http://dx.doi.org/10.5012/bkcs.2012.33.4.1285

Effects of a Phosphomimetic Mutant of RAP80 on Linear Polyubiquitin Binding Probed by Calorimetric Analysis

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Received January 12, 2012, Accepted January 20, 2012

RAP80 plays a key role in DNA damage responses by recognizing K63-linked polyubiquitin moieties through its two ubiquitin-interacting motif (UIM) domains. The linker between the two UIMs possesses a phosphorylation site, but the relationship between phosphorylation and polyubiquitin recognition remains elusive. We investigated the interaction between a phosphorylation-mimic RAP80 mutant S101E and linear polyubiquitins, structurally equivalent to the K63-linked ones, using isothermal titration calorimetry (ITC). ITC analysis revealed differential binding affinities for linear tetraubiquitin by otherwise equivalent UIMs in S101E. Mutational analysis supported such differential polyubiquitin recognition by S101E. Our results suggest a potential crosstalk between polyubiquitin recognition and phosphorylation in RAP80.

Key Words : RAP80, Polyubiquitin, Calorimetry, Phosphorylation, DNA damage response

Introduction

Polyubiquitination as non-degratory signals plays important roles in regulating activities of many cellular proteins. One example is found in DNA damage response pathway. RAP80 (receptor associated protein 80) is a nuclear protein involved in DNA double-strand break repair pathway.[1] Upon DNA damage, RAP80 recruits BRCA1 and its associated proteins to the DNA lesions by recognizing K63-linked polyubiquitin chains on histone proteins H2A and H2AX.[2-4] Central to the role of RAP80 in DNA damage response is the capability to bind K63-linked polyubiquitin chains.

RAP80 contains tandem ubiquitin-interacting motifs (UIMs) connected by a short, seven-residue long linker. Structural and biochemical studies have revealed that the two UIMs and the linker form a continuous helix to bind K63-linked di-ubiquitin in right geometry.[5] In this configuration, the length of the linker, not the sequence, is critical to polyubiquitin binding. Although RAP80 is known to preferentially bind K63-linked polyubiquitin over K48-linked one, it can also bind linear polyubiquitin which is structurally equivalent and functions similarly to K63-linked one in terms of forming foci upon DNA damage.[6]

Phosphorylation can affect ubiquitin binding by proteins involved in DNA damage response. NPM1, for example, binds K63-linked polyubiquitin only when Thr-199 located in UIM-like domain is phosphorylated.[7] Among multiple phosphorylation sites known in RAP80, Ser-101 is located in the linker between the two UIMs. Irradiation activates phosphorylation at Ser-101 *in vivo*.[2,3] However, no convincing data is available for potential crosstalk between phosphorylation at Ser-101 and polyubiquitin binding of RAP80. To assess whether the phosphorylation at Ser-101 affects polyubiquitin binding affinities of RAP80, we employed a phosphorylation-mimic mutant of RAP80, S101E, to measure affinities for linear polyubiquitin chains as a model system. Isothermal titration calorimetry (ITC) was used as a main tool for investigating the effects of phosphorylation on polyubiquitin binding by RAP80. Using two-site model for fitting ITC data, we found that one of the two UIMs shows differential affinities to linear tetraubiquitin in the presence of the phosphomimetic mutant S101E. Our results implicate a potential crosstalk between phosphorylation and polyubiquitin recognition in DNA damage response pathways.

Experimental Section

Cloning and Mutagenesis. Gene encoding RAP80(71-133) was amplified by PCR from full-length human RAP80 (courtesy of Dr. Hongtae Kim) and inserted between *BamH*I and *EcoR*I restriction enzyme sites of parallel GST2 vector.[8] GST-linear Ub_4 was constructed as described elsewhere.[6] GST-linear Ub_2 and GST-RAP80 mutants were generated by using Quikchange II site-directed mutagenesis kit (Agilent Technologies Inc.). The nucleotide sequences of all the mutated genes were confirmed by DNA sequencing.

