Carboxypeptidase Z (CPZ) modulates Wnt signaling and regulates the development of skeletal elements in the chicken

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Summary

Carboxypeptidase Z (CPZ) is a secreted Zn-dependent enzyme whose biological function is largely unknown. CPZ has a bipartite structure consisting of an N-terminal cysteine-rich domain (CRD) and a C-terminal catalytic domain. In the early chicken embryo CPZ is initially expressed throughout the somites and subsequently becomes restricted to the sclerotome. To initiate a functional analysis of CPZ, a CPZ producing retroviral vector was applied to the presomitic mesoderm at the level of the future wing. This resulted in a loss of the scapular blade and of rostral ribs. Such dysmorphogenesis is preceded by ectopic *Pax3* expression in the hypaxial part of the dermomyotome, a region from which the blade of the scapula normally derives. A mutant CPZ, lacking a critical active site glutamate, fails to induce *Pax3* expression and does not cause skeletal defects. The induction of *Pax3*, a Wnt-responsive gene in somites, and the presence of a CRD prompted us to examine whether CPZ affects Wnt signaling. In an in vitro assay we found that CPZ, but not its inactive mutant form, enhances the Wnt-dependent induction of the homeobox gene *Cdx1*. In addition, immunoprecipitation experiments suggest that the CRD of CPZ acts as a binding domain for Wnt. Taken together these data provide the first evidence for CPZ playing a role in Wnt signaling.

Key words: Carboxypeptidase Z, Somite, Sclerotome, Pax3, Pax1, Scapula, Rib, Wnt, Cysteine-rich-domain, CRD, AER, Paraxial head mesoderm, Chicken

Introduction

Carboxypeptidase Z (CPZ) is a member of the carboxypeptidase E subfamily of metallocarboxypeptidases (Song and Fricker, 1997). Although these Zn-dependent enzymes have generally been implicated in intra- and extracellular processing of proteins (Skidgel, 1988) (reviewed by Fricker, 1998: Reznik and Fricker, 2001) not much is known about the specific substrates of CPZ. Novikova and Fricker (Novikova and Fricker, 1999) found that CPZ cleaves a C-terminal arginine present in synthetic peptide substrates with maximal catalytic activity at neutral pH (Novikova and Fricker, 1999). This is consistent with an enzymatic function in the extracellular matrix. When expressed in cultured cells CPZ is secreted and associates with the matrix (Novikova et al., 2000).

CPZ harbors a cysteine-rich-domain (CRD) N-terminal to the catalytic domain (Song and Fricker, 1997; Xin et al., 1998). A CRD is characterized by a series of 10 cysteine residues and is found in several proteins including Frizzled, Frizzled related proteins, Smoothened, the receptor tyrosine kinase MuSK and CPZ. In the case of Frizzled and Frizzled related proteins the CRD has been shown to act as a ligand-binding domain for Wnts (Bhanot et al., 1996; Rattner et al., 1997). Wnt proteins are secreted molecules involved in many developmental processes (reviewed by Cadigan and Nusse, 1997) including patterning of somites and limb development. The presence of a CRD has implicated CPZ in Wnt signaling during development (Reznik and Fricker, 2001). However, experimental evidence has not yet been provided to support this proposal.

Somites are segmental units of the paraxial mesoderm. They form by epithelialization of mesenchymal cell clusters in the anterior region of the unsegmented paraxial mesoderm. Thereafter, epithelial somites are regionalized into a ventral compartment, the sclerotome, from which the axial skeleton forms, and a dorsal compartment, the dermomyotome giving rise to dermis and skeletal muscle (Keynes and Stern, 1988). Somite patterning is controlled by signals from adjacent tissues including the notochord, neural tube, surface ectoderm and lateral plate mesoderm (Brand-Saberi et al., 1993; Pourquie et al., 1993; Fan and Tessier-Lavigne, 1994; Kuratani et al., 1994; Spence et al., 1996). Several members of the Wnt family are expressed in these tissues and have been shown to induce the expression of dermamyotomal genes such as the paired-box transcription factor *Pax3* (Fan et al., 1997; Cossu and Borello, 1999). Sonic hedgehog is another major axial signal that is responsible for induction and differentiation of the sclerotome (Marcelle et al., 1999). Signaling activity of these secreted proteins may be regulated by proteolytic processing.

The present study uses a combination of strategies to unravel the developmental function of CPZ. In situ hybridization in chick embryos revealed regionalized expression of CPZ in somites, sclerotome, paraxial head mesoderm and the apical

ectodermal ridge. Retrovirus-mediated ectopic CPZ expression in the chick was used to investigate the role of CPZ during embryogenesis. Overexpression in the somites resulted in upregulation of *Pax3* in the hypaxial dermomyotome, in a downregulation of *Pax1* in cells fated to form the scapula and in a partial loss of the scapula and ribs. CPZ increased Wnt4-mediated induction of the homeobox gene *Cdx1* in vitro, and immunoprecipitation experiments showed that the CRD of CPZ can bind to Wnt4. Collectively, these experiments suggest that CPZ has a role in Wnt signaling.

Materials and methods

Full-length cDNA isolation and sequence comparison

A 750 bp cDNA fragment corresponding to chicken *CPZ* was used to screen a chick cDNA library. This 750 bp fragment had originally been isolated in a screen for retinoic acid-induced genes (Swindell and Eichele, unpublished data). Ten cDNAs were isolated, all of which lacked the predicted 5′ end of the coding region. To isolate the 5′ end, SMART 5′ RACE was carried out (Clontech). The murine CPZ ORF was isolated by RT-PCR starting with the amplification of a 300 bp fragment using the primer pair 5′CCCAGTACTGT-GCTC(C/T)GAGT3′; 5′CCGAATTTCTCTGTCACC(A/T)CAC3′.

The rest of the ORF was obtained by 5′ and 3′ RACE-PCR using the SMART RACE cDNA Amplification Kit (Clontech, USA). GenBank accession numbers: chicken CPZ AF351205; murine CPZ AF356844.

Whole-mount in situ hybridization

Whole-mount in situ hybridization (WMISH) and subsequent sectioning of embryos were carried out as described previously (Albrecht et al., 1997). The entire *cCPZ* coding region was used as template for riboprobe synthesis. In situ hybridization analysis on sections was performed as described previously (Swindell et al., 2001). For *Pax1*, *Pax3*, *myf5*, *myogenin* and *MyoD* full-length cDNAs were used as templates for riboprobe production.

Site directed CPZ mutagenesis

A single nucleotide change (G1405 to C1405) was inserted into cCPZ using the QuikChange site directed mutagenesis protocol (Stratagene). Primers: 5′GCTTTGAAGTTACTGTG**CAG**GTAG-GATGTG3′, 5′CACATCCTAC**CTG**CACAGTAACTTCAAAGC3′. This mutagenesis resulted in a single amino acid change (Glu469 to Gln469). The corresponding mutation was also inserted into the murine *CPZ* with the following primers: 5′GCTTTGAGATCACCGT-G**CAA**CTGGGCTGTGTGAAGTTC3′, 5′GAACTTCACACAGC-CCAG**TTG**CACGGTGATCTCAAAGC3′.

