The G-protein-coupled calcium receptor plays a key role in extracellular calcium homeostasis. To examine the role of the membrane-spanning domains and the ~200-residue cytoplasmic carboxyl terminus of the calcium receptor in cell-surface expression and signal transduction, we transfected HEK-293 cells with a series of truncation and carboxyl-terminal missense mutants and analyzed expression by immunoblotting, glycosidase digestion, intact cell immunoassay, and extracellular calcium-stimulated phosphoinositide hydrolysis assay. Two truncation mutants terminating at residues 706 and 802 within the second and third intracellular loops, respectively, were not properly glycosylated, failed to reach the cell-surface, and showed no calcium response, indicating that mutant receptors with the full extracellular domain but only three or five transmembrane domains are improperly folded and/or processed. Truncation mutants terminating at residues 888 and 903 within the carboxyl terminus were equivalent to the wild type in all assays, whereas mutants truncated at residues 865 and 874 showed no response to calcium, despite only ~25% reduction in cell-surface expression. Mutants with a full-length carboxyl terminus but with residues between positions 874 and 888 replaced with alanines showed either no (Ala875, Ala876, and Ala879) or significantly reduced (Ala881–Ala883) calcium response at levels of cell-surface expression equivalent to those of the wild-type receptor. These results indicate that deletion of the majority of the carboxyl terminus is compatible with normal processing, cell-surface expression, and signal transduction of the receptor. The truncation and alanine substitution mutants identify a small region between residues 874 and 888 critical for normal signal transduction by the receptor.

The calcium receptor (CaR), initially cloned as a cell-surface Ca\(^{2+}\)-sensing protein from bovine parathyroid glands (1), but also shown to be expressed in kidney, brain, and other tissues, is a member of the seven-transmembrane domain superfamily of G-protein-coupled receptors (GPCRs) (2). Extracellular calcium ion (Ca\(^{2+}\)) activates the CaR, leading to activation of phospholipase C-β via the G\(_{q}\) subfamily of G-proteins; this increases phosphoinositide (PI) hydrolysis, which in turn causes release of calcium from intracellular stores (1). CaR activation by Ca\(^{2+}\) is thought to control the rate of parathyroid hormone secretion from parathyroid cells and the rate of calcium reabsorption by the kidney (2). Thus, the CaR plays a critical role in the regulation of extracellular calcium homeostasis.

Although the CaR shares the GPCR-defining seven-transmembrane domain motif, it shows no sequence homology to GPCRs other than a unique subfamily composed of the metabotropic glutamate and y-aminobutyric acid type B receptors (1, 3). The cloned bovine (1), human (4), rat (5, 6), and rabbit (7) CaR cDNAs predict, in addition to the seven membrane-spanning domains, a very large (>800 residue) amino-terminal extracellular domain (ECD) and a large ~200-residue carboxyl-terminal tail. Unlike other members of the GPCR superfamily for which extensive mutagenesis studies have helped define regions and residues important for receptor synthesis and cell-surface expression, for ligand binding, and for G-protein coupling, relatively little is known about the structure-function relationships of the CaR. Several naturally occurring point mutations have been identified in the human CaR (hCaR) in subjects with autosomal dominant hypocalcemia and familial benign hypocalciuric hypercalcemia (FBHH) (2). A limited number of these putatively activating (autosomal dominant hypocalcemia) and inactivating (FBHH) mutations that occur primarily in the ECD have been expressed and tested for function in cell transfection studies (8, 9). With the exception of a frameshift mutation (P747fs) involving the second extracellular loop (9) and an Alu insertion mutant at threonine 876 in the carboxyl terminus (10), presumptively inactivating truncation mutations involving the seven transmembrane domains or the carboxyl terminus of the hCaR have not been identified in FBHH.

Given the unique sequence and predicted topology of the hCaR and the dearth of naturally occurring mutations involving the transmembrane domains and the carboxyl terminus of the receptor, we sought to define the importance of these regions in receptor biosynthesis, cell-surface expression, and signal transduction. For this purpose, we created a series of mutants that truncate or alter the receptor sequence within the second and third intracellular loops and within the carboxyl-terminal tail. Our results indicate that all seven transmembrane helices may be necessary for proper folding, processing, and cell-surface expression of the hCaR and define a membrane-proximal portion of the carboxyl-terminal tail that contains residues critical for cell-surface expression and signal transduction.

