BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 806–810 (1997)
ARTICLE NO. RC977352

Immunochemical Characterization of a Novel Mitochondrially Located Protein Encoded by a Nuclear Gene within the DFNB8/10 Critical Region on 21q22.3

Kai Krohn,*,† Vladimir Ovod,* Pekka Vilja,* Maarit Heino,* Hamish Scott,‡ Despina S. Kyriakou,‡ Stylianos Antonarakis,‡,§ Howard T. Jacobs,* Jorma Isola,*,† and Pärt Peterson*

**Institute of Medical Technology (IMT), University of Tampere, and †Tampere University Hospital, Tampere, Finland; and ‡Laboratory of Human Molecular Genetics, Department of Genetics and Microbiology, University of Geneva Medical School and §Division of Medical Genetics, Cantonal Hospital of Geneva, 1211 Geneva 4, Switzerland*

Received August 7, 1997

A novel protein encoded by the C21ORF2 gene in chromosomal locus 21q22.3 was characterized by immunochemistry. This chromosomal region is known to contain genes for human diseases such as non-syndromic autosomal recessive deafness (DFNB8/10) and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). Polyclonal murine antisera were produced against the multivalent peptides deduced from the amino acid sequence of the polypeptide. Immunological reactivity of the obtained antisera was tested with primary cells or established cell lines. On western blotting, the polyclonal sera recognized a single protein product of 25 Kd expressed in cell lines of epithelial and lymphoid origin. Subsequent immunochemistry of several human tissues indicated the ubiquitous expression of the protein. Immunofluorescence studies and co-staining with a mitochondrial-specific dye suggest the subcellular localization of the protein to mitochondria. Mitochondrial localization is also predicted by computer analysis of the polypeptide sequence. As deafness is known to be caused in some instances by defects in mitochondrial function, C21ORF2 is a plausible candidate gene for DFNB8/10.

Key Words: **chromosome 21; mitochondria; deafness; immunohistochemistry.**

The terminal part of human chromosome 21, between MX1 and the 21q telomere (HC21q22.3) contains yet unidentified genes linked to hereditary diseases such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, OMIM#240300) (2, 3, 6) well as gene(s) for one (or two) form(s) of non-syndromic hereditary deafness (OMIM#601072) (4, 5).

Linkage analysis of a large Pakistan kindred with nonsyndromic deafness mapped the autosomal recessive deafness gene designated DFNB8 between the markers D21S212 and D21S1225 (4). Analysis of a similar disorder in a large Palestinian family presented evidence for a defective gene (DFNB10) close to the marker D21S1259 (5). It thus remains unclear whether there are one or perhaps two autosomal recessive deafness genes in this region.

We have recently characterized the genomic structure and cDNA sequence of the gene C21ORF2 from this critical region, located telomericly from PFKL. In the present work, we have characterized the expression of the C21ORF2 protein product, using an antiserum which was produced by immunizing mice with synthetic multiple antigen peptides (MAP) that represented predicted immunogenic B cell epitopes. Our results demonstrate that the HC21ORF2 product is a novel protein with ubiquitous expression, that appears to be targeted to mitochondria. Since mutations in components of the mitochondrial genetic system have been implicated in hearing impairment (7, 8, 9, reviewed in 10) as well as in a mechanosensory defect in an animal model (16), C21ORF2 would appear to be a plausible candidate for the non-syndromic deafness gene(s) DFNB8/10.

MATERIALS AND METHODS

Peptide synthesis. Six peptides (Table 1) were chosen according to the antigenicity prediction by the Pepsort program (GCG package, University of Wisconsin, USA). Each peptide was synthesized onto a branched lysine core (Fmoc8-Lys4-Lys2-Lys-betaAla-Wang resin, Calbiochem-Novabiochem, La Jolla, CA, USA) resulting in an octameric multiple antigen peptide (MAP) (11, 12). Syntheses were performed by Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on a simultaneous multiple peptide synthesizer (SMPS 350, Zinsser Analytic, Frankfurt, Germany). Purity of MAPs was analyzed by reversed-phase HPLC (System Gold, Beckman Instruments Inc, Fullerton, CA, USA).

