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# Characterisation of monoclonal antibodies directed against the N-terminus of human mu and delta opioid receptors

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## Abstract

Opioid receptors play an important role in the control of nociceptive pathways. They are involved in drug dependence as well as in a number of pathologies related to pain and inflammation. A better understanding of the underlying cellular and molecular mechanisms constitutes therefore a critical issue to decipher the role of opioid receptors. Such studies often require to acknowledge the presence of the receptor or to localize it within a cell or a tissue. In this context we generated monoclonal antibodies directed against the amino-terminus of human mu and delta opioid receptors. In immunofluorescence experiments, the antibodies detected recombinant opioid receptors expressed in baculovirus-infected insect cells or mammalian cells as well as endogenous opioid receptors expressed in a human neuroblastoma cell line. Also they immunoprecipitated the unglycosylated form of recombinant opioid receptors. Finally anti-mu opioid receptor antibodies could be used to identify non glycosylated receptor in Western blotting experiments.

# 1. INTRODUCTION

Genes coding for delta, kappa and mu opioid receptor types have been identified and isolated from different vertebrates (Li *et al.*, 1996). Analysis of their sequences shows that the receptors belong to the G protein-coupled receptor (GPCR) superfamily. The opioid receptors and endogenous opioid peptides form a neuromodulatory system that plays a major role in the control of nociceptive pathways. Indeed mu opioid receptors are the sole target of morphine (Matthes *et al.*, 1996). The opioid system also modulates affective behaviour, neuroendocrine physiology, and controls autonomic functions such as respiration, blood pressure, thermoregulation and gastrointestinal motility. Importantly the receptors are targets for exogenous narcotic opiate alkaloids, such as heroin, that constitute a major class of drugs of abuse (Bodnar *et al.*, 2003).

The three opioid receptor types show distinct pharmacological profiles but share very similar amino acid sequences especially in the transmembrane regions. The amino- and carboxy-termini are both rather short (about 50 amino acids) as well as the loops connecting the transmembrane $\alpha$ helices (20-25 amino acids). As a consequence, most of the opioid receptor sequence is buried within the membrane which renders the protein poorly immunogenic. Polyclonal antibodies have been raised against various extracellular epitopes located at the amino- as well as carboxy-termini or within the loops connecting the transmembrane domains. We tested several commercial ones directed against extracellular portions of the human receptors but failed to identify any that gave a specific signal in recombinant systems under our experimental conditions. In addition several antibodies raised against a common epitope to human and murine opioid receptors were tested in knock out mice deficient for the corresponding opioid receptor type. All produced patterns in immunohistochemistry and immmunoblot that were identical in knock out and wild type mice.

The lack of specific detection led us to generate monoclonal antibodies on our own. Epitopes located in the amino terminal part of the receptors were chosen since they present several advantages. First this region is very divergent among receptor types and also between species minimising risks of cross-reactivity. Also antibodies directed to the N-terminus could be potentially used for FRET experiments with fluorescently labelled ligands to bring insight into receptor activation mechanisms (Ilien

*et al.*, 2003). Similarly such antibodies could be used to establish spatial proximity between mu and delta opioid receptors, a question relevant to possible modulation of receptor function by heterodimerisation (Daly *et al.*, 2003). In addition the amino-terminus of opioid receptors being located extracellularly, antibodies directed against this region allow detection of the receptors on intact cells. One major drawback however is raised by the possibility that N-glycosylations introduce a steric hindrance that prevents receptor recognition. Opioid receptors are known to be glycosylated (Garzon *et al.*, 1995; Moon *et al.*, 2001) but the glycosylation sites have not yet been unambiguously identified. Several putative N-glycosylation sites of the N-X-S/T types are indeed present within the first 50 amino acids of the sequence.

Here we describe the characterisation of monoclonal antibodies raised against the first 15 amino acids of the human mu and delta opioid receptors in immunofluorescence, immunoprecipitation and immunoblotting experiments using recombinant receptors produced in *E.coli*, baculovirus-infected insect cells or mammalian cells as well as endogenously expressed receptors.

## 2. MATERIAL AND METHODS

*Antibody generation*

Peptides with a sequence corresponding to M-E-P-A-P-S-A-G-A-E-L-Q-P-P-P-C for the human delta opioid receptor (hDOR) and to M-D-S-S-A-A-P-T-N-A-S-N-C for the human mu opioid receptor (hMOR) were synthesised and used as antigens. The two peptides were coupled to Imject maleimide activated ovalbumin (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations then injected with complete Freund's adjuvant to Balb/c mice according to standard procedure (Harlow *et al.*, 1998).

*Receptor expression in E.coli and purification*

hDOR and hMOR were cloned in fusion with a C-terminal hexahistidine tag in a pGEX2a vector (Amersham, Arlington Heights, IL, USA). Expression in *E.coli* BL21

was induced by IPTG 50mM for 6 hours. hDOR and hMOR were purified from inclusion bodies by affinity chromatography using a $Ni^{2+}$ chelating matrix (Amersham, Arlington Heights, IL, USA) following thrombin digestion (Sigma, St Louis, MO, USA).

*Receptor expression in baculovirus-infected insect cells*

*Spodoptera frugiperda* Sf9 cells were maintained in serum-free Insect Xpress medium (Cambrex Bio Science, Walkersville, USA). Generation of recombinant gp-hMOR-C-His baculoviruses was described previously (Massotte *et al.*, 1999). gp-hDOR-C-His recombinant baculoviruses were generated similarly. Briefly the hDOR coding sequence was cloned in frame as a 5'Bam HI - Eco RI 3' fragment in pACgp67A (BD Biosciences, Franklin lakes, NJ, USA) using standard cloning procedures. Recombinant baculoviruses were generated as described for gp-hMOR-C-His. Cells were infected at MOI= 2 and harvested 48 hours post infection.