**EXPERIMENTAL PROCEDURES**

Cloning hCaR cDNA from Human Pituitary—The entire coding region of the hCaR was amplified by polymerase chain reaction (PCR)
Calcium Receptor Carboxyl Terminus Mutagenesis

from a pituitary Marathon cDNA library (Promega, Madison, WI) using specific primers (nucleotides -14 to 6 to 5′-sense primer) and nucleotides 3256–3277 (3′-antisense primer) of the hCaR cDNA (4) and the Advantage KlenTaq polymerase mixture (CLONTECH). The PCR-amplified product was cloned into the pCR2.1 mammalian expression vector (Invitrogen, San Diego, CA) as a SphI restriction fragment and recloned as a hCaR coding fragment was cut out from the pCR3.1 expression vector (Stratagene, La Jolla, CA) as a HincII-XbaI restriction enzyme sites. Truncation mutant hCaRs are shown schematically in Fig. 1. Truncation mutants are designated by a “T” followed by the codon number corresponding to the first mutant stop codon. T706 was prepared by replacing a 356-base pair NsiI-SphI restriction fragment of the hCaR with a synthetic duplex constructed by PCR mutagenesis that substituted sequencing codon at amino acids 706 and 707. The DNA sequence showed no differences from a pituitary Marathon cDNA library (4) (GenBank™/EMBL Data Bank accession number U20759). The protein on the gel were electrotransferred to nitrocellulose membrane and incubated with 0.1 μg/ml protein A-purified monoclonal anti-hCaR antibody (raised against a synthetic peptide corresponding to residues 214–235 of the hCaR protein). Subsequently, the membrane was incubated with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Kirkgaard & Perry Laboratories, Gaithersburg, MD) at a dilution of 1:5000. The hCaR protein was detected with an enhanced chemiluminescence system (ECL, Amersham Corp.).

Intact Cell Enzyme-linked Immunoassay to Determine Cell-surface Expression—To compare cell surface expression of receptors in transiently transfected HEK-293 cells, 48 h after transfection, confluent transfected HEK-293 cells on 24-well plates were detached with 1 mM EDTA in PBS containing 0.5% bovine serum albumin. Cells were incubated with 0.5 μl of DEMEM containing 10% fetal bovine serum and 1 μg/ml monomeric anti-hCaR antibody T88 (raised against the purified extracellular domain of hCaRα) at 4 °C for 1 h. Following incubation, cells were pelleted by low-speed centrifugation (1000 rpm, 4 °C) and washed three times with PBS. Cells were then incubated with 5% (v/v) peroxidase-conjugated goat anti-mouse IgG (Kirkgaard & Perry Laboratories) in 10% fetal bovine serum for 1 h at 4 °C. After three washes with PBS, peroxidase substrate was added to each sample, and the color reaction was followed for 5–10 min; absorbance was measured at 405 nm using a Thermo max microtiter plate reader (Molecular Devices, Menlo Park, CA).

Phosphoinositide Hydrolysis Assay—Phosphoinositide hydrolysis was assayed as described (15, 14). Briefly, cells were incubated with 3 μCl/ml [3H]inositol (NEC Life Science Products) in DEMEM for 16–24 h, followed by a 30-min preincubation with PI buffer (120 mM NaCl, 0.5 mM CaCl2, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl2, and 20 mM LiCl in 25 mM PIPES (pH 7.2)). After removal of PI buffer, cells were incubated for an additional 30 min with different concentrations of Ca2+ in PI buffer. The reactions were terminated by addition of 1 ml of acid methanol (167 μl of HCl in 120 ml of methanol). Total inositol phosphates were purified by chromatography on Dowex 1×8 columns.

Statistical Analysis—The mean EC50 for each wild-type and mutant receptor in response to [Ca2+]i was calculated from EC50 values from all individual experiments and expressed as the mean ± S.E. Comparison of EC50 values was performed using analysis of variance to determine significance.

