Loss-of-function and Dominant-negative Mechanisms Associated with Hepatocyte Nuclear Factor-1β Mutations in Familial Type 2 Diabetes Mellitus*

(Received for publication, November 25, 1998, and in revised form, February 19, 1999)

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Hepatocyte nuclear factor (HNF)-1β, a homeodomain-containing transcription factor, regulates gene expression in a dimerized form in pancreas, liver, and some other tissues. Recent genetic studies have identified two HNF-1β mutations, R177X and A263fsinsGG, in subjects with a monogenic form of type 2 diabetes. Despite the defects being in the same gene, diverse severities of disease are observed in the affected subjects. To investigate the molecular mechanism by which mutations might cause various phenotypic features, wild type and mutant proteins were transiently expressed in insulin-producing (MIN6) and hepatic (HepG2) cells. Luciferase reporter assay showed that both mutations resulted in a marked reduction of transactivation activity. Because their dimerization activity was found to be intact by the yeast two-hybrid system, it was possible that they were dominant-negative to wild type activity. When co-expressed with wild type, both of the mutants significantly decreased wild type activity in HepG2 cells. In contrast, although A263fsinsGG functioned similarly in MIN6 cells, R177X failed to affect wild type activity in this cell line. Immunohistochemical analysis of the mutations suggests that this functional divergence might be generated by the modification of nuclear localization. These results suggest that HNF-1β mutations may impair pancreatic β-cell function by loss-of-function and dominant-negative mechanisms.

Noninsulin-dependent (type 2) diabetes mellitus is a genetic disorder characterized by elevated plasma glucose levels due to an absolute or relative deficiency of insulin. Recent progress in modern genetics research makes precise localization of disease genes within the genome possible, and this is especially relevant in the study of monogenic diseases.

Maturity onset diabetes of the young (MODY)1 is a monogenic form of type 2 diabetes characterized by onset usually under 25 years of age and autosomal dominant inheritance (1). Genetic linkage studies first localized three genes responsible for the development of MODY on chromosomes 20, 7, and 12 (2–4). The third form of MODY was found to result from mutations in the gene encoding hepatocyte nuclear factor (HNF)-1α (5), a homeodomain-containing transcription factor (6–8). HNF-1α forms a homodimer or heterodimer with structurally related HNF-1β (9–11), and they function together to regulate gene expression in liver, pancreas, and some other tissues.

In this context, the HNF-1β gene was also screened for mutations in subjects with early onset type 2 diabetes/MODY, and two mutations of R177X and A263fsinsGG were found in two Japanese families (12, 13). HNF-1β is a protein of 557 amino acids comprising three functional domains: a dimerization domain (residues 1–32), a DNA-binding domain with a POU subdomain and a homeosubdomain (residues 88–178 and 229–299, respectively), and a transactivation domain (residues 314–557) (9–11). The R177X nonsense mutation generates a truncated protein of 176 amino acids with the N-terminal dimerization and POU domains. The A263fsinsGG frameshift mutation due to insertion of a GG dinucleotide also generates a truncated mutation of 264 amino acids that lacks a third helix structure at the C terminus of the homeodomain and the entire transactivation domain. Interestingly, although the same gene is mutated, differing clinical features including diverse severity and inconsistency of onset age of diabetes are observed in these families (12, 13). In this study, the molecular mechanisms by which mutations in the HNF-1β gene might cause diabetes and generate the diverse phenotypic features were addressed by functional analysis of the mutant proteins.

MATERIALS AND METHODS

Wild Type and Mutant Plasmid Constructs—The mutations were generated by polymerase chain reaction-based site-directed mutagenesis and cloned in pSP72 (Promega, Madison, WI) to generate the pSP-R177X and pSP-A263fsinsGG and also in the expression vector pCMV-6b to generate the pCMV-R177X and pCMV-A263fsinsGG. Wild type HNF-1β was also cloned in pSP72 and pCMV6b to generate pSP-WT and pCMV-WT, respectively. For verification of dimerization activity, yeast two-hybrid analysis (15) was performed to monitor interaction of the mutant protein with wild type. The entire coding region of the wild type and mutant proteins

* This study was supported by a Grant-in-Aid for Creative Basic Research from the Japanese Ministry of Science, Education, Sports and Culture and by the Uchida Memorial 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.

