated CHO cell clones. The plasmids rescued from one CHO clone were radiolabeled by random priming and used as a probe for Southern blots of the other two clones. Two plasmids were found to be common to all three clones.

To determine which of these two plasmids encoded the 7C8 antigen, we transfected each plasmid separately into COS cells, and probed Western blots of cell homogenates with anti–7C8 antibody. Only plasmid 112 showed an immunoreactive protein of the expected size (Fig. 4 A). Analysis of the 1.6-kb insert showed sequence identity to rat Thy-1 (Moriuchi et al., 1983; Seki et al., 1985) beginning 16 nucleotides upstream of the initiation codon and extending through the entire protein-coding regions as well as an additional 1,053 base pairs of 3’ untranslated DNA. Thy-1 is a GPI-linked protein known to be on the surface of T lymphocytes and mature neurons, and has an apparent molecular weight of ~27 kD; it has been reported to run occasionally as a doublet due to heterogeneity in its N-linked glycosylation (Barboni et al., 1995).

To confirm the specificity of the identification procedure, we further subcloned the 1.6-kb insert into another plasmid expression vector (pcDNA I-Amp) in both sense and antisense orientations. After transfection into COS cells, Western blot analysis with the 7C8 antibody confirmed the presence of an immunoreactive protein only from the construct in the sense orientation (Fig. 4 B).

Biochemical experiments confirmed that the 7C8 antigen is Thy-1. Fig. 5 A shows that the 7C8 antibody binds to the same protein recognized by a commercially available monoclonal antibody against Thy-1 (OX7). We immunoprecipitated Thy-1 from a detergent extract of a PC12 cell postnuclear supernatant. As shown in the first panel, illustrating an immunoblot probed with the same OX7 anti-

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**Figure 4. Expression of the 7C8 antigen in COS cells.** (A) Homogenates of COS cells transfected with two of the pcDM8 expression plasmids isolated from immunoreactive CHO cell clones were stained on a Western blot with the 7C8 antibody. Clone 112 expressed a protein that comigrated with the 7C8 antigen expressed by PC12 cells, but untransfected COS cells (–) and cells transfected with plasmid 11 did not. (B) The cDNA insert from plasmid 112 was subcloned into the expression vector pcDNA I-Amp. Homogenates from COS cells transfected with the cDNA insert in the sense (S) but not the antisense (A) of the Thy-1 cDNA reacted with the 7C8 antibody.

Thy-1 antibody used for the immunoprecipitation, all of the Thy-1 in the homogenate was pelleted in the immunoprecipitation; in contrast, normal mouse serum leaves all the Thy-1 in the supernatant. The second panel in Fig. 5 A illustrates that probing these samples with the 7C8 antibody gives exactly the same pattern as for OX7; i.e., the 7C8 antigen is precipitated by the OX7 antibody, but not by control serum. A comparison of the two panels in Fig. 5 A also illustrates the identical mobility of the proteins recognized by these two antibodies.

To demonstrate even more conclusively that 7C8 recognizes Thy-1, we made use of the fact that mice express two different allotypes of Thy-1, known as Thy-1.1 and Thy-1.2. Thy-1.1, which is expressed by the AKR strain of mouse, is nearly identical to rat Thy-1; BALB/c mice, on the other hand, express Thy-1.2, which is different enough that it does not cross-react with anti–Thy-1 antibodies (Marshak-Rothstein et al., 1979). Fig. 5 B shows a panel of homogenates from PC12 cells, rat brain, BALB/C mouse brain, and AKR mouse brain probed with the OX7 or 7C8 antibodies. As was shown in Fig. 1, Fig. 5 B shows that the 7C8 antigen was present in both PC12 cells and rat brain; the same was true of Thy-1 visualized by the OX7 antibody. Most strikingly, there was no immunoreactivity in BALB/c mouse brain with either OX7 or 7C8 antibodies, although in brain tissue from AKR mice, immunoreactivity was present that comigrated with rat Thy-1. These experiments demonstrate unequivocally that the 7C8 antibody recognizes a protein that is identical to Thy-1.

**Cell Surface Versus Internal Thy-1**

Because Thy-1 has previously been considered to be only a marker for the plasma membrane, it was important to ask why we did not observe 7C8 binding to the cell surface as well as to vesicles. It was also essential to determine unambiguously that the Thy-1 we observed as a component of vesicles was not contamination from the plasma membrane. To address the former question, we carried out immunofluorescent staining of nonfixed, nonpermeabilized PC12 cells to determine whether we could observe surface staining. As shown in Fig. 6, binding of the antibody to the cells before fixation and detergent treatment resulted in intense staining around the periphery of the cell, consistent with staining of the plasma membrane. Our standard procedure for immunofluorescence involved fixing cells and then permeabilizing with 0.1% Triton X-100 for 2 min.
Although we occasionally observed such surface staining with conventionally fixed and permeabilized cells (data not shown), we routinely observed only the vesicular staining illustrated in Figs. 2 and 3. Why the plasma membrane Thy-1 is preferentially lost during standard fixation and permeabilization, even as the intracellular staining is retained, is not completely clear at this point. However, perhaps because of its GPI anchor, Thy-1 is known to present unusual difficulties for morphological localization (Morris and Grosveld, 1989).

