DDC-4, an apoptosis-associated gene, is a secreted frizzled relative

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Abstract The differential display method has been used in our laboratory as a coincidence analysis to isolate genes expressed in common in each of three different rat tissues undergoing physiological apoptosis: mammary gland, ovarian corpus luteum and ventral prostate. The most interesting of these isolates, DDC-4, shows a clear association with apoptosis, its expression being confined to these three organs, and only during their involution. Using DDC-4 as probe, we screened a rat ovarian cDNA library to obtain full-length isolates. One isolate, Y81 clone 40, gives rise to a protein of approximately 40 kDa with coupled in vitro transcription/translation. Sequencing of this clone indicates an open reading frame of 1044 nucleotides encoding a protein of 39.7 kDa with a putative signal sequence. This clone exhibits a high homology with the cysteine-rich domain, i.e. the ligand-binding domain, of the *frizzled* gene family originally defined as tissue polarity genes in *Drosophila*. The homology of Y81 clone 40 is most extensive with the newly described secreted frizzled relatives, the *frzb* subfamily.

Key words: *Frizzled*; Apoptosis; Mammary gland; Prostate; Corpus luteum

1. Introduction

Involution processes like those occurring in the mammary gland following forced weaning of young, in the ovarian corpus luteum just before parturition or in the prostate after castration are three examples exhibiting an ordered program of cell death. In each case the process is typical of apoptosis, essential features being DNA fragmentation with condensation and an early phagocytic disposal of the still intact dying cells [1–4]. Dividing the processes of physiological apoptosis into phases [5], one can anticipate a precoded stimulus which is detected and transduced as a signal, activating cell death effectors, thus bringing about an irreversible state in which death must be the outcome. Recent progress has improved our understanding of death signalling and of the effectors, the caspases, which proteolytically cleave target proteins essential for cell viability [5]. Apoptosis in vivo is apparently more complex, however, since a pre-condition for apoptosis in many cases is the requirement for new mRNA and protein synthesis in response to death signalling [6,7]. The newly synthesized gene products are presumably responsible for the characteristic preparations for apoptosis, of which the best documented are the 'flags' presented on the cell surface to facilitate an early phagocytic disposal [8].

Involution comprises much more than just cell death, however, since cyclical tissue remodelling is also a feature of the physiology of organs such as those mentioned [2]. Remodelling includes proteolytic degradation of extracellular matrix [9,10] and proliferative responses in precursor cell pools. Our laboratory has focused in recent years on the problem of identifying genes participating in physiological in vivo apoptosis. An initial differential screening approach used to isolate genes upregulated in mammary involution revealed mainly genes involved in tissue remodelling [11]. We then reasoned that a differential display approach [12] would allow a better selection of apoptosis-associated genes if a direct comparison could be made between gene expression in the mammary gland at different developmental stages and some other tissues undergoing apoptosis, but without significant tissue remodelling. Massive apoptotic cell death occurs in the ventral prostate as a consequence of castration, representing a kind of physiological response to testosterone ablation, as a one-time irreversible event fundamentally different from the cyclical involution of the mammary gland. Prostate regression fulfilled these criteria, since tissue remodelling as measured by metalloproteinase expression was minimal in the prostate [13]. A differential display coincidence (DDC) analysis was performed to isolate genes specifically expressed in apoptosis both in mammary gland and in prostate [13]. DDC-4 is one of the most interesting of these isolates, being uniquely expressed in mammary gland, ovarian corpus luteum and prostate, specifically at an early stage, only in apoptotic cells [13,14].

Several DDC-4 clones have been obtained from a rat ovarian cDNA library, of which one, Y81 clone 40, gives rise to a 40 kDa protein with coupled in vitro transcription/translation. This communication presents a detailed sequence characterization of Y81 clone 40 establishing it as a secreted relative of the *frizzled* family, sharing a homologous cysteine-rich domain (CRD) with perfect structural homology in the spacing of all 10 conserved cysteines [15,16]. DDC-4 detects three different transcripts in Northern blots, which share a common sequence region, but are distinct in their 3′ sequences. Evidence based on polymerase chain reaction is presented suggesting that separate genes must encode the different transcripts.

2. Materials and methods

2.1. Animals

The ovarian corpora lutea and abdominal mammary glands derived from female Sprague-Dawley rats representing different stages of development were isolated: pregnant, lactating and different involution stages after forced weaning. The ventral prostate glands of mature

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male rats of the same line were collected from normal and post-castration animals.

