We examined possible defects of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 2b (SERCA2b) associated with its 51 mutations found in Darier disease (DD) pedigrees, i.e. most of the substitution and deletion mutations of residues reported so far. COS-1 cells were transfected with each of the mutant cDNAs, and the expression and function of the SERCA2b protein was analyzed with microsomes prepared from the cells and compared with those of the wild type. Fifteen mutants showed markedly reduced expression. Among the other 36, 29 mutants exhibited completely abolished or strongly inhibited Ca\(^{2+}\)-ATPase activity, whereas the other seven possessed fairly high or normal ATPase activity. In four of the aforementioned seven mutants, Ca\(^{2+}\) transport activity was significantly reduced or almost completely lost, therefore uncoupled from ATP hydrolysis. The other three were exceptional cases as they were seemingly normal in protein expression and Ca\(^{2+}\) transport function, but were found to have abnormalities in the kinetic properties altered by the three mutations, which happened to be in the three DD pedigrees found by us previously (Sato, K., Yamasaki, K., Daiho, T., Miyauchi, Y., Takahashi, H., Ishida-Yamamoto, A., Nakamura, S., Iizuka, H., and Suzuki, H. (2004) J. Biol. Chem. 279, 35595–35603). Collectively, our results indicated that in most cases (48 of 51) DD mutations cause severe disruption of Ca\(^{2+}\) homeostasis by the defects in protein expression and/or transport function and hence DD, but even a slight disturbance of the homeostasis will result in the disease. Our results also provided further insight into the structure-function relationship of SERCA2b and revealed critical regions and residues of the enzyme.

Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCA)\(^{2}\) catalyze Ca\(^{2+}\) transport coupled with ATP hydrolysis (Fig. 1) and play an essential role in maintaining Ca\(^{2+}\) homeostasis in the cytoplasm and endoplasmic reticulum lumen of cells (1–7). SERCA2b has three cytoplasmic domains: phosphorylation (P), nucleotide binding (N), and actuator (A) and 10 transmembrane helices (M1–M10 or 11 in the SERCA2b isoform, M11). In the Ca\(^{2+}\) transport cycle, the ATPase is activated by the binding of two Ca\(^{2+}\) ions from the cytoplasm to the transport sites composed of M4, M5, M6, and M8 (E2 \textrightarrow E1Ca\(_{2}\), step 1). Asp\(^{857}\) in the P domain is then phosphorylated with MgATP to form the phosphorylated intermediate (EP) (step 2). During dephosphorylation of EP, the Ca\(^{2+}\) ions are released into the lumen. In the detailed mechanism, the dephosphorylation process includes the conformational transition of EP associated with Ca\(^{2+}\) release (step 3) and the subsequent hydrolysis of the acylphosphate bond (step 4).

The three human SERCA genes encode SERCA isoforms (8–10). Mutations in the SERCA2 gene (ATP2A2) and the resulting defects in the SERCA2b housekeeping isoform cause an autosomal dominant genetic skin disease, Darier disease (DD) (11, 12). Over 100 mutations have been found with the DD pedigrees (11–24). They include many nonsense mutations, and also substitution and deletion mutations of amino acid residues. The mutations are located throughout the SERCA2b molecule and show no “hot spots” on the primary sequence. To understand how each of the substitution and deletion mutations affects SERCA2b protein, a limited number of mutations had been explored (9 by Ahn et al. (25), 10 by Dode et al. (23), 3 by Sato et al. (26) (a total of 20 because of overlap in Refs. 23 and 25)). To provide a comprehensive insight into the molecular basis of DD, as well as to understand the basis for each case of the DD pedigrees, it is necessary to analyze further the many unexplored substitution and deletion mutations. We therefore carried out in this study a comprehensive analysis of the expression and function of most of the DD causing substitution and deletion mutations reported, i.e. the 51 mutations shown in Fig. 2. Our results showed that most of the mutations (48 of the 51) cause severe defects in protein expression and/or Ca\(^{2+}\) transport function. The loss of the transport function was ascribed to markedly reduced ATP hydrolysis or uncoupling from ATP hydrolysis. The remaining three mutations were exceptional in that they exhibited seemingly normal protein expression and...
Ca\textsuperscript{2+} transport function but with altered kinetic properties. Results therefore indicated diverse molecular defects as the cause of DD in the 51 pedigrees. On the basis of the atomic structures of SERCA1a, our results also provided further insight into the structure-function relationship of SERCAs.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis and Expression*—The Stratagene QuikChange\textsuperscript{TM} site-directed mutagenesis method (Stratagene, La Jolla, CA) was utilized for the substitution and deletions of residues in human SERCA2b cDNA in plasmid pGEM7-Zf(+) (Promega). Appropriate restriction fragments with the desired mutation were excised and ligated back into the corresponding region of the full-length SERCA2b cDNA in the plasmid. The full-length SERCA2b cDNA was then excised and ligated into the pMT2 vector. Appropriate restriction fragments with the desired mutation were excised and ligated back into the corresponding region of the full-length SERCA2b cDNA in the plasmid.

*RNA Preparation and Northern Blot Analysis*—Total RNA was extracted by the RNeasy Mini Kit (Qiagen) from COS-1 cells transfected with the pMT2 vector containing wild-type or mutant SERCA2b cDNA. The amount of expressed SERCA2b protein was determined by the real time reverse transcriptase-PCR with the LightCycler\textsuperscript{TM} system and the LightCycler-RNA Master SYBR\textsuperscript{®} Green I (Roche Molecular Biochemicals). Melting curve analysis was performed to enhance specificity of the amplification reaction, and LightCycler software version 3.5 was used to evaluate the amplification efficiency and thus quantify the relative mRNA level in comparison with the internal standard curve obtained with the cells transfected with wild-type SERCA2b. The mRNA levels of the mutants relative to the wild-type level thus obtained were corrected by the mRNA level of glucose-6-phosphate dehydrogenase. The primers used for SERCA2b were GGCAATCTACAACACATGAAC (forward) and GTAAGAATGACTACGTCTGG (reverse), and for glyceraldehyde-3-phosphate dehydrogenase, CATGTTCTGTACGGGTGTAGA (forward) and AGTGAGCTTCCGTTCAGCCTC (reverse).

