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A Fully Active Nonglycosylated V2 Vasopressin Receptor

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SUMMARY

The human V2 vasopressin receptor belongs to the superfamily of G protein-coupled receptors believed to be anchored to the plasma membrane by seven transmembrane regions. The extracellular portion of the human V2 vasopressin receptor contains one site susceptible to *N*-linked glycosylation. Metabolic labeling and immunoprecipitation of the receptor expressed in transfected cells were applied to examine whether the protein was indeed glycosylated. The V2 vasopressin receptor expressed transiently was glycosylated, but glycosidase treatment to test the complexity of the sugar moiety linked to asparagine revealed that the majority of the receptor protein lacked complex carbohydrates, an indication of an improperly processed protein. This immature protein displayed a tendency to form aggregates. In contrast with these data, testing of the sugar complexity of the receptor protein synthesized in stably transfected cells identified the predominant form as an appropriately processed receptor protein. Mutagenesis of asparagine 22 to glutamine produced on expression in transfected cells a nonglycosylated receptor with ligand binding affinity and coupling characteristics almost identical to those of the wild-type form. After exposure to elevated concentrations of AVP (100 nM), the nonglycosylated form desensitized to the same extent as the wild-type receptor.

The V2R expressed in the principal cells of the kidney collecting duct mediates the antidiuretic action of AVP (1). Occupation of the receptor by AVP promotes activation of the G_s/adenylyl cyclase system. The increased intracellular level of cAMP activates protein kinase A and starts a phosphorylation cascade that promotes the translocation of the AVP-regulated water channel, aquaporin 2, to the apical membrane of the cell, thus increasing water permeability (2, 3). In confirmation of the role these proteins play in the mechanism of water homeostasis, mutations affecting the V2R and aquaporin 2 have been described in individuals with hereditary defects in urine-concentrating capability (4–6). The cDNAs encoding the V2R from humans, rat, and mouse as well as that encoding the homologous pig LVPR have been cloned. Comparison of the deduced amino acid composition reveals an overall 85% similarity among them and the presence in all V2Rs of one predicted acceptor site for *N*-linked glycosylation in the extracellular amino-terminal segment (7–10).

Significant changes in receptor binding and trafficking have been observed in the pig LVPR expressed in the $LLCPK_1$ cell line when glycosylation was prevented or altered by the use of the antibiotic tunicamycin or the alkaloid castanospermin (11). Synthesis of the LVPR was reduced when cells were treated with these agents, and ligand-induced internalization of the receptor was diminished. These findings led to the conclusion that glycosylation plays a major role in the transport and function of V2Rs. It has also been reported that rhodopsin lacking the glycosylation site at Asn15 is transported poorly to the cell surface and is defective in stimulating G_t (12).

To verify whether the findings described for the pig LVPR could be applied to the human V2R, we examined the impact of glycosylation on the synthesis and function of this protein. The wild-type and a mutant receptor lacking the asparagine required for glycosylation were expressed, and their functional properties compared. Experiments were performed both in COS.M6 and HEK 293 cells expressing the receptor transiently and in stable form. The human V2R was tagged with the influenza HA epitope to allow for immunoprecipitation from cell extracts (13). Here, we report that the functional properties of the nonglycosylated human V2R we tested are unaltered; these include hormone binding, coupling to G_s, desensitization, and internalization. We also found that the receptor protein produced multiple bands in the expression systems that we analyzed.

Experimental Procedures

Materials. DMEM HG, HBSS, D-PBS, penicillin/streptomycin, 0.5% trypsin/5 mM EDTA, geneticin (G-418), and FBS were from

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ABBREVIATIONS: V2R, V2 arginine vasopressin receptor; HA, hemagglutinin; LVPR, lysine vasopressin receptor; DMEM, Dulbecco's modified Eagle's medium; HG, high glucose; HBSS, Hanks' balanced salt solution; D-PBS, Dulbecco's phosphate-buffered saline; PBS, phosphate-buffered saline; FBS, fetal bovine serum; AVP, arginine vasopressin; VIP, vasoactive intestinal peptide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RIPA, radioimmunoprecipitation assay.

GIBCO (Grand Island, NY); methionine/cysteine-free DMEM was purchased from ICN Biochemicals (Costa Mesa, CA); cell culture plasticware was from Costar (Cambridge, MA); AVP was from Peninsula Laboratories (Belmont, CA); VIP, (−)isoproterenol, and isobutylmethylxanthine were from Sigma Chemical (St. Louis, MO); and forskolin was from Calbiochem (San Diego, CA). All other reagents were from Sigma. [^{3}H]AVP (specific activity, 60–80 Ci/mmol), ^{35}S-Express Protein Labeling Mix (specific activity, >1000 Ci/mmol), [α-^{32}P]ATP (specific activity, 3000 Ci/mmol), and Amplify were purchased from DuPont-New England Nuclear (Boston, MA); [^{3}H]cAMP was from ICN Biochemicals (Irvine, CA); and PN-glycosidase F and endoglycosidase H were from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Construction of mutant V2Rs. The HA epitope (YPYDVPDYA) and the N22Q mutation were introduced into the V2R using a polymerase chain reaction-based approach (14). The resulting constructs were sequenced fully according to the dideoxy chain termination method of Sanger *et al.* (15). For expression in eukaryotic cells, the cDNA bearing the N22Q mutation was cloned into the expression vector pcDNA3 (InVitrogen, San Diego, CA). The HA epitope was inserted between amino acids 1 and 2 added at the amino terminus or after amino acid 370 at the carboxyl terminus of the receptor. Commercially available monoclonal antibody 12CA5 was used to immunoprecipitate receptor tagged with the HA epitope.

