

INTERACTION OF HYDROXYL-CYTOPLASMIC
DOMAINS WITH CAK1 PDB DOMAIN

A THESIS SUBMITTED TO

THE DEPARTMENT OF MOLECULAR BIOLOGY AND CHEMISTRY

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OF BIRMINGHAM UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

ANDREW J. HARRIS

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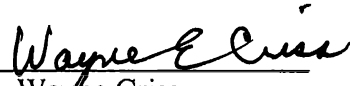
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**INTERACTION OF SYNDECAN-1 CYTOPLASMIC
DOMAIN WITH CASK PDZ DOMAIN**

**A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTERS OF SCIENCE**

**BY
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SEPTEMBER 2000**

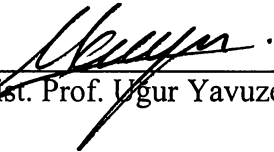
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to my beloved family

ABSTRACT

Syndecans are integral membrane proteoglycans which have important cellular functions. They are thought to function as co-receptors for various extracellular ligands like extracellular matrix molecules, cell-cell adhesion molecules, and growth factors. It is shown that they modulate cell proliferation, cell adhesion, cell motility, and cell fate upon such interactions. Recent studies indicated that they also modulate signal transduction events by their core protein interactions. Most of these interactions are through their highly conserved cytoplasmic domains. They contain PDZ domain binding site at their carboxy terminal end, and identification of PDZ protein interactions of Syndecans may be important to explain their role in signaling. We aimed to identify a protein-protein interaction between Syndecan-1 cytoplasmic domain and PDZ domains of CASK and AF-6 proteins with yeast two-hybrid technique. We showed that there is a detectable interaction between CASK and Syndecan-1 through these domains. Our results indicated that CASK PDZ domain binds to Syndecan-1 cytoplasmic domain when expressed in yeast, and this interaction may have important functions in signaling events.

ÖZET

Sindekan proteinleri önemli hücresel faaliyetlerde görev alan hücre zarı proteoglikanlarından. Sindekanların, hücre dışı matriks molekülleri, hücreler arası bağlantı molekülleri, ve bazı büyüme hormonları gibi değişik hücre dışı ligandları için yardımcı reseptör olarak görev aldıkları düşünülmektedir. Önceden bu tip fonksiyonları sayesinde, hücre çoğalmasında, hücreler arası bağlantılarda, hücre hareketlerinde, ve hücre kaderinin belirlenmesinde ayarlayıcı rolleri gösterilmiştir. Yakın zamandaki çalışmalarda ise bu moleküllerin hücre içindeki protein zinciri bölümlerinin görev aldığı etkileşmeler sayesinde sinyal iletimi fonksiyonlarını ayarladıkları gösterilmiştir. Bu etkileşmelerin büyük bir kısmı bu moleküllerin büyük oranda korunmuş olan sitoplasmik bölgeleri yardımıyla gerçekleşmektedir. Sindekanların karboksil uçlarında sinyal iletimi olaylarındaki rollerini açıklamada çok önemli olabilecek PDZ bölgesi bağlanma alanları bulunmaktadır. Biz, mayada melezleme tekniği ile sindekanların sitoplasmik bölgelerinin görev aldığı yeni protein etkileşmelerini ortaya çıkarmayı amaçladık. Bunun için CASK proteini ve AF-proteininin PDZ bölgeleri ile sindekanların sitoplasmik bölgeleri arasındaki etkileşmeleri mayada melezleme tekniği ile denedik. Çalışmalarımızın sonunda CASK proteini ile sindekan-I proteini arasında bir etkileşme olduğunu gösterdik. Sonuçlarımız gösterdi ki CASK proteini PDZ bölgesi sayesinde sindekan-I proteininin sitoplasmik bölgesine, maya hücresi içerisinde eksprese edildikleri zaman bağlanabilmektedir. Ve bu etkileşmenin sinyal iletimi olaylarında önemli fonksiyonları olması muhtemeldir.

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ABBREVIATIONS

amp.	amplification
APS	ammonium persulfate
bisacrylamide	N, N, methylene bis-acrylamide
bp	base pairs
c-terminus	carboxyl terminus
cDNA	complementary deoxyribonucleic acid
kb	kilobasepairs
kD	kilo daltons
DMSO	Dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
EDTA	diaminoethane tetra-acetic acid
EtBr	ethidium bromide
HSPG	heparan sulfate proteoglycan
MCS	multiple cloning site
ml	milliliter
mg	milligram
MQ	MilliQ water
nm	nanometer (1/10 ⁹ of a meter)
N-terminus	amino terminus
MW	molecular weight
OD	optical density

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
TAE	tris-acetic acid-EDTA
TE	tris-EDTA
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
Tris	tris (hydroxymethyl)-methylamine
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

1- INTRODUCTION

1.1- SYNDECANS

Syndecans are members of cell surface heparan sulphate proteoglycans (HSPG) consisting of a single membrane spanning domain, a short cytoplasmic domain, and a highly variable extracellular domain. Heparan sulphate chains attachment sites are localized on the extracellular domain of Syndecans. These attachment sites are highly variable among the four members of the Syndecan family whereas their locations and numbers are conserved through evolution for each single member of the family.

1.1.1- Syndecan core protein

Syndecans are long unbranched proteoglycans consist of a core protein, to which long unbranched carbohydrate polymers namely glycosaminoglycans are covalently attached (Saunders et al. 1989, Mali et al. 1990). According to a common nomenclature Syndecans are designated numerically (from 1 to 4) with respect to their cloning order (Carey et al. 1992).

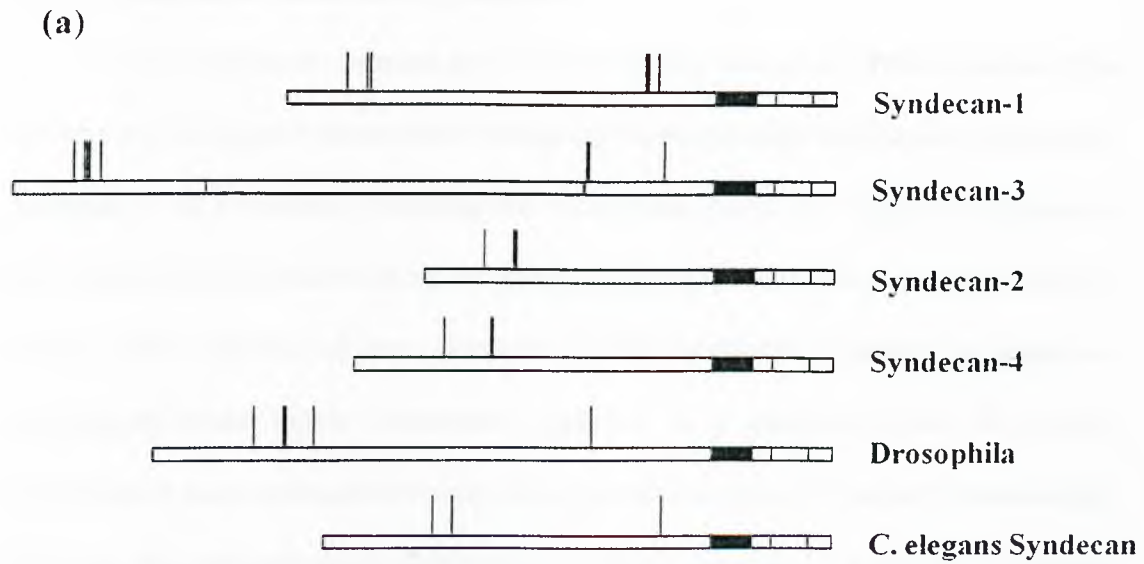
Syndecans are also cloned from some basic organisms such as *Drosophila melanogaster*, and *Caenorhabditis elegans* (Spring et al. 1994). In mice and humans, each of the four syndecan genes is located on a different chromosome. Genes coding for Syndecans 1, 3 and 4 have been cloned (Carey et al. 1997). They show a strikingly

similar exon–intron organization, which supports the idea that the Syndecans arose by gene duplication from a single ancestral gene (Carey 1997). A schematic representation of all Syndecans is shown in Figure 1.1.

1.1.2- Functions of Syndecans

Large number of ligands have been identified that bind to Syndecans at their extracellular domains. These encompass an amazing variety of molecules, including growth factors, extracellular-matrix proteins, cell–cell adhesion receptors, enzymes and other proteins (Schlessinger et al. 1995). In most cases the specificity of binding of these ligands to Syndecans appears to be relative and not absolute. For example, although bFGF binds to specific sugar sequences within heparan sulphate chains, every syndecan that has been examined appears to contain these sequences and binds bFGF (Lopez Casillas et al. 1993). On the other hand, bFGF has its own receptor bFGFR. These features appear to rule out the notion that Syndecans function as ligand-activated signaling receptors in the usual sense.

According to these notions Syndecans have been thought to function as co-receptors that bring extracellular ligands into close proximities to their specific receptors. For a long time these membrane proteins are considered to be fine tuners of ligand-receptors interactions, and they are conceived to be modulators of cell adhesion, cell migration, and cell differentiation.



(b)

Syn-3 KSILERKE VLVAVIVGG VVGALFAAFLVTL LIY RMKKKDEGSYTLEEPKQASVT YQ KP DKQEEFYA
 Syn-1 QGLLDRKE VLGVIAGG LVGLIFAVCLVAF MLY RMKKKDEGSYSLEEPKQANGG AYQ KP TKQEEFYA
 Syn-4 DNLFKRTE VLAAVIAGG VIGFLFAIFLILL LVY RMRKKDEGSYDLGERKPSS AAYQ K APTK EFYA
 Syn-2 SNIFERTE VLAALIVGG VVGILFAVFLILL LVY RMKKKDEGSYDLG KKPI Y KK APTN EFYA
 Dr. SSFFSQPG ILAAVI GGAVVGLLCA ILVVMFIVY RMRKKDEGSYALDEPKRSPANNSY AKNA NNREFYA
 C. e FHETLANG FYAA IAGGVLVAVITA ILLVLEVFV RIRKKDEGSYALDEPKQARPYASYGYTK ASTK EFYA

Figure 1.1: Structure of Syndecan core protein

(a) Diagram illustrating the structures of the four vertebrate and two invertebrate Syndecans. The black boxes represent the transmembrane domains. Vertical lines above the boxes indicate positions of attachment of heparan sulphate chains. (b) Amino acid sequences of syndecan transmembrane and cytoplasmic domains.

1.1.3- Cytoplasmic domains of Syndecans

The cytoplasmic domains are short but highly conserved. The sequence of the 13-amino-acid segment immediately following the transmembrane domain is essentially identical in all Syndecans, including the invertebrate forms (C1 region). All syndecan core proteins also contain an identical tetrapeptide sequence at their C-terminal ends (C2 region). The function of these portions of the cytoplasmic domain is unknown. Interrupting these highly conserved segments is a variable region that shows considerably less sequence similarity among syndecan types (V region). Interestingly, however, the variable-region sequences are highly conserved for specific Syndecans types in different species. The sequence of syndecan-1 in this region is identical in human, mouse, rat and hamster; the corresponding region in syndecan-2 is identical in human, rat and *Xenopus*. Thus, once the four vertebrate syndecan genes diverged during evolution, there was strong selective pressure to maintain their particular cytoplasmic domain primary structures. This, plus the structural diversity of the ectodomains, suggests that different Syndecans have evolved to carry out similar, but non-identical, functions.

The conserved characteristic of cytoplasmic domain of syndecan proteins indicate that these domains have important cellular functions and may bind to different cytoplasmic proteins. These possible interactions may be important for the determination of cell fate. Most probably Syndecan proteins regulate cell-matrix and cell-cell interactions outside the cell membrane and they also modulate cell signaling by signaling receptors through protein-protein interactions inside the cell via their highly conserved cytoplasmic domains.

1.1.4- Core protein interactions of Syndecans

N-syndecan (Syndecan-3) was previously isolated as a cell surface receptor for heparin-binding growth-associated molecule (HB-GAM) and suggested to mediate the neurite growth-promoting signal from cell matrix-bound HB-GAM to the cytoskeleton of neurites. However, it was unclear whether N-syndecan would possess independent signaling capacity in neurite growth or in related cell differentiation phenomena. Later it was reported that Syndecan-3 binds a protein complex containing Src family tyrosine kinases and their substrates and that Syndecan-3 acts as a neurite outgrowth receptor via the Src kinase-cortactin pathway (Kinnunen et al. 1998).

In 1995 it was shown that Syndecan-4 but not other Syndecans can be phosphorylated by Protein kinase C (PKC) which is an important protein in cell signaling events (Prasthofer et al. 1995, Baciuc et al. 1995). It was shown that Syndecan-4 can be phosphorylated by PKC and is possibly a physiological substrate for PKC in cells. The core protein of syndecan-4 can directly bind the catalytic domain of PKC α and potentiate its activation by phospholipid mediators. It can also directly activate PKC α in the absence of other mediators. This activity resides in the sequence LGKKPIYKK in the center of the short cytoplasmic domain, and other Syndecans lack this sequence and PKC regulatory properties. Syndecan-4 is a focal adhesion component, and this interaction may both localize PKC and amplify its activity at sites of forming adhesions (Oh et al. 1997). This was the first report suggesting direct transmembrane signaling through cell surface proteoglycans.

Another novel cellular protein was identified that interacts with the cytoplasmic domain of syndecan-4 but not with those of the other three syndecan family members. The interaction involves both the membrane proximal and variable central regions of the

cytoplasmic domain. The protein is named as syndesmos. Syndesmos is ubiquitously expressed and can be myristylated. Consistent with its myristylation and syndecan-4 association, syndesmos colocalizes with syndecan-4 in the ventral plasma membranes of cells plated on fibronectin (Baciu et al. 2000). When overexpressed in NIH 3T3 cells, syndesmos enhances cell spreading, actin stress fiber and focal contact formation in a serum-independent manner.

A third protein which was identified by yeast two-hybrid screening that binds to the cytoplasmic domains of the Syndecans, is syntenin (Grootjans et al. 1997). It contains a tandem repeat of PDZ domains that reacts with the FYA C-terminal amino acid sequence of the Syndecans. Cells that overexpress eGFP-syntenin show numerous cell surface extensions, suggesting effects of syntenin on cytoskeleton-membrane organization. It may be proposed that syntenin may function as an adaptor that couples Syndecans to cytoskeletal proteins or cytosolic downstream signal-effectors. This study was the first report suggesting PDZ proteins as interaction counterparts of Syndecan proteins.

CASK, the rat homolog of a gene (*LIN-2*) required for vulval differentiation in *Caenorhabditis elegans*, is expressed in mammalian brain, but its function in neurons is unknown (Hata et al. 1996). It is another member of PDZ protein family. Again by yeast two-hybrid screening, a specific interaction was identified between the PDZ domain of CASK and the COOH terminal tail of syndecan-2, a cell surface heparan sulphate proteoglycan (HSPG). The interaction was confirmed by coimmunoprecipitation from heterologous cells (Hsueh et al. 1998). In brain, syndecan-2 localizes specifically at synaptic junctions where it shows overlapping distribution with CASK, consistent with an interaction between these proteins in synapses. Cell surface HSPGs can bind to

extracellular matrix proteins, and are required for the action of various heparin-binding polypeptide growth/differentiation factors. The synaptic localization of CASK and syndecan suggests a potential role for these proteins in adhesion and signaling at neuronal synapses (Cohen et al. 1998).

