The syndecan proteoglycans are an ancient class of receptor, bearing heparan sulfate chains that interact with numerous potential ligands including growth factors, morphogens, and extracellular matrix molecules. The single syndecan of invertebrates appears not to have cell adhesion roles, but these have been described for mammalian paralogues, especially syndecan-4. This member is best understood in terms of interactions, signaling, and structure of its cytoplasmic domain. The zebrafish homologue of syndecan-4 has been genetically linked to cell adhesion and migration in zebrafish embryos, but no molecular and cellular studies have been reported. Here it is demonstrated that key functional attributes of syndecan-4 are common to both zebrafish and mammalian homologues. These include glycosaminoglycan substitution, a NXIP motif in the extracellular domain that promotes integrin-mediated cell adhesion, and a transmembrane GXXXG motif that promotes dimer formation. In addition, despite some amino acid substitutions in the cytoplasmic domain, its ability to form twisted clamp dimers is preserved, as revealed by nuclear magnetic resonance spectroscopy. This technique also showed that phosphatidylinositol 4,5-bisphosphate can interact with the zebrafish syndecan-4 cytoplasmic domain, and that the molecule in its entirety supports focal adhesion formation, and complements the murine null cells to restore a normal actin cytoskeleton.

Syndecans are heparan sulfate bearing type-1 transmembrane proteins intimately associated with cell adhesion and linkage to the cytoskeleton. In mammals there are four syndecan family members, syndecan-1, -2, -3, and -4. These are characterized by a short highly conserved cytoplasmic domain, a transmembrane domain, and a less conserved ectodomain that is substituted with heparan sulfate chains (for reviews, see Refs. 1–3). Syndecan-4 is expressed in nearly all cell types and tissues and is a focal adhesion component (4–5). In fibroblasts seeded on individual fibronectin domains there is a requirement for the engagement of syndecan-4 through its heparan sulfate chains for focal adhesion formation. Cells seeded on the RGD containing integrin-binding domain of fibronectin only form focal adhesions after the addition of the Hep II fibronectin heparin-binding domain (6, 7). In the syndecan-4 knock-out mouse vascular, wound healing, and migration defects have been reported and the ability of syndecan-4 null fibroblasts to form focal adhesions in response to fibronectin fragments is compromised (8).

The syndecan-4 cytoplasmic domain shares two highly conserved regions, the C1 and C2, with the other syndecan family members and they flank a variable region (V region) unique to syndecan-4. The membrane distal C2 region interacts with PDZ proteins such as syntenin, CASK, and GIPC (9–11). Contained within the V region is the KXXXXX motif that binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2)3 and this interaction causes shape change and promotes oligomerization of full-length syndecan-4 peptides (12). The V region of syndecan-4 also interacts with protein kinase Ca and in the presence of PtdIns(4,5)P2, the activity of this enzyme is up-regulated and this interaction is the basis of syndecan-4 signaling (13, 14). The actin bundling protein α-actinin also associates with this region of the syndecan-4 cytoplasmic domain (15). Peptides corresponding to the syndecan-4 cytoplasmic domain form intertwined dimers with a symmetric clamp topology as revealed by the solution structure of these molecules (12). All syndecan core proteins including syndecan-4 form SDS-resistant dimers, which is mediated by the transmembrane domain (16, 17). The substitution sites for the heparan sulfate chains of syndecan-4 are situated toward the N-terminal end of the mature protein. In addition, the ectodomain has a cell binding domain that when expressed as a bacterial fusion protein can support integrin-mediated cell adhesion (18, 19).

Syndecan-4 signaling and its role in cell adhesion is understood in mammalian cell culture model systems. However, little...
is understood in terms of its role in development. Syndecan-4 has been identified as being important for convergent extension movements in developing *Xenopus* embryos and has recently been shown to be essential for neural crest migration in zebrafish (20, 21). Only three syndecan homologues are present in the zebrafish genome, these are syndecan-2, -3, and -4 (22). Syndecan-2 is necessary for the efficient formation of angiogenic sprouts in zebrafish embryos and is also important for left-right axis formation in *Xenopus* (23, 24). In invertebrates, syndecans are mainly associated with growth factor interactions rather than cell adhesion. Although syndecan-4 is important for cell movements in lower vertebrates little is known about its structure and function. This is made more relevant given that the zebrafish syndecan-4 cytoplasmic domain has altered sequence composition compared with mammals. Here zebrafish syndecan-4 is shown to possess many of the cell adhesion properties of its mammalian homologue. We also show that the solution structure of the zebrafish syndecan-4 cytoplasmic domain also shows considerable similarities to mammalian syndecan-4 and can interact with PtdIns(4,5)P₂ despite sequence differences.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—CHO-K1 cells and Swiss 3T3 cells were grown in Ham’s F-12 (Cambrex) and Dulbecco’s modified Eagle’s medium (Cambrex), respectively. Both media were supplemented with 10% fetal bovine serum. Syndecan-4 modified Eagle’s medium (Cambrex), respectively. Both media were grown in Ham’s F-12 (Cambrex) and Dulbecco’s modified Eagle’s medium (Cambrex) with 10% fetal calf serum (8).

