Caveolin (Cav) proteins in the plasma membrane have numerous binding partners, but the determinants of these interactions are poorly understood. We show here that Cav-3 has a Small Ubiquitin-like Modifier (SUMO) consensus motif (Ψ-K-X-[D/E]) near the scaffolding domain and that Cav-3 is SUMOylated in a manner that is enhanced by the SUMO E3 ligase Protein Inhibitor of Activated STAT-y (PIASy). Site-directed mutagenesis revealed that the consensus site lysine is the preferred SUMOylation site but that mutation of all lysines is required to abolish SUMOylation. Co-expression of a SUMOylation-deficient mutant of Cav-3 with β-adennergic receptors (βARs) alters the expression level of β2ARs but not β1ARs following agonist stimulation, thus implicating Cav-3 SUMOylation in the mechanisms for β2AR, but not β1AR, desensitization. Expression of eNOS (NOS3) was not altered by the SUMOylation-deficient mutant. Thus, SUMOylation is a covalent modification of caveolins that influence the regulation of certain signaling partners.

The caveolin (Cav) family has three members: Cav-1 and -2 are co-expressed in most cell types, while Cav-3 is muscle-specific (1). Cavps are the principal protein component of caveolae membrane microdomains and function as scaffolds that compartmentalize and regulate numerous receptors, channels, transporters and signaling proteins that are enriched in caveolar membranes (e.g. G-proteins, GPCRs, RTKs and eNOS) or undergo rapid translocation to or from caveolae in response to stimuli (2-5). Proteins interact with the caveolin-scaffolding domain (CSD, Fig. 1A-B) via a “caveolin-binding motif” but how this interaction is dynamically regulated is poorly understood. Because of their rapid kinetics and reversibility, control by post-translational modifications (PTMs) is a likely mechanism, however no regulatory PTMs have been identified on Cav-3. We searched for motifs important for PTMs in the amino acid sequence of Cav-3 and found a conserved, SUMO consensus motif in Cav-3 and -1, but not Cav-2 (Fig. 1A). SUMOylation is the reversible modification of proteins by small ubiquitin-like modifier (SUMO) proteins and can regulate protein-protein interactions by masking or creating binding surfaces on target proteins (6-7). SUMOylation, initially thought to occur only in nuclear or perinuclear compartments (8), also regulates cytoplasmic and plasma membrane proteins (9-15). TGFβ induces SUMOylation of the Type I TGFβ receptors (16), which localize to caveolae and interact with Cav (17-18). Other SUMOylated signaling proteins that interact with Cavps or localize to caveolae include Kv1.5, PTP1B, RGS-Rz, and PDE4D5 (19-24). Thus, modification by SUMO may contribute to the binding and regulation of proteins that interact...
with Cavs. Here we define properties of the SUMOylation of caveolin-3 and the contribution of this SUMOylation to the expression of β-adrenergic receptors (βARs).

**Experimental Procedures**

**Materials and chemicals-** Reagents and their sources were as follows: Protein-G-agarose (Roche; Cat. 11243233001), MG-132 (Calbiochem; Cat. 474790), N-Ethylmaleimide (NEM) and (S)-(−)-Propanolol (Sigma; Cat. E3876 and P8688), Halt Protease Inhibitor Cocktail, EDTA-Free (Pierce; Cat. 87785), Ni-NTA agarose resin (Qiagen; Cat. 30210), goat anti-V5-agarose (Bethyl Labs; Cat. S190-119). Multi-Site and Lightning Mutagenesis kits (Stratagene; Cat. 200515 and 210518) and BL21(DE3) competent cells (Novagen; Cat. 70235-3), Isopropyl-β-D-1-thiogalactopyranoside (IPTG, Invitrogen; Cat. 15529019). 1-Octyl-β-D-glucopyranoside (A.G. Scientific; Cat. 0-1036). For SDS-PAGE, NuPAGE Bis-Tris 4-12% 12-well gels (Invitrogen) and PVDF membranes (Millipore) were used. SeeBlue Plus2 pre-stained standard (Invitrogen; Cat. LC5925) was used for visualization of protein molecular weights.

**Antibodies-** Antibodies and their sources were as follows: anti-FLAG M2 Affinity Gel and mouse monoclonal anti-FLAG M2 antibody (Sigma; Cat. A2220 and F1804), anti-FLAG-HRP and anti-Myc (Cell Signaling; Cat. 2044 and 2276), mouse monoclonal anti-V5 and anti-V5-HRP (Invitrogen; Cat. R960-25 and R961-25), rabbit polyclonal anti-PIASy, anti-Cav-3, anti-eNOS, anti-SUMO-1 and anti-rabbit secondary antibodies (Abcam; ab58416, ab2912, ab66127, ab11672 and ab6013), anti-SUMO-2/3, anti-Ube9 and anti-ubiquitin (Santa Cruz; sc-32873, sc-10759 and sc-47721), rabbit polyclonal anti-SUMO-1 (Invitrogen; Cat. 38-1900).

**Plasmids-** The mammalian expression plasmids for eNOS, SUMO-1, SUMO-3, Ube9, PIAS3 and PIASy were obtained from Origene (Cat. SC108440, SC118050, SC115792, SC109867, SC11632 and SC114580) and the FLAG-β1AR and FLAG-β2AR mammalian expression plasmids were obtained from Addgene (Cat. 14697 and 14698). Myc-SUMO-1, Myc-SUMO-3 and PIAS1 were provided by Dr. Serena Ghisletti. The E. coli expression plasmids pKRSUMO and pBADE12 were from Dr. Mario Menecia. The Cav-3-V5/His plasmid was provided by Dr. Brian Head and was constructed by cloning the rat caveolin-3 gene (AC_NM019155) into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Cat. K4800). The E. coli expression plasmid pST39-Cav-3-V5/His (referred to as pST39-Cav-3-V5) was generated by subcloning the Cav-3-V5/His coding region from the pcDNA3.1-V5/His expression vector and inserting it into cassette 2 (EcoRI/HindIII) of the polycistronic vector pST39. The following primers were used to amplify Cav-3-V5/His and introduce EcoRI and HindIII restriction sites by PCR: Cav3Fw 5′-GATCGAATTCATGATGACCGACACAC-3′, Cav3Rv 5′-GATCAAGCTTTCAATGGTGATGGATGATG-3′. Site-directed mutagenesis- The single and multiple ‘KR’ mutations were introduced into WT Cav-3-V5 using Multi-Site and Lightning QuikChange Site-Directed Mutagenesis Kits (Stratagene). The sequences for primers designed for creation of the ‘KR’ mutants are available upon request. Cav-3-V5-K7R was used to create a series of Cav-3-V5 ‘RK’ mutants. The sequences for primers designed for creation of the ‘RK’ mutants are available upon request. All plasmids were sequenced to validate presence of desired mutations.

**Cell culture and transfection-** Human embryonic kidney cells (HEK AD-293,) were obtained from Stratagene (Cat. 240085) and cultured in a 37°C, 5.0% CO2 incubator. Cells were grown in “HEK medium” (DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate, supplemented with 10% FBS)
without antibiotics. HEK AD-293 cells are derived from HEK 293 cells. Confluent HEK AD-293 cells were transfected 2-3 days post-plating in 6-well plates. 4.0 µg plasmid DNA and 9 µl Lipofectamine 2000 (Invitrogen) were each diluted separately in 250 µl OptiMEM (Invitrogen) before being combined, incubated for 20 min at room temperature and diluted with 500 µl HEK medium. Transfection complexes (1 ml total volume) were pipetted onto cells in 6-well plates to which 2 ml fresh HEK medium had been added. Cells were incubated with transfection complexes overnight and medium was replaced with 3 ml fresh HEK medium the following day. Except where stated, cells were analyzed 48 h post-transfection. For co-expression of the SUMOylation machinery with Cav-3-V5 the following ratios were used: Cav-3-V5 : SUMO-3 : Ubc9 : PIASy = 1 : 1 : 0.5 : 0.15-0.25. For the experiments that assessed the dose dependency of PIASy (Fig. 3B, lanes 3-7) the range was 0-400 ng plasmid. FLAG-β2AR, FLAG-β1AR or eNOS were co-expressed in a 1:1 ratio with Cav-3-V5 using the same ratios of SUMOylation machinery components. The total DNA for all transfections was normalized to 4.0 µg with empty pcDNA3.1 vector.