This nucleotide mutation resulted in the single amino acid change Glu477 to Gln477.

Viral overexpression

Full-length chicken *CPZ* and mutant chicken *CPZ* (*cCPZ*^{E469Q}) were cloned into the RCAS-BPA vector. The virus was produced and concentrated as described by Logan and Tabin (Logan and Tabin, 1998). The virus was injected into the segmental plate of HH stage 10 embryos at the level of the presumptive wings (Chaube, 1959). Injection of the virus to other sites had no effects. Embryos were then collected for WMISH or Alcian Blue staining at the times noted.

Skeletal preparations

Day-10 chick embryos were collected and fixed in 5% TCA. Embryos were then stained with 0.1% Alcian Blue, unspecifically bound dye was washed off with 1% HCl/70% ethanol, followed by dehydration in 100% ethanol and clearing in methyl salicylate in order to visualize the skeleton.

Generation of CPZ-expressing cell lines

HEK-293 cells were transfected by lipofection (Effectene, Qiagen) using linearized pcDNA3.1/myc-HisA (Invitrogen) containing the full-length coding region of the murine *CPZ* cDNA either in its native form or carrying a glutamate to glutamine mutation. Cells were split 24 hours after transfection and grown in 6-well plates under selective conditions (DMEM, 10% FCS, and 1 mg/ml G418). Clones were tested for native and mutant CPZ expression by western blot analysis using mouse anti-myc antibody (Invitrogen). CPZ-containing extracellular matrix (ECM) was prepared as described previously (Novikova et al., 2000).

Co-culture assay for detection of Wnt activity and quantitative RT-PCR

Analyses are based on multiple experiments using different CPZ and CPZ^{E477Q} cell lines. CPZ-expressing HEK-293 cells or wild type HEK-293 cells were seeded into 6 cm tissue culture dishes and grown for 1 day. These cultures were always done in duplicate. To generate plates coated with normal ECM or ECM spiked with CPZ, normal HEK-293 cells or CPZ-producing HEK-293 cells were detached with 1 mM EDTA in PBS. Into these conditioned plates were placed either untransfected HEK-293 cells or CPZ-expressing HEK-293 cells and NIH-3T3 fibroblasts stably transfected with different *Wnt* cDNAs (Kispert et al., 1998). Equal numbers of HEK-293 and NIH-3T3 cells were used to give a total cell number of 3×10^6 cells per plate. HEK-293 and NIH-3T3 cells were cultured for ~4 hours after which time ES cells were seeded on top of these cells as previously described (Lickert et al., 2000). Depending on the Wnt-expressing cell line used and the passage number of the ES cells, the co-cultures were grown for between 6 and 12 hours. Thereafter, RNA was isolated with RNAzol (WAK Chemie) and cDNA was generated using Superscript II Reverse Transcriptase (Invitrogen). *Cdx1* expression levels were detected with quantitative RT-PCR as described (Fruman et al., 2002) with the housekeeping gene *elongation factor 1 alpha* (*EF1α*) to standardize *Cdx1* expression levels. The following primer pairs were used: *EF1α*-forward 5′GTCC-CCAGGACACAGAGACTTCA3′, *EF1α*-reverse 5′AATTCACCAA-CACCAGCAGCAA3′, *Cdx1*-forward 5′TACAGCCGGTACATCAC-TAT CCG3′, *Cdx1*-reverse 5′CTGTTTCTTCTTGTTTAC-TTTGCGC3′. Co-cultures of *lacZ*-NIH-3T3, untransfected HEK-293 cells and ES cells display basal levels of *Cdx1* expression (Lickert et al., 2000). Hence the increase of *Cdx1* expression in the presence of inducers (Wnts, CPZ) was calculated as the ratio of expression in the presence of inducers and basal level of expression resulting in a ‘fold-induction’. The co-culture experiment were repeated multiple times and data shown in Fig. 6B are typical.

Co-immunoprecipitation

2.4×10^6 HEK-293 cells were plated on 10 cm dishes. 24 hours later they were transfected with 4 μg of a plasmid mixture using Effectene in a standard reaction set-up. This mixture contained 2.4 μg of pcDNA3.1-Wnt4-HA (Lescher et al., 1998) and 1.6 μg of either the positive control pcDNA3.1-sFRP2-myc (Lescher et al., 1998) or one of the CPZ constructs. 48 hours after transfection, cells were washed twice with PBS at 37°C and harvested in 350 μl of lysis buffer. After cell lysis the cotransfected proteins were precipitated with a monoclonal mouse anti-HA antibody (Babco) and protein-G agarose (Roche) using standard protocols. The coprecipitated proteins were detected with a monoclonal mouse anti-myc antibody (Invitrogen).

Results

Cloning of the chicken ortholog of mammalian *CPZ*

A 750 bp chicken cDNA fragment with homology to human and rat *CPZ* was used to screen a Hamburger Hamilton (HH) stage 14-17 chick cDNA library (Hamburger and Hamilton, 1951). Ten putative chicken *CPZ* cDNAs (*cCPZ*) were isolated,

but all lacked the 5′ end of the protein coding region. The missing 5′ end of the *cCPZ* cDNA was obtained using a 5′ RACE-PCR procedure. The assembled cDNA encodes a protein with 77% sequence similarity (identity 64%) to human and rat *CPZ* (Fig. 1). Similar to its putative mammalian orthologs, cCPZ harbors an N-terminal signal peptide, a cysteine-rich-domain (CRD) and a carboxypeptidase domain (Fig. 1). The CRD and carboxypeptidase domain of cCPZ show similarities of 84% and 86% (identity: 69% and 73%), respectively, with the human ortholog. Like the rat and human CPZs, the CRD of the chicken enzyme contains 10 conserved cysteine residues. Amino acid residues important for substrate binding and catalytic activity (His245, Glu248, Arg320, His377, Glu469) are also fully conserved. Amino acid sequence conservation to CRD domains of other proteins amounts to 25% and 32%, supporting the notion that the isolated cDNA represents the chicken ortholog of mammalian CPZ.

cCPZ is expressed in somites, paraxial head mesoderm and the apical ectodermal ridge

In situ hybridization analysis was used to determine the expression pattern of c*CPZ* during early developmental stages of the chick (Fig. 2). Weak *cCPZ* expression was first observed at HH stage 7 in the developing somites (not shown). In subsequent stages, when progressively more somites form and differentiate, *cCPZ* expression is maintained in these structures (Fig. 2. A-D,F,H). Transverse sections demonstrate the presence of *cCPZ* transcripts throughout the entire epithelial somite (Fig. 2D,H). As somites differentiate into sclerotome and dermomyotome c*CPZ* expression becomes restricted to the sclerotome (Fig. 2F). *cCPZ* transcripts are present throughout the sclerotome unlike, for example, *Pax1* that is expressed in the ventromedial portion of the sclerotome (Müller et al., 1996). By HH stage 22, when somite formation ceases, all sclerotomes express *cCPZ*. With condensation of the sclerotomes CPZ expression is lost in a rostral to caudal progression (not shown). Additionally, *cCPZ* is expressed in the unsegmented paraxial head mesoderm surrounding the notochord (Fig. 2E). *cCPZ* was also detected in the apical ectodermal ridge (AER, Fig. 2C,G), a transient signaling tissue mediating limb outgrowth.

