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13. ABSTRACT *(Maximum 200*
Amphiphysin, a neuronal protein first identified in chicken synaptic membranes, is the autoantigen of Stiff-Man Syndrome (SMS) associated with breast cancer. During the second year of this fellowship, we have cloned a new isoform of amphiphysin, termed amphiphysin II. This isoform is not restricted to the brain and may represent a form of amphiphysin that could be involved in breast cancer. Polyclonal antibodies have been raised to amphiphysin I and II in addition to monoclonal antibodies that recognize various portions of amphiphysin I. Muscle amphiphysin II is represented by several isoforms which run at about 65 kDa in SDS-PAGE and is concentrated around the I band, i.e. in close proximity of plasmalemmal T-tubules. Neurons express predominantly an 85 kDa isoform which is concentrated under the plasmalemma of axon hillocks and nodes of Ranvier. Similar localizations in both muscle and neurons were described for certain ankyrin isoforms and indicate that amphiphysin II is a component of specialized submembranous cytomatrices. In addition, a Chinese Hamster Ovary (CHO) cell line which overexpresses amphiphysin I was established. These cells had a normal morphology and growth, ruling out the possibility that amphiphysin I alone is directly involved in tumor development.

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Carol Ward 10/7/96

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INTRODUCTION

Stiff-man syndrome (SMS) is a rare neurological disease characterized by rigidity of the body musculature with superimposed painful spasms (Layzer, 1988). Most patients with this disease exhibit autoimmunity to GABA-ergic neurons. It has been found in Prof. De Camilli's laboratory that, to date, all patients diagnosed with breast cancer and SMS, have autoantibodies against a novel synaptic-associated protein, amphiphysin. This research project is to further define the role of anti-amphiphysin antibodies in the development and/or detection of breast cancer and to further understand the function of this protein and how the autoimmunity may arise.

In the early stages of the work on SMS, it was noticed in Prof. De Camilli's lab that two patients with this condition, but without GAD-antibodies or associated organ-specific autoimmune diseases, had high titers of autoantibodies directed against a 128 kDa protein. Immunocytochemistry suggested a synaptic localization of the autoantigen. Interestingly, both patients were women with breast cancer (ductal adenocarcinoma). Subsequently, the same antibodies were detected in a third patient with SMS without apparent breast cancer. On Prof. De Camilli's indication, a search of breast cancer in this patient was performed by ultrasonography and a small infiltrating ductal carcinoma was found and removed. A summary of the caseload of amphiphysin autoimmunity is indicated in table 1. In at least three of these cases (Folli et al, 1993; Meinck, personal communication) a remission of the neurological symptoms was documented after removal of the cancer and steroid therapy, supporting the hypothesis that no major degeneration of brain tissue occurs in SMS.

Table 1 : Paraneoplastic Stiff-man Syndrome Cases

	Anti-GAD Autoantibodies	Anti-amphiphysin Autoantibodies	Cancer	Source
Patient 1 England	Negative	Positive	Breast Cancer	Our caseload (Folli et al. 1993)
Patient 2 Italy	Negative	Positive	Breast Cancer	Our caseload (Folli et al. 1993)
Patient 3 Italy	Negative	Positive	Breast Cancer	Our caseload (Folli et al. 1993)
Patient 4 Germany	Negative	Positive	Breast Cancer	Our caseload (De Camilli et al. 1993)
Patient 5 U.SA.	Negative	Positive	Breast Cancer	Our caseload (David et al. 1994)
Patient 6 Germany	Negative	Positive	Breast nodule	Our caseload
Patient 7 U.SA	Negative	Positive*	Breast Cancer	D. Kaufman, personal communication
Patient 8 Italy	Negative	Positive*	Colon Cancer	Grimaldi et al. 1993
Patient 9 Japan	Negative	Positive	Breast Cancer	Tsutsui et al, 1995

*not tested in our lab

These findings raise the possibility that in some cases SMS may have an autoimmune paraneoplastic origin. Other autoimmune paraneoplastic neurological diseases have been described and characterized in recent years (Posner and Furneaux, 1990; Hetzel et al, 1990). These conditions are characterized by neurological symptoms which appear to follow the development of a cancer, and by the presence in the serum and CSF of high titer antibodies directed against specific brain autoantigens. The type of antibodies generally correlate with the type of neurological symptoms, but the pathogenic role of these antibodies remains unclear. It was proposed that ectopic expression of brain antigens by cancer cells triggers the immune response (Furneaux et al, 1990).

