Purification and Identification of G Protein-coupled Receptor Protein Complexes under Native Conditions*

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G protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors and are of major therapeutic importance. The identification of GPCR-associated proteins is an important step toward a better understanding of these receptors. However, current methods are not satisfying as only isolated receptor domains (intracellular loops or carboxy-terminal tails) can be used as “bait.” We report here a method based on tandem affinity purification coupled to mass spectrometry that overcomes these limitations as the entire receptor is used to identify protein complexes formed in living mammalian cells. The human MT1 and MT2 melatonin receptors were chosen as model GPCRs. Both receptors were tagged with the tandem affinity purification tag at their carboxyl-terminal tails and expressed in human embryonic kidney 293 cells. Receptor solubilization and purification conditions were optimized. The method was validated by the co-purification of Gi proteins, which are well known GPCR interaction partners but which are difficult to identify with current protein-protein interaction assays. Several new and functionally relevant MT1- and MT2-associated proteins were identified; some of them were common to both receptors, and others were specific for each subtype. Taken together, our protocol allowed for the first time the purification of GPCR-associated proteins under native conditions in quantities suitable for mass spectrometry analysis. Molecular & Cellular Proteomics 6:835–844, 2007.

With more than 800 members, GPCRs constitute the largest family of membrane receptors (1, 2). They respond to a wide variety of extracellular stimuli and are targeted by about half of the drugs prescribed for human diseases (3). GPCRs are key controllers of physiological processes such as neurotransmission, cellular metabolism, secretion, cell differentiation, and growth. The prototypic topology of GPCRs consists of an extracellular amino-terminal segment, a hydrophobic core of seven transmembrane (7TM) α-helices that interact together to form a three-dimensional barrel within the plasma membrane, and a cytosolic carboxy-terminal tail (C-tail). Whereas amino acids within the extracellular and/or hydrophobic 7TM core of the receptor are involved in ligand binding, the intracellular domain of the receptor composed of three loops and the C-tail is important for signal transduction (4).

Several strategies have been used to identify GPCR-associated complexes. Early work used affinity columns with biotinylated ligands to purify somatostatin receptor-G protein complexes from tissues (5). Subsequently based on the pioneering work of Husi et al. (6), more systematic proteomics analysis of GPCR-associated protein complexes was conducted using receptor-specific antibodies (7). However, the general application of these approaches was limited by the availability of adequate tools (labeled ligands, antibodies, etc.) for each GPCR. Later on, isolated intracellular domains were widely used to identify GPCR-associated proteins either as bait in yeast two-hybrid screens or to generate affinity matrices for the purification of interacting proteins from cell extracts (8–11). Using the entire C-tail of the 5HT2c receptor expressed as GST fusion protein, more than 15 proteins have been identified (12). Furthermore isolated protein-protein interaction motifs such as PDZ domain recognition motifs of GPCRs have been successfully used to identify interacting partners of the PDZ domain recognition motifs of the 5HT2a, 5HT2c, and 5HT4 receptors (13, 14).

Although several GPCR-interacting proteins could be identified, these methods obviously have important limitations as isolated receptor subdomains 1) do not mimic the GPCR topology (7TM domain, arrangement of intracellular loops and dem affinity purification; TEV, tobacco etch virus; HET, human embryonic kidney; [125I]MLT, 2-[125I]iodomelatonin; NT, non-transfected; MAPK, mitogen-activated protein kinase; IRS, insulin receptor substrate; PP2, protein phosphatase 2; ER, endoplasmic reticulum.

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the C-tail, and receptor oligomerization), 2) do not provide an adequate membrane environment, and 3) do not allow the recruitment of protein complexes upon agonist activation. Not surprisingly, well known interaction partners of GPCRs such as heterotrimeric G proteins are difficult to identify using these techniques.

