Structure of the Bradykinin B_2 Receptors' Amino Terminus†

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Received January 17, 1996; Revised Manuscript Received April 3, 1996⊗

ABSTRACT: The peptide hormone bradykinin exerts important biological functions by binding to and activating bradykinin B_2 receptors. B_2 receptors belong to the seven transmembrane domain (7TM) receptor family. Cloning of the cDNA sequences for the rat, human, and mouse bradykinin B_2 receptor revealed several in-frame AUG triplets as potential initiation sites for translation. Due to "Kozak-like" consensus nucleotides, the AUG codon closest to transmembrane domain 1 was assumed the preferred initiation site for translation, but the real amino terminus of the B_2 receptor protein was unknown. The amino terminus of several 7TM receptors has been shown to be essentially involved in receptor activation and/or ligand binding. Therefore we determined the amino terminus of the human and of the rat B_2 receptor using domain-specific antipeptide antibodies, amino acid sequence analysis, and *in vitro* transcription/translation. We report that the human and rat B_2 receptor protein start with the methionine which is translated from the first in-frame AUG. This start site extends the known amino terminus of the human and rat B_2 receptors by 27 or 30 amino acid residues, respectively. Antibodies raised against a peptide of the initial 27 amino acids of the human B_2 receptor stained B_2 receptors on intact cells. This finding excludes the existence of a signal sequence for this receptor.

Receptors for the peptide hormone family of kinins (Müller-Esterl et al., 1986) are classified into B_1 and B_2 type. The receptors belong to the 7TM receptor family which have seven hydrophobic domains assumed to form α-helical structures (Schertler et al., 1993). They transmit their signal by activation of a G protein-mediated signal transduction cascade (Bascands et al., 1993; Jones et al., 1995). B_1 receptors bind carboxy-terminally-truncated derivatives of bradykinin, e.g., des-Arg^9-bradykinin, desArg^{10}-kallidin (Regoli & Barabé, 1988), whereas the agonists of B_2 receptors are kinins with intact carboxy terminus (Bhoola et al., 1992). The cDNA sequences of the rat (McEachern et al., 1991), human (Hess et al., 1992), and mouse (Yokoyama et al., 1994) B_2 receptors display several in-frame ATG triplets. Due to "Kozak-like" consensus nucleotides, the AUG closest to the beginning of transmembrane domain 1 was assumed to be the preferred initiation site for translation. According to this prediction the coding region of the rat (Pesquero et al., 1994), human (Ma et al., 1994a), and mouse (Ma et al., 1994b) B_2 receptor protein was localized entirely on the last exon, i.e., exon 3 for the human and mouse B_2 receptor and exon 4 for the rat B_2 receptor. The amino terminus of various G protein-coupled receptors has been found important for ligand binding and/or signal transduction (Oppermann et al., 1993; Yokota et al., 1992). We analyzed the amino-terminal structure of denatured and native B_2 receptors using domain-specific antibodies, amino acid sequence analysis, and *in vitro* transcription/translation. In agreement with the scanning model developed by Kozak (1989), we found that the first in-frame AUG is used as start site of translation localized on exon 2 of the human and on exon 3 of the rat B_2 receptor gene without any evidence for context-dependent leaky scanning or the existence of a signal peptide.

EXPERIMENTAL PROCEDURES

Materials. The chemiluminescence detection kit and [^{35}S]-methionine were from Amersham; [2,3-prolyl-3,4-^{3}H]bradykinin (specific activity 98 Ci/mmol) was from NEN Dupont; 1,5-difluoro-2,4-dinitrobenzene (DFDNB) was from Pierce; wheat germ agglutinin (WGA from *Triticum vulgaris*), *N*-acetylglucosamine, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin were from Sigma; PVDF[1] sheets were from Millipore; Affi-Gel 10 and nonfat dry milk were from Bio-Rad; HOE140 was from Hoechst; the TNT reticulolysate kit was from Promega; the first strand cDNA synthesis kit was from Pharmacia; Vent-(exo+)DNA polymerase was from New England Biolabs; Centricon 30 filters were from Amicon. All other chemicals were of analytical grade.

Cell Culture. Human foreskin fibroblasts, HF-15 (Roscher et al., 1983), were grown to confluency in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf

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† This work was supported in part by the Deutsche Forschungsgemeinschaft Grant Mu 598/3-2.
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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

[1] Abbreviations: PVDF, polyvinylidenedifluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; E64, *N*-[*N*-(L-3-transcarboxirane-2-carbonyl)-L-leucyl]agmatine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride.

