A Cypher/ZASP Mutation Associated with Dilated Cardiomyopathy Alters the Binding Affinity to Protein Kinase C*

Received for publication, October 29, 2003, and in revised form, December 2, 2003
Published, JBC Papers in Press, December 3, 2003, DOI 10.1074/jbc.M311849200

Takuro Arimura†§, Takeharu Hayashi‡, Hajime Terada‡, Su-Yeoun Lee‡‡‡, Qiang Zhou‡‡, Megumi Takahashi‡, Kazuo Ueda‡, Tatsuhito Nouchi‡, Shigeru Hohda‡, Makoto Shibutani‡, Masao Hirose‡, Ju Chen‡‡, Jeong-Euy Park‡, Michio Yasunami‡, Hideharu Hayashi‡, and Akinori Kimura‡§§

From the †Department of Molecular Pathogenesis, Medical Research Institute, and Laboratory of Genome Diversity, School of Biomedical Science, Tokyo Medical and Dental University, Tokyo 101-0062, Japan, the ‡Division of Pathology, National Institute of Health Sciences, Tokyo 158-8501, Japan, the ‡‡Third Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan, the ‡‡‡Division of Cardiology, Samsung Medical Center, Seoul 135-230, Korea, and the ‡§§Institute of Molecular Medicine and Department of Medicine, University of California at San Diego, La Jolla, California 92039

Dilated cardiomyopathy is characterized by ventricular dilatation with systolic dysfunction of cardiac muscle. Recent genetic studies have revealed that mutations in genes for cytoskeleton proteins distributed in the Z-disc and/or intercalated discs of the cardiac muscle are major predictors of cardiomyopathy. However, as mutations in these genes can account for only a part of the patient population, there should be another disease-causing gene(s) for cardiomyopathy. Cypher/ZASP appears to be an ideal candidate for the cardiomyopathy causative gene, because Cypher/ZASP encodes a Z-disc associated protein, and recent studies have demonstrated that Cypher/ZASP knock-out mice develop cardiomyopathy. In this study, we searched for sequence variations in Cypher/ZASP in 96 unrelated Japanese patients with dilated cardiomyopathy. A D626N mutation located within the third LIM domain was identified in a familial case but not found in the unrelated controls. A family study of the patient showed that all affected siblings tested had the same mutation. Clinical information of the affected family members suggested that the mutation was associated with late onset cardiomyopathy. To reveal the biochemical changes due to the mutation, we performed a yeast two-hybrid assay and a pull-down assay. It was demonstrated by both assays that the D626N mutation of Cypher/ZASP increased the affinity of the LIM domain for protein kinase C, suggesting a novel biochemical mechanism of the pathogenesis of dilated cardiomyopathy.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

* This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Culture, Science and Technology of Japan, a research grant from the Ministry of Health and Welfare, Japan, a grant for Japan-Korea collaboration research from Japan Society for the Promotion of Science, and a research grant from Mitsui Life Social Welfare Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** A short-term visiting fellow to the Medical Research Institute, Tokyo Medical and Dental University and supported by a Grant-in-Aid for Scientific Research on Priority Area (C) Medical Genome Science from Ministry of Education, Culture, Sports, Science and Technology, Japan.

†§ To whom correspondence should be addressed: Dept. of Molecular Pathogenesis, Medical Research Inst., Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan. Tel.: 81-3-5280-8056; Fax: 81-3-5280-8055; E-mail: akitis@mri.tmd.ac.jp.

† Integrative Genetics Institute, National Institute of Health Sciences, Tokyo 158-8501, Japan.

‡ Division of Pathology, National Institute of Health Sciences, Tokyo, Japan.

‡‡ Division of Pathology, National Institute of Health Sciences, Tokyo, Japan.

‡‡‡ Division of Cardiology, Samsung Medical Center, Seoul, Korea.

†† Institute of Molecular Medicine and Department of Medicine, University of California at San Diego, La Jolla, California 92039.

‡§§ Institute of Molecular Medicine and Department of Medicine, University of California at San Diego, La Jolla, California 92039.

