Neurobeachin: A Protein Kinase A-Anchor, beige/Chediak-Higashi Protein Homolog Implicated in Neuronal Membrane Traffic

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We describe the identification and initial characterization of neurobeachin, a neuron-specific multidomain protein of 327 kDa with a high-affinity binding site (K{iota}, 10 nm) for the type II regulatory subunit of protein kinase A (PKA RII). Neurobeachin is peripherally associated with pleomorphic tubulovesicular endomembranes near the trans sides of Golgi stacks and throughout the cell body and cell processes. It is also found in a subpopulation of synapses, where it is concentrated at the postsynaptic plasma membrane. In live cells, perinuclear neurobeachin is dispersed by brefeldin A (BFA) within 1 min, and in permeabilized cells a recruitment of neurobeachin from cytosol to Golgi-near membranes is stimulated by GTP-S and prevented by brefeldin A. Spots of neurobeachin recruitment are close to but distinct from recruitment sites of COP-I, AP-1, and AP-3 coat proteins involved in vesicle budding. These observations indicate that neurobeachin binding to membranes close to the trans-Golgi requires an ADP-ribosylation factor-like GTPase, possibly in association with a novel type of protein coat. A neurobeachin isoform that does not bind RII, beige-like protein (BGL), is expressed in many tissues. Neurobeachin, BGL, and ~10 other mammalian gene products share a characteristic C-terminal BEACH-WD40 sequence module, which is also present in gene products of invertebrates, plants, protozoans, and yeasts, thus defining a new protein family. The prototype member of this family of BEACH domain proteins, lysosomal trafficking regulator (LYST), is deficient in genetic defects of protein sorting in lysosomal biogenesis (the beige mouse and Chediak-Higashi syndrome). Neurobeachin’s subcellular localization, its coat protein-like membrane recruitment, and its sequence similarity to LYST suggest an involvement in neuronal post-Golgi membrane traffic, one of its functions being to recruit protein kinase A to the membranes with which it associates.

Key words: AKAP; ARF; BEACH domain; BGL; coat protein; Golgi complex; LYST; membrane traffic; neurobeachin; protein kinase A; scaffolding protein; synapse; TGN

The progression of membranes and proteins through the stages and compartments of the secretory and endocytic pathways is a highly organized and regulated process. The maintenance of the overall architecture of endomembranes and of the plasma membrane requires a balance of lipid flows into and out of the various compartments, and proteins destined for diverse organelles or plasma membrane domains must be appropriately sorted and targeted, whereas resident proteins of specific pathway stages must be retained or retrieved. These events require the interplay of lipids, membrane proteins, soluble cytosolic and luminal proteins, and cytoskeletal and motor proteins. Their internal coordination and external regulation is known to involve protein phosphorylation and small and heterotrimeric G-proteins.

In neurons, the mechanisms for the trafficking of membranes and membrane proteins must be particularly active and complex. Because of their many and long cell extensions, neurons have to build up and maintain a very large plasma membrane area that is organized not only into the somatodendritic and axonal macrodomains but additionally into an elaborate mosaic of microdomains with specific protein compositions, particularly at presynaptic and postsynaptic sites (Foletti et al., 1999).

Received April 25, 2000; revised Aug. 11, 2000; accepted Aug. 28, 2000.

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the University of Bochum Medical School (FoRUM intramural research funding program) to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to M. Robinson and W. Huttner for discussions. We are indebted to J. and C. Theiss for fluorescence video microscopy, G. Papoutsoglou and N. Opitz for program (to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to the University of Bochum Medical School (FoRUM intramural research funding program) to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to M. Robinson and W. Huttner for discussions. We are indebted to J. and C. Theiss for fluorescence video microscopy, G. Papoutsoglou and N. Opitz for program (to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to the University of Bochum Medical School (FoRUM intramural research funding program) to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to M. Robinson and W. Huttner for discussions. We are indebted to J. and C. Theiss for fluorescence video microscopy, G. Papoutsoglou and N. Opitz for program (to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to the University of Bochum Medical School (FoRUM intramural research funding program) to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to M. Robinson and W. Huttner for discussions. We are indebted to J. and C. Theiss for fluorescence video microscopy, G. Papoutsoglou and N. Opitz for program (to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to the University of Bochum Medical School (FoRUM intramural research funding program) to M.W.K. and F.W.H., and from the Fonds der Chemischen Industrie to M. Robinson and W. Huttner for discussions. We are indebted to J. and C. Theiss for fluorescence video microscopy, G. Papoutsoglou and N. Opitz for program.

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The Journal of Neuroscience, December 1, 2000, 20(23):8551–8565
BEACH-D40 domains. The prototype of this family is lysosomal trafficking regulator (LYST), a cytosolic protein important for lysosomal biogenesis and implicated in protein sorting between endosomes, lysosomes, and the plasma membrane.

MATERIALS AND METHODS
cDNA cloning and Northern blot analysis. The chicken cDNA clone, 10.2, was isolated by immunoscreening a brain cDNA library in Agt11 as described by Lichte et al. (1992). It contained an uninterrupted coding sequence of 2379 nucleotides (nts) and was used as a hybridization probe to screen a mouse cDNA library in λZAP-II (Stratagene, La Jolla, CA). Drosophila expressed sequence tags (ESTs) from the Berkeley Drosophila Genome Project were used to query the database to define the mouse neurobeachin sequence and obtained through Genome Systems, Inc. Clone LD07020 was found to overlap with the published DAKAP550 sequence and extend it farther downstream. Clone HL00087 did not overlap displayed 90% predicted amino acid sequence identity in the downstream half of the neurobeachin BEACH sequence. We bridged the interval between the two clones by RT-PCR from Drosophila head RNA and thus determined the complete C-terminal coding sequence of DAKAP550.

Total and poly(A ) RNA preparation from chicken and mouse tissues and Northern blot analysis with 32P-labeled hybridization probes were performed according to conventional procedures. To analyze developmental expression of the neurobeachin mRNA in mouse brain, Northern blots on 20 μg total RNA from postnatal days 1, 5, 10, 15, 20, 25, 30, 56, and 150 (Kutzbach et al., 1998). Forskolin/3-isobutyl-1-methylxantine (IBMX) treatment and Northern blot analysis of NS20Y mouse neuroblastoma cells were performed as in Hoesche et al. (1995). Chicken xanthine (IBMX) treatment and Northern blot analysis of NS20Y mouse neuroblastoma cells were performed as in Hoesche et al. (1995).

