Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8715–8719, October 1991
Biochemistry

The receptor kinase family: Primary structure of rhodopsin kinase reveals similarities to the β-adrenergic receptor kinase

(guanine nucleotide-binding protein-coupled receptors/desensitization/serine/threonine protein kinase/polymerase chain reaction)

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Contributed by Robert J. Lefkowitz, July 1, 1991

ABSTRACT **Light-dependent deactivation of rhodopsin as well as homologous desensitization of β-adrenergic receptors involves receptor phosphorylation that is mediated by the highly specific protein kinases rhodopsin kinase (RK) and β-adrenergic receptor kinase (βARK), respectively. We report here the cloning of a complementary DNA for RK. The deduced amino acid sequence shows a high degree of homology to βARK. In a phylogenetic tree constructed by comparing the catalytic domains of several protein kinases, RK and βARK are located on a branch close to, but separate from, the cyclic nucleotide-dependent protein kinase and protein kinase C subfamilies. From the common structural features we conclude that both RK and βARK are members of a newly delineated gene family of guanine nucleotide-binding protein (G protein)-coupled receptor kinases that may function in diverse pathways to regulate the function of such receptors.**

Recent investigations have revealed previously unappreciated and pervasive similarities between the components of a wide variety of transmembrane signaling systems. Many such systems consist of specific receptors, functional coupling proteins called guanine nucleotide-binding regulatory proteins (G proteins), and effector enzymes or ion channels. These elements form a chain that links an extracellular stimulus to the perturbation of intracellular metabolic events. Such systems mediate signals as diverse as those carried by neurotransmitters, hormones, and sensory signals, such as photons of light or odorants (1). Receptors in such systems show a highly conserved structure featuring seven membrane-spanning domains linked by hydrophilic extra- and intracellular loops. Not only are the structural features of such receptors highly conserved, it appears that, even in disparate signaling systems, similar mechanisms may have evolved to regulate the functioning of such receptor molecules. Evidence from several systems suggests that phosphorylation of the receptor protein by highly specific receptor kinases may represent an important and unifying mechanism serving to dampen receptor function in the presence of persistent or excessive stimulation (2, 3). Moreover, biochemical data have suggested that an important property of such receptor kinases is their ability to recognize and phosphorylate only the stimulus-modified or activated forms of these receptors (4–7).

Previously, Lefkowitz and coworkers (8) cloned and sequenced cDNAs for the first member of this proposed receptor kinase family, the β-adrenergic receptor kinase (βARK) (8). This kinase phosphorylates and regulates the function of β-adrenergic (and possibly other G-protein-coupled) receptors by catalyzing phosphorylation of the receptor on a serine/threonine-rich cluster found at its carboxyl terminus (9). Functionally, a very analogous enzyme is rhodopsin kinase (RK). Discovered almost two decades ago (4–6), this enzyme is known to phosphorylate rhodopsin, thereby initiating its deactivation. The biochemical and functional properties of RK are such as to suggest that it might be closely related to βARK (10). Accordingly we set out to clone cDNAs for RK so as to elucidate its primary structure and thereby clarify structural, evolutionary, and functional relationships with βARK, as well as to understand the nature and diversity of the proposed family of receptor kinase molecules.††

MATERIALS AND METHODS

Protein Purification and Peptide Sequencing. RK was purified from bovine retina as described (11). The enzyme was extracted from photobleached rod outer segment membranes with 60 mM KCl. The kinase was further purified with a DEAE-cellulose step, and pooled activity was then applied to a hydroxyapatite column. RK was eluted in a buffer of 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane, 1 mM $Mg(OAc)_2$, and 95 mM KCl. Fractions (1 ml) were concentrated to 300 μl under reduced vacuum (Speed-Vac, Savant) in individual Eppendorf tubes. To each of the 10 tubes was added 700 μl of CNBr in formic acid (99%) to give a final CNBr concentration of 10 mM; the digestion was allowed to proceed for 24 hr in the dark at room temperature. Each sample was dried under reduced pressure and rehydrated with 500 μl of 90% water/10% acetonitrile/0.1% trifluoroacetic acid. The 10 samples were individually applied to an Aquapore C_4 reverse-phase column (Applied Biosystems; 2.1 × 30 mm) at a flow rate of 200 μl/min; the column was washed with 1.5 ml of 0.1% trifluoroacetic acid in water between each sample application. Once the last sample was loaded, a gradient of 0–70% acetonitrile was developed. Fractions were collected at 1-min intervals. A control digest, consisting of 1 ml of buffer handled exactly as the kinase-containing samples and subjected to CNBr treatment, was chromatographed under identical conditions. Peaks unique to the RK-containing digest were submitted for gas-phase peptide sequencing (R. Randall, Howard Hughes Medical Institute Biopolymer Lab, Durham, NC).

