Specificity and Autoregulation of Notch Binding by Tandem WW Domains in Suppressor of Deltex

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WW domains target proline-tyrosine (PY) motifs and frequently function as tandem pairs. When studied in isolation, single WW domains are notably promiscuous and regulatory mechanisms are undoubtedly required to ensure selective interactions. Here, we show that the fourth WW domain (WW4) of Suppressor of Deltex, a modular Nedd4-like protein that down-regulates the Notch receptor, is the primary mediator of a direct interaction with a Notch-PY motif. A natural Trp to Phe substitution in WW4 reduces its affinity for general PY sequences and enhances selective interaction with the Notch-PY motif via compensatory specificity-determining interactions with PY-flanking residues. When WW4 is paired with WW3, domain-domain association, impeding proper folding, competes with Notch-PY binding to WW4. This novel mode of autoinhibition is relieved by binding of another ligand to WW3. Such cooperativity may facilitate the transient regulatory interactions observed in vivo between Su(dx) and Notch in the endocytic pathway. The highly conserved tandem arrangement of WW domains in Nedd4 proteins, and similar arrangements in more diverse proteins, suggests domain-domain communication may be integral to regulation of their associated cellular activities.

The Nedd4 protein family constitute a major class of E3 ubiquitin ligases implicated in a variety of processes including neuronal degeneration (1), blood pressure regulation (2), cell–cell communication and differentiation (3–5), and viral budding (6). Nedd4-like proteins are defined by a modular architecture: an N-terminal C2 domain, two to four WW domains, and a C-terminal catalytic HECT domain. The proteins exert their effects though the regulated trafficking and degradation (via ubiquitination) of targets, usually located in the plasma membrane or nucleus, that are recognized by the WW domains (7).

The Drosophila Nedd4-like protein Suppressor of Deltex, Su(dx),6 has been characterized as a negative regulator of Notch receptor signaling (8–11), an intercellular signaling pathway of fundamental importance for multiple cell fate decisions (12–15). Su(dx) colocalizes with Notch at the adherens junction either at the cell membrane or in vesicles close to the membrane and has been detected in a complex with Notch, a direct or indirect association that requires the WW domains. The apparent influence of Su(dx) is to guide Notch into a post-endocytic down-regulatory pathway (5). A notable feature of the action of Su(dx) on Notch is that the interaction is transient during the time course of Notch endocytosis, thus providing a good model for understanding the regulated assembly and disassembly of WW domain-mediated interactions.

WW domains are well characterized triple-stranded antiparallel β-sheet protein-protein interaction modules containing two conserved tryptophan residues (16–19), the second of which is in a ligand binding pocket. The domains have been categorized into five groups showing affinity for various proline-containing sequences (20–24). The WW domains of the Nedd4 ubiquitin ligase family and, therefore, of Su(dx), are generally in the largest group I category, which bind the PY motif (L/PPXY) (20, 25). Group I WW domains adopt a common recognition mechanism for the L/PPXY core sequence (19, 26–29) in which the highly conserved binding site Trp plays a key role in recognition of the first Pro (or Leu). A small subset of WW domains, including the fourth domain in Su(dx), exist in which this Trp is substituted by Phe or Tyr. Although mutagenesis of the native Trp in Yes kinase-associated protein to Phe abrogates ligand binding (30), the structure of Smurf2 WW3 (which naturally possesses a Phe) bound to a Smad7 PY motif shows the Phe adopting much the same role as the canonical Trp in other WW domains (26).

A significant feature of group I WW domain binding activity is their apparent ability, when isolated from the parent protein, to bind several PY sequences, often with small (e.g. 2-fold) differences in binding affinity (31, 32). An important question, therefore, is how this potential promiscuity of interactions is overcome in an in vivo context. Interactions N- and/or C-terminal to the core PY motif occur in some cases (19, 26, 28), presumably critical for specificity of target selection within the
cell. Adjoining domains might assist binding affinity and specificity, such as the EF hand in dystrophin (27). Interestingly, studies with Nedd4 family members, including mNedd4 and yeast Rsp5 (33–36), fail to separate the functions of two WW domains located in a pair just prior to the catalytic HECT domain. Indeed, WW domains frequently exist in multiple numbers and, perhaps significantly, this arrangement in a protein can be modified by alternative splicing (37). Multiple WW domain modules may function by binding separate targets or act together to increase specificity as demonstrated with tandem SH2 (38–40) and PDZ domains (41, 42). Alternatively they may work together in an autoregulatory manner. The solution structures of paired WW domains for two examples: yeast Prp40 (43) and Drosophila Su(dx) (44), reveal a rigid \( \alpha \)-helical linker in the former case and a flexible linker in the latter. In both examples, the nature of the linker and resultant relative domain orientation has functional implications; Prp40 mediating a bridging interaction and Su(dx) likely undergoing regulated, transient interactions with a number of ligands. The Su(dx) tandem pair is in actual fact poorly structured in its apoform, in part because of a domain-domain association that the flexible linker does not prohibit. Previous NMR data (44) show that both domains attain a folded state upon binding of a PY ligand specific for the first domain. In this study, we further examine the structure and function of the four (paired) tandem WW domains in Su(dx), focusing on their interaction with Notch and revealing unique ligand specificity features. In addition, we address the issue of whether the affinity and specificity of ligand binding by a WW domain located in tandem is influenced by its neighboring WW domain, of potential importance for the co-operative assembly and disassembly of transient regulatory complexes, such as those likely formed by Su(dx).

