

Chapter 2

The Discovery of the Brain Immunomodulators

Thymosin β_4 (1-39)

The term “thymosin” was introduced by Goldstein et al. (48,49) and Hooper et al. (50) and was used for a biologically active fraction isolated from bovine thymus tissue (51). Among the members of T β -thymosin (T β) family, T β_4 is the major peptide in different cells of human, calf, rat, or mouse. Thymosin β_4 (T β_4) has been reported to induce terminal deoxynucleotidyl transferase activity in vivo and in vitro, inhibit the migration of guinea pig peritoneal macrophages, stimulate the hypothalamic secretion of luteinizing hormone-releasing hormone (51), or induce phenotypic changes in the Molt-4 leukemic cell line (52). Although several biological activities of T β_4 have been reported in the literature, its physiological role and biochemical mechanisms of action on the brain and immune cells still remain unknown. We discovered T β_4 (1-39) in the hypothalamus and elucidated its primary structure and fundamental biochemical mechanisms of action (53). T β_4 (1-39) is the primary activator of calcium-calmodulin-dependent enzymes (cAMP PDE, myosin light chain kinase (MLCK), etc.) in concentrations of 10^{-9} – 10^{-12} M without participation of Ca^{2+} and CaM. This polypeptide is a Ca^{2+} -CaM-replacing molecule for the regulation of Ca^{2+} -CaM-dependent enzymes in the hypothalamus. Thus, a new level of Ca^{2+} -CaM-activated enzyme regulation was discovered. We determined the epitopes in the structure of T β_4 (1-39), which are responsible for the activity. T β_4 (1-39) as well as fragments 25-31, 11-19, and 16-38 are strong activators of cAMP PDE, MLCK, cAMP-dependent protein kinase, etc. However, T β_4 derived peptides Ac-SDKP and Ac-ADKP are inhibitors of cAMP PDE (54).

The primary structure of T β_4 (1-39)

ACETYL-SDKPDMAEIEKFDSKLKKTETQEKNPLPSKETIEQEKK

These peptides caused incomplete competitive inhibition of phosphodiesterase (PDE) activation by calmodulin. A 20-fold increase in the constant for PDE activation by calmodulin was accompanied by an insignificant elevation of the maximum rate of cAMP hydrolysis. In this case, the value of the inhibition constant (K_i) was about 600 nM. In the absence of calmodulin, the saturation concentration of these peptides reduced the enzyme activity nearly 2-3-fold. The effect of the peptides

on PDE was noncompetitive with respect to cAMP ($K_i \sim 100$ nM). The data is characteristic of non-competitive binding of both peptides to the enzyme, with respect to changes in the enzyme properties during its interaction with the peptides. All the reactions of the enzyme mentioned here were reversible. Activating effects of these fragments can be explained by the presence of 5, 2, and 6 lysine residues in each peptide, respectively (3). Together with my coworkers, Gurvits and Sharova, we demonstrated that cyclic AMP PDE and 5'-N (5'-nucleotidase) make a coupled system (3,55). This system promotes quick conversion cAMP-5'-AMP-adenosine. The latter is of extreme importance for the regulation of vascular smooth muscle tone and for the formation of new cAMP in the brain through adenylate cyclase activation. We found that 5'-nucleotidase isolated from hypothalamus had the PDE activity (55). The existence of functional interrelation between PDE and 5'-nucleotidase (5'-N) was demonstrated. The first-order rate constants were determined for adenosine formation using the two substrates during 2-min incubation. It was found in kinetic studies of 5'-N that the first-order rate constant for 5'-AMP formed from cAMP under the action of PDE exceeded the first-order rate constant for exogenous 5'-AMP hydrolysis, by a factor of 100. This can be a result of directed transfer of intermediate product (5'-AMP in this case) from one active center of the enzyme to another, if they are conjugated. It was found that the first-order rate constant for cAMP is 2.0 min^{-1} , and that for 5'-AMP is 0.02 min^{-1} . The data shows that adenosine is formed mainly from cAMP. Moreover, neither cAMP (3–60 μM) nor N^{6-} , O^{2-} dibutyryl cAMP (3–60 μM) can activate the hydrolysis of (^{14}C) 5'-AMP if the latter is the substrate. This data indicates the existence of functional interrelations between cAMP, PDE, and 5'-N. The Lineweaver-Burk plots show that both cAMP PDE and 5'-N are characterized by two K_m and V_{max} values; the facts indicating the existence of two catalytic sites on both enzymes, with high and low affinities for the substrates. For cAMP PDE, the values of K_{m1} and K_{m2} are 2.5×10^{-6} and 3.7×10^{-5} M, respectively, and those of V_{max1} and V_{max2} are 6.3×10^{-6} and $3.7 \times 10^{-5} \text{ mol min}^{-1} \text{ mg}^{-1}$. If 5'-AMP is the substrate, the values of K_{m1} and K_{m2} for 5'-N are 5×10^{-6} and 1×10^{-4} M, and those of V_{max1} and V_{max2} are 5×10^{-6} and $7 \times 10^{-9} \text{ mol min}^{-1} \text{ mg}^{-1}$, respectively. cGMP PDE and 5'-GMP 5'-N are characterized by the same K_m values (55). We showed that cAMP PDE isolated from hypothalamus and also 5'-N are Ca^{2+} , CaM-activated enzymes. It was demonstrated that cAMP PDE and 5'-N activities are the properties of the same protein. The fact that the two enzymatic activities are associated with the monomer isolated from bovine hypothalamus provides evidence that the same molecule possesses cAMP PDE and 5'-N activities. TP_4 (1-39) (1 $\mu\text{g/ml}$) stimulated both cAMP PDE and 5'-N activities.