To determine the tissue distribution of neurobeachin, tissues were homogenized in 0.3 m sucrose, 1 mm EDTA, 10 mm Tris, pH 7.4, 0.5 mm PMSF, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, with a glass-Teflon homogenizer or, for muscle and heart, a turning-knife homogenizer. cDNA of neurobeachin and the ECL kit (Amersham, Arlington Heights, IL). The minor bands visible below the main band on Figure 5 were subjected to SDS-PAGE (5% polyacrylamide) and transferred to nitrocellulose, and the blot was developed with affinity-purified rabbit anti-neurobeachin antibodies, and neurobeachin scattering by BFA in live PC12 cells was performed as described previously (Kutzleb et al., 1998). For pre-

To Western blot analysis, COS-7 cells were grown until just confluent in 10 cm tissue culture dishes. They were washed with cytosol buffer and then frozen by floating the dish on liquid nitrogen for 10 sec. After thawing, the cells were washed with cytosol buffer, fixed with 4% paraformaldehyde in PBS and permeabilized with 0.02 or 0.04% saponin. For double-labeling experiments, cells were incubated simultaneously with both primary antibodies, which were then visualized with Cy3-labeled donkey anti-rabbit and with Cy2-labeled donkey anti-horseradish peroxidase. The sections were washed briefly with cytosol buffer and then incubated for 10 min at pH 7.0, 0.25 mg M GOAc, 100 μM EDTA, 1 mm dithiothreitol) in a glass-Telfon homogenizer. The homogenate was spun at 110,000 g for 20 min. The supernatant was collected as the cytosol (fraction 1).

Immunfluorescence microscopy. Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 and 0.05% Tween 20. Small gels were washed again with cytosol buffer, fixed with 4% paraformaldehyde in cytosol buffer, and processed for immunofluorescence. The characteristic immunofluorescence patterns of recombinant proteins were not seen if permeabilized cells were incubated with cytosol buffer only and were seen only in sparsely cells, presumably perforated by handling, if unfrozen cells were incubated with cytosol buffer plus GTPγS (negative controls). Copicapsulation of RI and neurobeachin by CAM-agarose. Rat brain cytosol was prepared as described above, but with rubin will be supplemented with 0.1% Triton X-100 and 0.05% Tween 20. CAM-agarose (Sigma A7396, 11-atom spacer with N-6 attachment) was preblocked with 3% BSA in cytosol buffer at 4°C for 1 h and then washed in cytosol buffer with 0.1% Triton X-100 and 0.05% Tween 20. CAM-agarose (50 μl) was incubated
with 1 ml cytosol, either with or without addition of 10 mM cAMP (Biolog, Bremen, Germany), for 5 hr at 4°C with gentle mixing. Beads were spun down, washed with 6 × 1.5 ml cytosol buffer, 0.1% Triton X-100, 0.05% Tween 20, and resuspended in SDS-PAGE sample buffer, and aliquots were analyzed by SDS-PAGE and immunoblotting.

**RESULTS**

Identification and primary structure of neurobeachin, a BEACH-WD40 domain protein

To identify new proteins of neuronal synapses, a chicken brain cDNA expression library in λgt11 was immunoscreened with a serum raised against synaptic plasma membranes (Lichte et al., 1992). One of the immunopositive clones hybridized to almost brain-specifically expressed mRNA in Northern blot analysis (see Fig. 5). By several rounds of plaque hybridization rescreening beginning with this chicken cDNA as a probe, a mouse cDNA contig of 10949 nt encompassing a complete reading frame was established. The putative start codon is preceded by 466 nts of GC-rich 5′-untranslated sequence with several in-frame stop codons, and the end of the reading frame is followed by 1675 nts of a 3′-untranslated sequence with multiple in-frame stop codons but no poly(A) tail. The mouse cDNA encodes a polypeptide of 2936 amino acids (327 kDa) that is moderately acidic (pI 6.0) and has a high percentage of hydrophobic (38%) and aromatic (7.7%) amino acids.

The C-terminal region of this protein, which we name neurobeachin, contains a BEACH domain (Figs. 1, 2). The BEACH domain has been defined as a sequence motif of ~280 amino acids that the LYST gene product, mutated in the beige mouse and human Chediak-Higashi syndrome, shared with several anonymous sequences in the database (Nagle et al., 1996). It is present in multiple gene products and is evolutionarily ancient. In a database screen, we found 10 distinct BEACH sequences from mammalian sources (human and/or mouse), 6 from Caenorhabditis elegans, and 5 in the Drosophila genome. BEACH sequences are also found in fish (Fugu), plants (Arabidopsis), slime molds (Dictyostelium), and yeasts (Saccharomyces cerevisiae and Schizosaccharomyces pombe).

**BEACH domain proteins have a common overall architecture (Fig. 1).** In all 10 sufficiently long sequences currently available (5 distinct mammalian molecules and 1 each from fly, worm, plant, slime mold, and yeast), BEACH domains are followed by a WD40 repeat and then by the C terminus of the polypeptide. Upstream of the BEACH domain, the sequences of most proteins containing this sequence module are unrelated. Among these are the only three BEACH proteins about which any functional information is available: mammalian LYST and FAN and protozoan LvsA. The LYST gene product is involved in the biogenesis of lysosomes and lysosome-related secretory granules (Burkhardt et al., 1993; Nagle et al., 1996; Perou et al., 1996, 1997; Dell’Angelica et al., 2000). LvsA is implicated in the plasma membrane dynamics of Dictyostelium cell division (Kwak et al., 1999). FAN is a signal transduction protein that interacts with the cytoplasmic domain of the 55 kDa tumor necrosis factor receptor (Adam-Klages et al., 1996). YCS2, the only yeast gene product with a BEACH domain, is functionally uncharacterized. A mammalian gene product named BGL aligns with neurobeachin and shares its domain organization over its entire length and may therefore represent an isoform. The BGL sequence, apparently incomplete at the N terminus, was found in a genetic study (Feuchter et al., 1992), and no biochemical or other functional information on this protein is available.
Figure 2. Predicted amino acid sequences of mouse neurobeachin (mNbea), partial chicken neurobeachin (cNbea), and DAKAP550 (DAKAP). The partial DAKAP550 sequence in regions A–D (short variant, DAKAP550S, without the facultative insert in region A) was taken from Han et al. (1997) and completed by us in regions D–G. Approximate borders between regions A and G, based on additional sequence comparisons with BGL, CEF10F2.1, LYST, FAN, YCS2, LvsA, and BEACH-containing ESTs, are indicated above the neurobeachin sequence by diamond symbols and letters. The sequence database accession numbers for mouse and chicken neurobeachin and for the completed DAKAP550 sequence are Y18276–Y18278.
Full-length neurobeachin homologs also exist in Drosophila (DAKAP550) and C. elegans (CEF10F2.1) (Fig. 1).