Amphiphysin is a synaptic-vesicle-associated protein that was discovered by the screening of a λGT11 library of chicken brain with antibodies to synaptic proteins (Lichte et al, 1992). Its sequence (total of 682 amino-acids) includes a stretch of about 20 amino-acids which could potentially form a transmembrane span. However, most of the protein is cytosolic and only a pool of the protein interacts with the cytoplasmic surface of synaptic vesicles. Its function is unknown. The properties of amphiphysin suggested a possible identity with the 128 kDa antigen, a hypothesis that was tested and confirmed (De Camilli et al, 1993).

We have been able to clone human amphiphysin and found the N- and C-terminal domains of the protein to be highly conserved between chicken and human (David et al, 1994). 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 (David et al, 1994). Portions of chicken and human amphiphysin are also homologous to portions of Rvs167 and Rvs161 (David et al, 1994), two yeast proteins whose mutant phenotype includes a striking

endocytic defect (Munn et al, 1995) in addition to growth and polarity defects (Crouzet et al, 1991; Bauer et al, 1993; Desfarges et al, 1993).

We have demonstrated a specific, SH3 domain-mediated, interaction between amphiphysin and dynamin by gel overlay and affinity chromatography (David et al, 1996). In addition, we showed that the two proteins are colocalized in nerve terminals and are coprecipitated from brain extracts consistent with their interactions in situ. We also reported that a region of amphiphysin distinct from its SH3 domain mediates its binding to the α_C subunit of AP2 adaptin, which is also concentrated in nerve terminals (David et al, 1996). These findings support a role of amphiphysin in synaptic vesicle endocytosis.

The current work was aimed at understanding more about amphiphysin function in the brain and to test the possibility that amphiphysin or a related protein may play a role in the biology of breast cancer.

BODY

Task 1. Preparation of recombinant amphiphysin and specific antibodies to it

a. Recombinant amphiphysin will be injected into rabbits and mice for production of polyclonal and monoclonal antibodies

After cloning amphiphysin (now referred to as amphiphysin I) from a human cDNA library (David et al, 1994) and noticing its striking homology to two yeast proteins, Rvs161 and Rvs167, we proceeded to search for other amphiphysin isoforms that may be present outside the brain. It is possible that characterization of non-neuronal isoforms of amphiphysin would help us in the understanding of amphiphysin autoimmunity in breast cancer. Using sequence data from the neuronal form of amphiphysin I (David et al, 1994) in homology searches on the database, we retrieved several partial sequences from the database that have significant homology to amphiphysin I. One of these sequences (acc # Z24784; 240 base pairs) was from a human muscle library and was 76% homologous to the C-terminal of amphiphysin. We performed polymerase chain reaction (PCR) using primers from this sequence and amplified an identical 240 b.p. piece from a human muscle cDNA library. This piece was used as a probe to screen the same library under high stringency conditions and yielded many positives. (In parallel, this 240 b.p. piece was subcloned into pGex2T to yield a GST fusion protein for antibody production - see below). Two of these clones were fully sequenced and yielded overlapping sequences with an open reading frame that would correspond to a protein of 454 amino acids. This protein has significant homology to brain amphiphysin (55% identity, 70% similarity), including a conserved SH3 domain in the C-terminus.

A further search of the database, using the muscle amphiphysin II sequence as a query, revealed three partial ESTs (expressed sequence tag) from a human fetal brain library that were identical to b.p. 350-800 of the muscle clone. The clones from the human fetal library were

obtained through the I.M.A.G.E. Consortium (Research Genetics, Inc.) and fully sequenced (Keck Biotechnology, Yale University). Two clones, of 1.8 kB, were identical to each other and one clone of 2.0 kB was identical except for a 93 b.p. insert at the N-terminal. All three sequences were identical to the muscle sequence of amphiphysin II except for a 381 b.p. insert in the latter half of the clone. Furthermore, this 381 b.p. sequence was 72% identical to amphiphysin I, indicating that the additional sequence probably represents a true insertion in the gene and not a cloning artifact.

