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Autoimmunity in Stiff-Man Syndrome with breast cancer is targeted to the C-terminal region of human amphiphysin, a protein similar to the yeast proteins, Rvs167 and Rvs161

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Abstract

Amphiphysin, a neuronal protein first identified in chicken synaptic membranes, is the autoantigen of Stiff-Man Syndrome (SMS) associated with breast cancer. We have now cloned human amphiphysin and found the N- and C-terminal domains of the protein to be highly conserved between chicken and human. Patient autoantibodies have a distinct pattern of reactivity with amphiphysin, and the dominant autoepitope is located in its C-terminal region, which contains an SH3 domain. Portions of chicken and human amphiphysin are also homologous to portions of Rvs167 and Rvs161, two yeast proteins which are involved in cell entry into stationary phase upon exposure to unfavourable growth conditions.

Key words: Stiff-Man Syndrome; Amphiphysin; Breast cancer; Synaptic vesicle; Rvs161; Rvs167

1. Introduction

Stiff-Man Syndrome (SMS) is a rare neurological disease characterized by rigidity of the body musculature with superimposed painful spasms [1–4]. SMS is one of the few human diseases for which evidence of an autoimmune process directed against CNS neuronal antigens has been found. High titer antibodies directed against neuronal autoantigens are found both in the serum and in the cerebrospinal fluid of the majority of SMS patients [5].

Two main targets of humoral autoimmunity have been identified in two populations of SMS patients which have similar neurological characteristics but different associated conditions. In 50–60% of the cases, autoantibodies are primarily directed against the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD). In these patients, insulin-dependent diabetes mellitus and other organ-specific autoimmune diseases are frequently present [5–7].

In five of the more than 100 cases of SMS patients whose sera we have tested, autoantibodies are directed against another neuronal protein of 128 kDa [8]. This protein was recently identified as amphiphysin [9], a synaptic vesicle-associated protein originally cloned from chicken brain [10]. Strikingly, all five patients with amphiphysin autoimmunity are women with breast cancer. In fact, in two of these patients, an infiltrating ductal adenocarcinoma of the breast was searched for, and found, only after the identification of the anti-amphiphysin antibodies ([8] and our most recent case (patient 1 of this study – see section 2)). A remission of the neurological symptoms was documented in three of the five patients after removal of the cancer and steroid therapy ([8,9,11] and this study (Dr. M. Myers, Jackson, MI, personal communication)) supporting the hypothesis that the condition results from a functional rather than structural damage of the CNS. Other cases of SMS associated with cancer have been described [12,13]. These findings raise the possibility that in some cases SMS may have an autoimmune paraneoplastic origin. As in the case of autoantigens of other autoimmune paraneoplastic diseases of the CNS, as well as of GAD, amphiphysin is an intracellular protein and the link between humoral autoimmunity directed against the autoantigen and the clinical symptoms remains to be explained.

In other neurological autoimmune paraneoplastic conditions, expression of the neuronal autoantigen in the tumor was reported [14,15]. It was proposed that the ectopic expression of the brain antigen by cancer cells may trigger the autoimmune response [16]. In some cases, the autoantigen is thought to play a role in neoplastic growth [17]. As a first step to investigate mechanisms of amphiphysin autoimmunity and a possible role of amphiphysin in the biology of human breast cancer, we have now cloned human amphiphysin and mapped the autoepitopes. By Western blotting, a stereotypic humoral autoimmune response to amphiphysin was detected similarly to what we have previously shown for

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Abbreviations: a.a., amino acid; cAmph, chicken amphiphysin; CNS, central nervous system; GAD, glutamic acid decarboxylase; hAmph, human amphiphysin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SMS, Stiff-Man Syndrome.