*Receptor expression in mammalian cells*

pcDNA plasmids encoding the signal sequence -FLAG-DOR and signal sequence -FLAG -MOR constructs were generated using standard cloning procedures. COS cells were maintained in DMEM containing 10% (vol/vol) foetal calf serum and 2 mM glutamine (Invitrogen, Carlsbad, CA, USA). For transient expression, cells were transfected with Jet-PEI reagent (Polyplus transfections, Illkirch, France) according to the manufacturer's recommendations and collected 48 hours later.
Neuroblastoma SHSY5Y cells were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum (Invitrogen, Carlsbad, CA, USA).

*Immunofluorescence on intact cells*

Sf9 cells were harvested, washed with PBS containing 0.32 M sucrose and resuspended in Eppendorf tubes at a concentration of $2x10^8$ cells $ml^{-1}$ then fixed. Mammalian cells were seeded at $2x10^5$ cells on cover slips and let to attach overnight before transfection. 48 hours later cells were fixed. All cell types were washed three times with cold PBS

then fixed in PBS containing 4 % paraformaldehyde for 30 minutes, followed by three washes with PBS containing 0.1% Tween 20. Subsequently, cells were incubated overnight at 4°C in PBS containing 0.1% Tween 20 and 1% BSA with anti-hDOR or anti-hMOR ascitic fluids at a 1:2000 and 1:1000 dilution respectively. Incubation with monoclonal anti-FLAG M1 antibodies was performed at a 1:1000 dilution. Cells were then washed three times with PBS containing 0.1% Tween 20 and incubated for 4 hours at room temperature in PBS containing 0.1% Tween 20 and 1% BSA with secondary anti-mouse antibody (Fab)'$_2$ fragments coupled to FITC (Beckman Coulter, Fullerton, CA, USA) (1:200 dilution). Cells were washed with PBS containing 0.1% Tween 20, nuclei were stained with DAPI (Sigma, St Louis, MO, USA) and cells were washed with PBS. Coverslips were mounted with Mowiol (Sigma, St Louis, MO, USA). Fluorescence was observed using Leica DMLB microscope and confocal images were recorded using a Leica TCS SP1 microscope.

*Preparation of membranes*

Cells were collected, washed twice with PBS and stored at -80°C in PBS containing 320 mM sucrose. Cell pellets were resuspended in ice-cold 50 mM Tris-HCl, 1 mM EDTA, pH 7.4, disrupted using a glass homogeniser and centrifuged at 2000 g for 10 min. The pellet was homogenised in ice-cold 50 mM Tris-HCl, 1 mM EDTA, pH 7.4 and centrifuged at 1000 g for 5 min. Both supernatants were combined and ultracentrifuged at 100 000 g for 40 min at 4°C. The pellet was resuspended in 50 mM Tris-HCl, 1mM EDTA, 320 mM sucrose, pH 7.4 then homogenised through a 26-gauge needle and stored in aliquots at -80°C before use.

*Immunoblotting*

Samples were heated in loading buffer (62.5 mM Tris-HCl, pH6.8, 5% (wt/vol) β-mercaptoethanol, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.1% (wt/vol) Bromophenol blue) for 5 min at 95°C and loaded onto a 12% SDS-PAGE gel. Proteins were transferred onto Immobilon P polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Alternatively for dot blot analysis, 1, 5, 10, 50,100,

500 and 1000 ng of hDOR or hMOR purified from *E.coli* were directly spotted on a PVDF membrane. Following blocking with 3% (wt/vol) non-fat dry milk in 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% (vol/vol) Tween 20 (TBST) for 1 hour, PVDF membranes were incubated overnight at 4°C with anti hDOR or anti hMOR ascitic fluid. PVDF membranes were washed three times for 10 min with 5% (wt/vol) non-fat dry milk in TBST, incubated for 2 h with a 1: 20 000 dilution of HRP-conjugated horse anti-mouse antibody (Vector) in 3% (wt/vol) non-fat dry milk in TBST. PVDF membranes were washed three times for 10 min in TBST. Detection of the hexahistidine tag was performed according to the manufacturer's instructions using INDIA HisProbe (Pierce, Rockford, IL, USA), a nickel activated derivative of horseradish peroxidase optimised for direct detection of recombinant hexahistidine-tagged proteins. Chemiluminescence was detected using $ECL^+$ (Amersham, Arlington Heights, IL, USA) according to the manufacturer's instructions.

*Immunoprecipitation*

Membrane preparations (500 μg) were solubilised in Tris-HCl 50 mM pH7.4, 100 mM NaCl, 10% CHAPS for 1hour at 4°C, immunoprecipitated with either 2 μg anti-his or 1 μl anti-hDOR or anti-hMOR ascitic fluid for 1 hour at 4°C and isolated by incubation with G protein-Sepharose for 1 hour at 4°C. Samples were washed three times with Tris-HCl 50 mM pH7.4 and prepared for electrophoresis on SDS-PAGE gels.

