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The polyadenylated RNA directing the synthesis of the rat myelin basic proteins is present in both free and membrane-bound forebrain polyribosomes

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Free and membrane-bound polyribosomes were isolated from the forebrain of actively myelinating 25-day-old rats. The poly(A)$^+$ RNA (polyadenylated RNA) extracted from both fractions was translated *in vitro* in reticulocyte lysates [Hall & Lim (1981) *Biochem. J.* **196**, 327–336] in the presence or absence of a heterologous microsomal membrane fraction from dog pancreas. The rat myelin basic proteins synthesized *in vitro* were isolated by CM-cellulose chromatography and by immunoprecipitation with purified anti-(myelin basic protein) antibody. The large (mol.wt. 18500) and small (mol.wt. 16000) myelin basic proteins were translational products of poly(A)$^+$ RNA from both free and membrane-bound polyribosomes. The identity of the myelin basic proteins was verified by analysis of peptides generated by the cathepsin D digestion of the immunoprecipitated proteins synthesized *in vitro*, in comparison with authentic rat myelin basic proteins. Although several other translational products of membrane-bound polyribosomal poly(A)$^+$ RNA were modified when microsomal membranes were present during translation, molecular weights of the myelin basic proteins themselves were unchanged. The myelin basic proteins synthesized *in vitro* also did not differ significantly in size from the authentic myelin basic proteins, indicating that these membrane proteins are unlikely to be synthesized as substantially larger precursor molecules. The presence of the specific mRNA species on both free and membrane-bound polyribosomes is compatible with the extrinsic location of the myelin basic proteins on the cytoplasmic surface of the myelin membrane.

The process of myelination involves the interaction of oligodendrocytes with neuronal axons and results in the formation of a multi-lamellar membrane sheath surrounding the axon. Myelin basic protein is an integral component of the myelin membrane, comprising 30% of the total protein. The sequence of this protein is known (Carnegie, 1971; Eylar *et al.*, 1971), and there is extensive sequence homology between diverse species (Dunkley & Carnegie, 1974). Rodents are exceptional in possessing two major myelin basic proteins; the large myelin basic protein (mol.wt. 18500), corresponding to that of other species, and the small myelin basic protein (mol.wt. 15000–16000), which has an internal deletion of 40 amino acids within the *C*-terminal half of the molecule (Dunkley & Carnegie, 1974). Two minor forms of myelin basic protein (representing about 10% of the content of the large and small basic protein) have also been reported to occur in mouse myelin (Barbarese *et al.*, 1977, 1978). These proteins, termed pre-large (mol.wt. 21500) and pre-small (mol.wt. 17000), differ from the large and small basic proteins respectively in that they have additional sequences located at the *N*-terminus. The structural relationship between these proteins suggested the possibility that, like other membrane proteins, the myelin basic proteins may be synthesized as larger precursor molecules and subsequently modified (see Wickner, 1979). The relationship of mRNA to poly(A)$^+$ RNA (Lim & Canellakis, 1970) is now clearly established. We have previously reported that the rat large myelin basic protein is synthesized in response to the introduction of brain microsomal poly(A)$^+$ RNA

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; poly(A)$^+$ RNA, polyadenylated RNA.

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into *Xenopus* oocytes (Lim *et al.*, 1974), in which processing of heterologous proteins is known to occur (Colman, 1981).

The free and membrane-bound polyribosomes from rat brain contain different populations of poly(A)$^+$ RNA (Hall & Lim, 1981). Certain translational products *in vitro* were found to be specific to membrane-bound polyribosomal poly(A)$^+$ RNA (e.g. proteins of mol.wt. 47000, 33000, 24000, 21000), whereas other proteins were predominantly products of the free-polyribosomal fraction. There were changes in the poly(A)$^+$ RNA populations during development, particularly in the membrane-bound fraction, with the appearance of certain translation products coincident with the onset of myelination (Hall & Lim, 1981; Davison & Dobbing, 1968). Many membrane and secretory proteins are synthesized by membrane-bound polyribosomes [e.g. Ca^{2+}-transport ATPase (Chyn *et al.*, 1979), albumin (Yap *et al.*, 1977)]. However, certain membrane proteins have been found to be synthesized by free polyribosomes (e.g. cytochrome b_5; Rachubinski *et al.*, 1980). Myelin basic proteins have been purported to be synthesized solely on free polyribosomes (Campagnoni *et al.*, 1980); we now present evidence that poly(A)$^+$ RNA directing the synthesis of myelin basic proteins is present on both free and membrane-bound forebrain polyribosomes and that the proteins are not derived from substantially larger precursors.

Materials and methods

Materials

Oligo(dT)–cellulose type T3 was from Collaborative Research (supplied by Uniscience, Cambridge, U.K.). The reticulocyte lysate translational system was obtained from New England Nuclear, Southampton, U.K., and was supplied together with L-[^{35}S]methionine (10.6mCi/ml; sp. radioactivity 1032Ci/mmol). [^{14}C]Methylated protein mixture (molecular-weight markers) and Na^{125}I (100mCi/ml; 13–17mCi/μg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Protein A–Sepharose and CNBr-activated Sepharose 4B were from Pharmacia, Uxbridge, U.K., and bovine spleen cathepsin D (EC 3.4.23.5) and aprotinin were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Dog pancreas microsomal membranes were generously given by Dr. Mike Owen, Imperial Cancer Research Fund, Lincoln's Inn Fields, London.

Animals

Male New Zealand white rabbits (2.5kg) obtained from Brock Rabbits, Sholden, Deal, Kent, U.K., were used to produce antiserum against myelin basic protein. Wistar rats (Porton strain) were bred in our own laboratory.