cDNA Library Screening and Polymerase Chain Reaction (PCR). Screening of a bovine Okayama–Berg cDNA library (12) with the peptide-derived oligodeoxynucleotide 5′-

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Abbreviations: RK, rhodopsin kinase; βARK, β-adrenergic receptor kinase; RACE, rapid amplification of cDNA ends.

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††The sequence reported in this paper has been deposited in the GenBank data base (accession no. M73836).

CTGGGCAGTGTAGAAGATGGCICIGGGCTCCTG-GAAGCCAGGGTTGTCCTCGTCCACGTTGTAGAT-GTGGTAICIGATGTCACCACCGTTCAT-3′ (where I is deoxyinosine) yielded the partial clone N-9. A random-primed bovine cDNA library in λZAPII (Stratagene) was hybridized with a 520-base-pair (bp) *Pst* I fragment derived from the 5′ region of clone N-9 and clones S-1 and S-2 were isolated. To obtain the amino-terminal sequences of RK the RACE procedure (rapid amplification of cDNA ends; ref. 13) was used. Briefly, a first-strand cDNA was synthesized from bovine retina poly(A)-selected RNA by using Superscript reverse transcriptase (BRL) and the RK-specific primer 5′-TCTGGCAGGCGAACACCT-3′ at 45°C. The cDNA was treated with RNase H, excess primer was removed by two centrifugations through Centricon 100 filters (Amicon), and the cDNA was tailed with poly(dA) by terminal deoxynucleotidyltransferase (BRL). Subsequent PCR amplification was performed with Replinase (NEN/DuPont), 1 μM RK-specific primer 5′-CCAGTCCTCGCCCATGGGCTGG, 0.5 μM $(dT)_{17}$-adapter primer 5′-GACTCGAGTCGACATCGA-$(T)_{17}$-3′, and 1 μM adapter primer 5′-GACTCGAGTCGA-CATCG-3′. Forty cycles of 40 sec at 95°C, 60 sec at 55°C, and 90 sec at 72°C were performed. PCR products were size-selected in an agarose gel and cloned in the plasmid vector pCR1000 (Invitrogen, San Diego). All cDNA and PCR clones were sequenced in both strands by the dideoxynucleotide chain-termination method (14) and with 7-deaza-2′-deoxyguanosine 5′-triphosphate or 2′-deoxyinosine 5′-triphosphate when necessary.

***In Vitro* Translation of the RK Construct.** For *in vitro* translation experiments, the construct pRK (Fig. 1) was linearized with *Bam*HI or *Hin*dIII and was then used for *in vitro* transcription with either T7 (*Bam*HI) or T3 (*Hin*dIII) RNA polymerase (Stratagene). Capped RNA was translated *in vitro* with a rabbit reticulocyte lysate (BRL) in the presence of 50 μCi (1.85 MBq) of [^{35}S]methionine before samples were run in an SDS/10% polyacrylamide gel. After fixation in 40% methanol/10% acetic acid for 30 min, the gel was dried and subjected to autoradiography for 36 hr at room temperature.

