Characterization of the Kremen-binding Site on Dkk1 and Elucidation of the Role of Kremen in Dkk-mediated Wnt Antagonism[*]

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Wnt signaling is involved in a wide range of developmental, physiological, and pathophysiological processes and is negatively regulated by Dickkopf1 (Dkk1). Dkk1 has been shown to bind to two transmembrane proteins, the low density lipoprotein receptor-related proteins (LRP) 5/6 and Kremen. Here, we show that Dkk1 residues Arg^{197}, Ser^{198}, and Lys^{232} are specifically involved in its binding to Kremen rather than to LRP6. These residues are localized at a surface that is at the opposite side of the LRP6-binding surface based on a three-dimensional structure of Dkk1 deduced from that of Dkk2. We were surprised to find that the Dkk1 mutants carrying a mutation at Arg^{197}, Ser^{198}, or Lys^{232}, the key Kremen-binding residues, could antagonize Wnt signaling as well as the wild-type Dkk1. These mutations only affected their ability to antagonize Wnt signaling when both LRP6 and Kremen were coexpressed. These results suggest that Kremen may not be essential for Dkk1-mediated Wnt antagonism and that Kremen may only play a role when cells express a high level of LRP5/6.

The Wnt family of secretory glycoproteins is one of the major families of developmentally important signaling molecules and plays important roles in embryonic induction, generation of cell polarity, and specification of cell fate. The members are also involved in regulation of a variety of physiological and pathophysiological processes, including bone development, neuronogenesis, adipogenesis, myogenesis, organogenesis, and lipid and glucose metabolism. The canonical Wnt signaling pathway regulates gene transcription by stabilization of β-catenin, which is otherwise degraded by a proteasome-mediated mechanism. This canonical Wnt/β-catenin signaling pathway is initiated by the binding of Wnts to their two coreceptors, LRP5/6[5] and frizzled proteins. LRP5 and LRP6 are single transmembrane proteins that contain four YWTD-epidermal growth factor repeat domains and three low density lipoprotein receptor repeat domains in their extracellular domains. The frizzled proteins are seven transmembrane proteins. Genome sequencing projects have identified 19 Wnt genes in mammals (1–8).

Wnt signaling is also regulated by a number of naturally occurring antagonists that include the Dkk molecules. The first Dkk (*Xenopus* Dkk1) was initially discovered as a Wnt antagonist that plays an important role in head formation (9). To date, four members of Dkk have been identified in mammals (10, 11). However, only Dkk1, 2, and 4 have been documented to function as antagonists of canonical Wnt signaling (12–15). The Dkk molecules contain two conserved cysteine-rich domains (15). Work from our laboratory and others further demonstrated that the second, but not the first, Cys-rich domains of Dkk1 and Dkk2 inhibit canonical Wnt signaling (16, 17). Both Dkk1 and Dkk2 have been shown to bind LRP5/6 with high affinities (12–14). However, they do not appear to inhibit Wnt signaling by directly competing with Wnt proteins because Wnt signaling requires the first two YWTD-epidermal growth factor repeat domains of LRP5/6, whereas Dkk inhibition depends on the third YWTD repeat domain (12, 18).

In addition to LRP5/6, Dkk molecules were also found to bind to another cell surface protein called Kremen (19). It was shown that Dkk was able to simultaneously bind to LRP5/6 and Kremen and that the ternary complex was rapidly endocytosed, thus preventing the Wnt-LRP interaction. However, it is not known how significant endogenous Kremen is in Dkk-mediated Wnt antagonism in mammalian cells, as most of the observations were made with overexpression systems. In this report, we mapped the LRP6- and Kremen-binding surfaces to the opposite sides of the Dkk1 molecules and revealed that Kremen was not required for Dkk-mediated antagonism in 3T3 and HEK293T cells.

MATERIALS AND METHODS

Cell Culture, Luciferase Assay, and Preparation of Conditioned Medium (CM)—The human embryonic kidney cell line HEK293T and the mouse fibroblast cell line NIH3T3 were maintained and transfected as described previously (18, 20). For luciferase assays, 3T3 cells in 24-well plates were seeded at 5×10^4 cells/well and transfected with 0.5 μg of DNA/well, containing 75 ng of luciferase reporter, 25 ng of LEF-1, 150 ng of green fluorescent protein, and 250 ng of LacZ plasmid, by using

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[5] The abbreviations used are: LRP, low density lipoprotein receptor-related protein; CM, conditioned medium; AP, alkaline phosphatase; RLU, relative light unit.

