Agonist-independent Nuclear Localization of the Apelin, Angiotensin AT1, and Bradykinin B2 Receptors*

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Signaling of the apelin, angiotensin, and bradykinin peptides is mediated by G protein-coupled receptors related through structure and similarities of physiological function. We report nuclear expression as a characteristic of these receptors, including a nuclear localization for the apelin receptor in brain and cerebellum-derived D283 Med cells and the AT1 and bradykinin B2 receptors in HEK-293T cells. Immunocytochemical analyses revealed the apelin receptor with localization in neuronal nuclei in cerebellum and hypothalamus, exhibiting expression in neuronal cytoplasm or in both nuclei and cytoplasm. Confocal microscopy of HEK-293T cells revealed the majority of transfected cells displayed constitutive nuclear localization of AT1 and B2 receptors, whereas apelin receptors did not show nuclear localization in these cells. The majority of apelin receptor-transfected cerebellum D283 Med cells showed receptor nuclear expression. Immunoblot analyses of subcellular-fractionated D283 Med cells demonstrated endogenous apelin receptor species in nuclear fractions. In addition, an identified nuclear localization signal motif in the third intracellular loop of the apelin receptor was disrupted by a substituted glutamine in place of lysine. This apelin receptor (K242Q) did not exhibit nuclear localization in D283 Med cells. These results demonstrate the following: (i) the apelin receptor exhibits nuclear localization in human brain; (ii) distinct cell-dependent mechanisms for the nuclear transport of apelin, AT1, and B2 receptors; and (iii) the disruption of a nuclear localization signal sequence disrupts the nuclear translocation of the apelin receptor. This discovery of apelin, AT1, and B2 receptors with agonist-independent nuclear translocation suggests major unanticipated roles for these receptors in cell signaling and function.

The apelin receptor was discovered as an orphan G protein-coupled receptor (GPCR) known as APJ, sharing highest identity with the angiotensin II AT1 receptor, although no binding to the receptor was observed with angiotensin II (1). The apelin peptide was subsequently discovered as the endogenous ligand for this receptor (2). At least two isoforms of the apelin peptide are known to exist, apelin-36 and apelin-13, both of which act as agonists of the apelin receptor with distinct pharmacological properties (2–4). The apelinergic system has a widespread pattern of distribution in the brain and periphery as shown for the apelin peptide (3, 5–8) and receptor (6, 9–11). Apelin has been shown to lower blood pressure (6, 12) and modulate contractility of cardiac tissue and blood vessels (13, 14), pituitary hormone release (15), fluid consumption (6, 15, 16), and cytokine suppression (17) and may have a role as a co-receptor for human immunodeficiency virus entry into cells (18, 19). Overall, the apelinergic system is most closely related to the angiotensin and bradykinin systems, as observed through peptide and receptor structural and sequence similarities, expression patterns, and physiology (6, 20). Apelin, angiotensin II, and bradykinin all have blood pressure-modulating effects, expression in cardiovascular tissue, and are cleaved by the human endothelial angiotensin I-converting enzymes (21). Recently, the AT1 receptor was found to form a heterodimer with the bradykinin B2 receptor, with consequent alterations in G protein activation and internalization (20). Thus, there is considerable evidence to support the notion that the apelin, angiotensin and bradykinin receptors make up a subfamily of GPCRs with shared ligand and receptor characteristics.