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Plasmids were constructed with pGEX-6P-1 (GE Healthcare) as the parent vector to enable expression of the following regions of Su(dx): WW1 (amino acids 352–398), WW2 (amino acids 392–431), WW3 (amino acids 471–514), WW4 (amino acids 515–557), WW1–2 (amino acids 352–438), and WW3–4 (amino acids 474–554). Insertion of a DNA fragment encoding Notch region 2319–2333 (NTGAKQPPSYECD1K), prepared by annealing complementary synthetic oligonucleotides, into pGEX-6P-1 (BamHI/XhoI sites) enabled expression of the N3 Notch PY peptide. The Su(dx) WW3 (W505F), WW4 (F549W), and WW3–4 (W505F) mutations were performed by QuikChange (Stratagene) site-directed mutagenesis. Regions of the Drosophila Notch gene encoding fragments, N-PpPS (amino acids 1763–2333) and NAPPS (amino acids 1763–2324), were amplified and cloned into the in vitro translation vector, pSPUTK (Stratagene), between ApaI and XmaI, incorporating a stop codon.

**Protein Sample Preparation**—pGEX-6P-1 transformed *Escherichia coli* BL21 cells were grown at 37 °C until 0.7 \( \text{A}_{600} \) at which stage protein expression was induced by the addition of 0.15 mm isopropyl 1-thio-\( \beta \)-D-galactopyranoside (overnight, 16 °C). Cells were lysed in BugBuster (Novagen) or using a French Press. Protein was affinity-purified using glutathione-Sepharose 4B (GS4B) beads (GE Healthcare) in phosphate-buffered saline, followed by cleavage using PreScission Protease (GE Healthcare). For ITC experiments, the cleaved WW domains were dialyzed against ITC buffer overnight. WW4 and Notch peptide (N3) samples for NMR were produced in a similar manner but were further purified by reverse phase chromatography (PepRPC HR 10/10, Pharmacia) with a gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid before lyophilization and resuspension in NMR buffer (20 mM sodium phosphate, pH 6.75, 50 mM NaCl, 50 mM arginine/glutamate mixture, 1 mM EDTA, 5% D2O). To prepare \( ^{15}\text{N} \)-labeled and \( ^{13}\text{C} \)-labeled NMR samples, cells were grown in M9 minimal medium containing \( ^{15}\text{NH}_4\text{Cl} \) and/or \( ^{13}\text{C} \) glucose and induced for 4 h at 30 °C. Typically, 1 liter of culture would yield 10 mg of purified labeled protein/peptide. All WW protein samples were checked for structure (one-dimensional \( ^1\text{H} \) NMR). \( ^{15}\text{S} \) Methionine-labeled Notch fragments were in vitro translated using the TnT SP6-coupled rabbit reticulocyte lysate system (Promega).

**GST Pull-down Assay**—Recombinant GST (control) and GST-WW domain fusion proteins, immobilized on GS4B beads, were equilibrated in interaction buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 10 mM MgCl2, 10 mM ZnCl2, 5% glycerol, 0.1% Nonidet P-40). Concentrations of immobilized proteins were adjusted by dilution with 50% GS4B slurry in interaction buffer to approximately equivalent levels as observed by SDS-PAGE. Immobilized GST or GST fusion protein (45 \( \mu\)l) was mixed and incubated with reticulocyte extract (45 \( \mu\)l) in 1 ml of interaction buffer (4 °C, overnight). The beads were washed 3 times in 1 ml of interaction buffer before analysis by SDS–PAGE with phosphorimaging (Typhoon 9410, GE Healthcare).

**Isothermal Titration Microcalorimetry**—Concentrations of recombinant proteins and synthetic peptides N1, N2, and WBPI (purchased from Pepceuticals Ltd., United Kingdom) were determined spectroscopically, under denaturing conditions, using Trp and/or Tyr UV absorbance. ITC experiments were performed using a VP-ITC system (MicroCal) at 25 °C with a reference power of 20 \( \mu\text{cal/s} \). Test samples were prepared in 50 mM phosphate buffer, 150 mM NaCl, pH 7.4, and degassed before use.

WW domain protein (15–35 \( \mu\)M) was placed in the 1.4384 ml of sample cell and 1–1.5 mm peptide was titrated in using the automated microsyringe while mixing at 310 rpm. Typically, 1 injection of 2 \( \mu\)l, 4 injections of 5 \( \mu\)l, and 22 injections of 10 \( \mu\)l were performed. These were performed at an injection speed of 0.5 \( \mu\text{l/s} \) with 240 s spacing and initial delay of 60 s. The integrated heat data were corrected for effects of heats of dilution and fitted to a theoretical curve using Origin software (MicroCal), with \( \Delta H \) (enthalpy change in kcal mol\(^{-1}\)), \( K_a \) (association constant in M\(^{-1}\)) as adjustable parameters, and \( n \) (number of binding sites per monomer) fixed at 1. Allowing \( n \) to float during fitting resulted in varying and unrealistic values due to the shape of the binding curves (characteristic of \( K_a < [P] \)), which ill defines \( n \). Where there were potentially two binding sites (for the case of Notch peptide binding to the apo form of WW3–4), the fitting procedure was identical, because fixing \( n = 1 \) makes least assumptions about the binding events and results in the most accurate \( K_a \). However, the value will not be absolutely
unambiguous. For binding of two peptides sequentially to WW3–4, a two-stage titration was performed. At the end of titration 1, the WW3–4 concentration was recalculated (taking into account volume of peptide injected) and the sample volume reduced back down to the cell volume (1.4384 ml) as done at the start; by withdrawing protein solution until at the “lip” level of the cell. Titration 2 was then performed.