2.1 Isolation of a Fragment of MBP from Bovine Hypothalamus

We isolated several biologically active compounds from bovine hypothalamus, which proved to be stimulators of the basal activity of calmodulin-dependent cyclic nucleotide PDE. One of these stimulants has been purified to homogeneity by reverse phase HPLC. Amino acid sequence analysis showed that the sample was indeed

a peptide containing 14 amino acid residues corresponding to residues 72–85 of MBP with one exception: Glu was found in place of Gin (72) (56). The following amino acid sequence was obtained for this peptide: Glu-Lys-Ala-Gln-Gly-His-Arg-Pro-Gln-Asp-Glu-Asn-Pro-Val. The full 169 amino acid residue sequence of bovine spinal cord myelin MBP is shown here (57).

AAQKRPSQRSKYLASASTMDHARHGFLPRHRDTGILDSLGRFFGSDRGAPKRGSGKDG
HHAARTHIGSL**PQKAQGH**RPQDENPWHFFKNIVTRPTPPPSQGKGRGLSSRFSGWGA
GQKPGFGYGGRASDYKSAHKGLKGHDAQGTLSKIFKLGGDRDSRSGSPMARR

The fragment 72–85 of the protein (in bold letters) is similar to the sequence of the isolated peptide, with the exception of glutamine in position 72, where, according to our data, glutamic acid occurs. This substitution could take place because of in vitro glutamine desamidation, since our protocol included the homogenation of fresh tissue in an acidic medium (the solution of 0.25–0.5 % acetic acid). It should be pointed out that in this peptide, we obtained two other glutamines, which, despite this treatment, retained their amidated form and corresponded to glutamines 75 and 80 in the MBP sequence.

It was shown in previous studies that glutamine and asparagine residues neighboring basic amino acids (for example, lysine or arginine) are subjected to desamidation. In our case, glutamine-72 is followed just by lysine-73. MBP is one of the major myelin structural proteins. Properties laid in MBP structure can determine its function as a phospholipid acceptor protein. Basic amino acids are distributed randomly in primary structure of the protein, without evident periodicity; they can interact with phosphate groups of phospholipids. Among 14 amino acid residues of the sequenced peptide, lysine, histidine, and arginine are present, which can participate in such interactions. Enzymes of the brain and other tissues also methylate MBP. It has been previously shown that only arginine-106 is methylated. In our peptide, arginine is present in a position corresponding to arginine-79 in MBP, and it is not yet known whether it can be methylated. Besides, it was shown that threonine-98 of MBP is glycosylated as a result of N-acetylgalactosamine transferase action. The question arises of whether MBP glycosylation and deglycosylation take place during its functioning as a component of the myelin membrane. There are no threonine residues in our peptide 72–85. It should be noted, that the bovine MBP structure 1–169 is highly homologous to the structure of rabbit and guinea pig MBPs, though some differences exist. In rabbit and guinea pig MBP, the amino acid residues glutamine-75 and histidine-77 are absent, and serine is substituted for alanine-74 and proline-79, respectively. These substitutions are of importance because MBP is known to be one of the best substrates for cAMP-dependent protein kinases and Ca^{2+} -phospholipid-dependent protein kinases. The important discovery was that MBP participates in the induction of experimental encephalomyelitis. The studies of the immunopathological role of MBP revealed that peptide fragments of MBP are also immunogenic 57–59. It was shown that guinea pig MBP fragment 114–123, which namely includes tryptophan residue, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-Pro, is the most effective of all peptides studied as inducers of the disease. In the case of rabbit MBP, the fragments 114–123 and 44–89 possess the same property. The amino acid sequence