*ATPase Activity*—The rate of ATP hydrolysis was determined at 37 °C in a mixture containing 20 μg/ml microsomal protein, 1 mM ATP, 1 μM A23187, 7 mM MgCl\textsubscript{2}, 0.1 mM KCl, 5 mM Na\textsubscript{2}HPO\textsubscript{4}, 50 mM MOPS/Tris (pH 7.0), and 1.84 mM CaCl\textsubscript{2} with 2 mM EGTA (3.2 μM free Ca\textsuperscript{2+} (pCa 5.5)). When the Ca\textsuperscript{2+} concentration dependence was determined, the CaCl\textsubscript{2} concentration was varied in the presence of 2 mM EGTA. The reaction was terminated by the addition of ice-cold trichloroacetic acid, and the amount of Pi released was quantified by the Youngburg and Youngburg method (29). Total Ca\textsuperscript{2+}-ATPase activity of the microsomes was obtained as above, but by subtracting the Ca\textsuperscript{2+}-independent ATPase activity, determined in the presence of 5 mM EGTA without added CaCl\textsubscript{2}. The Ca\textsuperscript{2+}-ATPase activity of the expressed SERCA2b was then obtained by subtracting the Ca\textsuperscript{2+}-ATPase activity of the control microsomes (background level) from that of the microsomes expressing SERCA2b. This background level was as low as 3% of the activity of those microsomes expressing the wild-type SERCA2b from its cDNA. The Ca\textsuperscript{2+}-ATPase activity of the expressed wild type in the microsomes thus obtained was 122.6 ± 3.7 nmol/min/mg of microsomal protein (n = 5). The Ca\textsuperscript{2+}-ATPase activity of each of the mutants was normalized to its protein expression level relative to that of the wild type (determined as above), thus the specific Ca\textsuperscript{2+}-ATPase activity of each of the mutants relative to that of the wild type was obtained.

*Ca\textsuperscript{2+} Transport Activity*—Oxalate-dependent and thapsigargin-sensitive Ca\textsuperscript{2+} transport was assayed as described previously (30) at 25 °C in the presence and absence of 0.5 μM thapsigargin in a mixture containing 20 μg/ml microsomal protein, 1 mM ATP, 7 mM MgCl\textsubscript{2}, 0.1 mM KCl, 20 mM MOPS/Tris (pH 7.0), 5 mM potassium oxalate, and 0.462 mM 45CaCl\textsubscript{2} with 0.5 mM EGTA (3.2 μM free 45Ca\textsuperscript{2+} (pCa 5.5)). The Ca\textsuperscript{2+} transport activity of the SERCA2b expressed from its cDNA in the microsomes was obtained by subtracting the thapsigargin-sensitive activity of the control microsomes (background level) from that of the microsomes expressing SERCA2b from its cDNA. This background level was as low as 1% of the activity of

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**FIGURE 1. Reaction sequence of sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase.**

![Diagram](image-url)
microsomes expressing the wild-type SERCA2b from its cDNA. The Ca\(^{2+}\) transport activity of the expressed wild type in the microsomes thus obtained was 68.1 ± 4 nmol/min/mg of microsomal protein (n = 4). The Ca\(^{2+}\) transport activity of each of the mutants was normalized to its protein-expression level relative to that of the wild type (determined as above), thus the specific Ca\(^{2+}\) transport activity of each of the mutants relative to that of the wild type was obtained.

**Formation of EP**—Phosphorylation of SERCA2b in microsomes with [γ-\(^{32}\)P]ATP or \(^{32}\)Pi was performed under conditions described in the figure legends and Table 1. The reaction was quenched with ice-cold trichloroacetic acid containing Pi. The precipitated proteins were separated by 5% SDS-polyacrylamide gel electrophoresis at pH 6.0 according to the Weber and Osborn method (31). The radioactivity associated with the separated SERCA2b was quantitated by digital autoradiography as described previously (32). The amount of EP formed with the expressed SERCA2b was obtained by subtracting the background radioactivity with the control microsomes. This background level was less than 5% of the radioactivity of EP formed with the expressed wild-type SERCA2b.

**Miscellaneous**—Protein concentrations were determined using the method of Lowry et al. (33) with bovine serum albumin as the standard. Free Ca\(^{2+}\) concentrations were calculated as described previously (34). Data were analyzed using Origin software (Microcal Software, Inc., Northampton, MA). Three-
RESULTS

Effects of DD Mutations on Protein Expression and ATPase Activity—Each of the 51 DD-causing substitution and deletion mutations was introduced into SERCA2b cDNA, and the mutant cDNA or wild-type cDNA was transfected into COS-1 cells. The expression level of the mutant protein in microsomes prepared from the cells was determined and compared with that of the wild-type protein (Fig. 3a). The amount of intrinsic wild-type SERCA2b protein in the control microsomes (prepared from the control cells transfected with the vector without having the SERCA2b cDNA) was less than 1% of the wild-type protein expressed with the cDNA. Depending on the mutations introduced, the expression of mutants varied significantly from undetectable levels to those comparable with the wild-type level. Expression levels of the 15 mutants were less than 30% of the wild-type level, thus very low. There were no hot spots on the primary and tertiary structures for the markedly reduced expression (see Figs. 2b and 3b). Such mark-

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dimensional models of the enzyme were reproduced by the program VMD (35).

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FIGURE 2—continued

Defects in 51 SERCA2b Mutants of Darier Disease
edly reduced expression occurred with the specific mutations in the cytoplasmic, transmembrane, and lumenal regions of the enzyme.

