Specific 14-3-3 isoform detection and immunolocalization in prion diseases

H. C. Baxter*†, J. R. Fraser‡, W.-G. Liu†, J. L. Forster*, S. Clokie*, P. Steinacker‡, M. Otto*, E. Bahn‡, J. Wiltfang† and A. Aitken*

*Department of Biomedical Sciences, University of Edinburgh, George Square, Edinburgh EH8 9XD, U.K., †Institute for Animal Health, Neuropathogenesis Unit, Edinburgh EH9 3JF, U.K., and ‡Departments of Neurology, Neuropathology and Psychiatry, University of Gottingen, D-37075, Gottingen, Germany

Abstract

14-3-3 proteins are involved in signalling processes in neuronal cells. Using isoform-specific antibodies we have examined the variation in 14-3-3 isoform neurolocation in normal and scrapie-infected murine brain and show that in defined areas of the brain there are significant changes associated with the pathology of the disease process. The appearance of 14-3-3 proteins in the cerebrospinal fluid (CSF) is a consequence of neuronal disease and the detection of specific isoforms of the 14-3-3 proteins in the CSF is characteristic of some neurodegenerative diseases. In this study, monitoring specifically for the γ 14-3-3 isoform in the CSF by both Western-blot analysis and ELISA we can show a level of correlation between the assays.

Introduction

Transmissible spongiform encephalopathies are a group of diseases which affect both humans and animals. These are commonly called prion diseases [1] and include the human diseases sporadic Creutzfeldt-Jakob disease (CJD), variant CJD and iatrogenic CJD and the animal diseases bovine spongiform encephalopathy (BSE) and natural scrapie in sheep [2]. All of these involve rapid neurodegeneration and are invariably fatal. Definitive diagnosis of prion diseases is currently only carried out post-mortem by neurohistopathological examination for the detection of abnormal form of the prion protein.

Concern over the link between BSE and variant CJD and the requirement for early or pre-mortem diagnosis of CJD has resulted in the evaluation of a number of proteins identified in the cerebrospinal fluid (CSF) of patients with neurodegenerative diseases for uses as surrogate markers. These include 14-3-3 proteins [3,4], neuron-specific enolase [5], S100β [6] and Tau protein [7], and of these proteins 14-3-3 has the highest specificity. At present only Western-blot analysis of 14-3-3 has been advanced by the World Health Organization as a pre-mortem biochemical indicator to aid early diagnosis of clinically characterized sporadic CJD.

The 14-3-3 proteins are a highly conserved family of multifunctional proteins that are primarily found in neurons but which are expressed in a wide range of other cells and tissues. 14-3-3 proteins have a typical monomeric molecular mass of approx. 30 kDa and have shown to form homo- and hetero-dimeric structures which complex with other proteins. There are seven distinct isoforms (β, γ, ε, ζ, τ, η and σ), named from their reversed-phase HPLC elution profile [8]. The β and ζ forms have also been isolated as phosphorylated forms, which are named γ and δ respectively [9]. Multiple isoforms of 14-3-3 have been shown to interact with distinct regulatory proteins of the signalling, regulatory and apoptotic pathways [10]. Many of these protein–protein interactions have been shown to be modulated by phosphorylation of the interacting 14-3-3 partner [11,12]. This is exemplified in the regulation of the Bcl-2 apoptotic pathway; here sequestration of phosphorylated BAD (Bcl-2-associated death promoter) in the cytosol by 14-3-3 proteins results in inhibition of the pro-apoptotic action of BAD [13].

Early immunohistochemical studies of the human brain showed that the 14-3-3 proteins are predominantly localized in neuronal cells [14]. Gene expression studies have shown that the ε, γ, η, β and ζ isoforms are expressed in neuronal cells in the grey matter area of the brain, including the hippocampus, thalamus and the cerebellar cortex [15,16], but that the τ isoform (also known as θ) is also expressed in white matter areas of the brain [17]. Characterization of the subcellular location of 14-3-3 [18] had shown the ε, γ, η, β and ζ isoforms to be present in synaptic vesicle mem-

Key words: CSF, ELISA, immunocytochemistry, neurodegeneration.

Abbreviations used: BAD, Bcl-2-associated death promoter; BSE, bovine spongiform encephalopathy; CSF, cerebrospinal fluid; CJD, sporadic Creutzfeldt-Jakob disease; LGN, lateral geniculate nucleus.

To whom correspondence should be addressed (e-mail hbaxter@ed.ac.uk).
branes whereas η, ε and γ isoforms are located at the synaptic junction and that the ε isoform binds to the synaptic plasma membrane through its N-terminal domain [19].