Recently a tandem affinity purification (TAP) method has been described (15). Importantly this method overcomes the aforementioned limitations and can be potentially applied to any protein. The full-length protein of interest can be expressed in mammalian cells where subcellular localization and post-translational modifications are conserved. In addition, the recruitment of protein complexes may be induced by treatment of the cells with different hormonal or pharmacological compounds. To perform a two-step affinity chromatography purification of complexes formed in intact cells, the TAP method relies on the presence of a TAP tag composed of two IgG binding domains, a tobacco etch virus (TEV) protease cleavage site, and a calmodulin binding domain (16). The TAP method has been successfully used for high throughput identification of soluble proteins engaged in interacting complexes in yeast and mammalian cells (17, 18). However, purification of membrane protein complexes in general and of GPCR-associated complexes in particular has been unsuccessful.

In the present study, we developed a modified TAP method suitable for the purification of GPCR-associated complexes. The human MT₁ and MT₂ melatonin receptors were chosen as model GPCRs. Both receptors were tagged with the TAP tag at their C-tails, and corresponding stable clones were established in HEK 293 cells. Receptor solubilization and purification was optimized in small scale experiments, and receptor-associated complexes were purified from large scale experiments and subsequently identified by nano-ESI MS/MS.

**EXPERIMENTAL PROCEDURES**

**Receptor Constructs**—MT₁-Rluc and MT₂-Rluc constructs have been described elsewhere (19). To obtain the MT₁-TAP and MT₂-TAP constructs, the TAP tag cassette from the pCDNA3-CMV-TAP plasmid (a gift from Nicolas Goardon, Institut Cochin, Paris, France) was fused in frame to the 3’-end of the MT₁ and MT₂ coding region.

**Cell Culture and Transfection**—HEK 293 cells were grown and transfected as described elsewhere (19). Stable cell lines were selected with G418 (Invitrogen).

**Crude Membrane Preparation, Radioligand Binding Assay, and Solubilization**—Crude membranes were prepared as described previously (20, 21) from non-transfected, MT₁-TAP-, or MT₂-TAP-expressing HEK 293 cells. Membranes were labeled with a saturating concentration (500 pm) of 2-[¹²⁵I]iodomelatonin ([¹²⁵I]MLT), and [¹²⁵I]MLT binding sites were determined on crude membranes, solubilized extracts, or at different steps of the TAP procedure. 0.5% CHAPS, 0.25% Brij96V, 0.5% digitonin, 0.5% Nonidet P-40 (all from Sigma) and 0.5% dodecylmaltoside (Roche Applied Science) were used for overnight solubilization at 4 °C in solubilization buffer (75 mM Tris, 2 mM EDTA, 5 mM MgCl₂, pH 8.0).

**Luminescence Measurements**—Crude membranes were prepared from HEK 293 cell lines stably expressing MT₁-Rluc or MT₂-Rluc and solubilized in solubilization buffer supplemented with increasing concentrations of CHAPS, Brij96V, dodecylmaltoside, or digitonin. The soluble fraction was separated from the insoluble fraction by centrifugation at 40000 × g. The insoluble fraction (pellet) was resuspended in the same buffer, and luciferase activity was measured in the soluble and resuspended insoluble fraction by adding coelenterazine h (Interchim, Montluçon, France) at a final concentration of 5 μM. Readings were performed with a luminometer at 488 nm (Fusion™, Packard Instrument Co.). Solubilization yields were defined as the percentage of luciferase activity in the supernatant over total luciferase activity (pellet + supernatant). Use of Nonidet P-40 was not compatible with the luciferase activity assay.

**Immunofluorescence Microscopy**—HEK 293 cells were grown on sterile coverslips and fixed and permeabilized for 20 min in ethanol at −20 °C. After blocking in PBS, 3% BSA for 20 min, cells were incubated with polyclonal anti-MT₁ or anti-MT₂ antibodies for 1 h at room temperature. Coverslips were washed three times with PBS and incubated with a FITC-coupled secondary antibody at 1:1000 dilution in PBS, 3% BSA (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. Coverslips were mounted and analyzed by confocal laser microscopy (Leica TCS SP2 AOBS).

**SDS-PAGE and Western Blotting**—Whole cells (ERK activation) or crude membranes (receptor detection) were denatured overnight at room temperature in SDS-PAGE loading buffer (62.5 mM Tris/HCl, pH 6.8, 5% SDS, 10% glycerol, 0.5% bromophenol blue). Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose. Immunoblot analysis was carried out with polyclonal anti-MT₁, anti-MT₂ (22), anti-phospho-ERK, or anti-ERK2 antibodies (Santa Cruz Biotechnology). Immunoreactivity was revealed using secondary antibodies coupled to horseradish peroxidase and the ECL reagent (Perbio).

