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Direct binding of visual arrestin to a rhodopsin carboxyl terminal synthetic phosphopeptide

Peng Liu,[1] Eric D. Roush,[2] JoAnne Bruno,[2] Shoji Osawa,[1] Ellen R. Weiss[1]

[1]Department of Cell and Developmental Biology, The University of North Carolina at Chapel Hill, Chapel Hill, NC; [2]Biacore, Inc., Piscataway, NJ

Purpose: The phosphorylated carboxyl terminus of rhodopsin is required for the stable binding of visual arrestin to the full length rhodopsin molecule. Phosphorylation of the carboxyl terminus has been shown to induce conformational changes in arrestin, which promote its binding to the cytoplasmic loops of rhodopsin. However, it has not been determined whether phosphorylation is also responsible for the direct binding of the rhodopsin carboxyl terminus to arrestin. To further investigate the role of rhodopsin phosphorylation on arrestin binding, surface plasmon resonance was used to measure the interaction between a synthetic phosphopeptide corresponding to the carboxyl terminus of rhodopsin and visual arrestin in real time.

Methods: Synthetic peptides were generated that correspond to the phosphorylated and nonphosphorylated carboxyl terminus of bovine rhodopsin. These peptides were immobilized on a biosensor chip and their interaction with purified visual arrestin was monitored by surface plasmon resonance on a BIAcore 2000 or 3000.

Results: A synthetic peptide phosphorylated on residues corresponding to Ser-338, Thr-340, Thr-342 and Ser-343 of bovine rhodopsin was sufficient for direct binding to visual arrestin. In contrast, a second phosphopeptide phosphorylated on Thr-340 and Thr-342 and a nonphosphorylated synthetic peptide were not able to bind arrestin. A peptide fully substituted at all serine and threonine residues with glutamic acid was unable to substitute for phosphorylation.

Conclusions: Surface plasmon resonance is a sensitive method for detecting small differences in affinity. We were successful in using this technique to detect differences in the affinity of phosphorylated and nonphosphorylated rhodopsin peptides for visual arrestin. The data suggest that these are low-affinity interactions and indicate that phosphorylation is responsible for the direct binding of the rhodopsin carboxyl terminus to visual arrestin. Four phosphorylated residues are sufficient for this interaction. Because the affinity of the synthetic phosphopeptide for arrestin is substantially lower than the full length rhodopsin molecule, the cytoplasmic loops and rhodopsin carboxyl terminus appear to interact in a cooperative manner to stably bind arrestin.

Arrestin binding to rhodopsin is a multisite interaction requiring the participation of the cytoplasmic loops and the phosphorylated carboxyl terminus of rhodopsin. The carboxyl terminus of mammalian rhodopsin contains six to eight serine and threonine residues that serve as substrates for phosphorylation by GRK1 (rhodopsin kinase). A number of studies, in vitro and in vivo, have examined the preference for phosphorylation of specific sites by GRK1 [1-4] and the role of site specific phosphorylation in visual arrestin binding [5,6]. Using site directed mutagenesis, our laboratory has determined that multiple phosphorylation sites enhance the binding of arrestin to rhodopsin in vitro [5]. These results have recently been confirmed by studies that have measured phosphorylation in vivo; the use of rapid quench methods and sensitive mass spectrometry indicates that phosphorylation of rhodopsin occurs at multiple sites within 1 min following light exposure [7]. Transgenic mice expressing rhodopsin mutants with alanines substituted for the carboxyl terminal serines and threonines demonstrate a correlation between the loss of phosphorylation sites and a delay in recovery of light sensitivity [8]. Under the conditions of their experiments, these investigators reported that all seven serines and threonines in the carboxyl terminus can be phosphorylated in vivo [8].

Although the level of arrestin binding can be correlated with the number of phosphorylated residues on the carboxyl terminus of rhodopsin, the individual mutation of two residues, Thr-340 and Ser-343, to alanine had the most severe effect, causing a 50% reduction in arrestin binding and suggesting a more direct role for these residues in the interaction between rhodopsin and arrestin [5]. Using a centrifugation binding assay, the reduced binding appeared to result from a change in B_{max} rather than K_d. This was an unexpected result, but could be explained by considering that centrifugation assays generally measure only relatively high affinity binding interactions [9]. If the interaction of arrestin with the carboxyl terminus of rhodopsin is a low affinity interaction, changes in this interaction that occurred as a result of mutagenesis may not be detectable as a change in K_d.

In the present report, we use surface plasmon resonance (SPR) to measure the binding of visual arrestin to synthetic peptides corresponding to the rhodopsin carboxyl terminus. SPR is a sensitive method for measuring molecular interac-

Correspondence to: Ellen R. Weiss, Ph.D., Department of Cell and Developmental Biology, The University of North Carolina at Chapel Hill, CB# 7090, 108 Taylor Hall, Chapel Hill, NC, 27599-7090; Phone: (919) 966-7683; FAX: (919) 966-1856; email: erweiss@med.unc.edu

tions in real time without the need for radiolabeling or other modifications of the binding partners and can be used effectively to measure low affinity binding events [10]. We demonstrate the direct binding of visual arrestin to a synthetic peptide corresponding to the phosphorylated rhodopsin carboxyl terminus and we have determined that phosphorylation at four sites on the peptide, Ser-338, Thr-340, Thr-342 and Ser-343, is sufficient for this interaction.

