Expression of short peptides *in vivo* to modulate protein interactions

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Gutachter:

1..

2..

3..

Tag der Doktorprüfung...

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Introduction... 1
1.1 Classes of protein-protein interactions
1.1.1 Homo-oligomerization.. 2
1.1.2 Heterologous protein interactions... 3
1.1.3 Non-obligate and obligate complexes.......... 3
1.1.4 Transient and permanent complexes.. 3

1.2 Protein interactions in signal pathways
1.2.1 Receptor tyrosine kinases... 4
1.2.2 Protein tyrosine phosphatase... 6

1.3 Protein interactions at domain level
1.3.1 SH2 domain in protein interaction.. 7
1.3.2 PTB domains.. 8
1.3.3 Domains recognizing phosphoserine/ threonine............................ 9

1.4 Transcription factors in protein interaction
1.4.1 Nuclear receptors as transcription factors...................................... 9
1.4.2 Coactivator recruitment by nuclear receptors................................. 10
1.4.3 Coactivators in chromatin remodeling... 13

1.5 Modulation in protein–protein interaction
1.5.1 By use of synthetic molecules... 14
1.5.2 By use of naturally organic molecules... 15
1.5.3 By use of peptides.. 15
1.5.4 Low molecular weight modulators
(Identified by screening of chemical libraries).............................. 16
1.5.5 By Mutation... 16

Aim of the study... 17

2.1 Material and methods
2.1.1 Chemicals...18
2.1.2 Enzymes...18
2.1.3 Kits and other materials...18

2.2 Medium and buffers
2.2.1 Medium for E.coli.. 19
2.2.2 Cell culture media.. 19
2.2.3 Stock buffers.. 19

2.3 Bacterial strains and cell lines
2.3.1 Bacterial strains.. 20
2.3.2 Cell lines... 20

2.4 Methods in molecular biology
2.4.1 Plasmid preparation for analytical purpose.................................... 21
2.4.2 Digestion of DNA samples with restriction
endonucleases... 21

2.4.3 Dephosphorylation of DNA 5´-termini with calf intestine alkaline phosphate (CIAP) 21
2.4.4 DNA insert ligation into vector DNA 21
2.4.5 Phosphorylation of DNA by T4 polynucleotide kinase (T4PNK).... 22

2.5 Agarose gel electrophoresis.... 22
2.5.1 Isolation of DNA fragments using low melting temperature agarose gels.... 22
2.5.2 Polymerase chain reaction (PCR).... 22
2.5.3 PCR product purification 23
2.5.4 Phenol chloroform precipitation.... 23

2.6 Introduction of plasmid DNA into E.coli cells
2.6.1 Preparation of competent cells.... 23
2.6.2 Transformation of competent cells.... 23

2.7 Vectors.... 24
2.7.1 Vector constructs.... 24
2.7.2 Oligonucleotides coding for short peptides.... 25
2.7.3 Annealing of oligonucleotides 25
2.7.4 Addition of self-annealing flanking clamp sequence.... 25

2.8 LXXLL motif peptides
2.8.1 Short LXXLL peptide with random amino acid residues.... 26
2.8.2 LXXLL peptides with varying number of motifs.... 27

2.9 General cell culture technique
2.9.1 Transfection with effectene reagent.... 28
2.9.2 Transfection by lipofectamine.... 28

2.10 Retrovirus
2.10.1 Retrovirus production.... 29
2.10.2 Retroviral infection 29

2.11 Cell proliferation.... 29
2.12 Fluorescence activated cell sorting (FACS) analysis.... 29
2.13 Secreted alkaline phosphate (SEAP) chemiluminescence detection 30

2.14 Protein antibody array
2.14.1 Lysis of cells.... 30
2.14.2 Determination of protein concentration.... 30
2.14.3 Protein detection by array.... 30

2.15 Random peptide library construction.... 31
2.15.1 Peptide rescue by PCR.... 32

Results
3.1 Targeting protein-protein interactions by short peptide expression.............. 33
3.1.1 Ros tyrosine phosphorylation domain peptide.............................. 34
3.1.2 Effect on NIH3T3TrkA Ros cell growth in absence and presence of SHP-1PTP... 36
3.1.3 Stimulation of TrkA Ros by NGF is not influenced by peptides... 38

3.2 Nuclear receptor coactivator interaction specificity; Effect of short peptides with LXXLL motif on transcription activation......................... 42
3.2.1 Suppression of vitamin D and 9-cis retinoic acid induced transcription 44
3.2.2 Efficiency of peptide mediated suppression is concentration dependent... 46
3.2.3 Suppression of dexamethasone and forskolin induced transcription... 48
3.2.4 Adjacent amino acids are major determinants of efficiency... 49
3.2.5 Pattern of efficiency is different for nuclear mediated transcription and PKA (forskolin) mediated transcription........... 51
3.2.6 LXXLL peptides are active in different cell types......................... 52
3.2.7 Influence of LXXLL peptides on cell proliferation and signal transduction.. 54

3.3 Peptide expression library to search novel bio active peptide........................ 57
3.3.1 Design and preparation of random peptide libraries....................... 58
3.3.2 Identification of peptide conferring resistance to dexamethasone toxicity in fibroblasts.. 58

4 Discussion.. 64
5 Summary.. 72
6 Zusammenfassung... 75
7 References.. 78

Abbreviations

AP-1	Activator protein-1
AR	Androgen Receptor
AF-1	Activation function 1
AF-2	Activation function 2
atc	Anhydrotetracycline
ATP	Adenosine –triphosphate
CREB	cAMP response element binding protein
CRE	cAMP response binding element
CAIP	Calf intestine alkaline phosphate
cAMP	Cyclic adenosine mono phosphate
DRIP	Vitamin D receptor interacting protein
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
DBD	DNA binding domain
Dex	Dexamethasone
DMEM	Dulbecco´s modified eagle medium
ES	Embryonal stem cells
EDTA	Ethylenediaminetetraacetic acid
EYFP	Enhanced yellow fluorescent protein
FCS	Fetal calf serum
Fks	Forskolin
FACS	Fluorescence activated cell sorting
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GRE	Glucocorticoid response element
GRIP-1	Glucocorticoid receptor interacting protein-1
Hsp70	Heat shock promoter
HAT	Histone acetyltransferase
HAT	Histone acetyl transferase
HRE	Hormone response element

HMG	High mobility group protein
IRES	Internal ribosomal entry site
IVS	Synthetic intron
LBD	Ligand binding domain
LTR	Long terminal repeats
MCS	Multiple cloning site
MHC	Multiple histocompatibilty complex
MAPK	Mitogen activated protein kinase
NGF	Nerve growth factor
NFkB	Nuclear factor–kappa B
NR	Nuclear receptors
NSP	Non specific peptide
Neg	Negative peptide
NC	Negative peptide with clamp
nRTKs	Non-receptor tyrosine kinases
OPLs	Oriented peptide libraries
Pos	Positive peptide
PC	Positive peptide with clamp
PTP	Protein tyrosine phosphatase
PI3 kinase	Phosphatidylinositol 3´-kinase
PCR	Polymerase chain reaction
PTB	Phoshphotyrosine binding
PI	Phosphoinositol
PBS	Phosphate buffer saline
PIC	Preinitiation complex
PIs	Proteinaceous inhibitors
RTKs	Receptor tyrosine kinases
RT	Room temperature
PSLs	Positional scanning libraries
RXR	Retinoid X receptor
RA	9-cis retinoic acid
rpm	revolutions per minute
RAR	Retinoid A receptor

SH2	Src homology domain
SRC-1	Steroid receptor coactivator
SRF	Serum response factor
SEAP	Secreted alkaline phosphate
TRAP	Thyroid hormone receptor associated protein
T4PNK	T4 polynucleotide kinase
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VD	Vitamin D

Introduction

Protein–protein interactions are central to virtually every cellular process, like DNA replication, transcription, translation, splicing, secretion, cell cycle control, signal transduction, intermediary metabolism, in the structure of sub-cellular organelles, transport machinery across the various biological membranes, packaging of chromatin, network of sub-membrane filaments, muscle contraction and regulation of gene expression comprise list of processes in which protein complexes have been implicated as essential components. Due to importance of these interactions in the growth and development intense research has been done in recent years. It has emerged that nature has employed in many instances a strategy of mixing and matching of specific domains that specify particular classes of protein–protein interactions, modifying the amino acid sequence in order to confer specificity for particular target proteins.

Protein-protein interactions have a number of different measurable effects some of them are mentioned as; First, they can alter the kinetic properties of proteins that can be reflected in altered binding of substrates, altered catalysis and altered allosteric properties of the complexes. Second, protein-protein interaction is one common mechanism to allow for substrate channeling. The paradigm for this type of complex is tryptophan synthetase from Neurospora crasa. Many similar metabolic channeling have been demonstrated, both between different subunits of a complex and between different domains of a single multifunctional polypeptide (Srere *et al.,*1987). Third, protein-protein interactions can result in the formation of a new binding site. Fourth, protein-protein interactions can inactivate a protein as in case of interaction of phage p22 repressor with its antirepressor (Susskind *et al.,*1983), interaction of trypsin with trypsin inhibitor (Vincent *et al.,*1972). Fifth, protein-protein interactions can change the specificity of a protein for its substrate, e.g. interaction of transcription factors with RNA polymerase directs the polymerase to different promoters.

Protein interactions may be mediated at one extreme by a small region of one protein fitting into a cleft of another protein and at another extreme by two surfaces interacting over a large area. Example for the first case, include protein-protein interactions that involve a domain of a protein interacting tightly with a small peptide, like interaction of SH2 domain with a specific small peptides containing a phosphotyrosyl residue. The paradigm for the second case *i.e.,* surfaces that interact with each other over large areas is that of the leucine zipper in which a stretch of ∝ helix forms a surface that fits almost perfectly with another ∝ helix from another subunit protein (Ellenberge *et al.,*1992; O´Shea *et al.,*1991).

Regulation of cell functions is delicately balanced by the relative affinities of various protein partners, modulation of their affinities by the binding of ligands, other proteins, nucleic acids, ions such as Ca^{2+} and by covalent modifications like specific phosphorylation or acetylation reactions. However, within a cell many intracellular and physico-chemical factors like temperature, ionic strength and pH also play a critical role in protein-protein interactions. For instance, at high temperature heat shock protein 90 oligomerizes and shows a new chaperone activity (Yonehara *et al.,*1996). Ionic strength of a solution affects oligomeric state of the protein (Brazil *et al.,*1998; Shima *et al.,*1998) and also influences the kinetics of protein interactions. pH plays an important role in stability of protein complexes (Gibas *et al.,* 1997; Xie *et al.,*1998).The covalent modification like phosphorylation is well known phenomenon of regulating protein-protein interaction in signal transduction cascade (Eyster, 1998).

