

## The Synaptic Vesicle-associated Protein Amphiphysin Is the 128-kD Autoantigen of Stiff-Man Syndrome with Breast Cancer

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### Summary

Stiff-Man syndrome (SMS) is a rare disease of the central nervous system (CNS) characterized by progressive rigidity of the body musculature with superimposed painful spasms. An autoimmune origin of the disease has been proposed. In a caseload of more than 100 SMS patients, 60% were found positive for autoantibodies directed against the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD). Few patients, all women affected by breast cancer, were negative for GAD autoantibodies but positive for autoantibodies directed against a 128-kD synaptic protein. We report here that this antigen is amphiphysin. GAD and amphiphysin are nonintrinsic membrane proteins that are concentrated in nerve terminals, where a pool of both proteins is associated with the cytoplasmic surface of synaptic vesicles. GAD and amphiphysin are the only two known targets of CNS autoimmunity with this distribution. This finding suggests a possible link between autoimmunity directed against cytoplasmic proteins associated with synaptic vesicles and SMS.

**S**tiff-Man syndrome (SMS) is a rare human central nervous system (CNS) disease, characterized by chronic rigidity of the body musculature with superimposed painful spasms (1). It is thought to result from an impairment of inhibitory pathways that control motor neuron activity (2–4). It was previously suggested that SMS may have an autoimmune pathogenesis. 60% of the patients affected by this condition are positive in their serum and cerebrospinal fluid (CSF) for autoantibodies directed against the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) (5–8). In our current caseload of 119 patients referred to us with a clinical diagnosis of SMS, 72 were found positive for these autoantibodies (Guarnaccia, J., M. Solimena, K. Marek, and P. De Camilli, unpublished observations). In the SMS patient subpopulation positive for GAD autoantibodies, a frequent occurrence of insulin-dependent diabetes mellitus and of other organ-specific autoimmune diseases is observed (6, 7).

We have recently described three female patients with SMS

and breast cancer and no signs of organ-specific autoimmune diseases. These three patients (referred to henceforth as patients BC-SMS 1, 2, and 3, respectively) were negative for GAD autoantibodies but were positive for autoantibodies directed against a 128-kD neuronal antigen concentrated at synapses (9). We have now identified a fourth patient with both breast cancer and SMS syndrome. This patient (BC-SMS 4), as well, was positive for autoantibodies to the 128-kD protein (this study). The characterization of this autoantigen is crucial to the elucidation of pathogenetic mechanisms in SMS with breast cancer.

Recently, a novel synaptic vesicle-associated protein, amphiphysin, was described (10). This protein was identified by screening a chicken brain  $\lambda$ gt11 library with antibodies raised against chicken brain synaptic proteins. Amphiphysin, an acidic protein present in chicken as well as in mammalian nervous tissue, was shown by immunocytochemistry to be concentrated in nerve terminals. A large fraction of am-

piphysin is present in cytosolic fractions of brain homogenates but a pool of the protein is recovered in a tightly bound form in highly purified synaptic vesicles, although the protein is not enriched on these organelles (10). Some of the properties of amphiphysin were reminiscent of the properties of the 128-kD antigen (10). Therefore, a detailed comparison of the characteristics of the two proteins was performed. We report here that the 128-kD antigen is amphiphysin.

## Materials and Methods

**Materials.** Three sera of SMS patients affected by breast cancer were previously described (9). These sera, defined BC-SMS 1, 2, and 3, correspond to sera of patients 1, 2, and 3, respectively, of reference 9. Serum BC-SMS 4 is from a new patient with both conditions. A GAD autoantibody-positive human serum is from our caseload of SMS sera (6, 8). Rabbit polyclonal antibodies directed against a  $\beta$ -galactosidase chicken amphiphysin fusion protein were previously described (10). These antibodies were shown to recognize rat amphiphysin (10). Rabbit sera directed against synaptophysin (11) and GAD (serum no. 6799 (12)) were the kind gift of Dr. R. Jahn (Yale University, New Haven, CT), and Drs. Z. Katarova and G. Szabo (Hungarian Academy of Sciences, Szeged, Hungary), respectively. Small-scale lysates of bacteria expressing  $\beta$ -galactosidase fusion proteins were prepared according to reference 13.  $\beta$ TC3 and  $\alpha$ TC9 cell lines were the kind gift of Drs. D. Hanahan (University of California, San Francisco, CA), S. Efrat (Albert Einstein College of Medicine, New York, New York), and E. Leiter (Jackson Laboratories, Bar Harbor, ME), and PC12 cells were the gift of Dr. L. Greene (Columbia University, New York).