Lipofectamine Plus (Invitrogen). Twenty four h, after transfection, the cells were treated with Wnt3a and Dkk1 CM for 6 h and then lysed directly. Luciferase activity was measured as described previously (20). Luminescence intensity was normalized against fluorescence intensity of green fluorescent protein. For preparation of conditioned medium of wild-type Dkk1 and its mutants, HEK293T cells were seeded in 6-well plates at 4×10^5 cells/well and transfected with 1 μg of DNA/well. Conditioned medium was collected 48 h after transfection. Alkaline phosphatase (AP) activity was then measured and adjusted to the same by adding the control CM, which only transfected with LacZ plasmid. Wnt3a CM was generated from cells stably expressing Wnt3a (ATCC).

Plasmids Construction—The wild-type and mutant forms of mouse DKK1 were generated by PCR using the high fidelity thermostable DNA polymerase *PfuUltra* (Stratagene) and verified by DNA sequencing. AP and FLAG tags were introduced to the N termini of DKK1 and its mutants between the signal peptide and Dkk coding sequence. The expression of these molecules was driven by a cytomegalovirus promoter. The LEF-1 reporter gene constructs were kindly provided by Dr. R. Grosschedl (21).

DKK1-AP Binding Assay—HEK293T cells seeded in 24-well plates at 4×10^5 cells/well were transfected with LacZ or 20 ng/well LRP6 or 50 ng/well Kremen1 by using Lipofectamine Plus; another LacZ was used to make the total DNA amount to 0.25 μg/well. 24 h later, cells were washed once with cold washing buffer (Hanks' buffered salt solution containing 1% bovine serum albumin, 20 mM HEPES, and 0.5% NaN$_3$) and incubated with the washing buffer containing same amount of DKK1-AP or AP-fused DKK1 mutant CMs on ice for 2 h. The cells were then washed three times with the washing buffer and lysed with 1% Triton X-100 and 20 mM Tris-HCl, pH 7.5. The lysates were heated at 65 °C for 10 min to inactivate endogenous AP, and AP activity was measured by using a Tropix luminescence AP assay kit.

Western Blot—Cells were lysed with 30 μl of 2× SDS loading buffer/well in 24-well plates. The lysates were heated at 100 °C for 5 min and then centrifuged at 13,000 rpm for 10 min. Then protein samples were separated by electrophoresis gel and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (135 mM NaCl, 2.7 mM KCl, 24.8 mM Tris-HCl, and 0.05% Tween 20, pH 7.4) and then probed with antibody in Tris-buffered saline/Tween 20 containing 0.25% bovine serum albumin for 1 h at 25 °C. Mouse green fluorescent protein (Covance) was used to probe enhanced green fluorescent protein, and mouse FLAG (Sigma) was used to probe DKK1-AP protein. Blots were then rinsed with Tris-buffered saline/Tween 20, incubated with the rabbit anti mouse IgG-horseradish peroxidase second antibody (Pierce) for 1 h, rinsed with Tris-buffered saline/Tween 20, and exposed with SuperSignal West Pico stable peroxide solution (Pierce).

