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The synaptic vesicle protein synaptotagmin associates with calcium channels and is a putative Lambert–Eaton myasthenic syndrome antigen

(ω-conotoxin receptor/autoimmunity/neurotransmitter release)

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ABSTRACT **Immunoglobulin G fractions from patients with Lambert–Eaton myasthenic syndrome (LEMS), an autoimmune disease of neuromuscular transmission, immunoprecipitate ^{125}I-labeled ω-conotoxin GVIA-labeled calcium channels solubilized from rat brain. A 58-kDa antigen was detected by probing Western blots of partially purified calcium channels with LEMS plasma and IgG and was shown to be the relevant antigen in ω-conotoxin receptor immunoprecipitation. Monoclonal antibody 1D12, produced by immunizing mice with synaptic membranes, has properties similar to these autoimmune IgGs in both immunoprecipitation and Western blotting assays. 1D12 antigen was purified by immunoaffinity chromatography and shown to bind LEMS IgG. The antigen was identified by screening a rat brain cDNA library with 1D12 and was found to have strong homology to the synaptic vesicle membrane protein synaptotagmin. Our results indicate therefore that these antibodies immunoprecipitate ω-conotoxin receptors by binding to synaptotagmin that is associated with calcium channels. We suggest that the interaction between synaptotagmin and the voltage-gated calcium channel plays a role in docking synaptic vesicles at the plasma membrane prior to rapid neurotransmitter release and that autoantibody binding to a synaptotagmin–calcium-channel complex may be involved in the etiology of LEMS.**

Lambert–Eaton myasthenic syndrome (LEMS) is an autoimmune disease of the neuromuscular junction in which pathogenic autoantibodies appear to downregulate presynaptic calcium channels, resulting in reduced acetylcholine release and consequent muscle weakness (1–5). In at least 60% of cases, LEMS is associated with small cell lung cancer (SCLC) and the initial stimulus for autoantibody production may be the expression of voltage-gated calcium channels by the tumor (5). Attempts to identify calcium channel-associated antigens using plasma from LEMS patients to probe immunoblots of crude membrane preparations have not been conclusive (6). However, N-type calcium channels, which play a major role in neuronal excitation–secretion coupling, can be labeled with the peptide antagonist ^{125}I-labeled ω-conotoxin GVIA (^{125}I-ωCgTx) (7–9). Plasma from LEMS patients contains antibodies that immunoprecipitate ^{125}I-ωCgTx–receptor complexes from solubilized human neuroblastoma and SCLC membranes (10–12), which suggests that the antigen is a subunit of the calcium-channel protein. The present report describes experiments to test this hypothesis by using a partially purified ωCgTx-sensitive calcium-channel preparation from rat brain synaptic membranes and a monoclonal antibody (mAb) with immunochemical properties remarkably similar to those of IgG from some LEMS patients.

MATERIALS AND METHODS

Materials. ωCgTx was obtained from the Peptide Institute (Osaka). Chelating Sepharose fast flow and protein A-Sepharose CL-4B were purchased from Sigma. Heparin Ultrogel was from IBF. Goat anti-human IgG and goat anti-mouse IgG alkaline phosphatase conjugates were from Zymed and Promega, respectively. Goat anti-mouse IgG peroxidase conjugate was supplied by Biosys. Na^{125}I (2000 Ci/mmol; 1 Ci = 37 GBq), goat anti-human IgG peroxidase conjugate, and enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham. The cDNA library was purchased from Clontech.