Protein Expression and Purification. GST-RAP80 and GST-linear polyubiquitin constructs were overexpressed in *Escherichia coli* BL21(DE3) strain. Cells were grown to OD_{600} of 0.8-1.0 at 37 °C, induced with 1 mM isopropyl D-thiogalactoside and further grown overnight at 20 °C. The cells were harvested, resuspended in buffer A (50 mM TrisHCl pH 7.5, and 150 mM NaCl) and lysed by sonication. Cleared supernatant fraction was applied to glutathione-Agarose resin (GE HealthCare) and eluted in buffer B (50 mM Tris-HCl pH 8.0 and 10 mM reduced glutathione). GST-linear polyubiquitins (Ub_2 and Ub_4) were cleaved using tobacco etch virus protease[9] to remove GST. The released GST was cleared using glutathione-Agarose resin. GST-

RAP80 proteins, linear Ub_2 and Ub_4 were further purified on Superdex200 size exclusion column (GE HealthCare) pre-equilibrated with the buffer A. Fractions containing pure protein were pooled and concentrated by centrifugation. Protein concentration was measured by absorbance at 280 nm and Bradford assay.[10]

GST Pulldown. 2 μg of GST-RAP80 was incubated with 20 μL of glutathione-Agarose resin for 30 min. at 25 °C in the buffer A followed by washing three times with the same buffer. Then 500 ng of either linear Ub_2 or Ub_4 was added. The mixture was incubated for 2 hr. at 4 °C followed by washing with buffer C (50 mM Tris-HCl pH 7.5, 300 mM NaCl, and 0.5% NP-40). Bound linear Ub_2 or Ub_4 was detected by immunoblot using anti-ubiquitin or anti-GST (Santa Cruz Biotechnology).

ITC Experiments. ITC experiments were carried out on a VP-ITC (MicroCal). 50 μM GST-RAP80 in the buffer A was placed in the sample cell and titrated with 500 μM linear Ub_2 or Ub_4 in the buffer A in a syringe at 22 °C. Total number of injections was 25 with each having 10 μL in volume. Equilibrium association constants were determined by fitting reference-corrected data using both a one-site and a two-site binding model provided by the manufacturer.

Results

Phosphomimetic and Polyubiquitin Binding-impaired Mutants of RAP80. To investigate a potential crosstalk between phosphorylation and polyubiquitin binding of RAP80 *in vitro*, we prepared a construct covering residues 71 through 133 (Fig. 1). We expressed human RAP80(71-133) as a GST fusion protein in bacteria and purified to homogeneity. Our construct contains tandem UIM domains connected by the seven-residue-long linker harboring Ser-101, a known phosphorylation site. To mimic constitutive phosphorylation on Ser-101 *in vitro*, we mutated Ser-101 to Glu (S101E). Phosphomimetic serine-to-glutamate mutation has been employed to study the effects of phosphorylation on biological functions of proteins *in vitro* and *in vivo*. For example, introduction of serine to glutamate mutation on a known phosphorylation site successfully mimicked phosphorylation-driven tau aggregation,[11] activated an activator of MAP kinases, Mek1,[12,13] and induced dimer dissociation of dynein light chain LC 8.[14] Therefore, it appears legitimate to investigate the effects of phosphorylation in the linker between the two UIMs on polyubiquitin recognition using S101E.

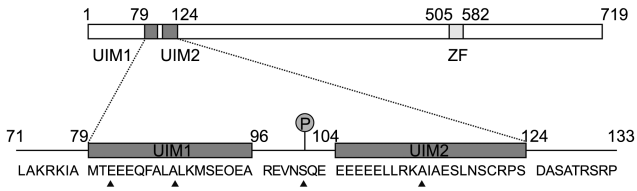

Figure 1. Construct used for this study. Residue numbers for the boundaries of domains and the construct are displayed. Amino acid sequence of the RAP80 construct is shown in one-letter code. Phosphorylation site between the two UIMs is shown. Residues mutated in this study are indicated by filled triangles.

To determine the contribution of each UIM to polyubiquitin binding in the absence and the presence of the phosphomimetic mutant S101E, we prepared a total of 10 mutants (Fig. 1). A88D and A113D are predicted to compromise polyubiquitin binding capability of UIM1 and UIM2, respectively, based on the crystal structure of RAP80 complexed with K63-linked diubiquitin (Fig. S1).[5] ΔE81, found in breast cancer families, reportedly reduces polyubiquitin binding affinity of RAP80.[15] E81A was prepared to examine the effect of ΔE81 at the side chain level. By combining the fore-mentioned mutations, we generated a battery of constructs for investigating the effects of phosphorylation on polyubiquitin recognition.

