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Article in Journal of Biological Chemistry · May 2001
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Novel Frameshift Mutations near Short Simple Repeats*

Received for publication, December 7, 2000, and in revised form, December 26, 2000
Published, JBC Papers in Press, January 3, 2001, DOI 10.1074/jbc.M011040200

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In patients with Alzheimer’s disease or Down’s syndrome, the cerebellar cortex exhibits protein deposits in neurofibrillar tangles and neuritic plaques. Recently, the deposits have been shown to contain protein fragments of ubiquitin-B and amyloid precursor protein (APP) with an aberrant carboxyl terminus resulting from frameshift mutations (dinucleotide deletions, ΔG, ΔA, or ΔGA) in or adjacent to GAGAG motifs in their mRNAs, a process referred to as molecular misreading. We have now used a bacterial expression system with the green fluorescent protein as a reporter to screen gene transcripts from aged controls, Alzheimer’s disease, and Down’s syndrome for molecular misreading. Novel frameshift mutations at a number of locations in the transcripts of the ubiquitin-B and APP genes were discovered (ΔGA, ΔG, ΔGU, ΔGG, ΔCA, ΔAU, ΔA, ΔAA, ΔC, ΔU, and insertion of an A). Interestingly, most mutations were in close proximity of short simple repeats (GAGAG, GGUGGU, GAGACACACA, UCAUCAUCA, CAAACAAA, and GAGAAGAGA), demonstrating that the GAGAG motif does not constitute the only hot spot for transcriptional errors. Unlike the previously detected aberrant APP fragments, some of the novel ones have the potential to generate the neurotoxic peptide β-amyloid. We conclude that during aging molecular misreading is a widespread phenomenon.

Alzheimer’s disease (AD) is a neurodegenerative disorder of the brain and accounts for the most frequent form of dementia at higher age. The disease is characterized by the presence of neurofibrillar tangles, neuritip hillocks, and neuritic plaques in the brain. A major constituent of the plaques is β-amyloid, a neurotoxic peptide generated by processing of the type I transmembrane β-amyloid precursor protein APP (1). Two different types of AD can be discriminated, namely the early-onset (familial) and the late-onset (sporadic) forms. The sporadic form accounts for more than 60% of all AD cases, but the cause of the development of these cases is unknown. A recent study provided a new view on the mechanism that may underly aging and that in particular may contribute to the development of the sporadic forms of AD and Down’s syndrome (2). The new view is referred to as molecular misreading and is based on the finding of a novel type of dinucleotide deletion in or adjacent to a GAGAG motif in the mRNA transcripts of neuronal genes encoding APP and the cytoplasmic garbage protein ubiquitin-B (Ubi-B) (2). These mutations were found at the RNA and not the DNA level and resulted in frameshifts and proteins with an aberrant carboxyl terminus (2–4). The same type of frameshift mutations may underlie other neuropathologies and age-related diseases.

To examine how widespread the phenomenon of molecular misreading is and whether hot spots other than GAGAG may occur, we have developed a bacterial expression system with the green fluorescent protein (GFP) as a reporter and successfully used this system for the screening of frameshift mutations in mRNAs even when the mutational rate is low. We discovered novel frameshift mutations at different sites in Ubi-B and APP gene transcripts isolated from cortical regions of aged individuals.

EXPERIMENTAL PROCEDURES

Isolation of RNA—Using a standard Trizol procedure (Life Technologies, Inc.), total RNA was extracted from cortical regions of aged individuals that had suffered from AD or DS (patients 92-080 (DS, 58), 93-048 (DS, 67), 92-50-529 (control, 96), 92-50-692 (control with plaques, 85), 94-52-312 (control, 85), 95-50-665 (control, 84), 88-50-322 (AD, 85), 88-50-120 (AD, 83), 92-51-371 (AD, 81), 95-51-242 (AD, 79), and 95-51-014 (AD, 81)) were obtained from the Department of Pathology, University of Nijmegen, The Netherlands.

