Volume 326, number 1,2,3, 135–139 FEBS 12675 July 1993

Expression of synaptotagmin and syntaxin associated with N-type calcium channels in small cell lung cancer

Pascale David[a], Oussama El Far[a], Nicole Martin-Mouto[a], Marie France Poupon[b], Masami Takahashi[c] and Michael J. Seagar[a]

[a]*INSERM U374, Faculté de Medecine Secteur Nord, Bd. Pierre Dramard, 13916 Marseille Cedex 20, France,* [b]*Institut Curie, Rue d'Ulm, Paris, France* and [c]*Mitsubishi Kasei Institute of Life Science, Machida-shi, Tokyo, Japan*

Received 9 April 1993

The presence of synaptic proteins involved in excitation/secretion coupling was examined in ten small cell lung cancer lines. N-Type calcium channels (ω-conotoxin receptors), synaptotagmin (p65) and syntaxin (HPC-1) were detected in eight. Co-immunoprecipitation experiments indicated that syntaxin can form a complex with synaptotagmin and calcium channels. The expression of synaptotagmin in small cell lung cancer may elicit an autoimmune response that reduces transmitter release at the nerve terminal.

ω-Conotoxin; Calcium channel; Synaptotagmin; Syntaxin; HPC-1; Small cell lung cancer

1. INTRODUCTION

Rapid exocytosis at nerve terminals is thought to require docking of synaptic vesicles at plasma membrane release sites in close proximity to calcium channels, a process that presumably involves protein–protein interaction [1–3]. Recent evidence suggests that docking is mediated by synaptotagmin (p65), a synaptic vesicle protein that can form a ternary complex with two plasma membrane proteins: the N-type calcium channel and syntaxin (alternatively termed HPC-1 or synaptocanalin) [4–9].

Small cell lung cancer (SCLC), the most malignant of human lung tumors, displays neurosecretory characteristics [10,11]. These include the expression of voltage-gated calcium channels [12–14] and the synaptic vesicle glycoprotein synaptophysin [11], and the release of neuropeptides such as bombesin/gastrin-releasing peptide, which are important autocrine growth factors [15]. In some cases the expression of neural antigens by the tumor initiates production of autoantibodies which then react with homologous targets in the nervous system, resulting in autoimmune disease. Lambert–Eaton myasthenic syndrome (LEMS) is the best-characterized neurological disorder of this kind. LEMS autoantibodies reduce presynaptic calcium influx and acetylcholine release at the neuromuscular junction, consequently leading to muscle weakness (reviewed in [16]).

Correspondence address: M.J. Seagar, INSERM U374, Faculté de Medecine Secteur Nord, Bd. Pierre Dramard, 13916 Marseille Cedex 20, France. Fax: (33) 91 09 05 06.

We have recently shown that LEMS IgG contains an anti-synaptotagmin specificity and hypothesized that these antibodies may inhibit neurotransmitter release by binding to a synaptotagmin/calcium channel complex [5]. If synaptotagmin is a relevant antigen in LEMS, one would predict its presence in SCLC. The present report examines the expression of synaptotagmin and syntaxin and their coupling to N-type calcium channels to form a putative docking complex in a panel of SCLC lines.

2. EXPERIMENTAL

2.1. *Materials*

ω-Conotoxin GVIA (ωCgTx) was purchased from the Peptide Institute (Osaka). The monoclonal antibodies, 1D12 and 10H5, were prepared and purified as previously described [4,7]. The monoclonal anti-synaptophysin antibody, 171B5, was provided by Dr. S.C. Fujita (Mitsubish Kasei Institute for Life Science). Goat anti-mouse IgG peroxidase conjugates were from Biosys, and enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham.

2.2. *Tissue preparation*

Crude rat brain synaptosomes (P2) were prepared by differential centrifugation. SCLC xenografts were prepared as previously described [17] and stored in liquid N_2. Minced fragments were homogenized in 10 mM Tris, 0.32 M sucrose adjusted to pH 7.4 with HCl containing the protease inhibitors 0.2 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, and 1 mM iodoacetamide, using an Ultraturrax apparatus. After a 10 min centrifugation at 600 × *g*, the resulting supernatant was centrifuged at 100,000 × *g* for 45 min and the membrane pellet collected. Protein was assayed by the BCA method (Pierce) with a bovine serum albumin standard.