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GAD autoimmunity in SMS [18]. The region of amphiphysin most highly conserved between chicken and human is also similar to two yeast proteins, Rvs161 [19] and Rvs167 [20] which are implicated in the transition from exponential cell growth to stationary phase upon exposure to nutrient starvation. The similarity of amphiphysin to yeast proteins which participate in stationary phase adaptation suggests the possibility that amphiphysin, or a closely related protein, has a role in the biology of breast cancer.

2. Materials and methods

2.1. Human sera

A new serum, belonging to a 75-year-old woman (patient 1) with SMS was referred to us (Dr. S. Songcharden, Dr. M. Myers, Jackson, MI) to be tested for the presence of anti-neuronal antibodies. The detection of anti-amphiphysin autoantibodies prompted a search for an occult breast cancer. A small ductal adenocarcinoma was found and surgically removed. Sera from four patients with SMS and breast cancer were previously described (patients 2–5) [8,9]. Control sera were from healthy subjects.

2.2. DNA manipulations

Molecular biological procedures were performed according to standard protocols [21]. Synthetic oligonucleotides were synthesized (Keck Biotechnology Resource Laboratory, Yale University) in order to amplify via PCR from a λZAP chicken brain cDNA library (courtesy of M. Bartkiewicz and R. Baron, Yale University) two fragments of chicken amphiphysin [10] corresponding to nucleotides 75–341 and 777–1384 (nt75–341, nt777–1384). [α-^{32}P]dATP was incorporated into purified PCR products via primer-direct labeling as described by Bogue [22] and used as probes at 2×10^6 cpm/ml for Northern blots and 10^5 cpm/ml for library screening under relatively high stringency conditions (hybridization – 50% formamide, 6 × SSC, 0.1% SDS, 2 × Denhardt's, 100 mg/ml salmon sperm DNA, 37°C, 20 h; wash – 2 × SSC, 0.05% SDS, 45°C).

1.5×10^6 plaques of a λgt11 human cerebellar cDNA library (random- and oligo(dT)-primed; Clontech, Palo Alto, CA) were screened with nt777–1384. Phage cDNA inserts of positive clones were purified and sequenced [23]. Sequence data, hydrophilicity, and antigenicity profiles were compiled and analyzed using MacVector (IBI Ltd., Cambridge, UK) and MacDNASIS Pro (Hitachi, San Bruno, CA) software. BESTFIT [24], BLAST [25] and PILEUP [26] analyses were programs of the Genetics Computer Group (Madison, WI). Secondary structure predictions were done by the Self Optimized Prediction Method [27].

2.3. Preparation of fusion proteins

The fragment corresponding to nt 89–2377 of clone 22–2 was ligated into *Sma*I-*Eco*RI sites of pGEX-2T vector (Pharmacia, Piscataway, NJ) to generate a glutathione *S*-transferase (GST)/full-length human amphiphysin fusion protein. Clone 22–2 was used as a cDNA template in PCR reactions with Vent$_R$ DNA polymerase (New England Biolabs, Beverly, MA), to obtain the amphiphysin fragments encoding amino acids (a.a) 1–161, 132–291, 262–435, 411–581, and 545–695 (designated I–V, see Fig. 4A). *Sma*I and *Eco*RI sites were added to 5′ and 3′ ends of the fragments respectively to allow unidirectional subcloning into the polylinker region of pGEX-2T. The sequences of all GST-amphiphysin fusion constructs were confirmed by DNA sequencing. The fusion proteins were produced in DH5a cells and purified on a glutathione-Sepharose 4B column (Pharmacia, Piscataway, NJ) essentially as described [28]. Constructs yielded recombinant proteins composed of GST (~28 kDa) fused to the N-terminus of the corresponding amphiphysin fragment.

2.4. Miscellaneous procedures

SDS-PAGE of 5–16% gradient gels and Western blotting were performed essentially as described by Laemmli [29] and Towbin [30], respectively. Western blots with patient sera was performed as described [18].

A serum directed against human amphiphysin (CD6) was obtained by injecting a rabbit with GST-human amphiphysin fusion protein (1 mg) which had been purified on a GTH-Sepharose column followed by preparative SDS-PAGE.