NMR Spectroscopy—NMR experiments were carried out at 293 K on a Bruker 600 MHz spectrometer equipped with a cryoprobe. Data were transformed/processed using nmrPipe/nmDraw (45) and CARA (www.nmr.ch) used for backbone and side chain resonance assignments. Two sets of triple resonance experiments were run, one with 13C,15N-labeled WW4 + unlabeled peptide, and another with 13C,15N-labeled N3 peptide and unlabeled WW4. In each case titrations were performed to ensure that the unlabeled component was sufficiently in excess before beginning triple resonance data collection. Standard experiments were run to allow backbone and side chain assignment. Short NOE experiments were run to determine the optimum mixing times for the long three-dimensional NOE experiments (both aliphatic and aromatic). The 15N-NOE experiments were run with and without 13C decoupling to assist in discriminating intermolecular NOEs from intramolecular NOEs. A subset of the experiments performed on the 13C,15N-labeled WW4 + unlabeled peptide were re-run in 99% D2O. To observe structural changes in 99% D2O, experiments were run with and without 13C decoupling to N3, 1H-15N HSQC spectra were recorded at increasing concentration of peptide, up to a molar ratio of 1:4 (WW3–4:peptide). Two-dimensional 1H-15N TROSY spectra were also recorded.

Structure Calculation and Refinement—The protein and peptide sequences were temporarily linked into a single polypeptide chain by an artificial linker sequence (subsequently removed from the final structures). All structural calculations were performed using CYANA (version 2.1) (46). A semiautomatic NOE assignment approach was adopted in which both unassigned NOE peak lists (generated using nmrview) and partially assigned peak lists (CARA) were used as input data. TALOS (47) was used to generate a list of 54 dihedral angle restraints, none of which were excluded from the final structure calculation (including four violations). A total of six interstrand hydrogen bonds were manually input based on D2O solvent exchange data and structural homology to other WW domains. A total of 30 unambiguous upper distance restraints (including 6 protein–peptide contacts) were also manually provided at the start of the structural calculations. The 20 lowest energy structures were selected out of 100 calculated. Each of these 20 structures was individually subjected to energy minimization by the generalized Born solvation model using AMBER (amber.scripps.edu). Non-native N-terminal residues encoded by pGEX vector sequences were assigned and present in the calculated structures but, for simplicity, have been deleted in the structures presented. The data have been deposited to RSCB (PDB code 2JMF; RCSB ID RCSB100015) and BMRB (entry code 15016).

**RESULTS**

**Su(dx) WW Domains Interact with a PPSY Motif in the Notch Intracellular Domain**—Previously, we observed that GST-Su(dx) WW domain fusion proteins can pull down full-length and extracellularly truncated Notch fragments from *Drosophila* S2 lysates (5). To determine which of the four WW domains of Su(dx) are involved in an interaction, whether the interaction is direct or via an intermediate, and whether it requires the single intracellular PY motif (a PPSY sequence) in Notch, further pull-down analyses were performed. In preliminary screens using seven *in vitro* translated Notch intracellular domain fragments (supplemental data), all four Su(dx) WW domains (as GST fusions, Fig. 1B) interacted with Notch fragments containing the PPSY sequence (located between the ankyrin repeats and PEST region). Fig. 1C shows the results of pull-downs using one of these Notch fragments (N-PPSY, Fig. 1A), which, although not strictly quantitative, reveal consistently stronger interactions of the individual WW4 domain with the N-PPSY polypeptide than WW1, WW2, or WW3. The WW3–4 tandem domain pair also demonstrates stronger interaction than the WW1–2 pair (because there are two domains present in the paired constructs, it is not straightforward to compare their pull-down results with those of single domains). All interactions were abolished when the experiment was repeated using the NΔPPSY polypeptide (Fig. 1D). Dependence on the PPSY sequence was further confirmed by making the same construct with a Y2328F mutation (creating a PPSF
motif), which greatly reduced all interactions (data not shown). The weaker interaction of the WW1–2 tandem pair with the PPSY region was complicated by an additional requirement for the Notch RAM domain, N-terminal to the ankyrin repeats. The nature of this complex interaction will be described elsewhere.

**WW4 Displays Greatest Affinity and Selectivity for the Notch PY Sequence**—Having identified direct interactions between Su(dx) WW domains and the Notch PY motif, isothermal titration calorimetry (ITC) was used to obtain quantitative measurements of binding affinity between the domains and Notch peptides containing the motif. The peptide sequences tested are shown in Tables 1 and 2 and representative interactions are detected. The data thus demonstrate that, compared with WW1, WW2, and WW3 domains all interacted with Notch PY motif peptide N1 with a $K_d$ of $\sim$200 $\mu M$, whereas WW4 showed a significantly stronger affinity ($K_d$ of $\sim$45 $\mu M$) (Table 1). WW4 bound peptides N2 and N3 with equivalent affinity to N1, demonstrating that no more than three residues on either side of the PPSY motif are required to achieve maximal binding.

