Pivotal Role of the C2 Domain of the Smurf1 Ubiquitin Ligase in Substrate Selection

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The C2-WW-HECT-type ubiquitin ligases Smurf1 and Smurf2 play a critical role in embryogenesis and adult bone homeostasis via regulation of bone morphogenetic protein, Wnt, and RhoA signaling pathways. The intramolecular interaction between C2 and HECT domains autoinhibits the ligase activity of Smurf2. However, the role of the Smurf1 C2 domain remains elusive. Here, we show that the C2-HECT autoinhibition mechanism is not observed in Smurf1, and instead its C2 domain functions in substrate selection. The Smurf1 C2 domain exerts a key role in localization to the plasma membrane and endows Smurf1 with differential activity toward RhoA versus Smad5 and Runx2. Crystal structure analysis reveals that the Smurf1 C2 domain possesses a typical anti-parallel β-sandwich fold. Examination of the sulfate-binding site analysis reveals two key lysine residues, Lys-28 and Lys-85, within the C2 domain that are important for Smurf1 localization at the plasma membrane, regulation on cell migration, and robust ligase activity toward RhoA, which further supports a Ca2+-independent localization mechanism for Smurf1. These findings demonstrate a previously unidentified role of the Smurf1 C2 domain in substrate selection and cellular localization.

Ubiquitin-proteasome-mediated protein degradation exerts critical roles in various biological processes (1–3). In this system, E3 ligases play a crucial role in the specific recognition of substrate proteins (4). E3 ligases mainly fall into two categories as follows: HECT-type E3s and RING finger and U-box E3s. Neuronal precursor cell-expressed developmentally down-regulated 4 (Nedd4) family proteins are representative of the HECT-type E3s. This family of E3 ligases has been characterized to possess similar C2-WW-HECT modular organizations (5). Smurf1 (Smad ubiquitination regulatory factor 1) and Smurf2 are two closely related members of the Nedd4 family that exert negative functions through down-regulation of Smads and membrane receptors in transforming growth factor-β (TGF-β) and BMP4 pathways (6–12). Smurf proteins also function in osteoblast differentiation and regulation by targeting Runx2 and MEKK2 for degradation (13–15). In addition, Smurfs are involved in the regulation of cell motility and polarity by targeting the small GTPases RhoA and Rap1B, as well as the core planar cell polarity protein Prickle1 for degradation (16–19).

E3 assembly and activity control are central issues in understanding their regulation mechanisms (20, 21). Recent research has shown that HECT-type E3s are subject to regulation either by phosphorylation or by adaptor proteins that could facilitate E2 or substrate recruitment (20, 22, 23). The autoinhibition of Smurf2 is achieved through an intramolecular interaction between the N-terminal C2 domain and the C-terminal HECT domain (23). This mode of autoinhibition also occurs in several other members of the Nedd4 family, including Nedd4-1 and WWP2 (WW domain containing E3 ubiquitin protein ligase 2), to regulate the E3 ligase activity and thus protect themselves and their substrates from futile degradation (23). Whether or not Smurf1 is also subject to this mode of regulation is not determined. Although Smurf1 is highly homologous to Smurf2 (sequence identity, 74%) (Fig. 1A), overlapping but distinct substrate and regulator specificity has been observed between Smurf1 and Smurf2 (24–26). Here, our research shows that Smurf1 does not exhibit the same mode of autoinhibition as Smurf2. The Smurf1 E3 activity is naturally maintained at an active status, demonstrating that there is no intramolecular interaction between the Smurf1 C2 and HECT domains. The free C2 domain is further found to be important for Smurf1 localization and determination of its selective activity toward RhoA, Smad5, and Runx2. The crystal structure of the Smurf1 C2 domain further reveals two key lysine residues that are critical for its cellular localization, E3 activity, and function on cell migration and RhoA signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs, Antibodies, and Reagents—Myc-RhoA, Myc-Smad5, and full-length FLAG-Smurfl expression plasmids have been described previously (26). FLAG-Smurfl C2 deletion and lysine mutants and His-C2 (Smurf1 13–150 amino acids) were all constructed by PCR. HA-ubiquitin was a gift from Dr. Debra Parkinson.**

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‡ This article was selected as a Paper of the Week.