1 The abbreviations used are: DCM, dilated cardiomyopathy; MLP, muscle LIM protein; PKC, protein kinase C; RT, reverse transcription; SSCP, single-stranded conformation polymorphism; Y2H, yeast two-hybrid; β-gal, β-galactosidase; ENH, Enigma homologue protein; CA, cerebellar ataxia; HA, hemagglutinin A; RACK, receptor for activated C kinase; RICK, receptor for inactivated C-kinase.

6746 This paper is available on line at http://www.jbc.org

This content is available online at http://www.jbc.org.
termius is required for binding to α-actinin in the Z-disc (25), and the LIM domains at the C terminus are involved in the binding to PKCs (24). In addition, Cypher/ZASP knock-out mice display disorganized and fragmented Z-discs in both skeletal and cardiac muscles, leading to a severe form of congenital myopathy and cardiomyopathy (27, 28). Moreover, human Cypher/ZASP is mapped on chromosome 10q22.3-q23.2 (26). We report here a Cypher/ZASP gene mutation, associated with late-onset familial DCM, that increases the LIM-PKC interaction. This is the first report suggesting that an abnormality in the anchoring-protein of PKC may play an important role in the pathogenesis of DCM.

EXPERIMENTAL PROCEDURES

Reverse Transcription (RT)-PCR Analysis of Cypher/ZASP—Expression of Cypher/ZASP was investigated by RT-PCR analyses using mRNAs from various human tissues. The combination of primers used in the RT-PCR are as follows: FIN (5′-GTGCCCCTCACCTCAACACTCT-3′) in exon 1 and R4 (5′-GAATTCAGCAGCTGGTGCTG-3′) in exon 8; R2 (5′-CACGAGACTCACTCCAGTG-3′) in exon 6 and R2 (5′-GGGCTTGAGAAGGCGGCTG-3′) in exon 11; 365P (5′-TTCCTT-CCACTCTGCGAGGG-3′) in exon 4 and R2 (5′-GCTATGATCTGTC-CCGCTCATCT-3′) in exon 7; and LIMF (5′-GAATTCAGAGCCGGACCTG-3′) and LIMR (5′-CTTTCCGAAGCTCTT-GTTGGCCAG-3′) in exon 16.

Subjects—Patients and family members were evaluated as described previously (12). Thirty-four proband patients with familial DCM and 62 sporadic DCM cases were the subjects. These patients had been examined before and had no mutation in the genes for dystrophin, α-cardiac actin, desmin, lamin a/C, β-cardiac myosin heavy chain, cardiac troponin T, α-tropomyosin, vinculin, MLP, Tcap/telethonin, and titin. When a sequence variation in Cypher/ZASP was found, family relatives and 400 unrelated healthy controls were examined. Blood samples were collected after obtaining informed consent from the subjects. All patients and controls were Japanese. The protocol for research on human materials was approved by the Ethics Reviewing Committee of the Medical Research Institute, Tokyo Medical and Dental University.

Genomic DNA Extraction and PCR-SSCP Analysis—DNA was extracted from peripheral blood leukocytes from each subject. Extracted DNA was subjected to PCR by using primer pairs specific to the analyzed regions. Sequences of primers are available upon request. The PCR fragment was sequenced on both strands. To confirm the amplified regions, Sequences of primers are available upon request. The protocol for research on human materials was approved by the Ethics Reviewing Committee of the Medical Research Institute, Tokyo Medical and Dental University.

