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Crosslinking Photosensitized by a Ruthenium Chelate as a Tool for Labeling and Topographical Studies of G-Protein-Coupled Receptors

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Summary

The purpose was to apply oxidative crosslinking reactions to the study of recognition and signaling mechanisms associated to G-protein-coupled receptors. Using a ruthenium chelate, $Ru(bipy)_3^{2+}$, as photosensitizer and visible light irradiation, in the presence of ammonium persulfate, we performed fast and efficient covalent labeling of the B_2 bradykinin receptor by agonist or antagonist ligands possessing a radio-iodinated phenol moiety. The chemical and topographical specificities of these crosslinking experiments were investigated. The strategy could also be applied to the covalent labeling of the B_1 bradykinin receptor, the AT_1 angiotensin II receptor, the V_{1a} vasopressin receptor and the oxytocin receptor. Interestingly, we demonstrated the possibility to covalently label the AT_1 and B_2 receptors with functionalized ligands. The potential applications of metal-chelate chemistry to receptor structural and signaling studies through intramolecular or intermolecular crosslinking are presented.

Introduction

The fast development of structural and proteomic approaches in the study of signaling mechanisms requires a panel of methods for the study of biological recognition processes. In this respect, biochemical strategies constitute powerful tools. They are currently applied in the field of G-protein-coupled receptors (GPCRs), to the topographical analysis of the receptor itself [1] or its interactions with protein partners [2–4].

The purpose of the present work was to extend the panoply of available crosslinking strategies by going deeply into previously reported chemistry [5–10] and proposing new applications in the field of ligand-receptor interaction investigations.

Specific covalent labeling of receptors with photoactivable hormone derivatives has been extensively used for characterization and mapping purposes. Many of these studies involved compounds possessing azido or benzophenone moieties which require UV irradiation for photoactivation and are often lacking specificity in their reactions with protein chemical functions. An alternative is based on ligand crosslinking to its receptor using homo- or hetero-bifunctional reagents. In spite of efforts devoted to the development of covalent labeling of the bradykinin B_2 receptor [11–13], no high yield labeling strategy is presently available. We decided to use a strategy previously applied to oxidative protein-protein crosslinking, using photosensitization by a ruthenium derivative, $Ru(bipy)_3^{2+}$ in the presence of ammonium persulfate (APS) [9, 10]: the underlying chemistry was proposed to involve the formation of radicals allowing tyrosine residues to give covalent bonds with another tyrosine or with a cysteine [9, 10]. Starting from this mechanistic proposal, we tried to photolabel the human B_2 bradykinin receptor with ligands possessing a radioiodinated phenol moiety at their N termini, [^{125}I]HPP-HOE 140 (radioiodinated hydroxyphenyl-propionyl-HOE 140) and [^{125}I]Tyr^0-BK, which behave as inverse agonist and agonist, respectively. The present work demonstrates that this strategy allows receptor photolabeling with a high yield and very short reaction times. We also endeavored to extend the method to other GPCRs belonging to the same subfamily (B_1 bradykinin, AT_1 angiotensin II receptors) or to the vasopressin/oxytocin receptor subfamily. The chemical and topographical specificities of the involved chemical reactions were investigated. The potential applications of the described strategies to topographical and signaling mechanism studies are presented; some of them take advantage of the very short reaction times required for efficient oxidative crosslinking photosensitized by ruthenium compounds.

Results

Receptor photolabeling using ligands possessing a radio-iodinated phenol was first carried out on the B_2 receptor. The work includes a rather exhaustive description of this pilot study. Then the strategy was extended to other receptor/ligand couples which are listed in Figure 1.

$Ru(bipy)_3^{2+}$ Photosensitized Covalent Labeling of the B_2 Receptor

Characterization of Covalently Labeled Receptor

COS-7 cells expressing the human B_2 receptor were incubated at 4°C with nanomolar concentrations of either the antagonist [^{125}I]HPP-HOE 140, which in fact behaved as an inverse agonist [14], or the agonist [^{125}I]Tyr^0-BK. After removal of the medium containing the radioligand, the dishes were supplemented with

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Receptor	Ligand name	Peptide sequence
B_2	L_1 [^{125}I]HPP-HOE 140	HO-(4-^{125}I-phenyl)-$(CH_2)_2$-CO-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg
	L_2 [^{125}I]Tyr^0-BK	Tyr(^{125}I)-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
	L_3 [^{125}I](Tyr^8)-BK	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Tyr(^{125}I)-Arg
	L_4 [^{125}I]Lys^0-(Tyr^8)-BK	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Tyr(^{125}I)-Arg
	L_{10} [^{125}I]Tyr^0-HOE 140	Tyr(^{125}I)-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg
	L_{12} [^{125}I]GGHY-HOE 140	Gly-Gly-His-Tyr(^{125}I)-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg
	L_{13} [^{125}I]Bio-Tyr^0-HOE 140	Biotinesulfone-6-Ahx-Tyr(^{125}I)-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg
B_1	L_5 [^{125}I]JMV 1109	HO-(4-^{125}I-phenyl)-$(CH_2)_2$-CO-Lys-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic
AT_1	L_6 [^{125}I]Tyr^0-AII	(Tyr(^{125}I)-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe // Tyr-Asp-Arg-Val-Tyr(^{125}I)-Ile-His-Pro-Phe
	L_7 [^{125}I](Sar^1)-AII	Sar-Arg-Val-Tyr(^{125}I)-Ile-His-Pro-Phe
	L_{11} [^{125}I]GGHY-AII	Gly-Gly-His-Tyr(^{125}I)-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
V_{1a}	L_8 [^{125}I]HO-LVA	HO-(4-^{125}I-phenyl)-CH_2-CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2
OT / V_{1a}	L_9 [^{125}I]OTA	CH_2-CO-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-NH-CH-CO-NH_2 (S—S; CH_2-(3-^{125}I-4-OH-phenyl))