RESULTS

Immunoblot Analysis of Expression of Truncation Mutant hCaRs—Six truncation mutant constructs of the hCaR (T706, T802, T865, T874, T888, and T903) were prepared as described under “Experimental Procedures.” The sites of the six truncation mutants are schematically shown in Fig. 1. The wild-type hCaR cDNA and each of the mutant constructs were transiently transfected into HEK-293 cells, and expression was assessed by immunoblotting using a monoclonal antibody (ADD) against a peptide epitope from the extracellular domain of the receptor that was not disrupted by the truncations (Fig. 2). Membrane preparations of cells transfected with vector containing the wild-type hCaR cDNA showed two major bands of ~150 and 130 kDa and a higher molecular mass band (>197-kDa marker) that may represent dimeric forms and/or

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shortest carboxyl-terminal tail truncation mutants, T865 and T903 ran at a slightly higher molecular mass. The two
shortest carboxyl-terminal tail truncation mutants, T865 and T874, showed relatively fainter bands compared with the wild-
type hCaR when equal quantities of membranes were loaded on each lane in three different immunoblot experiments; this may
indicate a lower level of synthesis and expression of these mutant receptors.

Deglycosylation of Expressed Mutant hCaRs with PNGase F

The wild-type hCaR has been shown to undergo N-glycosylation (8, 13). To evaluate whether the truncation mutants undergo this post-translational modification, a series of deglycosylation experiments were conducted. The enzyme PNGase F cleaves N-linked carbohydrates (both intermediate high mannose forms and fully processed forms) from glycoproteins (15). In contrast, Endo-H cleaves only high mannose forms (16). Membrane preparations of cells transfected with wild-type and truncation mutant receptors were treated with PNGase F and Endo-H, and immunoblotting was performed to visualize the products of digestion. As shown in Fig. 3, PNGase F treatment of membranes from HEK-293 cells transfected with the wild-type hCaR cDNA causes the disappearance of the 150-kDa band and a reduction in size of the 130-kDa band. In contrast, Endo-H digestion causes a minimal alteration of the 150-kDa band while, like PNGase F, reducing the size of the 130-kDa band. Unlike for the wild-type hCaR, in membranes expressing the T706 and T802 mutants, both PNGase F and Endo-H digestion reduced the size of the expressed band. The products of digestion corresponded roughly to the sizes predicted, 75 and 85 kDa, respectively, based on primary amino acid length (Fig. 3). For membranes expressing the carboxyl-terminal tail truncation mutants T865, T874, T888, and T903, which displayed a doublet pattern of immunoreactivity (Fig. 2), digestion with PNGase F caused a decrease in the size of both bands, but Endo-H digestion reduced only the size of the lower band (Fig. 4). A series of faster migrating bands seen after PNGase F digestion of T865, T874, and T888 may reflect incomplete digestion (Fig. 4).

Intact Cell Enzyme-linked Immunoassay to Assess Cell-surface Expression Level of Mutant hCaRs—Expression of the extracellular N-terminal domain of the hCaR should not be disrupted in any of the truncation mutants we made. We therefore took advantage of a monoclonal antibody that binds to the extracellular domain of the native receptor expressed on intact cells to quantitate cell-surface expression of the mutant recep-

FIG. 2. Immunoblot showing the expression pattern of different truncation mutant hCaRs transiently expressed in HEK-293 cells. Crude membrane extracts were isolated from 75-mm flasks of HEK-293 cells that were transiently transfected with the wild-type hCaR and different truncation mutant plasmid DNAs as described under “Experimental Procedures.” 20 μg of membrane protein from cells transfected with each receptor was loaded in each lane (labeled at the top; WT, wild type) and fractionated on a 5–15% linear gradient gel by SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed with monoclonal anti-hCaR antibody ADD. The blot shown here is representative in terms of sizes and expression patterns of the six truncation mutants with results seen in two independent transfections and three separate immunoblots. The positions of molecular size standards are indicated on the left. The two arrows indicate the locations of immunoreactive bands corresponding to 150- and 130-kDa N-glycosylated forms of the wild-type hCaR.
stopped by addition of an equal volume of buffer containing 0.25 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, and 20% (v/v) glycerol. The reaction mixtures were subjected to 5–15% linear gradient SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane for immunoblot analysis with monoclonal antibody ADD as described under “Experimental Procedures.”

The reaction was stopped by addition of an equal volume of buffer containing 0.25 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, and 20% (v/v) glycerol. The reaction mixtures were subjected to 5–15% linear gradient SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane for immunoblot analysis with monoclonal antibody ADD as described under “Experimental Procedures.”