In addition, we then screened a human brain cDNA library (Clontech, λgt11) with a 220 b.p. probe corresponding to a sequence upstream of the 381 b.p. insert discussed above. This probe recognizes a band of 4.5 kB in all tissues checked (human Multiple Tissue Northern blot, Clontech) and also a 2.2 kB band in skeletal muscle (probably representing the alternatively splice muscle form). Approximately $1x10^6$ clones were screened and 26 positive clones were isolated after tertiary screening. Of these, 19 clones were found positive with a 700 b.p. probe from the N-terminal of amphiphysin II. Three of these clones, representing inserts of approximately 1.8 - 2.1 b.p. were subcloned into pBluescript and sequence analysis confirmed the sequences obtained from the IMAGE clones. A contiguous sequence derived from these clones is shown in figure 1. Alternatively spliced regions are depicted by shaded amino acid residues.

Polyclonal antibodies to amphiphysin II were obtained by injecting rabbits with a GST fusion protein consisting of the C-terminal 70 amino acids of amphiphysin II. Western blots with these antibodies (CD7 and CD8) revealed a predominant 65 kDa band in muscle and an 85kDa band in brain (see figure 2). Upon longer exposure, lower levels of the 65kDa protein were present in most tissues tested.

Polyclonal anti-peptide antibodies were also developed which would recognize both amphiphysin I and II isoforms. These antibodies were made by injecting rabbits with a 22 amino acid peptide (RAQEKVLQKLGKADETKDEQFE; conjugated to KLH) from a sequence near the N-terminal that is identical in both forms. Indeed, these rabbit sera (CD9 and CD10) recognize both the 128 kDa (amphiphysin I) and 85kDa (amphiphysin II) bands on a western blot of rat brain. (see figure 3)