Data Analysis—Data are shown as the means ± S.E. The AP activity of wild-type Dkk1 in cells expressing Kremen or LRP6 was subtracted from that in cells expressing LacZ and taken as 100%. For Wnt activity data, the difference between the presence and absence of wild-type DKK1 was taken as 100%.
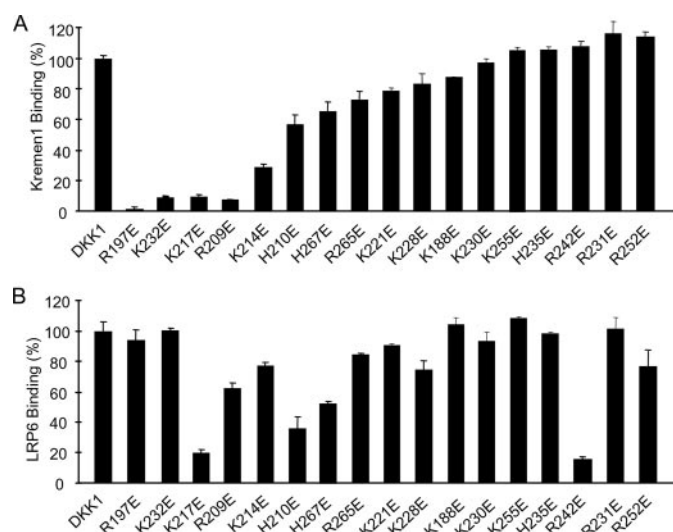
FIGURE 1. **Binding of DKK1 and its mutants to Kremen and LRP6.** HEK293 cells were transfected with LacZ, Kremen1, or LRP6 and incubated with conditioned medium containing Dkk1-AP or its mutants. The AP activity of the conditioned medium was adjusted to the same. The AP activities of the binding of wild-type DKK1 to cells transfected with LacZ, Kremen1, and LRP6 are 2150, 7670, and 17420 RLUs, respectively. The binding activity of the wild-type Dkk1 to cells expressing Kremen (*A*) or LRP6 (*B*) was subtracted by that to cells expressing LacZ and taken as 100%.