**Western Blotting of One- and Two-dimensional Gels.** Total rat brain homogenate was prepared as described (6, 14). Postnuclear supernatants of rat tissues and of cell lines were prepared by homogenization in 10 vol of ice-cold 10 mM Hepes buffer, pH 7.4, containing freshly added protease inhibitors (0.1 mM PMSF, 1  $\mu$ g/ml each of antipain, leupeptin, aprotinin, and pepstatin A) followed by centrifugation at 1,000 *g* for 10 min at 4°C. For two-dimensional gels, this supernatant (S1) was centrifuged at 170,000 *g* for 2 h at 4°C and the resulting supernatant was analyzed by the procedure described by O'Farrell et al. (15) and modified by Ames and Nikaico (16). Western blotting was performed as described (6, 14) using <sup>125</sup>I-protein A. Patient sera were used at the dilution of 1:250.

**Cell Fractionation and Triton X-114 Phase Separation.** A postnuclear supernatant of rat brain (S1) was prepared as described above. S1 was centrifuged at 36,000 *g* for 30 min at 4°C. The resulting supernatant (S2) was separated into particulate (P3) and cytosolic (S3) fractions by centrifugation at 170,000 *g* for 2 h at 4°C. P3 and S3 were extracted in 2% Triton X-114, and insoluble material was removed by centrifugation at 20,000 *g* for 30 min at 4°C. The soluble material was separated into detergent (D) and aqueous (A) phases as described (17). Volumes loaded in each lane were normalized so that corresponding aliquots of supernatants and pellets (or detergent and aqueous phases) were loaded for each pair of fractions.

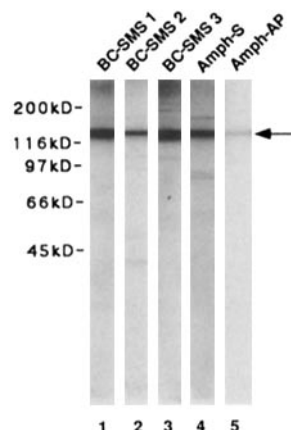
**Immunoprecipitation.** A soluble fraction of rat brain (S3) prepared as described above was extracted in ice-cold 2% Triton X-100, 150 mM NaCl, 10 mM Hepes, pH 7.4, for 2 h. Insoluble material was removed by centrifugation at 20,000 *g* for 30 min at 4°C. The resulting supernatant was diluted with an equal volume of 150 mM NaCl, 10 mM Hepes, pH 7.4 (buffer A), to a final protein concentration of 1 mg/ml. 900- $\mu$ l aliquots of extract were precleared as described (5) and used for each immunoprecipitation. For immu-

noprecipitation, the following additions were made in sequential order: (a) 25  $\mu$ l human sera (16 h); (b) 20  $\mu$ l rabbit anti-human IgGs (1.5 h); and (c) 125  $\mu$ l 50% protein A-Sepharose in buffer A (1.5 h). All incubations were performed at 4°C with rotation. Immunoprecipitated material was recovered and analyzed by Western blotting as described (14).

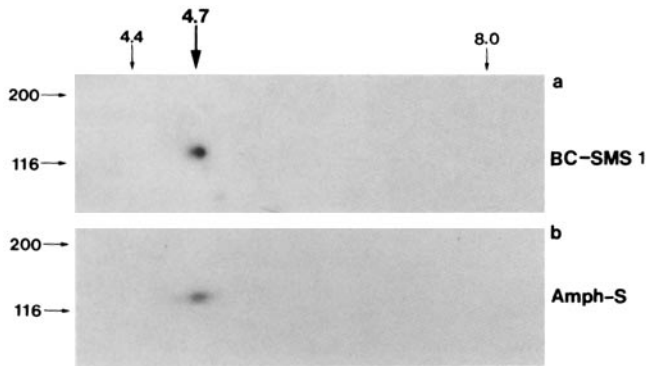
## Results and Discussion

Fig. 1 shows Western blots of total rat brain homogenate demonstrating that the 128-kD antigen, i.e., the protein recognized by the sera of BC-SMS patient (lanes 1–3), has the same electrophoretic mobility in SDS-PAGE as the protein recognized by rabbit serum (lane 4) and affinity-purified antibodies (lane 5) raised against a chicken amphiphysin fusion protein. Chicken amphiphysin was calculated to have a molecular mass of 75,204 daltons, but in SDS-PAGE runs, it had an apparent molecular mass of 115–125 kD (10). This molecular mass is very similar to the one of 128 kD estimated here for the rat protein. Fig. 2 shows that the 128-kD antigen and amphiphysin have an identical isoelectric point of 4.7 in two-dimensional gels. This isoelectric point is similar to the predicted isoelectric point of 4.4, calculated on the basis of the amino acid composition of chicken amphiphysin (10).