S0006-2960(96)00106-7 CCC: $12.00

serum for 2−3 weeks and used at passages 8−15. Chinese hamster ovary cells (CHO) and CHO cells transfected with the rat B_2 receptor cDNA, rB2CHO12/3 (AbdAlla et al., 1996), were grown in Ham's F12 medium containing 10% fetal calf serum. Cells were kept in a humidified 5% CO_2/95% air atmosphere at 37 °C.

Reverse Transcriptase PCR. Different human B_2 receptor cDNA fragments were obtained by reverse transcriptase PCR (RT-PCR). RNA was prepared from a human fibroblast cell line with the RNAzol B method (Wak Chemie, Germany). The following primers were used for cDNA synthesis: 5′ nucleotide position [according to Hess et al. (1992)] and primer direction are in parentheses: BKR1exC, 5′-CAAATGTTCTCTCCCTGGAAG-3′ (139, forward); BKR-18, 5′-ATGCTCAATGTCACCTTG-3′ (223, forward); BKR-16, 5′-AAGCAACTGTCCCTCAATC-3′ (1372, reverse). Primer BKR1exC directs DNA synthesis to start with the first in-frame ATG whereas BKR-18 will amplify B_2 receptor DNA beginning with the last, i.e., the third in-frame ATG. The reverse transcriptase-PCR products were cloned into the *Eco*RV site of the eucaryotic expression vector pcDNA3 (Invitrogen). Identity of the fragments created by PCR with the published human B_2 receptor cDNA sequence was confirmed by DNA sequencing.

In Vitro Transcription and Translation. In vitro transcription and translation of the various human bradykinin B_2 receptor expression clones was performed with the TNT reticulolysate kit (Promega) including T7 polymerase according to the manufacturer's instructions. One reaction was performed using approximately 400 ng of purified DNA. The polypeptides were labeled by [^{35}S]methionine.

Membrane Preparation. HF-15 cells and CHO cells were washed twice with ice-cold phosphate buffered saline (PBS). Cells were harvested and homogenized in PBS containing protease inhibitors, 1 mM phenylmethanesulfonylfluoride (PMSF), 1 μg/mL *N*-[*N*-(L-3-transcarboxirane-2-carbonyl)-L-leucyl]agmatine (E64), and 2 μM leupeptin. After centrifugation (3000*g*, 15 min), membranes were recovered from the supernatant, sedimented by another centrifugation step (15000*g*, 30 min), and washed twice with PBS. Crude membranes (protein concentration 1 mg/mL) were stored at −80 °C until use. The binding activities of the membranes for [^{3}H]bradykinin were 0.5−1 pmol/mg of protein for HF-15 cells and 1.5−3 pmol/mg of protein for rB2CHO12/3 cells.

Synthesis of Peptides and Production of Antipeptide Antibodies. Peptides derived from the rat or human B_2 receptor sequence were synthesized by solid phase peptide synthesis using the fluorenylmethyloxycarbonyl (Fmoc) or the *tert*-butyloxycarbonyl (tBoc) chemistry as described previously (AbdAlla et al., 1996). Peptides purified by high-performance liquid chromatography (HPLC) were routinely analyzed by Edman degradation and electrospray mass spectrometry. Peptides derived from extracellular domains $ED1_A$ and $ED1_B$ (Figure 1) were used for immunization in rabbit without prior coupling to a carrier protein. Antisera were tested for antigen specificity and cross-reactivity with human or rat peptides by the indirect enzyme-linked immunosorbent assay (ELISA) using microtiter plates (MaxiSorb, Nunc) coated with 2 μg/mL of the peptide.

Purification of Antipeptide Antibodies by Affinity Chromatography. Purification of antibodies to the B_2 receptor by affinity chromatography with the respective antigen was performed as previously described (AbdAlla et al., 1996).