All these interactions are indications of important cellular functions of Syndecan proteins other than their co-receptor functions. Some of the possible functions of such interactions may be association of focal adhesion complexes at cell to cell junctions, localizing membrane proteins or signaling molecules at specific membrane loci by communication with cytoskeletal proteins, or regulation of special cellular complexes. Some possible signaling pathways are Wnt-frizzled pathway, and CASK-reelin pathway which are summarized in the proceeding parts of this thesis.

Core protein interactions of Syndecans cytoplasmic domain are shown as a scheme in the Figure 1.2.

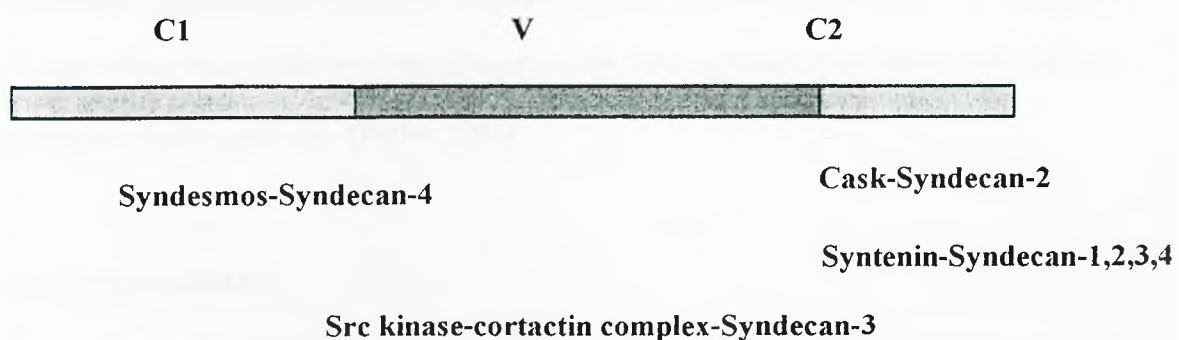


Figure 1.2: Core protein interactions of Syndecans

Proteins interacting with cytoplasmic domain of Syndecans and their interaction sites are shown.

1.2- WNT-FRIZZLED PATHWAY

Wnt proteins are large family of cystein rich glycoproteins that function as ligands controlling development in various organisms ranging from nematode worms to mammals. At the cellular level Wnt regulates cell proliferation, cell morphology, cell motility, and cell fate. Wnt proteins serve as short range chemical messengers, paracrine agents. Wnt proteins are associated with cell surface and extracellular matrix, such that their effects tend to be spatially localized. The first mammalian Wnt gene, originally termed Int-1 was identified at a site of murine mammary tumor virus (MMTV) integration in mammary tumors (Burrus et al. 1995). The Int-1 gene exhibited homology to wingless [wg], a *Drosophila* segment polarity gene, and subsequently the name Wnt was chosen for members of this family. At least sixteen mammalian Wnt genes have been identified so far. Wnt proteins elicit a variety of cellular responses, including proliferation, differentiation, and morphogenesis, and control of developmental decisions such as axis formation in *Xenopus* embryos. Indeed, Wnts are now recognized as one of the major classes of signaling proteins that regulate development and cell fate in multicellular organisms (Peifer 1996).

1.2.1- Wnt signaling

The action of Wnts on target cells is mediated by binding to the frizzled (Fz) group of transmembrane receptors, of which there are at least eight members in mammals. Structurally, Frizzled receptors have an extracellular Wnt-binding domain, seven-transmembrane-spanning sequences and an intracellular C-terminal tail (Bhanot et al. 1996). They form part of the seven-transmembrane-spanning (7TMS) superfamily of

receptors. The regulation of Wnt binding to Frizzled receptors is a key restriction point in Wnt signaling. The immediate downstream component of the signal transduction pathway is disheveled (Dsh), an intracellular protein that may directly interact with Fz receptors. Members of the Dishevelled family encode cytoplasmic proteins with no known enzymatic functions (Tsang et al. 1996). Sequence comparisons identified three regions of homology: an N-terminal Dsh homology domain, a central region containing a basic and a PDZ domain, and a DEP domain upstream of the non-conserved C-terminal sequence. The target of Dsh is glycogen synthase kinase-3 (GSK-3), which is homologous to the *Drosophila* protein encoded by *zeste-white3*. GSK-3 is believed to be constitutively active and functions to inhibit the Wnt pathway in the absence of Wnt signals. GSK-3 phosphorylates β -catenin, which enhances β -catenin turnover by a ubiquitin-mediated degradation pathway. β -Catenin is a member of a multigene family of proteins characterized by the presence of 'Arm' amino acid repeats that mediate a range of protein-protein interactions. Genetic analysis of the *Drosophila* homologue Armadillo (Arm), showed that it functions downstream of GSK-3/*zw3*. Considerable evidence suggests that the Wnt inhibition of GSK-3 activity prevents the turnover of β -catenin, leading to its accumulation. In response to Wnt signals, GSK-3 activity is inhibited, leading to stabilization of the β -catenin protein. One of the targets of β -catenin is the transcription factor TCF, an HMG-box DNA-binding protein. Multiple TCF family members exist in mammals, including TCF-1, LEF-1, TCF-3, and TCF-4. β -Catenin forms a complex with TCF, converting it from a transcriptional repressor to an activator and thereby stimulating the expression of target genes (Behrens et al. 1996). Although the genes in mammalian cells that are activated by TCFs in response to Wnt

signaling are not well characterized, the c-myc proto-oncogene is one target that may mediate proliferative responses to Wnt signals (He et al. 1998). In several versions of this model, the APC (adenomatous polyposis coli) gene product has been proposed to function as a signaling intermediate between GSK-3 and β -catenin. In this context, the finding that APC can bind both β -catenin and GSK-3 suggested that APC may function as a signaling scaffold (Cadigan & Nusse 1997, Siegfried et al. 1994). A schematic representation of Wnt signaling is shown in Figure 1.3.

1.2.2- Ligand binding

The 'Wnt-binding domain' found at the N-terminus of Frizzled family members was recently identified in genes of the Frzb family and in a splicing variant of collagen XVIII. The complexity of receptor–ligand specificity may be further increased by co-receptors such as proteoglycans and other putative 'Wnt receptors'. Proteoglycans consist of proteins linked to chains of disaccharide repeats called glycosaminoglycans. Proteoglycans are highly charged and are found predominantly at the cell surface, where they function as low-affinity cell-surface receptors for a variety of ligands, including TGF- β and FGF. Cell-culture studies showed that many Wnt family members bind glycosaminoglycans (Reichsman et al. 1996) and that cell-surface glycosaminoglycans are required for Wg stabilization of Arm in *Drosophila* CI-8 cells. Cell-surface proteoglycans may increase the local concentration of Wnts, leading to increased avidity and/or receptor clustering, as has been proposed in models for FGF–glycosaminoglycan interactions. Thus the loss of heparin-like glycosaminoglycans may allow Wnt proteins to abnormally diffuse and reach a concentration at which they are no longer effective.

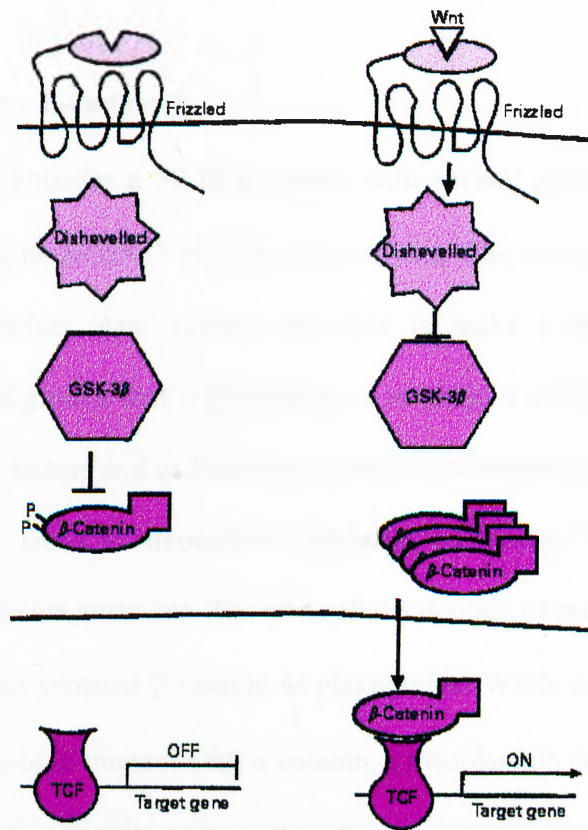


Figure 1.3: Wnt signaling

Following binding of the Wnt ligand to the Frz receptor, Dsh is recruited to the cell membrane, where it signals to inhibit the kinase activity of GSK-3. As GSK-3 normally promotes the instability of soluble β -catenin, Wnt signaling results in an increase of β -catenin levels. Soluble β -catenin then interacts with TCF-DNA-binding factors, forming a transcriptionally active complex. In several versions of this model, the APC (adenomatous polyposis coli) gene product has been proposed to function as a signaling intermediate between GSK-3 and β -catenin (Dale T. C. *Biochem J.* 1998).

Alternatively, the ability of Wnt–Frizzled complexes to form or transduce their signals may be directly regulated by glycosaminoglycan interactions.

1.2.3- β -catenin vs. E-cadherin

β -Catenin encodes a 90 kDa protein with several structural domains. The N-terminus contains the GSK-3 phosphorylation sites. The central domain of the protein contains 13 imperfect 'Arm' repeats that fold to make a superhelix containing a positively charged groove that is hypothesized to interact with acidic regions of APC, TCF transcription factors and cadherin cell adhesion molecules (Huber et al. 1997).

Cadherins are Ca^{2+} -dependent adhesion molecules that mediate cell–cell interactions at adhesive junctions. The intracellular domain of cadherins interact with the cytoplasmic adaptor proteins β -catenin or plakoglobin. While complexed to cadherins, β -catenin is also able to interact with α -catenin, a cytoplasmic protein with similarity to vinculin, which links actin filaments to the adherens junctions.

Levels of cadherin expression affected free β -catenin pools and signaling, in *Xenopus* embryos, ES cells and *Drosophila* embryos. However, as E-cadherin-deficient L-cells were able to respond to Wnt-1 by stabilizing β -catenin, it is unlikely that the reductions in E-cadherin expression were sufficient or were required for Wnt signaling. Cell adhesion may be a downstream target of Wnt signaling as the adhesive functions of cadherins are regulated by interactions with β -catenin.

E-cadherin and lymphocyte-enhancer factor-1 (LEF-1) form mutually exclusive complexes with beta-catenin; the association of beta-catenin with LEF-1 compete by the E-cadherin cytoplasmic domain. Similarly, LEF-1 and adenomatous polyposis coli

(APC) form separate, mutually exclusive complexes with beta-catenin. The potent ability of E-cadherin to recruit β -catenin to the cell membrane and prevent its nuclear localization and transactivation is demonstrated using SW480 colon carcinoma cells (Orsulic et al. 1999).

1.2.4- Wnt signaling vs. Syndecan-1

Syndecans bind to various different ligands including oncogenic proteins. But there were no direct evidence that Syndecans are involved in tumorigenesis. In a recent publication it was reported that they may have role in mammary tumor formation caused by ectopic Wnt-1 proto-oncogene. In this study, Syndecan-1-deficient mouse and transgenic mouse that express Wnt1 in mammary gland are crossed. Ectopic Wnt-1 expression is known to induce generalized mammary hyperplasia, followed by the development of solitary tumors (Ramakrishna et al. 1993). It is shown that in *Sdc1*^{-/-} mice, Wnt-1-induced hyperplasia in virgin mammary gland was reduced by 70%, indicating that the Wnt-1 signaling pathway was inhibited. These results provide both genetic and biochemical evidence that syndecan-1 can modulate Wnt signaling, and is critical for Wnt-1-induced tumorigenesis of the mouse mammary gland (Alexander et al. 2000).

1.3- CASK-TBR-1 PATHWAY

CASK is identified as a neurexin, a neuronal cell surface protein binding counterpart in synaptic junctions of neurons (Hata et al. 1996). It shows a great homology to Drosophila PSD95 protein. Later a *C. elegans* homolog of CASK LIN-2 is identified which co-localizes with EGF receptors in cell membranes (Kaech et al. 1998). It belongs to Membrane Associated Guanylate Kinase (MAGUK) protein family. Members of this protein family all share some characteristics like being composed of multiple protein domain such that C terminal calcium/calmodulin binding domain, SH3 domains and guanylate kinase domains. Guanylate kinase domain indicates that these proteins may bind to ATP through these regions and display kinase activities. Yet, no significant kinase activity could be shown to guanylate kinase domain of CASK.

MAGUK proteins are possibly involved in localization of multi protein complexes to cell surface proteins. These protein complexes may be involved in signal transduction events and are probably regulated by these scaffold proteins.

CASK is a membrane-associated protein that contains domains found in Ca^{2+} activated protein kinases and in proteins specific for intercellular junctions, suggesting that it may be a signaling molecule operating at the plasma membrane, possibly in conjunction with neurexins.

1.3.1- CASK and Syndecans

Another important domain of CASK is its PDZ like domain showing a similarity with drosophila PSD95 protein. This domain is also thought to be involved in protein-protein interactions of CASK. Later, CASK was reported to interact directly with

Syndecan-2 protein via its cytoplasmic domain through this PDZ domain. It is hypothesized that PDZ domain of CASK may interact with all other Syndecan proteins via their carboxy terminal EFYA sequence. In brain, Syndecan-2 localizes specifically at synaptic junctions where it shows overlapping distribution with CASK, consistent with an interaction between these proteins in synapses.

It is logical to think that other members of Syndecan family of proteins are also interacting with CASK through the same domain. Yet no such study is reported indicating that CASK is interacting with a syndecan protein other than Syndecan-2. Cell surface HSPGs can bind to extracellular matrix proteins, and are required for the action of various heparin-binding polypeptide growth/differentiation factors. The synaptic localization of CASK and syndecan suggests a potential role for these proteins in adhesion and signaling at neuronal synapses.

1.3.2- CASK in nucleus

A recent study reported that CASK interacts with a T-box protein (Tbr-1) which is a transcription factor. Tbr-1 binds DNA at a specific sequence (T-box) and activating the transcription of a protein named reelin (Hsueh et al. 2000). The interaction is through CASK's mysterious guanylate kinase domain. This was the first function attributed to guanylate kinase domain of MAGUK proteins. However the interaction was specific to CASK alone and other MAGUK proteins did not show any interaction with Tbr-1 protein.

