DEPARTMENT OF THE ARMY
U.S. ARMY CHEMICAL AND BIOLOGICAL DEFENSE COMMAND
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REPLY TO
ATTENTION OF

July 13, 1998

Freedom of Information and Privacy Act Office

Mr. John Greenewald, Jr.
[redacted]

Dear Mr. Greenewald:

In response to your June 23, 1998, Freedom of Information Act (FOIA) request, I have enclosed a copy of the following documents:

- "Progress In Cloning Of Enzymes For Detoxification of Organophosphorus Compounds"

- "Progress In Cloning Genes For Opiate Receptors"

- "Cloning, Sequencing And Characterization Of OPD Genes And Their Broad-Spectrum Organophosphate Hydrolases From Soil Bacteria"

- "Purification Of Opiate Receptors And Cloning Their Respective Genes"

Fees incurred while processing this request have been waived.

Sincerely,

Cheryl S. Fields
Freedom of Information and
Privacy Act Officer

Enclosures

PROGRESS IN CLONING OF ENZYMES FOR DETOXIFICATION OF ORGANOPHOSPHORUS COMPOUNDS

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ABSTRACT

The halophilic isolate, *Alteromonas sp.* JD6.5. possesses at least two DFP-hydrolyzing Organophosphorus Acid Anhydrases (OPAA). The predominant one, OPAA-2, was purified to homogeneity with a molecular weight of 60,000. In order to clone the genes that coded for OPAA in JD6.5, the 4-8 kb EcoRI partially digested DNA fragments and a λZAP expression phage were used to prepare a genomic library. Of $4x10^5$ plaques screened, 70 positive recombinants were detected by the monoclonal and polyclonal antibodies, and a non-radioactive biotinylated 20-mer oligodeoxynucleotide probe derived from the partial N-terminal sequence of OPAA-2. The pBluescript plamids that harbored OPAA inserts were excised from the recombinant phages and all were found to contain the same 6.7 kb DNA fragment. Various restriction endonucleases were used to generate the partial map of recombinant plasmids (pTC+ and pTC-) which have DNA inserted in the opposite orientation at the EcoRI site. The cloned gene of recombinant plasmids was expressed in *E. coli* and the expressed products had enzyme activity. Subcloning and Western blot analysis of the expressed products demonstrated that the cloned gene was located in a 4 kb KpnI fragment within the insert.

INTRODUCTION

Organophosphorus acid anhydrases (OPA anhydrases) are enzymes that are capable of degrading a variety of organophosphates including acetylcholinesterase inhibitors, pesticides and fluorine-containing chemical agents. These enzymes have been found in a number of organisms, both prokaryotic and eukaryotic (1-9). Studies of OPA anhydrases from these sources demonstrated that these enzymes were different in terms of substrate specificity, sensitivity to metal ions or inhibitors, molecular weight and structural properties. The natural substrates and functions of these enzymes have not yet been identified. Diisopropyl fluorophosphate (DFP) is a common substrate used to screen for OPA anhydrase activity.

Of all the organisms that have been under investigation, several halophilic bacterial strains isolated by Dr. DeFrank and a number of *Alteromonas* species obtained from the American Type Culture Collection (ATCC, Rockville, MD) have been found to have very high OPA anhydrase activity. Most of these bacteria possess at least two to several different enzyme activities against DFP. One of the halophilic bacteria, *Alteromonas* JD6.5, possesses at least two different OPA anhydrases that have activity against DFP. The predominant enzyme, OPA Anhydrase-2 (OPAA-2), can be separated from OPA Anhydrase-1 (OPAA-1) by DEAE-Sephacel chromatography following initial ammonium sulfate fractionation (10). The OPAA-2 which accounts for nearly 90% of total activity has been purified to homogeneity in an overall purification of over 1000 fold. This enzyme is composed of a single polypeptide with a molecular weight of 60,000. The polyclonal anti-serum and monoclonal antibody against OPAA-2 have been prepared and were shown to react specifically with both OPAA-2 and OPAA-1, suggesting that they may share a common immunological determinant. Only the first 10 out of 19 N-terminal amino acids of OPAA-2 have been positively determined. From the 5'-terminal, the amino acid sequence is as follows:

TrpProLeuIleAlaPheGlyGlnTyrLeuGln(or Trp)Gln(or Trp)Cys(?)ValPheSer(?)GluX(?)Met

In this paper, we described the cloning of the OPAA genes of JD6.5 by using an antibody probe. A lambda expression vector, λZAP II which can be expressed from the *lac Z* promoter as a fused protein, has been used to produce a recombinant DNA library. The cloned OPAA genes were identified by detecting lysate proteins from clones containing OPAA genomic DNA using rat anti-OPAA-2 monoclonal and polyclonal antibodies, and an oligodeoxynucleotide probe derived from the partial N-terminal sequence of OPAA-2.

EXPERIMENTAL PROCEDURES

Organism and Cultivation

JD6.5 was isolated from Grantsville Warm Springs, which is approximately 30 miles west of Salt Lake City, Utah. Cultures were grown on salt medium consisting of 50 g NaCl, 10 g $MgSO_4$, 10 g proteose peptone, 6 g yeast extract, 5 g casamino acids, 2.5 g HEPES per liter, pH 6.5. Inoculated flasks (4 or 6 liter) containing 1-1.5 liters of medium were incubated at 37 ° C, on a rotary shaker at 240 rpm, for 18-24 hours. Cells were then harvested by centrifugation (7,500x*g*) for 10 minutes and stored -20 ° C.

Enzyme Assay

OPA Anhydrase activity was routinely assayed by monitoring fluoride release by an ion-specific electrode (7). The reaction mixture contained, 500 mM NaCl, 50 mM Bis-Tris Propane, pH 7.2, 3 mM DFP (in isopropanol), 1 mM $MnCl_2$ and 5-50 μl of enzyme solution in a total volume of 2.5 ml. Assays were routinely run at 25 ° C in a thermostatically controlled vessel with stirring. The enzyme sample was preincubated in the reaction mixture for 1 minute before reaction was initiated by the addition of the DFP. The reaction was monitored for 4 minutes and the rate of fluoride release corrected for spontaneous DFP hydrolysis. One unit of OPA Anhydrase activity catalyzes the release of 1.0 μmole of F^- per minute at 25 ° C. Specificity activity is expressed as units per milligram of protein.

Enzyme Preparation and Protein Concentration

OPA Anhydrase-2 (OPAA-2) was purified from JD6.5 as previously described (10). The Coomassie Protein Assay Reagent (Pierce) was used for protein determination with bovine serum albumin (BSA) as the standard.

Preparation of Polyclonal and Monoclonal Antibodies

A Spraque-Dawley rat was immunized with HPLC-purified OPAA-2 (10). The rat was first given a footpad injection of 100 μg enzyme in complete adjuvant (Bacto). Three weeks later it received a subcutaneous booster of 20 μg enzyme in incomplete adjuvant (Bacto). One week later, a pre-fusion dose of 5 μg in sterile saline was administered intravenously. The withdraw of polyclonal serum and fusion procedure was started three days after the final injection.

The immunized rat spleen cells ($3.6x10^7$) were fused with an equal number of Sp2/O-Ag14 mouse myeloma cells (ATCC; #CRL1581) following the the procedure described by Kennet, McKearn and Bechtol (11). The fused cells were suspended in Dulbecco's minimum essential medium (DMEM) containing $1x10^{-4}$ M hypoxanthine, $8x10^{-7}$ M aminopterin, $1.6x10^{-5}$ M thymidine, and 10% iron-supplemented bovine calf serum (HAT medium) and distributed in 100 μl portions into 96-well tissue culture plates at a density of approximately $1.4x10^5$ cells/well.

A Rat ExTrAvidin Staining Kit (Sigma) was used to screen culture medium from wells containing macroscopic hybridoma colonies to determine the presence of antibodies specific for OPA Anhydrase. Populations of cells from positive supernatants were transferred to 24-well culture plates, expanded and subsequently cryopreserved for culture use.

Construction of a Genomic Library Expressing OPAA-2

High molecular weight genomic DNA from JD6.5 strain was prepared as described by Davis et al (12). Lambda ZAP II vector, helper phage R408, Gigapapck II Gold packaging extract, host strains BB4 and XL1-Blue were obtained from Stratagene. Briefly, the genomic DNA was first partially digested with EcoRI and the 4-8 Kb DNA fragments were size fractionated by preparative agarose gel electrophoresis followed by electroelution from the gel. These DNA fragments were ligated to EcoRI digested, phophatase-treated lambda ZAP arms, packaged *in vitro* and used to infect host strain BB4 to create a genomic library as described by Stratagene's protocol. A genomic library containing $1.5x10^6$ pfu/μg with less than 5% non-recombinant background plaques was obtained following plating with BB4 host cells on NZY plates. To verify that the genomic library contained OPAA-2 genomic DNA from JD6.5, it was screened for the presence of recombinant plaques using the monoclonal antibody (MAb#6). About $4x10^5$ plaques were plated at a density of $1.5x10^4$ plaques per 90 mm plate on BB4 cells (total 30 plates). After 4 hours of growth at 42 °C, the plates were overlaid with nitrocellulose filters saturated with 10 mM IPTG, and incubated for an additional 3.5 hours at 37 °C. The filters were then removed from the plates and blocked with TBS (10 mM Tris, pH 8.0, 150 mM NaCl) containing 2% BSA for 2 hours.

The immunodetection was carried out by first incubating with 1:5 (v/v) MAb#6 or 1:1000 (v/v)

polyclonal antiserum in TBS for 2 hours. The filters were then incubated subsequently for 1 hour with rabbit anti-rat IgG and ExtrAvidin peroxidase conjugate (Sigma's Rat ExtrAvidine Staining Kit) with three washes in TBS-0.05% Tween 20 (TBST) after each incubation. After the final wash with TBS, the bound peroxidase was assayed by incubating with 30 mg 4-chloro-1-naphthol in 50 ml TBS containing 0.03% hydrogen peroxide. When the color had developed to the desired intensity, the reaction was stopped by rinsing the filters in deionized water for several minutes, changing the water at least twice.

Detection of Cloned DNA with Photobiotinylated Deoxyoligonucleotide

A deoxyoligonucleotide of 20 residues TAICGIAAICCIGTIATIGA complementary to the 4-10 N-terminal amino acid sequence of OPAA-2 was synthesized on an Applied Bioxystems 380B synthesizer. This deoxyoligonucleotide probe contains deoxyinosine residues (I) at positions corresponding to ambiguous nucleotides. Following deprotection, the oligomer was desalted by gel filtration chromatography on a PD-10 column (Pharmacia) and subsequently lypholized. The purified deoxyoligonucleotide was converted via a photochemical reaction with a commercially available photobiotin (Sigma) to a biotinylated probe as described by Denman and Miller (13). The colony blot analysis with photobiotinylated deoxyoligonucleotide probe was performed as described (13). The presence of probe sequences in cloned DNA was detected by a simple colorimetric reaction using streptavidin-alkaline phosphatase (SA-AP), nitroblue tetrazolium (NBT) and 5-bromo-4-chloro- 3-indoxyl-phosphate (BCIP) system (13).

Excision of Plasmids from the λZAP Vector and Mapping of the cloned gene

The excision of plasmids from the λZAP clones were carried out as described (14). The plasmid DNA prepared following this protocol can then be used to infect *E. coli* cells (XL1-Blue) for production of large quantities of DNA and protein. The restriction enzyme digestion and subcloning of various inserted DNA fragments in recombinant plasmids were performed in similar procedures as described by Maniatis et al (15).

Western Blot Analysis of OPA Anhydrases

The protein samples were mixed with equal volume of 2x loading buffer (0.125 M Tris, pH 6.8, 4% SDS, 100 mM DTT, 10% glycerol and 0.025% bromophenol blue), incubated for 3 minutes at 100 ° C and layered on a 7.5% SDS-polyacrylamide gel (SDS-PAGE) according to Laemmli (16). The gel was run at 80 mA until the tracking dye was 1-2 cm from bottom of the gel. Prestained marker proteins (Bethesda Research Laboratory) were included on each gel. After electrophoresis, the proteins in the gel were blotted onto a nitrocellulose filter (MicronSep) using the polyblot transfer system (American Bionetics). The nitrocellulose blot was preincubated with TBS containing 2% BSA for 2 hours, then 1:1000 (v/v) polyclonal antiserum or 1:5 (v/v) monoclonal antibody (MAb#6) was added and incubated for additional 2 hours. The blot was then incubated sequentially for 1 hour with rabbit anti-rat IgG and ExtrAvidin peroxidase conjugate with washing in TBS-0.05% Tween-20 (TBST) between each incubation. After the final wash with TBS, the bound peroxidase was assayed by incubating with 30 mg 4-chloro-1-naphthol (dissolved in 10 ml methanol) in 50 ml TBS containing 0.03% hydrogen peroxide. When the color has developed to the desired intensity, the reaction was stopped by rinsing the filter in deionized water for several minutes, changing the water at least twice.

