

## **In vitro Interaction of Recombinantly Expressed Kringle 5 (rK5) with Ras Guanine Nucleotide Dissociation Stimulator-like Factor (Rgl2)**

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Kringle 5 (K5), located outside of angiostatin (K1-4) in human plasminogen, displays more potent anti-angiogenic activity on endothelial cell proliferation than angiostatin itself. Using a yeast two-hybrid system *in vivo*, we have recently identified Rgl2 (guanine nucleotide dissociation stimulator (RalGDS)-like factor 2) as a binding protein of human K5. In order to confirm *in vitro* protein interaction between K5 and Rgl2, we developed bacterial recombinant expression systems for them. K5 and Rgl2 proteins were expressed in high yields and purified into pure forms with His tags and GST fusion, respectively. GST-pull down experiments clearly demonstrated that K5 interacts specifically with Rgl2 *in vitro*. These results indicate that Rgl2 functions as a receptor protein for K5 *in vitro* as well as *in vivo*, leading to anti-angiogenesis through regulating Ras signaling pathways.

**Key Words :** Kringle 5, Rgl2, GST-pull down experiments

### **Introduction**

Angiostatin, an inhibitor of angiogenesis which is the process of formation of new capillaries from existing blood vessels, consists of four homologous triple disulfide-linked loops of plasminogen, called kringle domains (K1-4).<sup>1</sup> Among the four kringles, K1 is the most potent inhibitor on endothelial cell proliferation whereas K4 is the least effective domain.<sup>2</sup> Another kringle, K5, which is located outside of the angiostatin region in plasminogen, exhibits significant sequence homology with K1 up to 60%. Interestingly it is even more potent than K1 and K1-4 on inhibition of the growth factor-stimulated endothelial cell proliferation.<sup>3</sup>

In order to understand the molecular mechanisms by which each kringle exerts its anti-angiogenic effect, it is worthwhile investigating the structure-function relationship between each kringle domain based on a level of anti-angiogenic activity. Several groups have reported the structural data for kringle domains, such as angiostatin 1-3, K1 and K5.<sup>4,5,6</sup> The structure of angiostatin K1-3 was found to be triangular bowl-shaped producing a central cavity where K2 and K3 binds to the group A streptococcal surface protein, PAM.<sup>4</sup> The recombinant kringle 1 domain of human plasminogen (K1pg) binds with the ligands  $\epsilon$ -aminocaproic acid (EACA) judging from the X-ray crystallography results.<sup>5</sup> The crystal structure of the kringle 5 domain shows a substitution of Arg for Leu in the LSB-4 (lysine binding site-4) motif resulting in the formation of a less cationic center for EACA, which makes interaction with these types of ligands weaker.<sup>6</sup>

It has been proposed that angiostatin exerts anti-angiogenic effects via binding with some essential proteins

which play crucial roles on signal transduction pathways leading to cell proliferation. To substantiate this proposal, several groups have performed binding studies but only limited numbers of binding partners have been identified, including ATP synthase, integrin, and angiomin.<sup>7</sup> Little information has been available for binding proteins of individual kringle domain until recently. Our previous result with yeast two-hybrid shows that two distinct binding proteins, Ras guanine nucleotide dissociation stimulator (RalGDS)-like factor 2 (Rgl2)<sup>8</sup> and Myc-associated Zn finger-related factor (MAZR),<sup>9</sup> are implicated in specific binding with the K5 domain and angiostatin (K1-4), respectively (manuscript in press). Intriguingly, K5 only binds with Rgl2 whereas both K1 and K1-4 have strong interaction with both MAZR and Rgl2. From the fact that only two domains, K1 and K5, bind to Rgl2, we can suggest that the presence of a common binding motif near LBS-4 (the KRYDY sequence of K1 domain and the KLYDY sequence of K5 domain) may be a prerequisite for interaction with Rgl2, but not for interaction with MAZR. Correlated with our findings, several derivatives of this sequence, such as KLWDF, were synthesized using peptidomimetics and tested for angiogenesis inhibition.<sup>10</sup> Very recently, another small molecule mimetic resembling the KLYD sequence, lysyl 4-aminobenzoic acid derivatives were designed and reported as a potent inhibitor of HMVEC chemotaxis.<sup>11</sup>