**Production of zSDC-4 Ectodomain GST Fusion Proteins**—The plasmid pGSTz4EDANQP (1) was made using PCR mutagenesis of pGSTz4ED (19) with the primer pair gatgaaagggtccatttgct (forward) and attcacaaattcatcctcaatatc (reverse). Primers were designed to generate an in-frame deletion of the NQIP motif within the zebrafish syndecan-4 ectodomain. PCR products were digested with DpnI and ligated and transformed using procedures described in Ref. 19. Wild type GSTz4ED and the mutant GSTz4EDΔNQIP proteins were purified from *Escherichia coli* cell lysates as follows. Cells were grown to an A₆₀₀ of 0.6 prior to the addition of 0.25 ml/liter of 1-M isoprropyl 1-thio-β-D-galactopyranoside (Calbiochem) followed by a further 3 h of incubation. Bacteria were resuspended in PBS, lysed in a sonicator, and GST fusion proteins were purified on columns of glutathione-Sepharose 4 (GE Healthcare) using standard procedures. Attachment assays were performed as described in Ref. 19. Briefly, 24-well plates were coated overnight with 5 μg/ml human plasma fibronectin. After detachment using trypsin EDTA, cells were initially suspended in media containing serum prior to resuspension in serum-free medium at a concentration of 1 × 10⁵ cells/ml. 1 ml of cell suspension was plated into each well and the cells were photographed every 15 min for 1 h. Cell surface areas of at least 50 cells per time point were measured using the IMAGEJ software.

**Overexpression of Zebrafish Syndecan-4 in CHO-K1 Cells**—CHO-K1 cells were transfected with pIRE2-EGFP, pIRE2-Zeb4, pIRE2-Rat4 (25), and the HA tag inserted forms pIRE2-Zeb4HA or pIRE2-Rat4HA using the Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s instructions. Stable cell lines were obtained by selection with G418 (800 μg/ml).

**Scratch Wound Assays and Cell Spreading Assays**—Scratch wound assays were performed as previously described (26). Cell spreading assays were performed in six-well plates that had been coated overnight with 5 μg/ml human plasma fibronectin. After detachment using trypsin EDTA, cells were initially suspended in media containing serum prior to resuspension in serum-free medium at a concentration of 1 × 10⁵ cells/ml. 1 ml of cell suspension was plated into each well and the cells were photographed every 15 min for 1 h. Cell surface areas of at least 50 cells per time point were measured using the IMAGEJ software.

**In Vitro Transcription and Translation**—In vitro transcription and translation reactions were performed with the TnT quick coupled transcription/translation system (Promega) using linearized pBSzebSDC4pr as template. Reactions were performed in the presence of Redivue L-[³⁵S]methionine (Amersham Biosciences catalog number AG1094) and analyzed on 12.5% SDS-PAGE gels. Gels were dried and protein products visualized by autoradiography.

**TABLE 1**

Plasmids used in this study

| Plasmid                     | Description                                                                 | Reference |
|-----------------------------|-----------------------------------------------------------------------------|-----------|
| pBSzebSDC4pr                | Full-length zSyndecan-4 cDNA                                                | 19        |
| pIRE2-Zeb4                  | Full-length rat syndecan-4 cloned into pIRE2-EGFP                            | This study|
| pIRE2-Rat4                  | Full-length rat syndecan-4 cloned into pIRE2-EGFP                            | 25        |
| pIRE2-Zeb4HA                | HA-tagged zebrafish syndecan-4 derived from pIRE2Zeb4                      | This study|
| pIRE2-Rat4H4                | HA-tagged rat syndecan-4 derived from pIRE2Rat4                             | This study|
| pGSTz4ED                    | zSyndecan4 ectodomain sequence cloned in pET41(a) for expression of GST z4ED fusion protein | 19        |
| pGSTz4EDANQP                | Mutant of pGSTz4ED in which the NQIP motif has been deleted from z4ED       | This study|

