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Photoaffinity labeling of a 33 kDa protein subunit of the δ-opioid receptor in neuroblastoma and hybrid cell lines

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Tritiated DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr) binds with high affinity, specificity and saturability to neuroblastoma $N18TG_2$ and hybrid neuroblastoma × glioma NG108-15 and NG108-5 intact cells. The δ-opioid receptor density in cells cultured in chemically defined medium was increased about 2 times compared to that in cells cultured in 10% fetal calf serum. A major and a minor protein species covalently and specifically bound to [^{125}I]azido-DTLET (Tyr-D-Thr-Gly-pN_3Phe-Leu-Thr), photoactivatable ligand, migrated on SDS-gel electrophoresis with M_r values near 33000 and 58000, respectively.

Opioid receptor; Photoaffinity ligand; Defined medium; Gel electrophoresis; (Mouse neuroblastoma, NG108-15 cell line)

1. INTRODUCTION

Mammalian brain has been shown to contain at least three types (δ, μ, κ) of opioid receptors. The experimental complexity and heterogeneity of the system can be reduced by using neurotumor hybrid cell lines which possess specific membrane-associated receptors for neurotransmitters [1]. The neuroblastoma-glioma hybrid cell line NG108-15 carries only a single type of opioid receptor with δ-opioid receptor characteristics [2].

Characterization of the proteins carrying δ-binding sites in various tissues has been reported by several authors using selective ligands [3,4]. The photoactivatable opioid agonist azido-DTLET (Tyr-D-Thr-Gly-pN_3Phe-Leu-Thr) has been found to be a potent and specific probe for the irreversible labeling of δ-opioid receptors in rat brain membranes [5,6] (Bochet et al., submitted).

The purpose of this study was to grow tumorigenic cell lines of neural origin in various cell culture conditions to enhance the number of receptor sites, and to compare the use of the δ-selective opioid agonist DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr) and its photoactivatable iodinated derivative azido-DTLET to label the δ-opioid receptors of neuroblastoma and neuroblastoma × glioma hybrid cells. We found enhanced binding in cells grown in serum-free medium (SFM) to a protein whose apparent M_r has not been previously reported.

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2. MATERIALS AND METHODS

2.1. *Cell lines*

The initial stock of the mouse neuroblastoma $N18TG_2$ and rat glioma C_6BU-1 parent cell lines of the hybrid NG108-15 and NG108-5 cell lines, was a gift from Drs Reiser and Hamprecht (University of Tubingen, FRG). Cells were used between passages 20 and 25. The subclone NG108-15-H_2 was obtained by the glass-chip technique [7]. The C_6 rat glioma cells were supplied by Dr Faivre-Bauman (Collège de France, Paris, France).

2.2. *Cell culture conditions*

The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 4.5 g glucose/l, supplemented with 3.7 g/l sodium bicarbonate, 2 mM L-glutamine, with and without 10% Myo-clone fetal calf serum (FCS) (Gibco) and HAT (0.1 mM hypoxanthine, 10 μM aminopterine and 16 μM thymidine). Cultures were maintained without any use of an-

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tibiotics, in a humidified atmosphere of 5% CO_2 and 95% air and subcultivated in 75 cm^2 Corning flasks at a 1:2 split ratio. Cells were detached mechanically from their support and not enzymatically with trypsin which in some cases leads to a loss of opioid receptors [8].

When cells were grown in serum-free chemically defined medium, DMEM was supplemented with NCTC-135 medium (4%) (Gibco), 2 mM glutamine, 8 μg/l sodium selenite, 4 mg/l insulin, 3 mg/l transferrin and 4 μg/l epidermal growth factor (EGF).

Differentiation of the cells was induced 4 days after seeding either by removing the FCS or EGF and transferrin, by medium change, or by adding 200 mM ethanol. The flask was tightly closed after addition of the ethanol to minimize evaporation.