2.3. *[^{125}I]ω-Conotoxin binding*

Monoiodo[^{125}I]ωCgTx (2,200 Ci/mmol) was prepared and purified by HPLC [18]. Binding assays, containing 100 μg of SCLC membrane

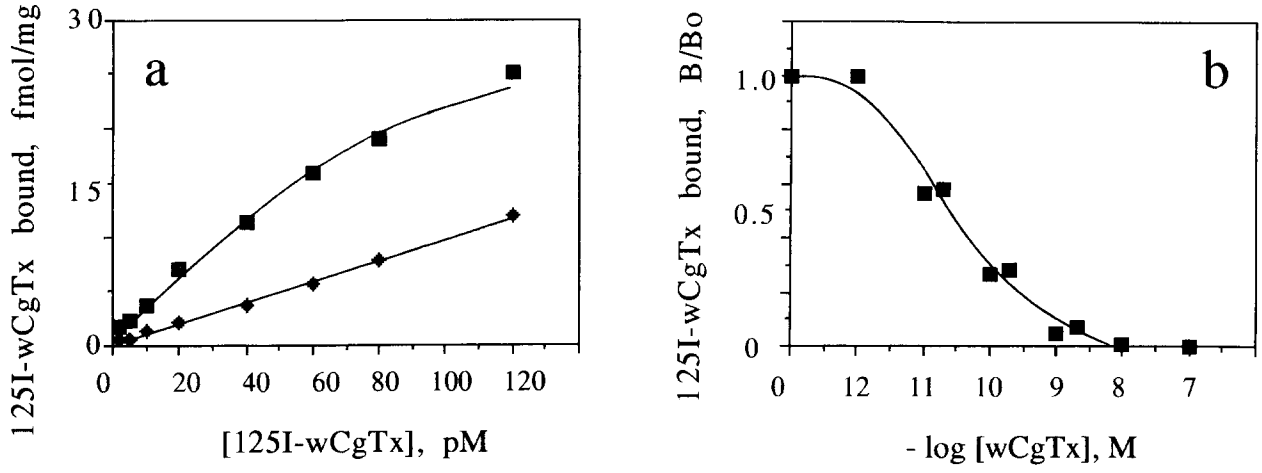

Fig. 1. [^{125}I]ω-Conotoxin binding to small cell lung cancer membranes. SCLC 82 membranes were incubated with (a) a range of [^{125}I]ωCgTx concentrations, in the presence (♦) and in the absence (■) of 0.1 μM unlabeled ωCgTx, or (b) 30 pM [^{125}I]ωCgTx in the absence and in the presence of a range of unlabeled ωCgTx concentrations. After filtration and three washes, bound [^{125}I]ωCgTx was measured by γ-counting.

protein in 0.5 ml of binding buffer (25 mM Tris, 0.15 M NaCl, 0.2% BSA adjusted to pH 7.4 with HCl), were incubated with the indicated concentrations of [^{125}I]ωCgTx ± unlabeled ωCgTx for 60 min at 37°C. Binding was terminated by rapid vacuum filtration over Whatman GF/C filters pretreated with 0.3% polyethyleneimine and 3 washes with 2 ml of ice-cold binding buffer. Bound ligand was measured by γ-counting.

2.4. *Immunoblotting*

Proteins were separated by SDS-PAGE on 10% acrylamide gels, transferred to a nitrocellulose membrane, blocked with 10% low-fat milk in Tris-buffered saline, and probed with the mAbs, 1D12, 10H5, or control mouse IgG (5 mg/ml). ECL detection was carried out with anti-mouse IgG peroxidase.