RESULTS

In addition to DFP, the purified OPAA-2 has been shown to possess activity against GD (Soman), GB (Sarin), paraoxon, and two chromogenic analogs of DFP, NPMPP (p-nitrophenyl [methyl]-phenylphosphinate) and NPEPP (p-nitrophenyl [ethyl]-phenylphosphinate) (17). NMR analysis revealed that all isomers of GD and GB were hydrolyzed at equal rates. Of all substrates tested, the highest activity was with GD and DFP (Table I). The enzyme was also shown to be highly specific toward the chromogenic substrate NPEPP. Paraxon was hydrolyzed at about 10% the rate of DFP. Similarly, the partial purified OPAA-1 also was shown to possess high activity against GD. These results clearly indicate that both OPAA-I and OPAA-2 can be powerful tools for use in decontamination of toxic organophosphorus compounds.

TABLE I. Substract Specificity of OPAA-1 and OPAA-2

Enzyme	Specific Activity (μmoles/min/mg)* Soman (GD)** [10mM]	Sarin (GB)** [10mM]	DFP [3mM]	NPEPP*** [0.1mM]
OPAA-2 (purified)	173.20	15.40	72.96	14.14
OPAA-2 (DEAE-Sephacel)	-	-	18.50	-
OPAA-I (DEAE-Sephacel)	10.89	-	4.60	-

*Activity determined in the absence of Mn^{++} at pH 7.2.
**Activity determined by NMR analysis (17).
***NPEPP (p-nitrophenyl [ethyl]-phenylphosphinate)
- (N/D)

The chromosomal DNA of JD6.5 was partially digested with EcoRI and the 4-8 kb DNA fragments were isolated by 0.7% agarose gel electrophoresis. The λZAP expression vector (Strategene) was used to construct a genomic DNA library. The site used for insertion of 4-8 kb DNA fragments on λZAP is the EcoRI site located within *lacZ*, 53 base pairs upstream of the β-galactosidase termination codon (18). After transfection of *E. coli* BB4 with the ligated 4-8 kb DNA fragments, OPAA DNA-containing clones were screened by immunodetection using MAb#6 probe. Of $4x10^5$ plaques examined, 70 positive clones were detected. These recombinant clones were further purified by replating the phages and repeating the screening procedure until all the plaques on the plate showed a positive signal. The intensity of the signals obtained with each of these clones was comparable regardless the presence or absence of inducer, IPTG. All positive clones also reacted with anti-OPAA-2 serum (data not shown).

To determine which clones contained OPAA 5' DNA sequences, a photobiotinylated deoxyoligonucleotide of 20 residues complementary to 4-10 N-terminal amino acid sequences was used as probe in colony hybridization. Following hybridization to the recombinant phage DNA on nitrocellulose membranes, the presence of probe sequences was detected by streptavidin-alkaline phosphatase and a simple colorimetric reaction (NBT and BCIP). All the clones were found to strongly hybridize with the probe. No signal was detected in DNA corresponding to λZAP phage alone. The results further confirm

the presence of OPAA DNA sequences in these clones.

To identify the OPAA polypeptide, the pBluescripts that harbored OPAA inserts were excised from the λZAP vector of each recombinant clone and infected by *E. coli* XL1-Blue. After isolation and analysis of recombinant plasmid DNAs by EcoRI digestion and agarose gel electrophoresis, all were found to harbor the same 6.7 kb DNA fragment at the EcoRI site. Various restriction endonucleases were used to generate a partial map of one of the recombinant plasmid DNAs which was designated pTC+. No restriction sites for BamHI, PstI, SacI or XbaI were found in the cloned gene. Various restriction endonucleases were used to generate a partial map of pTC+ (Fig. 1). Among the recombinant plasmids, one of them with DNA inserted in the opposite orientation was also found (pTC-).

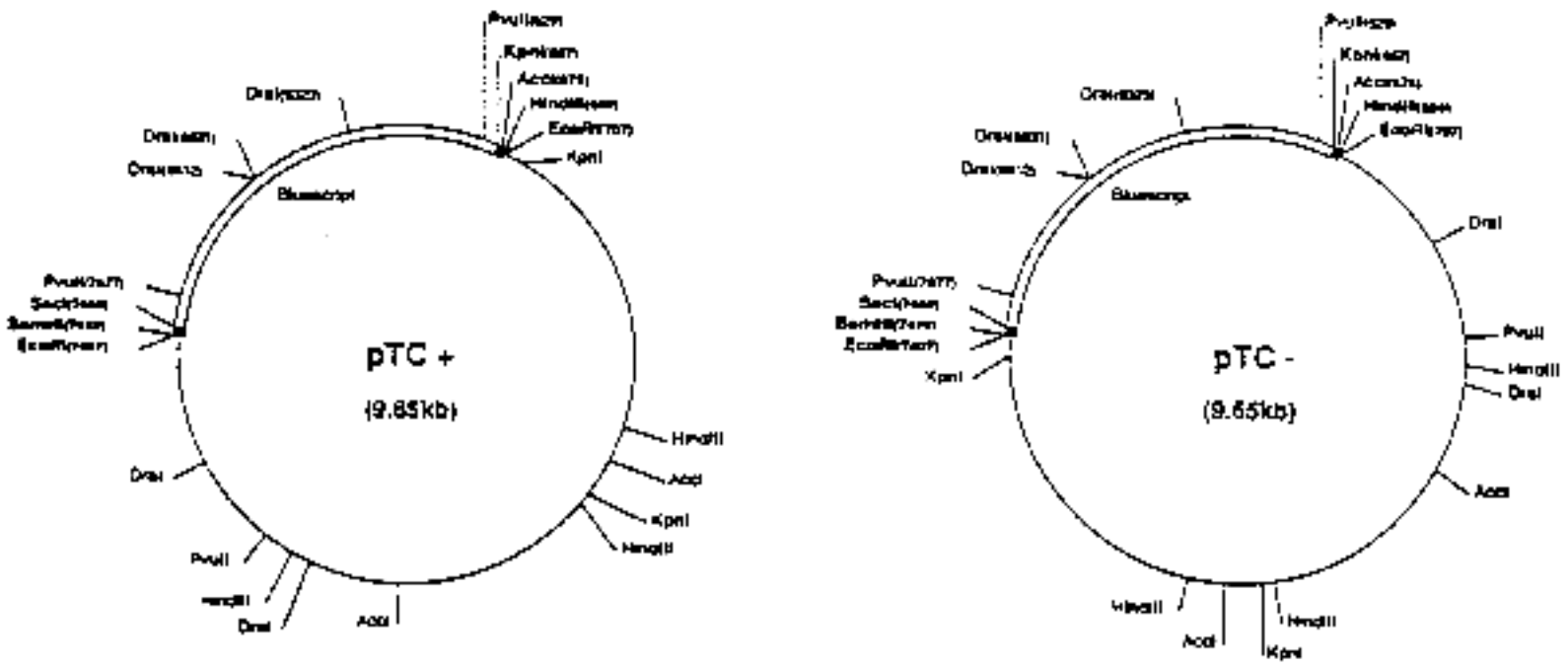

Fig. 1. Partial restriction endonuclease map of pTC+ and pTC-. The light line represents the insert DNA.

Protein lysates of each recombinant clone were subjected to SDS-PAGE and proteins were blotted from the gel onto a nitrocellulose membrane. The OPAA polypeptides were then detected by reaction with MAb#6. As shown in Fig. 2, on one of the blots, two equally intense proteins with MW of 74,000 and 78,000 were clearly observed from all three recombinant lysates tested but were absent for that of lysates transfected with pBluescript. The recombinant clone that carried pTC+ yielded signals comparable with those from pTC-. The molecular weights of these two proteins were in close agreement with that detected from the partially purified OPAA-1 of JD6.5 (10). In a parallel experiment, these lysates were subjected to SDS-PAGE and stained with Fast Stain (Zoion Research, MA). Indeed, two dense bands exhibiting the mobilities predicted for these two bands were observed, suggesting that significant amounts of the novel proteins accumulate (Fig. 3). In the above experiments, the protein lysates were obtained from the recombinant clones growing in the absence of IPTG.

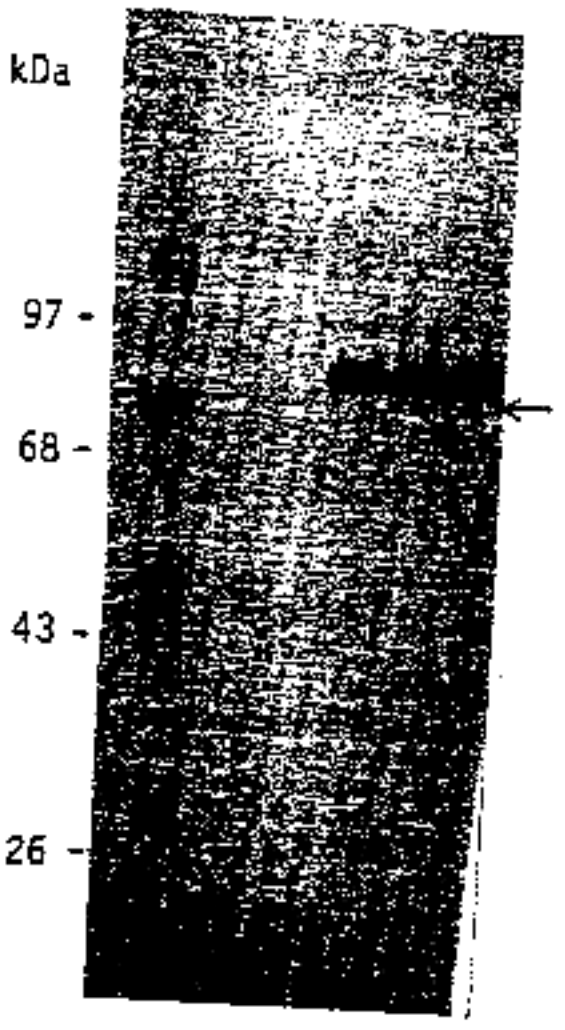

Fig. 2. Western blot analysis of protein lysates from various recombinants. The protein lysates of plasmid-bearing strains were analysed as described in "Experimental Procedures". Lanes are: 1. molecular weight standards; 2 and 3. λZAP alone; 4, pTC22; 5. pTC+; 6. pTC-.

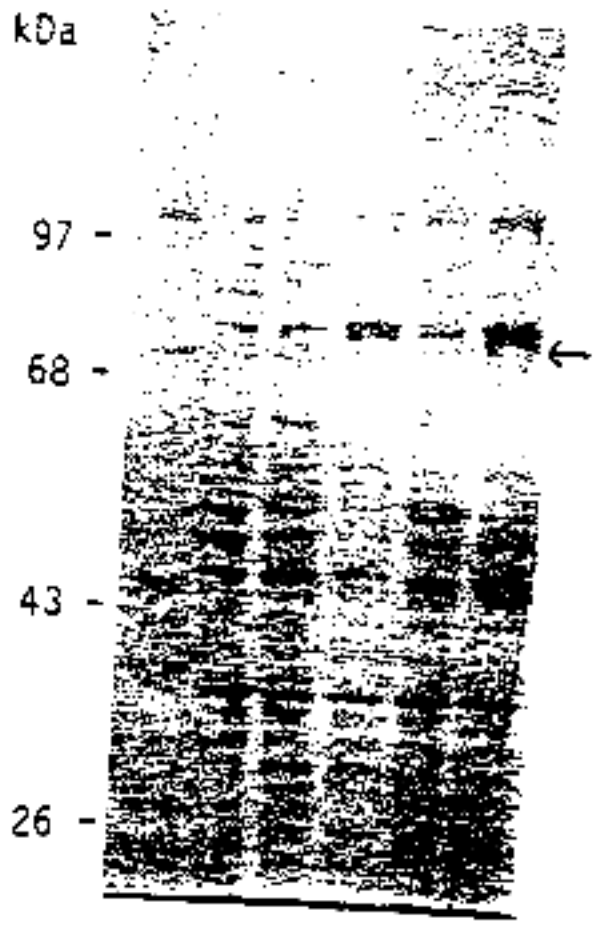

Fig. 3 SDS-polyacrylamide gel analysis of protein accumulation from various recombinants. Same amounts of lysate samples as in Fig. 3 were subjected to SDS-PAGE and stained with Fast Stain. Lanes are: 1. molecular weight standards; 2 and 3. λZAP alone: 4. pTC22; 5. pTC+; 6. pTC-.