Monoclonal antibodies to amphiphysin I have also been developed. After screening 350 wells by western blot with rat brain homogenates, over 90% were positive. 48 were taken for second screen and all were still positive. Of these, 29 were screened for their reactivity with different fragments of amphiphysin (as described for patient sera in David et al, 1994). In this manner we have isolated various polyclonal antibodies that recognize different portions of the amphiphysin molecule.

```
  1 MAEMGSKGVT AGKIASNVQK KLTRAQEKVL QKLGKADETK DEQFEQCVQN 50
 51 FNKQLTEGTR LQKDLRTYLA SVKAMHEASK KLNECLQEVY EPDWPGRDEA 100
101 NKIAENNDLL WMDYHQKLVD QALLTMDTYL GQFPDIKSRI AKRGRKLVDY 150
151 DSARHHYESL QTAKKKDEAK IAKPVSLLEK AAPQWCQGKL QAHLVAQTNL 200
201 LRNQAEEELI KAQKVFEEMN VDLQEELPSL WNSRVGFYVN TFQSIAGLEE 250
251 NFHKEMSKLN QNLNDVLVGL EKQHGSNTFT VKAQPRKKSK LFSRLRRKKN 300
301 SDNAPAKGNK SPSPPDGSPA ATPEIRVNHE PEPAGGATPG ATLPKSPSQL 350
351 RKGPPVPPPP KHTPSKEVKQ EQILSLFEDT FVPEISVTTP SQFEAPGPFS 400
401 EQASLLDLDF DPLPPVTSPV KAPTPSGQSI PWDLWEPTES PAGSLPSGEP 450
451 SAAEGTFAVS WPSQTAEPGP AQPAEASEVA GGTQPAAGAQ EPGETAASEA 500
501 ASSSLPAVVV ETFPATVNGT VEGGSGAGRL DLPPGFMFKV QAQHDYTATD 550
551 TDELQLKAGD VVLVIPFQNP EEQDEGWLMG VKESDWNQHK ELEKCRGVFP 600
601 ENFTERVP*
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Figure 1: Human amphiphysin II contiguous sequence obtained from a PILEUP analysis of the human clones. Alternatively spliced regions are depicted by shaded amino acid residues.

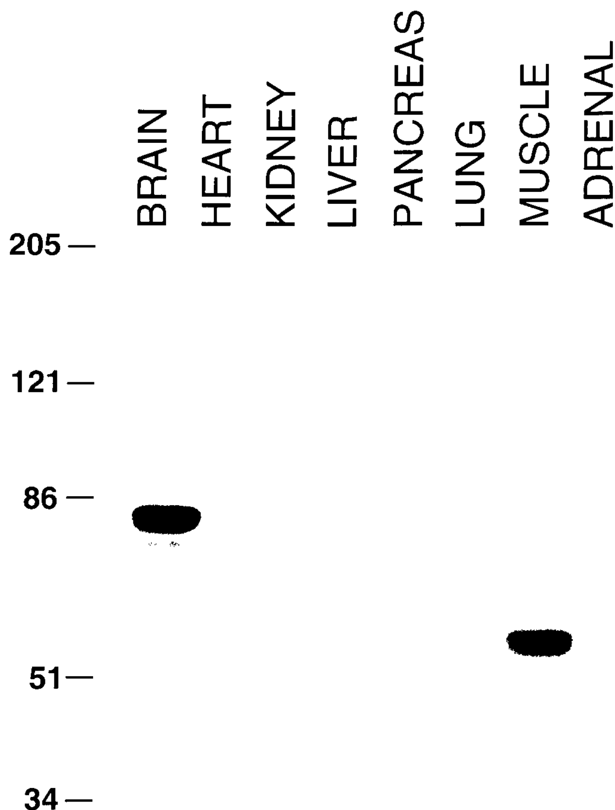


Figure 2: Tissue distribution of amphiphysin II as demonstrated by Western blotting. Amphiphysin II is expressed primarily in brain and skeletal muscle. Post nuclear supernatants of rat tissues were loaded in each lane and probed with the CD8 polyclonal rabbit serum specific for amphiphysin II. Bound antibodies were detected by ^{125}I Protein A.

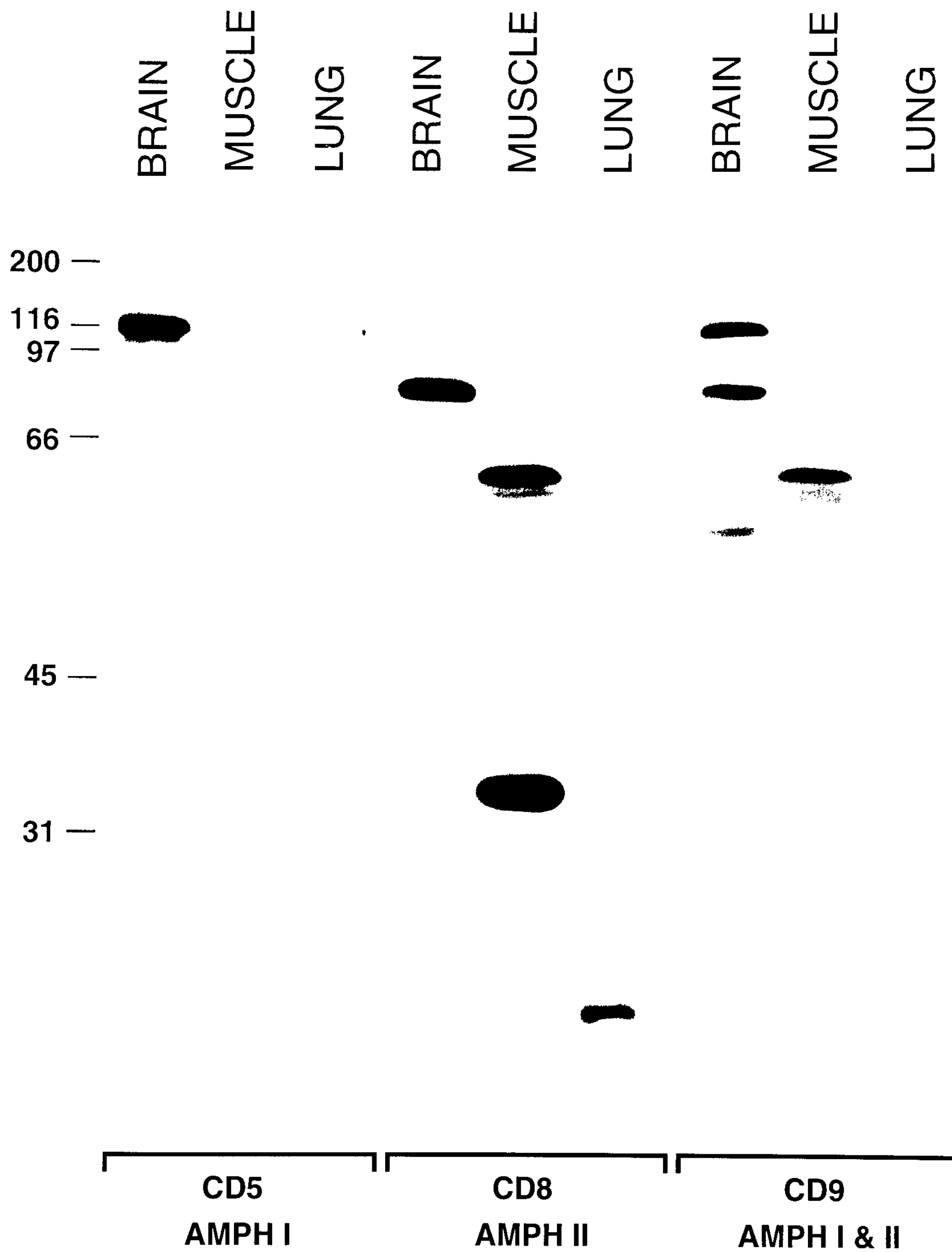


Figure 3: Comparison of amphiphysin I and amphiphysin II expression in rat brain, skeletal muscle and lung. Extracts of the three tissues were probed with an antibody specific for amphiphysin I (CD5), for amphiphysin II (CD8), and with an antibody that recognizes both amphiphysin I and II (CD9). Bound antibodies were detected by ^{125}I Protein A.