Immunological Techniques. Monoiodo ^{125}I-ωCgTx (2200 Ci/mmol) was prepared and purified by HPLC (13). Rat brain synaptic membranes were labeled with 0.1 nM ^{125}I-ωCgTx (13) and then solubilized in 1.5% Triton X-100. Immunoassays containing 10 fmol of ^{125}I-ωCgTx–receptor complex and plasma or purified IgG (14) in 0.1 ml of 0.1% Triton X-100/20 mM phosphate buffer, pH 7.4, were incubated for 1 hr at 20°C. Preswollen protein A-Sepharose CL-4B (dry weight, 4 mg) was added and samples were rotated for 1 hr at 4°C. After centrifugation at 10,000 × *g* for 2 min, the pellet was washed once with assay buffer and once with 0.1% Triton X-100/25 mM Tris·HCl/0.15 M NaCl/0.1% bovine serum albumin, adjusted to pH 7.5 with HCl. Immunoprecipitated radioactivity was measured by γ counting. Preparation of the mAb 1D12 and immunoaffinity purification of its antigen were as described (15). Partially purified ωCgTx receptors or purified 1D12 antigen were analyzed by 5–15% gradient SDS/PAGE and conventional Western blotting techniques. Blots were probed with 50-fold human plasma dilutions, 0.5 mg of human IgG per ml or 0.02 mg of mAb 1D12 per ml and stained with goat anti-human or anti-mouse IgG–alkaline phosphatase conjugate. ECL detection was performed with anti-IgG peroxidase according to the Amersham protocol. LEMS IgG was affinity purified by the method of Olmsted (16).

Partial Purification of ωCgTx Receptors. Synaptic membranes containing 150 mg of protein and 100 pmol of ωCgTx receptor were labeled with tracer amounts of ^{125}I-ωCgTx.

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Abbreviations: LEMS, Lambert–Eaton myasthenic syndrome; ωCgTx, ω-conotoxin GVIA; SCLC, small cell lung cancer; mAb, monoclonal antibody.

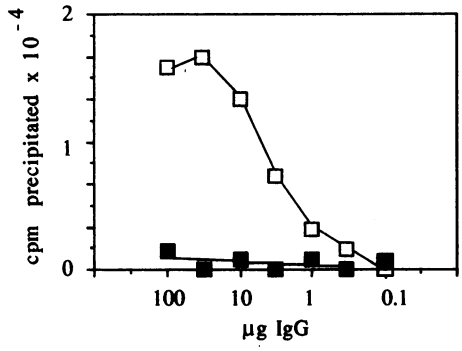

FIG. 1. Immunoprecipitation of ωCgTx receptors from rat brain by LEMS IgG. Calcium channels in rat brain synaptic membranes were labeled with ^{125}I-ωCgTx, extracted with Triton X-100, and incubated with IgG from a LEMS patient (□) or a healthy volunteer (■). Immune complexes were adsorbed to protein A-Sepharose and washed by centrifugation, and radioactivity was determined by γ counting.

Triton X-100-extracted ωCgTx receptors were purified by a two-step affinity chromatography procedure on chelating Sepharose fast flow loaded with cobalt ions and heparin Ultrogel, giving an ≈250-fold purification with a 15% yield (to be published in detail elsewhere). All the buffers used in the purification procedure contained 25 mM Hepes, 0.1% Triton X-100, 0.025% phosphatidylcholine, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, and 1 mM iodoacetamide adjusted to pH 7.4 with NaOH. For sucrose gradient centrifugation experiments, rat brain synaptosomes were labeled with 0.1 nM ^{125}I-ωCgTx and extracted with 1.5% Triton X-100. A 100,000 × *g* supernatant was layered over a 5%–20% linear sucrose gradient formed in 25 mM Hepes/0.15 M NaCl/0.1% Triton X-100/0.025% phosphatidylcholine, buffered to pH 7.4 with Tris base containing protease inhibitors, and spun for 19 hr at 33,000 rpm in a Kontron TST 41 rotor. The position of the ωCgTx receptor was determined by γ counting and the 58-kDa antigen was located by Western blotting with LEMS IgG.

Isolation of cDNAs and Sequencing. A rat brain cDNA library constructed in the expression vector λgt11 was screened with mAb 1D12. Positive clones were identified by using a mouse peroxidase-antiperoxidase system and both strands were sequenced entirely by the dideoxynucleotide chain-termination method (17). 1D12 antigen was isolated by immunoaffinity chromatography and SDS/PAGE. An endoproteinase Lys-C peptide was purified by HPLC and analyzed with an Applied Biosystems 477A gas-phase sequencer.