‡1 The atomic coordinates and structure factors (code 3PYC) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡4 The abbreviations used are: BMP, bone morphogenetic protein; TRITC, tetramethylrhodamine isothiocyanate; BRE, BMP response element.
**Pivotal Role of Smurf1 C2 Domain**

**A**

![Diagram showing the domain structure of Smurf1 and Smurf2 proteins.](image)

**B**

| Experiment | Description |
|------------|-------------|
| HA-Ub + E1/E2/ATP | + |
| Flag-Smurs | 1 WT, 1 CA, 2 WT, 2 CA |

**C**

| Experiment | Description |
|------------|-------------|
| HA-Ub + E1/E2/ATP | + |
| Flag-Smurs | 1 WT, △ C2, 2 WT, △ C2 |

**D**

| Experiment | Description |
|------------|-------------|
| Myc-RhoA | + |
| Flag-Smurf2 | WT, F29/30A, △ C2 |

**E**

| Experiment | Description |
|------------|-------------|
| Myc-RhoA | + |
| Flag-Smurf | 1 WT, 1 CA, 2 WT |

**F**

| Experiment | Description |
|------------|-------------|
| Myc-RhoA | + |
| Flag-Smurf | 1 WT, 1 CA, 2 WT |

**IB: HA**

**Poly-Ub**

**Input**

**IB: Flag**

**IB: Actin**
from Dr. Yue Xiong (University of North Carolina, Chapel Hill). Myc-Runx2 was provided by Dr. Zhijie Chang (Tsinghua University, Beijing, China). BMP-responsive luciferase plasmid was provided by Dr. Y-G Chen (Tsinghua University, Beijing, China). Smurf2 double phenylalanine mutant (F29A/F30A) was provided by Dr. Jeffrey L. Wrana (Samuel Lunenfeld Research Institute, Toronto, Canada). The proteasome inhibitor MG132 and antibodies used here have been described previously (26).

**Cell Culture, Immunoprecipitation, and Immunoblotting—**HEK293T, MCF7, HepG2, and C2C12 cells were cultured in DMEM supplemented with 10% FBS, penicillin, streptomycin, and glutamine. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Immunoprecipitation and immunoblotting were described previously (26).

**In Vitro Ubiquitination Assays—**Indicated Smurf proteins and truncates were expressed in HEK293T cells and immunoprecipitated for the assays, which were performed in 30 μl of ubiquitination assay buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol, 5 mM MgCl₂, 3 mM ATP), with the addition of 0.7 μg of E1, 1 μg of UbcH5c (E2), 15 μg of HA-ubiquitin (all from Boston Biochem, Cambridge, MA). Samples were incubated at 30 °C for 2 h before Western blotting with anti-HA antibody.

**In Vivo Ubiquitination Assays—**HEK293T cells were transiently transfected with HA-ubiquitin (HA-Ub), Myc-RhoA, Myc-Smad5, or Myc-Runx2 and full-length or deletion and mutant versions of Smurf proteins as indicated. Twenty-four hours after transfection, cells were lysed, and proteins were immunoprecipitated. Ubiquitinated substrates were detected by immunoblotting with an anti-HA antibody.

**Fluorescence Microscopy—**FLAG-tagged Smurf1 full-length, C2 deletion, or lysine mutants and Smurf2 full-length, phenylalanine mutant, and C2 deletion forms were transfected into MCF7 cells. After fixing and permeabilization, cells were incubated with mouse anti-FLAG (dilution 1:50; Sigma) for 1.5 h, followed by incubation with TRITC-conjugated goat anti-mouse IgG (dilution 1:50; Santa Cruz Biotechnology) for 1 h at room temperature. Nuclei were stained with DAPI. Images were acquired with a confocal microscope.

**Protein Expression, Purification, and Crystallization—**His-tagged C2 domain of Smurf1 was expressed in Escherichia coli BL21 (DE3). The harvested bacterial cells were lysed by sonication and purified with nickel-nitriiotriacetic acid beads (Qiagen) and a standard affinity purification procedure. The resultant protein was further purified with a Superdex 200 10/300 GL gel filtration column (GE Healthcare) and condensed at 20 mg/ml concentration in a buffer consisting of 20 mM Tris-Cl, pH 8.0, and 100 mM NaCl. The protein was crystallized using the sitting drop vapor diffusion method in 0.1 M HEPES, pH 6.5, and 2 M ammonium sulfate at 20 °C.

**Data Collection, Phasing, and Model Refinement—**Diffraction data from a single crystal were collected on beamline BL5A at the Photon Factory (Tsukuba, Japan). The diffraction images were integrated and scaled using HKL2000 (27). The structure was solved by the molecular replacement method using the program PHASER (28), and the Smurf2 C2 domain NMR structure (Protein Data Bank code 2QOZ) was employed as a search model. The structure model was built with the program COOT (29) and refined with REFMAC5 (30). All structural figures were drawn with the program PyMOL (DeLano Scientific, San Carlos, CA).

