# Phosphorylation of U24 from Human Herpes Virus type 6 (HHV-6) and its potential role in mimicking myelin basic protein (MBP) in multiple sclerosis

Andrew R. Tait, Suzana K. Straus*

*Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, BC, Canada V6T 1Z1*

**Abstract** Myelin basic protein (MBP) from multiple sclerosis (MS) patients contains lower levels of phosphorylation at Thr97 than normal individuals. The significance of phosphorylation at this site is not fully understood, but it is proposed to play a role in the normal functioning of MBP. Human Herpesvirus Type 6 encodes the protein U24, which has tentatively been implicated in the pathology of MS. U24 shares a 7 amino acid stretch encompassing the Thr97 phosphorylation site of MBP: PRTPPPS. We demonstrate using a combination of mass spectrometry, thin layer chromatography and autoradiography, that U24 can be phosphorylated at the equivalent threonine. Phospho-U24 may confound signalling or other pathways in which phosphorylated MBP may participate, precipitating a pathological process.

*Structured summary:*
MINT-6613181:
*MAPK* (uniprotkb:P28482) *phosphorylates* (MI:0217) *MBP* (uniprotkb:P02687) by *protein kinase assay* (MI:0424)
MINT-6613171, MINT-6613190:
*MAPK* (uniprotkb:P28482) *phosphorylates* (MI:0217) *U24* (uniprotkb:Q69559) by *protein kinase assay* (MI:0424)

© 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

*Keywords:* U24; Mimicry; Kinase; Phosphorylation; Myelin

## 1. Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the human central nervous system (CNS). While complex genetic traits may provide predisposition to MS, it is possible that an environmental factor, such as viral infection, triggers the disease [1]. Human Herpesvirus Type-6 (HHV-6), for example, has been shown to be associated with at least a subgroup of MS patients [2]. The exact mechanism by which HHV-6 may trigger or sustain disease pathology is as of yet unknown, but molecular mimicry is one proposed mode of action [3]. The concept of molecular mimicry is that a foreign protein (i.e.: viral or bacterial) triggers an immune response, and based on sequence or structural antigenic similarities with self-proteins, the self-proteins are degraded, leading to tissue damage such as demyelination. Myelin basic protein, a candidate autoantigen in MS, has a seven amino acid stretch identity with HHV-6 U24 protein, PRTPPPS ($MBP_{92-104}$ = IVT**PRTPPPS**QGK; $U24_{1-13}$ = MDP**PRTPPPS**YSE). It was demonstrated that greater than 50% of T-cells recognizing $MBP_{95-105}$ cross-reacted with and could be activated by a synthetic peptide corresponding to $U24_{1-13}$ in MS patients [4]. Based on this sequence similarity and the fact that MBP has two Thr that represent mitogen-activated protein kinase (MAPK) targets in vivo (underlined above) [5] within this segment, we propose a mechanism by which U24 may additionally contribute to the pathogenesis of MS: by representing an alternative kinase target, U24 might interfere with essential phosphorylation of MBP.

An excellent kinase substrate under both in vitro and in vivo conditions [6], MBP experiences a rapid turnover of its phosphate groups [7]. Regulation of phosphorylation in MBP is proposed to have both functional and structural implications for maintaining the efficiency of nerve conduction and physical integrity of the myelin sheath [8–10]. Thr94 (bovine numbering) is an in vivo phosphorylation site [5]. Thr97 is also an in vivo phosphorylation site [11] that is recognized by both glycogen synthase kinase (GSK) and MAPK under in vitro conditions. Phosphorylation at Thr97 attenuates the ability of MBP to polymerize and bundle actin, and to bind actin filaments to a negatively charged lipid membrane [9]. This phosphorylation site has also been proposed to play a role in cell signaling and myelin development, with the discovery that MBP that has been phosphorylated at Thr97 is specifically localized to lipid rafts [12,13].

In studies on humans with MS and on a spontaneously demyelinating mouse model of MS, there was much less phospho-Thr97 MBP detected compared to normal [5,14]. The full consequences of this absence of post-translational modification are as of yet unknown, but it has been shown that phosphorylation at Thr97 can protect against proteolysis of the Arg96-Thr97 peptide bond by at least three types of proteases [15]. Protection against MBP degradation would have a direct positive impact on the integrity of the myelin sheath, and

*Corresponding author. Fax: +1 604 822 2157.
E-mail address: sstraus@chem.ubc.ca (S.K. Straus).

should further help by preventing MBP epitopes from being released and exposed to the immune system. Here, in attempt to identify a factor which may contribute to the cause of low levels of phospo-Thr97 MBP in MS patients, we provide evidence that U24 protein from HHV-6 can be efficiently phosphorylated by MAPK, based on the sequence similarity between U24 and MBP.

