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Persistence of species variation and regional heterogeneity of the apparent molecular masses of benzodiazepine-binding proteins after deglycosylation

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Brain membrane preparations of different vertebrates were photoaffinity labeled with [^{3}H]flunitrazepam and subsequently deglycosylated with endoglycosidase F and peptide *N*-glycopeptidase. SDS-polyacrylamide gel electrophoresis followed by fluorography revealed that each benzodiazepine-binding protein is deglycosylated in two steps, indicating that each protein has two glycosylation sites. Species variation of the apparent molecular masses of the benzodiazepine-binding proteins and regional heterogeneity in avians persist after deglycosylation. These results indicate that the α-subunit(s) of the GABA/benzodiazepine receptor has undergone electrophoretically detectable changes in its amino acid composition during vertebrate evolution. The existence of at least two different α-subunits in avians is further substantiated.

GABA/benzodiazepine receptor; Deglycosylation; Photoaffinity labeling; Species variation; Isoreceptor

1. INTRODUCTION

The GABA/benzodiazepine receptor (GABA/BZR) presumably consists of two α- and two β-subunits [1]. The homologous genes are members of a superfamily of ligand gated ion channels [2]. The α-subunit, which encompasses the benzodiazepine (BZ) binding site [3], is a glycoprotein containing two possible sites for glycosylation at the large N-terminal domain [2]. The carbohydrate moieties are indispensible for the correct formation of the receptor subunits in membranes [4]. They also influence the binding properties of the GABA/BZR [5].

Photoaffinity labeling of crude synaptic membranes with [^{3}H]flunitrazepam (FNZ) followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography revealed regional heterogeneity of the BZ-binding proteins in rats [6]. A similar regional heterogeneity has been detected in avians: In cerebellum mainly a protein of 53 kDa is detectable whereas in telencephalon an additional 54 kDa band is labeled intensely [7,8]. Further phylogenetic studies have shown that a major BZ-binding protein of 55 kDa is labeled in several teleostean fishes [9]. Whereas we have hypothesized that multiple BZ-binding proteins within a single species represent different α-subunits of the GABA/BZR [10], different glycosylation states of a single subunit could also account for these observations [5]. In addition, species variation in the size of carbohydrate chains could also explain the phylogenetic differences observed.

Here we compared the molecular masses of deglycosylated BZ-binding proteins in different vertebrate species. For deglycosylation of BZ-binding proteins two enzymes were chosen, which reduce glycoproteins to carbohydrate-free proteins: endoglycosidase F (endo F) removes most high mannose and some hybrid and complex oligosaccharides from glycoproteins; peptide *N*-glycosidase F (PNGase F) hydrolyses at the glycosylamine linkage and cleaves most types of *N*-linked carbohydrates [11,12].

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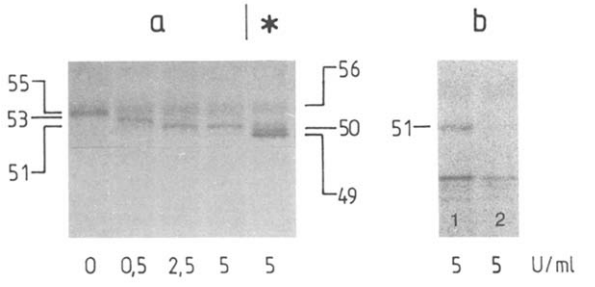

Fig.1. SDS-PAGE and fluorography of deglycosylated BZ-binding proteins in fish. (a) Brain membranes of mackerel were photolabeled with [^{3}H]FNZ and subsequently deglycosylated with various concentrations of endo F. The completely deglycosylated BZ-binding protein of 51 kDa in mackerel is of a slightly higher molecular mass than that of the two deglycosylated BZ-binding proteins of 50 and 49 kDa in avian telencephalon (*). (b) [^{3}H]FNZ binding in the absence (1) and presence (2) of 3 μM clonazepam at 5 U/ml Endo F. In all species a diffuse band of 56 kDa was visible when the incubation mixture contained endo F and to a considerably lesser extent when PNGase F was used. To a slight extent, this band is also labeled nonspecifically (figs 1c,2c,3c). Thus, it most likely does not represent a BZ-binding protein. In all figures numbers indicate apparent molecular masses (in kDa) which were calibrated with standard proteins.