2.2. Isolation and sequencing of DDC-4 clones

A rat corpus luteum cDNA λ Zap phagemid library was obtained from Stratagene (La Jolla, CA, USA). Phage plaque lifts were probed using the differential display DDC-4 clone [13]. Several independently derived cDNA isolates were rescued to pBluescript SK (Stratagene) for characterization. Clone 40 and clone 24 were analyzed in detail. Successive deleted fragments were prepared with the Exonuclease III Erase-a-Base system (Promega, Madison, WI, USA) in order to perform sequencing. Subcloned fragments were sequenced on both strands using the dideoxynucleotide chain termination method performed with the Sequenase 2.0 kit (United States Biochemicals, Cleveland, OH, USA). Sequence analysis was performed by comparing with the EMBL-GenBank database using the FastA program of the Genetics Computer Group (Madison, WI, USA).

2.3. RNA isolation and Northern blotting

Total RNA was prepared from different tissues described above using the guanidinium-thiocyanate extraction method [17]. 5 μg RNA samples were denatured with glyoxal, electrophoresed on a 1% agarose gel and blotted to nitrocellulose. Probes were prepared using the Random Primed Labeling kit (Boehringer Mannheim), or in a polymerase chain reaction using Taq polymerase as described below, including [^{32}P]dCTP (800 Ci/mM; Amersham International, Little Chalfont, UK) in the reaction.

2.4. In vitro translation

Using the TNT Coupled Reticulocyte Lysate System (Promega), 1 μg plasmid DNA was employed in parallel reactions with T3 or T7 RNA polymerases. Reactions were performed with [^{35}S]methionine for 2 h. The translation products were separated by SDS-PAGE, followed by drying and autoradiography.

2.5. Polymerase chain reaction

Polymerase chain reaction (PCR) with genomic rat DNA (250 ng) or plasmid DNA (2 ng) was employed using as a sense primer a sequence common between clone 40 and clone 24 (5′-GCC TTA GGA CCC ACT GTG GTT-3′), together with the clone 40-specific antisense primer (5′-TGG CCC AGC TCT CTT GAT GGA TTA ACC AAG ATG TC-3′) or the clone 24-specific antisense primer (5′-GGT CAT AGC ACT TAT AAG TAC TCT G-3′). PCR conditions were 94°C for 30 s, 59°C for 30 s and 72°C for 40 s. Fifty cycles were performed, using *Thermus aquaticus* polymerase obtained from Boehringer Mannheim (Penzburg, Germany).

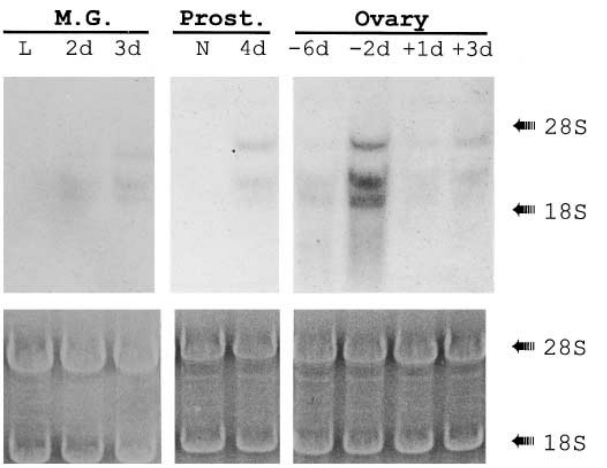

Fig. 1. A Northern blot of total RNA samples shows the relative expression of DDC-4 in rat mammary gland (M.G.) during lactation (L) and 2 and 3 days following forced weaning, in normal (N) prostate and at 4 days after castration, and in the ovary at different days pre (−6d, −2d) or post (+1d, +3d) parturition. The blots were hybridized with ^{32}P random primed probe prepared from the entire Y81 clone 40 insert. The lower panel shows the acridine orange stained ribosomal RNA bands from the same agarose gel electrophoresis as a loading control.

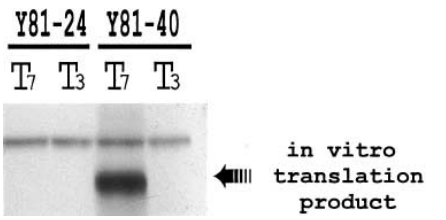

Fig. 2. In vitro translation was employed to demonstrate synthesis of a 40 kDa protein (see arrow) in coupled transcription/translation of the Y81 clone 40 insert. T7 polymerase gives rise to a sense transcript. Y81 clone 24 failed to generate translation product. The band at 53 kDa is an endogenous product of the reticulocyle lysate system.