The transcription levels were checked by the Northern blot analysis and more quantitatively by the real time reverse transcriptase-PCR method for these 15 mutants and three more mutants of the relatively low protein expression (30–40% of the wild-type level). We found, first of all, that the mRNA levels of all these mutants as well as of the wild type were ~500 to 800 times higher than the intrinsic wild-type SERCA2b mRNA level of the control cells, showing their extremely high transcription levels and suggesting high transfection efficiency. In the Northern blot analysis, the size of the mRNA of all these mutants and that of the wild type in the expression vector were shown to be exactly the same and their expression levels were very similar with no major reduction in the mutants (data not shown). The more quantitative comparison by the real time reverse transcriptase-PCR method in Fig. 3c actually showed no major reduction, although in some mutants, the mRNA levels were somewhat lower or moderately reduced as compared with the wild type. In any case, the mRNA reduction was not comparable or accountable to the marked reduction of the protein expression. The markedly reduced protein expression, likely due to the quality control of the misfolded SERCA (30) with some contribution of the possible moderate reduction in mRNA level, could be responsible for the development of DD in the pedigrees with these mutations.

The 36 mutants showed significant protein expression, over 30–100% of the wild-type level, and therefore we determined the Ca$$^2+$$-ATPase activity of these mutants at the optimum $\mathrm{pCa}$ 5.5. The specific activity of each of the mutants relative to that of the wild type was calculated and plotted versus the relative protein expression level (Fig. 4). Seven mutants (L321F, I274V, M719I, N767S, G807R, A803T, and V843F) among the 36 showed fairly high activity being 50–120% of the wild type activity. In these seven mutants, the expression levels were also fairly high. By contrast, the other 29 mutants showed markedly reduced or no activity regardless

FIGURE 3. Expression levels of DD mutants. a, the 51 DD mutants and wild type of SERCA2b were expressed in COS-1 cells and the amounts of expressed SERCA2b protein in the microsomal membranes were determined as described under “Experimental Procedures,” and normalized to the wild-type level. The values presented are the mean ± S.D. in three independent transfection/expression experiments. b, the arrowheads on the diagram show the approximate locations of the 51 DD mutations on the SERCA2b polypeptide chain. M1–M11, 1st to 11th transmembrane helices. The open and closed arrowheads indicate the expression levels lower (closed) and higher (open) than 30% of the wild-type level, respectively. c, the mRNA levels in the transfected COS-1 cells were quantified with the 18 DD mutants of the low protein expression level (~30% of the wild-type level or less, see the panel a) as described under “Experimental Procedures,” and normalized to the wild-type level. The values are the mean ± S.D. (n = 6).
of whether the expression level was high or low. It should be noted that there was no mutant displaying reduced expression with high ATPase activity (such mutant types, if present, would be plotted on the area far above the dotted line at the low expression range in Fig. 4). The findings may be clinically reasonable, because the high functional activity of the protein would compensate for the reduced protein expression.

**Ca**<sup>2+</sup> Transport Activity and Its Coupling with ATP Hydrolysis—**Ca**<sup>2+</sup> transport activity was determined with the seven mutants that were shown above to possess fairly high **Ca**<sup>2+</sup>-ATPase activity (L321F, I274V, M719I, N767S, G807R, A803T, and V843F) and also with eight other mutants that exhibited significantly reduced but still some activity (M699I, S916Y, S920Y, Q108H, A838P, S186P, P680L, and G310V, see Fig. 4). In Fig. 5, the specific **Ca**<sup>2+</sup>-transport activity of each mutant relative to that of the wild-type activity was plotted versus its relative protein expression level determined in Fig. 3. The expression range in Fig. 4). The findings may be clinically reasonable, because the high functional activity of the protein would compensate for the reduced protein expression.

**EP Formation from ATP and from P**<sub>i</sub>—To examine the phosphorylation ability at the catalytic site of the DD mutants and to understand the possible causes for the inhibition of ATP hydrolysis observed in Fig. 4, we determined EP formation from ATP and P<sub>i</sub> with the 36 DD mutants that were expressed high enough for the analysis (more than 30% compared with the wild type, see Fig. 3). The affinity for **Ca**<sup>2+</sup> in the ATP-induced EP formation was also determined in some cases and summarized in Table 1. In Fig. 6, the amount of EP formed from ATP at 0 °C and p**Ca** 5.5 at steady state was plotted versus **Ca**<sup>2+</sup>-ATPase activity. The mutants L321F, I274V, M719I, G807R, and A803T, which possess high ATPase activity, formed a fairly high amount of EP. Many of the other mutants, which exhibited the strongly or completely reduced ATPase activity, formed high levels of EP from ATP at a steady state, therefore the dephosphorylation process was probably inhibited in these mutants. These mutants include P680L, M699I, and C344Y in the P domain; P160H, P160L, G211D, N39D, N39T, S186P, and S186F in the A domain; and ΔL41 and ΔP42 on the A domain/M1 linker. In the transmembrane domain, they are L65S on M1, C318R on M4, A838P on M7, and Q108H on M2. On the other hand, EP formation from ATP and P<sub>i</sub> were almost completely inhibited with the mutants K683E at the catalytic site, D149N and G23E on the A domain, S765L and G769R on M5, and N809I at top of M6 (see Table 1 for the amounts of EP from P<sub>i</sub>). The mutant F487S showed strongly inhibited EP formation from ATP (thus no ATPase activity) but only a slight reduction in EP formation from P<sub>i</sub>, being consistent with the predicted function of Phe<sup>487</sup> in ring stacking for the adenine moiety (36–39).

**Figure 4.** **Ca**<sup>2+</sup>-ATPase activity of DD mutants and its relation with the protein expression level. The **Ca**<sup>2+</sup>-ATPase activity of the expressed SERCA2b in the microsomes was determined at **pCa** 5.5 and the specific activity of each of the mutants relative to that of the wild type was calculated, as described under “Experimental Procedures.” The relative specific activity of the mutant (the mean ± S.D. (n = 3)) thus obtained was plotted versus its relative protein expression level determined in Fig. 3. The dotted line was drawn from 0 through the wild-type level (100%). The values are also summarized in Table 1.