To demonstrate unequivocally that there is intracellular Thy-1 associated with secretory vesicles, and to quantitate the distribution of Thy-1 between intracellular and surface membranes, we used a procedure for immunopurifying secretory vesicles from PC12 cells with a monoclonal antibody that is directed against a cytoplasmic epitope of the SV2 protein (Schweitzer and Kelly, 1985). Figs. 7 and 8 show experiments using such an immunopurification procedure to demonstrate that Thy-1 is specifically and predominantly associated with vesicles.

For Fig. 7 A, a postnuclear supernatant from a PC12 cell homogenate was immunoprecipitated (in the absence of any detergent) with antibody against SV2, which should precipitate both the LDCVs and the SSVs, leaving nonvesicular membranes, including plasma membrane, in the supernatant. About half of the immunoreactive Thy-1 was recovered in the supernatant, and half in the immunoprecipitated pellet (Fig. 7 A, lanes 2 and 3). Quantitative analysis of these bands from three experiments gave a figure of 56 ± 9% vs. 44 ± 9% for the actual distribution of vesicular Thy-1 versus plasma membrane Thy-1. No Thy-1 was precipitated by normal mouse serum (lanes 4 and 5). To demonstrate that all the vesicles are precipitated by this procedure, the same samples were probed with an antibody against synaptophysin; essentially all of this vesicle marker precipitated with the SV2 antibody (lanes 7 and 8). To show that this procedure did not simply cause all the cellular proteins to precipitate nonspecifically, we also visualized the total protein in each of these samples by silver-staining a parallel gel (Fig. 7 B). It is clear that the vast majority of the protein remained in the supernatant after immunoprecipitation with SV2 antibody (lanes 2 and 3); in terms of total protein, the SV2-immunoprecipitated lane was barely distinguishable from the normal mouse serum control, except for the presence of the immunoglobulin bands (compare lanes 3 and 5). We therefore conclude that this immunoprecipitation procedure is a highly selec-

Figure 5. Identification of 7C8 antigen as Thy-1. (A) OX7 immunoprecipitation. Postnuclear supernatant of PC12 cells (PC12 S1) was solubilized with Det-HBS, and immunoprecipitated with OX7 coated-SAC (OX7) or with normal mouse serum coated-SAC (NMS). Supernatant (S) and pellet (P) fractions after low speed centrifugation were subjected to a nonreducing SDS-PAGE and immunoblotting with OX7 (lanes 1–5) or 7C8 (lanes 6–10) antibodies. (B) Immunoblots of OX7 and 7C8. Homogenates (100 μg total protein per lane) of PC12 cells, rat brain, BALB/c mouse brain, and AKR mouse brain were loaded on a nonreducing gel, transferred to nitrocellulose membranes, and probed with OX7 (lanes 1–4) or 7C8 (lanes 5–8) antibodies.

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Figure 6. Immunofluorescent staining of non-permeabilized PC12 cells with 7C8. NGF-treated PC12 cells were incubated with 7C8 antibodies at 0°C for 1 h before fixation. After fixation, cells were incubated with biotinylated sheep anti-mouse antibodies, followed by Texas red-streptavidin. Bar, 10 μm. A and B represent the phase contrast and fluorescence images of the same cells; C represents a single confocal optical section through a different cluster of PC12 cells.

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Thy-1 Is a Component of Synaptic Vesicles in Rat Brain