METHODS

Arrestin expression and purification: Wild type bovine arrestin was expressed in *E. coli* BL21 using the pTrcHisB vector (Invitrogen, Carlsbad, CA) and purified by FPLC with sequential Heparin-Sepharose and Mono-Q Sepharose (Amersham Biosciences, Piscataway, NJ) chromatography as described with some modifications [11,12]. Transformed BL21 cells were grown for 5-6 h at 30 °C in LB with 100 μg/ml ampicillin. Cells were induced with 25 μM IPTG for 16-18 h, harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM DTT, 1 mM PMSF, 1 mM benzamidine, 10 μM leupeptin, 0.7 μg/ml pepstatin A and 10 μM chymostatin). Complete lysis was achieved by freezing at -80 °C for 30 min, incubation with 0.1 mg/ml lysozyme for 30 min on ice followed by sonication. Cell debris was removed by centrifugation at 200,000x g for 20 min, followed by precipitation in 50% saturated ammonium sulfate. All procedures were performed at 4 °C. The precipitate was resuspended in CB buffer (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA plus protease inhibitors), filtered through a 0.8 μm filter (Millipore, Billerica, MA) and loaded onto a HiTrap heparin column equilibrated with CB buffer containing 150 mM NaCl (CB150 buffer). The column was washed with CB150 buffer until A_{280} absorbance was negligible, then eluted with a linear gradient of 150-350 mM NaCl in CB buffer. Arrestin eluted at 280-300 mM NaCl. Arrestin containing fractions, visualized by Coomassie Blue staining of 10% polyacrylamide gels, were pooled, concentrated (Centriprep YM-30, Millipore), diluted with CB buffer until the NaCl concentration was below 10 mM, and loaded onto a Mono Q Sepharose column equilibrated with CB buffer containing 100 mM NaCl (CB100 buffer). The column was washed with 10 column volumes of CB100 buffer and arrestin was eluted with a linear gradient of 75-200 mM NaCl in CB buffer. Fractions containing arrestin, which eluted at 100-120 mM NaCl, were pooled, concentrated (Centriprep YM-10, Millipore) and stored at -80 °C. The purity of arrestin, evaluated by SDS-PAGE and Coomassie Blue staining, was found to be greater than 95%. The concentration of purified arrestin was determined spectrophotometrically at 278 nm, using an extinction coefficient of 28,886 A/M/cm [13] and a molecular weight of 45,275 Da. The activity of arrestin was verified by demonstrating the light dependent binding of arrestin to phosphorylated urea stripped rod outer segment (ROS) membranes (data not shown).

Table 1. Synthetic peptides representing the carboxyl terminus of bovine rhodopsin

Name	Sequence
WT	CGKNPLGDDEASTTVSKTETSQVAPA
4P	CGKNPLGDDEASTTV(Sp)K(Tp)E(Tp)(Sp)QVAPA
2P	CGKNPLGDDEASTTVSK(Tp)E(Tp)SQVAPA
7E	CGKNPLGDDEAEEEVEKEEEEQVAPA
Scr	CTKDPGATEAKSEGLQTDSPVAVTSN

WT, amino acid residues 323-348 of wild-type, bovine rhodopsin; 4P, same sequence as the WT peptide phosphorylated on residues equivalent to Ser-338, Thr-340, Thr-342 and Ser-343; 2P, same sequence as the WT peptide phosphorylated on residues equivalent to Thr-340, Thr-342; 7E, contains glutamic acids substituted for Ser-334, Thr-335, Thr-336, Ser-338, Thr-340, Thr-342 and Ser-343 to provide negative charges in place of phosphorylated residues, testing whether the negative charge is sufficient for arrestin binding; Scr, contains the same amino acid composition as the WT peptide but in a random order. Residues in parentheses with a trailing "p" represent phosphorylated residues.

Peptide synthesis: A 26 amino acid peptide (WT) corresponding to amino acids 323-348 of wild type bovine rhodopsin, and the phosphopeptides 4P and 2P, containing phosphorylated serine and threonine residues (Table 1), were synthesized by Quality Controlled Biochemicals, Inc. (Hopkinton, MA). The peptide 7E, which contains glutamic acids substituted for all seven serine and threonine residues in the WT peptide, was designed to mimic the negatively charged, fully phosphorylated peptide. Scr contained the same amino acid composition as WT, but the amino acids were arranged manually in a random order. This peptide demonstrated no binding to arrestin. The 7E and Scr peptides were synthesized at the Peptide Synthesis Facility in the Department of Microbiology and Immunology at UNC-Chapel Hill.