**TABLE 2**

Plasmids used in this study

| Plasmid                     | Description                                                                 | Reference |
|-----------------------------|-----------------------------------------------------------------------------|-----------|
| pBSzebSDC4pr                | Full-length zSyndecan-4 cDNA                                                | 19        |
| pIRE2-Zeb4                  | Full-length rat syndecan-4 cloned into pIRE2-EGFP                            | This study|
| pIRE2-Rat4                  | Full-length rat syndecan-4 cloned into pIRE2-EGFP                            | 25        |
| pIRE2-Zeb4HA                | HA-tagged zebrafish syndecan-4 derived from pIRE2Zeb4                      | This study|
| pIRE2-Rat4H4                | HA-tagged rat syndecan-4 derived from pIRE2Rat4                             | This study|
| pGSTz4ED                    | zSyndecan4 ectodomain sequence cloned in pET41(a) for expression of GST z4ED fusion protein | 19        |
| pGSTz4EDANQP                | Mutant of pGSTz4ED in which the NQIP motif has been deleted from z4ED       | This study|
strates in serum-free media for 1 h at 37 °C after which time the media were removed and replaced with serum-free media containing 15 μM Calcein (Invitrogen) and incubated for a further 20 min. For β1 integrin blocking experiments cells were seeded on substrates as above in the presence or absence of 10 μg/ml of Ha 2/5 antibody (Chemicon). Wells were washed twice with PBS and cell incorporated fluorescence was measured using a Fluostar Galaxy Plate reader (BMG Lab technologies) at 485 nm excitation and 520 nm emission.

NMR Spectroscopy and Structure Calculation—Samples for NMR measurements were prepared by dissolving the z4L peptides in a 90% H2O, 10% D2O or 99.9% D2O solution in 50 mM potassium phosphate buffer, pH 7.0, and the final concentration was adjusted to 2–4 mM. PtdIns(4,5)P2 was purchased from Sigma and used directly for the preparation of the z4L-PtdIns(4,5)P2 complex. For NMR measurements of the z4L-PtdIns(4,5)P2 complex, 10–50 μl of concentrated PtdIns(4,5)P2 solution was added to free peptide solution up to a maximum ratio of 1:1 (PtdIns(4,5)P2:z4L). The pH of 7.0 was adjusted by adding 0.1 N HCl to the sample before NMR analyses.

All NMR experiments were performed on a Bruker DRX500 spectrometer in quadrature detection mode equipped with a triple resonance probe head with triple-axis gradient coils. All data were collected at 25 °C and the strong solvent resonance was suppressed by WATERGATE pulse sequence (27) combined with pulsed field gradient pulses. Mixing times of 100–400 ms were used in collecting two-dimensional NOESY spectra (28). Two-dimensional total correlation spectroscopy (two-dimensional TOCSY) data were also recorded in both H2O and D2O solutions using MLEV17 spin lock pulses with a mixing time of 69.7 ms (29). All data were recorded in the phase-sensitive mode using the time proportional phase increment method (30) with 2048 data points in the t2 domain and 256 in the t1 domain. All NMR data were processed using Bruker XWIN-NMR (Bruker Instruments) software and analyzed using Sparky 3.106 software on a SGI Indigo2 work station. Prior to Fourier transformation in the t1 dimension, the first row was half-weighted to suppress t1 ridges. The proton chemical shifts were referenced with internal sodium 4,4-dimethyl-4-silapentane 1-sulfonate (31).

Structure calculations were performed using CNS 1.1 (32) on a Linux work station with Intel Pentium-4 2.8 GHz CPU. NMR structures were calculated by hybrid distance geometry and simulated annealing calculations. For generation of the symmetric dimer, the method developed by Nilges and co-workers (32) was employed with minor modifications as described (33). Unambiguous and intersubunit NOEs constraints were used for calculating the structure of the symmetric dimer. All NOEs were classified and converted to distance constraints as strong (1.8–2.7 Å), medium (1.8–3.3 Å), and weak (1.8–5.0 Å) based on their intensities from NOESY spectra. A model of z4L-PtdIns(4,5)P2 complex was generated by molecular mechanics simulation using Discover3 (Accelrys). The dielectric constant was set to 80 for implicit water dynamics. The final structure of the syndecan z4L dimer was analyzed and displayed with Insight II program (Accelrys).