Cell culture. COS.M6 and HEK 293 cells were grown in DMEM HG supplemented with 10% heat-inactivated FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Transient expression in cells. COS.M6 or HEK 293 cells, kept below 75% confluence, were plated at a density of 1.0 or 2.8 × 10^6 cells/100-mm dish, respectively, and transfected the following day by a modification of the method of Luthman and Magnusson (16). Briefly, after rinsing with HBSS, each plate with cells received 800 μl of HBSS, pH 7.05, containing 3 μg of plasmid DNA mixed with 0.5 mg/ml DEAE-Dextran. After 20 min at room temperature, 100 μM chloroquine in DMEM containing 2% FBS was added. After 3 hr at 37°, the cells were exposed to 10% dimethylsulfoxide in HBSS for 2 min, rinsed twice with DMEM without additives, and returned to growth medium and 37°. A similar protocol was used to transfect HEK cells 293.

Stable expression in HEK 293 cells. The cells, maintained at subconfluency, were transfected according to the calcium phosphate precipitation technique of Graham and van der Eb (17). On the day before transfection, 1–2 × 10^6 cells were plated onto each of two 100-mm plates. The DNA/calcium phosphate coprecipitate containing 10 μg of pcDNA3, which contained the V2R cDNA, was prepared in a sterile hood immediately before use with all reagents at 37°. The reagents were mixed in a 15-ml sterile polystyrene tube in the following order: 10–100 μl of plasmid DNA in 1 mM EDTA/10 mM Tris·HCl, pH 7.5; sterile H_2O to bring the volume to 900 μl; 1 ml of 250 mM $CaCl_2$ and 100 μl of 15 mM Na_2HPO_4; 50 mM HEPES; 150 mM NaCl; and 5 mM KCl, adjusted to pH 7.05 with NaOH. All reagents were added dropwise and slowly, with gentle mixing after each addition. After 10 min at room temperature, half of the whitish suspension was added dropwise to each plate with cells and mixed by gentle swirling. After 18 hr in the incubator, the medium was removed, and cells were treated with 2 ml of 25% glycerol in HBSS at 37°. After 1 min, the glycerol/HBSS mixture was diluted with 10 ml of HBSS added slowly with continuous mixing. The solution was aspirated, and the cells were rinsed once with HBSS. Fresh medium was then added, and the plates were returned to the incubator. The next day, the cells were trypsinized and diluted with the selection medium containing 400 μg/ml G-418. Cells were then distributed into the wells of two 96-well microtitration plates (2000–4000 cells/well) using a Costar transplate device. G-418-resistant clones were picked after 16–18 days and expanded in six-well plates to assay for stimulation of adenylyl cyclase activity as described.

Metabolic labeling with ^{35}S-methionine and ^{35}S-cysteine and immunoprecipitation of V2R. Proteins were labeled in 100-mm dishes according to a modification of the method of Keefer and Limbird (18) at 48 hr after transfection or 24 hr after plating 2.8 × 10^6 stably transfected HEK 293 cells. After being starved for 1 hr in methionine-free DMEM HG, the cells were labeled for 2 hr with 2 ml of the same medium containing 100 μCi of ^{35}S-Express Protein Labeling Mix/plate. Cells were used at this point or incubated for an additional 2 hr in DMEM plus 10% fetal calf serum (chase). Cells were then rinsed, scraped from the plate, and collected by centrifugation in D-PBS. The cell pellet from one plate was homogenized in 500 μl of RIPA buffer (150 mM NaCl, 50 mM Tris·HCl, pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS containing protease inhibitors 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml soybean trypsin inhibitor, and 0.5 μg/ml leupeptin). Homogenization was achieved by drawing the cells through needles of decreasing gauge (20–25 gauge) fitted to a 1-ml plastic syringe. Cell extracts were then clarified by mixing them with 50 μl of a 50% slurry of prewashed Protein A-Sepharose in the same buffer. Prewashed Protein A-Sepharose was prepared by treatment for 1 hr with 25 mg/ml BSA in RIPA buffer, followed by two washes with RIPA buffer alone. For immunoprecipitation, monoclonal antibody 12CA5 was added to the clarified extracts at a concentration of 40 μg/ml and incubated overnight at 4°. The antigen/antibody complexes were then separated by incubation of the mixture with prewashed Protein A-Sepharose for 2 hr at the same temperature. The beads were centrifuged, incubated three times for 4 min at room temperature with RIPA buffer, and recovered each time by centrifugation. Proteins were then eluted for 20 min at room temperature with 80 μl of 2× Laemmli buffer (1× = 62.5 mM Tris·HCl, 1% SDS, 10% glycerol, 10% β-mercaptoethanol, pH 6.8) containing 10% β-mercaptoethanol. Alternatively, the proteins were eluted with 80 μl of RIPA buffer containing 100 μM HA peptide. After treatment with glycosidases, these aliquots were mixed with an equal volume of 2× Laemmli buffer containing 10% β-mercaptoethanol. The samples were electrophoresed in 10% polyacrylamide gels and visualized by treatment of the gel with Amplify and exposure of the dried gels to Kodak-Xomat film at −70° for the times indicated.