## 3. RESULTS AND DISCUSSION

*Detection of opioid receptors by immunofluorescence*

Antibodies giving a strong positive signal in ELISA using antigenic peptides were tested in immunofluorescence. In a first screen, Sf9 cells infected with a recombinant baculovirus encoding either hDOR or hMOR in fusion with a C-terminal hexahistidine tag (hDOR-C-His and hMOR-C-His respectively) were chosen because of the high level of receptor expression (respectively 8 $10^5$ or 5 $10^6$ receptors/cell). We identified one anti-hDOR and one anti-hMOR clones that generated a specific signal

(Figure 1). Using a 1:200 to 1:20 000 dilution range of ascitic fluid, optimal dilutions were determined as 1:2000 and 1:1000 for anti-hDOR and anti-hMOR antibodies respectively. No signal was present in non-infected cells or cells infected with a wild type baculovirus (data not shown). Also no cross-reactivity was detected when the anti-hDOR antibody was tested using Sf9 cells expressing hMOR-C-His or when the anti-hMOR antibody was tested using Sf9 cells expressing hDOR-C-His (Figure 1). As expected, labelling was clearly visible at the cell membrane. The presence of receptors at the cellular surface was confirmed by radioligand binding assay on intact cells. Immunofluorescence was then performed using COS cells expressing FLAG-tag-hDOR or FLAG-tag-hMOR. Once more, intense labelling was observed at the cell membrane (Figure 2a,b). An anti-FLAG antibody was used to confirm expression and membrane localisation of the opioid receptors. Patterns of labelling obtained with anti-opioid and anti-FLAG antibodies were similar (Figure 2c,d). No signal was detected using anti-opioid antibodies in non transfected COS cells. No cross-reactivity was observed when anti-hDOR antibodies were tested on COS cells expressing hMOR or when anti-hMOR antibodies were tested on COS cells expressing hDOR (data not shown). Also murine delta (mDOR) and mu (mMOR) opioid receptors were not recognised by anti-hDOR and anti-hMOR antibodies respectively confirming species specificity (data not shown).

Both anti-hDOR and anti-hMOR antibodies were also tested on SHSY5Y cells. This human neuroblastoma cell line endogenously expresses delta and mu opioid receptors each at a level of about 100 fmol/mg membrane proteins. Both anti-hDOR and anti-hMOR antibodies labelled the cell membrane as expected (Figure 3a,c). Upon preincubation with 10 mM antigenic peptide, labelling was significantly decreased (Figure 3b,d). This indicates that the signal results from a specific interaction with the antibody. Similarly preincubation with the corresponding antigenic peptide decreased anti-hDOR and anti-hMOR labelling in COS cells (data not shown).

To further assess the specificity of the labelling, cells were treated with opioid agonists. Agonists induce receptor internalisation leading to a drastic reduction of the amount of receptors still present at the cell surface (von Zastrow, 2003). Both COS and SHSY5Y cells were treated with 500 nM of the delta specific agonist deltorphin II for 30 minutes before fixation. In agonist treated cells, labelling at the cell surface disappeared whereas labelling appeared within the cytoplasm (Figure 4). Similarly in

