Mammalian brain contains a high mass protein (HMAP) that is unusually rich in atypical L-isoaspartyl (isoAsp) linkages. HMAP has now been purified from bovine brain by anion exchange, hydroxylapatite, and size exclusion chromatography. It is self-aggregating, acidic, and soluble in 5% trichloroacetic acid. Treatment with chondroitinase ABC eliminates the self-aggregation of HMAP and generates several distinct core proteins with estimated masses of 350–450 (doublet), 180, and 100 kDa, indicating that it is composed mainly of chondroitin sulfate proteoglycans (CSPGs). Most of the isoAsp residues in the 350–450 kDa core protein, which was identified by immunoblotting as phosphacan, a CSPG abundant in adult brain. The regional distribution and developmental profile of HMAP in rat brain support this identification. The 180-kDa core protein contains a tenascin-R-related molecule, consistent with recent observations that phosphacan forms a tight complex with tenascin-R. The average phosphacan molecule in adult brain contains at least seven isoAsp sites. Molecular heterogeneity due to isoAsp may explain some of the complex binding properties phosphacan exhibits with its natural ligands. Formation of isoAsp may be important in the roles that phosphacan and other CSPGs play in development of the nervous system.

We previously reported the presence in mammalian brain of an unusual protein termed high mass methyl-accepting protein (HMAP) discovered on the basis of its ability to serve as an effective substrate for protein-l-isoaspartyl O-methyltransferase (PIMT; EC 2.1.1.77) (1). PIMT selectively methylates proteins and peptides containing atypical L-isoaspartyl (isoAsp) residues that form when certain Asn-Xaa and Asp-Xaa sequences undergo a spontaneous intramolecular rearrangement resulting in an Asp-Xaa linkage that is made through the Asp side chain. The resulting peptide contains an extra methylene in the polypeptide backbone and a free internal ε-carboxyl group. IsoAsp seems to form most rapidly when Xaa is Gly or Ser and when the susceptible sequence falls within a highly flexible domain of the protein (2–6). The presence of isoAsp may result in altered biological function (7) as exemplified by the reduced ability of isoAsp-calmodulin to exhibit calcium-dependent enzyme activation (8) and by the reduced enzyme activities of isoAsp-containing forms of ribonuclease A (9) and the Escherichia coli phosphocarrier protein, HPr (10). Cellular proteins contain low levels of isoAsp, usually less than 0.05 mol/mol of protein. In contrast, we estimated that HMAP contains >1 mol of isoAsp/polypeptide chain (1).

Considerable evidence supports the current view that PIMT serves a repair function in cells. In vitro, purified PIMT has been shown to convert isoAsp-Xaa linkages to normal Asp-Xaa linkages (11–13) and has been shown to restore biological activity to age-damaged, isoAsp-containing forms of calmodulin (8) and the HPr phosphocarrier protein (10). In rat PC12 cells, inhibition of PIMT activity results in a rapid and significant accumulation of isoAsp sites that is reversed when the inhibition is withdrawn (14). Disruption of the PIMT gene in mice produces a phenotype characterized by elevated levels of isoAsp in all tissues examined and by premature death between the ages of 28 and 60 days that is preceded by epileptic seizures (15, 16). The emerging picture implicates isoAsp formation as a major source of spontaneous protein damage in vivo, and PIMT as an essential enzyme for preventing the accumulation of these atypical and deleterious structures.

The unusual level of isoAsp found in HMAP, its high affinity for PIMT, and its unique location in the brain prompted us to undertake a more detailed examination of its physical properties, regional distribution in brain, and developmental profile. Because of its high mass and heterogeneity, we previously speculated that HMAP might be an aggregate of cytosolic, age-damaged proteins that had become metabolically isolated, thereby eluding repair by PIMT (1). The properties of HMAP found in the present study lead, however, to a different conclusion. HMAP consists mainly of chondroitin sulfate proteoglycans (CSPGs) that reside in the extracellular matrix and play a key role in neural development by modulating the adhesive properties of neurons and glia. These new findings have important implications regarding a possible role for isoAsp in the development of the central nervous system.