2.3. *Binding assay on whole cells*

Cells at confluency were harvested by shaking them from the sides of the flask in the growth medium and centrifuging at 400 × *g* for 8 min. The pellets were washed twice with 50 ml of solution D_1 (137 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, 5.5 mM glucose, 0.22 mM KH_2PO_4) and resuspended in a Tris-sucrose solution (0.3 M sucrose, 0.01 M Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.75 M NaCl) containing protease inhibitors (50 mM EGTA, 5 μM leupeptin, 50 μM soybean trypsin inhibitor and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)), according to Diekmann-Gerber et al. [9]. The cell suspensions were kept on ice prior to the binding assay.

The incubation (500 μl total volume) was performed in 50 mM Tris-HCl, pH 7.4, at 37°C by adding 100 μl of cell suspension (3.5 × 10^5 cells) to 7 different concentrations (0.6, 1.3, 2.5, 4.8, 9.4, 18, 35 nM) of [^{3}H]DTLET (60 Ci/mmol) (CEA, Saclay, France). Experiments were performed in triplicate. The non-specific binding was determined in the presence of levorphanol 10 μM final concentration in the incubation mixture. 20 min later, the reaction was stopped by filtration over GF/B Whatman filters presoaked in 50 mM Tris-HCl, pH 7.4, containing 0.1 mg/ml bovine serum albumin (BSA). Dry filters were counted in aquasol (NEN) in an LKB scintillation counter.

2.4. *Photolabeling of the cells*

The cell suspension (5 × 10^5 cells) in 400 μl Tris-sucrose solution (see above) was incubated in the dark with 200 pM [^{125}I]azido-DTLET (2000 Ci/mmol) for 20 min at 37°C in a shaking waterbath. After washing the cell pellets twice in the Tris-sucrose buffer, by centrifugation at 800 rpm, cells were UV irradiated on ice for 10 min at 254 nm with a spectroline UV lamp (77 μW/cm^2). Radioactivity was determined using an LKB gamma counter.

2.5. *Membrane preparation*

Following the photoaffinity labeling of the receptors, the intact cells were incubated 30 min on ice in a hypotonic solution (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$), then homogenized in a Dounce glass potter (about 20 strokes) and centrifuged 10 min at 2000 rpm at 4°C. The supernatant was centrifuged 1 h at 160000 × *g* at 4°C. The pellets were kept frozen at −20°C prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein concentrations were determined by the amidoblack technique according to Schaffner and Weissmann [10].

2.6. *SDS-PAGE analysis*

The resolving gels, linear gradient 5–12% acrylamide, were performed according to a modified method first described by O'Farrel [11]. Samples (50 μl) in sample buffer (2.3% SDS, 5% β-mercaptoethanol, 5 M urea in 62.5 mM Tris-HCl, pH 6.8) were loaded on a stacking gel containing 4% acrylamide. After migration at 15°C, gels were fixed in cold trichloroacetic acid (10%), stained with Coomassie blue R 0.25% in methanol/acetic acid/water (9:2:9, v/v), destained in ethanol/acetic acid/water (3:1:6, v/v), then dried on What-

Table 1

Specific binding of [^{3}H]DTLET to neuroblastoma and hybrid cell lines

Cell lines	Passage no.	Culture conditions	K_d (nM)	B_{max} (fmol/mg prot.)	Receptor sites/cell (× 10^3)
NG108-15 (n = 5)	20–25	standard	1.04 ± 0.14	492 ± 77	105 ± 14
NG108-15	24	standard + 200 mM EtOH	1.21	373	71
NG108-15	23	SFM + EGF	1.57	610	105
NG108-15 (n = 3)	23–24	SFM	1.81 ± 0.06	661 ± 125	189 ± 47
NG108-15-H_2	4	standard	1.16	94	19
NG108-5	15	standard	0.54	103	27
N18TG_2	24	standard	1.40	545	41
C_6BU-1	28	standard	ND	ND	ND
C_6	26	standard	ND	ND	ND
C_6	25	SFM	ND	ND	ND

Values ± standard errors of the mean. EtOH, ethanol; SFM, serum-free medium; EGF, epidermal growth factor; ND, not detectable

man paper in a BioRad slab gel dryer. Dry gels were exposed to Kodak X-ray film for several days at −80°C, using intensifying screens (Dupont, Wilmington, DE, USA).

3. RESULTS

The time course of δ-opioid binding indicated that the maximal binding of [^{3}H]DTLET to NG108-15 cells for incubation at 37°C was observed at 20 min. Longer incubations led to a gradual decrease in binding.