Specificity of protein-protein interactions

Proteins generally reside in a crowded environment with many potential binding partners with different surface properties. Most proteins are very specific in interacting with their partners, although some are multispecific, having multiple (competing) binding partners on coinciding or overlapping interfaces. Protein complexes such as hormone-receptor and antigen-antibody complexes formed between protomers are initially not co-localized, where as functionally relevant interactions, such as enzyme-inhibitor assemblies are highly specific. Although, localization has a role to play, specificity clearly derives from the complementarity of shape and chemistry that determines the free energy of binding. For protein interactions multi specificity between two homologous families of proteins or between a homologous family can be distinguished. Multi-specific binding between two protein families is very common in regulatory pathways or networks such as in extracellular and intracellular signaling. However, the members of the protein family often recognize a specific pattern or surface patch on the target protein. For example, the SH2 and SH3 domains bind to proteins with phosphotyrosine and proline-rich sequences respectively.

Protein interactions are much more widespread as expected. To understand their significance in the cell it is necessary to identify the different interactions, understand the extent to which they take place and determine the consequence of the interaction.

1.1 Classes of protein-protein interactions

Proteins interact with other proteins in a number of ways involving number of forces predominantly non ionic like hydrophobic interactions, Van der Waals interactions. Although

weak, these forces contribute most to the stability of a protein complex. Protein interactions can be also classified as;

1.1.1 Homo-oligomerization

An enormous number of enzymes, carrier proteins, scaffolding proteins, transcriptional regulatory factors etc function as homo-oligomers. Incorporation of non-covalent interactions at the level of protein quaternary structure provides us with a number of regulatory possibilities that would not be possible if functional unit is comprised of a single polypeptide chain. Energy can be stored at the subunit interface that can serve to bind ligand, or modify the protein conformation in response to regulatory ligands. Modulation of subunit affinity in such a manner need not compromise the folded structure of the protein, yet provides a considerable energetic margin for modulation of activity.

1.1.2 Heterologous protein interactions

Communication at the level of the organism or the cell requires the translation of physical or chemical information signals from one compartment to another. In large part this communication relies on the specific interaction between particular heterologous proteins in response to particular chemical or physical signals. Clearly all of the mechanisms have not been elucidated that control cell functions.

1.1.3 Non-obligate and obligate complexes

As well as composition, two different types of complexes can be distinguished on the basis of whether a complex is obligate or non obligate. In an obligate protein–protein interaction, the protomers are not found as stable structures on their own *in vivo*. Such complexes are generally functionally obligate; for example, the Arc repressor dimer is essential for DNA binding. Many of the hetero-oligomeric structures in the protein data bank involve non-obligate interactions of protomers that exist independently, such as intracellular signaling complexes (e.g. RhoA-RhoGAP) antigen-antibody, receptor-ligand and enzyme inhibitor (e.g. thrombin-rodnin) complexes. The components of such protein-protein complexes are often initially not co-localized and thus need to be independently stable. However, some homo-oligomers, which by definition are co-localized can also form non-obligate assemblies.

1.1.4 Transient and permanent complexes

Interaction of proteins can also be distinguished on the lifetime of their complexes. In contrast to a permanent interaction that is usually very stable and only exists in its complexed form, a transient interaction associates and dissociates in vivo. A weak transient interaction features a dynamic oligomeric equilibrium in solution where the interaction is broken and

formed continuously and strong transient associations require a molecular trigger to shift the oligomeric equilibrium. For example, the heterotrimeric G protein dissociation into the G∝ and Gβγ subunits upon guanosine triphosphate (GTP) binding. Structurally or functionally obligate interactions are usually permanent, whereas non-obligate interactions may be transient or permanent.

1.2 Protein interactions in signal pathways

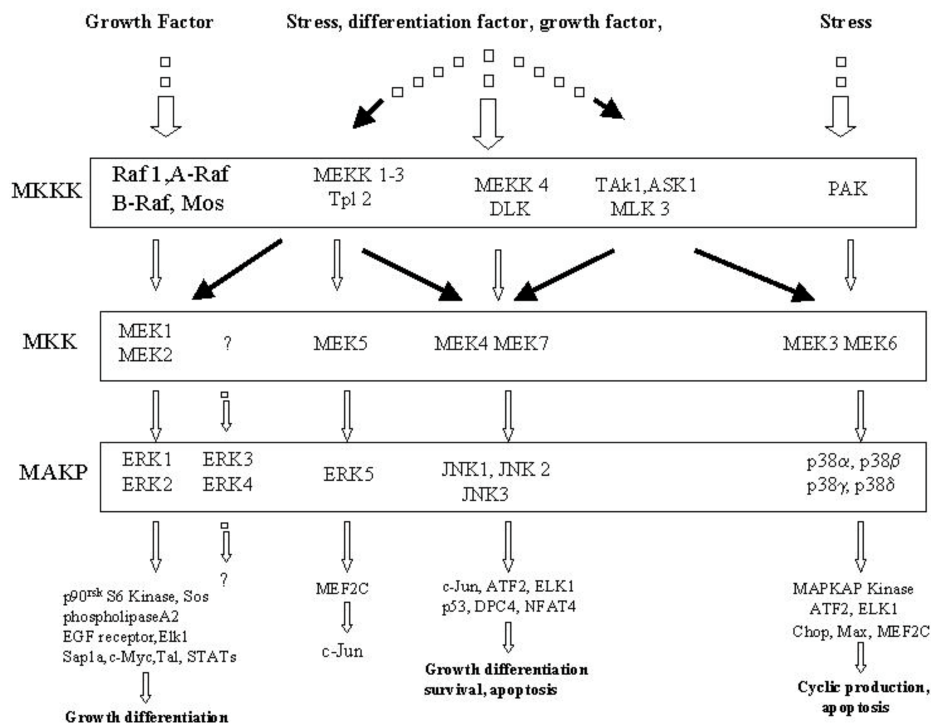
Ability of cells to respond various signals and culminating in multiple cascades of signal pathways involve interaction between various proteins. These responses are coordinated through signaling pathways that transduce and exchange information between different cells or inside the cell between different compartments. Apart from direct cell to cell contact, signaling to neighboring cells or to distant cells occurs by secreted messenger molecules such as growth factors and hormones. These molecules bind to their cognate receptor and there by transmit the signal inside the target cell to finally stimulate a distant biological response including cell proliferation, migration, differentiation or apoptosis. Consequently deregulated signal transduction events have been recognized as the underlying cause of many severe human diseases such as cancer, diabetes, immune deficiencies and cardiovascular diseases, among many others. Reversible protein phosphorylation has been identified as a key element in signal transduction processes. Tyrosine kinases and phosphatases are key proteins in regulating the signal pathway.

1.2.1 Receptor tyrosine kinases (RTKs)

RTKs are transmembrane enzymes responsible for transducing extracellular signals from peptide growth factors across the cell membrane. They are characterized by extracellular ligand binding domains, a single transmembrane helix and an intracellular portion containing tyrosine kinase activity (Robinson *et al.,*2000). The kinase domain is composed of ~300 conserved amino acid residues among kinases (Hubbard *et al.,*2000). It exhibits a two domain architecture consisting of an amino terminal lobe and a larger carboxy terminal lobe. The cleft formed by the two lobes harbors the reaction where the γ–phosphate from ATP is transferred to a hydroxyl group of the tyrosine in the protein substrate. Protein tyrosine kinases can be subdivided into two families; RTKs and non receptor tyrosine kinases (nRTKs), 58 RTKs and 32 nRTKs are reported (Manning *et al.,*2002).

Most RTKs are monomeric in their inactive state and dimerizes upon ligand binding (Hubbard *et al.,*1999). One exception is the insulin receptor family, members of which are dimerized also in the absence of ligands. Ligand binding induces autophosphorylation of

tyrosine residues in the so called activation loop and subsequently in the residues outside the catalytic domain. The phosphorylated non-catalytic tyrosine act as specific binding sites for



Mitogen activated protein kinase modules indicating signaling cascade; Each module consists of a Mitogen activated protein kinase kinase kinase (MKKK), Mitogen activated protein kinase kinase (MKK) and a MAPK (Mitogen activated protein kinase). While the MKKs are relatively specific for their target, MAPKs, MKKKs, can activate one or more MKK. Activation of MAPKs induces activation of different targets, comprising transcription factors but also for instance kinases such as EGFR (Johnson and Lapadat 200)

downstream signaling proteins containing phosphotyrosine binding (PTB) and Src homology (SH2) domains. The specificity of individual SH2 domains is determined by the three to five amino acid residues carboxy-terminal of the phosphotyrosine (Pawson *et al.,*1997), where as PTB domain binding specificity motif is conferred by five to eight amino acid residues amino–terminal of the phosphotyrosine (Songyang *et al.,*1993).

Activation and interaction of downstream signaling molecules is conferred by different mechanisms. The activation may involve conformational changes induced directly by the binding to the RTK and it can also be a consequence of tyrosine phosphorylation. The protein tyrosine phosphatase SHP-2 and the tyrosine kinase Src are examples of signaling proteins that undergo conformational changes and subsequent activation upon RTK recruitment. Activation may also involve translocation of the molecule in proximity to its substrate e.g. Phosphatidylinositol 3-kinase (PI3 kinase) is recruited to RTKs and thereby translocated to

the membrane where its substrate phophatidylinositol 4,5 bisphosphate is located. RTKs can also recruit and phosphorylate transcription factors for instance phosphorylation of STATs results in dimerization and nuclear translocation of the transcription factors (Xu *et al.,*1999). Another group of molecules recruited to activate RTKs is adapter molecules e.g. Shc and Grb2.

1.2.2 Protein tyrosine phosphatase (PTP)

Protein tyrosine phosphatase (PTP) is a family of enzymes regulating cellular phosphorylation state important for many cellular processes (Tonks *et al.,*1998). PTPs are biochemically and physiologically distinct from RTKs and are central to regulation of physiological processes (Hunter,1995) which depends on their subcellular localization (Mauro *et al.,*1994; Hunter,1995). PTP family is composed of about 100 enzymes that despite limited sequence similarity share a highly conserved catalytic signature motif (V/I HCSxGxGR(S/T) G, at the bottom of an active site cleft (Barford *et al.,*1998). The cleft confers specificity towards phosphotyrosine since hydrolysis of the shorter phospho-serine and phospho-threonine residues is prevented (Guan and Dixon, 1991). PTPs are divided into two major categories, transmembrane (receptor type) and cytoplasmic (non receptor type). Non receptor type PTPs contain one PTP domain carrying the PTP activity flanked by domains that are important for protein-protein interaction and enzyme activity. On the other hand receptor type PTPs contain one or two PTP domains in the intracellular region which are linked to a variety of extracellular domains through a transmembrane segment. Non receptor type PTPs undergo proteolytic cleavage that alter their subcellular localization and can result in their activation (Gu *et al.,*1996; Gurd *et al.,*1999; Nguyen *et al.,*1995; Rock *et al.,*1997). Src homology 2 domains of SHP1 and SHP2 mediate recruitment of PTPs to activated growth receptors (Frangioni *et al.,*1993; Stein-Gerlach *et al.,*1998). PTPs functions can be modulated by interaction between the non–catalytic segment of these enzymes and various binding proteins (Neel, 1993). Protein-protein interactions have the potential to modulate PTP activity either by altering enzyme directly or by controlling intracellular localization.