**Figure 5.** **Ca**<sup>2+</sup>-transport activity of DD mutants and its relation with **Ca**<sup>2+</sup>-ATPase activity. The **Ca**<sup>2+</sup>-transport activity of the expressed SERCA2b in the microsomes was determined at **pCa** 5.5 and the specific activity of each of the mutants relative to that of the wild type was calculated, as described under “Experimental Procedures.” The relative specific **Ca**<sup>2+</sup>-transport activity of the mutant (the mean ± S.D. (n = 3)) thus obtained was plotted versus its relative specific **Ca**<sup>2+</sup>-ATPase activity obtained in Fig. 4. The dotted line was drawn from 0 through the wild-type level (100%). The values are also summarized in Table 1.
Defects in 51 SERCA2b Mutants of Darier Disease

Inhibition of Ca²⁺-activated EP Formation at High Ca²⁺ Concentrations in K683R, D702N, and G703S—During the examination of EP formation from ATP and its Ca²⁺ depend-  

ece, we also found the unique behavior of mutants K683R, G703S, and D702N at the catalytic site on the P domain. As shown in Fig. 8, at 25 °C, the ATP-induced EP formation was activated by the high affinity Ca²⁺ binding but markedly reduced when the Ca²⁺ concentration was increased to over 10 μM. This biphasic behavior was more profound at 0 °C especially in K683R and G703S, and actually Ca²⁺ even at 1 μM was inhibitory. The behavior was in sharp contrast to that of the

the Ca²⁺-binding sites in the transmembrane domain show nearly no EP at 0 °C and pCa 5.5 but can form high levels of EP at a high temperature of 25 °C (see Fig. 7 for the Ca²⁺ dependence of EP formation from ATP). Results suggested the possible importance of the thermal motions of these regions for the high affinity Ca²⁺ binding and Ca²⁺ activation of the phosphoryla- 
tion site. It should be noted that N767S and V843F possessed high ATPase activity at the high temperature (but uncoupling in the Ca²⁺ transport), whereas G310V showed the completely inhibited Ca²⁺-ATPase activity probably because of a blockade in the dephosphorylation process.

### TABLE 1

Summary for functional properties of DD-causing mutants

The 36 DD mutants, whose protein expression levels were higher than 30% of that of the wild type, were subjected to functional analyses, and the data obtained are summarized. The specific Ca²⁺-ATPase activity of each of the mutants relative to that of the wild type (WT) was determined as described in the legend to Fig. 4, and shown in the fourth column. The mutants are placed from the top row in the table according to their activities thus obtained (i.e. from highest to lowest). The specific Ca²⁺ transport activity of each of the mutants relative to that of the wild type was determined in Fig. 5. The amount of EP formed from [γ-32P]ATP of the mutant was determined at steady state and at 0 °C, pCa 5.5, and normalized to its expression level relative to that of the wild type as described in the legend to Fig. 6. The Hill coefficient (n) were determined in the Ca²⁺ dependence of this EP formation at 0 °C by fitting the data to the Hill equation. EP formation from [γ-32P]ATP was also performed at 25 °C, 10 mM Ca²⁺ for 10 s in a mixture containing 40 μg/ml of microsomal protein, 0.1 mM MgCl₂, 80 mM KCl, 30 mM Tris-HCl (pH 7.5), and 10 mM CaCl₂, and the amount of EP in the mutant was calculated as above. The amount of EP formed with the wild type was 126 ± 6 (n = 6) pmol/mg of microsomal protein, and nearly the same as that at 0 °C and pCa 5.5. EP formation from [γ-32P]ATP, 5 mM MgCl₂, 80 mM KCl, 30 mM Tris-HCl (pH 8.0), and 5 mM EGTA, i.e. under the conditions previously demonstrated by Barrabin et al. (70) with SR Ca²⁺-ATPase to phosphorylate virtually all the phosphorylation sites. The amount of EP formed with the mutant was calculated as above. The amount of EP formed with the wild type was 134 ± 9 (n = 4) pmol/mg of microsomal protein. All the values are the mean values obtained in three to eight independent experiments (shown without the standard deviations for simplicity, but see figures for the deviations). In the second and third columns, the locations of the DD mutation in the gene and in the enzyme protein are indicated with the exon number and the region in the tertiary structure, respectively. The line below the mutant V843F was drawn to distinguish the first seven mutants (L321F–V843F) that possess the high specific ATPase activity (higher than 50% of that of wild type).

