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The Human β-Subunit of Rod Photoreceptor cGMP Phosphodiesterase: Complete Retinal cDNA Sequence and Evidence for Expression in Brain

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We have identified and sequenced cDNA clones that encode for the human β-subunit of rod cGMP phosphodiesterase (PDEB). A single 2565-bp open reading frame that codes for an 854-amino-acid protein was identified. The human β-subunit protein is 90% identical to the bovine β-subunit and 91% identical to the mouse protein. Northern blot analysis indicates that the gene is expressed as an abundant 3.5-kb transcript in retina and as a rare 2.9-kb transcript in brain. The isolation of cDNAs from human brain cDNA libraries confirms the brain as a site of expression for this gene. The molecular defect underlying retinal degeneration in the *rd* mouse has been found to be a nonsense mutation in the β-subunit of the mouse cGMP PDE, resulting in a truncated protein (Pittler *et al.*, 1991b, *Proc. Natl. Acad. Sci. USA.* 88: 8322–8326). The molecular cloning of the cDNA encoding for the PDEB represents the first step in establishing whether this gene plays a causative role in any one of the several human hereditary retinopathies or, based on its localization to chromosome 4p16.3, in the pathogenesis of Huntington disease.

INTRODUCTION

Rod cell cGMP phosphodiesterase (PDE) is a heterotrimeric peripheral membrane-bound protein composed of catalytic α- and β-subunits and two inhibitory γ-subunits (Baehr *et al.*, 1979; Deterre *et al.*, 1988; Fung *et al.*, 1990). Expression of the cGMP phosphodiesterase β-subunit gene has previously been described in rod cells of the mouse and bovine retina (Bowes *et al.*, 1990; Lipkin *et al.*, 1990). Rods are highly specialized retinal cells that function in the detection of photons and are able to convert a single photon into a neural signal. The rod proteins rhodopsin, transducin, and cGMP phosphodiesterase function together in modulating the phototransduction cascade. The absorption of a photon causes photoexcited rhodopsin to interact with the GTP binding protein transducin, which in turn disinhibits cGMP phosphodiesterase. Activated cGMP phosphodiesterase then hydrolyzes 3′, 5′-cyclic GMP to 5′ GMP, and intracellular cGMP levels decline. Reduced cytoplasmic cGMP results in the closure of rod plasma membrane cation channels and a transient hyperpolarization of the plasma membrane (Hurley, 1987; Stryer, 1986).

As part of our effort to identify genes on human chromosome 4p16.3 in a candidate region for the Huntington disease gene, we have identified the gene encoding for the human β-subunit of the rod photoreceptor cGMP PDE (PDEB). We report here on the isolation of multiple cDNAs spanning this gene and the full-length retinal cDNA sequence. Furthermore, we show for the first time that this gene is expressed in brain, suggesting that the encoded protein may play a role in cGMP-mediated signal transduction in the brain. Putative functional domains within the human protein have been identified by comparing the primary structure of the PDEB to the primary structures of other photoreceptor PDE proteins.

MATERIALS AND METHODS

cDNA library screening. Five cDNA libraries were utilized in this study. The fetal brain (14 week) and the fetal eye (14 week) cDNA libraries were the gift of Dr. David Kurnit (University of Michigan). The adult retina cDNA library was constructed by Dr. Jeremy Nathans (Johns Hopkins University) and the caudate and putamen cDNA libraries were purchased from Clontech. Approximately one million plaque-forming units of each library were plated onto five 25 × 25-cm petri plates (GIBCO), and four sets of replica filters were made using Hybond N^+ nylon filters (Amersham) by standard methods (Sambrook *et al.*, 1989). Hybridization probes were labeled by the method of Feinberg and Vogelstein (1984) and preannealed with 300 μg of sonicated total human DNA in TE, pH 7.5, at 65°C for 1 h prior to hybridization. cDNA library prehybridization and hybridization were performed in 0.5 *M* sodium phosphate buffer, pH 7.2, 7% sodium dodecyl sulfate, and 1 m*M* EDTA at 65°C (Church and Gilbert, 1984). Following overnight hybridization, the cDNA filters were washed in 0.1× SSPE, .1% SDS at 65°C. Autoradiography was performed for 24–72 h.