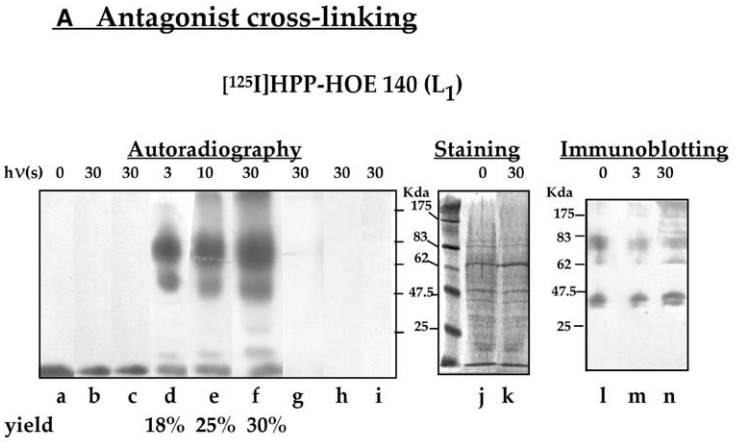
Figure 1. Structure of Synthetic Ligands Adapted to the $Ru(bipy)_3^{2+}$ Photosensitized Crosslinking of GPCRs

PBS medium containing $Ru(bipy)_3^{2+}$ (concentration range 0–2 mM) and ammonium persulfate (APS, concentration range 0-0.5 mM), then irradiated for short times (3-30 s) by a visible light source. Analyses of the SDS-solubilized cell samples showed labeling of a 76 kDa entity by both the antagonist (Figure 2A) and the agonist (Figure 2B). The rather heterogeneous aspect of this band and its molecular mass are those expected for intact receptor and the specificity of the labeling was assessed by its suppression when an excess of unlabeled ligands was present in the initial phase of receptor site saturation (Figure 2). No labeling was detected when the ruthenium derivative, or APS, or irradiation were omitted, as well as in nontransfected COS-7 cells (Figure 2A). Receptor labeling was also carried out on membrane preparations from COS-7 cells, with similar efficiency as in intact cells (not shown). As the B_2 receptor possesses a c-Myc tag at its N terminus, solubilized membranes from photolabeled cells were analyzed by SDS-PAGE and then transferred to nitrocellulose membranes: the major band detected by immunoblotting using anti c-Myc antibodies corresponded to the major radioactive photolabeled species (Figure 2A). The 40 kDa band probably represents immature receptor lacking glycosidic chains which has not reached the plasma membrane and is unable to bind ligands [15].

The minor labeled form, centered at 50 kDa in the autoradiograms, probably represents partially deglycosylated entities as its amount was independent from irradiation time and the presence of protease inhibitors. The differences between the patterns of these minor bands in immunoblots and autoradiograms is not surprising; if antibodies should recognize immature receptors which are unaccessible to ligand binding, one cannot rule out the possible proteolytic loss of the tag sequence.

Some labeling, possibly representing aggregates, was also detected at high molecular weights on the top of the gels: it was dependent upon the irradiation time, being negligible at 3 s.

The yields of [^{125}I]HPP-HOE 140 photolabeling, estimated from radioactivities associated with the 76 kDa and 50 kDa entities in SDS-PAGE gels, were 18%–25% for irradiation times as short as 3 s and slightly increased up to 30% when irradiation lasted 30 s. The radioactivities associated with the 76 kDa band were systematically found to be two times greater than 50 kDa radioactivity, irrespective of reaction times and Ruthenium chelate/APS concentrations. High photolabeling yields were also obtained for [^{125}I]Tyr^0-BK (16% and 18% for 3 and 30 s irradiation times, respectively). These determinations were in agreement with measurements of acid-wash-resistant radioactivities representing specific covalent association to intact cells : 24%–30% for [^{125}I]HPP-HOE 140 photolabeling (3–30 s irradiation times) and 21%–25% for [^{125}I]Tyr^0-BK photolabeling; the slightly higher values obtained using this protocol were probably due to the fact that they take into account all the photolabeled species, including the high molecular weight entities which are most often found at the top of electrophoresis gels; nevertheless they represent actual efficiencies of the photochemical pro-



Incidence of irradiation conditions on ligand binding properties

Receptor occupation during cell irradiation	[125I]HPP-HOE 140 binding to washed membranes from irradiated cells			
	Control		Irradiation	
	Kd (nM)	Bmax (pmol/mg)	Kd (nM)	Bmax (pmol/mg)
A / none	0.42 r = 0.95	11.4	0.27 r = 0.93	6.7
B / BK	0.43 r = 0.96	13.2	0.46 r = 0.96	13.1
C / HPP-HOE 140	0.45 r = 0.96	13.2	0.36 r = 0.98	8.7
D / HOE 140	0.52 r = 0.97	6.0	0.48 r = 0.99	6.1

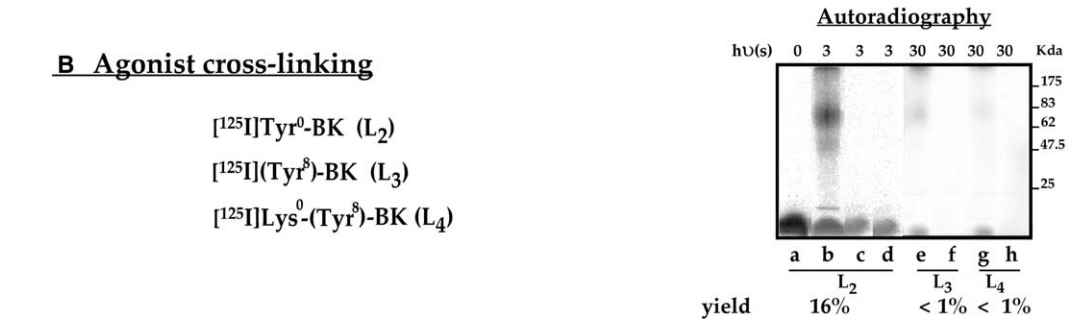


Figure 2. Photolabeling of the B_2 Bradykinin Receptor in Intact Cells by [^{125}I]HPP-HOE 140 and [^{125}I]Tyr^0-BK Using Ru(bipy)$_3^{2+}$ and APS

The B_2 receptor was transiently expressed in COS-7 cells, at a density of 5 × 10^5 sites/cell. Equilibrium binding of [^{125}I]HPP-HOE 140 (2 nM) or [^{125}I]Tyr^0-BK (8 nM) to intact cells, and photolabeling using Ru(bipy)$_3^{2+}$ (0.5 mM) and APS (0.5 mM) were carried out as described in Experimental Procedures, using the indicated irradiation times.