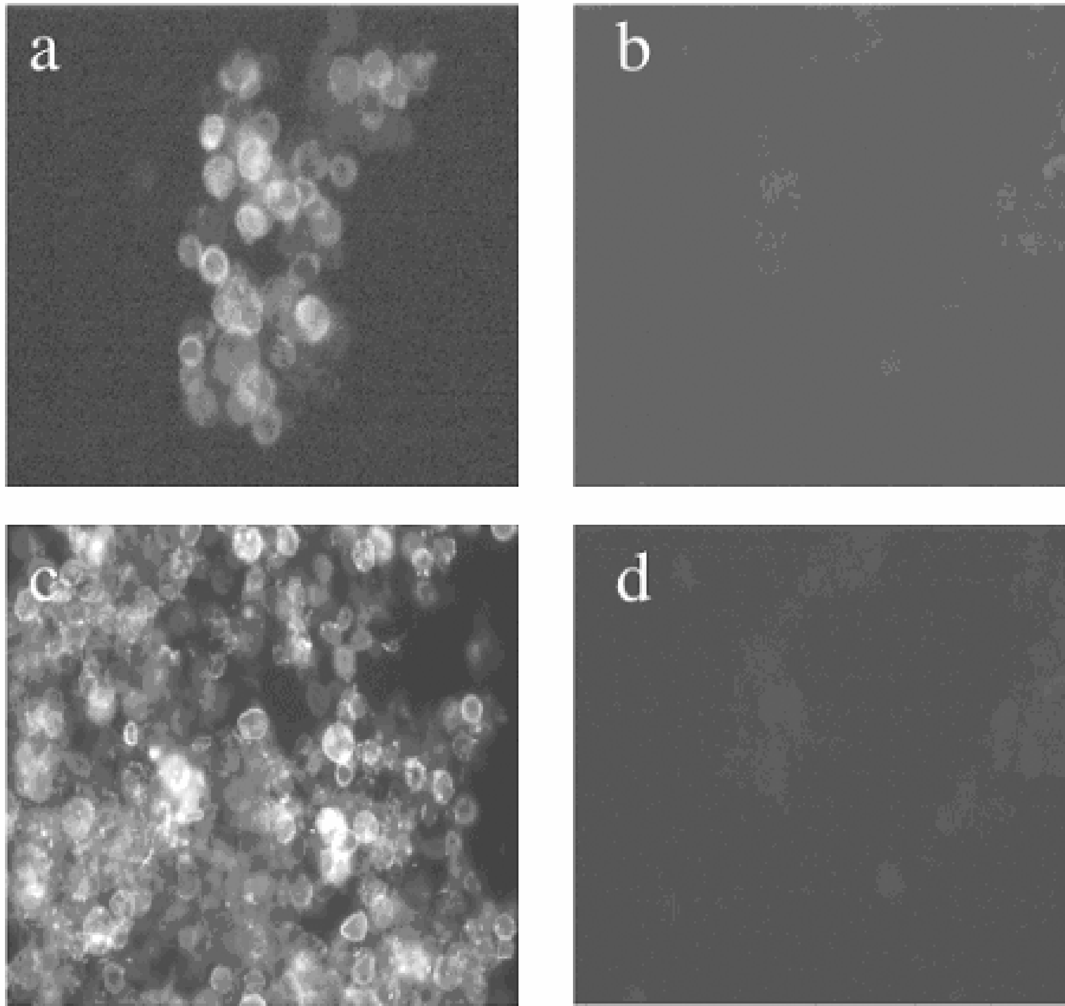


**Figure 1** : Labelling of Sf9 cells with anti-opioid receptor antibodies detected with fluorescein isothiocyanate-conjugated goat anti-mouse (Fab)'$_2$ fragments. Detection with the anti-hDOR antibody of cells infected with a recombinant baculovirus encoding hDOR-C-His (a) or hMOR-C-His (b). Detection with the anti-hMOR antibody using cells infected with a recombinant baculovirus encoding hMOR-C-His (c) or hDOR-C-His (d).

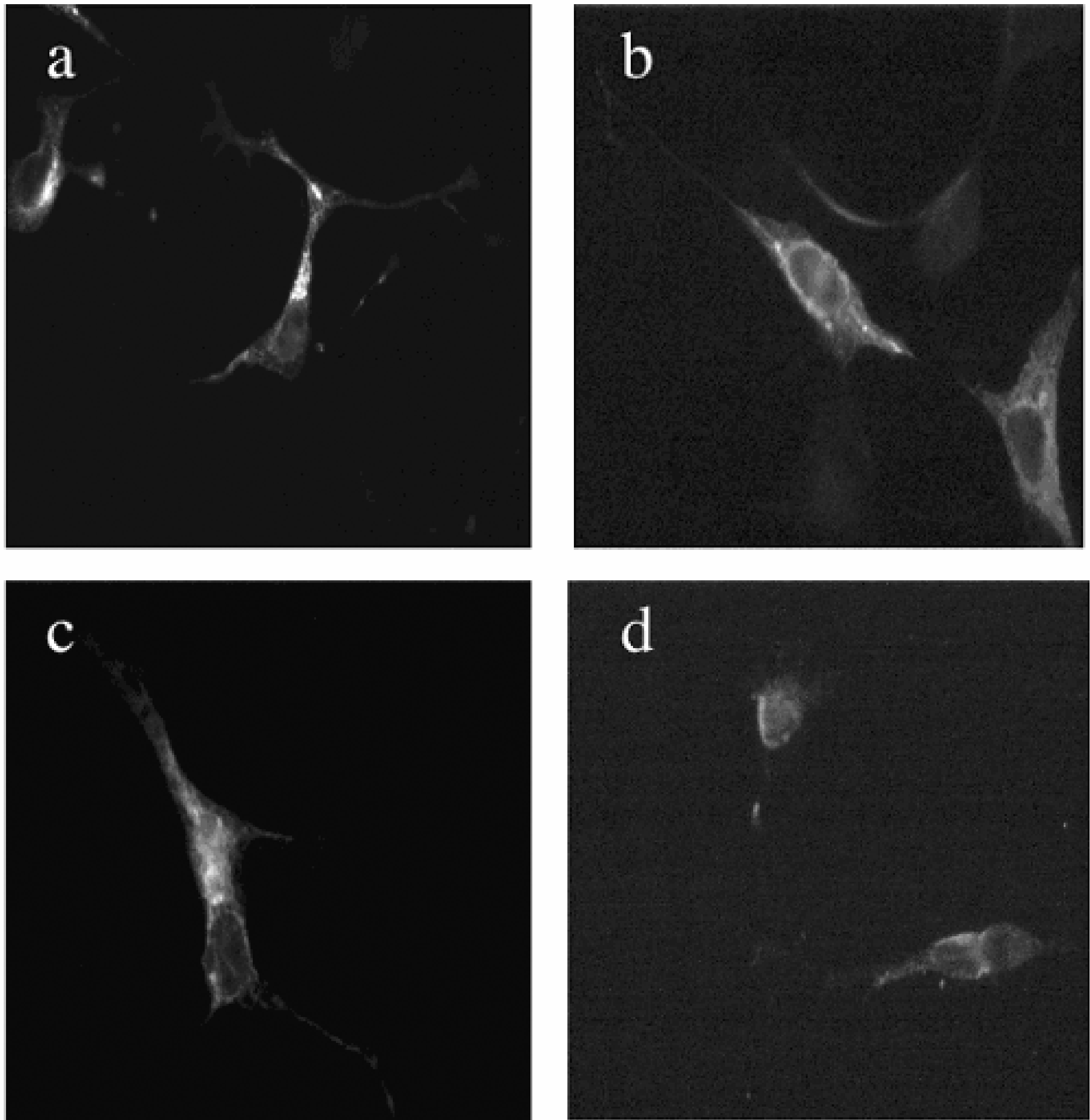


**Figure 2** : Immunofluorescent labelling of COS cells expressing FLAG-tag-hDOR with anti-hDOR antibodies (a) or anti-FLAG antibodies (c). Immunofluorescent labelling of COS cells expressing FLAG-tag-hMOR with anti-hMOR antibodies (b) or anti-FLAG antibodies (d). Detection with fluorescein isothiocyanate-conjugated goat anti-mouse (Fab)'$_2$ fragments.