The crude extract obtained from the plasmid pTC+ harboring XL1-Blue cells was assayed for DFP hydrolysis activity. As shown in Table 2, the cloned enzyme showed a specific activity corresponding to about 50% of the native enzyme. The result suggests that the OPAA gene of pTC+ is expressed in *E. coli* and that the expressed product is enzymatically active.

TABLE 2. Specific Activity on DFP Hydrolysis from Different Crude Extracts

Crude Extract	Specific Activity (μmoles/min/mg)*
JD8.5	0.150-0.270
XL1-Blue (pBluescript)	0.003-0.004
XL1-Blue (pTC+)	0.090-0.110

*Activity (3-4 preparations) determined in the presence of Mn^{++} at pH 7.2.

In order to localize the OPAA gene within DNA insert from the cloned gene, efforts were made to obtain several subclones with some of the non-essential DNA eliminated by different restriction

endonucleases. One of the recombinant plasmid, pTCk4, was derived from pTC+ after removal of smaller KpnI DNA fragment from the insert (Fig. 4A). Western blot analysis of the expressed products from this clone revealed that the cloned gene was located within the 4 kb DNA fragment of the insert (Fig. 4B).

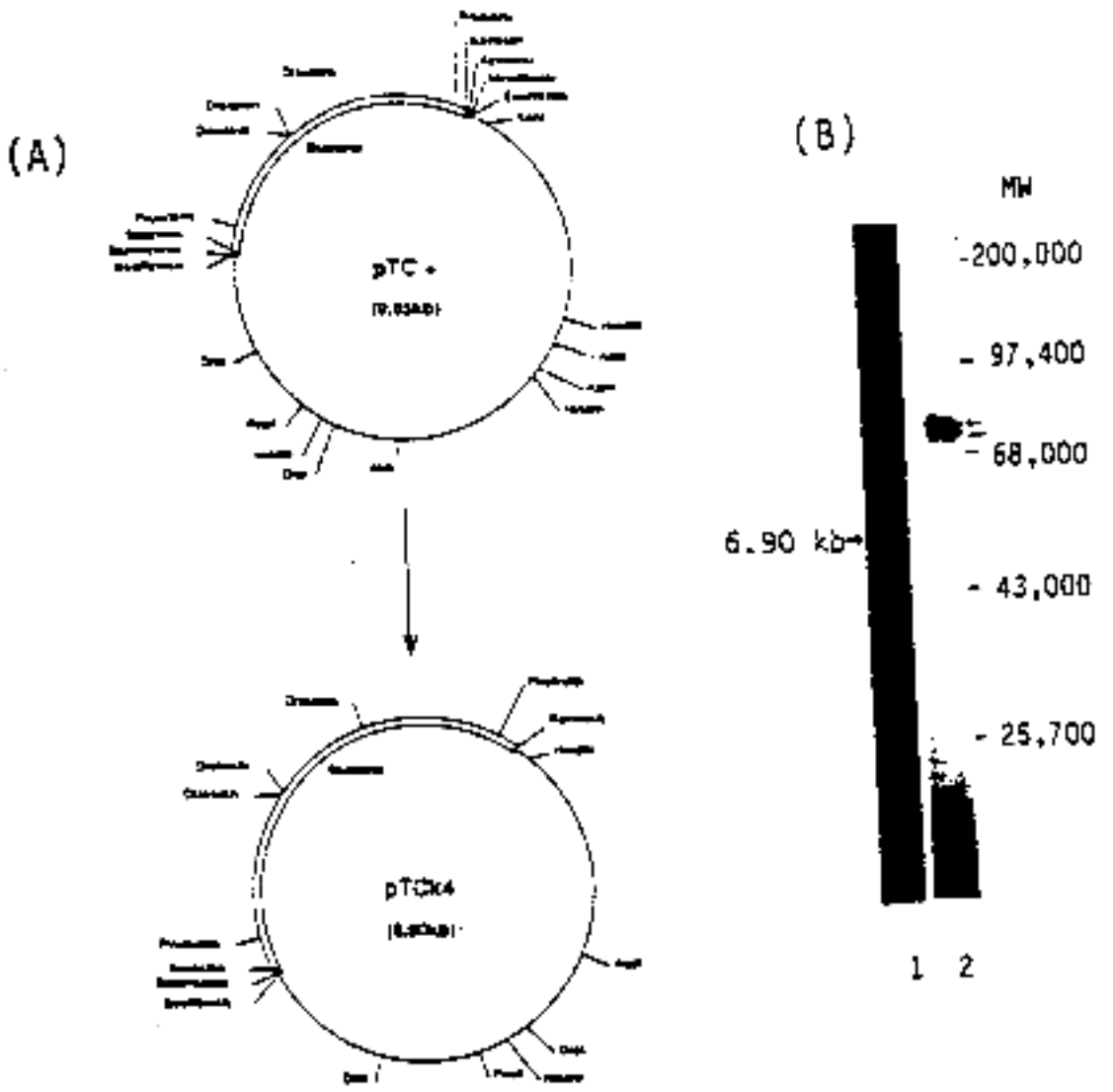

Fig. 4. Subcloning of 4 kb KpnI DNA fragment within the insert of pTC+ and Western blot analysis of its expressed product. (A) Construction of pTCk4. (B) Analysis of insert DNA of pTCk4 after digestion with KpnI and electrophoresis on a 0.8% agarose gel (lane 1) and Western blot analysis of its expressed product (lane 2).

DISCUSSION

JD6.5 contains at least two OPAA enzymatic activities, OPAA-1 and OPAA-2. In spite of the differences in their molecular weight, anti-serum and monoclonal antibody that were raised against OPAA-2 also recognize OPAA-1. Even though OPAA-2 exhibits higher enzymatic activity than OPAA-1 against DFP from the crude extract of JD6.5, the signals generated in the immunoassay suggest that the amount of OPAA-1 is significantly greater than OPAA-2 in the native cells (10). These antibodies were used as a tool to facilitate the identification of the cloned OPAA genes of JD6.5.

The cloned gene of pTC that was positive in the immunoassay contains a 6.7 kb DNA fragment. The evidence which indicating that this insert encoded OPAA-1 were the results of the Western blot analysis and the enzymatic activity against DFP. The cloned gene of pTC produced two protein bands which migrated at the same distance on SDS-PAGE as the native OPAA-1 enzyme fraction. Their molecular

weights, 74,000 and 78,000, correspond to approximately 2.00 and 2.10 kb of DNA coding sequences. These two proteins were expressed in recombinant plasmid transformed XL1-Blue cells at sufficiently high levels for easy detection. The expressed products had enzyme activity against DFP. IPTG was not required for their expression, suggesting that the complete OPAA-1 gene including its original promotor sequences was incorporated into the recombinant clones. It is not clear whether these two proteins are encoded from two separate but closely aligned genes or result from post-translational modification (phosphorylation, formylation, acetylation, hydroxylation etc.). It is also possible that they are two different subunits of OPAA-1. Sequencing of the cloned genes should allow the elucidation of their structures. The relatively low level of enzyme activity derived from the cloned gene could be accounted for by the fact that the condition for the cell growth and the recovery of the enzyme activity in crude extracts from *E. coli* and JD6.5 may be totally different. It must also be considered that the chemical properties of OPAA-1 have not yet been determined. Analysis of the expressed products from several subclones carrying various DNA fragments of the insert demonstrated that the cloned gene was located in a 4 kb KpnI fragment.

The present studies have demonstrated that some common structural domains are present in OPAA-1 and OPAA-2. Using the same polyclonal and monoclonal antibodies, numerous microorganisms with high levels of OPA Anhydrase activity can also be recognized by their unique binding properties (17). The results strongly suggest that these antibodies can be a powerful tool for later detection, purification and cloning of OPA anhydrases from these sources. In the past, OPA anhydrases were found in both prokaryotic and eukaryotic organisms (1-9). Some of the crude extracts obtained from these sources, such as proteins from *Tetrahymena, Pseudomonas diminuta* (Parathion hydroxylase), squid hepatopancreas, clam, hog and rabbit did not cross-react with these antibodies (data not shown). Enzymes that are found to have activity on chemical agents or their analogues may be improved through gaining an understanding of their structures. Efforts are now underway to sequence the cloned gene.

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PROGRESS IN CLONING GENES FOR OPIATE RECEPTORS

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ABSTRACT

Future detectors for chemical agents will contain generic sensors relying on biological receptors. Identification and large scale production of proteins that bind opiates and opiate analogs is an essential part of the program. Neuroblastoma cells, grown in culture, provide a reproducible supply of receptors. The NG 108-15 cells contain delta receptors while the SK-N-SH cells contain both mu and delta receptor. A photoaffinity label has been designed and used to covalently tag the mu receptors in the membrane. The radiolabeled tags will be used to identify the denatured protein separation from other membrane components. A mRNA enrichment technique has been employed to increase the concentration of mRNA's - particularly those present in low concentration. The mRNA's have been converted into double strand cDNA using reverse transcriptase and construction of a cDNA library nearing completion.

INTRODUCTION

Currently fielded detectors rely on a specific chemistry to identify the presence of threat agents. The next generation of detectors will be antibody based which will permit a general signal transduction mechanism but will still have the capability of detecting only preselected compounds. The long term goal is to develop a generic system that responds to classes of agents. The most promising approach is based on biological receptors. In order to develop these sensors, sufficient protein must be obtained. Also much larger quantities must become available for production, New techniques in molecular biology such as gene cloning offer the possibility of transferring genes from mammalian neurological cells into bacteria for large scale production. Also each batch of product would come from genetically identical organisms so that the detectors produced tody would be equal to those produced in later years. This might not be the case if receptor protein was isolated from laboratory animals.

APPROACH

DNA from bacteria (prokaryotic) is divided into discrete units called genes that consist of information that is translated into corresponding proteins. In addition there are sequences that provide sites for the ribosomes to bind, provide regions for regulation of expression, and provide punctuation so that protein synthesis starts and stops at the correct

amino acid. In contrast, DNA from higher organisms (eukaryotic) consists of regions that are expressed (exons) separated by nonexpressed (introns) regions. During transcription, the entire DNA region is converted into a messenger RNA (mRNA). The introns are enzymatically removed from the mRNA precursor before it migrates from the nucleus into the cytoplasm. Thus, it is usually not possible to obtain any useful genes from eukaryotic organisms by cutting their DNA into segments and inserting them into appropriate vectors.

The approach in this project is to isolate the precessed mRNA and then use it as a template for constructing a cDNA gene with the enzyme reverse transcriptase (RNA dependent DNA polymerase). In practice all the mRNA's are isolated and converted into a cDNA library from which the desired gene will be selected. Photoaffinity labeling is used to covalently tag to intact receptor on the cell membrane so that the specific protein can be identified when the entire membrane protein fraction is spearated by electrophoresis. The amino acid sequence of this protein will be used to design a short DNA probe that after chemical synthesis will be used to screen the cDNA library for the specific gene.

EXPERIMENTAL PROCEDURES

Cell cultures

Human neuroblastoma cells (SK-N-SH) were grown at 37°C in monolayer in RPMI-1640 medium supplemented with 10% fetal bovine serum. Mouse neuroblastomaxrat glioma hybrid cells (NG 108-15) were gran in monolayer in Dulbecco's modified Eagle's medium containing HAT buffer (0.1 mM hypoxanthine, 10 uM aminopterin and 17 uM thymidine) supplemented with 10% fetal bovine serum. These cells were grown in a humidified atmosphere of 10 per cent carbon dioxide.

Ligand binding experiments

For H^3-naloxone or H^3-diprenorphine binding experiments, the cells were grown in culture plate (16 mm) to confluency. The medium were then removed by aspiration and the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS). To each were, 0.5 ml of DPBS containing H3-tracer were added and incubated in various time as indicated at 25°C. Each experiment was done in triplicate or duplicate. After incubation, the reaction mixture were removed and the cells in each dish were washed four times with DPBS. One half ml of 0.5 NaOH was added to each dish and shaken gently for 1 hour. The protein concentration of cell lysates was determined by Bio-rad protein assay. The radioactivity was measured by liguid scientillation counting after adding Du Pont Formula-967. Non-specific tracer binding was measured in the presence of 1 uM naloxone or diprenorphine, usually accounted for less than 20% of total binding.