3. Results and discussion

The differential display clone DDC-4 encompassed approximately 400 bp and showed no significant homology to any gene in the GenBank [13]. In order to obtain additional sequence data, we first screened an ovarian λ Zap cDNA library (Stratagene), after rescue obtaining a collection of plasmids designated Y81, with inserts ranging from 1.9 kbp (clone 40) downward.

3.1. Y81 clone 40 detects apoptosis-associated gene expression

As an initial approach to validating Y81 clone 40 as a DDC-4 representative, we performed a Northern blot on total RNA samples from different involuting tissues, using as probe fragments obtained in a random primed reaction with Y81 clone 40. Fig. 1 shows the results of this experiment. Using total RNA, very weak signals could be obtained from involuting mammary gland and prostate samples. No signal was apparent in the lactating or the normal organs. Ovary, however, exhibited a strong signal 2 days before parturition, when previous experiments have shown luteolysis to be well established, and terminal transferase end-label reactions indicated significant apoptosis [14]. The three transcripts visible exhibit the same pattern previously observed with poly A$^+$-enriched mRNA using DDC-4 as probe [13,14].

3.2. Y81 clone 40 can be translated in vitro

A coupled transcription/translation reaction was performed since Y81 clone 40 is contained in a BlueScript vector (Stratagene) flanked by T3 and T7 polymerase initiation sites. Fig. 2 illustrates the results; a protein product of approximately 40 kDa was detected with clone 40 transcription/translation using T7, but not T3 polymerases. Y81 clone 24, with a smaller insert, failed to be transcribed/translated.

3.3. DDC-4 is a secreted relative of frizzled

The outcome of the preceding experiments suggested that Y81 clone 40 should reveal the coding sequence of this gene. Sequencing of deletion fragment clones was performed in both directions, and a sequence for Y81 clone 40 assembled and presented (GenBank AC: AF 012891). The sequence of the original DDC-4 isolate was found in Y81 clone 40 mainly overlapping with the 3′ untranslated sequence. An open reading frame of 1044 nucleotides was found, encoding a protein with a predicted molecular mass of 39.7 kDa. Homology of Y81 clone 40 to the *frizzled* family was immediately apparent from the nucleotide sequence. The deduced amino acid sequence is shown in Fig. 3a, aligned against several relatives