Task 2. Developing a screening assay to check for amphiphysin autoimmunity in a large population of breast cancer patients

Due to the need to characterize the new amphiphysin II isoform, this part of the project has been delayed and will be addressed in the final year of the fellowship.

Task 3. Studying the function of amphiphysin and how it relates to cancer

a. Human amphiphysin will be overexpressed in a variety of cell lines where its phenotype can be studied.

A Chinese Hamster Ovary (CHO) cell line which permanently overexpresses amphiphysin I was developed. To this end, human amphiphysin I (David et al, 1994) was cloned into the HindIII/XbaI sites of the pRC/RSV (Invitrogen) eukaryotic expression vector. The pRC/RSV Amph construct was transfected into CHO cells via lipofectAMINE (GIBCO) reagent according to the manufacturer's protocol. Stable incorporation of the pRC/RSV Amph construct was selected with 175 μg/ml G418 and expression of amphiphysin was verified by western blot analysis and immunocytochemistry. The cells did not exhibit any abnormal growth or morphology, ruling out the possibility that amphiphysin I alone is directly involved in tumor development.

b. The proteins that associate with amphiphysin will be identified

This aspect of the project was addressed in year 1 (refer to David et al, 1996).

c. The expression of amphiphysin in normal and neoplastic tissue will be studied

To determine whether amphiphysin II molecules predicted by cDNA cloning were indeed present in brain and muscle tissues, and possibly in other tissues, we raised polyclonal antibodies (CD7 and CD8) which recognize selectively amphiphysin II. To this aim we used as the immunogen a portion of the COOH terminus of amphiphysin II, which is substantially divergent from amphiphysin I and which is shared by all amphiphysin II clones. The anti-amphiphysin II specific serum recognizes a protein of about 85 kDa in brain extracts and 65kDa in muscle. Upon longer exposures, the 65kDa isoform can be seen in most tissues tested. It does not recognize the amphiphysin I doublet at approximately 128 kDa , which is instead recognized by the antibody CD5, which is specifically directed against amphiphysin I. To confirm the identify of the 85KDa