1.3 Protein interactions at domain level

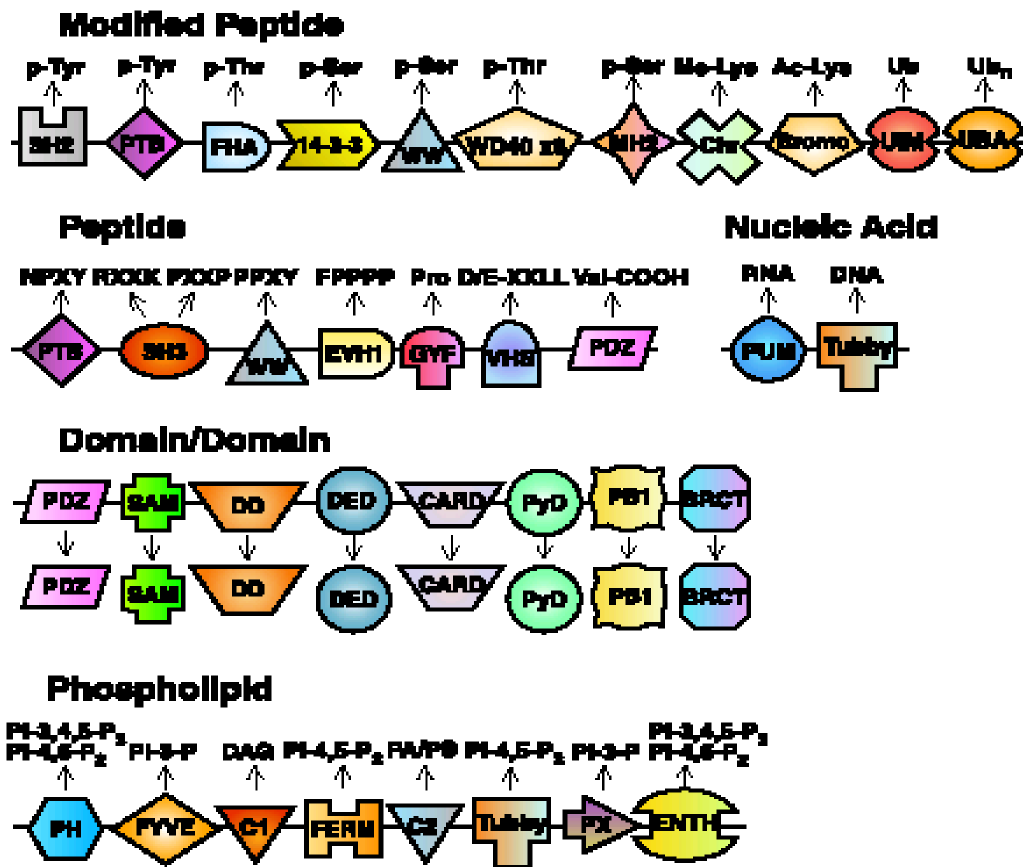
Many of the signaling pathways and regulatory systems in eukaryotic cells are controlled by proteins with multiple interaction domains that mediate specific protein-protein and protein-phospholipid interactions. In this way they determine the biological output of receptors for external and intrinsic signals. Cytoplasmic proteins conveying information from cell surface receptors to their intracellular targets are commonly constructed of modular domains that either have a catalytic function or mediate the interaction of proteins with one

another, or with second messengers (Pawson,1995). Protein interactions carried out by domains were originally identified in the context of phosphotyrosine signaling through the ability of Src homology 2 (SH2) domains of cytoplasmic proteins to recognize specific phosphotyrosine containing motifs on activated receptor tyrosine kinases (Anderson *et al.,*1990; Matsuda *et al.,*1990; Songyang *et al.,*1993). During protein–protein interaction domains not only recognize exposed features of binding partners but also post-translationally modified sequences (Blaikie *et al.,*1994; Kavanaugh *et al.,*1994; Van der Geer *et al.,*1996), phosphothreonine/serine–containing elements (14-3-3 proteins FHA,WD40 domains (Yaffe *et al.,*2001), phoshphoinositides (PI) (i,e PH, EYVE, PX, ENTH, Ferm) and Tubby domains (Cullen *et al.,*2001; Santagata *et al.,*2001).

1.3.1 SH2 domain in protein interaction

The ability of interaction domains to mediate the formation of protein complexes in a fashion that depends on protein phosphorylation is typified by the binding of SH2 domains to phosphotyrosine sites. SH2 domains are protein modules of 100 amino acids that recognize phosphotyrosine residues-containing peptides in the context of 3-6 carboxy terminal amino acids (Dilworth *et al.,*2001) such as those found in the non catalytic region of activated growth factor receptor, located either between the membrane and the kinase domain or in the C-terminal loop (Heldin *et al.,*1998). Such interactions link receptor autophosphorylation to the activation of specific cytoplasmic signaling pathways. SH2 domains serve as intracellular targets of RTK and more complex multi-subunit receptors, cytokines and extracellular matrix components (Schlessinger, 2000; Hunter, 2000). Binding energy for SH2 domain-phospho peptide interaction comes from its association with phosphotyrosine and also stabilizes the SH2 mediated complexes (Piccione *et al.,*1993). In addition, to recognition of phosphotyrosine SH2 domains recognize three to five residues immediately C–terminal to the phosphotyrosine in a fashion that varies from one SH2 domain to another (Reedijk *et al.,* 1992; Waksman *et al.,*1993; Pascal *et al.,*1994; Kay *et al.,*1998). Proteins with more than one SH2 domain bind with more specificity and affinity with their cognate partners. Thus, proteins with two tandem SH2 domains bind cooperatively to bisphosphorylated sites (Ottinger *et al.,*1998) and Src family kinases can potentially interact with their targets through both their SH2 domains and the covalently linked SH3 domain, which recognizes proline rich sequences (Kanner *et al.,*1991; Nakamoto *et al.,*1996; Pellicena *et al.,*2001). SH2 domain of SH2D1A protein shows apparent flexibility as it can interact not only a phosphotyrosine residue and more C-terminal amino acids, but also engages atleast two residues N-terminal to the phosphotyrosine (Poy *et al.,*1999; Li *et al.,*1999). There are 111 SH2 domains in the non-

reductant set of human gene products found in proteins with diverse functions, including regulation of protein/lipid phosphorylation, phospholipid metabolism, transcriptional regulation, cytoskeletal organization and control of Ras like GTPase.



Modulator interaction domains as building blocks in signal transduction. Interaction domains bind proteins, phospholipids of nucleic acids. A subset of such domains is illustrated and their general binding functions are indicated (Pawson and Nash 2003)

1.3.2 PTB domains

PTB domains are characterized through their ability to recognize phosphorylated Asn-Pro-X-Tyr ß–turn motifs such as found in the RTKs for Nerve growth factor, Insulin or Epidermal growth factor (Zhou *et al.*,1995; Trub *et al.*,1995) and show inherent flexibility for interaction. Scaffolding proteins with PTB domains like Shc, FRS2 or IRS-1 bind autophosphorylated receptors positioning these proteins for multisite phosphorylation and subsequent binding of SH2 domain targets such as Grb2 (for Shc and FRS2) or PI3-kinase (for IRS-1) (Rozakis-Adcock *et al.*,1992; Kouhara *et al.*,1997; Backer *et al.*,1992). PTB domains of FRS2 binds to a non phosphorylated peptide ligand found in the FGF receptors and this interaction is quite different from that exhibited by PTB domain for Asn-Pro-X-pTyr motifs (Ong *et al.*,2000; Dhalluin *et al.*,2000). This indicates that a PTB domain can bind

both phosphorylated and non phosphorylated motifs. Due to flexible scaffold structure proteins with PTB domains mediates a wide range of protein-protein and protein-phospholipid interactions (Blomberg *et al.,*1999; Forman-Kay *et al.,*1999; Prehoda *et al.,*1999; Pearson *et al.,* 2000).

1.3.3 Domains recognizing phosphoserine/ threonine

Large number of domains bind to phosphoserine/threonine containing motifs suggesting that protein phosphorylation is a rather general way of regulating protein-protein interactions (Yaffe *et al.,*2001), first recognized in the context of 14-3-3 proteins, which binds motifs such as Arg-Ser-X-pSer-X-Pro (Muslin *et al.,*1996; Tzivion *et al.,*2001) and has more recently been described for FHA domains,which are found in proteins that regulate the DNA damage response (*i.e.* Rad 53, Chk2), gene expression (Forkhead proteins) and protein trafficking (kinesins) (Durocher *et al.,*1999; Durocher *et al.,*2000). FHA domains bind preferentially to phosphothreonine motifs, and recognize the +3 residue relative to the phosphothreonine in a fashion that differs from one FHA domain to another any may impart biological specificity. FHA domain is similar to MH2 domains found at the C-terminus of Smad proteins, the targets of TGF ß-receptor serine /threonine kinases (Wu *et al.,*2000).

1.4 Nuclear receptors as transcription factors

Nuclear receptors (NR) as transcription factors play an important role in growth, development, homeostasis, reproduction and disease processes (Mangelsdorf *et al.,*1995; Whitfield *et al.,*1999). As, ligand activated transcription factors, NR provide a direct link between signaling molecules that control these processes and transcriptional responses. NR form a superfamily of phylogentically related proteins with 21 genes in the complete genome of Drosophila melanogaster (Adams *et al.,*2000), 48 in humans (Robinson-Rechavi *et al.,*2001) [but one more, FXRß, in the mouse (Robinson-Rechavi and Laudet, 2003)] and unexpectedly, more than 270 genes in Caenorhabditis elegans (Sluder *et al.,*1999). The superfamily includes the classic steroid receptors (androgen, estrogen, glucocorticoids, mineralocorticoids and progestrone receptors), thyroid receptors, vitmain D and retinoid receptors. NR share common functional domains that includes a ligand-binding domain, a DNA-binding domain (consisting of two zinc fingers) and two domains that are involved in transactivation of genes (Beato *et al.,*1995). NR regulate transcription by binding their cognate lipophilic ligands and subsequently undergo a conformational change that alters their ability to interact with regulatory proteins. which may lead to repression, depression or activation of transcription. In case of activation, NR recruit coactivators (discussed in detail separately), which lead to acetylation and hence condensation of the chromatin and

subsequently other coactivator protein complex bind the receptor and interact with the basal transcription machinery to initiate transcription (Lemon *et al.,* 2000; Rachez *et al.,* 2001; Glass *et al.,*2000; Freedman *et al.,*1999)**.** Ligand bound NR interact with cognate binding site called hormone response element (HRE) to affect the transcription of target genes. HREs are composed of two hexameric half–site core sequences (Aranda *et al.,*2001) and diversity among HREs is achieved by modifying the location of the half–sites relative to one another.

