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Multiple Effects of SERCA2b Mutations Associated with Darier's Disease*

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Darier's disease (DD) is an autosomal dominant disorder caused by mutations in the *ATP2A2* gene, encoding sarco/endoplasmic reticulum Ca^{2+}-ATPase pump type 2b isoform (SERCA2b). Although >100 mutations in the *ATP2A2* gene were identified, no apparent relation between genotype/phenotype emerged. In this work, we analyzed 12 DD-associated mutations from all of the regions of SERCA2b to study the underlying pathologic mechanism of DD and to elucidate the role of dimerization in SERCA2b activity. Most mutations markedly affected protein expression, partially because of enhanced proteasome-mediated degradation. All of the mutants showed lower activity than the wild type pump. Notably, several mutants that cause relatively severe phenotype of DD inhibited the activity of the endogenous and the co-expressed wild type SERCA2b. Importantly, these effects were not attributed to changes in passive Ca^{2+} leak, inositol 1,4,5-trisphosphate receptor activity, or sensitivity to inositol 1,4,5-trisphosphate. Rather, co-immunoprecipitation experiments showed that SERCA2b monomers interact to influence the activity of each other. These findings reveal multiple molecular mechanisms to account for the plethora of pathologic states observed in DD and provide the first evidence for the importance of SERCA2b dimerization in pump function *in vivo*.

Darier's disease (DD,[1] OMIM 124200), also known as keratosis follicularis, is an autosomal dominant skin disorder characterized by loss of adhesion between epidermal cells (acantholysis) and abnormal keratinization (1). DD is also associated with an increased prevalence of neuropsychiatric disorders including bipolar disorders, schizophrenia, epilepsy, and mental retardation (1). Recently, mutations in the *ATP2A2* gene encoding the sarco/endoplasmic reticulum Ca^{2+}-ATPase pump type 2b isoform (SERCA2b) have been identified as a cause of DD (2). SERCA2b is a housekeeping protein, which is expressed ubiquitously and is responsible for Ca^{2+} uptake into the ER, including the agonist-mobilizable Ca^{2+} pool (3, 4). By determining ER Ca^{2+} load, the SERCA2 pumps modulate several parameters of the Ca^{2+} signal. In resting cells, the SERCA2 pump provides the bulk of the dynamic Ca^{2+}-buffering capacity to prevent large fluctuation in $[Ca^{2+}]_i$ (5). In stimulated cells, SERCA2 controls the activity of the store-operated Ca^{2+} influx channel by preventing accumulation of Ca^{2+} at the mouth of the channels and their inhibition by $[Ca^{2+}]_i$ (6). SERCA2b reloads the ER with Ca^{2+} at the end of cell stimulation (5) and between Ca^{2+} spikes to determine the frequency of Ca^{2+} oscillations (7), and to control the diverse cellular functions regulated by $[Ca^{2+}]_i$ and Ca^{2+} oscillations (8, 9).

Alteration in SERCA2 pump function by overexpression in Chinese hamster ovary cells (10) or partial deletion in mice (7) lead to adaptation of the Ca^{2+} signaling machinery and Ca^{2+}-regulated cell functions that translates to nearly normal physiological response in the majority of organs and tissues. This correlates with the lack of consistent defect in DD patient platelet and heart function in which SERCA2 is the major pump in the endo/sarcoplasmic reticulum (11). By contrast, all of the DD-associated mutations cause moderate to severe skin disorders and several mutations cause neuropsychiatric disorders, suggesting particular susceptibility of these tissues to reduction in SERCA2 activity. These intriguing observations raise several questions, among them are noted as follows. (*a*) How mutations associated with DD affect SERCA2 pump activity? (*b*) Mutations in *ATP2A2* that cause DD are found all along the gene, and no phenotype-genotype could be deduced. Can phenotype-genotype be found based on SERCA2 pump activity? (*c*) Do selective mutants associated with DD affect native SERCA pump activity to provide a mechanism for the variable phenotypes? Partial answers to these questions can further our understanding of SERCA2 pump function and how mutations in the pump lead to DD.

In this work, we analyzed 12 SERCA2b mutants that cause DD in an attempt to address these questions. Mutants were selected from all of the regions of the protein. The clinical description of the disease for the vast majority of the mutants is incomplete and, for the most part, relies on a single patient of a single affected family. This made it difficult to attempt correlation between genotype and phenotype. Nevertheless, relying on published information, we attempted to select one group of mutants that was reported to affect only keratinocyte function and one group that was reported to also cause neurological deficiencies. An analysis of protein expression revealed a diverse effect of the DD-causing mutations on protein expression. Measurements of Ca^{2+} uptake and co-immunoprecipitation experiments showed interactions among SERCA2b monomers and highlighted the role of the dimers in pump regulation. Most notably, several mutants reduced the activity

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[1] The abbreviations used are: DD, Darier's disease; SECRA2b, sarcoendoplasmic reticulum Ca^{2+}-ATPase pump type 2b isoform; ER, endoplasmic reticulum; WT, wild type; IP_3, inositol 1,4,5-trisphosphate; HEK, human embryonic kidney; CMV, cytomegalovirus; TM, transmembrane; co-IP, co-immunoprecipitate; SLO, streptolysine O; BHQ, 2,5-di-*tert*-butylhydroquinone.

of the native and of co-expressed WT pumps. These findings provide the first evidence to indicate physical and functional interaction between SERCA2b monomers. Although no definitive correlations between the mutations and DD phenotype became apparent, probably because of poor clinical information available for this disease, our findings do provide a molecular mechanism to account for the disease pathogenesis and how diverse phenotypes can be manifested.

