Photoaffinity labeling of opioid-binding cell-adhesion molecule (OBCAM) by phenyldiazirine-integrated ligands.

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Abstract

An opioid-binding cell-adhesion molecule (OBCAM) protein, which is an immunoglobulin superfamily protein, recognizes various opioid ligands including enkephalin and β-endorphin. The molecular mechanisms of ligand-binding interface of the proteins are not yet clear. We prepared a bacterial expressed OBCAM protein using pGEX4T expression system, and photoaffinity ligand bearing diazirine and biotin, which was constructed using the partial sequence of β-endorphin, Tyr-Gly-Gly-Phe-Leu-Thr-Ser-Glu-Lys-Ser. Crosslinking formation between OBCAM protein and photoaffinity ligand was confirmed by SDS-PAGE analysis. Neither excess amount of [Leu]-enkephalin, [D-Ala^2, D-Leu^5]-enkephalin, nor β-endorphin inhibited the crosslinking formation. The diazirine-bearing peptide that had an identical amino acid sequence to that of the photoaffinity ligand only inhibited the crosslinking formation, and the removal of diazirine on the peptide by photolysis resulted in loss of the inhibitory activity. These results show that diazirine group on the opioid ligand enhanced its binding affinity to the OBCAM protein.

Key words : photoreactive group, β-endorphin, ligand-receptor interface

Introduction

The opioids are a highly diverse group of drugs, which are responsible for various actions including analgesic activities, autonomic and immune functions[1-3]. Opioid receptors are cell surface glycoproteins that constitute specific binding sites for variety opioid ligands[4,5]. Opioid-binding cell-adhesion molecule (OBCAM) was originally purified from rat brain based upon its ability to bind β-endorphin, one of the opioid ligand[6]. The cDNA encoding this protein was isolated from a bovine brain cDNA library, which encoded a 345 amino acid protein[7]. The OBCAM protein is an immunoglobulin superfamily protein, which contains three repeated Ig domains, and has a homology to adhesion molecules such as neural cell adhesion molecule and myelin associated glycoprotein[7]. A C-terminal hydrophobic sequence was characterized as transmembrane domain through a phosphatidylinositol linkage[8].

The OBCAM protein recognizes various opioid ligands including [Leu]-enkephalin, [D-Ala^2, D-Leu^5]-enkephalin, and β-endorphin[9]. However, the molecular mechanisms of ligand-binding interface of the protein are not yet clear. A photoaffinity labeling is useful for analyzing specific interactions between bioactive ligands and their receptor proteins[10]. A formation of covalent bond using photoreactive groups allows keeping the complex between the protein and ligand even in denature conditions. This feature facilitates the determination of

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peptides forming the ligand-binding domain, and these crosslinking peptides are analyzed by peptide sequencer or mass spectrometry because a tag is leaved on the receptor protein by photochemically crosslinking.

In this study, a bacterial expressed OBCAM protein was photoaffinity-labeled with the opioid ligand bearing photoreactive moiety, diazirine and biotin. We found that diazirine group on the opioid ligand enhanced its binding affinity to the OBCAM protein.

Materials and Methods

Materials

[Leu]-enkephalin (Tyr-Gly-Gly-Phe-Leu), [D-Ala^2, D-Leu^5]-enkephalin (Tyr-D-Ala-Gly-Phe-D-Leu), and β-endorphin (Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu) were obtained from Sigma Aldrich (Japan). The expriments using recombinant DNA techniques were performed under the permission of Recambinant DNA Adversary Committees in Toyama Mediacal and Pharmaceutical University.