2. MATERIAL AND METHODS

Mackerels were kindly provided by the Meeresbiologische Anstalt Helgoland. Jay, duck, pig and calf were obtained locally.

[^{3}H]FNZ (spec. act. 81.8 Ci/mmol) was purchased from NEN; clonazepam was a kind gift from Hoffmann-La Roche (Basle). The enzymes endo F (EC 3.2.1.96) and PNGase F (EC 3.2.2.18) were obtained from Boehringer (Mannheim).

Brains were removed immediately after death or after 2 (pig) or 4 h (calf), subsequently frozen and stored at −70°C. Membrane preparations including protease inhibitors and photoaffinity labeling with [^{3}H]FNZ were performed as described [9]. After photoaffinity labeling membranes were washed and resuspended in 25 mM Tris-HCl (pH 7.4), containing 0.2% SDS (protein content 15–30 mg/ml). Then endo F (0.25–10 U/ml) or alternatively PNGase F (4.8–60 U/ml) was added and the mixtures were incubated in a shaking water bath at 37°C for 18 h. Control samples without glycosidases were prepared under identical conditions. SDS-PAGE was performed on 10% polyacrylamide gels [9]. Gels were fixed, impregnated with Improved En^{3}Hance (NEN), dried and exposed to Kodak X-Omat AR films for 2–4 weeks at −70°C.

3. RESULTS

Addition of different concentrations of endo F to fish membrane preparations leads to degradation of the photolabeled 55 kDa protein in two steps (fig.1). The molecular mass of the BZ-binding protein first decreases from 55 to 53 kDa and then finally to 51 kDa.

In avian telencephalon deglycosylation of each of the two BZ-binding proteins seemingly also occurs in two steps. Both the 53 and 54 kDa proteins are first reduced to 51 kDa in jay and duck. This 51 kDa band clearly appears as a doublet (fig.2a). Upon addition of higher endo F or PNGase F concentrations two bands of 50 and 49 kDa are apparent, which cannot be further reduced (fig.2a,b). In duck and jay cerebella treatment with both glycosidases leads to a final degradation product of 49 kDa. Application of lower enzyme concentra-