RESULTS

Expression of Cypher/ZASP in Human Tissues—Several different cDNA isoforms of Cypher/ZASP were reported in human and mouse striated muscle (24–26). These isoforms are generated by alternative splicing of a single gene (Fig. 1A, Ensembl gene identification number ENSG00000122367). To investigate the alternative splice pattern in various human tissues, we performed RT-PCR analysis. As shown in Fig. 1B, human Cypher/ZASP expressed several transcripts. There were two different PCR products from exon 1 to 9; a short form (Cypher 2s; nomenclature is according to Ref. 27) was preferentially expressed in the skeletal muscle, whereas a long form (Cypher 2c) was abundant in the fetal heart. On the other hand, transcripts covering exons 4–7 were expressed preferentially in the fetal heart. Two different transcripts spanning exons 6–11 were generated; the longer form (corresponding to Cypher 1s) was expressed in the heart. Several other combinations of primers were used in addition to investigate the alternative splicing (for example, exons 4–11; data not shown). The RT-PCR products were sequenced to confirm that there were at least six different splice variants in human (Fig. 1C) as was reported in mouse (27). On the other hand, ZASP variant 2, which was reported to have a deletion of 51 base pairs (25), presumably as a result of a splice to a minor acceptor site in exon 11, could not be identified in this study with any combinations of primers (data not shown).

Identification of a Missense Mutation in the Cypher/ZASP Gene in a DCM Family—Sequence variations in the Cypher/ZASP gene were searched in 96 patients with DCM, and five different variations were identified (Fig. 1A). These include a T to C transition in an intron (–13 in intron 6) and four variations in the exons (GTC to ATC at codon 55 in exon 2, GTC to ATC at codon 588 in exon 14, GAT to AT at codon 626 in exon 15, and CAT to CAC at codon 644 in exon 15; codon numbers are from Cypher/ZASP 1c in Fig. 1C). Among the variations leading to amino acid replacement, V551 and V588 were polymorphisms because they were also found in unrelated healthy controls. In contrast, the D626N variation identified in a proband patient of familial DCM (designated II-6; Fig. 2, A and C) was not found in 400 unrelated healthy controls. A family study showed that the D626N mutation was present in all affected members tested (Fig. 2, B and D). The mutation was located at the fifth position next to a constant cytosine (29) in the third LIM domain, and this position was occupied exclusively by acidic residues in Cypher/ZASP and other PDZ-LIM proteins and in Enigma and the Enigma homologue protein (ENH) from various species (Fig. 2E).
The patients in this family developed DCM after age 50 (in the early (II-1 and II-9) or late (II-5 and II-6) fifties in male cases and at age 69 in a female case (II-2)), suggesting that the mutation was associated with late-onset DCM (Fig. 2A). Electrocardiogram findings of the affected individuals demonstrated no primary conduction defect. It was interesting to note that a sister (II-4) had the mutation but did not suffer from DCM, although she had been affected with cerebellar ataxia (CA). The CA was initially considered to be a clinical consequence of her carrying the mutation; however, this possibility was ruled out because another brother (II-3) who also suffered from CA did not have the mutation. No sign of skeletal muscle involvement was noted in the DCM patients, although a muscle biopsy could not be performed because consent was not obtained.

**Altered Interaction of LIM-LIM and LIM-PKC Binding Due to the D626N Mutation**—Because the D626N mutation changes the acidic residue conserved in the PDZ-LIM protein...
family, we tested the functional alteration using Y2H assays. Wild type or mutant bait plasmid containing the third LIM domain of Cypher/ZASP (Cypher-CB or Cypher-MB, respectively) was co-transformed with a prey plasmid containing wild type Cypher/ZASP (Cypher-CP), mutant Cypher/ZASP (Cypher-MP), PKC-α (PKCA-P), PKC-ε (PKCE-P), or PKC-ζ (PKCZ-P) (Fig. 3A). In the test for LIM-LIM homodimeric interaction, the β-gal activity of colonies containing Cypher-CB and Cypher-CP was 1.447 ± 0.094, whereas that of Cypher-MB and Cypher CP was significantly low (0.714 ± 0.075, p < 0.001) (Fig. 3B). Similarly, β-gal activity in colonies of Cypher-CB and Cypher-MP was low (0.510 ± 0.024, p < 0.001) (data not shown).