To demonstrate that the vesicular localization of Thy-1 is not unique to the PC12 cell line, we analyzed rat brain tissue for the presence and localization of Thy-1. Western blot analysis of various brain regions and other tissues demonstrated that 7C8 bound to virtually all synapse-containing regions of the central nervous system, but was undetectable in peripheral tissues, including liver, kidney, pancreas, and muscle (data not shown). Extensive immunohistochemical mapping of the distribution of 7C8 binding throughout the nervous system showed that 7C8 binding was prominent in the gray matter in synapse-rich areas, and that this staining paralleled that seen with other vesicle markers. One example of this general colocalization is shown in Fig. 9 A, in which the synaptic layers of the hippocampus stain prominently with 7C8 (A), SV2 (B), and synaptophysin (C). Although we can identify subtle differences in the relative staining of vesicles markers in some regions (for example, in the dentate gyrus of the hippocampus, there is relatively less SV2 staining in the vicinity of the granule cell layer as compared with 7C8 or synaptophysin); most areas show similar staining of all these vesicle markers. This result suggests that Thy-1 is somehow associated with synapses.
Figure 8. Biotinylation of surface but not vesicular Thy-1. Living PC12 cells were surface biotinylated by incubation of sulfo-NHS-LC-biotin for 30 min at 0°C. The cells were then harvested, rinsed, and homogenized. Supernatant and pellet fractions from SV2-immuniosolation procedures, performed exactly as described in Fig. 7, were solubilized with detergent and further immunoprecipitated with OX7-coated SAC. Samples were subjected to a non-reducing gel for probing with 7C8 antibodies (A) or HRPT streptavidin (B) to visualize total Thy-1 and biotinylated cell surface proteins.

Despite being consistent with a synaptic localization of Thy-1 in the central nervous system, immunohistochemical staining at the light microscopic level is inadequate to determine the subcellular localization of the Thy-1 visualized by the 7C8 antibody. To determine whether Thy-1 is an intrinsic component of synaptic vesicles, we analyzed purified synaptic vesicles prepared by a series of differential centrifugation, Nycodenz density gradient, and sizing column procedures (Floor et al., 1995). Fig. 7, D–F, shows the final step in this purification, consisting of a Sephacryl S-1000 sizing column. This separation not only produces highly purified synaptic vesicles, but can resolve the small synaptic vesicles from the larger, peptide-containing vesicles (Floor et al., 1988). The extent of plasma membrane contamination of these vesicles is very low; Na/K ATPase (ouabain-sensitive) activity accounts for <5% of the total ATPase activity found in the SSV fractions (Floor et al., 1990). When Nycodenz-enriched membranes were loaded onto the Sephacryl S-1000 column, very large membranes and membrane fragments passed through the column in the void volume (fractions 24–30); large, peptide-containing vesicles were included and eluted in fractions 33–37; and highly purified small synaptic vesicles eluted in fractions 41–47. As seen in Fig. 9 D,7C8 immunoreactivity co-purified with both the large and small vesicle peaks, consistent with its localization in PC12 cells. In contrast, the synaptic vesicle markers synaptophysin (Fig. 9 E) and SV2 (Fig. 9 F) were predominantly associated with the small synaptic vesicles in brain. Similar results were obtained with SSVs purified on controlled pore glass columns (data not shown). The observation that Thy-1 copurifies with highly enriched preparations of synaptic vesicles argues strongly that the vesicular localization observed in PC12 cells is also present in vivo in brain synapses.

We observed one notable exception to the colocalization of Thy-1 with other vesicle markers. In the retina, the difference between the staining patterns for Thy-1 and other synaptic vesicle markers was striking. As shown in Fig. 9 G, Thy-1 staining was intense in the retinal ganglion cells, but was completely absent from the outer plexiform layer, and very faint in the inner plexiform layer. The presence of other synaptic vesicle markers in these layers is illustrated by Fig. 7, H and I, in which SV2 and synaptophysin were visualized; both these vesicle proteins stained prominently in both the inner and outer plexiform layers. The absence of Thy-1 staining in the plexiform layers of the retina correlates with the presence in these locations of structurally unique and physiologically distinct synapses (ribbon synapses, see Discussion).

Functional Involvement of Thy-1 in the Release of Neurotransmitter

Because of its presence in synaptic vesicles and its localization in areas of synaptic contact, it was of interest to determine whether Thy-1 plays some demonstrable role in the release of neurotransmitter. Because Thy-1 on the inside of the vesicles is highly inaccessible, we chose to examine whether surface Thy-1 might contribute in some functional way to the regulated release of neurotransmitter. Using a semi-intact PC12 cell preparation (Walent et al., 1992), we examined the effect of anti-Thy-1 antibodies on Ca$^{2+}$-regulated neurotransmitter release. In this preparation, the plasma membranes of PC12 cells have been sheared to make them permeable to large molecules, as the organization of the synaptic vesicles and release sites remains sufficiently intact to observe regulated NE release that is dependent on Ca$^{2+}$ and cytosolic components (Hay and Martin, 1992; Walent et al., 1992; Hay et al., 1995). This preparation has been useful for identifying other molecular components of the regulated secretory apparatus (Banerjee et al., 1996).