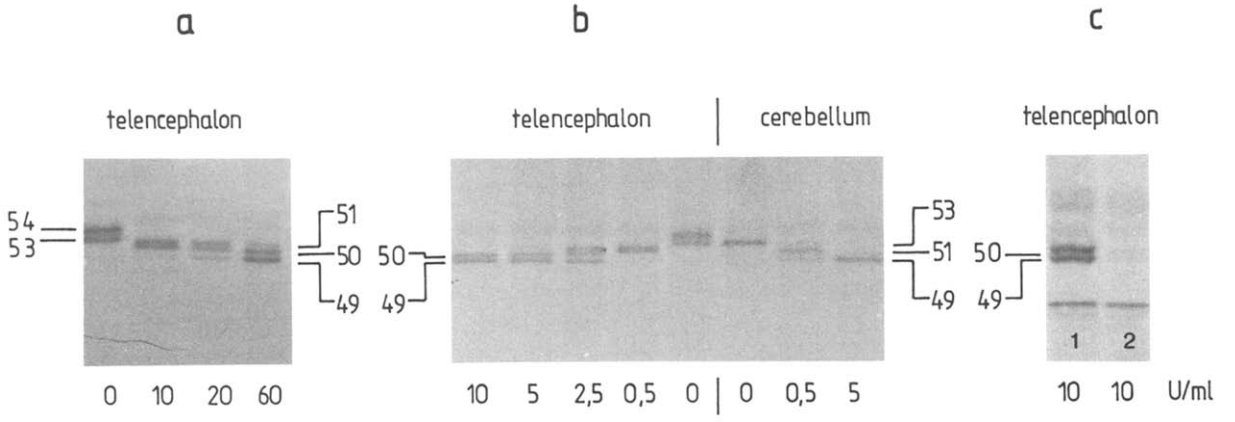

Fig.2. SDS-PAGE and fluorography of deglycosylated BZ-binding proteins in avians. (a) Deglycosylation patterns obtained with various concentrations of PNGase F in jay telencephalon. (b) Comparison of the deglycosylation patterns obtained with different concentrations of endo F in membranes of jay telencephalon and cerebellum. (c) [^{3}H]FNZ binding in the absence (1) and presence (2) of 3 μM clonazepam at 10 U/ml Endo F.

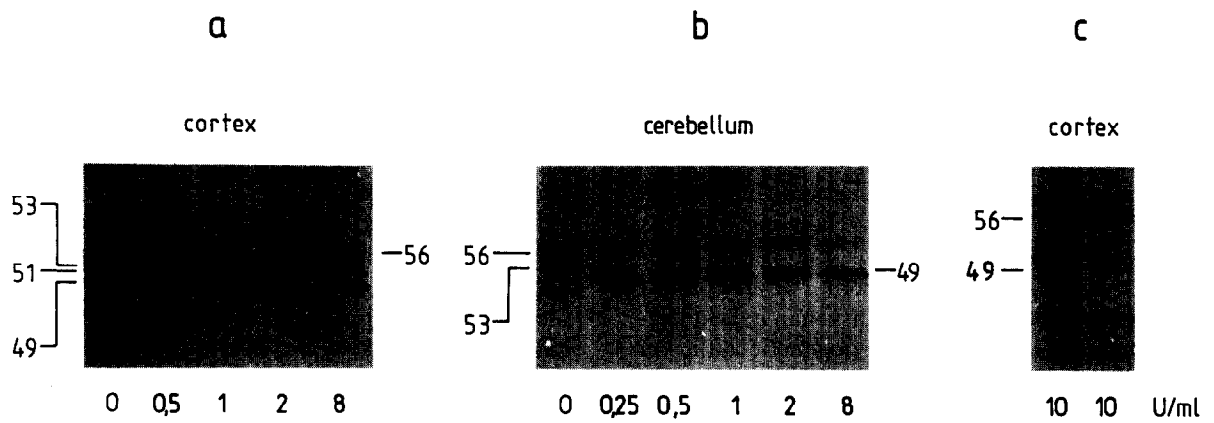

Fig.3. SDS-PAGE and fluorography of deglycosylated BZ-binding proteins in calf. Cortical (a) and cerebellar (b) membranes were photolabeled with ^{3}H-FNZ and treated with various concentrations of endo F. (c) [^{3}H]FNZ binding in the absence (1) and presence (2) of 3 μM clonazepam upon subsequent addition of 10 U/ml Endo F.

tions also demonstrates a deglycosylation of the cerebellar 53 kDa protein in two steps via the intermediate degradation product of 51 kDa (fig.2b).

In calf cerebellum a single BZ-binding protein of 49 kDa is visible after deglycosylation with endo F (fig.3b) or PNGase F. Similar results were obtained in pig cerebellum. An immunoblot performed with the monoclonal antibody bd-24 specific for the α-subunit [13] (kindly provided by Professor Möhler, Hoffmann-La Roche, Basle) revealed that at low glycosidase concentrations an intermediate deglycosylation product of 51 kDa also exists in pig cerebellum (unpublished). Thus, the degradation patterns in avian and mammalian cerebella are similar.

In cortices of pig and calf the main BZ-binding protein of 53 kDa is also deglycosylated in two steps by endo F and PNGase F to a final peptide of 49 kDa. Fig.3a also shows that a faint 51 kDa band is still detectable at high endo F concentrations in mammalian cortices. In some original fluorographics a BZ-binding protein of 59 kDa is weakly photolabeled in control samples. Upon deglycosylation a weak specific band of 55 kDa is discernible.