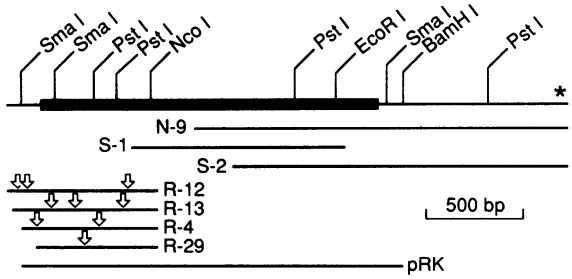

FIG. 1. Restriction map of the RK clones. The open reading frame of 1683 bp is indicated by a heavy line. The polyadenylylation signal at nucleotide 2879 is marked by a star. In clones N-9 and S-2 a poly(A) tail was found 15 bp downstream from the polyadenylylation signal. Clone N-9 was obtained by screening a bovine Okayama–Berg cDNA library (12) with a peptide-derived oligonucleotide. Clones S-1 and S-2 were isolated by hybridizing a random-primed bovine cDNA library in λZAPII (Stratagene) with a 520-bp *Pst* I fragment derived from the 5′ region of clone N-9. Clones including the amino-terminal sequence of RK were obtained by the RACE procedure (13). The clones R-12, R-13, R-4, and R-29 were derived from four independent PCR reactions. Arrows indicate point mutations in the nucleotide sequence of the respective PCR product. For further experiments, the *Hin*dIII–*Nco* I fragment (bp 93–746) from clone R-4, the *Nco* I–*Eco*RI fragment (bp 747–1705) from clone S-1, and the *Eco*RI–*Bam*HI fragment (bp 1706–2038) from clone N-9 were ligated into pT7T3 (Pharmacia), resulting in the construct pRK. Although the *Hin*dIII–*Nco* I fragment of clone R-4 contains two point mutations, neither of these nucleotide exchanges leads to an amino acid change in the translation product.

Expression of RK in COS-7 Cells. The *Hin*dIII–*Bam*HI fragment from the construct pRK was inserted into the *Hin*dIII and *Bam*HI sites of the expression vector pCMV5 (15). This fragment contains the complete coding block as well as 112 bp of 5′ untranslated sequence and 151 bp of 3′ untranslated sequence. COS-7 cells were grown to 60% confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 50 mg of gentamicin per ml and then were transfected with the vector pCMV5 or the construct pCMV-RK by the DEAE-dextran method (16). Forty-eight hours after transfection, cells from a 150-cm^2 dish were harvested in 0.4 ml of ice-cold buffer [10 mM Tris·HCl, pH 7.5/10 mM $Mg(OAc)_2$/5 mM dithiothreitol containing each of the following protease inhibitors at 30 μg/ml: aprotinin, benzamidine, leupeptin, and pepstatin] and were lysed with a Polytron homogenizer (Brinkmann) and centrifuged at 300,000 × *g* for 15 min. The supernatant was then analyzed for RK activity, using urea-treated rod outer segments (17, 18). The RK assays contained 75 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, 1 mM $Mg(OAc)_2$, 100 μM [γ-^{32}P]ATP (2000 dpm/pmol), 30 μM rhodopsin, and either 3 μl of pCMV5 supernatant or 3 μl of pCMV-RK supernatant. The reaction mixtures were incubated in room light or in the dark for 20 min at 25°C and terminated by addition of SDS sample buffer followed by electrophoresis in an SDS/10% polyacrylamide gel. The gel was dried and subjected to autoradiography for 15 min at room temperature.

Northern Blot Analysis. Following denaturation by glyoxylation, 5-μg samples of poly(A)$^+$ RNA from bovine tissues were fractionated by electrophoresis in 1.2% agarose gels and transferred to Biotrans membranes (ICN) as described (19). The filters were hybridized with a nick-translated probe, washed in 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% SDS, at 60°C, and subjected to autoradiography.

RESULTS AND DISCUSSION

RK was purified 1000-fold from bovine rod outer segments (11) and 60 μg of purified RK was digested with CNBr in 99% formic acid. The peptides resulting from this cleavage were resolved by reverse-phase HPLC. One of three peaks from the HPLC yielded a distinct sequence of 30 amino acids when subjected to gas-phase sequencing (NGGDIRYHIYNVD-EDNPGFQEPRAIFYTAQ). An oligo(dT)-primed cDNA library from bovine retina in the Okayama–Berg vector (12) was screened with an oligonucleotide probe (93 bp) derived from this amino acid sequence and cDNA clone N-9 was isolated (Fig. 1). Screening of a randomly primed cDNA library from bovine retina in λZAPII (Stratagene) with the 5′ portion of clone N-9 as a probe yielded two additional clones, S-1 and S-2 (Fig. 1). We were unable to obtain clones coding for the amino terminus of RK by screening of these and several additional cDNA libraries (as well as genomic libraries). Thus, we used the RACE strategy (13) to obtain sequences from the 5′ end of the RK mRNA. Several clones were obtained by amplifying specifically primed cDNA from bovine retina poly(A)$^+$ RNA.