**Optimized TAP Tag Procedure**—All purification steps were conducted at 4 °C in the presence of a protease inhibitor mixture (Roche Applied Science), 1 mM orthovanadate, and 2 mM NaF. For large scale experiments, crude membranes were prepared from ~10⁶ HEK 293 cells and solubilized overnight in solubilization buffer with 0.5% digitonin or 0.25% Brij96V at a concentration of 2 mg of protein/ml. The supernatant was recovered after centrifugation at 40000 × g for 30 min and incubated for 4 h with 400 μl of rabbit IgG-Agarose (Sigma). The resin was washed three times with 1 ml of solubilization buffer, resuspended in 500 μl of the same buffer, and incubated overnight with 100 units of TEV protease (Invitrogen). The supernatant was collected, mixed with 500 μl of calmodulin buffer (75 mM Tris, 5 mM MgCl₂, 50 mM CaCl₂, and 0.5% digitonin or 0.25% Brij96V, pH 8.0) and incubated for 2 h with 100 μl of calmodulin beads (Stratagene, La Jolla, CA). Beads were washed five times with 1 ml of calmodulin buffer, and retained proteins were eluted with SDS-PAGE loading buffer.

**Mass Spectrometry and Protein Identification**—Coomassie Blue-stained or silver-stained (23) bands were excised and subjected to in-gel tryptic digestion using modified porcine trypsin (Promega, Lyon, France) as described previously (24). The tryptic digest was analyzed by on-line capillary HPLC ( Dionex/LC Packings) coupled to a nanospray Qq-TOF mass spectrometer (QSTAR Pulsar XL, Applied Biosystems, Foster City, CA). Peptides were separated on a 75-μm inner diameter × 15-cm C₁₈ PepMap™ column after loading onto a 300-μm inner diameter × 5-mm PepMap C₁₈ precolumn (Dionex/LC Packings). The flow rate was set at 200 nl/min. Peptides were eluted using a 0–50% linear gradient of solvent B in 50 min (solvent A was 0.1% formic acid in 5% acetonitrile, and solvent B was 0.2% formic acid in 90% acetonitrile). The mass spectrometer was operated in positive ion mode at a 2.1- kV needle voltage. MS and MS/MS data were continuously acquired in an information-dependent acquisition mode consisting of a 10-s cycle time. Within each cycle, a MS
spectrum was accumulated for 1 s over the range m/z 300–2000 followed by three MS/MS acquisitions of 3 s each on the three most abundant ions in the MS spectrum. A dynamic exclusion duration was used to prevent repetitive selection of the same ions within 30 s. MS/MS data were acquired using a 3 m/z unit ion isolation window. Collision energies were automatically adjusted according to the charge state and mass value of the precursor ions. Peak lists of MS/MS spectra were created using Mascot.dll script (version 1.6b21) in Analyst QS software (version 1.1, Applied Biosystems). For generation of the peak lists, the default charge state was set to 2+, 3+, and 4+; MS/MS data were centroided and deisotoped with a threshold at 0% of the base peak; MS/MS spectra with less than five peaks were rejected; the precursor mass tolerance for grouping was set to 0.1; and the maximum number of cycles between groups and the minimum cycles per group were set to 60 and 1, respectively. MS/MS data were searched against a human sequence in-house database, a compilation of UniProt Swiss-Prot and UniProt TrEMBL databases (72,049 entries, versions 49.1 and 32.1, respectively) using the Mas-
cot® search engine (version 2.1.04). Up to two trypsin missed cleavages were allowed, and the mass tolerance for peptide and MS/MS fragment ions was 0.5 Da. Cysteine carbamidomethylation and propionamide and methionine oxidation were set as variable modifications. Identification was considered positive if the protein was identified with at least one peptide with an ion score greater than the Mascot significance threshold of 36 (p ≤ 0.05). For the protein with a score close to the threshold value, the identification was confirmed by manual interpretation of corresponding MS/MS data. To evaluate the false-positive rate in these large scale experiments, we repeated the searches using identical search parameters and validation criteria against a random database made of the same compilation in which the sequences have been reversed. These statistical analyses provided 2.1, 4.1, and 5.8% false-positive rate for MT1-TAP, MT2-TAP, and control experiments. See Supplemental Tables I and II for a detailed list of identified peptides.