Preparation of myelin basic protein

Human myelin basic protein was prepared from purified myelin in collaboration with Dr. Louise Cuzner, Department of Neurochemistry, Institute of Neurology, by using a chloroform/methanol extraction procedure (Banik & Davison, 1973).

Rat myelin basic proteins were prepared as previously described (Lim *et al.*, 1974) and radioiodinated as described by Hunter & Greenwood (1962) for use as markers for polyacrylamide-gel electrophoresis.

Myelin-basic protein–Sepharose affinity matrix

Human basic protein (1.4mg/ml) in 0.1M-$NaHCO_3$/0.2M-NaCl (11ml) was mixed with 1.5g of preswollen CNBr–Sepharose 4B for 1h at 25°C and then for 16h at 4°C. After washing with 0.1M-$NaHCO_3$/0.2M-NaCl, remaining active groups were inactivated by incubation in 1M-Tris/HCl (pH8.5) for 2h at 25°C. The myelin-basic-protein–Sepharose was washed sequentially with 1M-NaCl/0.1M-$NaHCO_3$ (pH8.3) and 1M-NaCl/0.1M-sodium acetate (pH4.0) several times to remove unbound protein. Only trace amounts of myelin basic protein were found in the supernatant fraction after binding or in the wash fractions. The matrix was stored at 0–4°C in 0.15M-NaCl/10mM-sodium phosphate buffer (pH7.2)/0.1% (w/v) NaN_3.

Preparation of antisera

Rabbit antiserum to human myelin basic protein was produced as described by Whitaker *et al.* (1976). Human myelin basic protein was coupled to rabbit serum albumin with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide in order to increase its antigenicity in the rabbit. Rabbits were injected subcutaneously with a mixture of 380μg of each protein in Freund's complete adjuvant. Subsequent injections of the same immunogen in Freund's incomplete adjuvant were administered at intervals of 4 weeks for a period of 6 months. With this protocol the myelin basic protein did not evoke any encephalitogenic response in the rabbits for at least 18 months. The antibody titre of sera obtained 7 days after each immunization was assayed by using a solid-phase radioimmunoassay developed by Dr. P. Glynn, Department of Neurochemistry, Institute of Neurology, which was similar to that described by Randolph *et al.* (1977). Briefly, tubes were treated with a solution of myelin basic protein (1μg/ml) for 16h at 4°C. After rinsing they were incubated with bovine serum albumin (0.5%, w/v) for a further 1h at 25°C. Serial dilutions of antisera were added to

the myelin-basic-protein-coated tubes and incubated for 3h at 30°C. ^{125}I-labelled protein A was added to each tube and the incubation continued for 1h before the tubes were washed and the amount of bound protein A was determined by radioactivity counting in a gamma counter. Anti-(basic protein) activity of the sera was detectable 1–2 months after the initial injection and continued to increase until the 5th month. The antibody titre of the serum from one rabbit was 16-fold higher than that of the others and was approx. 5 times less than that of the KK42 anti-(human myelin basic protein) serum of Palfreyman *et al.* (1978) measured by the same assay. This serum was used for the purification of anti-(myelin basic protein) IgG.

Purification of anti-(myelin basic protein) IgG

IgG was purified by precipitation with 40%-satd. $(NH_4)_2SO_4$. The precipitated IgG was redissolved in 15mM-NaCl/10mM-phosphate buffer (pH7.2), dialysed against 0.15M-NaCl/10mM-phosphate buffer (pH7.2) and finally centrifuged at 1000*g* for 15min to remove any insoluble material. An equal volume of 0.15M-NaCl/10mM-phosphate buffer (pH7.2)/0.1% (w/v) Triton X-100/0.2% (w/v) bovine serum albumin containing calf thymus histones (1mg/ml) was added and the IgG passed slowly through a myelin-basic-protein–Sepharose affinity column equilibrated in the same buffer. The IgG was recycled through the column for 30min and the column was then washed extensively with 0.5M-NaCl/0.1M-sodium phosphate buffer (pH7.2). The IgG specifically bound to the column was eluted with 2ml of 1.0M-acetic acid/0.5M-NaCl and immediately dialysed against 0.5M-NaCl/0.1M-sodium phosphate buffer (pH7.2) and subsequently against 0.15M-NaCl/10mM-sodium phosphate buffer (pH7.2). Purified anti-(myelin basic protein) IgG was stored at −70°C in small batches.

Preparation of polyribosomes and isolation of poly(A)+ RNA

Free and membrane-bound polyribosomes from rat forebrain were isolated by a modification of the method of Ramsey & Steele (1976) as described previously (Hall & Lim, 1981). Phenol extraction of polyribosomal RNA and isolation of poly(A)+ RNA were as described previously (Hall & Lim, 1981; Lim *et al.*, 1974).

Translation of poly(A)+ RNA and analysis of products

The reticulocyte-lysate translational system was used as previously described with the addition of saturating amounts (20μg/ml) of free or membrane-bound poly(A)+ RNA (Hall & Lim, 1981). The purified dog pancreas microsomal membranes (50A_{260} units/ml) were added to certain translation assays. The presence of dog pancreas microsomal membranes during translation (2μl in a 25μl assay) resulted in a 30% (approx.) decrease in the amount of [^{35}S]methionine incorporated into protein in response to membrane-bound polyribosomal poly(A)+ RNA, and there was slightly greater inhibition with free polyribosomal poly(A)+ RNA. Equivalent amounts of [^{35}S]methionine-labelled protein were used in all analyses of the translation products.

SDS/polyacrylamide-gel electrophoretic analysis of translation products was as described by Laemmli (1970), in 2.7mm-thick vertical slab gels (either 7cm × 14cm or 14cm × 20cm) with a linear acrylamide gradient of 5–15% or 10–20% (w/v). Gels were subsequently fluorographed as described by Bonner & Laskey (1974), by using Kodak XRP5 X-ray film, or more recently by the method of Laskey & Mills (1975), with Kodak XS-1 X-ray film.