The paradoxical finding of interaction of a membrane associated MAGUK protein CASK, interacting with a transcription factor which normally resides in nucleus is solved when it is demonstrated that CASK localizes to nucleus in Tbr-1 over

expressing cells. It was also shown that CASK activates the transcriptional activating function of Tbr-1 protein through this interaction.

Tbr-1 is a T-box transcription factor involved in forebrain development. CASK enters the nucleus and binds to a specific DNA sequence (the T-element) in a complex with Tbr-1. CASK acts as a coactivator of Tbr-1 to induce transcription of T-element containing genes, including *reelin*, a gene that is essential for cerebrocortical development. This findings show that a MAGUK which is usually associated with cell junctions has a transcription regulation function.

Another important finding is regulation of this pathway by syndecan expression. Syndecan-3 over expression diminished the translocation of CASK to nucleus. This type of regulation can be observed in Wnt pathway where β -catenin translocation to nucleus is regulated by E-cadherin expression. Probably in CASK pathway Syndecans have similar functions to E-cadherin functions in Wnt pathway. Syndecans are known to have important functions in developmental processes. This phenomenon may be a good example how these membrane glycoproteins are involved in developmental events.

CASK pathway resembles Wnt pathway in many ways indicating that both may have similar components and are regulated by similar mechanisms (Bredt 2000). A comparative diagram of both pathway is presented in Figure 1.4.

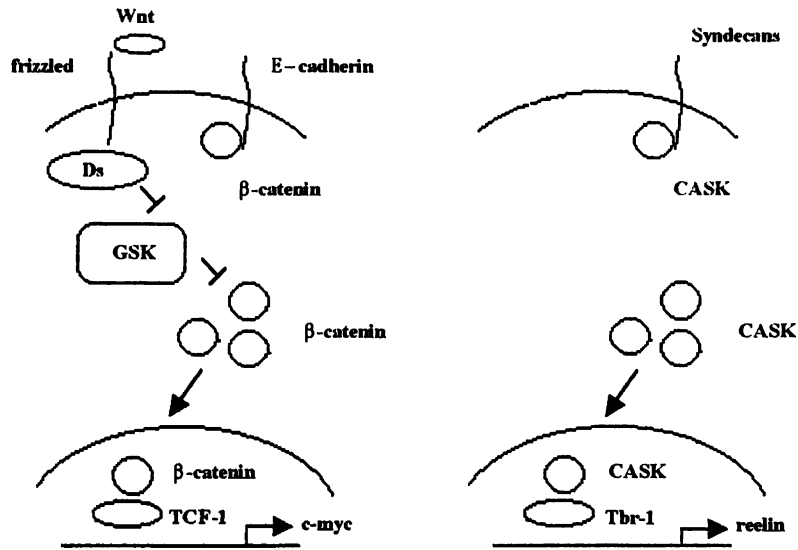


Figure 1.4: Wnt pathway and CASK pathway

Wnt pathway is shown as a diagram in the left picture. The CASK pathway is shown in the right picture. The involvement of β -catenin in the pathway resembles the involvement of CASK in the other pathway. The other components of the CASK pathway are not characterized yet, but may have similar characteristics to Wnt pathway.

1.4- PDZ DOMAIN PROTEINS

Many cytosolic signaling proteins and cytoskeletal proteins are composed of modular units of small protein-protein interaction domains like SH2 domains, SH3 domains or PDZ domains. As the name implies, PDZ domain have high homology with the drosophila PSD95 proteins, disc-large gene encoded Dlg protein, and ZO-1 protein. The first mammalian homolog identified was p55 protein which show a silent G/T polymorphism. PDZ domain containing proteins bind directly to the carboxy terminal residues of transmembrane proteins (Ruff et al. 1996). For example PSD95 and Dlg bind to similar carboxy terminal sequences on Shaker type K⁺ channels (Kim et al. 1995). So PDZ domain proteins are thought to be necessary for clustering of transmembrane proteins to specific locations in cells.

In a peptide library screening assay it was identified that there is a consensus peptide sequence for PDZ domain binding (Songyang et al. 1997). In this consensus it was identified that PDZ domains of most of the proteins tend to bind to a hydrophobic amino acid at the carboxy terminal end. It was concluded that an internal consensus sequence can not bind to PDZ domains even they match perfectly with the obtained consensus sequence. However the most important finding in this study is the PDZ domains can be divided into two major groups according to their interaction partner specificity. According to this finding it was shown that type II PDZ domains which are the PDZ domains of p55, CASK, Tiam-1, and also AF-6 prefer a hydrophobic residue especially a phenylalanine residue at the -2 location from the carboxy terminal end. This was against the general belief that PDZ domains prefer a hydroxyl group aminoacid like serine, threonine, and tyrosine at this specific location. The optimal residues predicted

by peptide library screening can be rationalized on the basis of crystal structure studies performed to identify PDZ domain binding (Doyle et al. 1996).

Structures and binding consensus sequences of some PDZ domain proteins are shown in Figure 1.5.

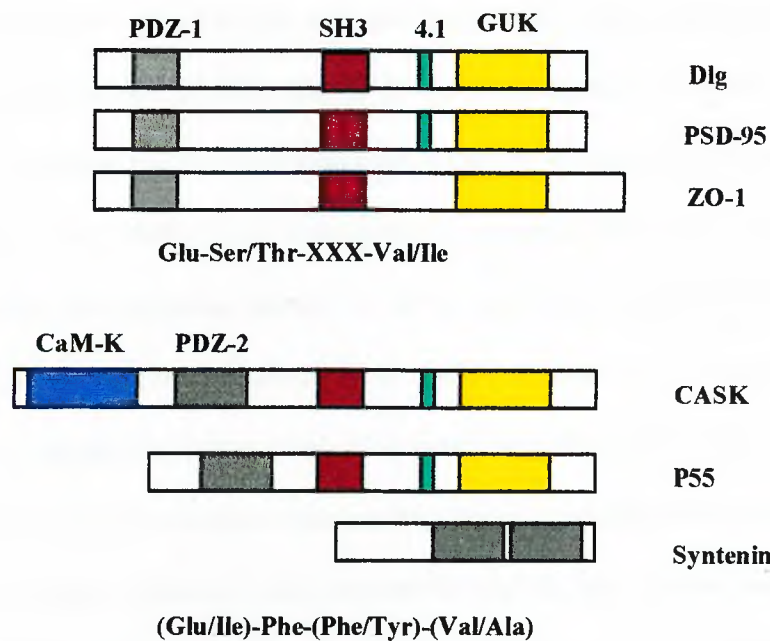


Figure 1.5: Binding consensus sequences of some PDZ domain proteins

Domain structures of some PDZ domain proteins are shown in the figures. **CaM-K:** Calcium/Calmodulin kinase. **4.1:** 4.1 protein binding domain. **GUK:** Guanylate kinase homology domain. Type I PDZ domains bind to a C Terminal consensus sequence of Glu-Ser/Thr-XXX-Val/Ile, whereas Type II PDZ domain proteins bind to a C Terminal consensus sequence (Glu/Ile)-Phe-(Phe/Tyr)-(Val/Ala) that match perfectly to C Terminal amino acid sequences of Syndecan proteins.

1.5- AF-6 AS A PDZ DOMAIN PROTEIN

AF-6 protein was identified and cloned as a proto-oncogene in acute myeloid leukemia (AML). It was identified to be the fusion partner protein of ALL-1 protein caused by a chromosomal translocation [t(6,1)(q23,q27)](Prasad et al. 1993, Saito et al. 1998). It was observed that this protein contains shared motifs with proteins involved in signal transduction, namely PDZ motifs. It was also mapped to minimal deletion region in epithelial ovarian cancer but it was shown that AF-6 sequences maps distal to these region (Saha et al. 1995). Later drosophila, *C. elegans*, and mouse homolog of AF-6 were identified and named as canoe, Ce-AF-6, and afadin respectively (Kuriyama et al. 1996, Watari et al. 1998, Mandai et al. 1997). Canoe is an important protein for drosophila development and is involved in Notch signaling (Miyamoto et al. 1995).

AF-6 contains a putative Ras binding domain, which shows great homology to Raf-Ras activating domain. It was shown to bind to Ras protein and another PDZ domain protein, ZO-1 in vivo. Unlike β -catenin, AF-6 is localized to tight junctions of epithelial cells and ZO-1 is known to be an important component of this type of cell-cell junctions (Yamamoto et al. 1997). Ras binding property of AF-6 is regulated with its ZO-1 interaction and activated Ras disturbs AF-6 localization to tight junctions. Its localization at junctional cell-cell contact sites is also confirmed by showing AF-6 interaction with JAM (Junctional Adhesion Molecule) (Ebnet et al. 2000).

AF-6 was shown to bind to GTP-activated Ras but not to mutant Ras which lacks its GTP binding domain. All these data indicate that AF-6 is a potential regulator of Ras

pathway which is an important pathway for determination of cell fate (Yamamoto et al. 1999).

AF-6 was also shown to bind to cell surface receptor tyrosine kinases (RTK) epinephrine receptors, EphB3 via their PDZ like domain. Their interaction with Eph receptors is regulated by the phosphorylation of the receptors. It was shown that they are phosphorylated *in vivo* upon binding to epinephrine receptors. Receptor tyrosine kinases are important integral membrane proteins involved in signal transduction processes. This result also show that PDZ like domains modulate signal transduction like other protein-protein interaction domains SH3 and PTB (Hock et al. 1998).

A very important finding about AF-6 came only recently reporting that AF-6 co-localizes and interacts with deubiquitinating enzyme FAM, the human homolog of *Drosophila* *faf* protein (Taya et al. 1998, Kanai-Azuma et al. 2000). *Drosophila* homolog of FAM, *faf* (fat facets) is known to be an important protein in developmental processes. This enzyme regulates the signal transduction events by regulating ubiquitination of some proteins. FAM and *faf* probably are involved in stabilization of proteins by inhibiting the ubiquitination and therefore degradation. The importance of ubiquitination pathway in signaling events is reported for Wnt pathway. It is shown that β -catenin is stabilized by deubiquitination enzyme FAM and it can be protected from GSK induced degradation (Taya et al. 1999). This may be a potential pathway by which these type of signaling events are modulated. This shows that AF-6 is also regulated by a similar pathway.

1.6- HYPOTHESIS

PDZ domain containing proteins play an important role in signaling events as cytoplasmic modulators of signaling pathways. They all localize to specific peripheral membrane locations like post-synaptic complexes, cell to cell adhesion locations, and tight junctions via protein-protein interactions of their PDZ domains. It is known that they bind to carboxy terminal of different integral membrane proteins via these domains. They are thought to modulate the formation of such complexes and localize integral membrane proteins to such locations. Many important PDZ domain containing proteins are known to involve in signaling events, Dsh, PSD95 and AF-6 are some of them.

Syndecans are integral membrane proteoglycans that are involved in signaling events. They interact with various cellular proteins via their core proteins. Some of these proteins are PKC and CASK. We hypothesize that, Syndecan proteins which are known to bind to two PDZ domain containing proteins (CASK and syntenin) may bind to other similar proteins such as Dsh and AF-6, since their consensus binding sequence match perfectly with the carboxy terminal of Syndecan family of proteins (EFYA).

It was recently reported that Syndecan-1 is involved in Wnt induced tumorigenicity in mammary cancer (Alexander et al. 2000). This report was the first finding suggesting that a Syndecan protein is involved in tumorigenesis. It is obvious that core protein interactions of Syndecan-1 may be important in this and other possible functions of Syndecan-1. CASK is a recently identified transcriptional activator (Hsueh et al. 2000). Although terminal cytoplasmic region of Syndecan-2, which is responsible for the CASK-PDZ domain interaction is identical to the same region of Syndecan-1 there is no direct evidence yet, that Syndecan-1 binds to CASK protein. AF-6 is shown

there is no direct evidence yet, that Syndecan-1 binds to CASK protein. AF-6 is shown to localize to nucleus as a fusion partner of MLL gene product but the importance of this localization is not identified yet (Joh et al. 1997). AF-6 protein has type II PDZ domain like CASK and syntenin which is the Syndecan-2 protein interacting domain. According to our hypothesis that all Syndecan proteins bind to type II PDZ domain containing proteins, we predicted that Syndecan-1 interacts with PDZ domains of CASK and AF-6 through its cytoplasmic domain. To test this hypothesis we used Yeast two-hybrid assay to identify Syndecan-1 cytoplasmic domain with PDZ domains of CASK and AF-6.

2- MATERIALS AND METHODS

2.1- Recombinant DNA manipulation techniques

2.1.1- Polymerase Chain Reaction (PCR)

Syndecan-1 cytoplasmic domain, CASK-PDZ domain, and AF-6-PDZ domain were amplified by polymerase chain reaction using Human Fetal Brain cDNA as template.

A standard 100 ul reaction set up in a 0.2 ml PCR tube

primers 0.1-1 uM of each primer (reverse and forward)

dNTP 0.2 mM diluted from the 10 mM stock

buffer 1X diluted from the 10X stock

template 10 ng of genomic or plasmid DNA

Made up to 100 ul with MilliQ water

enzyme 2.5 unit of Taq DNA polymerase

Tubes were placed on ice. To perform a hot-start PCR the thermal cycler was preheated to approximately 90°C before placing the tubes into it. Reaction conditions were as follows: Denaturation: 30 seconds at 94 °C, Annealing: 30 seconds at 60 °C, Extension: 30 seconds at 72 °C, 35 cycles.

List of the synthetic oligonucleotide primer sets used in this study are summarized in Table 2.1.

Table 2.1: List of the synthetic oligonucleotide primer sets used in this study

name of the primer	sequence (5'-3')	size
Sn-1026	CATGGCCATGGGGCTGTACCGCATGAAGAAGA	32
Sn-1143	CCGGGTCGACCGCGTCAGGCATAGAATTCC	30
Cs-1476	TCTCCGGATCCCCAATGTGACCAGAGTTCGGCT	33
Cs-1761	TCTCCCTCGCGACAGGACGAAGACTGAGTGC	31
Af-2925	TAGCCGGATCCCGCGTGAGAACACAGAGCTGG	32
Af-3268	AAAAAGAGCTCGCTGATTGAGAAGGGTGG	29

2.1.2- Purification of DNA

Purification of PCR products and linearized plasmids were done either as described below or by the MBI DNA extraction kit (#K0513) according to the manufacturer's instructions.

PCR products were purified by precipitating the DNA with ammonium acetate. 1/10 volume of 10X STE buffer, equal volume of 4M ammonium acetate and 2.5 volume of 100% (v/v) ethanol were added to the PCR products sequentially. The reaction tube was centrifuged at 13000 rpm for 20 minutes at room temperature to pellet DNA. The supernatant was removed carefully and discarded. The pellet was washed

with 200 ul of 70% (v/v) ice-cold ethanol. The pellet was dried and DNA was resuspended by using TE buffer.

2.1.3- Restriction enzymes

DNA restriction reactions were carried out in a total volume of 20-100 ul with 5-10 units of restriction enzyme. 1ul of boiled RnaseA (1mg/ml) was used where appropriate. The volume of the reaction buffers were arranged to be 1X and the enzyme volume was never greater than 1/10th of the reaction volume so as to prevent star activity due to the high glycerol concentration. Restriction enzymes used in this study are shown in Table 2.2.