It has been shown for several genes including *Pax1* that sclerotomal expression depends on signals released by the notochord and/or neural tube (Fan and Tessier-Lavigne, 1994; Marcelle et al., 1999); this is, however, not the case for *cCPZ*. When presomitic mesoderm not expressing *cCPZ* was separated from axial structures and the operated embryos were harvested 14 h later, all somites, even those deprived of axial signaling still expressed *cCPZ* (Fig. 2I). We conclude that *cCPZ* expression is not regulated by signals emanating from the notochord or neural tube but by factors intrinsic to the somitic mesoderm and/or derived from the surface ectoderm.

Ectopic expression of CPZ induces *Pax3* in the hypaxial dermomyotome and evokes dysmorphogenesis of scapula and ribs

The expression of *cCPZ* in somites suggests a role for this enzyme in the development of the axial skeleton. In order to test this we reasoned that ectopic expression of CPZ in the chick embryo might specifically affect the development of these structures. RCAS virus containing the *cCPZ* open reading frame was injected into the segmental plate of HH

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cCPZ    1 MVPSLLLLLTGLFRATEPAPRCETGQETLG------QCQTAQKAKCVDISLSSCTDVTYT
hCPZ    1 .P.PP....LTVLVVAAAR.G..FER--------------NPA.T...LQ.RT.S.AA.N
rCPZ    1 .PTTP...AALAAL.ALAVAAYSSCSPGPDPSGKCQRLASTHS.T...LH.RT.A.AA.N

          ------------------- CRD -----------------------------------
cCPZ   55 QTMYPNFLDQKSREVIEYSSEYILISVLHNLLQGECNPDLRLLGCSVLAPQCEKDKVIKP
hCPZ   47 H.TF..L.QHR.W..V.A......L....Q..E.Q..........A....R..GGW.RR.
rCPZ   61 H.SF.TP.EHR.W.AV.A.P..T.LG...F..E.Q...............R.QGGHTQR.

cCPZ  115 CRHVCENLKKNCLSAFDAIDMAWPYFLDCDRFFAGEEEGCFDPLAKLRGEVAVEEDLPSD
hCPZ  107 ...I..G.REV.QP...............H.Y.TR.D...Y...E....GLEAD.A...G
rCPZ  121 ..R...G.REA.QP...............TQY..P.....Y...EQ....LD...A...G

cCPZ  175 FPATFIQFKHHSYSQMVSTLKKTASRCSHIATTYSIGRSFEGKDLFVIEFSTKPGHHELL
hCPZ  167 L.P...R.S....A...RV.RR.....A.V.R........D.RE.L.....SR..Q...M
rCPZ  181 L.P...R.A....A...RV..R..A...QV.K.............V.....SR..Q...M

cCPZ  235 KPEFKYIGNMHGNEVVGKELLYTLRSICVQKYLLGNPRIQTLINNTRIHLLPSLNPDGYE
hCPZ  227 E..V.L...I.....A.R.M.IY.AQYLCSE.........R.L.T........I......
rCPZ  241 E..V.L...I.....A.R.I.IY.AQYLCSE.........R.L.T........M......

cCPZ  295 RAAEEGAGYNGWVIGRQTAQNLDLNRNFPDLTSEAYRRAGIRGARLDHIPIPQSYWWGKV
hCPZ  287 V..A........TS...N................Y..L.ET....S.......H......
rCPZ  301 V..A........TS...N................Y..L.ST..V.T.....S.Y......

          ————————————— carboxypeptidase domain ———————————————
cCPZ  355 APETKAVMKWMRSIPFVLSASLHGGELVVTYPYDYSRHPMEEKEFSPTPDEKMFKMLAKA
hCPZ  347 ......I....QT............D...S..F.F.K..Q...M...........L.SR.
rCPZ  361 ......I...IQT............D...S..F.F.K..H...M...........L..R.

                                                              ↓
cCPZ  415 YADAHPVISDRSEHRCGGNFVKRGGIINGAEWYSFTGGMADFNYLHTNCFEVTVEVGCEK
hCPZ  407 ...V..MMM....N......L...S.....D........S...........I...L..V.
rCPZ  421 ...V..MMM....N......L...S.....D........S...........I...L..V.

cCPZ  475 FPLEEELFTIWHENRDALLNYMEMVHRGIKGIVSDKFGNPIKNARISVRGIQHDITTAAD
hCPZ  467 ..P..A.Y.L.QH.KES...FV.T.......V.T....K.V.......K..R......P.
rCPZ  481 ..P..A.YGL.QH.KEP...FL.........V.T..Y.K.V.....L.K..R..V...P.

cCPZ  535 GDYWRLLPPGTYIVTAQAMGYTKVMKRVTLPIKMKRAGRVDFVLRPIEIWPNKLLRRPME
hCPZ  527 ..........IH..I...P..A..I.K.II.AR.........I.Q.LGMG.KNFIHGLRR
rCPZ  541 ..........SH..I...P..S.......I.LR..........I.Q.LGTG.KNF.PG.SR

cCPZ  595 DMYDQYDPLELFDPHAQHAQARGGS---QQVREKPWWWSYFSSLDLHKPLWLLKQH
hCPZ  587 TGPHDPLGGASSLGE.TEPDPLRARRQPSADGS........T..ST.R.R....Y-
rCPZ  601 ALPRSL..QGAPAQLDFEPPRARRQ---PASGS.....A..T..SP...R....Y-
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Fig. 1. Comparison of the predicted amino acid sequence of chicken CPZ with its putative human and rat orthologs. A dashed line marks the cysteine-rich-domain (CRD) in which bold and underlined residues indicate conserved cysteines, and a solid line indicates the carboxypeptidase domain. Catalytic important residues in this domain are marked in bold and underlined. An arrow indicates Glu^{469}, the residue required for catalytic activity of cCPZ.

stage 10 chick embryos in the presumptive wing region. Embryos were harvested 48-60 hours after injection. In most cases they showed a high level of *cCPZ* expression across 2-4 somites and in the lateral plate at the level of the wing bud (Fig. 3A). Expression of *cCPZ* was not seen in the somites on the non-injected side of the embryo (Fig. 3B,C). Transverse sections through whole mounts showed that virally mediated *cCPZ* expression occurred in epaxial, central and hypaxial dermomyotome but not in the sclerotome (Fig. 3C). Such targeted expression to dermomyotome by RCAS virus injected into the segmental plate has also been reported for *sonic hedgehog* (Johnson et al., 1994). The ectopic expression of *cCPZ* in the dermomyotome prompted us to search for changes of expression of dermamyotomal marker genes. Expression of *myoD*, *myf5* or *myogenin* was not changed (n=10 for each gene, data not shown), but the expression of *Pax3* was markedly affected. At the wing level, *Pax3* is normally expressed in the epaxial portion of the dermomyotome (Fig. 3F). Overexpression of *cCPZ* in the dermomyotome resulted in ectopic expression of *Pax3* in the hypaxial dermomyotome (Fig. 3D,F; 16 out of 31 injected embryos). Of note, overexpression of *cCPZ* in dermomyotome did not alter either the normal expression of *Pax1* in the sclerotome or, at this stage, induce *Pax1* in the dermomyotome (n=10, data not shown).