CM-cellulose chromatography

CM-cellulose (CM23; Whatman) chromatography was as described by Campagnoni *et al.* (1978) with minor modifications. The column (1.6cm × 5cm) was equilibrated in 0.005M-glycine/HCl (pH2.5) at 5–8°C and myelin basic protein (2mg) dissolved in the same buffer was applied to the column. After extensive washing the column was eluted with a two-phase linear gradient (0–0.15M-NaCl/0.005M-glycine/HCl, pH2.5) by using an Ultrograd 11300 gradient mixer (LKB, Croydon, U.K.). [^{35}S]Methionine-labelled translation products (2×10^6c.p.m.) were extracted with 0.5ml of 0.005M-glycine/HCl (pH2.5) (20min at 4°C), and after centrifugation at 10000*g* for 15min at 4°C the supernatant was loaded on the CM-cellulose column. Serial fractions (3ml) were collected and the radioactivity in 200μl portions was determined after precipitation with 10% (w/v) trichloroacetic acid. Peak fractions were pooled, dialysed against acetic acid (7%, v/v) and freeze-dried.

Immunoprecipitation of myelin basic protein

Anti-(myelin basic protein) IgG (4.5μg) was added to [^{35}S]methionine-labelled translation products (3×10^6c.p.m. in protein per assay) in 200μl of immunoprecipitation buffer, containing 10mM-Na_2HPO_4 (pH8.3)/0.15M-NaCl/0.1% (w/v) Triton X-100/0.1% (v/v) aprotinin/0.5% (w/v) bovine serum albumin. Incubation was for 18h at 4°C. IgG-bound [^{35}S]methionine-labelled protein was isolated by chromatography on mini-columns of protein A–Sepharose (7mg) in plastic pipette tips (200μl) attached to a syringe. After washing with 20ml of immunoprecipitation buffer, the antigen–antibody complexes were eluted with the SDS/polyacrylamide-

gel electrophoresis sample buffer ($2 \times 50\,\mu l$) of Laemmli (1970).

Cathepsin D digestion of immunoprecipitated myelin basic protein

The translation products of free and membrane-bound polyribosomal poly(A)$^+$ RNA were immunoprecipitated as described above, with the modification that the IgG was first bound to protein A–Sepharose and then incubated with translation products in immunoprecipitation buffer (final vol. $200\,\mu l$) with end-over-end mixing for 18h at 4°C. The protein A–Sepharose was transferred to pipette tips and washed repeatedly with immunoprecipitation buffer by centrifugation at 1300 *g* for 15s. The IgG-bound [^{35}S]methionine-labelled translation products were eluted from protein A–Sepharose in 1.0M-acetic acid containing rat myelin basic protein (0.2mg/ml) and freeze-dried. The protein was redissolved in 0.1M-ammonium acetate (pH3.5) and digested with bovine spleen cathepsin D as described by Whitaker & Seyer (1979). The enzyme concentration was 0.2mg/ml and digestion was for 1h at 37°C. The reaction was terminated by the addition of an equal volume of SDS/polyacrylamide-gel-electrophoresis sample buffer and boiling. The products of digestion were analysed by electrophoresis in SDS/polyacrylamide gradient gels (10–20%, w/v) as described above.

Results

Processing with dog pancreas microsomal membranes

Translation of poly(A)$^+$ RNA isolated from membrane-bound polyribosomes in the presence of dog pancreas microsomal membranes resulted in modification of several protein products (Fig. 1*a*). The most marked modification was that of the 33000-mol.wt. membrane-specific protein, which

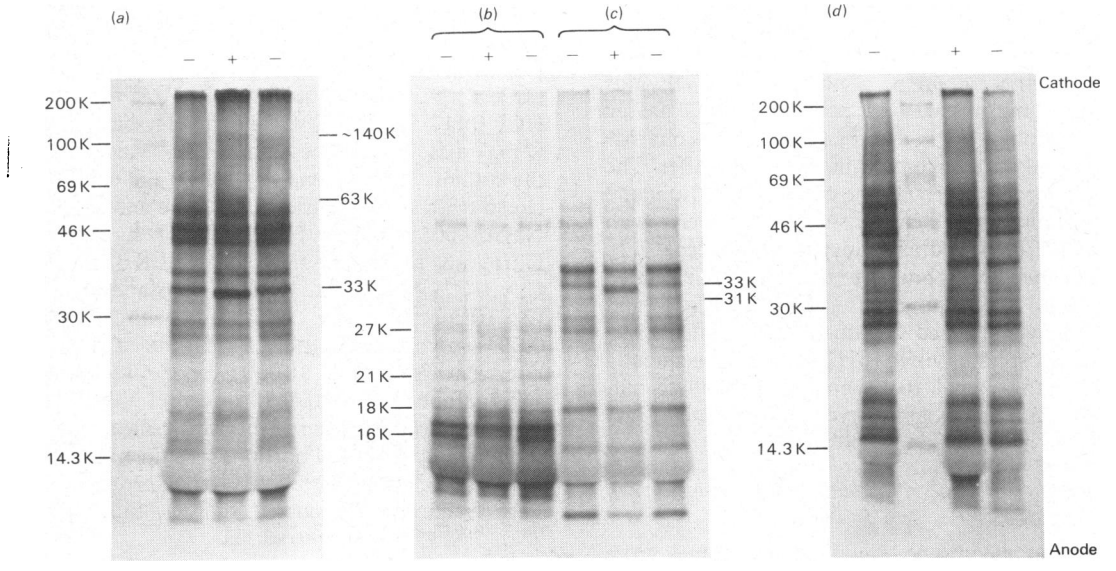

Fig. 1. *Translation products of polyribosomal poly(A)$^+$ RNA synthesized in the presence or absence of dog pancreas microsomal membranes*