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∥ The abbreviations used are: MODY, maturity onset diabetes of the young; GLUT2, glucose transporter type 2; HNF, hepatocyte nuclear factor; HA, hemagglutinin; DOTAP, N-[1-(2,3-dioleoyloxypropyl)-N,N,N-trimethylammonium methylsulfate.
Functional Analysis of HNF-1β Mutations—To investigate the molecular mechanism by which HNF-1β mutations cause impaired glucose tolerance, transactivation activities of R177X and A263fsinsGG were analyzed in liver cell and pancreatic β-cell lines by luciferase reporter assay using the promoter of the human gene for GLUT2, which mediates facilitative glucose transport in these tissues. Wild type HNF-1β bound to the cis-element and efficiently increased reporter gene activity directed by transcription from the GLUT2 gene promoter in HepG2 and MIN6 cells (Figs. 1 and 2A). When the region from −1056 to −1026 containing the cis-element for binding was deleted from the promoter segment, the reporter activity was significantly reduced (85 ± 4%, p < 0.001). However, the deleted reporter construct still represented a significant increase of activity mediated by wild type HNF-1β, suggesting that GLUT2 gene expression could also be indirectly regulated by other transcription factors whose expression is up-regulated by HNF-1β. When the HNF-1β muta-

![Fig. 1. In vitro transcription and translation and electrophoretic mobility shift assay. A, pSP-WT, pSP-R177X, and pSP-A263fsinsGG were in vitro transcribed and translated in the presence of [35S]methionine. The products were separated on a 10% SDS-polyacrylamide gel. B, in vitro translated products were incubated with a reaction solution containing 32P-labeled DNA fragments. The binding complex was separated on a 5% nondenaturing polyacrylamide gel. The complex formation was inhibited by adding excess unlabeled oligonucleotide (1, 0.2, and 2 pmol), whereas the complex formation was not affected by addition of mutated oligonucleotides of 5'-ACCTCAGGTAA-GAGGAAACATCA-3'. In contrast, when R177X and A263fsinsGG were examined with the same oligonucleotides, no shift of binding complex was observed. C, interaction of A263fsinsGG with wild type. The DNA binding activity of wild type was significantly reduced when mutant proteins were added in the reaction.]

![Fig. 2. A, transactivation activity of wild type (WT) and mutant proteins. pGL3-GT2 and each test plasmid were cotransfected into MIN6 and HepG2 cells. The relative luciferase activity (Firefly/Renilla luciferase) of each construct at 0, 6, 12, and 24 ng for MIN6 cells and at 0, 24, 48, and 96 ng for HepG2 cells was measured by four independent experiments. Mean ± S.D. is shown. **, p < 0.01. B, co-expression of wild type and each mutant protein. pGL3-GT2, wild type, and each mutant plasmid were co-transfected into MIN6 and HepG2 cells. The effect of increasing amounts (12 and 24 ng for MIN6 cells and 24, 48, and 96 ng for HepG2 cells) of each test plasmid on the wild type activity was examined. The open bar on the left side indicates the endogenous transcription activity. The other bars indicate the activities of the WT (wild type) alone (open bar), WT + R177X (hatched bar), and WT + A263fsinsGG (filled bar). ***, p < 0.01.]

**RESULTS**

Transactivation Activity of Wild Type and Mutant HNF-1β Proteins—To investigate the molecular mechanism by which HNF-1β mutations cause impaired glucose tolerance, transactivation activities of R177X and A263fsinsGG were analyzed in liver cell and pancreatic β-cell lines by luciferase reporter assay using the promoter of the human gene for GLUT2, which mediates facilitative glucose transport in these tissues. Wild type HNF-1β bound to the cis-element and efficiently increased reporter gene activity directed by transcription from the GLUT2 gene promoter in HepG2 and MIN6 cells (Figs. 1 and 2A). When the region from −1056 to −1026 containing the cis-element for binding was deleted from the promoter segment, the reporter activity was significantly reduced (85 ± 4%, p < 0.001). However, the deleted reporter construct still represented a significant increase of activity mediated by wild type HNF-1β, suggesting that GLUT2 gene expression could also be indirectly regulated by other transcription factors whose expression is up-regulated by HNF-1β. When the HNF-1β muta-
Functional Analysis of HNF-1β Mutations

Dimerization activity of the HNF-1β mutants was examined using yeast two-hybrid system. – indicates no growth of cotransformants in the selection media (SD/-Trp/-Leu/His). + indicates the efficient growth of the cotransformants in the selection media and the generation of positive β-galactosidase activity. ± indicates the efficient growth of the cotransformants in the selection medium but extremely weak generation of β-galactosidase activity. pAS2–1 and pACT2 are bait and prey plasmid vectors, respectively. ND, not done.