(A) Left panel, antagonist photolabeling. Lanes d–f, [^{125}I]HPP-HOE 140 labeling. No receptor labeling was detected when Ru(bipy)$_3^{2+}$ (b) or APS (c) were omitted or when an excess of unlabeled HPP-HOE 140 (g) or HOE 140 (h) was present in the initial step of receptor site saturation. No labeling was observed in control nontransfected COS-7 cells (lane i). Lanes j and k, protein staining of solubilized irradiated (3 s) or nonirradiated cells corresponding to assays a and d. Lanes l, m, and n, solubilized membranes from photolabeled cells were submitted to SDS-PAGE, transfered onto nitrocellulose membranes before analysis by immunoblotting using an anti-c-Myc antibody. The figure represents a typical experiment, representative of five separate experiments.

Right panel, incidence of irradiation conditions on ligand binding properties. COS-7 cells transiently expressing the B_2 receptor were incubated for 3 hr at 4°C in the absence or presence of 5 nM unlabeled BK, HPP-HOE 140, or HOE 140. After removal of unbound ligand and rapid washing, the cells were irradiated for 3 s in the presence of Ru(bipy)$_3^{2+}$/APS. Membranes were then prepared from these cells, and submitted to a ligand dissociation treatment consisting of a 30 min incubation at room temperature followed by centrifugation. The binding of [^{125}I]HPP-HOE 140 to membrane preparations was checked as described in Experimental Procedures. A, membranes from cells irradiated without receptor occupancy. B, membranes from cells irradiated after receptor occupancy by BK. C, membranes from cells irradiated after HPP-HOE 140 receptor occupancy. D, membranes from HOE 140 occupied receptor. The incomplete recovery of binding sites in the D section probably results from incomplete HOE 140 dissociation as compared to other checked ligands. The Figure represents a set of data obtained within the same experiment, each binding determination being carried out in triplicate. Similar results were reproduced twice.

(B) Agonist photolabeling. Lanes b and c, [^{125}I]Tyr^0-BK labeling. No receptor labeling was detected in the absence of irradiation (lane a) or when an excess of unlabeled BK (lane c) or Tyr^0-BK (lane d) was present in the initial step of receptor site saturation. Negligible crosslinking was observed when cells were irradiated after saturation with [^{125}I](Tyr^8)-BK (2 nM) without and with an excess of unlabeled ligand (lanes e and f) or with [^{125}I]Lys^0-(Tyr^8)-BK (16 nM) without and with an excess of unlabeled ligand (lanes g and h). The figure represents a typical experiment representative of three separate experiments. The indicated yields represent the mean of at least three experiments, and relative variations between experiments never exceed 10%.

cedure. These values were also corroborated by the detailed analysis of the binding properties of membranes prepared from photolabeled cells (see Figure 2 and related paragraph in the text).

Chemistry and Topographical Requirements of Covalent Labeling

A strong indication of the chemical and topographical specificities of the B_2 receptor photolabeling was the possibility of keeping excess free ligand up to 2 nM concentrations during the irradiation phase without labeling proteins other than the receptor (not shown).

The presence of a phenol moiety at the ligand N-terminal ends was required for efficient covalent labeling. [^{125}I](Tyr^8)-BK and [^{125}I]Lys^0-(Tyr^8)-BK, which display agonist properties, could not be significantly crosslinked to the receptor (Figure 2B). These negative results imply that their N-terminal amino group (and the amino group of the lysine side-chain of [^{125}I]Lys^0-(Tyr^8)-BK) does not participate in receptor labeling, in agreement with the failure of lysine to inhibit ligand crosslinking. They do indicate that [^{125}I]Tyr^0-BK crosslinking involves its phenol moiety. That [^{125}I]HPP-HOE

140 crosslinking involves its phenol moiety was corroborated by other experiments (see next section and Figure 2A).

Preservation of Receptor Binding and Signal Transduction Properties upon Photochemical Treatment

Taking into account the rapidity and diversity of chemical reactions catalyzed by the ruthenium chelate/APS/hν system, it appeared necessary to verify that undesired reactions accompanying ligand crosslinking to the receptor do not essentially perturb the pharmacological behavior of the system. COS-7 cells expressing B_2 receptors were submitted to irradiation treatment, with or without previous saturation of the binding sites by BK, HOE 140, or its crosslinkable analog HPP-HOE 140 (Figure 2A). When the cells were preirradiated in the presence of ruthenium chelate/APS without any ligand, subsequent binding of [^{3}H]BK and [^{125}I]HPP-HOE 140 to membrane preparations was significantly impaired (30% decrease in binding capacities for 3 s irradiation time, with no significant changes in affinity). The minimal treatment (3 s irradiation, 0.5 mM ruthenium chelate, 0.5 mM APS) of cells expressing moderate receptor amounts (10^5 sites/cell) induced about 30% decreases in the basal and BK-stimulated IP accumulation, consistent with the above mentioned loss of binding sites (not shown). Although one cannot exclude a possible destabilization of receptor structure, the slight loss of binding sites upon irradiation of unoccupied receptor might result from intramolecular receptor crosslinking which would cause limited accessibility for ligand binding.

Interestingly, ligand occupation of cell binding sites upon irradiation exerted a protective effect. Membrane preparations from liganded irradiated cells were washed so as to dissociate noncovalently bound ligand and checked for their ability to rebind [^{125}I]HPP-HOE 140. Irradiation of cells in the presence of BK induced no change in subsequent binding of the radioiodinated ligand to membrane preparations as compared to control nonirradiated cells (Figure 2A). As expected, irradiation of cells previously incubated with unlabeled crosslinkable HPP-HOE 140 induced a decrease in subsequent ligand binding to membrane preparations, consistent with the photolabeling yields determined from electrophoresis gels. No irradiation-induced decrease was observed for cells previously saturated with unmodified HOE 140, confirming that the phenol moiety of HPP-HOE 140 is required for ligand crosslinking; the difference observed between nonirradiated cells previously saturated with HOE 140 and HPP-HOE 140 should result from incomplete dissociation of the former ligand from the corresponding membrane preparations before the final radioligand binding. One can conclude that the photolabeling treatment has no overall deleterious effect and that the strategy is suitable for future structural investigations.