**Immunoprecipitation and immunoblotting:** Transfected HEK AD-293 cells were washed with ice-cold PBS and scraped into 500 – 800 µl ice-cold “in vivo SUMOylation” lysis buffer (20 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 0.5% Triton X-100, 1 mM EDTA) supplemented with 20 mM NEM (prepared fresh) and Halt protease inhibitor cocktail (Pierce) and pipetted into pre-chilled 1.5 ml Eppendorf tubes. Cells were incubated on ice for 30 min and sonicated in an ice water bath (3 x 10 sec). Lysates were clarified by centrifugation (5,000 x g for 15 min at 4°C) and protein concentrations were normalized by dilution with lysis buffer after performing BCA protein assays (Pierce). Clarified lysates were either directly resolved by SDS-PAGE and immunoblotted with appropriate antibodies or subjected to immunoprecipitation. Lysates were diluted with 4x LDS sample loading buffer (Invitrogen) supplemented with 100 mM DTT and heated (except where stated) to 80°C for 5 min before being resolved by NuPAGE Bis-Tris 4-12% 12-well gels. Proteins were then transferred for 1.5 - 2.5 h at 55V onto PVDF membranes that were immediately blocked for 1 h at room temperature in 4% milk/0.1% TBST. In general, primary antibodies were diluted at 1:1000 in 4% milk/0.1% TBST except for V5-HRP (1:5000), Cav-3 (1:2000) and PIASy (1:2000). For anti-SUMO-1 and anti-SUMO-2/3 antibodies 5% BSA/0.1% TBST was used for blocking and dilution instead of milk to reduce background signal. PVDF membranes were incubated overnight at 4°C with primary antibodies and washed at least 3 x 10 min with 0.1% TBST before incubation with secondary antibodies for 45-50 min at RT. Membranes were then washed (4 x 10 min) with 0.1% TBST and detected by Amersham ECL (enhanced chemiluminescence, GE Heathcare; Cat. RPN2135V2) or SuperSignal West Dura (Pierce; Cat. 34075). Images were collected with a Sensicam QE High Performance CCD camera (Cooke Corporation) mounted on an Epi Chemi II Darkroom (UVP BioImaging Systems) and analyzed using LabWorks 4.0 image acquisition and analysis software (UVP BioImaging Systems).

For immunoprecipitations, 300-500 µl clarified lysates were pre-cleared with 40 µl Protein G-agarose (Roche), incubated overnight at 4°C with primary antibodies (1-2 µg) followed by incubation with 40 µl Protein G-agarose for 1 h. Complexes were centrifuged at 10,000 x g at 4°C for 1 min; supernatants were transferred to pre-chilled 1.5 ml Eppendorf tubes. The beads were washed 4 times with lysis buffer and eluted by heating to 80-90°C for 5 min in 50 - 100 µl 2x LDS buffer with 100 mM DTT.
Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with appropriate antibodies. Immunoprecipitations with anti-FLAG M2 and anti-FLAG M2-agarose (Sigma) were performed per manufacturers instructions with FLAG IP lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with 20 mM NEM, 10% glycerol and Halt protease inhibitor cocktail. Isolation of adult rat cardiac myocytes for immunoprecipitation with anti-Cav-3, anti-SUMO-1 and anti-SUMO-2/3 antibodies was performed as described (61). Myocytes were washed with ice cold PBS and incubated on ice with lysis buffer (0.2% SDS, 0.5% NP-40, 15 mM MgCl₂, 1 mM DTT) supplemented with Halt protease inhibitor cocktail and 20 mM NEM. Lysates were sonicated, clarified by centrifugation and incubated overnight with primary antibodies at 4°C and processed by the Protein G-agarose method as described above.

In vitro SUMOylation assay- An in vitro protein synthesis kit (Qiagen EasyXpress Insect Kit II) was used to express recombinant Cav-3-V5 per the manufacturer’s instructions. 2 µl of protein synthesis reactions containing Cav-3-V5 were used directly in 20 µl in vitro SUMOylation reactions (BioMol International) consisting of 2 µl of 10x SUMOylation buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 2 mM ATP), 1 µl each of 20x SUMO E1 and SUMO E2 enzyme solutions and recombinant SUMO-1 or SUMO-3 proteins. Reactions were incubated at 37°C for 1 h and quenched with 2x LDS sample buffer with 100 mM DTT. 10 µl of the reaction product was analyzed by immunoblotting with anti-V5-HRP antibodies. Expression and purification of SUMOylated caveolin-3 in Escherichia coli- For production of SUMOylated Cav-3, BL21(DE3) competent cells were sequentially transformed with three E. coli expression plasmids; pKRSUMO, pBADE12 and pST39-Cav-3-V5 as described (28-29). For negative controls, cells were transformed with pST39-Cav-3-V5 alone or with pKRSUMO and pBADE12. Transformed cells were stored as glycerol stocks at -80°C. For protein expression, transformed cells were grown at 37°C in LB supplemented with antibiotics (e.g. Amp, Cm and Kan). The OD₆₀₀ was monitored and protein expression was induced at an OD₆₀₀ of 0.5 - 0.6 for 3 h. Cultures were pelleted and resuspended in TNE buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA). Purifications were performed with Ni-NTA agarose (Qiagen) and anti-V5-agarose (Bethyl Labs) resins per the manufacture’s instructions.

RESULTS

Caveolin-3 has a conserved SUMO consensus motif and is SUMOylated in vitro and in vivo. Cav-3 and -1, but not -2, have conserved, core and extended SUMO consensus motifs in their N-termini near the CSD (Fig. 1A), suggesting they are potential substrates for SUMOylation. The extended, ‘negatively charged amino acid-dependent SUMOylation motif’ (NDSM [25-26]) specifies the presence of negatively charged amino acids distal to the core motif and Cav-3 and -1 have motifs similar to other SUMO substrates (Supplemental Data, Fig. S1A-B). In vitro, the SUMO E1, E2 (Ubc9) and Mg²⁺/ATP are sufficient to SUMOylate targets (27). To determine if Cav-3 undergoes SUMOylation, we performed in vitro SUMOylation assays using a dual, affinity-tagged construct (Cav-3-V5/His, which we refer to as Cav-3-V5; Fig. 4A) expressed in a transcription/translation system and analyzed by immunoblotting with anti-V5-HRP antibodies.
immunoprecipitated with anti-Cav-3 (*) (Fig. 1D) or anti-SUMO antibodies (Fig. 1E). Immunoblotting for Cav-3 and SUMO-2/3 revealed bands at ~40 kDa corresponding to SUMOylated Cav-3.

Production of SUMOylated caveolin-3 in E. coli. To identify and purify SUMOylated caveolin proteins, we used an E. coli system (28-30), which has the advantages of scalability and absence of SUMO-specific proteases (SENP). Cav-3-V5/His, cloned into a bacterial expression vector (pST39-Cav-3-V5 [31]), was co-transformed into BL21(DE3) cells along with plasmids expressing SUMO-1 (pKRSUMO) and the SUMO E1 and E2 enzymes (pBADE12) (Fig. 2A, [29]). We tested the ability of BL21(DE3) cells sequentially transformed with: all 3 plasmids, pST39-Cav-3-V5 alone or pKRSUMO and pBADE12 to produce SUMOylated Cav-3. We analyzed the supernatant (Fig. 2B) and insoluble pellet (data not shown) and found that the majority of SUMOylated Cav-3-V5 was soluble. BL21(DE3) cells transformed with all 3 plasmids, but not with Cav-3-V5 alone, showed electrophoretic mobility shifts at 40 and 50 kDa (*, Fig. 2B); the 40 kDa band is consistent with in vitro and in vivo data (Fig. 1C-E).

We purified SUMOylated Cav-3-V5 using Ni-NTA resin (Fig. 2C). Elution fractions (E3-E5) contained unmodified Cav-3-V5 (25 kDa) and multiple slower migrating bands corresponding to SUMOylated Cav-3-V5 (*, Fig. 2C) that likely represent poly-SUMOylated Cav-3-V5. To confirm that these bands were a result of SUMOylation, the elution fractions were incubated with the catalytic domain of SENP1 (SENP1CD; Fig. 2D). The bands at 40, 50 and 60 kDa disappeared with SENP1 treatment; however it is unclear whether the multiple bands are indicative of multiple sites of SUMOylation or modification by poly-SUMO chains.

PIASy interacts with caveolin-3 and stimulates SUMO-3 modification. To validate the in vitro results and further investigate the determinants of Cav-3 SUMOylation, we performed SUMOylation assays in eukaryotic cells. Although SUMO-1 and SUMO-3 covalently modified Cav-3 in vitro (Fig. 1C), only SUMO-3 did so when co-expressed in HEK cells (Fig. 4B). The SUMO E1 and E2 enzymes (SAE1/2 and Ubc9) SUMOylate Cav-3 in vitro, however, in vivo a specific SUMO E3 ligase is often required. To identify a SUMO E3 ligase specific for Cav-3, we co-transfected PIAS1, PIAS3 or PIASy with Cav-3-V5, Ubc9 and Myc-SUMO-3 (Fig. 3A). When PIASy was co-expressed, bands at 40 and 50 kDa were enhanced in V5-HRP and Myc immunoblots. The 45 kDa band in anti-V5-HRP immunoblots between these Cav-3-V5-SUMO conjugates is likely dimerized Cav-3-V5 as it does not vary with co-expressed SUMO, Ubc9 or PIASy (Fig. 3A-D).