Surface plasmon resonance (SPR): Peptides were immobilized on either a CM5 or a F1 sensor chip via the ligand thiol coupling method according to the instrument manufacturer's manuals. The binding of arrestin to synthetic peptides immobilized on these two surfaces gave very similar results. The carboxylated dextran matrix on the sensor chip surface was first equilibrated with HBS (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween-20), and then activated with 50 mM N-hydroxysuccinimide (NHS) and 200 mM N-ethyl [(dimethylamino)propyl]carbodiimide (EDC) for 2 min at a flow rate of 5 μl/min. The activated dextran matrix was modified by injection of 80 mM 2-(2-pyridinyldithio)-ethaneamine hydrochloride (PDEA) in 0.1 M Na borate, pH 8.5 for 4 min at a flow rate of 5 μL/min. Peptides were immobilized at the N terminal cysteine residue via thiol-disulfide exchange by manual injection of the peptides (2 μg/ml) in 50 mM Na citrate, pH 3.0. The reaction was terminated by injection of buffer containing 50 mM cysteine and 1 M NaCl for 4 min at 5 μl/min.

The activity of the WT and 4P peptides immobilized on a CM5 sensor chip was assessed by measuring the binding of

two antibodies, 1D4 and A11-82, directed against the nonphosphorylated and phosphorylated rhodopsin carboxyl terminus, respectively, on the BIAcore 2000 (Biacore Inc., Piscataway, NJ). The monoclonal antibodies 1D4 (Cell Culture Center, Minneapolis, MN) and A11-82 (a gift from Dr. Paul Hargrave, University of Florida, Gainesville, FL) recognize the last eight amino acids of the unphosphorylated and phosphorylated rhodopsin carboxyl terminus, respectively. The sensor chip was equilibrated with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 7.9 mM Na_2HPO_4) prior to antibody binding. The antibodies were diluted to a concentration of 50 μg/ml and injected at a flow rate of 20 μl/min. The surfaces were regenerated by injection of 10 mM glycine-HCl, pH 2.0, and reequilibrated with PBS. The activity of the surface was evaluated by comparing the experimental R_{max}, determined by measuring the binding of the antibodies, and theoretical R_{max}. The R_{max} is the maximum binding of a given ligand density surface. The theoretical R_{max} is equal to the MW of analyte divided by MW of ligand times the surface density and binding valence, where the binding valence is the number of binding sites of the ligand for the analyte. The binding of both ligand and analyte to the surface of the sensor chip is measured in resonance units (RU). 1,000 RU is approximately equal to 1 ng/mm^2 for most proteins.

The interaction between arrestin and the rhodopsin carboxyl terminal peptides was monitored by SPR using the BIAcore 2000 and 3000 instruments. CM5 or F1 sensor chips were prepared as described above and equilibrated in arrestin binding buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.0 mM EDTA, 0.02% Tween-20). Arrestin was diluted to various concentrations in arrestin binding buffer and injected at a flow rate of 20 μl/min. To correct for nonspecific binding, arrestin binding to Scr peptide, immobilized at an equivalent ligand density, was compared. After each cycle, the sensor chip was regenerated by three sequential 30 s injections of 1 M NaCl, 6 M guanidine HCl, 0.01% SDS, followed by two 60 s injections of arrestin binding buffer. BIAevaluation 3.01 software (Biacore, Inc.) was used to determine whether the resulting data fit a 1:1 binding model and whether kon, koff or Kd could be defined. Although we observed lower nonspecific binding on the F1 chip (data not shown), the results of arrestin binding were essentially the same as with the CM5 chip.

For solution competition experiments, 4P and Scr peptides were immobilized on an F1 sensor chip as described above. Peptides Scr, WT, 4P and 7E, were added to 4 μM arrestin in arrestin binding buffer at the indicated concentrations. This mixture was incubated at 37 °C for 20 min before loading onto the sensor chip. The binding of arrestin under these conditions to the immobilized peptides was monitored during a 6 min association and 6 min dissociation time period at room temperature. The report point was taken at 10 s prior to the start of the dissociation phase. Regeneration of the surface was performed as described for the arrestin binding assay. The data were analyzed by nonlinear regression analysis using the computer program GraphPad Prism (version 4; GraphPad Software, Inc., San Diego, CA).