Metallocarboxypeptidases are characterized by a conserved glutamic acid residue that is required for enzymatic activity. Substitution of this Glu residue with a Gln abolishes the activity of CPE, but does not affect the binding of synthetic peptide substrates (Qian et al., 1999). If cCPZ functions as an enzyme, one would expect that loss of its catalytic activity should manifest itself by an inability to induce *Pax3* expression. We therefore mutated the corresponding residue Glu^{469} of cCPZ to a Gln, and produced an RCAS virus capable of expressing this mutant form of *CPZ*. Virus overexpressing CPZ^{E469Q} was injected in the same site as the normal *CPZ* virus. 48-60 hours after injection, we observed levels of mutant *CPZ* expression similar to those described for the wild-type virus (not shown). However, we did not detect ectopic expression of *Pax3* in hypaxial dermomyotome (n=15, data not shown).

The appearance of *Pax3* mRNA in the hypaxial dermomyotome may reflect the possibility that ectopic CPZ evokes a change in the fate of hypaxial mesodermal cells. In turn, this may affect the development of the scapula blade known to derive from this tissue (Huang et al., 2000). In the chicken, the scapula consists of a head (acromium) and a blade that are connected by the 'neck' of the scapula (Baumel and Witmer, 1993; Ede, 1964). When *cCPZ*-injected embryos were examined at day 10, 55% (12 out of 22 injected embryos) showed a truncation of the scapular blade (Fig. 4A,B). We also noticed that ectopic expression of cCPZ causes truncation or loss of rostralmost ribs (10 out of 22 injected embryos; Fig. 4A,D). Injection of a retrovirus encoding alkaline phosphatase as a control had no effect on morphogenesis of the scapula or ribs (n=22, not shown). When embryos injected with CPZ^{E469Q} were allowed to develop to day 10, we observed a much lower frequency and severity of skeletal malformations (3 out of 22 injected embryos). One embryo had a partial loss of the distalmost part of the blade of the scapula and the other two embryos exhibited a slight outward bending of the scapula (not shown).

At HH stage 26, *Pax1* is expressed in a stripe of mesenchymal cells located lateral to somites 17-20. These *Pax1*-positive cells derive

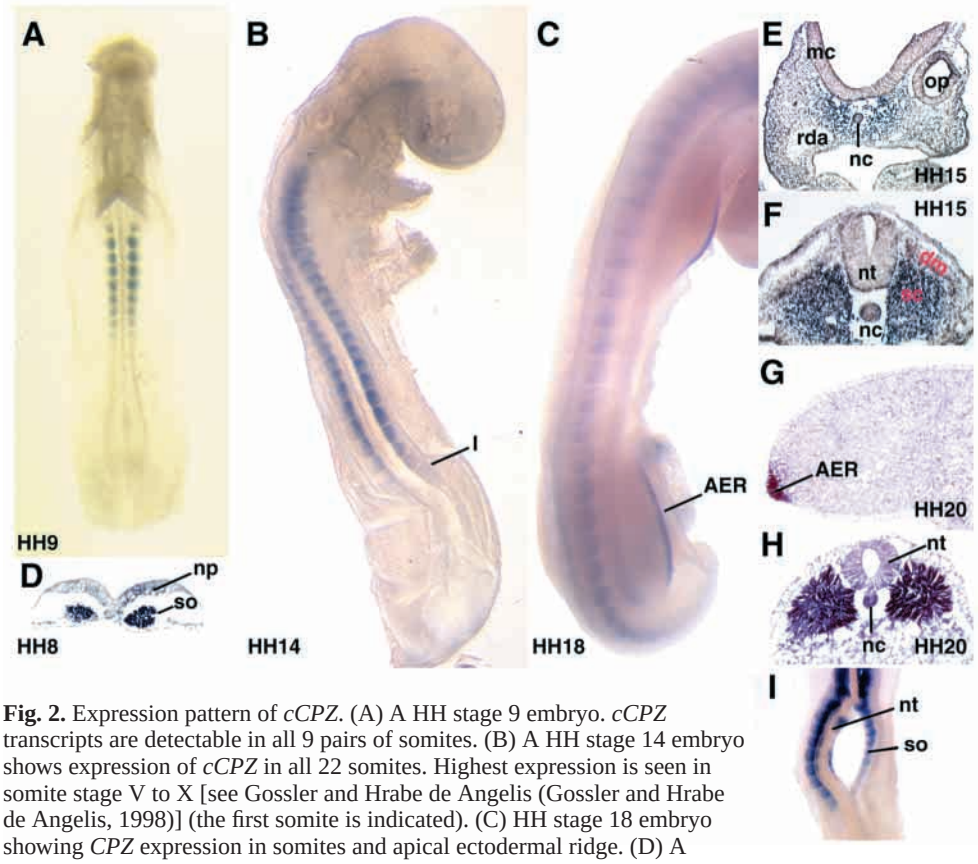

Fig. 2. Expression pattern of *cCPZ*. (A) A HH stage 9 embryo. *cCPZ* transcripts are detectable in all 9 pairs of somites. (B) A HH stage 14 embryo shows expression of *cCPZ* in all 22 somites. Highest expression is seen in somite stage V to X [see Gossler and Hrabe de Angelis (Gossler and Hrabe de Angelis, 1998)] (the first somite is indicated). (C) HH stage 18 embryo showing *CPZ* expression in somites and apical ectodermal ridge. (D) A transverse section through a HH stage 8 embryo at the level of the second pair of somites. *cCPZ* is expressed throughout the entire epithelial somite. (E) A transverse section through the head of a HH stage 15 embryo at the level of the otic placode. *cCPZ* is expressed in paraxial head mesoderm that surrounds the notochord. (F) Section through a HH stage 15 embryo at the interlimb level. Note *cCPZ* expression is restricted to the sclerotome. (G) Section through a leg bud of a HH stage 20 embryo. *cCPZ* is strongly expressed in the AER. (H) Transverse section through a HH stage 20 embryo at the tailbud level. The entire epithelial somite expresses *cCPZ*. (I) *cCPZ* is not regulated by signals from the neural tube and/or notochord, because this gene is expressed in somites derived from segmental plate that had been separated from midline structures by HH stage 10. AER, apical ectodermal ridge; dm, dermomyotome; mc, myelencephalon; nc, notochord; np, neural plate; nt, neural tube; op, otic placode; rda, right dorsal aorta; sc, sclerotome; so, somite.

