Volume 224, number 2, 272–276 November 1987

Immunoaffinity-purified opiate receptor specifically binds the δ-class opiate receptor ligand, *cis*-(+)-3-methylfentanylisothiocyanate, SUPERFIT

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Received 19 September 1987

Using an antibody generated against the opiate receptor on NG108-15 cells, we recently purified the putative receptor from this hybrid cell line. We herein report that the purified receptor complex specifically binds tritiated *cis*-(+)-3-methylfentanylisothiocyanate (SUPERFIT), with the predominant binding associated with a 58 kDa polypeptide chain. Consistent with these findings is the in situ labeling of a 58 kDa protein with [^{3}H]SUPERFIT on NG108-15 cells.

Complementary peptide; *cis*-(+)-3-Methylfentanylisothiocyanate; Opiate receptor

1. INTRODUCTION

Opiate receptor heterogeneity has, for the most part, complicated the isolation and characterization procedures of the receptor complex. However, with the advent of highly specific opiate receptor ligands, it has become possible to identify binding subunits of the receptor(s) on various tissues. One such ligand, *cis*-(+)-3-methylfentanylisothiocyanate (SUPERFIT) [1] has been used to isolate the binding polypeptide chain of the δ-class opiate receptor from the neuroblastoma × glioma hybrid cell line, NG108-15 [2]. The apparent molecular mass of this binding protein, 58 kDa, is in agreement with what other investigators have observed [3,4]. Although it is evident that the predominant binding chain of the δ-class opiate receptor is the 58 kDa polypeptide chain, other chains which are not responsible for recognition of ligands but have other intrinsic values to the receptor complex would not necessarily be isolated by this procedure. However, by making an antibody to the receptor complex, it would be possible to isolate all the constituents of the complex and determine which chain(s) is(are) responsible for ligand recognition.

Recently, we have developed an antibody to the opiate receptor complex from NG108-15 cells [5] by the use of the complementary peptide methodology [6]. This methodology is based on the Molecular Recognition Theory which states that binding sites of interacting proteins can be encoded by complementary strands of DNA and has been employed by ourselves and others [7,8] to investigate ligand-receptor interactions. We reported that in addition to a 58 kDa polypeptide (pertaining to the opiate receptor) not unlike that described by others, we isolated three other polypeptide chains (68, 45, 30 kDa) which appeared to be non-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

covalently associated. In order to determine if the immunoaffinity purified receptor still possessed opiate-specific characteristics as well as to determine the binding chain, we employed SUPERFIT.

2. MATERIALS AND METHODS

2.1. *Crosslinking cell surface proteins in NG108-15 cells*

The crosslinking procedure used has been previously described [9]. Briefly, 5×10^7 NG108-15 cells were pelleted and the reducible homobifunctional crosslinker, dithiobisphenylazide (Sigma, St. Louis, MO) was added to a final concentration of 15 mM in PBS-1.0% dimethyl sulfoxide (DMSO) at 4°C. The crosslinker was activated for 2 min using an ultraviolet light source (Ultraviolet Illuminator) with peak emission being 350 nm. After irradiation, cells were washed in PBS and subsequently solubilized with lysis buffer which consists of 1.0% 3-[3-cholamidopropyldimethylammonio]-1-propanesulfonate (CHAPS, Sigma)/0.5 M NaCl/0.1 mM phenylmethylsulfonyl fluoride.

2.2. *Immunoaffinity chromatography of NG108-15 cells*

The method for coupling ligands to CNBr-activated Sepharose 4B is as described in Pharmacia Fine Chemicals publication (affinity chromatography) (Uppsala). Dithiobisphenylazide-crosslinked NG108-15 cell lysates were spun at $700 \times g$ for 10 min to remove cell debris. The supernatant fluid was added to phosphate-buffered saline (PBS)-0.05 M NaN_3 (pH 7.4) at a 1:6 volume ratio and passed over an anti-opiate receptor antibody affinity column. Material which bound to the column was eluted with PBS-0.05 M NaN_3 (pH 2.0–2.5) and collected in 50 ml Falcon tubes (Fisher, Norcross, GA). The pH of the eluted solution was adjusted to 7.0 by the addition of 2 N NaOH. The material was then hydroextracted with polyethylene glycol (molecular mass range 15–20 kDa) in dialysis tubing (molecular mass cutoff 12–14 kDa, Fisher, Norcross, GA). Material was subsequently analyzed by polyacrylamide gel electrophoresis.

