Communication

Temperature-sensitive ZAP70 Mutants Degrading through a Proteasome-independent Pathway

RESTORATION OF A KINASE DOMAIN MUTANT BY Cdc37*

(Received for publication, August 26, 1999, and in revised form, September 29, 1999) Satoshi Matsuda‡, Tomoko Suzuki-Fujimoto‡, Akiko Minowa‡, Hideki Ueno‡, Kenji Katamura§, and Shigeo Koyasu¶¶

From the ‡Department of Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan and the ¶¶Department of Pediatrics, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

CD8 deficiency is an autosomal recessive form of severe combined immunodeficiency diseases characterized by the absence of CD8 T lymphocytes and impaired T cell functions. We identified two novel missense mutations in the zap70 genes of a CD8-deficiency patient. One mutation (P80Q) affects a residue in the SH2 domain and another (M572L) in the kinase subdomain XI. Both mutations cause a degradation of ZAP70 protein in a temperature-sensitive manner through an ATP-dependent and proteasome-independent pathway. We further demonstrated that Cdc37, a protein kinase-specific chaperone, bound to M572L but not P80Q mutant and restored the expression of the M572L mutant when overexpressed. The restoration of M572L mutant by Cdc37 required the function of HSP90. These results indicate that Cdc37 in conjunction with HSP90 functions as a molecular chaperone for a temperature-sensitive kinase domain mutant of ZAP70.

CD8 deficiency is an autosomal recessive form of severe combined immunodeficiency diseases (SCIDs) and is associated with defects in the ZAP70 protein tyrosine kinase (PTK), which plays a pivotal role in signal transduction through the T cell receptor (TCR) (1–5). Upon TCR stimulation, ZAP70 is recruited to tyrosine-phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic domains of TCR subunits; this is an essential step for ZAP70 activation and subsequent cellular signaling pathways (6–8). Association of ZAP70 with the TCR is mediated by an interaction between the two SH2 domains of the ZAP70 molecule arranged in tandem and the two phosphorylated tyrosine residues in the ITAM (9–13).

All of the mutations in the ZAP70 molecule that have been reported to cause SCID in humans cluster around the kinase subdomain VIII, resulting in instability of the ZAP70 protein (3–5). In general, misfolded proteins are recognized by cellular proteins, chaperones and proteases. Molecular chaperones assist in the proper folding of misfolded polypeptide and render it functional, whereas proteases eliminate such a polypeptide (14). It is thus likely that the counterbalance between chaperones and proteases determines the stability of mutant ZAP70 molecules. With respect to proteases, it is widely accepted that the ubiquitin-proteasome system is involved in degradation of abnormal proteins (15, 16). However, the contribution of proteasomes to the instability of mutant ZAP70 proteins is yet to be determined.

We show here that two novel missense mutations in the zap70 genes of a CD8-deficiency patient cause degradation of ZAP70 protein in vivo in a temperature-sensitive (ts) manner through an ATP-dependent and proteasome-independent pathway. We also show that overexpression of Cdc37 (17, 18), a protein kinase-specific molecular chaperone, preferentially restores the expression of a kinase-domain mutant of the ZAP70 molecule even at the nonpermissive temperature.

EXPERIMENTAL PROCEDURES

Cell Culture and Immunoblot Analysis—The HTLV-1-transformed T cell lines SN, established from a CD8 deficiency patient, and KO, derived from a healthy donor, were maintained in RPMI 1640 medium supplemented with antibiotics, 5 × 10⁻⁵ M 2-mercaptoethanol, 10 mM Hepes, 10% fetal calf serum, and IL-2 (10 units/ml). A cDNA library was constructed from mRNA of SN cells using λ-ZAP II (Stratagene) and screened with a probe comprising the N-terminal 74 residues of ZAP70. A cDNA clone for wild-type ZAP70 tagged with the Myc-epitope (EQLLISEDL) at its C terminus was obtained from Jurkat cells using reverse transcriptase-PCR and cloned into the HindIII and XhoI sites of pcDNA3.1+ (Invitrogen). P80Q and M572L mutants were created by PCR-based site-directed mutagenesis (Quick Change™, Stratagene). Dominant-negative ZAP70 (ZAP70ΔC) molecules were constructed by subcloning the SH2 domains (HindIII and HincII fragment) into the HindIII and EcoRI sites of oxidase; mAb, monoclonal antibody.

