Integrin-associated Protein Is a Ligand for the P84 Neural Adhesion Molecule*

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P84 (also known as SHPS-1, BIT, and SIRP) is a heterophilic adhesive membrane protein involved in receptor tyrosine kinase signaling that is found at synapses in the mammalian central nervous system and in non-neural tissues. We have identified a binding partner for P84 using an expression cloning strategy. Here we report that integrin-associated protein (IAP/CD47) is a predominant binding partner of P84. Immunohistochemistry reveals a virtually identical distribution of P84 and IAP in a variety of adult brain regions. Because IAP has been implicated in cell signaling in cells of the immune system, P84 and IAP represent a heterophilic binding pair that is likely to be involved in bi-directional signaling at the synapse and in other tissues.

A number of adhesive molecules have been shown to be important for formation and maintenance of synaptic connections in the CNS.1 Immunoglobulins, cadherins, integrins, as well as proteoglycans and their receptors appear to contribute to synapse formation (1–4). We originally identified the immunoglobulin family member P84 by virtue of its adhesive properties when tested with cerebellar and neocortical neurons (5). The ability of P84 to promote neurite outgrowth and stimulate robust filopodial extension in growth cones suggested that its downstream effects of P84 activation are, and what extracellular molecules are involved in initiating signaling via P84.

To address this last question, we undertook an expression-cloning approach to identify ligands that bound to the extracellular segment of P84. We have discovered that a predominant ligand for P84 is the integrin-associated protein (IAP/CD47). In addition to its expression in lymphocytes and other extraneuronal tissues, IAP was known to be expressed in the brain, and its expression has recently been associated with memory formation (11). Antibodies against IAP blocked the adhesion of cerebellar neurons, erythrocytes, and thymocytes to P84-coated substrates. We also showed that the distribution of P84 and IAP in the cerebellum and retina were very similar and consistent with their participation in a heterophilic synaptic adhesion complex.

EXPERIMENTAL PROCEDURES

Production of Alkaline Phosphatase Fusion Proteins—Alkaline phosphatase (AP)-tag2 and AP-tag4 plasmids were a generous gift of J. Flanagan (Harvard University, School of Medicine). The sequences corresponding to the signal peptide and extracellular segment of murine P84 were amplified by polymerase chain reaction, and cloned into the BglII site of the AP-tag 2 vector. This plasmid was transfected with LipofectAMINE (Life Technologies Inc.) into 293T cells (Edge Biosystems), and the supernatant was collected between days 4 and 8. The supernatant was monitored for phosphatase activity using a synthetic substrate, p-nitrophenyl phosphate (Sigma 104 phosphatase substrate) (12). The fusion protein was affinity purified and analyzed by SDS-polyacrylamide gel electrophoresis. As a control, 293T cells were transfected with the AP-tag4 plasmid, which directed the secretion of AP alone. For P84 ligand purification, the P84-transfected culture supernatant was incubated with tissue sections or cells (receptor alkaline phosphatase (RAP) in situ) following the method of Cheng and Flanagan (13). The extracellular domain of IAP was cloned into the AP-tag2 vector, and an IAP-AP fusion protein was produced as described above for P84-AP. Immunopurified P84, L1, and neural cell adhesion molecule (N-CAM) were spotted on nitrocellulose, blocked, and incubated with IAP-AP for 30 min. After washing, AP activity was detected with NBT/BCIP.

Expression Cloning—An adult mouse brain cDNA library was obtained from Edge Biosystems Inc. (Gaithersburg, MD). This library was constructed in the pEAK5 plasmid vector. Forty pools of about 2500 colonies each were plated. These were harvested into liquid medium, and an aliquot of each was saved. DNA was extracted from the remainder of each sample (Perfect Prep Kit, 5 Prime–3 Prime, Inc.). DNA from each pool (0.3–0.5 μg) was transiently transfected into COS-7 cells in 6-well plates using LipofectAMINE. After 48 h, cells were incubated with P84-AP fusion protein and stained. From a single positive pool, a single cDNA clone was purified by sib selection. Briefly, the culture corresponding to the positive pool was replica plated onto nylon membranes, and one of these membranes divided into ten segments. DNA was extracted from each of the subpools and tested for staining with the P84-AP fusion protein. This process was iterated until a single positive cDNA clone was obtained. As a control, transfected and untransfected cells were stained with P84-AP and AP alone. The positive clones were sequenced using vector-specific primers (pEAK5.for -gatctctggatctgat) and with gene-specific primers.