On the other hand, the Y2H assays showed that the β-gal activity in the colonies of Cypher-MB and PKCA-P was significantly higher than that of Cypher-CB and PKCA-P (1.072 ± 0.108 versus 0.747 ± 0.094, p < 0.05). In addition, the β-gal activity obtained from the mutant LIM and PKC-ε interaction was significantly higher than that of the normal LIM and PKC-ε interaction (0.872 ± 0.054 versus 0.562 ± 0.036, p < 0.001). Similarly, the β-gal activity for the mutant LIM-PKC-ζ interaction was significantly higher than that for the normal
We investigated, through an independent approach, whether the mutation would affect the LIM-PKC interaction. In the pull-down experiments, a mutation equivalent to human D626N was introduced into mouse Cypher/ZASP (24). Western blot analysis of immunoprecipitates of wild type or mutant Cypher with HA-tagged PKCs (PKC-α, PKC-β, and PKC-ε) revealed that, despite equal expression of genes, mutant Cypher had a higher affinity to the PKCs than the normal Cypher (1.96 ± 0.03-fold, p < 0.01 for PKC-α; 1.38 ± 0.12-fold, p < 0.02 for PKC-β; and 1.50 ± 0.29-fold, p < 0.04 for PKC-ε) (Fig. 3, C–E).

**DISCUSSION**

In this study, a Cypher/ZASP mutation in the third LIM domain was found in a DCM family, and the mutation altered the function of the LIM domain (i.e. a decrease in dimeric binding while the binding to PKCs was increased). All affected members had the mutation, but one female carrying the mutation did not suffer from DCM (II-4, 65 years old). She might develop DCM later in life, because the DCM phenotype was...
late-onset and the eldest sister (II-2) developed the disease at the age of 69. Another reason why II-4 may not develop DCM might also be because she could not exercise for several years due to CA, since DCM due to the Z-disc abnormality can be exacerbated by cardiac stress, as demonstrated in MLP-deficient mice (13).

The Cypher/ZASP isoforms have a PDZ domain and three LIM domains. These structures are highly homologous to other PDZ-LIM proteins such as Enigma and ENH (30, 31). The ability of Cypher/ZASP isoforms to bind to α-actinin through the PDZ domain and various PKC subtypes via the LIM domains in the Z-discs of the cardiac muscle imply that Cypher/ZASP might play a role in stretch response. The LIM domain consists of 50–60 amino acid residues and participates in protein–protein interactions (29). The mutant LIM showed increased binding affinity to PKCs in both Y2H assays and pull-down assays, although the extent of the increased affinity was different between these assays. This could be because one human LIM domain was used in the Y2H assays, and three mouse LIM domains were used for the pull-down assays. Nevertheless, our results demonstrated that the interaction of the Cypher/ZASP LIM domain to PKC, especially the ε subtype, was augmented by the mutation.

PKCs localize in nucleus, perinucleus, cytosol, and Z-discs in the cardiomyocytes (32, 33). Upon activation by lipid-derived second messengers, PKCs are known to translocate from one cell component to another via the function of anchoring proteins, and the proteins anchoring the inactivated PKCs are referred to as RACK-like functions. On the other hand, it has been shown that the N-terminal part of PKC-ζ was augmented by the mutation.