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.
mutant ZAP70 proteins were incubated with or without S500 fraction at 37 °C for 30 min and analyzed by SDS-PAGE (Fig. 2B), suggesting that the proteasome-independent manner—specific inhibitors for proteasomes such as lactacystin and the peptide aldehyde MG132 failed to block the proteolysis of ZAP70 mutants, whereas these inhibitors blocked degradation of β-catenin, a known target of the ubiquitin-proteasome pathway (Fig. 2C). When incubated with total cell lysates in an in vitro degradation assay system, both M572L and P80Q mutant proteins prepared by an in vitro translation system were degraded in an ATP-dependent manner, whereas WT ZAP70 protein remained stable (Fig. 2D). The proteolytic activity was recovered from the S500 fraction (Fig. 2E, upper panel), which was devoid of proteasomes (Fig. 2E, lower panel). Other protease inhibitors, including E64 (10 μM), TPCK (100 μM), TLCK (100 μM), leupeptin (20 μg/ml), and pepstatin A (20 μg/ml), had no effect on the degradation of ZAP70 mutants (data not shown). Although tripeptidyl peptidase II (TPPII) activity is ATP-dependent and non-ATP-dependent proteasome activities, the proteolytic activity of ZAP70 in cell lysates was resistant to these inhibitors. This indicates that the degradation of ZAP70 by proteasome is not substrate-specific and that other protease activities are also involved in the degradation of ZAP70.

RESULTS AND DISCUSSION

Novel Missense Mutations in the zap70 Genes in a CD8 Deficiency Patient—An HTLV-1-transformed T cell line, SN, established from a CD8 deficiency patient (22) expressed no Zap70 protein (Fig. 1A) even though the cell line expressed a normal size zap70 mRNA at the usual levels (data not shown). Molecular cloning and sequencing of zap70 cDNAs from SN cells uncovered two mutations (Fig. 1B): a C to A transition at position 448, resulting in a substitution of glutamine for proline at residue 80 (P80Q) in the N-terminal SH2 domain (numbering according to Ref. 6), and an A to T transition at position 1923, causing the substitution of leucine for methionine at residue 572 (M572L) in the kinase subdomain XI (23). This methionine residue is well conserved among PTKs. Genomic DNA analysis showed that the patient was heterozygous for these mutations; the C to A mutation was paternal, and the A to T mutation was maternal (Fig. 1C). A healthy sibling of the patient carries the paternal mutation.

Both Mutations Render ZAP70 Protein Unstable in Vivo—Protein expression was not detectable in a human T cell line Jurkat transfected with Myc-tagged mutant cDNAs, whereas transfection of the wild-type (WT) cDNA resulted in expression of a Myc-tagged ZAP70 protein of the expected size (Fig. 2A). However, these mutant Zap70 proteins were produced at a level comparable to the WT protein in an in vitro reticulocyte translation system (see Figs. 2C and 3C), suggesting that the mutant proteins are unstable and degraded in vivo. In fact, pulse-chase experiments showed that whereas WT Zap70 protein was stable with a half-life of >4 h, P80Q as well as M572L mutant proteins were rapidly degraded with a half-life of approximately 1 h (Fig. 2B).

Mutant Zap70 Proteins Are Degraded in an ATP-dependent and Proteasome-independent Manner—Specific inhibitors for proteasomes such as lactacystin and the peptide aldehyde MG132 failed to block the proteolysis of ZAP70 mutants, whereas these inhibitors blocked degradation of β-catenin, a known target of the ubiquitin-proteasome pathway (Fig. 2C). When incubated with total cell lysates in an in vitro degradation assay system, both M572L and P80Q mutant proteins prepared by an in vitro translation system were degraded in an ATP-dependent manner, whereas WT ZAP70 protein remained stable (Fig. 2D). The proteolytic activity was recovered from the S500 fraction (Fig. 2E, upper panel), which was devoid of proteasomes (Fig. 2E, lower panel). Other protease inhibitors, including E64 (10 μM), TPCK (100 μM), TLCK (100 μM), leupeptin (20 μg/ml), and pepstatin A (20 μg/ml), had no effect on the degradation of ZAP70 mutants (data not shown). Although tripeptidyl peptidase II (TPPII) activity is ATP-dependent and
Temperature-sensitive zap70 Mutations

