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Research Articles

Limulus hemocyanin, an unusual ligand for myelin basic protein from central nervous system

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Abstract. *Limulus polyphemus* hemocyanin binds with high affinity to the 18.5 and 21.5 kD isoforms of rat central nervous system myelin basic protein.

Key words. Myelin basic protein; Limulus hemocyanin; protein/protein interaction; fluorescent labelling; Western blot.

Hemocyanins from the arthropod *Limulus polyphemus* and the mollusc *Megathura crinulata* are frequently used in immunizations as carriers for labile and/or poorly immunogenic substances. When we raised antibodies to the brain-specific 21 kD neurocalcin [1] coupled to the more soluble *Limulus polyphemus* hemocyanin (LimHC) we noticed, more by chance than by intention, that the hemocyanin itself bound with high avidity to the white matter of the rat central nervous system. We show here that LimHC interacts with the 21.5 and the 18.5 kD isoforms of myelin basic protein (MBP).

Material and methods

Brains, spinal cord, sciatic nerve and small samples of liver, kidney and muscle were removed from decapitated rats. Tissue was either quick-frozen in 2-methylbutane precooled in liquid nitrogen to prepare 6–8 μm cryosections, or (brain only) used for the extraction of myelin basic protein [2].

Hemocyanins from *Limulus polyphemus* and *Megathura crinulata* (KHLHC); FITC-labelled Limulus hemagglutinin; MBP from rabbit and bovine brain were purchased from Sigma Chemical Co (Deissenhofen, Germany). Hemocyanins and antibodies to LimHC (raised in this laboratory) were labelled with FITC [3] or with horseradish-peroxidase [4].

For histology, unfixed cryostat sections were incubated for 30 min with the FITC-labelled hemocyanins (0.2–1 mg/ml) directly or with unlabelled LimHC (0.1 mg/ml) followed by FITC-labelled anti-LimHC (0.1 mg/ml), washed with PBS and mounted in 70% buffered glycerol at pH 8.6.

SDS-PAGE on 12 or 15% gels was performed by the procedure of Laemmli [5]. The protein bands were transferred from the gels to 0.1 μm nitrocellulose filters by diffusion [6]. After blocking with 1% blocking reagent (Boehringer, Mannheim), the strips were incubated with 50 μg/ml of the LimHC-peroxidase conjugate for 1 h and the reactive bands were then visualized with 4-chloro-1-naphthol/H_2O_2. The affinity matrix was prepared by impregnating nitrocellulose with 1 mg/ml LimHC in PBS, followed by extensive washing to remove unbound material. The strips were then incubated with the isolated rat MBP (1 mg/ml in PBS) for 1 h, and washed thoroughly to remove unbound MBP. The bound MBP fraction was then released by elution with 0.2 M glycine/HCl buffer at pH 2.5 and, after neutralization, analysed by SDS-PAGE and blotting.

Results

Fluorescent labelling of central nervous system myelin. In fresh frozen tissue sections of rat, we observed intense fluorescent labelling of myelinated fibre bundles in the white matter of cerebrum, cerebellum, pons and spinal cord after application of either fluorescently labelled LimHC or unlabelled LimHC followed by labelled anti-LimHC (fig. 1a + b). The same staining pattern was observed in sections of mouse, gerbil, chicken and pig brains (not shown). No reaction was seen with labelled *Limulus* hemagglutinin (a lectin component of hemolymph), hemocyanin of keyhole limpet (fig. 2c), or the FITC-labelled antibody alone. No other rat tissue tested: liver (fig. 2d), kidney, muscle, including the sciatic nerve (fig. 2a,b) gave a positive result.

Identification of MBP as the binding partner of LimHC. When MBP isolated from rat brain was analysed on SDS-PAGE, at least four protein bands were seen. The most prominent one was in the 18.5 kD range, followed in intensity by a 21.5 kD and a 14 kD band, and two minor bands below the 21.5 and 18.5 kD proteins

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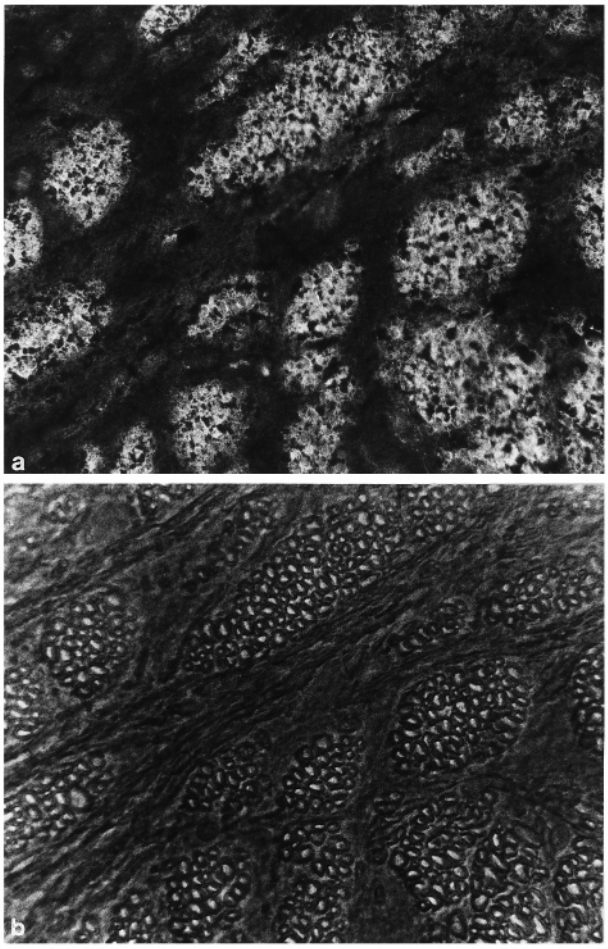

Figure 1. Cryostat section of rat brain (pons). (*a*) incubated with FITC-labelled LimHC, (*b*) phase contrast micrograph of the same section. The positive reaction corresponds to the location of the myelin sheaths (× 400).