The specific binding of [^{3}H]DTLET was tested with various cell lines derived from the nervous system as shown in table 1. Saturable and specific binding was obtained in whole cells. K_d and B_{max} values were derived from saturation experiments. Labeling specificity of the peptide was defined as labeling prevented when 10 μM of levorphanol was present during the binding assay. Fig.1 shows a typical experiment on NG108-15 cells cultured in standard conditions, i.e., with 10% fetal calf serum in the culture medium. Specific binding sites for [^{3}H]DTLET were found with the hybrid cell line NG108-15 originating from the fusion of the neuroblastoma N18TG$_2$ and the rat glioma C$_6$BU-1 with an average of 105 × 10^3 sites/cell. Culturing in serum-free medium in the presence of epidermal growth factor (EGF) did not alter the opioid affinity for the receptor nor the maximum binding capacity with values for K_d = 1.57 nM and B_{max} = 610 fmol/mg protein, versus averages of 1.04 and 492, respectively, for cells cultured in the presence of 10% fetal calf serum. The number of [^{3}H]DTLET binding sites/cell increased 1.8-fold when the NG108-15 cells were maintained in defined medium. However, ethanol-treated cells, in our culture conditions, behaved like the controls in that they did not gain receptor sites as reported by Charnass et al. [12]. The subclone NG108-15-H$_2$ had a reduced number of receptors but of the same affinity as the original clone NG108-15. Relatively few specific enkephalin binding sites were detected under these conditions using a sister neuroblastoma × glioma hybrid cell line NG108-5. δ-Opioid receptors were not detected on C$_6$BU-1 or C$_6$ cells.

Iodinated azido-DTLET was used to label the δ-opioid receptor. Covalent binding of the [^{125}I]azido-DTLET-receptor complex was obtained following UV irradiation. Approx. 2% of the

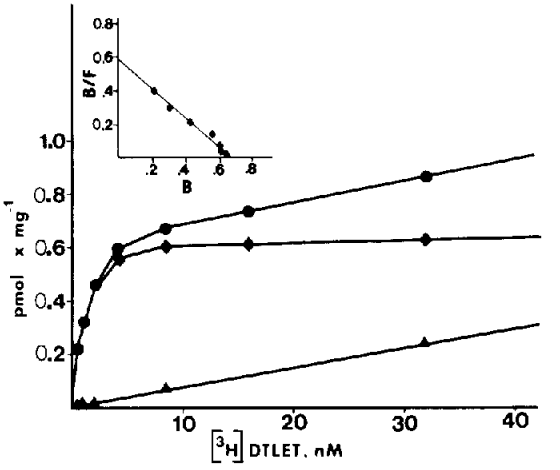

Fig.1. Saturation curve and Scatchard analysis for the binding of [^{3}H]DTLET at concentrations between 0.6 and 35 nM to NG108-15 cells at passages 23. Total binding (●), non-specific binding (▲) determined in the presence of 10 μM levorphanol and specific binding (◆) are shown. In this typical experiment, Scatchard analysis of specific binding yielded K_d = 1.14 nM and B_{max} = 669 pmol/mg protein. *B*, bound pmol × mg protein^{-1} × nM^{-1}; *B/F*, ratio bound/free pmol × mg protein^{-1}.

radioactivity of the irradiated ligand remained associated to the membranes after trichloroacetic acid precipitation. There was a good correlation (coefficient = 0.95) between the [^{125}I]azido-DTLET labeling of the cells and the B_{max} calculated from the [^{3}H]DTLET binding to neuroblastoma and hybrid cell lines (fig.2).