REFERENCES

1. Grunig, E., Tasman, J. A., Kucherer, H., Franz, W., Kucher, W., and Katus, H. A. (1998) J. Am. Coll. Cardiol. 31, 186–194
2. Seidman, J. G., and Seidman, C. (2001) Cell 104, 557–567
3. Schonberger, J., and Seidman, C. E. (2001) Am. J. Hum. Genet. 69, 249–260
4. Olson, T. M., Michels, V. V., Thibodeau, S. N., Tai, Y. S., and Keating, M. T. (1998) Science 280, 750–752
5. Li, D., Tsatsopoulou, T., Gonzalez, O., Burch, P. E., Quinones, M. A., Zoghbi, W. A., Hill, R., Bachinski, L. L., Mann, D. L., and Roberts, R. B. (1999) Circulation 100, 461–464
6. Patkin, D., Madlao, C., Sasaki, T., Wolf, M. R., Porco, M., Frenneaux, M., Atherton, J., Vidaillot, H. J., Jr., Spadich, S., De Girolami, U., Seidman, J. G., Seidman, C., Muntoni, F., Muehle, G., Johnson, W., and McDonough, B. (1999) N. Engl. J. Med. 341, 1715–1725
7. Tsuha, S., Bowles, K. R., Vatta, M., Zintz, C., Titus, J., Muhonen, L., Bowles, N. E., and Towbin, J. A. (2000) J. Clin. Invest. 106, 655–662
8. Kamisago, M., Shabana, S. R., Solomon, S., Sharma, P., McDonough, B., Smoot, L., Mullin, M. P., Wolf, P. K., Wigle, E. D., Seidman, J. G., and Seidman, C. E. (2000) N. Engl. J. Med. 343, 1688–1698
9. Olson, T. M., Kishimoto, N. W., Whitby, F. G., and Michels, V. V. (2001) J. Mol. Cell. Cardiol. 33, 725–732
10. Olson, T. M., Illenberger, S., Kishimoto, N. Y., Huttelmaier, S., Keating, M. T., and Jockisch, M. B. (2002) Circulation 105, 431–437
11. Gerull, B., Gramlich, M., Atherton, J., McNab, M., Trombitas, K., Klaassen, S. S., Seidman, J. G., Seidman, C. G., Gruner, H., Labeit, S., Frenneaux, M., and Thierfelder, L. (2002) Nat. Genet. 30, 201–204
12. Ishikawa, M., Hayashi, T., Nishi, H., Koga, Y., Azuma, T., Koyanagi, T., Takahashi, M., Hohda, S., Ueda, K., Ueno, T., Hiroe, M., Marumo, F., Imai, T., Yasunami, M., and Kimura, A. (2000) Biochem. Biophys. Res. Commun. 281, 385–393
13. Knoll, R., Hoshijima, M., Hoffman, H. M., Person, V., Freilich, S., and Chien, K. R. (2002) Cell 111, 943–955
14. Schmitt, J. P., Kamisago, M., Asahi, M. Li, G. H., Ahmad, F., Mende, U., MacLennan, D. H., Seidman, J. G., and Seidman, C. E. (2003) Science 299, 1410–1413
15. Haghighi, R., Kolokatidis, F., Pater, L., Lynch, R. A., Asahi, M., Grimaldi, A. M., Fan, G. C., Tsapogas, K., Hahn, S. M., Hageman, G., Dorn, G. W., MacLennan, D. H., Kremastinos, D. T., and Kranias, E. G. (2003) J. Clin. Invest. 111, 869–876
16. Bowles, K. R., Gajarski, R., Porter, P., Gotya, V., Bachinski, L., Roberts, R., Pignatelli, A., and Towbin, J. A. (1996) J. Clin. Invest. 98, 1355–1360
17. Arber, S., Hunter, J. J., Ross, J. R., Hongo, M., Sanzio, G., Berg, J., Periard, J. C., Chien, K. R., and Caron, P. (1997) Cell 88, 393–403
18. Negri, V., Okazaki, Y., Belitto, A., Pihan, G., Matsuda, Y., Piana, L., Nigro, G., Ventura, C., Abbondanza, C., Molinari, A. M., Acanporo, D., Nishimura, M., Hayashisakirzaki, Y., and Puca, A. G. (1997) Hum. Mol. Genet. 6, 601–607
19. Pashmforoush, M., Pomies, P., Peterson, K. L., Kubalak, S., Ross, J. R., Hefi, A., Aebi, U., Beckerle, M. C., and Chien, K. R. (2001) Nat. Med. 7, 591–597
20. Towbin, J. A., and Bowles, N. E. (2000) Curr. Cardiol. Rep. 2, 475–480
21. Steinberg, S. F., Goldberg, M., and Rybin, V. B. (1998) J. Mol. Cell. Cardiol. 27, 141–153
22. Bowling, N., Walsh, R. A., Song, G., Eistridge, T., Sandusky, G. E., Fouts, R. L., Mintze, K., Pickard, T., Roden, R., Bristow, M., Acampora, D., Nishimura, J. L., Grom, G., King, R. L., and Vlahos, C. J. (1999) Circulation 99, 384–391
23. Jalili, T., Takeishi, Y., Song, G., Ball, N. A., Howles, G., and Walsh, R. A. (1999) Am. J. Physiol. 277, H2304–H2309
24. Zhao, Q., Ruiz-Lopez, P., Martone, M. E., and Chen, J. (1999) J. Biol. Chem. 274, 18907–18913
25. Paulkner, G., Pallavincini, A., Formentin, E., Comelli, A., Ievolella, C., Tresivian, S., Bortolotto, G., Scannappeo, P., Salamon, M., Volpe, A., and Landranchi, G. (1999) J. Clin. Cardiol. 146, 465–475
26. Pretorius, R., Richardson, J. A., and Olson, E. N. (2000) Mech. Dev. 89, 277–284
27. Huang, C., Zhou, Q., Liang, P., Hollander, M. S., Shekhi, F., Li, X., Greaser, M., Shelton, G. D., Evans, S., and Chen, J. (2003) J. Biol. Chem. 278, 7360–7365
28. Zhou, Q., Chu, P. H., Huang, C., Cheng, C. F., Martone, M. E., Knoll, G., Shelton, G. D., Evans, S., and Chen, J. (2001) J. Clin. Cell Biol. 155, 655–662
29. Sanchez-Garraca, I., and Rabbits, T. H. (1994) Trends Genet. 10, 315–320
30. Wu, Y. T., and Gill, G. N. (1994) J. Biol. Chem. 269, 25985–25996
31. Kuroda, S., Tokunaga, C., Kiyohara, Y., Higuchi, O., Kunihi, H., Mizuno, K., Gill, G. N., and Kikikawa, U. (1996) J. Biol. Chem. 271, 31029–31032
Cypher/ZASP Mutation in Dilated Cardiomyopathy