Protein expression and purification

A DNA fragment containing partial human OBCAM gene (Acc. No. NM_002545) was obtained by PCR amplification from first-stranded cDNAs of human fetal brain (CLONTECH, USA) with a sense primer of 5'-GGATCCCCCGTGCGCAGCGG-3' and an antisense primer of 5'CTCGAGTCAAAACTTGATGAAGAAGTGGGC-3'. The each primer was designed to include the *Bam* HI or *Xho* I site, which was indicated by the underlining in the nucleotide sequence. The amplified DNA was ligated into *Bam* HI - *Xho* I site within pGEX 4T-3, and transformed into the *E. coli* strain DH5α. Isopropyl thio-β-D-galactoside (IPTG) was added to the *E. coli* culture at a final concentration of 1 mM. The harvested *E. coli* cells were resuspended in 50 mM Tris/HCl (pH 7.8) containing 30 mM NaCl, and then disrupted by sonication. The supernatant of cell suspension was obtained by the centrifugation at 20000 g for 10 min.

A recombinant protein was absorbed with glutathione column (10 mm i.d. x 20 mm, GE Healthcare, USA) equilibrated with 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, and washed with the same solution. The resultant OBCAM-GST protein was eluted by an addition of 10 mM reduced glutathione in 50 mM Tris/HCl (pH 9.6), and dialyzed against 50 mM Tris/HCl (pH 7.8) containing 30 mM NaCl. The purified protein was digested by specific protease, thrombin, which removed GST protein from OBCAM-GST fusion protein. The GST protein was absorbed with glutathione column, and the pass-through fraction was used as sample of OBCAM protein.

Preparation of photoaffinity ligand and competitors

N-(9-Fluorenylmethoxycarbonyl)-3-(4-[trifluoromethyl]-3*H*-diazirin-3-yl)-L-phenylalanine (Fmoc-tmd(Phe)) was synthesized according previous report[11]. The physical data was $[\alpha]_D$ + 22.4 ° (c = 0.52, EtOH), + 24.84 ° (c = 0.338, EtOH). The optical purity was determined by HPLC using a chiral support (Sumichiral OA-3300 column (4.6 x 250 mm), 0.01 M ammonium acetate in MeOH, flow rate 1 mL/min, λ = 360 nm). The D- and L- isomers were eluted at 11.7 and 12.4 min, respectively. The enantiomeric excess was calculated as 97% from area ratio of the signals.

The peptides were synthesized in a 10 mmol scale on Wang-PEG resin by a standard methodology using a Shimadzu PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan) with Fmoc amino acid derivatives. A biotinylated peptide was synthesized using the resin prepared as follows. Fmoc-Ser(Bu^t)-resin was treated by 30 % piperidine in dimethylformaide (DMF), and was connected with Fmoc-Lys(Mtt)-OH (Watanabe Chem, Japan). The Fmoc-Lys(Mtt)-Ser(Bu^t)-resin was treated by 1 % trifluoroacetic acid (TFA) and 5 % triisopropylsilane in dichloromethane for 30 min at room temperature to remove Mtt group on side chain of Lys. After washing, 3-time excess amount of biotin Osu (Bachem, Germany) was reacted in DMF for 12 h at room temperature. The resultant biotinylated resin was used as starting resin for peptide synthesis. The synthetic peptide was cleaved from the resin by treatment with 5 % ethanedithiol and 5 % thioanisole in TFA for 2 h. The product were purified by reverse-phase HPLC, and confirmed by the amino acid sequence and by mass spectrometry.

Photoaffinity labeling

The purified OBCAM protein (6 μg) were incubated with 1 μM photoaffinity ligand (biotinylated-Tyr-Gly-Gly-tmd(Phe)-Leu-Thr-Ser-Glu-Lys-Ser) in phosphate buffer saline (PBS, pH 7.4) at 37 ° C for 10 min in the dark. In the case of competitor experiments, the competitors were pre-incubated with the solution of OBCAM protein for 15 min at 37 ° C before the addition of photoaffinity ligand. Specimens were then irradiated at 0 ° C for 2 min with a 30-watt long-wavelength UV lamp (Funakoshi) to crosslink each other. The irradiated samples were subjected to SDS-PAGE (10 or 13% polyacrylamide gel) and transferred to a PVDF membrane, and then biotinylated ligands on the membrane were visualized by chemiluminescent detection with avidin-horseradish peroxidase (HRP) conjugate.