RESULTS AND DISCUSSION

Functional Expression of MT1-TAP and MT2-TAP Fusion Proteins in HEK 293 Cells

The human MT1 and MT2 melatonin receptors were tagged with the TAP tag at their C-tails, and corresponding stable clones were established in HEK 293 cells. HEK 293 cells of human origin, are extremely well characterized in terms of GPCR signal transduction, can be produced in quantities that are compatible with proteomics analysis, and do not express significant amounts of endogenous melatonin receptors. Western blot analysis with receptor-specific antibodies revealed immunoreactive bands at the expected molecular size of 85 kDa in an HEK-MT1-TAP clone expressing 1.0 ± 0.2 pmol of MT1-TAP/mg of protein (n = 3) and in an HEK-MT2-TAP clone expressing 340 ± 41 fmol of MT2-TAP/mg of protein (n = 3) (Fig. 1, a and b). The affinity constants (Kd) of the melatonin receptor agonist [125I]MLT were 85 ± 53 (n = 3) and 267 ± 78 pm (n = 3) for MT1-TAP and MT2-TAP, respectively, which are in agreement with previously reported values for wild-type receptors (25). Expression of receptors at the cell surface was assessed by fluorescence microscopy using receptor-specific antibodies (Fig. 1, c–f). TAP-tagged and wild-type receptors were all expressed at the plasma membrane of stably transfected HEK 293 cells.
of ERK1/2 phosphorylation, which was comparable to that obtained for wild-type receptors (Fig. 1g). Altogether these data demonstrate that the addition of the TAP tag does not affect the subcellular localization and the functionality of the melatonin receptor.

Optimization of Receptor Solubilization and Purification—
The crucial step for successful purification of GPCRs and associated proteins consists of mild but efficient solubilization of cells to extract maximal amounts of intact membrane-bound complexes. To quantify the amount of solubilized MT₁ and MT₂, we used cells expressing Rluc (Renilla luciferase)-tagged MT₁ and MT₂ receptors (19). These cells were incubated overnight with varying concentrations of detergents, and the solubilization yield was determined by measuring luciferase activity in the soluble and non-soluble fractions (Fig. 2a). The amount of solubilized receptor increased as a function of the detergent concentration and reached maxima at 50% (CHAPS), 65% (Brij96V), 70% (digitonin), or 80% (dodecylmaltoside). Data obtained with Nonidet P-40 could not be analyzed in this assay as Nonidet P-40 inhibits the luciferase activity. Comparable results were obtained for MT₁-Rluc and MT₂-Rluc. Minimal detergent concentrations, which gave maximal receptor solubilization, were used to study the solubilization kinetics (Fig. 2b). Both receptors solubilized progressively with time reaching a plateau after 15 h. For further experiments, receptors were solubilized with 0.5% CHAPS, digitonin, dodecylmaltoside, or Nonidet P-40 or with 0.25% for Brij96V for 15 h.

To evaluate the topological integrity of solubilized MT₁-TAP and MT₂-TAP, their ability to bind [¹²⁵I]MLT was used. Membranes prepared from HEK-MT₁-TAP and HEK-MT₂-TAP cells were labeled with [¹²⁵I]MLT. The receptors were solubilized and immobilized on IgG-coated beads via the IgG binding modules of the TAP tag (Fig. 2c). Digitonin and Brij96V were chosen for further experiments as 40–50% of [¹²⁵I]MLT bind-
ing sites were routinely retained on IgG beads under these conditions. Using these optimized solubilization conditions, the entire TAP procedure was carried out. The purification of functional receptors was monitored at each step with the $^{[125]}$I MLT binding assay. The overall yield of $^{[125]}$I MLT-labeled receptors varied from 27 ± 3 (digitonin) to 15 ± 2% (Brij96V) and from 33 ± 2 (digitonin) to 25 ± 5% (Brij96V) (n > 5) for MT$_1$ and MT$_2$, respectively (Fig. 3a and b).

