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Molecular characterization of a new immunoglobulin superfamily protein with potential roles in opioid binding and cell contact

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A purified opioid-binding protein has been characterized by cDNA cloning. The cDNA sequence predicts an extracellularly located glycoprotein of 345 amino acids. This protein does not possess a membrane-spanning domain but contains a C-terminal hydrophobic sequence characteristic of membrane attachment by a phosphatidylinositol linkage. It displays homology to the immunoglobulin protein superfamily, featuring three domains that resemble disulfide-bonded constant regions. More specifically, the protein is most homologous to a subfamily of proteins which includes the neural cell adhesion molecule (NCAM) and myelin-associated glycoprotein (MAG) and one subgroup of the tyrosine kinase growth factor receptors comprising the platelet-derived growth factor receptor (PDGF R), the colony-stimulating factor 1 receptor (CSF-1 R) and the c-*kit* protooncogene. These sequence homologies suggest that the protein could be involved in either cell recognition and adhesion, peptidergic ligand binding or both.

Key words: cDNA cloning/cell adhesion molecule/immunoglobulin superfamily/opioid receptor/phosphatidylinositol membrane linkage

Introduction

The opioids are a highly diverse group of drugs, which include both a large series of plant-derived alkaloids and many peptides in the mammalian brain. Opioid receptors are likewise heterogeneous, with at least three different classes, differing in their selectivities for alkaloids, or the opioid peptides (Hollt, 1986). Numerous reports of the solubilization and purification by affinity chromatography of opioid binding sites from mammalian brain have been made (Bidlack *et al.*, 1981; Cho *et al.*, 1983; Gioannini *et al.*, 1985; Maneckjee *et al.*, 1985). However, few have reported purification of opioid-binding proteins to apparent homogeneity (Gioannini *et al.*, 1985; Simonds *et al.*, 1985; Cho *et al.*, 1986) and, to date, no molecular characterization of these purified proteins has been achieved.

Cho *et al.* (1986) purified a 58-kd opioid-binding protein to apparent homogeneity. Unlike the opioid-binding preparations described by others, the purified protein (Cho *et al.*, 1986) bound ligands only when reconstituted with acidic lipids (Hasegawa *et al.*, 1987). Neither the protein alone, nor the lipids bound opioid ligands to a significant degree, but in combination high affinity binding and selectivity for alkaloid opioids was observed. The binding affinities of ligands to this reconstituted material are lower than values seen for brain membranes, but the rank order of ligand affinity to both preparations is highly correlated (Cho *et al.*, 1983; Hasegawa *et al.*, 1987).

We have undertaken a molecular characterization of this protein and report its primary sequence as deduced from cDNA clones derived from bovine brain. The sequence is homologous to various members of the immunoglobulin (Ig) protein superfamily, especially to those molecules involved in cell adhesion.

Results

Peptide sequences and identification of cDNA clones

The affinity-purified protein failed to yield any N-terminal protein sequence, presumably due to a blocked N terminus. Therefore, cyanogen bromide digestion was used to obtain peptide fragments. Four HPLC-purified peptides were subjected to gas-phase microsequencing and the sequences obtained were:

1. M I Q N V D V Y D E G P Y T X S V Q T
2. M *A* I E N K G H I S T L T F F (X V S E K D Y G)
3. M *A* E F Q W F K E D T R L A T
4. M X X V T V X Q G E S A T

Sequences 1, 2 and 3 were used for oligonucleotide probe design. Duplicate filters of 1.5×10^6 clones of a bovine brain cDNA library were screened at low stringency with ^{32}P-labelled oligonucleotides designed against peptides 1 and 3, respectively. Clones hybridizing to both sequences were re-screened with an oligonucleotide probe designed against peptide 2. A single clone λBOM106 was positive to all three probes and its 1.8 kb insert was sequenced. A large open reading frame encoding a 318 amino acid polypeptide contained the three peptide sequences used for probe construction as well as the fourth peptide sequence that was obtained from the purified protein (Figure 1).