Photoaffinity experiments

In photoaffinity experiments, various concentrations of photoreactive fentanyl compound (Fig. 1) in 0.9 mM K-phosphate buffer (pH 7.0) containing 1.3 mM $CaCl_2$ and 110 mM KCl were added to cells in each petric dish and incubated for 20 minutes at 25°C in the dark. For photolysis, open culture

plates were photolyzed with a hand-held UV lamp (Buchler Instruments/min 300 nM) at a distance of n2 cm for various time periods as indicated in the text. After photolysis, the supernatant were removed and the cells were washed 3 times with PBS. H^3-tracer binding assay was then performed as described above.

Isolation of total cellular RNA and polyadenylated mRNA

Confluent cells in 2 flasks (75 cm) were detached and lysed with the buffer containing 7 m urea, 2% SDS, 0.35 m NaCl, 1 nM EDTA and 10 mM Tris, pH 8.0 (1). After extraction with phenol-choloform-isoamyl alcohol, 1 gr/ 4 ml of solid CsCl were added to the aqueous solution. The solution were then layered on to 4 ml of 5.7 m CsCl containing 0.1 M EDTA (pH 7.5) in a Beckman Ti 80 centrifuge tube and centrifuged at 290,000xg for 7 hours at 20°C. The RNA pellets were resuspended in 0.3 M NaOAc, pH 5.6 and extracted with phenol. The total cellular RNA were then precipitated with ethanol.

The polyadenylated mRNA was purified from total cellular RNA by fractionated twice through oligo-(dT) cellulose chromatography (2).

Synthesis of double-stranded cDNA (ds-cDNA).

The cDNA synthesis kit and protocol from NEN Research Products were used to snythesis the ds-cDNA. Briefly, the snythesis steps involve: 1) synthesis of first stranded cDNA from polyadenylated mRNA template using reverse transcriptase and oligo dT, 2) utilizing a primer in the form of hairpin loop found at the 3' end of first stranded cDNA to synthesis ds-cDNA by E. coli polymerase I, and 3) digestion of the hairpin loop with S1 nuclea to produce the final ds-cDNA.

Electrophoresis of RNA and DNA

The precipitated RNA was resuspended and subjected to electrophoresis in a horizontal 2.2 M formaldehyde-1,1% agarose gel (2). The cDNA fragments were subjected to electrophoresis on on a 6% polyacrylamide slab gel containing 7 M urea (3). The gel that contain ratioactive cDNA fragments was directly exposed to x-ray film for 1-2 days at -80°C.

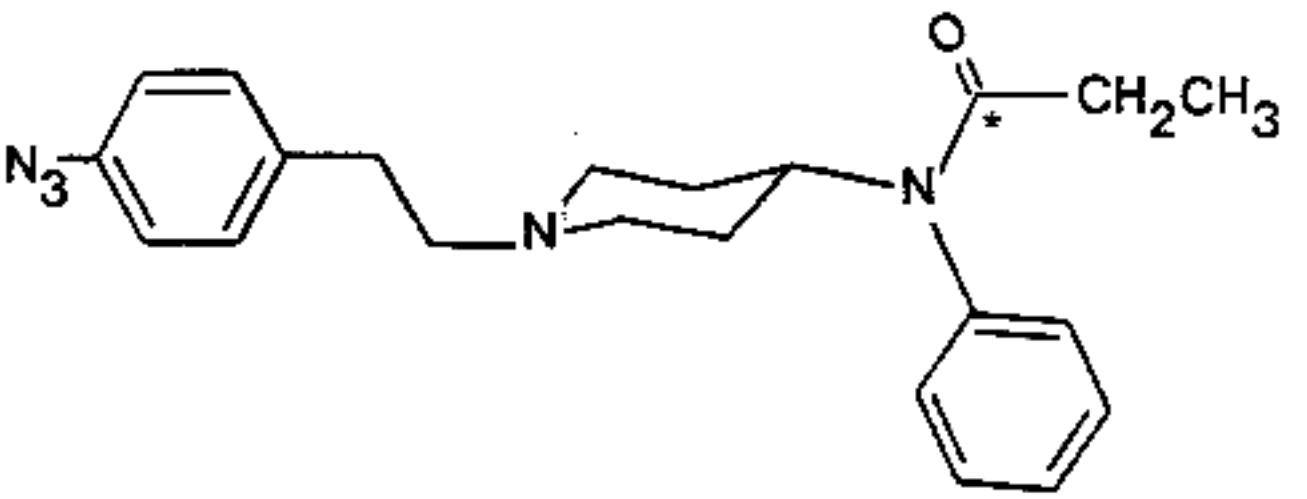
RESULTS

1. Binding of Opiates to cellular receptors

The results of binding assays for both (H^3)-nalxone and (H^3)-dipren-2. orphine in various cell lines are summarized in following Table. The results clearly demonstrated that SK-N-SH and NG 108-15 are well maintained in our lab and they are enriched with specific opiate receptor sites delta+mu and delta respectively. In addition, the binding experiments of human Schwann cells (SFP-6) suggested that these cells also express some delta and mu receptor sites. The hepatoma cells (liver) served as a control. There was no evidence of opiate binding to these cells.

2. Design of Photoaffinity Label

A fentanyl ligend (figure I) was designed for use in identifying the appropriate protein. Fentanyls are among the most potent opiate agonists



N – (1 – [2 – (4-azidophenyl)ethyl] – 4 – piperidinyl) –
N – phenyl – propanamide

1. PHOTOAFFINITY LABEL
2. FENTANYL
3. RADIOLABELED

FIGURE 1

and are considered to have potential threat as incapacinants. Because several opiate receptors have been identified, it is advantageous to use the compounds of interest in selecting the particular receptor. This analog has three distinct features. The compound itself is a fentanyl in order to provide highly specific, high affinity (though reversible) binding to opiate receptors. The azido function attached to the phenyl ring is chemically inert; however upon photolysis, it forms a highly reactive nitrene upon liberation of molecular nitrogen. The technique is to bind the ligand reversibly to the receptors in the intact cells (where the receptors are not denatured) and then covalently attach it by irradiating with visible light. When the membrane is subsequently solubilized, the ligand will not fall off even though the protein is denatured. The third feature is the incorporation of a C-14 isotope that permits the identification of the tagged protein by radioactivity.

3. Synehesis of the photoaffinity label

The photoaffinity label N-{1-[2-(4-azidophenyl)ethyl]-4-piperidinyl]-N-phenylpropanamide was synthesized by K. Narasimhan, M. Sasner, and L. Wingard at the Biotechnology Center at the University of Pittsburgh.

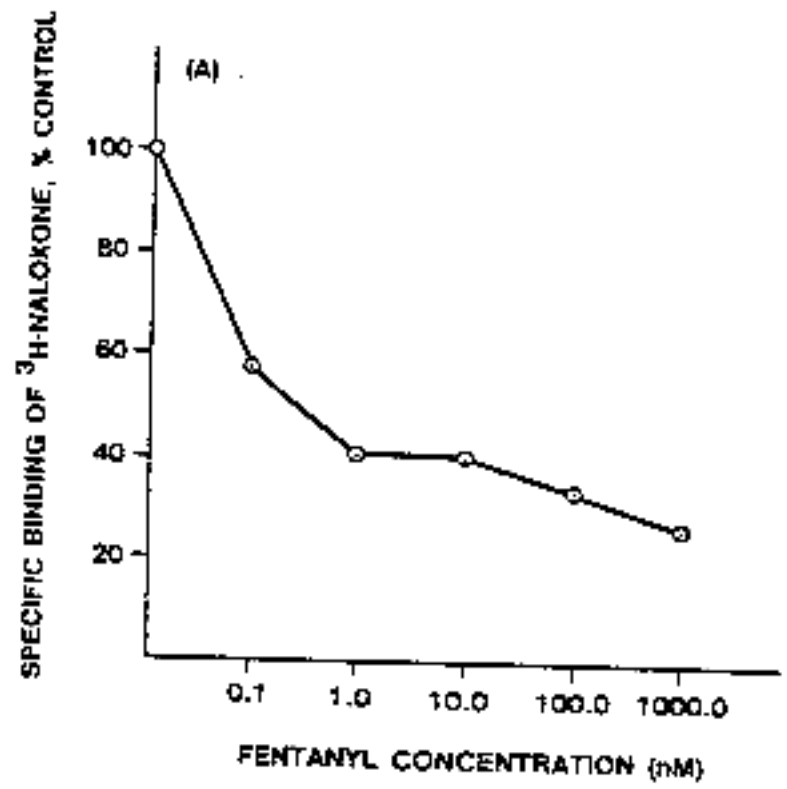
4. Labeling of the mu opiate receptor by photoaffinity labeling.

Figure 2 illustrates the effect of photolabeling on subsequent opiate binding. After attachment, the cells were washed to remove all non-covalently bound label. Binding studies with H^3-naloxone (mu specific) snowed a 60% loss of specific binding in the SK-N-SH cells labeled with I nM fentanyl. In contrast, H^3-diprenophine binding decreased by only 10% in the NG-108-15 cells (delta). Control experiments in which the cells were photolyzed alone under the same conditions resulted in an 5% loss of opiate binding. Ligand binding was not reduced when the fentanyl was incubated with the cells and washed out prior to photolysis. These results suggest that the binding sites of the fentanyl are similar to those for naloxone which are specific for the mu receptor.

DISCUSSION

The critical feature of this approach is the specific labeling of the receptor protein. If many proteins are labeled, then it will not be possible to identify which is the desired one because binding studies will not be possible after the protein has been denatured and removed from the membrane. The strong preference for the mu receptor is consistant with high specific binding; however the crucial experiments await the delivery of the radiolabeled material.

The construction of the cDNA library is progressing smoothly. Efforts have been taken to obtain sufficient quantities of mRNA in order to insure the presence of the messenger that codes for the mu receptor binding protein which is probably present in minute amounts. The isolated mRNA's have been used to generate double stranded cDNA. The average sizes of the cDNA's from the SK-N-SH and the NG-108-15 cells are 113 kilobases.



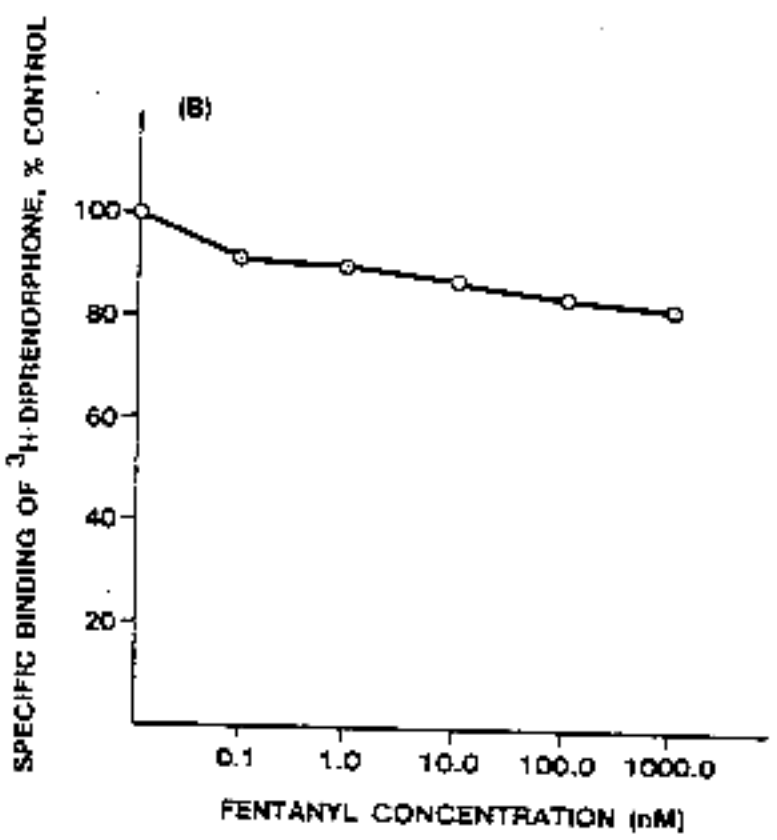


Figure 2. The H^3-ligand binding on SK-N-SH (A) and NG 108-15 (B) after photoaffinity labeling of fentanyl compound at different concentration. The cells in open culture plates were photolysed at 25°C for 90 seconds in the dark. After washing with PBS, the H^3-tracer binding assays were then performed with naloxone and diprenorphine concentration at 1.5×10^{-9} M and 2.0×10^{-9} M respectively.