To date, there have been many reports of the intracellular localization and trafficking of GPCRs both in vivo and in vitro (22–24). Whereas GPCRs have been best characterized as cell-surface mediators of signal transduction, there are few reports of GPCRs capable of nuclear translocation. In the large family I rhodopsin-like family of GPCRs, the AT1 receptor has been best characterized to have nuclear localization. Early studies revealed angiotensin II-binding sites were present in nuclei with angiotensin II-induced transcription of renin and angiotensinogen mRNA (25), and the nuclear localization of the AT1 receptor was reported to be induced by angiotensin II (26, 27). In addition, a family II GPCR, the parathyroid hormone receptor, was observed to localize to the nucleus both in tissues and cultured cells (28), and a recent study observed a family III GPCR, the metabotropic glutamate mGlur5 receptor, in nuclear membranes (29). The prostaglandin EP3, EP4, and EP2 receptors (31) were observed to have nuclear membrane localization, whereas a recent report (32) described the endosomal essential medium; mRFP, monomeric red fluorescent protein; NLS, nuclear localization signal; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.
thein ETA and ETB receptors to be localized in the nuclear membrane, with ETB receptors present in the interior of the nucleus as well. In the present study we report for the first time a nuclear localization of the apelin receptor in brain. Following our observations of immunocytochemical detection of apelin receptors in cell nuclei, we carried out experiments involving immuno-blot analyses of subcellular fractionated cells and confocal microscopy of cells to verify the nuclear distribution. Previously, the AT1 receptor was reported to traffic to cell nuclei by the presence of a nuclear localization signal (NLS) sequence (27). Identification of a similar sequence in the closely related ETB receptor suggested comparable nuclear localization of this receptor. In addition, the presence of an NLS-like sequence in an alternate position in the apelin receptor suggested distinctive patterns of nuclear localization of the apelin receptor compared with the AT1 receptor. We have utilized confocal microscopy analyses to (i) compare subcellular distributions of the apelin, AT1, and B2 receptors in various cell lines and (ii) to confirm the identity of the apelin receptor NLS sequence using a site-directed mutagenesis strategy. Together, our results provide evidence of an additional commonality between the apelin, AT1, and B2 receptors which is that of nuclear expression due to the presence of NLS motifs.

MATERIALS AND METHODS

Construction of cDNA Encoding Apelin, AT1, and B2 Receptors—DNA encoding the human and rat apelin receptors were obtained as described previously (1, 6). The cDNA encoding AT1 receptor was a gift from Dr. Fred Hess (Merck). The cDNA encoding arrestin1-GFP was a gift from Dr. Stephen Hess (Merck). The cDNA encoding monomeric red fluorescent protein mRFP1 was a gift from Dr. Roger Tsien (University of California, San Diego). The cDNA encoding arrestin1-GFP was a gift from Dr. Stephen Ferguson (University of Western Ontario, London, Ontario, Canada). The stop codons of cDNAs encoding the human apelin, angiotensin II AT1, bradykinin B2, and dopamine D1 receptors were modified to contain BamHI or KpnI sites by PCR amplification from vectors containing these full-length coding regions. These fragments were subsequently cloned in-frame into the pEGFP-N1 vector (BD Biosciences Clontech, Palo Alto, CA) or the mRFP-1 vector. For epitope-tagged human and rat apelin receptors, a C9 epitope tag sequence (encoding the nine carboxyl-terminal amino acids of rhodopsin) was inserted just prior to the stop codon by PCR mutagenesis.

Cell Culture and Transfection of Cells—COS-7 monkey kidney, human embryonic kidney (HEK-293T), and human cerebellum D283 Med cells (American Type Culture Collection, Manassas, VA) were maintained as monolayer cultures at 37 °C. COS-7 and HEK-293T cells were maintained in minimum essential medium (MEM) and the D283 Med cells in MEM with 2 mM l-glutamine, Earle’s balanced salt solution with 1.5 g/liter sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. All media were supplemented with 10% fetal bovine serum and antibiotics. COS-7 and HEK-293T cells were transiently transfected with DNA encoding pEGFP-N1 vector (BD Biosciences Clontech, Palo Alto, CA) or mRFP-1 vector. For epitope-tagged human and rat apelin receptors, a C9 epitope tag sequence (encoding the nine carboxyl-terminal amino acids of rhodopsin) was inserted just prior to the stop codon by PCR mutagenesis.

Confocal Microscopy—For the live cell fluorescence activation assay, HEK-293T cells were transfected with DNA encoding arrestin1-GFP and GRK2 with or without DNA encoding the apelin receptor-mRFP constructs for 48 h. 10 μM apelin-13 was administered to these cells, and confocal images were taken every minute. HEK-293T cells were transiently transfected with a nuclear marker, DsRed2-Nuc (BD Biosciences Clontech), and either the apelin receptor-GFP, AT1 receptor-GFP, or B2 receptor-GFP construct for 48 h. D283 Med cells were transfected with either apelin receptor-GFP, apelin receptor (K242Q)-GFP, AT1 receptor-GFP, or the dopamine D1 receptor-GFP. Live cell confocal microscopy was performed with a Zeiss LSM 510 microscope. Cell counts for nuclear localization were obtained from at least 100 and 250 cells for D283 Med cells and HEK-293T, respectively. Images were acquired and processed with Zeiss LSM Image Browser software.