Comparison of the neurobeachin sequence with its full-length and partial homologs defines regions of high or low sequence similarity (Fig. 1). The BEACH domain is highly conserved between all these proteins. In the WD40 regions, sequence similarity of neurobeachin is high to BGL, moderate to DAKAP550 and AKAP Ht31, and low to CEF10F2.1, but very low in comparison with LYST, FAN, LvxA, and YCS2 such that only the overall WD40 architecture is conserved between the partial homologs. Regions B and D (corresponding to amino acids 951–1311 and 1576–1872, respectively, of mouse neurobeachin) diverge in sequence or length among neurobeachin, BGL, DAKAP550, and CEF10F2.1. These two regions of neurobeachin have a distinctively lower percentage of hydrophobic amino acid residues (27–28%) than the other regions (36–43%). Between mouse and chicken neurobeachin, sequence similarity is very high (88% amino acid identity) also in regions B and D where neurobeachin and BGL diverge (Fig. 2), and several human ESTs in the sequence database have 100% amino acid sequence identity with mouse neurobeachin, including sequences in region B. This indicates very high phylogenetic sequence conservation of neurobeachin in vertebrates, and that mouse neurobeachin and human BGL are no species orthologs but distinct paralogs in region B. This indicates very high phylogenetic sequence conservation of neurobeachin in vertebrates, and that mouse neurobeachin and human BGL are no species orthologs but distinct paralogs in region B.

We found neurobeachin and BGL to be colinear with a partial cDNA from Drosophila, DAKAP550 (Han et al., 1997), with sequence similarity in regions A and C but not in B and D. We have completed the C-terminal DAKAP550 sequence, showing that DAKAP550 is a full-length homolog of neurobeachin and BGL (Figs. 1, 2). The DAKAP550 sequence is slightly more related to neurobeachin than to BGL (56% identity with neurobeachin in the C-terminal part of region A, 63% in region C, 59% in region E, 78% in region F, and 44% in region G, and low similarity at the beginning of region B; as opposed to 52% (A), 61% (C), 55% (E), 76% (F), and 45% (G) identity with BGL).

Two binding sites for the RII of PKA were identified by Han et al. (1997) in region B of DAKAP550. Neither these sites nor other
parts of DAKAP550 region B have sequence similarity to region B sequences of neurobeachin or BGL. However, the similarity of domain organization prompted us to investigate RII binding to regions B of neurobeachin and BGL. The RII binding sites of most established AKAPs do not share sequence similarity. Their common feature is the propensity to form an amphiphilic a-helix (Carr et al., 1992; Nauert et al., 1996; Han et al., 1997; Fraser et al., 1998; Gray et al., 1998; Colledge and Scott, 1999).

Domains B of mouse neurobeachin and human BGL, and a region of similar size (~350 amino acids) centered around the RII binding sites of Drosophila DAKAP550 as a positive control, were amplified by RT-PCR and subcloned into a His-tag expression vector. The interaction of the recombinant proteins with recombinant bovine RII was analyzed by SPR (Fig. 3). We found that domain B of neurobeachin binds RIIa with high affinity (K_d, 10 nM) and slow dissociation kinetics, in a fashion very similar to the Drosophila molecule, in which the affinity for RIIa is higher yet (K_d, 2 nM). In contrast, no significant RII binding could be detected with domain B of BGL (Fig. 3A,B). Neurobeachin region B binds only the RII isoforms, preferring RIIa (K_d, 10 nM) slightly over RIIb (K_d, 30 nM), whereas no detectable binding was found with RIIa and RIIb (Fig. 3C). A synthetic 22-residue peptide, Ht31 (493–513), derived from the RII binding site of the human thyroid AKAP, Ht31 (Carr et al., 1992), efficiently competed with neurobeachin region B for RII binding, with an IC_{50} of 24 nM at a region B concentration of 1 μM (Fig. 3D). This indicates that the same site on RIIa binds Ht31 and neurobeachin and is additional evidence for the specificity of the RII–neurobeachin interaction. Binding measurements with a series of deletion constructs delineated the RII binding site of neurobeachin region B to an interval between amino acids 1022–1107 (Fig. 3E). This interval contains a sequence with a high potential to form an amphiphilic a-helix (amino acids 1081–1099) (Fig. 3F) that is a good candidate for the core binding site.

From rat brain cytosol, neurobeachin could be coprecipitated with RIIa by cAMP-agarose, suggesting the existence of a complex of both native proteins. Pulldown of both proteins was blocked by an excess of free cAMP (Fig. 4).

**Figure 4.** cAMP-agarose coprecipitates neurobeachin with RII from rat brain cytosol. Immunoblots were developed with antibodies against neurobeachin (Nbea), EEA1 (negative control), and RIIa as indicated: cytosol, lane 1; cAMP-agarose beads after incubation in cytosol without (lane 2) or with (lane 3) 10 μM competitor cAMP. γ-Adaptin as a second negative control (data not shown) gave the same result as EEA1. The RIIa band of the cytosol sample is displaced downward by the thick, unlabeled tubulin band migrating above it.