1.4.1 Coactivator recruitment by nuclear receptors

Coactivators are different from the general transcription factors in that most of them do not directly bind to the DNA but are associated with the promoter region via a gene specific activator molecule like NR. Over 30 potential coactivators have been identified by their ability to bind and alter the transcriptional activity of ligand activated NR (Wallberg *et al.,* 2000). Steroid receptor coactivator (SRC-1) is the founding member of SRC family of coactivators (Onate *et al.,*1995), which also includes transcriptional intermediary factor–2 (TIF2, 42,43 M9 and receptor associated coactivators-3 (Li *et al.,*1997; Anzivk *et al.,*1997; Chen *et al.,*1997; Torchia *et al.,*1997).

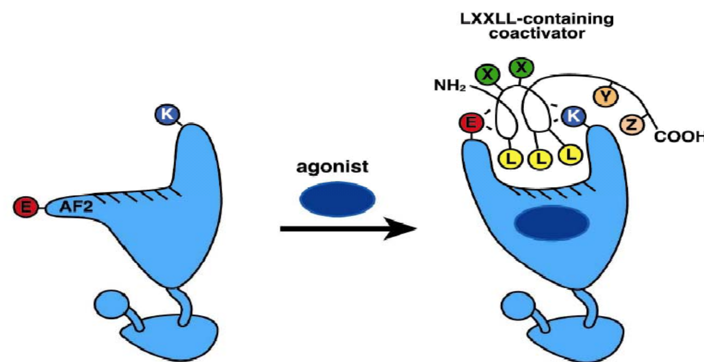
Two distinct steps in target gene activation turn up to be regulated by coactivators. Firstly, coactivators remodel the chromatin structure of the promoter region in order to facilitate binding of other activators and the component of the RNA polymerase II transcriptional machinery. Secondly, coactivators recruit protein complexes (mediator complex) that interact with one or more subunits of the RNA polymerase II and enhance the initiation of transcription by stabilizing the preinitiation complex (PIC) (Naar *et al.,*2001).

There are two general classes of enzymes complexes that appears to a play critical role in nucleosome remodeling mediated transcriptional activation. These are;

1. Histone acetyl transferase (HAT) which regulate nucleosome structure by altering the histone acetylation pattern of core histone tails (Sterner *et al.,*2000)
2. ATP-dependent chromatin remodeling factors (Sudarsanam and Winston, 2000; Varga–Weisz, 2001).

Histone acetyltransferases are the best characterized group of enzymes that covalently modify the structure of chromatin. They acetylate basic lysine residues located at the N-terminal tail of histones (Sterner and Berger, 2000). The acetylation of histones is thought to reduce electrostatic interactions between histones and DNA (Hong *et al.,*1993) and between separate nucleosome particles leading to the destabilization of the higher-order folding of chromatin (Tse *et al.,1998*). Acetylation might also disrupt the secondary structure of histone N-termini, which might further destabilize interaction with DNA and the nucleosome itself

(Hansen *et al.,*1998). Additionally, it has been shown that acetylation of specific lysine residues in the core histones provides novel recognition surface for proteins having bromodomain structures. This lead to the conclusion that, histone acetylation may enhance the initiation of transcription by two distinct mechanisms: by remodeling the structure of nucleosomes which leads to increased access of transcription factors to the promoter and by

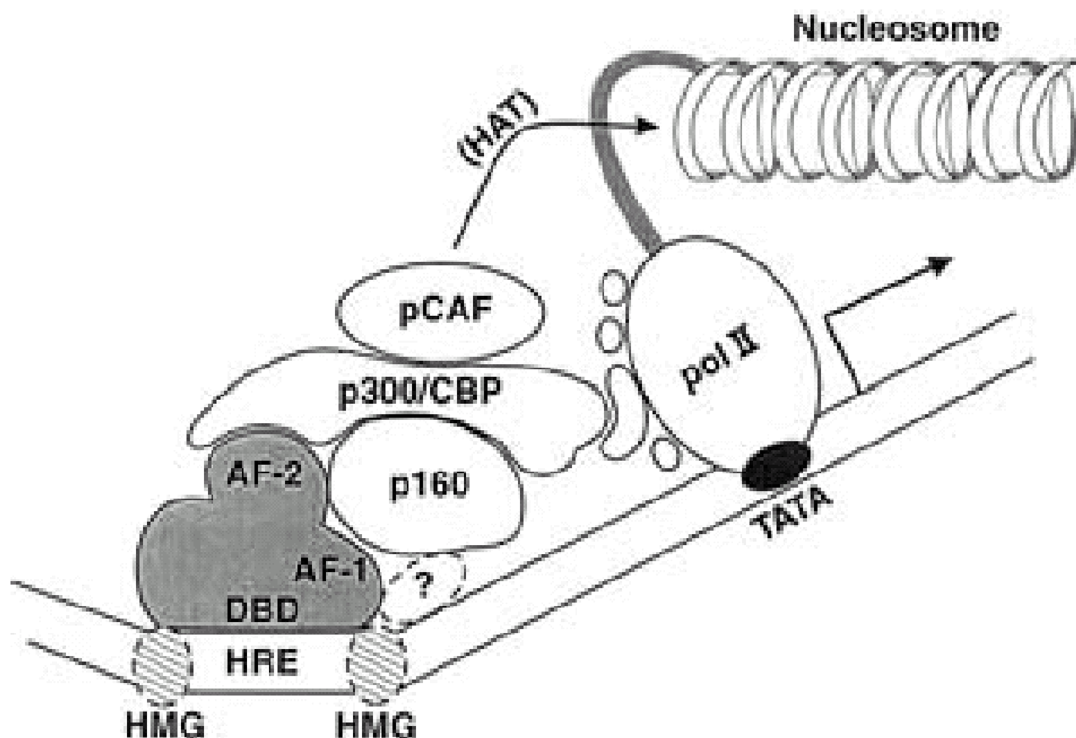


Coactivator recruitment; Ligand binding induces conformational changes in the ligand binding domain (LBD) of receptor and exposes coactivator docking site on LBD. Coactivators interact with the receptor on these exposed sites via specific LXXLL motifs present in coactivators (Pike *et al.,*2002)

creating the specific binding sites for bromodomain containing transcriptional co regulators such as TFIID and SWI/SNF (DiRenzo *et al.,* 2000; Hassan *et al.,*2000; Jacobson *et al.,*2000; Syntichaki *et al.,*2000). A number of coactivators that are recruited by activated NRs contain intrinsic HAT activity including the p160 family of coactivator's also known as steroid receptor coactivators (SRC) (Leo and Chen, 2000) and general coactivators CBP/p300 and PCAF (Yao *et al.,*1996). The p160 family coactivators communicate with virtually all NR in a hormone dependent manner, suggesting a common pathway of hormone-induced gene activation among the NR family (McKenna *et al.,*1999; Leo *et al.,*2000). The p160 proteins bind to the LBD of nuclear receptors via receptor interacting domain, which contains three short LXXLL binding motifs (where L is leucine and X is any amino acid). These motifs are conserved in both sequence and spacing and their number varies from one coactivator to another (Heery *et al.,*1999; Le Douarin *et al.,*1996; Rachez *et al.,*1998;Torchia *et al.,*1997). Analysis of these motifs has revealed that they form amphipathic α-helices with the leucine residues forming a hydrophobic surface on the face of the helix. Although the different receptors bind the common LXXLL motif in coactivators, there is receptor specific differential utilization of these motifs. Whereas a single motif of SRC-1 coactivator is sufficient for activation by ER, different combinations of two appropriately spaced motifs are

required for activation by TR, RAR and androgen receptor (AR). LXXLL binding motifs are needed for cooperative interaction with NR dimer (Darimont *et al.,*1998; Shiau *et al.,*1998; Wisely *et al.,*1998). The mechanism of interaction seems to be receptor specific and several combinations of LXXLL binding motifs are differently required for interaction with the different composition of NR dimers (Darimont *et al.,*1998; Mak *et al.,*1999; Heery *et al.,*2001). Recently, Zor *et al.,*2004 and Razeto *et al.,*2004 reported that there are differences in the binding mode of the LXXLL motif with the NR and non nuclear receptor based complexes.

In addition to having enzymatic HAT activity p160 family of coactivators have an important role as platform molecule which recruits other proteins such as CBP/p300 and PCAF complexes. CBP/p300 is one of the most potent acetyl transferases. Unlike p160 family members, CBP/p300 is able to acetylate all four histones within nucleosomes and it is able to communicate with numerous promoter–binding transcription factors such as CREB, NRs, STATs, Ets, c-Fos, c-Jun and c-Myb. Therefore, CBP/p300 could be seen as a global coactivator in higher eukaryotes (Ogryzko *et al.,*1996; Yang *et al.,*1996).



Mechanism of action of p160 nuclear receptor coactivators. A two-step mechanism has been proposed for p160 proteins mediate nuclear hormone receptor transcriptional activation. As an initial step, HAT activity of the recruited coactivator complex modulates local chromatin structure resulting in general transcription factors gaining access to DNA at the promoter. This step is followed by recruitment or stabilization of the RNA polymerase II holoenzyme (pol II) through direct or indirect binding of coactivators with general transcription factors associated with pol II. The high mobility group protein HMG-1/-2 enhances transcription by facilitating steroid receptor binding to specific hormone response elements and stabilizing the receptor–DNA complex (Edwards, 1999). [Abbreviations; DBD, DNA binding domain, AF-1/2, activation factor 1/2, HAT histone acetyltransferase, (HMG), high mobility group, HRE, hormone response elements]

In addition to histone acetylation CBP /p300 can also acetylate non-histone proteins such as p160 family of coactivators, transcription factors such as p53 and components of general transcription machinery such as TFIIE and TFIIF (Sterner and Berger, 2000). This acetylation mechanism is thought to mediate the autoregulation of coactivation process e.g. it is documented that the acetylation of lysine residues of p160 proteins in the vicinity of the LXXLL motif abolishes p160 coactivator interaction with NR, which in turn causes the dissociation of coactivator complex including p300/CBP from the receptor and target gene promoter leading to the attenuation of transcription (Chen *et al.,*1999). Thus CBP/p300 could have a dual role firstly to catalyze histone acetylation required for gene activation and secondly to attenuate the process by acetylating p160 proteins (Bevan and Parker„1999).