EXPERIMENTAL PROCEDURES

Materials, Antibodies, and Solutions—Chelex, ATP, phosphocreatinine, creatinine phosphokinase, antimycin A, and oligomycin were purchased from Sigma. Fluo-3 pentaammonium salt was from Molecular Probes, Inc. (Eugene, OR). D-*Myo*-inositol 1,4,5-trisphosphate (IP_3) hexasodium salt was from Alexis Biochemicals (San Diego, CA). Streptolysin O was from BD Diagnostic Systems (Sparks, MD). Lactacystin and ionomycin were from Calbiochem. Fetal bovine serum and 0.05% trypsin/EDTA were from ATLANTA Biologicals (Norcross, GA). Restriction enzymes were obtained from New England Biolabs (Beverly, MA) and Roche Applied Sciences.

Cell Culture and Transfection—HEK 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10 mM glucose supplemented with 10% fetal calf serum. The SERCA constructs were transfected using LipofectAMINE reagent (Invitrogen). The cells were used for immunoprecipitation or immunoblotting 48 h after transfection.

Site-directed Mutagenesis—The pcDNA3.1 plasmid containing human SERCA2b was a generous gift from Dr. Jonathan Lytton (University of Calgary, Calgary, Canada). Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to instructions provided by the manufacturer. All of the mutations were verified by the sequencing of four separate clones from each mutant. The mutants generated for this work are listed in Table I.

Preparation of HA-tagged and Myc-tagged Human SERCA2b Constructs—HA- and Myc-tagged human SERCA2b constructs were generated by PCR amplification of human SERCA2b using the 5′-oligonucleotide primer 5′-AC GTC AAT GGG TCG ACT ATT TAC G-3′ containing the underlined *Sal*I and 3′-oligonucleotide primer 5′-AGA AGG CAC AGT CGA GGC TG-3′ located after a *Not*I site. The amplified DNA was gel-purified, digested with appropriate enzymes, and ligated into the pCMV-HA or Myc vectors (Clontech Laboratories, Inc., Palo Alto) that had been digested with *Sal*I and *Not*I and gel-purified. The constructs were verified by DNA sequencing.

Immunoprecipitation and Immunoblotting—Transfected HEK 293 cells were washed with phosphate-buffered saline and lysed with buffer containing 135 mM NaCl, 0.1% Triton X-100, 20 mM Tris-HCl, pH 7.4, and a proteinase inhibitor mixture (Roche Applied Sciences). Precleared lysates (up to 500 μg of protein) were mixed with 3 μl of anti-HA antibodies or anti-Myc antibodies and incubated for 2 h at 4 °C in lysis buffer. Immune complexes were collected by overnight incubation with 50 μl of protein G-agarose at 4 °C under gentle agitation. Immunoprecipitated proteins were washed four times with lysis buffer prior to electrophoresis. The immunoprecipitates or lysates were suspended in SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, the proteins were detected with the appropriate primary and secondary antibodies.

Measurement of Ca^{2+} Uptake and Release in Permeabilized Cells—HEK 293 cells were released, washed, and re-suspended in serum-free Dulbecco's modified Eagle's medium containing 10 mM glucose. The cells then were counted, and 3×10^6 cells were washed twice with a high potassium solution (130 mM KCl and 10 mM HEPES, pH 7.4, with NaOH). The cells were suspended in 50 μl of Chelex-treated high potassium solution and added to 0.45 ml of SLO-permeabilization medium. The SLO-permeabilization medium was composed of resin-treated 130 mM KCl and 10 mM HEPES solution supplemented with 3 mM ATP, 5 mM $MgCl_2$, 10 mM phosphocreatinine, 30 units/ml creatinine phosphokinase, 5 μM antimycin A, 5 μM oligomycin, 2 μM Fluo-3, and 4.5 mg/ml SLO. The recording of Fluo-3 fluorescence was initiated upon the addition of cells to a warm (37 °C) permeabilization medium and was recorded at excitation and emission wavelengths of 488 and 530 nm, respectively. The cells were allowed to take up and reduce [Ca^{2+}] of the permeabilization medium to 50–100 nM before the addition of IP_3 or BHQ. The Fluo-3 fluorescence signals were calibrated by the addition of 1 mM $CaCl_2$ to the medium (F_{max}), which was followed by the addition of 5 mM EGTA and 30 mM NaOH to obtain F_{min}. [Ca^{2+}] was calculated using a K_d of 370 nM at 37 °C.