The open reading frame of λBOM106 lacked an initiation codon. Therefore, the 1749 bp insert of this cDNA clone was ^{32}P-labelled by random priming and used to re-screen the entire cDNA library at high stringency. No new hybridizing phage were obtained. A specifically primed cDNA library was therefore constructed from the same bovine brain mRNA using two different anti-sense

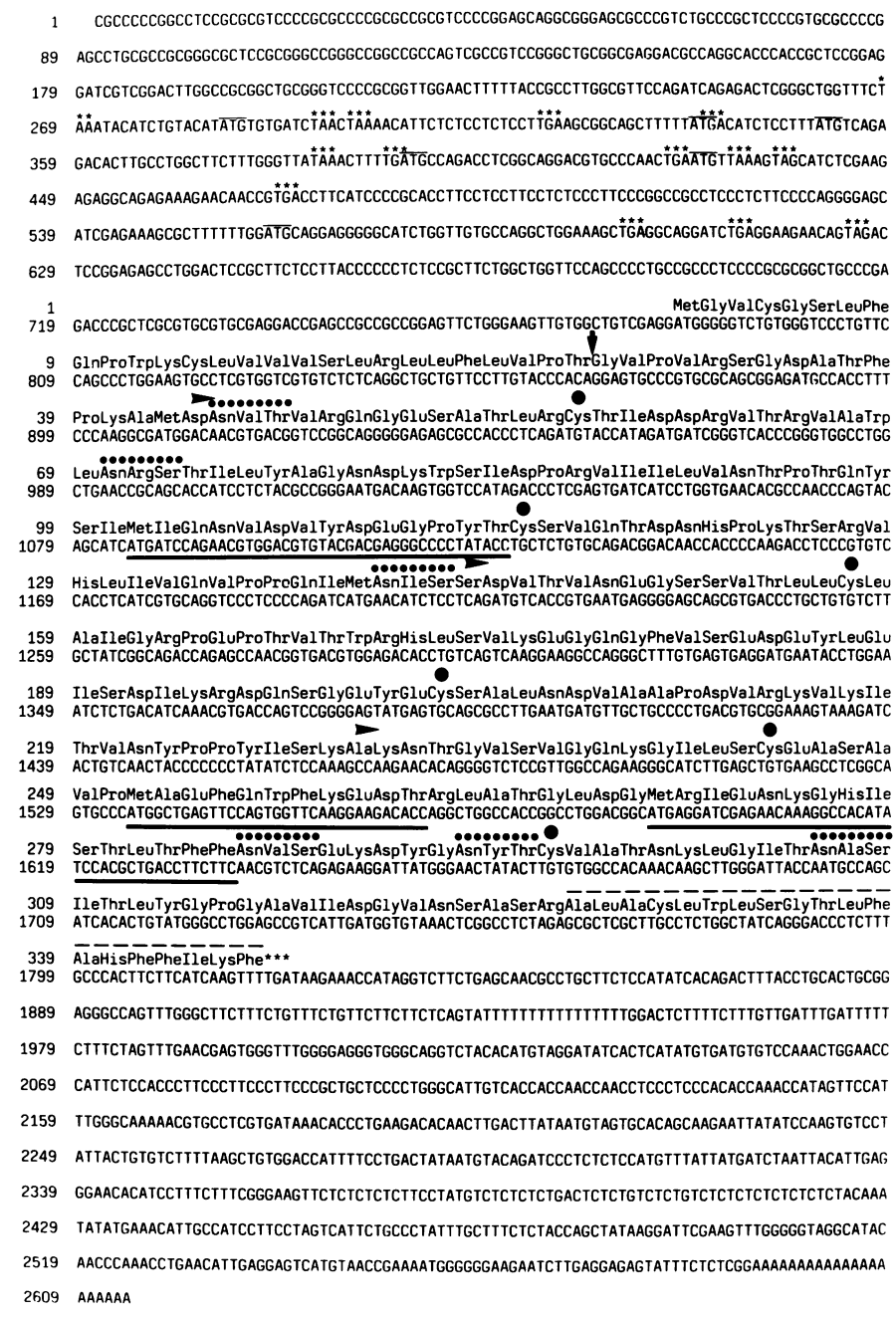

Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA clones. The sequence is derived from the two cDNA clones, λBOM159 (nt 1−1165) and λBOM106 (nt 865−2614). Potential initiation codons (ATG) in the 5′-untranslated sequence are overscored and stop codons marked with asterisks. Within the amino acid sequence the putative position of signal sequence cleavage is indicated by an arrow, potential N-linked glycosylation sites by filled dots and the peptide sequences used for probe construction are underlined. The positions of the three repeating Ig-like domains are indicated by arrowheads and the conserved cysteine residues are indicated by filled circles. The C-terminal hydrophobic sequence, characteristic of PI-linked proteins is indicated by a dashed line.

oligonucleotide primers. This library was screened unamplified with the ^{32}P-labelled 1749 bp insert and a single positively hybridizing clone, λBOM159, obtained. The sequence of this 1165 bp cDNA extended the open reading frame of λBOM106 a further 27 amino acids including a putative initiation codon (Figure 1).