Our yeast two hybrid results indicate that K5 binds with the C-terminal region of Rgl2 *in vivo*, which was previously known to specifically bind to Ras and Rap1A in the COOH terminal region. Upon binding with Ras and Rap1A, Rgl2 activates the Ras pathway and in turn participates in diverse signaling pathways leading to many cellular responses, such as cell proliferation.<sup>12</sup> Even though *in vivo* binding between K5 and Rgl2 was confirmed by yeast two-hybrid, it has not

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been demonstrated yet that they interact with each other *in vitro* as well. In this study we have therefore developed recombinant expression systems using bacteria for both K5 and Rgl2 with some modifications and examined their binding phenomena using GST-pull down experiments. Our result constitutes evidence that K5 is directly involved in anti-angiogenesis by specific interaction with Rgl2, which is a distinct effector of Ras signaling pathways leading to angiogenesis.

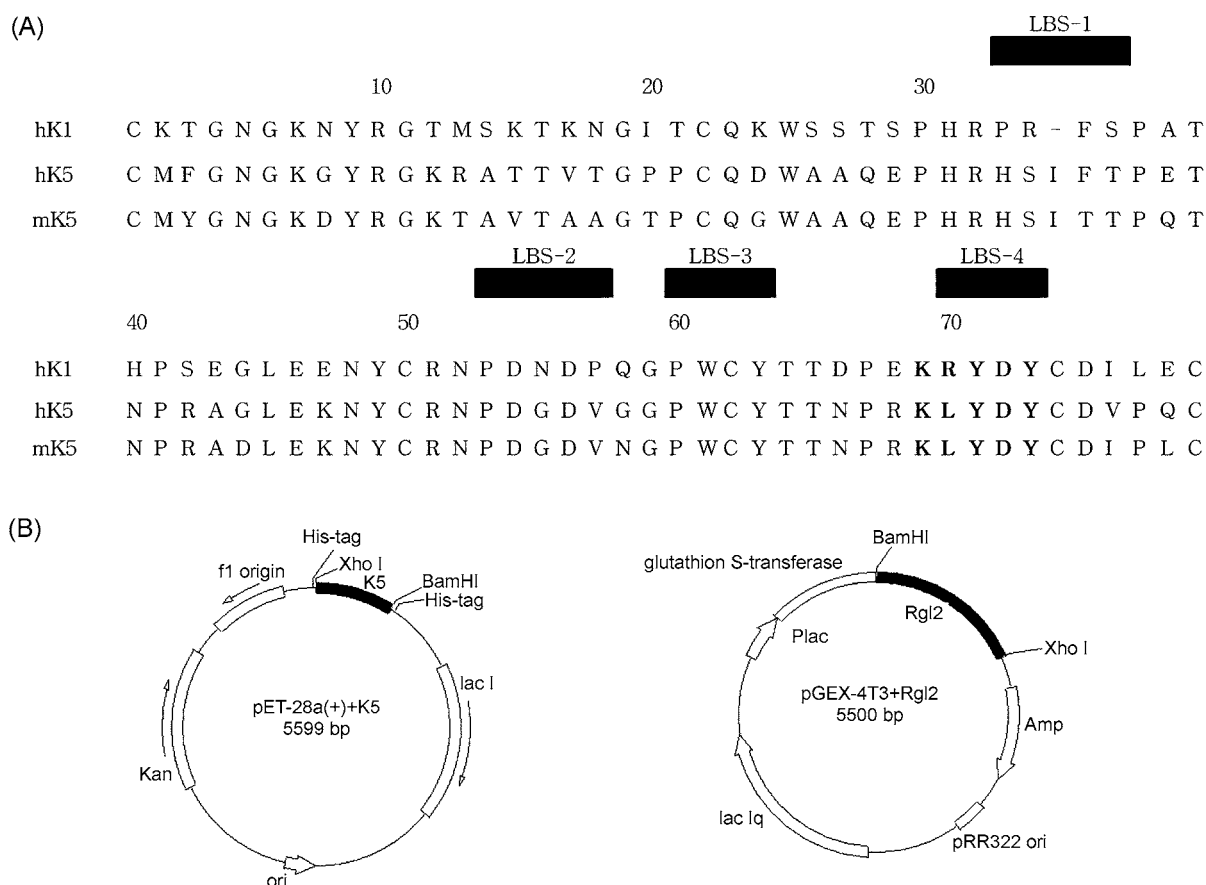
## Results and Discussion

### Expression and purification of recombinant K5 (rK5).

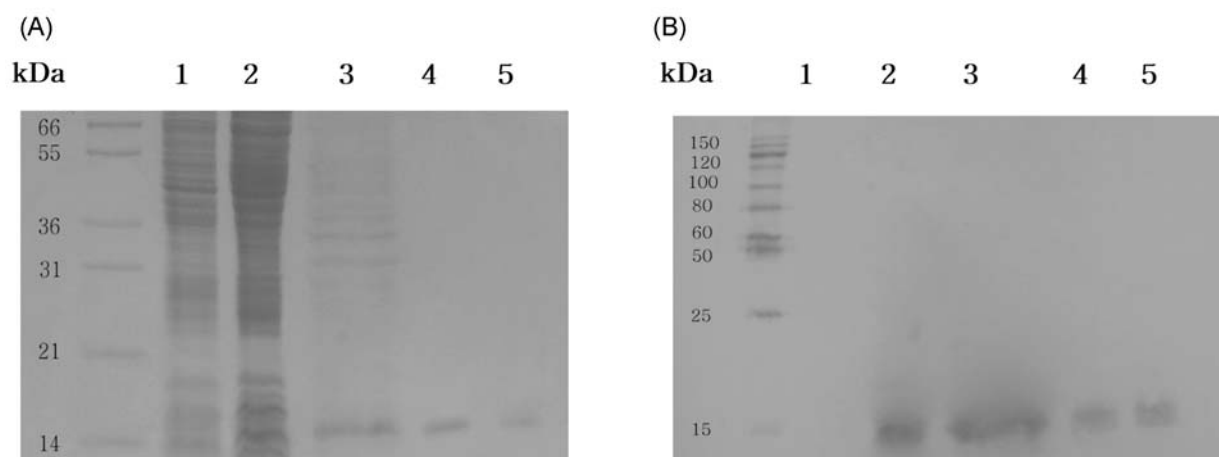
Purification of human K5 has been achieved by conventional chromatography methods following treatment of human plasminogen by appropriate enzymes, such as elastase.