3. Results and discussion

In order to clone human amphiphysin, two fragments of chicken amphiphysin [10], nt75–341 and nt777–1384, encoding for amino acids (a.a.) 1–81 and 228–430, respectively, were generated and tested for reactivity in Northern blot before proceeding to screen a λgt11 human cerebellar cDNA library. As expected, both fragments hybridized very strongly to a band of approximately 4.5 kb in chicken brain poly(A)$^+$ mRNA. They also labeled more weakly a band of similar size when tested under high stringency conditions on rat brain polyA+ mRNA (data not shown).

The nt777–1384, corresponding to the central region of chicken amphiphysin [10], was then used as a probe to screen 1.5×10^6 plaques of a λgt11 human cerebellar cDNA library. Eighteen positive clones were isolated, four of which (designated 22–2, 24, 27 and 34) were also positive when hybridized with the chicken fragment nt75–341, which encompassed the N-terminal. Sequence analysis revealed that clone 22–2 contained an ~2.4 kb insert, which had an open reading frame of 2088 nt (nt 111–2198) encoding a protein of 695 a.a. (Fig. 1, top line). The nucleotide sequence encoding the putative protein was 73% identical to the nucleotide sequence of chicken amphiphysin. The sequence surrounding the first ATG (gcagccatgg), at position 111 of clone 22–2, conformed very well to the initiation consensus sequence as defined by Kozak [31]. No polyadenylation signal was detected at the 3′ end of the clone (nt2199–2377). However, a stretch of 10 A's was found (nt2365–2374), suggesting that internal annealing of the oligo(dT) primer occurred at this site. Clones 24, 27 and 34 were identical in sequence to smaller portions of clone 22–2 except for clone 27 which differed by 20 nucleotides in the 5′ noncoding region. Whether this represents a cloning artifact or evidence of a transcript alternatively spliced at the 5′ region, remains to be determined.

The predicted molecular weight (76.25 kDa) of the protein encoded by clone 22–2 was considerably smaller than the apparent molecular weight of rat amphiphysin in SDS-PAGE gels which is approximately 128 kDa [8,9]. However, a similar aberrant electrophoretic mobility was previously reported for chicken amphiphysin [10]. Injection of a GST-human amphiphysin fusion protein in rabbits elicited the production of antibodies which reacted very strongly with the 128 kDa antigen recognized by patient sera in brain tissue (Fig. 2). Additionally, the GST-fusion protein had an apparent molecular