44–89 includes the sequence 68–74, which is structurally similar to the fragment 114–123. It was also found that even such amino acids of MBP as tryptophan, glutamine, and lysine are important for encephalogenic activity. It is worth noting that the peptide we studied was enriched enough in glutamine and glutamic acid and contained lysine. Such amino acid composition is favorable for the peptide immunogenicity. It is quite probable that under certain conditions various MBP peptide fragments can be formed by brain proteases, including the peptide under consideration, which possesses the encephalogenic activity. Studying functional properties of such peptides can help us to better understand pathogenesis of the disease and to suggest some methods of its treatment. MBP is a major component of myelin of all mammals. Much is known about the encephalotogenic epitopes of MBP (60). The portion of the molecule (residues 116–124) that causes EAE in strain 13 guinea pigs is conserved among various species. On the other hand, the portion of the molecule (residues 74–87) that produces EAE in Lewis rats varies among different species. I believe that residues 72–85 also can produce EAE in different species.

2.2 Hypothalamic Immunophilin-Receptor of FK-506 Immunosuppressor: FK-506 Binding Protein (FKBP-12—Peptidyl-prolyl-cis-trans isomerase

In 1992, we discovered immunophilin (Iph) in bovine hypothalamus peptidyl-prolyl cis-trans isomerase; its primary structure was determined by mass-spectral analysis and Edman degradation (61,62). The molecular weight of FKBP isolated from hypothalamus and that of FKBP earlier isolated from bovine thymus was 11,778 Da. This protein takes part in brain biochemical processes by changing the conformation of various enzymes and synaptic proteins; it participates in the formation of regulatory mechanisms of the immune system of the brain and of the organism as a whole. All proteins of this family (FKBPs of 12, 12.6, 14, 33, 38, 52, and 51 kDa) contain at least one domain that is homologous to FKBP12 (the FKBP-domain) (63). FKBPs have distinct structures, cellular localization, functions, and other properties. The 12 kDa FKBP has been initially isolated from human erythrocyte and lymphocyte membranes and from bovine hypothalamus (3,62). It effectively binds inositol-1,4,5-triphosphate ($K_d \sim 96$ nM) and inositol-1,3,4,5-tetrakisphosphate ($K_d \sim 14$ nM) and the inositolpolyphosphate binding inhibits the peptidyl-prolyl cis-trans isomerase activity of this FKBP. FKBP (107 amino acid residues) exhibits 85 % identity with the amino acid sequence of FKBP12; in mammalian tissues it is associated with one ryanodine receptor isoform (64).

2.3 The Primary Structure of Hypothalamic Immunophilin

GVQVETISPGDGRTPFKRGQTCWHYTGMLDGGKKFDDSSRDPVNKPFFKMLGKQEVIR
GWEEGVAQMSVGORAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE

Taking into account the existence of Iph in the neurosecretory cells of the hypothalamus, one can suggest that multiple forms of peptidyl-prolyl-cis-trans-isomerase (PPIase), and particularly of Iph, are synthesized by the hypothalamic neurosecretory nuclei and play a role in protein folding; they also can take part in the regulation of biosynthesis of interleukins in the same nuclei as well as in the metabolism of secondary messengers (Ca^{2+} , cyclic nucleotides) in the brain. There are endogenous activators and inhibitors of Iph, among which are $\text{T}\beta_4$, 1-39 and $\text{T}\beta_1$. Iph can take part in the mechanisms of signal transduction in neurosecretory neurons of hypothalamus. The effect of Iph and of its fragments 1-9; 1-15 on the brain and immunocompetent cells was investigated. By an immuno-histochemical method (avidin—biotin peroxidase complex) using antisera to 1-15 fragments of Iph, we observed Iph immunoreactivity (Iph-IR) in the NSO of frog and rat hypothalamus (65,66). The immunoreactivity of Iph fragment was detected also in NPV varicosities. Iph-IR was revealed in several groups of cells concentrated particularly in the medulla oblongata where the cross and the longitudinal sections of myelinated single nerve fibers and bundles were also found. They extended for a long distance through the Iph-IR neurons mainly in the paramedian giganto and parvicellular reticular nuclei. Calcineurin is a target for FK-506-FKBP complexes in cells. Precisely by that mechanism, the transcription of interleukin-2 gene is inhibited in lymphocytes, suppressing the immune response. It can be suggested that Iph and its endogenous ligands could form a similar complex and this complex could regulate the level of formation of interleukins in neurosecretory cells of hypothalamus.