**Analysis of Signal Transduction by Truncation Mutant Receptors Using Phosphoinositide Hydrolysis Assay—**The CaR responds to increases in [Ca\textsuperscript{2+}]\textsubscript{o}, in the millimolar range by activating G\textsubscript{q} family proteins to stimulate phospholipase C-\beta activity with resultant PI hydrolysis. We (13) and others (17) have used this assay in transfected HEK-293 cells to measure signal transduction by expressed wild-type and mutant CaRs. Two intracellular loop truncation mutants (T706 and T803) showed greatly attenuated or absolutely no PI response even at 20 mM Ca\textsuperscript{2+} (data not shown). Likewise, the two shortest carboxyl-terminal tail truncation mutants (T865 and T874) showed no detectable PI hydrolysis response to Ca\textsuperscript{2+} (Fig. 6). In contrast, the T888 and T903 mutants both showed PI hydrolysis responses to Ca\textsuperscript{2+} similar to that of the wild-type hCaR. Both mutants showed similar dose-response curves with EC\textsubscript{50} values of 3.7 ± 0.18 and 3.0 ± 0.53, respectively (Fig. 6).

**Analysis of Cell-surface Expression and Signal Transduction by Ala\textsuperscript{875–879} and Ala\textsuperscript{881–883} Mutant hCaRs—**Since the T888 mutant behaved comparably to the wild-type hCaR, whereas the T784 mutant showed no PI hydrolysis response to Ca\textsuperscript{2+}, we sought to evaluate the role of the 14 amino acid residues between T874 and T888 (Table I). We generated two site-directed mutants in which alanine residues were substituted for residues 875, 876, and 879 (Ala\textsuperscript{875–879} mutant; residues 884, 885, and 887 are alanines in the wild-type hCaR) and for residues 877 and 878 (Ala\textsuperscript{877–878} mutant; residues 877 and 878 are already alanines in the wild-type hCaR) and for residues 881, 882, and 883 (Ala\textsuperscript{881–883} mutant; residues 884, 885, and 887 are alanines in the wild-type hCaR) in the context of the full-length receptor. Because we sought to study the signaling properties of these receptors at similar expression levels, we first determined the levels of cell-surface expression for each mutant receptor after transfecting HEK-293 cells with varying amounts of plasmid DNA (0.5–2 μg). Results of the enzyme-linked immunosassay showed that cell-surface expression differed among the mutant and wild-type receptors when the same amount of plasmid DNA was used for transfection (Fig. 7). Similar levels of expression were achieved by transfecting HEK-293 cells with half the amount of wild-type hCaR cDNA compared with mutant
Our results indicate that for the CaR, up to five membrane-spanning domains are insufficient, whereas seven membrane-spanning domains are sufficient for normal processing and cell-surface expression. It is likely that mutant hCaRs lacking a full seven membrane-spanning domains are misfolded or lack cDNA as shown in Fig. 7. Next, we compared the \([Ca^{2+}]_o\) response of the alanine mutant receptors under conditions in which cell-surface expression was equivalent to that of the wild-type hCaR using the P1 hydrolysis assay (Fig. 8). The Ala^{875}–Ala^{879} mutant under transfection conditions in which cell-surface expression was equivalent to that of the wild-type hCaR showed no dose-dependent stimulation up to 20 mM Ca\(^{2+}\). The Ala^{881}–Ala^{883} mutant showed a 60–70% decrease in maximal response compared with the wild-type receptor and a right-shifted concentration-dependent increase in \([Ca^{2+}]_o\) response with an EC\(_{50}\) value of 3.8 ± 0.8.

**DISCUSSION**

This study provides new information on the role of membrane-spanning domains and the carboxyl-terminal tail in hCaR processing, cell-surface expression, and signal transduction. We created two truncation mutants, T706 (within the second intracellular loop) and T802 (within the third intracellular loop), predicted to generate receptor proteins with the full ECD and three (T706) or five (T802) transmembrane domains. Upon transfection in HEK-293 cells, immunoblotting of cell membranes showed that both T706 and T802 are expressed and glycosylated. Unlike transfection with wild-type receptor cDNA, which yields fully processed (Endo-H-resistant) and incompletely processed (Endo-H-sensitive) glycosylated proteins, T706 and T802 are expressed primarily as incompletely processed proteins. The latter form of the protein is thought to represent a high mannose form that fails to transit properly from the endoplasmic reticulum to the Golgi apparatus (8, 16). Failure of the T706 and T802 mutant receptors to reach the cell surface as shown by intact cell immunoblotting is consistent with defective processing and trafficking. A P747fs mutation (predicted to create a truncation at residue 775 at the start of the fifth transmembrane domain) identified in a subject with endoplasmic reticulum to the Golgi apparatus (8, 16).