Hormone binding to intact cells. Cells were plated onto 12-well plates at a density of 0.5–1.0 × 10^5 cells/well. Binding assays were performed the following day. Cells were washed twice with ice-cold D-PBS, after which each well received 0.5 ml of ice-cold D-PBS with 1 mM tyrosine, 1 mM phenylalanine, 0.2% glucose, 2% BSA, and the appropriate dilution of [^{3}H]AVP. Plates were incubated for 2 hr on top of crushed ice in a cold room before removal of the binding mixture by aspiration. After being quickly rinsed twice with ice-cold D-PBS, 0.5 ml of 0.1 N NaOH was added to each well to extract bound radioactivity. After 30 min at 37°, the fluid from the wells was transferred to scintillation vials containing 3.5 ml of Beckman ULTIMA-FLO M (Packard, Meriden, CT) scintillation fluid for radioassay. Nonspecific binding was determined under the same conditions in the presence of 10 μM unlabeled AVP (14). Replicate plated wells were trypsinized, and their cell content was determined to normalize the results as binding sites per cell. Binding experiments were performed at least three times, and the data are reported as mean ± standard error.

Adenylyl cyclase activity in cell homogenates. Adenylyl cyclase activity was assayed as previously described (14). The medium contained (in a final volume of 50 μl) 0.1 mM [α-^{32}P]ATP (1–5 × 10^6 cpm), 1.6 mM $MgCl_2$, 10 μM GTP, 1 mM EDTA, 1 mM [^{3}H]cAMP (~10,000 cpm), 2 mM isobutylmethylxanthine, a nucleoside triphosphate regenerating system composed of 20 mM creatine phosphate, 0.2 mg/ml (2,000 units/mg) creatine phosphokinase, 0.02 mg/ml myokinase (448 units/mg), and 25 mM Tris·HCl, pH 7.4. Incubations were at 32° for 20 min. Hormones (diluted in 1% BSA) were present at the concentrations indicated on the figures. Reactions were stopped by the addition of 100 μl of a solution containing 40 mM ATP, 10 mM cAMP, and 1% SDS. The cAMP formed was isolated by a modification of the standard double chromatography over Dowex-50 and alumina columns (19, 20).

Under these assay conditions, cAMP accumulations were linear

with time of incubation for ≤40 min and proportional to the amount of homogenate. The activities were expressed as pmol of cAMP formed/min/mg of homogenate protein. Protein was determined according to the method of Lowry (21) using BSA as standard. The experiments were performed at least three times. The values for adenylyl cyclase activity are expressed as the mean ± standard error.

Results

Glycosylation of the V2R in COS and HEK 293 cells. To examine whether the wild-type V2R is indeed glycosylated, the HA epitope was added to either the amino-terminal or the carboxyl-terminal end of the receptor to isolate the protein from cell extracts by immunoprecipitation. In either location, the presence of the epitope did not alter the level of receptor expression, ligand binding, or coupling to G_s (data not shown). As shown in Fig. 1A, after immunoprecipitation, only the extracts of cells transfected with the wild-type receptor cDNA produced radioactive bands that were detectable by autoradiography. A predominant radioactive band at 37 kDa was observed for the wild-type receptor as well as two less-intense bands: one at ≈74 kDa and a second broad, weak band between 45 and 55 kDa. The intensity of these two bands varied significantly from one experiment to another. As illustrated in Fig. 1B, expression of the N22Q mutant protein also produced three predominant bands: a strong band of 33 kDa and two weaker bands at ≈40 and ≈66 kDa. As expected, treatment of the wild-type receptor with PN-Glycosidase F, an enzyme that cleaves the *N*-glycosidic bond between the sugar moiety and asparagine, reduced the size of the 37 kDa band to ≈33 kDa, indicating that the faster migration correlated with the absence of *N*-linked sugar and not with a conformational change induced by the mutation. Surprisingly, treatment of the immunoprecipitated receptor with endoglycosidase H also reduced the size of the wild-type 37-kDa band to 33 kDa, as illustrated in Fig. 1C. The susceptibility to endoglycosidase H identified the 37-kDa band as a glycosylated protein containing high mannose sugar substitution, revealing that the majority of the isolated receptor protein had not been processed through the Golgi network as expected from a mature plasma membrane protein. A similar distribution of radioactive bands was observed when metabolically labeled V2R expressed transiently in HEK 293 cells was examined by the use of SDS-polyacrylamide gel electrophoresis and fluorography (not shown). As illustrated in Fig. 1, B and C, transfection of the N22Q mutant cDNA always produced a protein of 40 kDa, close in size to the predicted nonglycosylated V2R. Assuming that the glycosylated protein would have migrated as a slower and less-focused band, we considered the possibility that the 45–55-kDa protein corresponded to the mature glycosylated human V2R.

To examine the size of the receptor proteins synthesized in stably transfected cells, metabolic labeling and immunoprecipitation were then carried out with the HEK 293-derived HV2-HA_N cell clone expressing the tagged V2R. As illustrated in Fig. 2, after pulse labeling of the cells, proteins isolated before a 2-hr chase showed the presence of a broad 45–50-kDa radioactive band and of two well-defined bands at 37 and 30 kDa. At variance from what was observed in COS cells, the 74-kDa band was not apparent in multiple experiments performed in stably transfected cells. After isolation by immunoprecipitation from cell extracts and elution from the immunoprecipitate with HA peptide, the receptor was treated with glycosidases. As it has been seen in COS.M6 cell extracts, treatment with endoglycosidase H reduced the size of the 37- and 30-kDa bands seen before the chase period, but it did not alter the 45–55-kDa band. After a 2-hr chase period, the smaller bands almost disappeared, indicating that they may represent immature receptor proteins. On the other hand, the broad band at 45–50 kDa remained after the