Expression of the cloned genes will produce the unprocessed protein. It is unlikely that this material will be indentical to a naturally functioning neuroreceptor that is probably glycosolated. It is possible that the new material will bind opiates specifically and will therefore be useful in sensors.

TABLE 1

Specific (H^3)-opiate binding

(Counts/min/mg protein)

	(H^3)-Naloxone mu receptors			(H^3)-Diprenophine mu, delta, and kappa receptors		
Cell Line	Total Binding	Non-specific Binding	Specific Binding	Total Binding	Non-specific Binding	Specific Binding
SK-N-SH	8439	920	7519	10409	1135	9274
NG 108-15	1014	278	736	5790	1112	4628
SFP-6	243	5	238	1248	549	699
MEP-G2	57	29	28	50	38	18

For binding experiments, the cells were grown to confluency in 10 x 35 mm petri dishes. The medium was removed by aspiration and the cells were washed twice with DPBS. To each petri dish, 1.0 ml of DPBS containing 0.2 uCi (H^3)-naloxone (181 mCi/mg) or diprenorphine (85.8 mCi/mg) were added and incubated for one hour at room temperature. Each experiment was done in triplicate. After incubation, the reaction mixture was removed and the cells washed four times with DPBS. One half ml of 0.5 N NaOH was added to each dish which was then shaken for one hour to lyse the cells. Protein concentration was determined by the Bio-Rad protein assay. Radioactivity was measured directly by liquid scintilation counting.

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CLONING, SEQUENCING AND CHARACTERIZATION OF OPD GENES AND THEIR BROAD-SPECTRUM ORGANOPHOSPHATE HYDROLASES FROM SOIL BACTERIA

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Two homologous organophosphate degrading genes (opd) have been identified and subcloned from nonhomologous plasmids isolated from divergent soil bacteria. These genes produce broad-spectrum organophosphate hydrolases which appear to have a membrane-associated somanase activity (see Albizo and White, this symposium). During late stages of growth, the enzyme activity is released into the growth media as a membrane-independent complex of 60Kd or associate. Characterization of the substrate specificity of the enzymes indicate that the nature of the triester linkage is critical to hydrolytic cleavage. While the substituent at the para-position has no effect on binding affinity, it does have a dramatic effect on catalytic activity. The genes have been subcloned and expressed in various bacterial backgrounds. The nucleotide sequences of the open reading frames of the two genes are identical even though neighboring regions show restriction site polymorphism and sequence divergence.

Synthetic organophosphorus compounds have been used as xenobiotic pesticides, insecticides, fungicides, herbicides and as other biological control agents. Although some of the modern organophosphates are thought to be among the safest of all chemical pesticides, compounds of dangerously high mammalian toxicity are also found in this group. Some of the more toxic organophosphates are potent agonists for mammalian acetylcholinesterase (AChE) and pose important detoxification challenges. A variety of organophosphate hydrolyzing enzymes have been identified by their ability to detoxify the powerful cholinesterase (Che) inhibitor and neurotic agent DFP (diisopropyl-phosphonofluoridate) (1). The "squid type" DFPase has been extensively purified (2) and it hydrolyzes DFP more rapidly than Soman (1,2,2-trimethyl propylmethylphosphonofluoridate). The enzyme has a molecular weight of 26 Kd and is narrowly distributed in specific organs of cephalopods (3). The "Mazur-type" DFPase has a larger molecular weight (45 - 65 Kd) and hydrolyzes Soman many times faster than DFP. Recent studies on the inhibition of DFPases by the insecticide Mipafox have suggested that Escherichia coli may contain a third species of DFPase (4). The "Mazur-type" DFPase is an ubiquitous class of enzymes which have been identified in various organisms from bacteria to the mammalian kidney; however, the enzymes are not identical. Studies with the bacterial DFPase or DFPases compared DFP and paraoxon specificity and concluded that the enzyme was not an A-esterase (5).

It has been observed that a variety of microbial systems may be involved in the degradation of organophosphate insecticides in the soil and associated water systems. Parathion has been reported to be readily degraded in the soil by a variety of bacteria, fungi and algae. Furthermore, a broad spectrum of other organophosphates (including phorate, dialkyl phenyl phosphates, carbophenothion, dichlorovos, and fenthion) appear to be substrates for microbial degradation (6). A variety of parathion-degrading microorganisms possess phosphotriesterases which can hydrolyze a broad spectrum of organophosphates including parathion and amino-parathion. The esterase hydrolyzes parathion to diethylthiophosphate and p-nitrophenol. Several of these parathion hydrolases have been partially characterized (7,8). In two cases, these enzymes have been demonstrated to be carried on specific bacterial plasmids (Pseudomonas diminuta, 9; and Flavobacterium, 10). The genes have been designated opd (organophosphate degrading) and have were transferred to plasmid or phage cloning vehicles and expressed in various bacterial hosts (10-12). The enzymes were observed to be membrane-associated and the F1-enzyme was reported to have a molecular weight of approximately 60,000 d (7,8).

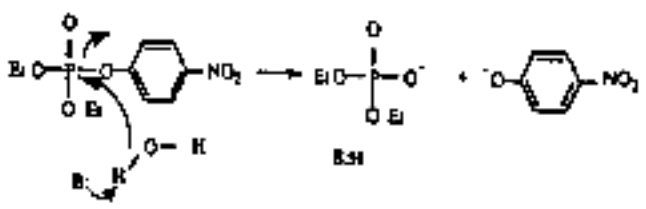
Phosphotriesterase substrate characterization. The substrate specificity of an acetone-extracted, cell-free extracts of the OP phosphotriesterase from P. diminuta MG (obtained from C. Serdar) was evaluated by gas chromatography analysis. The catalysis of 31 dialkoxy, organophosphates obtained from the Environmental Protection Agency was evaluated under gentle shaking at 37°C, pH 8.5 for 30'. The reaction was stopped with 100 microliters of 2-mercaptoethanol, extracted with hexane, dried and concentrated (13). Five microliters was analyzed in a Tracor gas chromatograph at various temperatures on a 6' x 2mm glass column with 3% OV101 and monitored in a nitrogen-phosphorus detector. The results summarized in Table 1 indicate that the nature of the triester linkage is critical to its susceptibility to hydrolytic cleavage. Alterations to the acid leaving moiety appears to stabilize the ester linkage (compare the effect of the addition of an electrophilic halogen substituent such as parathion vs bromophos ethyl). Thiol esters seems to be much less susceptible although there may be slight cleavage of some of these (e.g. malathion). Dimethyl substitution of the phosphorate and thiophosphorate moieties dramatically reduces hydrolysis. In addition, there may be small differences attributable to the shift between sulfones and oxones.

TABLE 1
Degradation of Organophosphates by Pd-phosphotriesterase

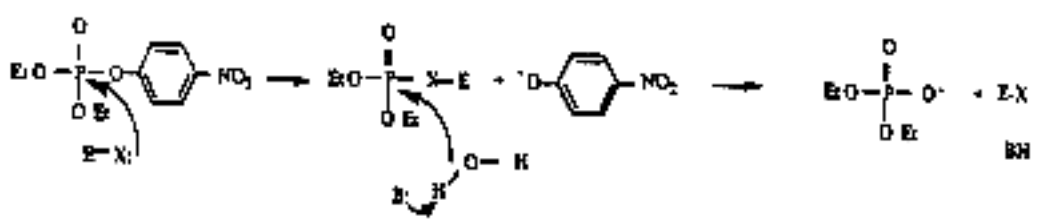
GROUP I		GROUP II		GROUP III
Bromophos-ethyl	53.2	Chlorfenvinphos	7.4	Chlorpyrifos-methyl
Coumaphos	100.0	Chlorothiophos	11.4	Ethoprop
Diazinon	72.1	Chlopyrifos	17.2	Etrimifos
Fensulfothion	100.0	Crufomate	0.9	Famphur
Leptophos-oxon	100.0	Dichlofenthion	32.6	Fenthion
Parathion	98.4	EPN	29.3	Isofenphos
Paraoxon	100.0	Fenitrothion	7.9	Jodfenphos
Pirimiphos-ethyl	94.3	Malathion	16.4	Ronnel
Pyrazophos	89.1	Parathion-methyl	31.3	Sulfopros
Quinalphos	99.3	Pirimiphos-methyl	17.1	Sulfotepp
		Trichloronate	19.8	

More detailed studies have been performed in order to describe the mechanism of catalysis and describe the nature of substrate specificity. At least three mechanisms can be written for hydrolytic cleavage of paraoxon (chosen as the model substrate for better aqueous solubility).

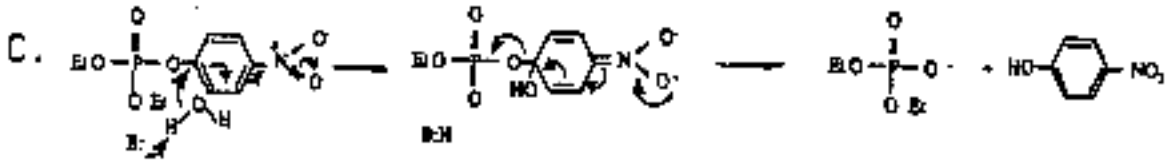
A.



B.



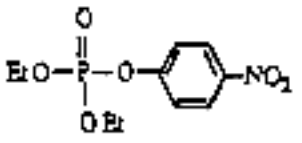
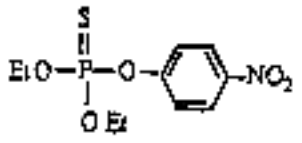
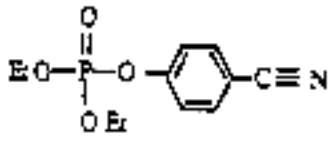
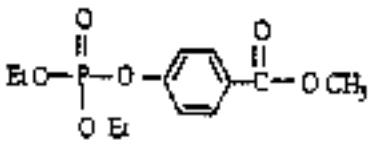
C.



These three mechanisms can be distinguished by appropriate labelling experiments in oxygen-18 H_2O. If mechanism C is operating, hydrolysis in the presence of water will result in the O-18 exclusively associated with p-nitrophenol, whereas mechanism A or B will leave the label associated with diethylphosphate. The 31P-NMR spectra of diethylphosphate was determined after enzyme-catalyzed hydrolysis of paraoxon in the presence of 70% oxygen-16 and 30% oxygen-18 water. The diethylphosphate clearly showed the incorporation of oxygen-18 water as indicated by the 0.03 ppm upfield chemical shift relative to standards containing only oxygen-16. Mechanism C can thus be eliminated. It will be possible to differentiate between mechanisms A and B through the analysis of a chiral phosphotriester (studies in progress, F.M. Raushel). A detailed examination of the pH profile of the Pd-enzyme reveals that there is a single break at pH 6.2 relative to both Vmax and Vmax/Km. This would appear to represent the titration of the basic residue responsible for activation of the water molecule implicated in the reaction mechanism. There is no indication of an acidic group at high pH that could be used to protonate the phenol (data presented, not published here).

A series of phenol substituted phosphotriesters have been recently synthesized and the relative Km and Vmax values are indicated in Table 2.

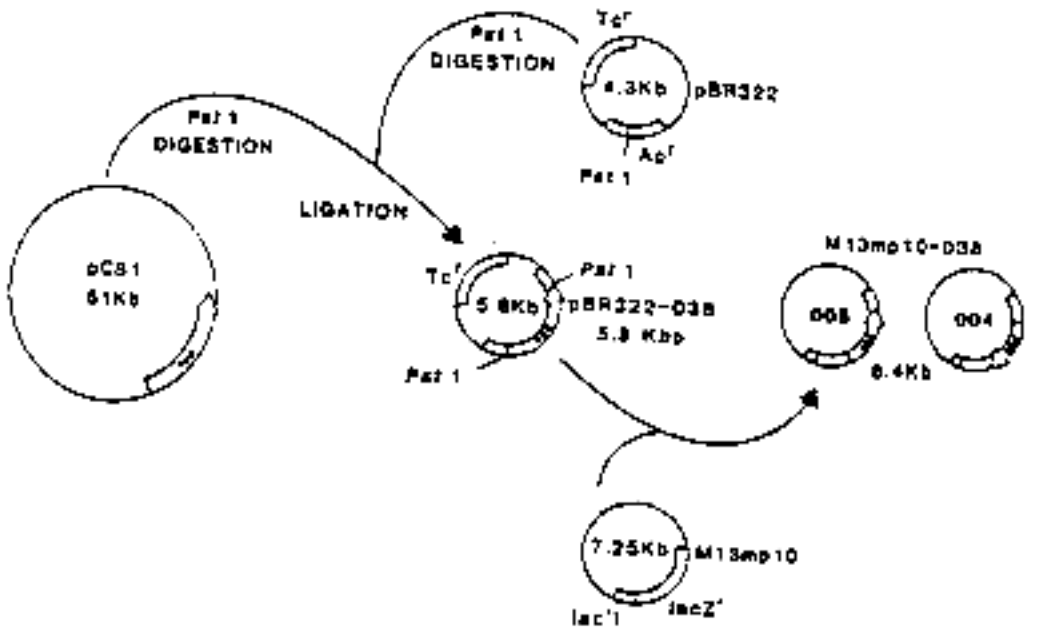
TABLE 2
Comparison of Phenol Substituted Phosphotriesters.