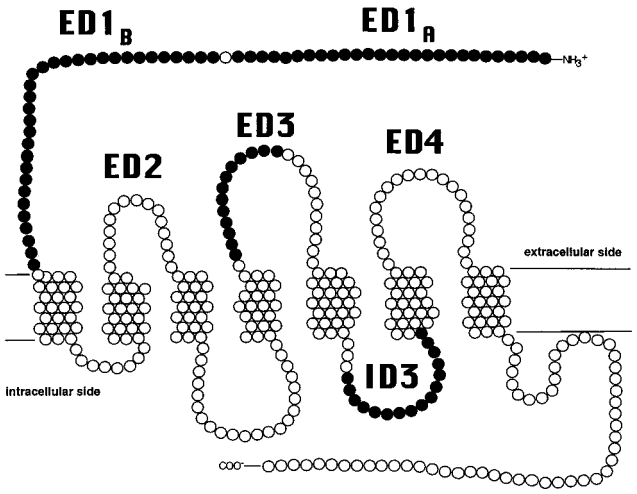
Western Blotting and Immunoprinting. Proteins were resolved by SDS−PAGE (Laemmli, 1970) and transferred to polyvinylidenedifluoride (PVDF) sheets using semidry blotting (Kyhse-Anderson, 1984). The sheets were treated with 50 mM Tris and 0.2 M NaCl, pH 7.4 (buffer A), containing 5% (w/v) nonfat dry milk and 1% (w/v) Tween 20 for 1 h. Antisera were diluted 1:1000 in buffer A containing 2% (w/v) bovine serum albumin (BSA). After a 30 min incubation at 37 °C, the PVDF sheets were washed 5× for 15 min each with buffer A and incubated for 30 min with peroxidase-labeled $F(ab')_2$ fragments of goat anti-rabbit antibody (Sigma, 1:5000). After extensive washing, bound antibody was visualized using the ECL chemiluminescence detection kit (Amersham).

Lectin Affinity Chromatography of B_2 Receptor. Enrichment of B_2 receptor from human foreskin fibroblasts by wheat germ agglutinin chromatography was done exactly as described (AbdAlla et al., 1996).

Immunoaffinity Chromatography and Amino Acid Sequence Analysis of the B_2 Receptor. Affinity-purified domain-specific antibodies were covalently coupled to Affi-Gel 10 according to the manufacturer's instructions (15 mg/mL gel). Membranes of rB2CHO12/3 (100 pmol of B_2 receptor/40 mg of total protein) were solubilized with 1% (w/v) sodium deoxycholate in PBS including 1 mM PMSF, 1 μg/mL E64, and 2 μM leupeptin. The deoxycholate was diluted to 0.1% (w/v) by the addition of 20 mM HEPES, pH 7.4, containing 150 mM NaCl and 1 mM EDTA (buffer B). Human B_2 receptors of HF-15 cells (100 pmol of B_2 receptor/80 mg of total protein) were partially enriched by WGA-affinity chromatography. Eluates from the lectin affinity column were diluted by buffer B with protease inhibitors. Then 10% glycerol and 0.1% (w/v) Triton X100 were added to the solubilized rat or human B_2 receptor preparation, and the solution was applied to the immunoaffinity matrix for an overnight incubation. The affinity matrix was extensively washed with buffer B, and bound proteins were eluted with 0.2 M glycine, pH 2.5, supplemented with 10% (v/v) 1,4-dioxane. The eluted protein fraction was neutralized with 1 M Tris, pH 8.0, and concentrated and desalted by Centricon filtration (exclusion limit 30 000 Da). The purity and identity of the enriched B_2 receptor was assessed by SDS−PAGE followed by silver staining and immunoprinting. For amino-terminal sequencing, proteins from three experiments were pooled, applied to a ProSpin sample preparation cartridge, and sequenced on a 477 A Protein Sequencer equipped with an on-line 120A PTH Analyzer (Applied Biosystems).

Affinity Cross-Linking of the B_2 Receptor. B_2 agonist bradykinin or antagonist HOE140 were cross-linked to the B_2 receptor by 1,5-difluoro-2,4-dinitrobenzene as previously described (AbdAlla et al., 1993).