Poly(A)$^+$ RNA isolated from membrane-bound (*a*, *b* and *c*) or free (*d*) polyribosomes was added to reticulocyte lysate {$25\,\mu l$, containing L-[^{35}S]methionine (2mCi/ml)} in either the presence (+) or absence (–) of dog pancreas microsomal membranes ($2\,\mu l$; 50 A_{260} units/ml). The poly(A)$^+$ RNA concentration was $20\,\mu g$/ml and incubation was at 37°C for 60min. [^{35}S]Methionine-labelled translation products were analysed by SDS/polyacrylamide-gel electrophoresis [on 10–20% (w/v) polyacrylamide gels] and fluorography (see the Materials and methods section). Equivalent amounts of trichloroacetic acid-precipitable radioactivity (3×10^5 c.p.m.) were loaded on each gel slot, and products synthesized in the absence of microsomal membranes were electrophoresed on either side of those synthesized in the presence of microsomal membranes to facilitate detection of molecular-weight changes. (*a*), Translation products of total poly(A)$^+$ RNA from membrane-bound polyribosomes; (*b*) and (*c*), translation products of size-fractionated poly(A)$^+$ RNA from membrane-bound polyribosomes. Poly(A)$^+$ RNA was centrifuged on 15–30% (w/v) sucrose density gradients as previously described (Hall & Lim, 1981): (*b*), translation products of 11S (approx.) poly(A)$^+$ RNA; (*c*), translation products of 14S (approx.) poly(A)$^+$ RNA. [^{14}C]Methylated protein molecular-weight markers were electrophoresed on the same gel ('200K' etc. represent mol.wt. 200000 etc.). Electrophoresis was at 150V for 16h.

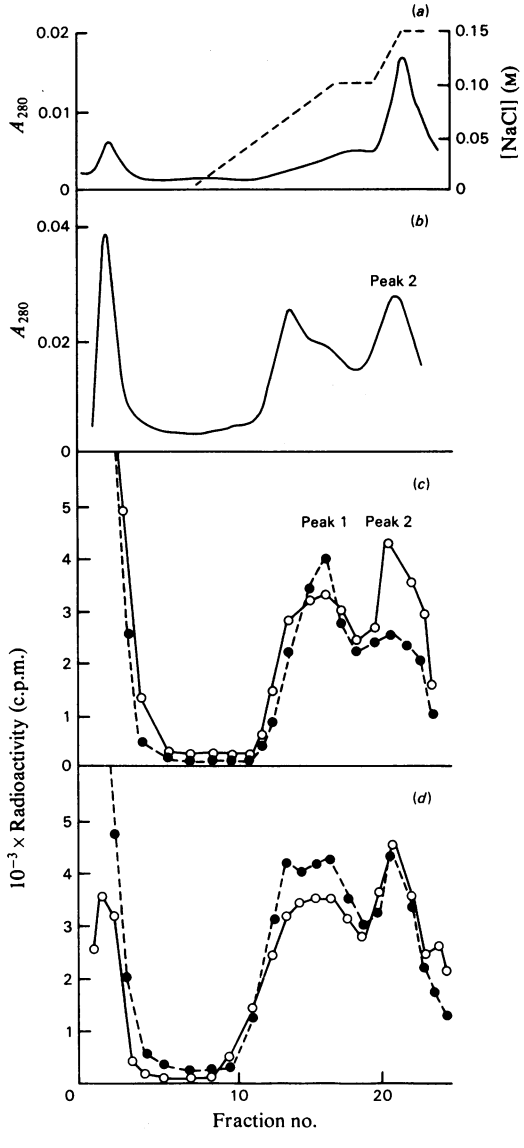

Fig. 2. *Analysis of [^{35}S]methionine-labelled translation products of poly(A)+ RNA by CM-cellulose chromatography*

CM-cellulose chromatography of human myelin basic protein (*a*) was as described in the Materials and methods section. The column was eluted with a two-phase linear gradient (----) of 0–0.15 M-NaCl/0.005 M-glycine/HCl (pH 2.5) (*a*) and the A_{280} of the eluate was monitored with an ISCO UA5 continuous absorbance monitor. Reticulocyte lysate (150 μl) (*b*) was extracted (20 min at 4°C) with 0.005 M-glycine/HCl (pH 2.5) and centrifuged at 10000 *g* for 15 min. The supernatant fraction was loaded on a CM-cellulose column and eluted with an identical gradient by using an Ultrograd 11300 gradient mixer. Equivalent amounts of [^{35}S]methionine-labelled translation products (2×10^6 c.p.m. in protein) of poly(A)+ RNA from membrane-bound (*c*) or free (*d*) polyribosomes synthesized in either the presence (●) or absence (○) of dog pancreas microsomal membranes were chromatographed on CM-cellulose as described above. Fractions (3 ml) of the eluate were collected and the radioactivity in 200 μl portions was determined after precipitation with trichloroacetic acid. Peak-2 fractions were pooled, dialysed against 7% (v/v) acetic acid and freeze-dried.

was previously found to be present at 10 days, but not at 3 days, after birth (Hall & Lim, 1981). There was a decrease in the molecular weight of this protein by approx. 1000; the relative intensity of labelling of the protein band was also increased, possibly owing to its co-migration with a second processed translation product. Additional bands of proteins of higher molecular weight (63000 and 140000) were observed when microsomal membranes were present during translation (Fig. 1*a*). The translational modification of lower-molecular-weight protein products was more clearly demonstrated after fractionation of the poly(A)+ RNA on sucrose density gradients (Figs. 1*b* and 1*c*). RNA molecules corresponding to 14S and 11S were used separately for translation. There were modifications of translation products of mol.wts. 31000, 27000, 21000, 18000 and 16000. The translation products of the free-polyribosomal poly(A)+ RNA were modified to a lesser extent by the presence during translation of dog pancreas microsomal membranes (Fig. 1*d*).