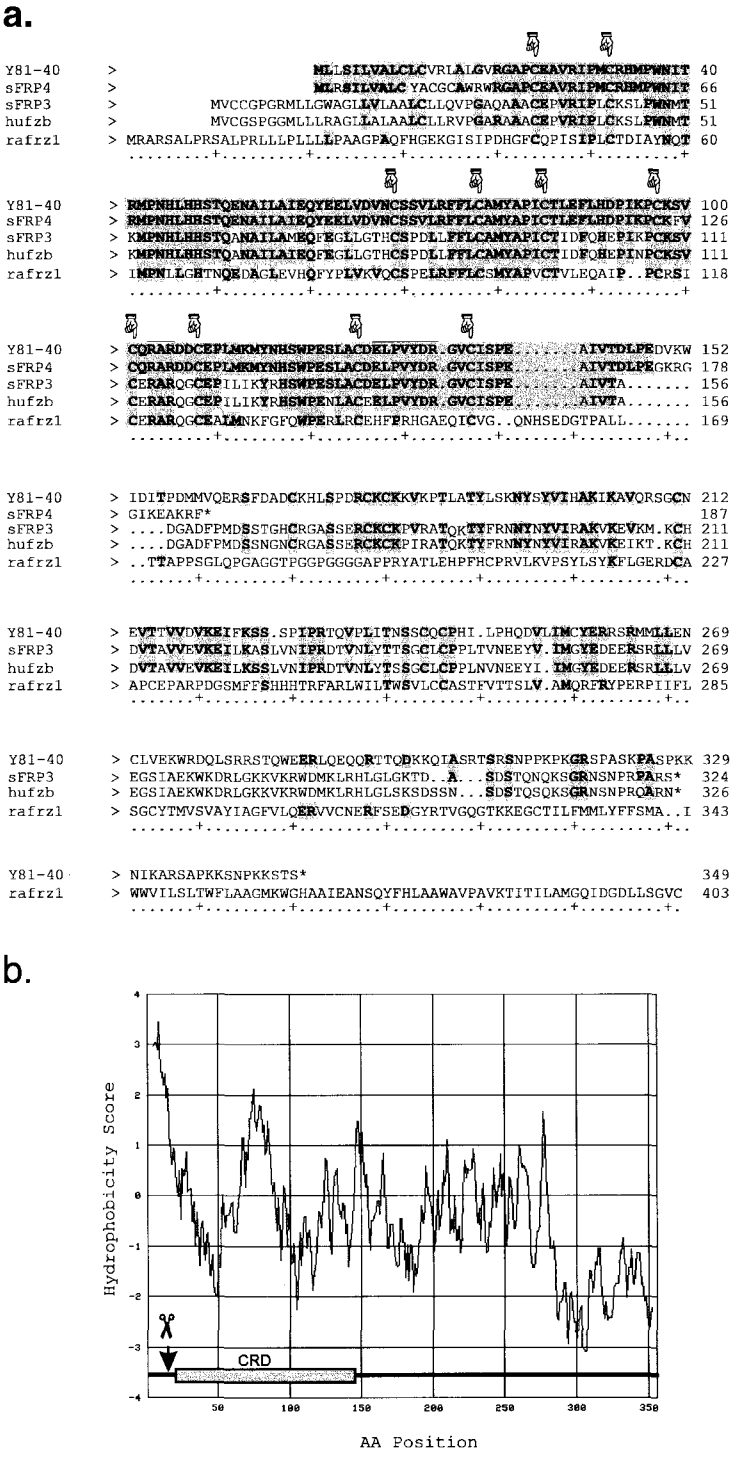

Fig. 3. a: An alignment of the deduced amino acid sequence of Y81 clone 40 is shown in relation to several other *frizzled* family members. Shadowed amino acids are identical to Y81 clone 40. The 'hands' point to the 10 conserved cysteines from the cysteine-rich domain (CRD). Y81 clone 40 (GenBank accession number AF012891, 347 aa), sFRP-3 [16] (GenBank accession number U88568, 323 aa), sFRP-4 [16] (GenBank accession number U88569, 159 aa), hufzb [20] (GenBank accession number U24163, 325 aa), and rafrz1 [15] (GenBank accession number L02530, 566 aa). Asterisks indicate the carboxy termini, shown for all sequences except rafrz1. sFRP denotes a mouse 'secreted, *frizzled* related protein'; hufrzb defines the secreted 'human *frizzled* of bone', while rafrz1 refers to rat *frizzled* 1, a seven-pass transmembrane protein. b: A hydrophobicity plot of the Y81 clone 40 deduced amino acid sequence was generated by peptide analysis tools on the Internet site (www.expasy.ch) using the paradigm of Kyte and Doolittle. Hydrophobic residues have positive values. The predicted cleavage site of the signal sequence (aa 18/19) is indicated with the scissors symbol. CRD is the cysteine-rich domain typical of all *frizzled* family members.

of *frizzled*. Most noteworthy are the 10 conserved cysteines in the amino-terminal third [15,16], the region comprising the CRD, which is known to be the ligand binding region of *frizzled* [18]. In the carboxy-terminal half of the sequence, the homology between Y81 clone 40 and *frizzled* genes of different species, seven-pass transmembrane receptors [19] is

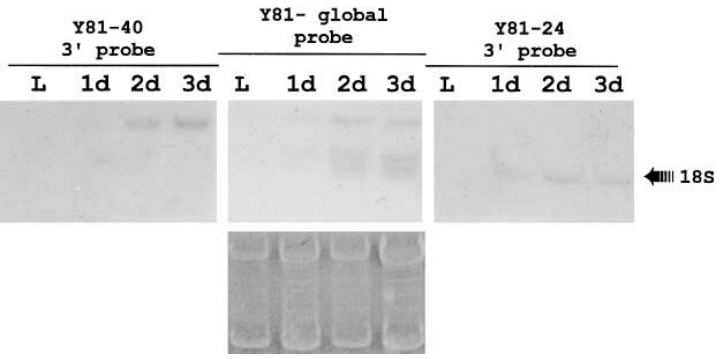

Fig. 4. A Northern blot was performed with four total RNA samples from the rat mammary gland. L = lactating, 1d, 2d and 3d = days after forced weaning. The same blot was probed three times sequentially in the following order. Firstly (left panel), using a probe prepared in PCR with Y81 clone 40 DNA using a sense primer common to all DDC-4 sequences together with a unique clone 40 antisense primer. Secondly (right panel), the blot was hybridized with probe prepared in the same way using a shared sense primer together with a unique clone 24 antisense primer. Thirdly (middle panel), the blot was probed with the global random prime product obtained from the entire Y81 clone 40 insert (as described in the legend to Fig. 1). The lower panel shows the acridine orange stained ribosomal RNA bands as a loading control.

minimal. Y81 clone 40 protein exhibits no transmembrane regions, but does show a typical signal sequence with a cleavage site between amino acids 18/19. The homology to secreted relatives such as *frzb* [20] is higher, showing 38% identity even in the carboxy half. Fig. 3b summarizes these data and illustrates a hydrophobicity plot for the clone 40 encoded protein.