RESULTS AND DISCUSSION

Antibodies from LEMS Patients Recognize a 58-kDa Protein That Can Associate with ω-Conotoxin-Sensitive Calcium Channels. Rat brain synaptic membrane preparations were labeled with ^{125}I-ωCgTx, a specific and irreversible antagonist of high-threshold voltage-dependent calcium channels. After Triton X-100 extraction, the channel–ligand complex was immunoprecipitated by an IgG fraction prepared from the serum of a LEMS patient but not by control IgG (Fig. 1), confirming that LEMS IgG cross-reacts with the appropriate rat antigen as expected from electrophysiological studies in murine cells (18, 19). Rat brain synaptosomes contain ≈100 times more ωCgTx receptors per mg of protein (13) than human neuroblastoma membranes (20) and are therefore a more abundant source for antigen identification.

We have used LEMS IgG to probe Western blots of ωCgTx receptor purified ≈250-fold from rat brain. "Blind" experiments were initially performed in Marseille, with coded samples prepared in Oxford from patients with LEMS, patients with other neurological or autoimmune diseases, and healthy volunteers. Of 19 samples initially tested, 5 labeled a single major immunoreactive protein of 58 kDa (Fig. 2, lanes 5, 8, 12, 15, and 18). These samples were from 3 LEMS patients, 2 of which, from DA (plasma lane 5 and IgG lane 18) and from CO (plasma lane 8 and IgG lane 15), had a high plasma titer of anti-ωCgTx receptor antibodies in the immunoprecipitation assay (see Table 1). Studies with a larger group of LEMS patients showed a good correlation between the ability of plasma to immunoprecipitate solubilized ωCgTx receptors and to react with the 58-kDa antigen in Western blots of purified ωCgTx receptor (Table 1). Samples from four patients with a titer >700 pM also reacted with a 58-kDa protein in Western blots of crude synaptosomes (data not shown).

Human autoantibody responses are typically polyclonal, and the possibility that the antibody specificity recognizing the ^{125}I-ωCgTx-labeled channel complex in the immunoprecipitation assay is different from that staining the 58-kDa band in immunoblots must be considered. LEMS IgG was therefore affinity purified on the 58-kDa protein and shown to immunoprecipitate ωCgTx receptors in a concentration-dependent manner (Fig. 3*A*), confirming that this antigen is physically associated with the calcium channel. However, although LEMS antibodies only react specifically with the 58-kDa band in immunoblots containing denatured ωCgTx receptor, we cannot yet rule out the possibility that LEMS sera also contain other antibodies that only recognize calcium channel-associated polypeptides in their nondenatured form. The 58-kDa antigen is not thought to be the ωCgTx binding polypeptide, as arylazide derivatives of ^{125}I-ωCgTx cova-