To determine whether the lower-molecular-weight processed proteins included myelin basic proteins, the translation products were further characterized by CM-cellulose chromatography and by immunoprecipitation. The translation products of free and membrane-bound polyribosomal poly(A)+ RNA derived from 25-day-old animals were examined in all subsequent experiments, since myelination proceeds maximally at about this age (Davison & Dobbing, 1968).

Isolation of basic proteins synthesized in vitro by CM-cellulose chromatography

The use of CM-cellulose in the isolation and tentative identification of myelin basic proteins has previously been described (Campagnoni *et al.*, 1978; Matthees & Campagnoni, 1980). By a modification of this method (see the Materials and methods section), myelin basic proteins were bound to CM-cellulose and eluted in a 0–0.15 M-NaCl gradient in 0.005 M-glycine/HCl buffer (pH 2.5) (Fig. 2*a*). Acid-soluble proteins of the reticulocyte lysate were also bound to CM-cellulose and fractions of the reticulocyte proteins were co-eluted with myelin basic protein (Fig. 2*b*). During CM-cellulose

chromatography of [^{35}S]methionine-labelled translation products, the use of unlabelled myelin basic proteins as carriers was avoided because of possible interactions between this basic protein (pI > 10.6) and other [^{35}S]methionine-labelled proteins.

Analysis of the translation products from both free and membrane-bound polyribosomal poly(A)$^+$ RNA, synthesized in either the presence or the absence of microsomal membranes, is shown in Figs. 2(*c*) and 2(*d*). The translation products of both fractions were bound to CM-cellulose and were eluted in two major peaks, one of which (peak 2) co-chromatographed with myelin basic protein. When poly(A)$^+$ RNA was translated in the presence of microsomal membranes the products were also bound to CM-cellulose (Figs. 2*c* and 2*d*).

The peak-2 fractions were further analysed by electrophoresis on SDS/polyacrylamide gels (Fig. 3). In addition to the putative large (18500 mol.wt.) and small (16000 mol.wt.) basic proteins, which were products of both polyribosomal fractions, there were other proteins present. Although the molecular weight of the rodent small basic protein is conventionally quoted as 14000–14400 (Kies *et al.*, 1972; Barbarese *et al.*, 1977), it actually migrates in Laemmli (1970) and other SDS-containing gels as mol.wt. 15500–16000 (Sulakhe *et al.*, 1980; Campagnoni & Magno, 1974; Fig. 4), which is close to the value predicted by the sequence data (Dunkley & Carnegie, 1974). The putative large and small myelin basic proteins were not altered in size when microsomal membranes were present during translation, although there were changes in several of the other protein bands (e.g. those of mol.wts. 11000–14000).

Since many proteins co-chromatograph with myelin basic protein on CM-cellulose at low pH (Figs. 2 and 3), this isolation procedure does not represent a very stringent criterion for the identification of myelin basic proteins synthesized *in vitro*. Further identification of the myelin basic protein synthesized *in vitro* was therefore undertaken by using specific anti-(myelin basic protein) serum.

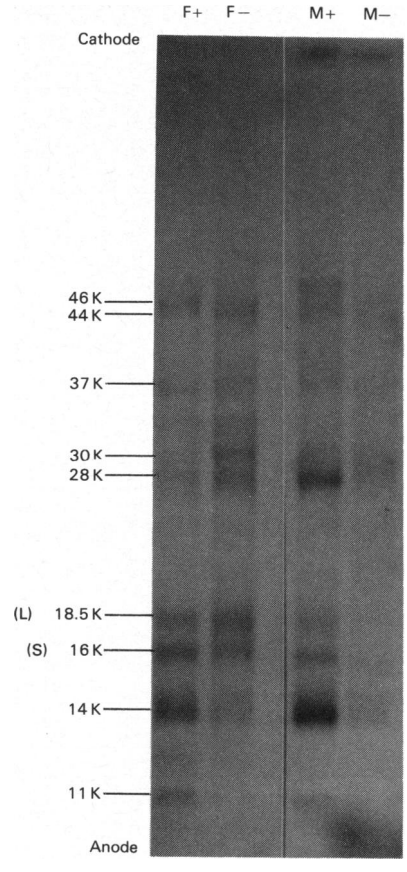

Fig. 3. *SDS/polyacrylamide-gel-electrophoretic analysis of translation products of poly(A)$^+$ RNA which co-chromatographed on CM-cellulose with myelin basic protein*

Poly(A)$^+$ RNA from free (F) and membrane-bound (M) polyribosomes was translated in the presence (+) or absence (−) of dog pancreas microsomal membranes. Translation products were chromatographed on CM-cellulose as described in the Materials and methods section and in the legend to Fig. 2. The freeze-dried peak-2 fractions were redissolved in SDS sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis in 10% polyacrylamide gels and fluorography. Exposure was at −70°C for 18 days, on Kodak XRP 5 X-ray film. L and S represent the large and small myelin basic proteins respectively.

Immunoprecipitation of myelin basic proteins synthesized in vitro

Antiserum to the human myelin basic protein was produced in rabbits as described by Whitaker *et al.* (1976) (see the Materials and methods section). The antiserum bound approx. 60μg of human basic protein/ml when measured by a competitive binding assay with ^{125}I-labelled basic protein and also cross-reacted with rat myelin basic protein (result not shown). Specific anti-(basic protein) IgG was purified by $(NH_4)_2SO_4$ precipitation and antigen-affinity chromatography (see the Materials and methods section for details). Approx. 130μg of specific IgG was purified from 1 ml of serum.