Membrane Preparation—COS-7 cells were washed extensively with PBS. P2 pellets were prepared by Polytron disruption in ice-cold 5.2 lysis buffer (containing 5 μM Tris-HCl, 2 mM EDTA buffer, containing 5 μg/ml leupeptin, 10 μg/ml benzamidine, and 5 μg/ml soybean trypsin inhibitor) as described previously (30).

Subcellular Fractionation—Cell fractionation of D283 Med cells was performed by a modified method of the previously reported hypotonic/Nonidet P-40 lysis (34). Briefly, washed and pelleted cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 5 μg/ml leupeptin, 10 μg/ml benzamidine, and 5 μg/ml soybean trypsin inhibitor), homogenized by several short bursts of low-force Polytron disruption on ice, and then centrifuged at 700 × g for 10 min at 4 °C. The supernatant and nuclei pellets were separated for recovery of P2 and nuclear fractions, respectively. The supernatant was centrifuged at 35,000 × g for 15 min at 4 °C to collect the P2 fraction. The nuclear pellet was resuspended in lysis buffer layered over a discontinuous sucrose gradient of 2.0 and 1.8 M sucrose containing 1 mM MgCl2, and centrifuged at 100,000 × g for 1 h at 4 °C. The morphological integrity and purity of the samples were assessed by light microscopy after creosyl violet staining and with a plasma membrane marker 5'-nucleotidase assay kit (Sigma). Protein levels were determined by the Bradford assay according to the manufacturer’s instructions (Bio-Rad).

Nuclear fractionation of unfixed human cerebellum tissue of a control 22-year-old Caucasian male (Brain and Tissue Banks for Developmental Disorders, University of Maryland, Baltimore, MD) was performed by a modified version of the Gorski method (35). Briefly, 4 g of cerebellum tissue was minced in ice-cold homogenization buffer containing sodium chloride (10 mM), sodium orthovanadate (0.1 mM), aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml antipain, chymostatin, pepstatin, 5 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor. Samples were homogenized on ice using a motor-driven Teflon-glass homogenizer (5 strokes), diluted to 75 ml with homogenization buffer, and layered over cushions of homogenization buffer. These samples were then centrifuged at 76,000 × g for 30 min at −3 °C. The nuclear pellet was resuspended in 2 ml of nuclear lysis buffer, left on ice for 30 min, gently homogenized (5 strokes), and lysed further for 30 min. The sample was further diluted to yield ~0.5 mg/ml DNA concentration (determined by UV absorbance at 260 nm), and 0.1 times the total volume of 4.0 × (NH4)2SO4 was added dropwise. The sample was then centrifuged for 30 min at 14,000 × g to yield the supernatant, shaken 1 h on ice, centrifuged at 90,000 × g for 25 min at 0 °C, and pellets stored overnight at 4 °C. Sample purity and protein levels were determined as for D283 Med cells.