![Figure 6](image6.png) **FIG. 6.** Concentration dependence for calcium stimulation of phosphoinositide hydrolysis in transiently transfected HEK-293 cells expressing wild-type and carboxyl-terminal tail truncation mutant hCaRs. Transfections were performed, and calcium-stimulated phosphoinositide hydrolysis was assayed as described under "Experimental Procedures." Each data point is the mean value of three independent experiments. For each experiment, calcium concentrations were assayed in triplicate. Results (counts/min of labeled inositol phosphates generated) are expressed as a percent of the maximal response of the wild-type hCaR (WT). The mean EC\(_{50}\) for each curve is presented as mean ± S.E. The mean EC\(_{50}\) values for the wild-type and mutant hCaRs were not significantly different (p > 0.05). NR, no response.

| Carboxyl-terminal tail amino acid sequence from residues 862 to 886 |
|---------------------------------------------------------------|
| **Wild-type hCaR**                      | FKFSRTNEEVGSTAAHAFFKVAAR |
| **Ala^{875}–Ala^{879}**                  | ------------------------AA-A-- |
| **Ala^{881}–Ala^{883}**                  | --------------------------AAAA--- |

**TABLE I**

Amino acid sequence of the proximal carboxyl-terminal tail of the CaR

All 25 residues are identical in different mammalian species (i.e., human, bovine, rat, and rabbit) except Cys874 (Ser in rat). The locations of alanine substitutions are indicated below. For the Ala^{875}–Ala^{879} mutant, codons for Phe^{881}, Lys^{882}, and Val^{883} are replaced with Ala.

![Figure 7](image7.png) **FIG. 7.** Relationship between amount of plasmid DNA used for transfecting HEK-293 cells and cell-surface expression of the wild-type hCaR and the Ala^{875}–Ala^{879} and Ala^{881}–Ala^{883} mutants using enzyme-linked immunosorbent assay. 24-well plates containing HEK-293 cells were transfected with varying amounts of plasmid DNA (0.25–2 μg/well) containing cDNAs encoding wild-type (WT) and mutant receptors. Cell-surface expression of the hCaR was measured by enzyme-linked immunosorbent assay with monoclonal antibody 7F8 and peroxidase-conjugated anti-mouse immunoglobulin secondary antibody as described under "Experimental Procedures." Absorbance (optical density (O.D.)) was measured at 405 nm, and data shown here are from one of two representative experiments performed in duplicate. The inset shows immunoblot results after transfecting 75-mm flasks containing 5 μg of wild-type and 10 μg of mutant receptor plasmid DNAs. 20 μg of membrane was loaded in each lane. Lane 1, wild-type hCaR; lane 2, Ala^{875}–Ala^{879} mutant; lane 3, Ala^{881}–Ala^{883} mutant.
The effects of carboxyl-terminal tail truncation on signal transduction differ in different GPCRs. For some, such as the glucagon receptor, most of the carboxyl-terminal tail can be truncated without reducing agonist-stimulated second messenger production (16). For others, e.g. the CCR5 chemokine receptor, truncation of the carboxyl-terminal tail from 52 to either 8 or 20 residues did not impair cell-surface expression, but for the shorter mutant, abolished signal transduction response (19). For the metabotropic glutamate receptors, which are more closely related to the CaR than other GPCRs, studies of chimeric receptors have defined a role for the carboxyl-terminal tail in determining specificity of G-protein coupling (20). A preliminary study of carboxyl-terminal tail truncation mutant hCaRs expressed in Xenopus oocytes showed that truncation at residue 896 or 922 did not affect the response to [Ca$^{2+}$]$_i$, whereas truncation at residue 867 abolished the response (21). An Ala insertion mutation identified in a kindred with FBHH results in a CaR with missense sequence and eventual truncation after residue 876. This mutant showed decreased cell-surface expression (measured by immunocytochemistry with an epitope tag-specific antibody) and was unresponsive to [Ca$^{2+}$]$_i$; an artificially created Ala$^{877}$ stop mutant also failed to respond to [Ca$^{2+}$]$_i$, but showed increased cell-surface expression compared with the wild-type receptor (10).