Fluorescence-Activated Cell Sorting (FACS) Analysis. Confluent HF-15 cells (1 pmol of B_2 receptor per mg of protein) were detached with PBS and 0.5 mM EDTA, pH 7.4, and washed twice with ice-cold RPMI 1640 medium containing 0.1% (w/v) bovine serum albumin and 20 mM Na^+-HEPES, pH 7.4 (incubation medium). Cells (1×10^6) were suspended in the incubation medium containing the affinity-purified antibodies (10 μg/mL) and incubated for 1 h at 4 °C. After washing three times, fluorescein isothio-



Peptides used for immunization in rabbit

$ED1_A$	MFSPWKISMFLSVREDSVPTTASFSAD
$ED1_B$	MLNVTLQGPTLNGTFAQSKCPQVEWLGWLNTIQ
ED3	KEYSDEGHNVTAC
ID3	RNNEMQKFKEIQTERR

FIGURE 1: Topology of the human B_2 receptor. A model of the B_2 receptor topology based on hydrophobicity plots and a transmembrane α-helix hypothesis is presented. Positions of the peptides from extracellular domain 1_A, 1_B, and 3 ($ED1_A$, $ED1_B$, ED3) or from intracellular domain 3 (ID3) used to raise antisera are marked in black. The amino acid sequence of these peptides is given in the lower panel. The single-letter code for amino acids is used.

cyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Sigma), diluted 1:80 (v/v), was added to the cells. The cells were incubated for 1 h at 4 °C, washed, fixed with 2% (v/v) formaldehyde, and analyzed on a FACScan (Becton Dickinson) using the Lysis program.

RESULTS AND DISCUSSION

Staining of Denatured Human B_2 Receptors by Anti-$ED1_A$ and Anti-$ED1_B$ Antibodies in Immunoprinting. To determine the structure of the B_2 receptor's amino terminus, we raised antibodies against a peptide corresponding to extracellular domain 1_B ($ED1_B$) which is the predicted amino terminus of the B_2 receptor and to a peptide corresponding to extracellular domain 1_A ($ED1_A$) which is a B_2 receptor sequence located on exon 2 of the human B_2 receptor gene (Ma et al., 1994a) (Figure 1). We first asked whether denatured human B_2 receptors of human foreskin fibroblasts (HF-15) are recognized by the antibodies to $ED1_A$ or to $ED1_B$. Human B_2 receptors partially enriched by wheat germ agglutinin affinity chromatography were separated by SDS−PAGE and transferred to PVDF membranes, and the Western blot was probed by antibodies to $ED1_B$ and to $ED1_A$. Both antisera recognized a major protein band of 69 kDa (Figure 2, lanes 1 and 3), which is in good agreement with the apparent molecular weight of B_2 receptors from HF-15 cells (AbdAlla et al., 1993). Preabsorption of the antisera on their cognate peptides abolished the staining, confirming the specific interaction of the anti-$ED1_A$ and anti-$ED1_B$ antisera with the denatured B_2 receptors (exemplified for anti-$ED1_A$ antiserum: Figure 2, lane 2). As an additional control B_2 receptor was detected by anti-bradykinin antibodies after cross-linking of the agonist to B_2 receptors (Figure 2, lane 4). A 1000-fold excess of the antagonist HOE140 suppressed the cross-linking of bradykinin to the B_2 receptor (Figure 2, lane 5).

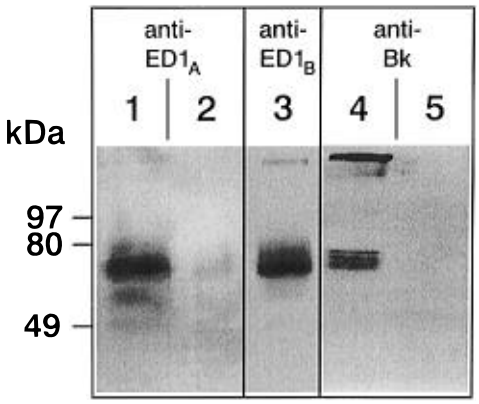


FIGURE 2: Immunoblotting of human B_2 receptors with domain-specific antisera to the amino terminus. Western blots of partially enriched B_2 receptors from HF-15 cell membranes were probed by antisera to $ED1_A$ (lane 1) and to $ED1_B$ (lane 3). As a control, the antiserum to $ED1_A$ was preabsorbed on the cognate antigen (lane 2). For comparison, bradykinin was cross-linked to the B_2 receptor in the presence (lane 5) or absence (lane 4) of a 1000-fold excess of HOE140 and detected by anti-bradykinin (anti-Bk) antiserum. Antiserum dilution was 1:1000, and bound antibodies were visualized using the chemiluminescence detection method.