## 2. Materials and methods

### 2.1. Isolation of HHV-6 U24 protein

U24 protein was expressed and purified based essentially on the method by Pryor et al. [16] with some changes. Full details of the expression and purification will be presented elsewhere [17]. Briefly, the gene for U24 from HHV-6A was cloned into plasmid pMAL-p2x (New England Biolabs) and expressed in *Escherichia coli* as a *C*-terminal fusion to maltose binding protein and a hexahistidine tag. Based on primary sequence analysis, U24 has a hydrophobic *C*-terminus that is possibly a transmembrane domain. Addition of Triton X-100 detergent was thus a requirement to solubilize the fusion protein from the *Escherichia coli* pellet. The expressed fusion protein was purified using an immobilized metal affinity chromatography column, Histrap HP (GE Healthcare) charged with Ni$^{2+}$. Buffers contained 20 mM Na$^+$/ K$^+$ phosphate, 1 mM dithiothreitol, 0.5% Triton X-100 and 0–500 mM imidazole, pH 7.4. The fusion protein included a thrombin cleavage site between the hexahistidine tag and U24 protein. After cleavage with bovine thrombin (GE Healthcare), the digested protein was reapplied to the column and the flow-through was applied to an anion exchange column, Hitrap FF Q-Sepharose (GE Healthcare) to remove thrombin, liberated maltose binding protein with histag, undigested fusion protein, and residual protein impurities. U24 was collected from the flow-through and its purity was determined by Tris–Tricine SDS–PAGE and found to be >95% pure. The protein concentration was determined using the bicinchoninic acid method (Pierce) which included a procedure for removing interfering substances [18]. The yield of U24 was found to vary between 1 and 2 mg per L of minimal media M9 culture. The molecular mass of U24 was verified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS); in agreement with the theoretical mass, MALDI-TOF MS data yielded an experimental mass of 10 235 Da (theoretical mass = 10 235 Da, i.e. U24$_{1-87}$ plus two additional residues at the N-terminus after thrombin cleavage).

### 2.2. Preparation of $^{32}$P-phosphate labeled U24 and MBP

Bovine MBP (Sigma) was dissolved in water and its concentration was measured by the bicinchoninic acid method or by absorbance, using an extinction coefficient of $\varepsilon_{276.4} = 10\,300$ cm$^{-1}$ M$^{-1}$ [19]. MAPK (Erk2) was obtained (New England Biolabs) and assays were set up for MBP and U24 based on previously described methods [20]. In separate microfuge tubes, 480 pmol each of U24 and MBP were diluted to a total volume of 60 μl with MAPK assay buffer containing 100 μM ATP (New England Biolabs) and 0.04 μCi/μl [γ-$^{32}$P]ATP. Reactions tubes were placed in a water bath or hotplate set at 30 °C, and reactions were started by the addition of 200 U of MAPK.

### 2.3. SDS–PAGE/ autoradiography analysis of $^{32}$P-labeled proteins

At various time points, 40 pmol of protein were removed from the kinase reaction, and the reaction was quenched by addition of an equal volume of Novex 2× SDS buffer (Invitrogen) supplemented with 5 mM of 2-mercaptoethanol (Sigma) and heated to 95 °C for 5 min. Samples were loaded on Tris–tricine polyacrylamide gels (13.5%) and run for 2 h at 100 V. The gels were then wrapped in thin plastic film and exposed for varying lengths of time to a phosphoimager screen before being scanned with a Typhoon 9200 phosphoimager (GE Healthcare). Relative $^{32}$P incorporation was quantified using ImageQuant 5.2 software (Molecular Dynamics).

### 2.4. Phosphoamino acid analysis

After an overnight kinase reaction, $^{32}$P-phosphate labeled MBP and U24 were precipitated by addition of tricholoracetic acid (20% w/v final) and the pellet was washed with three volumes of ice cold acetone.