In assessing the binding of Su(dx) WW domains to other PY sequences, in the absence of known in vivo target motifs, we used a WBP1 peptide shown to have high affinity for group I WW domains (48), in particular for the human Nedd4 protein, WWP1. Using NMR and fluorescence spectroscopy (44), the same peptide has been shown to interact with Su(dx) WW3, but not WW4. Using ITC, the WW1, WW2, and WW3 domains showed similar affinities for WBP1 ($K_d$ values 125, 117, and 115 $\mu M$, respectively, Table 1). The $K_d$ for binding of this peptide to WW3 differs to that previously reported (44) using fluorescence methods, primarily due to different buffer conditions (pH and salt concentration). We note that the reproducibility of ITC experiments was exceptionally good and superior to fluorescence detection. Significantly, in our ITC experiments, as with fluorescence assays, no measurable WW4/WBP1 interaction was detected. The data thus demonstrate that, compared with WW1, WW2, and WW3, the greater affinity of WW4 for the Notch PY motif (also indicated in the pull-down experiments, Fig. 1) is specific to that peptide and is not reflected in its interaction with WBP1. Thus WW4 exhibits greater apparent selectivity than WW1, WW2, or WW3.

Given that WW4 has the strongest affinity of all the individual domains for the Notch PPSY sequence, and also possesses

**TABLE 1**

<table>
<thead>
<tr>
<th>Peptide ligands</th>
<th>WW domain</th>
<th>WBP1</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
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<tr>
<td>WW1</td>
<td>125 ± 10</td>
<td>214 ± 3</td>
<td>214 ± 3</td>
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<tr>
<td>WW2</td>
<td>116 ± 12</td>
<td>199 ± 12</td>
<td>199 ± 12</td>
<td>199 ± 12</td>
<td>199 ± 12</td>
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<td>WW3</td>
<td>114 ± 6</td>
<td>208 ± 22</td>
<td>208 ± 22</td>
<td>208 ± 22</td>
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<tr>
<td>WW4</td>
<td>NBa</td>
<td>45 ± 2</td>
<td>45 ± 2</td>
<td>45 ± 2</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>WW3 (W505F)</td>
<td>NB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WW4 (F549W)</td>
<td>31 ± 2</td>
<td>15 ± 1</td>
<td>15 ± 1</td>
<td>15 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>WW4 (F549A)</td>
<td>NB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WW3–4 (W505F)</td>
<td>NB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>

<table>
<thead>
<tr>
<th>Peptide ligands</th>
<th>WW domain</th>
<th>WBP1 then N1</th>
<th>N1 then WBP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WW3–4</td>
<td>185 ± 10</td>
<td>42 ± 1</td>
<td>95 ± 1, NBa</td>
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</table>

| $a$ No data acquired. |
| $b$ NB, no observable binding. |
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of interest here is the observation that the WW4 (F549W) mutant domain was able to bind the WBP1 peptide with a strong affinity (31.0 μM), contrasting with lack of binding by wild-type WW4. The data further imply that wild-type WW4 shows selectivity for the Notch PY region, enhanced by the presence of a Phe, rather than the usual Trp in its binding site, albeit at the expense of binding affinity. The lack of binding of the WW4 (F549A) mutant domain to either the N1 or WBP1 peptides indicates that the Phe does, however, maintain a role in ligand binding. The inverse mutation in WW3 (W505F) abrogates binding to both the N1 peptide and WBP1, implying that the role of the conserved Trp is central to ligand recognition by this domain. Because the WW3 (W505F) mutation is not sufficient to mimic the ligand interactions of WW4, we anticipate that the sequence context of the Trp to Phe replacement in WW4 and interactions with the Notch peptide in regions flanking the core PY may make significant contributions to its ligand selectivity.

The Solution Structure of the WW4-Notch Peptide Complex—A high resolution solution structure of the Su(dx) WW4 domain in complex with a Notch peptide (the N3 peptide, Tables 1 and 2) has been determined using a combination of NMR data obtained for complexes between 13C,15N-labeled WW4 and unlabeled N3, and 15N-labeled N3 and unlabeled WW4 (the 1H,15N HSQC spectrum for labeled WW4 is shown in the supplemental data). Twenty NMR structures were subjected to energy minimization and the final structure statistics are presented in Table 3. Both the WW domain (Su(dx) amino acids 523–552) and the N3 peptide (Notch amino acids 2323–2333) are very well defined, with low backbone root mean square deviations. The interaction between them is also well defined due to a good intermolecular NOE density (Table 3 and supplemental data).

As with other WW domains, WW4 folds into a triple-stranded anti-parallel β-sheet, stabilized by a hydrophobic cluster on its non-binding face (Fig. 3). The peptide adopts an extended PP1I-type helix with a short α-helix at the C terminus that projects away from the binding site (Fig. 3). A similar C-terminal helix has been previously observed for PY ligands of the dNedd4 WW3 and rNedd4 WW4 domains (19, 28). The structure of the complex allows dissection of the Su(dx) WW4 ligand recognition mechanism, separating intermolecular contacts into two categories: those between WW4 and the core PY motif (PPXY) and those with residues flanking the PY core.