Functions of ZAP70 ts mutants. A. Jurkat cells were transfected with the expression vector for the WT, P80Q (PQ), or M572L (ML) mutants. After 24 h, the cells were split into three cultures and incubated for an additional 24 h at 37, 33, or 30 °C. ZAP70 proteins were immunoprecipitated and blotted with anti-Myc antibodies. *, indicates the migration position of the endogenous Myc protein. B. Jurkat cells transfected with the expression vector for WT, M572L (ML), or P80Q (PQ) mutants or for control vector alone (V) were cultured at 30 °C. The cells were then stimulated with pervanadate (PV) for 10 min at 30 °C and subjected to immunoprecipitation and in vitro ZAP70 kinase assay (23) at 30 °C using tubulin as a substrate (upper panel). Arrows indicate autophosphorylated ZAP70 (auto-P) and phosphorylated tubulin (tubulin). An aliquot of the immunoprecipitate was also probed with a mAb against phosphotyrosine (4G10) (lower). C. WT, M572L (ML), and P80Q (PQ) mutants were in vitro translated at 30 °C in the presence of [35S]methionine (upper panel). Samples were incubated with streptavidin-agarose (Sigma) bound to the phosphorylated biotinylated ITAM peptide (peptide +) or vehicle alone (peptide −) for 1 h at the indicated temperature and then subjected to SDS-PAGE and image analysis (lower panel). D. Jurkat cells were transfected with either ZAP70ΔC (WT) or ZAP70ΔC containing P80Q mutation (PQ) along with NFAT-Luc. To normalize the transfection efficiency, pRL-SV40 plasmid was also transfected. After 16 h, the cells were split into two cultures and incubated for an additional 24 h at the indicated temperature. The cells were then stimulated via the TCR using an SV40 plasmid was also transfected. After 16 h, the cells were split into three cultures and incubated for an additional 24 h at 37, 33, or 30 °C. ZAP70 proteins were immunoprecipitated and blotted with anti-Myc antibodies.

Importance of the conserved methionine residue at the kinase subdomain XI for PTK activity as well as the stability of the ZAP70 protein. We next examined the SH2 function of the mutant ZAP70 proteins. The [35S]-labeled P80Q mutant ZAP70 protein produced in vitro at 30 °C bound to the phosphorylated ITAM peptide with an affinity comparable to the WT and M572L mutant in an in vitro pull-down assay (Fig. 3C). Note that the P80Q mutant synthesized in vitro bound to the phosphorylated ITAM peptide even at 37 °C, the nonpermissive temperature, indicating that the protein is stable at 37 °C once synthesized and properly folded. To examine whether the SH2 domains of the P80Q mutant bind to ITAMs in vivo, we constructed a dominant-negative form of ZAP70 (ZAP70ΔC), consisting of two SH2 domains, that sequesters endogenous ZAP70 from phosphorylated ITAMs (26). When expressed at 30 °C, the ZAP70ΔC containing the P80Q mutation blocked the TCR-induced signaling pathway as well as the ZAP70ΔC consisting of the WT SH2 domains (Fig. 3D). However, such a blocking effect by the P80Q mutant was not observed at 37 °C. Thus, the defect resulting from the P80Q mutation is likely due to the rapid degradation of mutant polypeptide and not to a lack of SH2 function.

