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Identification of the G Protein-coupled Receptor Kinase Phosphorylation Sites in the Human β_2-Adrenergic Receptor*

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Rapid desensitization of G protein-coupled receptors is mediated, at least in part, by their phosphorylation by the G protein-coupled receptor kinases (GRKs). However, only in the case of rhodopsin have the actual sites of receptor phosphorylation been unambiguously determined. Although previous studies have implicated the cytoplasmic tail of the β_2-adrenergic receptor (β_2AR) as the site of GRK-mediated phosphorylation, the identities of the phosphorylated residues were unknown. Here we report the identification of the sites of GRK2- and GRK5-mediated β_2AR phosphorylation. The phosphorylation sites of both serine/threonine kinases reside exclusively in a 40-amino acid peptide located at the extreme carboxyl terminus of the β_2AR. Of the seven phosphorylatable residues within this peptide, six are phosphorylated by GRK5 (Thr-384, Thr-393, Ser-396, Ser-401, Ser-407, and Ser-411) and four are phosphorylated by GRK2 (Thr-384, Ser-396, Ser-401, and Ser-407) at equivalent phosphorylation stoichiometries (~1.0 mol P_i/mol receptor). In addition to the GRK5-specific phosphorylation of Thr-393 and Ser-411, differences in the distribution of phosphate between sites are observed for GRK2 and GRK5. Increasing the stoichiometry of GRK2-mediated β_2AR phosphorylation from ~1.0 to 5.0 mol P_i/mol receptor increases the stoichiometry of phosphorylation of Thr-384, Ser-396, Ser-401, and Ser-407 rather than increasing the number of phosphoacceptor sites. The location of multiple GRK2 and GRK5 phosphoacceptor sites at the extreme carboxyl terminus of the β_2AR is highly reminiscent of GRK1-mediated phosphorylation of rhodopsin.

Desensitization is the process by which prolonged exposure of cells to stimuli such as a hormone or neurotransmitter results in a decreased cellular response despite continued stimulation. The complex mechanisms involved in this process have been extensively studied using the β_2-adrenergic receptor (β_2AR)-mediated[1] stimulation of adenylyl cyclase as a model system. One of the most rapid events implicated in cellular desensitization of β_2AR function is phosphorylation of the receptor. Two distinct classes of kinases participate in this rapid desensitization process: the second messenger-regulated kinases (cAMP-dependent protein kinase and protein kinase C) and the G protein-coupled receptor kinases (GRKs 1–6).[2] The family of GRKs possess the unique ability to specifically recognize and phosphorylate the agonist-occupied form of the β_2AR. GRK-mediated phosphorylation of β_2AR leads to the binding of the cytosolic βarrestin protein and subsequent agonist-dependent functional uncoupling of the receptor from adenylyl cyclase stimulation. One important regulatory feature that distinguishes members of the GRK family is the mechanism by which these enzymes are targeted to their membrane incorporated receptor substrates (1, 2). Of particular note, GRK2 and 3 have been demonstrated, in the presence of a specific lipid co-factor (phosphatidylinositol-4,5-bisphosphate) to bind G protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) at a carboxyl-terminal pleckstrin homology domain (3, 4). Binding of prenylated $G_{\beta\gamma}$ promotes membrane association of these kinases and enhances both the rate and extent of receptor phosphorylation (3, 4). In contrast, GRK5 does not possess a pleckstrin homology domain, and the activity of this enzyme is not enhanced by $G_{\beta\gamma}$ (5).

Although the β_2AR system is one of the most thoroughly studied of the G protein-coupled receptors, the identities of the sites of GRK-mediated agonist-induced phosphorylation have not previously been determined. Mutational and proteolysis studies indicate that the sites of GRK2-mediated β_2AR phosphorylation lie potentially within the carboxyl-terminal cytoplasmic tail (C-tail) of this receptor. Thus, removal of the β_2AR C-tail by limited proteolysis with trypsin and carboxypeptidase Y (6), truncation of the C-tail at position 365 (7), and site-directed mutations at serine and threonine residues within the C-tail (7–9) result in the loss of phosphoacceptor sites. Additionally, carboxypeptidase Y digestion of β_2AR phosphorylated by GRK5, indicates that like GRK2, GRK5 phosphorylates C-tail serine and threonine residues (5).

The only G protein-coupled receptor in which the sites of GRK-mediated phosphorylation have been unambiguously identified is rhodopsin. Rhodopsin, the visual light perception receptor, is phosphorylated in a light-dependent manner by GRK1. Both *in vitro* and *in vivo* studies have demonstrated that GRK1 phosphorylates rhodopsin on specific residues located at the distal portion of the carboxyl-terminal C-tail (10–16). Additionally, *in vitro* GRK2, GRK3, and GRK5-mediated phosphorylation sites on rhodopsin have recently been identified (11). Interestingly, although the serine residues phosphorylated by these different GRKs are essentially identical, principally Ser-338 and Ser-343, GRK1 and GRK5 preferentially phosphorylate Ser-338, whereas GRK2 and GRK3 preferentially phosphorylate Ser-343.

This study was designed to identify and compare the specific β_2AR residues phosphorylated by the GRKs. To address whether the GRKs preferentially phosphorylate distinct or

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[1] The abbreviations used are: β_2AR, β_2-adrenergic receptor; C-tail, cytoplasmic tail; $G_{\beta\gamma}$, G protein $\beta\gamma$ subunits; GRK, G protein-coupled receptor kinase; HPLC, high performance liquid chromatography.

[2] There are six identified subtypes of GRKs: rhodopsin kinase or GRK1; β-adrenergic receptor kinase 1 or GRK2; β-adrenergic receptor kinase 2 or GRK3; GRK4; GRK5; and GRK6.

identical amino acid residues on the receptor, two members of the GRK family (GRK2 and GRK5) were utilized. In addition, the effect of increasing phosphorylation stoichiometry on the nature of the β_2AR phosphoacceptor sites was examined utilizing either GRK2 alone or in the presence of $G_{\beta\gamma}$.

MATERIALS AND METHODS

Purification and Reconstitution of the β_2AR—Recombinant human β_2AR was expressed and purified from baculovirus-infected *Spodoptera frugiperda* (Sf9) cells as described previously (3, 17). Purified receptor was reconstituted into crude soybean phosphatidylcholine (~20%; Sigma) vesicles (18), and the receptor concentration was determined by radioligand binding using [^{125}I](−)-iodocyanopindolol (19).