Recognition of the PY Motif Core Sequence—As observed in other WW domain complexes, the tyrosine side chain (Tyr2326) of the core PY motif is accommodated in a binding pocket on the WW surface where it primarily contacts Val540, His542, Glu528, and Arg530. A contact of this Tyr with Arg2324, commonly observed in other WW domain complexes (19, 27–29), is not observed. Arg543 in the β2-β3 loop of WW4 is instead making electrostatic interactions with the two acidic residues immediately C-terminal to this Tyr (also see below). The second proline (2326) of the PY motif is accommodated in a hydrophobic pocket (termed the XP groove) formed by a conserved phenylalanine residue (Phe538), the aliphatic portion of Thr547, and Phe549 (the substitutised conserved Trp position). The substitution of the exposed Trp for Phe (Pro549) appears to affect its ability to interact with ligand because the close association of this residue with the first Pro of the PY motif, seen in all other group I WW domain–PY motif complex structures, does not occur. Uniquely, in the WW4-Notch PY complex, Phe549 is situated much further away (5.1 Å) from the first Pro in the PPSY motif, in an orthogonal rather than stacked arrangement (see Fig. 3B). The reduced contribution of Phe549 to the interaction might explain the lack of binding of the WBP1 peptide to WW4, and suggests that residues flanking the PPSY region play an important role in Notch recognition by WW4.

Recognition of Residues Flanking the PY Motif—In the Su(dx) WW4-Notch PY structure, key contacts are made with peptide both N- and C-terminal to the core PY motif, as observed in just one other WW domain–PY motif complex structures, does not occur. Specifically, these involve the N-terminal residues Lys2323 and Glu2324 and the C-terminal residues Glu2329 and Asp2330. The charged group of Lys2323 interacts with that of Glu2356 in the WW4 β1–β2 loop, an electrostatic interaction that would not be able to occur in other Su(dx) WW domains because the equivalent residue is Arg rather than Glu. The aliphatic portion of Lys2323, is accommodated in a shallow hydrophobic groove in WW4 formed by Ala534 and the aliphatic portion of Glu536. The aliphatic portion of Glu2324, in the N3

<table>
<thead>
<tr>
<th>TABLE 3</th>
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<tbody>
<tr>
<td>NMR and refinement statistics for the WW4-N3 complex</td>
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<tr>
<td>Statistics</td>
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<tr>
<td>Distance and dihedral constraints</td>
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<td>Total meaningful NOE constraints</td>
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<td>Total dihedral angle restraints</td>
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<td>Structure statistics</td>
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<td>Violations (mean and S.D.)</td>
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<td>Distance constraints (Å)</td>
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<td>Root mean square deviations from idealized geometry</td>
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<td>Bond lengths (Å)</td>
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<td>Bond angles (°)</td>
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<td>Average root mean square deviations to mean structure (Å)</td>
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<td>Protein (WW4 523–552)</td>
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<td>Complex (WW4 523–552, N3 2323–2333)</td>
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<td>Average pairwise root mean square deviation to lowest energy structure (Å)</td>
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<td>Complex (WW4 523–552, N3 2323–2333)</td>
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<tr>
<td>Ramachandran statistics (%)</td>
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<tr>
<td>Residues in most favoured regions</td>
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<tr>
<td>Residues in additional allowed regions</td>
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<td>Residues in generously allowed regions</td>
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<tr>
<td>Residues in disallowed regions</td>
</tr>
</tbody>
</table>

* Supplementary material includes a list of intermolecular NOEs.
* Calculated for an ensemble of the 20 lowest energy structures.
* No distance restraint in any ensemble structure was violated by more than 0.2 Å.
* After CYANA calculations.
* After AMBER energy minimization.
* Generated using PROCHECK on the full ensemble after energy minimization.
peptide interacts with Ala\textsuperscript{533}, Ala\textsuperscript{534}, and Thr\textsuperscript{532}, again situated in the WW4/H9252\textsubscript{1–2} loop. The C-terminal peptide residues Glu\textsuperscript{2329}/H11032 and Asp\textsuperscript{2330}/H11032 both interact with Arg\textsuperscript{545} in the WW4/H9252\textsubscript{2–3} loop. The more extreme N-terminal (NTG) and C-terminal (CIK) residues of the N3 peptide do not contribute to binding, the peptide backbone extending away from the domain in these regions, in agreement with the equivalent binding affinities observed for the shorter N1 and N2 peptides (Tables 1 and 2). The aforementioned residues in the WW4/H9252\textsubscript{1–2} loop (Thr\textsuperscript{532}, Ala\textsuperscript{533}, Ala\textsuperscript{534}, and Glu\textsuperscript{536}) and Arg\textsuperscript{545} in the WW4/H9252\textsubscript{2–3} loop undergo a negative change in solvent accessible area upon forming a complex with N3 (Fig. 4A), consistent with a contribution to binding affinity and specificity. Furthermore, chemical shift changes observed in WW4 upon titration of a WW3–4 paired domain construct (see below) with N3 peptide (Fig. 4B) implicate the same set of residues in peptide binding. Peptide residues outside the core PY motif thus extend the recognition interface, making interactions with WW4 loop regions that compensate for the reduced interaction of the core PY sequence with the XP binding pocket (see “Discussion”).