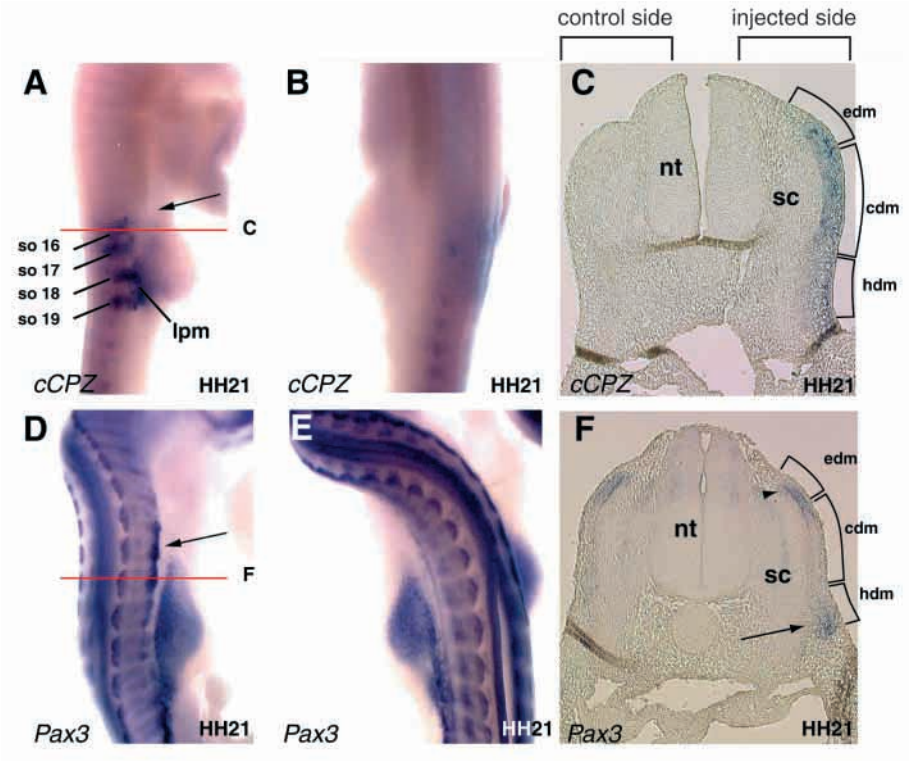

Fig. 3. Overexpression of CPZ in somites induces *Pax3*. (A) Whole-mount in situ hybridization with a *cCPZ* probe reveals strong ectopic *cCPZ* expression in somites 16-19 on the injected side of the embryo. (B) Absence of ectopic expression of *cCPZ* on the uninjected side of the same embryo. (C) Transverse section at level of red line in A. Overexpression (blue) of *cCPZ* is restricted to the dermomyotome. (D) Ectopic expression of *Pax3* in the hypaxial dermomyotome (arrow). (E) On the uninjected side of the same embryo as in D no ectopic *Pax3* expression is seen. (F) Transverse section at level of red line in D. Note ectopic *Pax3* expression in hypaxial dermomyotome (arrow); also note native epaxial Pax3 expression which is not altered by *cCPZ* overexpression (arrowhead). Embryos are at HH stage 21 at which time the endogenous expression of *cCPZ* has already declined at the axial levels depicted. Brackets in C and F delineate the subdivisions of the dermomyotome. cdm, central dermomyotome; edm, epaxial dermomyotome; hdm, hypaxial dermomyotome; lpm, lateral plate mesoderm; nt, neural tube; dm, dermomyotome; sc, sclerotome; so, somite.

from hypaxial dermomyotome and are thought to give rise to the blade of the scapula (Huang et al., 2000). Because in *cCPZ*-treated embryos the hypaxial dermomyotome expresses *Pax3* and the blade of the scapula is missing, one would predict that the *Pax1*-positive stripe may also be affected. Indeed, we noted absence of the stripe of *Pax1* expression in 50% of injected embryos (n=12) (Fig. 4C,D), a frequency similar to that seen for skeletal defects.

CPZ promotes Wnt4 based gene induction

The above experiments demonstrate striking effects of ectopic CPZ expression on *Pax3* expression in hypaxial dermomyotome. *Pax3* had previously been shown to be regulated by Wnt signals (Fan et al., 1997). This finding and the presence of a CRD in CPZ, which in other proteins was shown to bind Wnt ligands (see Introduction), prompted us to hypothesize that CPZ plays a role in Wnt signaling. To assess whether CPZ can influence Wnt signaling, we adapted a paracrine in vitro Wnt assay (Lickert et al., 2000). In this assay transfected Wnt secreting NIH-3T3 feeder cells were cocultured with murine ES cells. Wnts secreted by the feeder cells induce the homeobox gene *Cdx1* in ES cells. In order to test whether CPZ modulates Wnt signaling we added HEK-293 cells stably expressing murine CPZ to the culture and measured *Cdx1* induction. All CPZ HEK-293 cell lines generated exhibited similar levels of CPZ protein expression (see Fig. 5A for five representative cell lines). Immunolocalization studies of CPZ-producing HEK-293 cells showed that CPZ localizes to the endoplasmic reticulum (not shown). Cell extraction further demonstrated that CPZ is present in the extracellular matrix (Fig. 5A).

Next, CPZ HEK-293 or control HEK-293 cells were grown in culture dishes for a day. Cells were washed off with 1 mM EDTA in PBS leaving ECM on the plates. This created two types of conditioned plates, one containing ECM with attached CPZ (Fig. 5A), and a second type with unmodified ECM. The former plates were seeded with Wnt4-expressing NIH-3T3 cells, CPZ HEK-293 cells and ES cells, while the latter plates were seeded with Wnt4-expressing NIH-3T3 cells, HEK-293 cells and ES cells. After 8 hours of co-culture *Cdx1* induction was quantified by quantitative PCR. We found that *Cdx1* induction was increased by as much as 50 percent in the presence of CPZ-containing ECM and CPZ producing HEK-293 cells (Fig. 5B, compare bars 4 and 5). We also seeded CPZ HEK-293 conditioned plates with Wnt-4 producing NIH-3T3 cells and ES cells. This still resulted in *Cdx1* induction (Fig. 5B, bars 4 and 7). These results indicate that CPZ protein present in the extracellular matrix is sufficient to enhance Wnt4 signaling. Cells expressing CPZ bearing an active site glutamate to glutamine substitution did not potentiate *Cdx1* induction (Fig. 5B, bars 4 and 6) suggested that the catalytic activity of CPZ is required for this effect. *Cdx1* induction in ES cells strictly depends on Wnt4. No *Cdx1* induction was observed in ES cells cocultured with CPZ HEK-293 cells and NIH-3T3 cells containing *lacZ* instead of *Wnt4* (Fig. 5B, compare bars 1 and 2). Similarly, ECM generated by CPZ HEK-293 cells did not induce *Cdx1* in this system (Fig. 5B, compare bars 1 and 3). Wnt1 and Wnt3a were also tested in this assay but *Cdx1* induction was not potentiated by CPZ (data not shown).