Receptor Phosphorylation by GRK2 and GRK5—Recombinant GRK2 and GRK5 expressed in Sf9 cells, and G protein $\beta\gamma$ subunits from bovine brain were purified to near homogeneity (>90%) as previously reported (3, 5, 20). Phosphorylation of purified and reconstituted β_2AR was performed in final volumes of 500 μl consisting of 200–300 nM receptor and 125 nM kinase ± 320 nM $G_{\beta\gamma}$ in 20 mM Tris-HCl (pH 8), 2 mM EDTA, 10 mM $MgCl_2$, and 1 mM dithiothreitol containing 100 μM [γ-^{32}P]ATP (~3000 cpm/pmol) and 100 μM (−)-isoproterenol. The reactions were incubated at 30 °C for 20 min and stopped by placing the sample on ice. At this point, 10 pmol of phosphorylated receptor was removed for two-dimensional phosphoamino acid analysis and determination of phosphorylation stoichiometry. Following electrophoresis of 1 pmol of phosphorylated receptor on 10% SDS-polyacrylamide gels, the stoichiometries of phosphorylation were quantitated using a Molecular Dynamics PhosphorImager. Under the conditions described, GRK5 and GRK2 phosphorylate the β_2AR to stoichiometries of 0.7–1.1 and 0.7–1.3 mol of phosphate/mol of β_2AR, respectively. As previously reported (3, 4), the addition of $G_{\beta\gamma}$ significantly enhances GRK2-mediated β_2AR phosphorylation. In the presence of 320 nM $G_{\beta\gamma}$, GRK2 phosphorylated the receptor reconstituted in this crude phosphatidylcholine environment to a stoichiometry of 4.0–5.0 mol P_i/mol β_2AR. Phosphorylated receptor was subsequently pelleted by centrifugation at 350,000 × *g* for 20 min at 4 °C and resuspended in 2 ml of buffer (20 mM Tris-HCl, 2 mM EDTA, pH 8). To remove unbound [γ-^{32}P]ATP, this washing procedure was repeated at least four more times. The phosphorylated receptor was finally resuspended in digestion buffer consisting of 2 mM dithiothreitol, 6 M urea, and 50 mM Tris-HCl (pH 8).

The resuspended receptor was heat denatured at 95 °C for 20 min, briefly cooled on ice, and diluted with 50 mM NH_4OAc (pH 4) buffer until the urea concentration was below 2 M. To this mixture was added 1 μg of sequencing grade endoproteinase Glu-C (Promega), and digestion was allowed to proceed at 30 °C for approximately 17 h. Proteolysis was stopped by freezing the sample. Subdigestions of isolated phosphopeptides were carried out using 1 μg of modified, sequencing grade trypsin (Boehringer Mannheim). These digestions were allowed to proceed at 30 °C for approximately 17 h (after heat denaturing the peptide as described above) followed by dilution with buffer (50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6). Phosphorylated and cleaved receptors and phosphopeptides were applied to an Aquapore C18 column (220 × 2.1 mm) attached to an Applied Biosystems reverse-phase HPLC system consisting of a 140B solvent delivery system and a 1000S diode array multiwavelength detector. Peptides were eluted using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid over 180 min followed by a linear gradient of 60–90% acetonitrile in 0.1% trifluoroacetic acid over 15 min. The column was run at a flow rate of 150 μl/min with fractions collected every minute. Protein elution was monitored at 220, 230, 260, and 280 nm and by radioactivity detection of every fraction using a scintillation counter. Phosphopeptides were detected by plotting radioactivity counts (cpm) *versus* fraction number, and fractions were stored at −20 °C for subsequent protein sequencing analysis and two-dimensional phosphoamino acid analysis.

Protein Sequence Analysis and Two-dimensional Phosphoamino Acid Analysis—A pool of several fractions within each peak or the middle fraction of each radioactive peak was applied to an Applied Biosystems Model 477A protein sequencer with an in-line 120A PTH-analyzer. 50% of the sample was subjected to PTH-derivative analysis, and 50% was used for phosphopeptide sequencing. The amount of ^{32}P released after each Edman degradation cycle was counted for 5 min using scintillation counting. To determine the percentage of phosphoserine, phosphothreonine, and phosphotyrosine in each phosphopeptide, each peptide was also subjected to two-dimensional phosphoamino acid analysis. First, a small aliquot of each phosphopeptide was hydrolyzed in 5.7 M HCl for 2 h at 110 °C. Each sample was then cooled on ice, dried down using a speedvac concentrator, and resuspended in 10 μl of pH 1.9 buffer (2.5% (v/v) formic acid/7.8% (v/v) glacial acetic acid) along with 20 nmol of cold phosphoamino acid standards (phosphoserine, phosphothreonine, and phosphotyrosine). The phosphoamino acids were resolved by two-dimensional electrophoresis on thin-layer cellulose plates (Kodak). In the first dimension, electrophoresis was carried out at pH 1.9 for 1.5 h at 900 V and in the second dimension at pH 3.5 (0.5% pyridine/5.0% glacial acetic acid) for 45 min at 900 V. The phosphoamino acid standards were visualized using Sigma Ninhydrin spray reagent, and phosphopeptides were visualized by autoradiography for 1–4 days. The same phosphopeptides were analyzed quantitatively with a PhosphorImager.

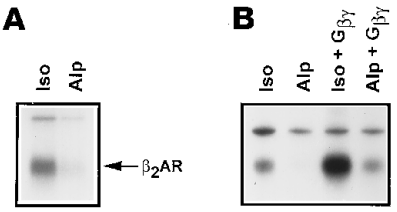

FIG. 1. **Phosphorylation of β_2AR by GRK2 or GRK5.** A representative autoradiogram of β_2AR phosphorylated by GRK5 (*A*) or GRK2 (*B*) in the absence or the presence of 320 nM $G_{\beta\gamma}$. Phosphorylations were performed as described under "Materials and Methods" in the presence of either a β-agonist (100 μM isoproterenol, *Iso*) or an antagonist (100 μM alprenolol, *Alp*) at 30 °C for 20 min. The β_2AR was phosphorylated to stoichiometries of approximately 1.0, 1.0, and 5.0 mol P_i/mol receptor by GRK5, GRK2, and GRK2 + $G_{\beta\gamma}$, respectively.

RESULTS

Identification of Phosphopeptides—To determine the location of the GRK2 and GRK5-mediated agonist-specific phosphorylation sites on the β_2AR, 400-1000 pmol of purified and reconstituted β_2AR was phosphorylated with either enzyme in the presence of receptor-saturating concentrations of the β-agonist isoproterenol (Fig. 1). The β_2AR was phosphorylated by GRK5 to stoichiometries of 0.7–1.1 mol of phosphate/mol of β_2AR (~1 mol/mol). The β_2AR was phosphorylated by GRK2 to stoichiometries of 0.7–1.3 and 4.0–5.0 mol P_i/mol β_2AR, respectively, in the absence and the presence of $G_{\beta\gamma}$ subunits. Autoradiography of SDS-polyacrylamide gels loaded with completed phosphorylation reactions revealed the presence of only two phosphorylated proteins; the phosphorylated β_2AR and autophosphorylated GRKs (Fig. 1). Phosphorylated β_2AR was subsequently digested with the proteases Glu-C, trypsin, or Asp-N and applied to an Aquapore C18 column to separate and purify phosphopeptides. Digestion of phosphorylated β_2AR with trypsin or Asp-N followed by HPLC purification proved to be ineffective methods for separating phosphorylated peptides. The radiolabeled peaks derived from these digestions were insufficiently separated and contained multiple peptides, making it impossible to distinguish between phosphorylated and unphosphorylated residues (data not shown). In contrast, chromatography of the peptides obtained following endoproteinase Glu-C digestion of the β_2AR revealed the presence of relatively few radiolabeled peaks, which were subsequently shown by amino acid sequence analysis to be separated from all other peptides.