The pellets were then dissolved in 200 μl 6 M HCl and incubated for 1 h at 110 °C. Acid was removed from the hydrolysate by speed-vac lyophilization, and the dried residue was suspended in 10 μl of water. A few microliters of hydrolyzed sample were spotted on a silica thin layer chromatography (TLC) plate (Merck). A small amount of a phosphoamino acid mixture containing of 1 mg/ml each of *O*-phospho-L-serine, *O*-phospho-L-threonine, and *O*-phospho-L-tyrosine (Sigma) was also spotted for reference. In a solvent composed of absolute ethanol:25% ammonia solution, 3.5:1.6 [21], the plate was developed by three rounds of thin layer chromatography. Phosphoamino acid standards were visualized with ninhydrin spray; identities of the $^{32}$P-labeled phosphoamino acids were determined after the TLC plate was exposed to a phosphoimager screen overnight and scanned with a phosphoimager reader.

### 2.5. Characterization of phosphorylated U24 by MALDI-TOF MS

Phosphorylation reactions were set up as mentioned in Section 2.2, with the omission of [γ-$^{32}$P]ATP, and unlabeled ATP concentration was either 0.1 or 1 mM. Control reactions did not have kinase added. All reactions were carried out for a minimum of 3 h to overnight. After, U24 protein was precipitated by addition of tricholoracetic acid (20% w/v final) and the pellet was washed with three volumes of ice cold acetone. The MALDI matrix used was sinapinic acid, dissolved in 50% acetonitrile with 0.1% TFA. The protein was also solubilized in 50% acetonitrile with 0.1% TFA. Sample solution (1 μl) was spotted on a MALDI target plate, sandwiched between two layers of matrix solution (2 × 1 μl), with air drying between applications. A Bruker Biflex IV (Bruker Daltonics) MALDI-TOF mass spectrometer was calibrated with bovine ubiquitin and horse heart cytochrome c (Sigma), and the samples were analyzed in positive linear ion mode.

## 3. Results

### 3.1. MAPK-mediated phosphorylation kinetics of U24 and MBP

Fig. 1 demonstrates the time course of protein phosphorylation, illustrating that U24 and MBP can both be efficiently phosphorylated by MAPK. Under these conditions, we take 100% phosphorylation of MBP to be approximately 1 mol phosphate per mol protein [20], with the major phosphorylation site as Thr97 or TPR(phospho)TP [11]. Others have observed that Thr94 also represents a minor phosphorylation site in vitro [22], one that can be also be phosphorylated with
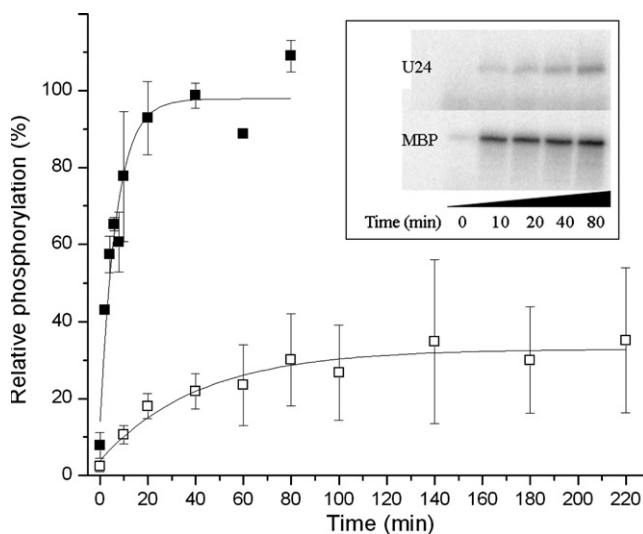


Fig. 1. Phosphorylation kinetics data for U24 (open squares) relative to MBP (closed squares), derived from SDS–PAGE/autoradiography results (inset). The error bars were obtained by repeating the measurements three times.

increased kinase concentration and incubation time [9]. Our results suggest that U24 can by phosphorylated up to approximately 50% that of MBP or 0.5 mol phosphate per mol protein.

## 3.2. Phosphoamino acid analysis

U24 has two potential MAPK target sites, Thr6 within the optimal PX(T/P)S consensus sequence, and Ser25 within the minimal (T/S)P consensus sequence [23]. To determine which one or if a mixture of both sites are phosphorylated, we performed a TLC analysis of the $^{32}$P-labeled phosphoamino acids that were released by acid hydrolysis (Fig. 2). Using phosphoamino standards dyed with ninhydrin and the $^{32}$P-labeled phospho-threonine from MBP acid hydrolysis, we confirmed that it is Thr6 in U24, part of the PRTPPPS sequence shared with MBP, and not Ser25 that is phosphorylated. It should be noted
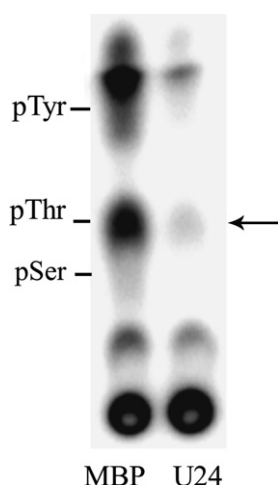