Effects of Phosphorylation on Polyubiquitin Recognition by Pulldown. To assess whether S101E alters polyubiquitin binding of GST-RAP80(71-133), we performed GST pulldown probed by immunoblotting (Fig. 2). We used linear polyubiquitin chains (tetra- and di-ubiquitin, denoted as Ub_4 and Ub_2, respectively) as a model system because we previously showed that RAP80 binds linear tetraubiquitin.[6] It has been known that linear and K63-linked polyubiquitin chains are structurally equivalent.[16] First, we checked whether

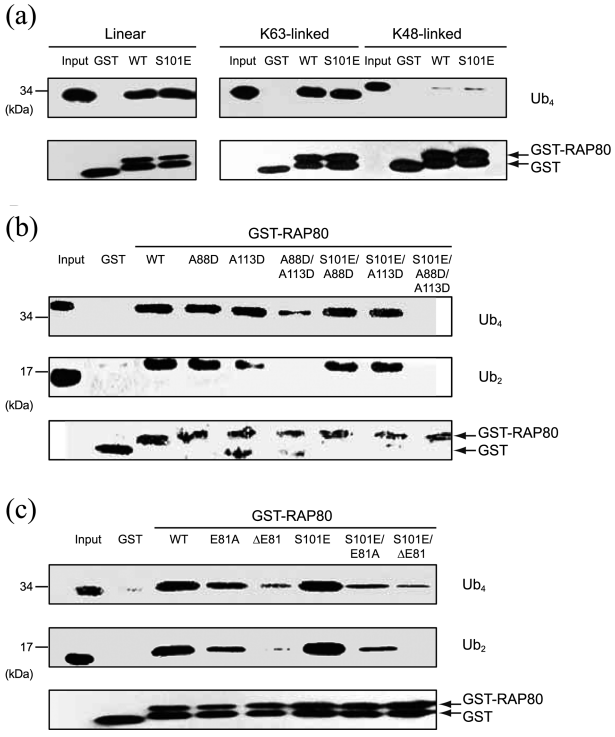

Figure 2. Pulldown assays for the interaction between RAP80 and polyubiquitins. (a) Interaction of wild-type (WT) and S101E mutant of RAP80 with three kinds of tetra-ubiquitin (Ub_4) and di-ubiquitin (Ub_2) chains. GST-RAP80(71-133) was used for pull-down in both WT and S101E. Upper panels, immunoblots after pulldown probed by ubiquitin antibodies; lower panels, immunoblots after pulldown probed by GST antibodies. (b, c) Interaction of RAP80 mutants with linear polyubiquitin chains. Immunoblots after pulldown were probed by ubiquitin antibodies. Input controls are shown in the lowest panels probed by GST antibodies.

S101E shows the same linkage-specific polyubiquitin selectivity as the wild-type (WT) (Fig. 2(a)). As expected, GST-RAP80(71-133) does bind both linear and K63-linked Ub_4, but not K48-linked one. The selectivity of S101E on linkage-specific polyubiquitin chain recognition appears to be the same as WT, suggesting that phosphorylation on S101 imposes no critical impact on polyubiquitin recognition of RAP80. We also found no significant difference in band intensities for binding of GST-RAP80 WT and S101E to linear, K63-linked and K48-linked Ub_4. It seems that S101E does not alter the linkage-specific polyubiquitin chain recognition of RAP80.

Next, we investigated the contribution of each UIM domain in RAP80 to linear polyubiquitin binding and the effects of phosphorylation on linear polyubiquitin recognition (Fig. 2(b)). For this purpose, we prepared mutants known to compromise polyubiquitin binding: A88D for UIM1 and A113D for UIM2. We tested linear polyubiquitin binding of the aforementioned mutants to linear Ub_4 and Ub_2 in the backgrounds of WT and S101E. A88D or A113D alone did not abolish linear polyubiquitin recognition significantly, suggesting that there may be compensatory mechanisms in binding polyubiquitin chains between the two UIM domains. Double mutant A88D/A113D ablated polyubiquitin binding completely for linear Ub_2 but retained weak affinity for linear Ub_4, which appears to be due to avidity. S101E/A88D and S101E/A113D showed virtually the same results as A88D and A113D, respectively. Triple mutant S101E/A88D/A113D completely disrupted linear Ub_2 binding just as A88D/A113D did. Interestingly, A88D/A113D exhibited a very weak interaction with linear Ub_4 but S101E/A88D/A113D did not, implicating that a potential avidity effect on linear Ub_4 binding may be deteriorated by phosphorylation on S101.