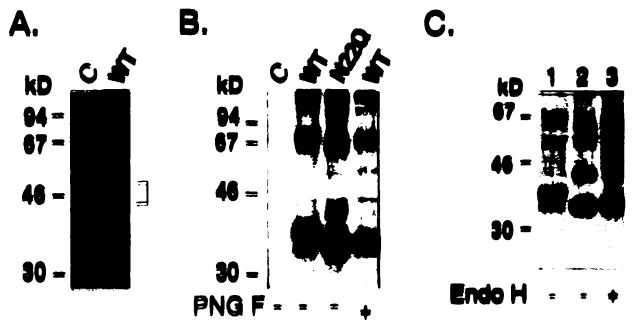

Fig. 1. Molecular mass of the V2R expressed in COS.M6 cell wild-type and N22Q HA-tagged mutant receptors were expressed in COS.M6 cells, metabolically labeled with ^{35}S-Express mixture 48 hr after transfection, and extracted for immunoprecipitation after a 2-hr chase. The eluates from the Protein A-Sepharose were analyzed as described in Experimental Procedures. A, Immunoprecipitated extracts from control (*C*) COS.M6 cells and cells expressing the wild-type (*WT*) V2R. B, Immunoprecipitated extracts from control COS.M6 cells, cells expressing the wild-type V2R, cells expressing the N22Q mutant receptor, and wild-type V2R plus PN-glycosidase F (*PNG F*) treatment. C, Immunoprecipitated extracts from cells expressing the wild-type V2R (*lane 1*), cells expressing the N22Q mutant V2R (*lane 2*), and cells expressing the wild-type V2R plus endoglycosidase H (*Endo H*) treatment (*lane 3*). Enzymatic treatments were carried out as described in the legend of Fig. 2. Samples were analyzed by electrophoresis in 10% SDS-polyacrylamide gels, and the gels were treated with Amplify, dried, and exposed to Kodak X-Omat for 24 hr.

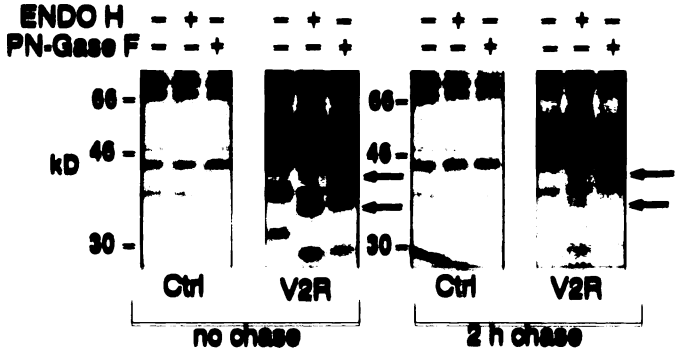

Fig. 2. Molecular mass of wild-type V2Rs expressed in HEK 293 cells. Proteins from control (*Ctrl*) and from the HEK 293-derived clone HV2-HA_N were metabolically labeled with ^{35}S-Express mixture for 2 hr at 24 hr after plating. Extracts prepared before and after a 2-hr chase were examined. Protein A-Sepharose bound immunoprecipitates were extracted with 100 μM HA peptide, and the eluates were treated with endoglycosidase H (*ENDO H*) or PN-glycosidase F (*PN-Gase F*) at room temperature for 1.5 hr at a concentration of 15 and 2500 munits/ml for endoglycosidase H and PN-glycosidase F, respectively. Samples were mixed with 2× Laemmli buffer and analyzed by electrophoresis in 10% SDS-polyacrylamide gels. The gels were treated with Amplify, dried, and exposed to Kodak X-Omat for 24 hr. Extracts from control HEK 293 cells and from clone HV2-HA_N, expressing 2.8×10^6 V2Rs/cell, were analyzed after the treatments indicated in the figure. *Arrows*, position of the bands generated by treatment with the glycosidases before and after the 2-hr chase.

chase and displayed resistance to endoglycosidase H treatment. Treatment with PN-glycosidase F reduced the size of the 37-kDa (Fig. 2, *bottom arrow*) and 30-kDa bands and gave rise to a 40-kDa band (Fig. 2, *top arrow*) while reducing the intensity of the broad band. Extracts from control HEK cells treated in a similar way did not undergo changes in their radioactive pattern.

Functional properties of the nonglycosylated V2R. The wild-type and N22Q mutant V2Rs were expressed transiently in COS.M6 and HEK 293 cells to assess the binding affinity of the receptor to AVP and the level of receptor expression. In the representative experiment illustrated in Fig. 3, the binding affinity for [^{3}H]AVP was 2.2 and 2.7 nM for the wild-type and the nonglycosylated receptors, respectively. The B_{max} was 5.9 × 10^6 sites/cell and 4.6 × 10^6 sites/cell for the wild-type and the nonglycosylated receptors, respectively. The mean values of these parameters calculated from three independent experiments were a K_D value of 2.5 ± 0.3 and 2.6 ± 0.3 and a B_{max} value of 4.9 ± 1.4 and 4.5 ± 1.7 × 10^6 sites/cell for the wild-type and nonglycosylated receptors, respectively. Thus, we concluded that these parameters were not affected by the absence of sugar.

Fig. 4 illustrates a representative experiment in which the ability of the nonglycosylated receptor protein to mediate AVP-dependent stimulation of adenylyl cyclase activity was examined and found to be very similar to the activity of the wild-type receptor. The EC_{50} for stimulation of adenylyl cyclase activity was 0.2 ± 0.07 and 0.4 ± 0.14 nM for the wild-type and N22Q mutant receptor, respectively.