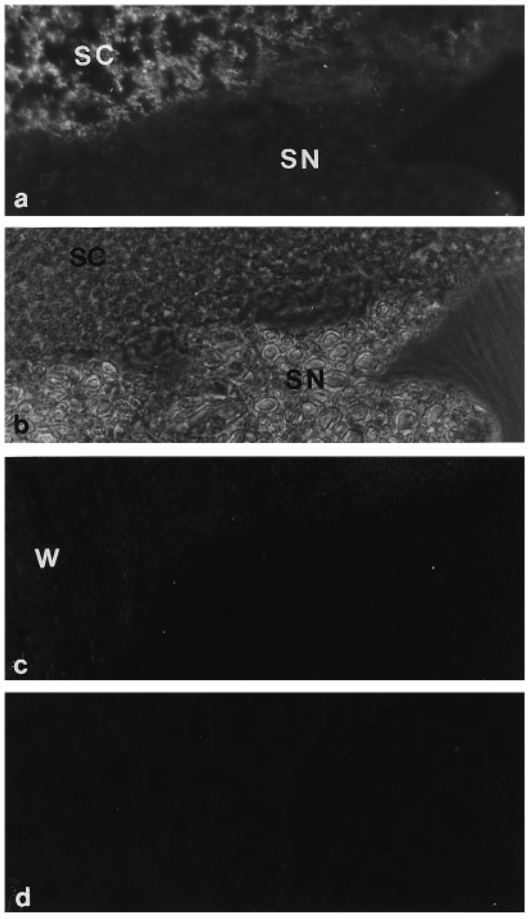

Figure 2. Cryostat sections of a combined tissue block of rat spinal cord (SC) and sciatic nerve (SN) (*a*, *b*) of rat cerebellum (*c*) and rat liver (*d*). a = incubated with FITC-labelled LimHC; b = phase micrograph of the same section. Positive reaction is seen in the white matter of the spinal cord, but not in the peripheral nerve. c = incubated with FITC-labelled KHLHC (w = white matter); d = incubated with FITC-labelled LimHC. No reaction is seen in these negative controls (× 250).

(fig. 3). In the bovine and rabbit MBPs, the 21.5 kD band was most prominent (fig. 3b). After transfer to nitrocellulose, the 21.5 and 18.5 kD bands from rat brain were labelled with horseradish peroxidase-coupled LimHC (fig. 3d), as were the 21.5 kD bands from ox and rabbit (fig. 3a, only ox shown). The same two bands from rat brain bound tighty to immobilized LimHC, whether purified MBP or crude brain extracts were applied, and were only released under extreme conditions, such as 0.2 M glycine/HCl at pH 2.5 (fig. 3e).

Discussion

Myelin basic protein, isolated from delipidated brain matter, is a very soluble protein. Four isoforms, with molecular masses of 21.5, 18.5, 17 and 14 kD, are found in the central nervous system of rodents; the same four isoforms are also found in the peripheral nervous system, although the content is quite low [7]. Rodent MBPs are encoded by one gene containing at least seven exons, and the message containing all seven exons codes for the

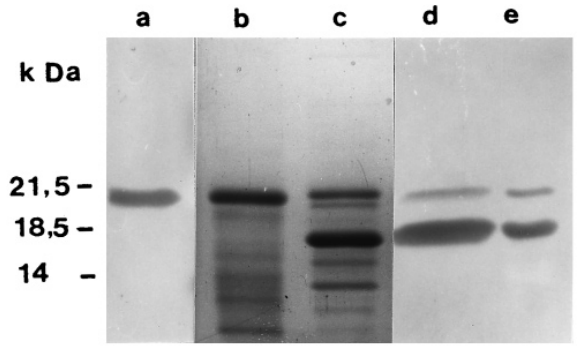

Figure 3. SDS-PAGE of myelin basic proteins (lanes b + c); b = MBP from ox brain; c = MBP from rat brain. Modified Western blot of these proteins in lane a (ox), d (rat) and e the MBP released from the LimHC affinity matrix. The 21.5 and 18.5 kDA isoforms are visualized with horseradish peroxidase-labelled LimHC and substrate.

21.5 kD form; the others are products of alternative splicing.

The sequence encoded by exon 6 (amino acid residues 141–181) is only found in the 21.5 and 18.5 kD isoforms, and since only these two were shown to bind LimHC, it seems feasible that the binding site resides in this region, allowing the detection of MBP both in situ and in vitro. This may also explain why MBP in peripheral nerve, where the 14 kD isoform predominates and the concentrations of the 18.5 kD and especially the 21.5 kD isoforms are quite low [7], cannot be visualized with labelled LimHC (fig. 2). The high affinity of the interaction between LimHC and MBP, only dissociable under the severe conditions used to separate antigen/antibody complexes, is very specific (although it is not known if it has any physiological meaning): we did not find it in other tissues nor in E 13 chicken spinal cord (not shown), where the myelination process has not yet begun [8]. In addition, it is not given by structurally unrelated protein molecules like mollusc hemocyanins or a lectin from Limulus hemolymph.

Unusual and unphysiological as this interaction may be, it offers a high affinity probe and ligand for the detection and isolation of brain MBP isoforms.

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