**EXPERIMENTAL PROCEDURES**

*Materials—S-Adenosyl-l-[methyl-3H]methionine (l-[methyl-3H]AdoMet), 15 Ci/mmol, was purchased from NEN Life Science Products and, when necessary, adjusted to lower specific activity by dilution with unlabeled AdoMet (Sigma) that had been purified prior to use (17). Rat recombinant PIMT was prepared as described (18) and had a specific activity of 9,600–15,200 units/mg, where one unit is defined as 1 pmol of methyl transferred to bovine γ-globulin per min at 30 °C. Protease-free chondroitinase ABC was purchased from Seikagaku America, Inc. Ponceau S, Stains-all, and bovine serum albumin, fraction V, were purchased from Sigma. The Asp 7 and isoAsp 8 forms of 5-sleep-inducing peptide (Trp-Ala-Gly-Asp-Ala-Ser-Gly-Glu) were purchased from Bachem California, Inc. Anti-phosphacan 1, a rabbit polyclonal antiserum to the PTP1 region (19) of rat phosphacan was provided by Dr. Yu Yamaguchi (Burnham Institute, La Jolla, CA). Anti-phosphacan 2 (20) and anti-neurocan (21), rabbit polyclonal antibodies to...*
purified rat phosphacan, and the C-terminal nonhomologous region of recombinant rat neuronan were provided by Dr. Richard U. Margolis (New York University). Anti-versican, a rabbit polyclonal antiserum to recombinant human versican V1 that contains epitopes common to all forms of versican (22), was provided by Dr. Richard LeBaron (University of Texas at San Antonio). Anti-neurocan, anti-neurocan, and anti-complex proteoglycan (CSPG), purified after gel filtration chromatography (23), was provided by Dr. Richard U. Margolis.

**Rat Brain Extracts**—Fresh rat brains used in the animal comparison and regional distribution studies were obtained from two 8-week-old anesthetized Sprague-Dawley male rats (Charles River). All subsequent steps were done at 4 °C using chilled equipment. The brains were homogenized in four volumes of homogenization buffer (5 mM sodium PIPES, pH 7.0, 2 mM EDTA, 10% (w/v) sucrose, 6.5 mM 2-mercaptoethanol, 90 μM phenylmethylsulfonyl fluoride, 0.8 μg/ml leupeptin) by six strokes in a glass Teflon homogenizer at 3/4 motor speed using a Contour Power-Stir (Eberbach Corp.). The homogenates were centrifuged at 100,000 g for 1 h. Supernatants were recovered and stored at -70 °C until used.

For studies on the developmental profile of HMAP accumulation, one Wistar male rat (Charles River) was used for each time point. Extracts were prepared as described above.

**Bovine and Human Brain Extracts**—Bovine and human brain extracts were prepared from frozen cerebral cortex in the same manner as described above for rat brain. The human sample, stored at -70 °C, was from a male who died of myocardial infarction with no evidence of brain disease. In a pilot study of human brain samples assayed for PIMT activity and protein isoAsp content, this sample placed closest to the group average (n = 11) for both of these parameters. The human brain sample was obtained from the Irvine Research Unit on Brain Aging and Alzheimer’s Disease at the University of California, Irvine. Bovine brains, obtained from a local slaughterhouse, were transported to the laboratory on ice. After removing the meninges and the bulk of the white matter that lines the cerebral cortices, the brains were stored at -70 °C until used.

**Purification of HMAP from Bovine Brain**—All operations were done at 4 °C unless otherwise noted. Frozen cerebral cortex (500 g) was thawed slowly and then homogenized in 2 liters of buffer A (20 mM BisTris-Cl, pH 7.0, 2 mM EDTA, 150 mM NaCl, 7.5 mM 2-mercaptoethanol, 100 μM phenylmethylsulfonyl fluoride, using an Ultraturrax model STD Tissuemizer (Tekmar Co.). After centrifuging the homogenate for 1 h at 25,900 g x g, the supernatant was recovered through four layers of cheesecloth and combined with 700 ml of wet, packed DEAE-PIPES, pH 7.0, 2 mM EDTA, 150 mM NaCl, 7.5 mM 2-mercaptoethanol. The chromatography and ultrafiltration steps were then repeated with the second half of the dialyzed HMAP, and the concentrated solutions were prepared as described above.

**Rat Brain Extracts**—Approximately 1 mg of HMAP in a volume of 200 μl were injected per run until all of the HMAP was chromatographed. A flow rate of 0.75 ml/min was used. HMAP eluted near the void volume and was concentrated by ultrafiltration, first with the Amicon ZM-500 membrane and then with the YM-10 membrane.