**Figure 5.** Brain-specific expression of neurobeachin. A, Chicken neurobeachin mRNA [10 μg poly(A)^+ RNA per lane]. R, Mouse neurobeachin protein (50 μg protein of tissue homogenate per lane). Tissue abbreviations are as follows: A, adrenal gland; B, brain; BS, brain stem; C, cerebellum; FB, forebrain; H, heart; I, small intestine; K, kidney; Li, liver; Lu, lung; M, muscle; O, ovary; Pa, pancreas; Sp, spleen; St, stomach; Te, testis. Smears and minor bands below the main bands are attributed to partial degradation of these very long mRNA and protein molecules.

**Neurobeachin is a brain-specific protein**

A Northern blot with mRNAs from several chicken tissues was hybridized with the chicken neurobeachin cDNA, 10.2 (Fig. 5A). A large mRNA of ~12 kilonucleotides hybridized with high intensity in forebrain and cerebellum, hybridized weakly in the endocrine tissues, adrenal gland, testis, and ovary, was barely detectable on longer exposures of the blot in heart and lung, and was undetectable in skeletal muscle, liver, spleen, and pancreas.

Analysis of the National Center for Biotechnology Information UniGene database showed that, in good agreement with the chicken Northern blot, most ESTs representing human neurobeachin (UniGene cluster Hs.3821) are from neuronal and endocrine tissues. Among 11 neurobeachin cDNAs from tissue-specific libraries, 7 are from brain and 1 each from retina, germ cell, testis, and kidney. In contrast, human BGL ESTs (UniGene cluster Hs.62354) come from a large variety of tissues but few from brain (56 cDNAs: one each from brain and retina, others from liver, spleen, colon, ear, germ cell, heart, kidney, lung, lymph node, pancreas, parathyroid, placenta, prostate, skin, stomach, testis, uterus, whole blood).

To determine the tissue specificity of neurobeachin at the protein level, a Western blot of homogenates from various mouse tissues was probed with an affinity-purified antibody that had been raised against recombinant neurobeachin region B (Fig. 5B). A strong protein band of molecular size far above the 206 kDa standard was detectable only in brain lysates, with similar intensities in forebrain, cerebellum, and brainstem; a very faint band was seen in stomach. This band could be specifically immunoprecipitated from brain lysate, and its labeling is blocked by preincubation of the antibody with neurobeachin region B but not by BGL region B (data not shown). Thus, Western blot analysis of mouse tissues indicates a highly brain-selective expression of neurobeachin protein. The low mRNA levels in endocrine tissues seen by Northern blot analysis may not be translated into protein.

During mouse postnatal brain development, neurobeachin mRNA abundance is highest in neonatal brain and declines to
reach adult levels (~50% of neonatal) at postnatal day 25. Neurobeachin mRNA expression in NS20Y neuroblastoma cells is not affected within 24 hr by raising the intracellular cAMP level through treatment with forskolin/IBMX (data not shown).

Neurobeachin is associated with tubulovesicular neuronal endomembranes near the trans-Golgi and throughout the cell

Immunoperoxidase staining of rat brain sections prominently visualizes the cell bodies and thick processes of many neuronal populations throughout the brain (Fig. 6). In light microscopy, immunoreaction product typically appears as coarse granules in the cytoplasm of neurons and their proximal dendrites, whereas the nuclei are spared (Fig. 6D,E). Neuropil-rich regions are stained in a diffuse or finely grained fashion, whereas myelin-rich regions are poorly stained or unstained.

In electron microscopy, neurobeachin immunoreaction product decorates the cytosolic faces of vesicular and tubular intracellular membranes, often in clusters (Fig. 7A). Neurobeachin-positive vesicle clusters are often adjacent to the ends and the concave faces, i.e., the trans sides, of Golgi stacks (Fig. 7B). Also the dilated ends of Golgi cisternae are sometimes decorated (Fig. 7C). Occasionally, extensive neurobeachin-positive endomembrane fields were observed (Fig. 7D). Neurobeachin immunoreactivity was also seen on buds of the plasma membrane (Fig. 7A, arrows). In the neuronal periphery, patches of immunoreaction product were found throughout thick and thin processes, at some postsynaptic sites, and, rarely, presynaptically (data not shown). These characteristic immunoperoxidase staining patterns in light and electron microscopy were seen with two different affinity-purified sera against region B. They were not observed if the antibodies were incubated
with an excess of the recombinant antigen or if preimmune antibodies were used as negative controls.

By pre-embedding immunogold electron microscopy of neurobeachin-positive subcellular structures in rat brain neurons. A, The electron-dense neurobeachin immunoreaction product decorates polymorphic tubulovesicular endomembranes (arrowheads) and plasma membrane buds (arrows) of a cerebellar Purkinje cell. B, Neurobeachin immunolabeling of a vesicle cluster next to the concave face of a Golgi stack (arrowhead) in a Purkinje cell body. A multivesicular body (mb) is immunonegative, as generally observed. C, Labeling of the dilated ends of Golgi-like membrane cisternae in a pyramidal neuron of the hippocampus. D, An extensive field of immunopositive tubulovesicular endomembranes surrounded by several Golgi stacks (arrows) in a Purkinje cell. er, Endoplasmic reticulum; n, nucleus. Scale bar (shown in A): A, 1 μm; B, 0.6 μm; C, 0.5 μm; D, 0.8 μm.

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Neurobeachin immunofluorescence puncta concentrate near the Golgi complex but do not colocalize with any of numerous endomembrane marker proteins

Neurobeachin is detectable in NS20Y mouse neuroblastoma cells and PC12 rat neuroendocrine cells by both immunoblotting (data not shown) and immunofluorescence (Fig. 9). Immunofluorescence visualizes small puncta, which are densest around the nucleus where they often form clusters or strings but also scatter...
throughout the cytoplasm. Diffuse immunolabeling is additionally observed.