band as amphiphysin II, we tested antibodies which we had generated against a 22 mer peptide from domain A of amphiphysin I, which is highly conserved in amphiphysin II. This antiserum (CD9) recognizes both the amphiphysin I band and the 85 kDa band, confirming its identify as amphiphysin II. A molecular weight of 85KDa is higher than the molecular weight predicted for brain amphiphysin II by a.a. sequencing, however we had already shown that amphiphysin I has an aberrant mobility in SDS-PAGE due to its high hydrophilicty (David et al, 1994) and amphiphysin II could be expected to have similar properties.

Considering the substantial similarities of amphiphysin I and II, they may have an overlapping function in brain. We investigated therefore, whether the two proteins have a similar cellular and subcellular distribution. To this aim we performed double labeling immunofluorescence of rat brain frozen sections with antibodies specific for amphiphysin I or amphiphysin II. As previously reported amphiphysin II immunoreactivity was found to be highly concentrated in nerve terminals, where it was strikingly colocalized with dynamin I (David et al, 1996). Amphiphysin II immunoreactivity had a strikingly different distribution and was primarily concentrated at axon hillocks and at nodes of Ranvier. (see figure 4)

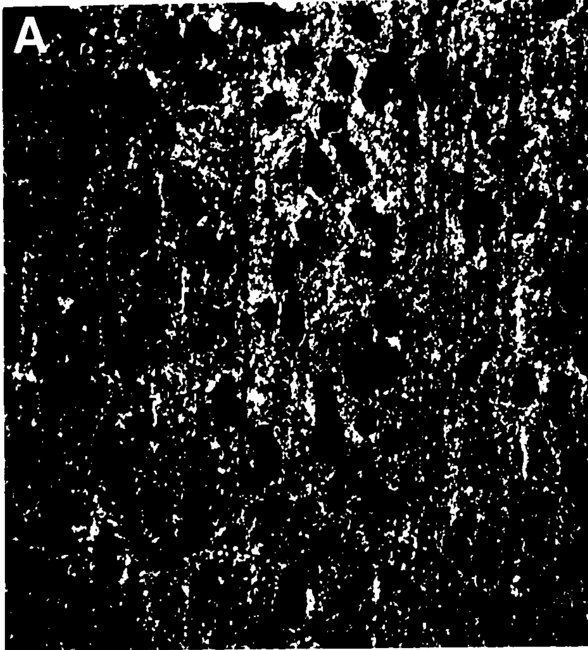
Clathrin coated vesicles were previously described at both axon hillocks and nodes of Ranvier, which is consistent with the occurrence of exo-endocytosis at these sites. However, neither axon hillocks not nodes of Ranvier are sites specialized for endocytosis. A unique characteristics of the axonal membrane at these two sites is the presence of a specialized submembranous cytomatrix, suggesting the possibility that amphiphysin may be part of a specialized actin cytomatrix.

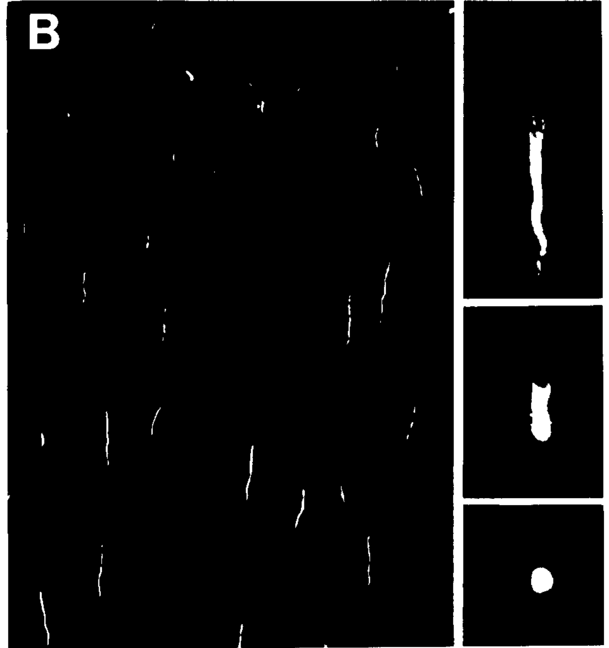
The distribution of amphiphysin II in skeletal muscle, was also investigated. In longitudinal sections amphiphysin II immunoreactivity appears to be widely localized throughout the muscle fiber with a striated pattern reflecting muscle striations. Amphiphysin II immunoreactivity overlaps with I bands, as demonstrated by double immunofluorescence for actin and by the overlap with