We also examined the potential changes in polyubiquitin recognition in the presence of phosphorylation by mutation at Glu-81 (E81) of RAP80 (Fig. 2(c)). E81, located in the UIM1, is reportedly deleted in familial breast cancer patients. ΔE81 reduces polyubiquitin binding and therefore compromises DNA damage response pathway.[15] Simply mutating Glu-81 to Ala (E81A) reduces affinities to linear ubiquitin chains to some extent. By contrast, ΔE81 showed a ultra-weak interaction with linear Ub_4. With linear Ub_2, ΔE81 showed virtually no interaction. Taken together, it seems that phosphorylation and the length of linear polyubiquitin chains play a combined role in modulating polyubiquitin binding affinity of ΔE81. The pulldown results indicate that phosphorylation on S101 apparently causes subtle changes in linear polyubiquitin binding of RAP80(71-133).

Differential Effects of Phosphorylation on Polyubiquitin Binding of Tandem UIMs. To further investigate changes in polyubiquitin binding affinities of RAP80 mutants caused by phosphorylation, we performed quantitative binding analysis using isothermal titration calorimetry (ITC). Initially, we fitted ITC data using a simple 1:1 binding model termed one-site model. However, close inspection of quality of

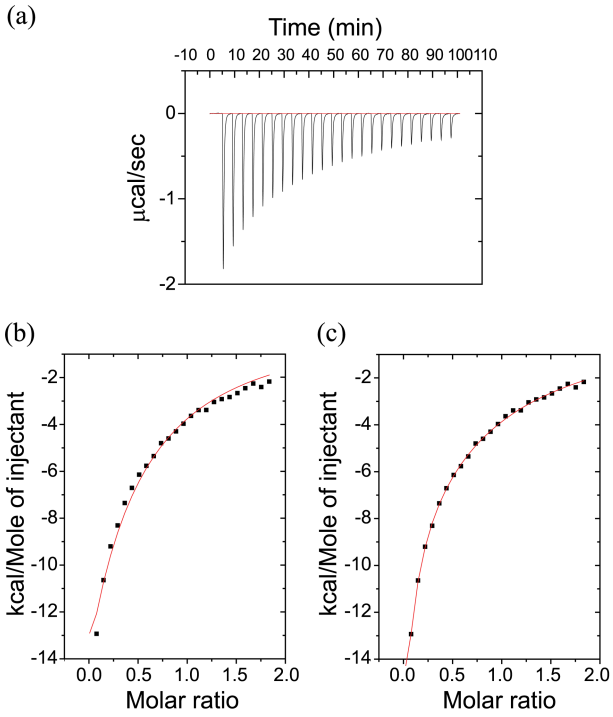

Figure 3. ITC data and fitting with one-site and two-site models. (a) A representative binding isotherms of ITC measurement for the interaction between GST-RAP80(71-133) and linear Ub4. (b) ITC data fitting using one-site model (left) and two-site model (right). Red lines indicate fitted lines by each model.

fitting revealed that a 2:1 binding model, termed two-site model, consistently produced better fitting results (Fig. 3). The employment of the two-site model in fitting ITC data can be justified considering that there are two binding sites on GST-RAP80(71-133), namely UIM1 and UIM2. Another advantage of using the two-site model is that such analysis can reveal the contribution of each UIM of GST-RAP80(71-133) in polyubiquitin binding and thereby that of phosphorylation in polyubiquitin binding.

Quantitative binding analysis using the two-site model on ITC data revealed that phosphorylation at S101 can affect polyubiquitin binding affinity at least in one UIM domain out of two (Figs. 4 and S2-S5; Tables 1 and 2). Data analysis using the one-site model produced no significant difference in polyubiquitin binding affinities of RAP80 by phosphorylation for both linear Ub_4 and Ub_2. Although apparent K_d values of S101E are slightly higher than WT for both linear Ub_4 and Ub_2 (110 *vs.* 78 μM for linear Ub_4 and 140 *vs.* 98 μM for linear Ub_2, respectively), no statistically significant difference judged by *t*-test was observed (Fig. 4(b) and 4(b), left panels). However, ITC data analysis using the two-site model indicated that S101E reduced K_d value for one of the two UIM domains when binding linear Ub_4 (Fig. 4(a), right panel). Such difference in the $K_{d,1}$ values for linear Ub_4 (9 *vs.* 2.7 μM for WT and S101E, respectively; see Table 1) was statistically significant ($P < 0.05$) while the $K_{d,2}$ values statistically remained the same for both WT and S101E, In contrast, no such statistically significant differences in both