| DD mutant | Exon | Location | Ca²⁺-ATPase activity | Ca²⁺-transport activity | EP from ATP at 0 °C, pCa 5.5 | EP from ATP at 25 °C, pCa 2.0 | EP from Pᵣ |
|-----------|------|----------|----------------------|-------------------------|----------------------------|----------------------------|------------|
| WT        |      |          | 100                  | 100                     | 100                        | 5.7E-07ᵃ      | 100        |
| L321F     | 8    | M4/P domain junction | 85                    | 67                      | 121                        | 1.5E-07ᵇ      | 111        |
| M719I     | 15   | P domain | 90                    | 82                      | 84                         | 4.5E-07ᵇ      | 75         |
| N767S     | 15   | M5       | 67                    | 28                      | 5                           | 5.9E-07ᵇ      | 115        |
| G807R     | 15   | M5       | 63                    | 28                      | 47                          | 1.5E-07ᵇ      | 79         |
| A838T     | 16   | M7       | 56                    | 0                       | 36                          | 1.4E-07ᵇ      | 106        |
| V843F     | 17   | M7       | 55                    | 4                       | 2                           | 6.9E-07ᵇ      | 65         |
| M699I     | 14   | P domain | 26                    | 12                      | 73                          | 1.4E-07ᵇ      | 100        |
| S916Y     | 19   | L8–9     | 24                    | 0                       | 50                          | 4.9E-07ᵇ      | 76         |
| Q108H     | 2    | M2       | 22                    | 12                      | 63                          | 4.9E-07ᵇ      | 68         |
| A838P     | 16   | M7       | 19                    | 1                       | 60                          | 4.9E-07ᵇ      | 78         |
| S920Y     | 19   | L8–9     | 19                    | 0                       | 12                          | 8.9E-07ᵇ      | 55         |
| S186P     | 7    | A domain | 28                    | 14                      | 84                          | 1.1E-07ᵇ      | 105        |
| P680L     | 14   | P domain | 53                    | 1                       | 104                         | 1.9E-07ᵇ      | 128        |
| G310V     | 8    | M4       | 9                     | 0                       | 3                           | 2.9E-07ᵇ      | 78         |
| N39D      | 1    | A domain | 5                     | 1                       | 57                          | 5.4E-07ᵇ      | 55         |
| D702N     | 15   | P domain | 4                     | 0                       | 79                          | 2             |
| S765L     | 15   | M5       | 4                     | 0                       | 0                           | 8             |
| F487S     | 12   | N domain | 3                     | 0                       | 21                          | 6             |
| N809I     | 16   | M6       | 2                     | 0                       | 0                           | 0             |
| C344Y     | 8    | P domain | 1                     | 1                       | 184                         | 182           |
| S318R     | 8    | M4       | 1                     | 0                       | 94                          | 175           |
| G23E      | 1    | A domain | 1                     | 0                       | 0                           | 40            |
| N39T      | 1    | A domain | 0                     | 0                       | 77                          | 85            |
| ΔP42      | 2    | A domain/ M1 linker | 0                     | 0                       | 110                         | 118           |
| L655      | 3    | M1       | 0                     | 0                       | 79                          | 87            |
| D149N     | 5    | A domain | 0                     | 0                       | 1                           | 16            |
| P160H     | 6    | A domain | 0                     | 0                       | 67                          | 71            |
| P160L     | 6    | A domain | 0                     | 0                       | 0                           | 75            |
| S186F     | 7    | A domain | 0                     | 0                       | 61                          | 66            |
| G211D     | 8    | A domain | 0                     | 0                       | 80                          | 81            |
| K683E     | 14   | P domain | 0                     | 0                       | 1                           | 0             |
| K683R     | 14   | P domain | 0                     | 0                       | 2                           | 43            |
| G703S     | 15   | P domain | 0                     | 0                       | 10                          | 66            |
| G769R     | 15   | M5       | 0                     | 0                       | 2                           | 3             |

ᵃ Data taken from our previous analysis on EP formation from ATP (26).
ᵇ Determined at 25 °C. K₉₅ of the wild type was 1.3E-07M and essentially the same as that at 0 °C.
FIGURE 6. EP formed from ATP at steady state and its relation with Ca\(^{2+}\)-ATPase activity. Expressed SERCA2b in microsomes was phosphorylated with [\(^{32}\)P]ATP at 0 °C for 10 s in a mixture containing 20 μg/ml of microsomal protein, 10 μM [\(^{32}\)P]ATP, 1 μM A23187, 7 mM MgCl\(_2\), 0.1 M KCl, 5 mM NaN\(_3\), 50 mM MOPS/Tris (pH 7.0), and 1.836 mM CaCl\(_2\) with 2 mM EGTA (3.2 μM Ca\(^{2+}\)). The amount of EP formed was determined as described under “Experimental Procedures,” and was 126 ± 9 (n = 4) pmol/mg of microsomal protein with the expressed wild type. The amount of EP of each of the mutants was normalized to its protein expression level relative to that of the wild type determined in Fig. 3. The amount of EP of the mutant relative to that of the wild type was thus obtained and plotted versus its relative specific Ca\(^{2+}\)-ATPase activity as determined in Fig. 4. The values presented are the mean ± S.D. (n = 3). The dotted line was drawn from 0 through the wild-type level (100%). The values are also summarized in Table 1.

FIGURE 7. Temperature-sensitive Ca\(^{2+}\) activation of EP formation from ATP in G310V, N767S, and V843F. EP formation of the expressed wild-type SERCA2b and DD mutants G310V, N767S, and V843F were performed at 25 (closed symbols) and 0 °C (open symbols) and at various concentrations of free Ca\(^{2+}\), as otherwise described in Fig. 6, and shown with the symbols indicated in the figure. The maximal amount of EP obtained at 25 °C was normalized to 100% in each of the mutants and wild type. K\(_d\) for the Ca\(^{2+}\) activation (μM) obtained by fitting the data to the Hill equation was 0.29 in G310V, 0.59 in N767S, 0.69 in V843F, and 0.13 in the wild type at 25 °C, and 0.11 in the wild type at 0 °C. The values are also listed in Table 1. The maximal EP levels in pmol/mg of microsomal protein (i.e. the values without correcting by the protein expression levels) obtained at 25 °C with the mutants were 85.6 (N767S), 50.8 (V843F), and 86.2 (G310V), and the levels with the wild type at 25 and 0 °C were both 126.

wild type, in which the Ca\(^{2+}\)-activated EP formation remained at maximum at 25 and 0 °C even with 10 mM Ca\(^{2+}\). Furthermore, none of the other mutants exhibited such inhibition at high Ca\(^{2+}\) levels, for example, C344Y, P680L, and M699I on the P domain, C318R on M4, F487S at the adenine binding pocket on the N domain (although this mutant required very high concentrations of ATP for EP formation), and S186P and G211D on the A domain (data not shown). Thus the unique feature of Ca\(^{2+}\)-induced inhibition seems to be confined to the mutants examined here for Lys\(^{683}\), Asp\(^{702}\), and Gly\(^{703}\).

It should be noted that mutants K683R, G703S, and D702N showed completely abolished ATPase activity (see Fig. 8). The loss of activity is probably due to blocking of the dephosphorylation process because EP was formed from ATP at high levels at steady state under the almost same conditions as those for ATPase assay, i.e. the high temperature, pCa 5.5. Actually, inhibition of the dephosphorylation process (and also the observed inhibition of EP formation from P, in the mutants for Lys\(^{683}\) and Asp\(^{702}\), see Table 1) is consistent with previous mutation studies for these residues with SERCA1a (Lys\(^{684}\) and Asp\(^{705}\) in SERCA1a) (36, 40, 41). The importance of Gly\(^{703}\) (in the D\(^{703}\)G后再循环) loop in the ATPase was revealed here for the first time. It should also be noted that EP formation from ATP was completely lost in K683E (in contrast to K683R), being consistent with the critical role of the positive charge of this residue for the phosphoryl transfer from ATP (36).