RESULTS

Sequence and Structural Conservation in Syndecan-4—We have previously reported the cloning and sequencing of the full-length zebrafish syndecan-4 and compared the primary amino acid sequences of zebrafish, amphibian, avian, and mammalian syndecan-4. All possess three Ser-Gly motifs in an acidic amino acid environment suitable for heparan sulfate substitution (Ref. 19 and Fig. 1A). The ectodomain of zebrafish syndecan-4 shares only 37% identity and 43% similarity with the ectodomain from rat syndecan-4, although the NXIP motif within the cell-binding domain identified as being important in integrin-mediated adhesion appears in both fish and mammalian syndecan-4 sequences (19). As is characteristic of the family, the transmembrane and cytoplasmic sequences of syndecan-4 are highly conserved (71.4% identity and 83.9% similarity between zebrafish and rat syndecan-4).

The presence of a GXXXG motif in syndecan transmembrane domains is compatible with the formation of SDS-resistant dimers (16, 17). This feature is present in zebrafish syndecan-4 (Fig. 1A), although the putative transmembrane domain is 28 amino acids, as compared with avian and mammalian syndecan-4 with 25 (19). The predicted mass of zebrafish syndecan-4 core protein (including signal peptide) is 20 kDa on the basis of the amino acid sequence. In vitro transcription and translation of the zSDC-4 coding sequence produced a protein that migrated with an apparent mass of ~40 kDa, indicative of stable dimer formation (Fig. 1B).

Homology searches of the zebrafish genome reveal that all of the transferases associated with heparan sulfate biosynthesis are present in the zebrafish (34–37). To test whether zebrafish syndecan-4 is expressed as a proteoglycan, an HA-tagged form of zSDC-4 was expressed in CHO-K1 cells and lysates were immunoblotted with an HA-specific antibody (Fig. 1C). This confirmed that the core protein forms SDS-resistant dimers
and that it could be substituted with glycosaminoglycan chains as evidenced by heterogeneous high molecular weight material. This was very similar to HA-tagged rat syndecan-4 immuno-blotted in the same way. We confirmed that the glycosaminoglycan chains on zebS4C were composed of heparan sulfate by performing Western blots on anti-HA immunoprecipitates from lysates with antibodies specific for heparan sulfate (supplemental Fig. S1).

Zebrafish Syndecan-4 Promotes Integrin-mediated Cell Adhesion—The syndecan-4 ectodomain contains cell adhesion activity that is mediated by β1 integrins (19, 38). Comparison of the avian, fish, amphibian, and mammalian cell-binding domain sequences revealed little sequence homology, but three conserved motifs were identified and subsequent mutagenesis experiments revealed that a conserved NQIP motif was important for the cell binding properties of the human form of syndecan-4 ectodomain (19). In zebSDC4 the corresponding motif is NQIP and the importance of this sequence was tested in deletion mutants of the ectodomain. Glutathione S-transferase fusion proteins with either the full-length of the zebrafish syndecan-4 ectodomain (zS4ED) or an in-frame deletion mutant lacking this motif (zS4EDΔNQIP) were purified as recombinant proteins (Fig. 2A). Swiss 3T3 fibroblasts attached and spread on the wild type zS4ED but completely failed to adhere to zS4EDΔNQIP at a range of protein coating concentrations (Fig. 2B). This contrast in cell attachment properties between wild type and deletion mutant forms of syndecan-4 was more marked than seen in the human form of syndecan-4 ectodomain where NHIP was deleted (19). At high protein coating concentrations this deletion had minor effects on cell attachment although cell spreading was diminished. Those few cells that attached to zS4EDΔNQIP spread very little (Fig. 2C). Adhesion to zS4ED could be compromised if cells were treated with β1 integrin blocking antibodies (Fig. 2F). Consistent with a
Zebrafish Syndecan-4