RESULTS AND DISCUSSION

We have previously shown that Glu[721] on the third YWTD-epidermal growth factor repeat domain of LRP5 plays a critical role in DKK1 binding (18). Thus, we postulated that there might be an opposite charged residue, namely a basic amino acid residue, on the DKK1 molecule that may form a salt bridge with Glu[721] and hence be required for LRP5/6 binding. Because the second cysteine-rich domain of DKK1 is necessary and sufficient for Wnt inhibition (16, 17), we decided to mutate all of 17 conserved basic amino acid residues within this domain to Glu one by one and test the ability of these mutants to bind to LRP6. We have previously used the Dkk-AP fusion proteins to measure the binding of Dkk to LRP5 and LRP6 (18). Thus, we generated all of these Dkk1 mutants as AP fusion proteins. In addition to LRP5 and LRP6, Dkk1 can also bind to Kremen with a high affinity via its second cysteine-rich domain, and this interaction could also be detected by using the Dkk-AP fusion proteins (19). Therefore, we tested these Dkk1-AP mutants for their ability to bind to both LRP6 and Kremen1 expressed in HEK293T cells compared with wild-type Dkk1-AP fusion proteins. Fig. 1*A* shows the ability of these 17 Dkk1 mutants to bind to Kremen1 relative to the wild-type Dkk1, whereas Fig. 1*B* shows their binding to LRP6. Because we have discussed the LRP-binding surface of Dkk in the accompanying article (23), we focused on Kremen binding in this study. Five Dkk1 mutants, including R197E, R209E, K214E, K217E, and K232E, showed significant loss (>50% reduction) in their binding to Kremen compared to the wild-type DKK1 (Fig. 1*A*). Among these five mutants, R197E and K232E retained the full ability to bind to LRP6, whereas R209E and K214E show weakly compromised (<40% reduction) and K217E strongly compromised (~90% reduction) LRP6 binding (Fig. 1*B*).
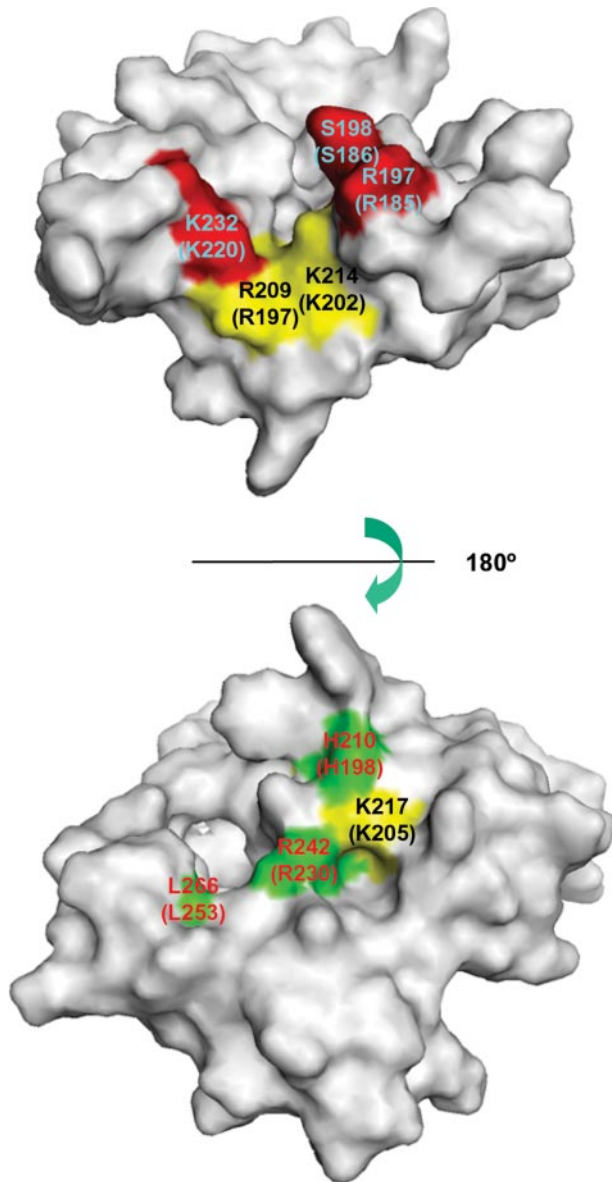
FIGURE 2. **Schematic representation of a deduced Dkk1 structure.** The residues specific for Kremen binding are highlighted in *red*, whereas those for LRP6 binding are in *green*. Those that, when mutated, affect both LRP and Kremen binding are highlighted in *yellow*. The corresponding Dkk2 residues are shown in the *parentheses*.
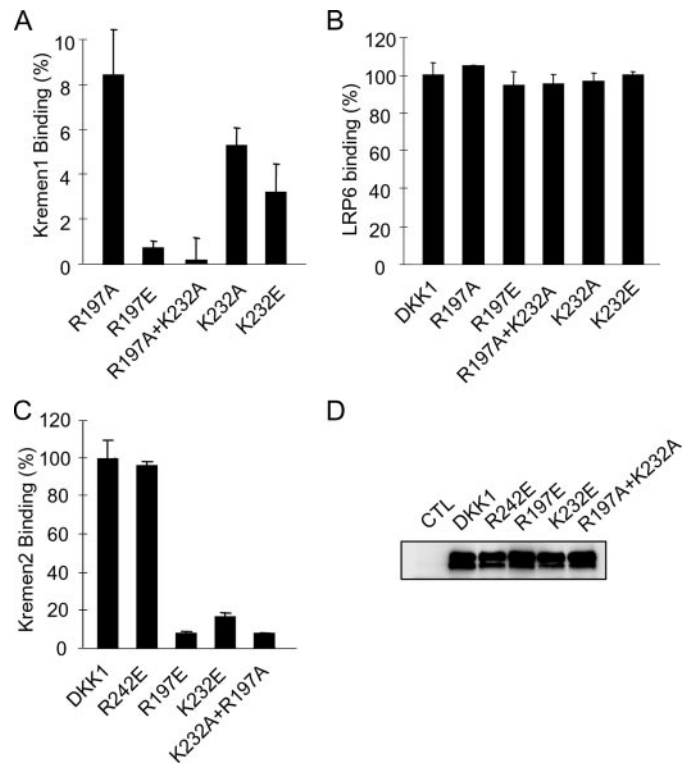

FIGURE 3. **Effect of the substitution of Ala for Arg[197] and Lys[232] on Kremen and LRP6 binding.** HEK293 cells were transfected and assayed as described in Fig. 1. These mutations markedly affected the binding to Kremen1 (*A*) and Kremen2 (*C*) but not to LRP6 (*B*). Dkk levels in the CMs were analyzed by Western blotting (*D*). *CTL*, control.