0006-291X/97 $25.00

Production of murine polyclonal antibodies. Eight-week-old Balb/c mice were immunised with an intraperitoneal injection of 25 mg of each peptide in 0.4 ml of a 1:1 mixture of Freund's Complete Adjuvant (Difco Laboratories, Detroit, MI, USA) and physiological saline (NaCl, 0.15M). One month later the animals were boosted with an intramuscular injection of 35 mg of antigens in Freund's incomplete adjuvant and saline (1:1) (0.2 ml were distributed into four sites). Three weeks later the peptides in a dose of 50 mg/mouse were administrated intravenously and sera were obtained 7 days later. For analysis of polyclonal sera, microtitre ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with peptides (1 mg/well in PBS, pH 7.5) at 4 oC overnight and blocked with 2% of BSA in PBS. The plates were then incubated with titrated mouse immune and normal (control) sera at room temperature for 4 h. Finally the bound peptide-specific antibodies were detected by using anti-mouse HRP-labelled immunoglobulins (Dako A/S, Denmark) essentially as previously described (13).

Production of EBV transformed B-cell lines. Peripheral blood leukocytes were obtained from 5 healthy control persons. The B-cells were transformed with Epstein-Barr virus and the cell lines were maintained in RPMI 1640, supplemented with 10% FCS (Gibco, Life Technologies, Paisley, UK).

Western blotting and immunofluorescence. EBV transformed cells from APECED patients or from control subjects, as well as established T cells (H9, MT4) and HeLa cells were boiled for 10 minutes in 2× sample buffer (one milion cells/100 microliter of buffer) and analysed by western blotting as described (15). For immunofluorescence, cytospin preparations were produced from the EBV blasts (150 000 cells/slide). These were air-dried at room temperature, permeabilized with Saponine (Permeabilization solution A, Epithelial cell detection kit, Epimet*, Micromet Gmbh, Planegg, Germany), fixed for 15 minutes with 5% buffered formaldehyde, rinsed in PBS and stained with the mice antisera. The dilution of primary serum was 1:1000. Biotinylated anti mouse IgG in 1:500, followed by Avidin-Fluorescein (Vector laboratories Inc, Burlingame, CA, USA) in 1:100 dilution, was used to demonstrate bound antibodies. The stained preparerates were mounted with Vectashield mounting medium (Vector laboratories) containing DAPI (4′,6-diamidine-2-phenylindole, Sigma, St. Louis, MO, USA) for nuclear staining, and viewed with a Zeiss Axioplan 2 epifluorescence microscope. Digital images were captured using Hamamatsu model C5985 "cooled CCD video camera."

Mitochondrial localization assay. EBV transformed B-cells were pre-stained in vitro with Mitotracker Red CMXRos (Molecular Probes Inc., Eugene, OR, USA) and washed twice with Hank's. Cytospin preparations of the pre-stained cells were then stained with the mouse anti-C21ORF2 serum as described above.

Sequence analysis. The predicted subcellular localization of the C21ORF2 polypeptide (Genbank Accession U84569) was deduced using the program PSORT (http://psort.nibb.ac.jp/) (14). BLAST searches of the public databases used the facilities provided by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/index.html).

RESULTS

Predicted subcellular localization of the C21ORF2 polypeptide. Computer prediction of the subcellular compartment to which the C21ORF2 polypeptide is targeted strongly indicates that the protein is mitochondrial. The compartments giving the top four scores on PSORT analysis were all mitochondrial, with the highest probability score of 0.636 for matrix targeting. Polypeptides independently demonstrated to be mitochondrially targeted typically show scores in this range, both for yeast and metazoans (L. A. Grivell, pers. comm.). The predicted boundary of the mitochondrial targeting sequence is at residue 31, with the consensus arginine residue at position -2 (see Figure 1). A splice variant of the gene has also been reported from cDNA sequencing (Genbank U84570), which creates a polypeptide lacking the initial 39 amino acids.

