bind I-E^d in our assay, and two bound I-E^d only weakly [dissociation constant (K_d) in the 2 to 10 μM range]. Three of the synthetic peptides, 5, 6, and 11, bound I-A^d with an affinity that was several times higher than that observed with the reference peptide ovalbumin (residues 323 to 339) (*15*).

Because most of the peptides associated with class I MHC contain only nine residues (*4–7*), we determined if the longer class II peptides were of optimal length for high-affinity binding to the I-A^d molecule. Truncated analogs of these peptides were synthesized and tested for their capacity to bind purified class II molecules (Table 2). The first two COOH-terminal residues of the peptide derived from the transferrin receptor, peptide 12, could be removed without any loss of binding capacity. Removal of the next residue yielded a peptide with 4% of the binding capacity for I-A^d. At the NH_2-terminal end of the peptide, removal of the first five residues caused a 66 to 80% reduction in I-A^d binding capacity, whereas removal of the sixth residue induced a greater than 98% decrease. Thus, the crucial I-A^d binding core region is contained within the ten-residue sequence QMVRTAAEVA (*27*).

Removal of each of the six, COOH-terminal residues from peptide 9 derived from apolipoprotein-E resulted in an approximately 50% decrease in binding capacity. Thus, an exact COOH-terminal boundary of the region crucial for I-A^d binding could not be clearly defined. Similar results have been obtained in the case of other class II peptide binding studies and have been attributed to the presence of more than one potential binding site in the epitope analyzed (*28*). At the NH_2-terminal end of the peptide, removal of the first seven residues caused a 66% reduction of binding affinity, but removal of the next residue, Arg^{243}, generated a peptide whose affinity was reduced by approximately 95% compared to that of the full-length determinant. More than one I-A^d binding site may therefore be contained within residues 236 to 252. The main binding site is contained within residues 243 to 251, RLQAEIFQA (*27*), as predicted by the presence of two potential canonical I-A^d binding motifs (*25, 26*).

Binding data on truncated synthetic peptides indicate that the length of the naturally processed peptides is not the minimum required for strong binding. Unlike class I peptides, class II peptides can be shortened without loss of binding activity. Crucial binding regions within the class II peptides are positioned variably within the observed sequences relative to their NH_2- and COOH-termini. In some cases, only two residues can be removed without a reduction of binding capacity, whereas in other cases, up to seven residues can be removed. These data are also at odds with a model in which the COOH-terminal end of the peptide is variable, but in which the NH_2-terminal end is fixed at a constant distance from the crucial binding region (*12*). Our results suggest that the binding groove of the I-A^d molecule is probably open at both ends.

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Neurotransmitter Release from Synaptotagmin-Deficient Clonal Variants of PC12 Cells

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Synaptotagmin (p65) is an abundant synaptic vesicle protein of neurons and contains regions similar to the regulatory domain of protein kinase C. These domains are thought to be involved in calcium-dependent interaction with membrane phospholipids during exocytosis. To assess the functional role of synaptotagmin, synaptotagmin-deficient clonal variants of PC12 cells were isolated. All of the variant cells released catecholamine and adenosine triphosphate in response to elevated intracellular concentrations of calcium, which suggests that synaptotagmin is not essential for secretion of catecholamine and adenosine triphosphate from PC12 cells.

Neurotransmitters and hormones are released from neurons and endocrine cells by exocytosis, but little is known about the molecular mechanisms involved (*1*). Synaptotagmin (p65) is an integral membrane glycoprotein found in secretory vesicles in a variety of neurons and some endocrine cells (*2, 3*). It has a single transmembrane segment with the NH_2- and COOH-termini located in the intravesicular and cytoplasmic spaces, respectively (*4, 5*). The primary structure of the cytoplasmic region is conserved in various animal species (*4–7*) and may be functionally important in secretion. This cytoplasmic portion contains two copies of a region similar to the C2 domain of Ca^{2+}-phospholipid–dependent protein kinase (protein kinase C), which is essential for the expression of Ca^{2+} and phospholipid dependency of the enzyme (*8*). In fact, synaptotagmin binds to phospholipid through this region, and this characteristic feature has led to the hypothesis that synaptotagmin participates in Ca^{2+}-dependent docking of secretory vesicles at the plasma membrane or in the fusion of secretory vesicles with plasma membrane during neurotransmitter release (*3, 4*).