In the accompanying article (23), we described the solution of the structure of the second Cys-rich domain of Dkk2 and characterized the LRP6 interaction surface. Because Dkk1 and Dkk2 are highly conserved (64.5% identity), we were able to deduce the Dkk1 structure by molecular modeling (Fig. 2) and found that Arg[197], Arg[209], Lys[214], and Lys[232] are located at the opposite side of the LRP6-binding surface that is centered at residues Arg[242] and His[210]. The localization of the LRP-binding and Kremen-binding surfaces to opposite sides of the Dkk molecule is consistent with the observation that both LRP and Kremen can bind to Dkk simultaneously (19). It is interesting to note that Arg[197], Arg[209], Lys[214], and Lys[232] form a valley, with Arg[197] and Lys[232] being the opposing walls and Arg[209] and Lys[214] at the bottom of the valley. Probably because of the location of residues Arg[209] and Lys[214] near the center of the mole-

cule, their mutations may cause some minor alterations in the LRP6-binding surface, which results in the small reduction in LRP6 binding. However, there appears to be no simple answer to the effect of Lys[217] mutation, which resulted in marked reduction in both LRP6 and Kremen binding by >80% (Fig. 1). The structure shows that the amine group of this residue is exposed at the LRP6-binding surface, which is predicted to form a hydrogen bond with Asp[887] of the third YWTD repeat domain of LRP5 (the accompanying article; Ref. 23). However, the rest of the molecule is buried inside. Thus, it is possible that its mutation may also affect overall structural integrity and thus disrupt the binding to Kremen.

Next, we wanted to better understand how mutation of Arg[197] or Lys[232] to Glu resulted in the loss of Kremen binding. The fact that these two mutants still retain their full ability to bind to LRP6 indicates that there should not be a significant global conformational change. Nevertheless, mutation from a basic residue to an acidic residue represents a rather extreme change. Thus, we mutated these two residues to Ala. Although R197A shows slightly more Kremen1 binding than R197E, it is still much weaker than the wild-type Dkk1 in binding to Kremen1 and has only 10% of Kremen-binding ability compared with the wild-type Dkk1 (Fig. 3*A*). On the other hand, K232A showed no difference from K232E at all. Mutation to Ala, like to Glu, did not affect LRP6 binding either (Fig. 3*B*). We also generated a mutant carrying the substitution of Ala for both Arg[197] and Lys[232]. This double mutant showed little binding to Kremen1 while retaining the full binding to LRP6 (Fig. 3). We then tested some of the mutants for their ability to bind to Kremen2.
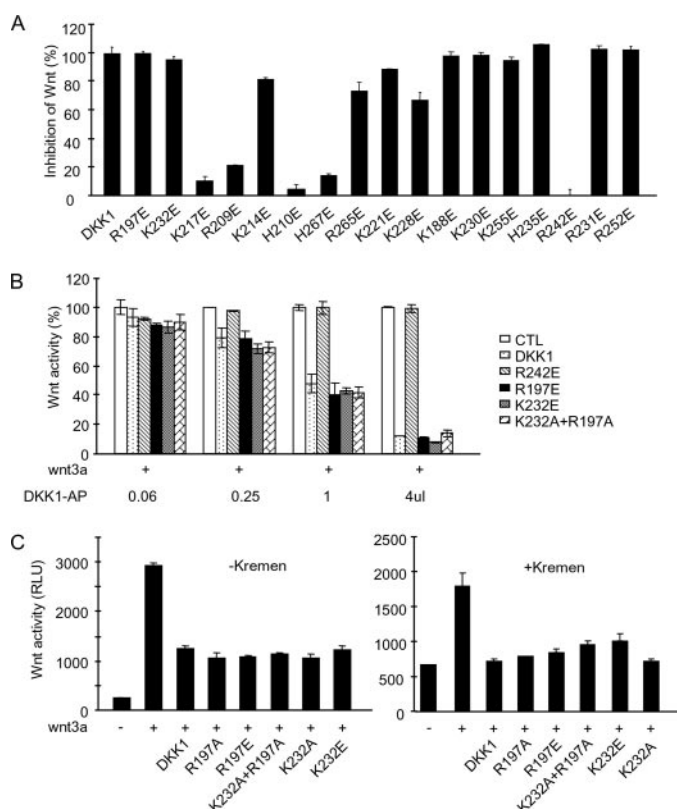
FIGURE 4. Ability of Dkk1 and its mutants to antagonize Wnt activity. *A*, cells were transfected with the LEF-1 reporter gene plasmids for 24 h and treated with Wnt3a CM in the presence of wild-type or mutant DKK1-AP CMs for 6 h. The basal luciferase activity is 265 RLU. The Wnt3a conditioned medium increased the activity to 3210 RLU, and the presence of wild-type DKK1 conditioned medium reduced it to 1220 RLU. The difference between the presence and absence of wild-type DKK1 is taken as 100%. *B* and *C*, cells were transfected and assayed as in *A*. The luciferase activities in cells treated with Wnt3a CM and control CM (*CTL*) are defined as 100% in *B*.