The bovine cDNA contains a very long 5′-untranslated region of 784 bp beginning with an extremely GC-rich sequence (90% of the first 128 nucleotides). This untranslated region contains six potential initiation codons (ATG), each preceding a small open reading frame of <35 amino acids. The seventh ATG triplet (nt 785−787), which conforms most closely to the consensus initiation sequence (Kozak, 1987), is preceded by an in-frame stop codon (TGA at nt 611−613) and starts the long reading frame encoding the four chemically determined peptide sequences. Thus, the protein is apparently initiated at the seventh ATG codon, whereas most eukaryotic proteins are initiated at the first such codon in the mRNA sequence. Kozak (1987) has suggested that such RNAs, with multiple potential initiation codons, may contain an unspliced intron. However, the rat cDNA homologue (P.R.Schofield and P.H.Seeburg, unpublished) contains an initiation codon in an analogous position, indicating a correct initiation codon assignment and

the absence of unspliced introns. The DNA sequence of clone λBOM106 terminates with a short A-rich sequence. However, the absence of a polyadenylation addition signal (AATAAA) suggests that there are further 3′-untranslated sequences that are not encoded by this cDNA clone.

Northern blot analysis

The cDNA encoding the bovine protein is rare, since only one clone was identified in a high complexity bovine brain cDNA library. This result is confirmed by Northern blot analysis (Figure 2). The mRNA is expressed within whole brain and hypothalamus but not in the liver. Two distinct mRNA species of 4500 and 7200 nt were detected, as were some minor species. Their size indicates that the cloned bovine cDNA does not contain the full mRNA sequence but was primed at an internal A-rich sequence (Figure 1). We do not know if the two mRNA species seen by Northern analysis encode proteins characterized by alternate exon usage or differ in the length of their 5′- or 3′-untranslated sequences.

Structural interpretations

The 345 amino acid long polypeptide encoded by this cDNA has a predicted mol. wt of only 38 kd, considerably less than the 58 kd of the purified protein. The presence of six potential N-linked glycosylation sites (Asn-X-Ser/Thr) (Figure 1) suggests that carbohydrate attachment may account for the size discrepancy. Thus, Asn_{285} is a particularly strong candidate site for glycosylation, as this residue alone was not identified in the chemically determined sequence of peptide 2. By similar criteria, Asn_{44} may also be glycosylated.

The protein is largely hydrophilic but has hydrophobic sequences at both ends. The N-terminal sequence has the characteristics of a signal peptide, with a putative cleavage site occurring after Thr_{27} (von Heijne, 1986). The presence of a signal sequence and of N-linked glycosylation sites suggest that this protein is extracellularly located.

The hydrophobic C-terminal sequence constitutes the last 19 amino acids, but its proximity to the end of the protein and the absence of a stop transfer or anchoring sequence (Blobel, 1980) suggests it is not membrane spanning. However, one class of membrane-anchored proteins is characterized by the presence of C-terminal hydrophobic sequences which lack charged anchoring residues. These are the phosphatidylinositol (PI)-linked proteins (Cross, 1987; Low and Kincade, 1985) which include the rodent Thy-1 antigen (Seki *et al.*, 1985), trypanosome variant surface glycoprotein (VSG) (Boothroyd *et al.*, 1980), chicken (Hemperly *et al.*, 1986) and mouse (Barthels *et al.*, 1987) $NCAM_{120}$, human decay-accelerating factor of complement (DAF) (Caras *et al.*, 1987), Qa-2 antigen of the major histocompatability complex (Stroynowski *et al.*, 1987), and T cell-activating protein (TAP) (Reiser *et al.*, 1986), reviewed by Low and Saltiel (1988). For each of these proteins, the C-terminal hydrophobic sequence is processed in the coupling of the protein to the phosphatidylinositol moiety. Lipid attachment occurs at a residue located within or adjacent to the hydrophobic sequence (Low and Kincade, 1985; Low and Saltiel, 1988; Tse *et al.*, 1985; Ferguson *et al.*, 1985). Apart from this hydrophobicity no strong PI-linkage consensus sequence is apparent. However, in the cases of NCAM, Qa-2 and DAF, the PI-linkage domain is