By double-immunofluorescence microscopy of PC12 cells with various marker antibodies, we explored the membrane compartment(s) with which neurobeachin is apparently associated (most data not shown; see Fig. 9 for examples). Clearly different patterns of no notable overlap with neurobeachin were produced by antibodies against Lamp-1 (lysosomes), rab7, and the cation-independent 300 kDa mannos-6-phosphate receptor (late endosomes), transferrin receptor (recycling endosomes), EEA1 (early endosomes), PDI (endoplasmic reticulum), and synaptophysin (synaptic-like microvesicles), and with Mitotracker (mitochondria). Patterns qualitatively similar to neurobeachin but without notable overlap of puncta were obtained for secretogranin II (secretory granules), Rab1A [ER-Golgi intermediate compartment (ERGIC)], Rab4, synaptobrevin 2, and cellubrevin (early endosomes and transport vesicles). The coat proteins involved in the generation of transport vesicles at the trans-Golgi network (TGN), clathrin heavy chain, γ-adaptin (AP-1), δ-adaptin (AP-3), and β-NAP/β3B-adaptin (a neuron-specific AP-3 subunit isoform) also gave patterns qualitatively similar to, but little or no clear overlap of, individual puncta with neurobeachin. The markers β-COP (transport vesicles of the Golgi complex and ERGIC), KDEL receptor (ERGIC and cis-Golgi), mannosidase II (Golgi complex), and TGN38 (TGN) labeled broad perinuclear ribbons. Neurobeachin-positive perinuclear clusters and strings were sparser than the gross Golgi or TGN structures labeled by these markers, lying within or immediately adjacent to them (Fig. 9). Conversely, in the cell periphery, puncta positive for β-COP, KDEL receptor, mannosidase II, and TGN38 were much sparser than neurobeachin-positive puncta and did not overlap with them. In conclusion, none of the markers tested displayed significant colocalization with neurobeachin, particularly not in the cell periphery. Very close proximity or partial overlap, within the resolution of light microscopy, was seen between perinuclear neurobeachin clusters and proteins of the Golgi complex or adjacent structures, notably with β-COP, KDEL receptor, mannosidase II, and TGN38. Perinuclear neurobeachin also follows Golgi rearrangement induced by aluminum fluoride. In untreated PC12 cells, microtubules were seen by immunofluorescence to tangentially surround the nucleus in a symmetrical way. AlF₄⁻ treatment for 30 min caused their rearrangement to a polarized pattern that converged presumably on the microtubule-organizing center (MTOC) (data not shown). Concurrently, the markers β-COP (data not shown) and KDEL receptor (Fig. 9A) concentrated around the MTOC, and so did perinuclear neurobeachin (Fig. 9A). Under all conditions, neurobeachin-positive perinuclear structures were in close vicinity or partial overlap but sparser and not congruent with β-COP-positive and KDEL receptor-positive structures. In the cell periphery, neurobeachin-positive puncta were more numerous than and did not overlap with β-COP-positive and KDEL receptor-positive puncta (Fig. 9A). The AlF₄⁻-induced redistribution of perinuclear neurobeachin toward the MTOC region, concurrent with the microtubule cytoskeleton and Golgi markers, suggests a physical or functional linkage between perinuclear neurobeachin-positive membranes and the Golgi complex or the microtubule cytoskeleton.

Neurobeachin association with Golgi-near membranes is stimulated by GTPγS and dispersed by brefeldin A

The proximity of a subpopulation of neurobeachin-positive endomembranes to the trans-Golgi/TGN suggested that neurobeachin

Figure 8. Pre-embedding immunogold electron microscopy of neurobeachin-positive endomembranes in rat cerebellar neurons. A, Immunolabeling concentrating at the concave side of a Golgi stack in the cell body of an unidentified neuron. B, Labeling of the dilated ends of an unidentified endomembrane tubule or cisterna in a Purkinje cell body. C, Labeling of the postsynaptic membrane of a granule cell dendrite in contact with a Golgi cell axon terminal. D, Labeling of small vesicle or tubule cross sections (arrows) in unidentified cell processes in the granule cell layer. a, Axon terminal; d, dendrite; er, endoplasmic reticulum; m, mitochondrion; n, nucleus. Scale bar (shown in A): A, 0.50 μm; B, 0.37 μm; C, 0.20 μm; D, 0.27 μm.

Wang et al. • BEACH Domain and AKAP Protein on Neuronal Endomembranes J. Neurosci., December 1, 2000, 20(23):8551–8565 8559
might associate with these membranes in an ADP-ribosylation factor (ARF) GTPase-dependent fashion, as do coat proteins involved in vesicle budding at the Golgi and TGN. First, we studied the effect of BFA on the subcellular distribution of neurobeachin in live PC12 cells (Fig. 9B). The circumnuclear clustering of punctate neurobeachin immunofluorescence dispersed very quickly, within 1 min, whereas diffuse neurobeachin increased and punctate neurobeachin in the cell periphery could still be seen. This BFA effect on circumnuclear neurobeachin was as fast as the dispersion of the coat protein \( \beta \)-COP (Fig. 9B), whereas the overall structure of the Golgi membrane system as visualized by the intrinsic membrane proteins, mannosidase II (Fig. 9B) and KDEL receptor (data not shown) was still largely intact after 1 min. However, although perinuclear neurobeachin dispersed rapidly, neurobeachin-positive puncta in the cell periphery remained visible through 30 min of BFA treatment. During this time the Golgi disintegrated, as visualized by mannosidase II (Fig. 9B) and KDEL receptor (data not shown) immunofluorescence. Circumnuclear clustering of punctate neurobeachin and the normal distributions of \( \beta \)-COP, mannosidase II, and KDEL receptor were fully reversible within 30 min after BFA was washed out (data not shown).

Next, we could demonstrate that neurobeachin is recruited in vitro from cytosol to perinuclear endomembrane structures of freeze-permeabilized cells in a fashion stimulated by GTP\( \gamma \)S and antagonized by BFA. In this experimental setup (Robinson and Kreis, 1992; Seaman et al., 1993), antibodies detect proteins newly recruited from exogenous cytosol (prepared from rat brain), whereas they give little or no background signal with corresponding endogenous proteins of the acceptor cells (monkey COS-7 cells), either because they do not cross-react with the COS-7 proteins, because these cells do not express the particular protein (as in the case of neurobeachin), or because the endogenous protein is washed out after permeabilization. This allows the selective detection of newly recruited proteins.