<sup>3</sup> For a recombinant protein expression, mouse kringle 5 (mK5) using His tag at the C-terminus was first expressed in a bacterial expression system and soluble mK5 thus expressed was purified to homogeneity using Ni-NTA agarose column.<sup>13</sup> This recombinant mK5 exhibited remarkable biological activities of inhibition for proliferation and migration of endothelial cells up to levels comparable to those of native human K5. DNA sequence alignment indicates that human K5 shares over 80% homology with mouse K5 and nearly

60% with human K1, respectively (Figure 1A). In order to obtain K5 protein with high purity and in large quantity, we have subcloned the PCR-amplified human K5 insert into pET-28a-c(+)/K5 to attach two His tags at both the N-terminus and C-terminus of K5 (Figure 1B). The resulting rK5 was expressed by IPTG induction at a high level and was fractionated into an insoluble inclusion body. After resolubilization by 8 M urea, the inclusion fraction was further purified by cobalt resin and dialyzed to remove salt and promote renaturation without additional refolding process. The presence of rK5 was monitored in SDS-PAGE (Figure 2A) and western blotting with anti-6His antibody (Figure 2B) to follow up successful purification. The purification procedures are summarized in Table 1, indicating that the yield of rK5 from 1 L bacterial culture is 13 mg after cobalt binding affinity chromatography. The molecular weight of rK5 was 14 kDa as anticipated from the amino acid sequence.