**Luciferase Reporter Assays—**Luciferase reporter assays in HepG2 cells were performed in 24-well plates by transfecting BMP-responsive BRE-luciferase and firefly luciferase reporter plasmid and the control pRL-CMV Renilla luciferase plasmid, together with the indicated Smurf1 expression plasmids. BRE-luciferase activity was determined after treatment with BMP-2 (100 ng/ml; PeproTech) for 12 h using the Dual-Luciferase assay system (Promega) as described previously (26).

**Quantitative Real Time PCR Analysis—**C2C12 cells were transfected with indicated Smurf1 expression plasmids (WT, C2 deletion form, and K28A/K85A mutants) and treated with BMP-2 (100 ng/ml) for 24 h after starvation in serum-free DMEM. Total RNA was extracted from cells with TRIzol reagent (Invitrogen). Reverse transcription was performed using 1 μg of RNA and the TaKaRa RNA PCR kit (AMV) Version 3.0 (TaKaRa). Quantitative PCR was performed using the IQ5 system (Bio-Rad). PCRs were carried out in 25-μl volumes using SYBR Green PCR master mix (Bio-Rad) and 0.4 μM specific primers. Primer sequences have been described previously (see Refs. 14 for alkaline phosphatase; Ref. 15 for osteocalcin and Hprt; and Ref. 40 for Smad6). The level of mRNA expression was normalized to that of Hprt.

**Transwell Migration Assays—**HEK293T cells were transfected with empty vector or various Smurf1 expression plasmids, trypsinized 24 h later, and plated in triplicate on Transwell filters (8-μm pore size, Costar). After 20 h, filters were fixed with methanol for 15 min, washed, and then stained with crystal violet. Nonmigrating cells were scraped off the upper layer, and the bottom layer of the filter was photographed. To quantitate cell motility, the filters were then cut and solubilized in 10% acetic acid, and absorbances at 595 nm were measured.

**Statistical Analysis—**Statistical evaluation was conducted with Student’s t test.

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**FIGURE 1. E3 ligase activity of Smurf1, unlike Smurf2, is not restricted by autoinhibition.** A, schematic diagram of Smurf1 and Smurf2. aa, amino acids. B, in vitro Smurf1 and Smurf2 ubiquitylation assays. Purified HA-ubiquitin, E1, E2 (UbcH5c), and FLAG-Smurf wild types and mutant types were mixed for in vitro ubiquitylation assays and immunoblotted with anti-HA. Input Smurfs are also shown. Ub, ubiquitin; I8, immunoblot. C,-self E3 activity of Smurfs of full-length or C2 deletion mutants was determined in in vitro ubiquitylation assays as in B. D, Smurf2 promotes the degradation of RhoA after its autoinhibition is disrupted. HEK293T cells were transfected with a constant amount of Myc-RhoA and increasing amounts of FLAG-Smurf2 (WT, C2 double phenylalanine mutant, or C2 deletion form). After 24 h, cells were harvested, and aliquots of total lysates were immunoblotted to detect the protein levels of RhoA and Smurf2. E, full-length Smurf1 decreases RhoA steady-state levels, although Smurf2 could not. HEK293T cells were transfected with a constant amount of Myc-RhoA and increasing amounts of FLAG-Smurf1 (WT or CA) and FLAG-Smurf2 (WT). Aliquots of total lysates were immunoblotted to detect the protein levels of RhoA and Smurfs. F, in vivo RhoA ubiquitylation by Smurfs. HA-ubiquitin, FLAG-Smurf1, and Myc-RhoA were transfected together into HEK293T cells. 24 h later, cells were treated with MG132 (10 μM) for 6 h and then harvested. Polyubiquitinated RhoA was precipitated by anti-Myc antibody followed by immunoblotting with anti-HA monoclonal antibody. IP, immunoprecipitation.
Pivotal Role of Smurf1 C2 Domain

A

Myc-RhoA + + + + + + + + + + + +
Flag-Smurfl - - - - - - WT - CA - Δ C2 - Δ C2/CA
IB: Myc
IB: Flag
IB: Actin

B

Myc-RhoA + + + + + + + + + + + +
Flag-Smurfl - - - - - - WT - CA - Δ C2 -
CHX (h) 0 4 8 12 0 4 8 12 0 4 8 12
IB: Myc
IB: Flag
IB: Actin