**The Tandem Arrangement of WW3 and WW4 Facilitates Cooperative Effects between Ligand Binding Sites**—Previous NMR studies (44) have established that binding of the WBP1 peptide to WW3 within the tandem WW3–4 pair relieves a probable interaction between WW3 and WW4 and facilitates proper folding of both domains. These prior observations led us to postulate that WW4 is consequently better able to bind ligand. Having now identified a WW4 ligand (Notch PY), we can test this hypothesis using a combination of NMR and ITC.

Using NMR, we again assessed changes to fingerprint spectra upon titration of the WBP1 peptide into the poorly structured apo-form of WW3–4 and followed this with titration of the Notch N3 peptide. The first titration confirmed structural changes to both domains and provided evidence of WBP1 binding only to WW3 (in agreement with ITC measurements of peptide binding to the single domains). The details of the changes observed are as previously reported (44) and include, for WW3, chemical shift changes to residues both on (e.g. Tyr\textsuperscript{198}, His\textsuperscript{498}, Thr\textsuperscript{203}, and Trp\textsuperscript{505}) and remote to (Leu\textsuperscript{279} and Trp\textsuperscript{483}) the binding surface, consistent with global conformational change and concomitant peptide binding. Differential line broadening, affecting a number of WW3 β-sheet residues (44), was observed, consistent with structural change and possible interaction of WW3 with WW4. Global chemical shift changes also occurred for residues in WW4 upon WBP1 titration, stopping when saturated. These included a large chemical shift change for Trp\textsuperscript{527}, a crucial part of the hydrophobic core that stabilizes the domain. Notably, residues that would normally partake in binding a PY peptide ligand in contrast exhibit
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FIGURE 4. Individual residue analysis of the WW4-Notch interaction.
A, change in accessible surface area upon interaction between WW4 and Notch. β1, β2, and β3 indicate the position of the WW4 β-sheets. WW4 residues are indicated and represent Su(dx) residues 515 to 557. The N3 peptide is shown above and the N3-WW4 interactions are indicated with lines. B, plot of the WW3–4 chemical shift changes upon peptide N3 binding versus residue numbers. The changes are upon addition of N3 subsequent to saturation binding of WBP1 peptide. Chemical shift changes were calculated using the equation: \( \Delta \delta = \sqrt{0.17(\Delta \delta_{1H})^2 + 6(\Delta \delta_{15N})^2} \). Negative bars indicate residues for which a chemical shift change could not be determined due to unsignaled signals in the HSQC spectrum for the WBP1-bound form of WW3–4, or exchange broadening upon binding of N3 to WW4.

FIGURE 5. Fragment of superposition of two-dimensional \(^{1}H,^{15}N\) HSQC spectra upon sequential titration of WW3–4 with WBP1 and N1 peptides. WW3–4 was overtitrated with WBP1 (GPPPPYPYG) and HSQC spectra recorded at each peptide addition (blue amide signals) followed by N3 (NTGAKQPPSYEDCIK, red signals) titration. Free WW3–4 amide signals are indicated in pale violet (obscured in some cases). With each peptide, titration continued until no further change in chemical shift values were observed. Residues and numbers are indicated next to the final position of their amide signals. Note that the changing protein-peptide molar ratio during titrations may lead to differing degrees of chemical exchange, likely accounting for the observed oscillations in signal intensity. All signals are for WW4 residues, with the exception of His\(^{598}\) (WW3).

relatively small (or no) changes. Line shape changes in WW4 particularly affected residues in the third β-strand, implicating this strand in an interaction with WW3 prior to WBP1 binding. New evidence that the structure of WW4 is affected by domain-domain interaction also comes from a comparison of the fingerprint spectra for the apo forms of WW3–4 and WW4; WW4 amide signals differing significantly between the two spectra (see supplemental Fig. S3A).

We have thus confirmed that domain-domain interaction occurs in WW3–4 (impeding proper folding of both domains) that is relieved upon binding of WBP1 to WW3. Upon subsequent titration of a Notch peptide (N3), changes in amide signals were restricted to WW4 residues that contact peptide in the WW4-Notch PY motif complex structure (Fig. 4B, also compare with supplementary Fig. S3B). Overlaid \(^{1}H,^{15}N\) TROSY spectra before and after this N3 titration are also provided (supplementary Fig. S4), emphasizing the point that only WW4 amide signals are affected. The same figure additionally shows that in a reverse order titration, binding N3 peptide first, WBP1 peptide can subsequently only compete N3 from the WW3 domain, further demonstrating the N3 and WBP1 peptide binding preferences for WW4 and WW3, respectively.

NMR titrations thus support our hypothesis that binding of peptide to WW3 allows WW4 to adopt a “ligand ready” conformation. An additional interesting observation is that some WW4 amide signals moved in two different directions during the sequential titrations, implying that they experience two very different environments. Most marked of these are the changes observed for His\(^{542}\) in the Tyr binding pocket of WW4 (Fig. 5). His\(^{542}\) is just N-terminal to β3 of WW4. Changes in its chemical shift are thus consistent with the proposal that, in its apoform, the β3 strand of WW4 is associated with WW3 and is displaced on WBP1 binding (changing the H542 environment), allowing WW4 to fold and subsequently bind the Notch PY motif (further changing the His environment through interaction with the PY motif Tyr).