1.4.2 Coactivators in chromatin remodeling

SWI/SNF, ISWI, CHD and MI-2 complexes form another important class of coactivators involved in NR mediated chromatin remodeling (Dilworth *et al.,*2000; DiRenzo *et al.,* 2000;Varga-Weisz *et al.,*2001). SWI/SNF and ISWI are the best characterized ATP-dependent remodeling complexes. Unlike HATs, these complexes do not carry out covalent modification of histones. Instead they catalyze the uncoupling of ionic interactions between histones and DNA using the energy supplied by ATP hydrolysis. They are able to alter nucleosome conformation by sliding histone octamers to another site on the DNA or by changing the helical torsion of the DNA twist (Havas *et al.,*2000; Sudarsanam and Winston , 2000; Fry *et al.,*2001). A novel multifunctional ATP-driven chromatin remodeling complex called WINAC that interacts with vitamin D receptor (VDR) was described by Kitagawa *et al.,*2003.

1.5 Modulation in protein–protein interaction

Precise protein–protein interaction is utmost important to carry out their normal functions. Any aberration in normal protein interaction leads to number of diseases and other abnormalities e.g. regulation of proteolysis is critical for the healthy function of the cell excessive proteolysis leads to diseases like emphysema, thrombosis, rheumatoid arthritis and hyper fibrinolytic hemorrhage (Stein *et al.,*1995; Whisstock *et al.,*1998), while incomplete proteolysis can be seen as a cause in Alzheimer's disease (Moir *et al.,* Caswell *et al.,*1999), psorisis (Abts *et al.,*1999) tumor development (Suminami *et al.,*2000) and infection by parasites and nematodes (Zhang *et al.,*2001). Many approaches have been developed to study and interrupt the abnormal interaction between proteins. Some of the approaches are as;

1.5.1 By use of synthetic molecules

Modulation of protein-protein interaction by synthetic molecules that can bind a protein surface is still a major challenge (Hartwell *et al.,*1997) due to difficulty in matching the unsymmetrical distribution of polar and non polar domains on the protein as well as covering a sufficiently large surface area to achieve high affinity. However, for some proteins with a cleft or cavity molecules have been designed e.g guanidine esters, bind to IL2 and block its interaction with its heterotrimeric receptor complex (Tilley *et al.,*1997). Small heterocylces bind to CD 4 and disrupt its binding to MHC class II proteins on the surface of antigen presenting T cells (Huang *et al.,*1997). Park *et al.,*2002 have developed a strategy to recognize protein surface by designing molecules that contains a large funtionalized and variable interaction surface (Hamuro *et al.,*1997) to disrupt the interaction between serine proteases and their proteinaceous inhibitors (PIs). Anionic polymers or oligomers such as aurintricarboxylic acids, heparin derivatives and oligophenoxyacetic are used to target charged regions on a protein surface.

1.5.2 By use of naturally organic molecules

Number of naturally occurring organic molecules have been used to target protein–protein interactions. Taxane agents like paclitaxel (Taxol) (Rowinsky *et al.,*1997), [a diterpenoid isolated from the bark of pacific yaw tree] and its semi-synthetic derivative docetaxol, bind to ß–subunit of the tubulin hetrodimer and there by stabilizes interaction between the tubulin heterodimers. They are used in a number of human cancers. Laulimalide (Moobery *et al.,*1999), epothilones A and B (Bollag *et al.,*1995), eleutherobin and discodermolide (ter Haar *et al.,* 1996) are among natural organic molecules used to stabilize microtubules. Brefeldin A, a fungal metabolite stabilizes protein interaction between guanidine diphosphatase bound proteins of Ark family and Sec7 domains (Peyroche *et al.,* 1999). FK1012 (Spencer *et al.,*1993), (a dimer form of naturally occurring small molecule FK506) and cyclosporin A (Belshaw *et al.,*1996) induce dimerization of genetically engineered receptors and consequently induce signal transduction and specific target–gene activation (Spencer *et al.,*1996). FK506 and rapamycin reconstitutes activity of transcriptional factors, whose functional domains had been separated and linked to the ligand–binding proteins of these organic molecules (Rivera *et al.,*1996).

1.5.5 By use of peptides

Peptides derived from the protein interaction surfaces have been reported by several workers to inhibit the protein interactions. Zhang *et al.,*1991 reported that the tetrapeptide Ac-Thr-Leu-Asn-Phe-COOH derived from the C-terminal of HIV-1 protease inhibits the protease

by dissociative mechanism. Zutshi *et al.,*1998 used a peptide derived from the N-termini of HIV-1 protease cross linked by a sequence from C-terminal to inhibit the protease. Ribonucleotide reductase is important for Herpes simplex virus for its virulence and reactivation from latency (Jacobson *et al.,*1989). This enzyme is active when its two subunits interact each other. Discovery of a hexapeptide Ala-Val-Val-Asn-Asp-Leu (Krogsrud *et al.,* 1993) inhibits enzymatic activity of ribonucleotide reductase by preventing association between the two enzymatic subunits. Tumor suppressor protein p53 is suppressed in majority of human tumors. In about 30% of sarcomas, Hdm2 protein interaction with p53 inhibits its activity by two different mechanisms; first Hdm2 binds to the transcriptional activation domain of p53 and thereby inhibits expression of p53 target genes, second a protein complex involving Hmd2 mediates nuclear export of p53 and subsequent degradation of p53 by cytoplasmic proteasomes (Freedman *et al.,*1999). Phage display has revealed that peptides of varying lengths (10-14 amino acid) could disrupt the interaction between p53 and Hdm2. In addition a cyclic nonapeptide composed of natural and unnatural amino acids also inhibits this interaction. Function of transcription factor E2F, a crucial cell cycle regulator controlling G1/S transition (Muller *et al.,* 2000) was effectively shown to be antagonized by the peptides by blocking its binding to DNA target sequence and there by inhibiting E2F–dependent transcription (Fabrizzio *et al.,*1999).

1.5.4 Low molecular weight modulators (Identified by screening of chemical libraries)

Screening chemical libraries could identify a number of modulators of protein interaction. Anti-apoptotic bcl-2 family genes bcl-2 and bcl-xl whose over expression provide resistance to the tumors to chemotherapy (Gutierrez-Puente *et al.,*2002) and prevent apoptosis by inhibiting the function of other pro-apoptotic members of the Bcl-2 family, such as Bax and Bak, by binding to their BH3 (Bcl-2 homology 3) domain. Degterev *et al.,* 2001 set up an *in vitro* assay based on fluorescence polarization to prevent this interaction by identifying small molecules by screening a chemical library comprising of 16320 chemicals. Three compounds termed as BH3I-1, BH3I-1′and BH3I-2 were identified to interrupt this interaction. Interaction of transcription factor c-Myc with Max (Amati *et al.,*1993) is known to be cause of one out of 7 human cancer deaths. On screening a chemicals library encompassing approximately 7000 compounds four active compounds were identified to modulate this interaction (Boger *et al.,*2000; Menssen *et al.,*2002; Brooks *et al.,*1996). Carter *et al.,* 2000 identified N-alkyl 5 arylalkylidine–2 thioxo-1,3 thiazolidine 4-ones as an antagonist for the TNFx/TNFRcI (tumor necrosis factor/Tumor necrosis factor receptor I)

interaction on screening chemical to interrupt this interaction which is known to be a cause of various autoimmune diseases like rheumatoid arthritis or Crohn´s disease (Risau, 1997).

1.5.5 By mutation

Protein-protein interactions are highly specific although some proteins are multispecific. A mutation in anyone of the interacting partners may disrupt their interaction. Shiu *et al.,* 1996 created a mutation in CREB that prevented its association with coactivator CBP. However, not all mutations lead to a disruption in the interaction, but also might enhance interaction between them. Human T-cell leukemia virus protein Tax does not interact directly with serum response factor (SRF) (Fujii *et al.,*1992; Suzuki *et al.,*1993). Mutations created in Tax activate c-fos promoter through SRE (Fujii *et al.,*1988) a process possible only from a direct interaction between Tax and SRF.

Aim of the study

Protein-protein interactions play a key role in cellular processes. Their specificity is instrumental for signal transduction and gene regulation, which govern cell growth, proliferation, differentiation and programmed cell death, as well as for basic metabolism and other biochemical processes. In all cases protein-protein interactions can be activating or inhibiting. They may be exclusive or with varying partners and may be the basis for the formation of large complexes comprised of many interaction partners. The latter is of particular interest because many proteins contain a range of domains suitable for different specific interactions enabling these proteins to interact with many partners and participate in various processes. Given this general importance, protein interactions are studied at various levels using a wide range of analytical methods, which mostly do not allow to assign specific activities to individual interactions or interaction domains *in vivo*.

The main aim of the project was to develop a tool to interfere with one specific protein interaction at a time *in vivo*. For this, a peptide expression vector system had to be be developed that allows the expression of small peptides in mammalian cells, which to ensure expression can be monitored with an unlinked fluorescent protein. This system should be so versatile that very small peptides can be presented with the same efficiency as somewhat larger peptides with and without flanking sequences for stabilization. In addition, the system should allow expression of random peptide libraries containing a large number of different unrelated peptides, which can be used for *in vivo* selection protocols.

This aim can be broken into the following developmental steps each representing an interesting scientific question of its own. These are: which vector system can be used for efficient expression of short peptides; disruption of known protein-protein interactions to establish the basic protocol and verify if the design of the expression vector system is appropriate; disruption of a protein-protein interaction in the nucleus to demonstrate that peptides also function in the nucleus; and finally selection of a novel bioactive peptide by *in vivo* selection from a random peptide library expression system.

Material and methods

2.1.1 Chemicals

Agarose	Roth, Karlsruhe
Agar	Roth, Karlsruhe
Ampicillin	Gibco/BRL, Karlsruhe
ATP (adenosine 3´-triphosphate)	MBI Fermentas
Anhydrotetracycline	Acros Organics, Leicestershire
Bromophenol blue	Roth, Karlsruhe
Chloroquine	Sigma, Deisenhofen
Deoxynucleotides (dG/A/T/CTP)	MBI Fermentas, St.Leon-Rot
Dimethyl sulfoxide (DMSO)	Sigma, Deisenhofen
Ethidium bromide	Sigma, Deisenhofen
Effectene	Qiagen, Heldin
Polybrene	Sigma, Deisenhofen
9-cisretinoic acid	Sigma, Deisenhofen
Penicillin/streptomycin	Gibco/BRL, Karlsruhe
Nerve growth factor	Biomol, Hamburg
Vitamin D	Biomol, Hamburg

2.1.2 Enzymes

Alkaline phosphate	MBI Fermentas, St.Leon-Rot
Restriction endonucleases	MBI Fermentas, St.Leon-Rot NEB, Frankfurt
T4 DNA ligase	MBI Fermentas St.Leon-Rot
Taq-DNA polymerase	Sigma, Deisenhofen
Trypsin	Gibco/ BRL, Karlsruhe

2.1.3 Kits and other materials

Qiagen mini prep kit	Qiagen, Hilden

Qiagen maxi prep kit	Qiagen, Hilden
Endofree plasmid kit	Qiagen, Hilden
Effectene transfection kit	Qiagen, Hilden
Gel extraction kit	Qiagen, Hilden
PCR purification kit	Qiagen, Hilden
Great EscAPe ™ SEAP chemiluminescence detection kit	Clontech, Heidelberg
Sterile filter 0.45µm, cellulose acetate free	Nalgene, Rochester
Luminometer	BMG, Offenburg

2.2 Media and buffers

2.2.1. Medium for E. coli

LB-Medium	1.0% Tryptone
	0.5% Yeast extract
	1.0% Nacl
	pH 7.2

Ampicillin 100µg/mL was added to the media after autoclavation.
LB-plates additionally contained 1.5% Agar.