Poly(A)$^+$ RNA isolated from free and membrane-bound polyribosomes was translated in either the presence or the absence of microsomal membranes, and [^{35}S]methionine-labelled translation products were specifically immunoprecipitated as described in the Materials and methods section. The

large and small myelin basic proteins were found to be products of both free and membrane-bound polyribosomal poly(A)$^+$ RNA whether or not microsomal membranes were present during the translation (Fig. 4*a*). The immunoprecipitated protein derived from the membrane-bound polyribosomal fraction contained two major bands of mol.wts. 16500 and 18500, closely corresponding to the molecular weights of the large and small myelin basic proteins. The small myelin basic protein was present in larger amounts than the large myelin basic protein. The presence of microsomal membranes during translation of the membrane-bound polyribosomal poly(A)$^+$ RNA did not alter the size of the myelin basic proteins immunoprecipitated.

When free-polyribosomal poly(A)$^+$ RNA was translated in the presence of microsomal membranes, the proteins immunoprecipitated also corresponded in molecular weight to those derived from the membrane fraction. In the absence of microsomal membranes during translation there was a selective loss of the small myelin basic protein, and the predominant band was that of the 18000-mol.wt. protein.

A third prominent protein band (mol.wt. 37500) present in all immunoprecipitates may correspond to a dimer of the large myelin basic protein, reported to be generated by the conditions of SDS solubilization (see Golds & Braun, 1978). A minor band of 21000 mol.wt. present may be 'pre-large' myelin basic protein. The relationships of minor lower-molecular-weight components (15500 and 14000) to the myelin basic proteins is uncertain, although there is some heterogeneity in the proteins of rat myelin and lower-molecular-weight proteins have been detected (Waehneldt, 1978). Alternatively these lower-molecular-weight immunoprecipitated proteins may be breakdown products of the larger proteins or incompletely synthesized basic-protein molecules.

The antibody binding of both the large (mol.wt. 18500) and small (16000) [^{35}S]methionine-labelled proteins was competed for by unlabelled myelin basic protein (Fig. 4*b*). The [^{35}S]methionine-labelled myelin proteins were also not recovered when

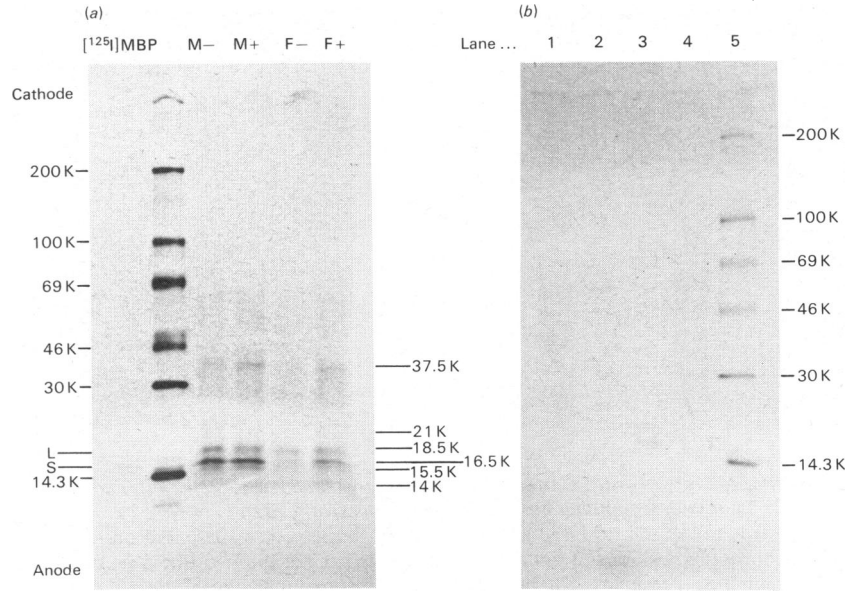

Fig. 4. *Electrophoretic analysis of immunoprecipitated myelin basic proteins synthesized in vitro*

Poly(A)$^+$ RNA from membrane-bound (M) and free (F) polyribosomes was translated in the presence (+) or absence (−) of dog pancreas microsomal membranes. Equivalent amounts of [^{35}S]methionine-labelled translation products (3×10^6 c.p.m. in protein) were immunoprecipitated with anti-(myelin basic protein) IgG as described in the Materials and methods section. IgG-bound proteins (50 μl) were analysed by electrophoresis on a 5–15% (w/v) polyacrylamide gel and fluorography (*a*). [^{14}C]Methylated protein molecular-weight markers (unlabelled lane) and ^{125}I-labelled myelin basic proteins ([^{125}I]MBP) were electrophoresed on the same gel. (*b*) Translation products of membrane-bound polyribosomal poly(A)$^+$ RNA synthesized *in vitro* in the presence of dog pancreas microsomal membranes were immunoprecipitated as described above (lane 3), with $(NH_4)_2SO_4$-precipitated pre-immune IgG (4·5 μg) in place of anti-(myelin basic protein) IgG (lane 1), with IgG omitted (lane 2) and with anti-(myelin basic protein) IgG in the presence of an excess (50 μg) of unlabelled rat myelin basic proteins (lane 4). The positions of the [^{14}C]methylated protein molecular-weight markers are shown in lane 5.

pre-immune IgG replaced the specific anti-(basic protein) IgG (Fig. 4*b*). These results further suggest that the myelin basic proteins synthesized *in vitro* are authentic myelin basic proteins. Verification of their identity was obtained by analysis of the products of cathepsin D digestion.