Desensitization of the nonglycosylated V2R. We previously showed that the human V2R is subject to ligand-induced sequestration and desensitization (22). After exposing cells expressing the wild-type or the N22Q mutant receptor to 100 nM AVP for 10 min at 37° in growth medium, we examined whether the absence of glycosylation affected the extent of receptor desensitization or internalization. As assessed by binding of [^{3}H]AVP to intact cells, the AVP-induced reduction in receptor sites was 50% for the wild-type and the mutant receptor (data not shown). As illustrated in Fig. 5, this was accompanied by a reduction in the maximal stimulation of adenylyl cyclase activity that was virtually the same for both receptors. In addition, the EC_{50} values for adenylyl cyclase activity stimulation for both receptors were right-shifted to the same extent. The EC_{50} values for AVP stimulation of adenylyl cyclase activity after desensitization were 2.8 ± 1.3 and 4.4 ± 1.7 nM for the wild-type and the nonglycosylated receptors, respectively. The EC_{50} values obtained in three independent experiments were used to calculate this value.

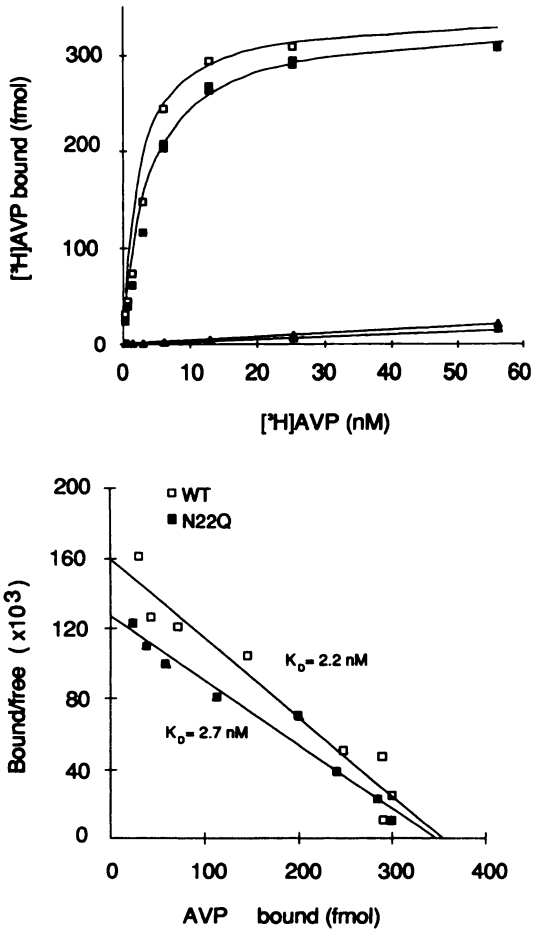

Fig. 3. Saturation binding of [^{3}H]AVP to COS.M6 cells. *Top*, [^{3}H]AVP saturation binding assays were carried out at 0° with cells transfected either with wild-type or N22Q V2R cDNA. Nonspecific binding was determined in the presence of 10 μM AVP. □ And △, total and nonspecific binding, respectively, in cells transfected with wild-type V2R cDNA; ■ and ▲, total and nonspecific binding in cells transfected with N22Q V2R cDNA. Bottom, Scatchard analysis of the binding data shown on the top. B_{max} values were 355 and 348 fmol/well for the wild-type (*WT*) and the N22Q mutant receptor, respectively.

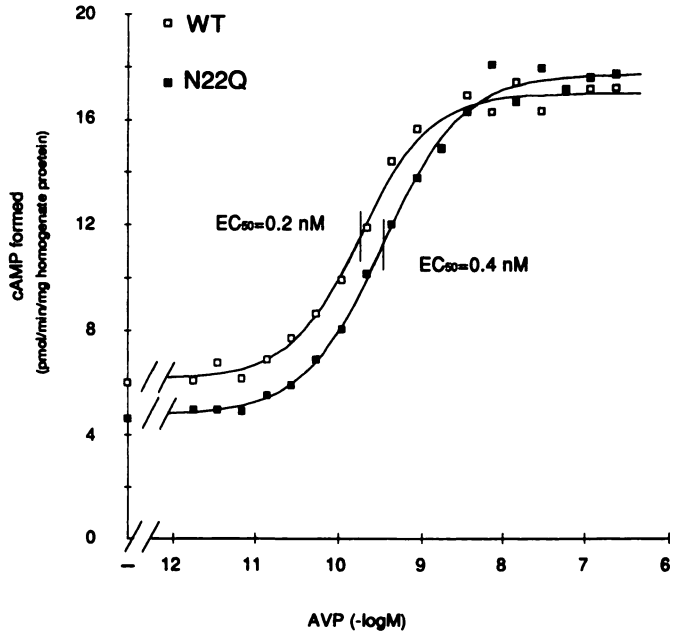

Fig. 4. AVP stimulation of adenylyl cyclase activity mediated by the N22Q mutant receptor HEK 293 cells expressing the wild-type (*WT*) or the N22Q mutant receptor were tested for AVP-stimulated adenylyl cyclase activity as described in Experimental Procedures; a representative experiment is shown. Adenylyl cyclase activities expressed as pmol of cAMP formed/min/mg of protein were 6.2 and 4.8 at base-line, 16 and 18 for 10 μM VIP, and 62 and 67 for 10 μM forskolin for the homogenate of cells expressing the wild-type and N22Q mutant cDNA, respectively. The VIP receptor is present in all HEK cells.