The distribution of the 128-kD antigen and of amphiphysin were compared in the course of subcellular fractionation of brain homogenates. As shown in Fig. 3, *a* and *b*, both the autoantigen and amphiphysin were present in roughly equal amounts in a cytosolic fraction (S3) and in a particulate fraction (P3) obtained from rat brain. Synaptophysin, an intrinsic membrane protein of synaptic vesicles, was present exclusively in the particulate fraction (P3) (Fig. 3 *c*), demonstrating that the centrifugation conditions used were sufficient to sediment all membranes. Membrane-bound amphiphysin was previously shown to be recovered in the aqueous phase after Triton X-114 extraction and phase separation (10). As shown in Fig. 3, *a* and *b*, both the 128-kD antigen and amphiphysin were completely solubilized by Triton X-114 and were recovered exclusively in the aqueous phase irrespectively of whether the extraction was performed on P3 or S3 (Fig. 3, *a* and *b*). The effectiveness of phase separation conditions was confirmed by the presence of the bulk of synaptophysin (a protein with



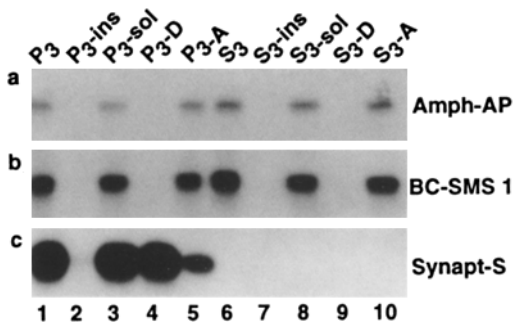
**Figure 1.** The 128-kD antigen and amphiphysin have identical electrophoretic mobilities in SDS gels. Western blots of total rat brain homogenate probed with sera of three BC-SMS patients and rabbit anti-amphiphysin antibodies (lane 4, crude serum [*Amph-S*]; lane 5, affinity-purified antibodies [*Amph-AP*]). The 128-kD antigen is indicated by an arrow. The single band of amphiphysin and of the 128-kD antigen visible in this gel can be resolved in a doublet of two closely spaced bands under different electrophoresis conditions (9, 10).



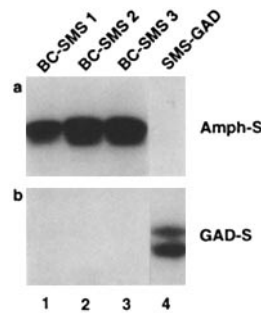
**Figure 2.** The 128-kD antigen and amphiphysin have identical electrophoretic mobilities in two-dimensional gels. Western blot of two-dimensional gels of a soluble rat brain fraction probed with BC-SMS 1 serum (a) and rabbit anti-amphiphysin serum (b). Numbers at the top indicate isoelectric points, numbers at the left indicate molecular mass markers (in kD).

four transmembrane regions (18)) in the detergent phase obtained from P3 (Fig. 3 c).

The 128-kD antigen and amphiphysin were found to be immunologically cross-reactive. To prove that anti-amphiphysin antibodies recognize the 128-kD antigen, BC-SMS patient sera were used to immunoprecipitate the 128-kD antigen from soluble extracts of rat brain. The presence of amphiphysin in the immunoprecipitate was then analyzed by Western blotting using anti-amphiphysin serum. An immunoreactive band with electrophoretic mobility of 128 kD was detected (Fig. 4 a, lanes 1–3) while no GAD was recovered in the same immunoprecipitates (Fig. 4 b, lanes 1–3) confirming previous