Transfection of HEK cells with increasing amounts of PIASy yielded increased modification of Cav-3-V5 by SUMO-3 (Fig. 3B, lanes 4-7). A ladder of bands corresponding to Cav-3 modified at multiple lysine residues or by poly-SUMO-3 chains was seen (*, Fig. 3B) at 65 kDa and higher in addition to the 40 kDa and 50 kDa bands observed in vitro (Fig. 2B-D). Transfecting >400 ng PIASy had no further effect on Cav-3-V5 SUMOylation and produced cell toxicity (data not shown).

HEK cells transfected with Cav-3-V5, SUMO-3, Ubc9 and PIASy were immunoprecipitated with anti-V5 and anti-Cav-3 antibodies. Immunoblotting revealed that Cav-3-V5 co-immunoprecipitates with PIASy (Fig. 3C). Reciprocal immunoprecipitation, performed with anti-PIASy antibodies, showed that PIASy interacts with unmodified Cav-3-V5 (25 kDa) and SUMOylated Cav-3-V5 (*) (Fig. 3D). Thus, PIASy interacts with Cav-3 and stimulates its modification at multiple lysine residues or by poly-SUMO-3 chains. PIASy expression in
cardiomyocytes was confirmed by immunoblotting of adult rat cardiomyocytes with anti-PIASy antibodies (Fig. 3E).

Mutation of the consensus site lysine reduced, but did not abolish, SUMOylation of caveolin-3. We next sought to identify the lysine(s) modified and to create a SUMOylation-deficient mutant that would facilitate identification of biological functions influenced by SUMOylation of Cav-3. We initially mutat ed the consensus site lysine in Cav-3-V5 (Fig. 4A) to arginine (K38R) and assessed SUMOylation (Fig. 4B). K38R reduced, but did not abolish, SUMOylation of Cav-3-V5 indicating either that: 1) multiple lysine residues are modified or 2) if the consensus site is mutated, a different lysine can be modified.

Identification of a SUMOylation-deficient mutant. We mutated Cav-3-V5 seven lysines to arginine one at a time (‘KR’ mutants) and assessed SUMOylation in transfected HEK cells (Fig. 4C). K38R reduced SUMOylation relative to WT Cav-3-V5 but SUMOylation of other ‘KR’ mutants was equal or greater than WT. It is unclear whether this is due to increased SUMOylation or differences in stability or expression of the mutants (e.g. K30R, K69R). K38 appeared to be the major site of SUMOylation; however, other sites can be modified, suggesting that two or more lysines might need to be mutated. Two rounds of multisite-mutagenesis were performed to generate multiple ‘KR’ mutants. None of these mutants completely prevented SUMOylation of Cav-3 (Fig. 4D, lanes 6-10). SUMOylation was abolished when all seven lysines were mutated (Cav-3-V5-K7R; Fig. 4C, lane 7; Fig. 4D, lanes 1-4), implying that unavailability of the preferred SUMOylation site(s) allows other lysines to be SUMOylated.

K38 is the preferred SUMOylation site. To define precisely which lysines are potential vs. preferred SUMOylation sites and determine their relative ability to be SUMOylated, we used Cav-3-V5-K7R (Fig. 5A) to create a series of ‘RK’ mutants. HEK cells were transfected with WT and the Cav-3-V5-K7R mutant in parallel with the ‘RK’ mutants (Fig. 5B). Immunoblotting with anti-V5-HRP antibodies detected SUMOylation at each lysine but re-introduction of the consensus site lysine in the R38K mutant most effectively restored the SUMOylation of Cav-3-V5 (Fig. 5B, lane 6). Moreover, R38K was the only mutant that, akin to WT Cav-3-V5, was poly-SUMOylated (*, Fig. 5B, lane 1 vs. 6), suggesting that PIASy stimulates SUMOylation by poly-SUMO-3 chains at this site.

To confirm that PIASy was required for SUMOylation at K38, we transfected the four N-terminal ‘RK’ mutants with and without co-expression of the SUMOylation machinery (SUMO-3, Ubc9 and PIASy). Immunoblotting with anti-V5-HRP, anti-PIASy and anti-GAPDH revealed that co-expression of the SUMOylation machinery had no effect on the low level of modification of R15K, R20K or R30K, but strongly enhanced modification of R38K (Fig. 5C, lane 6), which closely resembled WT (*, Fig. 5C, lane 2 vs. 6). Thus, PIASy is necessary for SUMOylation, including poly-SUMOylation, of K38.

WT Caveolin-3 increases, but the K7R mutant decreases, β2AR expression levels. We used the K7R mutant to probe the effect of SUMO modification on protein-protein interactions of caveolin with a known GPCR binding partner, the β2-adrenergic receptor (β2AR). β2ARs predominantly reside in caveolar microdomains in cardiac myocytes and interact with Cav-3 (32-34). To determine whether SUMOylation of Cav-3 modulates its interaction with β2ARs, we transfected FLAG-β2AR into HEK 293 cells either alone, or with WT or Cav-3-V5-K7R. Cells were grown in DMEM + 10 % FBS, harvested 48 h post-transfection and immunoprecipitated with anti-FLAG-agarose beads. Immuno-precipitates were analyzed by immuno-
blotting with anti-FLAG-HRP, ubiquitin, V5-HRP and PIASy antibodies (Fig. 6A). We anticipated that SUMOylation might change the ability of FLAG-β2AR to bind Cav-3-V5, perhaps by blocking access to the CSD (Fig. 1A; Fig. 8A). We found, however, that we were unable to determine the relative ability of FLAG-β2AR to interact with WT Cav-3-V5 or the K7R mutant because the level of FLAG-β2AR expression was increased or decreased depending on co-expression of WT or Cav-3-V5-K7R, respectively (Fig. 6A, lanes 1, 3 and 5). In addition, co-expression of the SUMOylation machinery (SUMO-3, Ubc9 and PIASy) increased the level of FLAG-β2AR expression (Fig. 6A, lanes 2, 4 and 6) independent of Cav-3-V5 expression.

Endogenous β2ARs have a half-life of 24 h if unstimulated (35). Thus, we repeated the experiment with cells harvested 24 h post-transfection. WT Cav-3-V5 increased expression levels of FLAG-β2AR when the SUMOylation machinery was co-expressed (Fig. 6B, lanes 1-2 vs. 3-4) while the K7R mutant decreased FLAG-β2AR (lanes 1-2 vs. 5-6). Moreover, there was no effect of co-expression of the SUMOylation machinery independent of Cav-3-V5. Thus, co-expression of the K7R mutant decreases expression levels of FLAG-β2AR.

The mechanisms of agonist-induced desensitization of the β2AR have been extensively studied in HEK 293 cells (36-37). β-adrenergic agonists (norepinephrine and epinephrine) are present in fetal bovine serum (FBS [38]). Therefore, during these experiments the FLAG-β2AR transfected HEK cells were exposed to β-agonists by use of FBS in the culture media. The data shown thus far suggest that modification of Cav-3 by SUMO-3 may alter FLAG-β2AR expression levels by inhibiting, while the SUMOylation-deficient Cav-3 mutant may promote, agonist-induced desensitization of β2ARs. To determine whether the effect of the K7R mutant of Cav-3 is specific for β2AR, we assessed impact of K7R on expression of eNOS (NOS3), a well-studied, caveolin-regulated protein (39-40). HEK 293 cells were transfected with eNOS alone or with WT or Cav-3-V5-K7R and with or without the SUMOylation machinery for 24 h (Fig. 6C). The expression level of eNOS was unaffected by expression of either WT Cav-3 or Cav-3-V5-K7R, suggesting that the effect on FLAG-β2AR is not a non-specific inhibition of protein expression caused by the Cav-3-V5-K7R mutant.

β1ARs and β2ARs are highly homologous both structurally and functionally (34) and a portion of β1ARs also localize to caveolae and interact with Cav-3, but do not undergo agonist-induced ubiquitination, internalization and desensitization (37,41). FLAG-tagged β1AR or β2AR were co-transfected into HEK 293 cells either alone or with WT or Cav-3-V5-K7R. Unlike FLAG-β2ARs, expression of FLAG-β1ARs was not altered by co-transfection of the Cav-3-V5-K7R mutant (Fig. 7A, lanes 1-3 vs. 4-6). Thus, SUMOylation of Cav-3 appears to influence the expression level of β2ARs but not β1ARs, and this effect may depend on agonist-induced desensitization of the receptor.