RESULTS & DISCUSSION

Expression of OBCAM-GST fusion protein in *E. coli*

Since the OBCAM protein has the signal sequence at N-terminus, we expressed OBCAM$^{27\text{-}345}$ protein with pGEX 4T vector, which generated fusion protein at C-terminal end of glutathione-*S*-transferase protein. The transfected *E. coli* after the induction of IPTG was disrupted and the supernatant and pellet of the cell were analyzed by SDS-PAGE (Fig. 1). The OBCAM-GST fusion protein, which was estimated at 65 kDa, was obtained in both fractions (Fig. 1 lanes 1 and 2). The affinity purification by glutathione column succeeded in preparing OBCAM-GST fusion protein (Fig. 1 lane 3). The coexisted protein around 30 kDa has the same mobility of GST protein alone (compare lanes 3 and 4). To separate the OBCAM protein from GST, the fusion protein was treated by thrombin protease for 2 or 16 h at room temperature (Fig. 1 lanes 7 and 8). The protein whose molecular weight was about 35 kDa appeared by protease digestion, and the longer treatment of thrombin caused decrease in 35 kDa protein by non-specific digestion (compare Fig. 1 lanes 7 and 8). The molecular weight of the protein was almost identical to the calculated weight of OBCAM protein (37 kDa).

Photoaffinity labeling of OBCAM protein

A photoaffinity ligand and competitors were summarized in Fig. 2. We used the partial sequence of β-endorphin, Tyr-Gly-Gly-Phe-Leu-Thr-Ser-Glu-Lys-Ser, as a binding peptide of OBCAM protein. The Phe was replaced by photoaffinity amino acid, tmd(Phe), which

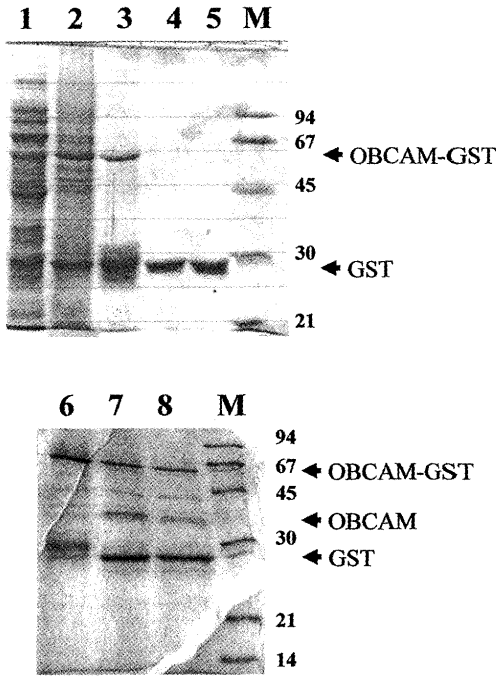
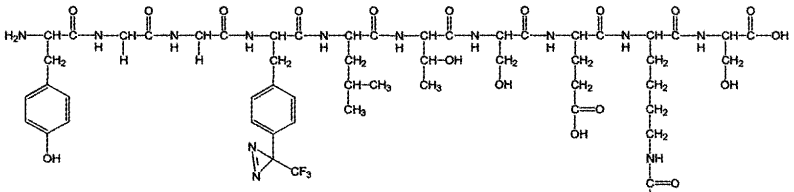


Figure 1 SDS-PAGE analysis of recombinant OBCAM protein in *E. coli*. The recombinant OBCAM protein was obtained by *E. coli* pGEX4T expression system, which generated a fusion OBCAM-GST protein. Lane 1, supernatant of the expressed cell lysate; 2, pellet of the lysate; 3, purified fraction by affinity column; 4 and 5, GST protein; 6, purified fraction by affinity column; 7, the fraction treated with thrombin for 2 h; 8, the fraction treated with thrombin for 16 h; M, molecular weight markers. SDS-PAGEs were performed with 10 % and 13 % polyacrylamide gel in lanes 1-5, and 6-8, respectively.