Cathepsin D digestion of myelin basic proteins

Cathepsin D selectively cleaves the two phenylalanine–phenylalanine (Phe–Phe) linkages located between residues 43–44 and 89–90 in bovine myelin basic protein (Benuck *et al.*, 1975; Whitaker & Seyer, 1979). Thus cleavage of the large myelin

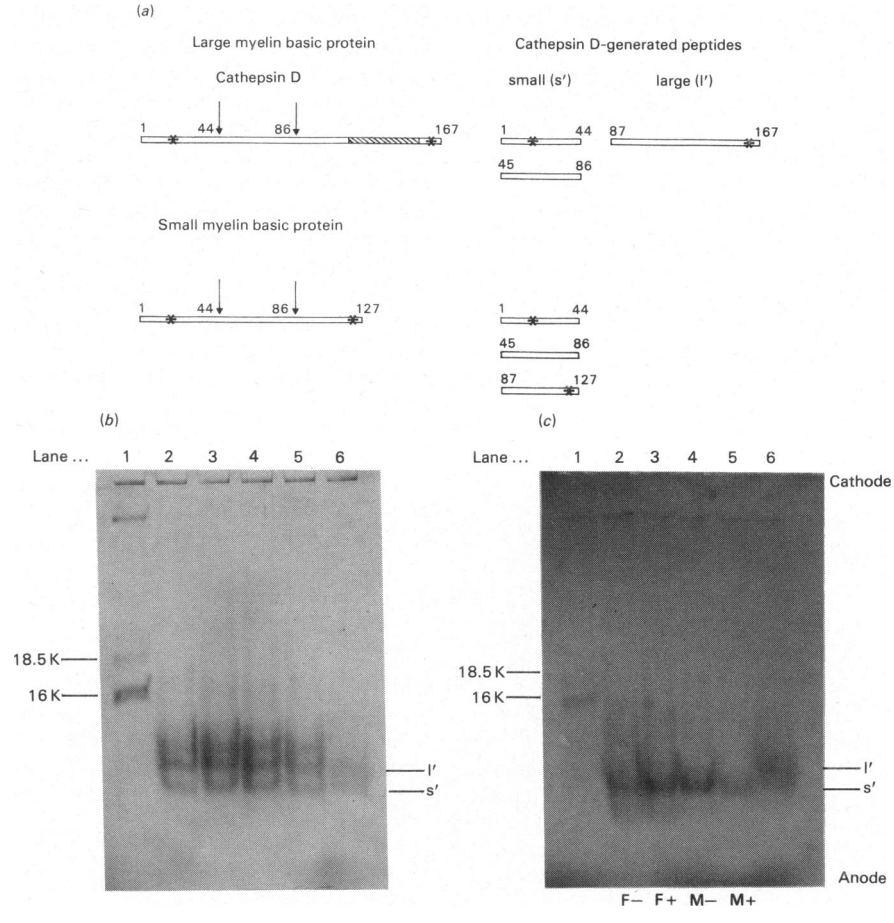

Fig. 5. *Schematic representation of cathepsin D digestion of the rat myelin basic proteins (a), and cathepsin D digestion of immunoprecipitated myelin basic proteins synthesized in vitro (b,c)*

(*a*) The outline of the structure of the rat large and small myelin basic proteins is based on the data of Dunkley & Carnegie (1974) and shows the positions of cathepsin D cleavage sites at the two Phe–Phe bonds (44–45; 86–87). The numbers refer to amino acid residues. The positions of the [^{35}S]methionine residues in the myelin basic proteins and in the cathepsin D-generated peptides are denoted by an asterisk (*). The sequence in the large myelin basic protein that is absent from the small myelin basic protein is shown by ▨. Note the generation of predominantly small peptides (41–44 amino acids) in terms both of amount and of radioactivity. (*b,c*), Cathepsin D digestion of immunoprecipitated [^{35}S]methionine-labelled translation products (3×10^6 c.p.m. in protein) was as described in the Materials and methods section. Total digests of [^{35}S]methionine-labelled immunoprecipitates containing 30μg of basic proteins extracted from crude myelin were analysed by SDS/polyacrylamide-gel electrophoresis in 10–20% (w/v) polyacrylamide gels. After staining with Coomassie Blue (*b*) the gel was processed for fluorography (*c*). Lane 1, undigested myelin basic proteins (^{125}I-labelled); lanes 2–5, digest of immunoprecipitated [^{35}S]methionine-labelled translation products plus crude myelin basic protein preparation; lane 6, digest of purified ^{125}I-labelled rat myelin basic protein. Key: F, digest of immunoprecipitates derived from translation products of free polyribosomal poly(A)$^+$ RNA; M, digests of immunoprecipitates derived from products of membrane-bound polyribosomal poly(A)$^+$ RNA; +, translation in the presence of dog pancreas microsomal membranes; –, translation without microsomal membranes. l', larger peptide; s', smaller peptide.

basic protein with cathepsin D generates three peptides, 43, 46 and 81 amino acid residues in length (Benuck *et al.*, 1975) (Fig. 5*a*). Since two of the peptides are approximately the same size, the cathepsin D-digestion products migrate in SDS/polyacrylamide gels as two bands (l′ and s′; Fig. 5*b*, lane 6). The two Phe–Phe linkages are also present in the rat small myelin basic protein, but, because of the internal deletion of 40 amino acid residues equivalent to residues 120–160 of the large myelin basic protein, cathepsin D digestion of this protein should generate three almost equal-sized peptides of approx. 40 amino acids. The myelin basic proteins contain two methionine residues, located near the *N*-terminal and near the *C*-terminal. Digestion of [^{35}S]methionine-labelled immunoprecipitates of myelin basic proteins synthesized *in vitro* should generate a preponderance of labelled small fragments (approx. 40 amino acids), since these peptides are derived from both large and small basic proteins, whereas the large peptide (81 amino acids) is derived only from the large basic protein (see Fig. 5*a*), and since in most cases the small basic protein is the predominant species synthesized *in vitro* (see Fig. 4*a*).