Digestion with Glu-C followed by HPLC purification of peptides was performed for the β_2AR phosphorylated with GRK5 or GRK2 ± $G_{\beta\gamma}$. Fig. 2*A* shows a representative absorbance trace at 220 nm for the Glu-C digestion of β_2AR phosphorylated by GRK2. The figure shows material eluted from the C18 column between 0 and 80% acetonitrile corresponding to retention times of 0 and 190 min, respectively. No further absorbant material was eluted between 80 and 90% acetonitrile. ^{32}P-Labeled peptides were located by subjecting fractions to scintillation counting (Figs. 2, *B–D*). In this manner two ^{32}P-labeled peaks were detected for the Glu-C-digested GRK5-phosphorylated β_2AR (Fig. 2*B*), and three ^{32}P-labeled peaks were detected for the Glu-C-digested GRK2-phosphorylated β_2AR ± $G_{\beta\gamma}$ (Figs. 2, *C* and *D*). These peaks represent the only

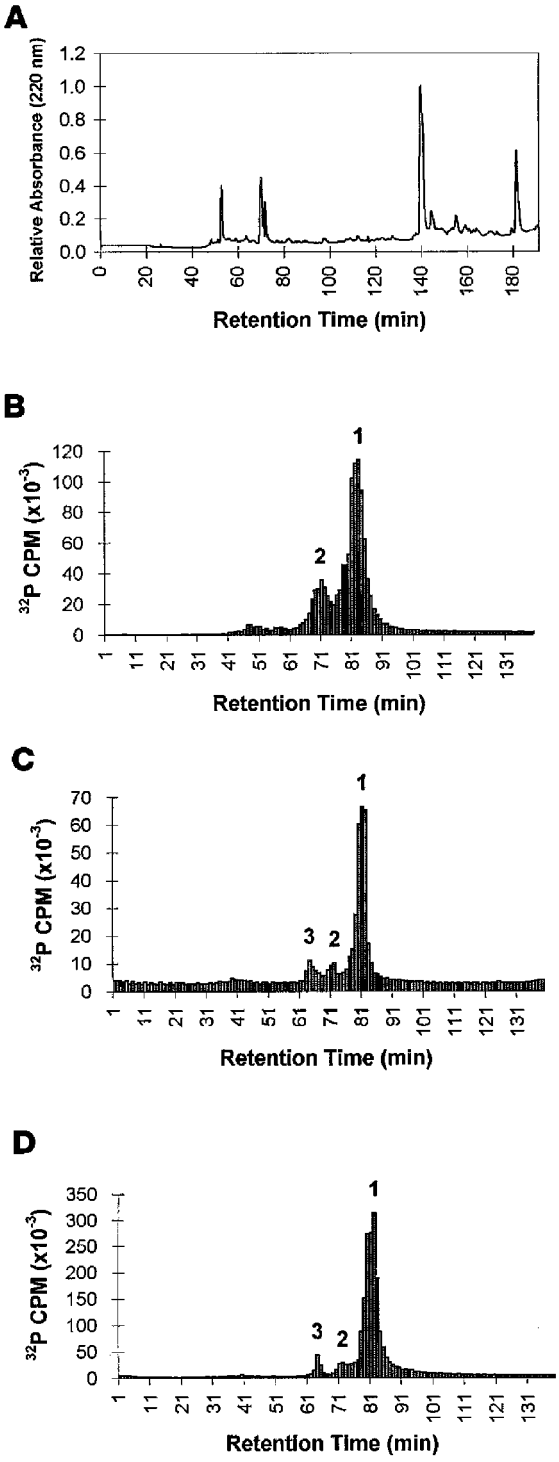

FIG. 2. **Reverse-phase HPLC analysis of phosphopeptides obtained by endoproteinase Glu-C digestion of phosphorylated β_2AR.** β_2AR was phosphorylated in the presence of 100 μM isoproterenol, digested by the endoproteinase Glu-C, and applied to an Aquapore C18 column attached to a reverse-phase HPLC system as described under "Materials and Methods." Peptides were separated using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid for 180 min and 60–90% acetonitrile in 0.1% trifluoroacetic acid for 15 min. Fractions were collected every minute, and phosphopeptides were detected using a scintillation counter. *A*, representative absorbance trace at 220 nm obtained upon chromatography of Glu-C-digested GRK5-mediated phosphorylated β_2AR. *B–D*, ^{32}P cpm *versus* retention time (0–45% acetonitrile) plots for β_2AR phosphorylated by GRK5 (1 mol P_i/mol β_2AR; n = 6 experiments) (*B*), GRK2 (1 mol P_i/mol β_2AR; n = 5 experiments) (*C*), and GRK2 in the presence of 320 nM $G_{\beta\gamma}$ (5 mol P_i/mol β_2AR; n = 5 experiments) (*D*). The radioactive peaks eluting at approximately 70 and 82 min for GRK5-mediated phosphorylations are termed, respectively, peaks 2 and 1 (*B*). The radioactive peaks eluting at 63, 70, and 80 min for GRK2 phosphorylations are termed, respectively, peaks 3, 2, and 1 (*C* and *D*). No other ^{32}P-labeled fractions were detected.

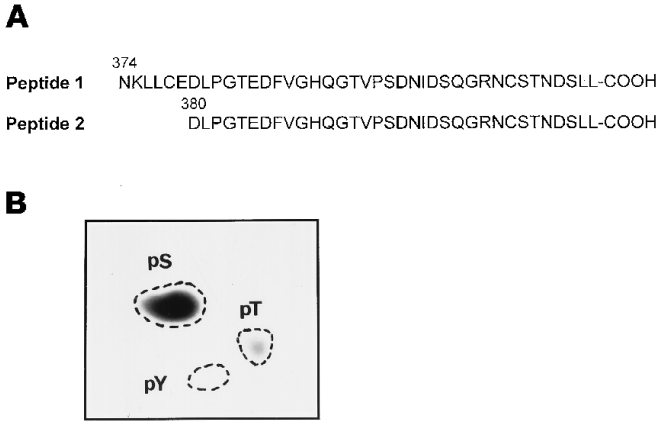

FIG. 3. **Amino acid sequences of peptides 1 and 2 and phosphoamino acid analysis of peptide 1.** *A*, amino acid sequences of peptides 1 and 2. These peptides were purified by reverse-phase HPLC and correspond to peaks 1 and 2, respectively, as shown in Fig. 2. Peptides from at least three independent digestions were subjected to amino acid sequence analysis for each experimental condition. *B*, peptide 1 derived from the GRK2-mediated β_2AR phosphorylation was prepared for phosphoamino acid analysis and separated in two dimensions as described under "Materials and Methods." Radioactive phosphoamino acids were visualized by exposing the cellulose TLC plates to film and quantitated using a PhosphorImager. The positions of phosphoserine (*pS*), phosphothreonine (*pT*), and phosphotyrosine (*pY*) standards are indicated. This figure is representative of three independent experiments. Similar results were obtained using peptide 1 derived from GRK5 and GRK2 + $G_{\beta\gamma}$-mediated β_2AR phosphorylations (discussed in the text).