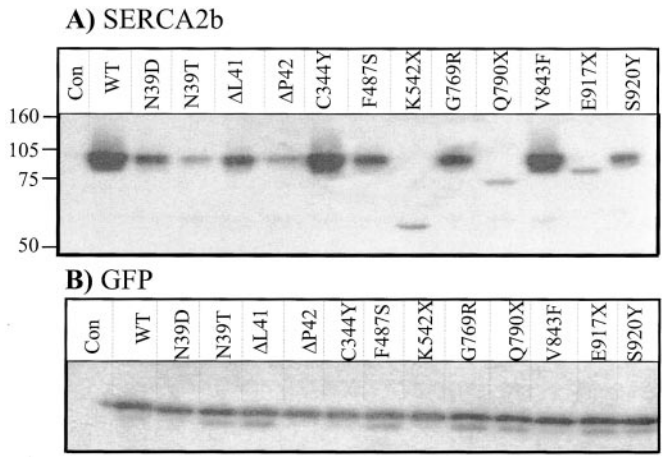

FIG. 1. **Effect of mutations associated with DD on SERCA2b protein expression.** HA-tagged WT or mutant SERCA and green fluorescent protein (*GFP*) plasmids were transiently transfected in HEK 293 cells. 5 μg of protein/lane were separated on 8% HA (*A*) or 10% GFP (*B*) SDS-polyacrylamide gels. *Con*, control.

RESULTS

Protein Levels of SERCA2b Mutants—Because haploinsufficiency cannot explain the variable phenotype of DD patients (12), our initial hypothesis was that the severity of the disease may correlate with protein expression levels. This hypothesis was refuted by the finding that most DD mutants examined affected SERCA2b expression and/or stability (Fig. 1*A*). With the exception of two mutants (C344Y and V843F), all of the mutants markedly reduced protein expression. The possibility of variable SERCA2b expression because of variable transfection efficiency was excluded by the analysis of co-transfected green fluorescent protein (Fig. 1*B*).

Effect of the N-terminal mutants is consistent with previous work with SERCA1. Deletion of the N-terminal domain of SERCA1 reduced protein expression (13). Accordingly, mutation of SERCA2b Asn-39 to Asp or Thr reduced protein expression, suggesting that the integrity of the N terminus is important for pump expression. Interestingly, all of the truncation mutants including E917X markedly reduced protein expression, suggesting increased degradation of these pump products (see below). No concrete conclusion emerged from the other mutations. Thus, a mutation in the P domain (phosphorylation domain), C334Y, had minimal effect on protein expression, whereas another mutation, F487S, markedly reduced protein expression. Similarly, G769R in TM5 reduced protein expression, whereas V843F in the TM8-TM9 loop had minimal effect. Hence, the overall significance of the protein expression findings to DD is that the mutations can affect pump activity by multiple mechanisms. Some mutants affect pump activity because of reduced protein expression, whereas others such as C344Y and V843F must act by a different mechanism. The results in Fig. 1 together with the phenotypic information listed in Table I indicate a lack of correlation between protein expression and disease phenotype.

Proteasome-mediated Degradation of Mutant SERCA2b Protein—Previous work showed that lactacystin, a specific proteasome inhibitor, enhanced degradation of WT SERCA1 and prevented the small degradation of ΔAla3-SERCA1 but had no effect on several other deletion or mutations between Glu2-Ala14-SERCA1 (14). Therefore, we tested the effects of lactacystin on SERCA2b protein levels. Several of the mutants that showed low protein expression exhibited enhanced protein degradation. However, as with protein expression, protein degradation followed a complex pattern with respect to the effect of individual mutants (Fig. 2). First, we confirmed the finding

TABLE I
DD-associated mutations analyzed in this work

Mutation	Nucleotide change	Consequence	Exon	Functional domain	Affected patients[a]	Skin features[b]	Neuropsychiatric features
N39D	115A→G	Missense	1	Upstream S1	1 family	Moderate	Hospitalization for violent behavior
N39T	116A→C	Missense	1	Upstream S1			None
ΔL41	121delTTA	In-frame deletion	2	S1		Severe	Suicide in family, emotional problems
ΔP42	124delCCG	In-frame deletion	2	S1		Severe	Depression
C344	1031G→A	Missense	8	M4/Phosphorylation	1 family	Mild-moderate	None
F487S	1460T→C	Missense	12	Phosphorylation	1 family (4)	Severe	Schizophrenia, seizures, depression
1625delAG (K542X)		Frameshift (PTC + 9aa)	13	ATP binding	1 family (2)	Moderate (2)	None
G769R	2305G→C	Missense	15	M5			
Q790X	2368C→T	Nonsense	16		12		Multiple neuropsychiatric features
V843F	2527G→T	Missense	17	M7			Epilepsy, major depressive disorder (single episode)
E917X	2749G→T	Nonsense	19	M8–M9 loop			None
S920Y	2759C→A	Missense	19	M8–M9 loop	2 families (5 + 3)	Mild (1), moderate (5), severe (2)	Epilepsy(1), dysmorphic facies, low-normal I.Q.

[a] The number in parentheses indicates number of patients.
[b] Skin severity described was adapted after the categorization of Ringpfeil *et al.* (38).

