Distinct Types of Abnormality in Kinetic Properties of Three Darier Disease-causing Sarco(endo)plasmic Reticulum Ca\(^{2+}\)-ATPase Mutants That Exhibit Normal Expression and High Ca\(^{2+}\) Transport Activity

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The possible functional abnormalities in three different Darier disease-causing Ca\(^{2+}\)-ATPase (SERCA2b) mutants, Ile\(^{274}\) → Val at the luminal end of M3, Leu\(^{271}\) → Phe on the cytoplasmic part of M4, and Met\(^{719}\) → Ile in P domain, were explored, because they exhibited nearly normal expression and localization in COS-1 cells and the high ATPase and coupled Ca\(^{2+}\) transport activities that were essentially identical (L321F) or slightly lower (I274V by ~35% and M719I by ~30%) as compared with those of the wild type. These mutations happened to be in Japanese patients found previously by us. Kinetic analyses revealed that each of the mutants possesses distinct types of abnormalities; M719I and L321F possess the 2–3-fold reduced affinity for cytoplasmic Ca\(^{2+}\), whereas I274V possesses the normal high affinity. L321F exhibited also the remarkably reduced sensitivity to the feedback inhibition of the transport cycle by accumulated luminal Ca\(^{2+}\), as demonstrated with the effect of Ca\(^{2+}\) ionophore on ATPase activity and more specifically with the effects of Ca\(^{2+}\) (up to 50 mM) on the decay of phosphoenzyme intermediates. The results on I274V and M719I suggest that the physiological requirement for Ca\(^{2+}\) homeostasis in keratinocytes to avoid haploinsufficiency is very strict, probably much more than considered previously. The insensitivity to luminal Ca\(^{2+}\) in L321F likely brings the luminal Ca\(^{2+}\) to an abnormally elevated level. The three mutants with their distinctively altered kinetic properties will thus likely cause different types of perturbation of intracellular Ca\(^{2+}\) homeostasis, but nevertheless all types of perturbation result in Darier disease. It might be possible that the observed unique feature of L321F could possibly be associated with the specific symptoms in the pedigree with this mutation, neuropsychiatric disorder, and behavior problems. The results also provided further insight into the global nature of conformational changes of SERCA for ATP-driven Ca\(^{2+}\) transport.

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¶The abbreviations used are: SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; Ca\(^{2+}\)-ATPase, SERCA; ADP-sensitive phosphoenzyme; TG, thapsigargin; DD, Darier disease; MOPS, 3-(N-morpholino)propanesulfonic acid.

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35995
who have been under careful examination of symptoms and treatment by our group for over 10 years. Kinetic analyses revealed that the three mutants, especially L321F, possess the distinctively altered properties and thus likely cause different types of perturbation in Ca\(^{2+}\) homeostasis, all of which nevertheless result in DD. The specific features in the symptoms of the patients of the L321F pedigree may possibly be associated with the unique property of the mutant. The results are also consistent with the global conformational changes of SERCAs for the energy coupling.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression**—Overlap extension PCR (36) was utilized for introducing mutations into human SERCA2b cDNA. The MunI-Csp45I restriction fragments (I274V and L321F) or PshAI-BamHI fragments (M719I) were excised from the PCR products and ligated back into the corresponding region in the full-length SERCA2b cDNA in the pMT2 expression vector (37). DNA sequence was confirmed by dyeoxy method. The pMT2 DNA thus obtained was transfected into COS-1 cells by the liposome-mediated transfection method, and microsomes were prepared from the cells as described (38). The “control microsomes” were prepared from the cells transfected with the pMT2 vector containing no SERCA2b cDNA. The amount of expressed SERCA2b was quantified by enzyme-linked immunosorbent assay by the use of a SERCA2b-specific monoclonal antibody IID8 (Affinity Bioreagents, Golden, CO), and the expression levels of mutants in the microsomes were obtained as values relative to that of wild type. The amount of intrinsic SERCA2b in the control microsomes was less than 1% of the amount of SERCA2b in the microsomes from the cells transfected with the wild-type cDNA.

**Immunofluorescence Analysis of COS-1 Cells**—Twenty-four hours after the transfection of the COS-1 cells, the localizations of the expressed SERCA2b and of the ER marker protein, protein-disulfide isomerase, were examined with immunofluorescence microscopy by the use of the affinity-purified rabbit polyclonal antibody against the peptide corresponding to the C-terminal Ser\(^{505}\)-Ser\(^{514}\) of human SERCA2b and the antibody against the mouse protein disulfide-isomerase (RL90; Affinity Bioreagents). For the secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG (Amersham Biosciences) were used. The fluorescence image was observed with a Fluoview FV500 confocal laser scanning microscope equipped with an argon ion laser (excitation line at 488 nm) and a helium-neon green laser (excitation line at 543 nm) mounted on an Olympus BX61 microscope (Olympus, Tokyo, Japan). Imaging was performed with Fluview version 2.0 software (Olympus America Inc, Melville, NY).

**Ca\(^{2+}\)-ATPase Activity**—The rate of ATP hydrolysis was determined at 37°C in a mixture containing 15 μM microsomal protein, 1 mM ATP, 1 mM AMG3187, 7 mM MgCl\(_2\), 0.1 M KCl, 50 mM MOPS/Tris, pH 7.0, and 2 mM EGTA with or without various concentrations of CaCl\(_2\). The reaction was terminated by the addition of ice-cold trichloroacetic acid, and the amount of P\(_i\) released was quantified by the method of Youngburg and Youngburg (39). The total Ca\(^{2+}\)-ATPase activity of SERCA2b expressed from its cDNA in the microsomes was obtained by subtracting the activity of the control microsomes (background level) from that of the microsomes expressing SERCA2b from its cDNA. This background level was as low as 5% of the activity of microsomes expressing the wild-type SERCA2b from its cDNA. The total Ca\(^{2+}\)-ATPase activity of the expressed wild-type protein in the microsomes thus obtained with a saturating 50 μM Ca\(^{2+}\) concentration was 87.2 ± 2.6 nmol/min/mg of microsomal protein (n = 6).