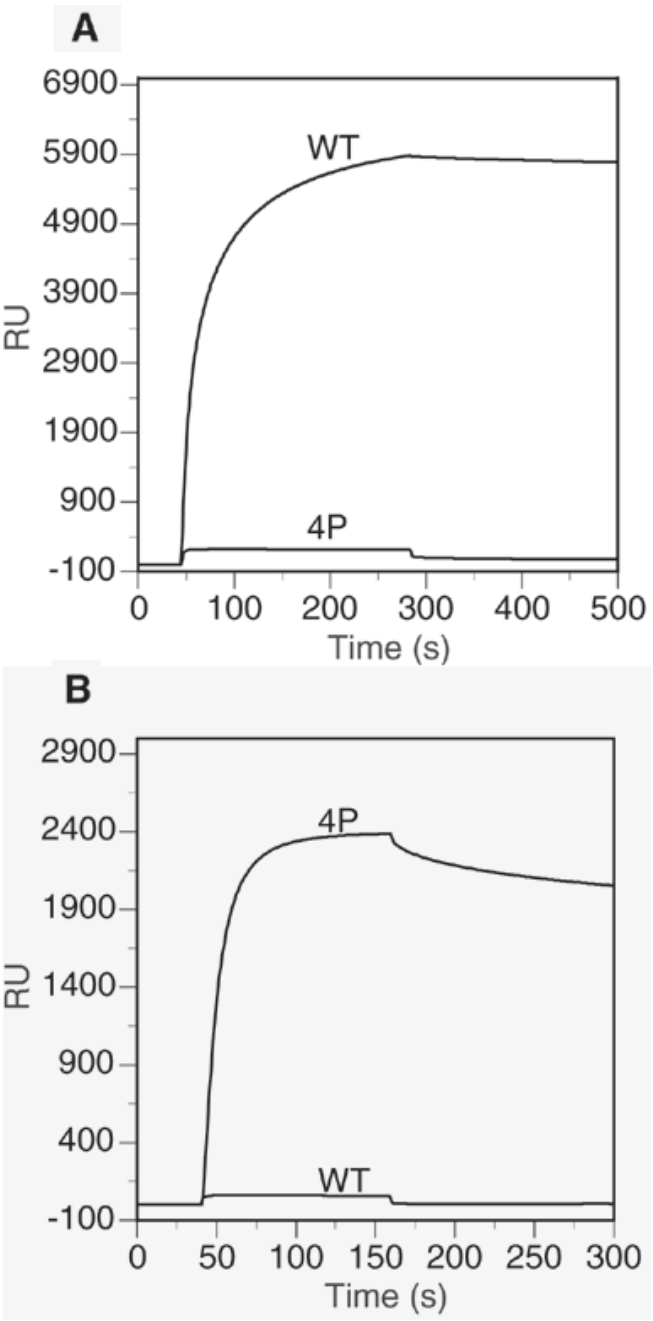
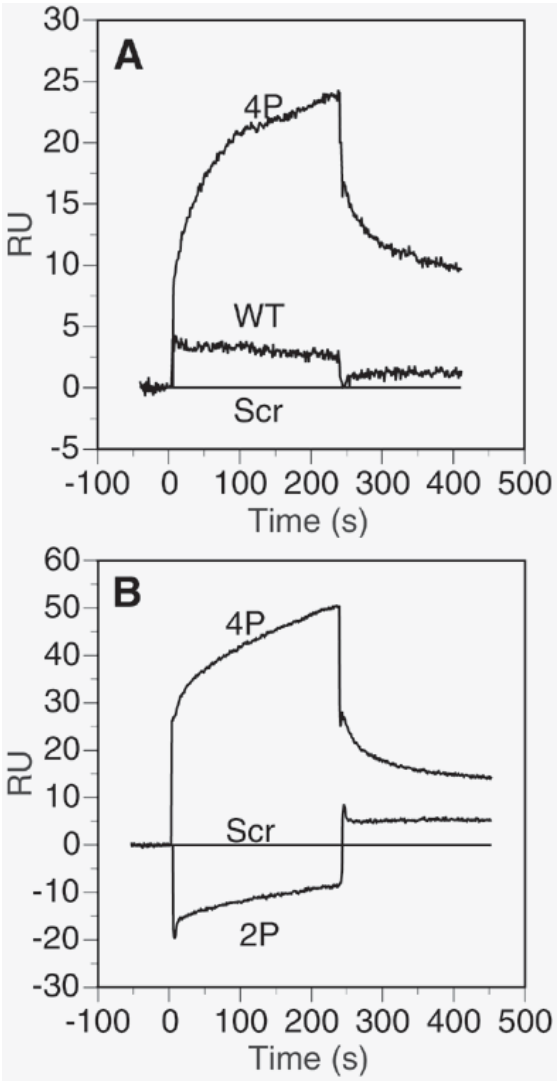


Figure 1. The binding of anti-rhodopsin antibodies to WT and 4P peptides. The peptides, WT and 4P, were immobilized on a CM5 sensor chip at approximately 400 resonance units (RU) using procedures described in Methods. **A**: 333 nM 1D4 antibody, which recognizes the unphosphorylated carboxyl terminus of rhodopsin, was injected onto the sensor chip from 40-280 s, followed by continuous flow of buffer alone beginning at 280 s to measure the dissociation phase. The level of binding was measured as a change in resonance units (RU). **B**: 333 nM A11-82P, which recognizes the phosphorylated carboxyl terminus of rhodopsin, was measured for binding to the sensor chip as described in **A**, except that antibody was injected from 40-160 s, followed by continuous flow of buffer alone beginning at 160 s to measure the dissociation phase. The data are representative of two independent experiments that gave very similar results.