Fig. 2. One-dimensional TLC, followed by autoradiography of $^{32}$P-phosphate labeled MBP and U24 acid hydrolysates. Positions of pTyr, pThr, and pSer were revealed by ninhydrin staining. Significant amounts of phosphothreonine only are detected in MBP and U24 (arrow).

because MAPK does not phosphorylate tyrosine, the artifacts at the top of the TLC plate are not from phosphotyrosine, but rather, they are likely from partially hydrolyzed peptides containing phosphothreonine.

## 3.3. Evidence of U24 phosphorylation by MALDI-TOF MS

A kinase reaction run overnight with 1 mM ATP substrate, using conditions analogous to those reported in [22], resulted in U24 being fully phosphorylated at a single position; with no unphosphorylated or multiply phosphorylated species detected (Fig. 3). Conversely, after only 3 h of reaction and 0.1 mM ATP, a mix of both unphosphorylated and monophosphorylated forms could be observed; no autophosphorylation activity was detected when kinase was not added (data not shown). The mass of unphosphorylated U24 was measured to be 10 235 Da, and monophosphorylated (+80 Da) was 10 313 Da. A control kinase reaction was also run on MBP and MALDI-TOF MS analysis of the data showed the addition of two phosphates (data not shown), as reported by Hirschberg [22].

## 4. Discussion

The concept that a foreign protein substrate could compete with MBP for phosphorylation was presented by Stoner et al. [24]. They noticed that the large T-antigen of papovaviruses JC shared a C-terminal subsequence with MBP that was also a phosphorylation site in MBP. Unfortunately, no cells expressing T-antigen were detected in plaque or periplaque regions of the MS brains or in control CNS tissue. However, a larger sample set later revealed that JC virus DNA could be detected in cerebral spinal fluid of 11 of 54 (9%) MS patients [25], but none in normal patients, thus indicating a possible role for JC virus in at least a small subset of MS patients. In another study [26], large T-antigen expression was detected in neurofilament-positive cells and astrocytes in the cortex juxtaposed to MS plaques, but not in the plaques themselves. While the molecular role of large T antigen as a phosphoacceptor
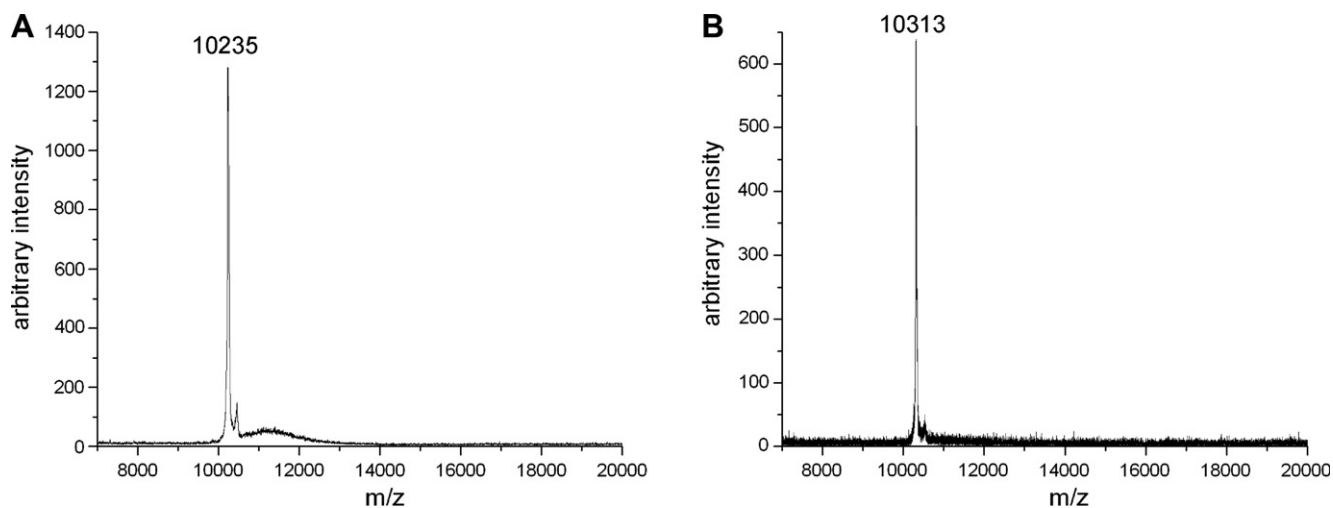