Immunostaining of Cells and Tissue—Animals were perfused with 4% paraformaldehyde in PBS, and dissected tissues were cryoprotected in 30% surose. Cryostat sections were collected on pretreated slides. The monoclonal P84 and IAP (miap301, PharMingen) antibodies were

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§The abbreviations used are: CNS, central nervous system; IAP, integrin-associated protein (CD47); BCP, 5-bromo-4-chloro-3-indolyl phosphate p-toluene sulfonic salt; NBT, nitro blue tetrazolium chloride; AP, alkaline phosphatase; RAP, receptor alkaline phosphatase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; N-CAM, neural cell adhesion molecule.
Fig. 1. P84 ectodomain fusion protein. A, diagram of the P84 ectodomain fused to human alkaline phosphatase. B, SDS-polyacrylamide gel electrophoresis of purified P84-AP (lane 2); Sigma high molecular weight markers (lane 1). The major band in lane 2 lies between the 116 and 205 kDa markers. C, postnatal mouse cerebellar neurons attach to P84-AP and extend neurites after 24 h in vitro. D and E, RAP in situ staining of sections of adult mouse cerebellum stained with P84-AP (panel D) and with AP alone (panel E). M indicates molecular layer, and G indicates granule cell layer. Bar in panels C and E indicate 0.1 mm.

Previously described (5, 14). Sections were stained with primary antibodies for 1 h and incubated with FITC-conjugated goat anti-rat secondary antibody (Cappel) for 30 min. The staining was examined with fluorescence microscopy.

Cell Adhesion Assays—Coverslips were first coated with nitrocellulose (Schleicher and Schuell, Inc.) as described previously (6). Purified proteins (P84, P84-AP, laminin, or miap301 antibody) were spotted at the center of the coated coverslips in 5-mm spots. The substrate solution was aspirated after 5 min. Coverslips were blocked with 1% BSA in PBS followed by medium containing 10% horse serum. Laminin was a generous gift of Dr. J. Hassel (University of Pittsburgh). Cerebellar cells were prepared as described previously (15). Blood was collected from adult mice in heparinized tubes, washed twice, and suspended in 1% BSA containing 0.5 mg/ml AP alone; Sigma high molecular weight markers and E indicate molecular layer and G indicates granule cell layer.

In the cerebellum, the molecular layer was heavily stained, and a pattern consistent with synaptic glomeruli in the granule cell layer was observed (Fig. 1D). This P84-AP cerebellar staining was very similar to that seen with P84 antibody staining (7), which suggested that the P84-AP probe detected an endogenous extracellular binding partner for P84. No staining was observed with AP alone (Fig. 1E).

Expression Cloning of a P84-binding Protein—To search for P84-binding proteins, we screened a mouse brain cDNA library with the P84-AP fusion protein. Transfected cells were screened for P84-AP binding using the procedure described by Cheng and Flanagan (13). AP without the P84 ectodomain was included as a negative control. From the 40 pools screened (about 2,500 colonies in each pool), two positive pools were identified. From one of these, a single positive cDNA clone was purified (Fig. 2). Sequencing of the cDNA derived from this clone revealed that it was the brain-specific form of mouse IAP (form 4) (16). This form of IAP has the longest cytoplasmic domain of all the forms of IAP. We also noticed that a region of 63 nucleotides that encode 21 amino acids in the extracellular domain is also lacking in some mouse, rat, and human IAP (like domain) is lacking in this particular clone. This 21 amino acid region is also lacking in some mouse, rat, and human IAP forms (Fig. 3A) (17). An IAP-AP fusion protein was tested for binding on purified P84, L1, and N-CAM. Binding was observed on P84 but not on the other molecules (Fig. 3B).

Anti-IAP Antibody Can Block Cell Binding to P84—Freshly dissociated cerebellar cells can bind to native P84 within 10 min. We tested whether IAP was present on trypsin-dissociated cerebellar cells. Anti-IAP (miap301) was bound to nitrocellulose-coated coverslips, and cerebellar cells were tested for their ability to bind to these coverslips. Cell binding to this antibody occurred within 10 min; with overnight incubation, the cells remained attached to the antibody substrate and extended neurites (data not shown). To determine whether IAP represented a major binding partner for P84, we attempted to block neuronal IAP with the miap301 antibody which is directed against the IgV domain of IAP. We then tested antibody-treated neurons for their ability to bind to purified brain P84. Freshly dissociated cerebellar cells were incubated with anti-IAP antibody or in antibody-free media for 30 min at 0 °C and then plated on P84-coated coverslips. Cells were allowed to attach for 2 h at 37 °C, washed with PBS, and photographed.