**Methylation Reactions**—Brain extracts and protein samples destined for analysis by SDS-PAGE and fluorography were methylelized with purified rat PIMT (1.2–3.0 μM) in 50-μl reactions incubated for 10 min at pH 6.2 as described previously (1). In these reactions, [methyl-3H]AdoMet was 36–50 μM and 12,000–30,000 dpm/μl. Prior to SDS-PAGE, disulfide bonds were reduced as described below.

**Immunodetection**—For the initial antibody screening, SDS-PAGE and protein samples were reduced at pH 6.2 for 10 min at 50 °C in the presence of 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS and then brought to the appropriate final sample compositions before loading the gels (29). Disulfide bond reduction at lower pH was found to be ineffective, whereas reduction at higher pHs promotes hydrolysis of the protein methyl esters. Fluorography was carried out as described previously (29).

**Chondroitinase Digestions**—Incubations were done at 37 °C for 45 min in 100 mM Tris-Cl, 30 mM sodium acetate, pH 8.0, with a final concentration of 0.5 milliunit chondroitinase ABC/μg HMAP or rat brain CSPGs and the following protease inhibitors: 1 mM EDTA, 1 μg/ml pepstatin, 0.25 mg/ml N-ethylmaleimide, and 0.4 mM phenylmethylsulfonyl fluoride.

**Immunodetection**—For the initial antibody screening, SDS-PAGE 5% acrylamide gels were used (26). After electrophoresis using a Bio-Rad Mini-PROTRAN II apparatus at a constant 120 V for 1 h, proteins were electrobotted onto nitrocellulose membrane in a Bio-Rad Mini Trans-Blot cell filled with Towbin (30) transfer buffer. Transfer was done at a constant 100 V for 1 h at room temperature with the cooling unit in place. After electroblotting, to visualize the protein bands and assist in cutting the lanes into strips, the nitrocellulose membrane was stained with 0.5% (w/v) Ponceau S dissolved in 1% acetic acid. The strips were blocked with 1% bovine serum albumin in 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TBST), washed, and probed with primary antibodies. After washing with TBST, the strips were incubated with goat anti-rabbit immunoglobulins conjugated with alkaline phosphatase (Vector Labs). Detection of antibody binding was monitored colorimetrically by the reaction of alkaline phosphatase with nitroblue tetrazolium and bromochloroindolyl phosphate (31).

**Immunoblots** used for comparison with fluorographs of [H-]methylated HMAP or rat brain CSPGs were exposed 14–120% using Chemiluminescence Reagent Plus on Whatman 3MM paper. Chemiluminescent bands were identified with a BioMax MR film and digitized by a Hokuto Denko gel documentation system.

**Protein Determination**—Protein concentration was determined by the method of Lowry et al. (32) following precipitation by 5% (w/v) trichloroacetic acid unless otherwise noted. Bovine serum albumin was used as a standard.

**Amino Acid Composition**—After hydrolysis in 6 N HCl, the amino acid composition of HMAP was determined on a Perkin-Elmer Applied

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2 J. Orpiszewski and D. Aswad, unpublished results.
Bioisosteric amino acid analyzer at the Protein/Peptide Micro Analytical Laboratory at the Beckman Institute, California Institute of Technology.

**Protein Sequencing—Chondroitinase-treated HMAP was subjected to SDS-PAGE (pH 2.4) in 10% acrylamide. Where indicated, α-sleep-inducing peptides (DSIP) were present at 400 μM, and PIMT was present at 1.2 μM. A 72-h fluorogram is shown.**

**RESULTS**

**HMAP in Rat, Bovine, and Human Brain—**Fig. 1 shows that soluble extracts from rat, bovine, and human brain contain similar levels of HMAP. Endogenous methylation (lanes 1, 5, and 9) carried out in the absence of added PIMT or alternative substrates results in the methylation of high mass material (HMAP) that barely enters the gel. Note that the acidic SDS-PAGE system used here does not employ a stacking gel. Lanes 2, 6, and 10 demonstrate that endogenous methylation of HMAP is mediated by PIMT because it is blocked by high levels of an isoAsp-containing nonapetide substrate (isoAsp-α-sleep-inducing peptide) but not by the normal Asp form of this peptide (lanes 3, 7, and 11). Maximal methylation of HMAP and of other lower mass substrates is seen when methylation is carried out with addition of purified PIMT (lanes 4, 8, and 12). Because of its size and ready availability, we chose bovine brain as the source for purification and characterization of HMAP.