Amino Acid Sequence Analysis of the Human B_2 Receptor. What is the amino-terminal sequence of the identified protein? We used a mixture of several domain-specific antibodies (AbdAlla et al., 1996) to isolate B_2 receptor protein from human foreskin fibroblasts by affinity chromatography. The purity of the isolated protein was assessed by silver staining after SDS−PAGE (Figure 3A, lane 1), and B_2 receptor identity was proven in immunoprinting (Figure 3A, lane 2). The isolated B_2 receptor protein was submitted to amino acid sequence analysis. The amino-terminal sequence obtained in two independent experiments is shown in Figure 3B. The isolated protein is the B_2 receptor. The amino-terminal sequence is derived from exon 2 of the B_2 receptor gene indicating that the first in-frame ATG located on exon 2 of the human B_2 receptor gene is used (after transcription) as initiation site (Figure 3B,I). This ATG precedes the predicted initiation site, i.e., the third in-frame ATG on exon 3. The B_2 receptor sequence of run II amino-terminally lacks 16 amino acids. This is probably due to partial proteolytic degradation of the protein during the purification as was similarly observed with the purification of, e.g., the endothelin receptor protein (Hick et al., 1995), though we cannot completely rule out the possibility that the sequence of run II resulted from initiation of translation at the second in-frame AUG.

Fluorescence-Activated Cell Sorting Analysis of Human B_2 Receptor's Amino Terminus. Do native B_2 receptors on the surface of intact cells also start with the first potential methionine or is the amino terminus identified in protein sequencing proteolytically cleaved as a signal peptide? A signal peptide has been hypothesized for a variety of G protein-coupled receptors, e.g., the endothelin (Sakurai et al., 1990) or the Ca^{2+} sensing (Brown et al., 1993) receptor. We stained B_2 receptors of intact HF-15 cells by antibodies to $ED1_A$ and to $ED1_B$ and did fluorescence-activated cell sorting (FACS) analysis. Anti-$ED1_A$ antibodies bound to human B_2 receptors on intact HF-15 cells (Figure 4A). Internalization and/or sequestration of B_2 receptors by a 40 min preincu-

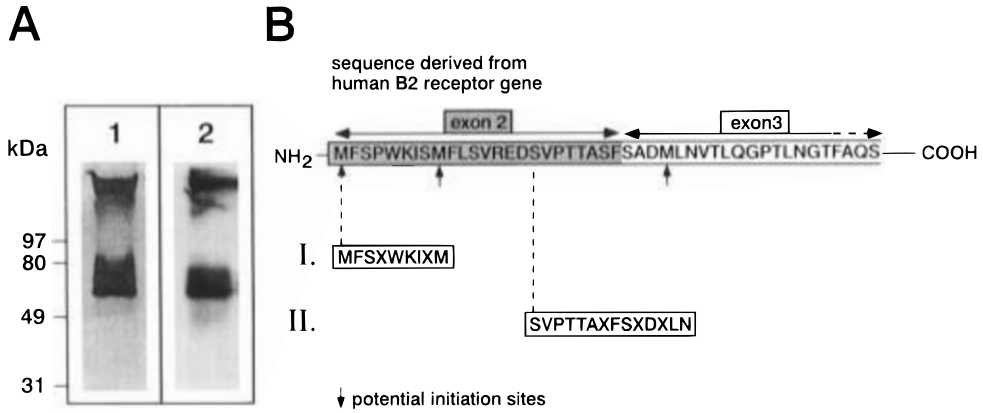


FIGURE 3: Amino acid sequence analysis of the human B_2 receptor. Human B_2 receptors of HF-15 cells were isolated by immunoaffinity chromatography. (A) Isolated proteins were separated by SDS−PAGE and visualized in silver staining (lane 1); enriched B2 receptors (10−20 fmol) were transferred to PVDF membranes and immunoblot was probed by anti-$ED1_A$ antiserum (lane 2). (B) Amino acid sequence analysis of two different B_2 receptor preparations (I, II) and alignment of the sequences to the protein sequence translated from the B_2 receptor gene. The single-letter code for amino acids is used; X stands for unidentified residue.

