been clearly demonstrated that MBP(s) in rabbit brain are phosphorylated on serine and threonine residues in vivo (Agrawal et al., 1981) and intriguing experiments have provided evidence that phosphate groups on MBP(s) in rat brain turn over within minutes even within the innermost lamellae of compacted myelin (Desjardins & Morell, 1983).

We have previously shown that in isolated myelin membranes from rabbit central nervous system there is a cyclic AMP and Ca\(^{2+}\) -stimulated phosphorylation of the larger subunit of 2',3'-CNPase in vitro (Bradbury et al., 1984; Bradbury & Thompson, 1986a,b). The present study demonstrates that the same subunit is rapidly phosphorylated in vivo.

Adult New Zealand White rabbits (2-2.5 kg) were used throughout this study. Animals were anaesthetized with Sagatal. \(^{32}\)POrthophosphate (in dilute HCl, carrier-free, Amersham International) was injected (1 mCi in 10 \(\mu\)l) into the left cerebral hemisphere under stereotaxic control. Animals were killed at varying time intervals after injection and brains were immediately removed into ice-cold sucrose. Myelin was prepared at 4°C essentially as described previously (Bradbury et al., 1984). Myelin pellets were resuspended in 0.32 m-sucrose, dissolved in sodium dodecyl sulphate sample buffer and electrophoresed in the same one-dimensional polyacrylamide-gel electrophoresis systems described previously (Bradbury et al., 1984). Gels were stained and destained, dried, and autoradiographed as described previously (Bradbury et al., 1984). In control experiments the same procedure was followed except that animals were killed immediately and 0.5 mCi of \(^{32}\)P was added to the 0.32 m-sucrose medium immediately before the brain was homogenized. Protein was measured by the method of Lowry (Lowry et al., 1951), phosphate by the method of Itaya & Ui (1965).

At 24 h after injection acid-soluble radioactivity/nmol of acid-soluble phosphate in brain homogenates fell to approx. 17% of the level seen at 30 min after injection presumably due to equilibration of \(^{32}\)POrthophosphate with the total body phosphate pool. Previous evidence has shown that injected \(^{32}\)POrthophosphate rapidly equilibrates with the y-phosphate of ATP in brain (Desjardins & Morell, 1983), and it is assumed that this also reflects the decay in specific activity of the ATP pool from which myelin membrane proteins become phosphorylated.

Different subcellular fractions (P1, nuclei, SN2, postmitochondrial supernatant, P2A, myelin, P2B, synaptosomes, and P2C, mitochondria) obtained at 1 h after injection were electrophoresed and autoradiographed. Prominent protein phosphorylation at this time-point was seen only in the myelin membrane fraction in proteins migrating with MBP itself and with the larger subunit of 2',3'-CNPase. Autoradiography of P2A myelin membrane fractions from rabbits killed at 1 h, 5 h, and 24 h after injection together with a control in which \(^{32}\)POrthophosphate was added to the medium immediately before homogenizing the brain showed that 2',3'-CNPase phosphorylation did not occur during homogenization but was maximal at approx. 1 h after injection and then decreased significantly at 24 h.

Although not specifically examined in the current study, it is unlikely that the peptide backbone of the enzyme is turning over rapidly since myelin membrane proteins appear to be metabolically stable (Desjardins & Morell, 1983). The rapid labelling of the enzyme and the subsequent decay suggest that the phosphate group(s) on the larger subunit of the enzyme are turning over in vivo and that the protein backbone is being rephosphorylated from a pool of ATP of decreasing specific activity.

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A DNA-sequencing approach to the structure of a key brain membrane endopeptidase

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The enzyme responsible for the inactivation of enkephalins and substance P at the synaptic junction has now been shown to be identical with endopeptidase-24.11 (for review, see Turner et al., 1983). This enzyme is found in the plasma membrane of a variety of cell types but is particularly abundant in kidney microvilli from which it was first purified (Kerr & Kenny, 1974; Fulcher & Kenny, 1983). Its precise distribution throughout the central nervous system has been mapped immunocytochemically using an affinity-purified rabbit polyclonal antibody and shown to correspond very closely with areas rich in neuropeptides such as substance P and [Leu]enkephalin (Matas et al., 1986). Endopeptidase-24.11 from kidney, intestine, lymph nodes and brain are all glycoproteins of Mr 87000–95000 which differ in the extent and pattern of glycosylation but appear identical in amino acid composition (Fulcher et al., 1983; Relton et al., 1983), and it seems likely that the polypeptide chain (Mr 77000) (Stewart et al., 1983) derives from the same gene in these different cell types. In order to study this possibility further and to gain information on the molecular structure of the enzyme and its mode of association with the lipid bilayer, the cloning of the endopeptidase-24.11 gene was undertaken using the expression vector \(\lambda gt11\).
Isolation of mRNA