**Ca\(^{2+}\) Transport Activity**—Oxalate-dependent Ca\(^{2+}\) uptake was assayed as described previously (35) at 25°C in the presence and absence of 0.5 μM thapsigargin in a mixture containing 20 μM/mg microsomal protein, 1 mM ATP, 7 mM MgCl\(_2\), 0.1 M KCl, 20 mM MOPS/Tris, pH 7.0, 5 mM oxalate, and 50 μM CaCl\(_2\). The total Ca\(^{2+}\) uptake activity of 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. The total Ca\(^{2+}\) uptake activity of the expressed wild-type protein in the microsomes thus obtained was 3.59 ± 0.03 nmol/min/mg of microsomal protein (n = 3).

**Formation and Hydrolysis of EP**—Phosphorylation of SERCA2b in microsomes with [γ-32P]ATP or [γ-32P]P, and dephosphorylation of 32P-labeled SERCA2b was performed under the conditions described in the legends to the figures and tables. The reactions were quenched with

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**Fig. 1. Reaction cycle of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase.**

**Fig. 2. Location of the three DD-causing mutations on crystal structure of SERCA1a E2(TG).** SERCAs have three cytoplasmic domains (P, N, and A) and ten transmembrane helices (M1–M10) with an an additional M11 in SERCA2b isoform at its long C-terminal region (8, 11, 66–68). The three DD-causing mutations of residues explored in this study, I274V at the luminal end of M3, L321F on the cytoplasmic part of M4, and M719I in the catalytic region in P domain of SERCA2b, were indicated with the autophosphorylation site Asp\(^{511}\) on the SERCA1a structure E2(TG), the enzyme without bound Ca\(^{2+}\) (E2) stabilized with thapsigargin (Protein Data Bank accession code IWOI (11)). The number of residues in SERCA2b is indicated in parentheses, if it differs from that in SERCA1a.
Summary for expression and functional properties of three DD-causing mutants and for symptoms in the patients

| Expression level in microsomes | EP from P<sub>m</sub> in microsomes | Total ATPase activity in microsomes | Total Ca<sup>2+</sup> transport activity in microsomes | Turnover rate | K<sub>Ca</sub> for Ca<sup>2+</sup> activation | Ionophore effect on ATPase (,<i>−[β(+)]</i>) | Symptoms on skin | Neuropsychiatric manifestations |
|-------------------------------|----------------------------------|-------------------------------------|-----------------------------------------------|--------------|---------------------------------|-----------------------------------|-----------------|---------------------------------|
| WT                            | 100                              | 71.8 (100)                          | 100                                           | 100          | 0.12                            | 61.3                               | Mild, onset at age 20, eruption mainly on frontal chest, no DD symptoms in his children | None |
| I274V                         | 97.4                             | 70.7 (99.0)                         | 63.7                                          | 65.0         | 0.11                            | 62.1                               | None            | None |
| L321F                         | 81.8                             | 67.2 (94.1)                         | 100.4                                         | 94.8         | 0.27                            | 99.0                               | Severe, onset at age 20, eruption diffusely on central trunk. Nearly the same symptoms in her daughter. | Neuropsychiatric disorder and behavior problems, also in her daughter. |
| M719I                         | 88.2                             | 65.3 (91.4)                         | 69.4                                          | 68.4         | 0.31                            | 73.0                               | Moderate, onset at age 20, eruption mainly on central trunk, no DD symptoms in his children | None |

Expression and Functional Activities of Mutants—The mutant and wild-type SERCA2b cDNA were transfected into COS-1 cells, and microsomes were prepared from the cells. The expression levels of the mutant protein I274V, L321F, and M719I in the microsomes (estimated by the enzyme-linked immunosorbent assay in the eight independent transfection experiments) were almost the same as or comparable with that of the wild type (Table I). The normal expression of mutants was also found with the maximum phosphorylation level of SERCA2b in the microsomes determined with P<sub>m</sub> in 35% Me<sub>2</sub>SO as described for SR Ca<sup>2+</sup>-ATPase (46), in which nearly all the phosphorylation sites are phosphorylated. Immunofluorescence microscopy for the COS-1 cells transfected with SERCA2b cDNA clearly demonstrated that all the three mutants as well as the wild type are co-localized with the protein disulfide-isomerase (ER marker) and localized on ER, whereas the cells transfected with the vector containing no SERCA2b cDNA exhibited very weak (or almost no) background signals (data not shown).