The rat pheochromocytoma cell line PC12 secretes catecholamines and acetyl-

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choline by exocytosis (9). Synaptotagmin is expressed in PC12 cells and is located in larger vesicles with a dense core—the so-called large, dense-core vesicles that contain catecholamines (2). After a large number of cell divisions, cell lines can spontaneously generate phenotypic and genotypic variants, and several PC12 subclones have been isolated (9, 10). We have selected synaptotagmin-deficient variants of PC12 cells and examined their secretory activity to elucidate the function of synaptotagmin in exocytosis.

To increase the probability of obtaining synaptotagmin-deficient cells, we eliminated most of the cells that expressed synaptotagmin by selectively killing those cells that reacted with an antibody to synaptotagmin. When cells are treated with antibodies that recognize antigens located on the cell surface and complement is added, the antigen-expressing cells are killed by immune cytolysis (11). Some synaptotagmin is localized in the presynaptic plasma membrane of neurons and in the plasma membrane of adrenal medullary cells (2, 12). Because of its intravesicular orientation, one would expect that the NH_2-terminal region of synaptotagmin would be exposed to the outside of the cell and that antibodies directed against this region could be used for the cell killing. From the cloning of cDNA, synaptotagmin appears to be present in multiple isoforms. At least two different synaptotagmin genes (synaptotagmin I and synaptotagmin II) are expressed in the rat nervous system, but only synaptotagmin I is expressed in the adrenal gland (7, 13).

We therefore produced polyclonal antibodies (PAb Stg1N) to a 20–amino acid peptide corresponding to the NH_2-terminal sequence of rat synaptotagmin I. When cerebral cortex neurons in monolayer culture were stained with PAb Stg1N without fixing or permeabilizing the cell membrane, dots of immunoreactivity were concentrated around cell bodies and along neurites, and the staining pattern was similar to that obtained with antibodies to synaptophysin and synapsin (14). These results indicate that the NH_2-terminus of synaptotagmin in the plasma membrane is exposed to the extracellular medium.

When PC12 cells were incubated with PAb Stg1N at a tenfold dilution and complement was added, about 80% of the cells were lysed. In contrast, only 10% of the cells died after treatment with nonimmune rabbit serum. We repeated the selection procedure with PAb Stg1N ten times and then checked the immunoreactivity of the surviving cells by indirect immunofluorescence microscopy with a monoclonal antibody to synaptotagmin (MAb 1D12) (12). Numerous cell colonies that were not stained with MAb 1D12 were detected (Fig. 1). After dissociation of the colonies, about 1000 cells were picked up individually with a micropipette and seeded separately in multi-well tissue culture plates, and three clones (PC12-B3, PC12-D6, and PC12-F7) were selected that were not immunoreactive with MAb 1D12. Two control clones (PC12-G11 and PC12-C10) that expressed synaptotagmin and had catecholamine-releasing activity comparable to that of PC12 cells before selection were also established from the original PC12 cells.

Membrane proteins (10 μg) from rat brain and the various PC12 subclones were separated on immunoblots and probed with MAb 1D12 (Fig. 2A). Synaptotagmin was detected in rat forebrain, cerebellum, and PC12 cells before recloning and in PC12-G11 cells but not in PC12-B3, PC12-D6, or PC12-F7 cells. Synaptotagmin was detected in PC12-G11 cells even when much less protein (0.01 μg) was loaded on the gel, which indicates that synaptotagmin is at least 1000 times less abundant in variant cells than in control cells.