Propranolol attenuates the effect of Cav-3-V5-K7R on FLAG-β2AR expression levels. We used the βAR antagonist propranolol to determine whether the decrease in FLAG-β2AR expression is a result of β-agonist stimulation of FLAG-β2AR by catecholamines present in the culture media (38). HEK 293 cells transfected with the SUMOylation machinery (SUMO-3, Ubc9 and PIASy) were co-transfected with either FLAG-β2AR alone, WT Cav-3-V5 or the K7R mutant in the absence or presence of 2 μM propranolol. Treatment with propranolol blunted the effect of the Cav-3-V5-K7R mutant on FLAG-β2AR expression (Fig. 7B, lanes 1-3). Incubation of cells in media with
reduced serum (1%) had a similar effect as did propranolol (Fig. 7B, lanes 4-6). In each case, WT Cav-3-V5 increased FLAG-\(\beta_2\)AR expression levels (Fig. 7B, lanes 2, 5), presumably by inhibiting the agonist-induced desensitization of the receptor.

To quantify this effect, HEK 293 cells were co-transfected with FLAG-\(\beta_2\)AR alone, WT Cav-3-V5 or the K7R mutant, and treated with or without 2 \(\mu\)M propranolol; FLAG-HRP immunoblots of anti-FLAG immunoprecipitates were then quantified by densitometry (Fig. 7C-D). Immunoprecipitates and protein samples are typically heated prior to analysis by SDS-PAGE, however this can cause self-aggregation of membrane proteins such as GPCRs (42). To achieve accurate quantification, we did not heat the anti-FLAG immunoprecipitates prior to SDS-PAGE to reduce the formation of >100 kDa FLAG-\(\beta_2\)AR aggregates (Fig. 7A-B). We found that K7R reduced FLAG-\(\beta_2\)AR expression levels by about 50% (P=0.005) in vehicle-treated cells and that treatment with propranolol returned the expression to control levels (Fig. 7C-D). WT Cav-3-V5 had the opposite affect, increasing FLAG-\(\beta_2\)AR expression by ~2 fold; expression was further increased by propranolol. Cav-3 and its ability to undergo SUMOylation thus appear to modulate the agonist-induced desensitization of \(\beta_2\)ARs.

**DISCUSSION**

These studies demonstrate for the first time that a caveolin, Cav-3, is post-translationally modified by SUMO proteins *in vitro* and *in vivo*. SUMOylation of Cav-3 appears to be a novel regulatory mechanism for agonist-induced desensitization of \(\beta_2\)ARs. *In vitro* SUMOylation assays and co-expression of recombinant Cav-3 in E. coli with the SUMOylation machinery showed that conjugation with SUMO-1 or SUMO-3 is possible. However, in mammalian cells, conjugation is specific for SUMO-3. In E. coli transformed with the SUMOylation machinery, Cav-3 was modified at multiple sites or by poly-SUMO-1 (Fig. 2B-D). There is debate regarding the ability of SUMO-1 to form chains *in vivo* (43); this possibility cannot be ruled out since SENPs (which reverse SUMO conjugation and edit poly-SUMO chains) are absent in E. coli. The tandem SUMO-Interacting Motifs (SIMs) of Cav-3 (Supplemental Data, Fig. S1C-D) may assist in the modification by poly-SUMO chains by recruiting Ubc9–thioesters containing polymerized SUMO. The specificity for modification by SUMO-3 in HEK 293 cells may result from its greater cytosolic localization or preferential interaction of Cav-3’s SIMs with SUMO-3 rather than SUMO-1 (44-46).

We find that PIASy preferentially enhances SUMOylation of Cav-3 at a consensus site but mutation of this site does not abolish SUMOylation. This may result from a lack of secondary structure of the N-terminus of Cav. Amino acids 79–96 in Cav-1, which comprise the CSD (aa55-72 in Cav-3), form an \(\alpha\)-helix while the remainder of the N-terminus lacks secondary structure (47). Perhaps non-consensus site lysines become accessible when K38 is mutated. Fernandez et al. proposed that the N-terminal tail region (aa1-54 in Cav-3) wraps back around the \(\alpha\)-helical region (Fig. 8). Mutation of Cav-1 aa66–70 (IDFED) to alanine dramatically affects oligomerization (47); Fernandez et al. speculated that this acidic patch binds basic residues in the \(\alpha\)-helix, thereby causing the tail to wrap around the CSD. The corresponding region in Cav-3 (aa39-43) is next to the SUMOylated residue in Cav-3, K38 (Fig. 8A; EDIV\(K\)(SUMO)VDFED). A helical wheel projection illustrates clustering of basic residues along one side of the \(\alpha\)-helical CSD (Fig. 8B). Thus, modification by SUMO at this site could block the interaction of the N-tail region with the CSD, acting as a

---

**Fig. 7B** Lanes 2, 4, 5: WT Cav-3-V5 increased FLAG-\(\beta_2\)AR expression levels (Fig. 7B, lanes 2, 5). Lanes 3, 6: Propranolol returned the expression to control levels (Fig. 7C-D). Immunoprecipitates and protein samples are typically heated prior to analysis by SDS-PAGE, however this can cause self-aggregation of membrane proteins such as GPCRs (42). To achieve accurate quantification, we did not heat the anti-FLAG immunoprecipitates prior to SDS-PAGE to reduce the formation of >100 kDa FLAG-\(\beta_2\)AR aggregates (Fig. 7A-B). K7R reduced FLAG-\(\beta_2\)AR expression levels by about 50% (P=0.005) in vehicle-treated cells and that treatment with propranolol returned the expression to control levels (Fig. 7C-D). WT Cav-3-V5 had the opposite affect, increasing FLAG-\(\beta_2\)AR expression by ~2 fold; expression was further increased by propranolol. Cav-3 and its ability to undergo SUMOylation thus appear to modulate the agonist-induced desensitization of \(\beta_2\)ARs.

**Fig. 7C-D** Immunoprecipitates and protein samples are typically heated prior to analysis by SDS-PAGE, however this can cause self-aggregation of membrane proteins such as GPCRs (42). To achieve accurate quantification, we did not heat the anti-FLAG immunoprecipitates prior to SDS-PAGE to reduce the formation of >100 kDa FLAG-\(\beta_2\)AR aggregates (Fig. 7A-B). K7R reduced FLAG-\(\beta_2\)AR expression levels by about 50% (P=0.005) in vehicle-treated cells and that treatment with propranolol returned the expression to control levels (Fig. 7C-D). WT Cav-3-V5 had the opposite affect, increasing FLAG-\(\beta_2\)AR expression by ~2 fold; expression was further increased by propranolol. Cav-3 and its ability to undergo SUMOylation thus appear to modulate the agonist-induced desensitization of \(\beta_2\)ARs.

**Fig. 7A-B** Immunoprecipitates and protein samples are typically heated prior to analysis by SDS-PAGE, however this can cause self-aggregation of membrane proteins such as GPCRs (42). To achieve accurate quantification, we did not heat the anti-FLAG immunoprecipitates prior to SDS-PAGE to reduce the formation of >100 kDa FLAG-\(\beta_2\)AR aggregates (Fig. 7A-B). K7R reduced FLAG-\(\beta_2\)AR expression levels by about 50% (P=0.005) in vehicle-treated cells and that treatment with propranolol returned the expression to control levels (Fig. 7C-D). WT Cav-3-V5 had the opposite affect, increasing FLAG-\(\beta_2\)AR expression by ~2 fold; expression was further increased by propranolol. Cav-3 and its ability to undergo SUMOylation thus appear to modulate the agonist-induced desensitization of \(\beta_2\)ARs.

---

**DISCUSSION**

These studies demonstrate for the first time that a caveolin, Cav-3, is post-translationally modified by SUMO proteins *in vitro* and *in vivo*. SUMOylation of Cav-3 appears to be a novel regulatory mechanism for agonist-induced desensitization of \(\beta_2\)ARs. *In vitro* SUMOylation assays and co-expression of recombinant Cav-3 in E. coli with the SUMOylation machinery showed that conjugation with SUMO-1 or SUMO-3 is possible. However, in mammalian cells, conjugation is specific for SUMO-3. In E. coli transformed with the SUMOylation machinery, Cav-3 was modified at multiple sites or by poly-SUMO-1 (Fig. 2B-D). There is debate regarding the ability of SUMO-1 to form chains *in vivo* (43); this possibility cannot be ruled out since SENPs (which reverse SUMO conjugation and edit poly-SUMO chains) are absent in E. coli. The tandem SUMO-Interacting Motifs (SIMs) of Cav-3 (Supplemental Data, Fig. S1C-D) may assist in the modification by poly-SUMO chains by recruiting Ubc9–thioesters containing polymerized SUMO. The specificity for modification by SUMO-3 in HEK 293 cells may result from its greater cytosolic localization or preferential interaction of Cav-3’s SIMs with SUMO-3 rather than SUMO-1 (44-46).