RT-PCR—Single stranded cDNA was synthesized by using 2 μg of total RNA, randomly primed with hexamers and reverse transcribed with RT Superscript II (Life Technologies, Inc.). The PCR was performed with PWO DNA polymerase (proofreading activity; Roche Molecular Biochemicals). Initially, we experienced a technical problem in that a substantial percentage (~1%) of the synthesized primers were designed to contain a deletion or an insertion of a nucleotide. Therefore, primers (Biologiek, Malden, The Netherlands) were purified by SDS-PAGE to diminish background signals caused by the mistakes made during primer synthesis. The primers used for Ubi-B mRNA analysis were 5'-gggggggaatctcagctgtaaaagt-3' as forward and 5'-gggggctagttcagctgtaaaagt-3' as reverse primer (255-bp PCR product), and for APP mRNA analysis, the following primer sets were used: APP-CT2 with 5'-gcttggctagctgtaaaagt-3' as reverse primer (112 bp product); APP-CT1 with 5'-gcttggctagctgtaaaagt-3' as reverse primer (141 bp product); 5'-gggggtctagattctgcatctgctcaaaagac-3' as reverse primer (242-bp product); APP-BA with 5'-gcttggctagctgtaaaagt-3' as reverse primer (255-bp PCR product); APP-BA with 5'-gcttggctagctgtaaaagt-3' as reverse primer (242-bp product); APP-BA with 5'-gcttggctagctgtaaaagt-3' as reverse primer (255-bp PCR product); APP-CT with 5'-gcttggctagctgtaaaagt-3' as reverse primer (242-bp product); and APP-CT with 5'-gcttggctagctgtaaaagt-3' as reverse primer (255-bp PCR product). Sequencing Strategy—PCR products were gel-purified, digested with HindIII and XbaI, and subcloned into the bacterial vector pGFPuv (6079–1; CLONTECH; GenBank™ accession number U62636; see Fig. 1). DNA was isolated from selected clones (Flexiprep kit, Amersham Pharmacia Biotech), and insert sizes were determined. During the development of this method we experienced a background signal caused by internal initiation of translation at the first AUG of the GFP coding region. To prevent this background signal, this AUG was removed via PCR with 5' primer gggtgctagctgtaaaagtggggcagctgtaaaagt-3' and 3' primer ctcctggctagctgtaaaagtggggctagctgtaaaagt-3' as reverse primer (APP-CT2; 112 bp product).
struct is referred to as pGFPuv and was used for the cloning of the PCR products to identify the frameshift mutations. DNA sequence analysis was performed according to the manufacturer’s protocol (ABI Prism 310; PerkinElmer Life Sciences). The screening strategy provides different ways to detect frameshift mutations. The Ubi-B primers were designed in such a way that clones containing a wild type insert would show no fluorescence, whereas clones containing an insert with a dinucleotide deletion would generate weak fluorescence. By changing the 3' primer one could also search for deletions of one nucleotide. For the detection of mutations in APP gene transcripts, the primers were designed in such a way that the wild type APP is in frame with GFP, whereas a frameshift would lead to nonfluorescent bacteria. However, one of the wild type APP PCR fragments (generated with primers APP-BA1 and APP-BA2) was found to result in a nonfluorescent GFP fusion protein because of the size of the insert. Surprisingly, in this case a number of fluorescent bacteria were still detected, and sequencing of these clones revealed the presence of APP frameshift mutations. These frameshifts caused an early termination of translation, and it is likely that reinitiation of translation occurred to generate a fluorescent GFP fusion protein.

RESULTS AND DISCUSSION

Age-related frameshift mutations in gene transcripts have been shown to occur in Brattleboro rats (5) and in patients who had suffered from AD or DS (2). These frameshift mutations are generated at the RNA and not the DNA level, and this process has been referred to as molecular misreading (6).
frameshifts were detected with an immunoscreening procedure implying the need for a specific antibody for each mutation to be investigated. To circumvent the necessity for the generation of an antibody for the detection of any new mutation, we developed a bacterial expression cloning strategy involving the use of GFP as a reporter protein to screen PCR fragments up to 300 nucleotides in length for the presence of frameshift mutations (see “Experimental Procedures”). To examine the sensitivity of the method and to show that the mutations are not generated as a result of a PCR artifact, constructs containing either wild type Ubi-B or Ubi-B with the dinucleotide deletion previously described (ΔGU; Ubi-B-Δ; see Ref. 2) were used in different ratios ranging from 1:1 to 1:10000 (wild type:ΔGU). Even at a ratio of 1:10000 the (weakly) fluorescent clones containing the frameshift mutation could still be detected at the expected frequency. Subsequently, the validity of the procedure was checked by searching for a frameshift that is known to occur in Ubi-B gene transcripts isolated from human DS brain tissue (2). A GU deletion following a GAGAG motif in the Ubi-B transcript identical to the one reported earlier (2) was indeed found (Table I). Because the PCR primers corresponded to regions located in separate exons of the Ubi-B gene, the size of the PCR fragment (255 bp) revealed that the mutations were detected at the RNA and not the DNA level. Interestingly, in addition to the ΔGU, we discovered several novel mutations (ΔGA, ΔG, AGG, and insertion of an A) (Table I). These novel mutations were also located adjacent to the GAGAG motif described earlier, except for the insertion of an A, which occurred adjacent to a GGUGGU motif. This latter observation indicates the importance of simple repeats for the process of molecular misreading.