To examine the expression of synaptotagmin subtypes in the PC12 subclones, we probed immunoblots with antibodies with different specificities. The PAb Stg1N gave essentially the same results as MAb 1D12, and a specific band was detected in forebrain, cerebellum, and PC12 cells before recloning and in PC12-G11 but not in the three variant cell lines (Fig. 2B). Because the degree of sequence identity between synaptotagmin I and II is low in the NH_2-terminus, we produced an antibody to a peptide for this region of synaptotagmin II (PAb Stg2N). This antibody stained a protein from cerebellar membranes (Fig. 2C). Immunoreactivity was much weaker in the

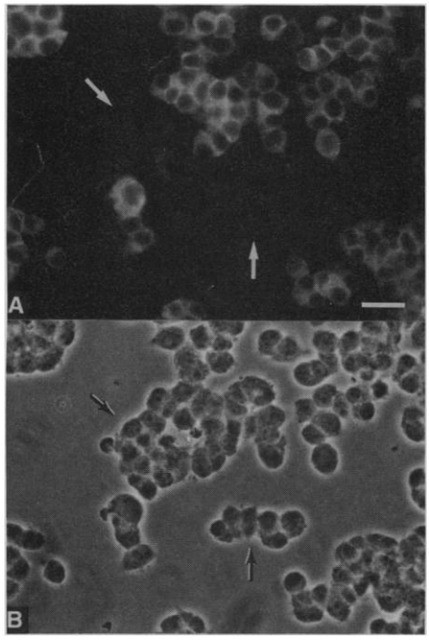

Fig. 1. Surviving PC12 cells after ten repeats of the selection procedure. (**A**) Immunofluorescence micrograph with an antibody to synaptotagmin, MAb 1D12. (**B**) Phase-contrast micrograph of the same field. Cells cultured on polyethylenimine-coated Lab Teck chambers (Nunc, Naperville, Illinois) were fixed with paraformaldehyde (4% w/v) and treated with ice-cold methanol. They were incubated with MAb 1D12 (2 μg/ml) followed by fluorescein-conjugated antibody to mouse immunoglobulin G (Cappel, West Chester, Pennsylvania) and observed with an Axioplan microscope (Zeiss). The colonies of cells not stained with MAb 1D12 are indicated by arrows. Scale bar, 20 μm.

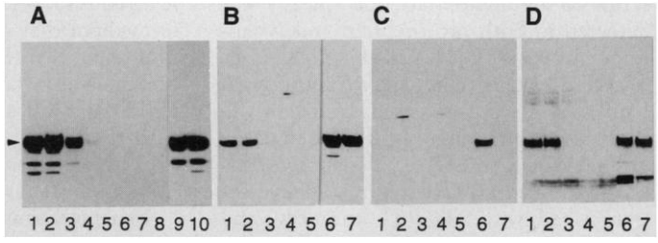

Fig. 2. Immunoblots of membrane proteins from rat forebrain, cerebellum, and PC12 cells probed with a battery of antibodies to synaptotagmin. Blots were probed with (**A**) MAb 1D12, (**B**) PAb Stg1N, (**C**) PAb Stg2N, and (**D**) polyclonal antibody to synaptotagmin. The migration position of synaptotagmin is indicated by an arrow. In (A): lane 1, PC12 before the recloning; lanes 2 to 5, PC12-G11; lane 6, PC12-F7; lane 7, PC12-B3; lane 8, PC12-D6; lane 9, cerebellum; and lane 10, rat forebrain. In (B) through (D): lane 1, PC12 before the recloning; lanes 2, PC12-G11; lane 3, PC12-F7; lane 4, PC12-B3; lane 5, PC12-D6; lane 6, cerebellum; and lane 7, rat forebrain. The MAb 1D12 was prepared as described (12). The PAbs Stg1N and Stg2N were raised to synthetic 20–amino acid peptides corresponding to the NH_2-terminal sequences of rat brain synaptotagmin I and II, respectively, coupled to keyhole limpet hemocyanin. The polyclonal antibody to synaptotagmin was raised to rat brain synaptotagmin purified on an immunoaffinity column of MAb 1D12 and was purified by binding to synaptotagmin adsorbed on nitrocellulose sheets (19). Membrane proteins [10 μg per lane except for (A), lane 3, 1 μg; lane 4, 0.1 μg; and lane 5, 0.01 μg; and (D), lanes 1 to 5, 50 μg] were separated by SDS–polyacrylamide gel electrophoresis (4 to 12% gradient gel; Tefco, Nogano, Japan), electrophoretically transferred to a nitrocellulose sheet (20), incubated with the various antibodies, and then incubated with horseradish peroxidase–conjugated antibody to mouse immunoglobulin G (Tago, Burlingame, California). The immunostained bands were visualized with an enhanced chemiluminescence-detection kit (Amersham, Buckinghamshire, United Kingdom).