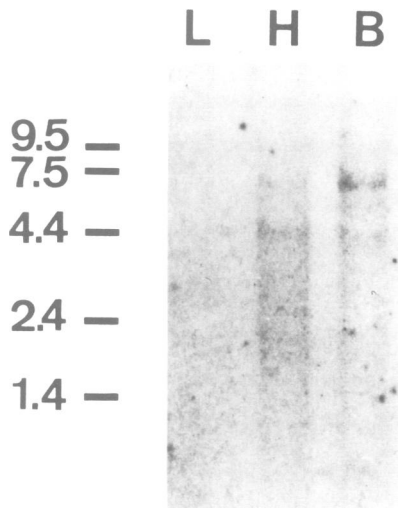

Fig. 2. Northern blot of bovine RNA. Poly(A)$^+$ mRNA (5 μg) from liver (L) hypothalamus (H) and whole brain (B) was subjected to Northern blot analysis using ^{32}P-labelled λBOM106 cDNA. Size markers (BRL) are shown in kb. Exposure was for 2 weeks.

encoded on a differentially spliced exon. Moreover, any eukaryotic protein that terminates in a short hydrophobic segment is a candidate for PI-linkage or glypiation (Cross, 1987). Such a proposal has been made for the carcinoembryonic antigen (CEA) (Oikawa *et al.*, 1987) which terminates with a characteristic C-terminal hydrophobic sequence (Williams, 1987). We suggest that this protein is similarly processed and attached to the cell membrane by glypiation. This conclusion is further supported by homologies to other PI-linked proteins (see below).

The hydrophilic portion of the amino acid sequence contains three internal repeating structures of ~100 amino acids (Figure 3). These three sequences share ~25% identity around two separate cysteine residues present in each of the repeatings.

Homologies with the immunoglobulin superfamilies

Comparison of the protein sequence to the Dayhoff protein database using the 'fastp' algorithm (Lipman and Pearson, 1985) revealed the highest and most significant homologies to be with two cell adhesion molecules, neural cell adhesion molecule (NCAM) (Cunningham *et al.*, 1987) (22% identity) and myelin-associated glycoprotein (MAG) (Arquint *et al.*, 1987; Salzer *et al.*, 1987; Lai *et al.*, 1987) (21% identity). Additionally, varying degrees of sequence identity were seen with members of the extended immunoglobulin protein superfamily (Williams, 1987). These homologies were centered around the conserved cysteine residues of the repeating structure identified above. The conserved motifs form the disulfide-bonded folded regions of the immunoglobulin superfamily of proteins. Other groups of proteins that belong to this superfamily include: (i), the cell adhesion molecules NCAM (Cunningham *et al.*, 1987), MAG (Arquint *et al.*, 1987; Salzer *et al.*, 1987; Lai *et al.*, 1987), the L1 glycoprotein, involved in fasciculation (Moos *et al.*, 1988), and the intercellular adhesion molecule, ICAM-1 (Simmons *et al.*, 1988); (ii) the carcinoembryonic antigen (CEA) (Oikawa *et al.*, 1987), which may be involved in cell adhesion, (iii) the type III tyrosine kinase growth factor receptors represented by the platelet-derived growth factor

