Identification of a single phosphorylation site within octopus rhodopsin
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[Headnote]
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ABSTRACT

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Light-dependent phosphorylation of rhodopsin (Rho) is a first step in the desensitization of the signaling state of the receptor during vertebrate and invertebrate visual transduction. We found that only 358Ser of the photoactivated octopus Rho (oRho*) was phosphorylated by octopus rhodopsin kinase (oRK). Tryptic truncation of the C-terminal PPQGY repeats of oRho that follow the phosphorylation region did not influence spectral or G-protein activation properties of oRho but abolished phosphorylation. Despite significant structural differences between oRK and mammalian RK, these results provide further evidence of the importance of singly phosphorylated species of Rho* in the generation of arrestin binding sites.

INTRODUCTION

Signaling via G-protein-coupled receptors is a widely used mechanism throughout biological systems. Studies on vertebrate phototransduction have been particularly useful in understanding principles of the amplification and desensitization steps. In photoreceptor cells, photoisomerization of the visual chromophore, Il-cis-retinal, activates rhodopsin (Rho*), initiating activation of hundreds of G-protein molecules during the course of visual signaling. The duration and amplitude of the light stimulation is inhibited by phosphorylation of Rho* (P-Rho*) by rhodopsin kinase (RK) and arrestin binding to P-Rho*. Arrestin contributes to desensitization by preventing the cytoplasmic loops of Rho* from interaction with a G-protein. Reduction of the photolyzed chromophore, all-trans-retinal, facilitates dissociation of arrestin. Subsequently, phospho-opsin is regenerated with 11-cis-retinal and dephosphorylated by a protein phosphatase (1). Similar to vertebrate systems, phosphorylation of invertebrate Rho* is also involved in the quenching and restoration of visual transduction (2). Much less is known, however, about the specific residues that are phosphorylated during desensitization of invertebrate Rho* or the enzymatic properties of invertebrate RK.

Several reconstitution studies in vertebrates have shown that Rho* is phosphorylated on Ser and Thr residues to a high stoichiometry. Analyses of phosphorylation sites have led to the identification of preferred sites at 334Ser, 338Ser, 336Thr and 343Ser (2-6). Phosphorylation at these multiple sites has been interpreted assuming that RK forms a stable complex with cytoplasmic loops of Rho*. In the complex, the RK active site loosely associates with the conformationally flexible C-terminus of Rho* and phosphorylates it at multiple sites (7). This model has been confirmed in several mutagenesis studies where mutation of a Ser or Thr residue to Ala led to phosphorylation of an alternative site (8,9). Under in vivo conditions, mostly singly phosphorylated species of Rho* were found at 334Ser, 338Ser and
343Ser, suggesting that one phosphate per Rho is sufficient for triggering binding of arrestin and converting Rho* into a quiescent state (10).

Octopus RK (oRK) has recently been identified by molecular cloning (11) and shown to belong to the family of G-protein-coupled receptor kinases (GRK). Because of a pleckstrin homology (PH) domain in the C-terminal region that interacts with 3γ subunits of G-proteins, oRK is more closely related to BETA-adrenergic receptor kinases (GRK2 and GRK3) than to mammalian RK (11). In fact, the [γ subunits enhance the rate of oRho* phosphorylation (11), as found for the [-adrenergic receptor system (12). In this study, we report sites within oRho* and bovine Rho* (bRho*) phosphorylated by oRK. Furthermore, we demonstrate a potential role for the C-terminal PPQGY repeat of oRho that is distinct from vertebrate Rho in oRho* phosphorylation.

RESULTS

Octopus microvillar membranes prepared in the dark contain most of the phototransduction proteins including oRho but lack soluble oRK (11). The membranes were reconstituted with purified oRK, and oRho phosphorylation was triggered by light and the addition of 1 mM [32P]-γ-ATP and carried out for 30 min. Next, microvillar membranes were digested with trypsin. During proteolysis, oRho (-50 kDa, multiple bands of unknown origin) was initially degraded into a 40 kDa form lacking the C-terminal proline-rich tail (17) that was then split into 24 kDa and 16 kDa fragments (Fig. 1A). Western blot analysis using a monoclonal antibody against the N-terminal region of oRho recognized native, 40 kDa and 24 kDa bands (Fig. 1B). To identify these three major fragments, tryptic digests were electroblotted to Immobilon and the corresponding band was excised. Edman sequence analysis revealed that the N-terminus of the 40 kDa (likely corresponding to IM-379K polypeptide) and 24 kDa fragments (lM-249K fragment) were not accessible for degradation, while the sequence of the 16 kDa fragment started with 25AQAGASEMKLAKI- (250A-379K fragment). Autoradiography showed that radioactivity comigrated with the 40 kDa and 16 kDa fragments (Fig. 1C), suggesting that the
phosphorylation site(s) are located within the 25A-379K fragment. To obtain smaller phosphopeptides, the mixture of 16 kDa and 24 kDa fragments was subdigested with thermolysin, and the radioactive peptide was isolated using reversed-phased HPLC on a C8 column (inset to Fig. 2). Edman degradation of the phosphopeptides identified the 356VASERGGES sequence, and MS/MS analysis located the 358Ser residue as the phosphorylation site (Fig. 2). The same site was phosphorylated when the activator of Gq, GTP(\gamma)S, was included in the assay (data not shown).