Cdc37 in Conjunction with Hsp90 Restores the Expression of M572L Mutant—It has been suggested that mutant proteins, which are incompletely or improperly folded when synthesized, are degraded unless folding intermediates are compromised by their cofactor(s), namely chaperone(s) (27, 28). One such chaperone is Cdc37, which stabilizes various PTKs by interacting with their kinase domains (17, 18). Indeed, co-transfection with Cdc37 increased the expression level of the WT ZAP70 protein in a dose-dependent manner (Fig. 4A). In contrast, only a slight effect was observed when the ZAP70ΔC lacking the kinase domain was co-expressed with Cdc37, indicating that Cdc37 functions as a kinase domain-specific chaperone for ZAP70. Expression levels of the M572L mutant at both permissive and nonpermissive temperatures were also remarkably augmented by co-transfection with Cdc37 (Fig. 4B). Although not shown, co-expression of Cdc37 did not affect the half-life of the M572L
mutant at the nonpermissive temperature, indicating that Cdc37 restored the expression of M572L mutant protein by assisting its folding process and not by blocking degradation pathways. In contrast, Cdc37 had a marginal effect on the P80Q mutant. It is possible that the P80Q mutation leads to conformational changes, preventing interaction between the kinase domain of the ZAP70 and Cdc37. In fact, whereas both WT ZAP70 and the M572L proteins bound GST-Cdc37, interaction of the P80Q mutant with GST-Cdc37 was marginal and observed only with an excess amount of GST-Cdc37 (Fig. 4C). Such differences in ability to bind Cdc37 would account for the preferential effect of Cdc37 on the M572L mutant.

Several studies have revealed that Cdc37 acts in concert with another chaperone, HSP90 (17, 18). Inhibition of HSP90 function with geldanamycin reduced the expression of both the WT and the M572L mutant even in the presence of Cdc37 (Fig. 4D). These results clearly indicate that HSP90 is required for the expression of ZAP70 protein. HSP90 is a highly abundant protein (28), but yet the expression of the M572L mutant depends on Cdc37. Therefore Cdc37 is likely the rate-limiting component and contributes to the proper folding of the M572L mutant with the aid of the HSP90 function.

With the exception of the ts-type I ocoulcuteaneous albinism (29), spontaneous ts mutations have rarely been reported in the human. We provide evidence here that ts mutations of the zap70 gene are involved in human SCIDs. The fact that these mutants have kinase activities at 30 °C, the permissive temperature, raises the possibility that the T cell defects could be rescued at a lower temperature. In fact, although the patient’s peripheral blood mononuclear cells failed to respond to antigenic stimulation in vivo, this patient autoreactive T cells that would have been negatively selected under normal conditions were actually positively selected. Because Syk expression is greatly reduced upon migration to the periphery, peripheral CD4− T cells in the patient would have become unresponsive to antigenic stimulation. Because local body temperature within the skin is lower, functional ZAP70 proteins may have been expressed with the help of endogenous Cdc37 and HSP90 chaperones, subsequently inducing T cell activation. It will be of interest to examine the possibility that Cdc37 in concert with HSP90 also rescues other mis-sense mutants reported in CD8 deficiencies (3–5) and other SCIDs involving Btk and JAK3 PTKs (30, 31).

Acknowledgments—We thank the family members of the patient for help with this study. We are grateful to M. Eck, K. Tanaka, Y. Kimura, and Y. Minami for stimulating discussions. Thanks are also due to G. Crabtree, E. L. Reinherz, T. Roberts, Y. Kimura, S. Tsukita, and K. Tanaka for various reagents and to I. Yahara, L. K. Clayton, D. Frucht, and J. J. O’Shea for critically reading the manuscript.