We find that PIASy preferentially enhances SUMOylation of Cav-3 at a consensus site but mutation of this site does not abolish SUMOylation. This may result from a lack of secondary structure of the N-terminus of Cav. Amino acids 79–96 in Cav-1, which comprise the CSD (aa55-72 in Cav-3), form an \(\alpha\)-helix while the remainder of the N-terminus lacks secondary structure (47). Perhaps non-consensus site lysines become accessible when K38 is mutated. Fernandez et al. proposed that the N-terminal tail region (aa1-54 in Cav-3) wraps back around the \(\alpha\)-helical region (Fig. 8). Mutation of Cav-1 aa66–70 (IDFED) to alanine dramatically affects oligomerization (47); Fernandez et al. speculated that this acidic patch binds basic residues in the \(\alpha\)-helix, thereby causing the tail to wrap around the CSD. The corresponding region in Cav-3 (aa39-43) is next to the SUMOylated residue in Cav-3, K38 (Fig. 8A; EDIV\(K\)(SUMO)VDFED). A helical wheel projection illustrates clustering of basic residues along one side of the \(\alpha\)-helical CSD (Fig. 8B). Thus, modification by SUMO at this site could block the interaction of the N-tail region with the CSD, acting as a

---

**Fig. 8** Helical wheel projection illustrates clustering of basic residues along one side of the \(\alpha\)-helical CSD (Fig. 8B). Thus, modification by SUMO at this site could block the interaction of the N-tail region with the CSD, acting as a...
switch to regulate binding of proteins to the CSD or oligomerization of caveolins.

The tandem SIMs in Cav-3’s N-terminus and the ability of caveolin to oligomerize may also contribute to the SUMOylation of other lysines, albeit at a much lower level than WT or R38K (Figs. 4-5). Proteins can be SUMOylated on non-consensus sites that are in close proximity to a SIM (e.g. USP25 [46]). This suggests that stabilization of the binding between Ubc9~SUMO thioesters and the target by a SUMO-SIM interaction or by substrate-E3 ligase interactions may facilitate the interaction of Ubc9 with non-consensus site lysines.

PIASy and other PIAS proteins have been considered nuclear proteins (48-49) while Cav-3 is primarily found in plasma membrane microdomains, with some cytosolic and perinuclear localization (50). A study in PIASy +/- MEFs showed that while PIASy was predominantly found in the nucleus, it could also be detected in the cytoplasm (51). PIASy accumulates in the cytoplasm and co-localizes with the E3 ubiquitin ligase TRIM32 when MG-132 is used to inhibit proteasomal degradation (49), which may explain why it is normally detected at low levels in the cytoplasm. Consistent with this idea, treatment of HEK 293 cells with MG-132 for 3 h enhanced SUMOylation of Cav-3 relative to untreated cells (Fig. 4D, lane 5), presumably by increasing the cytosolic expression of PIASy. This finding is also consistent with data obtained with HEK 293 cells in which we sought to manipulate the expression of PIASy. TGFβ stimulates a 6-8 fold increase in PIASy mRNA expression in Hep2B cells that peaks after 12 h (52). We incubated HEK 293 cells with TGFβ in the presence or absence of MG-132 (treatment for 12 hr) and found that cells treated with MG-132 have a 3-fold increase in PIASy protein expression and an increase in 40 kDa Cav-3-V5 (data not shown).

PIASy appears to be highly expressed in cardiac myocytes (Fig. 3E) and transfection of HEK 293 cells (which express low levels of PIASy (Fig. 3A)), with as little as 40 ng PIASy greatly enhances SUMOylation of Cav-3 (Fig. 3B). PIASy co-immunoprecipitates with Cav-3 in transfected HEK 293 cells (Fig. 3C-D) and immunofluorescence studies of HEK 293 cells transfected with Cav-3-V5 alone or with the SUMOylation machinery show that the affinity-tagged Cav-3 construct localizes in the cytosol and plasma membrane puncta, similar to endogenous Cav-3, consistent with the idea that PIASy co-localizes with Cav-3 in these compartments (data not shown).

PIASy interacts with the type I TGF-β receptor (TβRI) in MCF7 human breast cancer cells (53) and TβRIs localize to caveolae and interact with Cav-1 and eNOS in endothelial cells (18). Whether Cav-3 and PIASy co-localize in vivo has not been investigated. The E3 ubiquitin ligase TRIM32 can control cytosolic levels of PIASy and, akin to what has been observed for Cav-3, TRIM32 is mutated in Limb-Girdle Muscular Dysrophy. The latter mutation prevents TRIM32 from binding PIASy (49), suggesting that the regulation of cytosolic SUMOylation of Cav-3 and other targets by PIASy has functional consequences in terms of the pathophysiology of muscular dystrophy.

The findings presented here are consistent with recent data, that show PIASy-dependent SUMOylation of a phosphodiesterase subtype, PDE4D5, which is recruited to activated β2ARs (24,54,55). Caveolin and this PDE subtype share a high degree of similarity in their SUMOylation motifs (Supplemental Data, Fig. S1B). Perhaps Cav-3, β2AR, PDE4D5, and PIASy are part of a signaling complex localized in caveolae that regulates cellular responses to agonist stimulation.

The mechanism by which SUMOylation of caveolin affects β2AR
expression levels is not clear. Cav-3 might affect β2ARs through its ability to couple to the machinery that regulates the agonist-induced internalization and desensitization of the receptors. For example, β-arrestin-2 and Mdm2 are recruited to activated receptors. Cav-1 interacts with Mdm2 following H2O2 treatment (56) and oxidative stress caused by H2O2 can stimulate altered patterns and increases in SUMO-2/3 conjugation (57). Moreover, Mdm2 and β-arrestin-2 are targets of SUMOylation (36,58,59) and PIASy cooperates with Mdm2 to regulate SUMOylation of p53 (60). β-arrestin-2 preferentially interacts with the SUMOylated form of Mdm2 (36) (p90Mdm2). A search for SIMs similar to those in Cav-1 and -3 reveals the presence of tandem SIMs in β-arrestin-1 and β-arrestin-2 (Supplemental Data, Fig. S1C; ARRB1 and ARRB2), suggesting that these motifs may be responsible for the preferential binding of p90Mdm2. It will be thus be of interest to test if the decreased expression of β2AR caused by the Cav-3 K7R mutant results from SUMOylation-dependent alterations of Mdm2 / β-arrestin-2 interactions.

In summary, our findings are the first to show that Cavs are SUMOylated and that SUMOylation influences the expression level of a binding partner of Cav. These results thus define a previously unappreciated mechanism that contributes to the interaction of Cavs, presumably by the CSD, with such binding partners. It will be of interest to define the full range of binding partners whose interaction is influenced by SUMOylation and whether SUMOylation of Cavs is altered during physiological and pathophysiological settings.