2.2.2 Cell culture media

All cell culture media and addictives were from Gibco/BRL, Fetal calf serum (FCS), Dulbecco´s modified eagle medium (DMEM) with 4.5mg/mL glucose, 2mM L-glutamine, 1mM sodium pyruvate.
Freeze medium: 90% heat inactivated FCS, 10% DMSO

2.2.3 Stock buffers

DNA loading buffer (6x)	0.25% Bromophenol blue
	0.25% Xylencyanol
	30.0% Glycerol
	100.0mM EDTA pH 8.0
PBS	13.7mM Nacl
	2.7mM KCL
	80.9mM Na_2HPO_4
	1.5mM KH_2PO_4, pH 7.4
TAE (10x)	400mM Tris/acetate

	10mM EDTA
	pH 8.0 (Acetic acid)
PCR (10x)	100mM Tris-HCl, pH 8.8 at 25^0C
	500mM KCl
	0.8% Nonidet P40
	15mM $MgCl_2$
KCM (5x)	500mM KCl
	150mM $CaCl_2$
	250mM $MgCl_2$
Lysis Buffer	20mM Hepes pH7.5
	10mM EGTA
	40mM ß-glycerophosphate
	2.5mM MgCl2
	1% NP-40

* Protease inhibitor cocktail was added to the lysis buffer at the time of lysis of cells

Digestion Buffer	100mM Nacl
	10mM Tris-Hcl pH 8
	25mM EDTA pH 8
	0.5% (w/v) SDS
	0.1mg/mL Proteinase K

* Proteinase K was added fresh every time to buffer.

2.3 Bacterial strains and cell lines

2.3.1 Bacterial strains

E.coli	Description
Top 10 F´	F´{lacITn10 (Tet^R)}mcrAΔ(mrr-hsdRMmcrBC) φ80lacZΔM15 ΔlacX74recA1araD139 Δ(araleu) 7697galUgalK rpsLendA1nupG
DH5aF´	F´/endAI hsd 17 (rk-mk-) supE44, recAI, gyrA (NaI), thi-I,

2.3.2 Cell lines

Cell lines	Description
HeLa	Human cervix carcinoma, epithelial-like cells growing in monolayer

NIH3T3	Swiss mouse embryo, fibroblast, adherent monolayer
NIH3T3TrkA-Ros	Modified NIH3T3 cell line, expressing TrkA domain and SHP-1 (phosphatase)
Ecopack 293	Human embryonic kidney (HEK-293) fibroblast derived packaging cell line.

2.4 Methods in molecular biology

2.4.1 Plasmid preparation for analytical purpose

Small amounts of plasmid DNA were prepared as described by (Lee and Rashid, 1990). Plasmid preparation for mammalian cells, DNA of high quality was prepared using Qiagen maxi-kit and Qiagen Endofree maxi kit (Qiagen) according to manufacturer's protocol.

2.4.2 Digestion of DNA samples with restriction endonucleases

Restriction endonuclease cleavage was accomplished by incubating the enzyme(s) with the DNA in appropriate reaction condition. The amounts of enzyme, DNA, buffer, ionic concentrations and the temperature, duration of the reaction were adjusted to the specific application according to the manufacturer's recommendations.

2.4.3 Dephosphorylation of DNA 5´-termini with calf alkaline phosphatase (CIAP)

Dephosphorylation of 5´-termini of vector DNA in order to prevent self-ligation of vector termini was carried out by CIAP. For dephosphorylation required amount of DNA termini were dissolved in 44µL of deionized water, 5µL 10x reaction buffer (500mM Tris/HCL pH 8.0, 1mM EDTA pH 8.5) and 1µL CIAP (1U/µL). The reaction mixture was incubated at 37°C for one hour and stopped by heating at 65°C for 15 minutes.

2.4.4 DNA insert ligation into vector DNA

T4 DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5´-phosphate and 3´-hydroxyl termini in duplex DNA. In a total volume of 10µL the digested, dephosphorylated and purified vector DNA (200ng), the foreign DNA to be inserted, 1µL T4 DNA ligase (2U for sticky ends and 4U for blunt ends) were mixed. The reaction mixture was incubated at 16°C overnight. T4 DNA ligase was inactivated by heating the reaction mixture

at $65^{O}C$ for 10 minutes. The resulting ligation reaction mixture was directly used for bacterial transformation.

*Note: Concentration of DNA and insert in addition to mention above was varied in some cloning experiments.

2.4.5 Phosphorylation of DNA by T4 polynucleotide kinase

T4 polynucleotide kinase (T4PNK) is a polynucleotide 5'-hydroxyl kinase that catalyzes the transfer of the phosphate from ATP to the 5`-OH group of single and double stranded DNAs, RNAs, oligonucleotides or nucleoside 3'-monophosphates (forward reaction). In the presence of ADP, T4PNK exhibits 5'-phosphatase activity and catalyzes the exchange of terminal 5'-phosphate groups (exchange reaction).

In a total reaction mixture of 20µL DNA fragment to be phosphorylated, 2µL of T4PNK (10U/µL), 2µL (10x) T4PNK buffer (500mM Tris-HCl pH 7.6 at 25°C, 100mM $MgCl_2$, 50mM DTT, 1mM spermidine and 1mM EDTA), adenosine triphosphate (ATP) 1µL were mixed. Reaction mixture was incubated at $37^{O}C$ for half an hour. T4PNK was inactivated, by incubating at $68^{O}C$ for 10 minutes.

2.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separating and identifying DNA fragments. 0.5x TAE or TBE electrophoresis buffers were used for separation. The voltage was set typically to 1-10 V/cm of gel. Gels were stained by covering the gel in a dilute solution of ethidium bromide (0.5µg/mL in water) and gently agitating for 30 minutes or by adding ethidium bromide directly to the gel solution.

2.5.1 Isolation of DNA fragments using low melting temperature agarose gels

Following preparative gel electrophoresis using low melting temperature agarose, the gel slice containing the band of interest was removed from the gel. This agarose slice was then melted and subjected to isolation using the QIAquick gel extraction kit (Qiagen).

2.5.2 Polymerase chain reaction (PCR)

The following standard protocol was adjusted to the specific application;

In a total 50µL of reaction mixture DNA to be amplified, set of primers (sense and antisense 20pmoles each), dNTPs 1µL (10mM each), 10X PCR buffer (100mM Tris/HCl pH 8.8 at $25^{0}C$, 500mM KCl, 0.8% Nonidet P40, 15mM $Mgcl_2$) and 1µL of Taq polymerase (5U/µL) were mixed. The reaction was carried out as follows

95^0C	5 minutes	(first denaturation)
95^0C	30 seconds	(denaturation
56^0 C	40 seconds	(hybridization)
72^OC	45 seconds	(extension)
	Amplification 30 cycles	
72^OC	10 minutes	(last extension)

*Note: Temperature, time of hybridization and extension steps were adjusted as per the need of experiment.

10µL from each reaction were electrophoresed on an agarose gel appropriate for the PCR product size expected.

2.5.3 PCR product purification

DNA fragments obtained by PCR were purified by PCR purification kit (Qiagen) before cloning or sequencing to remove nucleotides and enzyme following the manufacturer's recommended protocol.

2.5.4 Phenol chloroform precipitation

Restriction enzyme digested DNA, PCR products were purified and concentrated by phenol/chloroform precipitation as follows;

To a reaction mixture, add equal amount of phenol/chloroform, vortex, and centrifuge at 12,0000rpm for 5 minutes at 4^0C. Supernatant carefully taken into another tube and first step repeated. Aqueous layer taken into new tube, one-tenth volume of 3M NaoAc pH 5.2 and two volume of 100% ethanol were added. Reaction mixture was kept at -20^0C for 30 minutes and centrifuged at 4^0C for 20 minutes at 13,000rpm, followed by washing with 70% ethanol. Dissolve the pellet in appropriate volume of water or TE buffer.

2.6 Introduction of plasmid DNA into E.coli cells

2.6.1 Preparation of competent cells

Competent cells were made according to the procedure described by (Chung *et al.,* 1988). For long term storage competent cells were frozen at -80^OC. Transformation frequency ranged between 10^6 and 10^7 colonies /µg DNA.

2.6.2 Transformation of competent cells

Reaction mixture comprising of 10µL ligation mixture, 20µL 5x KCM buffer (500mM KCL, 150mM $CaCl_2$, 250mM $MgCl_2$) 70µL of H_2O were added to 100µL of competent cells

and incubated on ice for 30 minutes, followed by incubation for 10 minutes at room temperature. 1mL LB medium with out antibiotic was added to cells and incubated for 1hour at 37^0C with mild shaking to allow expression of the antibiotic resistance gene. Transformants were selected on appropriate plates.

2.7 Vectors

pLNHX (Clontech) is a part of pantropic retroviral vector designed for efficient gene delivery and expression.

pIRES-EYFP (Clontech) is an IRES bi-cistronic vector with enhanced yellow fluorescent protein as a reporter

pCRE-SEAP (pCRE) vector has three copies of the cAMP response binding element (CRE) sequence fused to a TATA-like promoter (P_{TAL}) region from the herpes simplex virus thymidine kinase (HSV-TK) promoter and Secreted enhanced alkaline phosphatase (SEAP) gene as a reporter.

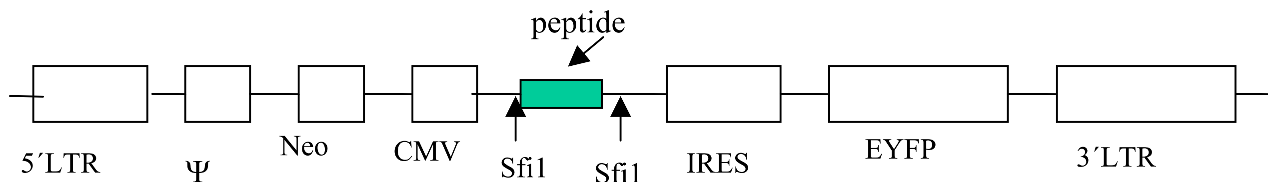
pGRE-SEAP (pGRE) vector has three tandem copies of Glucocorticoid response element (GRE) sequence fused to a TATA-like promoter (P_{TAL}) region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter and Secreted enhanced alkaline phosphatase (SEAP) gene as a reporter.

pTAL-SEAP vector was modified by introducing vitamin D response element (VDRE) in the multiple cloning site.

pOS IRESGFP, bi-cistronic retroviral vector with green fluorescent protein as a marker.