Table 2.2: Restriction enzymes used in this study

Enzyme	Catalog #	Optimized Buffer	% of Reactivity in Y⁺/Tango Buffer
<i>Bam</i> HI	MBI #ER0051	Buffer BamHI ⁺	50-100
<i>Nco</i> I	MBI #ER0572	Buffer Y ⁺ /Tango	100
<i>Sac</i> I	MBI #ER1132	Buffer SacI ⁺	20-50
<i>Sa</i> II	MBI #ER0641	Buffer O ⁻	50-100
<i>Xho</i> I	MBI #ER0691	Buffer R ⁻	100

2.1.4- DNA ligation

DNA fragments, that were cut from agarose gel and recovered by MBI DNA extraction kit (#KO513) were used for ligation. For cloning (where both vector and insert DNA has protruding ends) approximately 1:4 ratio of vector and insert DNA was mixed with the T4 DNA ligase buffer and 1 to 3 units of T4 DNA ligase (MBI #EL0011). The reaction was carried out either for 2 hours at room temperature or for 16 hours at 16°C. For protruding end ligation insert:vector ratio could be chosen as 1/1, 2/1, or 3/1. The reaction was stopped directly by heating at 65°C for 10 minutes or directly used to transform competent bacterial cells.

2.1.5- Culturing and handling bacteria

2.1.5.1- Bacterial strains

Strains of *E.coli* used in this study are summarized in Table 2.3.

Table 2.3: Strains of *E.coli* used in this study

Strain	genotype	usage	reference
DH5α	<i>supE44 ΔlacU169</i> (ϕ 80 <i>lacZΔM15</i>) <i>hsdR17 recA1</i> <i>endA1 gyrA96 thi-1 relA1</i>	Host for plasmid DNA	Hanahan (1983)
HB101	<i>supE44 hsdS20(r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	Host for plasmid DNA	Boyer and Rouland-Dussoix (1969); Bolivar and Backman (1979)
JM 109	F' [<i>tral</i> 36 <i>lacI</i> ^q <i>lacZΔM15 proAB</i> '] <i>recA1 endA1 gyrA96</i> (<i>Nal</i> ^r) <i>thi hsdR17(r_Km⁺K)</i> <i>supE44 relA1 thi Δ(lac-proAB)</i>	Host for plasmid DNA	Yanisch-Perron et al. (1985)

2.1.5.2- Storage of bacteria

Strains of *E.coli* were stored in glycerol (long term storage). Glycerol cultures of *E.coli* were prepared by adding 0.81 ml of fresh culture to 0.91 ml of sterile 80% glycerol in a sterile screw capped tube. The tubes were vortexed and were then frozen and stored at -70°C .

2.1.5.3- Growth of *E. coli* strains

All strains were grown in LB medium or on LA medium supplemented with the appropriate antibiotics.

Solid and liquid mediums:

LB medium : 0.5% Yeast extract, 1% Bacto-tryptone, 1% NaCl.

LA medium : 0.5% Yeast extract, 1% Bacto-tryptone, 1% NaCl and 1% agar.

Supplemented with appropriate antibiotics.

Antibiotics:

Ampicillin with a final concentration of 50 ug/ml was used in this study.

2.1.5.4- Preparation of competent bacteria

Cells were made competent using a modification of the CaCl_2 method described by Maniatis *et al.*, 1982.

5 ml LB was inoculated using a single colony from a freshly grown plate of the *E. coli* strain to be used, and was incubated at 37°C for approximately 2 hours, until the OD_{600} of the culture is 0.3-0.4. The culture was then cooled on ice for 5 minutes, 1 ml aliquots were added to microcentrifuge tubes and then the cells were pelleted by centrifugation (1 minute at 13,000 rpm). The cells were resuspended in 0.5 ml of 50 mM CaCl_2 by gentle vortexing, before being placed on ice for 30 minutes. The cells

were pelleted by centrifugation (1 minute at 13,000 rpm), and the supernatant was discarded. The pellet was resuspended in 0.1 ml of CaCl₂ with gentle vortexing. The cells were then stored on ice until required for transformation. Alternatively competent cells were prepared and stored at -70°C until required. 500 ml of LB was seeded with a 10 ml of overnight culture and grown to an OD₆₀₀= 0.6. Cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C, before being incubated on ice for 20 minutes. The cells were harvested as before, resuspended in 25 ml of 50 mM CaCl₂ /20% glycerol and aliquoted into microcentrifuge tubes before being frozen. Samples were stored at -70°C and were viable for at least 2 months. Cells were thawed on ice prior to the addition of DNA.

2.1.5.5- Transformation of plasmid DNA in bacterial cells

The DNA to be transformed (usually a 1ul of ligation mixture or approximately 100 ng of plasmid DNA) was added to the 100 ul of competent cells, mixed gently and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 90 seconds and chilled by placing on ice for 2 minutes. 1 ml of pre-warmed LB was then added and the suspension was incubated at 37°C for 1 hour. Each sample was pelleted by centrifugation at 13,000 rpm for 2 minutes, resuspended in 100-200 ul of LB and plated onto selective medium and incubated overnight at 37°C to allow the growth of the transformants.

2.1.6- Isolation of plasmid DNA from bacteria

2.1.6.1- Small scale preparation of plasmid DNA

This protocol is based on the alkaline lysis method of Birnboim and Doly (1979).

The transformed bacterial strain containing the plasmid of interest was grown at 37°C overnight in 5 ml of LB+antibiotic. 1.5 ml of the bacterial culture was pelleted by centrifugation for 1 minute (bench-top microfuge, 13,000 rpm) in a 1.5 ml microfuge tube. After the removal of the supernatant, the cells were resuspended in 0.1 ml of ice-cold solution I and stored for 5 minutes at room temperature. 0.2 ml of solution II was mixed by inversion, the tube was then stored on ice for 5 minutes. Bacterial chromosomal DNA and proteins were precipitated by the addition of 0.15 ml of ice-cold solution III. The mixture was left on ice for 5 minutes, then centrifuged at 13000 rpm in a bench-top centrifuge for 5 minutes to pellet the host DNA and proteins. The supernatant was mixed with an equal volume of phenol-chloroform (1:1) and centrifuged in a bench-top microfuge at 13000 rpm for 3 minutes to separate the two phases. The top phase was removed and plasmid DNA was precipitated by mixing it with 2.5 volumes of 95% ethanol, and pelleted by centrifugation for 10 minutes (bench-top microfuge, 13,000 rpm) after keeping the mixture at 4°C for 15 minutes. The supernatant was discarded and the pellet was left for 15-20 minutes at room temperature to dry and then resuspended in 20-30 ul of TE buffer containing 10 ug/ml RNaseA. Samples were stored at 4°C.

Solution I

50 mM glucose

25 mM Tris·Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 minutes at 10 lb/square in. on liquid cycle, and stored at 4°C.

Solution II

0.2 NaOH (freshly diluted from 10 N stock)

1% SDS

Solution III

5 M potassium acetate 60 ml

glacial acetic acid 11.5 ml

H₂O 28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

TE buffer

pH 7.4 10mM Tris·Cl (pH 7.4)

1mM EDTA (pH 8.0)

2.1.6.2- Medium scale purification of plasmid DNA

All midi-preparations were carried out by using the kit supplied by Macherey-Nagel (cat # 740 573) according to the manufacturer's instructions.

2.1.6.3- Large scale purification of plasmid DNA

The bacterial strain containing the plasmid of interest was first grown in a 30 ml culture with necessary antibiotic until late log phase (OD_{600} of 0.6) then inoculated into 500 ml LB medium for 10-12 hours. The cells were harvested by centrifugation at 4000 rpm for 15 minutes at 4°C in a Sorvall GS3 rotor (or equivalent). The supernatant was discarded and allowed to drain away in a upside down position.

2.1.6.3.1- Lysis by alkali

This protocol is based on the alkaline lysis method of Birnboim and Doly (1979) and the solutions were described above in the mini preparation section. The bacterial pellet was resuspended and washed in 18 ml solution I.

40 ml of freshly prepared solution II was added, the lid of centrifuge tube was closed and the bottle was inverted several times. The bottle was kept at room temperature for 5-10 minutes.

20 ml of ice cold solution III was added, the lid of the bottle was closed and shaken several times. The bottle was stored on ice for 10 minutes.

The cell lysate was centrifuged at 4000 rpm for 15 minutes at 4°C. The rotor was stopped without braking.

The supernatant was filtered through 4 layers of cheesecloth into a 250 ml centrifuge bottle. 0.6 volume of isopropanol was added and mixed well, in order to precipitate the nucleic acids. The bottle was kept at room temperature for 10 minutes.

Nucleic acids were recovered by centrifugation at 5000 rpm for 15 minutes at room temperature in a Sorvall GS3 rotor (or equivalent). Salt may precipitate if the centrifugation is performed at 4°C. The supernatant was poured off gently and the bottle

bottle was inverted so as to allow all fluid to drain away. The pellet and the walls of the bottle was washed with 70% ethanol at room temperature. After draining off ethanol, by the help of a Pasteur pipette attached to vacuum, beads of liquid that were attached to the walls of the bottle were removed. The inaccessible ethanol was left to evaporate at room temperature. The pellet was dissolved in 3 ml of TE (pH 8.0).

2.1.7- Quantification of double stranded DNA

The amount of DNA in samples were determined by reading the absorbency of the samples at 260 nm. An OD₂₆₀ of 1 corresponds to a concentration of 50 ug/ml for double stranded DNA, 40 ug/ml for single stranded DNA and 20 ug/ml for oligonucleotides (Maniatis *et al.*, 1982).

2.1.8- Plasmids

Plasmids used in this study are listed in Table 2.4. All plasmids are yeast vectors designed for expression of exogenous genes in yeast. They also contain β -lactamase gene serving as ampicillin resistance gene in order to amplify and store them in bacteria.

Table 2.4: List of Plasmids used in this study

pAS2-1	8.4-kb cloning vector; used to generate fusion of the bait protein with the GAL4 DNA-BD.
pACT2	8.1-kb cloning vector; used to generate fusion of a known protein (or a collection of random, unknown proteins) with the GAL4 AD.
pVA3-1	9.4-kb positive control plasmid used with pTD1-1; encodes a DNA-BD/murine p53 fusion protein in pAS2-1.
pTD1-1	9.9-kb positive control plasmid used with pVA3-1; encodes an AD/SV40 large T-antigen fusion protein in pACT2.
pCL1	15.3-kb positive control plasmid; encodes the full-length, wild-type GAL4 protein.
pLAM5'-1	9.1-kb false-positive detection plasmid; encodes a DNA-BD/human lamin C fusion protein in pAS2-1.

2.2- Yeast two-hybrid system

Yeast two-hybrid assay was used in this study to show Syndecan-1 interaction with PDZ domains of CASK and AF-6. Yeast two-hybrid assay was chosen since it is a recently developed, powerful, and easily used protein assay which is frequently used in similar studies (Fields & Song 1989, Frederickson 1998). MATCHMAKER Two-Hybrid System 2 (#K1604-1) was used which is a GAL4 based two-hybrid system that provide a transcriptional assay for detecting specific protein-protein interactions in yeast. Yeast two-hybrid system is either used to screen a cDNA library to identify unknown

interactions among the total population of cellular proteins, or it is used to detect specific protein-protein interactions between two known proteins.

Yeast two hybrid system is based on the fact that many eukaryotic transacting transcription activators are composed of separable, functionally distinct and independent domains. These domains are usually DNA binding domain (BD) that recruits the transcription factor to specific DNA sequences and transactivating domain (AD) that interacts with RNA polymerase II complex and initiates transcription. GAL4 is a yeast transcription factor composed of these two (BD and AD) domains. When these two domains are separated from each other by recombinant DNA techniques and expressed in the same cell they can not bind to each other and form a functional transcription factor. However these two domains can be placed in close proximity by the means of protein-protein interaction and functional transcriptional factor can be formed. Reporter gene expression can be obtained by such means, hence the reporter gene expression can be utilized as an indicator of protein-protein interaction. In MATCHMAKER system two functional domains of GAL4 transcription factor is supplied in two different yeast expression vectors such that two domains of GAL4 can be expressed as two fusion proteins in yeast. One of the vectors (pAS2-1) express DNA binding domain of GAL4 protein which binds to DNA at specific sequences. The other vector expresses transactivating domain of GAL4 protein which activates the transcription of the genes by interacting with transcription machinery.

Two different proteins can be cloned in these two vectors fused to BD and AD of GAL4. So that upon the interaction between these proteins reporter gene activation can be detected. A schematic representation of yeast two-hybrid assay is shown in the following figure. (Figure 2.1)

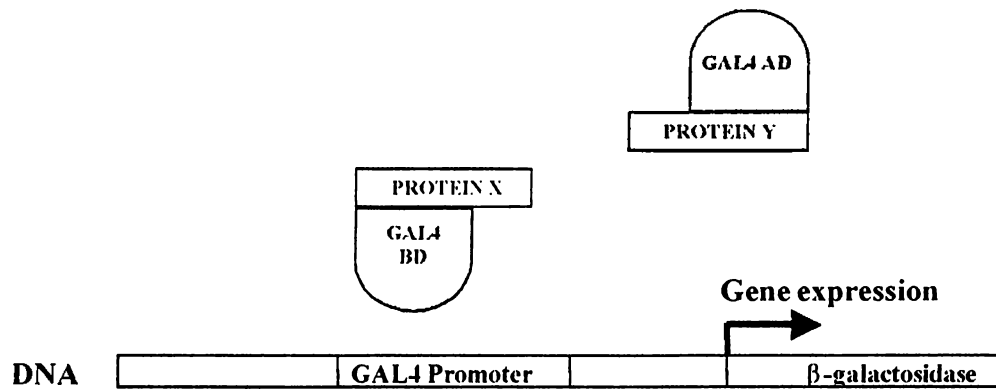


Figure 2.1: Yeast two-hybrid assay

Schematic representation of yeast two-hybrid assay. Protein-protein interaction between Protein X and Protein Y leads to construction of functional GAL4 transcription factor. Which can transactivate a reporter gene (β -galactosidase) under the control of GAL4 promoter sequence.

2.2.1- Strains of Yeast

The *Saccharomyces cerevisiae* strains used in this study and their specifications are listed in Table 2.5.

Table 2.5: Yeast strains used in this study

The GAL1 upstream activating sequence (UAS) and the three tandem copies of the GAL4 17-mer consensus sequence [GAL4_{17-mers(x3)}] are both responsive to the GAL4 transcriptional activator. The *trp1*, *leu2*, *his3*, *gal4* and *gal80* mutations are all deletions.