Immunoblot analysis (Fig. 10) shows that neurobeachin recruitment is strongly enhanced by GTP\( \gamma \)S but antagonized by BFA. Moreover, GTP\( \gamma \)S added after BFA fails to stimulate recruitment, whereas BFA after GTP\( \gamma \)S cannot block recruitment, as demonstrated previously for \( \gamma \)-adaptin (AP-1) and \( \mu \)3-adaptin (AP-3) recruitment (Robinson and Kreis, 1992; Simpson et al., 1996). This is in agreement with the current view of coat recruitment where BFA acts upstream of GTP by blocking GDP-to-GTP exchange on ARF (Springer et al., 1999). As positive controls in our experiment, both \( \gamma \)-adaptin (an AP-1 subunit) and \( \beta \)-NAP (a neuron-specific AP-3 subunit) show the same behavior. In contrast, as negative controls, neither the binding of exogenous RII\( \alpha \) subunit nor of HSB [a novel cytosolic protein peripherally associated with membranes; Kutzleb et al. (1998)] to acceptor cells is stimulated by GTP\( \gamma \)S or inhibited by BFA (Fig. 10).

Double-immunofluorescence microscopy demonstrates that neurobeachin is primarily recruited to perinuclear ribbons that are also positive for acceptor cell giantin, an intrinsic Golgi protein (Fig. 11). Neurobeachin labels all giantin-positive ribbons and in addition, but more faintly, numerous giantin-negative patchy structures...
all over the cell periphery. When inspected in fine detail (data not shown), giantin immunofluorescence forms continuous, sharp-edged threads, whereas neurobeachin immunofluorescence forms ribbons composed of many small puncta that lie over and immediately beside the giantin threads, suggesting neurobeachin recruitment to occur on many individual foci in the immediate vicinity of the Golgi complex. Giantin-negative patches of neurobeachin recruitment in the cell periphery are also clusters of small puncta and may represent endosomal or ERGIC membranes. However, double immunofluorescence for neurobeachin or γ-adaptin versus the early-endosomal marker EEA1 displayed only a low degree of overlap (data not shown). Only a minority (~10%) of EEA1-positive structures had recruited γ-adaptin or neurobeachin, and weakly so in comparison to EEA1-negative neurobeachin and γ-adaptin patches in the periphery, and an even smaller minority of neurobeachin- or γ-adaptin-positive patches were EEA1 positive. 

β-COP, γ-adaptin, β-NAP, and δ-adaptin are recruited to the same gross structures as neurobeachin, but with different preferences between the Golgi vicinity and the cell periphery. Like neurobeachin, β-COP recruitment (data not shown) occurs primarily on the perinuclear ribbons, whereas the labeling of peripheral patches is faint. γ-Adaptin (Fig. 11) densely labels all perinuclear giantin-positive ribbons, but with the same intensity also decorates the giantin-negative patches in the periphery, and it forms coarser puncta than neurobeachin and the other coat proteins. β-NAP, in contrast, is more sparsely recruited to the giantin-positive structures, producing scattered clusters of puncta rather than contiguous ribbons, thus displaying an even higher preference for the cell periphery (Fig. 11) in accordance with previous observations (Simpson et al., 1996). δ-Adaptin displays a distribution similar to β-NAP (data not shown). In double immunofluorescence, individual neurobeachin puncta within these gross structures are distinct from β-COP, γ-adaptin, or β-NAP puncta, respectively. 

Whereas neurobeachin and these coat proteins are recruited to distinct puncta on the same gross structures, the negative control proteins RIIα and HSB produce entirely different patterns. HSB immunofluorescence gives a pattern of fine puncta spread uniformly across the cell (data not shown). RIIα (Fig. 11) binds primarily to the centrosome and to mitochondria (identified, in separate double-immunofluorescence experiments not shown, with antibodies against γ-tubulin and cytochrome c oxidase, respectively). Separate subcellular compartments are known to carry AKAPs that may be responsible for this binding behavior (Chen et al., 1997; Schmidt et al., 1999; Takahashi et al., 1999; Witczak et al., 1999). Ribbons decorated intensely by neurobeachin occasionally also are labeled weakly by RIIα, suggesting corecruitment of RIIα to these structures via neurobeachin.

Immunofluorescence labeling of mitochondria by RIIα seems to be weaker in the presence than in the absence of GTPγS, in agreement with the slightly weaker signals also obtained by immunoblot analysis (Fig. 10). Centrosome labeling by γ-tubulin and RIIα differs at high magnification (data not shown). Whereas γ-tubulin immunofluorescence typically forms two round dots, the RIIα-positive structure is a short thread forming a circle or horse-shoe around them. These observations have no bearing on neurobeachin but may be of interest for the study of mitochondria- and centrosome-associated AKAPs.

Recruitment experiments analyzed by immunofluorescence microscopy were additionally performed in the presence of ATPγS or AlF4− (data not shown). ATPγS stimulated the recruitment of neurobeachin, γ-adaptin, and β-NAP to an extent similar to GTPγS, in agreement with observations made previously on γ-adaptin (Robinson et al., 1992). AlF4− was found to stimulate the recruitment of β-COP but not of γ-adaptin or neurobeachin, providing an additional criterion discriminating a putative neurobeachin-associated coat from the COP-I coat.

Subcellular fractionation indicates cytosolic and cytoskeletal-like subpools of brain neurobeachin

A 120,000 × g fraction of brain homogenate showed that approximately two-thirds of total neurobeachin was recovered in the supernatant and one-third was recovered in the pellet (Fig. 12, fractions S and P). The same result was obtained if 150 mM NaCl was omitted from the homogenization buffer or replaced by 320 mM sucrose (data not shown). From the pellet, brain neurobeachin could be extracted with neither 1 M NaCl nor 1% Triton X-100 but could be extracted with 0.1 M sodium carbonate, pH 11. In this behavior, neurobeachin differed from the intrinsic membrane protein, synaptophysin, which was almost completely solubilized by the detergent but not by sodium carbonate and was similar to the cytoskeletal control protein, tubulin (Fig. 12). Therefore, binding to salt- and detergent-resistant proteinaceous structures rather than directly to membranes seems to give rise to the sedimentable neurobeachin subpool. These observations suggest that brain neurobeachin is primarily a cytosolic protein that peripherally associates with the membranes that it decorates in immunomorphology, but a subpool is more firmly bound to a cytoskeletal-like subcellular fraction.