2.3. *In situ labeling of NG108-15 opiate receptor*

Approx. 1×10^7 cells/ml in 0.05 M K_2HPO_4 (pH 8.0) were incubated with *cis*-(+)-3-[^{3}H]-methylfentanylisothiocyanate (SUPERFIT) (1.02 μM, spec. act. 7.85 Ci/mmol) in the presence or absence of unlabeled SUPERFIT (0.11 mM) for 45 min at 37°C with agitation every 10 min. Cells were centrifuged for 5 min ($200 \times g$) and lysed in denaturing buffer (0.062 M Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) for 5 min. Samples were then boiled for 3 min and analyzed by electrophoresis on a 10% polyacrylamide gel. Gels were then dried and subjected to autoradiography (10 days).

2.4. *Specific opiate binding of [^{3}H]SUPERFIT by crosslinked, immunoaffinity-purified receptor*

Crosslinked, immunoaffinity-purified NG108-15 (40 μg) receptor was incubated with [^{3}H]-SUPERFIT (1.02 μM) in the presence or absence of unlabeled SUPERFIT (0.20 mM) or morphine (1.9 mM) at 37°C for 30 min in 0.05 M K_2HPO_4 (pH 8.0) containing 5 μg/ml bacitracin. The resulting sample was dialyzed for 18 h in running buffer to remove free [^{3}H]SUPERFIT and hydroextracted with polyethylene glycol. This material was electrophoresed on 10% polyacrylamide gels under reducing (0.5 M 2-mercaptoethanol) conditions. The fixed, dried gel was subjected to autoradiography (10–14 days).

2.5. *Polyacrylamide gel electrophoresis of purified receptor*

The discontinuous buffer system of Laemmli [10] was used for electrophoresis of proteins on SDS-polyacrylamide gels containing 10% acrylamide. Samples were boiled for 3 min in 2% SDS, 0.062 M Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue and applied to a 5% polyacrylamide stacking gel. Electrophoresis was carried out at a constant current of 25 mA for 4–5 h. After electrophoresis, the gels were fixed in 40% methanol-10% acetic acid and dried. Prestained molecular mass standards [myosin (200000), phosphorylase *b* (97400), bovine serum albumin (68000), ovalbumin (43000), α-chymotrypsinogen (25700), β-lactoglobulin (18400), and lysozyme (14300)] (Bethesda Research Laboratories, Gaithersburg,

MD) were run with each gel to generate a plot of log M_r vs R_f for determination of the M_r of the radiolabeled protein species.

3. RESULTS

3.1. *Purified receptor from NG108-15 cells specifically binds SUPERFIT*

Our previous results suggested that the anti-receptor antibody could recognize the opiate receptor complex from NG108-15 lysates. Since we had previously shown that the receptor was composed of more than one polypeptide chain, we sought to determine which polypeptide chain or chains were associated with the binding site. NG108-15 cells were in situ labeled with [^{3}H]SUPERFIT in the presence or absence of unlabeled SUPERFIT. Labeled cells were lysed and the lysates were subjected to polyacrylamide gel electrophoresis. The resulting gel was dried and specific binding of the ligand was determined by autoradiography of the gel. The results (fig.1A) indicate that the 58 kDa chain of the opiate receptor is responsible for the recognition of SUPERFIT. Furthermore, the binding is specific since unlabeled SUPERFIT competed with labeled SUPERFIT (fig.1B). The results we obtained confirm previous observations [2,3].