Extension of the Ru(bipy)$_3^{2+}$ Photosensitized Crosslinking Strategy to Other Receptors

We took advantage of the availability of phenolated ligands designed for radioiodination to extend the strategy to other GPCRs.

B_1 and AT_1 Receptors

We checked the possibility of performing covalent labeling of the AT_1 and B_1 receptors that belong to the same subfamily as the B_2 receptor, using respectively as ligands [^{125}I]Tyr^0-AII (which also possesses a Tyr residue at its position 4) and [^{125}I]JMV 1109, a synthetic B_1 receptor ligand which possesses a phenol moiety at its N terminus (Figure 1) and sufficient affinity for the receptor (Kd value of about 10 nM).

Figure 3 shows an efficient and rapid crosslinking of [^{125}I]Tyr^0-AII to the AT_1 receptor, thus providing an interesting alternative to the use of azido-phenylalanine derivatives [16, 17]. Much lower yields were obtained with [^{125}I](Sar^1)-AII (1% and 4% for 3 and 30 s irradiation times, respectively).

Interestingly, the human B_1 receptor photolabeling could also be achieved (Figure 3), with a quite satisfactory yield (10%–12%). These experiments constitute, to our knowledge, the first reported covalent labeling of this receptor.

Vasopressin and Oxytocin Receptors

The V_{1a}vasopressin receptor and the oxytocin receptor could also be efficiently labeled by their radioiodinated specific ligands, [^{125}I]HO-LVA and [^{125}I]OTA, respectively (Figures 1 and 3), the ability of [^{125}I]OTA to label both receptors being consistent with previously established pharmacological properties [18]. The labeling patterns, including the visualization of receptor proteolytic fragments, are quite superimposable to those previously obtained upon photolabeling by azido-derivatives which were shown to react with amino acids located in the upper parts of TM3, TM7, and the first extracellular loop [19, 20].

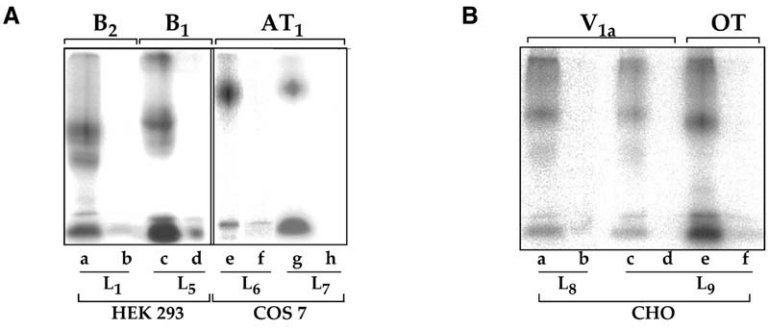
Extension of the Crosslinking Strategy to Receptor Photolabeling with Functionalized Ligands

Applications of receptor covalent labeling should include receptor purification for structural studies and adsorption of covalent ligand-receptor complexes to specific matrices for 2D crystallization (Figure 6). In this respect, it will be worth testing the possibility of adsorption of Gly-Gly-His-tagged ligand-receptor complexes to Ni^{2+} chelating supports.

These applications require receptor labeling with functionalized ligands. The B_2 could be photolabeled by the ligand L_{13} possessing, in addition to a Tyr residue, a biotin at its N terminus. The B_2 and AT_1 receptors could also be efficiently labeled by ligands L_{12} and L_{11}, N-terminally extended by the Gly-Gly-His-Tyr sequence (Figures 1 and 4) and displaying fairly preserved affinities (Kd values in the nanomolar range). The presence of this sequence at the ligand extremity should be helpful to target crosslinking to the ligand vicinity (possibly including ligand crosslinking itself), when required. We verified that the Gly-Gly-His/Ni^{2+} chelate [6–9], in the presence of magnesium monoperoxyphthalate (MMPP), could catalyze [^{125}I]HPP-HOE 140 covalent labeling of the B_2 receptor (8%–10% yield) (Figure 4).

Discussion

The present work constitutes an innovating alternative to existing strategies for GPCR covalent labeling by



Receptor	name	%crosslinking
B_2	L_1 [^{125}I]HPP-HOE 140	20%
B_1	L_5 [^{125}I]JMV 1109	11%
AT_1	L_6 [^{125}I]Tyr^0-AII	18%
AT_1	L_7 [^{125}I](Sar^1)-AII	4%
V_{1a}	L_8 [^{125}I]HO-LVA	21%
OT	L_9 [^{125}I]OTA	12%
V_{1a}	L_9 [^{125}I]OTA	17%