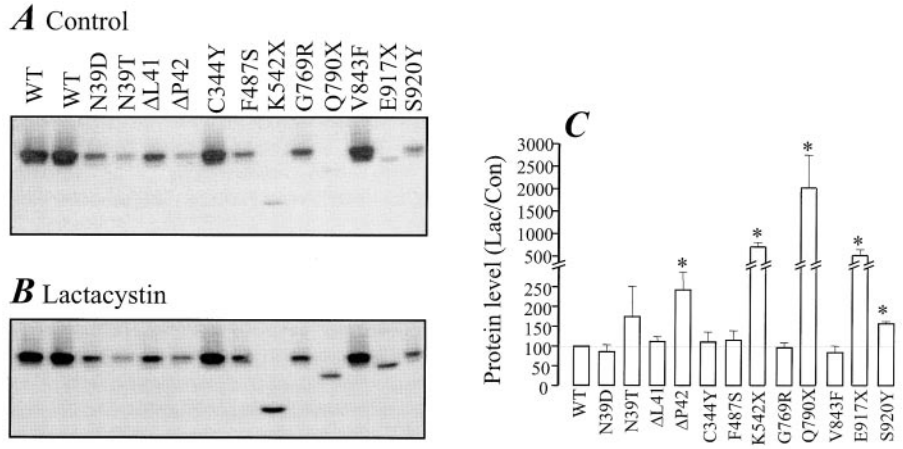

FIG. 2. **Proteasome-mediated degradation of SERCA2b mutants.** HA-tagged WT or mutant SERCA plasmids were transfected in two parallel sets of HEK 293 cells. After 24 h, the cells were incubated in media containing vehicle (*A*) or 10 μM lactacystin (*B*), and protein expression was determined 24 h later. *C*, a summary of the results of three experiments. *Lac*, lactacystin; *Con*, control.

made with SERCA1 by observing slight increased degradation of WT SERCA2b by lactacystin. Furthermore, lactacystin similarly increased degradation of the C344Y and V843F mutants that had minimal effect on protein expression. The group of mutants most susceptible to degradation by the proteasome is the nonsense and the premature translation termination codon-inducing frameshift mutations (1625delAG). Lactacystin increased the expression of these mutants between 5- and 20-fold (Fig. 2*C*). This finding indicates that the ER quality control-mediated degradation machinery recognizes the truncated SERCA2b mutants as misfolded proteins. Interestingly, the inhibition of the proteasome did not have a similar effect on all of the mutants. Furthermore, the mutants in the same region had an opposite effect. Thus, lactacystin had no effect on N39D but appeared to enhance expression of N39T, and it had no effect on expression of ΔL41 but enhanced the expression of ΔP42. Selective effect of lactacystin on the degradation of several SERCA2b (this work) and SERCA1 mutants (14) suggest that SERCA pumps are degraded by the proteasome and other proteases. However, as with protein expression, no obvious correlation between phenotype-genotype was apparent.

Ca^{2+} Uptake Activity—The most interesting results were obtained by measuring SERCA pump activity. Selective traces, the experimental protocols, and the parameters tested are illustrated in Fig. 3. A summary of pump activity of native cells, cells transfected with the WT pump, and all of the mutants is given in Fig. 4. Ca^{2+} uptake into the ER was initiated by adding cells to the SLO-containing permeabilization medium. The uptake attained constant rate after completion of permeabilization, and Ca^{2+} uptake rate was determined from the nearly linear portion of the slopes as indicated by the *dashed lines* in Fig. 3. At the end of the uptake period, the cells were used to measure either IP_3-mediated Ca^{2+} release (Fig. 3, *A–C*) or the rate of ER-passive Ca^{2+} leak by inhibition of SERCA2b with BHQ (Fig. 3, *D–F*). Residual Ca^{2+} in the stores was then discharged by the addition of 2 μM Ca^{2+} ionophore ionomycin. Expression of WT SERCA2b increased Ca^{2+} uptake rate by ~3-fold. Evaluation of Ca^{2+} uptake by all of the mutants revealed that only one mutant, V843F, which was expressed at levels comparable to the WT pump, retained activity that was approximately 50% that of the WT pump (Fig. 4). Six mutants had no activity and did not affect Ca^{2+} pumping by the native SERCA pumps. The most interesting mutants were the 5 of the 12 tested (42%) (N39D, N39T, C344Y, F487S, and S920Y) that reduced the activity of the native pumps.