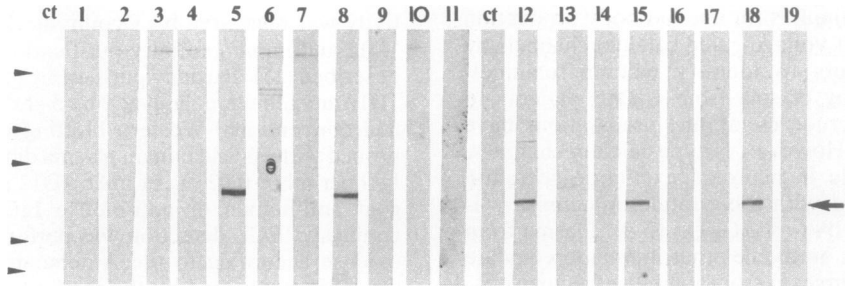

FIG. 2. Detection of a LEMS antigen. Western blots of partially purified calcium channels were probed with coded plasma and IgG fractions from healthy volunteers and patients with LEMS or other neurological and autoimmune diseases in a "blind" experiment. Lanes: 1, LEMS (SCLC); 2, healthy; 3, neuromyotonia; 4, LEMS (SCLC); 5, LEMS (NCD); 6, SCLC; 7, Guillain–Barré syndrome; 8, LEMS (NCD); 9, rheumatoid arthritis; 10, healthy; 11, LEMS (NCD); 12, LEMS (SCLC); 13, chronic inflammatory demyelinating neuropathy; 14, LEMS (SCLC); 15, LEMS (NCD); 16, healthy; 17, myasthenia gravis; 18, LEMS (NCD); 19, LEMS (SCLC). Lanes 1–11, plasma; lanes 12–19, IgG, each preceded by a control (lanes ct). NCD, no cancer detected. Arrowheads (from top to bottom) indicate molecular mass standards of 206, 110, 70, 43, and 28 kDa; arrow indicates 58-kDa immunoreactive bands.

Table 1. Anti-ωCgTx receptor antibody titer and anti-58-kDa immunoreactivity in a group of LEMS patients

Patient	Anti-ωCgTx receptor antibody titer, pM	58-kDa immunoreactivity in Western blots of purified ωCgTx receptor
DA	2000	+
FA	1400	+
CO	1400	+
AL	733	+
HN	602	±
HU	65	±
DU	58	±
BR	47	−
GR	45	−
BU	<40	−
AN	4	−
RI*	4	−
UN	2	±

Plasma samples from LEMS patients were assayed for their ability to immunoprecipitate ^{125}I-ωCgTx-labeled calcium channels (pM indicates pmol of ωCgTx receptor immunoprecipitated per liter of plasma) from SKN-SH neuroblastoma membranes (12) and to stain a 58-kDa band in Western blots (as in Fig. 2; +, positive; −, negative; ±, equivocal). Western blots probed with plasma from some of these patients are illustrated in Fig. 2.

*An apparent inconsistency should be pointed out. Plasma from RI was negative in Western blots, whereas in Fig. 2, lane 12, purified IgG from RI was positive. This may be due to the concentration of the relevant antibodies in RI plasma reaching the threshold for detection. An immunoprecipitation assay of the IgG from RI used in Fig. 2 indicated an ωCgTx receptor precipitating activity of ≈10% that in IgG from CO and DA.

lently label a 220- to 300-kDa protein in synaptic membranes (13, 21, 22), nor does it seem to be a channel subunit as most of the 58-kDa antigen detected in synaptosomes can be separated from ωCgTx receptor by sucrose gradient sedimentation (Fig. 3*B*). However, practically all calcium channels remain associated with at least one antigen as 86% of the ^{125}I-ωCgTx receptors in the sucrose gradient peak were immunoprecipitated with LEMS plasma (data not shown). These findings indicate that LEMS IgG immunoprecipitates ω-conotoxin receptors by binding to a 58-kDa synaptic terminal protein that can associate with calcium channels.

mAb 1D12 and LEMS IgG Recognize Closely Related 58-kDa Proteins. mAb 1D12 has properties remarkably similar to the LEMS IgGs: mAb 1D12 was produced by immunizing mice with chicken brain synaptic membrane preparations and subsequently selecting hybridomas secreting antibodies that immunoprecipitate ωCgTx receptors (15). This antibody also immunoprecipitates ωCgTx receptors solubilized from rat brain and recognizes a 58-kDa antigen in immunoblots of rat brain synaptic membranes (15). Furthermore, mAb 1D12 specifically labels the synaptic regions of both central and peripheral neurons showing, at the electron microscope level, the presence of antigen in both synaptic vesicles and presynaptic plasma membranes (15).

The experiments illustrated in Fig. 4 were carried out to determine whether LEMS IgG and mAb 1D12 interact with related antigens. The partially purified ωCgTx receptor preparation contains at least 20 polypeptides detected by silver staining (data not shown). The 58-kDa band that is stained by LEMS IgG (Fig. 4*A*, lane 3) also binds mAb 1D12 (lane 2) but not control mouse IgG (lane 1). In a parallel approach, 1D12 antigen was purified from detergent-solubilized rat brain synaptic terminals on a mAb 1D12-Sepharose CL-4B immunoaffinity column. Western blots of affinity-purified protein probed with LEMS IgG (Fig. 4*B*, lane 2) but not with control human IgG (lane 1) showed immunoreactivity at 58 kDa comparable to that obtained with mAb 1D12 itself (lane 3).