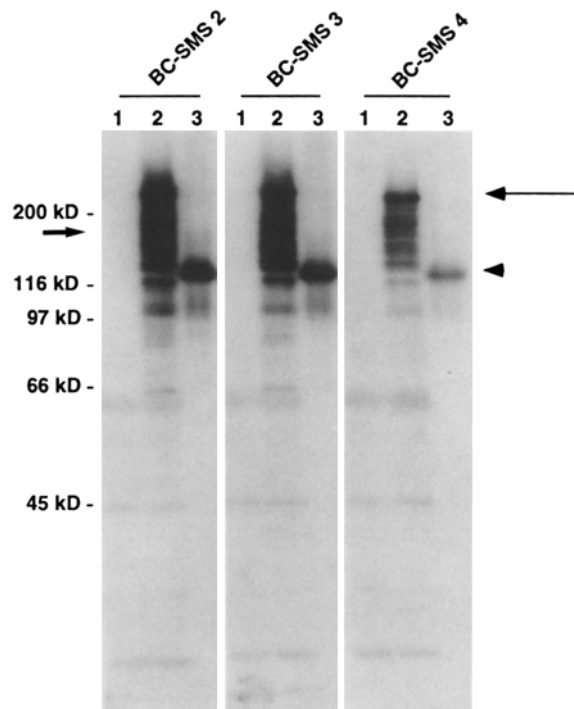


**Figure 3.** The 128-kD antigen and amphiphysin partition similarly in differential centrifugation and Triton X-114 phase separation. Particulate (P<sub>3</sub>) and cytosolic (S<sub>3</sub>) fractions of rat brain were extracted in Triton X-114 and centrifuged to obtain a soluble (sol) and an insoluble (ins) fraction. The Triton X-114-soluble material (lanes 3 and 8) was separated into detergent (D; lanes 4 and 9) and aqueous (A; lanes 5 and 10) phases. The fractions were probed by immunoblotting with anti-amphiphysin affinity-purified antibodies (Amph-AP), with BC-SMS 1 serum (BC-SMS 1), and with antisynaptophysin serum (Synapt-S). The 128-kD antigen and amphiphysin were recovered in both the cytosolic (S<sub>3</sub>) and in the particulate (P<sub>3</sub>) fractions, and exclusively in the Triton X-114 aqueous phase derived from these fractions. Synaptophysin, which is an integral membrane protein with four transmembrane spanning domains, is recovered only in P<sub>3</sub> and primarily in the detergent phase derived from the Triton X-114-soluble material obtained from P<sub>3</sub>.

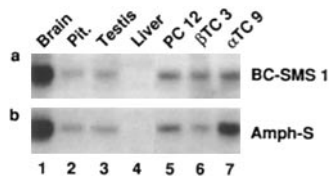


**Figure 4.** BC-SMS patient sera immunoprecipitate amphiphysin from rat brain extracts. Immunoprecipitates obtained with the sera of three BC-SMS patients (lanes 1–3) and with the serum of a SMS patient positive for GAD autoantibodies (SMS-GAD) (lane 4) were separated by SDS-PAGE. (a) Immunoblotted with rabbit anti-amphiphysin serum (Amph-S); and (b) immunoblotted with the rabbit anti-GAD serum no. 6799 (GAD-S) (12).

results (9). As a control, immunoprecipitation was performed with a SMS patient serum that contained high-titer anti-GAD antibodies (6, 8), but no antibodies against the 128-kD antigen. This immunoprecipitate was positive for GAD (Fig. 4 b, lane 4), but not for amphiphysin (Fig. 4 a, lane 4). Conversely, a protein of 128 kD was detected when an immunoprecipitate obtained with anti-amphiphysin serum was immunoblotted with BC-SMS patient sera (not shown). To



**Figure 5.** BC-SMS patient sera recognize recombinant amphiphysin. Western blots of three identical gel triplets reacted with sera BC-SMS 2, 3, and 4, as indicated. Lanes 1, bacterial lysate expressing a 165-kD fusion protein of  $\beta$ -galactosidase with a yet unidentified synaptic protein (clone 10.12.1; B. Lichte, and M.W. Kilimann, unpublished observation). Lanes 2, bacterial lysate expressing a  $\beta$ -galactosidase-chicken amphiphysin fusion protein (clone amphy-11.3; fusion protein size  $\sim$ 230 kD) (10). Lanes 3, chicken brain total homogenate. Identical results were obtained with serum BC-SMS 1 (not shown). Long and short arrows indicate the mobilities of the chicken amphiphysin fusion protein and of the control fusion protein, respectively. Bands below the major immunopositive bands of lanes 2 most likely represent proteolytic fragments of the fusion protein. An arrowhead points to chicken brain amphiphysin.