Reduction of native Ca^{2+}-pumping activity raised the ques-

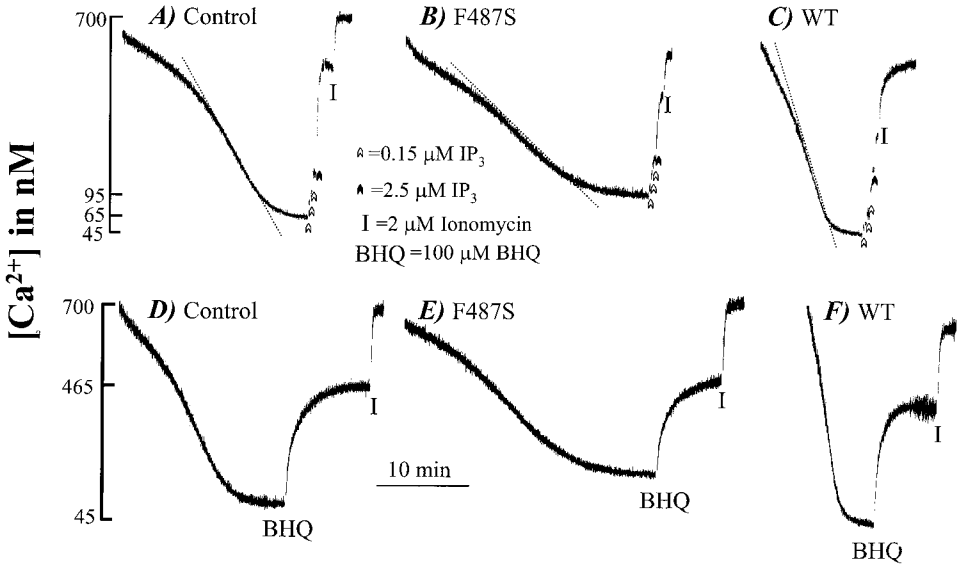

FIG. 3. **Ca^{2+} uptake, IP_3-mediated Ca^{2+} release and Ca^{2+} leak in cells expressing WT and mutant SERCA2b.** Approximately 48 h after transfection, cells were released from culture plates and counted, and 3×10^6 cells were washed and added to permeabilization medium to measure Ca^{2+} uptake into the ER as described under "Experimental Procedures." The rate of Ca^{2+} pumping was determined from the slopes of reduction in medium [Ca^{2+}]. Ca^{2+} uptake was measured in control non-transfected HEK 293 cells (*A* and *D*) and cells transfected with F487S (*B* and *E*) and WT SERCA2b (*C* and *F*). At the end of Ca^{2+} uptake, IP_3-mediated Ca^{2+} release was measured by the addition of increasing concentrations of IP_3 at 0.15, 0.30, 0.45, and 2.95 μM (*A–C*) and the rate of Ca^{2+} leak was determined by inhibiting the pump with 100 μM SERCA pump inhibitor BHQ (*D–F*). Residual stored Ca^{2+} was released by the addition of 2 μM ionomycin (*I*) at the end of each experiment. All traces were calibrated as described under "Experimental Procedures."

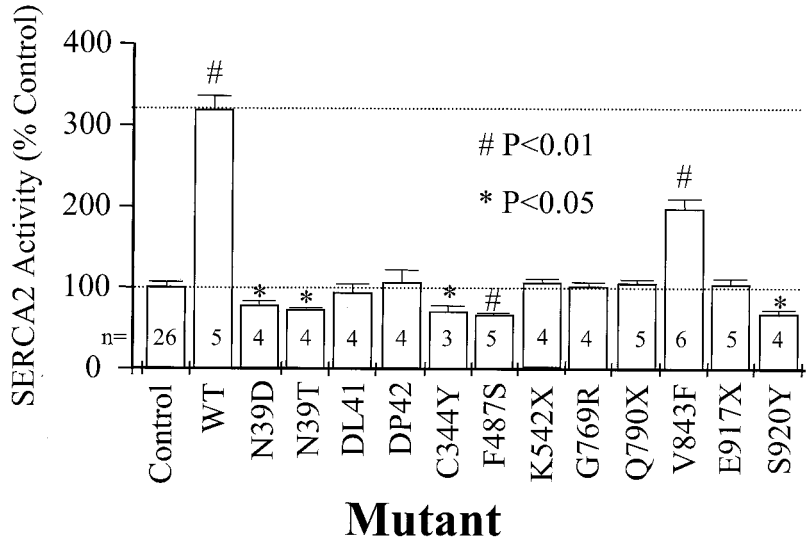

FIG. 4. **Ca^{2+} transport by WT and mutant SERCA2b pumps.** The protocol of Fig. 3 was used to evaluate pump activity of all of the mutants. The number of experiments is given in the columns. The results are plotted as the mean ± S.E. * and #, statistically significant difference of $p < 0.05$ and $p < 0.01$, respectively, compared with control.

tion of relevance to DD where the WT and most mutants mRNA are likely to be present at comparable levels. Therefore, to simulate the DD situation, a 1:1 mixture of WT and the mutant clones that reduced the activity of the native pump were transfected and the resulting pumping activity measured. The results are summarized in Fig. 5. The expression of WT SERCA2b again increased Ca^{2+}-pumping rate by ~3-fold, and this rate was taken as 100% control. Co-expression of WT and the G769R mutant that had no effect on the native pump (Fig. 4) was used as an expression control, and as expected, it had no effect on Ca^{2+} pumping by the expressed WT SERCA2b. Fig. 5 shows that all of the mutants that reduced the activity of the native pump similarly inhibited the activity of the expressed WT SERCA2b, indicating that the inhibition is inheritant to these mutants.

The poor clinical definition of DD phenotype makes it difficult to draw a precise correlation between the mutants that inhibited WT SERCA2 activity and the disease phenotype. However, the tendency is that mutants that cause a relatively severe phenotype such as F487S and S920Y noticeably inhibited endogenous and expressed WT SERCA2b activity. The finding of reduced WT pump activity by the mutants provides the first molecular mechanism that can account for the variable DD phenotypes.