Immunoblot Analyses—Nuclear fractions were treated with DNase and RNase (0.5 μg/ml each, MarlinGen Biosciences, Inc., Jamsville, MD) for 20 min at 37 °C. The protein samples were solubilized in sample buffer consisting of 50 mM Tris-HCl, pH 6.5, 1% SDS, 10% glycerol, 0.003% bromphenol blue, and 10% 2-mercaptoethanol. The samples were subjected to PAGE with 12% acrylamide gels and electrobotted onto PVDF transfer membrane as described previously (33). Apelin receptor immunoreactivity (IR) was revealed with a mouse monoclonal antibody at a dilution of 1:1000 (catalogue number MAB856, R&D Systems, Inc., Minneapolis, MN). This antibody was raised using membrane-embedded apelin receptors (36). Initial experiments with this antibody were made possible by a generous gift from Dr. Robert W. Doms (University of Pennsylvania Philadelphia, PA). C9 epitope-tagged receptor-IR was detected with the 1D4 antibody (National Cell Culture Center, Minneapolis, MN). In addition, immunoblot analyses to test the purity of the subcellular fractionated samples were performed using antibodies specific for nucleoporin p62 (nuclear fraction, number N43620), annexin II (plasma membrane and intracellular vesicles, number A14020), and GM130 (Golgi apparatus, number G65120) from BD Transduction Laboratories (Lexington, KY). This antibody was raised using monoclonal antibody at a dilution of 1:1000 (catalogue number MAB856, R&D Systems, Inc., Minneapolis, MN). This antibody was raised using membrane-embedded apelin receptors (36). Initial experiments with this antibody were made possible by a generous gift from Dr. Robert W. Doms (University of Pennsylvania Philadelphia, PA). C9 epitope-tagged receptor-IR was detected with the 1D4 antibody (National Cell Culture Center, Minneapolis, MN). In addition, immunoblot analyses to test the purity of the subcellular fractionated samples were performed using antibodies specific for nucleoporin p62 (nuclear fraction, number N43620), annexin II (plasma membrane and intracellular vesicles, number A14020), and GM130 (Golgi apparatus, number G65120) from BD Transduction Laboratories (Lexington, KY).
and mounted in Permount mounting medium via xylenes, viewed under a Zeiss Axioskop microscope, and photographed with Kodak ASA-160 film. Control experiments were also performed by using rat versus human cerebellum tissue or by the exclusion of primary antibody.

RESULTS

Nuclear Localization of the Apelin Receptor in Human Brain—The similarities between the apelin, angiotensin, and bradykinin systems are observed from the shared sequence identities between their respective receptors and ligands, expression distribution, and roles in comparable physiological functions. Our studies observed levels of sequence identity between the apelin and angiotensin receptors (1), as well as structural similarities between the apelin and angiotensin II peptides (6). We also reported comparable expression distribution patterns between the two systems, particularly in the choroid plexus, hippocampus, and hypothalamus in the brain and associated roles in the modulation of blood pressure and water consumption behavior (6). In this study, we continued to characterize the apelinergic system by investigating the distribution of the receptor.

By using a specific antibody for the human apelin receptor (36), apelin receptor IR was observed abundantly in the cerebellum and paraventricular nucleus of the hypothalamus (Fig. 1). Apelin receptor distributions were performed on these two regions as they were determined previously (6) to express high levels of apelin receptor mRNA. Virtually all neuronal cell nuclei in the granular layer of the cerebellum was densely labeled by the apelin receptor antibody, with abundant levels also observed in the cellular nuclei of the molecular layer (Fig. 1A). Purkinje cells displayed apelin receptor-IR in both the nucleus and cytoplasm. Cerebellar sections labeled for apelin receptor-IR (brown stain) were subsequently Nissl-stained with cresyl violet (purple stain), which confirmed the dense nuclear localization of the apelin receptor in the granular layer and demonstrated a heterogeneous population of nuclei with some nuclei negative for apelin receptor-IR in the molecular layer (Fig. 1B). In addition, some neurons had an exclusive cytoplasmic distribution of apelin receptor-IR, as seen in large pyramidal cells (Fig. 1C). In the paraventricular nucleus of the hypothalamus, there were two populations of apelin receptor-IR neurons, smaller neurons (parvo cellular) with dense labeling in the nucleus and larger neurons (magnocellular) with labeling in the nucleus and cytoplasm (Fig. 1D). Control sections of human cerebellum processed without the apelin receptor antibody (Fig. 1E) or from rat cerebellum processed with both apelin receptor and secondary antibodies (Fig. 1F) were absent of immunoreactive signals. These results revealed specificity of the unique nuclear expression of the apelin receptor between different cell types in human brain.