RESULTS

A series of synthetic peptides (Table 1) were analyzed for their ability to bind arrestin. The peptide WT was synthesized to represent the bovine rhodopsin carboxyl terminal amino acid residues 323-348. Two phosphorylated peptides were also generated. The 4P peptide was phosphorylated on residues equivalent to amino acids Ser-338, Thr-340, Thr-342 and Ser-343. A second synthetic phosphopeptide, 2P, was phosphorylated at residues corresponding to Thr-340 and Thr-342. The peptide 7E represents the carboxyl terminus of rhodopsin with glutamic acid substituted for all seven serines and threonines. The final peptide, Scr, contains the same amino acid composition as the rhodopsin carboxyl terminus but in a random order. This peptide served as a negative control in all our experiments. The peptides were coupled to a CM5 or F1 sensor chip at the amino terminal cysteine using thiol coupling. This cysteine corresponds to Cys-323 in bovine rhodopsin, which is palmitoylated in the native protein and provides a natural anchorage point to the phospholipid bilayer. Therefore, the anchoring of the rhodopsin carboxyl terminus has been reproduced as closely as possible. By linking the peptides to the surface through the amino terminal cysteine, the peptides are also oriented in an identical fashion, providing a uniform surface for arrestin binding. The anti-rhodopsin antibodies, 1D4 and A11-82, were tested for their ability to bind the WT and 4P peptides to determine whether these peptides are properly oriented on the surface (Figure 1). The 1D4 antibody, which recognizes the carboxyl terminal eight amino acids of bovine rhodopsin [14], bound preferentially to the WT peptide compared to the 4P peptide (Figure 1A). This antibody bound approximately 94% of the WT peptide, indicating that virtually all of the peptide is available for binding. In contrast, the A11-82 antibody, which recognizes only phosphorylated rhodopsin [15], preferentially bound the 4P peptide and demonstrated very little binding to the WT peptide. The A11-82 antibody bound approximately 89% of the immobilized 4P peptide, demonstrating that this peptide is also properly displayed on the surface and available for binding. In contrast, bovine serum albumin did not bind to either peptide (data not shown).

The binding of visual arrestin to 4P and WT peptides was evaluated. When arrestin binding to the Scr peptide is used as a measure of nonspecific binding and subtracted from the bind-



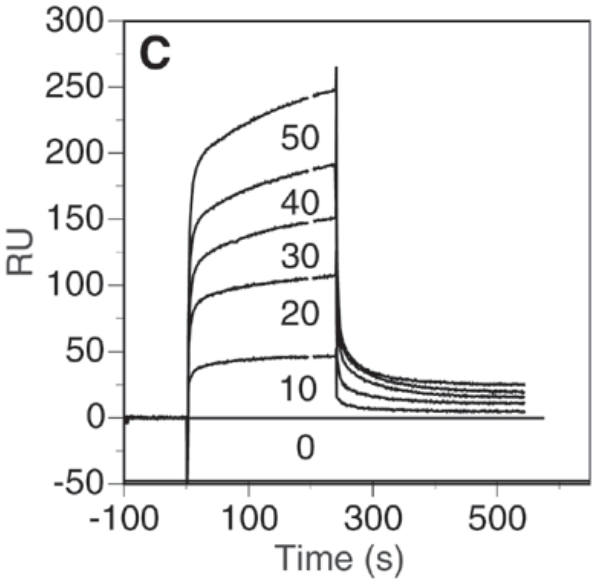


Figure 2. Arrestin binding to WT, 4P, and 2P peptides. Peptides were immobilized on the surface of a CM5 sensor chip using methods described in Methods. Arrestin was injected in buffer over the surface for 240 s to measure association, followed by continuous flow of buffer alone to measure dissociation. **A**: WT, 4P and Scr peptides were immobilized on the surface of the sensor chip at 400 RU. Arrestin (2 μM) was injected over the surface of the sensor chip. The binding of arrestin to Scr peptide was subtracted from arrestin binding to WT and 4P. **B**: The peptides (2P, 4P, and Scr) were bound to the sensor chip at 200 RU each. 10 μM arrestin was injected over the surface. The binding of arrestin to Scr peptide was subtracted from arrestin binding to 2P and 4P. **C**: The binding of arrestin (0-50 μM) to 4P and Scr peptides immobilized on the sensor chip surface at 200 RU. The binding of arrestin to Scr is subtracted from the binding to 4P at each concentration of arrestin. These results are from a minimum of four experiments.