CPZ binds to Wnt4 via its cysteine-rich domain

The potentiation of Wnt4 signaling by CPZ raised the question of whether CPZ can directly bind to Wnt4 and if so, which part of the protein may mediate this interaction. To answer this questions we performed co-immunoprecipitation experiments

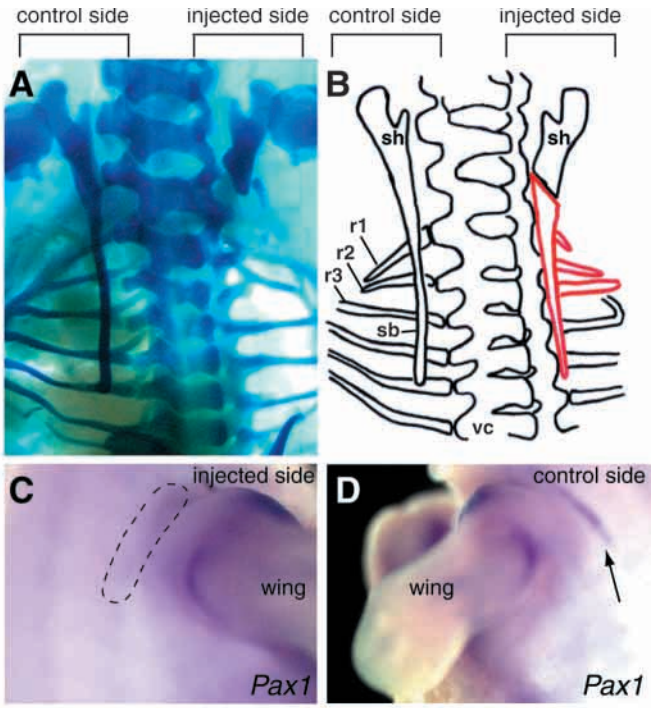

Fig. 4. Overexpression of CPZ in somites leads to scapular and rib defects on the injected side of the embryo. (A) Dorsal view of a 10-day old embryo injected with RCAS-*cCPZ* stained and cleared for visualizing skeletal structures. The scapula blade and the first three ribs of the embryo are severely truncated on the injected right side. (B) Line drawing of the skeletal preparation in A. (C) Injected side of a 5.5-day old embryo. *Pax1* expression is absent in a region that normally contains the developing scapula (dashed outline). (D) Untreated control side of the same embryo shows a *Pax1*-expressing stripe of mesenchymal cells (arrow) that will give rise to the blade of the scapula. sb, scapula blade; sh, scapula head (acromium); r1, 2, 3 , first, second and third rib; vc, vertebral column.

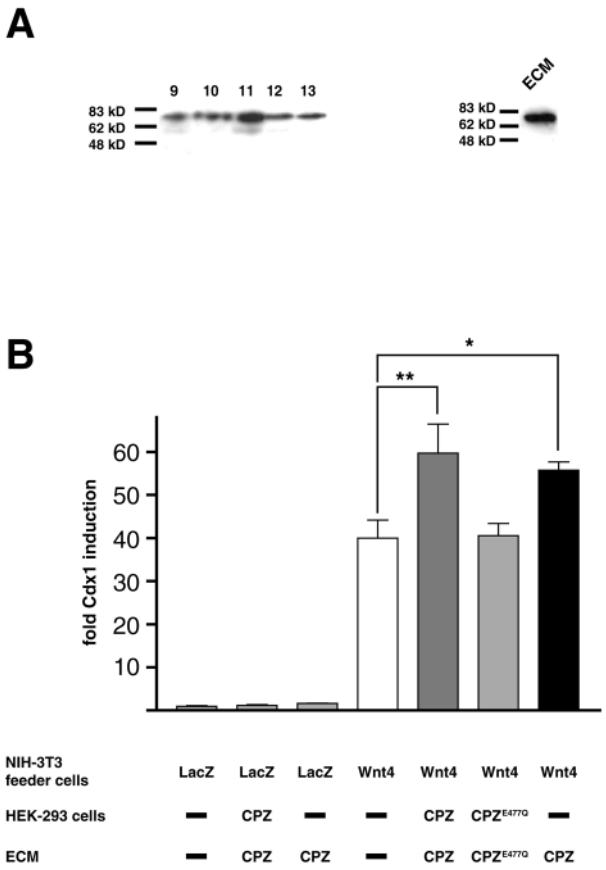

Fig. 5. *CPZ*-expressing cells promote *Cdx1* induction by Wnt4. (A) Cell lysates of five representative stably transfected HEK-293 cell clones express similar amounts of CPZ protein of the appropriate size (~75 kDa). The protein is found in extracts of the ECM. (B) Induction of *Cdx1* in ES cell co-cultured with different mixtures of Wnt4- and CPZ-producing cells (for experimental details see Materials and methods and Results). The matrix below the diagram indicates the components present in the cocultures. ECM deposited on the culture dish was generated by untransfected HEK-293 (–) or by cells expressing wild type (CPZ) or mutant (CPZ^{E477Q}) protein. Subsequent co-culture used NIH-3T3 cells that produced either β-gal (LacZ) (negative control) or Wnt4 together with HEK-293 cells transfected with wild-type (CPZ), mutant (CPZ^{E477Q}) CPZ protein or untransfected HEK-293 cells (–). Error bars represent standard deviation, *P* values smaller than 0.05 (bars 4 and 7) are indicated by one asterisk, *P* values smaller than 0.01 by two asterisks (bars 4 and 5) (Student's *t*-test).

from cells co-expressing HA-tagged $Wnt4^{HA}$ (Lescher et al., 1998) and myc-tagged CPZ (Fig. 6A, CPZ^{myc}). pcDNA3.1 expression vectors containing the appropriate ORFs were cotransfected into HEK-293 cells and complexes were precipitated using anti-HA antibody. Precipitated proteins were separated by PAGE, western blotted and an antibody directed against the myc-epitope was used to detect CPZ. Analyses of cell lysates demonstrated that CPZ^{myc} of the correct size was expressed (Fig. 6B, lane 2). Importantly, analysis of the co-precipitate demonstrated the presence of a $CPZ^{myc}/Wnt4^{HA}$ complex (Fig. 6C, lane 2). A myc-tagged sFRP-2 (Fig. 6A) served as a positive control [specific binding of sFRP-2 to Wnt4 had been described previously (Lescher et al., 1998)]. As shown in Fig. 6B,C sFRP-2 was produced (lane 1) and co-precipitated with Wnt4 (lane1). $CPZ^{myc/E477Q}$, bearing the above described inactivating point mutation, co-precipitated with $Wnt4^{HA}$ suggesting that the catalytic activity of CPZ is not required for Wnt4 binding (Fig. 6B,C, lanes 3). However, the CRD of CPZ is required for the interaction with Wnt4. In the absence of this domain, as in the constructs $CPZ^{\Delta CRD/myc}$ (Fig. 6A) and $CPZ^{\Delta CRD/myc/E477Q}$, CPZ could not be co-precipitated with $Wnt4^{HA}$ (Fig. 6C, lanes 4 and 5) although both mutant proteins were present in the lysate (Fig. 6B, lanes 4 and 5). In contrast, $Wnt4^{HA}$ co-precipitated with a CPZ lacking the carboxypeptidase domain ($CPZ^{\Delta CPD/myc}$) (see Fig. 6B,C, lanes 6). None of the co-immunoprecipitated proteins was unspecifically bound to Protein-G agarose or was unspecifically precipitated by the anti-HA antibody alone (data not shown).