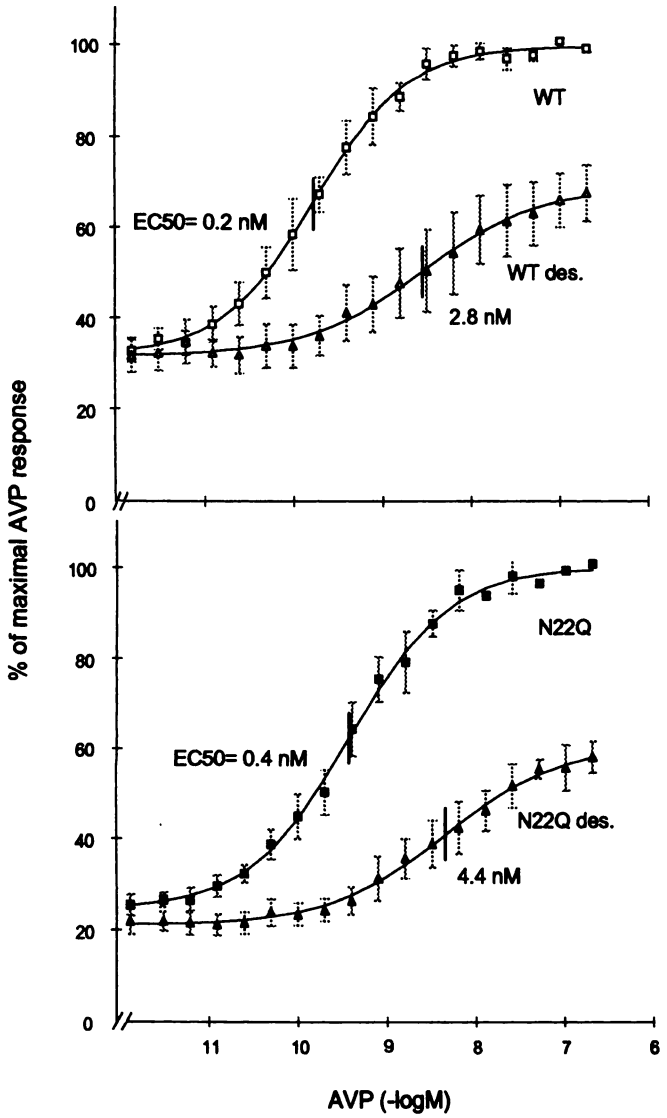

Fig. 5. Desensitization (*des.*) of nonglycosylated V2R. Cells expressing the wild-type (*WT*) and N22Q mutant receptors were exposed to 100 nM AVP for 10 min at 37° in growth medium. The cells were rinsed twice with D-PBS, scraped from the dish, and homogenized. AVP-stimulated adenylyl cyclase activity was determined as described in Experimental Procedures. The stimulation of adenylyl cyclase activities mediated by each receptor protein is expressed as percentage of the maximal AVP response obtained with the nondesensitized transfected cells. □ And △, activity of control and desensitized cells expressing the wild-type V2R cDNA; ■ and ▲, activity of control and desensitized cells expressing the N22Q mutant V2R cDNA.

Discussion

The cDNA encoding the human V2R predicts a protein of 371 amino acids with an expected molecular mass of 41,000 Da. The predominant form of ^{35}S-labeled V2R expressed transiently in COS.M6 or HEK 293 cells migrated as a 37-kDa protein, significantly smaller than expected. Of the two additional bands observed, the weak broad band at 45–55 kDa could have represented the glycosylated receptor, whereas the 74-kDa band could have represented a receptor aggregate or dimer. The intensity of both bands was variable in COS.M6 cells, and we believe that the 74-kDa band is an artifact created during processing of the cell extracts. Treatment with endoglycosidase H reduced the size of the 37- and 74-kDa bands. Because this enzyme does not cleave glycosidic bonds of complex carbohydrates, this result demonstrated that most of the receptor extracted from COS.M6 and HEK 293 cells expressing the protein transiently was inappropriately processed. The faster migration observed is presumably the result of a combination of inappropriate folding of the protein and incomplete maturation of the sugar moiety, which may alter the attachment of sodium dodecyl sulfate and interfere with complete unfolding of the peptide backbone. The immature proteins also show a marked tendency to aggregate, probably via interaction of their hydrophobic regions, and form the apparent dimers seen sometimes when the extracts from cells labeled in transient expression experiments are analyzed (Fig. 1A). These anomalies are probably a consequence of the high rate of protein synthesis from the transfected plasmid that prevails in these cells; we observed a different pattern of labeled proteins in stably transfected HEK 293 cells. The existence of dimers for other G protein-coupled receptors expressed in COS cells has been hypothesized because of their migration in gel electrophoresis. In view of our results, it seems necessary to verify the extent of maturation of those proteins before attempting to attach a functional correlate to their appearance. The ligand binding and coupling properties of the receptors analyzed in transient expression experiments point to the existence of a significant pool of mature protein, that migrating at 45–55 kDa, that is present on the cell surface and mediates those effects. As seen from our results, detection of the mature receptor in transient expression systems is impaired by the accumulation of immature protein.

The HA-tagged V2R labeled and extracted from a stable cell clone derived from HEK 293 cells was predominantly composed of mature protein, and as illustrated in Fig. 2, when this mature receptor protein predominated, the "dimeric" aggregates were not apparent. The carbohydrate of this mature protein had the resistance to endoglycosidase H expected of complexes that have completed their processing through the Golgi network. The high rate of protein accumulation in transient expression experiments is unlikely to be a consequence of the promoters that were used because the plasmid construct was the same; rather, it may reflect the different rates of mRNA synthesis from episomal opposite to integrated DNA templates.