Another transcript previously shown to contain dinucleotide deletions at a GAGAG motif is produced by the APP gene (2). In exons 9 and 10 of the APP gene, three GAGAG motifs are clustered. When applying our strategy on RNA derived from aged individuals, we were again able to confirm the previously observed GA deletion in the second GAGAG motif in exon 9 (Table I). Furthermore, we detected a CA deletion in exon 11 that, remarkably, occurred in a GAGACACACA motif, again pointing to the involvement of simple repeats in molecular misreading. Molecular misreading in neither exon 9 nor exon 11 of the APP gene can, however, by itself lead to an increase in the production of Aβ, because the APP fragments generated by these frameshift mutations do not contain the Aβ peptide region (encoded by exons 16 and 17 of the APP gene). In contrast, the occurrence of a frameshift mutation in a region of the mRNA corresponding to the C-terminal cytoplasmic region of APP would lead to an aberrant protein with the potential to give rise to Aβ (Fig. 1). We therefore searched for mutations in the mRNA region encoding the C-terminal part of APP, and this analysis revealed a number of novel frameshift mutations, namely ΔA and ΔAA (both in UCAUCAGAAAUUGG), ΔΔ (UUCAAAAGAGUGCAACAUUCU), ΔΔΔ (UGUGGCAUGGUUGGCGGCGGUUGUUGU; UUCAAACAAGGUGUACAUUCU; GAAGAGAAACAGUACACA), ΔΔ (UUGAGUCAUCAGACCU), and ΔC (CTGGTCAACAGGAGG) (Table I). Some of these mutations occurred in close proximity of short simple repeats (UCAUCAUCA, CAAACAAA, and GAAGAAGAA). The mutations were not only detected in mRNAs derived from patients who had suffered from AD or DS but also in temporal cortex mRNAs of aged controls. Interestingly, the control that showed the highest mutation frequency exhibited plaques in the brain.

The aberrant proteins resulting from molecular misreading might affect cell physiology. For instance, the previously published Ubi-B-Δ protein, lacking Glycine residue 76 that is essential for recognition of the target protein (7), is no longer able to ubiquitinate proteins in vitro (2). The above-mentioned novel frameshift mutations in the Ubi-B gene transcripts probably also result in ubiquitin molecules unable to function properly. Concerning the APP protein one should consider its cytoplasmic tail that contains two sorting signals (YTSI and YENPTY), characterized as internalization and endosomal/lysosomal targeting signals, and necessary for intracellular trafficking of APP. Deleting or mutating these signals indeed affects APP routing (8–13). The frameshift deletions ΔG at nucleotide 2103 and ΔC at nucleotide 2153, in the part of the APP transcript encoding the cytoplasmic region, result in proteins with an aberrant and elongated cytoplasmic tail lacking one or both of the targeting signals of APP (Fig. 1). Because an affected APP routing may lead to an increased production of Aβ (14), the novel APP proteins with the aberrant tails may give rise to this neurotoxic peptide.

Although the frameshift mutations were found at a relatively low rate (1:950 to 1:8600 transcripts; see Table I), at the single cell level the frequency may be considerably higher. Immuno-cytochemical analysis has revealed that only a small percentage of cells contain the aberrant frameshift proteins, indicating that molecular misreading is indeed restricted to a minority of neuronal cells (2). In an affected cell, the percentage of mutated transcripts may well be relatively high, which could significantly contribute to the malfunctioning of such a cell, possibly eventually even promoting its degeneration. We conclude that during aging molecular misreading is a widespread event occurring at a variety of short simple repeats.

Acknowledgments—We gratefully acknowledge the Netherlands Institute for Brain Research (Amsterdam, The Netherlands) and the Department of Pathology (University of Nijmegen, The Netherlands) for providing human brain material. Furthermore, we thank J. Eygensteyn for technical assistance and Dr. F. van Kuppenfeld, R. P. M. Vloet, Dr. F. W. van Leeuwen, and Dr. N. H. Lubsen for contributions.

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F. A. A. Weijts, W. H. van den Hurk, and G. J. M. Martens, unpublished data.
RNA: STRUCTURE METABOLISM AND CATALYSIS:
Novel Frameshift Mutations near Short Simple Repeats

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J. Biol. Chem. 2001, 276:11496-11498.
doi: 10.1074/jbc.M011040200 originally published online January 3, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011040200

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