In zebrafish the second lysine (Lys\textsuperscript{194}) is substituted with a threonine residue (KTPIYKK). Finally in the C2 domain the EFYA motif that is involved in the interaction between syndecan and PDZ proteins appears as an EIYA in the zebrafish (9–11). This prompted us to question whether the structure of the zebrafish syndecan-4 cytoplasmic domain, and therefore its function, would be affected by these changes. However, the solution structure of the dimeric cytoplasmic domain of the zebrafish syndecan-4 revealed a twisted clamp topology, which is very similar to that reported for the rat homologue (12, 39). Most intermolecular NOE interactions were detected in residues from Ser\textsuperscript{188} to Lys\textsuperscript{199}, consistent with a symmetric dimer of the twisted clamp shape (Fig. 3B). The presence of Thr\textsuperscript{194} in place of lysine appeared to have little impact on the three-dimensional structure, based on intermolecular interactions between this residue on one strand of the dimer, with the Gly\textsuperscript{192} and Lys\textsuperscript{193} residues of the opposing strand. As in rat syndecan-4 many of the intermolecular interactions are in the IYKKA residues in the second half of the V region, which is well conserved. The C1 and C2 regions of zsyndecan-4 were more flexible, consistent with the mammalian structure.

NMR structure also showed that PtdIns(4,5)\textsubscript{2} interacted with the zsyndecan-4 cytoplasmic domain, and caused conformational changes in the protein (Figs. 3C and 4, A and B). Proton one-dimensional spectra of the zsyndecan-4 cytoplasmic domain mixed at three different ratios with PtdIns(4,5)\textsubscript{2} are shown in Fig. 3, D–G. As with the mammalian homologue, intermolecular NOE interactions between the two strands of the dimer were enhanced by the presence of the phospholipid particularly in the V region (Figs. 3C, and 4, A and D). The residues in the V region interact with both the head group and fatty acyl chains of the phospholipid, and as in the mammalian molecule, possible hydrogen bonding between the 4-phosphate oxygen and Lys\textsuperscript{199} was observed (Fig. 4C). Therefore, despite three amino acid changes in sequence between zebrafish and mammalian syndecan-4 cytoplasmic domains, one each in the C1, V, and C2 regions, dimer formation, topology, and interaction with PtdIns(4,5)\textsubscript{2} are conserved.

Overexpression of Zebrafish Syndecan-4 Results in Reduced Cell Migration and Spreading—Overexpression of rat syndecan-4 in CHO-K1 cells leads to a reduction in cell motility as compared with vector only controls (26) and occurs as a result of excessive promotion of focal adhesion formation. We tested whether overexpression of zebrafish syndecan-4 had the same effect on CHO-K1 cell motility. Cell lines stably expressing either the rat or the zebrafish syndecan-4 were grown to confluence. Scratch wound cell migration assays showed that after 24 h both cell lines had impaired wound closure as compared with cells containing the vector only, and this was still evident even after 48 h (Fig. 5, A–I). A second effect of overexpressing rat syndecan-4 in CHO-K1 cells was that the average cell surface area of the stably transfected cells when grown under normal culture conditions was increased. CHO-K1 cells overexpressing zSDC4 did not exhibit this phenotype (data not shown).

The rate of CHO-K1 cell spreading on fibronectin is also affected by syndecan-4 (Fig. 5J). The CHO-K1 cell lines...
expressing either ratSDC4 or zebSDC4 were plated onto dishes coated with fibronectin in the absence of serum and the spread area was measured at time points up to 45 min. The rate of cell spreading was much reduced in cells overexpressing either species of syndecan.

zSyndecan-4 Localizes to Focal Adhesions and Can Restore smooth Muscle Actin Incorporation in Syndecan-4 Null Fibroblasts—Syndecan-4 is a focal adhesion component (5). To determine whether zSDC4 localized to focal adhesions we expressed an HA-tagged form of the protein in CHO-K1 cells and seeded the cells in serum-free conditions on cover-slips coated with fibronectin. Prominent focal adhesion structures were evident on cells expressing both rat syndecan-4 and to a lesser extent zebrafish syndecan-4 (Fig. 6, E and F). In addition, previous work where syndecan-4 was overexpressed in CHO-K1 cell showed that this resulted in thicker and more profuse actin stress fibers (Fig. 6, A–C). This was also the case when zSDC-4 was expressed in the same cell line.