RESULTS

Expression and Functional Activities of Mutants—The mutant and wild-type SERCA2b cDNA were prepared from COS-1 cells transfected with the cDNA, and the expression level of the mutant in the microsomes relative to that of the wild type was determined as described under “Experimental Procedures.” E2P was formed from P<sub>i</sub> at 25 °C for 10 min in a mixture containing 10 μg of microsomal protein, 0.1 mM 32P<sub>i</sub>, 10 mM MgCl<sub>2</sub>, 35% (v/v) Me<sub>2</sub>SO, 50 mM MOPS/Tris, pH 7.0, and 5 mM EGTA; the conditions that were previously demonstrated with SR Ca<sup>2+</sup>-ATPase to phosphorylate virtually all the phosphorylation sites and thus to reveal the content of the site (46). The amounts of E2P thus formed with the expressed SERCA2b were determined as described under “Experimental Procedures.” The total Ca<sup>2+</sup>-ATPase activity and the total Ca<sup>2+</sup> transport activity of the expressed SERCA2b in the microsomes were determined as described under “Experimental Procedures” and shown as the percentages of the respective values of the wild type. The turnover rate of ATPase in the mutants relative to that in the wild type was calculated with the total ATPase activity in the microsomes of the mutant in the microsomes relative to that of the wild type was determined as described under “Experimental Procedures.”

The Ca<sup>2+</sup>-ATPase activity of the microsomes was determined at 37 °C and at a saturating 50 μM Ca<sup>2+</sup> concentration. As compared with the total activity of the expressed SERCA2b wild type in the microsomes, the total activity of the expressed L321F was essentially identical, and there were 36 and 31%
reductions in activity for I274V and M719I, respectively (Table I). In each of three mutants, the total Ca\(^{2+}\) uptake activity relative to that of the expressed wild type was essentially the same as the relative total ATPase activity. Therefore the three mutants perform the Ca\(^{2+}\) transport coupled with the ATP hydrolysis as the wild type. The relative turnover rate calculated with the total ATPase activity and relative expression level in the microsomes was slightly higher in L321F (23\%) or lower in I274V (35\%) and M719I (21\%) than that of the wild type.

Formation of EP from ATP and Accumulation of E2P at Steady State—EP was formed from ATP with a saturating 100 \(\mu\)M Ca\(^{2+}\) concentration at 0 \(^{\circ}\)C under the conditions otherwise similar to those for the ATPase assay (Fig. 3). All three mutants as well as the wild type accumulated EP (with the steady-state level somewhat lower than the maximum phosphorylation level determined with P\(_i\) in Table I, as expected (46)). In L321F, the amount formed with ATP was notably lower, suggesting (together with the observed high turnover) possible rapid EP decay (as in fact so, Table II).

The amount of ADP-insensitive EP (E2P) accumulated at steady state in the wild type was low (19 \pm 2\% of the total amount of EP (n = 6)), which is consistent with the rate-limiting E1P to E2P transition in the transport cycle (5, 32). It was also low in M719I (10 \pm 3\%) but significantly larger in L321F (55 \pm 2\%) and in I274V (43 \pm 6\%). The large E2P accumulation can be brought about by the accelerated E1P-E2P transition (the shift toward E2P in the equilibrium) or the slowed E2P hydrolysis. The kinetic analysis indicated that the former is likely the case in L321F, but the latter is likely in I274V (as will be shown in Table II).

Kinetics of EP Formation with ATP from E1Ca\(_2\) and from E2—Time course of EP formation from E1Ca\(_2\) and ATP in step 3 was determined otherwise as above. The first order rate obtained was nearly the same in I274V and L321F as in the wild type and faster in M719I by ~3-fold (Table II). The rate of the E2 to E1Ca\(_2\) transition in steps 1 and 2 was determined by the simultaneous addition of saturating Ca\(^{2+}\) and ATP to the enzyme preincubated in the absence of Ca\(^{2+}\) at pH 6 where the equilibrium between E1 and E2 is most shifted to E2 (as shown with SR Ca\(^{2+}\)-ATPase; Ref. 47). The apparent first order rate in EP formation, which reflects the rate-limiting E2 to E1Ca\(_2\) transition, was almost the same in L321F and M719I as in the wild type or somewhat faster in I274V (Table II).

Ca\(^{2+}\) Concentration Dependences of ATPase and of EP Formation from ATP—K\(_{0.5}\) for the Ca\(^{2+}\) activation of the total ATPase activity of the expressed SERCA2b in the microsomes was nearly identical in I274V and the wild type but was slightly higher in L321F and M719I (Fig. 4A and Table I). Essentially the same results were obtained with the EP formation from ATP (Fig. 4B). The ATPase activity dramatically de-

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**Table II**

Rates for partial reaction steps and K\(_{0.5}\) for Ca\(^{2+}\)-induced feedback inhibition of E2P hydrolysis

|         | E2 to E1Ca\(_2\) | E1Ca\(_2\) to E1PCa\(_2\) | Decay of EP formed from ATP | Hydrolysis of E2P | K\(_{0.5}\) for Ca\(^{2+}\)-induced feedback inhibition of E2P hydrolysis |
|---------|------------------|------------------------|-----------------------------|------------------|--------------------------------------------------|
| Wild type | 0.075 (100)     | 4.2 (100)              | 0.016 (100)                 | 0.097 (100)      | 4                                                  |
| I274V   | 0.129 (172)     | 3.9 (93)               | 0.018 (112)                 | 0.027 (28)       | 4                                                  |
| L321F   | 0.085 (113)     | 5.1 (121)              | 0.028 (175)                 | 0.077 (79)       | >50                                               |
| M719I   | 0.083 (111)     | 11.5 (274)             | 0.016 (100)                 | 0.156 (161)      | 6                                                  |

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![Fig. 4A](image1.png)

![Fig. 4B](image2.png)