Taken together these data suggest that Wnt4 and CPZ can interact, that this interaction is mediated by the CRD of CPZ and that catalytic activity is not required for binding per se (although such activity is obviously required for CPZ function, see above). Since co-precipitation experiments were performed from cell lysates we cannot rule out the possibility that CPZ-

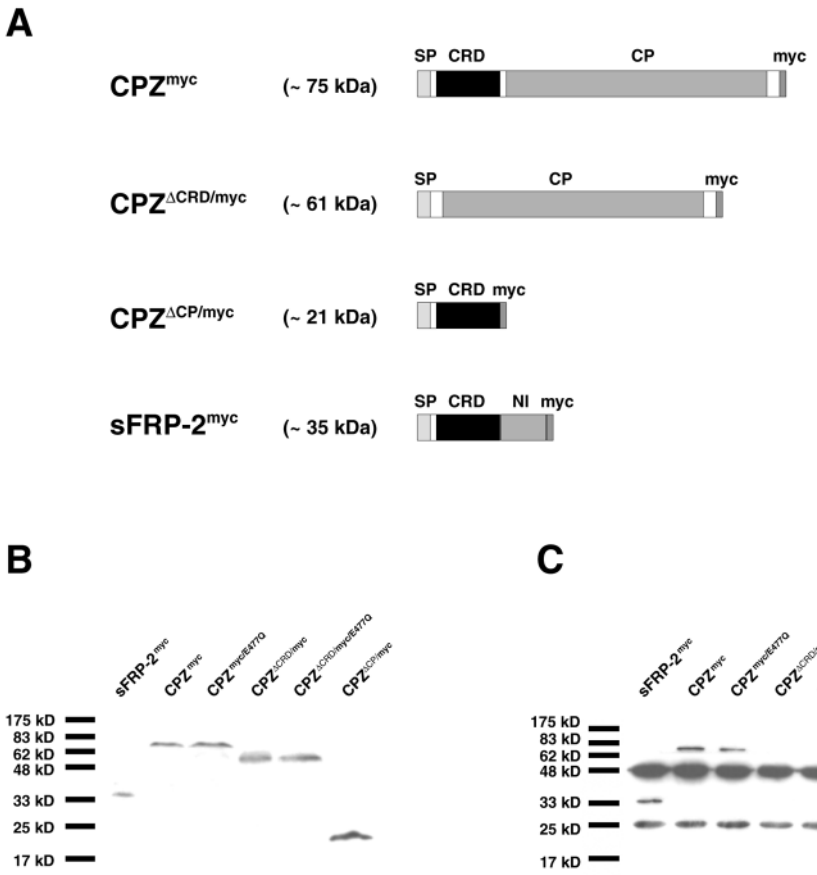

Fig. 6. Evidence for binding of Wnt4 to the CRD of CPZ. (A) Schematic illustration of the constructs tested in co-immunoprecipitation assays. The domains of the proteins are indicated (for abbreviations see below). The CPZ constructs bearing the point mutation of Glu^{477} to Gln^{477} ($CPZ^{myc/E477Q}$ and $CPZ^{\Delta CRD/myc/E477Q}$) are not depicted. (B) Western blots of lysates of cells co-transfected with $Wnt4^{HA}$ and the constructs depicted in A detected with an anti-myc antibody. All proteins were expressed and had the expected molecular mass (see A). (C) A western blot of proteins of the lysates in B after co-precipitation with $Wnt4^{HA}$, detected with an anti-myc antibody. sFRP-2^{myc} (positive control), CPZ^{myc}, $CPZ^{myc/E477Q}$ and $CPZ^{\Delta CP/myc}$ co-precipitated with $Wnt4^{HA}$. In contrast, the CRD deletion mutants, $CPZ^{\Delta CRD/myc}$ and $CPZ^{\Delta CRD/myc/E477Q}$, did not bind to $Wnt4^{HA}$. Note that the two bands at 25 kDa and 50 kDa in C are the immunoglobulin heavy and light chain of the primary antibody, which are also detected by the secondary antibody which is derived from the same species as the primary antibody. Since the sizes of these bands differ from those of the tested proteins, they do not interfere with detection of these proteins, but serve as a loading control. CP, carboxypeptidase domain; CRD, cysteine-rich-domain; Ni, netrin-like domain; SP, signal peptide.

Wnt4 complexes contain additional factors mediating CPZ-Wnt4 binding.

Discussion

We report a series of experimental studies that investigated the developmental and biochemical function of carboxypeptidase Z (CPZ). We show that *cCPZ* expression is restricted to epithelial somites, sclerotome, paraxial head mesoderm and the AER of the limb bud. Ectopic expression of *cCPZ* in the dermomyotome at the wing level has dramatic effects on the expression of *Pax1* and *Pax3*, and on the morphogenesis of scapula and rostral ribs.

It has previously been shown that hypaxial dermomyotome-derived *Pax1*-expressing cells give rise to the scapular blade (Huang et al., 2000). In embryos expressing *cCPZ* in the dermomyotome, ectopic *Pax3* expression was induced in presumptive scapula cells of the hypaxial dermomyotome, concomitant with a loss of *Pax1* expression in the descendants of these cells. These changes in the gene expression program are likely to underlie the severe dysmorphogenesis of the blade of the scapula and of the distal portion of the ribs. *Pax3* expression in paraxial mesoderm is known to be regulated by Wnt signals (Fan et al., 1997). Such a Wnt signal may be affected by ectopically expressed cCPZ. Support for this possibility comes from our observations that CPZ enhances Wnt4 signaling and that it binds to Wnt4 via its cysteine-rich domain (CRD). Except for binding to Wnt4, all effects described in this study require CPZ to be catalytically active.

CPZ and Wnt signaling

It has recently been suggested that CPZ processes Wnt signals (see Reznik and Fricker, 2001). This view is based on the fact that CPZ harbors a CRD domain. Such a domain is found in several other proteins (including Frizzled and sFRPs) known to directly interact with Wnts. The present study provides three lines of experimental evidence for a distinct role of CPZ in Wnt signaling. First, it is shown that CPZ potentiates the activation of a Wnt reporter gene, *Cdx1*, in an in vitro assay. In addition, ectopic expression of CPZ in dermomyotome induces *Pax3*, a Wnt response gene (Fan et al., 1997; Lee et al., 2000). Finally, evidence is provided that CPZ binds to Wnt4 and that this interaction occurs through the CRD of CPZ.