Sequence analysis of four of the RACE clones revealed an extremely G+C-rich 5′ region with a G+C content of 71.2% in the 680 most 5′ bases (73.2% in 205 bp of 5′ untranslated region). We assume that this base composition accounted for the difficulties in cloning the 5′ portion of the RK mRNA. Another feature of the nucleotide sequence of the clones we obtained by the RACE procedure is the high number of point mutations due to the relatively high infidelity of the DNA polymerase under the conditions used (arrows in Fig. 1).

The nucleotide sequence from the clones analyzed gave a total of 2898 bp (GenBank data base, accession no. M73836) and contains an open reading frame of 1683 bp (561 amino acids; Fig. 2) starting with a Kozak consensus sequence (22).

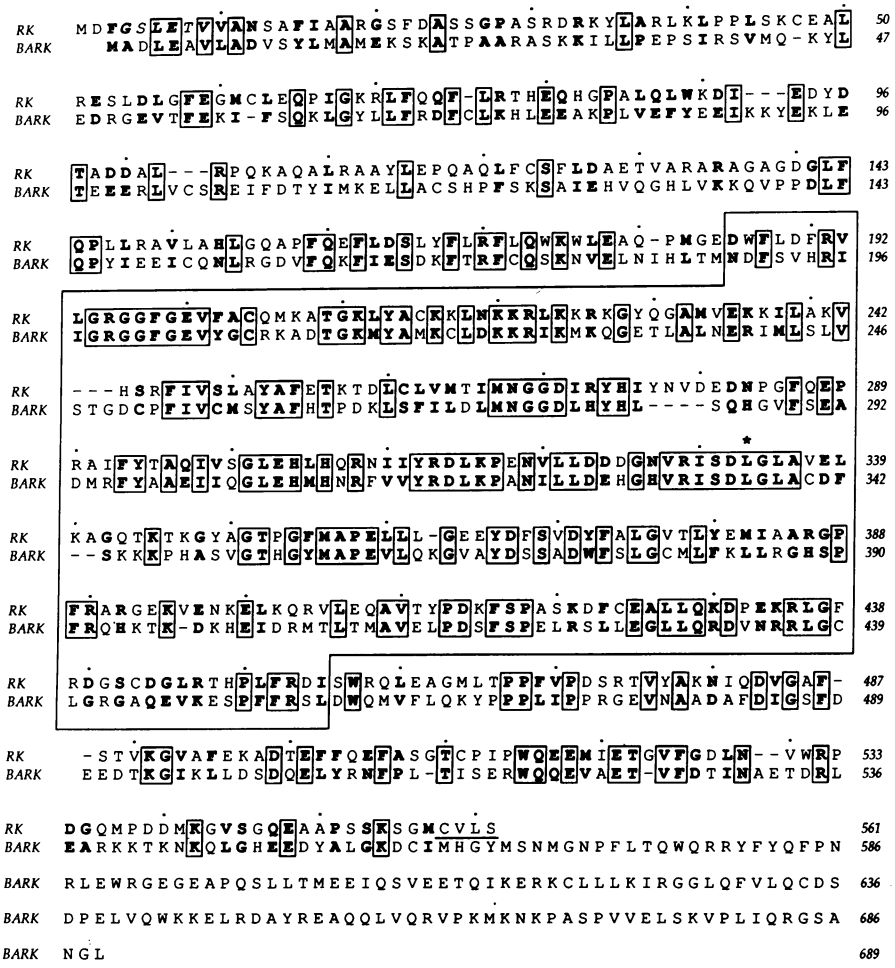

FIG. 2. Alignment of the deduced RK amino acid sequence with the βARK (BARK) sequence. Identical amino acids are shown by bold letters in boxes; bold letters indicate substitutions by amino acids with close evolutionary relationship as defined by Dayhoff and normalized by Gribskov and Burgess (20). The large box encompasses the catalytic domain. The star denotes the substitution of leucine for phenylalanine in the Asp-Phe-Gly consensus (21). The myristoylation consensus is shown in italics and the CAAX box (where C is cysteine, A is an aliphatic amino acid, and X is the carboxyl-terminal amino acid) is underlined in the RK sequence.