32. Mochly-Rosen, D., and Gordon, A. S. (1998) *FASEB J.* **12**, 35–42
33. Mackay, K., and Mochly-Rosen, D. (2001) *J. Mol. Cell. Cardiol.* **33**, 1301–1307
34. Robia, S. L., Ghanta, J., Robu, V. G., and Walker, J. W. (2001) *Biophys. J.* **80**, 2140–2151
35. Mochly-Rosen, D., Wu, G., Hahn, H., Osinska, H., Liron, T., Lorenz, J. N., Yatani, A., Robbins, J., and Dorn, G. W., II (2000) *Circ. Res.* **86**, 1173–1179
36. Pass, J. M., Zheng, Y., Wead, W. B., Zhang, J., Li, R. C., Belli, R., and Ping, P. (2001) *Am. J. Physiol. Heart Circ. Physiol.* **280**, H946–H955
37. Johnson, J. A., Gray, M. O., Chen, C. H., and Mochly-Rosen, D. (1996) *J. Biol. Chem.* **271**, 24862–24866
38. Liu, G. S., Cohen, M. V., Mochly-Rosen, D., and Downey, J. M. (1999) *J. Mol. Cell. Cardiol.* **31**, 1937–1948
A Cypher/ZASP Mutation Associated with Dilated Cardiomyopathy Alters the Binding Affinity to Protein Kinase C

Takuro Arimura, Takeharu Hayashi, Hajime Terada, Su-Yeoun Lee, Qiang Zhou, Megumi Takahashi, Kazuo Ueda, Tatsuhito Nouchi, Shigeru Hohda, Makoto Shibutani, Masao Hirose, Ju Chen, Jeong-Euy Park, Michio Yasunami, Hideharu Hayashi and Akinori Kimura

J. Biol. Chem. 2004, 279:6746-6752.
doi: 10.1074/jbc.M311849200 originally published online December 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M311849200

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 37 references, 13 of which can be accessed free at http://www.jbc.org/content/279/8/6746.full.html#ref-list-1