The specificity of the apelin receptor antibody was verified. DNA encoding the human or rat apelin receptors or empty vector DNA was transfected into COS-7 cells. Immunoblot analyses of P2 membrane fractions revealed specific bands in cells expressing the human apelin receptor, correlating to an expected molecular mass of ~42 kDa for the unglycosylated, monomeric receptor as well as bands of higher molecular mass consistent with glycosylated, monomeric (~45 kDa (36)), and dimeric receptor species (80–90 kDa) (Fig. 2A). Apelin receptor-specific bands were not detected in COS-7 cells expressing the rat apelin receptor, indicating the antibody to be species-specific despite the receptors having a high degree of sequence similarity (>90%). To confirm expression of rat apelin receptors, human and rat apelin receptors were fused with a rhodopsin-derived C9 epitope tag at their carboxyl-terminal ends and detected by immunoblot analyses using the 1D4 antibody. Immunoblot analyses of P2 membrane fractions revealed specific bands in cells expressing either the human or rat apelin receptors correlating to the unglycosylated (42 kDa) and glycosylated (~45 kDa) monomers and dimers (80–90 kDa) (Fig. 2B), confirming that the apelin receptor antibody was highly specific for the human apelin receptor.

The discovery of apelin receptors with nuclear expression suggested another shared trait with the angiotensin AT1 receptor. The NLS motif KKKFR described by Lu et al. (27) was observed to be located in the AT1 receptor within the recently identified eighth helix, positioned between the seventh transmembrane domain and the carboxyl-terminal tail (38). Sequence analysis revealed a similar sequence KRFRK located in an equivalent position in the bradykinin B2 receptor. In addition, the apelin receptor was observed to have an NLS motif RKRRR in the third intracellular loop (Fig. 3). Thus, we predicted that the B2 receptor also exhibited nuclear localization, and a functional NLS motif was located in the third intracellular loop of the apelin receptor.

Subcellular Apelin, AT1, and B2 Receptor Distributions in HEK-293T Cells—To examine the mechanisms for GPCR nuclear trafficking in cell culture, the apelin, AT1, and B2 receptors fused to green fluorescent proteins (GFP) were used in live cell confocal microscopy. AT1 receptor-GFP revealed signals at
the cell surface and in the cytoplasm, with nuclear localization in ~70% of cells counted (Fig. 4). The B₂ receptor-GFP was also localized at the cell surface and in the cytoplasm with nuclear localization in ~65% of cells counted. These observations revealed the AT₁ and B₂ receptors as GPCRs with agonist-independent trafficking to the cell nucleus. The majority of cells revealed apelin receptor-GFP expression in the cytoplasm and a clear cell surface distribution, with nuclear localization in less than 10% of cells. As a control, the distribution of dopamine D₁ receptor-GFP was also examined. Similar levels of nuclear localization (~10%) were observed with D₁ receptor-GFP (data not shown). These results reveal a receptor-specific nuclear localization for the AT₁ and B₂ receptors in HEK-293T cells.

Although there exists a wealth of evidence that GPCR-GFP chimeras present themselves as fully functional GPCRs in heterologous systems, it was conceivable that the apelin receptor fused to a fluorescent protein precluded nuclear expression in HEK-293T cells. Previous studies (26, 39–42) have shown the AT₁ and B₂ receptors as functional receptors when fused to such fluorescent proteins. To evaluate the cell surface functional consequences of a carboxyl-terminal fusion of a fluorescent protein onto the apelin receptor, the apelin receptor-monomeric red fluorescent protein (mRFP) construct was co-transfected into HEK-293T cells with βarrestin1-GFP and GRK2. By using live cell confocal microscopy, the subcellular distributions of the apelin receptor and βarrestin1 were observed before and after the administration of apelin-13 peptide (10 μM). Under basal conditions, βarrestin1-GFP displayed cytoplasmic and nuclear localization, whereas the apelin receptor-mRFP was observed at the plasma membrane and in cytoplasmic vesicles indicative of the endoplasmic reticulum-Golgi complex (Fig. 5). The administration of apelin-13 initiated a rapid movement within 1 min of βarrestin1-GFP from the cytoplasm to co-localize with the apelin receptor-mRFP at the cell surface. As a control, HEK-293T cells transfected with βarrestin1-GFP and GRK2 alone were unresponsive to apelin-13 (data not shown). These results show for the first time co-visualization of the apelin receptor and βarrestin1 in cells and confirm that a carboxyl-terminal fusion of a fluorescent protein does not disrupt apelin signaling through the apelin receptor.