**Fig. 4. Ca\(^{2+}\) dependence of ATPase activity and EP formation from ATP.** The total ATPase activity (A) and the amount of EP formed from ATP (B) of the expressed SERCA2b in the microsomes were determined at various concentrations of Ca\(^{2+}\) (CaCl\(_2\) with 2 mM EGTA) otherwise as described under “Experimental Procedures” (ATPase) and in the legend to Fig. 3 (EP formation). The wild type (○), I274V (○), L321F (△), and M719I (□) were indicated. In A, K\(_{0.5}\) for the Ca\(^{2+}\) activation and Hill coefficient obtained by fitting of the data below 10 \(\mu\)M Ca\(^{2+}\) to the Hill equation (solid lines) were 0.12 \(\mu\)M and 1.5 (wild type), 0.11 \(\mu\)M and 1.4 (I274V), 0.27 \(\mu\)M and 1.3 (L321F), and 0.31 \(\mu\)M and 1.2 (M719I), respectively. In the fitting of the data above 10 \(\mu\)M Ca\(^{2+}\) to the Hill equation for the apparent Ca\(^{2+}\)-induced inhibition, the Ca\(^{2+}\) concentrations to give 50\% of the maximal activity and Hill coefficient were 0.6 \(\mu\)M and 1.2 (wild type), 0.7 \(\mu\)M and 1.2 (I274V), and 0.8 \(\mu\)M and 1.2 (M719I), respectively. In L321F that showed the significantly lower extent of the apparent inhibition, the values were estimated (roughly) to be 2.8 \(\mu\)M and 1.1. In B, K\(_{0.5}\) for Ca\(^{2+}\) to activate phosphorylation and the Hill coefficient obtained were 0.18 \(\mu\)M and 1.5 (wild type), 0.15 \(\mu\)M and 1.3 (I274V), 0.57 \(\mu\)M and 1.5 (L321F), and 0.45 \(\mu\)M and 2.2 (M719I), respectively (note that the maximal EP level was normalized to 100\%).
increased at high Ca\(^{2+}\) concentrations with the wild type, I274V and M719I, but this inhibitory effect was much less in L321F. The effect is likely due to the feedback inhibition by Ca\(^{2+}\) binding at the luminaly oriented low affinity transport sites in E2P (and also Ca\(^{2+}\) binding at the catalytic Mg\(^{2+}\) subsite, which strongly reduce the E1P to E2P transition rate (48–51)).

Effect of A23187 on ATPase—To actually accumulate Ca\(^{2+}\) in the lumen and thus reveal the effects of accumulated luminal Ca\(^{2+}\), the Ca\(^{2+}\)-ionophore A23187 was omitted from the above ATPase assay medium as performed previously (32) at the optimum 10 \(\mu\)M Ca\(^{2+}\) (hence to avoid the possible Ca\(^{2+}\)-substitution of Mg\(^{2+}\) at the catalytic subsite). The activity was determined during a prolonged 120-min incubation period (in which ~15–20% of ATP in the medium was hydrolyzed (and the possible inhibition by the formed ADP was thus avoided)). The ATPase activities of the wild type and I274V were significantly reduced to ~60% of the value obtained in the presence of A23187 (Table I). The reduction in M719I (to ~70%) was slightly less. In contrast, in L321F the activity was not reduced, and thus the fully activated state was attained even in the absence of A23187. Because L321F performs the coupled Ca\(^{2+}\) transport as the wild type (Table I), the results indicate that L321F is significantly less sensitive to the feedback inhibition by the accumulated luminal Ca\(^{2+}\). The results also indicate that M719I may be (only) slightly less sensitive than the wild type. The luminal Ca\(^{2+}\)-induced feedback inhibition was more specifically explored in the following kinetic analyses for the decay of EP formed from ATP and from P\(_{i}\).

Decay of EP Formed from ATP—The decay of EP formed from ATP was determined at 0 °C by first phosphorylating the enzyme with \(\gamma\)-32P[MgATP in the presence of saturating 10 \(\mu\)M Ca\(^{2+}\) and A23187 and then diluting the samples 20-fold with a solution containing nonradioactive ATP and excess EGTA (data not shown). As summarized in Table II, the single exponential decay rate was almost the same in I274V and M719I as in the wild type but slightly faster in L321F, which is consistent with its observed slightly faster turnover in this mutant than the wild type. It should be noted that the EP decay is obviously rate-limiting in the transport cycle in the mutants as well as in the wild type, because the decay was much slower than the E2 to E1Ca\(_{\text{a}}\) transition and E1P formation (Table I). As also demonstrated previously in the extensive kinetic analyses on SERCA2b (32).

When various high concentrations of Ca\(^{2+}\) (0.3–50 mM) were included in the above dilution medium in place of EGTA, the EP decay became biphasic and almost completely inhibited at 10–50 mM Ca\(^{2+}\) in the wild type, I274V, and M719I (data not shown). The profile essentially agreed with the previous detailed kinetic analyses on the effects of luminal Ca\(^{2+}\) on SR Ca\(^{2+}\)-ATPase (SERCA1a) by Nakamura et al. (52), in which the amplitude and the rate of the second phase were dramatically increased and decreased, respectively, with increasing Ca\(^{2+}\) to 1 or 2 mM.\(^{3}\) In contrast, the EP decay in L321F was much less sensitive to Ca\(^{2+}\), and the significant fraction of EP (~40%) decomposed rapidly in the first phase even at 50 mM Ca\(^{2+}\) (data not shown).