DISCUSSION

Neurobeachin binds protein kinase A

Neurobeachin binds RII with high affinity and specificity. Thus, neurobeachin qualifies as an AKAP, and like classical AKAPs it does not bind the type I regulatory subunit. RII binding was previously identified in the protein product of a partial Drosophila cDNA (DAKAP550) (Han et al., 1997) homologous to neurobeachin, and we show here that neurobeachin harbors an RII binding site of similar functional properties in a region colinear with the DAKAP550 RII binding site although not conserved in sequence. Vice versa, we demonstrate by completing the Drosophila cDNA sequence that it encodes a full-length neurobeachin homolog. DAKAP550 is the only full-length counterpart of both neurobeachin and BGL in the Drosophila genome. Its sequence is almost equally distant to neurobeachin and BGL, and although it is 10-fold more abundant in fly heads than bodies, it does not seem to be as highly tissue specific as mouse neurobeachin. Like neurobeachin, it behaves as a cytosolic protein in cell fractionation but
gives a granular pattern in immunolight microscopy (Han et al., 1997).

A rapidly growing number of AKAPs are recently emerging that recruit PKA to its diverse subcellular sites of action. Some AKAPs are very small and may consist only of kinase anchoring and subcellular targeting domains (Fraser et al., 1998; Gray et al., 1998), whereas others such as gravin/AKAP250 [1780 amino acids (Nauert et al., 1996)], mAKAP/AKAP100 [2319 amino acids (Kapiloff et al., 1999)], and the yotiao/AKAP350/AKAP450/CG-NAP family of splice variants [1645–3908 amino acids (Schmidt et al., 1999; Takahashi et al., 1999; Witczak et al., 1999; Westphal et al., 1999)] are much larger and can be expected to have functions in addition to PKA anchoring. Such additional functions can include the possession of binding sites not only for PKA but also for other regulatory enzymes, as known for AKAP79 (Klauck et al., 1996), gravin (Nauert et al., 1996), and yotiao and CG-NAP (Takahashi et al., 1999; Westphal et al., 1999), making these molecules act as scaffolding proteins. Also, neurobeachin is a large multidomain protein and probably ties the recruitment of PKA, and thus a regulatory input by this kinase, into the context of its additional functions. The existence of the putative isoform, BGL, which presumably is functionally similar to neurobeachin but has no RII binding site in region B, emphasizes that RII binding is only one functional facet of neurobeachin.

Neurobeachin is recruited in coat protein-like fashion to trans-Golgi-near membranes: a role in membrane trafficking?

Neurobeachin is peripherally associated with the cytoplasmic faces of tubulovesicular endomembranes, which concentrate in trans-Golgi-near locations but also distribute throughout the neuronal soma and dendrites, and additionally with the postsynaptic plasma membranes of some synapses. The trans-Golgi-near location suggests an association with or a possible involvement in the generation of transport organelles. Indeed, association of neurobeachin with the perinuclear, Golgi-near compartment is stimulated by GTP

Figure 11. Recruitment of neurobeachin from cytosol to Golgi-near membranes is stimulated by GTPγS and antagonized by BFA: double-immunofluorescence analysis. The localization of newly recruited neurobeachin in the presence of BFA versus GTPγS (left/right) is shown in comparison to the reference proteins (top/bottom) giantin (Golgi membranes), γ-adaptin and β-NAP (AP-1 and AP-3 coats), and RIIα (negative control). It can be seen that neurobeachin and the two coat proteins are recruited in a BFA/GTPγS-sensitive fashion to giantin-positive ribbons and, with different relative preferences, additionally to patches in the cell periphery. Recruitment experiments analyzed by immunofluorescence were also performed under the additional incubation conditions of Figure 10 (buffer only, cytosol only, sequential BFA and GTPγS treatments), producing relative recruitment intensities in agreement with Figure 10 (data not shown).
This putative neurobeachin-linked coat involves a BFA-sensitive GTPase but is apparently distinct from COP-I, AP-1, AP-3, or the neuronal AP-3 variant with β-NAP, because in permeabilized COS-7 cells neurobeachin and markers for these four coat types are recruited to distinct foci and with different regional preferences, albeit on the same gross endomembrane structures close to the Golgi and in the cell periphery. Neither was significant colocalization of neurobeachin with β-COP (COP-I), γ-adaptin (AP-1), δ-adaptin (AP-3), or β-NAP observed at steady state in double-immunofluorescence microscopy of PC12 cells. The recruitment of neurobeachin to membranes of COS-7 cells suggests that it interacts with molecular receptors that may normally, in these non-neuronal cells, bind BGL or other neurobeachin homologs.

Various membrane trafficking events are known to be influenced by PKA, including the generation of vesicles at the TGN for both constitutive and regulated secretion in neuroendocrine PC12 cells (Ohashi and Huttner, 1994) and for constitutive secretion in non-neuronal NRK cells (Muniz et al., 1997). The Golgi complex and TGN are major subcellular locations of RII, both in neurons (De Camilli et al., 1986) and in non-neuronal cells (Griffiths et al., 1990). In neurons, neurobeachin presumably contributes to the concentration of RII in the trans-Golgi region, but particularly in non-neuronal cells, the bulk of RII at this location is probably bound by other AKAPs such as the yotiao/AKAP350/AKAP450/CG-NAP family. In our own double-immunofluorescence experiments with neurobeachin versus RIIα or RIIβ in PC12 cells (data not shown), both regulatory subunits gave punctate patterns with perinuclear clustering similar to, but no obvious colocalization of individual puncta with, neurobeachin. This also suggests that neurobeachin is responsible for the recruitment of only a fraction of RII present in the trans-Golgi region, probably targeting it selectively to specific substrate proteins and events regulated by PKA.

Many, probably most, neurobeachin-positive membrane profiles lie in the neuronal cell periphery. However, only a small proportion of GTPγS-stimulated recruitment occurs to Golgi-distant structures in permeabilized COS-7 cells; in live PC12 cells BFA rapidly disperses perinuclear neurobeachin, whereas punctate immunofluorescence remains visible in the cell periphery. Perhaps neurobeachin is recruited to nascent membrane organelles primarily in the trans-Golgi region by an ARF-dependent, BFA-sensitive mechanism but remains associated with them after completion of the ARF GTPase cycle and translocation of the organelles out to the cell periphery.