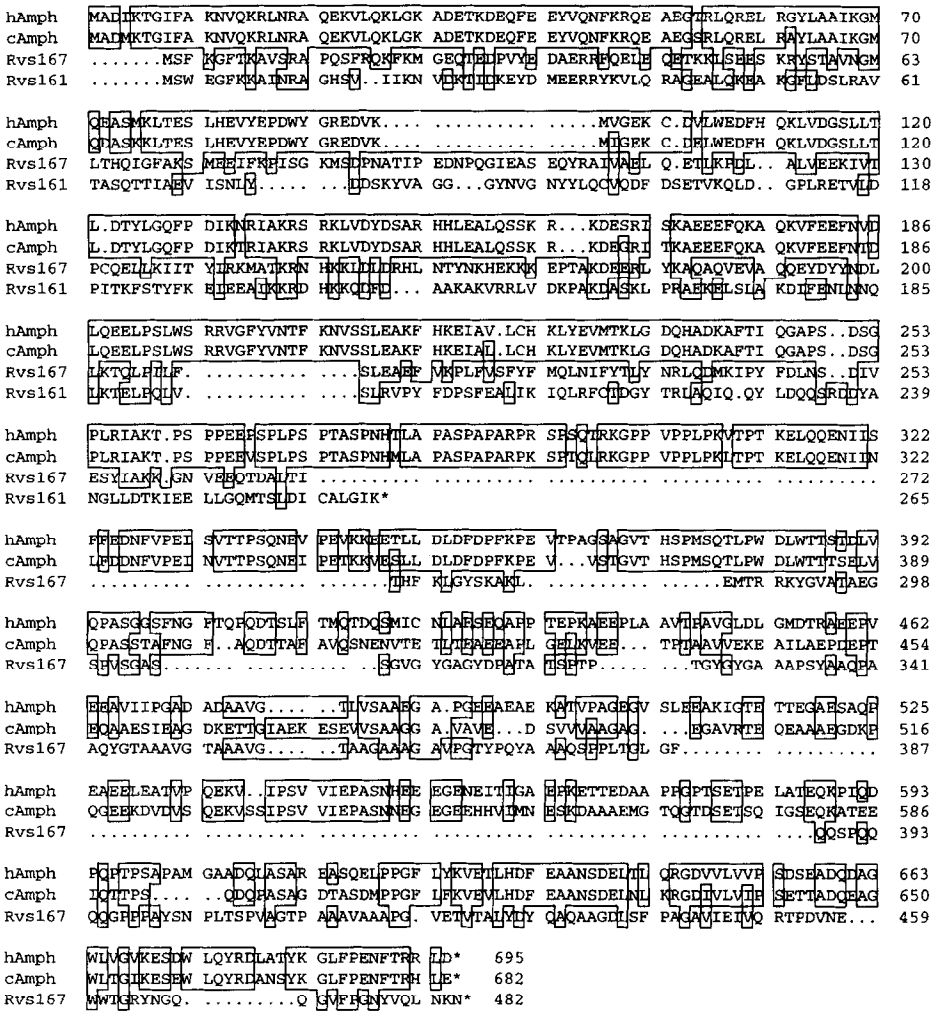


Fig. 1. Amino acid sequence alignment of human amphiphysin (hAmph) with chicken amphiphysin (cAmph) and the yeast proteins, Rvs167 and Rvs161. Amino acids are shown in single letter code and identity with the human sequence at a given position is boxed. First the sequences of the two amphiphysins and of Rvs167 were aligned to each other using the program PILEUP. The sequence of Rvs 161 was then compiled with the other three sequences according to its alignment with Rvs 167. The complete cDNA sequence of hAmph (clone 22–2) is available from GenBank under accession number U07616.

weight of approximately 160 kDa, of which only about 28 kDa could be attributed to GST (see figure 4B, panel CB, lane H). Finally, when the human and chicken sequences were aligned, the N- and C-termini of the two molecules were in precise register (Fig. 1, top two lines). In conclusion, the above data indicates that we had isolated a full-length clone of human amphiphysin.

A comparison of the a.a. sequences of human and chicken amphiphysin [10] revealed a high degree of similarity with the exception of a region of approximately 180 a.a. in the second half of the molecules (Fig. 1 and Fig. 3A domain C). Overall the two proteins are 75% identical and 84% similar at the a.a. level. As was previously reported for chicken amphiphysin [10], the human protein is very hydrophilic, has an acidic pI of 4.4, and contains many putative phosphorylation sites for protein kinases A and C and for casein kinase II. The first

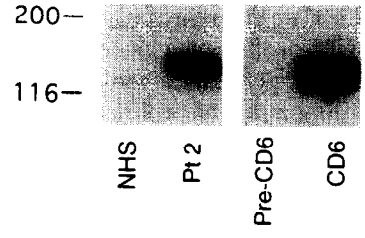
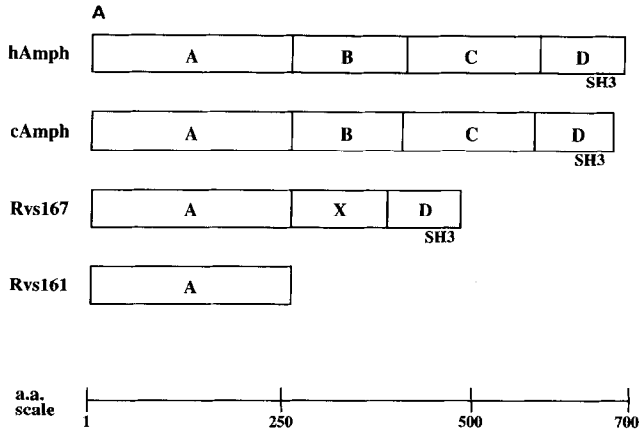