**Purification of HMAP from Bovine Brain—**Purification of HMAP from bovine brain is summarized in Table I, and the details are described under “Experimental Procedures.” Results of the anion exchange chromatography, which provided the greatest purification of any single step, are shown in Fig. 2. The majority of HMAP eluted as a broad peak between 0.27 and 0.36 M NaCl, suggesting that HMAP is highly acidic. As previously reported (1), HMAP eluted near the void volume during size exclusion chromatography on a BioSil SEC-250 column, indicating an apparent mass of ≈700 kDa.

SDS-PAGE in 10% acrylamide was used to monitor the purification of HMAP (Fig. 3). When gels were stained with Coomassie Blue R-250, the intensity of protein staining at the expected position of HMAP (at the top of the gel lanes) remained low, and very little protein staining was evident anywhere in the most highly purified material (lane 6). Because of the highly acidic nature of HMAP, we tested Stains-all (27, 33) as an alternative. As shown in the right panel of Fig. 3, Stains-all revealed a staining pattern quite different from that obtained with Coomassie Blue. Most notable was the presence of intense purple-blue staining coincident with the expected position of HMAP, which is especially evident at the tops of lanes 10–12.

Initial attempts to quantitate the specific activity and methylation stoichiometry of HMAP during its purification were hampered by problems with accurate protein determination. Our lab typically uses a Lowry assay (32) preceded by precipitation of the protein sample with 5% trichloroacetic acid to remove potentially interfering substances such as reducing agents and chelators. When this method was used with the most purified fractions of HMAP, we obtained protein levels that seemed unusually low. To investigate this further, we compared the trichloroacetic acid-Lowry method with protein estimated spectrophotometrically from the absorbance at 280 and 260 nm and with quantitative amino acid analysis. The latter indicated that purified HMAP has an amino acid composition similar to that of the “average” protein (data not shown), and the 280/260 ratio indicated that HMAP contains little or no nucleic acid. The protein content of purified HMAP estimated by amino acid analysis agreed well with the estimate obtained by absorbances at 280 and 260 nm, and both estimates were 4–7 times higher than that obtained by the trichloroacetic acid-Lowry method. Upon further study, we found that HMAP is highly soluble in 5% trichloroacetic acid. Elimination of the trichloroacetic acid precipitation step, afforded by the use of appropriate buffer blanks, resulted in a Lowry assay that agreed well with the other methods. The direct Lowry assay allowed us to calculate a final specific activity of 17.7 nmol of methyl acceptor sites/mg protein, a value 80 times that obtained with the 26,000 × g soluble supernatant fraction of brain. 3 Considering that HMAP constitutes ≈50% of all the methylation capacity seen in soluble brain extracts (Fig. 1), the isoAsp content of HMAP is calculated to be 150–200 times that of the average protein in brain cytosol. This methylation stoichiometry corresponds to 1 isoAsp residue/60 kDa of polypeptide mass.

**HMAP Is Composed Mainly of Chondroitin Sulfate Proteoglycans—**The tissue distribution, high mass, heterogeneity, solubility, acidic nature, and high affinity for Stains-all of HMAP suggested that it might consist of one or more CSPGs, a group of proteins that is enriched in the extracellular matrix of brain. This hypothesis was borne out by the results shown in Fig. 4. Treatment of purified HMAP with chondroitinase ABC largely eliminated the heterogeneity and self-aggregating tendency of HMAP, resulting in the presence of several distinct protein bands that could be detected on SDS-PAGE gels with Coomassie Blue or, more effectively, with Stains-all. Most notable were bands with apparent masses of approximately 350–450 (doublet), 180, and 100 kDa.

The high stoichiometry of HMAP methylation suggested that...
"authentic" soluble brain CSPGs prepared by standard methods might also be rich in isoAsp. As shown in Fig. 5, this is indeed the case. Equal amounts of HMAP and a preparation of rat brain CSPGs purified by anion exchange and gel filtration chromatography (23) were methylated in the presence or absence of PIMT and then subjected to SDS-PAGE. The electrophoretic behavior and methylation capacity of the two samples were nearly identical.