Mutants that have low affinities for Kremen1 also show low binding to Kremen2 (Fig. 3, *C* and *D*). The fact that the double mutant showed less Kremen binding than either K232A or R197A further suggests that both of these residues contribute significantly to Dkk binding to Kremen. Mutating either of them would significantly affect, and mutating both residues would completely abrogate, the binding. The fact that R197E behaves similarly to the double mutant rather than R197A suggests that Arg^{197} may interact with an acidic amino acid on Kremen. When Arg^{197} is mutated to Glu, the mutation would not only prevent the interaction with the presumed acidic residue on Kremen but also might yield an electrostatic repulsion that would further block the interaction. On the contrary, the fact that K232A and K232E showed similar binding to Kremen suggests that Lys^{232}, unlike Arg^{197}, may not interact with an acidic amino acid. Lys^{232} may contribute to the Dkk-Kremen interaction by forming hydrogen bonds with polar residues on Kremen.

Kremen has been previously reported to be involved in Dkk-mediated antagonism of Wnt signaling (19). To test how our Dkk mutants with varying ability to bind to Kremen and LRP6 behave in inhibiting Wnt activity, we examined the ability of all of the Dkk1 mutants to inhibit Wnt activity using a Wnt reporter gene assay that we (16, 20, 22) and others (21) have
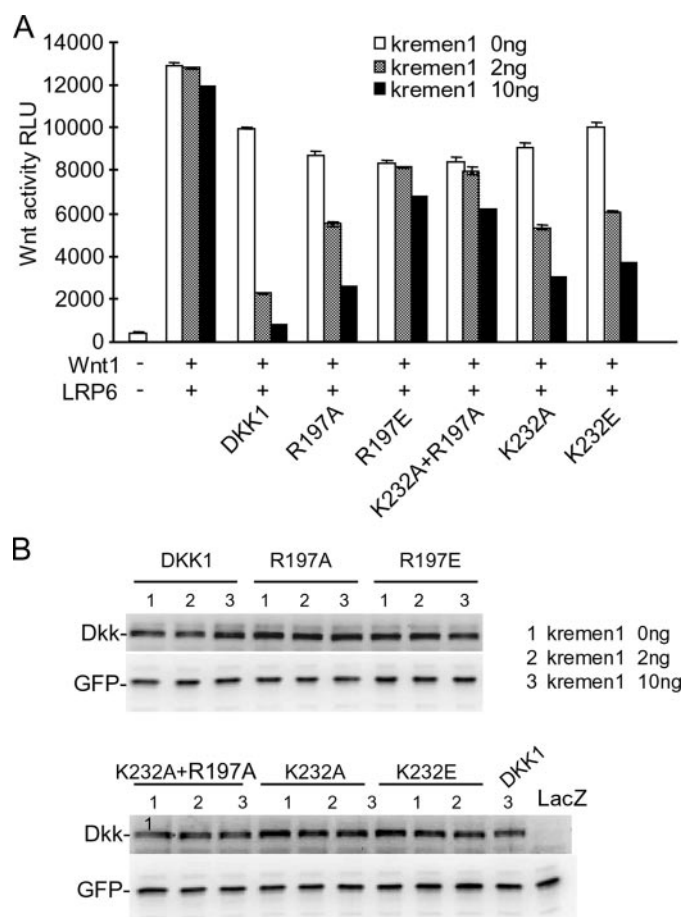
FIGURE 5. Effects of Arg^{197} and Lys^{232} mutation on Wnt antagonism in the presence of overexpressed LRP6. Cells were transfected with the LEF-1 reporter gene plasmids, 20 ng of LRP6, 10 ng of Wnt1, 30 ng of wild-type or mutant DKK1, and varying amounts of the Kremen1 cDNA as indicated in the figure for 24 h. An aliquot of the cell lysate was used to determine the luciferase activity (*A*), and the other was used to determine the expression level (*B*). *GFP*, green fluorescent protein.