**DISCUSSION**

**Summary of Defects in 51 DD Mutants**—We have examined here the expression and function of 51 DD mutants of SERCA2b, and compared them with those of the wild type. The results showed that, among the 51 mutants, 15 are severely defective in protein expression (Fig. 3), 29 severely defective in ATP hydrolysis (Fig. 4), and four severely defective in Ca\(^{2+}\) transport due to its uncoupling from ATP hydrolysis (Fig. 5). Thus, cellular Ca\(^{2+}\) transport function must be considerably disrupted by these mutations (in total 48) to result in an increase in cytoplasmic Ca\(^{2+}\) and a decrease in endoplasmic reticulum lumenal Ca\(^{2+}\), hence DD. This is consistent with notions (23, 25) that DD is caused by haploinsufficiency in Ca\(^{2+}\) homeostasis or by a possible intermolecular interaction of the defective mutant with the wild type, which may possibly inhibit the function of the wild type or cause its protein degradation. On the other hand, exceptions among the 51 DD mutants were found in the three mutants I274V, L321F, and M719I as they exhibited seemingly normal protein expression and Ca\(^{2+}\) transport function coupled with ATP hydrolysis (Fig. 5). Because these three mutations happened to be in the cases of the three
DD pedigrees found by us (16), we carried out a detailed kinetic analysis and found (26) that the three mutants possess altered kinetic properties, each exhibiting distinct types of abnormality, i.e. a slightly decreased Ca\(^{2+}\) transport rate (I274V and M719I as also shown in Fig. 5), a slightly decreased Ca\(^{2+}\) affinity at the transport sites (L321F and M719I, see Table 1), and a markedly reduced sensitivity to the feedback inhibition by luminal Ca\(^{2+}\) (L321F). The possible increase in luminal Ca\(^{2+}\) due to L321F abnormality was suggested to be related to the specific symptoms of this DD pedigree, neuropsychiatric disorder and behavior problems (26). Thus, for the 51 DD pedigrees, our results revealed the molecular defects of SERCA2b as the causes of disease. Taken together, our findings indicate that the DD mutations in most cases cause severe disruption of Ca\(^{2+}\) homeostasis and hence DD but even a slight disturbance still results in DD, and further points to the possible relation between the specific molecular defects of SERCA2b and the specific symptoms associated with DD. It is also important to note that uncoupling in the Ca\(^{2+}\) transport and the reduced sensitivity to feedback inhibition by luminal Ca\(^{2+}\) in the SERCA2b mutants may reduce the energy charge of cells by wasting or consuming more ATP, which then could be an additional and important pathogenic mechanism for the development of symptoms.

In the following discussions, we further inspected the functional defects of the DD mutants on the basis of the atomic structures of SERCA1a to learn more about the structure and function of SERCAs from the DD mutations. We focused on the three findings with the DD mutations in the transmembrane domain (N767S, A803T, G807R, and V843F) causing uncoupling in the Ca\(^{2+}\) transport, those in the catalytic site (K683R, D702N, and G703S) uniquely exhibiting Ca\(^{2+}\)-induced inhibition of \(E_p\) formation from ATP, and those in the A domain revealing two critical regions (at Pro\(^{160}\) and Asp\(^{149}\)) of this domain. Other interesting findings are discussed in detail under the supplemental discussion, which cited additional references (Refs. 71–78). It is important to note that the residues of DD mutations and those described in the supplemental discussion are all conserved in SERCA2b and SERCA1a (as the two are highly homologous), enabling such inspection on the basis of the SERCA1a atomic structures.

**Mutations N767S, A803T, G807R, and V843F, Causing Uncoupling**—The DD mutants N767S on M5 and V843F on M7 at or near the high-affinity Ca\(^{2+}\)-binding sites, and A803T and G807R on the cytoplasmic side of M6 have never been explored except V843F, for which Ca\(^{2+}\) transport was previously reported to be significantly slowed (25). We found here that these mutations cause complete loss (A803T and V843F) or significant reduction (N767S and G807R) of Ca\(^{2+}\)-transport activity due to strong or partial uncoupling from ATP hydrolysis (Fig. 5). The results indicate the importance of these residues for the formation of the proper transport pathway. Interestingly, no change in Ca\(^{2+}\) affinity at the high-affinity binding
Defects in 51 SERCA2b Mutants of Darier Disease

sites was observed in Ca\(^{2+}\) activation of the enzyme with A803T and G807R, and a ~6-fold reduction with N767S and V843F at 25 °C (Fig. 7 and Table 1). The reduction with N767S is consistent with the function of this residue as a Ca\(^{2+}\) ligand predicted with SERCA1a (42–44). In V843F, the bulky side chain introduced in the vicinity of Ca\(^{2+}\) ligands Asn\(^{767}\)/Glu\(^{770}\) and the critical residue Gly\(^{769}\) of M5 likely affected the Ca\(^{2+}\) affinity (in SERCA1a, Asn\(^{768}/\)Glu\(^{771}\) and Gly\(^{770}\)).

In all the atomic models of SERCA1a, each of the four residues, Asn\(^{767}\), Ala\(^{803}\), Gly\(^{807}\), and Val\(^{843}\), is involved in interactions between transmembrane helices (see Fig. 9 for E1-AlF\(_{2}\)/ADP, and in the SERCA1a numbering they are Asn\(^{768}\), Ala\(^{804}\), Gly\(^{808}\), and Val\(^{844}\)). Asn\(^{767}\) on M5 not only coordinates Ca\(^{2+}\) at site I (44) but also forms hydrogen bonds with Ala\(^{305}\) and Ala\(^{306}\) on M4. Ala\(^{803}\) on M6 is between the bound Ca\(^{2+}\) and cytoplasmic surface of the membrane, and its side chain is situated at the center of the hydrophobic residues clustered from M5 (Phe\(^{759}/\)Tyr\(^{762}/\)Leu\(^{763}\)), M6 (Pro\(^{802}/\)Leu\(^{806}\)), and M4 (Val\(^{814}\)). Therefore Ala\(^{803}\) on M6 likely functions as a hydrophobic spacer for the proper packing of M6/M4/M5. Gly\(^{807}\) at the top of M6 forms hydrogen bonds with Cys\(^{318}\) on M4 and Asn\(^{795}\) on M5, which also forms hydrogen bonds with residues on L6–7, thus Gly\(^{807}\) is involved in interactions of M6 with M4/M5 at the cytoplasmic surface of the membrane. Asn\(^{767}\), Ala\(^{803}\), and Gly\(^{807}\) therefore probably function to stabilize the properly packed M4/M5/M6 and to prevent the possible formation of a leakage path from the Ca\(^{2+}\)-binding sites to the cytoplasm. The DD mutations N767S, A803T, and G807R probably disrupted the interaction networks or caused a steric clash between the helices. It might also be possible that these DD mutations disrupted the function of Glu\(^{309}\) on M4, the cytoplasmic gate to occlude Ca\(^{2+}\), because the three residues of the DD mutations are close to Glu\(^{309}\).