C

Myc-RhoA + + + + + + + + + + + +
Flag-Smurfl - - - - - - WT - CA - Δ C2 - Δ C2/CA
HA-Ub + + + + + + + + + + + +
IP: Myc
IB: HA
IB: Flag

D

Myc-Smad5 + + + + + + + + + + + +
Flag-Smurfl - - - - - - WT - CA - Δ C2 - Δ C2/CA
IB: Myc
IB: Flag
IB: Actin

E

Myc-Smad5 + + + + + + + + + + + +
Flag-Smurfl - - - - - - WT - CA - Δ C2 - Δ C2/CA
HA-Ub + + + + + + + + + + + +
IP: Myc
IB: HA
Lysate
IB: Myc
IB: Flag

F

Myc-Runx2 + + + + + + + + + + + +
Flag-Smurfl - - - - - - WT - CA - Δ C2 - Δ C2/CA
IB: Myc
IB: Flag
IB: Actin

G

Myc-Runx2 + + + + + + + + + + + +
Flag-Smurfl - - - - - - WT - CA - Δ C2 - Δ C2/CA
HA-Ub + + + + + + + + + + + +
IP: Myc
IB: HA
Lysate
IB: Myc
IB: Flag
RESULTS

E3 Activity of Smurf1 Is Not Restricted by Autoinhibition—
Smurf1 shares high homology and a similar modular architecture with Smurf2 (Fig. 1A). To investigate whether Smurf1 is also regulated by autoinhibition mediated by the C2-HECT interaction, as observed in Smurf2 (23), we first compared the in vitro autoubiquitination of Smurf1 with that of Smurf2. The autoubiquitination of Smurf2 is quite low because of the intramolecular interaction, although Smurf1 on the contrary exhibits much higher autoubiquitination activity (Fig. 1B). We further found that the autoubiquitination of Smurf1 does not change markedly with or without the C2 domain, although the Smurf2 autoubiquitination is indeed dramatically enhanced following truncation of the C2 domain (Fig. 1C). Thus, Smurf1 exhibits natural E3 activity on itself or so-called cis-E3 activity without autoinhibition.

It has been reported that Smurf2 could not promote the degradation of RhoA if its autoinhibition is not disrupted (23). Indeed, we observed that the Smurf2 F29A/F30A mutant, which dissociates the C2-HECT intramolecular interaction and relieves Smurf2 E3 activity, decreased the protein levels of RhoA effectively (Fig. 1D, 5th to 7th lanes), although the wide-type Smurf2 did not (2nd to 4th lanes), consistent with a previous report (23). Surprisingly, if the C2 domain was deleted, the truncated form ΔC2 could not promote the degradation of RhoA (Fig. 1D, 8th to 10th lanes). This result suggested that Smurf2 activity on RhoA (or called trans-E3 activity) not only needs the autoinhibition to be disrupted but also needs to keep the C2 domain.

In contrast to Smurf2, wild-type Smurf1 could effectively decrease the protein level of RhoA, although its E3 ligase-inactive mutant (C699A) could not (Fig. 1E). Further in vivo ubiquitination assays showed that wild-type Smurf1 could promote the ubiquitination of RhoA but Smurf2 could not (Fig. 1F). All of these observations demonstrate that Smurf1 possesses E3 activity naturally, both on itself and on RhoA, indicating that its activity is not restricted by autoinhibition.

C2 Domain Is of Pivotal Importance for Smurf1 E3 Activity on RhoA—The observation that Smurf1 activity is not subject to intramolecular restriction precludes the C2-HECT inhibitory interaction in Smurf1. This then raises the following question. Is there any functional specificity of the Smurf1 C2 domain in substrate selectivity? Following deletion of the C2 domain, we detected Smurf1 activity on RhoA. In contrast to the fact that C2 domain has no influence on Smurf1 autoubiquitination (Fig. 1C), Smurf1 was unable to decrease RhoA protein levels once its C2 domain had been deleted (Fig. 2A). The acceleration of RhoA turnover by full-length Smurf1 was not observed for the Smurf1 C2 deletion mutant (Fig. 2B). In vivo ubiquitination assays also demonstrated that the ubiquitination of RhoA by Smurf1 was reduced after the C2 domain was deleted (Fig. 2C). These results indicate that although the C2 domain is not required for Smurf1 cis-activity on itself, it plays a pivotal role on Smurf1 trans-E3 activity toward its substrate RhoA.