This open reading frame codes for a protein of 62.9 kDa, which is in agreement with the molecular weight of purified RK as reported by Kelleher and Johnson (23). An exact amino acid match was found with the peptide sequence determined by gas-phase sequencing in the middle of the open reading frame (Fig. 2, residues 268–297). Initiation of translation at the first ATG in the sequence was shown by *in vitro* translation. The coding region of the RK cDNA was cloned into pT7T3 (pRK, Fig. 1). Capped RNA was synthesized and *in vitro* translated in a rabbit reticulocyte lysate (BRL), giving rise to a major protein of 63 kDa (data not shown).

The deduced amino acid sequence of RK was compared with the sequences of several serine/threonine protein kinases. Similarities to other protein kinases were mostly found within the putative catalytic domain of RK, whereas the degree of homology was lower when the amino and carboxyl termini of RK were compared with those of other protein kinases. RK is most closely related to βARK, showing 33.6% amino acid identity overall and 44.2% identity in the catalytic domains (boxed area in Fig. 2). The degree of amino acid identity of RK with the amino terminus of βARK is 22.7% in an overlap of 180 amino acids and 27.0% with the carboxyl terminus in an overlap of 102 amino acids. Comparisons of the amino and carboxyl termini of RK with members of the cyclic nucleotide-dependent protein kinase or protein kinase C subfamilies revealed no significant similarities.

To further explore the sequence relationship of RK to other protein kinases, a phylogenetic tree was constructed by comparing the catalytic domain with those of several other protein kinases (Fig. 3). RK and the βARK isozymes βARK-1 and βARK-2 form a separate branch that is most closely related to the cyclic nucleotide-dependent protein kinase and protein kinase C subfamilies. These results suggest that RK and βARK are members of a distinct gene family of G-protein-coupled receptor kinases.

Both RK and βARK are acidotrophic serine/threonine protein kinases phosphorylating serine/threonine clusters on the carboxyl-terminal tail of rhodopsin and the β-adrenergic receptor, respectively (29, 30). The primary amino acid sequence of RK places the catalytic domain of 270 amino acids approximately in the center of the sequence flanked by a 185-residue amino-terminal domain and a 106-residue carboxyl-terminal domain (Fig. 2). In terms of domain structure, βARK differs from RK only by having a longer carboxyl-terminal domain (125 amino acids). The similarities between the amino-terminal domains of RK and βARK in length and amino acid sequence suggest that this region of the kinases has a common functional role, perhaps bestowing upon these enzymes their ability to recognize and phosphorylate only the activated forms of their respective receptors.

The catalytic domain of RK contains the essential landmarks of a serine/threonine protein kinase: the motif Gly-Xaa-Gly-Xaa-Xaa-Gly involved in ATP binding at position 193 (31), the conserved catalytic residues Asp-332 and Lys-216 implicated in phosphoryl transfer (32–34), and the other conserved features of protein kinase catalytic domains described by Hanks *et al.* (21). A significant difference noted earlier (8), however, between βARK and the other protein kinases was the substitution in βARK of a leucine for phenylalanine in the otherwise highly conserved Asp-Phe-Gly consensus sequence implicated in ATP binding and catalysis. This same replacement is also found in RK (Fig. 2) and may possibly reflect a point of common evolutionary divergence of these receptor kinases. This mutational change may provide a marker, in addition to amino-terminal resem-