ing to the 4P and WT peptides, the binding of 2 μM arrestin to WT peptide is approximately 3 Resonance Units (RU) at the end of the association phase (Figure 2A). Arrestin binding to the 4P peptide is approximately 8 fold higher (25 RU). A second phosphopeptide, 2P, which is phosphorylated on residues equivalent to Thr-340 and Thr-342 (Table 1) was compared with 4P peptide for its ability to bind arrestin (Figure 2B). In contrast to the binding of arrestin to the 4P peptide, arrestin binding to 2P peptide was not significant, even at five times higher concentration (10 μM), appearing as a negative deflection when binding to Scr peptide is subtracted. The results suggest that phosphorylation of Thr-340 and Thr-342 is not sufficient for arrestin binding under these conditions. Since arrestin demonstrated significant binding to 4P peptide at 2 μM (Figure 2A), a series of arrestin concentrations was tested for the ability to bind to 4P and Scr peptides to measure the kinetics of phosphorylation specific binding. After subtracting the binding of arrestin to Scr from the binding to 4P at each arrestin concentration up to 50 μM, the results demonstrate that increasing concentrations of arrestin results in increased binding specifically to the 4P peptide (Figure 2C). Since the binding curve is complex, potentially composed of multiple on and off phases, it was not possible to determine a single K_d, k_{on} or k_{off}, for arrestin binding. A second feature of these curves is that the slow binding phase appears to increase with increasing arrestin concentration. Binding could not be saturated even at an arrestin concentration of 60 μM, which was the highest concentration we were able to achieve in our assay (data not shown), suggesting that this is a low affinity interaction. It should be noted that less peptide was linked to the sensor chip surface for arrestin binding in Figure 2B and Figure 2C compared with the antibody binding experiments in Figure 1. However, the smaller number of resonance units observed for arrestin binding is due mostly to the lower affinity of arrestin for the peptides and to the lower molecular weight of arrestin compared to the molecular weight of the antibodies.

Because determination of affinity constants was difficult under our experimental conditions, the relative affinities of different peptides for arrestin were compared using a solution competition assay. Increasing concentrations of the 4P peptide in solution reduce arrestin binding to 4P peptide linked to the surface of the sensor chip (Figure 3A). An IC_{50} of 256 μM was calculated from these data. Peptides Scr, WT, 4P and 7E, a synthetic peptide containing glutamic acid residues substituted for the seven serine and threonine residues in the wild type rhodopsin carboxyl terminus (Table 1), were preincubated with arrestin at a concentration of 256 μM before injecting