Using a large number of protein markers for a wide range of endomembrane compartments, we have been unable to detect clear colocalization of any of them with neurobeachin by double-immunofluorescence microscopy. According to their electron microscopic morphology, neurobeachin-positive membranes in the neuronal cell periphery might be ERGIC or endosomal subcompartments or transport organelles (Hirschberg et al., 1998; Nakata et al., 1998; Burack et al., 2000; Kaether et al., 2000). The neuron-specific expression of neurobeachin could reflect a role in the trafficking of neuronal membrane proteins such as neurotransmitter receptors or ion channels. Besides the identification of neurobeachin-binding proteins in addition to RII, genetic approaches are also expected to shed light on the functions of neurobeachin and its homologs. The chromosomal locations of the human and mouse neurobeachin genes have been determined, but no loci of neurogenetic defects that map to their vicinities are known at present in man or mouse (Gilbert et al., 1999).

**Postsynaptic neurobeachin**

We initially identified the neurobeachin cDNA by immunoscreening with a serum raised against synaptic plasma membranes, and indeed we observe by immuno-electron microscopy an association of neurobeachin with the postsynaptic plasma membranes of some synapses. The identity of most neurobeachin-positive synapses is unclear, but among them are GABAergic synapses formed between Golgi cell terminals and granule cell dendrites in the glomeruli of the cerebellum. Within a population of morphologically similar synapses, only a few percent were neurobeachin positive. It will be of interest to clarify which type or functional states of synapses a concentration of neurobeachin at the postsynaptic membrane is correlated.

Neurobeachin may arrive at the postsynaptic membrane in escort of transport organelles from the perikaryon or it could be part of the machinery of local postsynaptic membrane traffic. Local de novo synthesis of some neurotransmitter receptor subunits and other postsynaptic proteins (Angenstein et al., 1998; Gardiol et al., 1999; Schuman, 1999; Huber et al., 2000; Sigrist et al., 2000), the regulated exocytosis and re-endocytosis of neurotransmitter receptors (for review, see Lüsher et al., 2000; Turrigiano, 2000), and shape remodeling of the postsynaptic compartment [Okabe et al., 1999; Toni et al., 1999; Sigrist et al., 2000 (and references therein)] are recently emerging as important mechanisms in the ontogeny and plasticity of synapses. Independently of a possible role in membrane protein trafficking, neurobeachin may add to the group of postsynaptic scaffolding proteins like AKAP79, yotiao, and spinophilin, which are believed to recruit PKA and other protein kinases and phosphatases for the regulation of neurotransmitter receptors and ion channels by reversible phosphorylation (Fraser and Scott, 1999).

**The BEACH domain**

Neurobeachin is the fourth full-length member to be characterized of the emerging family of BEACH-WD40 proteins. The members of this family share a common architecture in which the BEACH and WD40 repeat modules are positioned at the C terminus, whereas the upstream sequences of most of them are dissimilar. There are at least 10 gene products with BEACH sequences in mammals, 5 in *Drosophila*, 6 in *C. elegans*, and several in *Arabidopsis*, but only 1 in *S. cerevisiae*. This suggests that BEACH-WD40 proteins exist in all eukaryotes but that the expansion to a family is a hallmark of multicellular organisms. It remains to be seen whether additional full-length homologs of neurobeachin, BGL, or LYST will be found among the as yet uncharacterized mammalian BEACH-WD40 proteins.

The function of the BEACH domain is unknown. As pointed...
out earlier (Nagel et al., 1996), its size of ~280 amino acids is much larger than a site for protein–protein interaction. It might be a protein module with, e.g., an enzymatic activity of its own. Although BGL appears to be an isoform of neurobeachin, LYST has no explicit sequence similarity with neurobeachin upstream of the BEACH domain. However, the upstream sequence of LYST is similar to neurobeachin in length and amino acid composition, including numerous hydrophobic stretches that in LYST were noted by Nagle et al. (1996) to resemble HEAT or armadillo repeats. LYST, like neurobeachin, is a cytosolic protein peripherally associated with endomembranes and the cytoskeleton (Faigle et al., 1998). It is possible, therefore, that there is a distant relationship in structure and function beyond the BEACH-WD40 region. In both proteins, intramembrane protease cleavage results in membrane proteins between endosomes, lysosomes, and the plasma membrane (Faigle et al., 1998; Barrat et al., 1999), and an analogous role for neurobeachin in another pathway of membrane traffic is conceivable, e.g., in the sorting, routing, or targeting of neuron-specific plasma membrane proteins. It will be of interest to see whether other BEACH domain proteins, or other AKAPs, are recruited to membranes in a GTP- and BFA-sensitive fashion like neurobeachin.

Also Dictyostelium LvsA (Kwak et al., 1999) and the functionally uncharacterized yeast BEACH protein, YCS2/BPH1 (SWISS-PROT accession no. P25356, gene designation YCR032w), are large proteins of 3619 and 2167 amino acids, respectively, that have no clear sequence similarities with neurobeachin or LYST upstream of the BEACH domain but similar amino acid composition. A short, degenerate sequence motif centered around RRYL-QNTALEVF in neurobeachin, is detectable ~60–100 amino acids upstream of the BEACH domain also in LYST, FAN, YCS2, and LvsA (Fig. 1). A genetic defect of LvsA perturbs plasma membrane dynamics, causing an arrest during the course of cytokinesis and instead the formation of a large plasma membrane bulge (Kwak et al., 1999). The molecular mechanisms underlying this phenotype are unknown. FAN, which mediates receptor-induced activation of neutral sphingomyelinase, has only a short upstream sequence, and there are no indications for a role of FAN in membrane protein trafficking. An involvement, directly or indirectly, in the modulation of local membrane lipid composition may be a common denominator of neurobeachin, LYST, LvsA, and FAN.

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Wang et al. • BEACH Domain and AKAP Protein on Neuronal Endomembranes J. Neurosci., December 1, 2000, 20(23):8551–8565