Cardenas et al. (67) showed the possibility of complex formation between FKBP 12 and calcineurin (with catalytic subunits), both in the presence and absence of immunosuppressor FK-506. Three Iph families, FK506/rapamycin-binding proteins (FKBP), cyclosporin A-binding proteins (cyclophilins, CyP), and parvulins, are recognized based on their structure and sensitivity to inhibitors.

2.4 Iphs as Cytokines

Some Iphs are secretory proteins that may potentially be involved in distant cell communication. During bacterial lipopolysaccharide-induced stimulation, macrophages secrete CyPA (68). Exogenous CyPA exerts a chemotactic effect on eosinophils and neutrophils. The maximal chemotactic effect was observed at 10 nM CyPA, and cyclosporin A blocked this effect. CyPB is also a secretory protein, which has been found in blood and milk. Its secretion is stimulated by fibroblast growth factor (FGF) and by epidermal growth factor (EGF) (69). The plasma membrane of human lymphocytes and the Jurkat cells contain CyPB receptor-binding sites, $\sim 35,000$ per cell, $K_d \sim 12$ nM. The N-terminal 24-amino acid fragment of CyPB interacts with this receptor (70). After binding, the ligand-receptor complex internalizes, and then CyP dissociates and undergoes proteolysis. Cyclosporin does not influence CyPB binding to its receptor and its translocation into the cell. Stimulation of mast cells with anti-IgE causes FKBP12 secretion (71). Extracellular FKBP12 exerts a chemotactic effect on neutrophils, and this FKBP12-induced activation is blocked by FK506 and

rapamycin. In spite of structural and functional heterogeneity, Iphs form a group of enzymes that share one common property: they all possess peptidyl-prolyl cis-trans isomerase (rotamase) activity. The involvement of PPI rotamase activity in protein folding is now well recognized. However, the nature of functional coupling between Iph native proteins, which form joint structural—functional complexes, is still unclear. As stated earlier, reversible cis-trans isomerization of imide bonds in oligopeptides and native proteins may have a regulatory role. It is logical to suggest that the regulatory effect of Iphs as components of various protein complexes is due to its rotamase activity. However, there is not enough experimental evidence that could confirm the involvement of Iph rotamase activity in such regulatory reactions.

2.5 Targets of the Iph-Immunosuppressant Complexes

In the course of search of target proteins for Iph-immunosuppressor complexes, which are responsible for immunosuppression, the calcium and calmodulin-dependent protein phosphatase known as calcineurin (CaN) has been identified (72,73). FK506 and cyclosporin A, when bound to Iph, inhibit CaN activity, and CaN inhibition might mediate immunosuppression: IL-2 gene transcription is regulated by the nuclear factor of activated T cells (NF-AT). The cytoplasmic form of NF-AT is phosphorylated, and it must be dephosphorylated in order to be translocated into the nucleus and activate the IL-2 gene transcription. Thus, FK506 and cyclosporin A appear to regulate immunosuppression by increasing the phosphorylation level of transcription factors required for IL-2 formation.

Rapamycin acts via a different mechanism. It influences a later, calcium independent stage in the T-cell cycle. Rapamycin inhibits the ability of IL-2 to stimulate the transition of T cells from G1 to S phase by blocking several steps in growth factor action on translational regulators. The proteins referred to as TOR1 and TOR2 (targets of rapamycin), RAFTs or FRAPs (rapamycin and FKBP targets) were found to be involved in the phosphorylation of cytoplasmic protein kinases (74,75).

