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Structure of the myelin membrane enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase: Evidence for two human mRNAs

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2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNPase, EC 3.1.4.37) is found in high concentrations in central- and peripheral-nervous-system myelin, as well as in the retinal-photoreceptor cell membrane. It is an enzyme capable of hydrolysing 2',3'-cyclic nucleotides to the corresponding 2' derivatives in vitro, however, its physiological role has remained unknown for over 30 years. The native CNPase protein is a dimer of approx. 100 kDa and on SDS/PAGE it can be separated into 46 kDa (CNPI) and 48 kDa (CNPII) polypeptides [1, 2]. More recently, it has been suggested that there are as many as three or four CNPase polypeptides in the mouse.

Partial amino-acid and early nucleotide sequences from various species suggested that there was a single CNPase message transcribed from a simple gene of just three exons [2]. More recently a fourth exon (exon 0) has been described after the discovery of a second and shorter CNPase cDNA in mice [3]. This additional exon encodes an initiator methionine only and therefore it has been suggested that an alternative splicing mechanism may operate for the CNPase gene [3]. Transcription of exons 1–3 would result in a long message encoding CNPI, whereas inclusion of exon 0 and its splicing 60 bp upstream of exon 1 gives a shorter CNPII mRNA. Such CNPase transcripts would differ only at their 5' ends and would make use of alternative initiation codons from exons 1 and 0, respectively. The smaller CNPII message, with its shorter 5' end, would make use of the upstream initiation codon and therefore encodes a polypeptide with an additional 20 amino acids at its amino-terminal end. Antibodies have been produced now that can distinguish between the CNPI and CNPII polypeptides [4, 5], confirming the difference at their amino terminus. Being predominantly basic, this N-terminal extension could explain the more basic migration of rabbit-brain CNPII on 2-dimensional gel electrophoresis [6], as well as providing the cyclic AMP-dependent phosphorylation site specific to CNPII in rabbit-brain myelin [6]. It is of some importance therefore to establish whether this alternative splicing mechanism operates within the CNPase genes of other species.

In the human a single CNPase gene has been identified on chromosome 17 by somatic-cell hybrid studies [7–9] and has been localized further to band 17q21 by in situ hybridization analysis [7, 8]. This gene spans 9 kb, has four exons and has a sequence [8] identical in all but one base to that described previously for the human cDNA [10]. Of the various human CNPase cDNAs isolated so far [8, 10] only two contain considerable 5' non-coding sequence and both represent CNPII-type mRNAs, that is, a transcript spliced from all four human CNPase exons. There has been no evidence as yet for a human CNPI mRNA and the existence of only a single message is supported by Northern-blot analysis, where a single CNPase-mRNA band of 3 kb has been observed in human-brain RNA [10].

In the present study we have identified the transcriptional start site of the human CNPII-type mRNA (Figure 1). Primers complementary to sequences both within exons 0 and 1 gave reverse-transcription products ending 87, 88, 89 and 90 bp upstream of the initiation codon in exon 0. This start site 30–33 bp downstream of the TATA box previously identified in the human CNPase gene [8], corresponds almost exactly with the mouse CNPII cDNA transcriptional start site [3]. However, as the human CNPase message, at 3 kb [10],

Abbreviation used: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase.
Proposed alternative splicing mechanism at the 5' end of the human CNPase gene

Two promoter regions exist upstream of exons 0 and 1, producing CNPll and CNPI mRNAs, respectively. The restriction sites BamH1 (B), HindIII (H), BglII (G) and PstI (P) are labelled on the genomic sequence only. For the mRNAs, solid boxes represent coding region, empty boxes non-coding region. The shaded box highlights the position of the CNPI PCR product.

is longer than the 2.28 and 2.53 kb mRNAs of the mouse [3], the 5' end of the human CNPll mRNA is actually surprisingly short. This suggests a very long 3' non-coding region, already known to be at least 1.1 kb long from previous human CNPase cDNA studies [8, 11].