To investigate the influence of this domain-domain cooperativity on ligand binding affinities, peptides N1 and WBP1 were used in ITC binding studies with the WW3–4 pair and the data compared with that for individual WW3 and WW4. The N1 peptide reproducibly binds more weakly to WW3–4 than to isolated WW4 (Tables 1 and 2, Fig. 2) (although we note that the \( K_d \) for N1 binding to WW3–4 cannot be determined as precisely as other values in Tables 1 and 2, see “Experimental Procedures”). However, preloading WW3–4 protein with WBP1 (which only binds WW3) increased the subsequent affinity for N1 (Tables 1 and 2) to that observed for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM). Significantly, this increase in affinity is not observed for the WW3–4 (W505F) mutant, in which WW3 cannot bind WBP1, unambiguously demonstrating that ligand binding to one domain does influence ligand binding by the other. A peptide sequence that binds WW4 but not WW3 is therefore required for binding to isolated WW4 (42 μM).
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the apparent $K_d$ for WBP1 binding will always depend on the concentration of N1 present.

The cumulative evidence (NMR and ITC) is thus congruent with a model whereby domain-domain association competes with proper folding and peptide binding. Upon peptide binding to WW3, WW4 is "released" from this inter-domain association and assumes an active, ligand ready, conformation, subsequently binding the Notch peptide with equal capacity to the isolated domain.

**DISCUSSION**

**Su(dx) Interacts Directly with Notch**—Su(dx) interacts with Notch transiently and regulates its endosomal sorting, usually leading to Notch down-regulation (5). Here we have shown that the ability of Su(dx) to interact with Notch is complex, context-dependent and likely involves multiple mechanisms. All four Su(dx) WW domains, whether individual or paired, were found to have the potential to interact directly with Notch via its single PY motif located between the ankyrin repeats and PEST region.

A weaker interaction of the WW1–2 pair may require Notch sequences distant to the PY motif (within the RAM domain), whereas the interaction of the WW3–4 pair depends only on the PY motif and is probably primarily mediated by a strong association of WW4. The multiple interactions of Su(dx) WW domains with Notch may reflect the requirement for regulation of Notch trafficking in different cellular and subcellular contexts involving dynamic assembly and disassembly of complexes. Furthermore, cleavage of Su(dx) has been observed between the WW1–2 and WW3–4 pairs7 and thus it is possible that the truncated forms of Su(dx) can retain an interaction with Notch through independently interacting motifs.

**The Structure of the WW4-Notch PY Complex Reveals Unique Specificity Features**—It is interesting that Su(dx) WW4, which has a replacement of the usually highly conserved binding site Trp with a Phe (Phe549), exhibits the strongest and most selective interaction with the Notch PY motif; the selectivity lost upon mutation of the Phe to Trp. The solution structure of the Su(dx) WW4-Notch PY motif complex reveals the molecular basis behind these observations, particularly on comparison with other solved WW domain complexes. Fig. 6 shows an alignment of Su(dx) WW domains with WW domains of all other group I WW domains for which structures in complex with a peptide ligand are available (brackets are used to emphasize the aligned domains with available complex structures). Residues are color highlighted according to the regions of the PY peptide ligand with which interactions are observed: interaction with core PY residues, pink; interaction with specific residues N-terminal to the core PY motif, yellow; interaction with specific residues N-terminal to the core PY motif, blue. Residues that interact with C-terminal peptide residues and core PY motif residues are indicated in pink with yellow highlighting.

**FIGURE 6.** A comparison of WW domain residues involved in ligand binding. A, the top section of the alignment shows all four WW domains in Su(dx) with native residue numbering as used in the main text. Below the Su(dx) WW domains are aligned all other members of Group I WW domains for which structures in complex with a peptide ligand are available (brackets are used to emphasize the aligned domains with available complex structures). Residues are color highlighted according to the regions of the PY peptide ligand with which interactions are observed: interaction with core PY residues, pink; interaction with specific residues N-terminal to the core PY motif, yellow; interaction with specific residues N-terminal to the core PY motif, blue. Residues that interact with C-terminal peptide residues and core PY motif residues are indicated in pink with yellow highlighting. Weak interactions are highlighted in a paler color and boxed. Residues in the β–β loop are shown in blue text. B, the PY motif peptides bound to the Su(dx), Smurf2, dNedd4, nNedd4, YAP65, and dystrophin WW domains, respectively. Residues that interact with WW domain residues are highlighted and colored according to the description of their location in the peptide: core PY residues (pink); C-terminal to the core motif position (yellow); N-terminal to the core PY motif (blue).

7 M. Baron, unpublished data.
maintain an interaction with the canonical Trp, whereas the peptide also interacts with the $\beta_1$–$\beta_2$ loop (Fig. 7B). $\beta_1$–$\beta_2$ loop residues of Smurf2 WW3 are also crucial for ligand recognition but contact peptide residues C-terminal to the PY core motif rather than N-terminal via a looping back of the C-terminal region of the Smad7 peptide, almost antiparallel with the PY core (26). Thus, although the $\beta_1$–$\beta_2$ loop can undoubtedly provide a specificity determining binding pocket, how an interaction occurs is less predictable.