In order to determine whether immunoaffinity-purified receptor had the same characteristics once isolated from the NG108-15 cells, the above procedure was conducted on such purified material. Thus, cell surface proteins from NG108-15 cells were crosslinked with the reducible crosslinker, dithiobisphenylazide as in [5] and the putative opiate receptor was purified by immunoaffinity chromatography. Isolated receptor was then incubated with [^{3}H]SUPERFIT in the presence or absence of nonlabeled SUPERFIT or morphine (μ-receptor ligand). The resulting material was analyzed by electrophoresis on polyacrylamide gels and the resultant gels were dried and subjected to autoradiography to determine which chain(s) was(were) responsible for the recognition of SUPERFIT. The results (fig.2) indicate that the 58 kDa chain is the major binding chain of the δ-class opiate receptor from NG108-15 cells. In addition, some radioactivity was also associated with the 30 kDa polypeptide chain in a relatively specific manner.

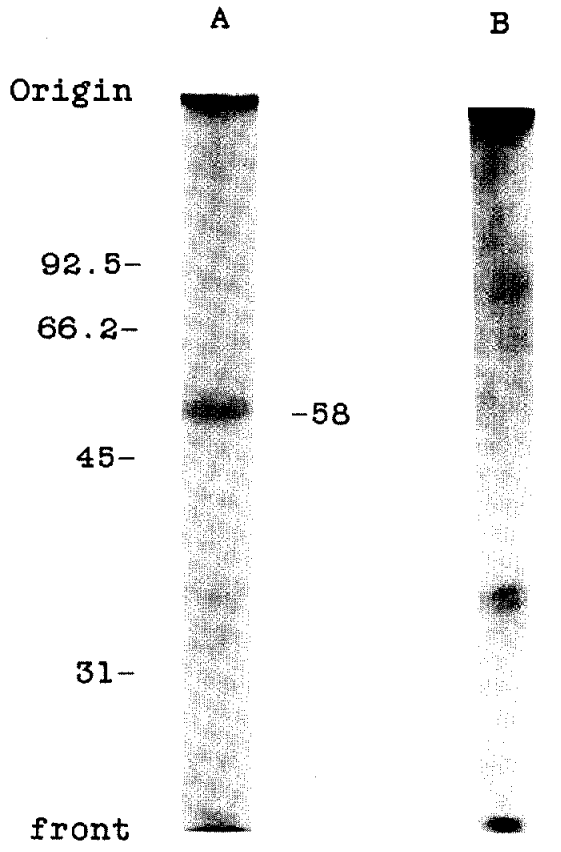

Fig.1. [^{3}H]SUPERFIT-labeled opiate receptor from NG108-15 neuroblastoma × glioma hybrid cells. Autoradiogram of a 10%, reduced (2-mercaptoethanol) polyacrylamide gel exposed for 10 days. (A) [^{3}H]SUPERFIT-labeled NG108-15 cells, (B) [^{3}H]-SUPERFIT-labeled NG108-15 cells in the presence of excess unlabeled SUPERFIT.

In order to establish whether it was necessary to crosslink the receptor complex prior to immunoaffinity chromatography to maintain the integrity of the receptor and its binding chain, noncrosslinked purified opiate receptor was analyzed for its ability to bind the ligand, SUPERFIT. The results (not shown) indicate that like crosslinked, purified receptor, noncrosslinked, purified receptor had the capability of binding SUPERFIT with specificity although the efficiency of binding was less (20–30%). This strongly suggests a role for the intact receptor complex with regard to maximal binding capacity.

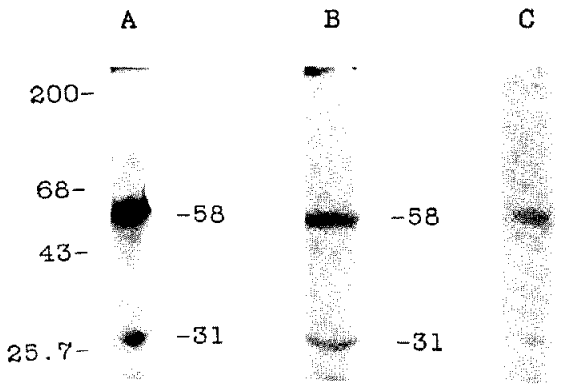

Fig.2. Autoradiography of electrophoresed, immunoaffinity-purified [^{3}H]SUPERFIT-labeled opiate receptors from NG108-15 cells. Crosslinked (DTBP) immunoaffinity-purified receptors (40 μg) were incubated with 1.02 μM [^{3}H]SUPERFIT in the presence or absence of unlabeled SUPERFIT (2×10^{-4} M) or morphine (1.9×10^{-3} M) for 45 min in 0.05 M K_2HPO_4 (pH 8.0). The resultant material was dialyzed overnight in phosphate-buffered saline (pH 7.4) to remove free radiolabeled ligand. Samples were hydroextracted with polyethylene glycol and applied to polyacrylamide gel electrophoresis. The resultant gels were dried and subjected to autoradiography for 96 h. (A) Labeled SUPERFIT only, (B) lane A + morphine, (C) lane A + SUPERFIT.