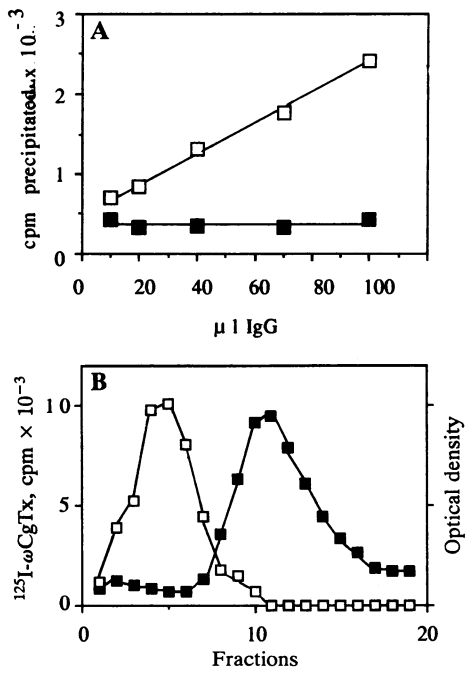

FIG. 3. Relationship between the 58-kDa LEMS antigen and the ωCgTx receptor. (*A*) LEMS IgG was affinity purified on a nitrocellulose strip containing immunoreactive 58-kDa synaptosomal proteins (□) or an identically sized control strip (■) cut from the 70-kDa region of the same Western blot. Eluted IgG was tested for its ability to immunoprecipitate solubilized ^{125}I-ωCgTx-labeled receptors. (*B*) Rat brain synaptosomes were solubilized in Triton X-100 and relative rates of sedimentation of ^{125}I-ωCgTx-labeled calcium channels (■) and the 58-kDa LEMS antigen (□) through a 5–20% sucrose gradient were compared.

These observations suggest that mAb 1D12 and the anti-ω-conotoxin antibodies from LEMS patients recognize closely related proteins.

The mAb 1D12 Antigen Is Synaptotagmin, a Synaptic Vesicle Protein That Can Associate with Calcium Channels. We have immunoscreened a rat brain cDNA library constructed in the expression vector λgt11 with mAb 1D12 and we isolated four overlapping clones. The sequence obtained from these clones

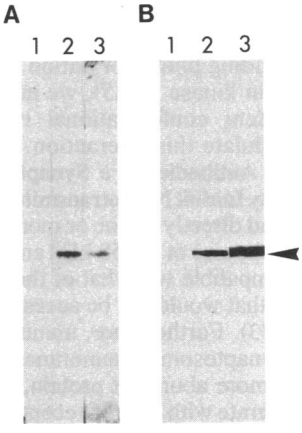

FIG. 4. mAb 1D12 and LEMS IgG recognize closely related proteins. (*A*) Western blots of partially purified ωCgTx-sensitive calcium channel from rat brain were probed with control mouse IgG (lane 1), mAb 1D12 (lane 2), or LEMS IgG (lane 3). (*B*) Western blots of 1D12 antigen immunoaffinity purified from rat brain synaptic membranes on mAb 1D12-Sepharose were probed with healthy human IgG (lane 1), LEMS IgG (lane 2), or mAb 1D12 (lane 3). Arrowhead indicates immunoreactive 58-kDa bands.

was virtually identical to nucleotides 577–2673 of synaptotagmin (previously designated p65), a membrane protein of synaptic vesicles (23–26). Two differences were observed in the coding region at bases 1089 and 1646, changing Glu-188 to Asp and Asp-374 to Gly. Nine bases (positions 875–883) were missing in one of our clones, a deletion already reported (24). Our cDNA clones do not, however, cover the 5′ region including the nucleotide sequence encoding the first 17 amino acids of the N terminus of synaptotagmin. The amino acid sequence of an endoproteinase Lys-C fragment isolated from purified 1D12 antigen was shown to be Asn-Ala-Ile-Asn-Met-Lys corresponding to rat synaptotagmin residues 99–104 (24). Identification of this antigen therefore implies that mAb 1D12 immunoprecipitates ωCgTx receptors by binding to synaptotagmin that is associated with calcium channels. The possibility that immunoprecipitation is due to a common epitope being shared by synaptotagmin and calcium channels may be ruled out as antibodies that recognize other synaptotagmin epitopes also immunoprecipitate ωCgTx receptors (data not shown).