Since endopeptidase-24.11 is more abundant in kidney than any other tissue (about 100 × greater than in striatum, Matsas et al., 1986), this tissue was chosen for the extraction of mRNA. Early attempts to isolate the endopeptidase-24.11-specific mRNA from intact poly-somes using polyclonal and monoclonal antibodies were unsuccessful, due to the presence of large amounts of ribonuclease (RNase) activity in this tissue. Hence, total kidney poly(A+) RNA was isolated using a method devised to minimize RNA degradation. Current methods used with other tissues rich in RNase activity (e.g. Chirgwin et al., 1979) gave low yields with pig kidney, though this was not the case with rat kidney or pig intestinal mucosa. The purification procedure is outlined in Scheme 1. Precipitation with lithium chloride was essential to remove a low-Mₐ nucleic acid fraction that was strongly inhibitory to the translation of poly(A+) RNA in vitro using a rabbit reticulocyte system. Immuno-precipitation of the primary translation product with affinity-purified polyclonal antibodies revealed a polypeptide of similar Mₐ (77 000) to that of the chemically deglycosylated kidney endopeptidase (Stewart et al., 1984). The poly(A+) RNA fraction represented about 1% of the total cellular RNA.

(1) Cortex homogenized in 5 M-guanidinium isothiocyanate
(2) Hot/cold phenol/chloroform extraction and ethanol precipitiation (1 vol.)
(3) 2 M-Lithium chloride precipitation
(4) Oligo d(T)-cellulose chromatography

Scheme 1. Isolation of poly(A+) RNA from pig kidney

(1) First strand cDNA synthesis (human placental RNase inhibitor, reverse transcriptase)
(2) Second strand synthesis (RNase H, E. coli DNA ligase, DNA polI–Klenow fragment)
(3) EcoRI methylation of cDNA (EcoRI methylase)
(4) Ligation of EcoRI linkers followed by EcoRI digestion
(5) Removal of excess linkers and size fractionation of cDNA (h.p.l.c.)
(6) Ligation of cDNA into EcoRI expression site of λgt11 (T4 DNA ligase)
(7) Packaging of λgt11 recombinant DNA into phage particles
(8) Screening of λgt11 cDNA library with oligonucleotide probes and antibodies

Scheme 2. Cloning of cDNA into λgt11 expression vector

Cloning of poly(A+) RNA in λgt11 expression vector

The cloning procedure is outlined in Scheme 2. Using the RNase H method (Gubler & Hoffman, 1983), double-stranded complementary DNA (ds-cDNA) with an average size of 2.5 kb (max. 5 kb) was synthesized, a range which would include the predicted size of the endopeptidase-24.11 mRNA. EcoRI linkers were ligated to the ds-cDNA and cleaved with EcoRI to generate cohesive termini. However, to avoid the possibility that the endopeptidase-24.11 gene may be cleaved internally by EcoRI, the ds-cDNA was first methylated. Excess EcoRI linkers were separated by a novel h.p.l.c. method using a Waters Protein Pak I-60 column, which also allowed the cDNA to be fractionated. The cDNA corresponding to 1–4 kb was ligated into the EcoRI expression site of λgt11 using the 'Protoclone' system (Promega Biotec) and packaged into phage particles using 'Packagene' (Promega Biotec). The cloning efficiency was 1.0 × 10⁷ p.f.u./μg of ds-cDNA. The library containing 3.5 × 10⁶ p.f.u., of which less than 0.01% were non-recombinant as determined by plaque morphology.

Synthesis of endopeptidase-24.11-specific oligonucleotide probes

Limited proteolysis of the kidney enzyme using either proteinase V8 or trypsin (after acetylation) generated a number of peptides, of which four contained suitable amino acid sequences from which to synthesize mixed oligonucleotide probes. These were synthesized using cyanoether phosphoramidite chemistry and comprised three 17mers and one 15mer corresponding to amino acid sequences (1) -KEDEYF-, (2) -KKQRWT-, (3) DNEGNP- and (4) -NQNKQ-. The second of these is believed to be at or near the N-terminus of the endopeptidase polypeptide chain. Southern analysis showed that least two of the probes (2 and 3) are cross-hybridized strongly to human DNA.

Screening of recombinants

Phage recombinants were screened in E. coli Y1090 (rif^-) using the oligonucleotide probes and both monoclonal (GK7C2 and GK3C7; Gee & Kenny, 1985) and affinity-purified polyclonal antibodies to detect endopeptidase-24.11-specific recombinants. To date, from an initial screening of 200 000 p.f.u., 40 independent clones have been isolated each of which is positive with both polyclonal antibodies and at least one oligonucleotide probe. These are currently being characterized by restriction mapping.

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