RESULTS

Production of a Soluble P84 Ectodomain Fusion Protein—To facilitate the cloning of P84-binding proteins, a cDNA encoding the P84 ectodomain was inserted into the AP-tag2 vector (13). This plasmid directs expression of a soluble fusion protein with the P84 ectodomain was inserted into the AP-tag2 vector (13). This plasmid directs expression of a soluble fusion protein with human alkaline phosphatase at the carboxyl-terminal end (Fig. 1). 293T cells transiently transfected with this construct secreted the recombinant P84-AP, and this fusion protein was purified with an anti-P84 affinity column (Fig. 1B). Because native P84 was known to be a good substrate for cerebellar cell attachment and neurite outgrowth, we tested the purified P84-AP fusion protein in a cell adhesion assay. The purified P84-AP was immobilized on Petri dishes, and mouse cerebellar cells were allowed to attach and grow for 24 h. There was no obvious difference in the attachment of neurons or pattern of neurite growth on native P84 or P84-AP (Fig. 1C, and see Ref. 5). RAP in situ staining (13) was done to examine P84 binding activity in the brain regions which were known to contain P84.
The antibody-blocking effect was dramatic; virtually no antibody-treated neurons attached, in contrast to the large number of cells that attached in untreated controls (Fig. 4, A and B). To be sure that the blocking effect was specific, we tested the ability of anti-IAP to interfere with neuronal binding to laminin. As shown in Fig. 4, D and E, anti-IAP had no effect on neuronal binding to laminin. Both P84 and IAP are found on a number of non-neuronal tissues, with IAP found on lymphocytes and erythrocytes (as well other tissues) and P84 found on dendritic cells and macrophages (18, 19). To determine whether any of the other IAP expressing cells could bind to P84, we tested thymocytes and erythrocytes for their ability to bind to brain P84. Both erythrocytes and thymocytes attached rapidly to P84-coated coverslips, and this binding could be completely blocked by anti-IAP antibody (Fig. 4, G–L). These experiments support the idea that IAP might be a ubiquitous receptor for P84.

**P84 and IAP Have Nearly Identical Distributions in Synapse-rich Regions of the Brain—**To be a functional ligand-receptor binding pair in the brain, P84 and IAP must be expressed on adjacent membranes of interacting cells. Previous studies with a P84 antibody demonstrated an apparent synapse-associated distribution of P84 in cerebellum and retina. We stained adjacent sections from these two structures with anti-IAP and anti-P84 antibodies for comparison. The overall distribution of P84 and IAP was strikingly similar. In the cerebellum, the molecular layer exhibited the most intense P84 and IAP staining, and in the granule cell layer, staining was observed that appeared to be associated with synaptic glomeruli (Fig. 5, A and B; see also Ref. 6). In the retina, both P84 and IAP were found in the synapse-rich inner plexiform and outer plexiform layers, with little or no staining outside of these synaptic layers (Fig. 5, C and D). This co-localization of P84 and IAP is consistent with an adhesive association between these two molecules in vivo.

**DISCUSSION**

P84 is a synapse-associated cell adhesion molecule that is a member of the immunoglobulin superfamily (5, 7). P84 is identical to SHPS-1, BIT, and SIRP-α, molecules that are known to bind the tyrosine phosphatase SHP-2 and modulate cell signaling (8–10). The human SIRPs comprise a large family of signaling molecules, encoded by different genes, with multiple isoforms generated from these genes by alternative splicing (10). Because P84 has been associated with synapse-rich re-

**FIG. 4. Inhibition of cell attachment to P84 by anti-IAP.** Cerebellar neurons attach to immobilized P84 in the absence of anti-IAP (A), but attachment is inhibited by antibody (B). Neuronal attachment to laminin is unaffected by absence (D) or presence (E) of anti-IAP. Panel G demonstrates binding of erythrocytes, and panel J demonstrates binding of thymocytes to P84 in the absence of anti-IAP. This binding is completely blocked by anti-IAP as shown in panels H and K, respectively. Cell counts for each experiment are shown to the right in panels C, F, I, and L. Asterisk indicates values that differ significantly. Bar indicates 20 μM.