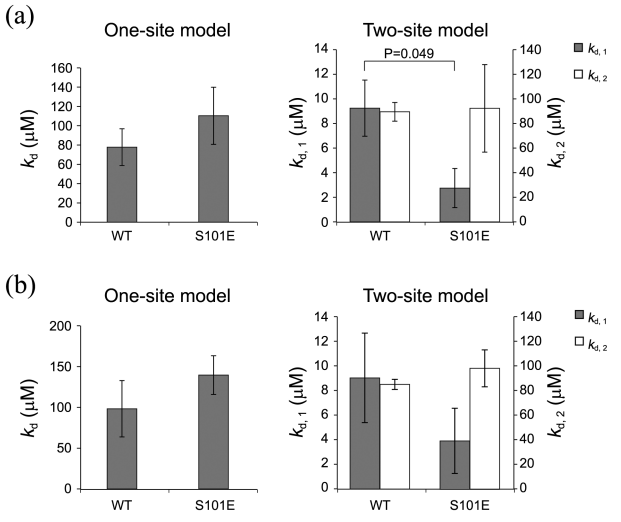

Figure 4. Binding affinities of GST-RAP80(71-133) to linear polyubiquitins. (a) Binding affinities derived by one-site and two-site models for linear Ub4. (b) Binding affinities derived by one-site and two-site models for linear Ub2.

Table 1. Binding affinities of GST-RAP80(71-133) with linear Ub_4

GST-RAP80	One-site model K_d (μM)	Two-site model	
		$K_{d,1}$ (μM)	$K_{d,2}$ (μM)
WT	78 ± 19	9.3 ± 2.3	90 ± 7.6
S101E	110 ± 30	2.7 ± 1.6	92 ± 36
A88D	85 ± 4.3	21 ± 0.21	1,080 ± 25
A113D	54 ± 3.6	14 ± 1.4	350 ± 20
A88D/A113D	n.d.[a]	n.d.[a]	n.d.[a]
E81A	v.w.[b]	v.w.[b]	v.w.[b]
ΔE81	n.d.[a]	n.d.[a]	n.d.[a]
S101E/A88D	45 ± 14	9.0 ± 3.8	113 ± 52
S101E/A113D	73 ± 5.0	7.0 ± 0.74	197 ± 13
S101E/A88D/A113D	n.d.[a]	n.d.[a]	n.d.[a]
S101E/E81A	v.w.[b]	v.w.[b]	v.w.[b]
S101E/ΔE81	n.d.[a]	n.d.[a]	n.d.[a]

[a]K_d was not determined because no binding was observed. [b]Very weak interaction was observed but K_d couldn't be determined reliably.

K_d values were observed for linear Ub_2 (Table 2 and Fig. 4(b), right panel). Thus, our data analysis using the two-site model revealed that two UIMs are differentially affected in their polyubiquitin recognition capability by phosphorylation on S101 and that such effects depend on the length of polyubiquitin chains. Such results suggest that a subtle crosstalk between phosphorylation and polyubiquitin recognition in RAP80 exists and that such crosstalk may be influenced by the length of polyubiquitin chains. Since polyubiquitin chain length is usually longer than two, our results are relevant to the situation *in vivo*.

We then analyzed how polyubiquitin chain recognition by each UIM in RAP80 may be affected by phosphorylation at S101 using the RAP80 mutants mentioned earlier (Tables 1 and 2). A88D and A113D, which are supposed to abolish ubiquitin binding capability of UIM1 and UIM2, respectively, virtually induced no change in K_d values for binding to linear Ub_4 while both mutants decreased affinities to linear Ub_2 about two- to three-fold. For linear Ub_4, each of A88D and A113D decreased affinity of the affected UIM while increasing the affinity of the other, intact UIM (Table 1). In A88D, $K_{d,2}$ value was greatly increased in comparison to WT while for A113D, the extent of increase in $K_{d,2}$ was not so large as for A88D. Such a relatively large increase in $K_{d,2}$ values of A88D and A113D was counteracted in S101E/A88D (1,080 *vs.* 113 μM) and S101E/A113D (350 *vs.* 197 μM). It appears that UIM1 is affected by phosphorylation more in linear Ub_4 binding. Such differential polyubiquitin binding by the two UIMs was also observed in binding to linear Ub_2. Taken together, our results reveal that phosphorylation differentially affects polyubiquitin binding of the tandem UIMs in RAP80.