Our results are consistent with these limited studies of carboxyl-terminal tail truncation mutant hCaRs and help to define more clearly a region of the carboxyl-terminal tail critical for normal signal transduction. While all of the carboxyl-terminal tail truncation mutant hCaRs we made are expressed at the cell surface, these mutants differ significantly in their ability to mediate increased PI hydrolysis in response to [Ca$^{2+}$]$_i$. The T888 and T903 mutants, lacking 192 and 177 carboxyl-terminal residues, respectively, show responses to [Ca$^{2+}$]$_i$, equivalent to that of the wild-type receptor. This indicates that most of the hCaR carboxyl-terminal tail is dispensable for a normal signal transduction response in transiently transfected HEK-293 cells. While the amino acid sequence of the CaR is extraordinarily conserved (>92%) among human (4), bovine (1), rat (5, 6), and rabbit (7), the region of greatest sequence divergence is the carboxyl-terminal tail from approximately residue 920 to the end. Lack of sequence conservation of this region is consistent with lack of a critical role in signal transduction response. Our results, however, should not be interpreted to indicate that this region of the hCaR has no biologic function. This region contains several consensus sites for phosphorylation by protein kinase C (4) and could play a role in receptor desensitization and/or internalization.

Interestingly, the T903 mutant showed higher expression than the wild-type receptor, suggesting the possibility that determinants in the truncated carboxyl-terminal tail could impede receptor expression or increase degradation. Indeed, Bai et al. (10) found that C-terminal truncation at residue 877 leads to increased receptor expression compared with the wild type. Their result contrasts with our finding that T887 mutant cell-surface expression is slightly reduced compared with and that T888 mutant cell-surface expression is comparable to that of the wild type. We cannot definitively explain this difference between our results and those of Bai et al., but they may relate to the differing methods used to assess cell-surface expression. Bai et al. employed immunocytochemistry using a monoclonal antibody against an epitope tag inserted in the receptor extracellular domain (10), whereas we used a more quantitative method, enzyme-linked immunosorbent assay, with a monoclonal antibody against an epitope within the endogenous extracellular domain.

Recently, the extreme carboxyl-terminal tail of the related metabotropic glutamate receptors (types 1 and 5) has been shown to interact specifically with a protein termed “homer” that serves to localize the receptor to discrete areas of neuronal membranes (22). It is conceivable that a similar protein interacts with the CaR carboxyl-terminal tail to localize this recep-
tor within specific areas of neurons (6) or other cells in which the receptor is expressed. This and other hypotheses concerning the function of the carboxyl terminus can now be tested by appropriate studies comparing wild-type and carboxyl-terminal tail truncation mutant CaRs.

A comparison of the $[\text{Ca}^{2+}]_o$ response of the T874 and T888 mutants indicates that residues between positions 874 and 888 are critical for a normal signal transduction response. At present, lack of a high-affinity ligand binding assay for the CaR prevents us from determining whether loss of signal transduction response reflects a defect in agonist binding, receptor activation, or G-protein coupling. Since the ECD, which has been suggested to be the site of agonist binding in the CaR (2), prevents us from determining whether loss of signal transduction responsiveness does not simply reflect a requirement for a minimum length of carboxyl-terminal tail is shown by the Ala$^{875}$–Ala$^{879}$ mutant (substituting alanines for Ser$^{875}$, Thr$^{876}$, and His$^{879}$), which shows no response despite having a full-length carboxyl-terminal tail. The Ala$^{881}$–Ala$^{883}$ mutant (substituting alanines for Phe$^{881}$, Lys$^{882}$, and Val$^{883}$) shows significantly reduced response compared with the wild type. Differences in signal transduction response by the Ala$^{870}$–Ala$^{879}$ and Ala$^{881}$–Ala$^{883}$ mutants do not reflect differences in cell-surface expression compared with the wild type since transfections were performed with amounts of plasmid DNA shown to give equivalent cell-surface expression. Since both the Ala$^{875}$–Ala$^{879}$ and Ala$^{881}$–Ala$^{883}$ mutants show significantly reduced signal transduction response, several residues between positions 874 and 888 are likely to contribute to normal signal transduction responsiveness. Further studies are needed to define the precise contribution of individual residues in this critical region and to determine their specific structural and functional roles.

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