2.7.1 Vector constructs

pLNHX is a part of pantropic retroviral vector designed for efficient gene delivery and expression. Retroviral vector constructs are based on the pLNHX vector. Drosophila heat shock promoter ^{P}hsp70 was replaced by human cytomegalovirus (CMV) major immediate early promoter, excised from pIRES-EYFP vector.



Schematic structure of pLNHX retroviral vector indicating site of cloning short peptides, restriction enzymes employed and various vector constituents.

Nsi1 and BsrG1 restriction sites were introduced into the multiple cloning site of pLNHX, employed to insert Internal Ribosome Entry Site (IRES) and Enhanced yellow fluorescent protein (EYFP) gene as a single fragment, vector was renamed as pLNHX IR-EY. Two Sfi1 sites with different overhangs were introduced in the multiple cloning sites (MCS) of pLNHX IR-EY vector before the IRES sequence to allow efficient directional one step cloning of peptide coding oligonucleotides.

2.7.2 Oligonucleotides coding for short peptides

Various oligonucleotides coding for short peptides were cloned in the pLNHX IR-EY vector .Two sequences were chosen. One is called as Pos peptide sequence (Pos) it is 13 amino acid sequence derived from Ros tyrosine phosphorylation domain with specific tyrosine residue needed for interaction with SHP-1 protein tyrosine phosphatase (SHP-1PTP). The other peptide is called as Neg peptide sequence (Neg) having a single point mutation which replaces tyrosine by phenylalanine and thus making it no more a binding partner for SHP-1PTP. In addition to this, another peptide of random sequence was taken as a control peptide having no specific sequence similarity with Ros tyrosine domain. This peptide was referred as nonspecific peptide (NSP).

Oligonucleotides used for Pos peptide sequence (Pos)

5´aggccatggagggtcttaatt***a***tatggttcttgctactaaatcttcctaaggcctgct 3´

5´ aggccttaggaagatttagtagcaagaaccata***t***aattaagaccctccatggcctgag 3´

Oligonucleotides used for Neg peptide sequence (Neg)

5´aggccatggagggtcttaatt**t**tatggttcttgctactaaatcttcctaaggcctgct 3´

5´ aggccttaggaagatttagtagcaagaaccata***a***aattaagaccctccatggcctgag 3´

2.7.3 Annealing of oligonucleotides

For annealing 50pmoles from each oligonucleotide were mixed in 50µL of annealing buffer or water, incubated at 90^{0}C for 10 minutes, cooled and subsequently phosphorylated before cloning in pLNHX IR-EY vector at Sfi1 restriction site. Vector with Pos was renamed as pLNHX IR-EY Pos, vector with Neg was renamed as pLNHX IR-EY Neg and vector with NSP was renamed as pLNHX IR-EY NSP.

2.7.4 Addition of self-annealing flanking clamp sequence

A self-annealing clamp sequence was added to Pos and Neg peptide sequences on both N and C termini. Self-annealing flanking sequence (EFLIVIKS) as reported by (Gururaja *et al.,* 2000) forms a stable dimer and protects the peptide from proteases.

Pos peptide with self-annealing clamp is designated as (PC) and Neg peptide with self-annealing clamp sequence as (NC).

Oligonucleotides used for PC

5´gatccggccactcaggccatgggcgagttcttgatcgtgataaagtcaggg 3´

5´gataaggaattctccggaagatttagtagcaagaaccata***t***aattaagaccctccatccctgactttatcacgat 3`

5´gatccggcctagcaggccaatcaggttttaaggaggccctgatttgatgacgataaggaattctcc 3 ´

Oligonucleotides used for NC

5´gatccggccactcaggccatgggcgagttcttgatcgtgataaagtcaggg 3´

5´gataaggaattctccggaagatttagtagcaagaaccataa***a***attaagaccctccatccctgactttatcacgat3

5´gatccggcctagcaggccaatcaggttttaaggaggccctgatttgatgacgataaggaattctcc 3´

PC and NC encoding oligonucleotides were cloned in pLNHX IR-EY vector and vectors were renamed as pLNHX IR-EY PC and pLNHX IR-EY NC respectively. Three oligos were used for PC and NC, each having atleast 15 to 18 bases complementary to one other. For PC, 10pmoles from all three oligonucleotides were mixed in 40µL of water used as a template for PCR. 10-15 cycles of PCR were carried out. Three bands were seen on the agarose gel, band of required size was excised, amplified using appropriate primers, digested with Sfi1 and cloned in pLNHX IR-EY vector, renamed as pLNHX IR-EY PC, similarly NC was cloned in pLNHX IR-EY vector and renamed as pLNHX IR-EY NC.

2.8 LXXLL motif peptides

2.8.1 Short LXXLL peptide with random amino acid residues

Transcriptional activation by nuclear receptors is achieved by the recruitment of coactivator proteins upon ligand binding. This recruitment involves an activation domain on the receptor surface and an LXXLL motif located with in the comodulator (McInerney *et al.,* 1998; Hall *et al.,* 2000).

LXXLL peptides with random residues are in the format M $X_7LX_2LLX_7$ *Ter*, L is leucine and X is any amino acid

Oligonucleotides used

5´gatcggccactcaggccatgnnknnknnknnknnknnknnkctgnnknnkctgctgnnknnknnkn nknnknnknnktaagtacaggcctgctaggccggatc 3´

5´gatccggcctagcaggcc 3´

(In nnk, n is any base, k is either g or t. Use of k at third position reduces the frequency of stop codons, while preserving the diversity of amino acids. It ensures occurrence of only one stop codon (uag).

Elongation reaction was carried out in a reaction mixture of 50µL containing 50 pmoles from each oligonucleotides, 1µL Taq polymerase (5U/µL), 1µL dNTP mix (10mM each), 5µL PCR buffer in an automated thermocycler using the following programme

95^0C	5 minutes
45^0C	55minutes
Hold	4^0C

Reaction product was purified by PCR purification kit, digested with Sfi1 and cloned in pIRES-EYFP vector at Sfi1 restriction enzyme site.

Four pIRES-EYFP vector constructs with this random sequence are pIRES-EYFP LX1 (pLX1), pIRES-EYFP LX2 (pLX2), pIRES-EYFP LX3 (pLX3) and pIRES-EYFP LX4 (pLX4). Vector were sequenced by Jena Biosciences GmbH (Germany)

Amino acid sequence of peptide LX1	MLGFFYDLLWFLLCVCVLHP
Amino acid sequence of peptide LX2	MTIAVVFRLMCLLVLGGRVS
Amino acid sequence of peptide LX3	MLQTYVVFLEPLLFDFSRDR
Amino acid sequence of peptide LX4	MRVSLLSLLLRLLQSIAVYR

2.8.2 LXXLL peptides with varying number of motifs

Three short LXXLL peptides varying in number of LXXLL motif and amino acid residues around the motif were chosen, named as LX 5, LX 6 and LX 7.

Peptide (LX 5) has one LXXLL motif with no additional amino residues on N and C termini of it.

Peptide (LX 6) has two LXXLL motifs separated with three amino acid residues.

Peptide (LX 7) has one LXXLL motif with two additional amino acid residues on C termini of motif.

Amino acid sequence of peptide LX5	MLHRLL *Ter*
Amino acid sequence of peptide LX6	MLHRLLAAALSRLL *Ter*
Amino acid sequence of peptide LX7	MHLRLLQL *Ter*

Oligonucleotides used for LX 5

5´ aggccatgttacatcgtctactgtaaggcctgct 3 ´

5´aggccttacagtagacgatgtaacatggcctgag 3´

Oligonucleotides used for LX 6

5´aggccatgttacaccgtctccttgctgccgcactaagtcgcctcctataaggcctgct 3´

5´aggccttataggaggcgacttagtgcggcagcaaggagacggtgtaacatggcctgag 3´

Oligonucleotides used for LX 7

5´aggccatgttacaccgtctccttcagttataaggcctgct 3´

5´aggccttataactgaaggagacggtgtaacatggcctgag 3´

Oligonucleotides for LX 5, LX 6 and LX7 were cloned in pIRES-EYFP vector at Sfi1 restriction site, vector was renamed as pIRES-EYFP LX 5 (pLX 5), pIRES-EYFP LX 6 (pLX 6) and pIRES-EYFP LX 7 (pLX7) respectively.

Vitamin D response element (VDRE) sequence

Oligonucleotides coding for one copy of VDRE sequence was cloned in the multiple cloning site of pTAL-SEAP vector employing Xba1 restriction sites. Vector was renamed as pTVE.

2.9 General cell culture technique

All cell lines were grown in a humidified 95% air, 5% CO_2 (Heraeus) at 37^0C routinely assayed for contamination. Before plating cells were counted by Coulter Counter. Cells were cultured in Dulbecco´s modified Eagle's medium (DMEM) supplemented with 2mM L-glutamine, 1.0mM sodium pyruvate and 10 % fetal calf serum /FCS).

2.9.1 Transfection with effectene reagent

Ecopack TM -293, NIH3T3 and HeLa cells were transfected transiently at about 75 % confluence using Effectene transfection reagent (Qiagen). Cells were seeded 24h before transfection. The following protocol as per the recommendation of manufacturer was followed

Culture format	DNA (µg)	Enhancer (µL)	Buffer EC (µL)	Effectene reagent (µL)
24 well plate	0.3	2	75	5
12 well plate	0.4	3	100	6
6 well plate	0.6	5	150	9
60mm dish	1.5	12	200	15

*Note: In addition to this protocol some times concentration of reagents and DNA were changed.

Co-transfection of HeLa cells with two vectors was carried out, by using Effectene transfection reagent.

2.9.3 Transfection by lipofectamine

HeLa cells were transiently transfected using lipofectamine (Gibco/BRL) essentially as described (Daub *et al.,* 1997). For transfection in 6-well plates 1.0mL of serum free

medium containing 7µL of lipofectamine and 1.0µg of plasmid DNA per well was used. After 4h, transfection mixture was removed and fresh medium was added.

2.10 Retrovirus

2.10.1 Retrovirus production

Ecopack TM-293 packaging cell lines ($2x10^5$ cells) were seeded in 6 well plates coated with collagen1 one day prior to transfection. Retroviral vector constructs were transfected by Effectene Transfection reagent according to the manufacturer's protocol. 25µM/mL chloroquine was added to cells 3h before transfection. Post 24h of transfection, medium was replaced with fresh medium lacking chloroquine. Forty eight hours later, conditioned medium from these cells was harvested, filtered through 0.45µm sterile cellulose acetate free filters The estimated titer of the retrovirus were 1-2 x 10^6 colony forming unit /mL based on the G418 resistant colony formation of the NIH3T3 cells.