2.5. *Immunoprecipitation*

Rat brain P2 or SCLC membranes were labeled by incubation with 0.1 nM [^{125}I]ωCgTx, washed by centrifugation and solubilized in 1% CHAPS, 0.32 M sucrose, 10 mM Tris adjusted to pH 7.4 with HCl. After centrifugation at 100,000 × g to eliminate insoluble material, the supernatant was incubated for 4 h at 4°C in the presence of 30 μg of mAb 1D12 of 10H5 in a final volume of 100 μl. Pre-swollen protein A-Sepharose CL-4B (4 mg dry weight) was added and samples were rotated for 1 h at 4°C. The tubes were centrifuged for 2 min at 10,000 × g and pellets were washed in 1 ml 0.4% CHAPS, 25 mM Tris, 0.15 M NaCl, adjusted to pH 7.4 with HCl. Immunoprecipitated radioactivity was then measured by γ-counting. Results were expressed as % immunoprecipitation, taking as 100% the cpm corresponding to the ligand/receptor complex, i.e. the cpm retained on Whatmann GF/B filters pretreated with 0.3% polyethyleneimine.

3. RESULTS AND DISCUSSION

N-Type calcium channels which are involved in excitation/secretion coupling at certain synaptic terminals are selectively blocked by ω-conotoxin GVIA (ωCgTx). N-Type Ca channels were detected in SCLC membranes as receptors for [^{125}I]ωCgTx. A specific binding component was demonstrated from saturation curves (Fig. 1a) and from competition with increasing concentrations of unlabeled ωCgTx (Fig. 1b). [^{125}I]ωCgTx binds irreversibly to its receptor on the Ca channel and conventional Scatchard analysis cannot therefore be used to measure the equilibrium dissociation constant or the receptor capacity [18]. The data presented in Fig. 1a and b indicate 50% receptor occupation at 30 pM ωCgTx. The binding site density estimated from the plateau level of the saturation curve (Fig. 1b) was 13 fmol/mg of protein in membranes of the SCLC 82 line. The expression of [^{125}I]ωCgTx receptors by ten different SCLC lines was examined by binding assays at a single saturating concentration (0.1 nM) of [^{125}I]ωCgTx in the presence and absence of 0.1 μM native ωCgTx (Table I). Although specific binding was

Table I

Co-expression of N-type calcium channels, synaptotagmin and syntaxin in small cell lung cancer lines

Cell line	ω-Conotoxin receptor (fmol/mg/protein)	Synaptotagmin	Syntaxin
SCLC 82	13.7	++	++
SCLC 10	13.6	++	++
SCLC 61	10.1	++	++
SCLC 74B	10	++	+
SCLC 91	9.5	++	+
SCLC 41	8.5	++	+
SCLC 74A	7.9	+	+
SCLC 6	4.7	+	+
SCLC 75	1.5	ND	ND
SCLC 95	ND	ND	ND

The relative intensity of immunoreactive bands in Western blots was scored as ++ or +; ND = not detected.

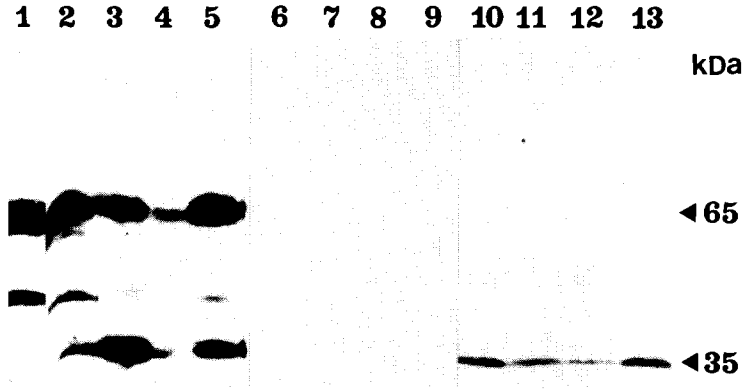

Fig. 2. Expression of synaptotagmin and syntaxin small cell lung cancer. Immunoblots of rat brain synaptosomes (lane 1, 50 μg protein) and SCLC membranes (lanes 2–13, 250 μg protein) were probed with anti-synaptotagmin mAb 1D12 (lanes 1–5), control mouse IgG (lanes 6–9) or anti-syntaxin mAb 10H5. (SCLC 61 = lanes 2, 6 and 10; SCLC 91 = lanes 3, 7 and 11; SCLC 6 = lanes 4, 8 and 12; SCLC 10 = lanes 5, 9 and 13).

clearly detected in 8 out of 10 lines, at similar levels to those reported in human neuroblastoma membranes [14], receptor density was about 50- times less than in rat brain nerve terminals [18].