**Phosphacan Is the Major Methyl-Acceptor in HMAP**—We used antibodies to three brain-enriched CSPGs (neurocan, phosphacan, and versican) in an attempt to identify the protein bands produced by chondroitinase treatment of HMAP. Fig. 6 shows a series of immunoblots obtained after SDS-PAGE of chondroitinase-treated HMAP. Immunoreactivity was seen with two different polyclonal antibodies raised against phosphacan (lanes 2 and 3) but was not seen with antibody raised against neurocan (lane 4) or versican (lane 5). The pattern of immunostaining suggests that both the 350–450-kDa doublet and the 180-kDa band contain phosphacan-like epitopes, whereas the absence of immunostaining bands in the anti-

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**TABLE I**

Purification of HMAP from 500 g of bovine brain cortex

| Step                        | Volume (ml) | Protein (mg/ml) | Activity (nmol/mg) | Purification fold | Yield (%) |
|-----------------------------|-------------|-----------------|--------------------|-------------------|-----------|
| Centrifugation at 25,900 × g| 1850        | 5.7             | 0.22               | 1.0               | 100       |
| DE-23 filtration           | 1325        | 2.6             | 0.51               | 2.3               | 73        |
| Ammonium sulfate precipitation | 192       | 9.4             | 0.94               | 4.3               | 72        |
| DE-52 chromatography       | 317         | 0.24            | 8.3                | 38                | 27        |
| ZM-500 ultrafiltration     | 25.8        | 2.5             | 9.8                | 45                | 27        |
| Hydroxylapatite chromatography | 3.7       | 7.6             | 13.2               | 60                | 16        |
| Size exclusion chromatography | 2.3        | 5.3             | 17.7               | 80                | 9         |

*a* Protein was determined as described under "Experimental Procedures" except that the trichloroacetic acid precipitation step was omitted for samples from the last three stages of purification. In these cases, corrections for the effect of interfering substances in the buffers was made by including the appropriate buffer blanks.

*b* Methyl accepting capacity expressed as nmol methyl groups accepted/mg protein.

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**FIG. 2. Chromatography of HMAP on DEAE-cellulose.** Solid line, protein content as determined by the Bio-Rad protein assay reagent. Dotted line, methylation of HMAP determined by incubating fractions with [methyl-3H]AdoMet and PIMT under the same conditions described in the legend to Fig. 1, followed by SDS-PAGE (pH 2.4) in 10% acrylamide and fluorography. The relative intensity of HMAP bands on the film was quantitated by scanning densitometry. Dashed line, conductivity.

**FIG. 3. Purification of HMAP from bovine brain.** Protein samples (10 μg) from the indicated stage of purification were subjected to SDS-PAGE (pH 8.8) in 10% acrylamide. Lanes 1–6 were stained with Coomassie Blue R-250, and lanes 7–12 were stained with Stains-all. SU, low speed supernatant; IE (at left), ion exchange batch adsorption (Whatman DE-23, DEAE cellulose); AS, ammonium sulfate precipitation; IE (at right), ion exchange chromatography (Whatman DE-52, DEAE cellulose); HA, hydroxylapatite chromatography; SE, size exclusion chromatography (Bio-Rad SEC-250).
neurocan and anti-versican lanes suggests that neither of these latter proteins is a major component of HMAP. It should be noted, however, that samples of neurocan and versican were not available to use as positive controls, so the ability of these antibodies to detect their respective antigens under the conditions used could not be assessed. We presume the lack of neurocan immunostaining of chondroitinase-treated rat CSPGs in lane 7 is due to a relatively low level of neurocan in this preparation and/or a lower affinity of the neurocan antibody compared with the phosphacan antibody.

Fig. 7 shows a direct comparison of the protein staining pattern, phosphacan immunoreactivity (using anti-phosphacan 2), and the methylation pattern obtained after SDS-PAGE of chondroitinase-treated HMAP. The strongest immunoreactivity and the strongest methylation both occur in the 350–450-kDa doublet, but immunoreactivity and methylation also occur in the 180-kDa band. A similar 180–185-kDa band observed by others in chondroitinase-treated phosphacan was assumed to be a proteolytic breakdown product of phosphacan (19, 34, 35). It appears that a phosphacan-like molecule is both a major component of our HMAP preparation and the major source of isoAsp sites.

Attempts were made to identify both the 350–450- and 180-kDa bands by N-terminal amino acid sequencing after blotting the SDS-PAGE-separated proteins onto a polyvinylidene difluoride membrane. No reliable sequence information was obtained from the 350–450-kDa region. In contrast, the 180-kDa band yielded the sequence ATGQLDY before the signal was lost amid a high background. A search of the combined protein data base at the Munich Information Center for Protein Sequence on February 2, 1998 using the ATLAS retrieval system revealed a perfect match of this sequence to residues 165–171 of the predicted sequence of rat janusin (J1–160/180) (36), also known as tenascin-R (37). The cDNA of rat tenascin-R codes for a protein of 1356 amino acids and is detected by SDS-PAGE as a 160/180-kDa doublet (36, 38). This finding suggests that the HMAP 180-kDa region contains the bovine form of tenascin-R and that a phosphacan-tenascin-R complex may comprise a significant portion of purified HMAP. This possibility is supported by recent evidence that phosphacan and tenascin-R form tight functional complexes in vivo (39, 40).
Isoaspartate in Brain Proteoglycans