To date, up to 10 substrates of Smurf1 have been identified, and they are either localized at the plasma membrane (for example, TGF-β receptor and RhoA), in the cytoplasm (Smad1/5 and MEKK2), or in the nucleus (Smad1/5, JunB, and Runx2). Among these substrates, RhoA is localized at cellular protrusions where Smurf1-targeted RhoA degradation occurs, and this regulation is functionally relevant to the epithelial to mesenchymal transition and cell migration (16, 18). We then asked whether the C2 domain also plays a similar key role in the Smurf1 E3 activity toward other substrates such as Smad5 and Runx2. Surprisingly, Smurf1 decreased the Smad5 protein levels more dramatically following deletion of the C2 domain (Fig. 2D). In vivo ubiquitination assays further confirmed the enhancement of E3 activity on Smad5 by the C2-truncated Smurf1 form (Fig. 2E). Similar to Smad5, the protein levels of Runx2 were more effectively decreased by Smurf1-ΔC2 than Smurf1-ΔC2.

TABLE 1

Summary of crystallographic statistics

| Data collection | P2, 2, 2, | a = 30.959, b = 47.048, c = 91.266, α = β = γ = 90
| Wavelength | 1.5418 Å |
| Resolution | 50 to 1.96 Å (2.01 to 1.96 Å) |
| Measured reflections | 73,595 |
| Unique reflections | 10,113 |
| Completeness | 99.6% (95.2%) |
| Rmerge | 0.040 (0.137) |
| Redundancy | 7.3 (6.5) |
| l/i | 56.5 (15.8) |

Refracture

| Rmerge | 0.206 |
| Root mean square deviation bond lengths | 0.018 Å |
| Root mean square deviation angles | 1.7° |
| Average B factor | 23.8 Å² |
| Ramachandran plot | 98.4%/1.56%/0% |

a Numbers in parentheses refer to the statistical data of the outer shell.

b Calculated using MolProbity. Numbers reflect the percentage of residues in the preferred, allowed, and disallowed regions, respectively.

c Calculated using MollProbity. Numbers reflect the percentage of residues in the preferred, allowed, and disallowed regions, respectively.

d Calculated using MolProbity. Numbers reflect the percentage of residues in the preferred, allowed, and disallowed regions, respectively.

FIGURE 2. Depletion of the C2 domain inhibits Smurf1 activity on RhoA while enhancing its activity on Smad5. A, expression of full-length Smurf1 decreases RhoA steady-state protein levels in vivo, although C2 deletion mutants lost the E3 activity. HEK293T cells were transfected with a constant amount of Myc-RhoA and increasing amounts of full-length Smurf1 (WT or CA) and C2 deletion mutants (WT or CA). Aliquots of total cell lysates were immunoblotted with anti-Myc antibodies. B, half-life analysis of RhoA in the presence or absence of overexpressed Smurf1 wide type and C2 deletion mutant. HEK293T cells were transfected with Myc-RhoA with or without FLAG-Smurf1 (WT and ΔC2). 24 h later, cells were treated with cycloheximide for the indicated times and then lysed for protein level analysis. CHX, cycloheximide. C, in vivo RhoA ubiquitination by Smurf1 (WT) and Smurf1 (ΔC2) was analyzed. Indicated plasmids were transfected into HEK293T cells. Polyubiquitinated RhoA was precipitated and determined. IP, immunoprecipitation. D, contrary to the effect on RhoA, Smurf1 C2 deletion mutant decreases the Smad5 protein levels more effectively than the full-length Smurf1. Similar experiments were carried out as in A. E, in vivo polyubiquitination of Smad5 is more effectively promoted by Smurf1 with C2 deleted. Similar in vivo ubiquitination assays were carried as in A. E, and F. 

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Pivotal Role of Smurf1 C2 Domain

A

Smurf1-C2

PKC-C2 +Ca\(^{2+}\) + PS

B

C

D

E

Smurf1-C2

PKC-C2

K28 S1 K85

β6 β5 β2
the wild-type Smurf1 (Fig. 2F, 8th to 10th lanes). In vivo ubiquitination assays showed that the Runx2 ubiquitination induced by Smurf1 was even enhanced when the C2 domain was deleted (Fig. 2G, 4th lane). The above results demonstrate that the C2 domain exerts differential effects on Smurf1 activity toward different substrates, such as RhoA, Smad5, and Runx2.