| Prey     | pAS2–1 | HNF-1β | R177X | A263fsinsGG |
|----------|--------|--------|-------|------------|
| pACT2    | –      | –      | –     | –          |
| HNF-1β   | –      | +      | +     | +          |
| R177X    | –      | +      | ±     | ND         |
| A263fsinsGG | –    | +      | ND    | +          |

The modified localization of R177X within the cells was found to be differently patterned and markedly modified among the cells. In MIN6 cells, 90% of the cells transfected with R177X showed strong signals only in the cytoplasm, whereas the other 6 and 4% of the cells showed relatively weak signals in both nucleus and cytoplasm or only in the nucleus, respectively; 50–100 cells in two experiments were counted to estimate the frequency of each pattern. On the other hand, 92% of the transfected HepG2 cells showed strong staining in the nucleus (nucleus only, 19%; nucleus and cytoplasm, 73%). The other 8% of the cells showed only cytoplasmic staining. Although the frequency of nuclear or cytoplasmic staining varied to some extent in two experiments, the general patterns of intracellular localization were similar. The modified localization of R177X within the cells might be due to lack of a short stretch of basic residues (residues 229–237) in the POU domain, which has been suggested as a nuclear localization signal (16, 17).
DISCUSSION

Liver and pancreatic \(\beta\)-cells play a central role in regulation of glucose homeostasis by glucose uptake and disposal and insulin secretion, respectively, in response to the levels of plasma glucose. Because expression of the genes involved in such liver- and \(\beta\)-cell-specific function is regulated by a tissue-specific subset of transcription factors, molecular defects of these factors could lead to the development of impaired glucose tolerance.

In this study, the HNF-1\(\beta\) mutations of R177X and A263fsinsGG were found to have markedly reduced activity of transactivation of the human GLUT2 gene in liver or pancreatic \(\beta\)-cell lines. These results are consistent with the contribution of the mutations in the development of diabetes in patients with the mutation. Because a functional loss of GLUT2 has been suggested to be responsible for hyperglycemia and relative hypoinsulinemia in the fed state in patients with Fanconi-Bickel syndrome and early onset diabetes in mice lacking GLUT2 (18, 19), the decreased expression of GLUT2, which could be generated by HNF-1\(\beta\) mutations, might be involved in the pathogenesis of hyperglycemia in patients in concert with other target gene defects. In this regard, further identification of the target genes of HNF-1\(\beta\), which are expressed in pancreatic \(\beta\)-cells and liver, is important to better understand the pathogenesis of HNF-1\(\beta\)-deficient diabetes. The present study also shows that the two mutations function differently in pancreatic \(\beta\)-cells, possibly due to the difference of intracellular localization of the protein. Because the DNA binding activity of wild type is found to be reduced in the presence of A263fsinsGG (Fig. 1C), mutation in pancreatic \(\beta\) cells could interfere with the wild type regulation of target gene expression. Accordingly, the differing functional properties of the mutations in pancreatic \(\beta\)-cells may explain, at least in part, the differing severity of insulin secretion deficiency in the affected subjects. However, because only two mutations have so far been reported, further identification of families with the mutation will be necessary to understand to what extent these distinct molecular behaviors could account for differing clinical features in patients.

Because the primary structures of HNF-1\(\beta\) and HNF-1\(\alpha\), which recognize the same DNA sequence, are highly related and the tissues that express these proteins are mostly overlapped except lung and ovary (6–11), the structure and function relationships in HNF-1\(\beta\) may also apply to HNF-1\(\alpha\). The possible nuclear localization sequence of HNF-1\(\beta\), which is absent in R177X, is also found in HNF-1\(\alpha\) (residues 197–205), so the modification of nuclear targeting could be observed in HNF-1\(\alpha\) mutations involved in this region (20). Accordingly, functional analysis of the HNF-1\(\beta\) mutations should be helpful to clarify the molecular mechanism of impaired insulin secretion not only in HNF-1\(\beta\)-deficient diabetes but also in the related form of HNF-1\(\alpha\)-deficient diabetes.

Acknowledgment—We thank Dr. J. Miyazaki (Osaka University, Osaka, Japan) for providing MIN6 cells.