Hydrolysis of E2P Formed from P\(_{i}\)—The feedback inhibition by Ca\(^{2+}\) binding at the low affinity transport sites was explored more directly with E2P formed from P\(_{i}\)/Mg without Ca\(^{2+}\) (Fig. 5). This is because the Ca\(^{2+}\) transport sites are luminaly oriented in E2P formed from P\(_{i}\)/Mg (as the Ca\(^{2+}\) released form of EP (16)), and thus the luminal Ca\(^{2+}\) ions are accessible to the sites (in contrast to the occluded transport sites in E1P), and because the catalytic Mg\(^{2+}\) subsite in E2P is occluded (in contrast to the nonoccluded subsite in E1P (48–51)), and thus Mg\(^{2+}\) bound at the subsite would not be substituted with Ca\(^{2+}\) unless E2P is converted to E1P. It is hence expected that the Ca\(^{2+}\) binding to the transport sites in E2P from the luminal side, if it occurred, will reverse the Ca\(^{2+}\) releasing step 5 and further the E1P-E2P transition step 4 (as demonstrated previously with SR Ca\(^{2+}\)-ATPase (53)) and thus inhibit the E2P hydrolysis. It is also likely that the decay of E1P (converted from E2P) would become very slow when Mg\(^{2+}\) at the catalytic subsite in E1P is substituted with the added Ca\(^{2+}\) (48–50, 52).

In the experiments, E2P was first formed from 32P/P/Mg in the absence of Ca\(^{2+}\) and presence of A23187 and Me\(_{2}\)SO without K\(^{+}\) (the conditions that extremely favor the E2P formation (54)), and then the hydrolysis of 32P-labeled E2P was initiated by diluting 20-fold at 0 °C with a solution containing nonradioactive P, and 100 mM KCl in the absence or presence of various concentrations of CaCl\(_{2}\) (Fig. 5). The conditions were thus made otherwise similar to those for the above decay of EP formed from ATP. As summarized in Table II, the single exponential hydrolysis rates in the absence of Ca\(^{2+}\) in L321F and M719I were almost the same as or slightly faster than that in the wild type. In I274V, the rate was strongly reduced by ~4-fold (note the time scale). This slowed hydrolysis likely resulted in the observed large accumulation of E2P from ATP at steady state in this mutant (Fig. 3).

The E2P hydrolysis was strongly inhibited by the added Ca\(^{2+}\) in the wild type, I274V, and M719I. The time courses in the wild type and M719I appeared to be biphasic in the presence of Ca\(^{2+}\), in which the first phase was as rapid as the hydrolysis without Ca\(^{2+}\) and the second phase was extremely slow (or virtually no decay in the periods of observation). In I274V, this feature was less apparent because of the considerably slowed E2P hydrolysis (in the first phase). Nevertheless, the amplitude in the first rapid hydrolysis phase was dramatically reduced with increasing Ca\(^{2+}\) and almost completely disappeared at 20 mM Ca\(^{2+}\) in the wild type and I274V. In M719I, the small fraction of EP decomposed rapidly even at 20–50 mM Ca\(^{2+}\), and thus this mutant may be slightly less sensitive to the feedback inhibition (being consistent with the observed slightly less effect of the omission of A23187 on the ATPase activity as compared with the wild type and I274V (Table I). In contrast, L321F was much less sensitive to the added Ca\(^{2+}\), showing a large fraction (more than 60%) of EP hydrolyzed even at 50 mM Ca\(^{2+}\) as rapidly as in the absence of Ca\(^{2+}\).