Crystal Structure of the Smurf1 C2 Domain—The C2 domain was first identified as the Ca\(^{2+}\) binding domain in conventional protein kinase C (PKC) to mediate protein membrane localization (31, 32). The above results showed that the C2 domain in Smurf1 does not exhibit an autoinhibitory role but is important for its substrate selectivity; so we speculated whether this function of Smurf1 C2 is related to its role as a localization mediator. Smurf1 has been reported to localize at the plasma membrane in a Ca\(^{2+}\)-independent manner (11). Smurf1 C2 may take a different way to bind membrane phospholipids without Ca\(^{2+}\) as a bridge. We crystallized the C2 domain (residues 13–150) of human Smurf1 and determined its structure at 2.0 Å resolution. Refinement of the protein structure resulted in a final model with crystallographic R-factor (R\(_{cryst}\)) of 0.204 and a free R-factor (R\(_{free}\)) of 0.241. Each asymmetric unit contained one C2 domain containing 132 amino acid residues, 54 water molecules, 1 chloride ion, and 2 sulfate ions. The final structural model has excellent stereochemical quality with no amino acid residues located in disallowed regions of the Ramachandran plot and a final average temperature factor (B-factor) of 23.8 Å\(^2\). Final coordinates of the Smurf1 C2 domain and experimental structure factors have been deposited in the Protein Data Bank with accession number 3PYC. Data collection and refinement statistics are summarized in Table 1.

The Smurf1 C2 domain folds into a typical type II β-sandwich composed of four-stranded anti-parallel β-sheets, plus an additional α-helix that connects β-strands 6 and 7, as shown in Fig. 3A. The C2 domain generally contains five aspartate residues that form a calcium-binding motif that mediates protein-membrane associations (33, 34). The equivalent region in the Smurf1 C2 domain binds one sulfate ion (S1, sulfate ion 1) (Fig. 3B, top panel). Analysis of the surface electrostatic potential shows that the Ca\(^{2+}\) binding region in PKC is negatively charged, and the corresponding region in Smurf1 C2 domain is positively charged (Fig. 3B, bottom panel). The different surface electrostatic potentials of Smurf1 C2 and PKC C2 domains may help to explain why they bind ions with opposing electrical charges. A structure-based sequence alignment of the Smurf1 C2 domain with other C2 domains is shown in Fig. 3C. The second sulfate ion (S2)-binding site, where phosphatidylinositol-glycercide generally locates, is well conserved (two residues in green) (Fig. 3C). Among the five aspartate residues involved in Ca\(^{2+}\) and phosphatidylserine binding in all other C2 domains, only two of them are conserved in Smurf1, although the remaining three residues are substituted by neutral or positively charged residues (Fig. 3C, asterisks). Furthermore, many positively charged residues are located around this region in the Smurf1 C2 domain, thus accounting for the positive electrostatic surface potential exhibited in this region. The structure of Smurf1 C2 domain is highly conserved with the Smurf2 C2 domain (Fig. 3D), which is consistent with the high homology between their C2 sequences (92% identity). Two lysine residues in this region, Lys-28 and Lys-85 (blue in Fig. 3C), are responsible for the sulfate ion binding (Fig. 3E). These two residues are also conserved in Smurf2 (Fig. 3C).

Lys-28 and Lys-85 in Smurf1 C2 Domain Play an Important Role for Smurf1 Cellular Localization and E3 Function—The sulfate-binding sites identified in the Smurf1 C2 domain are potential binding sites for phosphate(s) headgroups of phospholipids, based on the high similarity between sulfate and phosphate ions. We examined the cellular localization of Smurf1, with or without its C2 domain, and also a mutant in which the two sulfate-binding lysine residues were mutated to alanine (K28A/K85A). The full-length Smurf1 was mainly localized in a plasma membrane-associated manner (Fig. 4A), and the mutant lacking the C2 domain was distributed to the whole cytoplasm (Fig. 4A). Intriguingly, the K28A/K85A mutant exhibited a similar change in localization as the C2-truncated form (Fig. 4A). We further detected Smurf2 localization in cells and found that wild-type Smurf2 was localized in the whole cell, whereas the F29A/F30A mutant, which disrupts the autoinhibition, changed its localization to the plasma membrane where RhoA is localized (Fig. 4A). In contrast to the F29A/F30A mutant, Smurf2-ΔC2 lost the ability of the plasma membrane localization and redistributed to the cytoplasm and also the nucleus, where RhoA was not localized (Fig. 4A, bottom panel). The localization difference between ΔC2 and F29A/F30A should explain why they possess different abilities to regulate the stability of RhoA (Fig. 1D).