Figure 3. Extension of the Photolabeling Strategy to Other Receptors

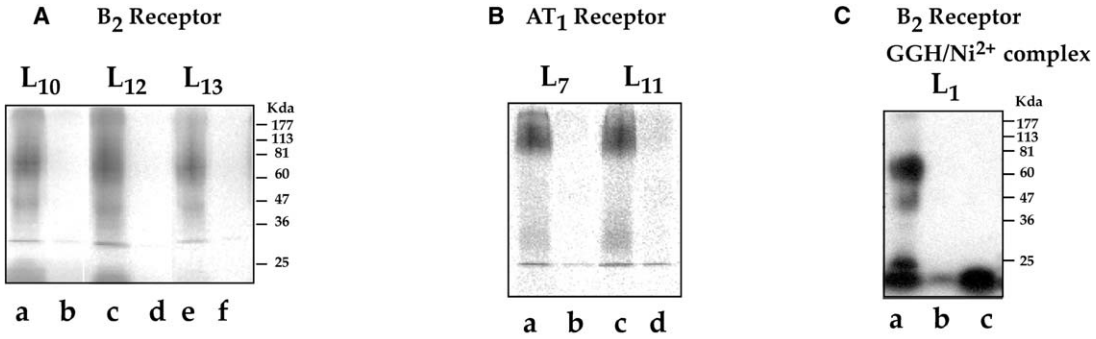
(A) B_1 and AT_1 receptors HEK 293 or COS-7 cells, transiently expressing the B_2 or B_1 bradykinin receptors, or the AT_1 angiotensin II receptor, were saturated by their respective radioiodinated ligands [^{125}I]HPP-HOE 140, [^{125}I]JMV 1109, [^{125}I]Tyr^0-AII, and [^{125}I](Sar^1)-AII. After removal of unbound ligand and rapid washing, the cells were irradiated for 3 s in the presence of $Ru(bipy)_3^{2+}$/APS. Cells were solubilized in electrophoresis sample buffer and analyzed by SDS-PAGE and autoradiography. Lanes a and b, photolabeling of B_2 receptor expressed HEK 293 cells (50 × 10^3 sites/cell, 1.8 × 10^6cells/well), after site saturation with [^{125}I]HPP-HOE 140 (2 nM) in the absence or presence of excess unlabeled ligand; notice that the B_2 receptor displays a decreased extent of glycosylation as compared to receptor expressed in COS-7 cells. Lanes c and d, photolabeling of the B_1 receptor transiently expressed in HEK 293 cells (40 × 10^3 sites/cell, 1.5 × 10^6 cells/well), an expression level higher than those routinely found in COS-7 cells [38], after site saturation with [^{125}I]JMV 1109 (10 nM) in the absence or presence of excess unlabeled ligand. Lanes e and f, photolabeling of the AT_1 receptor expressed in COS-7 cells, after site saturation with [^{125}I]Tyr^0-AII (3 nM). Lanes g and h, photolabeling of the AT_1 receptor expressed in COS-7 cells, after site saturation with [^{125}I](Sar^1)-AII (2 nM) in the absence or presence of excess unlabeled ligand.

(B) Vasopressin and ocytocin receptors. The human V_{1a} vasopressin and ocytocin receptors, stably expressed in CHO cells (expression levels: 3–4 × 10^5 and 1–1.5 × 10^5 sites/cell, respectively) were saturated by [^{125}I]HO-LVA (0.5 nM) or [^{125}I]OTA (0.5 nM and 3 nM for V_{1a} and ocytocin receptors, respectively). After removal of unbound ligand and rapid washing, the cells were irradiated for 5 s in the presence of $Ru(bipy)_3^{2+}$/APS. Cells were solubilized in electrophoresis sample buffer and analyzed by SDS-PAGE and autoradiography. Lanes a and b, V_{1a} receptor labeling by [^{125}I]HO-LVA, in the absence or presence of excess unlabeled ligand. Lanes c and d, V_{1a} receptor labeling by [^{125}I]OTA in the absence or presence of excess unlabeled ligand. Lanes e and f, OT receptor labeling by [^{125}I]OTA in the absence or presence of excess unlabeled ligand. As detailed for the B_2 receptor ligands (Figure 2), no labeling was observed with any of the ligands on control nontransfected cells. Receptor labeling was suppressed when irradiation was omitted (not shown). The indicated yields represent the mean of at least three experiments, and relative variations between experiments never exceed 10%.

specific ligands. Several receptors of the family could be photolabeled, in intact cells, by ligands possessing a radioiodinated phenol moiety, using $Ru(bipy)_3^{2+}$ in the presence of APS. The labeling yields were at least as high as those obtained using other methods, most often involving azido-derivatives [16, 17, 19, 20], with a substantial advantage in terms of reaction time course. Concerning the B_2 receptor which we studied in more detail, previous BK or analog crosslinking studies were carried out using classical bifunctional agents [11–13]; however, the labeling yields were only assessed by indirect evidence and the experimental protocols recently used for the characterization of B_2 receptor dimeric structures [21, 22] suggest rather low efficiencies. A requirement for the high efficiency of the ruthenium chelate/APS/hν procedure appears to be the localization of the ligand phenol moieties in the receptor extracellular loops or upper parts of transmembrane domains, which ensure their accessibility to the reagents which cannot penetrate into the cells. The pharmacology of the used ligands and previous mapping studies of the various receptors [11, 13, 19, 20] are consistent with this assumption; the much lower efficiencies in the crosslinking of [^{125}I](Tyr^8)-BK and [^{125}I](Sar^1)-AII to their respective receptors probably results from a more buried position of their Tyr residues in the receptor binding site [11–13, 23], although the lack of neighboring reacting partner might provide an alternative explanation.

The chemical mechanism of the ligand phenol reticulation to the receptor deserves a comment: it has been postulated that radical formation at ortho positions of the hydroxyl group are primarily involved in crosslinking reactions (Figure 5), without excluding alternative possibilities [9, 10]. Our data report attempts to crosslink a phenol moiety possessing an iodine atom at an ortho position of the hydroxyl group: obviously, this iodine does not inhibit the crosslinking reaction and is not eliminated. Moreover we showed that the diiodinated compounds [$^{125}I_2$]Tyr^0-BK and [$^{125}I_2$]HPP-HOE 140 could be crosslinked to the receptor with unchanged yields as compared to the monoiodinated compounds (not shown). It indicates that, as previously emphasized [9], the chemistry of phenol crosslinking is not exhaustively understood (Figure 5).