Fig. 3. MALDI-TOF MS of unphosphorylated U24 (A) and U24 that has been phosphorylated by MAPK (B) after overnight incubation with 1 mM ATP and 200 units of kinase. The mass shift of ~+80 Da signifies the addition of one phosphate to the protein.

competing with MBP has not been followed up on, we sought to investigate a similar role for U24 protein from HHV-6.

Transcription of HHV-6 genes has been detected in the brains of MS patients [27]. It is the HHV-6A strain that has been directly linked to MS, with a possible genetic factor placing those with certain MHC2TA gene polymorphisms at higher risk [2]. U24 expressed by HHV-6 has been shown to down-regulate surface expression CD3 receptors in T-cells [28], but the result of its expression in nervous tissue has yet to be examined. Here we propose that because of a stretch of sequence similarity between MBP and U24, PRTPPPS, there may be cases of mistaken identity, resulting in essential interactions with and post-translational modifications done on the viral as opposed to the self-protein. This sequence is also a PXXP SH3-target consensus sequence [29], and ultimately there may be more examples [30] in which U24 and MBP compete for molecular recognition based on their identity, perhaps additionally contributing to the destabilization of the myelin sheath and the pathogenesis of multiple sclerosis. Although there are clearly many membrane-associated phosphorylation targets in myelin (e.g. MBP, tau [31]), perhaps U24, with its putative transmembrane domain, sequesters phosphates to the membrane, thereby interrupting signalling or other pathways in which phosphorylated MBP may participate.

## References

[1] Sospedra, M. and Martin, R. (2005) Immunology of multiple sclerosis. Annu. Rev. Immunol. 23, 683–747.

[2] Martinez, A. et al. (2007) Environment–gene interaction in multiple sclerosis: human herpesvirus 6 and MHC2TA. Hum. Immunol. 68, 685–689.

[3] Wucherpfennig, K.W. and Strominger, J.L. (1995) Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell 80, 695–705.

[4] Tejada-Simon, M.V., Zang, Y.C., Hong, J., Rivera, V.M. and Zhang, J.Z. (2003) Cross-reactivity with myelin basic protein and human herpesvirus-6 in multiple sclerosis. Ann. Neurol. 53, 189–197.

[5] Kim, J.K., Mastronardi, F.G., Wood, D.D., Lubman, D.M., Zand, R. and Moscarello, M.A. (2003) Multiple sclerosis: an important role for post-translational modifications of myelin basic protein in pathogenesis. Mol. Cell Proteomics 2, 453–462.

[6] Steck, A.J. and Appel, S.H. (1974) Phosphorylation of myelin basic protein. J. Biol. Chem. 249, 5416–5420.

[7] DesJardins, K.C. and Morell, P. (1983) Phosphate groups modifying myelin basic proteins are metabolically labile; methyl groups are stable. J. Cell Biol. 97, 438–446.

[8] Atkins, C.M., Yon, M., Groome, N.P. and Sweatt, J.D. (1999) Regulation of myelin basic protein phosphorylation by mitogen-activated protein kinase during increased action potential firing in the hippocampus. J. Neurochem. 73, 1090–1097.

[9] Boggs, J.M., Rangaraj, G., Gao, W. and Heng, Y.M. (2006) Effect of phosphorylation of myelin basic protein by MAPK on its interactions with actin and actin binding to a lipid membrane in vitro. Biochemistry 45, 391–401.

[10] Ramwani, J.J., Epand, R.M. and Moscarello, M.A. (1989) Secondary structure of charge isomers of myelin basic protein before and after phosphorylation. Biochemistry 28, 6538–6543.

[11] Erickson, A.K., Payne, D.M., Martino, P.A., Rossomando, A.J., Shabanowitz, J., Weber, M.J., Hunt, D.F. and Sturgill, T.W. (1990) Identification by mass spectrometry of threonine 97 in bovine myelin basic protein as a specific phosphorylation site for mitogen-activated protein kinase. J. Biol. Chem. 265, 19728–19735.