Passive Ca^{2+} Leak and IP_3 Sensitivity—The next question we addressed is how the DD-associated mutations reduce WT SERCA2b-pumping activity. One possibility is that the mutant proteins increase passive Ca^{2+} leak across the ER membrane, resulting in an apparent reduced pumping rate. Increased passive leak was proposed to account for the inhibition of native Ca^{2+} pump activity by SERCA1 splice variants when expressed in heterogeneous systems (15). In this work, we used a SERCA pump inhibition protocol to estimate the Ca^{2+} leak rate at the end of the uptake period. Measurement of Ca^{2+} leak with all of the DD mutants used in this work (Fig. 3, *D–F*, *BHQ* addition) showed that this mechanism cannot account for the reduced

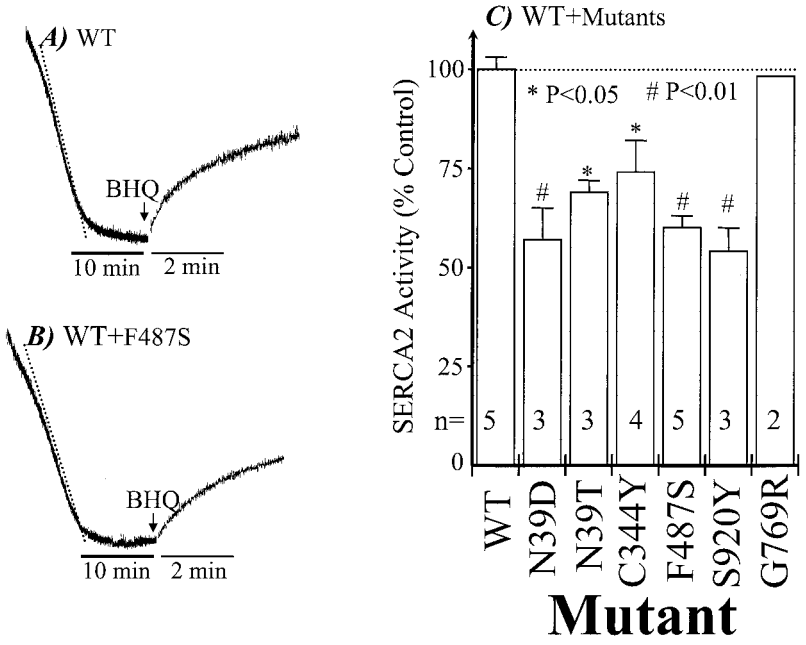

FIG. 5. **Effect of selective mutants on pump activity of co-transfected WT SERCA2b.** The protocol of Fig. 3 was used to assay pump activity. WT SERCA2b (*A*) alone or together with the indicated mutants (*B* and *C*) were co-transfected in HEK 293 cells. The effect of all of the mutants that reduced the activity of the native pump (see Fig. 4) was analyzed. The results are plotted as the mean ± S.E. * and #, denote statistically significant difference of $p < 0.05$ and $p < 0.01$, respectively, compared with control.

SERCA pump activity, because none of the mutants increased the passive Ca^{2+} leak. Because SERCA pumps determine Ca^{2+} content in the ER and the ER Ca^{2+} load affect sensitivity to IP_3 (5), another possibility is that the mutant pumps altered IP_3-mediated Ca^{2+} release. The measurement of the extent of Ca^{2+} release and its dependence on IP_3 concentration showed that none of the mutants had an apparent effect on IP_3-mediated Ca^{2+} release (Fig. 3, *A–C*), confirming the results obtained in the SERCA2b$^{+/-}$ mice (7).

Interaction between SERCA2b Monomers—The simplest mechanism by which the mutants can inhibit the activity of the WT SERCA2b is reducing the expression of the pump; however, this was found not to be the case (Fig. 6). The level of WT pump protein was not affected by co-expression of any of the mutant proteins. Equally, co-expression of the WT and mutant pumps had no effect on expression of the mutant pumps.

A second alternative is that SERCA2b exists as a dimer and the mutants, including those that are expressed at low levels, interact with the WT pump to reduce its activity. A clue to this possible mechanism is provided by earlier work suggesting that SERCA pump monomers sense and are influenced by their neighbors. Thus, the titration of Ca^{2+}-ATPase and Ca^{2+} uptake activities with fluorescein isothiocyanate (16), radiation inactivation analysis of pump activity and integrity (17, 18), freeze-fracture and deep-etching of sarcoplasmic reticular membranes (19, 20), and analysis with photoaffinity spin-labeled derivative of ATP (21) all suggested that the functional unit of the Ca^{2+}-ATPase is a dimer. Furthermore, an analysis of a purified 48-kDa SERCA1 fusion protein by small angle x-ray scattering suggested a requirement of the hinge domain (amino acids 670–728) region for self-association of the large hydrophilic domain into a dimer (22). Thus, it is possible that the mutant pumps affect the WT pump by way of protein interaction.

To test interaction between pump monomers, we attempted to co-immunoprecipitate (co-IP) co-expressed HA- and Myc-tagged SERCA2b. Initial attempts to do so failed. Fig. 7, *A* and *B*, shows that when the cells were lysed with a medium containing 1.0% Triton X-100 and 150 mM NaCl, it was possible to immunoprecipitate each of the tagged monomers but not to co-IP them. We then considered the possibility that interaction among the SERCA2b monomers is subtle and could not withstand the harsh solubilization conditions. This was confirmed by observing co-IP of the monomers when the cells were lysed in a medium containing 0.1% Triton X-100 and 135 mM NaCl (Fig. 7, *C* and *D*). Furthermore, co-IP of the SERCA2b monomers could be observed when the cells were lysed with 1% of the milder detergent Nonidet P-40 (data not shown). The findings in Fig. 7 provide the first direct evidence that SERCA2b monomers can interact with each other *in vivo*.