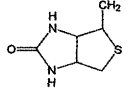
included diazirine as photoreactive moiety. We integrated biotin into the photoaffinity ligand for detection tag at the side chain of Lys because N-terminal modification of enkephalin had been reported to decrease in physiological activity[12]. The photoaffinity labeling experiments were performed with thrombin-digested

Photoaffinity ligand



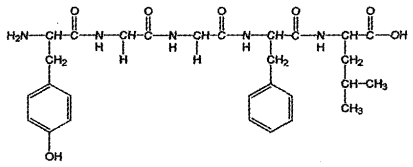
Tyr-Gly-Gly-tmd(Phe)-Leu-Thr-Ser-Glu-Lys-Ser

Biotin



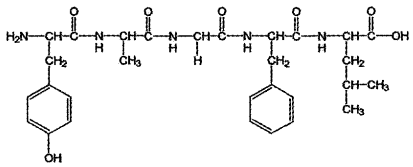
Competitors

L-ENK



Tyr-Gly-Gly-Phe-Leu

D-ENK



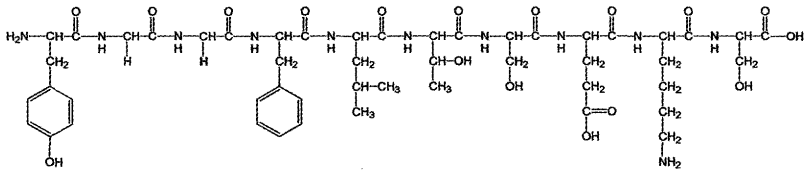
Tyr-D-Ala-Gly-Phe-D-Leu

β-END

Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-

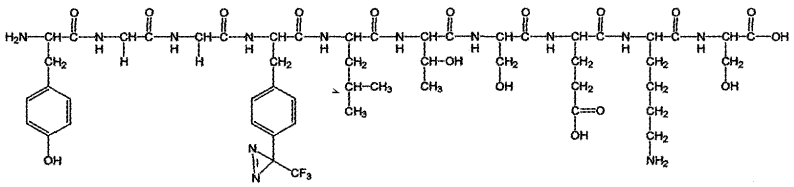
Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu

ID-COMP



Tyr-Gly-Gly-Phe-Leu-Thr-Ser-Glu-Lys-Ser

ID-COMP-TMD



Tyr-Gly-Gly-tmd(Phe)-Leu-Thr-Ser-Glu-Lys-Ser

Figure 2 A photoaffinity ligand and competitors used in this study.

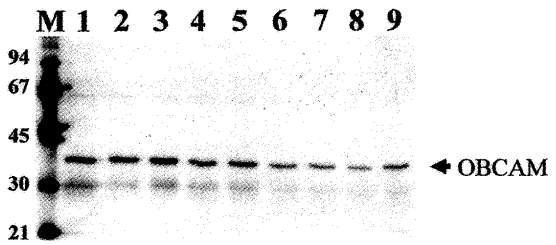


Figure 3 Photoaffinity labeling of OBCAM protein with photoaffinity ligand. The crosslinking complexes were visualized by chemiluminescent detection system. Lane 1, without competitor; lanes 2-4, 10-, 30-, and 100-time excess amount of D-ENK competitor, respectively; lanes 5-7, 10-, 30-, and 100-time excess amount of β-endorphin competitor, respectively; lanes 8 and 9, 10- and 100-time excess amount of L-ENK competitor, respectively.