Northern blot analysis. RNA was isolated using the single-step method of homogenization in guanidinium isothiocyanate as de-

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cDNA Libraries Hybridized	The Number of Hybridization Signals Identified Per 1x10^6 PFU Hybridized With Genomic Fragment A, B, C & D, E (shown below)			
	A	B	C & D	E
Adult Retina	35	0	35	153
Fetal Eye	-	0	0	30
Fetal Brain	-	0	0	2
Caudate	-	0	0	6
Putamen	-	-	0	0

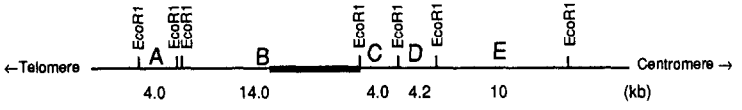

FIG. 1. The results of screening five human cDNA libraries with radiolabeled genomic fragments A, B, C+D, and E. The physical localization of the genomic fragments is described in Weber *et al.* (1991a). Libraries that were not screened with a particular probe are indicated by (—). Hybridization probe B, a 7.8-kb cosmid end fragment, is indicated by a bold line.

scribed (Chomczinski and Sacchi, 1987). Poly(A)$^+$ RNA was prepared using oligo(dT) drip columns (Stratagene) and fractionated on 1% agarose gels containing 2.2 *M* formaldehyde. Alkaline transfer was done to a Hybond N$^+$ membrane according to the manufacturer's instructions. The cDNA probe used in the Northern analysis was used immediately following denaturation without preannealling. Hybridization was performed in 0.5 *M* sodium phosphate buffer, pH 7.2, 7% sodium dodecyl sulfate, and 1 m*M* EDTA (Church and Gilbert, 1984). Following hybridization the Northern blot was washed in 0.5× SSPE, 0.1% SDS at 65°C for 1 h. Autoradiography was performed for 14 days.

Sequencing. All cDNAs sequenced were subcloned into the plasmid Bluescript KS(+) (Stratagene). Sequencing templates were prepared by the method of Lee and Rasheed (1991). Double-stranded DNA sequencing was carried out by the dideoxy method (Sanger *et al.*, 1977) using a Sequenase kit (USB). Initial sequencing was performed using T3 and T7 primers. Sequence obtained from these primers was then used to synthesize internal oligonucleotide primers for subsequent sequencing. All sequencing primers were synthesized in our laboratory with an Applied Biosystems, Inc. PCR-Mate 391 synthesizer. Sequence analysis was carried out using MacVector (IBI) sequence analysis software, and the amino acid alignments were performed using Hein's program (Hein, 1990).

RESULTS

We have previously cloned a 460-kb region of chromosome 4p16.3 (Weber *et al.*, 1991a). As part of the characterization of this cloned region, an extensive search was performed to identify polymorphic markers (Weber *et al.*, 1992). One of the genomic clones, a 10-kb *Eco*RI fragment probe (E) (Fig. 1), is located approximately 3 kb distal to a CpG island and was observed to be conserved on zoo blots. Therefore, this probe (E) together with a probe isolated from fragment (E) by the inter-*Alu*-PCR (Weber *et al.*, 1991b) was hybridized to a caudate cDNA library and six positive clones were isolated. One of these clones cDNA (K1) was used as a hybridization probe on a Northern blot of human RNA isolated from retina, frontal cortex, basal ganglia, caudate, skeletal muscle, lung, and adrenal gland. A prominent signal of 3.5 kb and a less abundant 4.5-kb transcript were observed following an overnight exposure in retinal RNA (Fig. 2). Following a 14-day exposure hybridization signals became evident in frontal cortex, basal ganglia, and caudate at approximately 2.9 kb. No signal was observed in RNA from skeletal muscle, adrenal gland (Fig. 2), or lung.