**Nuclear Localization of Apelin Receptor-GFP and AT₁, Receptor-GFP, and B₂ receptor-GFP fusion proteins in HEK-293T cells.** HEK-293T cells were co-transfected with a nuclear marker DsRed2-Nuc and either apelin receptor-GFP, AT₁ receptor-GFP, or B₂ receptor-GFP for 48 h. Shown are the representative live cell confocal microscopy images using a Zeiss LSM 510 microscope of each receptor-GFP (green) and a nuclear marker DsRed2-Nuc (red) from at least 250 cells counted. Live cell conditions were at room temperature immersed in serum-free MEM. Nuclear localization is shown in the overlay (yellow). These images are representative of at least four independent experiments. White bar, 10 μm.
roblastoma) cells were transfected with apelin receptor-GFP and observed to have cell surface and cytoplasmic distributions for the apelin receptor with only background levels of nuclear localization (less than 10% of counted cells revealed apelin receptors in the nucleus, data not shown). Based on our immunocytochemical studies of dense labeling of apelin receptor in cerebellum, we acquired a human cerebellum cell line, D283 Med. These cells revealed a majority of cells with apelin receptor-GFP expression in the nucleus, cytoplasm, and at the cell surface (Fig. 6), with nuclear localization in approximately 75% of cells counted. Furthermore, D283 Med cells revealed a striking level of nuclear localization of the AT$_1$ receptor-GFP, with nuclear levels exceeding cytoplasmic levels and little to no distribution at the cell surface (Fig. 6). As a control, D283 Med cells were transfected with dopamine D$_1$ receptor-GFP that revealed plasma membrane and cytoplasmic distributions and only background levels of nuclear localization of the receptor (Fig. 6). Together with the distributions seen in HEK-293T cells, these results confirm differential nuclear localization of apelin receptor in different cell types and recognize the cerebellum-derived D283 Med cell line as capable of facilitating nuclear localization of the apelin receptor.

**Apelin Receptor Distribution in Subcellular Fractions**—The apelin receptor antibody was used in immunoblot analyses of subcellular fractions from both human cerebellum and D283 Med cells. Nuclear and P2 fractions were observed as enriched in nucleoporin p62 and annexin II/GM130 proteins, respectively, confirming a high degree of purity between the two fractions (Fig. 7, A and B). Apelin receptor-IR was not detected in P2 membrane fractions of human cerebellum, whereas in nuclear membrane fractions bands correlating to monomeric (42–45 kDa) and dimeric (80–90 kDa) species were observed (Fig. 7A). Untransfected D283 Med cells were also fractionated, and immunoblot analyses revealed apelin receptor-IR in P2 and nuclear membrane fractions, which revealed endogenous levels of expression of this receptor in these cells (Fig. 7B). In both tissue and cultured cells, an additional band at 64 kDa was observed, and other bands were observed at ~105–120 kDa in the nuclear fraction. The sizes of these bands do not correspond to multiples of the monomer but may represent the apelin receptor coupled to other proteins. Together, these results revealed apelin receptors with nuclear localization in both human cerebellum tissue and endogenously expressed in a cerebellum-derived cell line.

**Absence of Apelin Receptor (K242Q)-GFP from D283 Med Cell Nuclei**—Given the high levels of nuclear expression of apelin receptor-GFP observed in D283 Med cells (Fig. 6 and Fig. 8), we assessed the putative NLS motif RKRRR found in the third intracellular loop of the apelin receptor by mutagenesis. We mutated the cDNA encoding apelin receptor-GFP to encode a substituted glutamine in place of lysine at amino acid position 242 of the apelin receptor. Apelin receptor (K242Q)-
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Fig. 8. Apelin receptor (K242Q)-GFP does not undergo nuclear localization in D283 Med cells. D283 Med cells transfected with either apelin receptor-GFP or apelin receptor (K242Q)-GFP for 48 h. Shown are the representative live cell confocal microscopy images using a Zeiss LSM 510 microscope of each receptor-GFP (green) against a brightfield image (shown as an overlay) from at least 100 cells counted. Live cell conditions were at room temperature immersed in serum-free MEM. These images are representative of at least four independent experiments. White bar, 10 μm.