OBCAM protein. The complexes of the ligand and protein generated by light irradiation were separated by SDS-PAGE, and visualized by chemiluminescence using avidin-HRP conjugate (Fig. 3). The biotin tag was mainly detected at about 37 kDa (Fig. 3 lane 1), which is corresponded to the sum of the molecular weights of the OBCAM protein (35 kDa, apparent) and the photoaffinity ligand (1.5 kDa, calculated). The minor band also appeared at 30 kDa, which was corresponded to the molecular weight of GST-ligand complex. Since [Leu]-enkephalin (L-ENK), [D-Ala^2, D-Leu^5]-enkephalin (D-ENK), and β-endorphin were reported to be bound to OBCAM protein[9], competition experiments were performed with these peptides (Fig. 3 lanes 2-9). Excess amount (10 to 100 times comparing to that of the ligand) of the competitors did not inhibit the crosslinking formation between the OBCAM protein and the ligand.

We performed further the competition experiments with the ID-COMP and ID-COMP-TMD peptides (Fig. 4A). These peptides had the identical amino acid sequence to that of the photoaffinity ligand, and the introduction of biotin is just difference between ID-COMP-TMD and photoaffinity ligand. The crosslinking formation between OBCAM protein and photoaffinity ligand was not inhibited by the addition of excess amount of ID-COMP competitor at even 100 or 500 times (Fig. 4A lanes 2 and 3), but was done by the addition of 100-time excess amount of ID-COMP-TMD. These results suggest that the trifluoromethyl diazirine group in the photoaffinity ligand is important to binding to the OBCAM protein. To determine whether diazirine group was necessary for inhibition of the crosslinking formation, we used the photo-decomposed ID-COMP-TMD as a competitor, which was prepared by light irradiation in H_2O. The diazirine group in the photo-

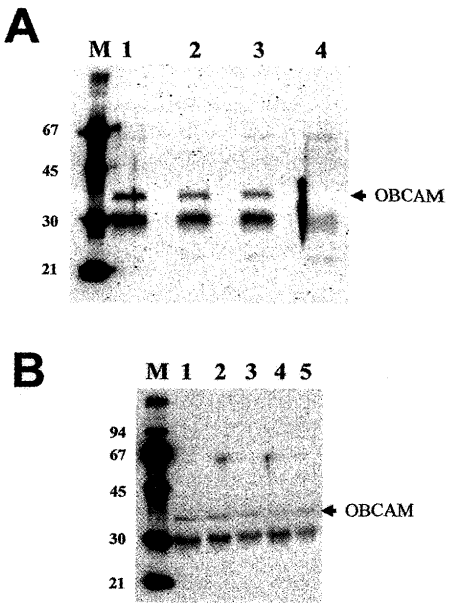


Figure 4 Competition experiments with specific competitors in photoaffinity labeling of OBCAM protein. The crosslinking complexes were visualized by chemiluminescent detection system. A) Lane 1, without competitor; lanes 2 and 3, 100- and 500-time excess amount of specific competitor, ID-COMP, respectively; lane 4, 100-time excess amount of specific competitor, ID-COMP-TMD. B) Lane 1, without competitor; lanes 2 and 3, 100- and 500-time excess amount of specific competitor, ID-COMP, respectively; lanes 4 and 5, 100- and 500-time excess amount of photo-decomposed ID-COMP-TMD competitor. The structural details of the competitors are described in Results and Discussion.

decomposed ID-COMP-TMD was almost broken down because the absorption at 360 nm, which is corresponded to the n to π^* absorption of diazirine, was disappeared (data not shown). The crosslinking formation between OBCAM protein and photoaffinity ligand was not inhibited by the addition of 100- and 500-time excess amount of the photo-decomposed ID-COMP-TMD (Fig. 4B). Since the photo-decomposed competitor

possesses the trifluoromethyl group even after the photolysis, this result strongly suggests that diazirine group is a key element for binding to the OBCAM protein.