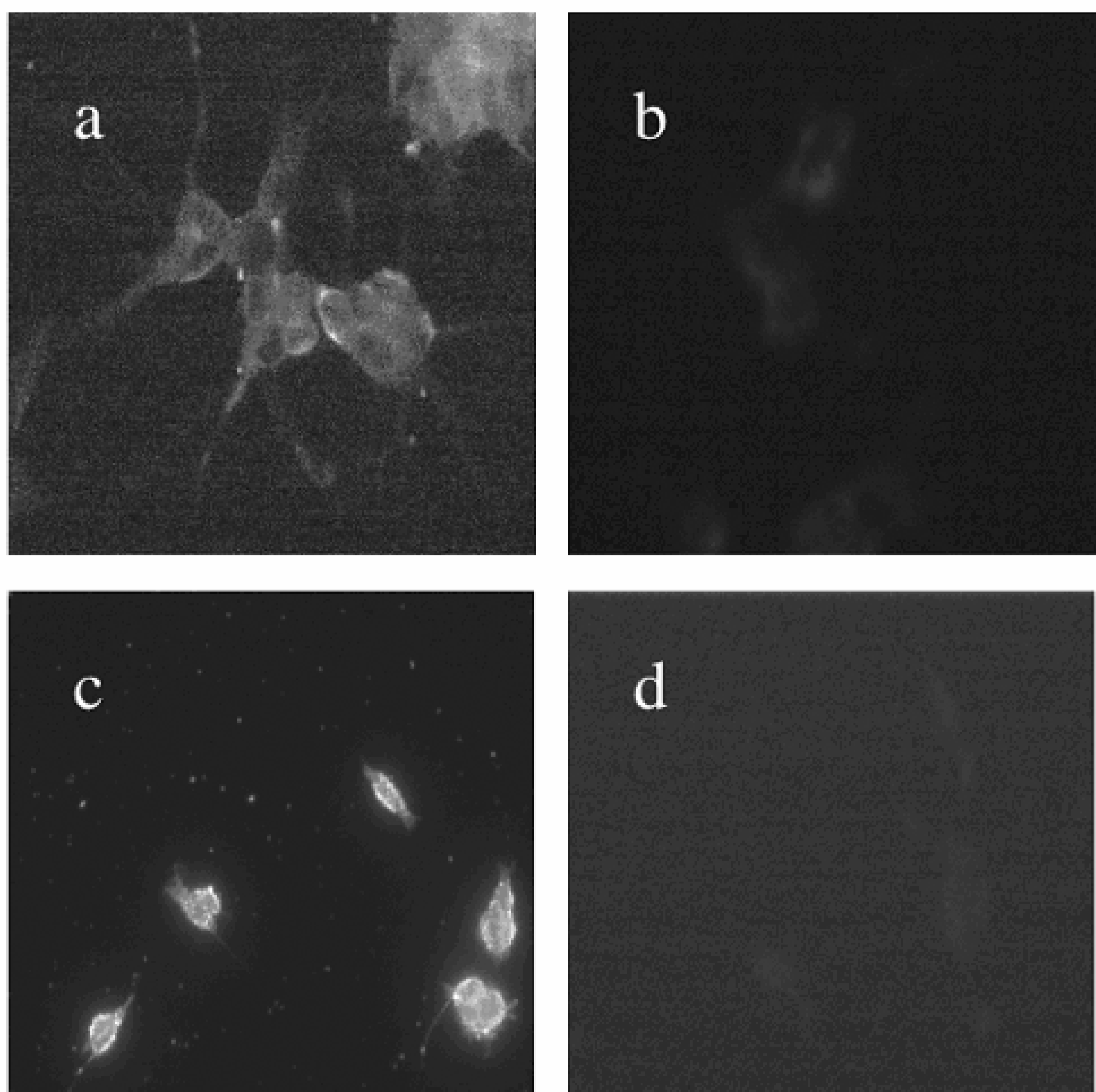


**Figure 3** : Imunofluorescent labelling of SHSY5Y cells with anti-hDOR (a) or anti-hMOR antibodies (c). Celles were preincubated with the corresponding antigenic peptide before labelling with anti-hDOR (b) or anti-MOR (d) antibodies. Detection with fluorescein isothiocyanate-conjugated goat anti-mouse (Fab)'$_2$ fragments