We have been able to establish the existence of a human CNPI-type mRNA also using PCR. Primers on either side of the exon 1 splice site (Figure 1) have been used to amplify a CNPll-mRNA PCR product of the correct size and restriction map, from both human-brain total RNA and from a human-brain cDNA library. This message is produced by means of a second CNPase promoter within intron 1. Exon 0 and the splice site 60 bp upstream of exon 1 are unused, so that the 5' end of the CNPI mRNA is encoded by intron 1 sequence. In the human the CNPI promoter elements remain to be found, although the primer-extension studies mentioned above suggest a start site 300-400 bp upstream of exon 1. It is intriguing that, like the mouse messages, the human mRNAs for CNPI and CNPll appear to have 5' ends varying by 200-300 bp and yet this size difference is not apparent from Northern-blot studies. Further analysis is required to establish the full length of these human CNPase mRNAs, as it remains a possibility that these messages differ at their 3' ends also and are, in fact, of similar size.

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Molecular studies on chemotactic receptors
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Introduction

Neutrophils detect invading micro-organisms by the formylated peptides (chemo-attractants) that they release. The formylated-peptide receptor (FPR), upon ligand binding, initiates chemotaxis towards the site of infection. The high concentration of chemo-attractant at the target site initiates degranulation, which destroys the infectious agents [1]. FPR has a high and a low affinity state, and this is probably related to its chemotactic response. FPR from human-leukaemic (HIl-60) and rabbit cells [2, 3] has been cloned and sequenced. The hydropathy profiles from the primary sequences indicate that there are seven putative transmembrane segments, while the secondary structure predictions suggest these transmembrane regions to be α-helical. For these reasons the FPR may be a member of the seven-transmembrane-helix 'super' family. To allow detailed characterization and structural studies of the FPR to be carried out we are undertaking the cloning and heterologous expression in *Xenopus* oocytes of the FPR from murine cells (FDCP; factor-dependent cells from the Paterson Institute), using the human FPR gene as a probe [4].

In this report we present results which indicate that the murine cell line FDCP-A4, differentiated to neutrophils [5], is a better physiological model for the study of FPR than the commonly used human-leukaemic cell line (HIl-60). In addition preliminary binding studies indicate a difference either in chemo-attractant peptide specificities or in binding affinities. The dissociation constants for the murine FPR with the peptide [3H]formyl-

MLP (fMLP) were 2–3 orders of magnitude lower compared with the human FPR. Murine-FDCP surface receptor numbers are similar to those in isolated human blood neutrophils, whereas HIl-60 cells have much lower receptor numbers. This may reflect the fact that FDCP cells are multipotent cells stimulated to differentiate by physiological growth factors, whereas HIl-60 cells are transformed cells stimulated to differentiate by non-physiological chemicals.

Methods

HIl-60 and FDCP cells were cultured and caused to differentiate as previously described [4, 5]. In outline HIl-60 cells were stimulated to differentiate with NO2-dibutyryladenosine-3′,5′-cyclic monophosphate (0.6 mM) and FDCP cells with interleukin-3 (IL-3) (1 unit/ml), granulocyte-macrophage-colony-stimulating factor (GM-CSF) (50 unit/ml) and granulocyte-CSF (G-CSF) (1000 unit/ml). Differentiation was carried out for 4 days for experiments to measure Nitro Blue Tetrazolium (NBT) reduction or for 6 days for binding studies.

NBT is reduced by superoxide radicals produced by fMLF-stimulated neutrophils [4]. After incubating the cells in an NBT solution with or without fMLF (6.67 × 10−5 M), the level of NBT reduction is calculated from the difference between the absorbances at 450 nm and at 620 nm. The data are expressed either as the percentage change between cells incubated with and without fMLF or as the difference in absorbance per 10^6 cells.

The binding study was carried out using [3H]fMLP. Cells (5 × 10^6) were incubated for an hour on ice with different concentrations of the radiolabelled ligand, pelleted and washed in Hanks balanced salt solution (with 0.1% w/v bovine serum albumin). Scintillant was added and the radioactivity was measured. Specificity of binding was measured using unlabelled fMLP.

Abbreviations used: f, formyl; FDCP, factor-dependent cells from the Paterson Institute; FPR, formylated-peptide receptor; HIl-60, human leukaemic-60; NBT, Nitro Blue Tetrazolium.