GFP revealed only background levels of nuclear localization in D283 Med cells, which strongly suggested the presence of a critical lysine residue in the RKRRR sequence for nuclear import (Fig. 8). These results demonstrate the presence of a functional NLS motif RKRRR in the third intracellular loop of the apelin receptor.

**Discussion**

We report for the first time agonist-independent nuclear localizations of the apelin, angiotensin AT₁, and bradykinin B₂ receptors. By using a highly specific antibody for the apelin receptor, we provide evidence of nuclear localization of this receptor in various human brain regions and in D283 Med cells, a human cerebellum-derived cell line that endogenously expresses the apelin receptor. The AT₁ and B₂ receptors demonstrated nuclear localization in HEK-293T cells, unlike the apelin receptor.

Immunocytochemical analyses of human cerebellum and hypothalamus revealed nuclear transport of the apelin receptor. In the molecular and granular layers of the cerebellum and the paraventricular nucleus of the hypothalamus, the predominant nuclear localization of the apelin receptor was evident. Quite notably, in the cerebellum there were three different subcellular distributions for the apelin receptor. The majority of neurons in both the molecular and granular layers had expression of the apelin receptor exclusively in the nucleus, whereas Purkinje cells exhibited expression in both the nucleus and cytoplasm. Alternatively, some neurons had only cytoplasmic immunoreactivity with little or no immunoreactivity detected in the nucleus. In the paraventricular nucleus of the hypothalamus, two different subcellular distribution patterns were observed, with smaller paravascular neurons showing apelin receptor expression in the nucleus and magnocellular neurons revealing expression in nuclei and cytoplasm. The high levels of apelin receptor nuclear localization, in particular neuronal subtypes, are therefore indicative of nuclear translocation of the apelin receptor occurring only in certain cerebellar and hypothalamic neurons.

Visualization of the subcellular distribution of the AT₁ and B₂ receptors revealed a high level of constitutive nuclear localization in ~70% of HEK-293T cells. In contrast to this, less than 10% of either HEK-293T, U-2, or NIE-115 cells expressing the apelin receptor demonstrated nuclear localization of this receptor. A similarly low percentage was also observed with the dopamine D₁ receptor, suggesting these levels to be background and not indicative of NLS motif-dependent trafficking.

The dense labeling of apelin receptor-IR in the nuclei of cerebellar neurons suggested a cerebellum-derived cell line would possess the proper cellular milieu for the nuclear transport of apelin receptors. Apelin receptor-GFP-expressing D283 Med medulloblastoma cells derived from human cerebellum revealed ~75% of transfected cells with nuclear localization of the apelin receptor, along with signals at the cell surface and in the cytoplasm. These results resembled a similar pattern of distribution as observed in the Purkinje cells of the cerebellum and magnocellular neurons of the hypothalamus. An even greater degree of nuclear localization was observed for AT₁ receptor-GFP in these cells. D₂ receptor-GFP revealed only background levels of nuclear localization in D283 Med cells, which confirmed the specificity of nuclear trafficking of the apelin and AT₁ receptors. Together, these results reveal distinct levels of agonist-independent nuclear localization of the apelin, AT₁, and B₂ receptors in specific cell types.

Further evidence for the nuclear localization of the apelin receptor was observed in subcellular fractions of both human brain tissue and cultured cells. In human cerebellum only the nuclear fraction revealed detectable levels of apelin receptor, corresponding to both monomeric and possible dimeric forms, with no receptors detected in the P2 membrane fraction. In D283 Med cells, these apelin receptor forms were detected in both P2 and nuclear membrane fractions. Taken together, our observations show a clear and novel nuclear expression of the apelin receptor in human brain tissue and the human cerebellum-derived D283 Med cell line, as well as evidence for the nuclear localization of B₂ receptors in HEK 293T cells.