Fig.3 shows the results obtained when membranes labeled with [^{125}I]azido-DTLET and UV irradiated were subjected to SDS-PAGE and autoradiography. Equal quantities of membrane proteins (about 100 μg/slot) were applied to the gel. The label was incorporated into several membrane components. A major species of 33 kDa and a minor species of 58 kDa were labeled only in the absence of levorphanol. Other bands of lower intensity were labeled both in the presence or absence of competing opiate (40, 44, 51, 72 and 100 kDa). The 33 kDa protein was always the major labeled membrane component and was comparatively more abundant in the cells cultured in serum-free medium (fig.3A, lane 4) compared to control cells (fig.3B, lane 2) cultured in 10% FCS. The results are reproducible and the patterns are the same whether or not protease inhibitors were present

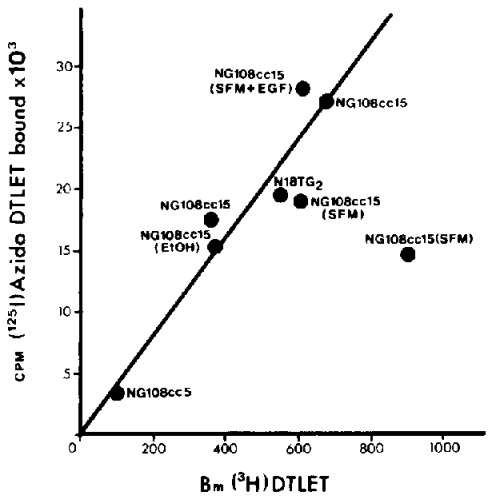

Fig.2. Correlation of cpm bound of [^{125}I]azido-DTLET and B_{max} obtained with [^{3}H]DTLET for different cell lines. NG108-15 (SFM) was not included in the linear regression. SFM, serum-free medium; EGF, epidermal growth factor. See section 2 for cell culture conditions.

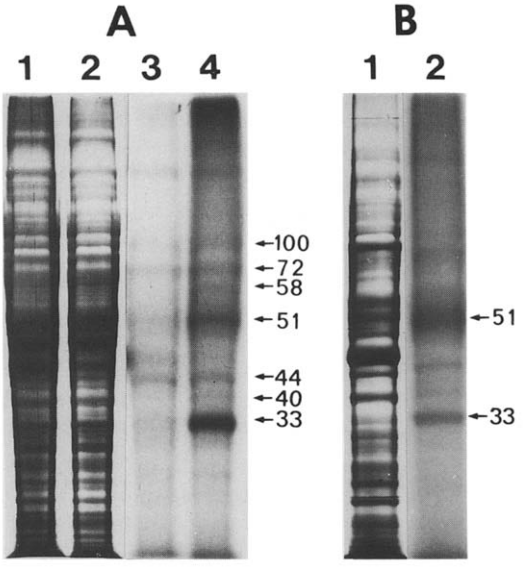

Fig.3. SDS-gel electrophoresis of membrane preparation from NG108-15 cells labeled with [^{125}I]azido-DTLET (5–12% gradient in polyacrylamide). Cells grown in serum-free medium (A), and in serum-containing medium (B). Coomassie blue-stained gels (A, lanes 1,2; B, lane 1) and corresponding autoradiograms (A, lanes 3,4; B, lane 2) after one week exposure. Cells labeled in the absence of competing opioid ligand (A, lanes 2,4; B, lanes 1,2) or in the presence of saturating concentration of levorphanol (10 μM) (A, lanes 1,3). Numbers to the right of the figures are expressed in kDa.

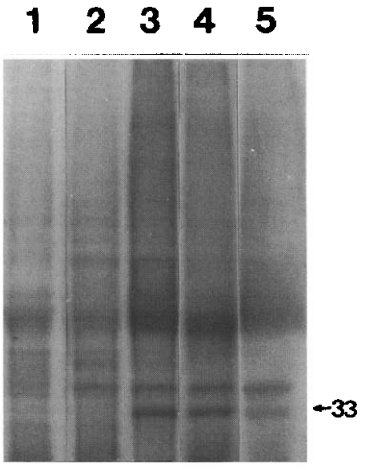

Fig.4. SDS-gel electrophoresis of membrane preparation from cells labeled with [^{125}I]azido-DTLET (5–12% gradient in polyacrylamide). C_6BU-1 rat glioma cells (lane 1); N18TG$_2$ mouse neuroblastoma cells in the presence (lane 2) and absence (lane 3) of 10 μM levorphanol; NG108-15 hybrid cells (lane 4); NG108-5 hybrid cells (lane 5). Numbers to the right of the figure are expressed in kDa. Autoradiograms after one week exposure.

during the preparation of membranes. Equivalent results were obtained with the neuroblastoma N18TG$_2$ cell line (fig.4, lane 3). Comparison between the NG108-15 cells and the sister cell line NG108-5 shows a weaker signal for the band at 33 kDa in the latter (fig.4, lanes 4 and 5, respectively). With the C_6BU-1 cells, we did not obtain any label at 33 kDa (fig.4, lane 1), as for the levorphanol-treated neuroblastoma cells (fig.4, lane 2).