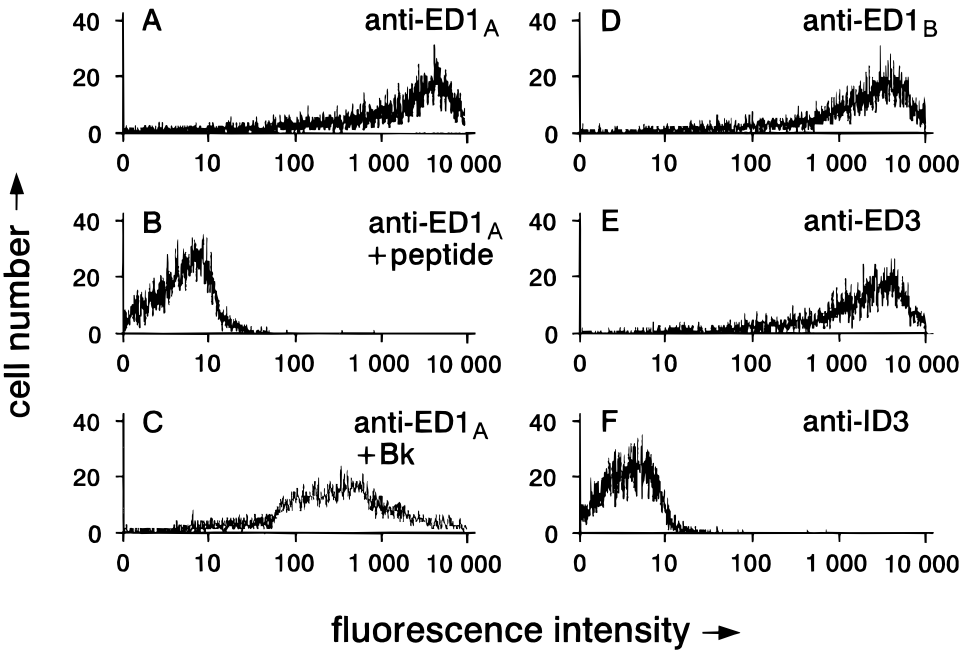


FIGURE 4: Fluorescence-activated cell sorting analysis of the B_2 receptor's amino terminus. Intact HF-15 cells containing about 1 pmol of B_2 receptor per mg of protein were labeled with affinity-purified antibodies to $ED1_A$ (A) and to $ED1_B$ (D). To internalize B_2 receptors, cells were pretreated with 1 μM bradykinin at 37 °C for 40 min, cooled to 4 °C, and stained by anti-$ED1_A$ antibodies (C). As controls antiserum to $ED1_A$ preabsorbed on the cognate peptide (B) or antiserum to intracellular domain 3, ID3 (F) were used. Staining of HF-15 cells by antibodies to extracellular domain 3 (ED3) is presented (E) to compare fluorescence intensities between different domain-specific antibodies. The ordinate specifies the relative cell number and the abscissa gives the log of fluorescence intensity.

bation at 37 °C with 1 μM bradykinin suppressed the staining of HF-15 cells by anti-$ED1_A$ antibodies (Figure 4C) confirming that anti-$ED1_A$ antibodies specifically interact with native B_2 receptors on the surface of intact cells. Preabsorption of the antibodies on their cognate peptide abolished the staining of HF-15 cells (Figure 4B). Antibodies to $ED1_B$ and to ED3 (AbdAlla et al., 1996) also bound to B_2 receptors of intact HF-15 cells (Figure 4D,E). The specific staining of B_2 receptors with anti-$ED1_A$, anti-$ED1_B$, or anti-ED3 antibodies was similar (cf. Figure 4A,D,E) indicating that equal amounts of B_2 receptors are recognized by each antibody. Domain-specific antibodies to intracellular domain 3 (ID3) served as a negative control (Figure 4F). This experiment clearly demonstrates that extracellular domain 1_A ($ED1_A$) is accessible to antibodies on intact cells indicating that this receptor region is not a signal peptide but an additional receptor domain of 27 amino acids located on the extracellular side of the cell.