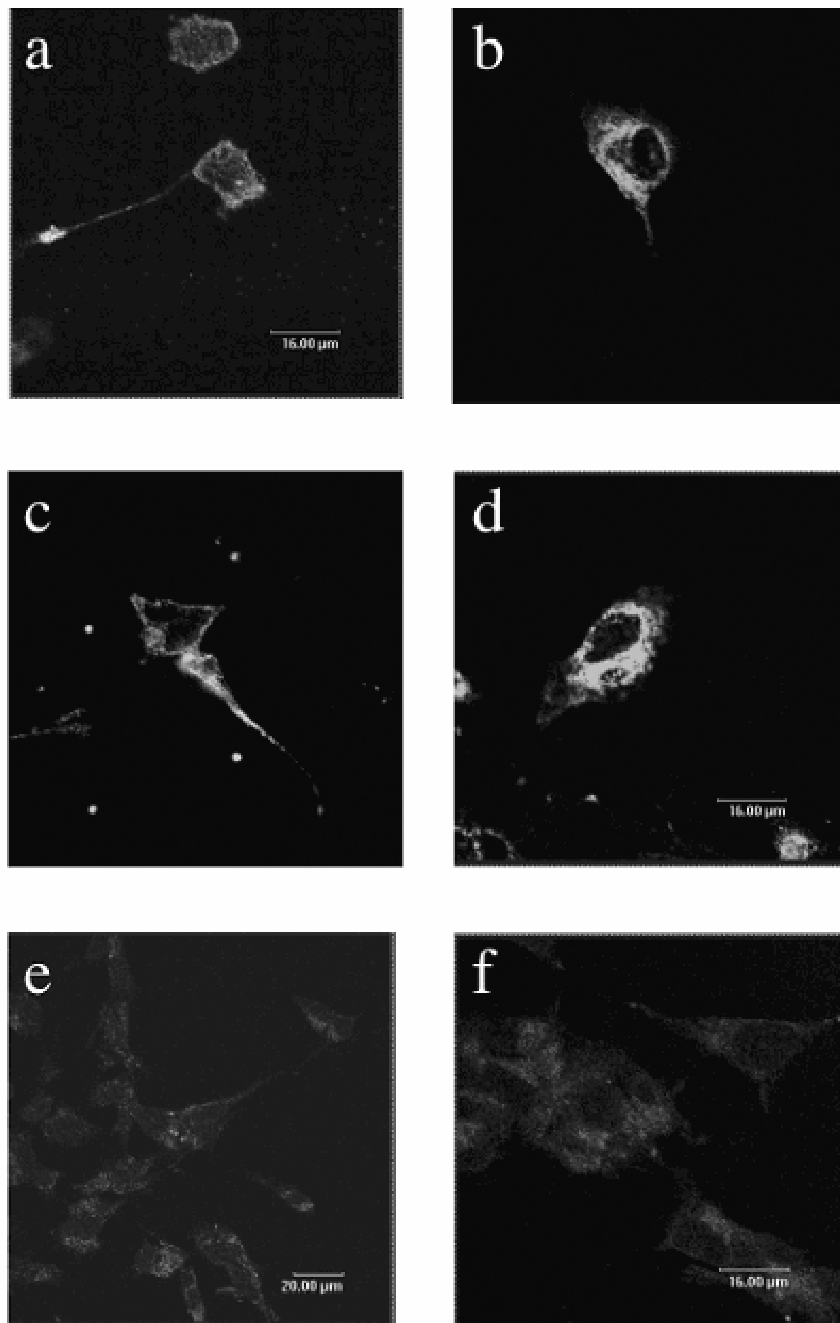


**Figure 4** : Confocal immunofluorescent microscopy. FLAG-tag-hDOR transfected COS cells labelled with the anti-hDOR (a, b) or anti-FLAG antibodies (c, d). SHSY5Y cells labelled with the anti-hDOR antibody (e, f). Untreated cells (a, c, e) or cells treated with 500 nM deltorphin II for 30 minutes (b,d,f). Detection with fluorescein isothiocyanate-conjugated goat anti-mouse (Fab)'$_2$ fragments

cells treated with DAMGO, a mu specific opioid agonist, labelling with the anti-hMOR antibody was absent from the cell surface and concentrated in the cytoplasm (data not shown). Such a change in the signal pattern is consistent with receptor internalisation. In a control experiment, an anti-FLAG antibody was used to monitor receptor redistribution in COS cells treated with the agonist deltorphin II. Similarly to what was observed with the anti-opioid antibodies, labelling at the cell surface was strongly decreased (Figure 4).

Altogether, immunofluorescence data indicate specific recognition of the opioid receptors by the monoclonal antibodies.

*Detection of opioid receptors by immunoblotting*

In order to determine optimal dilutions of antibodies, dot blots were performed using a 1 – 1000 ng range of *E.coli* affinity purified opioid receptors. The anti-hMOR antibodies allowed detection of 5 ng hMOR protein at a 1 :500 dilution of the ascitic fluid whereas the anti-hDOR antibodies only allowed to detect 50 ng hDOR protein at a 1 :200 dilution of the ascitic fluid. Purification of the IgG fraction by affinity on a protein G Sepharose column did not increase the detection sensitivity of the antibodies (data not shown).

Purified opioid receptors as well as membranes from baculovirus-infected insect cells and COS cells were used as test material in Western blotting experiments. Detection of *E. coli* purified hMOR by the anti-hMOR antibody revealed a band with a molecular mass comprised between 37 kDa and 50 kDa (Figure 5 lane 3). This is in good agreement with the expected 44 kDa theoretical mass based on the protein sequence devoid of post-translational modifications. The recombinant receptor expressed in *E. coli* is fused to a C-terminal hexahistidine tag. Detection of this tag in Western blot revealed a band with an size identical to the one detected using the anti-hMOR antibody confirming the protein identity (data not shown).

A band with a molecular mass comprised between 37 kDa and 50 kDa was also detected in membranes prepared from insect cells expressing hMOR in fusion with a C-terminal hexahistidine tag (figure 5 lane 1). This band likely corresponds to the unglycosylated form of the receptor since it has a similar size to the protein produced in

*E. coli*. Insect cells are able to perform glycosylation though mostly of the high-mannose type (Jarvis *et al.*, 1995). However overexpression at levels up to 20 pmol/mg membrane proteins much overwhelm the glycosylation capabilities of the cell resulting in a very large fraction of unglycosylated receptors that are visualised here. Bands with higher molecular masses (about 100 kDa and over 200 kDa) were also detected (Figure 5 lane 1). They come of the natural propensity of G protein-coupled receptors to oligomerise and/or aggregate likely due to their high hydrophobicity (George *et al.*, 2000; Salim *et al.*, 2002). Interestingly such aggregation phenomenon was also observed with the *E. coli* purified protein (Figure 5 lane 3).

We also tested the ability of the anti-hMOR antibody to detect the receptor in membranes prepared from COS cells. A single band with a molecular mass comprised between 37 kDa and 50 kDa was observed which corresponds to the unglycosylated form of the receptor (Figure 5 lane 4). No band with a higher molecular weight was visualised by Western blot. This result may be a little surprising because mammalian cells perform efficient post-translational modifications. However high levels of protein expression may exceed the capabilities of the cell system. Noteworthy, folded receptors expressed in eukaryotic cells (Figure 5 lanes 1 and 4) showed higher apparent mass compared to unfolded polypeptides produced in bacteria (Figure 5 lane 3).
Detection with the anti-hMOR antibody was also evaluated following immunoprecipitation with a monoclonal anti-his antibody performed on membranes from insect cells expressing hMOR in fusion with a C-terminal histidine tag. A band with a molecular mass between 37 and 50 kDa as well as a band with a molecular mass larger then 150 kDa were detected using the anti-hMOR antibody (Figure 5 lane 6). Bands with similar sizes were detected with a nickel activated derivative of horseradish peroxidase optimised for direct detection of recombinant hexahistidine-tagged proteins therefore supporting anti-hMOR specificity (not shown).