The third key feature of the Su(dx) WW4-Notch PY interaction is the presence of the electrostatic contacts N- and C-terminal to the core PY motif, which contrast with the predominantly hydrophobic interaction interface present in all other known WW domain complex structures. The interaction between Lys$^{2325}$ in the N-terminal region of the peptide and the $\beta_1$–$\beta_2$ loop residue, Glu$^{236}$, would appear to be crucial to specificity because the equivalent position is occupied by an Arg in all other aligned WW domains from Nedd4 family members (Fig. 6). Further electrostatic salt bridges occur between Arg$^{545}$ and the acidic residues in the helical C-terminal region of the Notch peptide (colored yellow, Fig. 6). The equivalent residue to Arg$^{545}$ in dNedd4 (see Fig. 6) and the other group I WW domains usually interacts (non-electrostatically) with the PY motif Tyr residue, whereas Arg$^{530}$ instead performs this function in Su(dx) WW4. The result is a slightly different positioning of the C-terminal helical portion of the PY peptide in the Su(dx) WW4-Notch PY complex. In this position, residues further to the C terminus of the peptide helix, which are observed to interact with the residue equivalent to Arg$^{530}$ in the dNedd4 and rNedd4 WW domains, cannot do so, in agreement with retention of full binding affinity by truncated Notch peptides.

The Competition between Domain-Domain Association and PY Motif Binding Observed for Su(dx) WW3–4 is the first demonstration that tandem arrangement can directly influence ligand targeting by WW domains. Although we cannot be sure that the tandem WW domains are similarly unstable in the

FIGURE 7. Superposition of Su(dx) WW4-Notch with other WW domain peptide complexes. A, structure superposition of the Su(dx) WW4-Notch PY motif complex with the Smurf2 WW3-Smad7 PY motif complex. The WW domains are superimposed to compare the relative positioning of bound peptide. Both domains are in green with the Smurf2 WW3 conserved binding Phe in pink and Su(dx) WW4 conserved Phe (549) in yellow. The Notch peptide is in red and Smad7 peptide is in blue. Key residues in the L/PPXY sequence (both prolines and tyrosine) are indicated. B, structure superposition of the Su(dx) WW4-Notch PY motif complex with the Smurf2 WW3-Smad7 PY motif and dNedd4-Comm PY motif complexes. All three domains are shown in green, Notch peptide in red, Smad7 peptide in blue, and Comm peptide in yellow. Key residues in the L/PPXY sequence (both prolines/leucine and tyrosine) are indicated. This stereo figure was generated using Swiss PDB (www.expasy.org/spdbv) and PyMol (www.pymol.org).
context of the full Su(dx) protein, their modular nature, small hydrophobic core and potentially "sticky" hydrophobic surface all favor the possibility. Certainly, a contribution of the autoinhibitory mechanism observed in vitro to regulation of the interaction between Su(dx) and Notch in vivo can be envisaged, whereby a ligand binds WW3 and subsequently increases the affinity of WW4 for Notch. Increased Notch ubiquitination by Su(dx) would presumably result. Alternatively, the reverse mechanism may operate, whereby disruption of a WW3-ligand interaction through its ubiquitination by Su(dx) subsequently leads to reduced affinity of WW4 for Notch. Our previous observation that a ubiquitin ligase-defective mutant of Su(dx) prevents onward trafficking of Notch from Su(dx) containing early endosomes (5), indeed suggests that the interaction of the Su(dx) WW domains with Notch is normally self-limiting. A further possibility is that the autoinhibitory mechanism might provide a switch-like response to Notch activation, requiring a threshold level of the Notch intracellular domain before Su(dx) binds effectively. An in vivo ligand for WW3 is yet to be confirmed, although the domain is less selective than WW4. Masking of its binding site by WW4 to reduce its potential promiscuity is another possible outcome of the domain-domain cooperativity observed. In vivo studies are now underway to explore such possibilities.

The mechanism of autoinhibition observed here for tandem WW domains in Su(dx) is unique for tandem intracellular signaling modules, in that it is caused by a direct intramolecular association between the domains that impede their proper fold, whereas tandem PDZ domains in the X11 protein family and tandem SH3 domains in p42phox require interaction with an autoinhibitory region, separate to the domains themselves (49, 50). Interestingly, the last two WW domains in the Nedd4-like family are in a conserved pair in almost all members and thus like family proteins have a conserved linker length (7–10 residues), together with three conserved linker residues (supplementary materials). The influence of linker flexibility and domain-domain proximity on ligand binding by Su(dx) could therefore be a conserved feature of the tandem pair in other Nedd4-like ubiquitin ligases and thus have wider significance. Tandem WW domains also occur in a wide range of other proteins, e.g. the human proteins HYP/C (Huntingtin yeast partners A/C), MAGI1/2/3 (membrane-associated guanylate kinase inverted-1/2/3), WWOX (WW domain containing oxidoreductase), and Yes-associated protein-2. Of these, the tandem WW domains in HYP/C have recently been shown to stabilize on ligand binding (51). Domain-domain communication might thus be a critical facet of selective regulation of the dynamic assembly and disassembly of interactions of WW domain proteins with targets in multiple signaling pathways. This cooperativity may be particularly important given the ability in vitro of isolated WW domains, in particular the Group I class, to bind more than one target PY motif.

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Specificity and Autoregulation of Notch Binding by Tandem WW Domains in Suppressor of Deltex
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