Because of the preponderance of fully processed receptor observed in the stably transfected cell after the 2-hr label/2-hr chase period, the impact of the chase period on the nature of the predominant receptor bands deserves close examination. As shown in Fig. 2, the labeled receptor immunoprecipitated from stably transfected cells after a 2-hr pulse/no chase period has both a prominent 37-kDa band and a diffuse band in the 45–55-kDa region. After the 2-hr chase, the 37-kDa band practically disappeared, and the mature receptor predominated. In experiments presented elsewhere, we determined that the mature receptor has a $t_{1/2}$ of 5 hr in transient and stably transfected cells, whereas the 37-kDa form has a $t_{1/2}$ of 8 hr and of 20 min in transient and stably transfected cells, respectively (23). We ascribe the prolonged apparent half-life to the accumulation of the immature form in the transient expression experiments. In addition to the increasing complexity of the sugar moiety, transit of the receptor protein through the endoplasmic reticulum and the Golgi network may be necessary to achieve appropriate fold-

ing of the peptide backbone. Even in the absence of a requirement for processing of the high mannose sugar, appropriate refolding may be required to translocate to the cell surface a protein that is fully functional. We concluded that the V2R undergoes this maturation from our observation that expression of the N22Q mutant receptor in COS.M6 cells produced two bands: the larger one at 40 kDa that migrates like the deglycosylated mature receptor, and the smaller one at 33 kDa that migrates like the deglycosylated immature receptor (Fig. 1, A and B).

An earlier report describing the impact of single amino acid changes on the level of expression of the human V2R contained immunoblots of HEK cell membranes expressing V2R tagged with a polyoma virus epitope (24). It was stated that the predominant band detected at 60 kDa corresponded to the AVP receptor protein, analogous to the value reported by Kojro *et al.* (25) for the photoaffinity labeled bovine AVP receptor. The receptor analyzed by Pan *et al.* (24) was expressed transiently, and the size of the bands shown differs significantly from our results. We have performed similar experiments with receptor tagged with the *c-myc* epitope and found by immunoblotting a weak poorly defined band of the same size as we obtained for the mature immunoprecipitated receptor (not shown). Thus, we propose that the mature human V2R has an apparent molecular mass of 45–55 kDa, which is similar to the size reported for rhodopsin, either purified from rod outer segments or from cells expressing the protein (26).

As expected, treatment with PN-glycosidase F, which cleaves the linkage between asparagine and the first attached sugar regardless of its composition, altered the size of all of the detected glycosylated bands. The immature forms of the receptor were reduced in size, and the mature form generated a much better focused protein band at 40 kDa. The presence of the *N*-linked sugar at the amino terminus was not required for efficient transport of the V2R to the cell surface or for receptor function. Similarly, the presence of the epitope at the amino-terminal end of the receptor protein did not alter expression levels, ligand binding affinity, or coupling to G_s, suggesting that this region does not contribute significantly to the formation of the binding pocket or the coupling structure. The lack of effect of the sugar moiety on V2R function that we report contradicts the report by Jans *et al.* (11), which described significant changes in expression, ligand binding activity, and internalization for the LVPR expressed in the $LLCPK_1$ cells when the cells were treated with drugs that interfere with normal *N*-glycosylation. It is possible that the results they observed were the result of alteration by the glycosylation inhibitors of other cell functions. These effects could be the results of previously unknown effects of the substances used to inhibit glycosylation. For example, recent reports describe the inhibitory effect of tunicamycin on the palmitoylation of proteins.

The role of glycosylation in expression and function of G protein-coupled receptors is variable. Both rhodopsin and the β_2-adrenergic receptor have two consensus sites for *N*-linked glycosylation, and although rhodopsin requires glycosylation of one of the sites for proper transport to the cell surface and coupling to transducin, only subtle changes in activity have been described for the nonglycosylated β_2-adrenergic receptor (12, 27). Other receptors of this superfamily (e.g., the human α_{2B}-adrenergic receptor and the adenosine A_2 receptor) lack consensus sites for glycosylation (28, 29) and therefore are fully active in the absence of glycosylation. The V2R, even though it is glycosylated, seems to belong to the class of receptors for which glycosylation does not play a role in function, although we reported that the half-life of the mature nonglycosylated receptor was 3.5 hr compared with 5 hr for the wild-type V2R (23). The observation that the mature nonglycosylated V2R protein is easier to detect in gel electrophoresis has practical implications because expression of this mutant receptor will facilitate the study of biochemical modifications. Finally, our results illustrate that the assumption that the most abundant protein form detected in transient expression experiments is the one responsible for the measured biological effects is not correct for the V2R, and the same may apply to other receptor proteins. Because transient expression is commonly used to produce proteins for characterization of antibodies or to examine the possible existence of receptor dimers, scrutiny of the extent of maturation of the proteins synthesized should play an important role in the interpretation of the data.