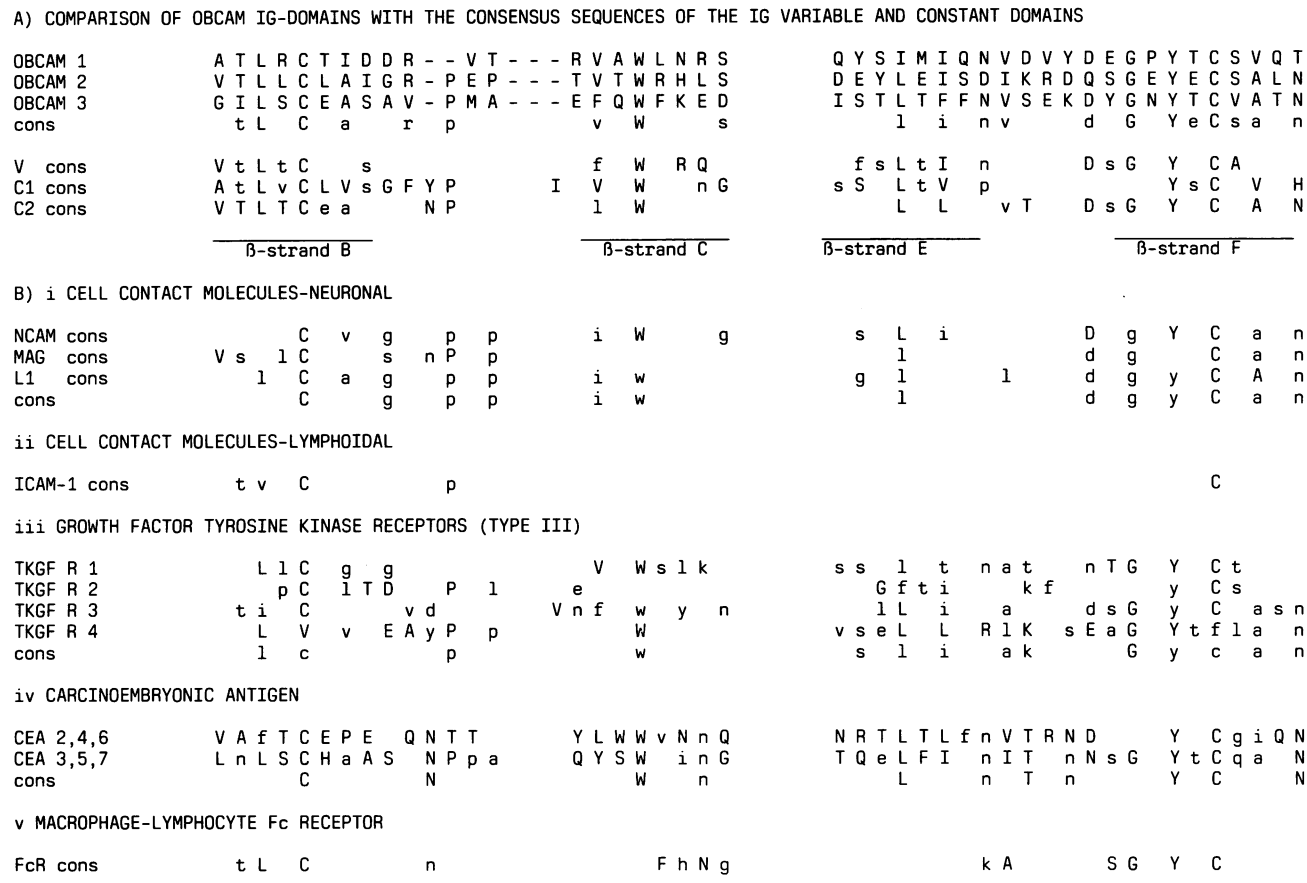

Fig. 3. Alignment of the Ig domains of the protein with members of the immunoglobulin protein superfamily. **(A)** The three Ig-like repeating domains are aligned and the derived consensus sequence compared with the three Ig domain consensus sequences. The positions of the relevant β-strands are indicated. Only portions of the respective protein sequences are shown. **(B)** Consensus sequences derived from functionally related groups of the C2 class of Ig-related molecules. Consensus sequences used are derived from the complete amino acid sequences. Except for the first three lines which contain the OBCAM protein sequence, captial letters denote an invariant residue while lower case letters denote a residue conserved in >50% of sequences. Numbers indicate the Ig domain number of the molecule. The consensus (cons) sequences presented in i, iii, and iv are derived from the date presented only.

receptor (PDGF R), the colony-stimulating factor-1 receptor (CSF-1 R) and the putative receptor and protooncogene *c-kit* (Yarden *et al.*, 1987); (iv) the leukocyte (macrophage) Fc receptors (Lewis *et al.*, 1986; Stengelin *et al.*, 1988). All contain immunoglobulin-like domains that display either seven anti-parallel β-strands, characteristic of constant domains, or nine anti-parallel β-strands, characteristic of variable domains. The seven β-strand constant domain-containing molecules have been divided into two classes; C1, which includes the immunoglobulin and T cell receptor constant domains, and C2 which includes the four other groups of Ig-related proteins, typified respectively by NCAM, CEA, PDGF R and the Fc R.