**Refolding and purification of rK5.** During cell lysis and solubilization, it is possible that rK5 can be denatured to become unfolded or misfolded. Once the disulfide bonds are disrupted, one can imagine that a kringle protein may not function as an angiogenesis inhibitor because the binding sites for receptor protein might have been altered. Therefore an approach to prevent misfolding or to induce refolding



**Figure 1.** Construction of recombinant plasmids. (A) Alignment of amino acid sequences of kringle 5 domain. Native human K1 (hK1), Native human K5 (hK5), and recombinant mouse K5 (mK5) were compared each other to display 4 lysine binding sites (LBS). (B) The plasmid maps for pET28a-c(+)/K5 and pGEX-4T3/Rgl2. rK5 was bound with two His tags at both termini and Rgl2 was fused with GST at the N-terminus.



**Figure 2.** Purification and characterization of rK5. rK5 was overexpressed in *E. coli* and analyzed in 10% SDS-PAGE for whole cell extract without induction (lane 1), whole cell extract with induction (lane 2), inclusion bodies (lane 3), eluted fraction from Cobalt column (lane 4) and refolded protein from Lys binding column (lane 5). Proteins were visualized by Coomassie brilliant blue staining (A) or Western blotting with anti-His antibody for PVDF membrane (B).

**Table 1.** Purification of expressed rK5 protein from 1 L *E. coli* culture

Purification step	Protein concentration (mg/mL)	Volume (mL)	Total protein (mg)
Whole cell extract	8.04	50	402
Soluble fraction	6.25	48	300
Inclusion body	26.0	3.0	80
Solubilized fraction	22.0	2.8	67
Cobalt column eluted	0.33	40	13
Lys binding column eluted	0.12	50	6

into a proper domain structure should be seriously considered in order to avoid loss of anti-angiogenic activity *via* maintenance of the normal mode of protein-protein interaction.

The degree of proper refolding was confirmed by purifying refolded rK5 protein through Lysine binding resin, SP-Sepharose FF. Once the purified rK5 underwent denaturation and refolding process, the yield of protein purification (6 mg) fell down to more than 50% compared to cobalt bound fraction (13 mg) as indicated in Table 2. To our disappointment, only a little rK5 was recovered by the refolding procedure, implying that the native structure of rK5 had not been recovered completely (Figure 2A). However, as demonstrated by McCance *et al.*, individual kringle domains with proper folding bind to EACA with a decreasing binding affinity in the order of K1, K4, and K5.<sup>14</sup> In the case of K5, the lack of a cation center formed by R34/R70 was attributed to the hampered interaction between rK5 and ligand.<sup>5</sup> Therefore using SP-Sepharose FF column for monitoring the presence of a lysine binding site was proven to be ineffective approach for kringle-specific affinity purification in our case.

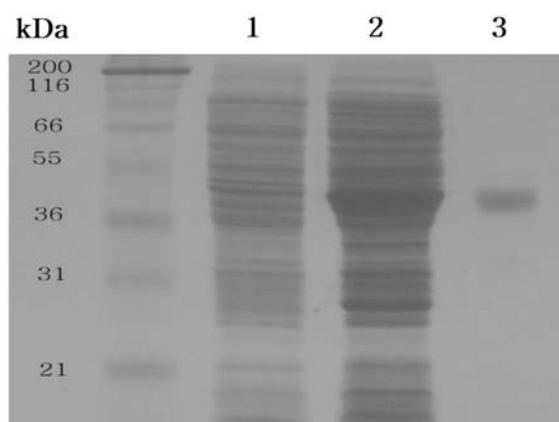
In addition, it was reported that the lysine binding site of rK5 was not absolutely required in BCE cell proliferation as