To isolate additional cDNAs corresponding to the transcripts observed on the Northern blot, an adult retinal cDNA library, a fetal eye (14 week) cDNA library, a

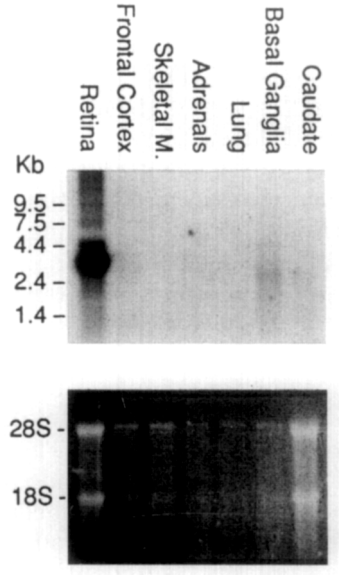

FIG. 2. Northern blot of total human RNA from retina (5 μg) and caudate (20 μg) and 2 μg each of poly(A)+ RNA from frontal cortex, skeletal muscle, adrenal glands, lung, and basal ganglia. The signals observed were obtained following a 14-day exposure. A photograph of the ethidium bromide-stained gel is shown below the autoradiograph.

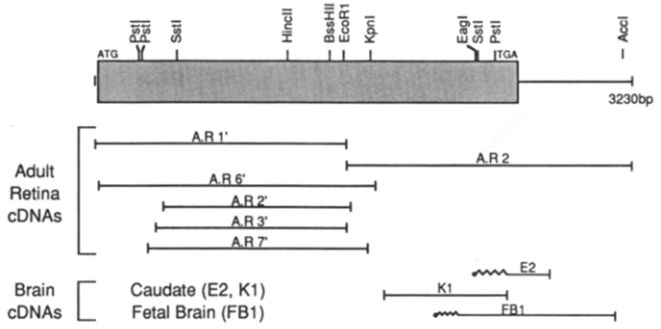

FIG. 3. Restriction map of the complete coding region (box) and the 5′ and 3′ untranslated regions (line) determined from overlapping adult retina cDNAs, and the relationship of the adult retina cDNAs, caudate cDNAs, and the fetal brain cDNA to the restriction map. The cDNA E2 is incompletely processed and contains an intronic sequence (Weber *et al.*, 1991b) indicated by the zig-zag line. The cDNA FB1 was mapped by sequencing both it's ends. One end maps to the 3′ untranslated region and one end represents cDNA cloning artifact (zig-zag).

fetal brain (14 week) cDNA library, an adult caudate cDNA library, and an adult putamen cDNA library were screened with the genomic probes shown in Fig. 1. The numbers of cDNAs identified upon screening all five cDNA libraries are shown in Fig. 1. Six cDNAs were isolated from the caudate cDNA library and five of these were determined to be identical (data not shown). Two cDNAs were isolated from the fetal brain cDNA library, and restriction mapping revealed that these were independent but similar clones. The largest of these was partially sequenced for mapping relative to the retinal cDNA sequence. Screening of the adult retina cDNA library with the indicated probes resulted in a set of six overlapping cDNAs that together span the PDEB gene (Fig. 3).

The sequence of the full-length cDNA for the PDEB gene is shown in Fig. 4. The translation initiation codon is underlined. Support for this being the true translation initiation site is derived from the alignment of the full-length human β-subunit cDNA sequence with the bovine and mouse β-subunit cDNA sequences, by the alignment of the deduced human β-subunit amino acid sequence with those of the mouse and bovine, and by the nucleotide context that conforms to the Kozak consensus sequence (Bowes *et al.*, 1990; Lipken *et al.*, 1990; Kozak, 1987). A single open reading frame extends from the proposed start codon to the first termination codon at position 2565. An *Alu* element is present 130 bp downstream from the translation termination codon and in the opposite orientation to the transcription of the PDEB gene. No direct repeats flanking the *Alu* element are discernable in the sequence. The *Alu* element is underlined in Fig. 4. A potential polyadenylation signal, AATAAA, at position 3047 is underlined; however, it is unlikely that this is a functional polyadenylation signal since no poly(A) tail has been identified. A computer-generated restriction map of the full-length PDEB cDNA is shown in Fig. 3.

A comparison of the sequenced PDEB cDNAs to each other and to the PDEB genomic sequence (Weber *et al.*, 1991b) has identified two polymorphisms in the coding portion of the cDNA. Two silent substitutions include a CGG to CGT transversion in codon 553, which codes for arginine, and a GTG to GTA transition in codon 835, which codes for valine. In the 3′ untranslated region three polymorphisms have been revealed. An insertion of a G occurs at position 2592, a G to A transition is found at position 2598, and the insertion of T occurs within the poly(T) tail of the *Alu* element.