radioactivity eluted from the HPLC column when developed with a 0–90% acetonitrile gradient. For GRK5-mediated phosphorylations, peak 1 eluted at approximately 82 min, and peak 2 eluted at 70 min (n = 6 experiments; Fig. 2*B*). For GRK2-mediated phosphorylations, peak 1 eluted at approximately 80 min, peak 2 eluted at 70 min, and peak 3 eluted at 63 min (n = 5 experiments; Fig. 2*C*). Peaks derived from phosphorylations by GRK2 in the presence of $G_{\beta\gamma}$ eluted at the same times as peaks derived from β_2AR phosphorylated by GRK2 alone (n = 5 experiments; Fig. 2*D*). For both GRK2- and GRK5-mediated phosphorylations, peak 1 is the main peak containing most of the radioactivity, whereas peaks 2 and 3 (GRK2-mediated phosphorylations only) are minor, containing less than 25% of the radioactive counts.

A pool of several fractions within a peak or the fraction containing the most radioactivity from each peak was subjected to amino acid sequence analysis. Peaks 1 and 2 contain distinct peptides. Comparison of the GRK2- and GRK5-derived peak 1 and similarly peak 2 revealed identical peptide sequences. Amino acid sequence analysis of fractions within peak 1 revealed the presence of a single peptide, a 40-amino acid phosphopeptide comprised of residues 374–413 of the β_2AR (peptide 1; Fig. 3*A* and Table I). Levels of extraneous amino acids at each sequencer cycle were negligible. In Table I, the residue identified, the percent yield for each residue, and the cpm released at each cycle are listed. Sequencer results obtained from the analysis of peak 1 from GRK5, GRK2, or GRK2 + $G_{\beta\gamma}$ phosphorylated β_2AR are shown (Table I). This peptide, located at the extreme C terminus of the carboxyl-tail of β_2AR, contains four serine and three threonine residues (Thr-384, Thr-393, Ser-396, Ser-401, Ser-407, Thr-408, and Ser-411) and is schematically represented in Fig. 3*A*. Amino acid sequence

TABLE I

Amino acid and phosphopeptide sequence analysis of peptide 1

The cycle number, amino acid residue, percentage of amino acid yield, and cpm are presented for each cycle of amino acid and phosphopeptide sequence analysis of peptide 1. The data listed are representative examples of sequence information obtained for GRK5 or GRK2 ± $G_{\beta\gamma}$-phosphorylated peptide 1. The amino acid identified in the first cycle of sequence analysis was assigned as 100% amino acid yield. The first amino acid yield was approximately 19 pmol for GRK5, 68 pmols for GRK2, and 33 pmol for GRK2 + $G_{\beta\gamma}$. Peptide 1, derived from Glu-C digestion of β_2AR phosphorylated by GRK5 or GRK2 ± $G_{\beta\gamma}$ was subjected to sequence analysis on at least three separate occasions.

Cycle	Residue	GRK5		GRK2		GRK2 + $G_{\beta\gamma}$	
		Yield	cpm	Yield	cpm	Yield	cpm
		%		%		%	
1	Asn	100	112	100	199	100	335
2	Lys	33	88	124	149	13	236
3	Leu	125	100	106	160	119	220
4	Leu	128	96	106	197	123	339
5			150		267		434
6	Glu	58	168	40	254	47	410
7	Asp	66	189	28	349	45	474
8	Leu	94	236	73	306	79	452
9	Pro	81	240	47	290	60	489
10	Gly	104	277	56	393	64	489
11	Thr	27	743	36	603	40	1325
12	Glu	47	561	25	483	32	1132
13	Asp	54	513	18	474	34	1004
14	Phe	54	442	41	421	45	788
15	Val	51	403	39	480	46	835
16	Gly	73	368	29	541	40	699
17	His	15	368	30	540	31	821
18	Gln	34	423	28	485	32	748
19	Gly	67	459	27	595	32	682
20	Thr	13	524	20	640	20	776
21	Val	31	449	26	660	29	863
22	Pro	31	434	15	657	20	811
23	Ser	4	716	4	1485	5	1872
24	Asp	24	658	5	1128	10	1612
25	Asn	59	628	11	905	15	1362
26	Ile	28	570	9	777	13	1208
27	Asp	24	532	6	728	11	1153
28	Ser	2	663	2	1625	2	1768
29	Gln	13	607	5	1354	7	1874
30	Gly	32	567	6	1093	8	1498
31	Arg	31	501	6	798	10	1224
32	Asn	24	495	7	673	9	1111
33			453		573		1057
34	Ser	2	569	1	1030	1	1472
35	Thr	5	556	1	877	2	1387
36	Asn	18	532	5	811	7	1276
37	Asp	9	508	3	556	3	1167
38	Ser	<1	478	<1	607	<1	1180
39	Leu	7	493	2	475	2	1176
40	Leu	9	464	3	383	4	1034

analysis of fractions within peak 2 revealed the presence of two amino acids at each sequencer cycle. These amino acids correspond to peptide 2 (major peptide) and peptide 1 (Fig. 3*A*). Peptide 2 is a fragment of peptide 1, comprised of residues 380–413 of the β_2AR (Fig. 3*A*). The molar ratio of peptide 2 to peptide 1 present in peak 2 was approximately 10:1, with no other amino acids detectable at any cycle. Peak 3, derived from GRK2-mediated phosphorylations, contains exclusively a phosphopeptide from autophosphorylated GRK2 (peptide 3; data not shown). Thus, all the agonist-induced GRK2- and GRK5-mediated sites of phosphorylation on the β_2AR appear to lie within a 40-amino acid peptide located at the C terminus of the C-tail.

Two ^{32}P-labeled β_2AR peptides were eluted from the HPLC C18 column following Glu-C digestion (Figs. 2 and 3*A*). To examine the possibility that additional phosphorylated peptides were loaded but did not elute from this column, the Glu-C-digested β_2AR was subjected to gel filtration chromatography under denaturing conditions. β_2AR phosphorylated by GRK2 was digested with Glu-C as described previously. Following digestion the lipid vesicles containing undigested β_2AR and regions of the receptor inaccessible to the proteinase were removed by centrifugation. Under these conditions, whereas judged by SDS-polyacrylamide gel electrophoresis approximately 75–90% of the receptor is digested (data not shown), approximately 80% of the total counts incorporated into the β_2AR are found in the supernatant. Gel filtration chromatography of these peptides in 50 mM NH_4OAc, 2 M urea (pH 4) revealed the presence of two ^{32}P-labeled peaks (data not shown). The estimated molecular weights of these peaks correspond approximately to the predicted molecular weights of peptides 1 and 2. Thus chromatography of the Glu-C-digested receptor on two different resins in two different buffer systems yields similar peptide profiles. These results suggest that the ^{32}P-labeled peptides identified by HPLC indeed constitute the major sites of GRK-mediated phosphorylation.