**Table 2.** Purification of expressed Rgl2 protein from 1 L *E. coli* culture

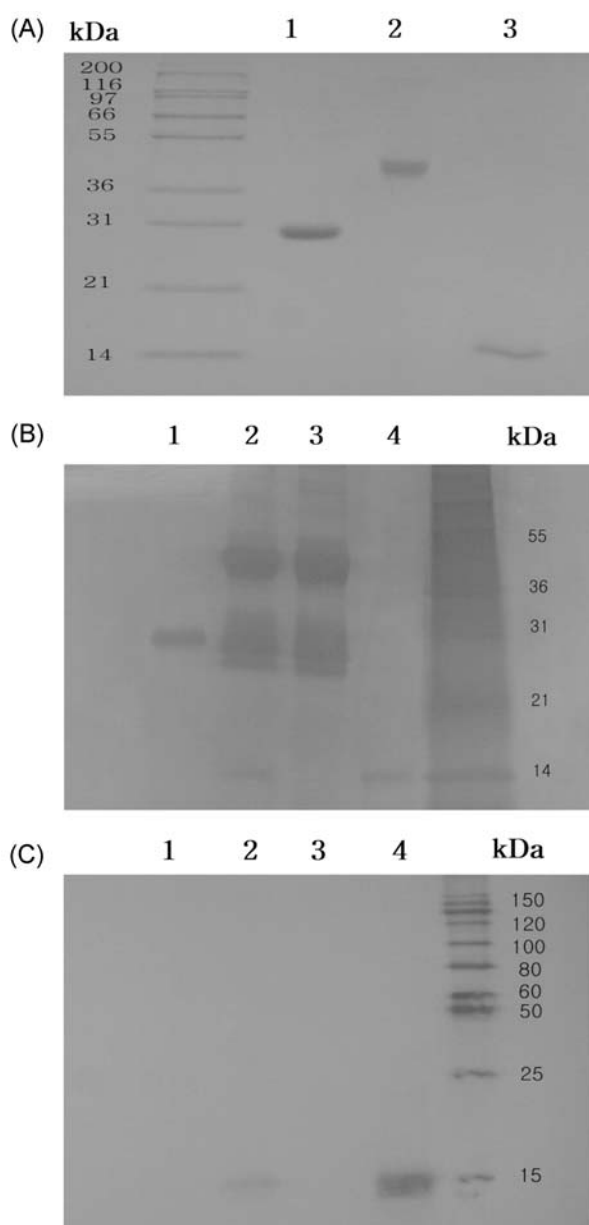
Purification step	Protein concentration (mg/mL)	Volume (mL)	Total protein (mg)
Whole cell extract	10.1	50	503
Soluble supernatant	9.25	40	370
Glutathione agarose column eluted	5.52	3	16.6

demonstrated with both ligand-free and ligand-bound K5.<sup>3</sup> The presence of a lysine binding site can be disregarded when the binding protein for kringle domain is a major concern.

**High level of expression of binding partner protein, Rgl2.** The full cDNA for Rgl2 was amplified by PCR to yield BamHI-XhoI insert which was then ligated into



**Figure 3.** Purification and characterization of Rgl2. Protein samples were taken each step during purification. Samples were subjected to electrophoresis and visualized by Coomassie brilliant blue staining. Samples were whole cell extract without induction (lane 1), whole cell extract with induction (lane 2), and eluted fraction after glutathione 4B agarose column (lane 3).