Using two-dimensional analysis an increase of the levels of the ε and η isoforms has been shown in the cortical region of the brain in both Alzheimer’s disease and Down’s syndrome [20].

Studies on the immunolocalization of 14-3-3 isoforms in neurodegenerative brain disease has shown 14-3-3 to co-localize with neurofibrillary tangles in the brain of patients with Alzheimer’s disease [21] and in the Lewy bodies in Parkinson’s disease and in Diffuse Lewy Body disease [22].

Immunocytochemistry

In a comparative analysis of the neurolocation of the individual 14-3-3 isoforms in normal and scrapie-infected murine brain we have shown that in areas where severe pathological changes occur there are distinct changes in the isoform labelling [23]. This is clearly shown in the hippocampus and in the thalamus. In the hippocampus there is a loss of labelling of the τ isoform and the number of pyramidal cell bodies in the CA1 decreases with a corresponding loss of labelling of the β, η, γ and ζ isoforms (Figure 1), yet in the molecular layer of the dentate gyrus there is a significant increase in the intensity of labelling. In the lateral geniculate nucleus (LGN) of the thalamus we observe a marked decrease in the isoform labelling in the dorsal LGN but this is not observed in the ventral LGN. As the 14-3-3 isoform labelling in the central nervous system in terminal scrapie is lost in some areas, but increases in others, this would suggest that the processing of these proteins during neurodegeneration may be much more complex than previously recognized.

Although the pathogenic role of the 14-3-3 isoforms in the prion diseases is unknown these proteins do modulate the apoptotic pathway by interaction with the Bcl-2 complex and by inhibiting the pro-apoptotic action of BAD.

Since changes in distribution pattern of various 14-3-3 isoforms is provoked by transmissible spongiform encephalopathy infection it is of intrinsic importance to determine the fate of these proteins in the disease process.

CSF

As 14-3-3 proteins are not normally detected in the CSF the appearance of small quantities of specific 14-3-3 isoforms in the CSF during the disease processes tends to suggest that the pathway from neuron to CSF may be isoform-specific. Here it is of importance to note that of the six isoforms, β, γ, ε, ζ, τ and η, that are normally found in the brain, only four of these, the β, γ, ε and η isoforms, are present in the CSF of patients with sporadic CJD [24,25]. It is also noteworthy that the only isoform consistently observed in the CSF of patients with Alzheimer’s disease is the η isoform [24] and that the ζ 14-3-3 isoform is not typically found in the CSF in either of these diseases. Currently the World Health Organization protocol for the use of 14-3-3 proteins as surrogate markers in CSF of sporadic CJD patients uses Western-blot analysis, which is not isoform-specific.

Figure 1

Immunolabelling of β14-3-3 isoform

β-Labelling of the cell bodies of pyramidal cells in CA1 hippocampus (a) in normal brain and (b) in ME7 scrapie-infected murine brain. Scale bar, 100 μm.
Western analysis

Of the $\beta$, $\varepsilon$, $\gamma$ and $\eta$ 14-3-3 isoforms typically identified in the CSF of patients with sporadic

**Figure 2**

Western-blot analysis of CSF from CJD patients using 14-3-3 isoform-specific antisera

(A) A standard mixture of 14-3-3 isoforms isolated from sheep brain and CSF samples from patients 2 and 4 were analysed using $\gamma$, $\varepsilon$, $\eta$ and $\beta$ 14-3-3 isoform-specific antisera. (B) CSF samples from CJD patients 1–4 were analysed using $\gamma$ 14-3-3 isoform-specific antisera with the following scoring regime: –, no detectable signal; +, very faint signal; ++, faint signal; ++++, moderate signal; +++++, strong signal.

ELISA

In developing our present ELISA procedures the capture antibody used was a monoclonal antibody raised against a heterogeneous 14-3-3 peptide. The detecting antibody used is a biotinylated form of the polyclonal anti-$\gamma$ 14-3-3 antibody used in the Western analysis, followed by streptavidin–horseradish peroxidase-conjugated secondary antibody and tetramethylbenzidine peroxide substrate. The absorbance was measured at 450 nm. The standard mixture of 14-3-3 isoforms used in the study was isolated from sheep brain and the isoform ratio in this mixture was quantified by calibration of the isoform HPLC profile using purified $\gamma$ 14-3-3 protein as a standard. The
coefficients of variation for the within-batch and between-batch precision of the assay were 25% and 13% respectively. Our current ELISA protocol has a working range of 10–100 ng/ml. A typical standard curve is shown in Figure 3(A).