Strain	Genotype	Reporter	Transformation markers
Y187	MAT α , <i>ura3-52</i> , <i>his3-200</i> , <i>ade2-101</i> , <i>trp1-901</i> , <i>leu2-3</i> , 112, <i>gal4</i> Δ , <i>met</i> ⁻ , <i>gal80</i> Δ , URA3::GAL1 _{UAS} -GAL1 _{TATA} -lacZ	lacZ (high)	<i>trp1</i> , <i>leu2</i>
Y190	MAT α , <i>ura3-52</i> , <i>his3-200</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1-901</i> , <i>leu2-3</i> , 112, <i>gal4</i> Δ , <i>cyh</i> ^{r2} , LYS2::GAL1 _{UAS} -HIS3 _{TATA} -HIS3 URA3::GAL1 _{UAS} -GAL1 _{TATA} -lacZ	HIS3 (leaky), lacZ (high)	<i>trp1</i> , <i>leu2</i> , <i>cyh</i> ^{r2}
CG1945	MAT α , <i>ura3-52</i> , <i>his3-200</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1-901</i> , <i>leu2-3</i> , 112, <i>gal4</i> Δ , <i>cyh</i> ^{r2} , LYS2::GAL1 _{UAS} -GAL1 _{TATA} -HIS3 URA3::GAL4 _{17-mers(x3)} -CYC1 _{TATA} -lacZ	HIS3 (less leaky), lacZ (low)	<i>trp1</i> , <i>leu2</i> , <i>cyh</i> ^{r2}

2.2.2- Storage of yeast

Yeast strains were stored in YPD medium with 25% glycerol at -70°C. Transformed yeast strains were stored in the appropriate SD medium to keep selective pressure on the plasmid. To recover frozen strains, a small portion of glycerol stock was streaked onto YPD or appropriate SD agar plate.

2.2.3- Growth of yeast

Yeast strains were grown in liquid YPD medium or appropriate SD medium for preparing liquid cultures. They were grown on solid media by using YPD-Agar plates or SD-Agar plates according to their transformation status.

YPD medium:

20 g/L Difco peptone

10 g/L Yeast extract

20 g/L Agar (for solid media only)

950 ml of H₂O is added and pH is adjusted to 5.8 and autoclaved.

After cooling glucose was added to a final concentration of 2% as carbon source.

SD medium:

6.7 g Yeast Nitrogen Base without amino acids

20 g Agar for solid media only

850 ml H₂O

100 ml of the appropriate sterile 10X dropout solution

pH is adjusted to 5.8 and autoclaved. After cooling glucose was added to a final concentration of 2% as carbon source.

10X Dropout (DO) supplements:

Filter sterilized solution of the following amino acids in H₂O (Table 2.6).

Table 2.6: Dropout (DO) supplements

L-Isoleucine	300 mg/L	I-7383
L-Valine	1500 mg/L	V-0500
L-Adenine hemisulfate salt	200 mg/L	A-9126
L-Arginine HCl	200 mg/L	A-5131
L-Methionine	200 mg/L	M-9625
L-Phenylalanine	500 mg/L	P-5030
L-Threonine	2000 mg/L	T-8625

2.2.4- Transformation of Plasmid DNA into Yeast using LiAc method

1 ml of YPD or SD was inoculated with several colonies of yeast, 2–3 mm in diameter and was vortexed for 5 minutes to disperse any clumps. For host strains previously transformed with another autonomously replicating plasmid, the appropriate SD medium was used. This was then transferred into a flask containing 50 ml of YPD or the appropriate SD medium. It was incubated at 30°C for 16–18 hr with shaking at 250 rpm to stationary phase ($OD_{600} > 1.5$). 30 ml of the overnight culture was then transferred to a flask containing 300 ml of YPD.

This culture was incubated at 30°C for 3 hr with shaking (230 rpm). At this point, the OD_{600} was 0.4–0.6. Cells were placed in 50-ml tubes and centrifuged at 1,000 x g for 5 min at room temperature (20–21°C). Supernatant was discarded and 25–50 ml of sterile TE or distilled H₂O was added to the tube. Resuspended thoroughly by vortexing. Cells were pooled in one tube and centrifuged at 1,000 x g for 5 min at room temperature. Supernatant was discarded.

Cells were resuspended in 1.5 ml of freshly prepared, sterile 1X TE/1X LiAc. 0.1 mg of plasmid DNA and 0.1 mg of Salmon testes carrier DNA (Sigma #D-9156) was added to a fresh 1.5-ml tube and mixed. 0.1 ml of yeast competent cells were added to each tube and mixed well by vortexing. Then 0.6 ml of sterile PEG/LiAc solution was added to each tube and vortexed at high speed for 10 sec to mix. Incubated at 30°C for 30 min with shaking at 200 rpm. 70 ul of DMSO was added. Mixed well by gentle inversion. Heat shocked for 15 min in a 42°C water bath. Cells were then chilled on ice for 1–2 min.

Cells were centrifuged for 5 sec at 14,000 rpm at room temperature. The supernatant was removed and were resuspended in 0.5 ml of sterile 1X TE buffer. Plated 100 ul on each SD agar plate that would select for the desired transformants.

PEG/LiAc Solution:

PEG 3350 (Sigma #P-3640)	40%
TE Buffer	1X
LiAc	0.1 M

pH : 7.5

2.2.5- Colony-lift filter assay

Fresh colonies were used (i.e., grown at 30°C for 2–4 days), 1–3 mm in diameter. Z buffer/X-gal solution was prepared freshly as described below.

For each plate of transformants that were assayed, a sterile Whatman #5 or VWR grade 410 filter was presoaked by placing it in 2.5–5 ml of Z buffer/X-gal solution in a clean 100- or 150-mm plate.

Using forceps, a clean, dry filter was placed over the surface of the plate of colonies that were assayed. The filter rubbed gently with the side of the forceps to help colonies cling to the filter. Holes poked through the filter into the agar in three or more asymmetric locations to orient the filter to the agar. When the filter had been evenly wetted, it was carefully lifted off the agar plate with forceps and transferred (colonies facing up) to a pool of liquid nitrogen. Using the forceps, it was completely submerged for 10 sec.

After the filter had frozen completely (~10 sec), it was removed from the liquid nitrogen and allowed to thaw at room temperature. (This freeze/thaw treatment is to permeabilize the cells.)

The filter was carefully placed, colony side up, on the presoaked filter. Trapping of air bubbles under or between the filters was avoided. The filters were incubated at 30°C (or room temperature) and checked periodically for the appearance of blue colonies. The time it took colonies producing β -galactosidase to turn blue varied, from 30 min to 8 hr.

The β -galactosidase-producing colonies were identified by aligning the filter to the agar plate using the orienting marks. The corresponding positive colonies from the original plates were picked to fresh medium. If the entire colony was lifted onto the filter, the original plate was incubated for 1–2 days to regrow the colony.

2.2.6- Liquid Culture Assay Using ONPG as Substrate

5-ml overnight cultures were prepared in liquid SD selection medium appropriate for our system and plasmids. On the day of the experiment, ONPG was dissolved at 4

mg/ml in Z buffer with shaking for 1–2 hr. The overnight culture tube was vortexed for 0.5–1 min to disperse cell clumps. 2 ml of the overnight culture was immediately transferred to 8 ml of YPD. The fresh culture was incubated at 30°C for 3–5 hr with shaking (230–250 rpm) until the cells were in mid-log phase (OD_{600} of 1 ml = 0.5–0.8). The exact OD_{600} when the cells were harvested was recorded. Before checking the OD, the culture tube was vortexed for 0.5–1 min to disperse cell clumps.

1.5 ml of culture was placed into each of three 1.5-ml microcentrifuge tubes. Centrifuged at 14,000 rpm (10,000 x g) for 30 sec. Supernatants were carefully removed. 1.5 ml of Z buffer was added to each tube and vortexed until cells were resuspended. Cells were centrifuged again and supernatants removed. Each pellet was resuspended in 300 ul of Z buffer. (Thus, the concentration factor is $1.5 / 0.3 = 5$ -fold).

0.1 ml of the cell suspension was transferred to a fresh microcentrifuge tube. The tubes were placed in liquid nitrogen until the cells were frozen (0.5–1 min). Frozen tubes were placed in a 37°C water bath for 0.5–1 min to thaw. Freeze/thaw cycle was repeated for two more times to ensure that the cells have broken open.

A blank tube was prepared with 100 ml of Z buffer. 0.7 ml of Z buffer- β -mercaptoethanol was added to the reaction and blank tubes.

160 ml of ONPG in Z buffer was added immediately to the reaction and blank tubes. Tubes were placed in a 30°C incubator.

After the yellow color developed, 0.4 ml of 1 M Na_2CO_3 was added to the reaction and blank tubes. Elapsed time was recorded in minutes. Reaction tubes were centrifuged for 10 min at 14,000 rpm to pellet cell debris. Supernatants were carefully transferred to clean cuvettes. The spectrophotometer was calibrated against the blank at

A_{420} and the OD_{420} of the samples were measured relative to the blank. β -galactosidase units were calculated. 1 unit of β -galactosidase is defined as the amount which hydrolyzes 1 mmol of ONPG to o-nitrophenol and D-galactose per min per cell (Miller, 1972; Miller, 1992):

$$\beta\text{-galactosidase units} = 1,000 \times OD_{420} / (t \times V \times OD_{600})$$

where: t = elapsed time (in min) of incubation

V = 0.1 ml x concentration factor

$OD_{600} = A_{600}$ of 1 ml of culture

Z Buffer:

$Na_2HPO_4 \cdot 7H_2O$	16.1 g/L
$NaH_2PO_4 \cdot H_2O$	5.50 g/L
KCl	0.75 g/L
$MgSO_4 \cdot 7H_2O$	0.246 g/L

pH adjusted to 7.0 and autoclaved

X-gal Stock solution:

5-bromo-4chloro-3-inodyl-b-D-galactopyranoside (X-GAL; Sigma #) was dissolved in N,N-dimethylformamide (DMF) at a concentration of 20 mg/ml. Stored in dark and at $-20^{\circ}C$.

Z Buffer/ X-gal solution:

Z buffer	100 ml
β -mercaptoethanol (b-ME; Sigma #M-6250)	0.27 ml
X-gal solution	1.67 ml

prepared freshly before use.

Z buffer- β -mercaptoethanol:

To 100 ml of Z buffer, 0.27 ml of β -mercaptoethanol was added.

ONPG (o-nitrophenyl β -D-galactopyranoside) solution:

Prepared freshly before use. 4 mg/ml o-nitrophenyl β -D-galactopyranoside (sigma #N-1127) was added to Z buffer. pH was adjusted to 7.0 and mixed.

2.2.7- Purification of total protein from yeast**2.2.7.1- Preparation of yeast cultures for protein extraction**

For each transformed yeast strain, a 5-ml overnight culture was prepared in SD selection medium. A single isolated colony (1–2 mm in diameter, no older than 4 days) was used. Overnight cultures were vortexed for 0.5–1 min to disperse cell clumps. For each clone to be assayed (and the negative control), the entire overnight culture was separately inoculated in 50-ml aliquots of YPD medium. Incubated at 30°C with shaking (220–250 rpm) until the OD₆₀₀ reached 0.4–0.6.

OD₆₀₀ (of a 1-ml sample) is multiplied by the culture volume (i.e., 55 ml) to obtain the total number of OD₆₀₀ units.

The culture was quickly chilled by pouring it into a prechilled 100-ml centrifuge tube halfway filled with ice. Tubes were immediately placed in a prechilled rotor and centrifuged at 1000 x g for 5 min at 4°C. Supernatants were discarded and cell pellets were resuspended in 50 ml of ice-cold H₂O. The pellets were recovered by centrifugation at 1,000 x g for 5 min at 4°C. The cell pellets were immediately frozen by placing the tube on dry ice or in liquid nitrogen. Cells were stored at –70°C until usage.

2.2.7.2- Preparation of protein extracts by Urea/SDS method

Complete cracking buffer was prepared and prewarmed to 60°C. 100 ml of cracking buffer was used per 7.5 OD₆₀₀ units of cells. Cell pellets were quickly thawed by separately resuspending each one in the prewarmed cracking buffer.

Each cell suspension was transferred to a 1.5-ml screw-cap microcentrifuge tube containing 80 ml of glass beads per 7.5 OD₆₀₀ units of cells. Samples were heated at 70°C for 10 min.

Samples were vortexed vigorously for 1 min. Cell debris and unbroken cells were pelleted in a microcentrifuge at 14,000 rpm for 5 min, at 4°C. The supernatants were transferred to fresh 1.5-ml screw-cap tubes and placed on ice (first supernatants).

Pellets were treated as follows:

- a. Tubes were placed in a 100°C (boiling) water bath for 3–5 min.
- b. Vortexed vigorously for 1 min.
- c. Debris and unbroken cells were pelleted in a microcentrifuge at 14,000 rpm for 5 min, at 4°C.
- d. This supernatant (second supernatant) was combined with the corresponding first supernatant.

The samples were boiled briefly. Immediately loaded on a gel. Alternatively, samples were stored on dry ice or in a –70°C freezer until they were run on a gel.

Protease inhibitor solution(stock):

Pepstatin A	Sigma #P4265	0.1 mg/ml	(for Carboxyl proteases)
Leupeptin	Sigma #L2884	0.03 mM	(for some thiol and serine proteases)
Benzamidin	Sigma #B6506	145 mM	(for trypsin, plasmin, and thrombin)
Aprotinin	Sigma #A6279	0.37 mg/ml	(for some serine proteases)

PMSF:

0.1742 g PMSF (Sigma #P7626) was dissolved in 10 ml isopropanol.

Glass Beads:

425-600 μ m acid washed glass beads (Sigma #G-8772)

Cracking Buffer (stock solution):

Urea	8 M
SDS	5% (w/v)
Tris-HCl [pH 6.8]	40 mM
EDTA	0.1 mM

Deionized H₂O to a final volume of 100 ml

Cracking Buffer (complete solution):

Cracking Buffer stock solution	1 ml
β -mercaptoethanol	10 μ l
Protease inhibitor solution	70 μ l
PMSF	50 μ l of 100X stock

used freshly.

2.3- Constructs

Syndecan-1 PCR product was cloned in pAS2-1 using Sall and NcoI enzymes, such that it is expressed as fusion protein with GAL4 BD. CASK PDZ and AF-6 PDZ PCR products were also cloned in pACT2 such that they are expressed fused to GAL4 AD. Enzymes used to clone CASK-PDZ domain were BamHI and XhoI. BamHI and

SacI enzymes were used to clone AF-6-PDZ domain. Schematic representation of recombinant proteins are presented in Figure 2.2.

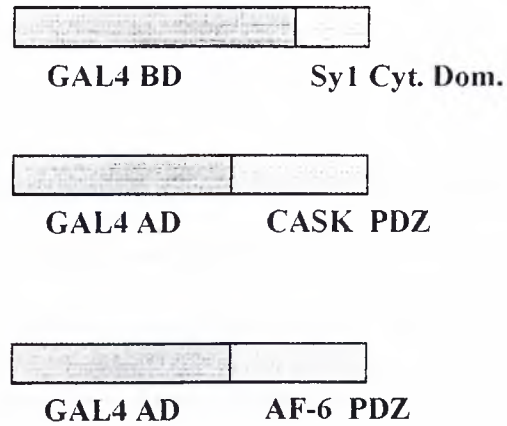


Figure 2.2: Schematic representation of recombinant proteins

2.4- Sequencing of DNA

Sequencing of plasmid DNA was performed by automated ABI PRISM™ 377 DNA Sequencer, Perkin Elmer. Sequencing experiments were performed by Birsen Cevher at Bilgen.