The stained gels (Fig. 5*b*) show the presence of the small and large peptides generated by cathepsin D digestion of purified ^{125}I-labelled rat myelin basic proteins (lane 6) and of the crude myelin basic protein preparation present in digestion mixtures of the [^{35}S]methionine-labelled basic proteins (lanes 2–5). In addition to the large and small fragments, other peptides were generated from the cathepsin D digestion of the crude preparation, used as carrier.

The predominant band in the radioactive peptides (Fig. 5*c*) derived from immunoprecipitated myelin basic protein products of both free and membrane-bound polyribosomal poly(A)+ RNA exactly co-migrated with the smaller of the two peptide bands (corresponding to approx. 40 amino acids) generated from rat myelin basic proteins (Fig. 5*b*). A minor band which co-migrated with the larger peptide could also be detected in the digests of the [^{35}S]methionine-labelled immunoprecipitates. A second minor band, which migrated ahead of the marker myelin basic protein peptides, was also present in digests of immunoprecipitates. Further experiments are required to determine the origin of this additional peptide, which may relate to the presence of lower-molecular-weight proteins (see Fig. 4). The size of the cathepsin D peptides was unaffected by the presence of dog pancreas microsomal membranes in the initial translations.

Discussion

On the basis of immunological identification, size distribution and peptide analysis we conclude that the mRNA species for both myelin basic proteins are present in both free and membrane-bound polyribosomes. Further, because the size of the proteins as synthesized *in vitro* corresponds to those of the appropriate authentic myelin basic proteins, and since the presence of microsomal membranes did not alter the size of the myelin basic proteins synthesized *in vitro* (Fig. 4), it appears that these proteins are not derived from substantially larger precursors.

The presence of the specific mRNA molecules on both free and membrane-bound polyribosomes accords with our previous report, based on the injection of brain poly(A)+ RNA into *Xenopus* oocytes (Lim *et al.*, 1974), and contrasts with those of Campagnoni *et al.* (1980), who reported that the mRNA molecules for myelin basic proteins were restricted to free polyribosomes. There are several points at issue, not least of which is the identification of the protein. As we have pointed out, the method used by Campagnoni *et al.* (1980), involving CM-cellulose chromatography, is not stringent enough to permit a positive characterization (see Figs. 2 and 3) and needs to be complemented by immunological and/or peptide analysis (Figs. 4 and 5). Another point concerns the isolation of free and membrane-bound polyribosomes. We have used the method of Ramsey & Steele (1976, 1977) for the preparation of polyribosomal fractions, since alternative methods utilizing post-mitochondrial supernatant result in a selective loss of polyribosomes bound to heavier membrane fragments which sediment with nuclei and mitochondria (Zomzely *et al.*, 1970; Venkatesan & Steele, 1972). Our previous results (Hall & Lim, 1981) have also shown that cross-contamination is minimal, since several of the translational products *in vitro* were exclusive to the poly(A)+ RNA isolated from one or the other polyribosomal fraction. In their study Campagnoni *et al.* (1980) reported that their membrane-bound polyribosomes were less aggregated and only half as active as free polyribosomes; this suggests that there may have been a selective loss of membrane-bound polyribosomes resulting from their use of a post-mitochondrial supernatant as the source of cytoplasmic polyribosomes.

Although the precise orientation of the myelin basic proteins in the membrane is uncertain, several reports suggest that it is an extrinsic protein associated with the cytoplasmic surface of the membrane (Podusla & Braun, 1975; Wood *et al.*, 1977). Many proteins which are inserted into membranes or secreted are synthesized with an *N*-terminal leader sequence (see Blobel & Dobberstein, 1975). This sequence is thought to be responsible for the attachment of the relevant polyribosomes to the membrane. Since initiation of this sequence occurs on free polyribosomes, the mRNA molecules for membrane or secretory proteins can

be found on free as well as membrane-bound polyribosomes, although in many cases their distribution is predominantly in favour of the membrane compartment. For some membrane proteins lacking an *N*-terminal leader sequence, internal hydrophobic sequences may play a role in the insertion of these proteins into the membrane (Wickner, 1979). The attachment of their polyribosomes to the membranes would thus occur later than those with *N*-terminal leader sequences on their nascent peptides.

Myelin basic protein contains several interspersed hydrophobic regions, which could facilitate its insertion into the myelin membrane, either post-translationally in the case of synthesis by free polyribosomes, or co-translationally. Those membrane-bound polyribosomes engaged in the synthesis of myelin basic proteins may be associated with membranes because of the lipophilic nature of the nascent polypeptides [myelin basic protein has been shown to interact with lipid vesicles *in vitro* (Stollery *et al.*, 1980; Vadas *et al.*, 1981)]. The presence of the myelin-basic-protein mRNA species in both free and membrane-bound polyribosomes suggests a requirement for the synthesis of a substantial portion of the nascent polypeptide in order for membrane attachment to occur. It is also possible that a proportion of the myelin-basic-protein mRNA species in the free polyribosomes may consist of newly synthesized mRNA molecules, since at 25 days *post partum* there is extensive myelination. The distribution of specific polyribosomes in the free and membrane-bound compartments may represent a dynamic process which has been shown to be influenced by the metabolic (Yap *et al.*, 1978) or hormonal status (Kurtz *et al.*, 1978) of the animal. In addition, the localization of the myelin-basic-protein mRNA species in both compartments may be a reflection of mRNA (and/or protein) heterogeneity. The family of myelin basic proteins (which differ both in size and in their relative proportions in the myelin membrane) do not appear to bear any precursor–product relationship to one another. The results suggest that they are products of several mRNA species. Heterogeneity in these various individual mRNA species could affect their distribution between free and membrane-bound polyribosomes and play a role in the myelin membrane assembly. The purification of the myelin-basic-protein mRNA species is required to clarify further the role of these proteins in the synthesis of the myelin sheath.

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