The three Ig domains of this protein were aligned and a consensus sequence derived. The consensus sequence was then compared (Figure 3A) to V, C1 and C2 consensus sequences (Williams, 1987). The sequence is assigned to the C2 set of proteins based on the number of conserved residues present, and the fact that the sequences around the second cysteine residue are more V- than C-like. Additionally, the spacings between the cysteine residues are consistent with the presence of C-like domains. Within this set, consensus sequences were derived for the various C2 protein subgroups (Figure 3B). Greatest conservation of the consensus sequence was detected with the molecules involved in cell adhesion, and to a lesser degree with the type III tyrosine kinase growth factor receptors. The similarities between the OBCAM protein and members of the NCAM family of proteins suggest that we have isolated a new member of the cell adhesion class of molecules. The putative PI-linkage of the protein to the cell membrane is another characteristic shared with the various Ig-related recognition and adhesion molecules, such as NCAM, Thy-1,Qa-2, TAP and perhaps CEA. Sequence similarities with the type III tyrosine growth factor receptors suggest a role of the protein in receptor-like peptidergic ligand binding, a function consistent with the purification and biochemical characterization of the opioid-binding protein.

Identity of cloned cDNA

To show that the cloned cDNA encodes the purified protein, we made use of an anti-peptide antibody raised against a portion of the cDNA-encoded amino acid sequence. An immunoaffinity antibody column was constructed using the IgG fraction of the anti-peptide antibody. Protein solubilized from rat brain membranes, that bound and was specifically eluted from this column, bound opioids when examined in the assays used for protein purification (Figure 4). Opioid binding was inhibited by the presence of a monoclonal antibody (3B4R11) (Roy *et al.*, 1988) which was raised against the purified protein (Figure 4). These results indicate that the purified protein is encoded by the cloned cDNA,

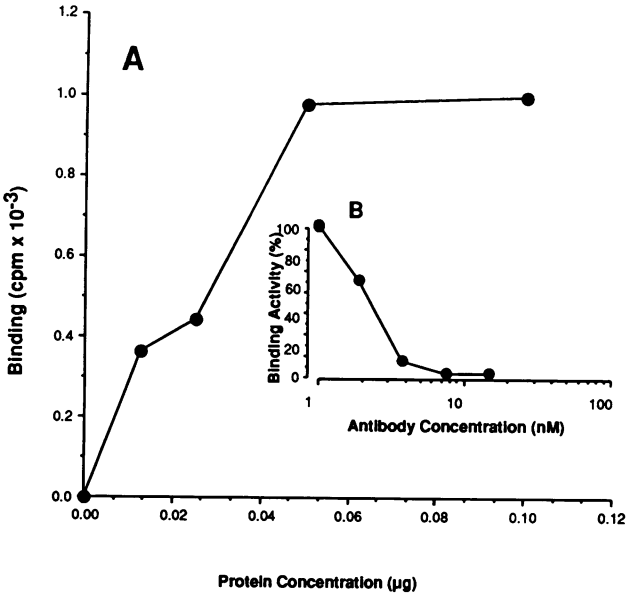

Fig. 4. Co-identity of purified protein and the cloned cDNA. (**A**) Solubilized rat brain membrane protein, purified on an anti-peptide antibody column, binds [^{3}H]diprenorphine. (**B**) Binding was inhibited by the presence of monoclonal antibody 3B4F11.

supporting the notion that the protein has a role in opioid binding.

Discussion

The primary sequence of the purified opioid-binding protein is characterized by the presence of a signal sequence, the lack of internal hydrophobic membrane-spanning sequences and by potential N-linked glycosylation sites, suggesting that this protein is extracellularly located. Of the six potential glycosylation sites, two, Asn_{285} and Asn_{44} appear to be glycosylated, as determined by chemical sequencing. This is in agreement with binding of the solubilized protein to lectin columns and a calculated mol. wt of the protein of ~20 kd lower than that observed for the purified protein.