2.6 Iphs in the Nervous System

After the discovery of Iph, the majority of researchers were studying their activity in tissues and cells of the immune system. However, a substantial level of ubiquitously distributed Iphs was also revealed in the nervous system. The discovery that Iphs are much more abundant in the brain than in lymphocytes suggested important roles for the Iphs in neural functions. A few selected examples of the evidence for the neurotropic Iph functions are presented below. First, FKBP and CyP protein and mRNA localizations are quite similar to those of CaN that suggest a physiologic link of Iphs to CaN. The limbic system is enriched in the Iphs and CaN, with high levels in the hippocampus. High levels of FKBP and CaN, but not CyP, were revealed in the caudate nucleus; the brain stem displays CyP, but neither CaN nor FKBP (76). The protein

whose phosphorylation level is enhanced by the treatment with immunosuppressors is nitric oxide synthase (NOS). NOS is a calcium—calmodulin-dependent enzyme, and the activation of NMDA receptors stimulates NOS activity because these receptors possess a calcium channel activity and allow calcium to enter the cell upon glutamate action (77). Being a physiologic neurotransmitter, glutamate elicits neurotoxicity via NMDA receptors when released in excess following cerebral vascular stroke. By enhancing NOS phosphorylation, the immunosuppressors might inhibit NO formation and block glutamate/NMDA neurotoxicity in the cerebral cortex and other brain regions.

In the nervous system, another protein that binds to Iph is the type I receptor for TGF β . TGF β induces the long-term synaptic facilitation, protects hippocampal neurons from ischemic damage and amyloid peptide-induced neurotoxicity, and promotes the axonal regeneration (41, 78–81). Iph is a releasing factor for serotonin from mast cells. FK506 inhibits this release (79). Potent effects of FK506 are inhibiting spontaneous and depolarization of induced release of dopamine and acetylcholine from PC 12 cells (80). The effects of FK506 on retinal ganglion cells after optic nerve crush were also reported. The purpose of the study was to determine the physiologic consequence of Iph (FKBP12) presence in rat retina, particularly in the retinal ganglion cells. It was demonstrated that FK506 confers neuroprotection on cells due to its ability to interfere with apoptotic mechanisms after optic nerve crush (81).

2.7 Thymosin β 1 (Ubiquitin) Isolated from the Neurosecretory Granules of Neurohypophysis is Calmodulin Binding Endogenous Protein, a Calmodulin Antagonist

By the use of HPLC chromatography, a new protein has been isolated from peptide-protein fraction of hypothalamus, and also from neurosecretory granules, which was eluted together with Iph, superoxide I dismutase, and parvalbumin group (acetonitrile gradient, 40–44 %). Two peptides registered at the wavelength of 210 nm were eluted in 41 and 42.1 % of acetonitrile at a flow rate of 1.0 ml/min (3,82). Employing mass-spectrometry analysis and micro sequencing, we succeeded in establishing their primary structures. We have identified 30 N-terminal amino acid residues of the Thymosin β 1/ubiquitin. The peptides contained 74 and 76 amino acid residues. The amino acid sequences of the obtained proteins were completely identical to those of T β 1, 1-74 and 1-76. Data obtained indicates that ubiquitin (T β 1) plays an important role in the proteolytic breakdown of interleukin precursors produced by hypothalamic neurosecretory nuclei. The primary structure of thymosin β 1 (ubiquitin):

MQIFVKTLTGKTTITLEVEPSDTIEDVKAKIQDKEGIPPDQQLIFAGKQLEDGRTLSDYNIQKESTLHLVLRRLR₇₄-GG₇₆

It has been shown that ubiquitin forms rather stable complexes with CaM in the presence of calcium ions (0.2 mM). Thus, one of the fundamental functional properties

of $T\beta_1$ (ubiquitin) has been established on the fact that at high calcium ion concentrations (resulting in Ca^{2+} -dependent conformational changes), it binds to CaM, not permitting the latter to activate the enzymes depending on it, such as cAMP PDE and MLCK. $T\beta_1$ inhibits the phosphorylation of myosin light chain and other substrates. Thus, we isolated it from bovine hypothalamus proteins, which in the presence of high calcium concentrations (0.2 mM), act as CaM antagonists and change the direction of metabolic shifts. Data obtained indicates that the immunocompetent cells and the pool of immunomodulators are located mainly in the hypothalamic neurosecretory neurons of NSO and NPV.