4. DISCUSSION

Previous studies with [^{125}I]azido-DTLET showed that this ligand is specific for the δ-opioid receptor and binds covalently after irradiation to rat brain membrane protein fractions (Bochet et al., submitted).

Our results show that all cell lines bind the opioid peptide with the same affinity but fewer specific sites were observed with parental N18TG$_2$ and sister NG108-5 cell lines than with the NG108-15 cell line. No specific binding was obtained with the other parental C_6BU-1 cell line (table 1). The subclone NG108-15-H$_2$ had a reduced number of receptors, but of the same affinity as the original clone (NG108-15), which may

indicate a lack of homogeneity in this cell population. The binding of DTLET in these cells was blocked by the opiate agonist levorphanol.

Neuroblastoma × glioma hybrid cell lines maintained in defined medium exhibited morphological changes, such as neurite extension as described by Bottenstein and Sato [13]. Serum removal was correlated with a more differentiated morphology along with an increase of specific δ-receptor binding sites. This suggests that we were able to induce differentiation (as defined by the expression of binding sites) of these cells by serum removal and that we can manipulate the abundancy of this receptor subtype on this cell line in the presence of SFM. SFM is a known medium for inducing receptors, like the GM_1 ganglioside [14], which is the receptor for the cholera toxin. The results obtained in serum-free culture conditions are in agreement with those of Griffin et al. [15] who obtained using etorphin as opiate ligand a $K_d = 1.29 \pm 0.22$ nM and $B_{max} = 830 \pm 44$ fmol/mg protein vs a $K_d = 1.75 \pm 0.28$ and $B_{max} = 760 \pm 88$ for serum-cultured NG108-15 cells.

Comparison between the tritiated probe [^{3}H]DTLET and the iodinated photoactivatable derivative [^{125}I]azido-DTLET showed that they both bind with high affinity in a specific manner to the same population of binding sites in these cell lines (fig.2). SDS-PAGE gels indicated that after photoirradiation, the irreversibly bound radioactivity was associated with proteins of approx. 33 and 58 kDa (fig.3). These bands were not detectable when the incubation was performed in the presence of levorphanol. The 58 kDa band, not labeled in levorphanol-treated cells, could be the subunit of the δ-receptor described by Klee et al. [16] and a polymer of the 33 kDa protein, since, according to Simonds et al. [4], the δ-receptor displays a strong tendency to aggregate. Furthermore, the parental neuroblastoma N18TG2 cell line had [^{3}H]DTLET binding sites (41000 sites/cell). When the N18TG2 membrane preparation labeled with [^{125}I]azido-DTLET was subjected to SDS-PAGE analysis, we obtained the same migration pattern as for the NG108-15 cells. With the other parental cell line C_6BU-1, there was no specific binding and no band at 33 kDa. This suggests strongly that proteins of an apparent M_r 33000 for the major species and 58000 for the minor species (in denaturing conditions) might be components of the δ-opioid receptor in mouse neuroblastoma and hybrid mouse neuroblastoma rat glioma cell lines.

Howard et al. [3] reported, using β-[^{125}I]endorphin and bifunctional cross-linking reagents, two species of labeled proteins of 25 kDa and 53 kDa in NG108-15 cells. Simonds et al. [4] reported, in this cell line, a glycosylated protein with an M_r near 58000 using the acylating agent [^{3}H]Super-FIT. We have found here that we can enhance by 2-fold the number of δ-receptor sites/cell, and that the photoactivatable azido-DTLET ligand labeled specifically two proteins of 33 and 58 kDa. To our knowledge, this is the first study to show that a protein of 33 kDa is part of the δ-opioid receptor in the mouse neuroblastoma cells and their derived hybrid cell lines.

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