Phosphopeptide Sequencing and Two-dimensional Phosphoamino Acid Analysis—For β_2AR phosphorylated by GRK5 or GRK2 ± $G_{\beta\gamma}$, phosphopeptide sequence and phosphoamino acid analysis data were collected for each of the phosphopeptides isolated. Only data from peptide 1 are reported here because peptide 2 is essentially the same as peptide 1, and peptide 3 is a kinase peptide not a receptor peptide. The only peptide detected within peak 1 is peptide 1, therefore phosphopeptide sequencing and phosphoamino acid analysis of these fractions are unambiguous. To determine whether serine,

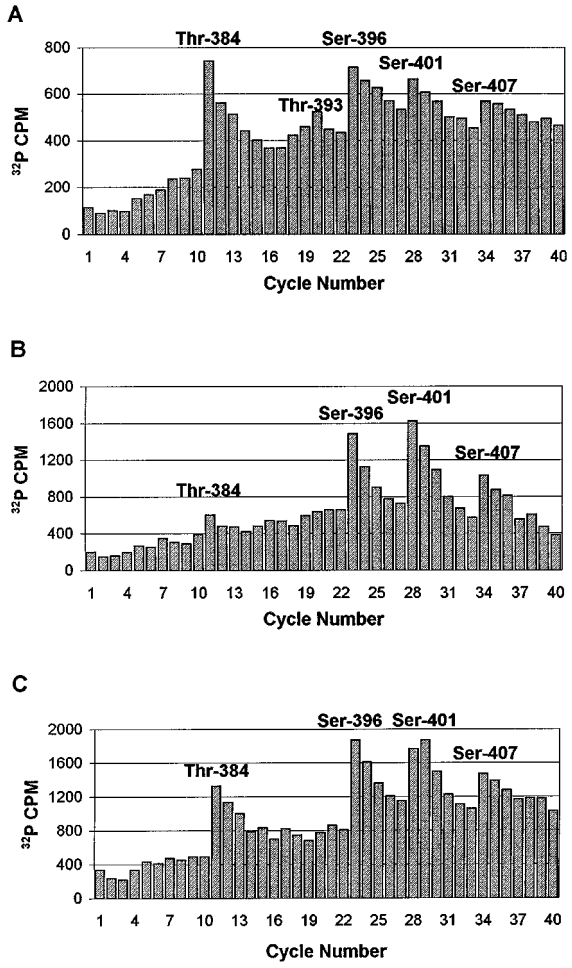

FIG. 4. **Release of ^{32}P radioactivity during Edman degradation of peptide 1.** Peptide 1 was subjected to phosphopeptide sequence analysis and the ^{32}P radioactivity in the eluate from each cycle was counted using a scintillation counter. Radioactivity plots are shown for peptide 1 derived from β_2AR phosphorylations mediated by GRK5 (*A*) and GRK2 (*B*) in the absence and the presence of $G_{\beta\gamma}$ (*C*). The amino acid residues corresponding to peaks of ^{32}P release are indicated on the figure. Phosphopeptide sequence analysis of peptide 1 was repeated at least two times for each experimental condition yielding similar results.

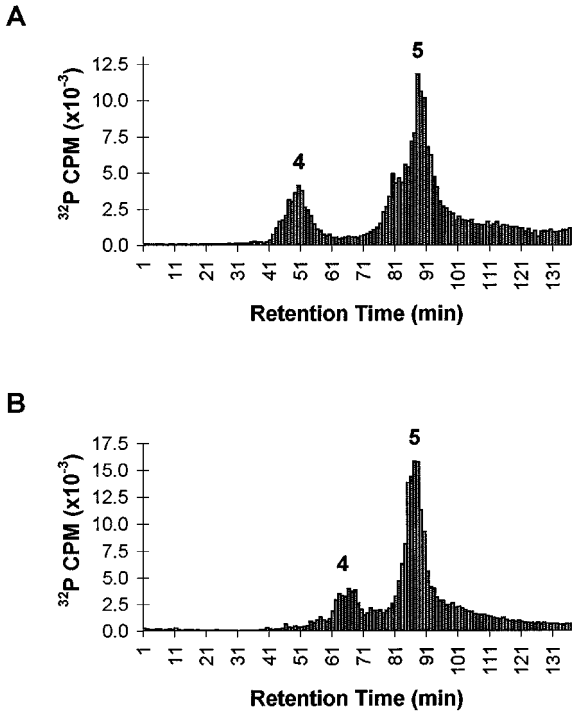

FIG. 5. **Reverse-phase HPLC and amino acid sequence analysis of phosphopeptides obtained by tryptic digestion of peptide 1.** Peptide 1 derived from β_2AR phosphorylated by GRK5 and GRK2 in the presence of $G_{\beta\gamma}$ was digested by trypsin and subjected to HPLC separation as described under "Materials and Methods." ^{32}P cpm *versus* retention time (0–45% acetonitrile) plots are shown for peptide 1 phosphorylated by GRK5 (*A*) and GRK2 (*B*) in the presence of $G_{\beta\gamma}$. Significant radioactive peaks eluted at approximately 50 and 88 min for GRK5-mediated phosphorylations corresponding, respectively, to peaks 4 and 5 (*A*) and at approximately 64 and 87 min for GRK2 phosphorylations in the presence of $G_{\beta\gamma}$ corresponding respectively to peaks 4 and 5 (*B*).

threonine, or tyrosine residues were phosphorylated by either GRK, two-dimensional phosphoamino acid analysis of peptide 1 was performed three times for each GRK. The results indicate that peptide 1 contains phosphoserine and phosphothreonine residues for both GRK2 and GRK5 phosphorylations (Fig. 3*B*). No phosphotyrosine residues were detected. Quantitation of the data using a PhosphorImager reveals that for GRK2-mediated ($\pm G_{\beta\gamma}$) phosphorylations ~90% of the total ^{32}P incorporated in peptide 1 was present on phosphoserine and ~10% on phosphothreonine residues. In contrast, for GRK5-mediated phosphorylations ~80% of the total ^{32}P incorporated was associated with phosphoserine and ~20% with phosphothreonine residues.