**Figure 4.** *In vitro* interaction of rK5 and Rgl2 confirmed by GST-pull down experiments. (A) GST alone (lane 1), Rgl2 with GST fusion (lane 2), and purified rK5 with two His tags (lane 3) were visualized in 15% SDS-PAGE gel stained with Commassie blue. (B) Glutathione 4B agarose beads were incubated with GST alone (lane 1), GST-Rgl2 and rK5 (lane 2), and GST-Rgl2 alone (lane 3). rK5 protein (1.2 g) was loaded as a control in lane 4. Bound proteins were eluted with glutathione and separated on 15% SDS-PAGE followed by silver staining. (C) The same gel was blotted onto PVDF membrane and analyzed by Western blotting with anti-His antibody.

pGEX-4T-3 to express a glutathione S-transferase (GST) fusion Rgl2 protein upon induction by IPTG (Figure 1B). Following bacterial cell lysis, a soluble fraction was applied to Glutathione agarose 4B column to affinity purify GST-fused Rgl2. Expression of GST-Rgl2 was also confirmed at every stage of purification (Figure 3). In our work the GST tag was not cleaved off by thrombin treatment since Rgl2

protein was precipitated out during the enzyme treatment. Finally, excess glutathione was removed by dialysis from the purified GST-Rgl2 fraction. The molecular weight of GST-Rgl2 was 47 kDa as indicated in SDS-PAGE. Table 2 indicates that 16.6 mg of recombinant GST-Rgl2 was obtained as a pure protein from 1 L bacterial culture.

**Specific binding of rK5 with its binding partner by GST-pull down experiment.** Having established that K5 interacts with Rgl2 *in vivo* by yeast-two hybrid, we examined whether they interact with each other *in vitro* as well. We utilized recombinant plasmids to express rK5 protein with His-tag attached and Rgl2 protein with GST fused whose purities were checked in Figure 4A. When glutathione agarose 4B beads were incubated with GST and His-tagged rK5, only GST was pulled down at 27 kDa in the silver stained gel (lane 1 in Figure 4B). No band was observed for rK5 in the western blot (lane 1 in Figure 4C). In case of incubation with GST-Rgl2 alone yielded the similar result (lane 3 in Figure 4B and 4C). However, when the beads pre-incubated with GST-Rgl2 were incubated with His-tagged rK5, rK5 was bound to the resin through GST-Rgl2 and finally eluted with competitive binding by GST. The eluted rK5 was clearly observed in the silver stained SDS-PAGE gel (lane 2 in Figure 4B). The presence of rK5 was also confirmed by immunoblot analysis with anti-His antibody as shown in Figure 4C (lane 2). The position of His-rK5 was demonstrated in lane 4 as a control. These results confirmed that the Rgl2 specifically binds with rK5 *in vitro* as well as *in vivo*.

It is often demonstrated that binding *in vivo* is not necessarily the same as binding *in vitro*. This rule is also applied to the biological activities of kringles, in which each kringle is associated with a different level of anti-angiogenic effect *in vivo* and *in vitro*. In the case of the anti-endothelial effect of each kringle, *in vitro* activity decreases as K5 > K1-4 > K1 > K4. On the other hand, *in vivo* study shows that K5 is less active than K1-4 in suppression of angiogenesis in the chicken chorioallantoic membrane assay.<sup>3</sup> Therefore it would be interesting to check if K1-4 also binds *in vitro* with Rgl2 since a stronger binding of K1-4 has been demonstrated *in vivo* (paper in press). Further experiments with K1-4 are in progress.

Developing rK5 as a candidate for a therapeutic application becomes a more fascinating prospect when one considers that the kringles selectively inhibit proliferating endothelial cells but not quiescent endothelial cells. So far, K1-3 has been the most attractive target in developing a new drug for clinical cancer therapy. However, several drawbacks have been reported with the use of K1-3, including the need for high dosages and repeated administration.<sup>15</sup> Our study clearly shows that rK5 is a better choice since it can be easily expressed, readily obtained in large quantities, and most importantly binds to one of the effectors on the Ras pathway leading to anti-angiogenic activity *in vitro* as well as *in vivo*. rK5 can therefore be considered as a more suitable target than K1-3 for development of a new drug for anti-angiogenesis.