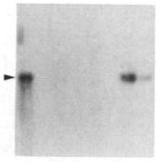

Fig. 3. Analysis of synaptotagmin I mRNA expression in rat brain and PC12 subclones. Lane 1, rat forebrain; lane 2, PC12-B3; lane 3, PC12-D6; lane 4, PC12-F7; lanes 5 and 6, PC12-G11. The migration position of synaptotagmin I mRNA (4.8 kb) is indicated by an arrowhead. Polyadenylated-enriched RNA was isolated by means of the acid guanidinium thiocyanate-phenol-chloroform method (*21*) and oligo(dT) centrifuged columns (Pharmacia). The samples were then electrophoretically separated on formaldehyde-agarose gels and transferred to nitrocellulose (lanes 1 through 5, 4.8 μg of RNA; lane 6, 0.48 μg of RNA). Filters were hybridized with ^{32}P-labeled DNA probes from random primers corresponding to the entire coding region of rat brain synaptotagmin I and washed at 65°C in 0.15 M NaCl, 15 mM sodium citrate, 0.1% SDS, and 0.1% sodium pyrophosphate.

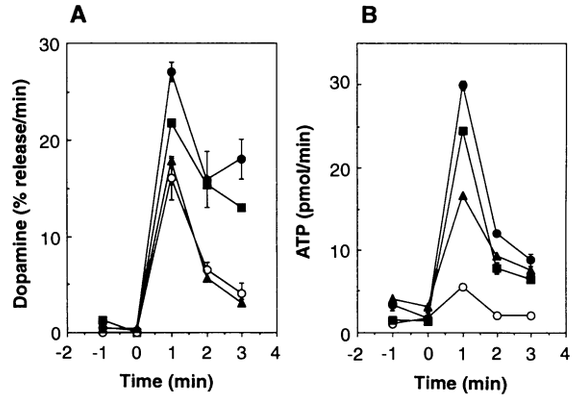

Fig. 4. Release of dopamine (**A**) and ATP (**B**) after depolarization of synaptotagmin-expressing and synaptotagmin-deficient subclones of PC12 cells. Two days before the experiments, the synaptotagmin-expressing cells (PC12-G11, ○) and synaptotagmin-deficient cells (PC12-B3, ▲; PC12-D6, ■; and PC12-F7, ●) were plated on polyethylenimine-coated plastic culture dishes (35 mm; Corning) at a density of 10^6 cells per dish. Catecholamine and ATP release were measured as described (*22, 23*). The cells were incubated for successive 1-min periods in assay medium [140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11 mM glucose, and 15 mM Hepes (pH 7.3 with tris)]. After 2 min, the K^+ concentration in the assay medium was increased to 60 mM, and the Na^+ concentration was decreased to 85 mM (time 0). The amounts of dopamine and ATP released in the assay medium were measured by high-pressure liquid chromatography with an electrochemical detector and by the luciferin-luciferase reaction, respectively. The amount of dopamine released into the medium was expressed as a percentage of the total amount of dopamine stored in the cells at the beginning of each period. All the values are means of duplicate determinations.

forebrain and absent in all of the PC12 cells. These results are consistent with Northern (RNA) blotting experiments, which have indicated that synaptotagmin II mRNA is abundant in the cerebellum but not detectable in the adrenal gland (*13*). No synaptotagmin was detected in the variant cells with a polyclonal antibody to rat brain synaptotagmin that had been purified by immunoaffinity chromatography with MAb 1D12 (Fig. 2D). These results with a battery of antibodies to conserved regions and variable isoform-specific domains of synaptotagmin indicate that the selected PC12 variants do not express synaptotagmin. The synaptotagmin I deficiency was shown to be stable for at least 30 passages of the cells over a period of 5 months.