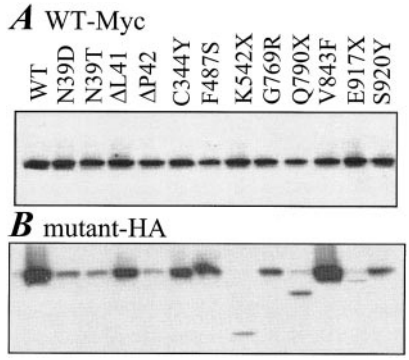

FIG. 6. **DD mutants do not affect expression of WT SERCA2b.** Myc-tagged WT and HA-tagged WT or mutant SERCA2b plasmids were co-transfected into HEK 293 cells. 5 μg of protein were analyzed for expression of both WT (*A*) and mutant pumps (*B*).

The functional consequence of dimerization of the SERCA2b monomers can be deduced from examining the interaction of the mutants with the WT pump. The results of such analysis are displayed in Fig. 7, *E* and *F*. Several findings are noticeable. First, most but not all SERCA2b mutants interact with the WT pump, providing a plausible explanation for inhibition of the native and expressed WT SERCA2b activity by the mutants. Second, all of the extreme N-terminal mutants showed reduced co-IP compared with their expression levels, implicating this region of the protein in dimerization. The same probably holds for the G769R mutation that showed good expression when expressed alone (Fig. 1 and 2) or together with the WT pump (Fig. 6) but showed very poor co-IP (Fig. 7). Because this mutation inserts an additional positive charge into TM5, it may result in misfolding of the protein to prevent dimerization. Third, all of the truncation mutants and the S920Y mutant

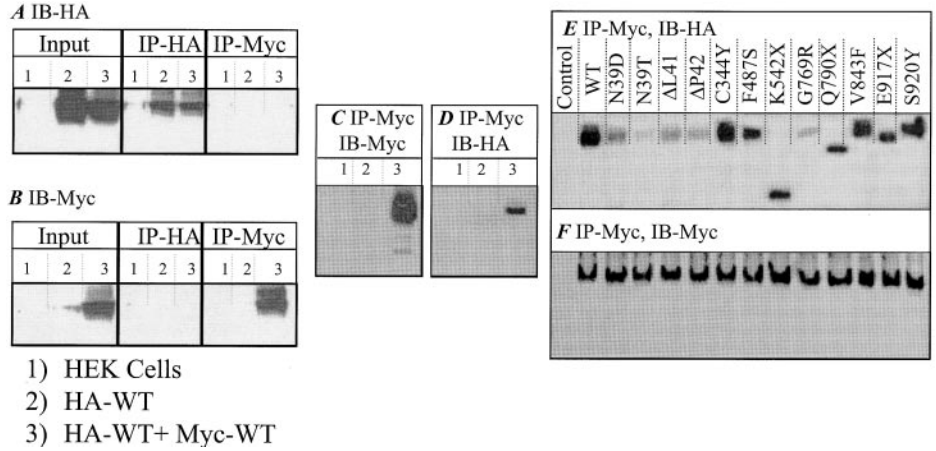

FIG. 7. **Interaction of SERCA2b monomers.** HEK 293 cells transfected with empty vector (*lane 1*), HA-WT (*lane 2*), or HA-WT and Myc-WT SERCA2b (*lane 3*) were used for the experiments in the blots (*A–D*). The cells were lysed 48 h after transfection with a buffer containing either 1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, and protease inhibitor mixture (*A* and *B*) or the same medium but with Tition X-100 reduced to 0.1% and NaCl reduced to 135 mM (*C* and *D*). The lysates were used to immunoprecipitate HA (*middle three lanes* in *A* and *B*) or Myc (*right three lanes* in *A* and *B* and in *C* and *D*) and blot for HA (*A* and *D*) or Myc (*B* and *C*). Note that only when the cells were extracted with 0.1% Triton X-100 and 135 mM NaCl, the SERCA2b monomers could be co-immunoprecipitated. *E* and *F*, cells were co-transfected with Myc-tagged WT SERCA2b and the HA-tagged mutants and used for extraction with 0.1% Triton X-100. The extracts were used to immunoprecipitate Myc and were probed for HA (*E*) and Myc (*F*). *IP*, immunoprecipitate; *IB*, immunoblot.

showed preferential co-IP with the WT pump, more than expected from their expression. Because the truncation mutants are the most susceptible to proteolysis (Fig. 3), the simplest explanation for this finding is that dimerization protects these mutants from proteolysis. However, the analysis of protein expression levels in Fig. 6 does not support such an explanation. Another possibility is that sequences C-terminal to position Glu-917 (the shortest truncation tested) function to reduce pump dimerization and their truncation resulted in enhanced association of the truncated mutants with the WT pump. This interpretation is supported by the finding that the only other mutation to induce preferential binding is the S920Y and that this portion of the protein between TM8 and the C terminus appears to be the most rigid because it shows the least movement upon transition from the E1 to the E2 conformation (23).