**Figure 6.** The 128-kD antigen and amphiphysin have the same tissue distribution. Western blot of postnuclear supernatants of rat tissues (brain, pituitary, testis, liver) and endocrine cell lines (PC12 cells [rat chromaffin cell line];  $\beta$ TC3 cells [mouse insulinoma];  $\alpha$ TC9 [mouse glucagonoma]) probed with either BC-SMS patient serum (BC-SMS 1) or anti-amphiphysin serum (*Amph-S*).

conclusively demonstrate that BC-SMS sera recognize amphiphysin, they were tested against bacterial lysates containing recombinant amphiphysin as shown in Fig. 5. BC-SMS sera labeled a  $\beta$ -galactosidase–chicken amphiphysin fusion protein (Fig. 5, lanes 2) and chicken brain amphiphysin (Fig. 5, lanes 3), but not a control fusion protein (Fig. 5, lanes 1).

Amphiphysin was previously shown to have a restricted tissue distribution (10). Amphiphysin mRNA was detected at high levels in brain and at lower levels in adrenal gland. By immunoblotting amphiphysin was detected in the anterior and posterior pituitary as well (10). The expression of amphiphysin was compared with that of the 128-kD antigen in a variety of tissues and cell lines using Western blotting. Fig. 6 shows that the serum of a BC-SMS patient and the rabbit serum directed against amphiphysin label a similar protein in brain, pituitary, and cell lines derived from adrenal chromaffin cells (PC12 cells) and from pancreatic  $\alpha$  and  $\beta$  cells ( $\alpha$ TC9,  $\beta$ TC3) (19, 20) but not in liver. Presence of amphiphysin in all cells of pancreatic islets was confirmed by immunocytochemistry (not shown). Endocrine cells of the anterior pituitary, adrenal medulla, and pancreatic islets have many biochemical and functional similarities to neurons and contain organelles closely related to neuronal synaptic vesicles (21, 22). In addition, both amphiphysin and the 128-kD antigen were detected in the testis (Fig. 6). By immunocytochemistry with BC-SMS sera, amphiphysin immunoreactivity in the testis was found to be confined to germ cells. All germ cells expressed amphiphysin irrespective of their stage of differentiation (from spermatogonia to mature spermatozoa) (not shown). Previously, amphiphysin mRNA could not be detected in chicken testis (10), but this was probably so because the RNA had been purified from immature testis of 7-d-old animals. It should be noted that GAD as well is expressed in male germ cells (23).

These data convincingly demonstrate that the 128-kD antigen is amphiphysin. Amphiphysin contains in its sequence a hydrophobic 21-amino acid region that would be competent to form a transmembrane helix (10). However, the recovery of amphiphysin both in soluble and membrane fractions (10) as well as the presence of a pool of the protein detectable by immunocytochemistry throughout the neuronal cytoplasm (9) indicate that amphiphysin is not an intrinsic membrane protein. Like amphiphysin, GAD, the other major autoantigen of SMS, is concentrated in nerve terminals where it interacts with synaptic vesicles but is not an intrinsic membrane protein (14, 24–26). While GAD is expressed only by GABA-secreting neurons, amphiphysin is not restricted to these neurons (9, 10).

The similar subcellular localization of GAD and amphiphysin is intriguing if one considers that they are the only two known targets of CNS autoimmunity with this distribution. This observation raises the possibility that pathogenetic mechanisms in SMS may be linked to CNS autoimmunity directed against presynaptic components that interact with synaptic vesicles. Autoantibodies directed against GAD and amphiphysin are not likely to be directly pathogenetic because antibodies are not thought to have access to the cytoplasmic compartment. They may represent the dominant autoantibody species in the context of an autoimmune response directed against multiple antigens (27), including proteins exposed at the cell surface that may be the pathogenetic targets of the autoimmune response. Alternatively, these autoantibodies may reflect an autoimmune reaction in which T cells are the primary players. GAD and amphiphysin autoantibodies segregate with two SMS patient subpopulations, each characterized by different associated diseases. GAD autoantibody-positive SMS is often associated with organ-specific autoimmune diseases and primarily insulin-dependent diabetes mellitus (3–7). Amphiphysin autoantibody-positive SMS occurs only in association with cancer (9) and has the characteristics of an autoimmune paraneoplastic syndrome (28, 29). Therefore, the two patterns of autoimmunity are likely to be related to these different associated conditions. Further studies of anti-amphiphysin autoimmunity and of the function of amphiphysin or amphiphysin-related molecules not only may shed some light on pathogenetic mechanisms in SMS, but may also help in the elucidation of the biology of at least some types of cancer. Although amphiphysin was not detected in breast cancer tissue (9), it will be of interest to determine whether amphiphysin-related proteins are expressed in breast cancer.

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