Fig. 2. The same protein band from rat brain is recognized by a rabbit antibody raised against human amphiphysin and by the serum of a patient with SMS and breast cancer. A total rat brain homogenate was subjected to SDS-PAGE and Western blotting as described in section 2. Sera used for western blotting are as follows: NHS = serum from control human subject, 1/500; Pt 2 = serum from patient with SMS and breast cancer, 1/500; pre-CD6 = pre-immune rabbit serum 1/50; CD6 = serum from rabbit immunized with human amphiphysin, 1/50. Molecular weight standards are indicated on the left.



B

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hAmph    TLHDPEAANS DELTLQRGDV VLVVPSDSEA DQDAGWLVGV KESDWLQYRD LATYKGLFPE NFTRRLD*   695
hsrc     A-Y-Y-SRTE TD-SFKK-ER LQI-...NNT EG-WWLAHSL STGQ ..... ....T-YI-S -YV        140
yabp1    A-Y-YD--ED N---FVEN-K IINI...EFV -D-WWLGELE -DGS..... ....K----S -YVSLGN    592
hGRB2-C  A-F--DPQED G--GFR---F IH-M...DNS -P..N-WK-A CHQQ..... ....T‡M--R -YVTPVNRNV- 217
Drk-C    A-Y--VPQE- G--DFR---- IT-T...DRS -E..N-WN-E IGNR..... .....-I--A TYVTPYHS   211
hspec    A-Y--Q-RSF R-VTMKK--- LTLL...-SI NK..D-WKVE AADH..... ....Q-IV-A -YV       1122
hGRB2-N  A-Y--K-TAD ---SFK---I LK-L...N-E CDQN.-YKAE LNGK..... ....D-FI‡K -YIEMKPHDW  60
Drk-N    A-H--S-TAD ---SFRKTQI LKIL...NME -DSN.-YRAE LDGK..... ....E--I-S -YIEMKNHDW  60
Rvs167   A-Y-YQ-QAA GD-SFPA-A- IEI-...QRT PDVNR-WT-R YNGQ..... ....Q-V--G -YVQINKN*  482

CONSENSUS ALYDF A  D  ELSF  GD  L   ...    DD   WW      G ..... .... G FP  NYV
          | || |     ||:   ||  :             |    |                  | ||  |:
hAmph     TLHDPEAANS DELTLQRGDV VLVVPSDSEA DQDAGWLVGV KESDWLQYRD LATYKGLFPE NFTRRLD*
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Fig. 3. (A) Domain diagram of amphiphysins and related yeast proteins suggested by blocks of a.a. similarity. The borders of the domains were based on sequence alignment and are drawn to scale (shown). (B) The last 60 a.a. of hAmph was aligned with the SH3 domains of human src (hsrc [42]), yeast abp1 (yabp1 [43]), human GRB2 (hGRB2 [35]), drosophila drk gene product (Drk [44]), human spectrin α chain (hspec [45]) and Rvs167 [20] using the PILEUP program. N and C represent N- and C-terminal SH3 domains where applicable. Numbers to the right of the sequence indicate the position of the last a.a. shown in the sequence of the protein and a (*) indicates a terminal a.a. Dashes indicate a.a. that are identical with human amphiphysin and periods represent gaps. A consensus SH3 sequence was constructed whenever the same a.a. was conserved in at least four of the sequences listed and an alignment of this sequence with amphiphysin is shown as was done by Bauer et al, [20]. G and P in bold refer to highly conserved residues and * indicates G_{203} and P_{49} of GRB2 (see text).