2.8 Isoforms of Macrophage Migration Inhibitory Factor in Bovine Brain

In the study of primary structures and molecular mechanisms of action in immunologically active compounds of the nervous system, we have isolated two thermostable proteins from the soluble fraction of total bovine brain. The purification procedure was mainly based on DEAE-servacel ion-exchange chromatography and reverse-phase HPLC. The proteins were identified by the N-terminal Edman degradation and database search as macrophage migration inhibitory factor (MIF). The N-terminal sequences for MIF1 and MIF2 were found to be identical. Molecular masses for MIF1 and MIF2 were determined as 12,369.21 and 12,299.7 Da by mass-spectrometry analysis. In addition, we have also isolated a third peptide with the same N-terminal sequence and M_r of 9,496.2 Da, probably a proteolytic fragment of MIF. Using *p*-hydroxyphenylpyruvate as a substrate, we have not revealed tautomerase activity of either MIF1 or MIF2. A comparatively simple purification procedure was worked out, which may widely be used for simultaneous isolation in one run of the MIF isoforms (83). The protein known as macrophage migration inhibitory factor (MIF) was originally described as one of the first cytokines to be secreted from activated lymphocytes and inhibited the random migration of macrophages *in vitro* (84,85). More recently, MIF was characterized as an immunological mediator that counter-regulates glucocorticoid action (86). A multi-functional nature of MIF was suggested in further studies that demonstrated its occurrence in a wide variety of tissues and cell types. However, despite the fact that MIF has been widely studied in relation to the immune system, its physiological functions in the immune response are far from being clear. In relation to the nervous system, the role of MIF seems even much more obscure. MIF was previously rediscovered as being an anterior pituitary gland hormone; its presence was demonstrated in other regions of the nervous tissue (87–89). However, the biological role of MIF in terms of “where and when” its activity is expressed remains to be elucidated. Moreover, although MIF also was found to exhibit a number of catalytic functions (D-dopachrome- and phenylpyruvate tautomerase activities), the physiological significance of MIF enzyme activity is unclear (90,91). The primary structure of the brain MIF is:

NH₂-PMFWNTNVP RASVPDGLLS ELTQQLAQAT GKPAQYIAVH WP-

By searching the database, the primary structure of the N-terminus of each of the isolated proteins was found to be the exact match for that of the known MIF from calf brain cytosol, which has 114 amino acid residues (87,92). Comparison of the MIF isolated from bovine brain with other MIFs (human, rat, murine, chicken, etc.) demonstrates a high degree of similarity in amino acid sequences with many homology motifs (93). As mentioned before, the molecular masses of isolated proteins of fractions 6 and 7 were estimated as 12,369.2 (MIF1) and 12,299.7 (MIF2) Da, respectively. Mass-spectrometry analysis showed another peak of $M_r = 29,568.9$ in both fractions. For example, fraction 6 showed a protein identical to bovine MIF revealed in two peaks (m/z of 12,369.2 and 6,184.6) and an additional single peak having m/z of 29,568.9. As Edman microsequence analysis of the MIF proteins demonstrated no additional meaningful N-terminal amino acid sequence, we concluded that the fractions contain a protein that either is acetylated at the N-terminus or has the N-terminal amino acid sequence identical to that of MIF. The latter suggestion is less likely, because this protein was not revealed by Western-blot analysis. In addition, it should be noted that the existence of two MIF isoforms characterized by pI values equal to 9.5 and 9.4 has been reported previously (87). Two MIF isoforms have also been demonstrated with the use of RP-HPLC system. Sequence analysis and Western blotting revealed that one isoform was identical to bovine MIF and the other was an N-terminally modified form of MIF (88). Based on the evidence presented earlier, we also concluded that at least two MIF isoforms existed in the bovine brain, and that the two proteins have identical N-terminal amino acid sequences. As both immunologic and enzymatic activities were reportedly expressed by the oligomeric structure of MIF, we suggested that our study might give additional information on MIF structure. Using *p*-hydroxyphenylpyruvate as a substrate, we have not revealed tautomerase activity of MIFs.

It still remains to be revealed whether the difference between MIF1 and MIF2 subunits found by RP-HPLC is of importance for MIF oligomeric structure. As MIF was found to be multifunctional, the results presented could contribute to further understanding of structural-functional relationships of MIF isoforms involved in the regulation of a variety of fundamental neuro-immunological processes. The precise physiological functions of MIF1 and MIF2 in relation to the immune response in the central nervous system remain to be elucidated.

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