The OBCAM protein have been reported to be specifically bound to various opioid ligands such as β-endorphin and [D-Ala^2, D-Leu^5]-enkephalin[9]. In this study, we show that diazirine group on the opioid ligand enhance its binding affinity to bacterial expressed OBCAM protein. Though the addition of diazirine group is an artificial modification for analyzing the ligand-binding site of OBCAM protein, the findings in this study may give new viewpoints for understanding the ligand-binding interface of OBCAM protein at molecular level. Further experiments are needed for the interpretation of these findings.

REFERENCES

1. Pasternak, G.W.: The Opiate Receptors, Humana Press, Clifton, NJ:pp499,1988.
2. Rouveix, B.: Opiates and immune function. Therapie, 47:503-512,1992.
3. Sabinga, N.E.S and Goldstein, A.: Opioid peptides and opioid receptors in cells of the immune system. Annu. Rev. Immunol. 6:219-249,1998.
4. Loh, H.H. and Smith, A.P.: Molecular characterization of opioid receptors. Annu. Rev. Pharmacol. Toxicol. 30:123-147,1990
5. Knapp, R.J., Malatynska, E., Collins, N., et al.: Molecular biology and pharmacology of cloned opioid receptors. FASEB J, 9:516-525,1995.
6. Cho, T.M, Hasegawa, J., Ge, B-L., et al.: Purification to apparent homogeneity of a μ - type opioid receptor from rat brain. Proc. Natl. Acd. Sci. USA 83:4138-4142,1986.
7. Schofield, P.R., McFarland, K.C., Hayflick, J.S., et al.: Molecular characterization of a new immunoglobulin superfamily protein with potential roles in opioid binding and cell contact. EMBO J. 8:489-495,1989.
8. Wick, M.J., Fan, G., and Loh, H.H.: Expression of OBCAM-related cDNA clones in Cos 1 cells: evidence for a phosphatidylinositol linkage to the cell membrane. Mol. Brain Res. 36:322-328,1996.
9. Lane, C.M., Elde, R., Loh, H.H. et al.: Regulation of an opioid-binding protein in NG108-15 cells parallels regulation of δ-opioid receptors. Proc. Natl. Acd. Sci. USA 89:11234-11238,1992.
10. Sadakane, Y., and Hatanaka, Y.: Photochemical fishing approaches for identifying target proteins and elucidating the structure of a ligand-binding region using carbene-generating photoreactive probes. Anal. Sci. 22: 209-218,2006.
11. Nakashima, H., Hashimoto, M., Sadakane, Y., et al. Y.: Simple and versatile method for tagging phenyldiazirine photophores. J. Am. Chem. Soc. in press, 2006.
12. Taylor, J.W. and Kaiser, E.T.: The structural characterization of β-endorphin and related peptide hormones and neurotransmitters. Pharmacol. Rev., 38:291,1986

ジアジリン型光反応性リガンドによるオピオイド結合細胞接着分子（OBCAM）の光アフィニティーラベリング

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要旨

オピオイド結合細胞接着分子（OBCAM）タンパク質は、イムノグロブリンのスーパーファミリーに属し、エンケファリンやβ-エンドルフィンなどのオピオイドリガンドを認識する。我々は、大腸菌で組換えOBCAMタンパク質と、β-エンドルフィンの部分配列をもとにジアジリンとビオチンをもつ光アフィニティーリガンドとを製作し、光アフィニティーラベル実験を行った。光照射により両者が共有結合で結ばれた複合体を形成した。しかしながら、[Leu]-エンケファリン, [D-Ala^2, D-Leu^5]-エンケファリンおよびβ-エンドルフィンを過剰に加えても、この複合体形成を阻害できなかった。光アフィニティーリガンドと同一の構造をもつ（ジアジリンをもつ）ペプチドアナログのみが複合体形成を阻害した。このペプチドアナログ中のジアジリンを光分解したものは阻害活性を失った。これらの結果は、オピオイドペプチド中のジアジリン構造がOBCAMタンパク質との親和性を増強させていることを示している。

キーワード：光反応基、β-エンドルフィン、リガンド－受容体インターフェイス