In Vitro Transcription/Translation. The third AUG triplet was assumed a preferred start site due to "Kozak-like" consensus nucleotides which would enable context-dependent leaky scanning (Kozak, 1995). We therefore asked whether the third methionine located on $ED1_B$ closest to transmembrane domain 1 can act as start codon. To this end, we truncated the B_2 receptor cDNA thus that only the AUG closest to TM1 was available for translation initiation. We did *in vitro* transcription/translation and compared the product obtained with the truncated cDNA with a product of the full-length B_2 receptor cDNA. The ^{35}S-labeled proteins were separated by SDS−PAGE: The truncated cDNA was translated into a B_2 receptor of 35 kDa (Figure 5, lane 2); the full-length clone resulted in a single protein

Table 1: Sequence Comparison[1] of B2 Receptors' Amino-Terminal Domains Derived from Different Species

	$ED1_A$ ⟵	⟶ $ED1_B$
human	MFSPWKISMFLSVREDSVPTTASFSADMLNVTLQ..GPTLNGTFAQSKCPQVEWLGWL[2]	
rat	MDTRSSLCPKTQAVVAVFWGPGCHLSTCIEMFNITTQALGSAHNGTFSEVNCPDTEWWSWL[2]	
mouse	MPCSWKLLGFLSVHEPMPTAASFGIEMFNVTTQVLGSALNGTLSKDNCPDTEWWSWL[2]	
consensus[3]	.....s...k.....v....pt.aSf.ieMfNvTtQ.lGsalNGTfs..nCPdtEWwsWL	

[1] The single letter code is used. [2] Beginning of TM-1. [3] Identical residues between human, rat, and mouse (Yokoyama et al., 1994) sequences are indicated by uppercase letters, and identities between two of three species are given by lowercase letters.

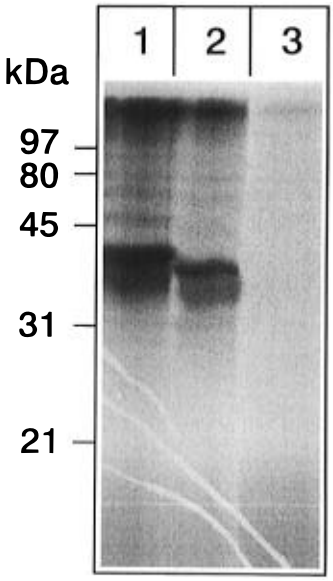


FIGURE 5: *In vitro* transcription/translation. The *in vitro* transcription/translation products of two different B_2 receptor cDNAs, which start three base pairs upstream of the first ATG in frame (lane 1) or with the third ATG in frame (lane 2) were labeled by [^{35}S]methionine and separated by SDS−PAGE. The autoradiography of a 15% SDS−polyacrylamide gel is presented. As a control the pcDNA3 vector without insert (lane 3) was used.

band of 38 kDa (Figure 5, lane 1). For comparison, the calculated molecular mass of the additional 27 amino acids is 3.5 kDa. The major differences in the electrophoretic mobility of B_2 receptors expressed in HF-15 cells and of B_2 receptors expressed with the reticulocyte lysate (69 versus 38 kDa) points to the fact that the B_2 receptor protein on the surface of eucaryotic cells, similarly to other G protein-coupled receptors, is heavily glycosylated (AbdAlla et al., 1993). We conclude that the third methionine acts as the start site if the cDNA is truncated. With the full-length cDNA, translation starts with the first in-frame AUG (Figure 5, lane 1). Under the experimental conditions applied, there is no evidence for context-dependent leaky scanning, as suggested. The vector pcDNA3 without insert served as a negative control. It did not lead to a labeled polypeptide chain (Figure 5, lane 3).

The Amino Terminus of the Rat B_2 Receptor. We demonstrated that the amino terminus of the human B_2 receptor is derived from the first in-frame AUG triplet. We asked whether, in analogy to the human B_2 receptor, it is also the first in-frame AUG which is the start site of the rat B_2 receptor. Two cDNA sequences for a rat B_2 receptor have been published differing in the absence (McIntyre et al., 1993) or presence (McEachern et al., 1991) of the nucleotides derived from exon-3 of the rat B_2 gene. These two cDNAs are probably derived from two different splice variants of rat B_2 receptor mRNAs (Pesquero et al., 1994). We stably expressed full-length rat B_2 receptor cDNA which contains the sequence derived from exon 3 (McEachern et al., 1991) in CHO cells. Recombinant rat B_2 receptor expressed in these cells is functionally active and displays characteristics similar to those of a B_2 receptor in primary cells (Quitterer et al., 1995). Antibodies to $ED1_B$ of the rat B_2 receptor sequence (AbdAlla et al., 1996) recognized a protein of 69 kDa in immunoblots (Figure 6A, lane 1). This protein was absent on mock-transfected cells indicating