400 a.a. (domains A+B) are 91% identical and the last 100 a.a. (domain D) are 67% identical.

Although the C domains are only 40% identical, in this region the a.a. profile of chicken and human amphiphysin is strikingly similar with a predominance of alanine and glutamate. A proline-rich region between amino acids 263–277 in domain B, which contains putative binding sequences for src-homology (SH3) domains [32,33] is also highly conserved. SH3 domains are protien modules of about 60 a.a. that are found in many signaling and cytoskeletal proteins [34]. Domain C of chicken amphiphysin was reported to contain the only hydrophobic stretch (a.a. 478–499) of the molecule, although this stretch does not appear to form a transmembrane region [9,10]. A short a.a. sequence with similar hydrophobic properties is present in domain C of human amphiphysin (a.a. 468–480).

Domain D, present in both human and chicken amphiphysin, comprises the C-terminal 100 a.a. and is approximately 30% identical with SH3 domains from a variety of proteins in its last 60 a.a.(Fig. 3B). Secondary structure analysis of this domain predicted the presence of β-sheets between a.a. 648–652 and 664–668, in agreement with the known crystal structure of SH3 domains [34]. The gaps needed to align the SH3 domain consensus with amphiphysin are in regions where there is considerable variability between SH3 containing proteins [34]. Furthermore, the putative SH3 domain of amphiphysin includes a glycine (position 684) and a proline (position 687), (Fig. 3B – in bold) which are conserved in all SH3 domains listed. In particular, mutations of G_{203} or $P_{49}$of GRB2 (Fig. 3B, asterisks) result in an inability of GRB2 to bind other proteins via its C-terminal and N-terminal SH3 domains, respectively [28,35] and correspond to loss of function mutants in the GRB2 homologue, sem-5 [36].

The availability of the human amphiphysin clone allowed us to start investigating mechanisms of autoimmunity to amphiphysin in SMS associated with breast cancer. Neurological symptoms of SMS patients with either GAD or amphiphysin autoimmunity are similar [8,9]. This observation, together with the intracellular localization of both GAD and amphiphysin, suggest that autoantibodies are closely related to, but not responsible for, the disease. In the case of GAD autoimmunity, we have shown a common autoreactive pattern of SMS autoantibodies. In all cases, autoantibodies predominantly recognize a single epitope in the C-terminal region of GAD65 [18]. To determine whether the humoral autoimmune response to amphiphysin was stereotyped, sera from all 5 breast cancer patients with SMS as well as control sera were tested by western blotting against five overlapping amphiphysin fragments expressed as GST-fusion proteins (Fig. 4A).

As shown in Fig. 4B, autoantibodies from all 5 pa-

Table 1
Comparison of domain A of amphiphysin related proteins

	Chicken amphiphysin 1–261	Rvs 167 1–272	Rvs 161 1–265
Human amphiphysin 1–261	95.4 (97.7)	27.3 (48.7)	25.2 (49.2)
Chicken amphiphysin 1–261	–	26.5 (48.7)	22.8 (46.8)
Rvs 167 1–272	–	–	26.9 (51.9)

Percent identities and similarities (in brackets) of the a.a. sequence of domains A (numbers represent the boundaries of each domain A, refer to Fig. 3) of the indicated proteins as obtained using the BESTFIT program.