The most likely receptor candidates for crosslinking to phenolated ligands (Figure 5B) appear to be Tyr, Cys, and Trp residues and the extracellular disulfide bridge(s). It was suggested by their ability to reverse the B_2 receptor photolabeling by [^{125}I]HPP-HOE 140 (which does not constitute a proof by itself [9]), by previous mechanistic studies relative to metal chelates [9, 10] or heme enzymes [24–28], and by recent data emphasizing cystine reactivity [29]. Preliminary experiments designed to investigate the role of extracellular Tyr residues (B_2 receptor mutated at Tyr^{174} and Tyr^{188}, V_{1a} receptor mutated at Tyr^{115}) were unsuccessful; how-



Receptor	name	%crosslinking
B_2	L_1 [^{125}I] HPP-HOE 140	9% (Gly-Gly-His/Ni^{2+})
AT_1	L_7 [^{125}I] Tyr^0-A II	18%
B_2	L_{10} [^{125}I] Tyr^0-HOE 140	19%
AT_1	L_{11} [^{125}I] GGHY- A II	15%
B_2	L_{12} [^{125}I] GGHY-HOE 140	22%
B_2	L_{13} [^{125}I]Bio-Tyr^0-HOE 140	21%

Figure 4. Photolabeling of AT_1 and B_2 Receptors by Functionalized Ligands

(A and B) The B_2 (A) and AT_1 (B) receptors, transiently expressed in COS-7 cells, were photolabeled for 5 s by the parent-specific ligands (L_{10}: 0.4 nM; L_7: 1.8 nM) or ligands possessing either a biotin (L_{13}: 0.4 nM), or a GGH sequence (L_{11}: 0.3 nM and L_{12}: 1.8 nM) at their N termini, in the presence of $Ru(bipy)_3^{2+}$/APS as described in the Experimental Procedures. Lanes b, d, and f, presence of an excess of unlabeled ligand. (C) B_2 receptor labeling by [^{125}I]HPP-HOE 140 in the presence (lane a) or absence (lane c) of 5 mM GGH/Ni^{2+} complex and 5 mM MMPP (reaction time 1 min). Lane b, presence of excess unlabeled ligand during site saturation. The indicated yields represent the mean of at least three experiments, and relative variations between experiments never exceed 10%.

ever, one cannot rule out possible compensatory mechanisms allowed by the multiplicity of chemical reactions and receptor loop flexibility. Future compared mapping studies of these receptors and determination of crosslinked receptor fragments by mass spectrometry should bring informations about the involved chemical reactions. Interestingly, they should help to understand the role of extracellular domains in GPCR ligand recognition and activation or inactivation processes. Such a role for the second extracellular loop in rhodopsin activation has recently been shown [30].

More generally the diversity and complexity of electron transfer reactions and generation of protein radicals is emphasized by the mechanism of action of heme enzymes [24]. Crosslinking reactions described in the present work obviously depend on fortuitous proximity between a ligand phenol and selected receptor amino acids. An essential point is that the diversity of appropriate electronic environments is not inconsistent with the specificity of the labeling of each receptor of interest, with negligible random reactions and no overall deleterious modifications of the signal transduction system. The diversity of possible chemical reactions probably explains the successful examples reported in this paper and allows us to predict applications to other signal transduction systems.

The ruthenium chelate photosensitized reactions should also be extended to intramolecular crosslinking between specific amino acids of wild-type or mutant receptors possessing Tyr, Cys, or Trp at appropriate locations; it might allow the stabilization of selective conformational states and thus be helpful for mechanistic and structural studies (Figure 6). Stabilization of rhodopsin extracellular domain through a salt bridge [31] or an additional disulfide bridge [32] underlines the interest of this goal.

Receptor photolabeling by functionalized ligands possessing a biotin or a Gly-Gly-His sequence, restricted at this stage to the AT_1 and B_2 receptors, open interesting applications such as receptor purification, targeted crosslinking in the ligand vicinity, and adsorption of covalent ligand-receptor complexes or larger supramolecular complexes to specific matrices for 2D crystallization (Figure 6). The initial goal proposed by Kodadek's group, i.e., protein-protein crosslinking [10], should be a matter of interesting applications in the GPCR field (Figure 6); studies dealing with receptor-cofactor interactions and receptor homo- or heterodimerization should take advantage of crosslinking strategies involving chemical reactions which display specificities other than those allowed by classical bifunctional agents or photolabels. The various structural applications will require taking into account the localization of desired crosslinking sites, the diffusion and cell penetration properties of metal chelates, and their action radius in terms of electronic transfer. In this respect, the possibility of using the Gly-Gly-His/Ni^{2+} chelate [7] is liable to extend the applications. When neces-