REFERENCES

1. Song, K. S., Scherer P. E., Tang, Z., Okamoto, T., Li, S., Chafel, M., Chu, C., Kohtz, D. S. and Lisanti, M. P. (1996) J Biol Chem 271(25), 15160-15165
2. Feron, O., Saldana, F., Michel, J. B. and Michel, T. (1998) J Biol Chem 273(6), 3125-3128
3. Lamb, M. E., Zhang, C., Shea, T., Kyle, D. J. and Leeb-Lundberg, L. M. (2002) Biochemistry 41(48), 14340-14347
4. Sabourin, T., Bastien, L., Bachvarov, D. R. & Marceau, F. (2002) Mol Pharmacol. 61(3), 546-553
5. Fecchi, K., Volonte, D., Hezel, M. P., Schmeck, K. and Galbiati, F. (2006) FASEB J 20(6), 705-707
6. Wilkinson, K. A. and Henley, J. M. (2010) Biochem J 428(2), 133-145
7. Gocke, C. B., Yu, H. and Kang, J. (2005) J Biol Chem 280(6), 5004-5012
8. Muller, S., Ledl, A. and Schmidt, D. (2004) Oncogene 23(11), 1998-2008
9. Seeler, J. S. and Dejean, A. (2003) 4(9), 690-699
10. Bossis, G. and Melchior, F. (2006) Cell Div 1:13
11. Benson, M. D., Li, Q. J., Kieckhafer, K., Dudek, D., Whorton, M. R., Sunahara, R. K., Iniguez-Lluhi, J. A. and Martens, J. R. (2007) Proc Natl Acad Sci USA 104(6), 1805-1810
12. Scheschonka, A., Tang, Z. and Betz, H. (2007) Trends Neurosci 30(3), 85-91
13. Giorgino, F., de Robertis, O., Laviola, L., Montrone, C., Perrini, S., McCowen, K. C. and Smith, R. J. (2000) Proc Natl Acad Sci 97(3),1125-1130
14. Martin, S., Wilkinson, K. A., Nishimune, A. and Henley, J. M. (2007) Nat Rev Neurosci 8(12), 948-959
15. Martin, S., Nishimune, A., Mellor, J. R. and Henley, J. M. (2007) *Nature* **447**(7142), 321-325
16. Kang, J. S., Saunier, E. F., Akhurst, R. J. and Derynck, R. (2008) *Nat Cell Biol* **10**(6), 654-264
17. Santibanez, J. F., Blanco, F. J., Garrido-Martin, E. M., Sanz-Rodriguez, F., del Pozo, M. A., Bernabeu, C. (2008) *Cardiovasc Res* **77**(4), 791-799
18. Schwartz, E. A., Reaven, E., Topper, J. N. and Tsao, P. S. (2005) *Biochem J* **390**(Pt 1), 199-206
19. Foster, L. J., De Hoog, C. L. and Mann, M. (2003) *Proc Natl Acad Sci USA* **100**(10), 5813-5818
20. Lee, H., Xie, L., Luo, Y., Lee, S. Y., Lawrence, D. S., Wang, X. B., Sotgia, F., Lisanti, M. P. and Zhang, Z. Y. (2006) *Biochemistry* **45**(1), 234-240
21. Martens, J. R., Sakamoto, N., Sullivan, S. A., Grobaski, T. D. and Tamkun, M. M. (2001) *J Biol Chem* **276**(11), 8409-8414
22. Rodriguez-Munoz, M., Bermudez, D., Sanchez-Blazquez, P. and Garzon, J. (2007) *Neuropsychopharmacology* **32**(4), 842-850
23. Dadke, S., Cotteret, S., Yip, S. C., Jaffer, Z. M., Haj, F., Ivanov, A., Rauscher, F. 3rd, Shuai, K., Ng, T., Neel, B. G. and Chernoff, J. (2007) *Nat Cell Biol* **9**(1), 80-85
24. Li, X., Vadrevu, S., Dunlop, A., Day, J., Advant, N., Troeger, J., Klussmann, E., Jaffrey, E., Hay, R. T., Adams, D. R., Houlsay, M. D. and Baillie, G. S. (2010) *Biochem J* **428**(1), 55-65
25. Blomster, H. A., Hietakangas, V., Wu, J., Kouvon, P., Hautaniemi, S., Sistonen, L. (2009) *Mol Cell Proteomics* **8**(6), 1382-1390
26. Yang, S. H., Galanis, A., Witty, J. and Sharrocks, A. D. (2006) *EMBO J* **25**(21), 5083-5093
27. Werner, A., Moutty, M. C., Möller, U. and Melchior, F. (2009) *Methods Mol Biol* **497**, 187-199
28. Saitho, H., Uwada, J. and Azusa, K. (2009) *Methods Mol Biol* **497**, 211-221
29. Mencia, M. and de Lorenzo, V. (2004) *Protein Expr Purif* **37**(2), 409-418
30. Uchimura, Y., Nakamura, M., Sugawara, K., Nakao, M. and Saitho, H. (2004) *Anal Biochem* **331**(1), 204-206
31. Tan, S. (2001) *Protein Expr Purif* **21**(1), 224-234
32. Ostrom, R. S., Gregorian, C., Drenan, R. M., Xiang, Y., Regan, J. W. and Insol, P. A. (2001) *J Biol Chem* **276**(45), 42063-42069
33. Xiang, Y., Rybin, V. O., Steinberg, S. F. and Kobilka, B. (2002) *J Biol Chem* **277**(37), 34280-34286
34. Shcherbakova, O. G., Hurt, C. M., Xiang, Y., Dell'Acqua, M. L., Zhang, Q., Tsien, R. W., Kobilka, B. K. (2007) *J Cell Biol* **176**(4), 521-533
35. Berthouze, M., Venkataramanan, V., Li, Y. and Shenoy, S. K. (2009) *EMBO J* **28**(12), 1684-1696
36. Shenoy, S. K., McDonald, P. H., Kohout, T. A. and Lefkowitz, R. J. (2001) *Science* **294**(5545), 1307-1313
37. Liang, W., Austin, S., Hoang, Q. and Fishman, P. H. (2003) *J Biol Chem* **278**(41), 39773-39781
38. Dibner, M. D. and Insol, P. A. (1981) *J Biol Chem* **256**(14), 7343-7346
39. Ostrom, R. S., Bundey, R. A. and Insol, P. A. (2004) *J Biol Chem* **279**(19), 19846-19853
40. García-Cardeña, G., Martasek, P., Masters, B. S., Skidd, P. M., Couet, J., Li, S., Lisanti, M. P. and Sessa, W. C. (1997) *J Biol Chem* **272**(41), 25437-25440

41. Liang, W. and Fishman, P. H. (2004) *J Biol Chem* **279**(45), 46882-46889

42. Ren, H., Yu, D., Ge, B., Cook, B., Xu, Z., Zhang, S. (2009) *PLoS One* **4**(2), e4509

43. Yang, M., Hsu, C. T., Ting, C. Y., Liu, L. F. and Hwang, J. (2006) *J Biol Chem* **281**(12), 8264-8274

44. Su, H. L., Li, S. S. (2002) *Gene* **296**(1-2), 65-73

45. Hay, R. T. SUMO-specific proteases: a twist in the tail. (2007) *Trends Cell Biol* **17**(8), 370-376

46. Meulmeester, E., Kunze, M., Hsiao, H. H., Urlaub, H. and Melchior, F. (2008) *Mol Cell* **30**(5), 610-619

47. Fernandez, I., Ying, Y., Albanesi, J. and Anderson, R. G. (2002) *Proc Natl Acad Sci USA* **99**(17), 11193-11198

48. Ihara, M., Yamamoto, H. and Kikuchi, A. (2005) *Mol Cell Biol* **25**(9), 3506-3518

49. Albors, A., El-Hizawi, S., Horn, E. J., Laederich, M., Frosk, P., Wroegemann, K. and Kulesz-Martin, M. (2006) *J Biol Chem* **281**(35), 25850-25866

50. Smythe, G. M., Eby, J. C, Disatnik, M. H and Rando, T. A. (2003) *J Cell Sci* **116**(Pt 23), 4739-4749

51. Martin, N., Schwamborn, K., Urlaub, H., Gan, B., Guan, J. L. and Dejean, A. (2008) *Mol Cell Biol* **28**(8), 2771-2781

52. Imoto, S., Ohbayashi, N., Ikeda, O., Kamitani, S., Muromoto, R., Sekine, Y. and Matsuda, T. (2008) *Biochem Biophys Res Commun* **370**(2), 359-365

53. Conrotto, P., Yakymovych, I., Yakymovych, M. and Souchelnytskyi, S. (2007) *J Proteome Res* **6**, 287-297

54. Lynch, M. J., Baillie, G. S., Mohamed, A., Li, X., Maisonneuve, C., Klussmann, E., van Heeke, G. and Houslay, M. D. (2005) *J Biol Chem* **280**(39), 33178-33189

55. De Arcangelis, V., Liu, R., Soto, D. and Xiang, Y. (2009) *J Biol Chem* **284**(49), 33824-33832

56. Bartholomew, J. N., Volonte, D. and Galbiati, F. (2009) *Cancer Res* **69**(7), 2878-2886

57. Manza, L. L., Codreanu, S. G., Stamer, S. L., Smith, D. L., Wells, K. S., Roberts, R. L. and Liebler, D. C. (2004) *Chem Res Toxicol*. **17**(12), 1706-1715

58. Meek, D. W. and Knippschild, U. (2003) *Mol Cancer Res* **1**(14), 1017-1026

59. Wyatt, D., Malik, R., Vesecky, A. C., Marchese, A. (2011) *J Biol Chem* **286**(5), 3884-3893

60. Carter, S., Bischof, O., Dejean, A. and Vousden, K. H. (2007) *Nat Cell Biol* **9**(4), 428-435

61. Head, B. P., Patel, H. H., Roth, D. M., Murray, F., Swaney, J. S., Niesman, I. R., Farquhar, M. G. and Insel, P. A. (2006) *J Biol Chem* **281**(36), 26391-26399
This work was supported by research grants from the National Institutes of Health (HL091071, GM066232 and HL066941) to P.A.I. S.R.F. was supported in part by the UCSD Graduate Training Program in Cellular and Molecular Pharmacology through an institutional training grant from the National Institute of General Medical Sciences, T32 GM007752, as well as a PhRMA Foundation Pre-Doctoral Fellowship in Pharmacology/Toxicology. We thank Dr. Mario Mencia (CSIC, Madrid) for the pKRSUMO and pBADE1E2 plasmids and Dr. Serena Ghisleti (Glass lab, UCSD) for the Myc-SUMO-1, Myc-SUMO-3 plasmids.