**FIG. 5. Co-localization of P84 and IAP in adult mouse CNS.** A, adult mouse retina was stained with anti-P84. Arrowhead indicates outer plexiform layer, and arrow indicates inner plexiform layer. B, retina stained with anti-IAP shows a similar pattern to that seen in panel A. C, mouse cerebellum shows intense staining with anti-P84 in the molecular layer (M). Structures within the granule cell layer (G) are also stained. D, cerebellum stained with anti-IAP shows a pattern similar to that seen in panel C. Bar indicates 0.1 mm.
regions in the CNS, and because P84 was known to bind to a heterophilic receptor, the intriguing possibility existed that P84 and its receptor were involved in establishment or regulation of synaptic function. Isolation of a membrane ligand that bound to the extracellular domain of P84 was critical for the analysis of mechanisms by which P84 and this ligand contributed to the formation of synapses.

We have cloned a ligand for the P84 molecule and identified it as IAP (CD 47). This identification is supported by the following observations. 1) The distribution of P84 binding activity (by RAP-in situ staining with P84-AP fusion protein) and IAP by immunohistochemistry are identical. 2) The distribution of P84 and its ligand, IAP, within the brain and retina are also identical, with both being strongly expressed in various synaptic regions. 3) A monoclonal antibody against IAP completely blocked the attachment of cerebellar neurons, erythrocytes, and thymocytes to P84-coated substrates. These findings support our conclusion that IAP was indeed a ligand for the P84 adhesion molecule.

IAP was initially described as a 50-kDa cell surface protein that was involved in the enhancement of neutrophil adhesion, chemotaxis, and phagocytosis triggered by extracellular matrix molecules (20, 21). IAP did not directly interact with integrin ligands (RGD-containing peptides) but could physically associate with certain integrins and could regulate integrin function (21). IAP appears to be a functional component of several processes including (a) transendothelial and transepithelial migration of neutrophils (23–25); (b) integrin-mediated activation of neutrophils (14, 21); (c) modulation of T-cell activation (26, 27); (d) modulation of binding between integrins and ligands (24); (e) direct interaction between IAP and thrombospondins (C-terminal cell binding domain) (28); and (f) stroma-supported erythropoiesis in spleen and other tissues (17).

IAP was widely expressed in hematopoietic cells (erythrocytes, lymphocytes, platelets, monocytes, and neutrophils) and other tissues (placenta, surface epithelia, liver, and brain), including cells that did not express integrins (29, 22). This suggested that there were integrin-independent functions of IAP. The structure of human and murine IAP was inferred from primary sequence of cloned cDNAs (30). The N terminus of IAP (extracellular) contains an immunoglobulin-like domain, most similar to members of the IgV family. Several alternatively spliced isoforms of IAP mRNA are known to exist, one of these (form 4) being most abundant in the brain and peripheral nervous system (16). The human SIRP gene family contains at least 14 members that differ in the amino acid sequence of the extracellular domains (10). Whether these different forms of P84/SIRP all bind to IAP isoforms or to other ligands, remains to be determined.

Recently, an interesting correlation has been noted between levels of IAP mRNA in the hippocampus and memory retention in rats (11). These authors suggest that IAP may have a role in hippocampal synaptic plasticity and memory formation, an idea consistent with our finding that IAP is a ligand for the synaptic neural adhesion molecule, P84.

P84 homologues SHPS-1 and SIRP are known to be involved in cell signaling via tyrosine phosphorylation in a variety of cell types. As noted above, IAP is also known to play critical roles in cell signaling in cells of the immune system. Our findings identify P84 and IAP as a bi-directional signaling pair that may be preserved across a variety of tissues, including both the nervous system and immune system. Although the details of the mechanisms of signaling of each of these molecules remains unclear, their distinct structures suggest that the signals are likely to be different in the cells that express P84 and those that express IAP. Based on their synaptic localization and apparent signaling capacities, we propose that P84 and IAP may also be involved in regulation of synaptic function and remodeling.