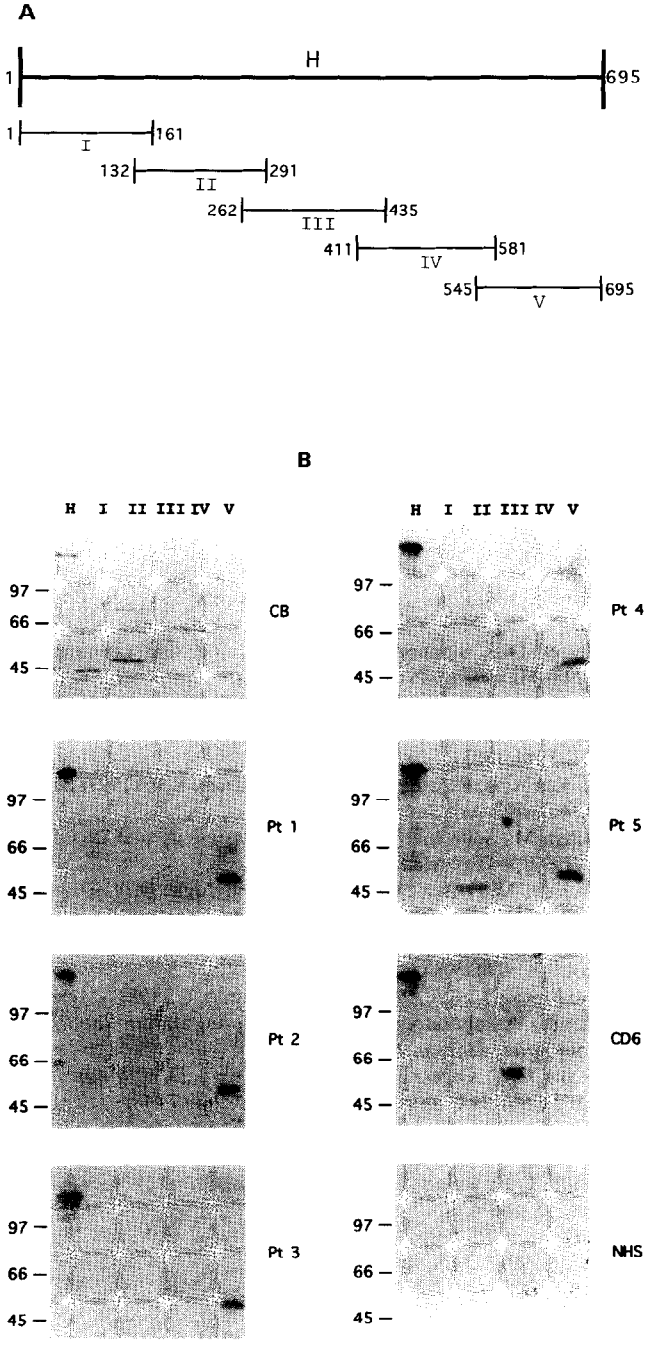

Fig. 4. Similar pattern of reactivity of patient sera with the C-terminal portion of human amphiphysin. (A) Schematic diagram of full-length human amphiphysin (H) and of its fragments (I–V) which were tested for reactivity with human sera. Boundaries of the fragments are indicated by a.a. numbers. (B) GST-fusion proteins of the constructs shown in A were subjected to SDS-PAGE and either Coomassie blue stained (CB) or Western blotted as described in section 2. The blots were reacted with either sera of patients with SMS and breast cancer (Pt 1–5), normal human sera (NHS), or CD6 (rabbit) sera at 1/500 as indicated. Approximately 1/3 of the amount loaded in the gel for panel CB was used for the Western blot analysis. These results are representative of at least 3 separate experiments. Molecular weight standards are indicated on the left. All fusion proteins (I–V) run between 45 and 66 kDa. The 80 kDa band seen in lane II (panel CB) was a protein from the host bacteria that was not related to the specific fusion protein, and never reacted with the antibodies.

tients were directed primarily, although not exclusively, against the amphiphysin C-terminal fragment (fragment V). Autoantibodies from patients 4 and 5 also recognized, but to a lower extent, fragment II. In contrast, a rabbit serum (CD6), raised against human amphiphysin, recognized primarily the central fragment III, suggesting that the stereotypic pattern of antibody reactivity against the C-terminal fragment is typical of the disease and does not simply reflect an unusually high antigenicity of this domain. We have previously reported that the neurological symptoms of SMS improve after removal of the cancer [8]. Interestingly, a similar improvement was noticed for the two patients identified after our original report (Dr. H.-M. Meinck, Heidelberg, Germany and Dr. M. Myers, Jackson, MI, personal communication). This observation strengthens the concept that SMS associated with breast cancer is an autoimmune paraneoplastic disease.