To identify the exact phosphorylation sites on the receptor, phosphopeptide sequencing of peptide 1 was performed in conjunction with PTH-derivative sequence determination. After each Edman degradation cycle, the amount of released radioactivity was determined by scintillation counting (see Table I). The release of radioactivity at a certain cycle indicates phosphorylation of the residue present at that same cycle of amino acid sequencing. The phosphopeptide sequencing plots for peptide 1 phosphorylated by GRK5 and GRK2 ± $G_{\beta\gamma}$ are shown in Fig. 4. For β_2AR phosphorylated by GRK5 to a stoichiometry of 1 mol P_i/mol receptor, radioactivity was released from peptide 1 at five cycles: 11, 20, 23, 28, and 34, corresponding to Thr-384, Thr-393, Ser-396, Ser-401, and Ser-407 residues, respectively (Fig. 4*A* and Table I). For β_2AR phosphorylated by GRK2 (1 mol P_i/mol β_2AR), significant radioactivity was released from peptide 1 at only four cycles: 11, 23, 28, and 34 corresponding to Thr-384, Ser-396, Ser-401, and Ser-407 residues, respectively (Fig. 4*B* and Table I). Interestingly, the addition of $G_{\beta\gamma}$ to GRK2-mediated phosphorylation reactions increased the stoichiometry of phosphorylation of peptide 1 (from ~1.0 to 5.0 mol P_i/mol receptor) but did not alter the nature or number of phosphorylated residues (Fig. 4*C* and Table I).

Technical limitations of phosphopeptide sequencing, arising due to the large size of peptide 1, make it difficult to analyze the most C-terminal of the potential phosphorylation sites. As the number of Edman degradation cycles increases, the background counts increase while the amino acid yield and ^{32}P signal decrease (Table I) due to incomplete and nonspecific peptide bond cleavage. These limitations obscure the release of radioactivity at cycles 35 and 38, which correspond to Thr-408 and Ser-411, respectively. Additionally, the "carry over" of ^{32}P signal observed after each cycle containing a phosphorylated residue in combination with decreasing ^{32}P signal may cause the signal at cycle 34 (Ser-407) to mask any radioactivity potentially released at cycle 35 (Thr-408).

To attempt to elucidate the sites of phosphorylation at the C terminus of peptide 1, this peptide was subjected to tryptic digest. Tryptic digest of peptide 1, derived from GRK5 (Fig. 5*A*) or GRK2 + $G_{\beta\gamma}$-mediated (Fig. 5*B*) phosphorylations of β_2AR, followed by HPLC purification yields two ^{32}P-labeled peaks

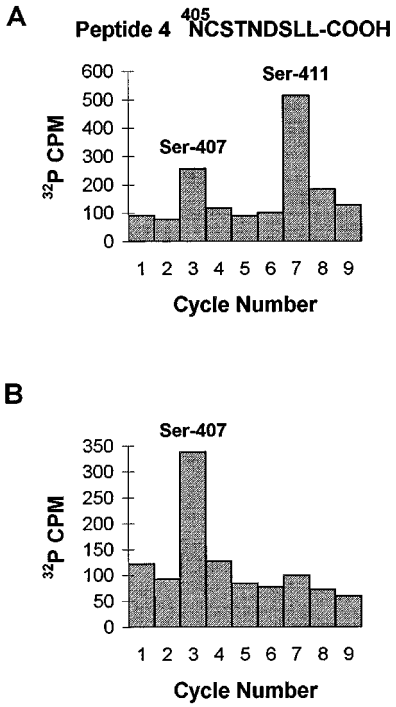

FIG. 6. **Release of ^{32}P radioactivity during Edman degradation of peptide 4.** A nine-amino acid phosphopeptide (peptide 4) eluting with peak 4 was isolated and subjected to phosphopeptide sequence analysis as described in the legend to Fig. 4. The amino acid sequence of peptide 4 obtained for both GRK-mediated phosphorylations is shown at the top (*A*). Radioactivity plots are shown for peptide 4 derived from β_2AR phosphorylations mediated by GRK5 (*A*) and GRK2 + $G_{\beta\gamma}$ (*B*). The amino acid residues corresponding to significant peaks of ^{32}P release are indicated. Phosphopeptide sequence analysis of peptide 4 was repeated at least two times for each experimental condition, yielding similar results.

(Fig. 5, *A* and *B*). Amino acid sequence analysis of peptides within peak 4 derived from either GRK phosphorylation reveals the presence of a nine-amino acid phosphopeptide corresponding to residues 405–413 of the receptor (peptide 4). The sequence of peptide 4 is shown in Fig. 6*A*, and the peptide sequence data are listed in Table II. Analysis of the fractions within peak 4 from either GRK5 or GRK2 + $G_{\beta\gamma}$-mediated phosphorylations revealed the exclusive presence of peptide 4. Amino acid sequence analysis of fractions within peak 5 revealed the presence of two peptides, a 29- and a 31-amino acid peptide corresponding, respectively, to residues 374–404 (peptide 5) and 376–404 (peptide 6) of the β_2AR. Phosphopeptide sequence analysis of peptide 4 suggests that both Ser-407 and Ser-411 are significantly phosphorylated by GRK5 but Thr-408 is not (Fig. 6*A* and Table II). In contrast, phosphopeptide sequence analysis of peptide 4 derived from GRK2 + $G_{\beta\gamma}$ phosphorylated peptide 1 suggests that GRK2 + $G_{\beta\gamma}$ significantly phosphorylates Ser-407 but not Thr-408 or Ser-411 (Fig. 6*B* and Table II).

A summary of the results obtained from the phosphopeptide sequence analysis is shown in Fig. 7. In Fig. 7*A*, the phosphate distribution within peptide 1 for both GRK5 and GRK2 ± $G_{\beta\gamma}$-mediated phosphorylations is illustrated as the percentage of phosphate incorporated at each potential phosphoacceptor site for which ^{32}P release could be accurately determined. The total counts incorporated in peptide 1 at positions 384, 393, 396, and 401 was taken as 100%. Positions 407, 408, and 411 were not included in Fig. 7*A* due to the high background ^{32}P release obtained upon phosphopeptide sequence analysis of this region of peptide 1. The phosphate distribution between these potential phosphoacceptor sites is instead illustrated in Fig. 7*B* for both GRK5 and GRK2 + $G_{\beta\gamma}$-mediated phosphorylations of peptide 4. The total counts incorporated in peptide 4 at positions 407, 408, and 411 was taken as 100%. Examination of Fig. 7 reveals differences in both the nature and number of

TABLE II

Amino acid and phosphopeptide sequence analysis of peptide 4

The cycle number, amino acid residue, percentage of amino acid yield, and cpm are presented for each cycle of amino acid and phosphopeptide sequence analysis of peptide 4 derived from tryptic digest of peptide 1. The data show a representative example of sequence information obtained for GRK5 or GRK2 + $G_{\beta\gamma}$ phosphorylated peptide 4. The amino acid identified in the first cycle of sequence analysis was assigned as 100% amino acid yield. The first amino acid yield was approximately 18 pmol for GRK5 and 7 pmol for GRK2 + $G_{\beta\gamma}$. Peptide 4, derived either GRK5 or GRK2 + $G_{\beta\gamma}$ phosphorylated β_2AR, was subjected to sequence analysis on at least two separate occasions.