	Vmax	Km
	100	0.0514
	34.3	0.177
	30.0	0.0177
	6.9	0.0252

Several analogues were completely inactive as substrates ($-CH_3$ and $-F$) and it appears that the determinative characteristic may be the pKa generated by the phenol complex. In addition, various para-substituted phosphotriesters have been evaluated as competitive inhibitors for paraoxon hydrolysis (data presented, not published here). The Ki values ranged from 0.039 for the cyano-substituted phenol to 0.222 for the non-substituted phenol. The relatively constant values indicate that the nature of the substituent at the para positon has no effect on binding affinity but it does have a dramatic effect on hydrolytic cleavage.

Cloning and sequencing of the opd genes. The plasmid CS1 carrying the opd gene from P. diminuta MG was isolated by a mild lysis procedure adapted from Berns and Thomas (14). Isolated plasmid DNA was restricted with a variety of restriction endonucleases and the resulting fragments were sized by agarose electrophoresis. The plasmid was approximately 50 Kb based on independent digests with 4 restriction endonucleases (PstI, BamHI, SmaI, and HindIII). This size differs from that reported earlier by electron microscope estimation (9) but similar EM-analysis in our laboratory (12) provided that same estimation. The entire degradative plasmid was subcloned into the PstI site of the bla gene of pBR322 and parathion-degradating transformants of E. coli HB101-4442 (15) were identified. Each of the active transformants were identical (pBR322-038) and carried a 1300 bp insert. This fragment was isolated and transferred into the polyclonal region of ML3mp10 where it became obvious that external promotion was necessary for expression. In one orientation (008) (Figure 1) opd was expressed and in the other (004) it was not. Subsequent studies have revealed that expression can be enhanced by removing approximately 250bp of the 5'-flanking sequence of the cloned fragment and introducing it into a tac expression vector (16). Upon induction with IPTG (isopropyl-thiogalactoside) the specific activities of the op-phosphotriesterase in E. coli are similar to those in the native host. The same procedure has been utilized to enhance the expression of the initially cloned Flavobacterium gene (10).

FIGURE 1
Cloning of opd from P. diminuta MG.



The DNA sequences for both of the opd genes are virtually identical through their 846 bp open reading frame and should produce an amino acid with 282 amino acids (data presented, not published here). An interesting feature of the homologous structural gene regions is that they are contained in plasmids of different size and possessing neighboring restriction site polymorphism (10). CS1 from P. diminuta is estimated to be 50Kb by restriction fragment analysis (60-70 Kb by other analyses) and pPDL2 from Flavobacterium is approximately 40Kb. The DNA sequence homology begins to diverge in the 5'-flanking region of the genes and there is no evidence of homology outside of the common 1300 bp regions.

Characterization of the OP-phosphotriesterases. The phosphotriesterases from both native bacterial hosts and their tac cloned opd genes expressed in E. coli are membrane-associated during active growth and expression is constitutive, unrelated to growth phase. However, at later growth phases, activity begins to appear in the supernatant. When chromatographed by molecular weight sieves, the activity was distributed between a large, heterogenous membrane associated fraction (Mr > 300,000 d) and a discrete molecular weight species of 60,000 d. It was possible to use detergents to chase OPD activity from its membrane-association into the free aggregate. This form of the enzymes appears to be a dimer of approximately 30,000 d (data not shown). Triton X-100 and Tween 20 (.1 - 1%) were effective for releasing the enzyme, while SDS, CETAB and laurylsarcosine disrupted activity. It is possible to release greater than 90% of the enzymatic activity associated with whole cells by a hypertonic wash (0.25 - 1.0M NaCl). Most of the activity is associated with a membrane component which is indistinguishable from that released into the supernatant. The enzyme, in its various forms, is relatively stable at a variety of temperatures if the pH is maintained between 8.5 and 9.0. In addition, the enzyme is active in various organic solvents (MeOH, Dioxane and DMF) up to 20-25% but then becomes irreversibly inactivated. Studies are underway to detail the stability of the free form of the enzyme. The membrane-associated form has been frozen and kept at room temperature for months without appreciable loss of activity.

CONCLUSIONS

Two genes which produce a broad-spectrum organophosphate hydrolase have been cloned from different plasmids isolated from soil bacteria. The DNA sequences of the two genes have been determined and the nature of the substrate specificity is being examined. The enzyme appears to have an extremely broad binding affinity for organophosphate but significantly more limited hydrolytic cleavage. The combination of these studies will permit the genetic manipulation of the opd genes in order to design specific catalysis. In studies that are more important for the nearer time frame, it has been possible to construct expression systems so that enzyme can be produced in high concentration in E. coli and other bacterial hosts. This will result in greater availability of the enzyme from bacterial systems that can grow more quickly and in more limited environments.

ACKNOWLEDGMENTS

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PURIFICATION OF OPIATE RECEPTORS AND CLONING THEIR RESPECTIVE GENES

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ABSTRACT

The photoaffinity label, a fentanyl analog, was synthesized and used to probe the opiate receptor of human neuroblastoma cells (SK-N-SH). Upon photolysis in situ, this photoaffinity ligand inhibited binding of naloxone to the mu opiate receptors but did not affect the binding of delta receptors. The digitonin-solubilised proteins were prepared from these cells and purified by wheat-germ agglutinin and affinity gel chromatography. The affinity gel was prepared by attaching the modified fentanyl to Affi-Gel 202 with carbodiimide. The protein fraction eluted by naloxone revealed a major band with molecular weight of ~58,000 as determined by SDS-polyacrylamide gel electrophoresis. The cDNA library for SK-N-SH cells was also prepared with λgt 10 cloning vector. The library can be used for future screening opiate receptor genes.

INTRODUCTION

The opiate receptor system consists of several types of binding sites with different biological functions (1-6). It has not been established whether one or more of these receptor types are different conformations of a single macromolecule, or whether each of them represent different molecular species. Several neuroblastoma cell lines have been identified as possessing specific membrane-associated opiate receptors (7-9). These clonal culture systems offer a clear advantange over brain and primary neural cell preparations in that intact viable homogeneous cell preparations may be obtained in relatively large quantitives without resorting to expensive cell separation procedures. They have proven to be of great value in elucidating the structure and function of opiate receptors.

Among cell lines that have been examined for the presence of opiate receptors, the δ (delta) receptor found in mouse neuroblastoma x rat glioma hybrid cell line (NG 108-15) has beenwell studied (9-13). Recently, a human neuroblastoma cell line (SK-N-SH) that expresses μ (mu) and δ opiate receptors has also been identified (8). These two receptors of SK-K-SH cells closely resemble the previously reported μ and δ sites in human (14,15) and rodent brain (1-4).

Fentanyl is one of the most potent opiate agonists with an estimated analgesic potency of 80 times that of morphine (16). The difference in potency between the two drugs probably results from an increased penetration of fentanyl into the brain (17). Despite the structural differences between fentanyl and morphine, binding and displacement studies (18,19) with preparations of opiate receptors have indicated a strong similarity between these two ligands. More important, the pattern of fentanyl binding

closely resembles naloxone binding and was stereospecific (19). The development of opiate affinity ligands fentanyl isothiocynate (^{3}H-FIT) and 3-methyl fentanyl isothiocynate (^{3}H-super FIT) allowed the identification and purification of a molecular weight 58,000 glycoprotein from NG 108-15 cells (12,13). Interestingly, the μ receptor of rat brain membranes has also been purified by biochemical approaches and shown to have a molecular weight of 58,000 (20,21). In this article, we report the use of a fentanyl analog for specific labeling and purification of the opiate receptor in SK-N-SH cells and the progress in construction of an SK-N-SH cDNA library.

EXPERIMENTAL PROCEDURES

Cell culture. Human neuroblastoma cells (SK-N-SE: ATCC HTB 11) were grown at 37 C in monolayer in RPMI 1640 medium supplemented with 10 % fetal bovine serum. Mouse neuroblastoma x rat glioma hybrid cells (NG 108-15) were grown in monolayer in Dulbecco's modified Eagle's medium containing HAT buffer (0.1 mM hypoxanthine, 10 μM aminopterin and 17 μM thymidine) supplemented with 10 % fetal bovine serum. These cells were grown in a humidified atmosphere of 10 % carbon dioxide.

Photoaffinity experiment. In photoaffinity experiments, the cells were grown into confluency in culture plate (16mm). After the medium was removed by aspiration, the cells were washed twice with 2 ml of 0.9 mM K-phosphate buffer (pH 7.0) containing 110 mM KCl and 1.3 mM $CaCl_2$. Various concentrations of the photo-reactive fentanyl compound (Fig.1A) in 0.5 ml of above buffer were then added to cells in each well and incubated for 30 min at 25 C in the dark. For photolysis, open culture plates were photolysed with a UV lamp (Buchler Instruments/min. 300 nM) at distance of 2 cm for selected intervals as indicated in the text. After photolysis, the supernatants were removed and the cells were washed 3 times with 2 ml of Dulbecco's phosphate buffer saline (DPBS).

Ligand binding experiments. (N-allyl-2,3-^{3}H)-Naloxone (specific activity 60 Ci/mmole) and (15,16[n]-^{3}H)diprenorphine (specific activity 36.6 Ci/mmole) were purchased from Amersham. For ^{3}H-naloxone or ^{3}H-diprenorphine binding experiments, 0.5 ml of DPBS containing ^{3}H-tracer were added to cells in each well and incubated for 1 hr at 25 C. Each experiment was done in triplicate. After incubation, the reaction mixture was removed and the cells in each well were washed four times with DPBS. One half ml of 0.5 N NaOH was added to each well and shaked gently for 1 hr. The protein concentration of a portion of cell lysates was determined by Bio-rad protein assay. The radioactivity of remaining cell lysates was measured directly by liquid scintillation counting after adding Du Pont Formula-967. Specific ^{3}H-tracer binding was defined as the difference between total binding in the absence and presence of 1 μM unlabeled drug. Non-specific binding usually accounted for 20-30 % of total binding.

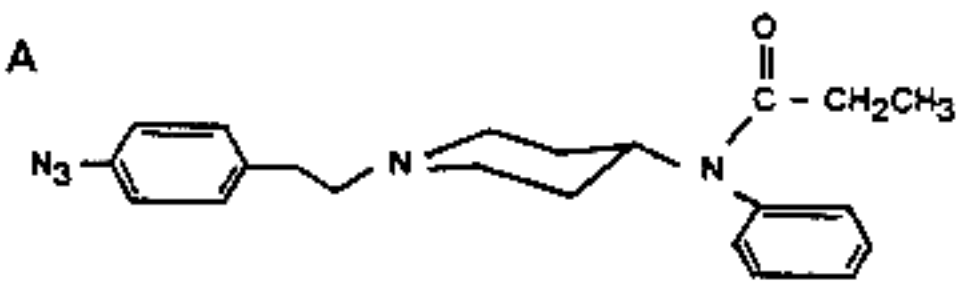
Solubilization. The cells were grown in four culture flasks (75 cm2) to confluency. After washing with phosphate buffered saline, the cells were solubilized with 6 ml/flask of 2 % digitonin in Mg-buffer (10 mM Tes-KOH, pH 7.5, 1mM EDTA, 10 mM $MgSO_4$, 1 mM benzanidine-HCl, 0.01 % bacitracin, 0.002 % soybean trypsin inhibitor, 1 μM pepstatin and 0.2 μM phenylmethanesulfonyl fluoride) (22). The cells were incubated at room temperature for 1 hr and the 105,000 x g (4 C, 70 min) supernatant was collected.