Western blots of SCLC membranes were probed with monoclonal antibodies against synaptotagmin and syntaxin. A representative experiment is illustrated in Fig. 2. Synaptotagmin was detected as a 65 kDa band in SCLC and rat brain synaptosomes (Fig. 2, lanes 1–5). Immunoreactive bands at approximately 46 and 37 kDa were consistently seen in both tissues. These are thought to be proteolytic fragments, produced either in vivo or during membrane preparation. No immunoreactivity was detected in control experiments with non-immune mouse IgG (Fig. 2, lanes 6–9). The same tumors also expressed syntaxin migrating as a 35 kDa band (Fig. 2, lanes 10–13).

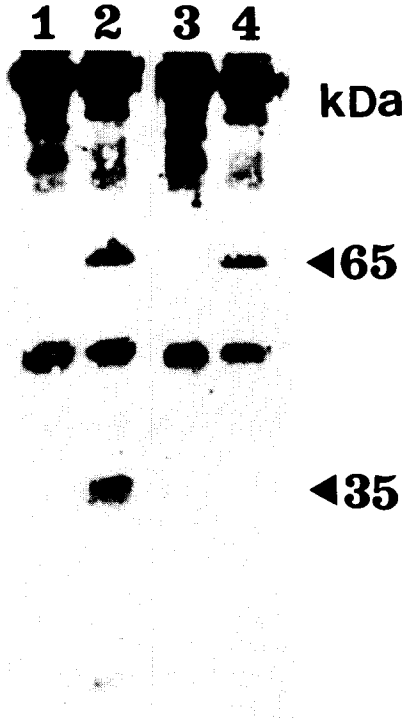

Fig. 3. Association of synaptotagmin with syntaxin-1 in small cell lung cancer. CHAPS extracts of SCLC 82 membranes were incubated with control mouse IgG (lanes 1, 3) or anti-syntaxin mAb 10H5. Immune complexes were recovered on protein A-Sepharose CL4B and the presence of syntaxin and synaptotagmin was examined by probing immunoblots of the immunoprecipitated proteins with mAb 10H5 (lanes 1, 2) and mAb 1D12 (lanes 3, 4). The intense immunoreactivity present in all lanes, both at the entry to the resolving gel and at approximately 50 kDa, corresponds to immunoblogulins used in the immunoprecipitation step that are subsequently detected by the secondary anti-mouse IgG antibody.

Eight out of ten SCLC lines expressed both synaptotagmin and syntaxin. These proteins were not detected in SCLC 75 and 95, which also had the lowest density of [^{125}I]ωCgTx binding sites. These two lines did, however, display some neuroendocrine characteristics, as both contained synaptophysin detected by Western blotting (not shown).

In nerve terminals syntaxin is thought to be involved in docking synaptic vesicles at exocytotic sites via its interaction with synaptotagmin. We therefore examined whether synaptotagmin associates with syntaxin in SCLC by co-immunoprecipitation experiments (Fig. 3). When CHAPS-solubilized SCLC 82 membranes were immunoprecipitated with mAb 10H5, syntaxin (Fig. 3, lane 2) and synaptotagmin (Fig. 3, lane 4) were identified in immunoblots of the recovered proteins. Note that a fraction of syntaxin in Fig. 3, lane 2, migrates at 67 kDa. This band presumably represents a dimeric form of the 35 kDa protein. Syntaxin contains four heptad motifs [6,7] that are thought to be involved in protein–protein interactions and the formation of homo-oligomers, even after SDS denaturation, has been reported [7]. Neither syntaxin nor synaptotagmin were detected in experiments in which the immunoprecipitating antibody was replaced by control mouse IgG (Fig. 3, lanes 1 and 3). Syntaxin can therefore associated with synaptotagmin in SCLC.