Cycle	Residue	GRK5		GRK2 + $G_{\beta\gamma}$	
		Yield	cpm	Yield	cpm
		%		%	
1	Asn	100	91	100	122
2			78		92
3	Ser	33	257	36	339
4	Thr	36	118	45	127
5	Asn	48	90	53	84
6	Asp	40	100	36	77
7	Ser	15	514	14	99
8	Leu	24	184	29	72
9	Leu	25	127	32	59

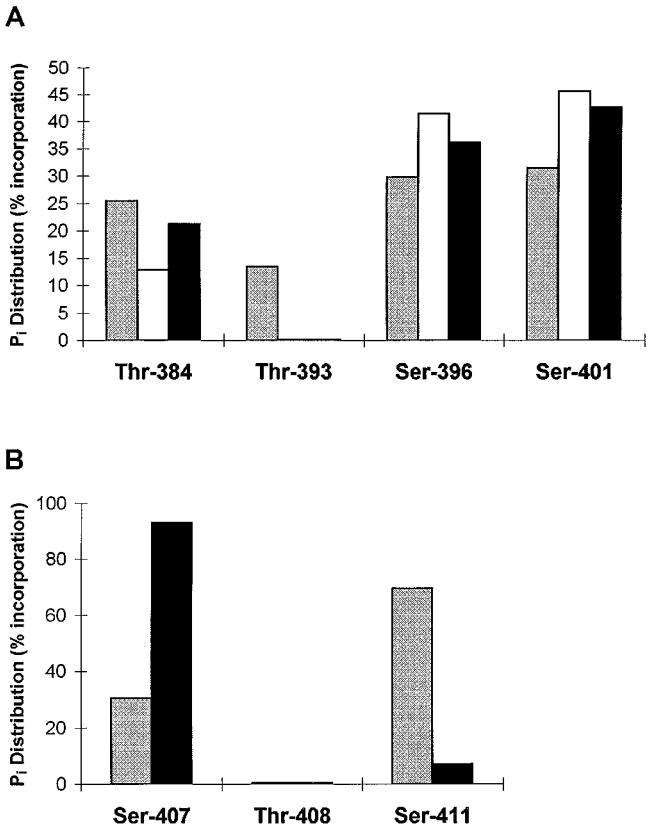

FIG. 7. **Distribution of phosphate within peptides 1 and 4.** The phosphate distribution at the potential sites of GRK2- and GRK5-mediated β_2AR phosphorylation is illustrated. Phosphate distribution values were derived from the phosphopeptide sequence data of Figs. 4 and 6 by subtracting background counts at each cycle. The total counts (*i.e.* 100% P_i incorporation) was determined by adding all counts above background for residues (*A*) 374–404 of peptide 1 or residues 405–413 of peptide 4 (*B*). The *gray*, *white*, and *black bars* respectively represent GRK5, GRK2, and GRK2 + $G_{\beta\gamma}$-mediated phosphorylations of the β_2AR.

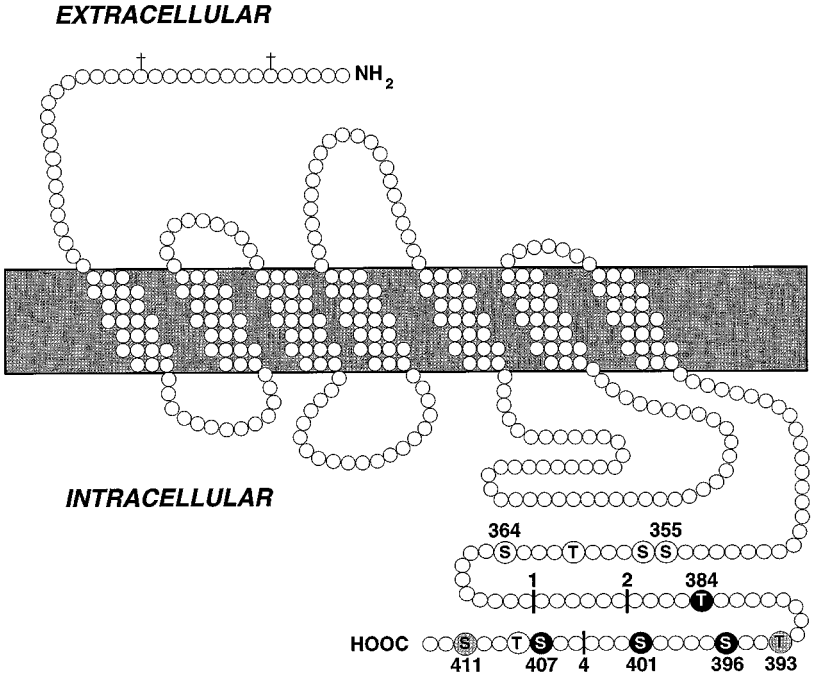

FIG. 8. **Sites of GRK2- and GRK5-mediated phosphorylation on the human β_2AR.** Schematic representation of the primary structure of the human β_2AR showing GRK-mediated sites of phosphorylation. The 11 potential sites of GRK-mediated phosphorylation on the C-tail of the β_2AR are labeled. Those residues phosphorylated by both GRK2 and GRK5 are shown in *black*, and GRK5-specific phosphorylation sites are shown in *gray*. The positions of peptide 1 (residues 374–413), peptide 2 (residues 380–413), and peptide 4 (residues 405–413) are also indicated.

the sites phosphorylated and the distribution of phosphate between the phosphoacceptor sites for these two enzymes. First, Fig. 7 (*A* and *B*) demonstrates that six of the seven potential phosphoacceptor sites in peptide 1 are phosphorylated by GRK5 (Thr-384, Thr-393, Ser-396, Ser-401, Ser-407, and Ser-411) and four of the seven are phosphorylated by GRK2 (Thr-384, Ser-396, Ser-401, and Ser-407). Interestingly, the addition of $G_{\beta\gamma}$ to GRK2-mediated phosphorylations increases the stoichiometry of phosphorylation but does not alter the nature or the number of the sites phosphorylated by this enzyme (Fig. 4, *B* and *C*). The phosphoacceptor sites of GRK5 and GRK2 overlap; however, GRK5, unlike GRK2, significantly phosphorylates both Thr-393 and Ser-411 (Fig. 7, *A* and *B*). Additionally, at equivalent phosphorylation stoichiometries (~1.0 mol P_i/mol receptor), GRK5, unlike GRK2, displays no marked preference for any particular phosphoacceptor site. The positions of the GRK-mediated phosphorylated residues within the β_2AR sequence are represented schematically in Fig. 8. Residues phosphorylated by both GRK2 and GRK5 are shown in *black*, GRK5 specific phosphorylation sites are shown in *gray* (Fig. 8).