PSORT analysis suggests this may also be targeted to the mitochondrial matrix, but with a lower probability score of 0.485. The location of an intron at or close to the boundary of the mitochondrial targeting sequence is an almost universal feature of nuclear-encoded mitochondrial proteins. BLAST searches revealed no convincing similarities with any functionally identified polypeptide, although weak, possible matches to unidentified proteins in various species were revealed. Since these were generally polypeptides of different sizes, none is likely to be the orthologue of C21ORF2. Eight reported human ESTs from a variety of tissues appear to correspond with this gene.

```
                .         .         .         .         .         .
1   MKLTRKMVLTRAKASELHSVRKLNCWGSRLTDISICQEMPSLEVITLSVNSISTLEPVSR   60
                                              |--> splice variant A2
                .         .         .         .         .         .
61  CQRLSELYLRRNRIPSLAELFYLKGLPRLRVLWLAENPCCGTSPHRYRMTVLRTLPRLQK  120

                .         .         .         .         .         .
121 LDNQAVTEEELSRALSEGEEITAAPEREGTGHGGPKLCCTLSSLSSAAETGRDPLDSEEE  180

                .         .         .         .         .         .
181 ATSGAQDERGLKPPSRGQFPSLSARDASSSHRGRNVLTAILLLLRELDAEGLEAVQQTVG  240

                .
241 SRLQALRGEEVQEHAE
```

FIG. 1. Amino acid sequence of the predicted polypeptide encoded by C21ORF2, showing the amino-terminal mitochondrial targeting sequence predicted by PSORT (bold, underlined). The start of the polypeptide encoded by splice variant A2 (Genbank Accession U84570) is as shown. Based on sequences in the database, the A2 polypeptide also lacks the amino acid SVN (underlined). The sequences from where the synthetic peptides were selected are marked with a dotted line.

TABLE 1

Sequence of the Peptides Used to Immunize Mice # 1-6 and Their Reactivity with the Peptide Used for Immunization in ELISA and with EBV Transformed B-Cells in Western Blotting

Mouse #	Peptide	ELISA	Western blot
1	CQRLSELYLRRNRI2.2	2.2	+
2	ITAAPEREGTGHGGP	<0.1	+
3	TGRDPLDSEEEATS	<0.1	−
4	SEEEATSGAQDERGL	<0.1	−
5	AQDERGLKPPSRGQ	0.8	−
6	LSARDASSSHRGRN	0.5	−

Antiserum against C21ORF2 gene product. We produced five branched polyvalent 16-20 mer peptides, linked to the poly-lysine core and used these for immunization of mice. In ELISA assay towards the immunogenic peptides, one mouse gave a strong and two gave weaker reactivity towards the peptide used for immunization (Table 1). In Western blotting, using either established T and B cell line or peripheral blood leukocytes, a strong band of 25 kD molecular weight was seen in activated T and B cell lines (Figure 2, lanes 2-4, 6) while a fainter reaction was observed with nonactivated PBLs (Figure 2, lanes 1, 5 and 12). The 25 kD protein band was also expressed in established HeLa and Cos-1 cells (Figure 2, lanes 7 and 8).

The intensity of the 25 kD protein band clearly increased T-lymphocytes, activated by PHA stimulation (Figure 2, lanes 9 - 11).

Immunofluorescence. EBV-transformed B cell line, when stained with sera from mice 1 and 2, showed a strong punctate cytoplasmic fluorescence, located most intensively in the perinuclear zone (Figure 3). No reaction in the nucleus was observed. This pattern is typical of mitochondria in a wide variety of cell types. Co-staining with the mitochrondial-specific dye Mitotracker Red confirmed that the C21ORF2 protein is localized in the mitochondria (Figure 4A and 4B).