Since amphiphysin is a synaptic vesicle-associated protein [10], its relation to a disease involving abnormal synaptic function is plausible. On the other hand, the connection between amphiphysin and breast cancer has been more elusive. A possible clue concerning this connection comes from an interesting homology we have identified by searching protein databases for a.a sequences similar to the regions of amphiphysin conserved between human and chicken. The conserved N-terminal region of amphiphysin has considerable homology to Rvs167 [20] and Rvs161 [19], two yeast proteins which were cloned by isolating mutants with a reduced viability to nutrient starvation (rvs). The alignment of Rvs167 and Rvs161 with human and chicken amphiphysin (Fig. 1) suggests a domain model of the four proteins shown in Fig. 3A. Domain A is shared by all four proteins and in all four has a high α-helix potential. The similarities among the various domains A are indicated in Table 1. Rvs161 is comprised exclusively of this domain, whereas Rvs167 shares an additional region of similarity with amphiphysin in domain D. Domain D of Rvs167 was previously shown to contain an SH3 domain [20]. Domain X of Rvs167 was defined as a GPA-rich region because of its high content in glycine, proline and alanine. While this region is substantially shorter than the central region of amphiphysin, it shares some features of both domains B and C of human and chicken amphiphysin which are rich in proline and alanine, respectively. These considerations suggest that Rvs167 is the yeast homologue of amphiphysin. While amphiphysin has been shown to have a very restricted tissue distribution (brain, endocrine tissues and testis)[9,10], the presence of a homologue in yeast strongly suggests that amphiphysin homologues are present in all cells.

The phenotype of *RVS167* and *RVS161* mutant cells is associated with abnormal morphology and alterations in the peripheral cytoskeleton. Cells appear to be unable to adapt to unfavorable growth conditions by an im-

paired link between the mechanisms which control cell proliferation and those which allow the cell to undergo stationary phase adaptation. Mutations of either one of the two genes produce a similar phenotype [19,20] and are suppressed by the same set of genes [37], indicating that they act in the same pathway. The function of amphiphysin in the nervous system remains to be elucidated. Amphiphysin was reported to be a synaptic vesicle-associated protein, although it is not enriched in these organelles [10]. The homology of amphiphysin to the two Rvs yeast proteins suggests a function of amphiphysin in controlling the properties of the membrane associated cytoskeleton and offers the possibility of using yeast genetics to further investigate the function of the protein.

It was proposed that neurological autoimmune paraneoplastic syndrome are triggered by the ectopic expression in the neoplastic tissues of a neuronal protein or a protein antigenically related to it, which then becomes an autoantigen [16,38]. The homology of amphiphysin to yeast proteins which have been shown to participate in the cell adaptation to stationary phase raises the possibility that amphiphysin or some related protein may be directly involved in at least some form of breast cancer. There is evidence to suggest that proteins of the peripheral cell cytoskeleton may be directly involved in the pathogenesis of some forms of cancer [39–41] In the breast cancer tissue of SMS patients which have been investigated, amphiphysin immunoreactivity was not detected using patient autoantibodies [8]. However, one should consider the possibility that the T-cell triggering autoepitope might belong to an amphiphysin-related molecule which similarly to Rvs161, contains only domain A. This protein would not be recognized by patient autoantibodies which we have now shown to be primarily directed against the C-terminus of amphiphysin. We note that Nova, another autoantigen of a paraneoplastic neurological autoimmune disorder, is a neuronal protein expressed in a truncated form (which does not include the dominant C-terminal autoepitope for humoral autoimmunity) in the neoplastic tissue [17].

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