In Northern blotting experiments with a synaptotagmin I probe, a 4.8-kb transcript of the appropriate size (*5, 13*) was detected in both rat forebrain and synaptotagmin-positive PC12-G11 cells (Fig. 3). On the other hand, the synaptotagmin I mRNA was not detected in any of the three variant clones. Polyadenylated mRNA from the PC12 cells before selection and from PC12-F7 cells was reverse-transcribed with oligo(dT) primers, and the cDNAs corresponding to the entire coding region of synaptotagmin I were amplified by the polymerase chain reaction (PCR). Only the mRNA isolated from the PC12 cells before selection contained the appropriate PCR-amplified product (*15*). These results indicate that the phenotypic defect in the variant cells occurred at a step before transcription.

No other differences between control and variant cells were detected, including the expression of tyrosine hydroxylase, dopamine β-hydroxylase, and synaptophysin, another synaptic vesicle protein. The catecholamine content, the morphology and numbers of large, dense-core vesicles, the extent of Ca^{2+} mobilization induced by membrane depolarization, and neurite extension induced by NGF-treatment were also unmodified.

Like normal PC12 cells, all of the variants released dopamine when treated with medium that contained a high concentration of K^+ (60 mM) (Fig. 4A). Although the time course of dopamine release was similar for each of the cell types, the maximum value of dopamine released was higher in the variants than in the PC12-G11 cells. Adenosine triphosphate (ATP) is stored in large, dense-core vesicles of adrenal medullary and PC12 cells and is released together with catecholamines during exocytosis (*16*). All of the variant cells released ATP when depolarized by exposure to medium that contained a high concentration of K^+ (Fig. 4B). The release of catecholamine and ATP from all of the PC12 cells was also induced by treatment with the Ca^{2+} ionophore ionomycin (0.1 to 1 μM) and was abolished by removing Ca^{2+} from the assay medium.

Synaptotagmin is localized in the catecholamine-containing large, dense-core vesicles of PC12 cells (*3, 5*). If synaptotagmin is essential for the exocytosis of these vesicles, then a substantial reduction in catecholamine release from synaptotagmin-deficient PC12 variant cells would be expected. However, we found that all the variant cells we obtained released catecholamines upon membrane depolarization. Several observations indicate that this catecholamine release was caused by exocytosis: (i) ATP, which is stored in the large, dense-core vesicles in adrenal medullary and PC12 cells, was also secreted by the variants during depolarization. (ii) The time course of release of catecholamines and ATP from the variants was similar to that in cells that contained synaptotagmin. (iii) Catecholamines and ATP were released from the variants in a Ca^{2+}-dependent manner (*1*). We therefore conclude that synaptotagmin is not essential for the exocytosis of the large, dense-core vesicles of PC12 cells.

Two distinct populations of secretory vesicles, small, clear vesicles and large, dense-core vesicles, have been described in PC12 and adrenal medullary cells. Because their protein composition and the processes that control their biosynthesis and recycling are likely to be different (*17*), it is possible that exocytosis of these two types of secretory vesicles occurs by different mechanisms. Synaptotagmin may be essential for secretion by small, clear vesicles but not for secretion by large, dense-core vesicles.

The possibility that synaptotagmin is not essential but has a regulatory function in exocytosis should also be considered. Synaptic transmission occurs rapidly and is complete within 1 to 2 ms after the action potential is propagated through the synaptic terminal. The Ca^{2+} ions that trigger exocytosis enter cells through voltage-sensitive Ca^{2+} channels in the presynaptic plasma membrane. However, during this very short period, the increase in Ca^{2+} concentration is sufficient to induce exocytosis only in a restricted zone close to the Ca^{2+} channel (*18*). Therefore, some synaptic vesicles may be docked in the vicinity of Ca^{2+} channels ready for a rapid neurotransmitter release. A portion of the synaptotagmin in cells is tightly associated with the ω-conotoxin receptor, a putative N-type Ca^{2+} channel that participates in neurotransmitter release in the brain (*12*).