Table 2. Binding affinities of GST-RAP80(71-133) with linear Ub_2

GST-RAP80	One-site model K_d (μM)	Two-site model	
		$K_{d,1}$ (μM)	$K_{d,2}$ (μM)
WT	98 ± 35	9.0 ± 2.2	150 ± 29
S101E	140 ± 24	3.9 ± 2.7	180 ± 27
A88D	227 ± 6.8	72 ± 17	3,856 ± 2,150
A113D	321 ± 97	83 ± 58	586 ± 286
A88D/A113D	n.d.[a]	n.d.[a]	n.d.[a]
E81A	v.w.[b]	v.w.[b]	v.w.[b]
ΔE81	n.d.[a]	n.d.[a]	n.d.[a]
S101E/A88D	130 ± 6.9	38 ± 9.0	90 ± 17
S101E/A113D	116 ± 8.4	10 ± 7.0	162 ± 36
S101E/A88D/A113D	n.d.[a]	n.d.[a]	n.d.[a]
S101E/E81A	v.w.[b]	v.w.[b]	v.w.[b]
S101E/ΔE81	n.d.[a]	n.d.[a]	n.d.[a]

[a]K_d was not determined because no binding was observed. [b]Very weak interaction was observed but K_d couldn't be determined reliably.

Finally, we attempted to measure K_d values for the rest of the RAP80 mutants with linear Ub_4 and Ub_2, but failed to determine the K_d value for A88D/A113D with linear Ub_4 despite the presence of a weak band on an immunoblot (Fig. 2(b) and Table 1). We interpret these apparent contradictions as the incapability of ITC detecting such a weak interaction. Likewise, we failed to determine K_d values for ΔE81 and S101E/ΔE81 with linear Ub_4 and for E81A and S101E/E81A with linear Ub_2. We detected weak interactions of E81A and S101E/E81A with linear Ub_4, consistent with the result from immunoblots. Nonetheless, we were not able to determine K_d values reliably, presumably due to the technical limitations in ITC. These results imply that differential effects of phosphorylation on polyubiquitin binding of RAP80 are valid only when the tandem UIMs retain basic polyubiquitin binding capabilities.

Discussion

In the present study, we investigated whether there is a crosstalk between phosphorylation and polyubiquitin binding of the tandem UIMs in RAP80 *in vitro* using linear Ub_4 and Ub_2 both qualitatively and quantitatively. Quantitative ITC analysis using the two-site model revealed that one of the two UIMs of RAP80 showed changes in binding affinities to linear Ub_4 by the phosphomimetic mutant S101E. Mutational analysis supported such differential affinities to linear Ub_4 in the presence of S101E. Taken together, our findings suggest that there may be a crosstalk between phosphorylation and polyubiquitin binding in DNA damage response pathways mediated by RAP80. It is not clearly documented whether phosphorylation does affect polyubiquitin binding affinities of RAP80 *in vivo*. A previous study noted that the mutation of Ser-101 to Ala (S101A) formed damage-induced nuclear foci, implicating that the polyubiquitin recognition may not be interfered by the absence of phosphorylation at S101.[2] However, the foci formed by S101A were more dispersive than those by the wild-type, which suggests that lack of phosphorylation in the linker between the two UIMs of RAP80 may have caused such morphological modulation. Thus, one would imagine that phosphorylation might modulate polyubiquitin recognition of RAP80 to the limited extent *in vivo*.

Structural information suggests that the phosphorylation on S101 does not interfere with polyubiquitin binding (Fig. S1). However, our ITC analysis implicates that the conformation around S101 may be subject to a local conformational change, possibly leading to the modulation of polyubiquitin binding capability. Homology modeling and circular dichroism measurements revealed that the linker region shows weaker helical propensity than either UIM and that a loop conformation is possible without ubiquitin binding.[17] In support for the possibility that the linker may possess non-helical conformation, an NMR study uncovered that the linker region can adopt a random coil conformation.[18] Phosphorylation can couple folding and binding of a partially unfolded protein. For instance, phosphorylation on Ser-133 in kinase inducible activation domain (KID) of CREP induces the folding of the C-terminal helix of the phosphorylated KID upon binding to CREP.[19] Therefore, it is plausible that the phosphorylation at S101 of RAP80, albeit no drastic conformational change upon phosphorylation may occur, can modulate local conformational environment in the linker region so that polyubiquitin recognition by the tandem UIMs of RAP80 is affected to some extent.

Acknowledgments. We thank Seunghyun Lee for his contribution in early stages of the project, Kyeongmin Kim for technical assistance in ITC measurements and Prof. Kyeong Kyu Kim for allowing us to access the ITC machine. This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0064219 and 2009-0074586).

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