The purified opioid-binding protein had been thought to be the μ opioid receptor (Cho *et al.*, 1986). However, the predicted primary sequence of the cDNA shows a lack of membrane-spanning domains. This is somewhat suprising, since receptors that interact with GTP-binding proteins, including the β-adrenergic receptors (Dixon *et al.*, 1986), the muscarinic cholinergic receptors (Kubo *et al.*, 1986), rhodopsins (Hargrave, 1982) and the substance K receptor (Masu *et al.*, 1987) share significant sequence and structural similarities including the presence of seven membrane-spanning domains. At least some types of opioid receptors are thought to associate with G-proteins, most notably the δ receptor of NG108-15 neuroblastoma-glioma cells (Chang and Cuatrecasas, 1979) and of mammalian striatum (Law *et al.*, 1981), but possibly also the μ receptor (Milligan *et al.*, 1987). G-protein interactions suggest that opioid receptor proteins would belong to the same seven membrane-spanning protein superfamily. The protein sequence predicted by the cloned cDNA thus suggests that the OBCAM molecule may not be a G-protein-coupled opioid receptor. The protein may, however, have a functional role in opioid binding, as suggested by the properties of the purified protein (Cho *et al.*, 1983, 1986; Hasegawa *et al.*, 1987).

The primary sequence of the protein is very highly conserved between bovine and rat (data not shown). The mRNA is expressed in neurons in the brain and not detected in other tissues (manuscript in preparation). However, the distribution of the mRNA does not co-localize with the known distributions of the μ or δ opioid receptors, as determined by *in vitro* autoradiography using radiolabelled opioid receptor ligands. The brain-specific expression of this highly conserved protein implies functional significance although its physiological role remains to be determined.

PI-linked membrane-anchoring mechanisms have been variously suggested as a primitive form of membrane attachment or as a mechanism that facilitates release of the protein component upon phospholipase cleavage (Low and Saltiel, 1988). For the trypanosome VSG, phospholipase cleavage may permit specific protein release from the cell surface during the parasitic life cycle (Ferguson *et al.*, 1985; Low and Kincade, 1985). The release of the protein component of PI-linked proteins may simultaneously release phosphatidylinositol, diacyglycerol or phosphatidic acid (Low and Kincade, 1985) which concomitantly effect cellular responses, in particular the mobilization of calcium (Berridge and Irvine, 1984) and, hence, the activation of protein kinase C (Nashizuka, 1984). The demonstration that cross-linking Thy-1 with antibodies increases the cytoplasmic free Ca^{2+} concentration (Kroszek *et al.*, 1986) suggests a role for PI-linked proteins in signal transduction. Recently, the major Fc receptor in blood has been shown to be PI-linked (Selvaraj *et al.*, 1988; Simmons and Seed, 1988). This receptor is capable of signal transduction, resulting in the triggering of cell-mediated killing, thus demonstrating that PI-linked proteins can act as true signal transducing receptors and not just binding proteins. By analogy, we suggest that cell contact or ligand binding of some PI-linked molecules, such as the protein characterized in this study, may activate membrane-bound phospholipases which could cleave the PI-linkage molecules and initiate the PI-mediated release of free cellular calcium and activation of protein kinase C.

The homology of the protein with the C2 class of the Ig superfamily suggests two possible functions. One is an involvement in cell recognition, contact or adhesion, demonstrated by the homology to NCAM, MAG and related molecules. The presence of a putative PI-linkage site for membrane attachment is consistent with this notion. The other function is as a peptidergic binding protein or receptor, suggested by the homology to the type III tyrosine kinase growth factor receptors. Since the primary sequence of the protein shows homologies with two functionally different classes of immunoglobulin-related proteins, we have termed the protein OBCAM (opioid-binding protein-cell adhesion molecule). Expression of the cDNA will help in the elucidation of its role in binding opioids and in cellular recognition and adhesion.