The amino acid sequence deduced from the PDEB cDNA sequence is shown in Fig. 4. The predicted protein is composed of 854 amino acids and has a predicted molecular mass of 98.4 kDa. The amino acid alignment of the β-subunit of the human rod cGMP PDE with the α-subunit of human rod cGMP PDE (Pittler *et al.*, 1990), the α-subunit of the bovine rod cGMP PDE (Ovchinnikov *et al.*, 1987; Pittler *et al.*, 1990), the β-subunit of bovine rod cGMP PDE (Lipkin *et al.*, 1990), and the β-subunit of the mouse rod cGMP PDE (Bowes *et al.*, 1990) is shown in Fig. 5. The amino acid sequence of the PDEB shares 90% identity with the β-subunit of the bovine rod cGMP PDE and 91% identity with the β-subunit of the mouse rod cGMP PDE. The least conservation occurs at the N- and C-terminal regions of the protein. The PDEB also shares 71% identity with the α-subunits of cGMP PDE of both human and bovine, respectively.

DISCUSSION

We have identified multiple overlapping cDNAs, from a retinal cDNA library, that together encode for the full-length PDEB. This gene is expressed as an abundant transcript in retina and as a rare transcript in brain, specifically in the frontal cortex and basal ganglia including the caudate nucleus. The deduced amino acid sequence of this protein has 91 and 90% identity with the β-subunits of cGMP PDE from mouse (Bowes *et al.*, 1990) and bovine (Lipkin *et al.*, 1990), respectively. This similarity strongly supports the conclusion that the identified cDNAs encode for the PDEB.

The overall amino acid identity between the α-subunit (Pittler *et al.*, 1990) and the β-subunit of the human rod cGMP PDE is 71%. However, the homologies between the proposed catalytic domain and the two proposed noncatalytic cGMP binding domains (Lipkin *et al.*, 1990) for these subunits are 86 and 76%, respectively. This similarity further supports the conclusion that the reported gene is part of the phosphodiesterase gene family and may suggest that the α- and β-subunit genes are homologous, having evolved from an ancestral gene through a gene duplication event (Haldane, 1932; Muller, 1935; Ohno, 1970). A comparison of the genomic organization between the genes coding for the α-subunit of bovine rod cGMP PDE and the β-subunit of mouse cGMP PDE suggests that these two genes did indeed evolve from a common ancestral PDE gene (Pittler and Baehr, 1991b).