## Experimental Section

**Materials.** *Escherichia coli* strain BL21(DE3)pLysS (Novagen, Madison, WI, USA) was used for expression of recombinant proteins. SP Sepharose FF, TALON cobalt resin, and glutathione 4B agarose beads were also from Novagen. PVDF membranes and DNaseI were purchased from Sigma. Protein DC assay kit was purchased from BioRad Laboratories (Hercules, CA, USA). IPTG was obtained from USB. Amicon-10, 50 were purchased from Millipore. Silver staining kit was from Amersham. Rabbit anti-6His antibody (BETHYL, USA) and Goat anti-rabbit conjugated antibody (KPL, USA) were used. All other reagents were of the highest pure grade.

**Construction of recombinant K5 expression plasmid.** Recombinant K5 (rK5) protein was expressed as His-tag attached at both termini using pET-28a-c(+)/K5 plasmid. Construction of pET-28a-c(+)/K5 was performed as follows. K5 insert with BamHI and XhoI sites were prepared from pET-15b/K5 (kindly provided by Dr. Yeom) by polymerase chain reaction (PCR) using two primers (forwarding primer: 5'-CGC GGA TCC ACT TCT GAC TTG GGC TTC CCT-3', reversing primer: 5'-CCG CTC GAG GTA CGG CTT CTC CCC CGA GTG-3'). After ligation with BamHI and XhoI end of pET-28a-c(+) by T4 DNA ligase (Bioneer, South Korea), the recombined plasmid was transformed into DH5. After selection of positive colonies, correct constructs were confirmed by re-amplification of the inserts by PCR using the above primers and subsequent nucleotide sequencing.

**Expression of rK5 protein.** Plasmid DNA was purified from pET-28a-c(+)/K5 construct by GeneMed Cleanup Kit and was transformed into BL21(DE3)pLysS. Cells were cultured in 1 L YTA (16 g trypsin, 10 g yeast, 5 g NaCl) at 37 °C overnight to reach O.D. of 1.0 and they continued to grow for another 3 hr after addition of IPTG to reach a final concentration of 1 mM. After centrifugation at 7,000 rpm for 25 min at 4 °C, pellets were resuspended with binding buffer (20 mM Tris-Cl (pH 8.0), 5 mM imidazole, 0.5 M NaCl). After freezing at -78 °C for 1 hr, the cells were thawed on ice. Kept at 4 °C, cells were treated with DNaseI and 1% Triton X-100 by shaking with a platform shaker for 20 min. Cells were centrifuged at 15,000 rpm for 20 min at 4 °C, supernatant fraction was decanted and inclusion body fraction was recovered. After resuspension with binding buffer supplemented by 8 M urea for 1 hr on ice, the resulting supernatant fraction was collected and applied into TALON cobalt resin pre-equilibrated with binding buffer. Bound proteins were washed with 5 vol of binding buffer followed by 5 vol of wash buffer (20 mM Tris-Cl (pH 8.0), 20 mM imidazole, 0.5 M NaCl). Finally, His-tagged rK5 protein was purified by eluting with elution buffer (20 mM Tris-Cl (pH 8.0), 300 mM imidazole, 0.5 M NaCl), dialysis against 1 mM Tris-Cl (pH 8.0) and 0.1 M NaCl and concentration by amicon filtration to give a final volume of 5 mL rK5. rK5 protein was separated in 15% SDS-PAGE and stained with coomassie blue staining. Alternatively, the

protein samples were visualized by Western blotting, where the membranes were blocked with 0.5 mg/mL BSA in TBS buffer (200 mM Tris (pH 7.5), 5 M NaCl), incubated with rabbit anti-6His antibody (1 : 1,000 diluted) as the first antibody and Goat anti-rabbit conjugated secondary antibody, and visualized with AP-conjugated substrate kit as a color developer.