REFERENCES
1. Gelfand, E. W., and Dosch, H.-M. (1983) Birth Defects: Original Article Series, pp. 65–72, Alan R. Liss, Inc., New York
2. Rinftman, C. M., Hummel, D., Martinez-Valdez, H., Thorner, P., Doherty, P. J., Pan, S., Cohen, F., and Cohen, A. (1989) J. Exp. Med. 170, 2177–2182
3. Monao, W. J., Polmar, S. H., Neudorf, S., Mather, A., and Filipovich, A. H. (1992) Clin. Exp. Immunol. 90, 390–393
4. Arpaia, E., Shahar, M., Dadi, H., Cohen, A., and Roifman, C. M. (1994) Cell 76, 947–958
5. Elder, M. E., Lin, D., Clever, J., Chan, A. C., Hope, T. J., Weiss, A., and Littman, D. R. (1994) Science 264, 1596–1598
6. Chan, A. C., Ishikawa, M., Turck, C. W., and Weiss, A. (1992) Cell 71, 649–662
7. Samelson, L. E., and Klausner, R. D. (1992) J. Biol. Chem. 267, 24913–24916
8. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
9. Wang, R. L., Malek, S. N., Desiderio, S., and Samelson, L. E. (1993) J. Biol. Chem. 268, 19797–19801
10. Koyasu, S., Tae, A. G. D., Moingeon, P., Hussy, R. E., Mildenian, A., Hannnissian, J. Clayton, L. K., and Reinherz, E. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6683–6697
11. Hatada, M. H., Liu, X., Laird, E. R., Green, J., Morganstern, J. P., Lou, M., Marr, C. S., Phillips, T. B., Ram, M. K., Theriault, K., Zoller, M. J., and Karas, J. L. (1995) Nature 377, 32–38
12. van der Oo, N. S., Tally, N., and Weiss, A. (1996) J. Exp. Med. 183, 1053–1062
13. Sunder-Plassmann, R., Lissios, F., Madsen, M., Koyasu, S., and Reinherz, E. L. (1997) Eur. J. Immunol. 27, 2001–2009
14. Gottesman, S., Wiinckner, S., and Maurizi, M. (1997) Genes Dev. 11, 815–823
15. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
16. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
17. Kupershmidt, Y., Rutherford, S. L., Miyata, Y., Yahara, J., Freeman, B. B., Yue, L., Morimoto, R. I., and Lindquist, S. (1997) Genes Dev. 11, 1775–1785
18. Grammatikakis, N., Lin, J.-H., Grammatikakis, A., Tsichlis, P. N., and Cochrane, B. H. (1999) Mol. Cell. Biol. 19, 1661–1672
19. Matsuda, S., Morriguchi, T., Koyasu, S., and Nishida, E. (1998) J. Biol. Chem. 273, 12373–12382
20. Ozaki, T., Irie, K., and Sakiyama, S. (1999) DNA Cell Biol. 14, 1017–1023
21. Gotoh, Y., Matsuda, S., Takenaka, K., Hattori, S., Iwamatsu, A., Ishikawa, M., Kosako, H., and Nishida, E. (1994) Oncogene 9, 1891–1898
22. Katamura, K., Tai, G., Tachibana, T., Yamabe, H., Ohmori, K., Mayumi, M., Matsuda, S., Koyasu, S., and Furusho, K. (1999) Clin. Exp. Immunol. 118, 124–130
23. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
24. Geier, E., Pfeifer, G., Wilm, M., Louchardi-Hartzt, M., Baumeister, W., Eichmann, K., and Niedermayer, G. (1999) Science 283, 973–981
25. Isakov, N., Wang, R. L., Watts, J. D., Aebersold, R., and Samelson, L. E. (1996) J. Biol. Chem. 271, 15753–15761
26. Qian, D., Mollenauer, M. N., and Weiss, A. (1996) J. Exp. Med. 183, 611–620
27. Morimoto, R. I., Kline, M. P., Bimston, D. N., and Cotto, J. J. (1997) Essays Biochem. 32, 17–29
28. Parsell, D. A., and Lindquist, S. (1993) Annu. Rev. Genet. 27, 437–496
29. Giesel, L. B., Tripathi, R. K., King, R. A., and Spritz, R. A. (1991) J. Clin. Invest 87, 1119–1122
30. Ochs, H. D., and Smith, C. I. (1986) Medicine (Baltimore) 75, 287–299
31. Leonard, W. J., and O’Shea, J. J. (1998) Annu. Rev. Immunol. 16, 283–322
32. Yonezawa, N., Nishida, E., Sakai, H., Koyasu, S., Matsuzaki, F., Iida, K., and Yahara, I. (1988) Eur. J. Biochem. 177, 1–7
Temperature-sensitive ZAP70 Mutants Degrading through a Proteasome-independent Pathway: RESTORATION OF A KINASE DOMAIN MUTANT BY Cdc37

Satoshi Matsuda, Tomoko Suzuki-Fujimoto, Akiko Minowa, Hideki Ueno, Kenji Katamura and Shigeo Koyasu

J. Biol. Chem. 1999, 274:34515-34518.
doi: 10.1074/jbc.274.49.34515

Access the most updated version of this article at http://www.jbc.org/content/274/49/34515

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

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

This article cites 31 references, 14 of which can be accessed free at http://www.jbc.org/content/274/49/34515.full.html#ref-list-1