This suggests that the anti-hMOR antibody only recognises non or poorly N-glycosylated forms of the receptor. Indeed a putative N-glycosylation site is present within the antigenic sequence which may explain impairment of recognition when it is post-translationally modified.

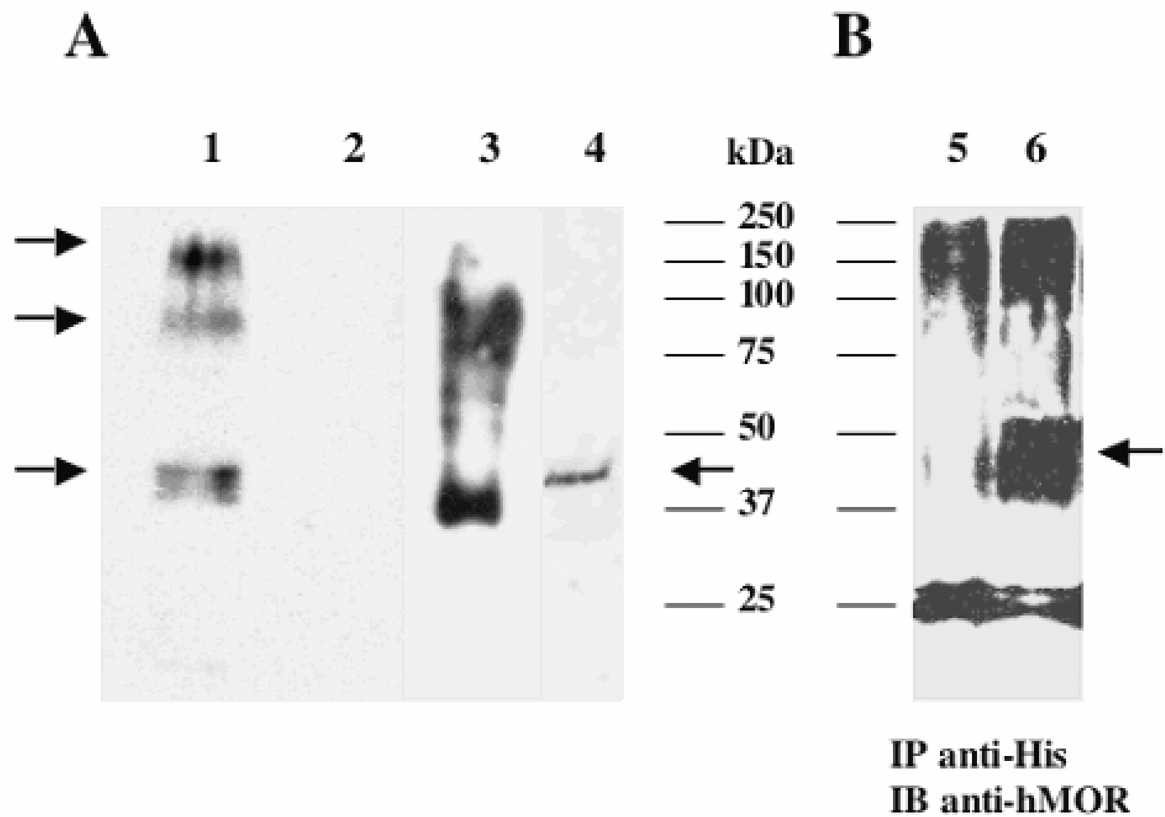


**Figure 5** : Detection in Western blot with anti-hMOR antibodies.

A. Membranes from Sf9 insect cells infected with a recombinant baculovirus encoding His-tagged hMOR (1), membranes from Sf9 insect cells infected with a wild type baculovirus (2), *E. coli* purified hMOR protein (3), membranes from COS cells transiently transfected with hMOR (4).

B. Detection with anti-hMOR antibodies following immunoprecipitation with an anti-histidine tag antibody. Membranes from Sf9 cells infected with a wild type baculovirus (5) or a recombinant baculovirus encoding His-tagged hMOR (6).