DISCUSSION

Ca^{2+} pools have a key role in shaping the spatiotemporal complexity of Ca^{2+} signaling and in controlling cellular processes of major physiological and pathological relevance (24). SERCA pumps are key participants in the Ca^{2+} signal as they determine ER Ca^{2+} content at rest and during stimulation (10). In addition, several ER functions such as the activity of several chaperones and that of the unfolded protein response are regulated by ER Ca^{2+} content (25, 26). Thus, it is not surprising that mutations in SERCA2 pump activity lead to DD. However, DD is characterized by variable phenotypes from very mild symptoms with dislodging of skin keratinocytes to severe manifestation with several neurological deficiencies.

The effect of DD-causing mutations on SERCA2b pump activity is unknown, let alone the underlying mechanism for the variable phenotype of the disease. In this work, we analyzed several mutations that cause DD in an effort to partially address these questions and to gain a further understanding of SERCA2b pump function. All of the mutations analyzed with the exception of one showed no Ca^{2+}-pumping activity, and the V843F mutation reduced pump activity by 50%. Hence, it is clear that the mutations that cause DD do so by reducing SERCA2b activity. Multiple biochemical mechanisms appear responsible for reduced pump activity. Thus, nonsense mutations resulted in truncated pump proteins that were susceptible for degradation by the proteasome. Several missense mutations caused a marked reduction in protein level that was partially attributed to degradation by the proteasome, whereas others had a minimal effect on pump expression. This variability probably contributes to the variable clinical phenotype, but it is not sufficient to account for all of the variability because 11 of the 12 mutants examined had no pumping activity.

The two most notable and related findings in this work are the biochemical evidence for interaction between SERCA2b monomers and the inhibition of the native and expressed WT SERCA2b activity by five of the mutants. The crystal structure of SERCA1 in the E1 (Ca^{2+}) (27) and E2 ($2H^{+}$) (23) conformations suggest that SERCA pumps can exist as monomers. Of course, this does not exclude the possibility that *in vivo* the monomers interact with each other or that the pump functions as a dimer. Indeed, several lines of functional and biochemical evidence (16–21) support this notion. Here we provided the first direct evidence that SERCA2b exists as a dimer by showing that HA-tagged and Myc-tagged pumps can be co-IPed under mild extraction conditions. Importantly, all of the DD mutants interacted with the WT pump.

The significance of interaction of the WT and mutant SERCA2b monomers *in vivo* is that it provides a mechanism to explain reduced Ca^{2+} pumping below 50% that is expected from the recessive nature of the disease. This provides a likely mechanism to account for the variable clinical features of DD. Hence, the essential role of SERCA2b in Ca^{2+} signaling makes it vital for life, and even a 50% reduction in pump activity is not tolerated, leading to the detachment of skin keratinocytes. Other cellular activities such as Ca^{2+}-dependent exocytosis (7) and myocytes contractility (28) undergo adaptation to the modified Ca^{2+} response. However, the interaction of WT and the DD-causing mutants that leads to a further reduction of SERCA2b pump activity is likely to affect additional cells and organs such as the nervous system.

The clinical features of DD point to particular vulnerability of keratinocytes and neuronal cells to mutations in SERCA2b and Ca^{2+} homeostasis in the ER. This suggests that a cellular function common to the two cell types is particularly sensitive to ER Ca^{2+} content. Such a potential function can be cell-cell and cell-matrix attachment that in keratinocytes are mediated by cadherins, a family of calcium-dependent adherent proteins. Cadherins also play a central role in the formation of neuronal connections (29, 30) and may contribute to neurite outgrowth

and pathfinding and to synaptic specificity in the central nervous system (31). Aberrant function of cadherins can explain the symptomatic skin disorders in DD and that the second most prevalent disorder in DD is neurological deficiencies. If aberrant function of cadherins is the underlying cause of DD symptoms, our findings point to a central role of ER Ca^{2+} in the function of cadherins.

Most studies attribute Ca^{2+} regulation of cadherins function to extracellular Ca^{2+} (32, 33). In fact, little is known regarding the role of $[Ca^{2+}]_i$ in the regulation of cadherins function. However, accumulating evidence points to such a role. Depletion of ER Ca^{2+} with thapsigargin and cyclopiazonic acid prevented accumulation of cadherins at cell-cell junctions (34) and increased endothelial permeability by increasing discontinuities in cadherins junctions (35). Conversely, increased ER Ca^{2+} load by overexpression of calreticulin, a Ca^{2+} storage protein and chaperone in the ER, enhances assembly of cadherins junctions in cell-cell contacts (36). Significantly, dissociation of cadherins was observed in the skin of patients with DD (37). Together, these findings point to the importance of ER Ca^{2+} in cadherins function and their possible aberrant function in DD.

In summary, this work demonstrates multiple effects of mutations in SERCA2b pump associated with DD. The mutations affect protein expression, degradation, and activity. The most significant finding is that several DD-associated mutants inhibit the activity of the native and the expressed WT pumps. The inhibition was not the result of altered protein expression nor increased passive leak but rather the inhibition of pumping activity by protein-protein interaction. These findings provide a plausible molecular mechanism for diverse phenotypes of DD and a framework to further understand the role of SERCA2b in specific cell functions.

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