Syndecan-4 null fibroblasts are compromised in their ability to incorporate smooth muscle actin into stress fibers and this effect is more pronounced under conditions of serum starvation (40). This defect was corrected when rat syndecan-4 was expressed in these cells (Fig. 7A). When zebrafish syndecan-4 was expressed in null fibroblasts it was again observed that smooth muscle actin was organized into stress fibers in contrast to the vector only control (Fig. 7A). The reconstitution of the wild type phenotype was mar-

FIGURE 4. The solution structure of the zebrafish syndecan-4 cytoplasmic domain. A, solution structure of the dimeric unit of free z4L and z4L in the presence of PtdIns(4,5)P2 (Bound). Ribbon drawings of REM average structure of dimeric z4L displaying two subunits and side chain (in stick model) orientation. B, view from the N-terminal face of zebrafish syndecan-4 showing the free dimer (left) and the conformational change induced by addition of PtdIns(4,5)P2 (right). The electrostatic potential surface of dimeric z4L in the presence of a single molecule of PtdIns(4,5)P2 is shown in C. Negative electrostatic potential is represented in red, positive in blue, and neutral in white.

FIGURE 5. Overexpression of zebSDC-4 or ratSDC-4 in CHO-K1 cells results in impaired cell migration and a reduction in cell spreading in response to fibronectin. Stable cell lines of CHO-K1 cells transformed with pIRE2-EFΔ (A–C), pIRE2-RatSDC4 (D–F), or pIRE2-ZebSDC4 (G–I) were seeded into 3-cm dishes and scratch wound assays were performed as described (26). Micrographs of scratch wounds were taken at 0 (A, D, and G), 20 (B, E, and H), and 44 h (C, F, and I) (bar = 50 μm). Overexpression of zebrafish syndecan 4 impairs CHO-K1 cell spreading on fibronectin-coated cell culture dishes (J). Vector (A) control, rat Syndecan-4 ( ), or zebrafish syndecan-4 ( ) expressing cell lines were plated onto fibronectin-coated dishes and photographed at the time points indicated. The mean spread cell surface area of cells was measured using IMAGEJ software and was calculated from at least 50 cells. The fold increase in mean cell surface area of cells after seeding was then calculated.
Syndecan-4 was shown here to have roles as an adhesion molecule in fish and many of the syndecan-4-mediated cellular and molecular processes are common to both mammals and fish. We show that syndecan-4 can affect the rate of cell spreading in response to fibronectin. Syndecan-4 null fibroblasts have defects in α-smooth muscle actin organization that can be corrected by the reintroduction of syndecan-4 cDNA from either rat or zebrafish. We also show the NMR solution structure of the zebrafish syndecan-4 cytoplasmic domain in the presence and absence of PtdIns(4,5)P2 to much greater resolution than has been possible previously. The structure is broadly similar to that of mammalian syndecan-4, however, we have identified more interacting residues within the syndecan-4 dimer.

In common with many receptors and extracellular matrix molecules, syndecans appear to have gone through two rounds of gene duplication at the invertebrate/primitive chordate boundary. All invertebrates examined so far have one syndecan gene, which is expressed as a heparan sulfate proteoglycan, at least in Drosophila (41). The Ser-Gly motif required for heparan sulfate substitution, usually in an acidic amino acid environment (42), is retained in all syndecans, and they are likely always substituted. Searches of the completed fish genomes including Danio rerio, Fugu rubripes, Tetraodon nigroviridis, Gasterosteus aculeatus, and Oryzias latipes reveal that each has only three syndecans, syndecan-1 appears to have been secondarily lost in teleost fish (Ref. 22 and data not shown). In contrast to vertebrates, Drosophila syndecan appears to have roles mostly associated with establishing growth factor or morphogen gradients and roles in cell adhesion have not yet been described. However, mammalian syndecans have been shown to associate with the actin cytoskeleton through their cytoplasmic domains, and syndecan-4 in particular, is present in focal adhesions (5). It has been shown in several different systems to be a co-receptor for selective integrins, and together, they promote focal adhesion assembly (6, 7). The cytoplasmic domain of mammalian syndecan-4 binds PtdIns(4,5)P2 and protein kinase Cα and signals downstream to RhoA and rho kinases for the assembly of microfilament bundles and focal adhesions (13, 14, 43). Since this work was completed, a very recent report suggests that zebrafish syndecan-4 has a cell adhesion role and is essential for persistent directional migration of neural crest cells (21). This process is Rac1 dependent and supports previous work demonstrating a role for syndecan-4 in maintaining directional cell migration in vitro (44). This timely report, did not, however, include any molecular studies of the zsyndecan-4 molecule, which are now reported here.