As illustrated in Fig. 10, preincubation of the semi-intact PC12 cells with purified 7C8 antibody reproducibly produced a partial inhibition of the Ca$^{2+}$-dependent portion of NE release. Fig. 10 shows that this inhibition (35% in this case) did not increase further with increased amounts of antibody. In contrast, the addition of similar concentrations of a different monoclonal antibody, directed against the cytoplasmic portion of the synaptic vesicle component SV2, had no effect on the stimulated release of NE. Because SV2 is present on the NE-containing vesicles (Schweitzer and Kelly, 1985; Blumberg and Schweitzer, 1992; also see above), this result argues that this assay is relatively insensitive to nonspecific steric hindrance effects. In addition, it seems unlikely that a nonspecific effect could reproducibly inhibit a constant fraction of the Ca$^{2+}$-stimulated release. Although the concentration of antibody required to produce this inhibition is quite high, we have observed similarly high requirements for other antibodies that exert an inhibitory effect in this semi-intact cell system (unpublished data). Because this system does not depend on Ca$^{2+}$...
channels to control the \([\text{Ca}^{2+}]\) in the vicinity of the release apparatus, the inhibition observed here is not mediated by an effect on the presynaptic \(\text{Ca}^{2+}\) channel.

The inhibitory effect of anti-Thy-1, taken together with the widespread distribution of Thy-1 and its presence in multiple populations of synaptic vesicles, strongly suggests a fundamental role for Thy-1 in the events that mediate regulated synaptic vesicle fusion in phasic synapses in the central nervous system.

**Discussion**

We were surprised to learn that Thy-1 is a component of synaptic vesicles, and even more so to find it a component of each of the regulated secretory vesicles that we have examined, suggesting that it plays some fundamental role in the structure and/or function common to various types of secretory vesicles. The existence of Thy-1 has been recognized for over 30 years; how can we explain the fact that its presence in secretory vesicles was not previously appreciated?

Thy-1 is an unusual membrane protein belonging to the class of GPI-linked integral membrane proteins whose transmembrane domain is cleaved during biogenesis. It exhibits somewhat unexpected biochemical and histological properties, as shown by its heterogeneous sensitivity to phospholipase C (Low et al., 1988; Wilkerson and Touster, 1993), and its unusual behavior after reduction, removal of its lipid tail (Barboni et al., 1995), or fixation (Morris and Grosveld, 1989). Despite these anomalies, its presence in the plasma membrane of neurons was well recognized, but was viewed mainly as simply a cell surface marker. It has, however, been reported to accumulate proximal to a nerve.
constriction (Morris et al., 1983), which is consistent with its presence in some sort of intracellular vesicle.

We report here that Thy-1 is not only a component of regulated secretory vesicles, but that a major portion (>50% in PC12 cells) of Thy-1 is found in the vesicles, as compared with the cell surface. We have confirmed the presence of Thy-1 in distinct populations of synaptic vesicles purified from brain, and observed that Thy-1 is distributed in virtually all regions of the central nervous system with a distribution that generally parallels that of other synaptic vesicle proteins. We do not yet know the detailed relationship between vesicular Thy-1 and cell surface Thy-1. Whether one serves as a precursor to the other, and whether the relative distribution between these two sites can be altered by nerve activity or other conditions will require detailed cell biological analysis to determine. It is clear, however, that a major portion of the Thy-1 in cells is associated with the secretory vesicles, where it is presumably attached to the luminal leaflet of the bilayer. In this orientation, Thy-1 would be able to interact with neurotransmitters, peptides, or other soluble components of the secretory vesicles. In addition, although we do not provide evidence for this here, we presume that this internal Thy-1 becomes exposed on the surface of the cell specifically at the synapse where these vesicles fuse with the presynaptic plasma membrane, i.e., in an activity-dependent manner. Although most synaptic vesicle proteins become externalized only transiently as a result of fusion of synaptic vesicles with the plasma membrane, it seems that a significant fraction of Thy-1 is retained on the cell surface, where it has the potential to interact with extracellular molecules in the synapt cleft.

The specific targeting of Thy-1 to the presynaptic terminal fits well with our understanding of sorting and target-

Figure 10. Effect of antibodies on NE release from semi-intact PC12 cells. ATP-primed, semi-intact PC12 cells were incubated with the indicated amount of purified antibody (▲, 7C8; or ●, anti-SV2) for 60 min at 0°C before conducting 5 min Ca2+-triggering incubations as described in Materials and Methods. Incubations were conducted in duplicate with ranges indicated by error bars. All values were adjusted by subtracting the amount of background release in the absence of Ca2+ and cytosol.
be observed in our cell culture systems. However, as we do observe an inhibition of neurotransmitter release after an acute application of antibody, it is clear that Thy-1 is involved in some short-term aspect of the process of synaptic activity. Because we observe such an inhibition both in intact cells and in cells whose plasma membranes have been disrupted, it is clear that this effect must be on the sequence of events between the increase in intracellular Ca++ and the fusion of the neurotransmitter-containing vesicles.

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