Val\(^{843}\) on M7 is situated at the slightly lower part (lumenal side) of the bound Ca\(^{2+}\), and very close to M5 (the Ser\(^{766}/\)Val\(^{772}\) region that includes the Ca\(^{2+}\) ligands Asn\(^{767}/\)Glu\(^{770}\) and the critical Glu\(^{769}\)), M8 (Leu\(^{905}/\)Ile\(^{906}/\)Glu\(^{907}\) and Val\(^{843}\)) within 3.4 Å to Gly\(^{769}\) and Val\(^{976}\) in E1-AlF\(_{2}\)/ADP. It is likely that the introduced bulky side chain in

\(5\) It should be noted that the mutation of Ala\(^{804}\) of SERCA1a (Ala\(^{803}\) of SERCA2b) to Val caused a complete loss of Ca\(^{2+}\) transport function but the mutation to Ser was less deleterious (43). The DD mutation A803T is thus similar to this Val mutant, and we further revealed here the complete loss of Ca\(^{2+}\) transport with A803T due to uncoupling from ATP hydrolysis. The size of the side chain at this position therefore is critical for the handling of Ca\(^{2+}\), being consistent with the view of the structural role of this residue as a hydrophobic spacer for the proper packing of M6/M4/M5.
Defects in 51 SERCA2b Mutants of Darier Disease

V843F caused a steric clash between these helices and disrupted their proper packing, and thus disrupted the formation of a proper Ca$^{2+}$ release pathway to the lumen. It is of interest to note that Ca$^{2+}$ transport in A803T and V843F was strongly uncoupled (i.e. no transport activity) but it was only partially uncoupled in N767S and G807R (i.e. the transport activity was 42–44% of the ATPase activity, see Fig. 5 and Table 1). The difference in the degree of uncoupling might be due to the structural facts described above that Ala$^{683}$ and Val$^{684}$ are extensively involved in the hydrophobic interactions between the transmembrane helices, whereas Asp$^{702}$ and Gly$^{807}$ are involved only for (a) few hydrogen bonds. It is possible that the disruption of the proper hydrophobic interactions have much more serious consequences on the proper packing of the transmembrane helices for formation of the proper transport pathway. Regarding G807R, it is also possible that the structural disruption at the cytoplasmic surface affected less transport activity. Regarding G807R, it is also possible that the disruption of the proper hydrophobic interactions become more serious at 0 °C than at 25 °C suggests the importance of possible thermal motions in the conformation of the enzyme and/or the bound ATP for relieving this inhibition. It is possible that the coordination of Ca$^{2+}$ became more favored as compared with Mg$^{2+}$ (especially at the lower temperature) in the mutated catalytic site and inhibited the phosphorylation. In phosphoserine phosphatase, a member of the haloacid dehalogenase superfamily of SERCAs, the coordination geometry of Ca$^{2+}$ at the catalytic site was previously revealed to be different from that of the native cofactor Mg$^{2+}$ and therefore not suitable for catalysis (46). For another possible cause of the Ca$^{2+}$-induced inhibition of phosphorylation observed here, it is of interest to note that there are two distinct binding sites for the phosphate moiety with the coordinated Mg$^{2+}$ (i.e. Asp$^{351}$ and Thr$^{441}$), and also two different conformations of ATP (i.e. extended and folded ones). In the E1-AMPPCP crystals (38, 39), the bound AMPPCP (ATP) adopts an uncommon extended conformation (Fig. 10, and also see Fig. 4 in Ref. 39) thereby γ-phosphate and Mg$^{2+}$ are bound at Asp$^{351}$, Lys$^{684}$, and Asp$^{703}$/Asp$^{707}$ in the native cofactor Mg$^{2+}$ coordinated by the β/γ-phosphate of ATP (47, 49, 67), which possibly adopts the folded conformation as depicted by the thin wire model for ATP with the small green sphere for Mg$^{2+}$ (the PDB code 1KAX (68)) according to Ref. 47. The binding of ATP in the folded conformation was also previously inferred with Na$^+$, K$^+$-ATPase (69) and most recently with Ca$^{2+}$-ATPase (48).
Defects in 51 SERCA2b Mutants of Darier Disease