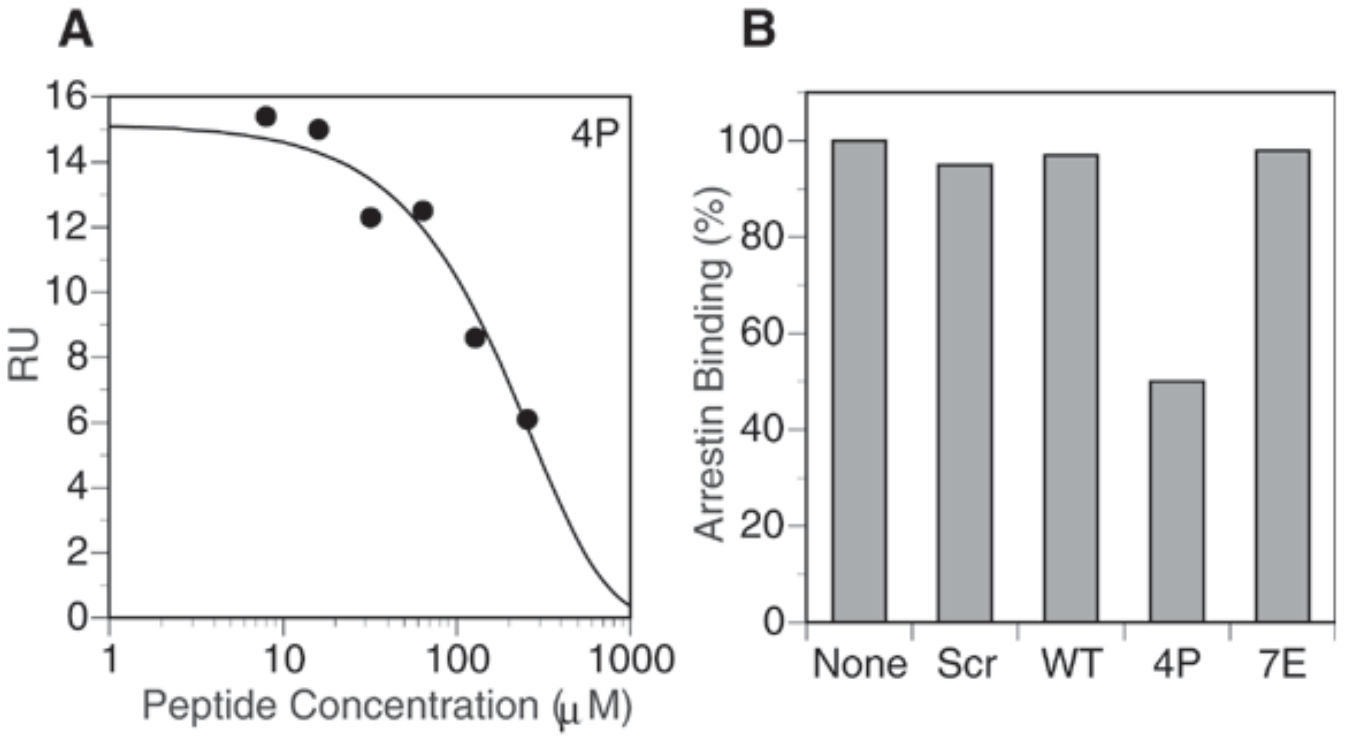


Figure 3. Solution competition analysis of arrestin binding to synthetic peptides. The peptides 4P and Scr were immobilized on the surface of an F1 sensor chip at 180 RU and 188 RU, respectively. **A**: Arrestin (4 μM) was mixed with varying concentrations of 4P peptide as described in the Methods and injected over the surface of the chip to measure arrestin binding. The level of arrestin binding to Scr was subtracted from the binding to the 4P peptide. The data were analyzed by nonlinear regression analysis using the computer program GraphPad Prism. **B**: Arrestin (4 μM) was preincubated with each peptide at a concentration of 256 μM and subjected to surface plasmon resonance to analyze the binding to 4P and Scr peptides immobilized as described in **A**. The binding of arrestin to Scr was subtracted from the binding to 4P peptide and the values normalized to the binding of arrestin to the surface in the absence of competing peptide. These results are from two independent experiments that gave very similar results.

the mixture onto the sensor chip and examining the binding of arrestin to 4P peptide linked to the surface (Figure 3B). The results demonstrate that only 4P peptide significantly inhibits arrestin binding to 4P peptide linked to the sensor chip, as expected, based on the observation that Scr and WT peptides do not bind arrestin at significant levels (Figure 2). The inhibition by 4P peptide was approximately 50%, which corresponds to the IC_{50} calculated from the data in Figure 3A. Interestingly, the negatively charged peptide, 7E, was not able to compete with 4P for arrestin, even at a concentration of 256 μM. Arrestin also did not bind to the 7E peptide attached to the sensor chip surface (data not shown). Our results demonstrate the direct, phosphorylation dependent binding of arrestin to a synthetic peptide representing the carboxyl terminus of rhodopsin. The presence of four phosphorylated residues, corresponding to amino acids Ser-338, Thr-340, Thr-342 and Ser-343, is sufficient for this interaction. In contrast, the negatively charged amino acid, glutamic acid was not able to substitute for phosphorylated residues.

DISCUSSION

The present work demonstrates the direct binding of arrestin to a synthetic phosphopeptide corresponding to the rhodopsin carboxyl terminus. Because most of the studies on the phosphorylation of rhodopsin and its influence on arrestin binding have used the bovine proteins, we selected bovine rhodopsin and arrestin as our model system. Previous studies have shown that a synthetic peptide phosphorylated on seven residues, corresponding to amino acids 330-348 of bovine rhodopsin, induces conformational changes in visual arrestin. These conformational changes increase arrestin's sensitivity to proteolysis and promote its binding to unphosphorylated, light activated rhodopsin [16]. However, direct binding between the rhodopsin carboxyl terminal peptide and visual arrestin has not been shown prior to the studies reported here. Because SPR is capable of detecting low affinity binding events, we were able to measure significant binding of arrestin to 4P peptide at concentrations ranging from 1 to 50 μM. In contrast, neither the 2P peptide, which contains two phosphorylated residues, nor WT, the nonphosphorylated peptide, demonstrate appreciable binding even at high concentrations of arrestin. These results were confirmed using a solution competition assay (Figure 3). The inability of the 2P peptide to bind arrestin at the concentrations we tested may be due either to insufficient numbers of phosphates or to not having the critical ones. We attempted to synthesize additional peptides containing phosphorylated residues at different positions, but none of them could be verified by mass spectrometry or other analytical methods. Therefore, we were unable to distinguish between these two possibilities. Nevertheless, our data directly show for the first time that phosphorylation itself contributes to the affinity of visual arrestin for the rhodopsin carboxyl terminus.