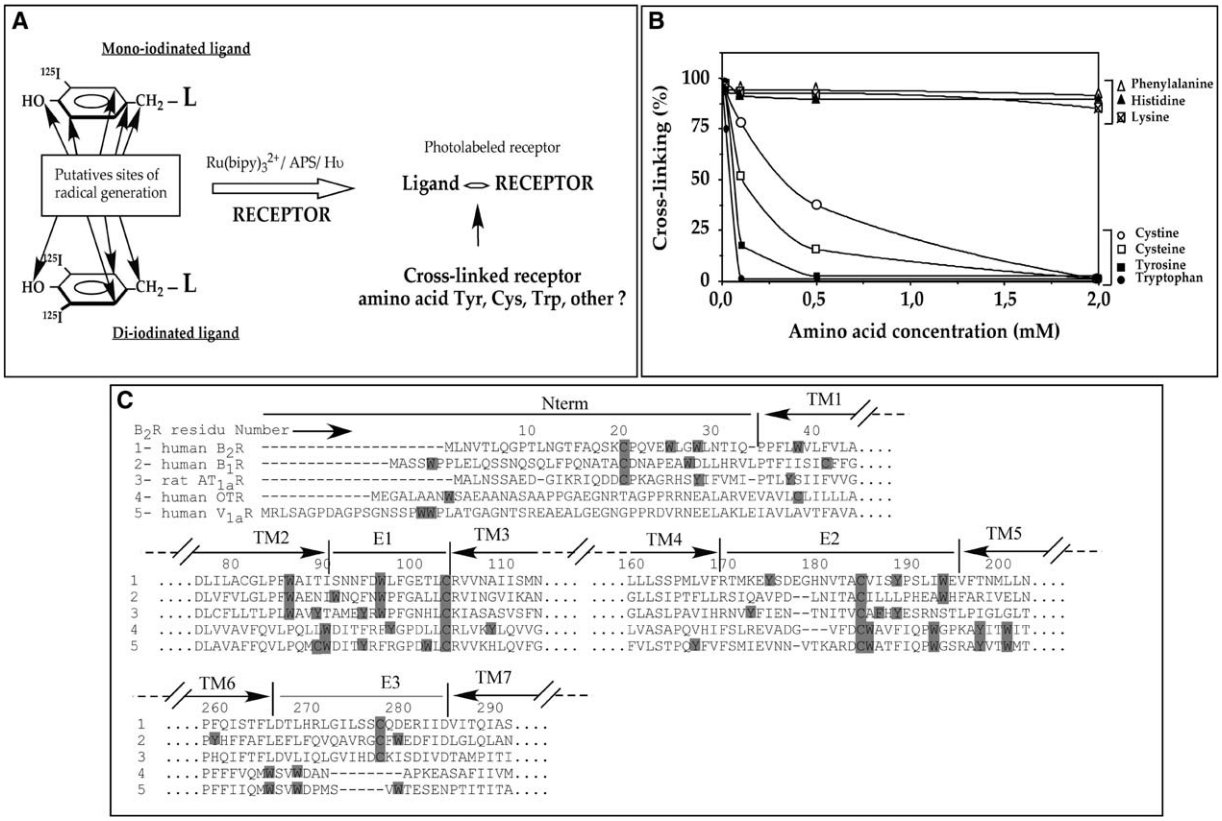


Figure 5. Putative Reactions and Crosslinking Sites Involved in Receptor Photolabeling

The common structural feature of the ligands which can be efficiently crosslinked to the various GPCRs is the presence of a parahydroxybenzyl group radio-iodinated at one of its ortho positions for routine experiments (A). The presence of a second iodine atom does not prevent crosslinking and the iodine atoms do not appear to be eliminated during the Ru(bipy)$_3^{2+}$/APS catalyzed reaction, indicating that the reactions are not restricted to the phenol ortho positions which have been suggested to be primarily involved [9, 10]. A detailed chemical study would be required to determine the effects of iodine atoms on the preferential generation of free radicals on the various positions.

(B) Effect of various amino-acids on B_2 receptor photolabeling by [^{125}I]HPP-HOE 140 using the Ru(bipy)$_3^{2+}$/APS system: The B_2 receptor transiently expressed in COS-7 cells was photolabeled as described in the legend of Figure 2 and Experimental Procedures. Various amino acids were added, at the indicated concentrations, to the Ru(bipy)$_3^{2+}$/APS containing medium immediately before irradiation (5 s). Results are expressed as % of photolabeling yield without any added amino acid. The figure represents a typical experiment representative of three separate experiments.

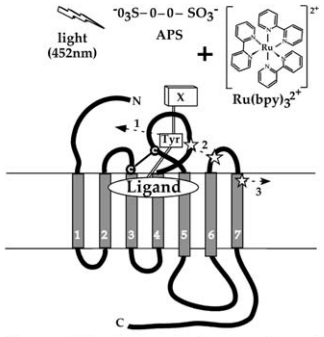
(C) Location of Tyr, Cys, and Trp residues in the GPCR extracellular domains or upper part of the TMs, as candidates to ligand crosslinking photosensitized by extracellularly added ruthenium chelate. The candidates to ligand crosslinking in the B_2 receptor are Tyr^{174}, Tyr^{188}, Trp^{25}, Trp^{28}, Trp^{96}, Trp^{193}, the conserved Cys^{103}-Cys^{184} disulfide, Cys^{20}, and Cys^{277} (possibly disulfide linked).

sary, the crucial problem of accessibility might be solved by replacing Ru(bipy)$_3^{2+}$ by metallic porphyrin derivatives [33] and APS by permeant oxidizing agents.

Finally, the short duration times allowed by the photochemistry of ruthenium compounds should give rise to applications in cell biology, for instance kinetic studies of signaling cascades, and thus help to better understand the dynamic aspects of scaffoldings in these pathways.

Significance

The present work reports, to our knowledge, the first applications of Ru(bipy)$_3^{2+}$ photosensitized reactions, previously initiated for protein-protein crosslinking [10], to the covalent labeling of GPCRs by appropriate ligands possessing a radioiodinated phenol. Based on a more extensive study of B_2 receptor labeling by agonist and antagonist ligands, which constitutes by itself a significant progress, we performed a covalent labeling of the B_1 bradykinin receptor and proposed the same strategy for the photolabeling of the AT_1 angiotensin II receptor, the V_{1a} vasopressin receptor, and the oxytocin receptor. The pharmacology of the used ligands, previous mapping studies of the various receptors [11, 13, 19, 20], and the likely difficulty of the ruthenium chelate to penetrate inside the cells, point to crosslinking sites located in the extracellular loops or the upper part of the transmembrane domains and probably involving Tyr, Cys, or Trp residues. Such crosslinkings, which display specificities other than those allowed by classical bifunctional agents or photolabels, is expected to stabilize receptors for structural studies and might be completed by additional intramolecular or intermolecular crosslinking. Protein-protein crosslinking should be a



X	Applications
Biotin-spacer arm	- Stabilization and purification of receptor or supramolecular complexes - Adsorption to streptavidin-derivatized surfaces
Gly-Gly-His	- Targeted cross-linking - Stabilization and purification of receptor or supramolecular complexes ? - Adsorption to metal-chelated lipids layers ?

(1) : Cross-linking between the ligand tyrosine and receptor residue(s)

(2) : Putative receptor intramolecular cross-linking

(3) : Putative cross-linking of receptor to an interacting partner

☆ : Tyr, Cys or Trp residue (other ?)

Figure 6. Potential Applications of Oxidative Crosslinking to GPCR Structural and Signaling Mechanism Studies

The photolabeling of several GPCRs with ligands possessing a phenol moiety probably involves the extracellular loops or the upper parts of the TMs, taking into account the lack of penetration of the reagents $Ru(bipy)_3^{2+}$ and APS. Future mapping studies will allow characterization of the receptor crosslinking sites. Stabilization of specific receptor conformational states through covalent binding can be expected, and might be completed by additional intramolecular crosslinking, using appropriate mutagenesis. The interest of this kind of biochemical engineering is emphasized by growing evidence for a concerted role of extracellular loops and transmembrane domains in GPCR activation. Intermolecular crosslinking might also occur between the receptor and interacting partners. The possibility of applying crosslinking to functionalized ligands should open the way to the purification of covalent ligand-receptor complexes or larger supramolecular edifices, as well as targeted crosslinking or adsorption to derivatized surfaces for structural studies.

matter of interesting applications in the GPCR field; studies dealing with receptor-cofactor and receptor homo- or heterodimerization should benefit from these new crosslinking strategies. The chemical design of fonctionalized crosslinkable ligands open interesting applications for the structural analysis of ligand-receptor complexes, or larger supramolecular edifices. The short reaction times required for ruthenium chelate photolabeling should allow kinetic studies of signaling pathways, thus extending biochemical approaches to cell biology. They might be associated with alternative oxidative crosslinking strategies using the Gly-Gly-His/Ni^{2+} chelate or porphyrins, and be applied to other signal transduction systems for assessing their general usefulness in molecular pharmacology.