Immunofluorence analysis of tissue sections with the anti-C21ORF2 antisera revealed strong cytoplamic staining in most adult organs, aspecially in heart, sceletal muscle, liver, kidney and pancreas.

DISCUSSION

In the present work, we analysed the expression and localisation of a hitherto unknown gene product (C21ORF2) encoded on human chromosome 21q22.3, recently cloned from this region which is known to contain genes for several hereditary diseases. A highly potent antiserum was generated by immunizing mice with polyvalent, branched peptides, predicted to be antigenic and to represent various parts of the putative protein encoded by C21ORF2.

The reactive antisera generated detected a protein that was expressed in a variety of primary cells or cell lines of epithelial (HeLa) and lymphoid (B- and T-cell lines) origin. Interestingly, a protein with similar molecular weight was also present in the COS-1 cells from African Green Monkey. The 25 kD size of the protein in Western blotting corresponds approximately with the predicted length of the coding region of C21ORF2 after intra-mitochondrial processing as inferred from PSORT analysis. The cDNA contains an ORF of 786 bp encoding a polypeptide of 256 amino acids (Fig. 1 and Scott et al., manuscript submitted), 225 of which would be in the predicted mature polypeptide. It is noteworthly, that we did not have an antiserum that would have reacted with the the first 31 amino acids predicted to represent the mitochondrial targeting sequence. By both computer prediction and immunohistochemistry, the localization of this newly detected protein was mitochondrial. The data thus provide strong support for the inference that the chromosomal gene C21ORF2 codes for a mitochondrial protein that is ubiquitously expressed in human cells. BLAST

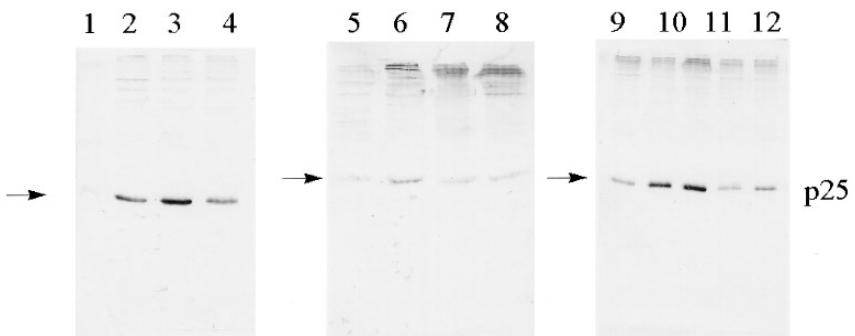

FIG. 2. Western blotting of primary lymphoid cells and established cell lines with antiserum from mouse # 1. Lane 1, peripheral blood leukocytes; lane 2, EBV transformed B-cells; lane 3, T-cell line H-9; lane 4, HTLV-1 infected T-cell line MT-4; lane 5, peripheral blood leukocytes; lane 6, EBV transformed B-cells; lane 7, HeLa cell line; lane 8, Cos-1 cells; lanes 9 - 11, peripheral blood leukocytes stimulated with PHA at days 1, 2, and 3, respectively; lane 12, unstimulated peripheral blood leukocytes.

searches revealed no convincing similarity with any functionally identified polypeptide in any species. Therefore, the function of this protein inside mitochondria remains completely unknown.

The finding of ubiquitous expression of C21ORF2 in several human tissues is in good correlation with the Northern blot data. The mRNA of C21ORF2 was found in all 16 adult and four fetal tissues studied, including heart, brain, sceletal muscle and pancreas (15), known to be a rich source for mitochondria. The Northern blot analysis showed the presence of two alternatively spliced transcripts 1.1 and 2.2 kb due to two separate polyadenylation sites in 3′UTR. Our western blotting results are consistent with a single major protein product of this gene, although the shorter polypeptide produced from the splice variant would be almost identical in size and structure with the putatively cleaved product of the mitochondrial import machinery.