2.5- Gel Electrophoresis

2.5.1- Agarose Gel Electrophoresis (AGE)

1 % Agarose (w/v) in TAE buffer was used in all Agarose gel electrophoresis experiments.

TAE buffer

Tris acetate 0.04 M

EDTA 0.001 M

pH: 7.0

2.5.2- SDS-Polyacrylamide gel electrophoresis of proteins

The glass plates were assembled according to the manufacturer's instructions (EC). The volume of the gel mold was determined according to the information provided by the manufacturer (EC). In an Erlenmeyer flask, the appropriate volume of solution containing the 15 % of acrylamide for the resolving gel was prepared.

Without delay, the mixture was swirled rapidly and the acrylamide solution was poured into the gap between the glass plates. Sufficient space (the length of the teeth of the comb plus 1 cm.) for the stacking gel was left. The acrylamide solution was overlaid by using a Pasteur pipette with isobutanol. The gel was placed in a vertical position at room temperature. After polymerization was complete, the overlay was poured off and the top of the gel was washed several times with deionised water to remove any unpolymerized acrylamide. As much fluid as possible was drained from the top of the gel and then any remaining water was removed with the edge of a paper towel.

Stacking gel was prepared in a disposable plastic tube at an appropriate volume and at 5% concentration. Without delay, the mixture was swirled rapidly and the stacking gel solution was poured directly onto the surface of the polymerized resolving gel. The comb was immediately inserted into the stacking gel, being careful to avoid trapping air bubbles. The gel was placed in a vertical position at room temperature. While the stacking gel was polymerizing, the samples to be loaded were prepared by

heating them to 100°C for 10 minutes in 1X SDS gel-loading buffer to denature the proteins. After polymerization was complete, the comb was removed carefully. By using a squirt bottle, the wells were washed with deionized water to remove any unpolymerized acrylamide.

The electrophoresis apparatus was attached to an electric power supply and the gel was run at a voltage that is approximately 8V/cm, After the dye front has moved to the resolving gel, the voltage was increased to 15 V/cm, until the bromophenol blue reaches the bottom of the resolving gel. Then the power supply was turned off.

The glass plates were removed from the electrophoresis apparatus and placed on a paper towel. By using a spatula, the plates were pried apart. Orientation of the gel was marked by cutting a corner from the bottom.

1X SDS gel-loading buffer

50 mM Tris·Cl (pH 6.8)

100mM dithiothreitol (DTT)

2% SDS (electrophoresis grade)

0.1% bromophenol blue

10% glycerol

1X SDS gel-loading buffer lacking DTT can be stored at room temperature.

DTT should be added, just prior to use, from 1 M stock.

Tris-glycine electrophoresis buffer

25 mM Tris

250 mM glycine (electrophoresis grade)

0.1% SDS

30% mix (Acrylamide and bis-acrylamide solution)

A stock solution of 29% (w/v) acrylamide and 1% (w/v) bis-acrylamide. Solution was stored in dark bottles at 4°C.

10% SDS

A 10% (w/v) stock solution was prepared in deionized water

APS

A small amount of 10% stock solution was prepared in deionized water and stored at 4°C.

2.6- Transfer of proteins from SDS-polyacrylamide gels to solid supports

As the SDS-polyacrylamide gel was approaching the end of its run, six pieces of Whatman 3MM paper and one piece of transfer membrane (Millipore-Immobilon-P) was cut to the exact size of the SDS-polyacrylamide gel by wearing gloves because oil and secretions from the skin may prevent the transfer of proteins from the gel. Matching of the sizes of these papers and transfer membrane is very important because if the paper or membrane is larger than the gel, the overhanging edges of the paper and the membrane will touch the charged plates, causing a short circuit that will prevent the transfer of protein from the gel. One corner of the membrane was marked with a soft-lead pencil.

The membrane was left in methanol for 5 minutes and then washed with deionized water and soaked into transfer buffer for 15-20 minutes. Meanwhile the

Whatman 3MM papers were soaked into a shallow tray containing a small amount of transfer buffer and kept shaking for 15-20 minutes.

Wearing gloves, the transfer apparatus was set as follows:

3 layers of Whatman 3MM paper that have been soaked in transfer buffer was put onto the plate which will be negatively charged (cathode). It is important to squeeze out any air bubbles.

The transfer membrane was placed onto the Whatman 3MM papers. (The transfer membrane should be exactly aligned and the air bubbles trapped between it and the Whatman 3MM paper should be squeezed out .)

The glass plates holding the SDS-polyacrylamide gel was removed from the electrophoresis tank, and the gel was transferred to a tray of deionized water.

SDS-polyacrylamide gel was placed onto the transfer membrane. Any trapped air bubbles were squeezed out with a gloved hand.

3 layers of Whatman 3MM paper were placed to the top of the sandwich (this side will be positively charged during the transfer (anode side).)

The upper plate of the apparatus which will be the cathode during the transfer. The electrical leads of the apparatus were connected to the power supply and the transfer was carried out at a current of 0.65 mA/square. cm of the gel for a period of 1.5-2 hours.

The electric current was turned off at the end of the run time and the transfer apparatus was disassembled from top downward, peeling off each layer in turn. The gel was transferred to a tray containing Coomassie Brilliant Blue and stained in order to check if the transfer is complete or not. The bottom left-hand corner of the membrane was cut as insurance against obliteration of the pencil mark.

2.7- Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue

Polypeptides separated by SDS-polyacrylamide gels were fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue R250.

0.25 g of Coomassie Brilliant Blue R250 was dissolved in 90 ml of methanol:H₂O (1:1 v/v) and 10 ml of glacial acetic acid. In order to remove any particulate material, the solution was filtered through a Whatman No. 1 filter. The gel was immersed in at least 5 volumes of staining solution and was placed on a slowly rotating platform for a minimum of 4 hours at room temperature.

At the end of staining, the stain was removed and saved for future use. The gel was destained by soaking in the methanol-acetic acid solution without the dye (90 ml of methanol:H₂O (1:1 v/v) and 10 ml of glacial acetic acid), on a slowly rotating platform for 4-8 hours at room temperature, changing the destaining solution three or four times. destaining for 24 hours allows as little as 0.1 ug of protein to be detected as a single band.

After destaining, gels can be stored indefinitely in water or can be dried.

2.8- Staining proteins immobilized on solid surfaces with Ponceau S

The membrane onto which proteins were transferred was washed with deionized water and then soaked into Ponceau S. When bands of proteins become visible, the

membrane was washed in several changes of deionized water at room temperature. The positions of proteins used as molecular-weight standards were marked.

Ponceau S

10% glacial acetic acid

5% Ponceau S

dH₂O

2.9- Immunological detection of immobilized proteins (Western Blotting)

After staining the proteins immobilized on transfer membrane with Ponceau S, the membrane was washed gently with deionized water and neutralized with the blocking buffer for 5 minutes. In order to inhibit non-specific binding sites, the membrane was immersed in the blocking solution for at least 60 minutes at room temperature. Mouse monoclonal antibodies are used in this study (Table 2.7). Primary antibody was diluted as recommended by the supplier in blocking solution and was kept at room temperature for at least one hour on a slowly rotating platform. Afterwards the membrane was washed for three times, once for 15 minutes and twice for 5 minutes, with blocking buffer. Following the washes the membrane was incubated in the blocking solution containing the secondary antibody (peroxidase-conjugated rabbit anti-mouse immunoglobulin) diluted as recommended by the supplier for 1 hour and then washed for three times, once for 15 minutes and twice for 5 minutes, with the blocking buffer. Finally the membrane was washed with deionized water and was ready for development.

Blocking Buffer

0.1 % Tween-20 in 1X PBS

Blocking Solution

5% milk powder in 0.1% Tween 20-PBS solution

1X PBS

8 g NaCl

0.2 g KCl

1.44 g Na₂HPO₄

0,24 g KH₂PO₄

Volume was adjusted to 1 liter

pH was adjusted to 7.4

Table 2.7: Antibodies used in this study

Antibody	Developer/Cat. #
Anti-GAL4 (DBD) mouse monoclonal IgG _{2a}	Santa Cruz Biotech. Cat. #Sc-510
Anti-[HA]-peroxidase mouse monoclonal IgG	Boehringer-Mannheim Cat. #1667 475
Anti-[mouse IgG (H+L)]-peroxidase	Boehringer-Mannheim Cat. #1814 168

2.10- Detection of proteins immobilized on membranes

Detection of proteins immobilized on membranes were done by using the ECL Western Blotting kit (Amersham Pharmacia Biotech.) (Catalog # RPN 2109) according to the manufacturer's instructions.

2.11- Equipment

Instruments which were routinely used in this study are as follows:

Automatic pipettes (Eppendorf), filter and 3MM paper (Whatman), programmable dry-block temperature cycler GeneAmp PCR System 9600 (Perkin Elmer), heating blocks (Stuarts Scientific), benchtop centrifuge (Heraus Instruments), avanti J-025I centrifuge (Beckman), gel tanks for agarose (E-C Apparatus Corporation), vertical protein gel tank (E-C Apparatus Corporation), spectrophotometer DU 640 (Beckman), power supply (BioRad), pH meter (Beckman), UV transilluminator (Herolab), transferblot SD (BioRad).

2.12- Consumables

All chemicals were purchased from Sigma, Difco, or Carlo-Erba. Plastic disposables were purchased from Costar, Nunc, or Greiner Scientific.

3- RESULTS

3.1- Cloning of Syndecan-1 cytoplasmic domain in yeast vector pAS2-1

Syndecan-1 cytoplasmic domain was amplified by polymerase chain reaction using Human Fetal Brain cDNA as template. Syndecan-1 cDNA sequence was obtained from the Genebank (Accession number: NM_002997). Primers were designed to amplify the DNA fragment between nucleotides 1026 and 1143. NcoI enzyme recognition sequence was attached as a linker upstream of sense primer. SalI site was attached upstream of antisense primer. Expected size of the PCR product was 138 base pairs. The result of PCR reaction is shown in Figure 3.1.

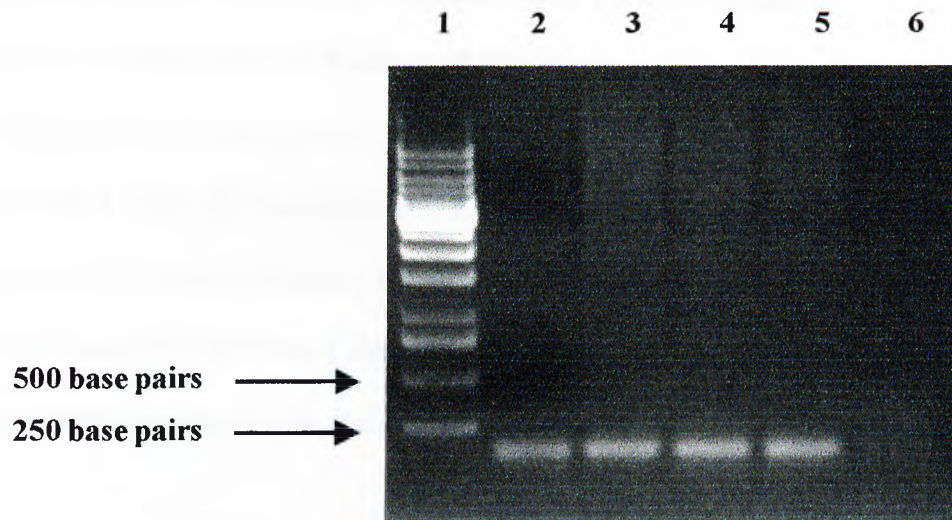


Figure 3.1: PCR amplification of Syndecan-1 cytoplasmic domain

Lane 1: 1 Kb DNA ladder, **Lane 2:** PCR Syndecan-1, **Lane 3:** PCR Syndecan-1,
Lane 4: PCR Syndecan-1, **Lane 5:** PCR Syndecan-1, **Lane 6:** PCR negative control

PCR product and pAS2-1 vector were double digested with NcoI and Sall enzymes. Double cut DNA fragments were then ligated and transformed in bacteria. Plasmid DNA was purified from the transformed clones and checked for the presence of the insert by double digestion with NcoI and Sall enzymes. Result of the digestion reaction is shown in the following figure (Figure 3.2).

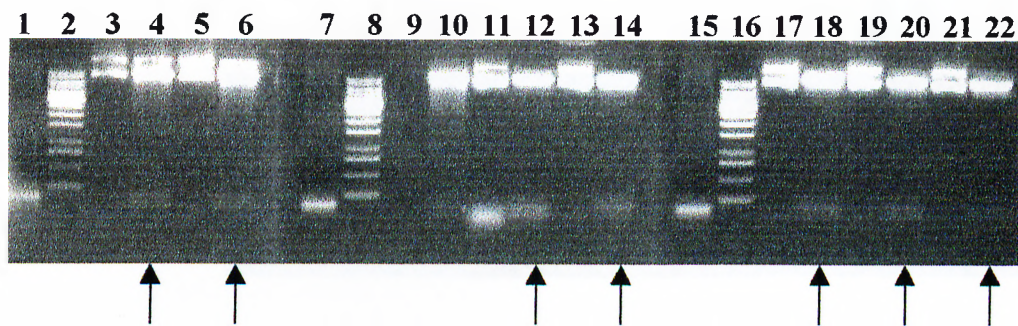


Figure 3.2: Restriction enzyme digestion of Sy1-pAS construct

Lane 1, 7, 15: PCR product Syndecan-1 Cytoplasmic domain, **Lane 2, 8, 16:** 1 Kb DNA Ladder, **Lane 3:** Uncut clone 1, **Lane 4:** Cut clone 1, **Lane 5:** Uncut clone 2, **Lane 6:** Cut clone 2, **Lane 9:** Uncut clone 3, **Lane 10:** Cut clone 3, **Lane 11:** Uncut clone 4, **Lane 12:** Cut clone 4, **Lane 13:** Uncut clone 5, **Lane 14:** Cut clone 5, **Lane 17:** Uncut clone 6, **Lane 18:** Cut clone 6, **Lane 19:** Uncut clone 7, **Lane 20:** Cut clone 7, **Lane 21:** Uncut clone 8, **Lane 22:** Cut clone 8 (Places of inserts are shown by vertical arrows)

Seven of the eight clones (clones 1,2,4,5,6,7, and 8) contained inserts exactly the same size with the PCR product of Syndecan-1 cytoplasmic domain (138 base pairs). These clones were further confirmed by expression studies.