Experimental Procedures

Reagents and Ligands

BK, Tyr^0-BK, (Tyr^8)-BK, Lys^0-(Tyr^8)-BK, and AII were purchased from Sigma, myo-[2-^{3}H]inositol and [^{3}H]BK (specific radioactivity about 100 Ci/mmol) from Amersham Pharmacia Biotech, and $Ru(bipy)_3^{2+}$ from Aldrich. The radioiodinatable B_2 receptor antagonist hydroxyphenyl-propionyl-HOE 140 (HPP-HOE 140) and B_1 receptor antagonist JMV 1109 (hydroxyphenyl-propionyl-Lys-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic) were kindly supplied by Professor J. Martinez (CNRS, Montpellier, France). Tyr^0-AII, Tyr^0-BK and related functionalized derivatives were synthesized by solid phase strategy, essentially as described in [34] with preoxidation of biotin to its sulfone derivative [35], the vasopressin and ocytocin receptor ligands synthesized as in [18]. The radioiodinated peptidic ligands (mono- or di-iodo derivatives) were prepared using $Na^{125}I$ and iodogen as oxidizing agent. COS-7, CHO cells and HEK 293 cells were from the European Cell Type Collection (Salisbury, United Kingdom). Anti c-Myc antibodies were from B. Mouillac.

Receptor Expression

The human B_2 receptor sequence has been determined by Hess et al. [36]. The WT or mutant receptors were systematically tagged through the addition of a 10 amino acid epitope (EQKLISEEDL) from the c-Myc oncogene at their N termini truncated at the Asn^3 residue [14, 37]. The cDNA encoding the human B_1 receptor was subcloned in the pcDNA3 vector [14]. The AT_1 receptor sequence encoded a receptor possessing a c-Myc epitope at the N and C termini as in [15]. Receptors were transiently expressed in COS-7 or HEK 293 cells using the electroporation transfection method [14]. The V_{1a} and oxytocin receptors were stably expressed in CHO cells (4–5 × 10^5 and 1–2 × 10^5 sites/cell respectively). Receptor characterization and crosslinking experiments were performed on cells cultured for 2 days at 37°C in Dulbecco's modified Eagle's medium, 4.5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin.

Crosslinking Experiments

Crosslinking experiments were routinely carried out on intact cells in 24-well plates. Ligand binding to intact cells expressing the B_2 receptor was carried out at 4°C in Dulbecco's phosphate-buffered saline supplemented with 140 μg/ml bacitracin, 1 mg/ml bovine serum albumin, 1 mM o-phenanthroline and 10^{-5} M captopril (pH 7.0), using a 3 hr incubation time, under gentle agitation [14, 37]. Incubation media used for the other receptors are those described in [19, 20, 23, 38]. The binding medium containing excess radioiodinated ligand was discarded and the cells were washed with ice-cold phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (PBS). Photolabeling in the presence of ruthenium chelate was carried out as follows: under red light, the cell wells were added with PBS supplemented with cold $Ru(bipy)_3^{2+}$ (0–2.5 mM) and APS (0–0.5 mM), then irradiated for 3–30 s, at 0°C, by visible light (two 100 watt tungsten lamps, located 15 cm from the samples, and separated from these latter by a water filter). The irradiation medium was removed and the cells were solubilized in 150 μl electrophoresis sample buffer (containing mercaptoethanol which stops any further crosslinking) and containing or not a protease inhibitor cocktail from Sigma (2.5 μl). The samples were sonicated before analysis.

The yields of covalent labeling were estimated by comparing the radioactivities associated to receptor bands in electrophoresis gels to the radioactivities representing specific binding of the ligand to an equivalent cell sample, measured immediately before the crosslinking step (systematic evaluation of total and nonspecific binding, and electophoretic control of samples from nonirradiated cells). The obtained values were in close agreement with those obtained by evaluation of radioactivities specifically (difference between binding in the absence or presence of excess unlabeled ligand) associated to cells after crosslinking followed by one PBS washing (in the presence of 2 mM tyrosine) and three 15–30 s acid washings (150 mM NaCl, AcOH 50 mM [pH 3]) to dissociate noncovalently bound ligand.

Ligand Binding to Membranes

Membranes from COS-7 cells (100 μg in 1 ml) expressing the B_2 receptor, prepared as described previously [14] were incubated with ligands for 1.5 hr at 25°C in 25 mM TES (pH 6.8), 140 μg/ml

bacitracin, 1 mg/ml BSA, and 1 mM o-phenanthrolin added with a protease inhibitor cocktail from Sigma (20 μl/ml). Unbound ligand was eliminated by centrifugation (4°C, 15 min, 20,000 × g), followed by PBS washing and centrifugation membrane pelleting. For immunoblotting analysis, the membranes were solubilized by addition of concentrated "electrophoresis sample buffer."

Electrophoresis, Electrotransfer, and Autoradiography

The samples were analyzed by SDS-PAGE (10% acrylamide) and the radioactivity revealed by autoradiography. Quantitative estimations of the radioactivity profiles were routinely carried out by storage phosphor autoradiography using a Fujix Bas 1000 apparatus. Proteins separated by electrophoresis of solubilized membrane preparations were transferred onto nitrocellulose membranes. Receptor detection was carried out using anti c-Myc antibodies and the SuperSignal West Pico Chemiluminescent kit from Pierce.

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