The difference in the stoichiometry between bRho and oRho could have resulted from the primary sequence of Rho or RK, or both. To distinguish between these possibilities, urea-washed bovine rod outer segment membranes were phosphorylated by oRK. The Rho* phosphorylation was triggered by light and [\sup{32}P] -y-ATP. Based on methodology employed previously (4), we found that oRK catalyzed phosphorylation of bRho at 334Ser (8.3%), 338Ser (63.4%) and 343Ser (28.3%) (data not shown). This distribution is almost identical to that determined in bRho phosphorylation by bRK (4,18). These data demonstrate that the differences in stoichiometry and sites of phosphorylation are due to differences in the structures of oRho and bRho.

There are additional PPQGY repeats in the C-terminal region of oRho compare to bRho (19). Do these repeats influence oRho* phosphorylation? A mixture of tryptic truncated forms of Rho were prepared containing a 40 kDa form (truncated at 379K, thus lacking C-terminal PPQGY repeats) and 24 kDa/16 kDa forms (split in V-VI loop at 249K and lacking the C-terminal repeats due to cleavage at 379K). No significant phosphorylation was observed when the membranes were bleached in the presence of oRK and 32P--y-ATP (Fig. 3A). Absorption spectra (data not shown) and Gq-protein activation of these forms, however, were unaffected by the trypsin digestion (Fig. 3A).

DISCUSSION

Similar to vertebrate Rho, oRho undergoes light-dependent phosphorylation (20). As shown in this study, only 358Ser of oRho* is phosphorylated. Apparently, the flexibility of the C-terminal region and the sequence of oRho may not allow for an alternative site to be phosphorylated in contrast to bRho, which is phosphorylated at multiple sites. An important implication of monophosphorylation is that a single phosphate on oRho* is sufficient to produce a high-affinity receptor for arrestin. The oRho also possesses a characteristic C-terminal tail composed of PPQGY repeats not present in vertebrate Rho (19,21). Similar motifs are also present in synaptophysin (22), synexin (23) and other integral membrane proteins. In cephalopods, this domain may be involved in formation of the highly organized structure of rhabdomorphic microvilli through Rho-Rho interactions (24). In this study, we found that truncation of the C-terminal tail of oRho abolished phosphorylation but had no effect on the absorption spectrum or Gq activation. These data suggest that oRho lacking PPQGY repeats is spectrally active and capable of activating Gq, but this domain is necessary for the receptor to be phosphorylated effectively.
importance of the C-terminal region may be a more general phenomenon. In the case of bRho, we found that truncation of the C-terminal four to nine amino acids led to changes in phosphorylation levels of Ser residues and unusual phosphorylation of 336Thr (25). In addition, transgenic mice carrying C-terminal truncation in Rho at 329Gly (26) or 344Glu (27) showed prolonged responses and retinal degeneration, and studies using peptides corresponding to the C-terminal region of bRho revealed that alteration within the sequence led to significant changes in phosphorylation (28). Thus, the C-terminus of oRho appears to be necessary for efficient phosphorylation.

Like GRK2 and GRK3, oRK contains a PH domain at the C-terminus that is involved in binding the \( \gamma \) subunits of G-proteins rather than the C-terminal isoprenylation present in vertebrate RK (29). The differences in vertebrate RK and GRK2 resulted in phosphorylation of distinct sets of residues within bRho by both kinases (18). Our present study showed that preferential phosphorylation sites within bRho by oRK (334Ser [8.3%], 338Ser [63.4%] and 343Ser [28.3%]) were identical to those by bRK and clearly different from those by GRK2 or GRK3 (334Ser [5-10%], 338Ser [8-14%] and 343Ser [80-85%]) (4,18). Therefore, oRK is structurally distinct from, but functionally similar to, vertebrate RK (GRK1).

In conclusion, only 358Ser located in the C-terminal of photoactivated native oRho* is phosphorylated by oRK, suggesting that a singly phosphorylated receptor is sufficient for providing a high-affinity binding site for arrestin (Fig. 4). Tryptic truncation of the C-terminal PPQGY repeat of oRho that is adjacent to 358Ser did not influence signaling properties of oRho but abolished phosphorylation. These studies illustrate divergent evolution of the biochemical steps of phototransduction among vertebrate and invertebrate species.

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Figure 2.
[Footnote]
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Abbreviations: bRho, bovine Rho; bRK, bovine RK; BTP, bis-Tris propane; ES/MS, electrospray mass spectrometry; GRK, G-protein-coupled receptor kinase; MS/MS, tandem mass spectrometry; oRho, octopus Rho; oRK, octopus RK; PAGE, polyacrylamide gel electrophoresis; PH, pleckstrin homology; P-Rho*, phosphorylated Rho*; Rho, rhodopsin; Rho*, photoactivated Rho; RK, rhodopsin kinase; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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