desmin immunoreactivity which is centered around the Z band and extend into the I band. In transverse sections, however, the distribution of amphiphysin II was clearly different from the distribution of actin, and was very similar instead to the distribution of desmin, which surrounds, but does not extent into, myofibrils. Its distribution was also very similar to the distribution of triadin, a marker of the terminal cisternae of the sarcoplasmic reticulum, which are concentrated around Z-lines and surround myofibrils. The high concentration of amphiphysin II throughout the muscle fiber and along the sarcomere strongly suggest an involvement of amphiphysin II in the contractile apparatus of the cell. (see figure 5).

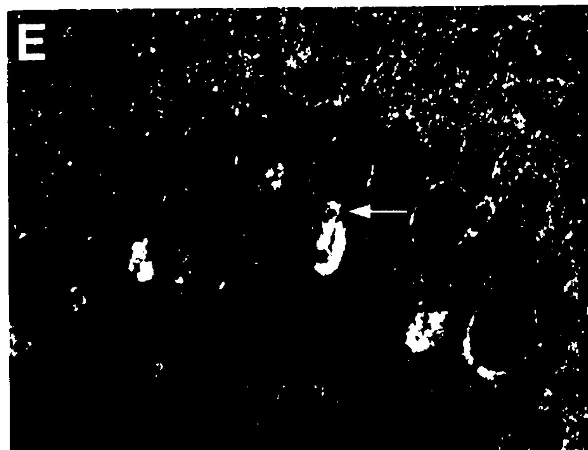
Figure 4: (next page): Comparison of the localization of amphiphysin I and amphiphysin II in rat brain. Double immunofluorescence micrographs. In all fields, amphiphysin I immunoreactivity (a, c, e) has a typical nerve terminal pattern represented by small puncta throughout the gray matter. Amphiphysin II (b, d, f) is primarily localized at initial axon segments. a and b: cerebral cortex. The inset of b shows high power views of two longitudinal section and one transverse section of initial axon segments. Note the concentration of immunoreactivity in the cortical region of the cytoplasm. c and d: CA1 region of the hippocampus demonstrating in field d the initial axon segments of pyramidal neurons visible in field c as negative images. e and f: cerebellar cortex. Arrows points to the amphiphysin II-positive initial segment of a Purkinje cells axon which is surrounded by amphiphysin I-positive nerve terminals of basket cells. Arrowheads in f point to initial axon segments of stellate cells. (Bar: 63mm, Insert: 126mm).

Amphiphysin I

Amphiphysin II







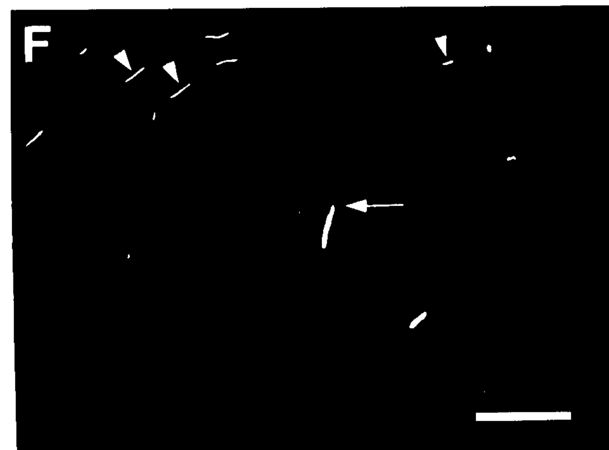


Figure 4

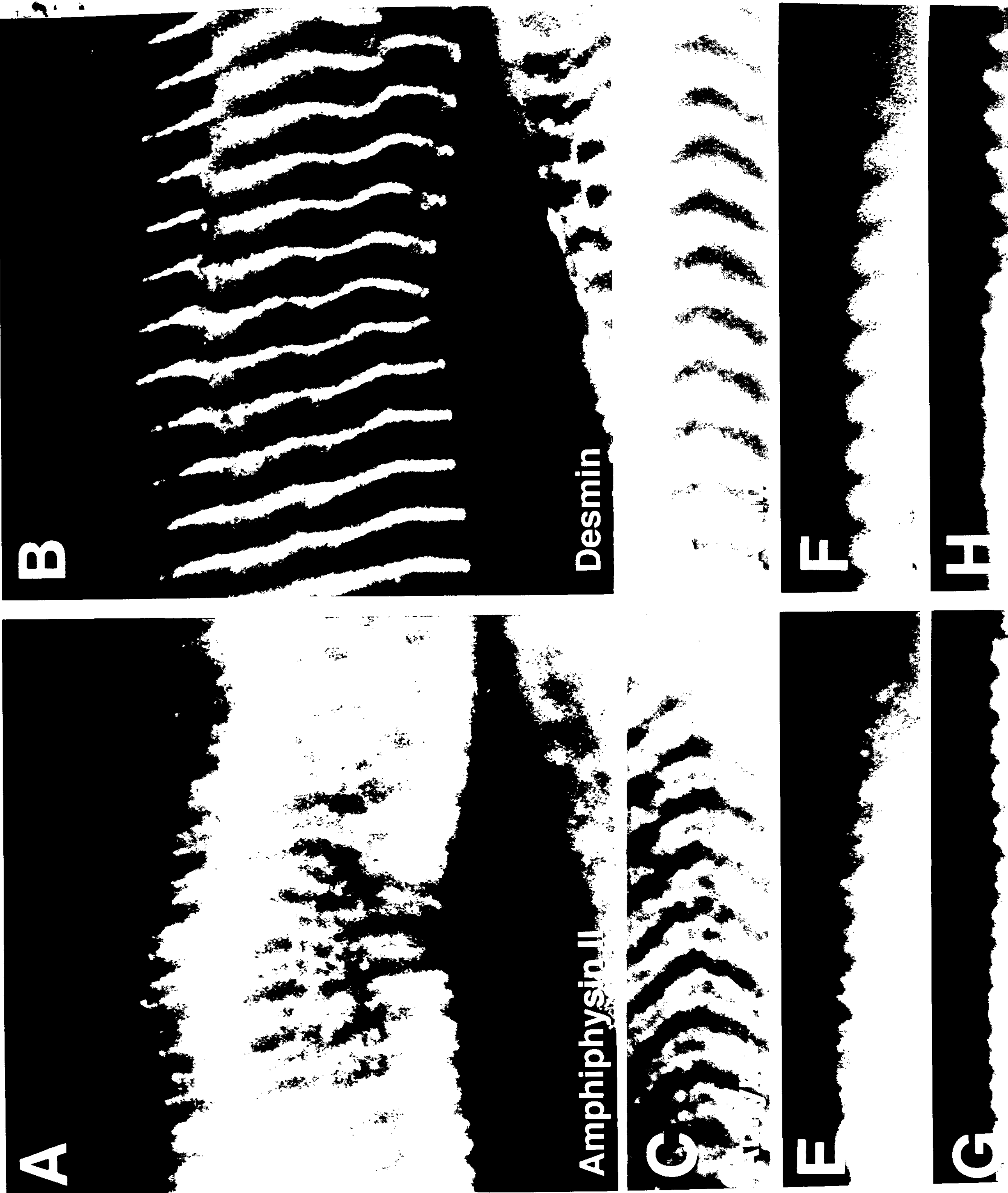