The mechanisms underlying nuclear transport of GPCRs are not well characterized. The number of GPCRs with a recognized nuclear localization is limited, with little evidence of known nuclear function as yet. Recently, there have been advances in our understanding of the mechanism of import and export of macromolecules through nuclear pore complexes (43–45). Generally, nuclear import requires the docking of the "cargo" protein to an importin α/β heterodimer. Importin β recognizes cargo proteins by binding to NLS motifs that usually consist of short stretches of basic amino acids, arginine and lysine (although many atypical NLS sequences have been determined) (46). As mentioned previously, the cytoplasmic tail of the AT₁ receptor contains an NLS motif KKKFR (27), positioned just downstream of the seventh transmembrane domain within the eighth helix. A similar NLS motif also exists for the B₂ receptor, KRFRK, in this same position. The similar levels of nuclear localization seen with both the AT₁ and B₂ receptors in HEK-293T cells suggest that this also is a functional NLS motif. A search of over 200 human rhodopsin family GPCR sequences maintained at the GPCRDB website (47) revealed 17 GPCRs with a clearly recognizable NLS motif in the eighth helix (Fig. 3), including adenosine, growth hormone secretagogue, motilin, purinergic and orphan receptors, in addition to the angiotensin, bradykinin, and endothelin receptors. Intriguingly, no such sequence appears in the apelin receptor at this position. However, a putative NLS motif RKRRR is located in the apelin receptor at the carboxyl-terminal region of the third intracellular loop. A mutant form of the apelin receptor, K242Q, demonstrated only background levels of nuclear localization in D283 Med cells, which strongly suggested the following: (i) RKRRR is a functional NLS motif located in the third intracellular loop of the apelin receptor, and (ii) the lysine residue is critical for the proper functioning of this NLS motif. Furthermore, these NLS position and motif differences may be responsible for the cell-specific nuclear localization of these receptors in brain or cultured cells. To-
gether, these results suggest that the position of the NLS motif in these receptors may play a role in nuclear transport. Our observations of GPCRs in cell nuclei revealed localization in the nucleoplasm and not restricted to the nuclear envelope. GPCRs in the interior of cell nuclei have been reported previously for the angiotensin II AT$_1$ (26, 27), parathyroid hormone receptor (28), and endothelin ETB receptors (32). Thus, the evidence for nucleoplasmic localization of GPCRs is compelling from a variety of experimental procedures including confocal microscopy, immunocytochemistry, and immunogold staining detection by electron microscopy. Several hypotheses have been proposed to elucidate the mechanism by which membrane-bound receptors are localized in the interior of cell nuclei, including nuclear import of cleaved receptor fragments and chaperone proteins facilitating the removal of the receptor from a membrane-embedded state (48). However, intact apelin receptors were detected in cell nuclei by immunoblot analyses. The hypothesis of chaperone proteins removing all seven transmembrane domains of a GPCR from a membrane is most unlikely. Presently, the most plausible explanation for GPCRs in the nucleoplasm is through transport within micelles, which would retain a membrane-embedded GPCR suitable for further signaling in cell nuclei.

The physiological functions of GPCRs in cell nuclei are presently poorly understood. However, proteins well known for their cell surface association with GPCRs are present in nuclei (including heterotrimeric G proteins (49), adenylyl cyclase (50), phospholipase A2 (51), and phospholipase C (52)), which suggests unanticipated and novel functions for each of these G protein-coupled receptors.

In conclusion, we report a novel agonist-independent nuclear localization of the apelin, angiotensin AT$_1$, and bradykinin B$_2$ receptors. The nuclear expression of these GPCRs appears to be cell-specific. Nuclear localization was observed for the AT$_1$ and B$_2$ receptors, but not the apelin receptor, in HEK-293T cells. In human cerebellum and hypothalamus, the apelin receptor was observed with a polyclonal antibody, nuclear localization with some neurons displaying solely a cytoplasmic localization or in both the nucleus and cytoplasm. Nuclear localization for the apelin and AT$_1$ receptors was observed in human cerebellum-derived D283 Med cells. We identified the RKKRR sequence in the third intracellular loop of the apelin receptor to be the functional NLS motif. Together with the observation of differently positioned NLS motifs in the AT$_1$ and B$_2$ receptors, these results suggest that various mechanisms of NLS-dependent GPCR nuclear transport may exist. In addition a nuclear location suggests unanticipated and novel functions for each of these G protein-coupled receptors.

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