DISCUSSION

The difficulties associated with obtaining sufficient quantities of purified G protein-coupled receptors has hampered attempts to unambiguously determine the sites of GRK-mediated phosphorylation. Previously, the only receptor for which this has been possible is rhodopsin, whose relative abundance in rod outer segment membranes has permitted identification of GRK1 phosphorylation sites both *in vitro* (10–13, 15, 16) and more recently *in vivo* (14). In this study, the problem of insufficient material was overcome by the expression and subsequent purification of human β_2AR in an Sf9 cell/baculovirus system. Purified β_2AR was reconstituted in lipid vesicles and phosphorylated *in vitro* by either GRK2 or GRK5. GRK-mediated phosphorylation sites were subsequently assigned by phosphopeptide sequencing of HPLC-purified peptides derived from proteolysis of phosphorylated β_2AR. All the sites of both GRK2 and GRK5-mediated phosphorylation were found to reside within a peptide comprising the carboxyl-terminal 40 amino acids of the β_2AR (Figs. 2 and 3). Phosphoamino acid analysis revealed that this peptide contained both phosphoserine and phosphothreonine but no phosphotyrosine residues when phosphorylated by either GRK5 (~1.0 mol P_i/mol receptor) or by GRK2 at either low (~1.0 mol P_i/mol receptor) or high (~5.0 mol P_i/mol receptor) stoichiometries of phosphorylation (Fig. 3*B*). Of the seven potential phosphorylation sites within this 40-amino acid peptide (4 serine and 3 threonine residues), GRK5 was shown to phosphorylate six sites and GRK2 was shown to phosphorylate four sites even at stoichiometries as low as 1.0 mol P_i/mol receptor (Fig. 4, *B* and *C*). These results are highly reminiscent of those obtained with rhodopsin in which three carboxyl-terminal residues (Ser-334,, Ser-338, and Ser-343) were shown to be phosphorylated by GRK1 (12). Additionally, our findings are consistent with those of Hausdorff *et al.* (8), who demonstrated that substitution mutants of the β_2AR in which all of the potential GRK2 phosphorylation sites in the C-tail were replaced with alanine residues fail to phosphorylate or undergo desensitization.

Phosphorylation studies using synthetic peptides have demonstrated that both GRK1 and GRK2 are acidotropic kinases, preferring acidic amino acids in proximity to phosphorylatable residues (21, 22). In this respect it is interesting to note that both the β_2AR and rhodopsin have pairs of acidic residues located at the amino-terminal side of the most amino-terminal phosphorylated residue (Fig. 9). Similarly, the putative GRK2 phosphorylation sites on the carboxyl-terminal domain of the *N*-formyl peptide receptor, deduced from studies in which a GST fusion protein encompassing the C-tail of this receptor was utilized, also possess a pair of acidic amino acids adjacent to its most amino-terminal phosphorylation site (23). The putative phosphorylation sites on the third loop of the α_2C10 adrenergic receptor are also similar in this respect (24) (Fig. 9). In the case of the α_2C10 receptor, the putative phosphorylation sites were assigned by point mutation of potential phosphorylatable residues and subsequent examination of the phosphorylation state of these mutant receptors upon exposure to agonist.

Interestingly, the use of peptides derived from the carboxyl terminus of rhodopsin (12), a *N*-formyl peptide receptor fusion protein (23) or mutant C5a receptors in which serine residues

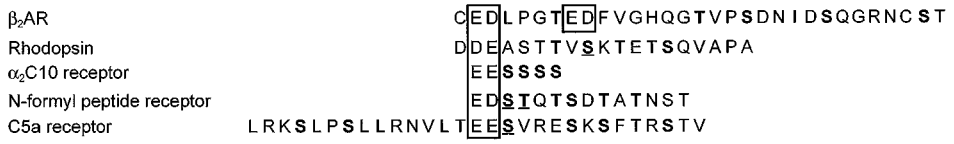

FIG. 9. **Alignment of known and postulated G protein-coupled receptor GRK phosphorylation sites.** The sequences surrounding the GRK-mediated phosphorylation sites of the human β_2AR and rhodopsin and the putative GRK-mediated phosphorylation sites of the α_2C10, *N*-formyl peptide, and C5a receptor are shown. The sites of phosphorylation are indicated in *bold* type, the proposed initial sites of phosphorylation are *underlined*, and pairs of acidic residues are *boxed*.

in the carboxyl terminus are substituted by alanines (25), indicates that GRK-mediated phosphorylation may proceed in a sequential manner. Thus mutation of a single residue, for example Ser-338 of rhodopsin, results in inhibition of phosphorylation of both Thr-336 and Ser-343. In all these examples a pair of acidic amino acids is located amino-terminal to the postulated initial site of phosphorylation (Fig. 9). Phosphorylation of the four serines in the third loop of the α_2C10 receptor, however, appears to occur independently because each of the four mutant receptors (EESSSA, EESSAS, EESASS, and EEASSS) are phosphorylated to approximately 75% of the wild type receptor (EESSSS) level (24). It remains to be determined if the nonsequential nature of GRK-mediated α_2C10 phosphorylation reflects differences between different GRKs, the close proximity of the α_2C10 serine residues, or their third loop location. In this respect it will be of interest to determine the sequential or nonsequential nature of GRK2- and GRK5-mediated β_2AR phosphorylation.

Both the β_2AR and rhodopsin systems have been observed to be phosphorylated *in vitro* to stoichiometries of greater than 7 mol/mol (3, 26, 27); however, agonist-induced phosphorylation of the β_2AR has been observed to be approximately 1–2 mol/mol in intact cells (28). High stoichiometries of *in vitro* phosphorylation are likely due to nonstringent conditions, including high concentrations of kinase and receptor and low concentrations of proteins that limit the extent of phosphorylation, such as β-arrestin and phosphatases. An important question to ask is whether the sites identified in our *in vitro* study represent the sites of *in vivo* phosphorylation. A recent study on mouse rhodopsin found the primary sites of *in vivo* phosphorylation by GRK1 to be Ser-338 and Ser-334 (14). These findings agree with several *in vitro* studies that found Ser-338 to be a major site of phosphorylation (11, 13–16, 29). Ser-338 and Ser-334 were also found to be the two major sites of *in vitro* phosphorylation in bovine rhodopsin under several experimental conditions (11, 13). Considering these findings, we suggest that the major *in vitro* sites of GRK-mediated β_2AR phosphorylation identified in this study potentially represent important sites of *in vivo* phosphorylation.

Increasing the stoichiometry of GRK2-mediated β_2AR phosphorylation from ~1.0 to 5.0 mol P_i/mol receptor had no effect on the nature or number of the sites phosphorylated by this kinase (Fig. 7*A*). These results suggest that the interaction with $G_{\beta\gamma}$ serves to facilitate the GRK2-receptor interaction without significantly altering the mode of action of the kinase. Similar results have recently been reported for GRK2 phosphorylation of rhodopsin (11). Interestingly, although GRK2 and GRK5 phosphorylate an overlapping series of residues, significant differences exist: the GRK5-specific phosphorylation of Thr-393 and Ser-411 and the differential distribution of phosphate between the phosphoacceptors. This specificity potentially reflects subtle differences in the nature of the GRK2 and GRK5 binding sites on the β_2AR.

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