Preparation of fentanyl-affinity gel. The procedure for preparing the affinity column was essentially that described by the Bio-rad protocol. Briefly, NH_2-fentanyl (10-50 μmoles) was coupled to Affi-Gel 202 at using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). The coupling was allowed to proceed for at least 5 hr in water (pH 4.7-5.0) at room temperature. The affinity gel obtained was packed and washed with Mg-buffer in a 5 ml syrange column.

Protein purification and SDS-PAGE analysis. The solubilized preparation (diluted with 3 volumes of buffer) was applied first to wheat germ agglutinin-sepharose column (2ml packed vol) pre-equilibrated with Mg-buffer. The column was washed with 25 ml of Mg-buffer and eluted with same buffer containing 0.5 M N-acetylglucosamine.

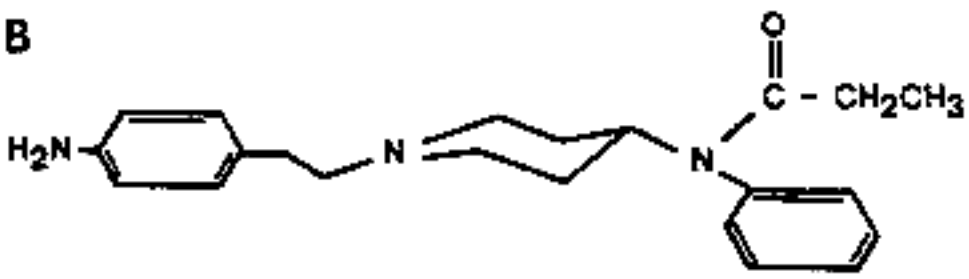
The pooled eluates in 1 mM DTT were incubated with the affinity gel (2 ml) at 25 C for 2 hr. The gel was packed in a 5 ml syrange column and washed extensively with Mg-buffer containing 1 mM DTT

A



N-(1-[2-(4-azidophenyl)ethyl]-4-piperidinyl)-N-phenyl-propanamide

B



N-(1-[2-(4-aminophenyl)ethyl]-4-piperidinyl)-N-phenyl-propanamide

Fig. 1. Structure of fentanyl derivatives.

and 0.05 % digitonin. The affinity gel was then removed and incubated with ~3 ml of Mg-buffer containing 100 μM naloxone, 1mM DTT and 0.05 % digitonin at 30 C for 30 min. The mixture was packed and washed twice with the same buffer. The combined eluation was concentrated by Amicon PM-30 to ~200 μl.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (23). The binding reaction of purified proteins were carried out according to the method of Cho et al (24), except the incubation time was 60 min at 25 C. In addition, prior to the binding assay, the detergent of solubilized proteins was removed by Extracti-Gel D (Pierce) following suggested protocol. Specific ^{3}H-naloxone binding was assayed as described above.

Isolation of total RNA and poly (A) RNA. Confluent cells in 4 flasks (75 cm^2) were detached and lysed in 4 M guanidine isothiocynate (25). The lysate was layered onto 4 ml of 5.7 M CsCl cushion containing 0.1 M EDTA (pH 7.5) in a Beckman ultracentrifuge tube and spun at 290,000 x g for 8 hr at 20 C. The RNA pellets were resuspended in 100 ul 10 mM Tris (pH 7.5) and 1 mM EDTA. Poly (A) RNA was purified from total RNA by oligo-(dT) cellulose chromatography (26).

Preparation of double-stranded cDNA and cloning into λgt 10 vector. The Amersham's cDNA synthesis system and protocol were used to synthesize the double-stranded cDNA (ds-cDNA) from poly (A) RNA. The basis of the system involves: 1) synthesis of first strand cDNA using reverse transcriptase and oligo-dT, 2) using E. coli ribonuclease H to produce nicks and gaps in the mRNA strand of RNA/DNA hybrid and 3) utilizing E. coli DNA polymerase I to replace the mRNA strand by DNA in a nick translation type reaction (27).

The ds-cDNA generated as described above was treated with T4 DNA polymerase to remove any 3' overhangs and then incubated with a large molar excess of EcoR I linker in the presence of T4 DNA ligase (27). After protecting the DNA by methylation with EcoR I methylase, the ds-cDNA molecules carrying EcoR I linker sequences at their ends were recovered by cleavage with EcoR I. cDNA libraries were then constructed in λgt 10 by using Promega Protoclone λgt 10 system and protocol.

Gel analysis of DNA. The ds-cDNA fragments were subjected to electrophoresis on a 1.4 % alkaline agarose gel (28). The gel that containing radioactive cDNA fragments were exposed to x-ray film for 1-2 days at -80 ° C.

RESULTS

Effect of fentanyl labelling on opiate receptor binding capacity. Photolysis of cells showed a slight loss of binding capacity due to photodestruction of receptor site (29). In our control experiments, a 10-15 % decrease of ^{3}H-naloxone or ^{3}H-diprenorphine binding was observed in SK-N-SH and NG 108-15 cells after 90 seconds exposure to UV light (Fig. 2). The loss of binding and the detachment of cells in culture plate increased significantly by 2 minute exposure. In order to reduce the photodestruction, the irradiation period was routingly less than 90 seconds.

To illustrate the effect of photolabeling on subsequent opiate binding, the following experiments were performed. Various concentrations of the N_3-fentanyl were added to cells prior to a 30 minute incubation in the dark. The cells in open culture plates were photolyzed at 25 C for 90 seconds. The presence of opiate receptor sites in cells was then determined with the tracer ^{3}H-naloxone (μ selective ligand) and ^{3}H-diprenorphrine (μ, δ, and κ (Kappa) ligand) (8). As shown in Fig.3, the specific binding of ^{3}H-naloxone was greatly reduced in the SK-N-SH cells photolysed with increased concentration of N_3-fentanyl (3A). It showed a ~65 % loss of specific binding in the SK-N-SH cells labeled with 1 nM fentanyl. In contrast, ^{3}H-diprenorphine binding in the same cells was reduced by ~15 % (3B). Likewise, the loss of ^{3}H-diprenorphine binding in NG 108-15 decreased by ~20 % under the same conditions (3C). In a control experiment, the binding of either ^{3}H-tracer in these cells decreased by only 5-10 % when the binding assays were performed in the presence of competitive fentanyl (data not shown). In addition, no photolabeling was observed when the fentanyl derivative was photolysed prior to incubating with the cells. Because the

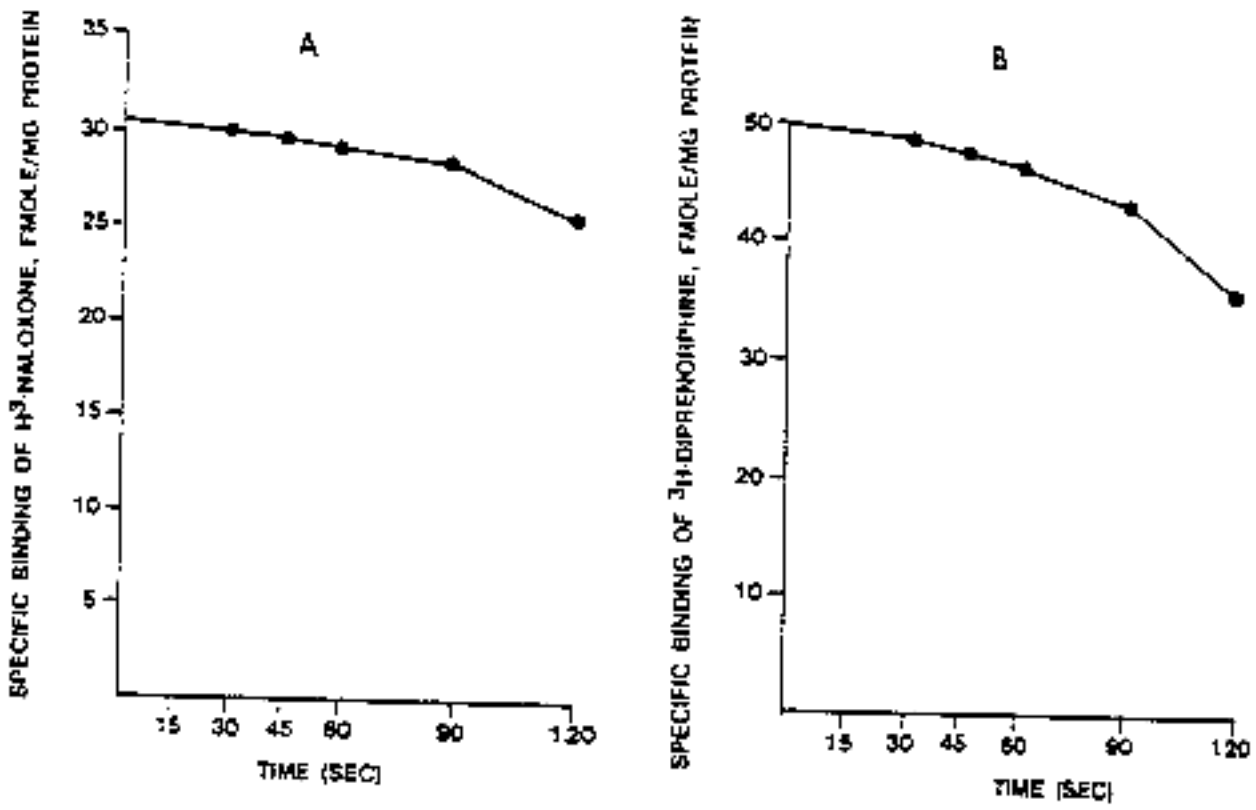


Fig. 2. The photodestruction of ^3H-naloxone and ^3H-diprenorphine binding sites in SK-N-SH (A) and NG 108-15 (B). The neuroblastoma cells were photolysed for various time periods without adding any drug. The ^3H-tracer binding assays were then performed after washing the cells with DPBS.

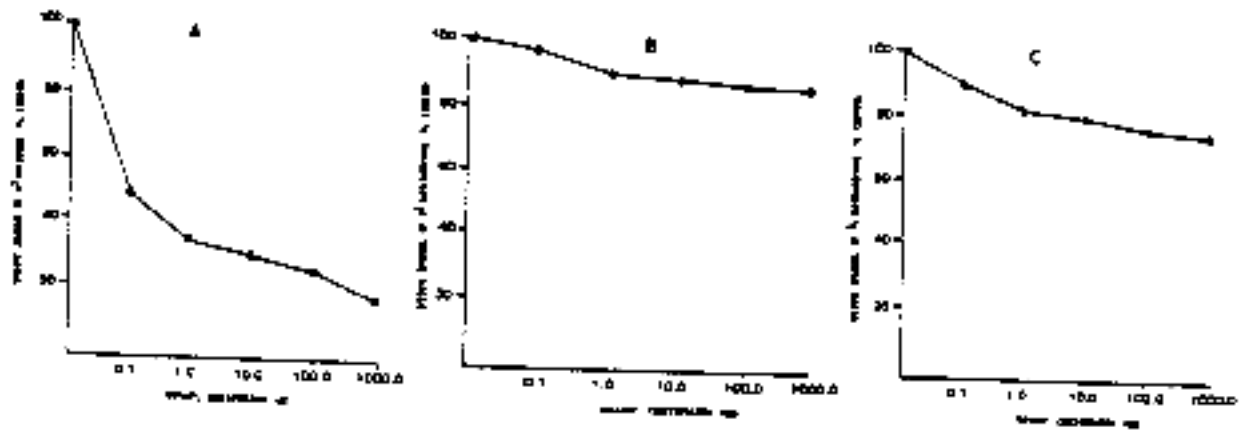


Fig. 3. The ^3H-ligand binding of SK-N-SH and NG 108-15 after photoaffinity labeling of fentanyl derivative at different concentration. The cells in open culture plates were photolysed at 25 C for 90 seconds in the dark. After washing with DPBS, the ^3H-ligand binding assays were then performed with naloxone and diprenorphine concentration at 1.5×10^{-9} and 2.0×10^{-9} M respectively. Specific tracer binding was defined as the difference between binding in the absence and presence of unlabeled drug. Panel A, the specific ^3H-naloxone binding of SK-N-SH; B, the specific ^3H-diprenorphine binding of SK-N-SH in the presence of 1 uM naloxone; C, the specific ^3H-diprenorphine binding of NG 108-15. Values represent the means of triplicate experiments. Specific activity (fmole/mg protein) of control cells (in the absence of fentanyl) in A, B and C were 35±5, 30±5 and 50±10 respectively.