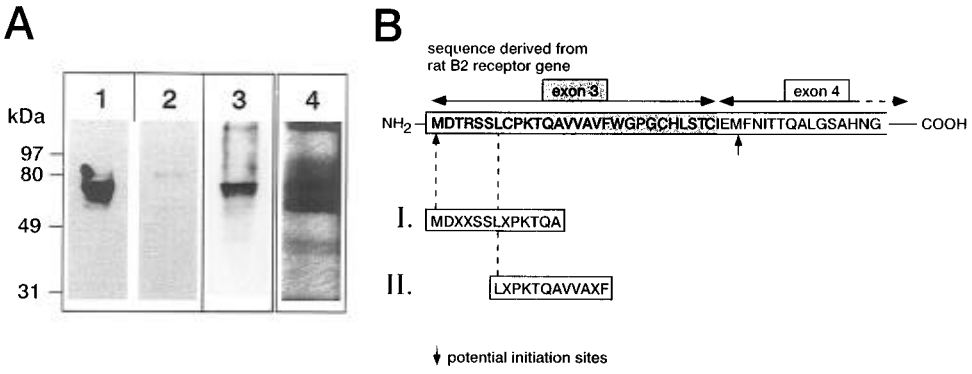


FIGURE 6: Rat B_2 receptor's amino terminus. (A) Immunoblot of rB2CHO12/3 cell membranes probed with anti-$ED1_B$ antiserum (lane1). As a control, mock-transfected cells were applied (lane 2). For comparison, HOE140 was cross-linked to the rat B_2 receptor and detected by anti-HOE140 antiserum (lane 3). Silver staining of rat B_2 receptors isolated by immunoaffinity chromatography (lane 4). (B) Amino acid sequence analysis of two different B_2 receptor preparations (I, II) and alignment of the sequences to the protein sequence translated from the rat B_2 gene. The single-letter code for amino acids is used; X stands for unidentified residue.

specific staining of B_2 receptors (Figure 6A, lane 2). As an additional control, we cross-linked the B_2 antagonist HOE140 to rat B_2 receptors and detected the cross-linked receptor by anti-HOE140 antibodies. In the cross-linking experiment a protein of similar molecular weight was identified (Figure 6A, lane 3). To determine the rat B_2 receptor's amino terminus, we isolated B_2 receptors by affinity chromatography using a mixture of domain-specific antibodies. A major protein of 69 kDa was isolated (Figure 6A, lane 4), and additional protein bands may represent differentially glycosylated and/or aggregated B_2 receptors as was similarly observed for human B_2 receptors [cf. Figure 3A and AbdAlla et al. (1993)]. The amino-terminal sequence starts with a methionine corresponding to the first in-frame AUG (Figure 6B, I). In a second experiment a truncated B_2 receptor lacking six amino acids was isolated (Figure 6B, II). Both experiments indicate that rat B_2 receptors expressed in CHO cells start with the first in-frame AUG. Thus additional 30 amino acids are added to the cloned sequence of the rat B_2 receptor protein (McEachern et al., 1991).

In analogy to the rat and human B_2 receptor, we postulate that for the mouse B_2 receptor it is also the first in-frame AUG that is used as start site for translation. We conclude that, in contrast to previous reports, the coding sequence of B_2 receptors from man, rat, and mouse is interrupted by an intron. Protein sequences encoded by different exons often constitute structural and functional entities (Go, 1981). Corresponding to the exon−intron boundaries of the B_2 receptor gene, we nominated the additional B_2 receptor domain $ED1_A$. A sequence comparison of the amino-terminal $ED1_A$ and $ED1_B$ domains of the rat, human and mouse B_2 receptor is given in Table 1. Compared to an overall sequence identity of more than 80% of the entire receptor sequences, the amino-terminal $ED1_A$ domains of these three receptors share less than 20% sequence identity. It will be interesting to learn whether these major differences on the protein level of the B_2 receptors' amino termini are important for the observed differences in receptor function (Félétou et al., 1994) and/or ligand binding (Burch et al., 1993) of B_2 receptors derived from different species.

ACKNOWLEDGMENT

We thank B. Welsch and I. Heidemann for technical assistance, M. Plenikowski for the art work, Dr. S. Bhakdi and S. Strauch (University of Mainz) for support with the FACS analyses, Dr. A. Maidhof (University of Mainz) for help with the immunizations, and Dr. K. Jarnagin, (Syntex, Palo Alto) for the rat B_2 cDNA.

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BI9601060