Testing of CSF samples from patients with neurodegenerative diseases

The CSF samples contain dimeric 14-3-3 proteins complexed with other interacting proteins. In this heterogeneous mixture the 14-3-3 proteins are not as readily detectable by ELISA as by Western-blot analysis and for this reason all of the protein samples were concentrated (× 5) and heat-treated before being assayed.

In our preliminary studies the ELISA protocol was used to determine the levels of γ 14-3-3 in the CSF of patients with two neurological disorders: (a) from patients with sporadic CJD and (b) from patients with multiple sclerosis. Elevated levels of 14-3-3 in the CSF of multiple sclerosis patients has previously been reported by Sathoh et al. [26].

As shown in Figure 2(B), each of the CJD patients tested by ELISA recorded positive for γ 14-3-3 with good correlation between the scoring analysis from Western blotting and the differential levels of 14-3-3 recorded by the ELISA. In contrast, the multiple sclerosis samples tested were all below the detectable limit for the γ 14-3-3 protein. However, it may be that the CSF of multiple sclerosis patients contains other isoforms of 14-3-3 protein.

Conclusion

Kenney et al. [27] have shown the total 14-3-3 protein content present in the CSF of sporadic CJD patients during neurodegeneration to be in the ng/ml range. If the appearance of small quantities of specific 14-3-3 isoforms in the CSF is consistent with the loss of neurons in neurodegenerative conditions, then the variation in the pattern of isoforms suggests that control of the pathway from neuron to CSF may be isoform-specific.

While our analysis indicates that at present the Western-blot analysis of 14-3-3 in CSF is more sensitive than the current ELISA, the positive correlation between the assays indicates that development of an isoform-specific ELISA using adaptations of the method is valid. The optimization of selectivity and sensitivity of our ELISA protocol is in progress.

References


© 2002 Biochemical Society
Effect of multiple phosphorylation events on the transcription factors FKHR, FKHRL1 and AFX

Y. L. Woods1 and G. Rena2

MRC Protein Phosphorylation Unit, School of Life Sciences, MSI/WTB Complex, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland, UK.

Abstract

The insulin-stimulated phosphoinositide 3-kinase (PI 3-kinase) pathway is believed to play a critical role in metabolic control and cell survival, largely mediated through PKB phosphorylation of many proteins. Recent findings demonstrate that the transcription factors FKHR (forkhead in rhabdomyosarcoma), AFX (ALL1 fused gene from chromosome X) and FKHRL1 (FKHR-like 1; termed FKHR isoforms) are phosphorylated by PKB in cells, leading to their exit from the nucleus. These exciting results suggest that FKHR isoforms may be critical effectors of PI 3-kinase/PDK1/PKB signalling in vivo.

Introduction

Many of insulin’s intracellular actions are transmitted through a conserved kinase cascade. In brief, insulin-stimulated autoposphorylation of the insulin receptor brings about the activation of phosphoinositide 3-kinase (PI 3-kinase), leading to the recruitment of protein kinase B (PKB) to the plasma membrane and its activation by 3-phosphoinositide-dependent kinase-1 (PDK1). The PI 3-kinase/PKB signalling pathway has a critical role in metabolic control and cell survival [1]. These effects are largely mediated through PKB phosphorylation of many proteins, of which the first to be identified was glycogen synthase kinase-3 (GSK3) [2].

Given that insulin alters the transcription of at least 100 genes [3] it is likely that many of the effects of PI 3-kinase/PKB signalling are due to altered gene expression. Until recently, however, no PKB-regulated transcription factor had been identified. This situation changed in 1999, when we and others reported that the transcription factors FKHR (forkhead in rhabdomyosarcoma), AFX (ALL1 fused gene from chromosome X) and FKHRL1 (FKHR-like 1) are phosphorylated directly by PKB in cells, preventing them from stimulating gene transcription and leading to their exit from the nucleus [4-10]. These findings have stimulated much more detailed analysis of the effect of phosphorylation on the regulation of FKHR isoforms, the results of which are the major focus of this review.

DAF16 regulation by an insulin-like signalling pathway

Mechanistic evidence suggesting that FKHR isoforms are regulated by PKB was initially obtained from genetic studies in Caenorhabditis elegans [11,12]. An insulin-receptor-like signalling pathway regulates dauer formation, a developmental stage of the nematode that causes animals to shift metabolism towards fat storage and to live longer [13,14]. Inactivating mutations in the insulin/insulin-like growth factor 1 (IGF-1) receptor homologue DAF-2 (where DAF stands for dauer arrest phenotype) [15], in the PI 3-kinase hom-