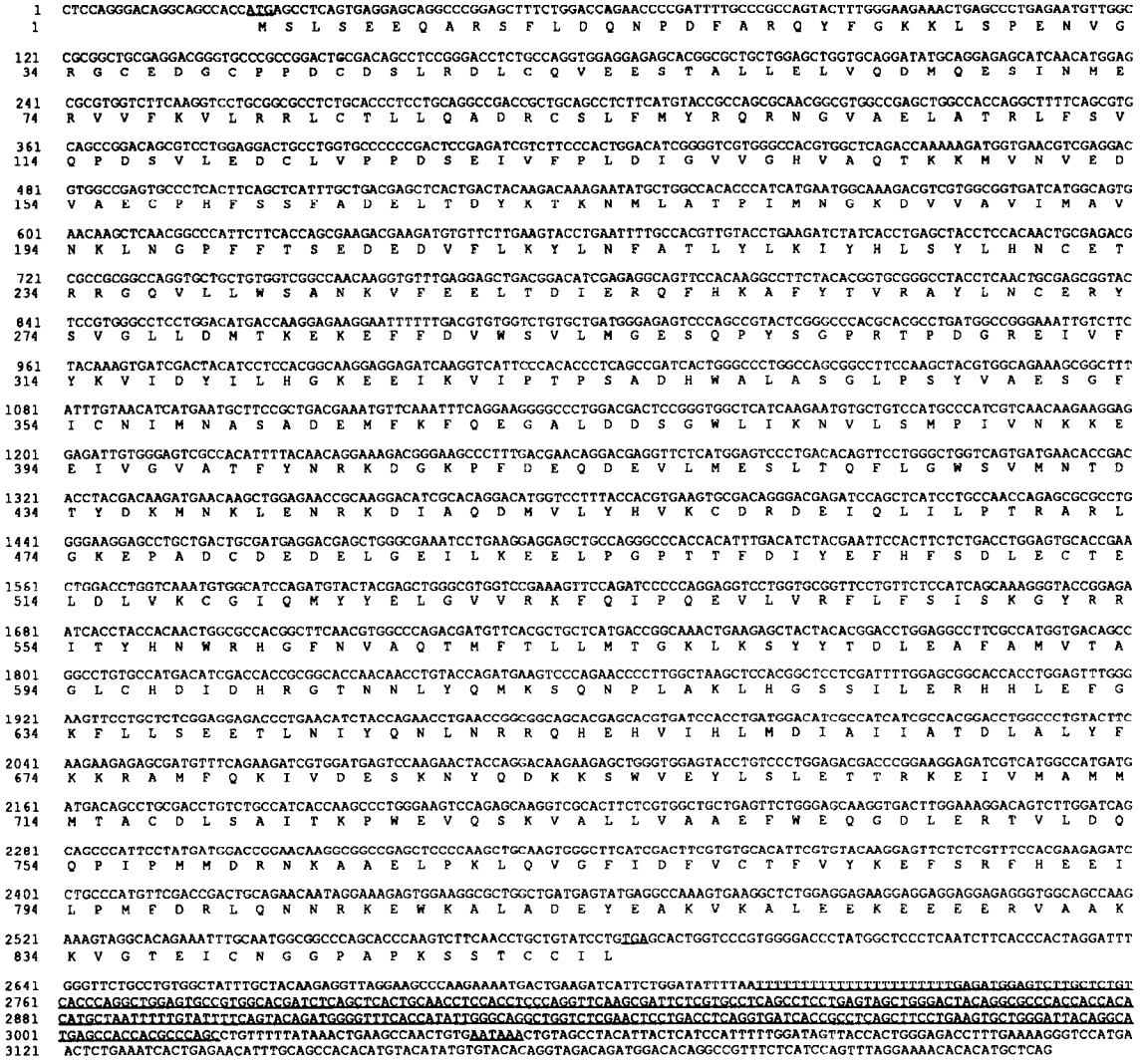

FIG. 4. Complete nucleotide sequence of the β-subunit of the human rod cGMP PDE cDNA with the encoded amino acid sequence shown below the nucleotide sequence. The translation initiation codon, termination codon, *Alu* element insertion, and potential polyadenylation signal are underlined.

The greatest dissimilarity between the α- and the β-subunits of the human rod cGMP PDE occurs at the N- and C-terminal regions (amino acid residues 1–85 and 824–854, respectively). A possible functional significance for the divergence at the N-terminus is suggested by the recent finding that the inhibitory γ-subunit, of the holoenzyme, interacts with this region of the α-subunit (Oppert *et al.*, 1991). The functional significance of the corresponding region in the β-subunit protein has not been biochemically defined.

The positions of the functional domains in the PDEB protein can be derived from their corresponding positions in the β-subunit of the bovine cGMP PDE protein (Lipken *et al.*, 1990). In this model there are two proposed noncatalytic cGMP binding domains, cGMP binding domain I and cGMP binding domain II (Charbonneau *et al.*, 1990; Li *et al.*, 1990), and a single proposed catalytic domain (Charbonneau *et al.*, 1986; Ovchinnikov *et al.*, 1987; Stroop *et al.*, 1989; Oppert *et al.*, 1991). The first noncatalytic binding domain is coded for by residues 89–251 and the second noncatalytic domain by residues 295–464. It has been suggested that the two noncatalytic domains are related as a tandem repeat (Charbonneau *et al.*, 1990; Li *et al.*, 1990), and in support of this we find a homology at the protein level, between the two domains, of 21%.

The position of the catalytic domain, which is the site of cGMP hydrolysis, has been assigned in the bovine protein to residues 555–790 (Lipkin *et al.*, 1990) based on strong sequence conservation between all members of the eucaryote PDE family (Charbonneau *et al.*, 1986; Li *et al.*, 1990; Ovchinnikov *et al.*, 1987; Sass *et al.*, 1986). The catalytic domain in the human homolog can thus be assigned to residues 555–790 in the human protein based on this homology.