In Fig. 5E, the fraction of remaining EP (not decomposed even after the long reaction periods) were replotted versus the concentration of added Ca\(^{2+}\). At 20–50 mM Ca\(^{2+}\), almost all of EP remained in the wild type and I274V, and the amount of remaining EP in M719I was slightly less. The Ca\(^{2+}\) concentration giving 50% of the amount of EP initially formed from P\(_{i}\) was ~4 mM in the wild type and I274V and 6 mM in M719I (Table II). We further observed that when the subsaturating 10 mM Ca\(^{2+}\) was added to E2P formed from P\(_{i}\), the major part of EP remaining at 30 s after the Ca\(^{2+}\) addition was ADP-sensi-
FIG. 5. Hydrolysis of EP formed from P<sub>i</sub> and effects of high concentrations of Ca<sup>2+</sup>. Microsomes expressing the wild type (WT, A) and mutant I274V (B), L321F (C), and M719I (D) of SERCA2b were phosphorylated with <sup>32</sup>P<sub>i</sub> at 25 °C for 10 min in 5 μl of a mixture containing 1 μg of microsomal protein, 0.1 mM <sup>32</sup>P<sub>i</sub>, 10 mM MgCl₂, 35% (v/v) Me₂SO, 50 mM MOPS/Tris, pH 7.0, and 5 mM EGTA. The mixture was then cooled and diluted at 0 °C by the addition of 95 μl of a mixture containing 2.1 mM nonradioactive P<sub>i</sub>, 10 mM MgCl₂, 105 mM KCl, and 50 mM MOPS/Tris, pH 7.0, without (G) or with various concentrations of CaCl₂ to give final Ca<sup>2+</sup> concentrations of 0.5 mM (E), 1 mM (F), 2 mM (G), 5 mM (H), 10 mM (I), 20 mM (J), and 50 mM (K), and the decay of <sup>32</sup>P-labeled EP was followed. At different times after the dilution, the decay reaction was quenched with trichloroacetic acid. The amounts of EP formed with <sup>32</sup>P<sub>i</sub> at zero time are normalized to 100%. The amounts of EP remaining after the rapid decay (first phase) increased with increasing Ca<sup>2+</sup> concentration, and almost no further decay was observed during the long periods of observation. Therefore, data were fitted to the single exponential decay (solid lines), $EP(t) = EP(0) \times e^{-kt} + C$, in which $k$ is the decay rate constant, and $C$ is the fraction of EP remaining at infinite time (nearly the value obtained at the longest period of observation). The decay rates thus obtained for the rapid phase without Ca<sup>2+</sup> were 0.097 s<sup>−1</sup> (wild type), 0.027 s<sup>−1</sup> (I274V), 0.077 s<sup>−1</sup> (L321F), and 0.156 s<sup>−1</sup> (M719I), as summarized in Table II. In each of the mutants and wild type, the rates obtained for the rapid hydrolysis phase in the presence of Ca<sup>2+</sup> were not significantly different from that in the absence of Ca<sup>2+</sup> (i.e., 0.06–0.1 s<sup>−1</sup> (wild type), 0.02–0.03 s<sup>−1</sup> (I274V), 0.06–0.09 s<sup>−1</sup> (L321F), and 0.1–0.3 s<sup>−1</sup> (M719I) (although as rough estimations especially at the very high concentrations of Ca<sup>2+</sup> in which the amplitude was very small)). In E, the remaining fraction of EP ($C$ in the above equation, nearly the value obtained at the longest reaction period) was replotted versus Ca<sup>2+</sup> concentration as the percentages of the amount of EP initially formed from P<sub>i</sub> at the zero time of decay reaction. The wild type ( ), I274V (○), L321F (△), and M719I (□) were indicated. The solid lines in E show least squares fit to the Hill equation, in which the Ca<sup>2+</sup> concentration to give half-maximal value, the Hill coefficient, and the maximal fraction of remaining EP, were 3.9 mM, 1.4, and 95.3% (wild type); 3.4 mM, 1.7, and 95.5% (I274V); and 4.2 mM, 1.9, and 83.1% (M719I). In L321F, the fitting (with the assumption for saturation at the low fraction of remaining EP) gave the respective values of 5.7 mM, 1.2, and 36.5%, but the fitting may not provide accurately the affinity for lumenal Ca<sup>2+</sup> because the fraction of remaining EP was very low even at the highest 50 mM Ca<sup>2+</sup>. In Table II, therefore, the Ca<sup>2+</sup> concentration ($K_{Ca}$) to give 50% of the amount of EP initially formed from P<sub>i</sub> was simply indicated for all of the mutants and wild type.
tive (E1P) in the wild type, I274V, and M719I (being 79 ± 4, 75 ± 3, and 75 ± 2%, respectively, of the total amount of remaining EP (n = 3)). Thus, E2P formed from P, was obviously converted to E1P by the added Ca\(^{2+}\) in the reversal of the Ca\(^{2+}\) releasing and E1P to E2P transition steps.

On the other hand, the increase in the remaining EP in L321F was much less and occurred at distinctly higher Ca\(^{2+}\) concentrations, indicating that the Ca\(^{2+}\)-released form of the phosphorylated intermediate (E2P) is strongly favored in this mutant. Consistently, we observed that at 30 s after the addition of 10 mM Ca\(^{2+}\), the major fraction (68 ± 6%) of the remaining EP in this mutant was ADP-insensitive (E2P) and thus not converted to E1P, being in sharp contrast to the above major conversion to E1P in the wild type, I274V, and M719I. The observed large accumulation of E2P from ATP at steady state in this mutant (Fig. 3) is also consistent with the above view. The remarkably reduced sensitivity to Ca\(^{2+}\) may be brought about by the reduced affinity for luminal Ca\(^{2+}\) in E2P (step 5), but it can also be accounted for by the possible large shift in step 4 toward E2P\(\Delta\)CAp followed by the subsequent rapid Ca\(^{2+}\) release and resulting increase of the Ca\(^{2+}\)-released form of E2P that is rapidly hydrolyzed. In any case, on the basis of the remarkably reduced sensitivity to Ca\(^{2+}\) of the EP decay kinetics and the A23187-insensitive (luminal Ca\(^{2+}\)-insensitive) full activation of ATPase, it is concluded that L321F has the dramatically reduced sensitivity to the feedback inhibition by the accumulated luminal Ca\(^{2+}\). Because this mutant performs the Ca\(^{2+}\) transport coupled with the ATP hydrolysis, it is likely that the luminal Ca\(^{2+}\) in ER of cells is elevated by this mutant to a level abnormally higher than the usual one, which is likely adjusted by the feedback inhibition (of the wild type) by the accumulated luminal Ca\(^{2+}\).

**DISCUSSION**

Possible Causes of DD by the Three Mutations—In the present study, we explored functional abnormalities in the three DD-causing SERCA2b mutants I274V, L321F, and M719I, because they exhibited nearly normal expression and localization in cells (thus escaping from degradation by the quality control) and the seemingly high specific ATPase and coupled Ca\(^{2+}\) transport activities. These mutations were in Japanese DD patients found by us (25). It was revealed that the mutants possess the distinct types of altered kinetic properties, especially L321F with its dramatically reduced sensitivity to the feedback inhibition by luminal Ca\(^{2+}\). The three mutations therefore likely cause different types of perturbation of intracellular Ca\(^{2+}\) homeostasis but nevertheless cause DD. Because DD is thought to be caused by haploinsufficiency in luminal Ca\(^{2+}\) homeostasis in keratinocytes and in fact the previous analyses on 22 different DD mutations revealed (32, 34) that the mutations resulted in the remarkably reduced or almost completely suppressed protein expression and/or function of SERCA2b, the present results on the three new types of mutants likely provide further insight into the haploinsufficiency and the possible consequent of the insensitivity to luminal Ca\(^{2+}\) found with L321F as discussed below.