As the localisation of C21ORF2, close to the gene for PFKL, suggested that it might be the defective gene in APECED, we have previously studied the coding region from patient and healthy control and found that they do not differ (Scott et al, manuscript submitted). C21ORF2 is therefore unlikely to be the APECED gene. Other hereditary diseases linked to the 21q22.3 region include two forms of non-syndromic deafness (DFNB8 and DFNB10). Hereditary deafness is often caused by mutations in genes coded by mitochondrial DNA. However, most mitochondrial proteins, including all of those that function as components of the mitochondrial genetic apparatus, are encoded by nuclear genes and mutations in these are also candidates in deafness (10). Although the function of its gene product remains unknown, C21ORF2 is a strong candidate gene in this disorder. The availability of a potent and specific antiserum, capable of detecting differences of the expression pattern of C21ORF2 should facilitate the functional analysis of C21ORF2 and its role in DFNB8/10 or other hereditary diseases.

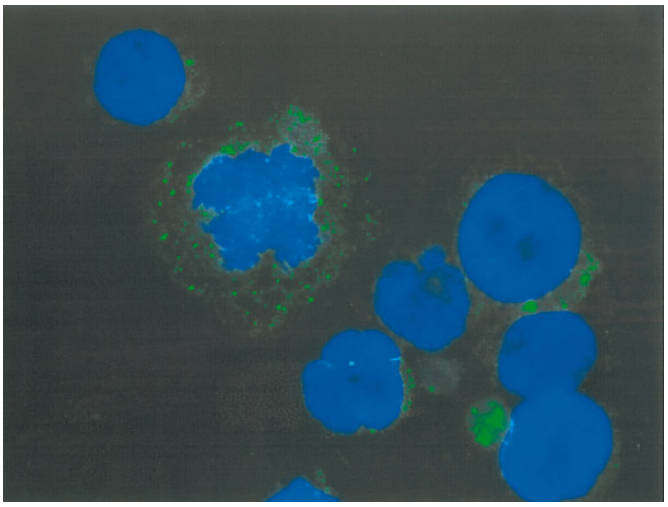

FIG. 3. Immunofluorescence of EBV transformed B-cells with mouse serum # 1. Note the granular staining of mitochondria. Primary magnification 600×.