The positive clones were transformed to the yeast strains and total protein was extracted from the transformed yeast strains as explained in the materials and methods

section. Expression of pAS2-1 based constructs (Sy1-pAS clones) were checked in yeast (strain Y187) by SDS-PAGE followed by western blotting using monoclonal antibody against the GAL4 BD. The size of the GAL4 BD is 21.5 kDa and expected size of the GAL4 BD-Syndecan-1 cytoplasmic domain fusion protein is 22.5 kDa. Result is presented in the following figure (Figure 3.3):

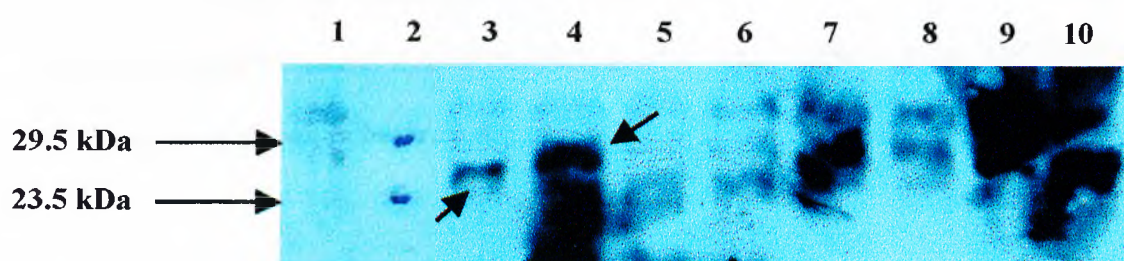


Figure 3.3: Expression of constructs cloned in pAS2-1 vector

Lane 1: Untransformed, **Lane 2:** Molecular Weight marker, **Lane 3:** pAS2-1 transformed, **Lane 4:** Clone 1 of Sy1-pAS transformed, **Lane 5:** Clone 2 of Sy1-pAS transformed, **Lane 6:** Clone 4 of Sy1-pAS transformed, **Lane 7:** Clone 5 of Sy1-pAS transformed, **Lane 8:** Clone 6 of Sy1-pAS transformed, **Lane 9:** Clone 7 of Sy1-pAS transformed, **Lane 10:** Clone 8 of Sy1-pAS transformed (places of fusion proteins are shown by arrows)

The constructs 1, 5 and 8 expressed expected size of proteins. These constructs were further confirmed by DNA sequencing analysis. All three constructs had correct DNA sequence. By this way cloning of Syndecan-1 cytoplasmic domain was verified.

3.2- Cloning of PDZ domain of CASK and AF-6 in yeast plasmid pACT2

PDZ domains of CASK and AF-6 were amplified by polymerase chain reaction using Human Fetal Brain cDNA as template. cDNA sequences were obtained from the Genebank (Accession numbers: AF032119 for CASK and U02478 for AF-6). Primers were designed to amplify the DNA fragments between nucleotides 1476 and 1761 for CASK, and 2925 and 3268 for AF-6. BamHI enzyme recognition sequence was attached as a linker upstream of sense primer of CASK. XhoI site was attached upstream of antisense primer. Expected size of the PCR product was 329 base pairs. The result of PCR reaction is shown in Figure 3.4.

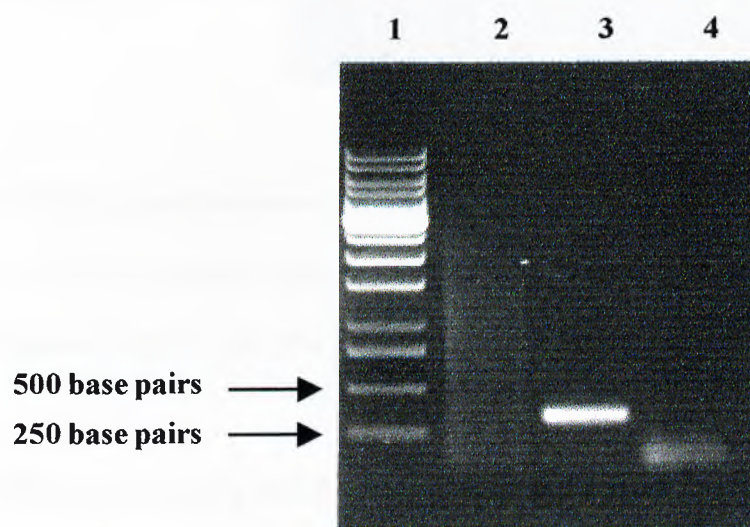


Figure 3.4: PCR amplification of CASK PDZ domain

Lane 1: 1 Kb DNA ladder, **Lane 2:** PCR negative control, **Lane 3:** PCR CASK PDZ domain, **Lane 4:** PCR positive control (PCR Syndecan-1)

BamHI enzyme recognition sequence was attached as a linker upstream of sense primer of AF-6. SacI site was attached upstream of antisense primer. Expected size of the PCR product was 367 base pairs. The result of PCR reaction is shown in Figure 3.5.

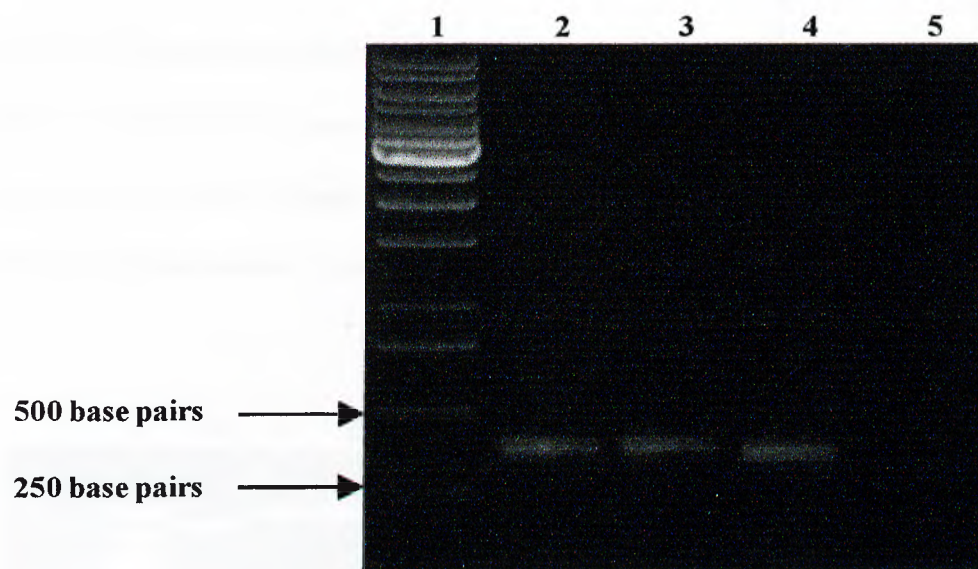


Figure 3.5: PCR amplification of Af-6 PDZ domain

Lane 1: 1 Kb DNA ladder, **Lane 2:** PCR Af-6 PDZ domain, **Lane 3:** PCR Af-6 PDZ domain, **Lane 4:** PCR CASK PDZ domain, **Lane 5:** PCR negative control

PCR products and pACT2 vector were double digested with BamHI and XhoI for CASK, and BamHI and SacI for AF-6. Double cut DNA fragments were then ligated and transformed in bacteria. Plasmid DNA's were purified from the transformed clones and checked for the presence of the insert by double digestion with corresponding enzymes. Results of the digestion reactions are shown in the following figures (Figure 3.6, Figure 3.7).

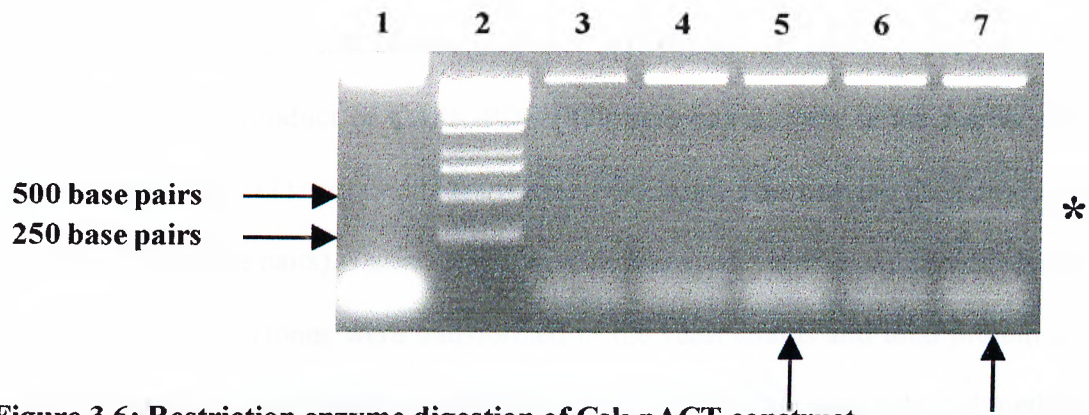


Figure 3.6: Restriction enzyme digestion of Csk-pACT construct

Lane 1: Uncut pACT2, **Lane 2:** 1 Kb Ladder, **Lane 3:** Cut clone 1, **Lane 4:** Cut clone 2, **Lane 5:** Cut clone 3, **Lane 6:** Cut clone 4, **Lane 7:** Cut clone 5, (Places of inserts are shown by vertical arrows) Asterisks: Place of insert

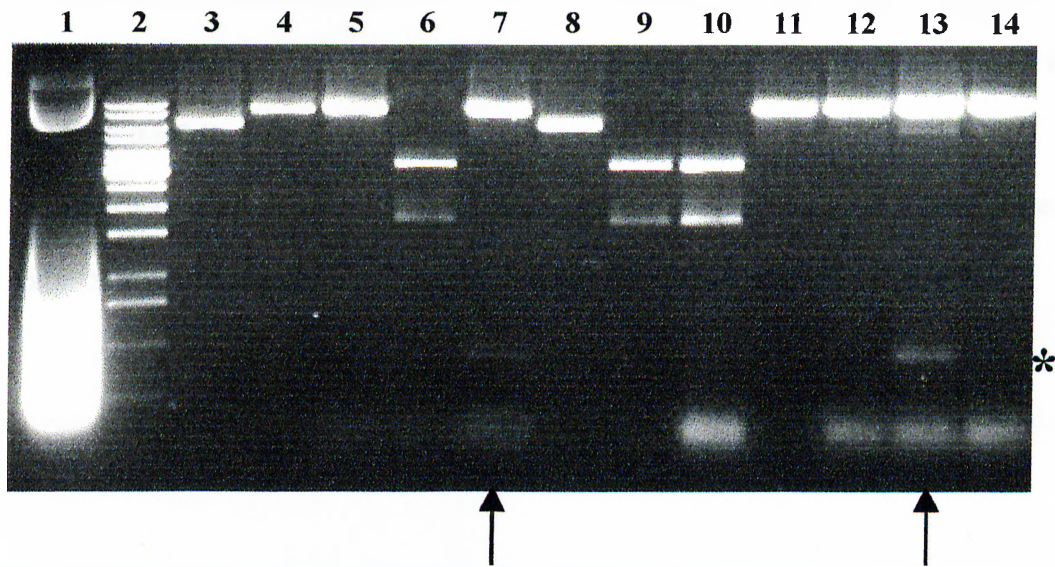


Figure 3.7: Restriction enzyme digestion of AF6-pACT construct

Lane 1: Uncut pACT2, **Lane 2:** 1 Kb Ladder, **Lane 3:** Cut clone 1, **Lane 4:** Cut clone 2, **Lane 5:** Cut clone 3, **Lane 6:** Cut clone 4, **Lane 7:** Cut clone 5, **Lane 8:** Cut clone 6, **Lane 9:** Cut clone 7, **Lane 10:** Cut clone 8, **Lane 11:** Cut clone 9, **Lane 12:** Cut clone 10, **Lane 13:** Cut clone 11, **Lane 14:** Cut clone 12 (Places of inserts are shown by vertical arrows) Asterisks: Place of insert

Two of the five CASK clones (clones 3 and 5) contained inserts exactly the same size with the PCR product of CASK-PDZ (329 base pairs). Two of the twelve AF-6 clones (clones 5 and 11) contained inserts exactly the same size with the PCR product of AF-6-PDZ (367 base pairs). These clones were further confirmed by expression studies.

The positive clones were transformed to the yeast strains and total protein was extracted from the transformed yeast strains as explained in the materials and methods section. Expression of pACT2 based constructs (Csk-pACT clones and Af6-pACT clones) were checked in yeast (strain Y187) by SDS-PAGE followed by western blotting using monoclonal antibody against the Hemagglutinin (HA). HA tag is present upstream of multiple cloning site of pACT2 cloning vector. The size of the GAL4 AD is 18.9 kDa and expected size of the GAL4 AD-CASK PDZ domain fusion protein is 29.7 kDa. Expected size of the GAL4 AD-AF-6 PDZ domain fusion protein is 30.6 kDa. Result is presented in the following figure (Figure 3.8):

Only a single construct Clone 5 of Csk-pACT expressed expected size of protein (μ kDa). DNA sequencing analysis was not performed for any of these constructs.

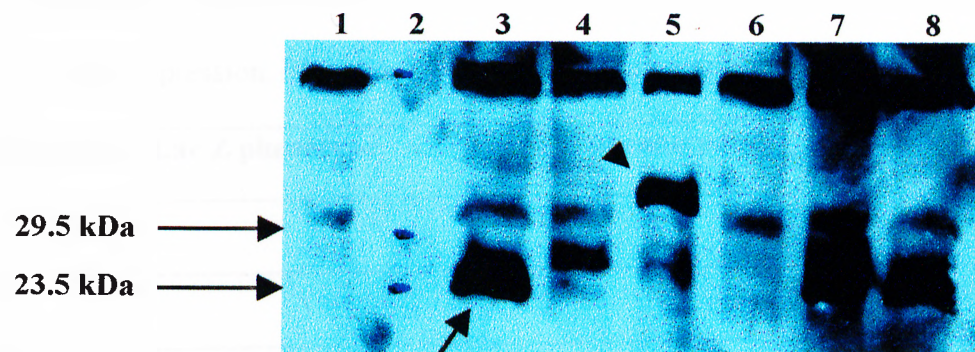


Figure 3.8: Expression of constructs cloned in pACT2 vector

Lane 1: Untransformed, **Lane 2:** Molecular Weight marker, **Lane 3:** pACT2 transformed, **Lane 4:** Clone 3 of Csk-pACT transformed, **Lane 5:** Clone 5 of Csk-pACT transformed, **Lane 6:** Clone 5 of Af6-pACT transformed, **Lane 7:** Clone 11 of Af6-pACT transformed, **Lane 8:** : pACT2 transformed (places of fusion proteins are shown by arrows, fusion protein is shown by arrow head)

3.3- Yeast two-hybrid assay

Positive clones were transformed in yeast strains (Y187 and Y190) and colony lift filter assay and liquid culture assay were performed to detect protein-protein interaction between Syndecan-1 cytoplasmic domain and CASK PDZ domain or AF-6 PDZ domain.

All negative and positive controls of the assay were performed to check the specificity of the interactions reported. Plasmids and strains were checked by control transformations and control yeast two hybrid assays. β -Gal assay was performed using these transformants to determine there was no spontaneous Lac Z activity of the vectors (Table 3.1).