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REFERENCES

1. Mayford, M., Barzilai, A., Keller, F., Schacher, S., and Kandel, E. R. (1992) Science 256, 638–644
2. Fannon, A. M., and Colman, D. B. (1996) Neuron 17, 423–434
3. Einheber, S., Schnapp, L. M., Salzer, J. L., Cappelli, Z. B., and Milner, T. A. (1996) J. Comp. Neurol. 370, 105–134
4. Husey, Y. P., Yang, F. C., Kharazia, V., Naisbitt, S., Cohen, A. R., Weinberg, R. J., and Sheng, M. (1995) J. Cell Biol. 132, 139–151
5. Chuang, W., and Lagenaur, C. F. (1990) Dev. Biol. 137, 219–232
6. Abosch, A., and Lagenaur, C. (1993) J. Neurobiol. 24, 344–355
7. Comu, S., Wong, W., Olinsky, S., Ishwad, P., Mi, Z., Hempel, J., Watkins, S., Lagenaur, C. F., and Narayan, V. (1997) J. Neuroscience 17, 8762–8780
8. Fujikura, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Touda, M., Takada, T., and Kasuga, M. (1996) Mol. Cell. Biol. 16, 6887–6899
9. Sano, S., Ohnishi, H., Omori, A., Hasegawa, J., and Kubota, M. (1997) FEBS Lett. 411, 327–334
10. Kharitonenkov, A., Chen, Z., Suess, I., Wang, H., Schilling, J., and Ulrich, A. (1997) Nature 386, 181–186
11. Huang, A-M., Wang, H. L., Tang, Y. P., and Lee, E. H. Y. (1998) Science 280, 6887–6899
12. Flanagan, J. G., and Leder, P. (1990) Cell 63, 185–194
13. Cheng, H.-J., and Flanagan, J. G. (1994) Cell 79, 157–168
14. Lindberg, F. P., Bullard, D. C., Caver, T. E., Gresham, H. D., Beaudet, A. L., and Brown, E. J. (1996) J. Neuroscience 16, 795–798
15. Schmutzer, J., and Schachner, M. (1984) J. Neuroscience 4, 429–456
16. Reinhold, M. I., Lindberg, F. P., Plas, D., Reynolds, S., Peters, M. G., and Brown, E. J. (1995) J. Cell Biol. 101, 101–106
17. Adams, S., van der Laan, L. J. W., Vernon-Wilson, E., de Lavallete, C. R., Dopp, E. A., Dijkstra, C. D., Simmons, D. L., and van den Berg, T. K. (1998) J. Immunol. 160, 1853–1859
18. Timms, J. F., Carlberg, K., Hu, H., Chen, H., Kamatkar, S., Nadler, M. J. S., Roehrsneider, L. R., and Neel, B. G. (1998) Mol. Cell. Biol. 18, 3838–3850
19. Senior, R. M., Gresham, H. D., Griffin, G. L., Brown, E. J., and Chung, A. E. (1992) J. Clin. Invest. 90, 2251–2257
20. Brown, E., Hooper, L., Ho, T., and Gresham, H. (1991) J. Cell Biol. 111, 2785–2794
21. Maewy, W. J., Holmes, C. H., Anstee, D. J., Spring, F. A., and Tanner, M. J. (1994) Biochem. J. 304, 525–530
22. Cooper, D., Lindberg, F. P., Gambale, J. R., Brown, E. J., and Vadas, M. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3978–3982
23. Lindberg, F. P., Gresham, H. D., Reinhold, M. I., and Brown, E. J. (1996) J. Cell Biol. 134, 1313–1322
24. Parkos, C. A., Colgan, S. P., Liang, T. W., Nusrat, A., Bascarra, E. E., Carnes, D. K., and Madara, J. L. (1996) J. Cell Biol. 132, 437–450
25. Wachowiak, M., Majdic, O., Stulnig, T., Berger, M., Baumruker, T., Knapp, W., and Pfeil, W. F. (1997) J. Immunol. 159, 5345–5354
26. Ticchioni, M., Deckert, M., Mary, F., Bernard, G., Brown, E. J., and Bernard, A. (1997) J. Immunol. 158, 677–684
27. Gao, A.-G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J., and Frazier, W. A. (1996) J. Biol. Chem. 271, 21–24
28. Rosaules, C., Gresham, H. D., and Brown, E. J. (1992) J. Immunol. 149, 2759–2764
29. Lindberg, F. P., Gresham, H. D., Schwarze, E., and Brown, E. J. (1993) J. Cell Biol. 123, 485–496