4. DISCUSSION

We had previously reported the purification of the opiate receptor from NG108-15 cells to apparent homogeneity with an antibody to the opiate receptor [5] developed by the application of the complementary peptide approach [6]. In that report, we demonstrated the ability of the antibody to compete for the same binding site as β-endorphin on NG108-15 cells by a radioreceptor assay and ELISA procedure. In addition, our data indicated that the receptor was a complex composed of four polypeptide chains with molecular masses of 68, 58, 45, and 30 kDa. In order to establish further that we had isolated the putative opiate receptor from NG108-15 cells, we determined whether purified receptor had the ability to recognize and bind the δ-class opiate ligand, SUPERFIT. The results indicate the purified receptor bound SUPERFIT with specificity and furthermore, the predominate binding polypeptide chain had a molecular mass of 58 kDa. That the 58 kDa chain of the immunoaffinity-purified receptor is the major binding subunit of the opiate receptor tends to confirm the in situ labeling of NG108-15 cells we (this work) and others [2,3] have observed.

In addition to the specific labeling of the 58 kDa protein subunit of the crosslinked, purified material, we also observed the labeling of a 30 kDa protein. That this protein is not labeled in situ suggests that the crosslinking procedure draws the 30 kDa chain in close enough proximity to the 58 kDa protein that it too is labeled with SUPERFIT in a specific manner. Therefore, we believe the labeling of this band is an experimental artifact but relatively important in that it suggests the 30 kDa chain is closely associated with the binding chain.

In addition to the 58 and 30 kDa proteins, we have also reported that the receptor consists of 68 and 45 kDa protein chains. Although we do not know the function of these subunits, they may play a role in binding other ligands or in mediating secondary events (i.e. phosphorylation or (de)activation of secondary messengers). Other investigators have reported opiate-binding proteins with similar molecular masses. For example, in 30% purified preparations of an opiate receptor-fentanylisothiocyanate (FIT) complex from NG108-15 cells recognized by an anti-FIT immunoglobulin, observation of 45 and 31 kDa proteins was described [3]. In addition, using an avidin-biotin system, other investigators have reported the purification of the δ-class opiate receptor to consist of proteins of 58, 65, and 71 kDa [11]. Recently, the demonstration of a 46 kDa protein to be common to all opiate receptor classes [12] (including μ, $\varkappa$, and δ) along with reports by others [13–18] suggesting that the opiate receptor(s) consist(s) of proteins ranging in molecular mass from 70 to 23 kDa, seems to indicate the probability that more than one constituent is associated with the opiate receptor binding chain and/or subunit structure.

Our results using morphine to block SUPERFIT binding to the receptor suggest that μ-specific ligands may recognize the same binding chain as the δ-specific ligand, but interact with a different portion. This hypothesis is not without precedent since it has recently been demonstrated that the μ-type opiate receptor binding site has an apparent

molecular mass of 58 kDa [19,20]. In addition, we suggest that crosslinked, purified receptor binds SUPERFIT more efficiently than noncrosslinked receptor. Such an observation may be due to the ability of the crosslinked complex to remain intact with its lipid moieties since it is believed that lipid plays an important role in opiate binding [21,22]. Finally, our data seem to substantiate the results of other investigators working on opiate receptor purification. That our results are similar and in some cases, identical with those of others, seems to demonstrate the fidelity of the complementary peptide methodology and its application in ligand-receptor interactions.

ACKNOWLEDGEMENTS

We thank Diane Weigent for typing this manuscript. This research was supported by Triton Biosciences, Inc.

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