The extracellular domains of vertebrate syndecans are not well conserved, and that of zebrafish syndecan-4 has only 37% identity with its rat homologue. Moreover, whereas the mammalian and avian syndecan-4 proteins have 100% sequence conservation of their cytoplasmic domains, four residues are different in the zebrafish, one in each of the C1 and V subdomains and two in the C2. Therefore, it was important to ascertain whether a lower vertebrate syndecan had cell adhesion supporting roles.
A key to understanding the cytoplasmic roles of syndecan-4 has emerged from structural studies by NMR spectroscopy. Mammalian syndecan-4 forms symmetric dimers, stabilized by both hydrophobic and hydrogen bonds; they are not covalently associated. The dimer has an unusual twisted clamp topology, which is altered, but not destroyed, by phosphorylation of the single serine residue in the C1 region (25). This event does, however, decrease PtdIns(4,5)P₂ binding affinity by ~100-fold, and so the ability to bind and activate protein kinase Ca is also compromised (25, 45). The zebrafish syndecan-4 cytoplasmic domain retains the overall twisted clamp motif, together with PtdIns(4,5)P₂ binding. It is interesting that the three non-conserved residues (Ile₁₈₁, Thr₁₉⁴, and Ile₂₉⁵) in zebrafish, replaced by Met¹⁷⁵, Lys²₈₈, and Phe¹⁹⁹ in the mammal, respectively, seem not to be essential for intermolecular association of the two strands of the dimer, nor for interactions with the inositide. In both rat SDC-4 and zebrafish SDC-4 the majority of intramolecular NOE interactions occur between residues contained within the V region. This region is of critical importance to syndecan-4 function because it is only this region that remains conserved. Syndecan-4 from Xenopus shows considerable sequence variation within its C1 and C2 domains and yet retains all of the residues characteristic of the syndecan-4 V region (20). The V region has a central KKXXXXK motif in mammals, which is known for a capacity to interact with inositides (46), yet even when replaced with KTXXXXK in zebrafish, binding is not compromised. Consistent with this, zebrafish syndecan-4 cytoplasmic domain dimers with inositol are capable of activating protein kinase Ca, just as was seen originally with the rat homologue (data not shown).

Given the structural similarity of fish and mammalian syndecan-4 cytoplasmic domains, it was consistent that the zebrafish syndecan-4 could be expressed on the cell surface and promote focal adhesion assembly. One of the hallmarks of syndecan-4 overexpression is slowed cell migration, commensurate with increased focal adhesion assembly (26). This was clearly seen where the zebrafish syndecan-4 was expressed in CHO-K1 cells. Microfilament bundle number and length was also increased in these cells. In syndecan-4 null cells, there are clear deficits in focal adhesion assembly, and in the incorporation of α-smooth muscle actin into microfilament bundles. These can be corrected by re-expression of rat syndecan-4. The zebrafish syndecan-4 can also complement the null fibroblasts, in a manner indistinguishable from the mammalian proteoglycan.

Another highly conserved feature of all syndecans is the presence of a GXXXXG motif within the putative transmembrane domain, indicative of homodimer formation (16, 17). It has been suggested that the dimeric state is probably constitutive in syndecans. A characteristic that follows from this self-association is that syndecan core proteins resolve as SDS-resistant dimers. In mammals has three Ser-Gly motifs suitable for substitution with glycosaminoglycan chains. All are close to the N terminus, and two are immediately adjacent. This feature is conserved across the vertebrates, and the zebrafish parologue can be identified in a glycanated form when analyzed in cell lysates. The heparan sulfate chains are a major site of ligand interaction, including many growth factors and extracellular matrix molecules.

In addition to the glycosaminoglycan chains, a region of the extracellular core protein can be itself promote β1 integrin-mediated adhesion. We previously identified a NXIP motif in human syndecan-4, which is essential for the adhesion process (19). In the zebrafish homologue the sequence is present as NQIP, and its role was established in adhesion assays. Although the wild type protein promoted cell attachment and spreading, in a β1 integrin-dependent manner, this property was almost entirely lost when the NQIP tetrapeptide was deleted. As with cytoplasmic determinants of cell adhesion, therefore, this property is probably conserved throughout the vertebrate syndecan-4 homologues. Our previous data also strongly suggest that the syndecan ectodomain does not directly interact with integrin, but with one or more intermediates that lead to integrin activation (19). This pathway is, perhaps, both ancient and an important regulator of cell adhesion.

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