The co-purification of associated proteins upon melatonin stimulation was evaluated by the presence of the G$_i$ subunit of the heterotrimeric G protein (G$_i$) (20, 21). Whereas G$_i$ was readily detected throughout the purification of digitonin-solubilized MT$_1$-TAP and MT$_2$-TAP (Fig. 3c), G$_i$ was rarely co-purified when using Brij96V (not shown) because this detergent apparently destabilized the receptor/G protein interaction. The integrity of the heterotrimeric G protein was further confirmed by the presence of the G$_b$ subunit at the final purification step in the presence of digitonin (not shown), which was used for further experiments.

**Purification and Identification of MT$_1$- and MT$_2$-associated Proteins**

The ultimate aim of the TAP tag procedure is the purification of sufficient amounts of receptor to identify associated proteins by mass spectrometry analysis. To reach this goal, crude membranes of $\sim 1 \times 10^9$ HEK-MT$_1$-TAP, HEK-MT$_2$-TAP, and non-transfected HEK 293 cells were prepared, and the digitonin-solubilized fraction was submitted to the TAP procedure. Eluates were separated by one-dimensional gel electrophoresis, and depending on the receptor expression levels, proteins were detected either by Coomassie Blue staining (MT$_1$-TAP, $\sim 1$ pmol/mg) or by silver staining (MT$_2$-TAP, $\sim 0.3$ pmol/mg) (Fig. 4, a and b). Whereas only a few bands were visible in non-transfected (NT) HEK 293 cells, several specific protein bands were reproducibly present in MT$_1$-TAP- and MT$_2$-TAP-expressing cells (n = 4). Lanes were systematically excised and digested with trypsin, and the resulting peptides were analyzed by nano-LC-nano-ESI MS/MS and identified with Mascot software in Swiss-Prot and TrEMBL databases. Several abundantly expressed proteins, mostly of mitochondrial or ribosomal origin, were detected in all three lanes (NT, MT$_1$-TAP, and MT$_2$-TAP) and were classified as nonspecific proteins. The proteins repeatedly present in the MT$_1$-TAP or MT$_2$-TAP lane but absent from the NT lane were considered to be specifically associated to MT$_1$ or MT$_2$, respectively (Tables I and II). Consistently both bait proteins (MT$_1$ and MT$_2$) were identified in the corresponding lanes. Each receptor was identified in two regions, at 45 and 90 kDa, corresponding to the well documented monomeric and SDS-resistant dimeric form of the receptors, respectively (26). This indicates that both receptors reached their fully functional quaternary structure.

Importantly the presence of heterotrimeric G proteins in MT$_1$- and MT$_2$-associated complexes was confirmed by mass spectrometry. Consistent with the known coupling of melatonin receptors to G$_i$ proteins, all three G$_i$ isoforms (specific peptides for G$_{i1-3}$) and two different G$_b$ isoforms (specific peptides for G$_{b1-3}$) were identified. Co-purification of G proteins and receptors validates our method and provides a major advantage compared with other currently available protein-protein interaction assays where the G protein/receptor interaction is generally lost. These results clearly demonstrate that our procedure can identify functionally relevant GPCR-interacting proteins that associate only with intact receptors expressed in the natural membrane environment.

Several previously unknown melatonin receptor-associated proteins were identified. Interestingly these proteins localized to different subcellular compartments (cytosol, plasma membrane, and different intracellular membrane compartments such as the endoplasmic reticulum). Melatonin receptor-associated proteins could be divided into three functionally distinct groups: proteins likely to be involved in receptor biosynthesis, intracellular trafficking, and signaling/regulation of GPCRs (Tables I and II). The function of remaining proteins, classified as "others," are unknown or appear not to relate directly to known GPCR function. This is the case for the MT$_2$-specific heterogeneous nuclear ribonucleoprotein A0 that is suspected to participate in mRNA maturation (27) and is a major substrate for MAPK-activated protein kinase 2, which is itself activated upon GPCR-promoted p38 MAPK stimulation (28).