Regional Distribution and Age Dependence of HMAP Levels in Rat Brain—Fig. 8 shows the regional distribution of HMAP in the brain of an 8-week-old rat. Relative to an extract of whole brain, HMAP is enriched in most areas of the forebrain (especially the superior and inferior colliculi) and is lower in the cerebellum and olfactory bulb. The age dependence of HMAP in whole rat brain is shown in Fig. 9. Methylation remained relatively low for the first postnatal week and then increased dramatically up through day 40, the latest day tested. The age dependence of HMAP resembles that of phosphacan as determined by Meyer-Puttlitz et al. (41) using a slot-blot radioimmunoassay.

DISCUSSION

We have purified HMAP 80-fold from a 26,000 × g supernatant fraction of bovine brain. It is soluble in 5% trichloroacetic acid, stains poorly with Coomassie Blue R-250 but intensely purple with the cationic dye Stains-all, and is highly acidic as revealed by its strong interaction with anion exchange material. Treatment with chondroitinase ABC converts HMAP from a self-aggregating and electrophoretically heterogeneous protein with an apparent mass ≥700 kDa into a series of well-defined protein bands at 350–450 (doublet), 180, and 100 kDa. The majority of the methyl-accepting capacity in chondroitinase-treated HMAP is associated with the 350–450-kDa doublet, whereas a lesser but still significant amount is associated with the 180-kDa region. These properties of HMAP, together with our previous observations on tissue specificity (1), indicate that HMAP is composed mainly of brain-specific, chondroitin sulfate proteoglycans that presumably reside in the extracellular matrix. A sample of soluble CSPGs from rat brain (23) displayed electrophoretic behavior and methyl-accepting capacity similar to that of our bovine HMAP. The presence of isoAsp in proteins of the extracellular matrix has not been recognized previously.

Soluble CSPGs that show highly selective expression in brain include phosphacan, a protein whose coding sequence is identical to the first 1616 amino acids of a larger receptor-type protein tyrosine phosphatase (RPTPβ/ζ), and three members of the versican/aggrecan family: neurocan, brevican, and the V2 form of versican (22, 42–46). Table II lists the masses of the core proteins reported for these four proteins as judged by SDS-PAGE after chondroitinase treatment. The apparent mass of the major methyl-accepting species in chondroitinase-treated HMAP is a doublet in the range of 350–450 kDa, a mass that is closest to that of phosphacan and the V2 variant of versican, two CSPGs shown to be abundant in mature brain. Our immunoblot results (Fig. 6) indicate a clear cross-reaction of the bovine 350–450-kDa doublet with antibody directed

![Image](32068)

**Fig. 7.** Identification of phosphacan as the major methyl-accepting protein in HMAP. Chondroitinase-treated HMAP was subjected to SDS-PAGE (NuPAGE, pH 6.4) in a 4–12% acrylamide gradient. Lane 1 shows 5.3 µg of HMAP stained with Stains-all; lane 2 shows an immunoblot of 4 µg of HMAP probed with anti-phosphacan 2 antibody; lane 3 shows 1 µg of HMAP stained with Stains-all; lanes 4 and 5 show fluorograms of 0.5 µg of HMAP methylated by PIMT with 50 µM [methyl-3H]AdoMet (12,000 dpm/µmol). Lanes 4 and 5 had exposures of 5 and 24 h, respectively.

**Fig. 8.** Regional distribution of HMAP in rat brain. Rat brain extracts prepared from rats approximately 60 days old were methylated with PIMT and [methyl-3H]AdoMet, then subjected to SDS-PAGE (pH 2.4) in 10% acrylamide. Gel slices containing HMAP were excised and soaked in 400 µl of 0.5 N NaOH, 5% Triton X-100, and 1% methanol overnight. The eluted radioactivity was quantified by liquid scintillation counting. OB, olfactory bulb; NC, neocortex; FB, remaining parts of the forebrain; ST, striatum; HC, hippocampus; SC, superior colliculus; IC, inferior colliculus; CE, cerebellum; HB, remaining parts of the hindbrain; WB, whole brain homogenate. The bars represent the mean of two experimental measurements on the same extract; error bars represent the range. Where no error bars appear the range was less than ± 0.5 pmol/mg compared with the mean.