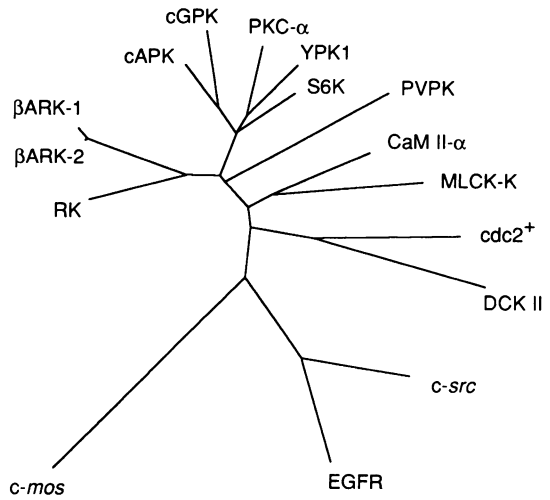

FIG. 3. Phylogeny of the receptor kinase catalytic domains. The catalytic domain of RK (amino acids 185–455) was compared with the catalytic domains of several other protein kinases (21) by construction of a dendrogram. The methodology used has been described (24). The enzymes used for the comparison were βARK-1 and βARK-2 (25); cAMP-dependent protein kinase (cAPK); cGMP-dependent protein kinase (cGPK); protein kinase C, α form (PKC-α); yeast (*Saccharomyces cerevisiae*) protein kinase (YPK1, ref. 26); rat S6 kinase (S6K, ref. 27); dicot bean (*Phaseolus vulgaris* L.) protein kinase (PVPK, ref. 28); Ca^{2+}/calmodulin-dependent protein kinase type II, α subunit (CaM II-α); myosin light-chain kinase (MLCK-K); "cell-division-cycle" gene product in yeast ($cdc2^+$); *Drosophila* casein kinase II, α subunit (DCK II); c-*src* protooncogene (c-src); epidermal growth factor receptor (EGFR); and c-*mos* protooncogene (c-mos).

blances, for protein kinases of the G-protein-coupled receptor kinase class.

The primary structure of RK suggests that several interesting posttranslational modifications may take place on the enzyme. (*i*) Two amino acids beyond the proposed initiator methionine (Fig. 2) lies the consensus sequence for myristoylation (35). Our inability to obtain protein sequence starting from the amino terminus of purified bovine retinal RK suggests that some type of modification resides at the amino terminus of the protein. (*ii*) Eight potential phosphorylation sites for acidotrophic protein kinases preferring serines and threonines to the amino-terminal side of the acidic group [e.g., RK (29, 30) and casein kinase II (36)] as well as four potential protein kinase C-sensitive serines and threonines (37) are present in RK. Some of the acidotrophic sites are most likely sites of autophosphorylation known to occur on RK (23, 38). (*iii*) The carboxyl terminus ends with a CAAX box (Cys-Val-Leu-Ser) where C is a cysteine, A is an aliphatic amino acid, and X is the terminal amino acid of the protein. This sequence has been shown to direct isoprenylation of the cysteine, which appears to be the first of three processing events that occur in proteins containing this sequence. Following isoprenylation, proteolytic cleavage of the carboxyl-terminal three amino acids (-AAX) and carboxymethylation of the isoprenylated cysteine has been shown to occur in several proteins with the CAAX motif (for review, see ref. 39 and references therein). Interestingly, the primary amino acid sequences as deduced from the cDNA of several retinal proteins involved in the visual signal transduction pathway have been shown to carry the CAAX box, including the γ subunit of transducin (40, 41) and the α subunit of the photoreceptor cGMP phosphodiesterase (42).

An expression plasmid for RK was constructed by inserting the *Hin*dIII–*Bam*HI fragment of pRK into the vector pCMV5 (15). The resulting construct, pCMV-RK, was transfected into COS-7 cells by the DEAE-dextran procedure (16).

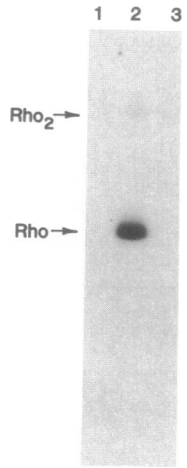

FIG. 4. Expression of RK in COS-7 cells. COS-7 cells were transfected with the expression construct pCMV-RK or the vector pCMV5 as described. Cytosol was analyzed for RK activity by using 30 μM rhodopsin with either 3 μl of pCMV5 supernatant (lane 1) or 3 μl of pCMV-RK supernatant (lanes 2 and 3). The reaction mixtures were incubated in room light (lanes 1 and 2) or in the dark (lane 3) for 20 min at 25°C, and the reactions were terminated by addition of SDS sample buffer followed by electrophoresis in an SDS/10% homogeneous polyacrylamide gel. The gel was dried and subjected to autoradiography for 15 min at room temperature. Rho, rhodopsin; Rho_2, rhodopsin dimer.