Other studies of GPCR interaction with arrestin family members have reached varying conclusions. For example, although desensitization and internalization of the CC chemokine receptor 5 (CCR5) are phosphorylation dependent [17], phosphorylated and nonphosphorylated synthetic peptides representing the CCR5 carboxyl terminus were found to bind β-arrestin equally well (3.6 μM vs. 5.5 μM for phosphorylated and nonphosphorylated peptides, respectively) [18]. The authors speculate that phosphorylation acts as a "molecular switch" to alter the conformation of β-arrestin. This conformational change induces stable binding to other domains within the full length receptor rather than directly contributing to the affinity of the receptor carboxyl terminus for arrestin. The role of phosphorylation is somewhat different for the D_1 dopamine receptor, which is phosphorylated on both the third cytoplasmic loop and the carboxyl terminus [19]. When all of the phosphorylation sites in this receptor are eliminated, dopamine dependent desensitization is impaired. However, if the carboxyl terminus is also truncated, desensitization is normal, suggesting that the function of the carboxyl terminus is to shield the β-arrestin binding domain on the third cytoplasmic loop. Phosphorylation appears to move the carboxyl terminus away and expose this binding site. Therefore, receptor phosphorylation is not directly required for binding or for inducing a conformational change in β-arrestin. In a third example, β-arrestin binding to a synthetic peptide corresponding to the carboxyl terminus of the *N*-formyl peptide receptor was enhanced by phosphorylation of the peptide [20]. This result is similar to ours with visual arrestin and demonstrates a direct role for the phosphates in the affinity of arrestins for at least these two GPCRs. Structural differences between arrestin family members may also contribute to variability in the mechanism of interaction observed for different GPCRs. Residues in β-arrestin form a cationic amphipathic helix, which is speculated to associate with the phospholipid bilayer after activation by GPCR [21]. This provides a mechanism of membrane association that is distinct from that proposed for visual arrestin. Another significant difference between visual and β-arrestin is that visual arrestin is likely to be a dimer at physiological concentrations [22,23]. It has been proposed that the dimer of visual arrestin is a storage form, since it is quite clear from different binding assays that the monomer is functional [24,25]. In contrast, β-arrestin is proposed to be a monomer [21,26]. Dimerization may occur only when it contacts phosphorylated receptor as a consequence of activation and may be necessary for β-arrestin's unique function in recruiting GPCRs to clathrin coated pits or activation of downstream signaling partners [21,27].

Our results are consistent with the hypothesis that multiple phosphates are necessary for optimal binding of visual arrestin. In vitro, increasing levels of phosphorylation have been shown to decrease the activity of rhodopsin towards transducin while increasing its binding to visual arrestin [28]. Similarly, work from our laboratory and others suggest that increasing the number of phosphorylated residues results in more binding of arrestin [5,6]. Although monophosphorylated rhodopsin predominates under high bleach conditions in vivo, when the levels of GRK1 are likely to be rate limiting [4,7], experiments under low bleach conditions indicate that all seven serines and threonines can be phosphorylated and that at least three phosphates are necessary to efficiently quench

phototransduction [8]. Interestingly, visual arrestin did not bind to the 7E peptide in our experiments, consistent with our previous results using mutagenesis [5] and studies from other laboratories [29]. The charge density or perhaps the conformation of the phosphorylated residues may be important in the direct binding of arrestin to the rhodopsin carboxyl terminus. This is likely, considering the complex spatial requirement necessary for the phosphorylated residues to interact with arrestin's carboxyl terminus and destabilize the polar core, resulting in the conformational changes in arrestin that promote high affinity, stable binding to rhodopsin [22,30]. A naturally occurring arrestin variant, p44, in which an alanine is substituted for the carboxyl terminal 35 amino acids of full length arrestin, binds forms of rhodopsin that do not normally interact with wild type arrestin, such as phosphorylated rhodopsin in the dark, phosphorylated opsin and light exposed, unphosphorylated rhodopsin [31-33]. This illustrates the importance of the carboxyl terminus of arrestin in maintaining the protein in its low affinity state until it interacts with the phosphorylated carboxyl terminus of rhodopsin.

Previously, we demonstrated that residues in cytoplasmic loops 1 and 2 of rhodopsin play a role in the binding of arrestin [34,35]. A mutation (R175E) was generated in arrestin to determine the role of the polar core in arrestin function. This mutation, which was found to destabilize the polar core, also results in equal binding of arrestin to nonphosphorylated and phosphorylated rhodopsin [23,36]. This mutant was used to define the cytoplasmic loop sequences in rhodopsin important for arrestin binding apart from the requirement for phosphorylation. Several mutations in the cytoplasmic loops of rhodopsin that disrupted its binding to R175E-arrestin were less effective when these rhodopsin mutants were phosphorylated [35]. This suggests that the phosphorylated carboxyl terminus does play an important role in determining the affinity of rhodopsin for arrestin. However, the affinity of arrestin for the carboxyl terminal phosphopeptides is clearly much lower than the affinity for the entire rhodopsin protein, based on an apparent K_d for the interaction between arrestin and rhodopsin of 0.74 nM [5]. These observations illustrate that interaction between rhodopsin and arrestin is cooperative in nature; the cytoplasmic loops and the phosphorylated carboxyl terminus of light activated rhodopsin both contribute to its affinity for arrestin [35]. In summary, the present results in combination with work from other laboratories indicate that rhodopsin phosphorylation has a dual role in arrestin binding: it increases the affinity of the rhodopsin carboxyl terminus for visual arrestin and induces conformational changes in arrestin that result in the stable binding of arrestin to other domains in the rhodopsin molecule.

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