**FIGURE LEGENDS**

Fig. 1. Caveolin-3 has a SUMO consensus motif and is SUMOylated in vitro and in vivo.

A. Caveolin-3 and -1, but not caveolin-2, have a core SUMO consensus motif (Ψ-K-X-[D/E], Ψ=VIL) in their N-termini. This motif also conforms to the extended, ‘negatively charged amino acid-dependent SUMOylation motif’ (NDSM). The SUMO consensus motif (aa37-40) lies near the caveolin scaffolding domain (CSD; aa55-74) and transmembrane domain (TM; aa75-104) which are highly conserved among the three caveolin family members.

B. Caveolin membrane topology. Caveolins form a hairpin loop in the plasma membrane such that both the N- and C-termini face the cytoplasm. Caveolin-3 (P51638 [CAV3_RAT]) has seven lysine residues that are potential SUMOylation sites. A putative SUMO consensus site at K38 in the N-terminus lies near the CSD.

C. In Vitro SUMOylation of caveolin-3: Recombinant expression of Cav-3-V5 in insect cell extracts. Reactions (in 20 µl) included SUMOylation buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 2 mM ATP), Mg²⁺/ATP, and recombinant SUMO E1 and E2 enzymes and were incubated at 30°C for 1 h with either SUMO-1 or SUMO-3. Reactions omitting Cav-3-V5 (lane 2), SUMO (lanes 3) or Ubc9 (lane 4) were negative controls. Reactions were analyzed by immunoblotting with anti-V5-HRP antibodies.

D. Detection of endogenously SUMOylated caveolin-3. Rat myocyte lysates were immunoprecipitated with anti-Cav-3 antibodies and immunoprecipitates (IP) and supernatants (SN) were analyzed along with whole cell lysates (WCL) by immunoblotting with anti-Cav-3 and anti-SUMO-2/3 antibodies. E. Rat myocyte lysates were immunoprecipitated with anti-SUMO antibodies and IP, SN and WCL were analyzed by immunoblotting with anti-Cav-3 antibodies.

Fig. 2. In vitro SUMOylation of caveolin-3: expression and purification of Cav-3-V5 in E. Coli.

A. Strategy for expression of SUMOylated Cav-3-V5 in E. coli. BL21(DE3) cells were sequentially transformed with 3 plasmids with compatible origins of replication and selection markers: pKRSUMO, pBADE12 and pST39-Cav-3-V5, which allow inducible expression by IPTG of SUMO-1, the SUMO E1 and E2 enzymes and Cav-3-V5 respectively (plasmid maps adapted from [29]).

B. Small scale expression to determine solubility of Cav-3-V5 and validate expression of each plasmid. BL21(DE3) cells transformed with all three plasmids (lanes 5-6) were lysed pre- and post-IPTG induction of protein expression. Cells transformed with pST39-Cav-3-V5 alone (lanes 1-2) or just pKRSUMO and pBADE12 (lanes 3-4) were negative controls. The soluble supernatant and insoluble pellet (data not shown) from cell lysates were analyzed by immunoblotting with anti-V5-HRP antibodies.

C. Ni-NTA purification of SUMOylated Cav-3-V5. Cells expressing all 3 plasmids were scaled up for Ni-NTA purification. Cav-3-V5 was eluted with 250 mM imidazole (lanes 8-12). Cells transformed with pST39-Cav-
3-V5 alone or with pKRSUMO and pBADE12 were processed in parallel as negative controls (data not shown) (IN=Input, SN=Supernatant, W=Wash, E=Elution).  

**D. Incubation of E. coli lysates with the catalytic domain of SENP1.** Purified lysates from BL21(DE3) cells transformed with all 3 plasmids (C) were incubated for 1 h at 37°C with and without the catalytic domain of SENP1 (SENP1\textsubscript{CD}) to validate that electrophoretic mobility shifts in anti-V5-HRP immunoblots were caused by covalent modification of Cav-3-V5 by SUMO-1.

**Fig. 3.** In vivo SUMOylation assays: SUMOylation of caveolin-3 is enhanced by interaction with the SUMO E3 ligase PIASy.  

**A. PIASy stimulates SUMOylation of caveolin-3.** HEK 293 cells were co-transfected with Cav-3-V5 (lanes 1-5), Myc-SUMO-3 and Ubc9 (lanes 1-4) and either PIAS1, PIAS3 or PIASy (lanes 2, 3 and 4 respectively). Cells transfected with empty vector DNA were included as a negative control (lane 6). Lysates were analyzed by immunoblotting with anti-Myc, anti-V5-HRP and anti-PIASy antibodies.  

**B. PIASy dose-dependently enhances caveolin-3 SUMOylation.** HEK 293 cells were co-transfected with Cav-3-V5, SUMO-3 and Ubc9 with or without increasing amounts of PIASy (lanes 4-7; 0, 40, 100 or 200 ng plasmid DNA). Cells transfected with just Cav-3-V5 and SUMO-3 or PIASy alone were negative controls (lanes 1 and 2 respectively). Lysates were analyzed by immunoblotting with anti-V5-HRP and anti-GAPDH antibodies.  

**C and D. PIASy co-immunoprecipitates with caveolin-3.**  

**C.** Lysates from HEK 293 cells co-transfected with Cav-3-V5, Ubc9, SUMO-3 and PIASy were immunoprecipitated with anti-V5 and anti-Cav-3 antibodies and immunoblotted with anti-V5-HRP and anti-PIASy antibodies.  

**D.** The reciprocal immunoprecipitation was performed with anti-PIASy antibodies and immunoblotted with anti-V5-HRP and anti-PIASy antibodies.  

**E. Endogenous expression of PIASy in adult rat cardiac myocytes.** Freshly isolated rat cardiac myocytes were lysed and analyzed by immunoblotting with anti-PIASy antibodies.

**Fig. 4.** Modification of Cav-3-V5 by SUMO-3 is reduced by mutation of the consensus site lysine (K38R), and abolished by mutating all lysines (K7R).  

**A. Schematic of the Cav-3-V5 construct.** Each lysine residue is shown (K15, K20, K30, K38, K69, K108, K144) in context with the N-terminus, scaffolding domain, transmembrane domain, C-terminus and V5/His affinity tags.  

**B. Mutation of consensus site lysine K38.** Wild-type Cav-3-V5 (WT, lanes 1-2) or the consensus site, Lys to Arg mutant (K38R, lane 3) were co-transfected into HEK 293 cells with Ubc9, PIASy (lanes 1-3) and either SUMO-1 (lane 2) or SUMO-3 (lanes 1 and 3). Lysates were analyzed by immunoblotting with anti-V5-HRP antibodies.  

**C. Single ‘KR’ mutants.** HEK 293 cells were co-transfected with Cav-3-V5 ‘KR’ mutants; K20R, K30R, K38R, K69R and K144R (lanes 2-6) and the SUMOylation machinery; SUMO-3, Ubc9 and PIASy (lanes 1-7). Lysates were analyzed by immunoblotting with anti-V5-HRP antibodies (long exposure (top panel) and a short exposure (bottom panel)). The WT (lane 1) and Cav-3-V5-K7R mutant (lane 7) are included as positive and negative controls for Cav-3-V5-SUMO-3 conjugates.  

**D. Multiple ‘KR’ mutants.** WT or the K7R mutant were transfected with and without the SUMOylation machinery (lanes 1-5). MG-132 was pre-incubated with cells 3 h prior to lysis (lane 5). Multiple ‘KR’ mutants were also co-transfected with the SUMOylation machinery (lanes 6-11). Labels indicate residues not mutated (i.e. K144 has a single Lys at position 144).

**Fig. 5.** Reversal of each arginine mutation in K7R back to lysine one at a time reveals that K38 is the preferred SUMOylation site when PIASy is co-expressed.  

**A. Schematic of the Cav-3-V5-K7R construct.** Each arginine mutation present in K7R (R15, R20, R30, R38, R69,
R108, R144) is shown in context with the N-terminus, scaffolding domain, transmembrane domain, C-terminus and V5/His affinity tags.  

**B. Identification of the preferred SUMOylation site: ‘RK’ mutants.** HEK 293 cells were co-transfected with each of the seven ‘RK’ mutants (lanes 3-9) along with the SUMOylation machinery (SUMO-3, Ubc9 and PIASy). WT and K7R (lanes 1 and 2 respectively) served as positive and negative controls for Cav-3-V5-SUMO-3 conjugates. Lysates were analyzed by immunoblotting with anti-V5-HRP antibodies. 

**C. PIASy-dependent SUMOylation of K38.** The four N-terminal ‘RK’ mutants; R15K, R20K, R30K and R38K (lanes 5-12) were transfected with and without the SUMOylation machinery. WT and Cav-3-V5-K7R (lanes 1-4) were included as positive and negative controls for Cav-3-V5-SUMO-3 conjugates. Lysates were analyzed by immunoblotting with anti-V5-HRP, anti-PIASy and anti-GAPDH antibodies.