REFERENCES

1. Fajans, S. S. (1990) Diabetes Care \textbf{13}, 49–64
2. Bell, G. I., Xiang, K.-S., Newman, M. V., Wu, S.-H., Wright, L. G., Fajans, S. S., Spielman, R. S., and Cox, N. J. (1991) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{88}, 1484–1488
3. Froguel, P., Vaxillaire, M., Sun, F., Velho, G., Zouali, H., Butel, M. O., Lesage, S., Vinnot, N., Clement, K., Foquierousse, F., Tanizawa, Y., Weissenbach, J., Beckmann, J. S., Lathrop, G. M., Passa, P., Pernott, M. A., and Cohen, D. (1992) \textit{Nature} \textbf{356}, 162–164
4. Vaxillaire, M., Bocco, V., Philippi, A., Vigouroux, C., Terwilliger, J., Passa, P., Beckmann, J. S., Velho, G., Lathrop, G. M., and Froguel, P. (1995) \textit{Nat. Genet.} \textbf{9}, 418–423
5. Yamagata, K., Oda, K., Kaisaki, P. J., Menzel, S., Furuta, H., Vaxillaire, M., Cox, R. D., Lathrop, G. M., Bosiraj, V. V., Chen, X., Cox, N. J., Oda, Y., Yano, H., LeBeau, M. M., Yamada, S., Nishigori, H., Takeda, J., Chevre, J.-C., Fajans, S. S., Hattersley, A. T., Iwasa, N., Pedersen, O., Polonsky, K. S., Turner, R. C., Froguel, P., and Bell, G. I. (1996) \textit{Nature} \textbf{384}, 455–458
6. Frain, M., Swart, G., Monaci, P., Nicosia, A., Stampfli, S., Frank, R., and Cortese, R. (1989) \textit{Cell} \textbf{59}, 145–157
7. Tronche, F., and Yaniv, M. (1992) \textit{BioEssays} \textbf{14}, 579–589
8. Mendel, D. B., and Crabtree, G. R. (1991) \textit{J. Biol. Chem.} \textbf{266}, 677–680
9. Mendel, D. B., Hansen, L. P., Graves, M. K., Conley, P. B., and Crabtree, G. R. (1991) \textit{Genes Dev.} \textbf{5}, 1042–1056
10. Rey-Campos, J., Chouard, T., and Yaniv, M. (1991) \textit{EMBO J.} \textbf{10}, 1445–1457
11. De Simone, De Magistris, L., Lazzaro, D., Gerstner, J., Monaci, P., Nicosia, A., and Cortese, R. (1991) \textit{EMBO J.} \textbf{10}, 1435–1443
12. Horikawa, Y., Iwasa, N., Hara, M., Furuta, H., Hinokio, Y., Cockburn, B. N., Linder, T., Yamagata, K., Ogata, M., Tomonaga, O., Kuroki, H., Kasahara, T., Iwamoto, Y., and Bell, G. I. (1997) \textit{Nat. Genet.} \textbf{17}, 384–385
13. Nishigori, H., Yamada, S., Kohama, T., Tomura, H., Sho, K., Horikawa, Y., Bell, G. I., Takeuchi, T., and Takeda, J. (1998) \textit{Diabetes} \textbf{47}, 1354–1355
14. Takeda, J., Kayano, T., Fukumoto, H., and Bell, G. I. (1998) \textit{Diabetes} \textbf{47}, 773–777
15. Field, S., and Song, O. (1989) \textit{Nature} \textbf{340}, 245–247
16. Treisman, J., Goncey, P., Vashisthata, M., Harris, E., and Desplan, C. (1989) \textit{Cell} \textbf{59}, 553–562
17. Dingwall, C., and Laskey R. A. (1986) \textit{Annu. Rev. Cell Biol.} \textbf{2}, 367–390
18. Santer, R., Schneppenheim, R., Dombrowski, A., Gotze, H., Steinmann, B., and Schaub, J. (1997) \textit{Nat. Genet.} \textbf{17}, 324–326
19. Guillam, M.-T., Hummler, E., Schaerer, E., Wu, J.-Y., Birnbauer, M. J., Beer- mann, F., Schmidt, A., Deriaz, N., and Thorens, B. (1997) \textit{Nat. Genet.} \textbf{17}, 327–330
20. Yamada, S., Tomura, H., Nishigori, H., Sho, K., Mabe, H., Iwatain, N., Takumi, T., Kito, Y., Moriya, N., Muroya, K., Ogata, T., Ogijata, K., Morikawa, A., Inoue, I., and Takeda, J. (1999) \textit{Diabetes} \textbf{48}, 645–648