Northern blot hybridization clearly demonstrates that the PDEB gene is predominantly expressed in the retina but also reveals a smaller size transcript expressed in frontal cortex and basal ganglia including caudate. Two transcripts are observed in the retina, one giving a prominent band at 3.5 kb and the other a minor signal at approximately 4.5 kb. This larger, less abun-

FIG. 5. Amino acid sequence alignment of the human β-subunit of rod cGMP PDE (humcgprb), the mouse β-subunit of the rod cGMP PDE (mmpde), the cow β-subunit of rod cGMP PDE (bovcgmp), the human α-subunit of rod cGMP PDE (humcgpra), and the cow α-subunit of rod cGMP PDE (bovcgmppra). Shaded regions indicate amino acid identities in three or more members of the cGMP phosphodiesterases. Dashes represent spaces introduced to facilitate alignments.

dant, transcript may represent a differentially or incompletely processed β-subunit transcript.

The observation, on a Northern blot, of a population of rare transcripts in brain RNA is supported by the identification of cDNAs in both caudate and fetal brain cDNA libraries. The caudate cDNA (E2) is identical at 59 of the last 60 coding nucleotides of the human PDEB gene. The single nucleotide difference corresponds to the aforementioned silent polymorphism in codon 835. In addition, this cDNA spans 153 bp of the 3′ untranslated region. A second caudate cDNA (K1) shares complete sequence identity with nucleotides 1767–2485 of the proposed catalytic domain. To our knowledge this is the first direct evidence for the expression of the PDEB gene in a tissue other than retina. The observation that the transcripts are smaller in brain than in retina may be due to alternate splicing, which has previously been documented for the mouse β-subunit gene of rod cGMP PDE (Baehr *et al.*, 1991) and other PDE genes (Davis *et al.*, 1989; Davis and Dauwalder, 1991). However, if the population of smaller transcripts observed in brain RNA is indeed the product of alternate splicing, then nucleotides 1767–2485 and 2524–2588 are not involved since the sequences of the two caudate-derived cDNAs are identical to the retinal PDEB sequence except for the single polymorphism. This would exclude most of the catalytic domain and the C-terminal domain from being alternately processed in the caudate. The low level of

expression observed on the Northern blot and reflected in the caudate and fetal brain cDNA libraries may be due to low levels of cellular expression. Alternatively, the low signal intensity may reflect a moderate to high level of expression in a very small subset of cells within a specific tissue.

It is noteworthy that the PDEB gene is expressed in both retina and brain. Recent indirect evidence that the PDEB gene may be expressed in the brain comes from the finding of a reduced number of granule cells in the hippocampal dentate gyrus of the *rd* mouse (Wimer *et al.,* 1991). The PDEB gene maps to one of the candidate regions that based on genetic analysis have been shown to likely contain the Huntington disease (HD) gene (Andrew *et al.,* 1992; MacDonald *et al.,* 1989). What significance could this have for the underlying pathogenesis of HD? In retina reduced PDE activity leads to the accumulation of cyclic GMP with subsequent neurotoxic effects culminating in selective photoreceptor cell death (Farber and Lolley, 1974; reviewed in Pittler and Baehr, 1991a). Theoretically, defective caudate PDEB activity could also lead to an increase in cyclic GMP and the subsequent selective neuronal death observed in HD (reviewed in Vonstattel *et al.,* 1985). One might then logically expect patients with HD to present with retinal problems, which does not occur. However, the absence of retinal pathology in patients with HD could reflect different tissue vulnerabilities or the effects of alternatively spliced gene products with differential cytotoxic effects. The role of this gene as a cause for HD will be further clarified following formal assessment of the structural integrity of this gene in affected persons with HD.

The mutation causing autosomal recessive retinal degeneration (*rd*) in the mouse has recently been mapped to the gene encoding for the β-subunit of rod cGMP PDE (Bowes *et al.,* 1990; Pittler and Baehr, 1991b). We have obtained the full-length cDNA sequence for the PDEB. The cloning of the human gene for the PDEB and the identification of polymorphisms within the cDNA will allow this gene to be assessed for its role in the molecular pathology of different retinal diseases.

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