*Fig. 6.* Wild-type Cav-3-V5 stabilizes, while the SUMOylation-deficient K7R mutant destabilizes, FLAG-β2AR.  

**A.** and **B.** *Co-expression of Cav-3-V5-K7R reduces the expression of FLAG-β2AR protein and WT has an opposite effect when co-transfected with the SUMOylation machinery.* HEK 293 cells were co-transfected with FLAG-β2AR (lanes 1-11) and either WT Cav-3-V5 (lanes 3-4 and 8-9) or the K7R mutant (lanes 5-6 and 10-11), both with and without the SUMOylation machinery; SUMO-3, Ubc9 and PIASy (even vs. odd lanes, respectively). The proteasomal inhibitor MG-132 was pre-incubated with cells 3 h prior to lysis (lanes 7-11). 48 h post-transfection, lysates were immunoprecipitated with anti-FLAG antibodies and analyzed by immunoblotting with anti-FLAG-HRP, anti-ubiquitin, anti-V5-HRP and anti-PIASy antibodies.  

**B.** HEK 293 cells were transfected identical to those in (A) lanes 1-6; however, cells were harvested 24 h, rather than 48h, post-transfection. Lysates were immunoprecipitated with anti-FLAG antibodies and analyzed by immunoblotting with anti-FLAG-HRP and anti-V5-HRP antibodies.  

**C.** eNOS expression is not effected by co-transfection WT or Cav-3-V5. HEK 293 cells were co-transfected with eNOS (lanes 1-6) and either WT Cav-3-V5 (lanes 3-4) or the K7R mutant (lanes 5-6) with and without the SUMOylation machinery; SUMO-3, Ubc9 and PIASy. Cells were harvested 24 h post-transfection and anti-eNOS immunoprecipitates were analyzed by immunoblotting with anti-eNOS and anti-V5-HRP antibodies.

*Fig. 7.* The SUMOylation-deficient K7R mutant does not affect the stability of β1AR and destabilization of β2AR by K7R is blocked by β-adrenergic antagonist.  

**A.** *FLAG-β1AR is not destabilized by the K7R mutant.* HEK 293 cells were co-transfected with FLAG-β1AR (lanes 1-3) or FLAG-β2AR (lanes 4-6), the SUMOylation machinery (lanes 1-6) and either WT Cav-3-V5 (lanes 2 and 5) or the K7R mutant (lanes 3 and 6). Cell lysates were immunoprecipitated with anti-FLAG antibodies and analyzed by immunoblotting with anti-FLAG-HRP and anti-V5-HRP antibodies.  

**B. - D.** The destabilization of FLAG-β2AR by Cav-3-V5-K7R is blocked by (-)-propranolol and reduced serum.  

**B.** HEK 293 cells were co-transfected with FLAG-β2AR and the SUMOylation machinery (lanes 1-6) with either WT Cav-3-V5 (lanes 2 and 5) or the K7R mutant (lanes 3 and 6). Cells were grown in media plus 2 µM (-)-propranolol for 48 h (lanes 1-3). Cells were also grown with reduced serum for 24 h prior to harvest (lanes 4-6). Anti-FLAG immunoprecipitates were analyzed by immunoblotting with anti-FLAG-HRP and anti-V5-HRP antibodies.  

**C.** HEK 293 cells were transfected as in (A) and (B) and were treated or not for 48 h with 2 µM (-)-propranolol. Lysates were immunoprecipitated with anti-FLAG antibodies but proteins were not heated prior to SDS-PAGE to reduce formation of FLAG-β2AR aggregates and aid in quantification. Immunoprecipitates and cell lysates were analyzed by immunoblotting with...
Fig. 8. A “SUMO Switch”: regulation by SUMOylation of protein-protein interaction by the caveolin scaffolding domain (CSD). Proposed interaction of basic residues in the α-helical CSD with an ‘acidic patch’ surrounding the SUMOylation site of caveolin-3. A. Membrane topology and α-helical secondary structure of the CSD of caveolin-3. The CSD (aa55-72) forms an α-helix while the remainder of the N-terminus lacks secondary structure; it has been proposed that the N-terminal tail region (aa1-54) wraps back around the CSD (47). This interaction might occur through electrostatic interactions of an acidic patch (\textsuperscript{34}EDIVKV\textsuperscript{43}DFED) with basic residues in the CSD (α-helix image adapted from [47]). B. Helical wheel projection of the Cav-3 CSD. The amino acid sequence of the CSD (\textsuperscript{55}VVKVSYTFTVSKYW\textsuperscript{72}C) is color coded by each residue’s side chain properties and represented linearly and as a helical wheel projection to illustrate the clustering of basic (blue), polar (purple) and non-polar (yellow) residues along different sides of the helix. (http://heliquest.ipmc.cnrs.fr/). The single letter amino acid codes in the helical wheel are proportional to amino acid volume.
Figure 2
Figure 4

| A | N-Terminus | $\Psi_{KxD/E}$ | Cav-3-V5 | C-Terminus |
|---|---|---|---|---|
| | K | K | K | K | CSD | K | TM | K | K | V5 | His6 |
| 1 | 15 | 20 | 30 | 38 | 69 | 108 | 144 | 151 | |

B

| SUMO-1 | SUMO-3 | PIASy | Cav-3-V5 |
|---|---|---|---|
| + | + | + | WT WT K38R |

C

| SUMO-3 | Ubc9 | PIASy | Cav-3-V5 |
|---|---|---|---|
| + | + | + | WT K20R K30R K38R K69R K144R K7R |

D

| SUMO-3 | Ubc9 | PIASy | Cav-3-V5 | MG-132 |
|---|---|---|---|---|
| + | + | + | WT WT K7R K7R WT |

(Short Exposure)
Figure 5

### A

| N-Terminus | Ψ_{KxD/E} | C-Terminus |
|------------|----------|-----------|
| R          | R        | R         |
| R          | 30       | 38        |
| R          | 69       | 108       |
| R          | 144      | 151       |
| V5         | His₆     |           |

### B

| SUMO-3 | Ubc9 | PIASy | Cav-3-V5 | Mₘ(K) |
|--------|------|-------|----------|-------|
| +      | +    |       |          |       |
| +      | +    |       |          |       |
| +      | +    |       |          |       |
| +      | +    |       |          |       |
| +      | +    |       |          |       |
| +      | +    |       |          |       |
| +      | +    |       |          |       |
| +      | +    |       |          |       |

---

**Anti-V5-HRP**

- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9

**SUMO-3**

- WT
- K7R
- R15K
- R20K
- R30K
- R38K
- R69K
- R108K
- R144K

---

### C

| SUMO-3 | Ubc9 | PIASy | Cav-3-V5 | Mₘ(K) |
|--------|------|-------|----------|-------|
| -      | +    |       |          |       |
| -      | +    |       |          |       |
| -      | +    |       |          |       |
| -      | +    |       |          |       |
| -      | +    |       |          |       |
| -      | +    |       |          |       |
| -      | +    |       |          |       |
| -      | +    |       |          |       |

---

**Anti-V5-HRP**

- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9

**Anti-PIASy**

- 10
- 11
- 12

**Anti-GAPDH**

- 10
- 11
- 12
Figure 7

(A) SUMO-3 | Ubc9 | PIASy | Cav-3-V5
---|---|---|---
+ | + | + | + | +
+ | + | WT | K7R | +
- | WT | K7R | - | +

M_{r}(K)

anti-FLAG

anti-V5-HRP

1 2 3 4 5 6

(B) SUMO-3 | Ubc9 | PIASy | Cav-3-V5
---|---|---|---
+ | + | + | + | +
+ | + | WT | K7R | +
- | WT | K7R | - | +

Treatment:
2μM Propranolol
1% FBS (24hrs)

M_{r}(K)

anti-FLAG

anti-V5-HRP

anti-PIASy

1 2 3 4 5 6

(C) SUMO-3 | Ubc9 | PIASy | Cav-3-V5
---|---|---|---
+ | + | + | + | +
+ | + | WT | K7R | +
- | WT | K7R | - | +

Propranolol:

anti-FLAG

anti-GAPDH

1 2 3 4 5 6

(D) Fold over vehicle-treated B2AR control (normalized GAPDH)

Vehicle • Propranolol

P=0.055

P=0.03

P=0.02

B2AR B2AR + WT B2AR + K2R
Caveolin-3 undergoes SUMOylation by the SUMO E3 ligase PIASy: SUMOylation affects GPCR desensitization
Stephen R. Fuhs and Paul A. Insel

J. Biol. Chem. published online March 1, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.214270

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/03/03/M110.214270.DC1