Lys-28 and Lys-85 are not only important for Smurf1 cellular localization but also exert key roles for the Smurf1 ligase activity toward RhoA, because the double mutant form (K28A/K85A) of Smurf1 could not decrease the RhoA protein levels as effectively as the wild type (Fig. 4B). The half-life of RhoA was dramatically longer in the presence of the Smurf1 K28A/K85A mutant than in the presence of wild-type Smurf1 (Fig. 4C). Nonetheless, the Smurf1 K28A/K85A mutation had no significant effects on the half-life of Smurf1 itself. We further observed the decrease of Smurf1 activity on RhoA through in vivo ubiquitination assays, which clearly showed that Smurf1 E3 activity on RhoA was largely inhibited by this double mutation (Fig. 4D).

**Figure 3. Structure of the Smurf1 C2 domain.** A, ribbon representation of the overall structure of the Smurf1 C2 domain. The structure is colored gray, and two sulfate ions bound to the Smurf1 C2 are indicated and rendered as sticks with oxygen atoms colored in red and sulfur atoms in yellow. B, oppositely charged ion binding of Smurf1 C2 domain and PKCa C2 domain (top panel), and different electrostatic potential surfaces are indicated with arrows (bottom panel). PS, phosphatidylserine. C, structure-based multiple sequence alignment of C2 domains in Smurf1 and other proteins. The top three C2 domains are from *Homo sapiens* and *Rattus norvegicus*, and the Smurf1 and Smurf2 are from *H. sapiens*; the other six are all from the C2A domains of *R. norvegicus* proteins: α, β, and γ subclasses of protein kinase C, three homolog forms of synaptotagmin-1, DODCA, DOC2B, and rabphilin-3A. Secondary structure elements according to the Smurf1 C2 domain are labeled at top of alignment. D, comparison of the crystal structure of Smurf1 C2 domain with the NMR structure of Smurf2 C2 domain. The C2 domains of Smurf1 and Smurf2 are colored yellow and blue, respectively. E, interactions between the sulfate ion and binding residues of the C2 domain are shown, and the binding residues are pointed out with numbers. PyMol was used to generate A, B, D, and E.
Pivotal Role of Smurf1 C2 Domain

**Panel A**

Flag-Smurfl (WT)

Flag-Smurfl (Δ C2)

Flag-Smurfl (K28,85A)

Flag-Smurfl2 (WT)

Flag-Smurfl2 (F29,30A)

Flag-Smurfl2 (Δ C2)

**Panel B**

Myc-RhoA + + + + + +

Flag-Smurfl WT K28,85A

IB: Myc

IB: Flag

IB: Actin

**Panel C**

Myc-RhoA + + + + + +

Flag-Smurfl1 WT K28,85A

CHX (h) 0 4 8 12 0 4 8 12

IB: Myc

IB: Flag

IB: Actin

**Panel D**

Myc-RhoA + + + + + +

Flag-Smurfl1 WT CA K28,85A

HA-Ub + + + + + +

IP: Myc

IB: HA

Lysate

IB: Flag

**Panel E**

Control

Flag-Smurfl1

Flag-Smurfl1 (Δ C2)

Flag-Smurfl1 (K28,85A)

Graph showing Q.D. 595nm

Flag-Smurfl1 – WT Δ C2 K28,85A

**Panel F**

Graph showing Relative BRE activity (fold)

BRE-luc + + + + + +

BMP-2 – – WT Δ C2 K28,85A

Flag-Smurfl1 – – WT Δ C2 K28,85A

**Panel G**

Graph showing Relative mRNA level

ALP

Osteocalcin

BMP-2 – – + + +

Flag-Smurfl1 – – WT Δ C2 K28,85A

Flag-Smurfl1 – – WT Δ C2 K28,85A

**Panel H**

Graph showing Relative mRNA level

Smad6

BMP-2 – – + + +

Flag-Smurfl1 – – WT Δ C2 K28,85A
Smurf1 was reported to promote cell migration and polarity through degradation of RhoA at cellular protrusions (18). To determine the role of the Smurf1 C2 domain on this process, we performed transwell assays by transfecting Smurf1 wide type and the indicated mutants in HEK293T cells. Cell migration was obviously enhanced by ectopic wild-type Smurf1 expression; by contrast, Smurf1 ΔC2 or K28A/K85A mutant had no significant effect on cell migration (Fig. 4E), indicating the requirement of Lys-28/Lys-85 within the C2 domain in migration enhancement. The results of this functional assay are consistent with the regulatory effects of Smurf1 mutants on RhoA levels.