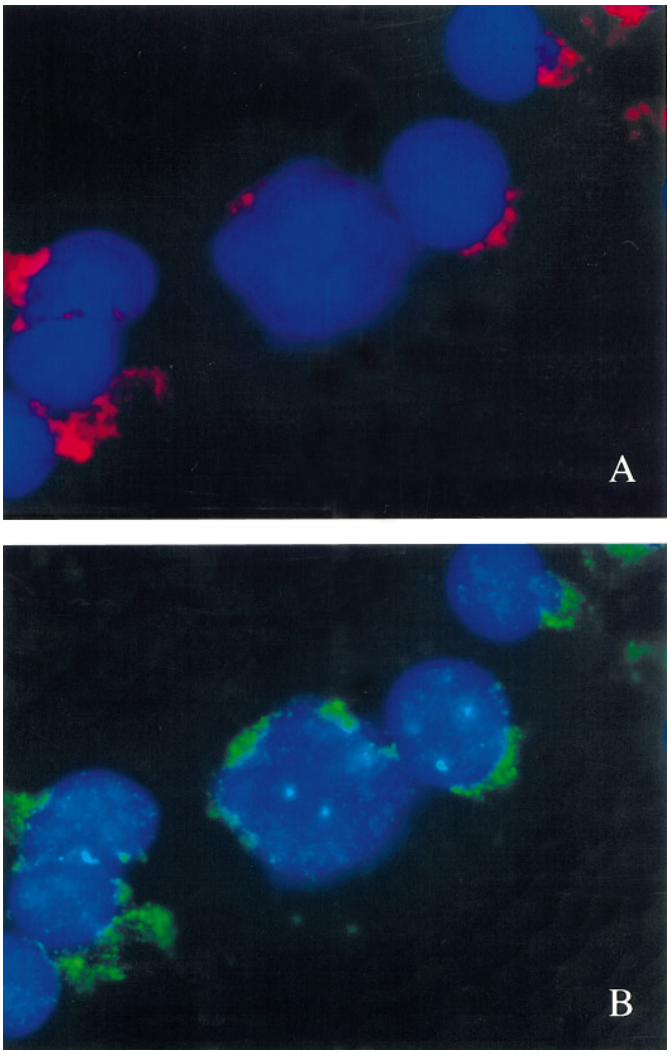

FIG. 4. Immunofluorescence of EBV transformed B-cells. A depicts the red fluorescence by the mitochondrial dye Mitotracker Red CMXRos. In B, the same preparate is viewed under green fluorescence, demonstrating the binding of antibodies to the C21ORF2 protein. Primary magnification 600×.

ACKNOWLEDGMENTS

This study is partly supported by EU Biomed2 programme CA action grants to K.K. and P.P. (BNH4-CT95-0729) and to H.T.J. (BMH4-CT96-0353). K.K., P.P., H.T.J., and M.H. are also supported by grants from Tampere University Hospital Medical Research Fund. H.S.S. is supported by a C. J. Martin Fellowship from the National Health and Medical Research Council of Australia (957348). This study was supported by grants from the Swiss FNRS 31.33965.92 and 31-40500.94 and the European Union/OFES CT93-0015 and funds from the University and Cantonal Hospital of Geneva.

REFERENCES

1. Lehesjoki, A. E., Koskiniemi, M., Norio, R., *et al.* (1993) *Hum. Mol. Genet.* **2,** 1229–1234.
2. Ahonen, P., Myllrniemi, S., Sipil, I., and Perheentupa, J. (1990) *N. Engl. J. Med.* **322,** 1829–1836.
3. Aaltonen, J., Bjorses, P., Sandkuijl, L., Perheentupa, J., and Peltonen, L. (1994) *Nature Genet.* **8,** 83–87.
4. Veske, A., Oehlmann, R., Younus, F., *et al.* (1996) *Hum. Mol. Genet.* **5,** 165–168.
5. Bonne-Tamir, B., DeStefano, A. L., Briggs, C. E., *et al.* (1996) *Am. J. Hum. Genet.* **58,** 1254–1259.
6. Björses, P., Aaltonen, J., Vikman, A., *et al.* (1996) *Am. J. Hum. Genet.* **59**(4), 879–886.
7. Van den Ouweland, J. M. W., Lemkes, H. H. P., and Ruitenbeek, W. (1992) *Nat. Genet.* **1,** 368–371.
8. Prezant, T. R., Agapian, J. V., Bohlman, M. C., *et al.* (1993) *Nat. Genet.* **4,** 289–294.
9. Reid, F. M., Vernham, G. A., and Jacobs, H. T. (1994) *Hum. Mutation.* **3,** 243–247.
10. Jacobs, H. T. (1997) Mitochondrial deafness. *Ann. Med.* (in press)
11. Tam, J. P. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5409–5413.
12. Adermann, K., Vilja, P., Kuhn, M., Austermann, S., and Forssmann, W.-G. (1994) *in* Innovation and Perspectives in Solid Phase Synthesis, Biological and Biomedical Applications, (Epton, R., Ed.), pp. 429–432, Mayflower Worldwide, Birmingham.
13. Ovod, V., Lagerstedt, A., Ranki, A., *et al.* (1992) *AIDS* **6**(1), 25–34.
14. Nakai, K., and Kanehisa, M. (1992) *Genomics* **14,** 897–911.
15. Scott, H. S., Kyriakoua, D. S., Peterson, P., *et al.* (1997) Submitted.
16. Shah, Z. H., O'Dell, K., Miller, S. C. M., An, X., and Jacobs, H. T. (1997) Submitted.