**Fig. 9.** Developmental profile of HMAP accumulation in rat brain. Extracts were methylated with PIMT and [methyl-3H]AdoMet and subjected to SDS-PAGE (pH 2.4) in 10% acrylamide. HMAP was quantified as described in the legend to Fig. 8. Data points represent the mean of two or three experimental measurements on the same extract; error bars represent the range. Where no error bars appear the standard deviation was less than 2% of the mean.
against rat phosphacan but not with antibody to rat neurocan or human versican.

The regional distribution of HMAP within the brain, as well as its developmental profile, supports the idea that phosphacan is a major constituent of HMAP and the major source of its isoAsp sites. Immunoblot analysis of rat phosphacan expression using phosphacan-specific monoclonal and polyclonal antibodies has shown that its level rises dramatically for the first 20 days after birth and remains high at least through day 75 (41). Our profile of HMAP expression in rat brain parallels that of phosphacan except that the rise in HMAP begins with a postnatal delay of about 1 week. This is consistent with the expectation that any newly synthesized protein will initially be devoid of isoAsp sites, which subsequently accumulate over a period of days or weeks as the protein ages. In model peptides, isoAsp has been shown to accumulate under physiological conditions at Asn-Xaa sites with half-lives ranging from 6 h to 500 days (4). In rat brain (8 weeks postnatal), we found HMAP to be highest in the cerebral cortex and other forebrain regions and lowest in the cerebellum and other hindbrain regions, although even these regions have significant levels of HMAP. This distribution is consistent with that reported for rat phosphacan, which was found by immunoblot analysis to be considerably higher in the cerebrum than in the cerebellum (47).

Amino acid sequencing of the 180-kDa band of chondroitinase-treated HMAP yielded a seven-residue sequence identical to residues 165–171 of rat tenascin-R. The cDNA of tenascin-R predicts a protein of 1356 amino acids (36), and the protein migrates as a 180/160-kDa doublet on SDS-PAGE (38). To our knowledge, the N terminus of mature rat tenascin-R has never been determined by direct sequencing. Alternative splicing, known in this region, increases the number of isoAsp at several different sites (47).

Because tenascin-R does not contain chondroitin sulfate, its apparent mass on SDS-PAGE should not be altered by chondroitinase treatment. In Fig. 4, a 180-kDa band is discernible in the untreated samples of HMAP (lanes 1, 3, 6, and 8), but the amount of material at this position increases significantly upon chondroitinase treatment (lanes 2, 5, 7, and 10). Much of the chondroitinase-dependent material at 180 kDa is apparently a proteolytic degradation product of phosphacan (19, 34, 35), a conclusion supported by staining of this region with anti-phosphacan polyclonal antibodies (lanes 2 and 3 of Fig. 6). In lanes 7 and 10 of Fig. 4, the 180-kDa band appears to contain two components, a lower one that stains more purple and an upper portion that stains more blue. Thus, the 180-kDa band apparently contains both a proteolytic fragment of phosphacan and a tenascin-R-related protein.

Purified bovine HMAP was able to accept 17.7 nmol of methyl groups/mg of protein. From Fig. 7 we estimate that the 350–450-kDa doublet is responsible for 40% of the protein in HMAP and 90% of the methyl incorporation. Given a polypeptide mass of 172.3 kDa for phosphacan, we estimate its isoAsp content at seven residues/polypeptide chain. Studies on in vitro aging of synthetic peptides and purified proteins indicate that the formation of isoAsp depends strongly on both sequence and flexibility of the polypeptide. The amino acid immediately following an Asn or Asp residue has a major influence on its propensity for isoAsp formation, with Asn-Gly, Asn-Ser, and Asp-Gly sequences appearing most frequently during in vitro aging of proteins (3, 5). Secondary structure disfavors isoAsp formation (6, 48), thus these atypical structures tend to occur when susceptible sequences fall within flexible domains where the α-nitrogen of the amino acid C-flanking to the Asx can make an appropriate angle of attack on the Asx carbonyl to form the requisite succinimide intermediate. Given the extraordinary level of isoAsp in HMAP, it was of interest to determine whether phosphacan contains susceptible sequences, and if so, the number and locations of these sequences. The results of such an analysis are summarized in Fig. 10, where the locations of predicted isoAsp-prone sites are indicated in the context of the domain structure of phosphacan. In choosing the indicated sites, we first considered all Asn-Gly, Asn-Ser, and Asp-Gly sequences, of which there are 18 in rat phosphacan, and then limited further consideration to those that occur in regions predicted to be highly flexible as judged by the flexibility plot of Ragone et al. (49). Seven of the eight predicted sites lie in the carboxyl half of the molecule and are clustered near two of the predicted glycosaminoglycan attachment sites.