Materials and methods

Purification and peptide sequencing

Bovine brain membranes were sonicated and solubilized in Triton X-100. Opioid-binding protein was purified by 6-succinyl morphine affinity chromatography, ultrogel filtration, wheat germ affinity chromatography and preparative isoelectric focussing as previously described (Cho *et al.*, 1986). A single 58-kd band was observed which was electroeluted and digested with cyanogen bromide overnight in 300 μl of 60 mg/ml CNBr in 70% formic acid. Individual peptides were resolved by separation on a Synchron C-4 reverse phase HPLC column, eluted with a *n*-propanol/

trifluoroacetic acid gradient and subjected to gas-phase microsequence analysis (Rodriguez, 1985). Peptides 2 and 3 were resolved by additional chromatography using a propanol/heptafluorobutyric acid gradient. A portion of sequence 2 was found in the mixture, indicated by parentheses, but was not identified as the extension of peptide 2 until confirmed by the DNA sequence. Unidentified residues are indicated by X and residues >30% below the expected yield are italicized. The proximal methionine residue is assumed.

cDNA cloning and DNA sequencing

Synthetic oligonucleotide probes based on codon usage (Lathe, 1985) were constructed. Three oligonucleotides, 1 (a 42-mer against peptide 1), 2 (two 45-mers against peptide 2) and 3 (two 45-mers against peptide 3) were used, their sequences being:

1:
5′ ATGATCCAGAACGTGGACGTGTACGACGAGGGCCCTTACC 3′

2:
5′ ATGGCCATCGAGAACAAGGGCCACATC$^{AGC}_{TCC}$AACCTGACCTTCTTC 3′

3:
5′ ATGGCCGAGTTCCAGTGGTTCAAGGAGACACC$^{AGA}_{CGC}$CTGGCCACC 3′

Total bovine brain RNA was prepared using guanidinium thiocyanate (Chirgwin *et al.*, 1979) followed by ultracentrifugation over a 5.7 M CsCl cushion. Poly $(A)^+$ mRNA was isolated by oligo d(T)-cellulose chromatography (Aviv and Leder, 1972) and 5 μg was used for library construction. Oligo d(T)-primed cDNA was prepared as previously described (Wood *et al.*, 1984). After *Eco*RI–*Xho*I adapters were ligated, cDNA >1500 bp in size was selected by elution from a 6% polyacrylamide gel. The cDNA (20 ng) was ligated to λgt10 (1 μg) and packaged in Gigapack (Stratagene) packaging extracts giving a library of 1.4×10^6 independent clones. To obtain full length cDNAs, a specifically-primed library was constructed using the same bovine brain poly$(A)^+$ mRNA by priming with two synthetic anti-sense oligonucleotides:

X1 5′ ACGGGAGGTCTTGGGGTGGTTGTCCGTC 3′
and
X2 5′ GGATGGTGCTGCGGTTCAGC 3′

cDNA >200 bp was size-selected by elution from a polyacrylamide gel and 20 ng was ligated to λgt10 (1 μg) and packaged, giving a library of 0.2×10^6 independent clones. cDNA inserts were subcloned into appropriate M13 derivatives and sequenced by the chain termination method (Sanger *et al.*, 1977; Messing *et al.*, 1981) using specific oligonucleotide sequencing primers.

Northern blot analysis

Bovine poly$(A)^+$ mRNA was isolated from whole brain, hypothalamus and liver as described above. RNA (5 μg) was electrophoresed in a formaldehyde–1.2% agarose gel and transferred to nitrocellulose prior to hybridization in 50% formamide at 42°C with the ^{32}P-labelled insert of λBOM106. The blot was washed at 65°C in 0.1 × SSC prior to exposure to X-ray film for 2 weeks.

Immunological analysis

A synthetic peptide corresponding to amino acids 188–202 (EISDIKRDQSGEYEC) was synthesized and used to raise rabbit polyclonal antibodies. An immunoaffinity antibody column was constructed using the IgG fraction of this antiserum (obtained by Protein-A binding). Rat P_2 membranes were solubilized with 0.5% *N*-octyl β-D-glucopyranoside, 1 mM dithiothreitol, 0.05M Tris, pH 7.4 for 1 h at 4°C and the supernatant passed through the immunoaffinity column and bound proteins eluted with a gradient of 0.1 M glycine HCl, pH 2.0. The eluate was immediately neutralized with Tris base (0.1 M) and dialysed against 0.05 M Tris, pH 7.4. Determination of the [^{3}H]diprenorphine binding was carried out as described (Cho *et al.*, 1986). The monoclonal antibody 3B4F11 (Roy *et al.*, 1988) raised against purified opioid-binding protein was used to study the inhibition of ligand binding to the immunoaffinity purified protein.

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