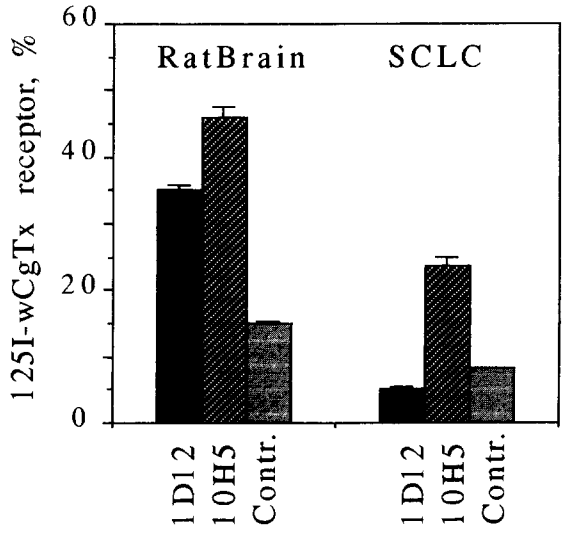

Fig. 4. Association of syntaxin with N-type calcium channels in small cell lung cancer. CHAPS-solubilized [^{125}I]ωCgTx-labeled calcium channels from SCLC 82 membranes were incubated with monoclonal anti-synaptotagmin (1D12), and anti-syntaxin (10H5) antibodies and control mouse IgG. Immune complexes were recovered on protein A-Sepharose, and precipitated [^{125}I]ωCgTx receptors were measured by γ-counting (± S.E.M., n = 3).

Immunoprecipitation experiments with solubilized [^{125}I]ωCgTx receptors from rat brain membranes have shown that a population of N-type calcium channels forms a ternary complex with synaptotagmin and syntaxin [9,10]. We therefore asked whether mAbs 1D12 and 10H5 immunoprecipitate N-type calcium channels from SCLC 82 membranes. Specific immunoprecipitation of a fraction of CHAPS-solubilized N-type calcium channels (about 20%) was consistently observed with anti-syntaxin antibodies but not with anti-synaptotagmin antibodies (Fig. 4). Low receptor density and solubilization yield in SCLC membranes imposed the use of relatively low concentrations of labeled antigen in these assays. Signal-to-background ratio was consequently poor, which may preclude detection of low levels of specific immunoprecipitation, however, parallel assays, performed with [^{125}I]ωCgTx receptors solubilized from synaptic membranes and diluted to the same antigen concentration, did indicate significant immunoprecipitation by both mAbs 1D12 and 10H5 (Fig. 4). Although we have not directly demonstrated the presence of ternary complex, the data presented in Figs. 3 and 4 allow us to conclude that both synaptotagmin and N-type calcium channels can associate with syntaxin in SCLC membranes.

Recent evidence suggests that in nerve terminals synaptotagmin and syntaxin may play a role in locating synaptic vesicles near to the voltage-gated calcium channels that control transmitter release [5,7,8]. Synaptotagmin is a transmembrane synaptic vesicle protein that is a putative calcium sensor in exocytosis [20,21]. Synaptotagmin associates with N-type calcium channels [5] in a complex with syntaxin [7,8], and this interaction may locate vesicles in a zone accessible to rapid calcium transients.

The detection of these key components of the neurosecretory pathway in SCLC may be relevant to certain paraneoplastic autoimmune diseases of the nervous system. Patients with LEMS show reduced acetylcholine release as a consequence of the production of autoantibodies directed against calcium channel-associated proteins [16,22]. We have recently reported that some LEMS IgG contain an anti-synaptotagmin specificity [5]. The intravesicular N-terminal domain of synaptotagmin is exposed at the cell surface during exocytosis, and LEMS IgG may inhibit neurotransmitter release by binding to a synaptotagmin/syntaxin/calcium channel complex via this region. Our present results demonstrate that a large number of SCLC lines co-express synaptotagmin, syntaxin, and N-type calcium channels. Furthermore, syntaxin interacts with synaptotagmin and associates with calcium channels, as described in synaptic terminals. These findings are compatible with the hypothesis that the immune response in some LEMS patients is directed against synaptotagmin expressed by SCLC.

The production of autoantibodies against components of the secretory pathway may have other implications. Many patients with paraneoplastic neurological disease of autoimmune origin show extended survival, and regression of SCLC has been documented [23]. The antibodies that produce the neurological syndrome may therefore have anti-tumoral activity. SCLC growth is sustained by autocrine secretion of neuropeptides, such as bombesin/gastrin releasing peptide [15]. An interesting possibility to explore is that autoantibodies that inhibit exocytosis in the nervous system by binding to synaptotagmin and/or calcium channels, may also interfere with the autocrine secretory pathway in the tumor itself.