used previously. To our surprise, neither R197E nor K232E, which lost most of their ability to bind to Kremen, lost any ability to inhibit Wnt activity (Fig. 4, *A* and *B*). The same is true with other Dkk1 mutants that fail to bind to Kremen but retain full LRP6 binding, including the double mutant with the substitution of Ala for both Lys^{197} and Lys^{232}, regardless of the presence of overexpressed Kremen (Fig. 4*C*). On the other hand, there is a clear correlation between the ability of the Dkk1 mutants to bind to LRP6 and to inhibit Wnt (Fig. 4). Mutants R242E, K217E, R209E, H210E, and H267E that showed lowest binding to LRP6 also showed the poorest ability to inhibit Wnt. We also tested these mutants in HEK293T cells expressing the Wnt reporter gene; the same results were observed (data not shown). These results suggest that Dkk-Kremen interaction is not required for Dkk to inhibit Wnt signaling in HEK293T and NIH3T3 cells.

After carefully reviewing previous work on Kremen (19), we realized that the role of Kremen in mammalian cells was mainly investigated when Kremen and LRP6 were overexpressed. In fact, we were able to reproduce the observations (19); when LRP6 was overexpressed, Dkk1 became inept in inhibiting Wnt signaling unless Kremen was also coexpressed (Fig. 5*A*). We
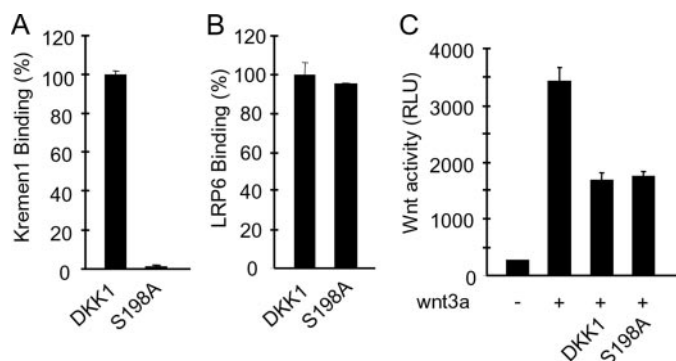
FIGURE 6. **Specific involvement of Ser^{198} in Kremen binding.** *A* and *B*, cells were transfected with Kremen1 (*A*) or LRP6 (*B*) and assayed as described in Fig. 1. *C*, cells were transfected with the Wnt reporter gene plasmid, and Wnt activity was assayed as described in Fig. 4.

also tested those Dkk mutants that show poor binding to Kremen but not to LRP6 for their ability to inhibit Wnt signaling in the presence of overexpressed LRP6. These mutants behaved similarly to the wild-type Dkk in the absence of coexpressed Kremen; they showed poor inhibition of Wnt activity. Of note, what was shown here were the maximal effects under the condition; adding more Dkk could not lead to further inhibition (data not shown). When Kremen was coexpressed, these Dkk mutants showed varying degree of resistance to Kremen-facilitated Wnt antagonism compared with the wild-type Dkk1, with R197E and K232A/R197A showing the most resistance (Fig. 5*A*). The resistance is inversely correlated with the ability of these Dkk mutants to bind to Kremen. Putting together all of the results shown thus far, it is reasonable to conclude that Kremen may not be essential for Dkk to inhibit Wnt signaling and that its role may depend on the levels of LRP6. It appears that only when there is a high level of LRP6, Kremen may be needed to assist Dkk-mediated Wnt antagonism.

To further corroborate our conclusion for the two binding surfaces on Dkk1, we generated a few additional mutants by mutating residues at the binding surfaces based on the three-dimensional structure depicted in Fig. 2. Ser^{198} and Ser^{234}, which are located at the Kremen-binding surface, and Leu^{237} and Gln^{241} at the LRP-binding surface were mutated to Ala to produce S198A, S234A, L237A, and Q241A. Although mutation of Ser^{198} resulted in a specific defect in Kremen binding (Fig. 6*A*), but not LRP6 binding (Fig. 6*B*), the other mutations did not affect either LRP6 or Kremen binding (data not shown).

In summary, our study delineated two surfaces on Dkk molecules, one involved in binding to Kremen and the other to LRP6. Our results also suggest that the interaction between Dkk and Kremen is not essential for Dkk-mediated Wnt antagonism in at least two cell lines (NIH3T3 and HEK293T), but this interaction can play a role in facilitating Dkk-mediated antagonism if the level of LRP5/6 is high.

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