Figure 5: Localization of amphiphysin II along the sarcomere at I bands of skeletal muscle. Amphiphysin II immunoreactivity (a, e, and g) forms transverse bands which flank the Z line, which is visualized by counterstain for desmin immunoreactivity in field h (semi-thin section). a and b: double labeling for amphiphysin II and clathrin. Clathrin is comprised between the two amphiphysin bands and overlaps with the Z line. (Bar: 48mm). c and d: double labeling for ankyrin and actin demonstrating the similarity of the ankyrin pattern to the amphiphysin II pattern shown in a, e and g. e and f: double labeling for amphiphysin II and actin showing the partial overlap of amphiphysin II with actin.

CONCLUSIONS

It was proposed that neurological autoimmune paraneoplastic syndromes 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 (Thirkill et al, 1989; Furneaux et al, 1990). The homology of amphiphysin to yeast proteins strongly suggested to us that there is an amphiphysin homologue present in all cells. Indeed, we have been able to clone and characterize a homologue of amphiphysin that is present primarily in muscle and brain and to a lesser extent in other tissues as well. It still remains to be seen exactly which (if any) isoform is expressed in the breast under either normal or cancerous conditions. Much of the work described in this report deals with the cloning of different alternatively spliced amphiphysin II isoforms and the development of specific polyclonal and monoclonal antibodies to amphiphysin I and II. These reagents are invaluable for studies on the localization of amphiphysin II in brain and muscle (described here) and the further analysis of both normal and cancerous breast tissue.

The immunohistochemical and western blot analysis of amphiphysin II in both muscle and neurons is similar to that described for certain ankyrin isoforms and indicate that amphiphysin II is a component of specialized submembranous cytomatrices. This is of interest since there is evidence to suggest that proteins of the peripheral cell cytoskeleton may be directly involved in the pathogenesis of some forms of cancer (Trofatter et al, 1993; Rubinfeld et al, 1993; Su et al, 1993).

Amphiphysin I stable cell lines were established in CHO cells. They did not display any abnormal morphology or growth which indicates that amphiphysin I alone is not directly causal in the cancer process. Even so, this cell line is an additional tool that we are using to further study the localization of amphiphysin and interacting proteins.

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