The molecular properties of synaptotagmin suggest that it plays a role in exocytosis. It is a transmembrane protein that binds calmodulin (27) and contains an internally repeated cytoplasmic sequence homologous to the regulatory C2 region of protein kinase C (24). Synaptotagmin interacts specifically with acidic phospholipids (24, 25) and, by analogy with protein kinase C isoforms that include a C2 region, may bind calcium (25). A recent report suggests that exocytosis may involve association with the receptor for α-latrotoxin, a plasma membrane protein of unknown physiological function (28).

We have now demonstrated that a mAb selected for its ability to immunoprecipitate ωCgTx receptors in fact binds to synaptotagmin. Furthermore, synaptotagmin remains associated with calcium channels throughout a conventional fractionation procedure that results in a 250-fold purification of the ωCgTx receptor (Fig. 4*A*, lane 2). These observations suggest that a tight physical interaction between synaptotagmin and the calcium channel can occur that may play a role in docking synaptic vesicles at the plasma membrane. At the frog neuromuscular junction, the ωCgTx-sensitive calcium channels that control transmitter release (29) are in fact known to be exclusively localized at the active zones where exocytosis occurs (30). The association of synaptotagmin with calcium channels would site synaptic vesicles in a zone that would be rapidly accessible to calcium transients. This interaction must be tightly controlled and, although synaptotagmin possesses an extensive panoply of potential sites for regulation (24), including phosphorylation by protein kinase C and Ca/calmodulin kinase II (15), we may also speculate that voltage-dependent conformational transitions of the channel protein modulate this interaction.

Binding of LEMS Antibodies to a Synaptotagmin–Calcium-Channel Complex May Inhibit Neurotransmitter Release. LEMS IgG is believed to bind directly to one or more calcium-channel subunits. However, the size of the 58-kDa antigen that we have identified is only compatible with that of the β subunit (31), a cytoplasmic protein that would not be accessible to circulating autoantibodies (32, 33). Furthermore, immunoblotting experiments with crude synaptosomal membranes suggest that this LEMS antigen is a more abundant protein, the major part of which does not comigrate with ωCgTx receptors during sucrose gradient centrifugation. Western blotting with anti-synaptotagmin antibodies has demonstrated a similar antigen distribution in velocity sedimentation experiments (data not shown). Synaptotagmin, which is known to be a relatively abundant component of synaptic terminals, is presumably present in considerable molar excess in relation to ωCgTx receptor, which may explain the preferential detection of the unassociated form in these experiments. Our data are consistent with the hypothesis that the 58-kDa LEMS antigen is an isoform of synaptotagmin and that LEMS IgGs immunoprecipitate ωCgTx receptors by binding to a synaptotagmin/calcium-channel complex.

Multiple synaptotagmin variants are expressed in the rat central nervous system (34) and in fish electric organ (35). In view of the suggestion that tumor cells trigger the initial immune response in LEMS (2), it will be interesting to determine whether SCLC cells also express this protein. Synaptotagmin has a putative transmembrane segment, with the N terminus orientated toward the interior of synaptic vesicles (24) but presumably exposed at the surface of the nerve terminal after exocytosis. Circulating autoantibodies could then bind to the calcium-channel–antigen complex and consequently disrupt neurotransmitter release mechanisms. Determination of the primary structure of the 58-kDa antigen detected with human autoimmune IgG will, however, now be required to confirm and extend these findings and examine whether antibodies directed against extracellular domains of this protein are pathogenic in LEMS.

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