4. DISCUSSION

Deglycosylation of BZ-binding proteins has previously been investigated in mammals only. Mamalaki and co-workers [1] also used endo F for deglycosylation of the purified bovine GABA/BZR; their labeling results with [^{3}H]FNZ are similar to ours in calf and pig cortex. Treatment of rat hippocampal membranes with endo F or PNGase F modified the apparent molecular masses of the two major BZ-binding proteins labeled in this region [14]. The results described by Sweetnam and Tallman [5] in rat cortex differ from ours, however, these investigators used neuraminidase and endo H, which removes *N*-linked mannose-rich carbohydrates and certain hybrid oligosaccharides from glycoproteins.

Our deglycosylation patterns are similar in all species and brain regions investigated. Each original BZ-binding glycoprotein is degraded in two steps: low glycosidase concentrations apparently cleave a carbohydrate moiety of 2–3 kDa, higher concentrations induce a further cleavage of an additional carbohydrate chain of similar molecular mass. Since the cleavage process is clearly dependent on glycosidase concentrations, it is essential to apply these enzymes generously in order to obtain carbohydrate-free BZ-binding proteins.

The degradation of BZ-binding proteins in two steps points to the presence of two glycosylation sites on each individual protein. Apparently, the respective cleavage sites differ in their accessibility for glycosidases. It thus appears that both potential glycosylation sites on the bovine α-subunit [2] are indeed glycosylated. Furthermore, the molecular mass of the final deglycosylation product of the 53 kDa protein in our experiments is very similar to that evaluated for the amino acid chain of the α-subunit (48.8 kDa).

The general accordance of our results with those obtained by Schofield et al. [2] facilitates the interpretation of our deglycosylation studies: the two glycosylation sites are phylogenetically conserved, since degradation occurs in two steps in each species examined. The phylogenetic differences in molecular mass of the original labeling patterns persist after deglycosylation, e.g. the carbohydrate-free BZ-binding protein in teleostean fishes has a higher molecular mass than its counterpart in mammals. Thus, the phylogenetic variation in molecular mass of the BZ-binding proteins is seemingly due to different amino acid compositions.

The presence of two or more photolabeled bands within a single species is of great interest, since they possibly represent additional α-subunits, thus rendering the GABA/BZR an isoreceptor complex [10]. Avians are especially suited for an assessment of regional and ontogenetic variation of labeling patterns [7,8]. The 54 kDa band was shown to be clearly distinct from the β-subunit in chickens [1]. Our deglycosylation studies apparently exclude the possibility that the 53–54 kDa bands are the result of different glycosylation states of a single subunit. Instead, the presence of two distinct deglycosylated BZ-binding proteins in avians further substantiates the existence of at least two α-subunit-specific genes, which are differentially expressed according to regional and ontogenetic requirements.

In mammals mainly the 53 kDa band is fluorographically visualized using our labeling conditions. In cortices additional faint bands of 56 and 59 kDa are apparent [15]. These bands are seemingly further decreased in intensity in the controls for the deglycosylation studies. Thus, the labeling patterns in mammalian cortices do not allow a clear-cut answer as to the degradation of the 56 and 59 kDa band upon glycosidase treatment. However, in hippocampus differences in the molecular mass of BZ-binding proteins also persist after deglycosylation [14].

In view of the labeling results obtained in avians, the 53 kDa band in mammals could conceivably consist of the combined labeling intensity of two proteins comigrating upon SDS-PAGE. If this is the case, one of the two proteins is possibly more readily totally deglycosylated than the other as suggested by the 4-fold difference in glycosidase concentrations required to degrade completely the cerebellar and cortical 53 kDa band. Immunoblots performed with the α-subunit specific monoclonal antibody bd-24 have revealed similar degradation patterns to those observed upon photoaffinity labeling (unpublished).

In summary, after complete deglycosylation of BZ-binding proteins species variation and regional heterogeneity are still detectable, thus supporting the hypothesis that multiple α-subunits exist which have evolved by gene duplication and subsequent divergence [10,16]. We conclude that phylogenetic and regional multiplicity of the photolabeled BZ-binding proteins are not based on different glycosylation states but on differences in the length of the respective amino acid chain.

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