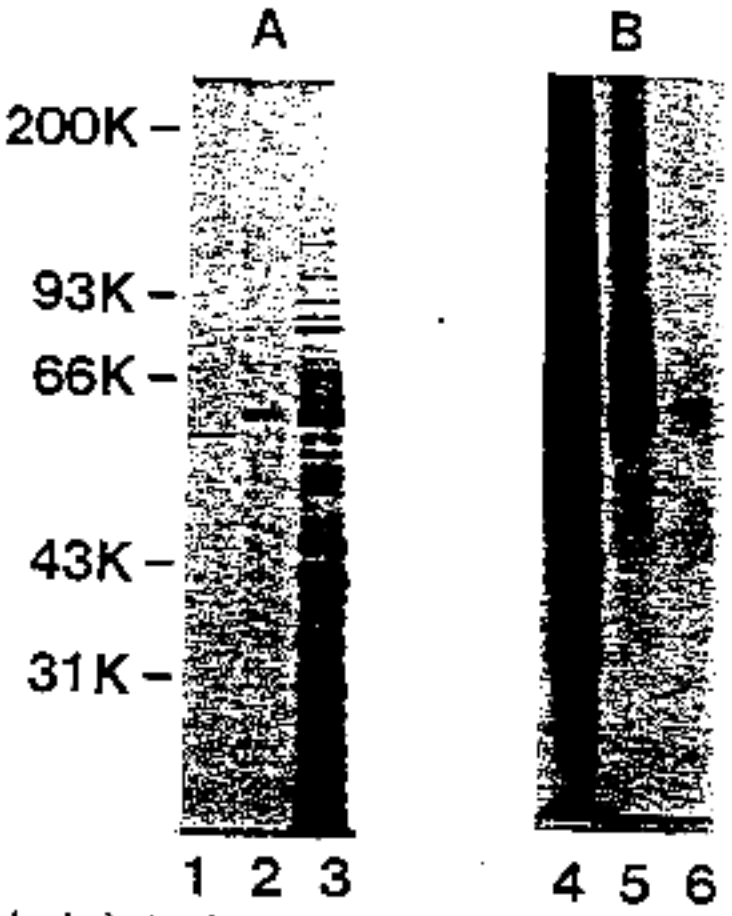


Fig. 4. Analysis of various purified protein fractions from SK-N-SH cells by polyacrylamide gelelectrophoresis. The digitonin-solubilized proteins and purified protein fractions were identified on 7.5 % SDS (A) and non-SDS (B) polyacrylamide gel. Lanes 1 and 6, total digitonin solubilized proteins; lanes 2 and 5, protein fractions eluted from WGA-sepharose 6B; lanes 3 and 4, protein fractions eluted from fentanyl-affinity gel.

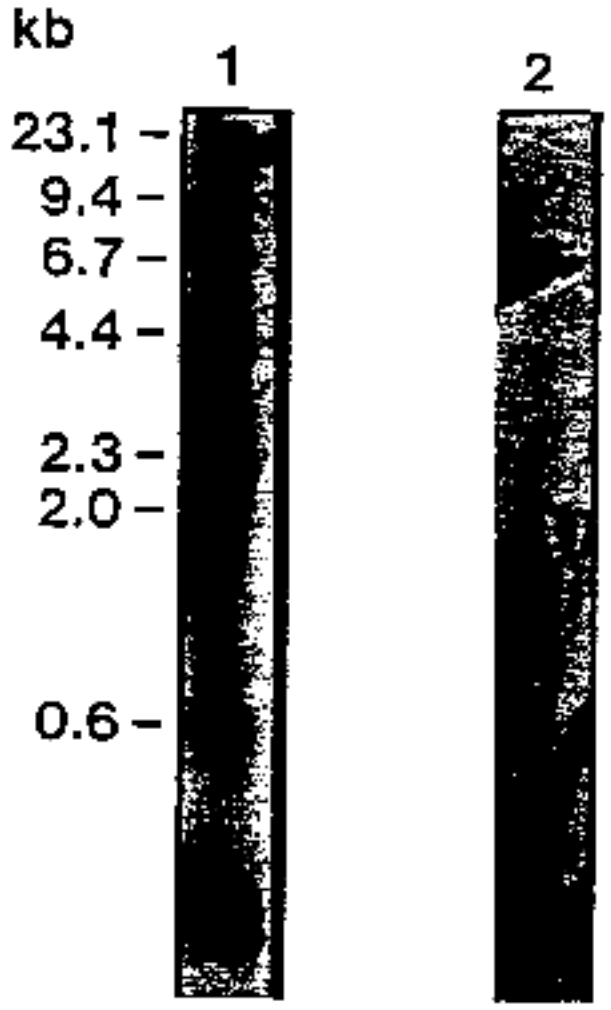


Fig. 5. Autoradiograph of ^{32}P-labeled double stranded cDNA synthesized from SK-N-SH poly(A) mRNAs. Electrophoresis was performed on an alkaline agarose (1.4 %) gel. Lane 1, molecular weight markers prepared from end-labeling of λDNA digested with Hind III; lane 2, double stranded cDNA synthesized from poly(A) mRNAs. Exposure of gel was for 18 hours without intensifying screen at -80 C.

fentanyl analog reduced predominantly the binding sites labeled with naloxone, these results clearly suggest that it is covalently attached to the μ opiate receptor.

Purification of fentanyl-binding proteins. Quantitative solubilization of proteins from SK-N-SH cells was achieved with 2.0 % digitonin. This detergent solubilized ~50 % of the total cellular proteins. Recent studies {12,21} suggested that the opiate receptor contains glycosylated residues that enabled the protein to bind to WGA- affinity column. The purification of opiate binding proteins from SK-N-SH cells is summarized in Table I. After solubilized proteins from SK-N-SH were subjected to fractionation on WGA-sepharose, a ~10 fold purification of protein with a 3.8 % protein recovery was routingly achieved on elution with 0.5 M N-acetylglucosamine. The eluated proteins were further purified by affinity gel containing a fentanyl derivative as an affinity ligand to the receptors. The bound receptors were recovered by elution with 10 μM naloxone. This procedure resulted greater than 10,500 fold protein purification and a yield of less than 0.001 %. The SK-N-SH samples at different purification steps were analysed by SDS-polyacrylamide gel electrophoresis (Fig. 4A). A major band with a MW of 58,000 was identified by silver staining. Several smaller minor bands which could have been arisen from proteolysis of the protein of 58,000 molecular weight, were also observed. In another control experiment, an electrophoretically homogeneous band was seen in a non-denatured gel electrophoresis (Fig. 4B). These results strongly suggest that the final purified protein is a distinct opiate binding material.

Table I. **Summary of Opiate Receptor Purification**

Step	Total Protein μg	Recovery %	Specific Binding pmol/mg protein	Purification (fold)
Digitonin-extract	50,000	100	0.026	-
WGA-sepharose eluate	1,900	3.8	0.245	9.6
Affinity column eluate*	<0.3	<0.001	>272.0	>10,500

^{3}H-naloxone (1.5×10^{-9} M) binding were carried out as described in "Experimental Procedures".
*Protein concentration was estimated from silver-stained, SDS-polyacryamide gel using BSA as a standard.

Construction of the cDNA library. Oligo-dT primed cDNA library of SK-N-SH was generated from poly (A) mRNA isolated in guanidine isothiocyanate. The first and most important step in this cDNA cloning procedure is the efficient synthesis of ds-cDNA representing as much of the original mRNA sequence as possible. The modified procedure of Gubler and Hoffman (27) offered by Amersham's cDNA system provided the convenient and efficient approach to obtain a high percentage of full length cDNA. Following this protocol, the second strand cDNA syntheis was initiated by RNase H digestion of the RNA:DNA hybrid formed during first strand synthesis. The second strand cDNA synthesis with radioactive label ^{32}P incorporated only in the second strand was then analysed on a 1.2 % alkaline agarose gel. As shown in Fig. 5, the size distribution of ds-cDNA obtained was around 0.5 to 6.0 kb with an average of 2 kb.

The ds-cDNA was cloned into λgt 10 cloning vectors to generate cDNA library through a long, extensive procedure. Briefly, the ds-cDNA was first methylated by EcoR I methylase to protect any internal EcoR I site. After EcoR I linker addition, the ds-cDNA was then inserted into EcoR I-digested, alkaline phosphatase-treated λgt 10 DNA and packed into E. coli c600 Hfl (see "Experimental Procedures"). By using the λgt 10 arms and packaging extracts purchased from "Promega", the packaging efficiencies of approximately 4×10^{6} inserts containing plaques per μg λgt10 arms were achieved.

DISCUSSION

Photoaffinity labeling techniques are widely used in mapping the ligand binding sites of receptors, enzymes and antibiotics (30). The photoaffinity reagents permit the measurement of reversible binding as well as covalent attachment of ligand to the binding site. Among these reagents, the photoreactive

carbenes have the advantage of higher energy of greater reactivity, whereas the nitrene precursors are usually photolysed by radiation at longer wavelength where damage to the target sites is less severe. In addition, azides, which are common precursors for nitrenes, are smaller and therefore less likely to interfere with biding of the ligand to the receptor site. The fentanyl analog designed in the present study has two distinct features. The analog itself is a fentanyl and provides high specific binding to opite receptors. The azide functional group attached to the phenyl ring is chemically inert but forms a highly reactive nitrene during photolysis. These characteristics permit the ligand to bind to the opiate receptor irreversibly.

The fentanyl analog was shown to bind reversibly only to the μ opiate receptor of SK-N-SH cells. After incubating the cells with the fentanyl analog, ^{3}H-naloxone effectively chased all the label from the receptors. Covalent attachment of the fentanyl to the receptors occured only when the fentanyl analog was reversibly bound to the receptors and photolysed subsequently. On the other hand, the binding of δ receptors on the NG 108-15 cells was not affected under the same condition. These results clearly suggest that this fentanyl analog blocks the opiate receptors of SK-N-SH for ^{3}H-naloxone and therefore is predominately labeling μ opiate receptors.

One important problem in photoaffinity experiment is photodestruction of opiate receptor. Because irradiated receptor cells were found to lose binding activity with time (Fig. 2A and 2B), the photoactivation must be completed within a short interval (90 seconds in present study) to minimize receptor destruction. The ability to regulate the labeling by controling the irradiation permits the effects of covalent attachment to be differentiated from those of reversible binding.

Recently, considerable progress in opiate receptor purification has been made by affinity chromatography (12,13,21,22). In our present study, solubilization of culture cells was perfomed to minimize the significant loss of receptors during repeated centrifugation of the membrane preparations. After WGA-sepharose chromatography to eliminate non-specific proteins, the receptor was further purified by an affinity chromatography. This major purification step utilized affinity gel that had a fentanyl derivative as an affinity ligand for the receptors. In this procedure, only the protein fraction from SK-N-SH cells that binds to the gel can be eluated with high concentration of naloxone. The purified preparation revealed a major band with a molecular weight of ~58,000 as determined by SDS-polyacrylamide gel electrophoresis. The result agrees well with that reported for a μ subtype opiate receptor from rat brain identified by other groups (20,21,31). The data thus suggested that the purified preparation contained genuine opiate receptors.

Although the purified opiate receptor from SK-N-SH cells has not been characterized completely, it should be useful for structural studies. Determination of amino acid sequence of a peptide fragment of this protein fraction will provide information for synthesis of a oligonucleotide probe with which to clone the cDNA coding for the opiate receptor. Furthermore, amino acid sequence data will allow synthesis of peptide fragment for use as antigens to generate antibodies of predefined specificity (32). In the present study, the cDNA library for the SK-N-SH cells was prepared with λgt 10 cloning vector. The size of cDNA sequence in this library was around 0.5 to 5.0 kb. This library will be used for future screening of opiate receptor genes with synthetic oligonucleotide probes. Cloning of such a gene will permit genetic manipulation of its expression in a suitable host so as to produce ample quantities of protein for subsequent development and production of a genetic sensor for fentanyls and other related incompacitants.

CONCLUSIONS

1. The photoaffinity ligand (azido-fentanyl) inhibited the binding of naloxone to the mu opiate receptors of SK-N-SH cells.

2. A ~58,000 MW protein fraction was purified from a digitonin-solubilized preparation of SK-N-SH cells by the use of affinity gel column, which has a fentanyl as an affinity ligand to the receptors.

3. The cDNA library for the SK-N-SH cells was prepared with λgt 10 cloning vector. This library will be used for future screening of opiate receptor genes.

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