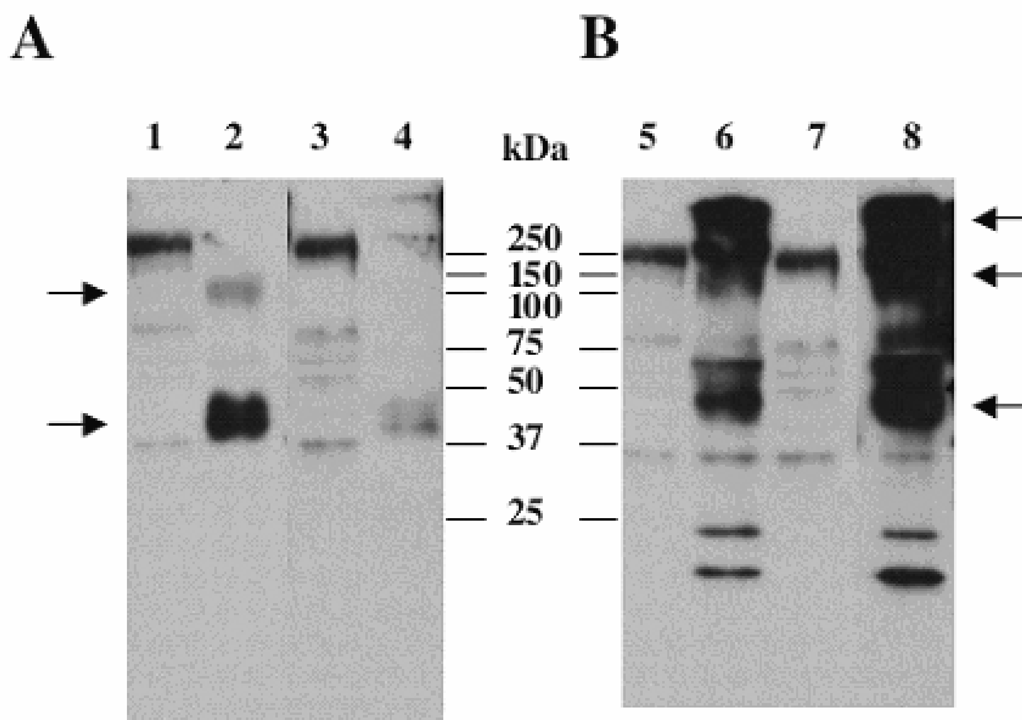


**Figure 6** : Immunoprecipitation using anti-opioid receptor or anti-his antibodies of membranes prepared from baculovirus-infected insect cells.

A Immunoprecipitation using anti-hDOR antibodies of membranes prepared from cells infected with a wild type baculovirus (lane 1) or a recombinant baculovirus encoding His-tagged hDOR (lane 2). Immunoprecipitation using antihistidine tag antibodies of membranes prepared from cells infected with a wild type baculovirus (lane 3) or a recombinant baculovirus encoding His-tagged hDOR (lane 4).

B. Immunoprecipitation using anti-hMOR antibodies of membranes prepared from cells infected with a wild type baculovirus (lane 5) or a recombinant baculovirus encoding His-tagged hMOR (lane 6). Immunoprecipitation using antihistidine tag antibodies of membranes prepared from cells infected with a wild type baculovirus (lane 7) or a recombinant baculovirus encoding His-tagged hMOR (lane 8)

In all cases, immunoprecipitated His-tagged proteins were detected following blot on PVDF membranes using a nickel activated derivative of horseradish peroxidase.

**Table 1**.: Overview of anti-hDOR and anti-hMOR properties in the different assays.

| Assay | Anti-hDOR | Anti-hMOR |
|---|---|---|
| Immunofluorescence | | |
| Sf9 insect cells | | |
| Non infected or mock infected | - | - |
| hDOR-C-His | + | - |
| hMOR-C-His | - | + |
| Cos cells | | |
| Non transfected | - | - |
| FLAG-tag-hDOR | + | - |
| FLAG-tag-mDOR | - | - |
| FLAG-tag-hMOR | - | + |
| FLAG-tag-mMOR | - | - |
| Preincubated with antigenic peptide | decreased signal | decreased signal |
| SHSY5Y | | |
| Endogenous receptors | + | + |
| Preincubated with antigenic peptide | decreased signal | decreased signal |
| Immunoblotting | | |
| protein produced in *E.coli* | - | + |
| protein produced in insect cells (Sf9) | - | + |
| protein produced in Cos cells | - | + |
| Immunoprecipitation | | |
| Membranes isolated from insect cells (Sf9) | + | + |

In Western blot, no specific detection was ever observed with the anti-hDOR antibody. Indeed a high background was always present that could not be reduced efficiently (data not shown).

*Receptor immunoprecipitation with anti-opioid antibodies*

We evaluated the ability of both anti-hDOR and anti-hMOR antibodies to immunoprecipitate receptors from membranes prepared from insect cells. Recombinant opioid receptors expressed in those cells are fused to a C-terminal hexahistidine tag and were detected by Western blot using a nickel activated derivative of horseradish peroxidase optimised for direct detection of recombinant hexahistidine-tagged proteins. Bands with a molecular mass corresponding to a non-glycosylated form of the delta or mu opioid receptors were detected (Figure 6 lanes 2 and 6). As expected the apparent molecular mass of hMOR was a little higher than the apparent molecular mass of hDOR (calculated masses:44 and 40 kDa respectively). In addition bands of high molecular masses were also present (Figure 6 lanes 2 and 6). A band of about 100 kDa could correspond to a dimer while higher molecular masses likely reflect aggregation (George *et al.*, 2000; Salim *et al.*, 2002). None of the bands mentioned above were detected in insect cells infected with a wild-type baculovirus (Figure 6 lanes 1 and 3). In a parallel experiment, opioid receptors were immunoprecipitated using a monoclonal anti-his antibody and detected as above using a nickel activated derivative of horseradish peroxidase. The resulting detection profile fully mirrored what we observed following immunoprecipitation with the anti-opioid antibodies confirming the specificity of the latter (Figure 6 lanes 4 and 8).

## 4. CONCLUSIONS

Characterisation of the monoclonal antibodies raised against the amino-terminal sequence of the human delta and mu opioid receptors showed their specificity towards hDOR and hMOR respectively. Both anti-hDOR and anti-hMOR antibodies can be used to detect the receptors by immunofluorescence in various cell types, or to immunoprecipitate unglycosylated recombinant receptors. Finally anti-hMOR

antibodies can also be used to identify the non glycosylated form of the hMOR receptor in Western blotting experiments (Table1).

These new antibodies represent important tools that will open interesting perspectives in the study of opioid receptors. In particular, we are currently testing their potential to selectively label endogenous receptors in immunohistochemical protocols .

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