**Refolding and purification of rK5.** Inclusion bodies were resuspended and solubilized in 40 mL of denaturation buffer (4 M guanidine hydrochloride, 20 mM sodium acetate (pH 5.6), 1 M NaCl, 0.01% Tween-80, 0.05%  $\beta$ -mercaptoethanol). The denatured fraction was rapidly diluted 25-fold with refolding buffer (20 M sodium acetate (pH 5.6), 1 M NaCl, 0.01% Tween-80) and was then added to 1 L pre-chilled refolding buffer without NaCl and gently stirred for 1 hr at room temperature. The refolded protein in 2 L volume was then applied to a SP Sepharose FF column, pre-equilibrated with equilibration buffer (20 mM sodium acetate (pH 5.6), 0.5 M NaCl, 0.01% Tween-80). The column was washed three times with equilibration buffer and the rK5 protein was eluted with 0.5 M-1.5 M NaCl gradient in 100 mL buffer. Protein concentrations of eluted fractions were measured by Protein DC assays and the purity of rK5 was confirmed in 15% SDS-PAGE with coomassie blue staining and western blotting with anti-His antibody as described above. Alternatively, the refolding procedure was carried out by simple dialysis of cobalt column eluted proteins against 10 mM Tris-Cl (pH 8.0), 0.1 M NaCl, and 0.01% Tween-80 at 4 °C for 24 hr. Refolded rK5 was applied into SP Sepharose-FF column and washed with equilibration buffer extensively. Bound fraction was finally purified with 0.5 M-1.5 M NaCl gradient elution.

**Construction of recombinant Rgl2 plasmid.** Rgl2 was expressed as a glutathione S-transferase (GST) fusion protein using pGEX-4T-3. Both inserts and vectors were prepared to contain BamHI/XhoI sites using PCR followed by restriction enzyme treatment and mutual ligation by T4 DNA ligase to generate pGEX-4T-3/Rgl2 plasmid. The primers for amplifying Rgl2 cDNA were 5'-CGC GGA TCCCAC CGT CGC TCA GCC TCC TGT-3' as a forwarding primer and 5'-CCG CTC GAG GAA CAG TGC CCG TGC AAT CTT-3' as a reversing primer.

**Expression and purification of Rgl2 protein.** pGEX-4T-3/Rgl2 was transformed into BL21(DE3)pLysS and plated on YTA. After incubation at 37 °C overnight, a positive colony was selected and cultured in 1 L of LB<sup>amp</sup> to OD<sub>600</sub> = 0.5-1.0, at which point protein expression was induced by 1 mM IPTG. Cells were centrifuged at 6,500 g for 15 min at 4 °C and the collected cells were resuspended in 25 mL Tris-HCl (pH 8.0). The cells were shaken for 20 min at 4 °C in the presence of DNase and 1% Triton X-100. After centrifugation at 15,000 rpm for 20 min at 4 °C, the supernatant fraction was loaded to Glutathione agarose 4B, to which GST-fused Rgl2 protein was subject to bind as described in Ahn *et al.* with a minor modification.<sup>16</sup> The column was washed with PBS buffer extensively and was released the expressed GST-fused Rgl2 protein by washing with elution

buffer (10 mM glutathione, 50 mM Tris-Cl (pH 8.0)). Dialysis was performed in 5 L of 1 mM Tris-Cl (pH 8.0) three times for 3 hr each. The purified protein was concentrated by Amicon-50 filtration to provide 3 mL of Rgl2.

**GST-pull down experiments.** To Glutathione agarose 4B beads, previously washed with PBS buffer in 1.5 mL E. tube, was added 500  $\mu$ L GST-fused Rgl2 (5.5  $\mu$ g/ $\mu$ L). The mixture was shaken for 2 hr at 4 °C and washed 5 times with PBS at 12,000 rpm for 5 min. Then 1 mL of His-tagged K5 (0.12  $\mu$ g/ $\mu$ L) was added and incubated for another 4 hr at 4 °C. The beads were washed 5 times with 1xPBS and the bound proteins were eluted using glutathione elution buffer (10 mM glutathione in 50 mM Tris-Cl (pH 8.0)). Bound K5 in supernatant was identified either by silver staining kit of 15% SDS-PAGE or western blotting of PVDF membrane using anti-6His antibody.

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