Filamin A and insulin receptor substrate 4 (IRS4) were identified as common members of MT$_1$- and MT$_2$-associated complexes. Consistent with these findings, filamin A has been shown previously to interact with several other members of the GPCR family, including dopamine D2/D3 (29, 30), calcium-sensing (31, 32), and $\mu$-opioid receptors (33). The role of
IRS4 is less well documented. The involvement of IRS4 in fibroblast growth factor receptor signaling (34) and interaction with the protein phosphatase 4 have been described (35).

We were also able to identify several MT1-specific signaling proteins such as Rac1, RAP-1A, and the 2',3'-cyclic-nucleotide 3'-phosphodiesterase and the protein elongation factor 1-\(\alpha\) (eEF-1\(\alpha\)). Interestingly the small GTPases Rac1 and RAP-1 have been shown to function downstream of 5HT4 receptors and the cAMP guanine nucleotide exchange factor Epac1 (36). The 2',3'-cyclic-nucleotide 3'-phosphodiesterase, belonging to the PDE3A family, is involved in the degradation of second messengers such as cAMP and cGMP (37). Activation of melatonin receptors is known to modulate both second messengers (25). eEF-1\(\alpha\) and other elongation factors have been reported to modulate GPCR function by direct interaction with the receptor (38, 39).

Catenin δ1 (p120) and the protein phosphatase 2C\(\gamma\) (PP2C\(\gamma\)) have been identified as MT2-specific signaling proteins. Whereas p120 is known to affect intracellular signaling by NFkB activation through regulation of Rho GTPases, its specific role in GPCR signaling is currently unknown (40). Several serine/threonine phosphatases participate in the dephosphorylation of activated GPCRs (41). Phosphatases of the PP2A and PP2B subfamilies have been reported to target GPCRs, whereas PP2C subfamily members have been shown to dephosphorylate the metabotropic glutamate receptor 3 (42). It will be interesting to determine whether PP2C\(\gamma\) participates in MT2 dephosphorylation.

Most of the identified proteins that are involved in receptor biosynthesis are present in both receptor-associated com-
complexes. This is expected because all GPCRs are suspected to follow the same biosynthetic pathway. Interestingly identified proteins interact with different distinct receptor domains including the cytoplasmic and the endoplasmic reticulum (ER) luminal receptor interface. In contrast, proteins involved in trafficking differ clearly between the two receptor subtypes indicating different trafficking behavior.

Apart from heterotrimeric G proteins, not much is known about the repertoire of melatonin receptor interaction partners. This makes it difficult to estimate the proportion of known interaction partners that are covered by our data set. However, a rough estimation can be made with the assumption that many interaction partners are likely to interact at least ubiquitously expressed interaction partners than cell type-specific (i.e., neuron-specific) partners. The repertoire of proteins identified for both melatonin receptors confirmed this prediction. The next step will be to identify cell type-specific interaction partners by expressing TAP-tagged melatonin receptors in neurons or endocrine cells that express endogenous receptors with well defined functions.

Taken together, our TAP protocol allowed for the first time the purification of GPCR-associated proteins under native conditions. This is expected because all GPCRs are suspected to follow the same biosynthetic pathway. Interestingly identified proteins interact with different distinct receptor domains including the cytoplasmic and the endoplasmic reticulum (ER) luminal receptor interface. In contrast, proteins involved in trafficking differ clearly between the two receptor subtypes indicating different trafficking behavior.

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Taken together, our TAP protocol allowed for the first time the purification of GPCR-associated proteins under native conditions.
conditions in quantities suitable for mass spectrometry analysis. Interaction partners from different cellular compartments recognizing extra- and intracellular receptor domains of monomeric and/or dimeric receptor species were identified. This presents a major methodological advance in the identification of GPCR-associated protein complexes. In addition, this method is relatively fast, generates a low number of nonspecific proteins, and needs no confirmation of the interaction by co-immunoprecipitation experiments. Similar results were obtained between MT1 and MT2 in terms of solubilization efficiency, complex stability, and purification yields with this protocol, suggesting a more general application. The increasing number of TAP tag variants, including the split TAP tag (16), demonstrates the flexibility of this method and allows the possibility of rapid optimization for any GPCR homo- and heterodimer.

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