The results on I274V suggest that the only ~35% reduced Ca\(^{2+}\) transport activity without change in the Ca\(^{2+}\) affinity is still not fast enough (even together with the full activity of wild type from the normal allele) to maintain or rapidly reduce the cytoplasmic Ca\(^{2+}\) level and fill up the internal Ca\(^{2+}\) store. M719I possesses an additional functional problem besides the ~20% reduced turnover: the ~3-fold reduced affinity for cytoplasmic Ca\(^{2+}\), which results in the slightly elevated cytoplasmic Ca\(^{2+}\) level. These observations suggest that the physiological requirement for Ca\(^{2+}\) homeostasis both in the transport...
rate and the cytoplasmic Ca\(^{2+}\) level in keratinocytes to avoid haploinsufficiency is very strict, probably much more than being recognized. Upon keratinization the Ca\(^{2+}\) level in epidermis becomes very high (55), and therefore such small defects in pumping cytoplasmic Ca\(^{2+}\) would still cause insufficiency. Interestingly, the patient with the M719I mutation (exhibiting the additional defect in the mutant) has been developing the significantly stronger skin symptoms than the patient with the I274V mutation (Table 1).

On L321F, the \(\sim\)2-fold reduced affinity for cytoplasmic Ca\(^{2+}\) despite its nearly normal maximal activity means slight elevation of cytoplasmic Ca\(^{2+}\)-level. Even more remarkable in L321F is the dramatically reduced sensitivity to the feedback inhibition by accumulated lumenal Ca\(^{2+}\), which will likely bring about an abnormally elevated Ca\(^{2+}\)-level in ER lumen. This is a dominant function of L321F over the wild type. It may be possible that this distinct feature of L321F is associated with some specific aspect of symptoms, and it is very interesting that the patients in the pedigree with this mutation possess (in addition to the severe DD skin problems) the severe neuropsychiatric disorders and behavior problems, which in contrast have not been present at all in the patients with the mutation I274V and M719I (Table 1) as observed during the careful examination and treatment of all these patients for over 10 years by our group. It was reported previously that Ser\(^{2920}\) \(\rightarrow\) Tyr mutation resulted in the reduced sensitivity to lumenal Ca\(^{2+}\) (32) and that the patients in one DD pedigree with this mutation also exhibited the neuropsychiatric symptoms (22, 23). It can be suggested at least in the case of the L321F mutation that the reduced sensitivity to lumenal Ca\(^{2+}\) in this housekeeping SERCA2b protein and the likely consequence, abnormally elevated lumenal Ca\(^{2+}\), severely affected the ER functions in brain and nerve cells as the Ca\(^{2+}\)-releaseable store and as the apparatus operating for newly synthesized proteins; the elevated lumenal Ca\(^{2+}\) would cause the larger amplitude of Ca\(^{2+}\) release, and the consequent possible changes in the finely tuned Ca\(^{2+}\) signals and also affect the lumenal events such as the modifications, folding, and trafficking of newly synthesized proteins and the postulated functional interactions between SERCA2b and calnexin/calreticulin (56, 57).

It is also important to note that the abnormally elevated lumenal Ca\(^{2+}\) by L321F would produce the strong feedback inhibition of the wild type on ER from the normal allele (thus dominant effect over the wild type), which may result in the severe haploinsufficiency in Ca\(^{2+}\)-homeostasis (more than that caused by the mutants I274V and M719I via the totally different mechanism) and therefore the severe skin symptoms in the L321F patients. It is also of interest to note that L321F likely consumes more ATP than the wild type because of the lack of feedback inhibition of the ATPase by the accumulated lumenal Ca\(^{2+}\) and therefore possibly reduces the energy charge in cells, which could be an additional and very important pathogenic mechanism of the mutation for the severe symptoms, particularly in brain and nerve cells to cause neuropsychiatric manifestation.

**Insight into Global Conformational Changes of Enzyme**—In the previous mutations of SERCA1a, Leu\(^{321}\) was stated as “retention of function” (5, 58), and Ile\(^{274}\) and Met\(^{720}\) (corresponding to Met\(^{719}\) in SERCA2b) were not explored. Because of their distinct locations on the enzyme molecule (Fig. 2) and the observed distinct effects of mutations on the kinetic properties, the results likely provide further insight into the global nature of conformational changes of SERCAs for ATP-driven Ca\(^{2+}\) transport, as discussed below.

The kinetic properties of L321F indicate that the Ca\(^{2+}\)-released forms, E2 and E2P, are favored in this mutant (because of the observed reduced affinity for cytoplasmic Ca\(^{2+}\) (shift in the E2-E1Ca\(_2\) equilibrium toward E2), the largely increased accumulation of E2P from ATP among E1P and E2P at steady state, and the remarkably reduced sensitivity to the feedback inhibition by luminal Ca\(^{2+}\)). On the atomic structure E2(TG) of SERCA1a (11), Leu\(^{321}\) on the cytoplasmic part of M4 is in close contact with Phe\(^{809}\) at the top of M6 (N terminus of Loop 6–7 connecting M6 and M7) (Fig. 6). Phe\(^{809}\) and thus Leu\(^{321}\) are therefore very close to Trp\(^{932}\) at the top of M9 in E2(TG), but Leu\(^{321}\) is more apart from these residues in E1Ca\(_2\) (6) because of the upward movement of Leu\(^{321}\) relative to the others. The introduced aromatic ring of phenylalanine in L321F therefore likely forms strong hydrophobic interactions with those of Phe\(^{809}\) and Trp\(^{932}\) as if clustering the top parts of M4, M6, and M9 and thus affecting the transmembrane and luminal domains so as to favor the Ca\(^{2+}\)-released forms. It is also possible that the mutation at Leu\(^{321}\) affected (via Phe\(^{809}\)) the predicted essential large motions of Loop 6–7 and its interactions with transmembrane helices (8, 59) or arrangements of M6/M7 directly connected to this loop.