After 48 hr, cytosol preparations from cells transfected with either pCMV5 or pCMV-RK were tested for RK activity. Cells transfected with pCMV5 showed no RK activity (Fig. 4, lane 1). Cytosol preparations from cells transfected with pCMV-RK phosphorylated rhodopsin in a light-dependent manner (lanes 2 and 3).

To examine the size and localization of RK mRNA we hybridized Northern blots of poly(A)$^+$ RNA from various bovine tissues with a radiolabeled 860-bp *Pst* I fragment from clone S-1. In RNA from retina only, an mRNA species of 3.1 kilobases (kb) and to a lesser extent a species of 5.8 kb hybridized to the probe used (Fig. 5). After longer exposures, the same hybridization pattern was observed in RNA from the pineal gland. The two major mRNA species detected in the retina and pineal gland may be due to alternative processing of the same transcript, but the exact nature of these mRNAs remains to be elucidated. The minor band of 5.0 kb appears to result from cross-hybridization with a different mRNA species. After longer exposures it was also detected in RNA preparations from lung, spleen, pineal, and heart (data not shown). The detection of RK mRNA in the pineal

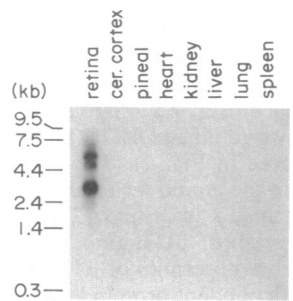

FIG. 5. Northern blot analysis of RK mRNA in bovine tissues. After hybridization with a nick-translated 860-bp *Pst* I fragment containing the 5′ portion of clone S-1, the blot was washed in 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% SDS at 60°C and subjected to autoradiography for 4 hr at room temperature. The quality of the RNA preparations was verified by rehybridization of this blot with a βARK probe (data not shown). cer., Cerebral.

gland is consistent with other biochemical similarities between the mammalian pineal gland and the retina (43, 44). The highly specific expression of the RK gene in restricted tissues underscores the specific function of this kinase in the phosphorylation of photoactivated rhodopsin. It remains to be determined whether RK is also expressed in the cone cells and whether it phosphorylates other (color) opsins *in vivo*.

Stimulus-dependent receptor phosphorylation occurring in association with attenuation of receptor signaling has been documented for diverse G-protein-coupled receptors. These include, in addition to the β_2-adrenergic receptor and rhodopsin, the M_2 muscarinic cholinergic receptor, the cAMP receptor of *Dictyostelium discoideum*, and the pheromone α-mating-factor receptor of *Saccharomyces cerevisiae* (45–48). Moreover, numerous members of the family of G-protein-coupled receptors contain within their deduced sequences clusters of serine and threonine residues disposed toward the carboxyl terminus or in the third cytoplasmic loop, which may serve as substrates for βARK- and RK-like enzymes. In the case of the α_2-adrenergic receptors, evidence suggests that such an enzyme mediates homologous desensitization (49).

The functional analogies and structural homologies between RK and βARK are mirrored by similarities between their cofactors arrestin and β-arrestin. These cytosolic proteins, which bind to and uncouple the phosphorylated forms of rhodopsin or β-adrenergic receptor, respectively, from their G protein, are 60% conserved in sequence (50). These relationships further underscore similarities in function and regulation of very disparate signaling systems.

Despite this evidence suggesting the generality of receptor kinases in modulating the function of G-protein-coupled receptors, βARK and RK are the only members of this enzyme family for which structural information is presently available. It is hoped that the availability of their sequences will facilitate the identification and cloning of the other putative members of this family, thereby expanding our understanding of the basic mechanism by which receptor function is regulated.

We thank A. M. Quinn for analyzing the sequence of the RK catalytic domain; S. Numa, T. Tanabe, J. Nathans, M. L. Applebury, and D. Oprian for providing cDNA libraries; J. Arriza for helpful discussions; J. Higgins for help with DNA sequencing; G. Irons for technical assistance; and R. Randall for peptide sequencing and oligonucleotide synthesis. This work was supported in part by a fellowship of the Deutsche Forschungsgemeinschaft (W.L.), National Institutes of Health Grant HL16037 (R.J.L.), and grants from the National Eye Institute (EY0806) and the Medical Research Foundation of Oregon (K.P.).

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