Table 3.1: Lac Z phenotype of the transformant yeast












Each transformant is tested for its Lac Z phenotype by colony lift filter assay. White: No β -galactosidase expression. Blue: positive β -galactosidase expression.

Transformants / Lac Z phenotype	Lac Z phenotype
Pas2-1	white
PaCT2	white
pVA3-1	white
pTD1-1	white
pLAM5-1	white
pCL1	blue
Sy1-PAS	white
Csk-pACT	white
Af6-pACT	white
Untransformant	white

Cotransformations of control vectors were performed. Colony lift filter assay is applied to test if the assay is working properly or not (Table 3.2).

Table 3.2: Control transformations for yeast-two hybrid assay

Yeast strains Y187 and Y190 are used in this experiment. **-L**: leucine minus, tryptophan and histidine plus medium, **-W**: tryptophan minus, leucine and histidine plus medium, **-H**: Histidine minus, leucine and tryptophan plus medium.

Plasmid1	Plasmid 2	Selection medium	Lac Z phenotype	Phenotype
-	pCL1	-L	blue	
pAS2-1	-	-W	white	
-	pACT2	-L	white	
pAS2-1	pACT2	-L, -W	white	
pVA3-1	-	-W	white	
pVA3-1	pACT2	-L, -W	white	
-	pTD1-1	-L	white	
pAS2-1	pTD1-1	-L, -W	white	
pVA3-1	pTD1-1	-L, -W	blue	
pLAM5-1	-	-W	white	
pLAM5-1	pTD1-1	-L, -W	white	

CASK PDZ domain and AF-6 PDZ domain interactions with Syndecan-1 cytoplasmic domain were determined by yeast-two hybrid assay using colony lift filter assay (Table 3.3).

Table 3.3: Results of Yeast two-hybrid assay

Transformants and their Lac Z phenotype were shown. Blue coloring indicates that two fusion proteins, expressed by the plasmid are interacting .

Plasmid 1	Plasmid 2	Selective medium	Lac Z phenotype
Sy1-Pas	Csk-pACT	-L, -W	blue
Sy1-pAS	Af6-pACT	-L, -W	white
Sy1-pAS	-	-W	white
-	Csk-pACT	-L	white
-	Af6-pACT	-L	white
Sy1-pAS	pACT2	-L, -W	white
pAS2-1	Csk-pACT	-L, -W	white
pAS2-1	Af6-pACT	-L, -W	white
Sy1-pAS	pTD1-1	-L, -W	blue
pLAM5-1	Csk-pACT	-L, -W	white
pLAM5-1	Af6-pACT	-L, -W	white

Syndecan-1 cytoplasmic domain and CASK PDZ domain interaction is demonstrated in the following figure (Figure 3.9).



Figure 3.9: Syndecan-1 cytoplasmic domain and CASK PDZ domain interaction

Colony lift filter assay is applied to Sy1-pAS and Csk-pACT cotransformed yeast. The small blue dots represent the expression of β -galactosidase gene indicating a protein-protein interaction among the fusion proteins, Sy1-BD and Csk-AD.

Syndecan-1-CASK interaction was further confirmed by comparing the results of yeast-two hybrid assay of different transformants (Figure 3.10).

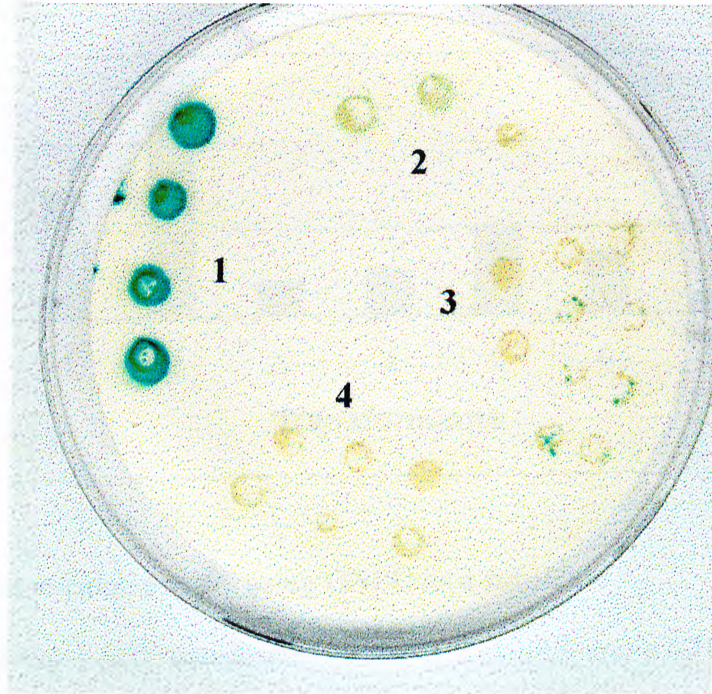


Figure 3.10: Comparison of β -galactosidase expressions in different transformants
1: pTD1-1 and pVA3-1 transformed yeast, 2: pTD1-1 and Sy1-pAS transformed yeast,
3: Sy1-pAS and Csk-pACT transformed yeast, 4: Csk-pACT and pLAM5-1 transformed yeast. Blue coloring indicates the β -galactosidase expression hence protein-protein interaction among fusion proteins.

Syndecan-1 cytoplasmic domain and CASK PDZ domain interaction was confirmed by liquid culture assay. ONPG was used as a substrate of β -galactosidase in the assay. The result is presented in the following figure (Figure 3.11).

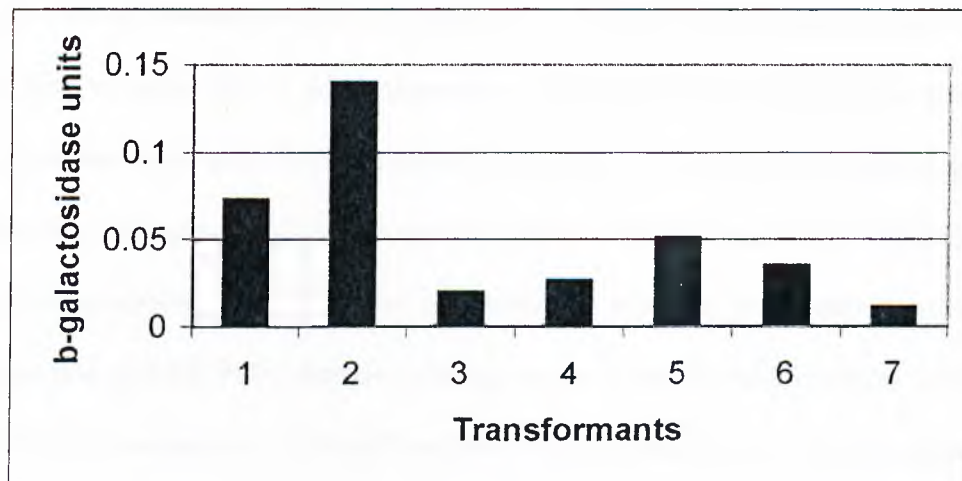


Figure 3.11: Liquid culture assay using ONPG as a substrate

1: Syl-pAS and Csk-pACT transformed yeast, **2:** Syl-pAS and pTD1-1 transformed yeast, **3:** Syl-pAS and pACT2 transformed yeast, **4:** Csk-pACT and pLAM5-1 transformed yeast, **5:** Csk-pACT and pAS2-1 transformed yeast, **6:** Syl-pAS transformed yeast, **7:** Csk-pACT transformed yeast. Higher β -galactosidase units mean stronger interaction.

4- DISCUSSION

In this study we aimed to show a direct interaction between Syndecan-1 cytoplasmic domain with PDZ domains of CASK and AF-6 proteins. In previous studies it was reported that CASK interacts with Syndecan-2 cytoplasmic domain. This interaction is through PDZ domain of CASK and C2 region of Syndecan-2 cytoplasmic domain. C2 region of all Syndecans are 100% identical in all family members (EFYA). So it is expected that a similar interaction is possible with other Syndecan family members. CASK PDZ domain belongs to the Type II PDZ domains which have a binding consensus of (Glu/Ile)-Phe-(Phe/Tyr)-(Val/Ala). This sequence match perfectly with the last four amino acid sequence of all Syndecans so it is logical to think that a similar interaction can be seen among CASK and other Syndecans.

AF-6 is another important PDZ containing protein, which localizes to cell to cell junctional sites. It interacts with receptor tyrosine kinases like epinephrine receptor EphB3 through its PDZ domain. It also contains a Ras binding domain through which binds to GTP activated Ras protein and modulates its signal transduction activities. AF-6 PDZ domain also belongs to Type II PDZ domains.

Syndecan-1 was chosen since it is known to have important regulatory functions in developmental processes. It probably regulates cell proliferation, cell motility, cell differentiation, and cell fate by modulating signaling events and cell to cell and cell to matrix adhesions. It is recently reported to be an important modulator of tumorigenicity induced by Wnt signaling pathway in mammary cancer.

To identify Syndecan-1 cytoplasmic domain interactions with PDZ domains of CASK and AF-6, we used yeast to hybrid assay. This system is a recently established protein -protein interaction assay, which is reported to be a powerful tool to identify novel protein interactions. We first cloned Syndecan-1 cytoplasmic domain into yeast expression vector pAS2-1 that it is expressed as a fusion protein with GAL4 DNA binding domain. CASK PDZ domain and AF-6 PDZ domain were cloned in yeast expression vector pACT2 as fusion proteins with GAL4 transactivating domain.

Constructs were checked by restriction enzyme digestion and it is confirmed that they all contain inserts of expected sizes. Seven syndecan-1 cytoplasmic domain containing clones, two CASK PDZ domain containing clones, and two AF-6 PDZ containing clones were obtained.

The reliability of the constructs were further confirmed by DNA sequencing analysis. But only the sequence of Syndecan-1 clones was obtained. Sequence of these constructs turned out to be perfect indicating the success of the cloning steps. The expression of fusion proteins in yeast were also checked by total protein extraction followed by a western-blotting. It was shown that three Syndecan-1 clones out of seven were expressing Syndecan-1 Cytoplasmic domain, GAL4 DB domain fusion protein. Only a single CASK clone were expressing PDZ domain, GAL4 AD fusion protein. Other clones were not expressing proteins of expected sizes.

Colony lift filter assay using X-Gal as substrate indicated that CASK PDZ interact with Syndecan-1 Cytoplasmic domain when expressed in yeast. A weak positive signal can be seen in all experiments. Interaction was not as strong as positive controls (p53 vs. SV40 LT Antigen) but this result is satisfactory enough for concluding that CASK protein binds to Syndecan-1 cytoplasmic domain.

AF-6 clones were not expressing a fusion protein at expected size. Sequencing data of these clones are also not available at the moment. The cause of expression profile of AF-6 clones can be clarified after sequencing results. In all occasions yeast two-hybrid assays gave no positive signal for AF-6 and Syndecan-1, probably because AF-6 was not expressed in yeast. According to our hypothesis AF-6 is also interacting with cytoplasmic domain of Syndecan-1. The cloning of AF-6 will be performed again in order to obtain AF-6 PDZ, GAL4 AD fusion protein expressing clones. The yeast two-hybrid assays will be repeated with these clones and possible Syndecan-1 AF-6 interaction will be identified.

Liquid culture assay using ONPG as substrate was also performed to confirm the results obtained from the colony lift assay. A detectable interaction is also observed in ONPG assay. The strength of interaction is determined using spectroscopic measurements. CASK-Syndecan-1 interaction is quantitated by comparison of β -galactosidase units of this interaction with the one used for positive control. It turned out that CASK-Syndecan-1 interaction is one thousand fold weaker than the p53-SV40 LTA interaction. But yet this result is significant enough for us to conclude that there is a physical interaction between CASK and Syndecan-1.

Our results indicated that Syndecan-1 can bind to CASK protein via its PDZ domain. This result is quite reasonable since a similar interaction is reported previously for Syndecan-2. The interaction domain of Syndecan-2 is identical to that of Syndecan-1 (EFYA). Syndecan-1 being an important integral membrane protein is known to be involved in signaling events. One example of such signaling processes is Wnt signaling pathway (Alexander C. M. et al. 2000). Dsh protein is an immediate target for activated

frizzled receptors. Dsh protein also contains Type II PDZ domain and it is highly possible that Syndecan-1 involvement in the pathway is through this PDZ protein.

CASK-Syndecan-1 interaction may be an important element of another signaling pathway, since CASK is known to translocate into nucleus and activate the transcriptional activities of some well known transcription factors (Tbr-1). That is why we hypothesize that Syndecan-1 CASK interaction have important cellular function in vivo.

In our studies we also identified that Syndecan-1 cytoplasmic domain bind to SV40 LTA. This interaction was observed both in colony lift filter assay and ONPG assay. The strength of interaction was two fold higher than Syndecan-1-CASK interaction.

Syndecan-1 SV40 LTA interaction was the unexpected result of our studies, but it is quite logical to think an interaction between an oncogenic antigen which transforms the infected cells, and an important integral membrane protein. Similar effects can be seen upon transfection of other oncogenic viral proteins. Increased tyrosine phosphorylation is reported for some insulin receptors caused by SV40 LTA transfection (Wang 1999). This is an example for the modification of cellular proteins by viral antigens. Most of these effects are indications of direct physical interactions of viral proteins and integral membrane proteins.

In another study it is shown that expression of Syndecan-1 increases in SV40 LTA and adenoviral Ea1 transfected 293 T cell lines but not in untransfected cells (Numa et al. 1995). Regulation of Syndecan-1 may be an important aspect of virus mediated alteration of cell proliferation and cell motility. Probably by such means viruses cause the transformation of normal cells easily.

4.1- Future Perspectives

We hypothesized that PDZ domains of CASK and AF-6 interact physically with cytoplasmic domain of Syndecan-1. We showed CASK Syndecan-1 interaction successfully but we could not show any interaction with AF-6 PDZ domain. This may be due to an error during the preparation of the constructs since we do not have the sequence of the AF-6 clones, this part of the project remains to be determined. The assay will be repeated with other AF-6 constructs of which their sequences and expression profiles are confirmed like the Syndecan-1 clones.

The protein-protein interaction may be confirmed by *in vitro* studies. AF-6, CASK, and Syndecan-1 cytoplasmic domain may be cloned in bacterial or mammalian expression vectors and immunoprecipitation experiments may be conducted. Purification columns may also be used to identify direct physical interactions. Interaction may be further tested in mammalian cells, *in vivo*. So co-localization of CASK or AF-6 with Syndecan-1 will be another evidence of interaction among these proteins.

Based on our hypothesis Dsh protein which also contains a Type II PDZ domain, may be another target protein. Protein-protein interaction of Syndecan-1 with Dsh may be identified by a similar approach.

Since interest subject of this study is just to identify novel protein interactions of Syndecan-1 core protein, screening of a cDNA library with yeast two-hybrid technique may be a useful tool to identify more of such interactions. Protein-protein interactions of C1 and V domain of Syndecan-1 protein may be identified by such techniques. Other important interactions may identify important signaling events that Syndecan-1 regulate or at least modulate.

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