[12] DeBruin, L.S., Haines, J.D., Bienzle, D. and Harauz, G. (2006) Partitioning of myelin basic protein into membrane microdomains in a spontaneously demyelinating mouse model for multiple sclerosis. Biochem. Cell Biol. 84, 993–1005.

[13] DeBruin, L.S., Haines, J.D., Wellhauser, L.A., Radeva, G., Schonmann, V., Bienzle, D. and Harauz, G. (2005) Developmental partitioning of myelin basic protein into membrane microdomains. J. Neurosci. Res. 80, 211–225.

[14] Yon, M., Ackerley, C.A., Mastronardi, F.G., Groome, N. and Moscarello, M.A. (1996) Identification of a mitogen-activated protein kinase site in human myelin basic protein in situ. J. Neuroimmunol. 65, 55–59.

[15] Medveczky, P., Antal, J., Patthy, A., Kekesi, K., Juhasz, G., Szilagyi, L. and Graf, L. (2006) Myelin basic protein, an autoantigen in multiple sclerosis, is selectively processed by human trypsin 4. FEBS Lett. 580, 545–552.

[16] Pryor, K.D. and Leiting, B. (1997) High-level expression of soluble protein in *Escherichia coli* using a His6-tag and maltose-binding-protein double-affinity fusion system. Protein Expr. Purif. 10, 309–319.

[17] Tait, A.R. and Straus, S.K. (in preparation) Purification and characterization of U24 – a membrane protein from human herpesvirus type-6. Protein Expr. Purif.

[18] Brown, R.E., Jarvis, K.L. and Hyland, K.J. (1989) Protein measurement using bicinchoninic acid: elimination of interfering substances. Anal. Biochem. 180, 136–139.

[19] Liebes, L.F., Zand, R. and Phillips, W.D. (1975) Solution behavior, circular dichroism and 22 HMz PMR studies of the bovine myelin basic protein. Biochim. Biophys. Acta 405, 27–39.

[20] McKenzie, J.A. and Strauss, P.R. (2003) A quantitative method for measuring protein phosphorylation. Anal. Biochem. 313, 9–16.

[21] Munoz, G. and Marshall, S.H. (1990) An alternative method for a fast separation of phosphotyrosine. Anal. Biochem. 190, 233–237.

[22] Hirschberg, D., Radmark, O., Jornvall, H. and Bergman, T. (2003) Thr94 in bovine myelin basic protein is a second phosphorylation site for 42-kDa mitogen-activated protein kinase (ERK2). J. Protein Chem. 22, 177–181.

[23] Davis, R.J. (1993) The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 268, 14553–14556.

[24] Stoner, G.L., Ryschkewitsch, C.F., Walker, D.L., Soffer, D. and Webster, H.D. (1986) Immunocytochemical search for JC papovavirus large T-antigen in multiple sclerosis brain tissue. Acta Neuropathol. 70, 345–347.

[25] Ferrante, P., Omodeo-Zorini, E., Caldarelli-Stefano, R., Mediati, M., Fainardi, E., Granieri, E. and Caputo, D. (1998) Detection of JC virus DNA in cerebrospinal fluid from multiple sclerosis patients. Mult. Scler. 4, 49–54.

[26] Del Valle, L., Delbue, S., Gordon, J., Enam, S., Croul, S., Ferrante, P. and Khalili, K. (2002) Expression of JC virus T-antigen in a patient with MS and glioblastoma multiforme. Neurology 58, 895–900.

[27] Opsahl, M.L. and Kennedy, P.G. (2005) Early and late HHV-6 gene transcripts in multiple sclerosis lesions and normal appearing white matter. Brain 128, 516–527.

[28] Sullivan, B.M. and Coscoy, L. (2008) Downregulation of the T-cell receptor complex and impairment of T-cell activation by human herpesvirus 6 u24 protein. J. Virol. 82, 602–608.

[29] Boggs, J.M. (2006) Myelin basic protein: a multifunctional protein. Cell Mol. Life Sci. 63, 1945–1961.

[30] Polverini, E., Rangaraj, G., Libich, D.S., Boggs, J.M. and Harauz, G. (2008) Binding of the proline-rich segment of myelin basic protein to SH3 domains: spectroscopic, microarray, and modeling studies of ligand conformation and effects of posttranslational modifications. Biochemistry 47, 267–282.

[31] Ekinci, F.J. and Shea, T.B. (2000) Phosphorylation of tau alters its association with the plasma membrane. Cell Mol. Neurobiol. 20, 497–508.