It should be noted that the observed kinetic properties of L321F are similar to those of the DD mutant S920Y previously reported by Dode et al. (32) with respect to the increased turnover, the reduced affinity for cytoplasmic Ca\(^{2+}\) and the reduced sensitivity to luminal Ca\(^{2+}\) (as revealed in the case of S920Y by the effect of A23187 on the ATPase activity), except the observed dominant accumulation of E1P among E1P and E2P in S920Y at steady state. They suggested (32) that the substitution of Ser\(^{2920}\) with a bulky tyrosine at the middle of cytoplasmic Loop 8–9 causes a steric clash and affects arrangements of M9, M10, and M11. The results on L321F and on S920Y are nevertheless both consistent with the long range effects from the cytoplasmic region to the Ca\(^{2+}\)-binding sites/release pathway in the transmembrane and luminal domains. With respect to the cause for DD in the S920Y mutation, Dode et al. (32) have indicated that the dominant phenotype produced by mutant S920Y could be caused by haploinsufficiency because the expression level of S920Y protein was significantly lower than its corresponding wild-type protein, and its contribution to the total cellular capacity for Ca\(^{2+}\) accumulation was remarkably reduced. Dode et al. (32) further suggested that the wild-type enzyme present in dimeric complexes with mutant S920Y becomes more susceptible to cellular degradation than in its monomeric state. Thus, for the cause of DD, the situation in L321F (with its normal expression) is distinct from that in S920Y.

On the valine substitution of Ile\(^{274}\) at the lumenal end of M3, the remarkably slowed hydrolysis of E2P formed from P\(_i\) (observed in the absence of Ca\(^{2+}\)) indicates the long range effect from the lumenal end to catalytic site in cytoplasmic region and thus the global conformational changes of enzyme to take place during the E2P hydrolysis in step 6. Actually, we have recently revealed (16) that the Ca\(^{2+}\)-release pathway and luminal gate are open in E2P but closed upon its hydrolysis to E2 and further that this change occurs concomitantly with the change in the catalytic site from its strongly hydrophobic (closed) to hydrophilic (open) state. It may be possible that the I274V mutation at the lumenal end of M3 retarded the structural changes for closing of Ca\(^{2+}\)-release pathway/gate and thus for the coupled changes in catalytic site required for the acylphosphate hydrolysis. Actually, luminal Loop 3–4 connected directly to M3 at the immediate C-terminal region of Ile\(^{274}\) (Fig. 2) have been predicted to form the lumenal Ca\(^{2+}\)-gate (11) with essential contribution of Loop 7–8 (11, 60). In the atomic structure E2(TG) (11), Ile\(^{274}\) and the neighboring Val\(^{271}\) on M3 are gathered into a hydrophobic cluster at the luminal end of M5 (Ile\(^{775}\)/Phe\(^{779}\)/Ala\(^{782}\)/Ala\(^{786}\)) and of M4 (Tyr\(^{294}\)/Tyr\(^{298}\)/Ile\(^{299}\))
(and such interactions are less significant in E1Ca structure (8)) (Fig. 6). The observed retardation of E2P hydrolysis by the conservative valine substitution in 1274V indicates the importance of terminal methyl group of Ile274 possibly in such hydrophobic interactions for the predicted motions of Ca\(^{2+}\) release pathway/gate during the E2P hydrolysis. It should be noted that the 1274V mutation, on the other hand, is not too drastic as to cause loss of transport function (or Ca\(^{2+}\) leakage) because the Ca\(^{2+}\) uptake was shown to be coupled with ATP hydrolysis in this mutant as in the wild type. The observed significantly accelerated process from E2 to E1Ca in 1274V (without change in the Ca\(^{2+}\) affinity) suggests that such hydrophobic interactions of Ile274 may be rather destabilizing the transition state of the process.

Met720 in SERCA1a (corresponding to Met719 in SERCA2b) is located on one of \(\beta\)-strands very close to Asp\(^{351}\) in the Rossin fold of P domain (Fig. 2), and its side chain actually points to the catalytically critical residues. These residues and regions (with the numbering of SERCA1a) include Cys\(^{493-496}\) Leu\(^{566}\) Lys\(^{664}\) Thr\(^{701}\) Asp\(^{702}\), Asp\(^{707}\), Ile\(^{118}\) Ser\(^{722}\), Met\(^{731}\), Ile\(^{744}\) (the region from the C-terminal part of P domain to the top part of M5), and Arg\(^{604}\) on the P/N domain-connecting loop. The observed accelerated phosphorylation from E1Ca and ATP in M719I may therefore be caused by the possibly increased conformational flexibility within catalytic site and/or at the P/N domain junction to accelerate the rate-limiting conformational change, which is likely the P/N domain closure in E1CaATP complex (10, 61) as revealed in earlier studies with a fluorophore bound to Cys\(^{677}\) in SR Ca\(^{2+}\)ATPase (64, 65). The observed reduced affinity for cytoplasmic Ca\(^{2+}\) in I274V (without change in the Ca\(^{2+}\) affinity) suggests that such hydrophobic interactions of Ile274 and Thr285 and Thr286 in M5, and Arg604 on the P/N domain-connecting loop may possibly be mediated via M5 directly linked with P domain (8, 11).

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