Acknowledgements: We thank Cecile Raymond for technical assistance and Dr. S.C. Fujita for the gift of 171B5 antibody. This research was funded by grants from Bayer Pharma, the Association Française contre les Myopathies, and the Assistance Publique de Marseille.

REFERENCES

[1] Almers, W. and Tse, F.W. (1990) Neuron 4, 813–818.
[2] Smith, S.J. and Augustine, G.J. (1988) Trends Neurosci. 11, 458–464.
[3] Kelly, R.B. (1993) Cell 72, Neuron 10 (Suppl.) 43–53.
[4] Takahashi, M., Arimatsu, Y., Fujita, S., Fujimoto, Y., Kondo, S., Hama, T. and Miyamoto, E. (1991) Brain Res. 551, 279–292.
[5] Leveque, C., Hoshito, T., David, P., Shoji-Kassai, Y., Leys, K., Omori, A., Lang, B., El Far, O., Sato, K., Martin-Moutot, N., Newsom-Davis, J., Takahashi, M. and Seagar, M.J. (1992) Proc. Natl. Acad. Sci. USA 89, 3625–3629.
[6] Inoue, A. and Akagawa, K. (1992) J. Biol. Chem. 267, 10613–10619.

[7] Yoshida, A., Oho, C., Omori, A., Kuwahara, R., Ito, T. and Takahashi, M. (1992) J. Biol. Chem. 267, 24925–24928.
[8] Bennet, M.K., Calakos, N. and Scheller, R. (1992) Science 257, 255–259.
[9] Morita, T., Mori, H., Sakimura, K., Mishina, M., Sekine, Y., Tsugita, A., Odani, S., Horikawa, H., Saisu, H. and Abe, T. (1992) Biomed. Res. 13, 357–364.
[10] Carney, D.N. (1992) Lancet 339, 843–846.
[11] Aletsee-Ulfrecht, M.C., Langley, K., Rotsch, M., Havemann, K. and Gratzl, M. (1990) FEBS Lett. 267, 295–300.
[12] Roberts, A., Perera, S., Lang, B., Vincent, A. and Newsom-Davis, J. (1985) Nature 317, 737–739.
[13] De Aizpurua, H.J., Lambert, E.H., Griesmann, G.E., Olivera, B.M. and Lennon, V.A. (1988) Cancer Res. 48, 4719–4724.
[14] Oguro-Okana, M., Griesmann, G.E., Wieben, E.D., Slaymaker, S.J., Snutch, T.P. and Lennon, V.A. (1992) Mayo. Clin. Proc. 67, 1150–1159.
[15] Cuttitta, F., Carney, D.N., Mulshine, J., Moody, T.W., Fedorko, J., Fischler, A. and Minna, J.D. (1985) Nature 316, 823–826.
[16] Vincent, A., Lang, B. and Newsom-Davis, J. (1989) Trends Neurosci. 12, 496–502.
[17] Thomas, F., Arvelo, F., Antoine, E., Jacrot, M. and Poupon, M.F. (1992) Cancer Res. 52, 4872–4877.
[18] Marquèze, B., Martin-Moutot, N., Levêque, C. and Couraud, F. (1988) Mol. Pharmacol. 34, 87–90.
[19] Sher, E., Pandiella, A. and Clementi, F. (1988) FEBS Lett. 235, 178–182.
[20] Perin, M.S., Fried, V.A., Mignery, G.A., Jahn, R. and Sudhof, T.C. (1990) Nature 345, 260–263.
[21] Brose, N., Petrenko, A.G., Sudhof, T.C. and Jahn, R. (1992) Science 256, 1021–1025.
[22] Sher, E., Gotti, C., Canal, N., Scopetta, C., Piccolo, G., Evoli, A. and Clementi, F. (1989) Lancet ii, 640–643.
[23] Darnell, R.B. and De Angelis, L.M. (1993) Lancet 341, 21–22.