The presence of isoAsp may affect the interaction of phosphacan with ligands such as tenascin-R for which binding is apparently mediated by the core polypeptide of phosphacan rather than the glycosaminoglycan chains or other carbohydrate moieties (40). Indeed, the glycosaminoglycan chains of phosphacan interfere with tenascin binding, suggesting that the polypeptide region involved in binding may be located in the vicinity of the glycosaminoglycan attachment sites. The presence of varying amounts of isoAsp at several different sites would introduce considerable heterogeneity into any molecule in which it occurs. Such heterogeneity could provide at least part of the explanation as to why the high affinity binding of rat phosphacan to tenascin-R is apparently limited to only a por-

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**TABLE II**

Reported apparent masses of brain CSPG core proteins detected by immunoblotting

| Protein   | Animal | Mass   | Antibody | Reference |
|-----------|--------|--------|----------|-----------|
| Phosphacan| rat    | 100    | 3F8 mAb  | 52        |
|           |        | 400    | Polyclonal| 35        |
|           |        | 300    | 6B4 mAb  | 47        |
| Neurocan  | rat    | 245/150| 1D1 mAb  | 52        |
| Brevican  | bovine| 145    | Polyclonal| 45        |
| Versican V2| bovine| 400    | Polyclonal| 46        |

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4 A five-residue moving sum of the HV product (hydrophobicity index times side chain volume index) was used to detect regions of high predicted flexibility (49). Most proteins have an average HV sum close to 9000, with approximate limits of 6000 on the low side (maximum flexibility) and 12,000 on the high side. In Fig. 10 we plotted any Asn-Gly, Asn-Ser, and Asp-Gly pair that fell within a five-residue window having an HV sum ≲ 8000.

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**Fig. 10. Location of predicted isoAsp-prone sites in rat phosphacan.** Inverted filled triangles indicate the proposed isoAsp sites. C and F indicate the carbonic anhydrase-like and type III fibronectin-like domains, respectively, and vertical hatched lines indicate likely glycosaminoglycan attachment sites (44).
tion of the phosphacan molecules and why this binding shows an anomalous inhibition at phosphacan concentrations only slightly above its apparent Kᵦₜ for tenasin-R (40). An understand-
ing of the effects of isoAsp on the ligand binding proper-
ties of phosphacan and other isoAsp-bearing CSPGs will likely prove to be important in interpreting such binding data and in understand-
ing the role these molecules play in development of the nervous system.

Although the data presented here implicate phosphacan as the major source of isoAsp in HMAP, we consider it likely that isoAsp also accumulates to a significant extent in other pro-
teins of the extracellular matrix. A survey of the amino acid sequences of neurocan, versican, brevican, and tenasin-R, for example, reveals that all of these molecules contain multiple isoAsp-prone sites. The dominance of phosphacan in bovine HMAP may simply reflect a high abundance of phosphacan, relative to other known CSPGs, in mature mammalian brain.

In this regard, it will be informative to directly assess the isoAsp levels in specific CSPGs isolated from rat brain at various stages of development.

Twenty-four years ago, Robinson and Rudd (50) hypothe-
sized that deamidation of asparagine residues, a process now known to be a major source of isoAsp in proteins, may serve as an intramolecular clock that functions to limit the lifetime of certain proteins by rendering them more susceptible to proteo-
lytic degradation. This idea is supported to some extent by the work of Rogers and Rechsteiner (51) who found an inverse correlation between the Asn and Gln content of proteins and their in uivo half-lives. The deamidation clock hypothesis stems from the observation that amino acid sequence plays a major role in determining the susceptibility of proteins to spontaneous 
deamidation and that such “programmed deamidations” can occur with half-lives extending from hours to years. The high levels of isoAsp we see in brain-specific CSPGs suggest an intriguing modification to this hypothesis. It may be that the formation of isoAsp in these proteins has been intentionally programmed to alter the time scale of isoAsp formation, these molecules would thus provide their natural ligands. By using sequence to alter the time

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