A tight interaction between synaptotagmin and the Ca^{2+} channel might lead to docking of synaptic vesicles at active zones. The efficiency of synaptic transmission could be controlled by modulation of the binding of synaptic vesicles to the active zone.

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Involvement of the *N*-methyl D-aspartate (NMDA) Receptor in Synapse Elimination During Cerebellar Development

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In many instances, the establishment of highly specific neuronal connections during development results from the rearrangement of axonal projections through the trimming of exuberant collaterals or the elimination of functional synapses or both. Although the involvement of the *N*-methyl D-aspartate (NMDA) subtype of the glutamate receptor has been demonstrated in the shaping of axonal arbors, its participation in the process of selective stabilization of synapses remains an open issue. In this study, the effects of chronic in vivo application of D,L-2-amino-5-phosphonovaleric acid (D,L-APV), a selective antagonist of the NMDA receptor, on the synapse elimination process that takes place in the developing cerebellum of the rat have been analyzed. D,L-APV treatment prevented the regression of supernumerary climbing fiber synapses in 49 percent of the recorded Purkinje cells, while the inactive isomer L-APV was ineffective. Thus, activation of the NMDA receptor is a critical step in the regression of functional synapses during development.

Since its first description by Ramon y Cajal (*1*), synaptogenesis between cerebellar Purkinje cells and olivary climbing fibers has been considered one of the best models to study selective stabilization of synapses during central nervous system development (*2*). In the rat, each Purkinje cell is innervated by several climbing fibers during the first week after birth. Then, a massive elimination of most synapses occurs through the withdrawal of axonal collaterals (*3*, *4*) without substantial cell death of olivary neurons (*5*). The innervation of each Purkinje cell by only one climbing fiber, typical of the adult stage, is attained on postnatal day (PN) 14 to 15.

Multiple innervation of the Purkinje cells persists in adulthood when development has occurred in the absence of their second main input, that is, the granule cells, as in several mutant mice (*6*), in rats irradiated with x-rays (*7*), and in ferrets infected with feline panleucopenia virus (*8*). The mechanisms that prevent synapse elimination in these models are unknown. However, these observations indicate that regression of the polyinnervation during normal development requires granule cell input. Impulse activity from these cells could be the critical signal for regression because neural activity is known to regulate synapse elimination at the neuromuscular junction (*9*) and is a prominent factor in shaping neuronal connectivity in the central nervous system (*10*, *11*).

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The NMDA subtype of the glutamate receptor participates in activity-dependent synaptic modifications underlying learning and memory in the adult hippocampus (*12*). During development, NMDA receptors are involved in the segregation of eye-specific stripes (*13*) and in the pruning of retinal axon arbors (*14*) in the central nervous system of amphibians. Moreover, the segregation of retinogeniculate fibers in ferrets (*15*) as well as the formation of ocular dominance columns in kittens (*16*) seem also dependent on the activation of the NMDA receptor, although the interpretation of these results is currently debated (*17*).

In the cerebellum, a transient sensitivity of Purkinje and granule cell responses to NMDA occurs during the period of synapse elimination in rodents (*18*). As both climbing fiber and parallel fiber afferents likely use excitatory amino acid transmitters such as glutamate (*19*), these observations led us to investigate whether the activation of NMDA receptors is involved in cerebellar synapse elimination.

D,L-2-amino-5-phosphonovaleric acid (D,L-APV), a selective NMDA receptor antagonist, was delivered chronically from an implant of Elvax polymer (*13*, *20*) applied onto the surface of the posterior vermis in 4- to 5-day-old rats (PN 4 to 5) just before the period of synapse elimination. The implants remained in situ until the number of climbing fibers innervating each Purkinje cell was determined by in vivo intracellular recording at 18 to 31 days of age (PN 18 to 31) (Fig. 1). In each Purkinje cell, this number was estimated (*3*, *4*) from the stepwise increase in amplitude of the climbing fiber excitatory postsynaptic potentials (CF-EPSP) that occurred spontaneously or that were elicited by gradually