References

1. Orloff, J., and J. S. Handler. The role of adenosine 3′,5′-phosphate in the action of antidiuretic hormone. *Am. J. Med.* **42**:757-768 (1967).
2. Birnbaumer, M., and L. Birnbaumer. Signal transduction by G protein coupled receptors, in *Membrane Functions in Health and Disease* (S. M. Schultz, T. E. Andreoli, A. M. Brown, D. M. Fambrough, J. F. Hoffman, and M. J. Welsh, eds.). Plenum Press, New York, 321–366 (1996).
3. Nielsen, S., S. R. DiGiovanni, E. I. Christensen, M. A. Knepper, and H. W. Harris. Cellular and subcellular immunolocalization of vasopressin-regulated water channel in rat kidney. *Proc. Natl. Acad. Sci. USA* **90**: 11663-11667 (1993).
4. Rosenthal, W., A. Seibold, A. Antaramian, M. Lonergan, M.-F. Arthus, G. N. M.-F., Hendy, M. Birnbaumer, and D. G. Bichet. Molecular identification of the gene responsible for congenital nephrogenic diabetes insipidus. *Nature (Lond.)* **359**:233-235 (1992).
5. Pan, Y., A. Metzenberg, S. Das, S., and J. Gitschier. Mutations in the V2 vasopressin receptor gene are associated with X-linked nephrogenic diabetes insipidus. *Nat. Genet.* **2**:103-106 (1992).
6. Deen, P. M., M. A. Verdijk, N. V. Knoers, B. Wieringa, L. A. Monnens, C. H. van Os, and B. A. van Oost. Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine. *Science (Washington D. C.)* **264**:92-5 (1994).
7. Birnbaumer, M., A. Seibold, S. Gilbert, M. Ishido, C. Barberis, A. Antaramian, P. Brabet, and W. Rosenthal. Molecular cloning of the receptor for human antidiuretic hormone. *Nature (Lond.)* **357**:333-335 (1992).
8. Lolait, S. J., A.-M. O'Carroll, O. W. McBride, M. Konig, A. Morel, and M. J. Brownstein. Cloning and characterization of a vasopressin V2 receptor and possible link to nephrogenic diabetes insipidus. *Nature (Lond.)* **357**: 336-339 (1992).
9. Gorbulev, V., H. Buechner, A. Akhundova, and F. Fahrenholz. Molecular cloning and functional characterization of V_2 [8-lysine]vasopressin and oxytocin receptors from a pig kidney cell line. *Eur. J. Biochem.* **215**:1-7 (1993).
10. Birnbaumer, M. ADH receptors: molecular biology and signal transduction, in *Molecular Nephrology* (J. V. Bonventre and D. Schlondorff, eds.). Marcel Dekker, New York, 517–524 (1995).
11. Jans, D. A., P. Jans, H. Luzius, and F. Fahrenholz. N-Glycosylation plays a role in biosynthesis and internalization of the adenylate cyclase stimulating vasopressin V2-receptor of LLC-PK1 renal epithelial cells: an effect of concanavalin A on binding and expression. *Arch. Biochem. Biophys.* **294**:64-69 (1992).
12. Kaushal, S., K. D. Ridge, and H. G. Khorana. Structure and function in rhodopsin: the role of asparagine-linked glycosylation. *Proc. Natl. Acad. Sci. USA* **91**:4024-4028 (1994).
13. Green, N., H. Alexander, A. Olson, S. Alexander, T. M. Shinnick, J. G. Sutcliffe, and R. A. Lerner. Immunogenic structure of the influenza virus hemagglutinin. *Cell* **28**:477-487 (1982).
14. Birnbaumer, M., S. Gilbert, and W. Rosenthal. An extracellular CNDI mutation of the vasopressin receptor reduces cell surface expression, affinity for ligand and coupling to the G_s/adenylyl cyclase system. *Mol. Endocrinol.* **8**:886-894 (1994).
15. Sanger, F., S. Nicklen, and A. B. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467 (1977).
16. Luthman, H., and G. Magnusson. High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* **11**:1295-1308 (1983).

17. Graham, F. L., and A. J. Van der Eb. A new technique for the assay of infectivity of human adenovirus S DNA. *Virology* **52**:456-467 (1973).
18. Keefer, J. R., and L. E. Limbird. The alpha 2A-adrenergic receptor is targeted directly to the basolateral membrane domain of Madin-Darby canine kidney cells independent of coupling to pertussis toxin-sensitive GTP-binding proteins. *J. Biol. Chem.* **268**:11340-11347 (1993).
19. Bockaert, J., M. Hunzicker-Dunn, and L. Birnbaumer. Hormone-stimulated desensitization of hormone-dependent adenylyl cyclase: dual action of luteinizing hormone on pig graafian follicle membranes. *J. Biol. Chem.* **251**:2653-2663 (1976).
20. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541-548 (1974).
21. Lowry, O. H., N. J. Rosebrough, O. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275 (1951).
22. Birnbaumer, M., A. Antaramian, A. P. N. Themmen, and S. Gilbert. Desensitization of the human V2 vasopressin receptor: homologous effects in the absence of heterologous desensitization. *J. Biol. Chem.* **267**:11783-11788 (1992).
23. Sadeghi, H. M., G. Innamorati, and M. Birnbaumer. Maturation of receptor proteins in eukaryotic expression systems. *J. Recept. Signal Transduct. Res.*, in press (1996).
24. Pan, Y., P. Wilson P, and J. Gitschier. The effect of eight V2 vasopressin receptor mutations on stimulation of adenylyl cyclase and binding to vasopressin. *J. Biol. Chem.* **269**:31933-31937 (1994).
25. Kojro, E., P. Eich, G. Gimpl, and F. Fahrenholz. Direct identification of an extracellular agonist binding site in the renal V2 vasopressin receptor. *Biochemistry* **32**:13537-13544 (1993).
26. Sung, C.-H., B. G. Schneider, N. Agarwal, D. S. Papermaster, and J. Nathans. Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. *Proc. Natl. Acad. Sci. USA* **88**:8840-8844 (1991).
27. Rands, E., M. R. Candelore, A. H. Cheung, W. S. Hill, C. D. Strader, and R. A. Dixon. Mutational analysis of *beta*-adrenergic receptor glycosylation. *J. Biol. Chem.* **265**:10759-10764 (1990).
28. Weinshank, R. L., J. M. Zgombick, M. Macchi, N. Adham, H. Lichtblau, T. A. Branchek, and P. R. Hartig. Cloning, expression, and pharmacological characterization of a human α2B-adrenergic receptor. *Mol. Pharmacol.* **38**:681-688 (1990).
29. Maenhaut, C., J. Van Sande, F. Libert, M. Abramowicz, M. Parmentier, J. J. Vanderhaegen, J. E. Dumont, G. Vassart, and S. Schiffmann. RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* **173**:1169-1178 (1990).

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