In the following section, we discuss mechanisms by which CPZ could participate in Wnt signaling. Wnt molecules are locally released from cells, diffuse into the extracellular space, bind to Frizzled transmembrane receptors and through a β-catenin or a 'non-canonical' pathway regulate gene expression (for reviews, see Wodarz and Nusse, 1998; Borycki and Emerson, 2000). In the extracellular space, Wnts also bind to soluble Frizzled related proteins (sFRPs) that sequester Wnt from binding to their cognate receptors (for reviews, see Wodarz and Nusse, 1998; Bejsovec, 2000; Pandur et al., 2002; Lee et al., 2000). Where in this complex inter- and intracellular signaling process could CPZ play a role? Three obvious, not necessarily mutually exclusive mechanisms of action can be envisaged. (1) CPZ could degrade components of the extracellular matrix, thereby enhancing the availability of Wnt molecules [for an example, see Dhoot et al., 2001)]. (2) CPZ

could proteolytically process sFRPs and thereby affect their affinity for Wnts. A precedent for such a mechanism is provided by the BMPs that bind to chordin which is proteolytically cleaved by the protease BMP-1/tolloid, allowing BMPs to bind to their cognate receptor (for review, see Nakayama et al., 2000). (3) Wnts and CPZ could directly interact. Our experiments provide evidence for the latter mechanism, as we show that Wnt4 and CPZ can be co-precipitated from mammalian cell extracts. Of course, we cannot rule out that other components are required for a Wnt4-CPZ-interaction to occur. A direct in vitro binding study could address this issue, but in vitro production or purification of Wnt proteins has remained elusive. Binding of Wnt4 to CPZ may result in quenching of Wnt signaling. We deem this less likely, because CPZ does not abolish, but enhances Wnt4 signaling in our in vitro assay. An additional argument against a quenching mechanism arises from our observation, that enzymatically inactive CPZ fails in our functional studies. Quenching would not depend on such a catalytic activity. Hence we favor a mechanism in which binding of Wnt4 to CPZ represents a first step followed by proteolytic processing of the Wnt4 ligand. Song and Fricker (Song and Fricker, 1997) have shown that CPZ effectively hydrolyses peptides carrying a C-terminal arginine. Intriguingly, among the three Wnt molecules tested in our in vitro assay (Wnt1, Wnt3a and Wnt4) only Wnt4 has a C-terminal arginine and only the combination of Wnt4 and CPZ potentiates *Cdx1* induction. Wnt8C from chicken is the only other Wnt carrying a C-terminal arginine residue Hume and Dood, 1993).

Ectopic CPZ expression and the formation of the blade of the scapula and of ribs

Most of the dermomyotome should give rise to muscles with exception of the hypaxial dermomyotome at somite levels 17 to 24 from which the blade of the scapula arises (Huang et al., 2000). It has been proposed that downregulation of *Pax3* expression in the hypaxial dermomyotome prevents this tissue from committing to a myogenic fate (Huang et al., 2000). Instead, signals from ectoderm would trigger a chondrogenic fate in the hypaxial dermomyotome, as shown by the finding that cells descending from this region switch on *Pax1* (Huang et al., 2000). In embryos expressing *CPZ* throughout the dermomyotome, *Pax3* is induced in the hypaxial portion of the dermomyotome suggesting that these cells do not acquire a chondrogenic fate. It has been shown that Wnt molecules, including Wnt4, induce Pax3 in the presomitic mesoderm (Fan et al., 1997; Lee et al., 2000). Ectopic expression of CPZ in the dermomyotome may thus lead to greater activation of a Wnt signal emanating from surrounding tissues, most probably the ectoderm. This subsequently causes a change in the developmental fate of this tissue and thus prevents morphogenesis of the blade of the scapula. At the molecular level this is reflected by ectopic activation of *Pax3* in earlier stages (HH 21-22) followed by the downregulation of *Pax1* in cells originating from the hypaxial dermomyotome and fated to form the scapula blade. It remains to be seen whether these changes in Pax gene expression are indeed causing the observed morphological defects. Of note, *Pax1*$^{-/-}$ mice lack part of the spine of the scapula, a structure homologous to the avian scapular blade (Wilm et al., 1998).

Ectopic CPZ expression also results in the partial (i.e. distal) or complete loss of the rostralmost ribs. Ribs are thought to either exclusively derive from the sclerotome (Huang et al., 2000b; Evans, 2003) or from sclerotome and dermomyotome (Kato and Aoyama, 1998). Sclerotome normally expresses *CPZ* and it is thus unlikely that ectopic *CPZ* per se causes the observed rib defects. Since ectopic CPZ is expressed in dermomyotome, one possibility is that this leads to an excessive or 'ectopic' activation of Wnt signaling. In turn, this may influence the differentiation of the lateral part of the sclerotome, resulting in partial or complete absence of ribs. If one assumes that part of the ribs derive from the dermomyotome (Kato and Aoyama, 1998), the rib defects can be readily explained along the lines discussed for the blade of the scapula. Ectopic *CPZ* may upregulate *Pax3* and thereby abolish rib chondrogenesis.

Function of native CPZ

So far the developmental function of CPZ has been discussed in the context of overexpression in the dermomyotome. However, *CPZ* transcripts are normally expressed in the epithelial somites and in the sclerotome. Because CPZ protein is secreted, it may also act in tissues adjacent to the sclerotome. In fact, our in vitro studies find CPZ in the extracellular matrix of CPZ-producing HEK-293 cells. Since Wnt molecules are also secreted factors acting over a certain distance (Fan et al., 1997) CPZ could encounter Wnts that are released from surrounding tissues, e.g. the surface ectoderm (see below) or it could interact with Wnt molecules that are directly expressed in the somites, such as *Wnt5a* and *Wnt11* (Cauthen et al., 2001). If one assumes that CPZ functions in tissue adjacent to the sclerotome, such as in the dermomyotome or mesenchymal cells in this region, CPZ could interact with a number of Wnt molecules. *Wnt4* is expressed in the dorsal neural tube of the mouse and chicken embryo as well as the surface ectoderm of the mouse embryo (Parr et al., 1993; Cauthen et al., 2001). In addition to *Wnt4*, several other Wnts are expressed in, and hence released by, the surface ectoderm, the dorsal neural tube [e.g. *Wnt6* and *Wnt7a*, (see Parr et al., 1993; Cauthen et al., 2001; Tajbakhsh et al., 1998)], and in the dermomyotome [e.g. *Wnt11* (see Tanda, 1995; Marcelle et al., 1997)].

A future challenge remains: the identification of endogenous substrates of CPZ. Although our data suggest that Wnt4 may represent such a substrate, definitive biochemical proof is still lacking. In addition, the relevance of proteolytic processing for binding of Wnts to their cognate receptors remains to be explored.

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