3.4. *The three transcripts detected with DDC-4 probably represent three distinct genes*

Since three transcripts were detected among mRNAs of involuting organs, an experiment was performed to determine the specificity of clone 40. When the entire Y81 clone 40 insert was used to prepare probe, all three transcripts were detected (Fig. 1, Fig. 4 center). When a PCR fragment of clone 40 encompassing approximately 25% of the 3′ sequence was labeled as probe, hybridization was observed only with the largest (2.9 kbp) transcript (Fig. 4). Similarly, a 200 bp PCR fragment from the 3′ terminal region of Y81 clone 24 detected only the smallest (2.0 kbp) of the Northern blot transcripts (Fig. 4). Partial sequence analysis of clone 24 shows an extensive central region of identity with clone 40 (data not shown).

Alternative splicing is the most likely explanation for the three transcripts detected by DDC-4. In order to obtain a preliminary indication whether this is indeed the correct explanation, a shared sense primer was employed in PCR reactions using two alternative antisense primers, specific for the 3′ regions of either clone 40 or clone 24. These pairs were employed using as templates either rat genomic DNA, clone 40- or clone 24-containing plasmids. To our surprise, the results presented in Fig. 5 indicate that the genomic structures are co-linear with the cDNA clones in each case. Thus, the different transcripts cannot be explained by the splicing-out of intron sequences. Most likely, the unique 3′ termini found in clone 40 and clone 24 represent unique sequences of distinct genes.

3.5. *A secreted frizzled associated with the process of apoptosis*

Recently the CRD regions of *frizzled* gene products have been shown to be ligand binding sites [18,21,22] for *Wnt* gene products, long recognized as having important signalling functions in adult and embryonic pattern formation in *Drosophila*, in the establishment of the dorso-ventral axis in *Xenopus* embryos and in development of the central nervous system of mice [23]. In the past few months, secreted forms of *frizzled* have been reported in *Xenopus*, mice and even in the human, which apparently compete with cell membrane receptors for *Wnt* ligands [20,22,24,25]. Recently, using RT-PRC, Blankesteijn et al. [26] reported the detection of a *frizzled* related gene in migrating myoblasts repopulating granulation tissue following myocardial infarction. Our finding of DDC-4 associated with three cases of physiological apoptosis suggest that the DDC-4 secreted *frizzled* relatives may be active in adults and in processes associated with apoptosis.

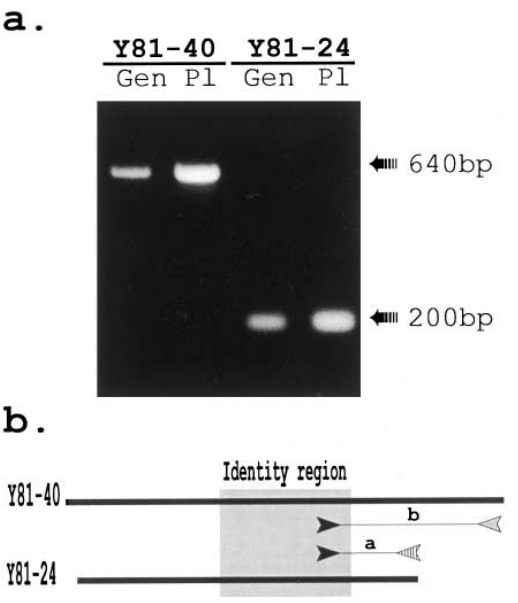

Fig. 5. Polymerase chain reactions were perfomed with the shared sense primer together with Y81 clone 40 or Y81 clone 24-specific 3′ antisense primers, using either rat genomic (Gen) or clone 40/clone 24 plasmid DNAs (Pl). a: The result is presented as an ethidium bromide stained agarose gel showing the characteristic bands obtained. No size difference between genomic DNA and cDNA inserts in plasmids was observed. b: A scheme showing the sequence relationships and the predicted relative sizes of PCR products.

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