We also detected the role of the C2 domain in Smurf1 function on the negative regulation of BMP signaling and Smad5 transcriptional activity. We performed luciferase reporter assays using a BMP-responsive reporter, BRE-luciferase, and quantitative real time PCR analysis. As shown in Fig. 4F, BMP-2 treatment remarkably stimulated BRE-luciferase activity. Smurf1 C2 deletion or K28A/K85A mutation inhibited the activity of BRE-luciferase as well as did the wild-type Smurf1 (Fig. 4F). Furthermore, we showed that Smurf1 C2 deletion or K28A/K85A mutation inhibited the expression of BMP-2-induced downstream target genes, including ALP, osteocalcin, and Smad5, as well as did the wild-type Smurf1 (Fig. 4G). All of the above results suggest the importance of the C2 domain for Smurf1 and reveal two lysine residues, Lys-28 and Lys-85, that play a key role in Smurf1 localization and selective ligase activity toward its substrates with different cellular distribution.

**DISCUSSION**

Smurf1 and Smurf2 share most of their substrates, although each possesses their own specific targets (7–19, 35, 36). The substrate overlap and specificity between Smurf1 and Smurf2 may partially lie in the WW domains, which adopt a similar coupled arrangement to bind substrates, although different linker lengths determine the binding affinity (37).

Recently, both Smurf1 and Smurf2 HECT domains were found to possess ubiquitin-binding surfaces, which promote substrate polyubiquitination (38). However, the regulation of Smurf activity also shows specificity, such as the CKIP-1 regulation on Smurf1 but not Smurf2 (26). Smurf2 is subject to an intramolecular C2-HECT interaction to keep its activity at a very low level. Although highly homologous (74% identity between full-length proteins and 92 and 85% identity for C2 and HECT domains, respectively), our results clearly show that Smurf1 E3 activity is not restricted by the same regulatory mechanism as Smurf2. Unlike Smurf2, Smurf1 is able to exert E3 activity on RhoA naturally. All of these observations preclude the possibility that the autoinhibition mechanism that exists in Smurf2 could also happen to Smurf1. The C2 domain in Smurf1 has no obvious effect on Smurf1 self-ubiquitination (Fig. 1C). However, we found that without the C2 domain, the Smurf1 E3 activity on RhoA is dramatically reduced (Fig. 2, A–C) but is enhanced on Smad5 and Runx2 (Fig. 2, D–G). These results indicate that the Smurf1 C2 domain provides some selectivity for Smurf1 to target its different substrates.

The Smurf1 activity toward hPEM-2, a guanine nucleotide exchange factor for Cdc42, is lost when the C2 domain is deleted (39). However hPEM-2 is the only known substrate of Smurf1 that binds to the C2 domain of Smurf1 instead of to the WW domains. We propose that the reason why the C2 domain exerts opposing effects on Smurf1 toward RhoA, Smad5, and Runx2 degradation may lie in their different cellular localizations. RhoA is localized at cell protrusions; Smad5 is primarily localized in the cytoplasm and also is translocated into the nucleus after activation; and Runx2 is a nuclear transcriptional factor. The C2 domain may function to recruit Smurf1 to the plasma membrane, especially cell protrusions. We found that the structure of the Smurf1 C2 domain adopts a classical C2 architecture, yet with many basic residues located on loops that in other C2 domains contain acidic residues that bind two or three Ca2+ ions. The Smurf1 C2 domain is therefore Ca2+-free, which may explain why Smurf1 membrane localization is not regulated by cytosolic Ca2+ (11). Two lysine residues in this region were found to be important for Smurf1 localization and activity on RhoA-related biological processes such as cell migration but not for Smad5-mediated BMP signaling. We speculate that the current notions should be extended to other membrane-associated substrates of Smurf1, such as the TGF-β receptor.

Taken together, this study shows that Smurf1 utilizes a different regulation model to regulate its ligase activity, despite its high similarity to Smurf2. The C2 domain in Smurf1 is not engaged in intramolecular interactions with the HECT domain as in Smurf2; instead, it exerts a pivotal role for Smurf1 in subcellular localization and activity on
Pivotal Role of Smurf1 C2 Domain

substrate selection. The information shown here not only provides new insight into knowledge on the E3 ligase regulation but also offers a useful starting point for the control of E3 activity by specific small molecular inhibitors for use in clinical studies.

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