FIGURE 11. Interaction networks at Pro^{160} and Asp^{149} on the A domain. The pictures of the A domain (a) and the three enlarged regions of the DD mutations (b, c, and d) were depicted with $E_1$-AlF$_4^-$·ADP, the $E_1$PC$_{2a}$·ADP model (PDB accession code 1WPE (53)). c, at the Pro^{160} region, residues Pro^{160}, Asn^{39}, and Gly^{211} having DD mutations form an extensive hydrogen-bonding network by themselves together with neighboring residues, i.e. the Pro^{160} main chain carbonyl with the side chain of Asn^{39}, the main chain carbonyl at the N terminus of Pro^{160} (Val^{159}) with the Gly^{211} amide, and the Pro^{160} carbonyl with the side chain of Ser^{186} at the N terminus of Gly^{211} (Ser^{186} is not depicted for simplicity). Pro^{160} and Gly^{211} are each on different layers of the bent-loop/β-strand, which are gathered to form the A domain structure at this region. Asn^{39} is the junctional residue of the A domain with the A domain/M1 linker. Thus, Pro^{160}, Gly^{211}, and Asn^{39} are stabilizing this region and properly fixing the A domain/M1 linker at Asn^{39} in this region. Asn^{39} with its main chain amide and side chain oxygen also forms a hydrogen-bonding network with the top part of the A domain/M3 linker (Asn^{99}–Gly^{227}–Thr^{230}) and that of the A domain/M2 linker (Asn^{99}–Ala^{142}–Glu^{225}) (not depicted for simplicity), thus stabilizing the top parts of these linkers. Gly^{211} is in the vicinity of Tyr^{122} and therefore it might be possible that the introduced side chain in G211D disrupted not only the above interaction network but also the proper formation of the Tyr^{122}–hydrophobic cluster for the A-P domain association in E2P (see supplemental Fig. IVa for the Tyr^{122} hydrophobic cluster in $E_2$-MgF$_4^2$-). b, at the Asp^{149} region, residues Asp^{149}·Gly^{23}·Arg^{131}, and Val^{223} having DD mutations form an extensive interaction network together with neighboring residues, i.e. the hydrogen bonds between the side chains of Asp^{149} and Arg^{131} and between the main chains of Asp^{149} and Val^{223}, and the van der Waals contact of Asp^{149} with Leu^{24} next to Gly^{23} (within 3.3–3.8 Å in the atomic models). The Val^{223} carbonyl forms a hydrogen bond with the Gly^{148} amide at the N terminus of Asp^{149} (not depicted for simplicity). Asp^{149}·Arg^{131}, and Val^{223} are each on different layers of the bent-loop/β-strand, which are gathered by the interactions to form the distorted jellyroll-like structure of the A domain (44). These residues are also at the top of the three linkers connecting the A domain to M1 (Gly^{7}–Leu^{6}), M2 (Arg^{131}), and M3 (Val^{223}), respectively, and therefore contribute to the proper positioning of the top of these linkers on the A domain by the interaction network at this Asp^{149} region. d, Ser^{186} forms a hydrogen-bonding network with Arg^{124} and Ser^{184} and therefore probably contributes to the stabilization of the 181TGES loop.

The 110°-DGVD loop seems to be designed toward the coordination of Mg$_2^{2+}$ and to avoid the possible inhibitory effect of Ca$^{2+}$.-ADP → $E_2$-ADP → $E_2$-MgF$_4^2$ (i.e. the $E_1$PC$_{2a}$ → E2P transition) (38, 39, 53–56). Three structural elements on the A domain, the 181TGES loop, Val^{200} loop (Asp^{196}–Asp^{203}), and Tyr^{122} hydrophobic cluster have been predicted to be critical for the A-P domain association (57–59), and Glu^{183} on the 181TGES loop participates in the hydrolysis (53, 54, 60). Interestingly, most of the DD mutations on the A domain are not in these regions, but located at two other regions: the Pro^{160} region (P160H, P160L, G211D, N39D, N39T) and the Asp^{149} region (D149N, G23E, R131Q, and V223M) (Fig. 11). An exception was at Ser^{186} in the immediate vicinity of the 181TGES loop. Mutations S186F and S186P inhibited the dephosphorylation process, and this inhibition was possibly via structural effects on the 181TGES loop because Ser^{186} of its side and main chains form a hydrogen-bonding network with Ser^{184} in the loop and Arg^{174} at the N-terminal region of the loop thus likely contributing to the proper structure and positioning of the loop (Fig. 11d). The results on S186F are consistent with those of Dode et al. (23).

The all the DD mutations of residues at the Pro^{160} region, Pro^{160}, Gly^{211}, and Asn^{39}, caused inhibition of the dephosphorylation process (i.e. $E_2$-ATP formation from ATP at a steady state but no Ca$^{2+}$-ATPase activity). These residues form an extensive hydrogen-bonding network by themselves within the A domain (see Fig. 11c and the legend for details), thus stabilizing this region of the domain and, importantly, fixing the A domain/M1 linker at Asn^{39}, the junctional residue of this linker. Deletions ΔL41 and ΔP42 on this linker also inhibited the dephosphorylation process.

These results are entirely consistent with our previous mutation study with SERCA1a and the prediction (61) that the proper structure of the A domain at the junctional residue Asn^{39} and the appropriate length of the A domain/M1 linker (Glu^{40}–Ser^{48}) are critical in the dephosphorylation process probably for inducing the A domain motion. Asn^{39} also forms a hydrogen-bonding network with the top part of the A
Defects in 51 SERCA2b Mutants of Darier Disease

domain/M3 linker, the strain of which has also been predicted to be critical as a moving force for the A domain rotation in the dephosphorylation process (53). The DD mutations of Pro160, Gly223, and Asn23 probably disrupted these structural bases of the A domain and the connected linkers, and possibly inhibited the proper motions of the A domain required for the dephosphorylation process. It should be noted that the Pro160 → Ala mutant in SERCA1a was previously found to possess normal Ca²⁺ transport function (62). The serious consequence of the DD mutations P160L and P160H observed here is therefore consistent with the importance of the DD mutations P160L and P160H observed here is therefore consistent with the importance of the interaction network at the Asp149 region therefore seem to be critical in the formation and stabilization of the fundamental structure of the A domain with the “distorted jellyroll-like structure” (44). The residues are also at the top of the three linkers that connect the A domain to M1 (Gly23/Leu24), M2 (Arg131), and M3 (Val223), respectively. The DD mutations D149N, G25E, R131Q, and V223M probably caused a sterical clash and disrupted the interactions in this region, and seriously affected the protein structure. The residues and their interaction network at the Asp149 region therefore seem to be critical in the formation and stabilization of the fundamental structure of the A domain with the properly positioned linkers. In a detailed kinetic analysis, the importance of Gly23 in function was also previously noted (23).

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AUGUST 11, 2006 • VOLUME 281 • NUMBER 32 • JOURNAL OF BIOLOGICAL CHEMISTRY 22895

22895

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