2.10.2 Retroviral infection

NIH3T3TrkA-Ros cells ($4x10^5$ cells) were seeded in 60mm dishes one day prior to infection. Conditioned medium from Ecopack TM 293 packaging cell line was harvested after forty eight hours, filtered through 0.45µm sterile cellulose acetate free filters and added to NIH3T3TrkA Ros cells. Cells were grown in presence of 8µg/mL polybrene for 24h. After 24h of infection medium was changed with fresh medium with out polybrene. Infected cells were analyzed as indicated.

2.11 Cell proliferation

Cell numbers were counted by using a Coulter Counter. Medium was removed followed by washing of cells with PBS. After washing with PBS, trypsin was added to cells to detach them from the surface. Cell numbers are displayed per mL.

2.12 Fluorescence activated cell sorting (FACS) analysis

Flow cytometry allows counting and analysis of physical and molecular attributes of particles in a liquid media. One of the most used applications is the analysis of cells and cell (sub) populations on single cell level with probes like (antibodies, receptors, fluorescent markers, streptavidin, etc).

Infected cells were harvested by trypsinization after 48h and were resuspended at a concentration of 10^6cells/mL in DMEM containing 10% fetal bovine serum. Samples were analyzed by FACS by using an argon laser to excite cells at 488nm and a 530 ± 15nm band pass filter to detect fluorescent emission. For FACS scans, 10,000 cells were typically

analyzed by using a FACScan flow cytometer (Becton Dickinson). FACS data were analyzed using CellQuest software.

2.13 Secreted alkaline phosphatase (SEAP) chemiluminescence detection

SEAP detection was done by using Great EscAPe ™ chemiluminescence detection kit (Becton Dickinson) following manufacturer's protocol. SEAP is a modified and heat resistant alkaline phosphatase secreted directly in the medium. SEAP detection was generally done after 24h of transfection of SEAP vectors using the following procedure. 110µL of cell culture medium was taken from the transfected cells, centrifuged at 12,000rpm for 30 seconds, and supernatant was taken in fresh micro centrifuge tube.

SEAP assay protocol (For 96 well plate format)

Pipette 15µl of supernatant to each well, add 45µL of 1x dilution buffer. Incubate at 65^0C for 30minutes in a water bath, cool samples on ice for 2-3 minutes. Add 60µL of Assay buffer to each sample; incubate for 5 minutes at room temperature (RT). Add 60µL of 1.25mM CSPD substrate diluted with chemiluminescent enhancer. Incubate for 30 minutes at RT. Detection was carried out by luminometer (BMG, Germany).

2.14 Protein antibody array

2.14.1 Lysis of cells

Medium was removed from the treated cells and washed with PBS. Cells were incubated in lysis buffer, scrapped off the plate and transferred into an eppendorf tube. Lysate was vortexed, centrifuged at 13000rpm at 4^0C for 20-25 minutes.

2.14.2 Determination of protein concentration

Protein concentration was determined according to Bradford, 1976 method.

2.14.3 Protein detection by array tube

Array tubes were purchased from Clondiag chip technologies (Germany). Array tube contains 19 antibodies; each antibody is spotted three times. The same antibodies diluted in 1:5 ratios are also spotted three times. In addition to this, array tube contains markers and controls. Proteins were spotted on the array tube following this protocol.

1. Biotinylation of proteins

50µg of protein was mixed with 0.5µL (100µg/µL) of Biotin in a reaction mixture of 50µL (volume was make up with PBS) Incubated at 30^0C in a thermo-mixer with mild shaking 500rpm for one hour.

2. Array Tube Blocking

250µL of 5% PBS-Milk powder was added to each array tube and incubated for 1h at 30^0C with shaking.

3. Array tube incubation with biotinylated protein.

Wash array tube with PBS, mix 50µg biotinylated protein from first step with 50µL 2% PBS-BSA and add to array tube. Incubate at 30^0C for 2h with constant shaking.

4. Washing

Wash array tube with PBS-T (Tween 0.05%) two times for 5 minutes each at 800 rpm.

5. Array tube incubation with streptavidin gold

0.5µL of streptavidin gold in 99µL PBS-T was added to array tube and incubated at 30^0C for half an hour and washed thrice with PBS-T.

6. Detection

50µL from Silver enhancing kit A and B were mixed and added to Array tube and the tube was put in the array tube reader (Clondiag) for detection.

Array data was analyzed by IconoClust software (Clondiag).

2.15 Random peptide library construction

Random library of 1x 10^6 peptides were synthesized employing pOS IRESGFP (gifted by Klingmuller) bi-cistronic retroviral vector. In order to make random peptide library nnk codons, where n denotes all four nucleotides and k denotes g and t were chosen for generation of randomized codons. This ensures a more equal distribution of the amino acids in the library and two out of three stop codons are omitted. Each peptide has 13 random amino acids. Two partly complementary oligonucleotide sequences coding for 13 random amino acid residues with restriction sites for Not1 and BamH1 at 5´and 3´ends respectively are;

5´ataagaatgcggccgctaaactatatgnnknnknnknnknnknnknnknnknnknnknnknnknnktaaggaattcgccg gatccgcg 3`

(In nnk, n is any base, k is either g or t. Use of k at third position reduces the frequency of stop codons, while preserving the diversity of amino acids. It ensures occurrence of only one stop codon (uag).

Second oligonucleotide sequence 5´cgcggatccgcggaattcctta 3´

Annealing and elongation was carried out in a reaction mixture of 50µL containing 50 pmoles from each oligonucleotides, 1µL Taq polymerase (5U/µL), 1µL dNTP mix (10mM each), 5µL PCR buffer in an automated thermocycler using the following programme

95^0C	5 minutes

45^0C	55 minutes
Hold	4^0C

Reaction product was purified by phenol chloroform precipitation, digested with BamH1 and Not1 and cloned in pOS IRESGFP retroviral vector.

Hundreds of transformations were carried out as described above. Colonies from agar plates were scrapped by adding 2-3mL of LB medium to each plate. Colonies were scrapped from plates (approximately 10^6 colonies) mixed, centrifuged, DNA was extracted and subsequently purified by Qiagen kit.

DNA was transfected in packaging cell line, viruses were collected after 48h of transfection. Target cells, were infected by viruses as described above.

2.15.1 Peptide rescue by PCR

Infected cells after treatment and sorting were allowed to grow in 96 well plate. Peptides, which provided resistance to the cells against dexamethasone, were obtained by using specific PCR primers to amplify them from isolated DNA. Cells after trypsinization and washing were incubated in digestion buffer overnight at 50^oC with mild shaking and DNA was precipitated with repeated phenol chloroform extraction.

PCR was carried out by using the primers with restriction enzyme sites for Not1 and BamH1. PCR product was purified, digested with Not1 and BamH1 for further recloning in pOS IRESGFP retroviral vector.

Primer used

Forward primer	5´ataagaatgcggccgctaaac 3´
Reverse primer	5´ ctggcggccgttactagt 3´

Vectors with recloned peptide were further used to infect cells in order to check if the observed resistivity was due to the peptide or not.

Results

3.1 Targeting protein-protein interaction by expressing short domain specific peptide

Proteins interact with their cognate partners to carry out different functions necessary for the growth and development of cell. Interactions are usually mediated by defined domains that recognize specific interaction partners. There can be complementary interaction domains of other proteins, nucleic acids or other molecular ligands. Domains can be highly specific for one potential interaction or may have the specificity to interact with various ligands permitting different interactions. In many cases proteins comprise more than one interaction domains, providing the potential to form multi-protein complexes. Domains through their interactions mediate the targeting of proteins to a specific sub-cellular location, nucleate the formation of multiprotein signaling complexes, control the conformation, activity and substrate specificity of enzymes. Studying protein interactions at the domain level gives a global view of the complexity of the protein interaction network and possibly of protein functions. Dissecting a particular signaling pathway by interrupting interaction between different proteins can reveal specific informations and their effect on the cell. Interaction between proteins can be disrupted by many ways like blocking interface of the partners, mutating a specific region, or by organic molecules. Interfering protein interactions by peptides as inhibitors is also a method of choice. However, instead of adding peptides directly to cells, peptides expressed within the cells to alter protein interactions could be a approach to regulate cellular processes. In the present study a strategy based on disruption of specific protein-protein interaction by expression of peptides that mimic protein binding domains was implemented. One way to accomplish this approach is, to select peptide sequences from one interacting partner where its cognate partner binds and express them *in vivo.*

For a cell to grow, proliferate and differentiate a number of processes involving different protein-protein interactions are involved to carry out these functions. However, in addition to protein interactions that enhance cell growth and differentiation, there are some inhibitory protein interactions that limit proliferation and growth of cell. One such known protein interaction inhibiting cell growth and differentiation occurs between tyrosine kinase Ros and protein tyrosine phosphatase SHP-1 (PTP SHP-1). PTP SHP-1 interacts with tyrosine kinase Ros at specific phosphorylated tyrosine residue and its dephosphorylation results in inhibition of cell growth. This particular interaction was chosen as a target to interrupt it by specific peptides derived from Ros tyrosine phosphorylation domain.

To target a known protein-protein interaction by peptides, an efficient tool is required to express peptides and monitor them *in vivo.* To start with, a retroviral expression vector

system was developed to express efficiently small specific peptides. pLNHX retroviral vector (Clontech) was employed for this purpose. It was extensively modified by cloning Internal Ribosomal Entry Site (IRES), Enhanced Yellow Fluorescent Protein (EYFP). hsp promoter was replaced by human cytomegalovirus (CMV) major immediate early promoter. Multiple cloning site was further modified to introduce restriction sites for two Sfi1 enzyme with different overhangs to make sure unidirectional cloning of oligonucleotides encoding for peptides, as a single cassette. Oligonucleotides were cloned in pLNHX vector at Sfi1 restriction sites. EYFP was used as a reporter and the presence of IRES makes sure that peptides and EYFP are translated from the same mRNA transcript.

To study a possible interference of a known protein interaction by peptides specially genetically modified cell line was used. For interrupting tyrosine kinase Ros and PTP SHP-1 interaction, domain specific peptides were expressed in modified NIH3T3 cell line referred as NIH3T3TrkA Ros cell line (gifted by F. Böhmer). These cells were expressing tyrosine kinase Ros and PTP SHP-1. However, expression of PTP SHP-1 was anhydrotetracycline (atc) regulated.

3.1.1 Ros tyrosine phosphorylation domain specific peptides

To influence interaction between Ros tyrosine kinase and PTP SHP-1 short specific peptides were selected from Ros tyrosine phosphorylation domain. Two peptide sequences were derived from Ros tyrosine phosphorylation domain named as Positive peptide (Pos) and Negative peptide (Neg). Pos has 13 amino acids identical to sequence of Ros tyrosine phosphorylation domain with specific tyrosine residue necessary for PTP SHP-1 interaction. Amino acid sequence of Pos is as; MEGLN***Y***MVLATKSS*Ter*. Neg is identical to Pos except tyrosine residue is replaced by phenylalanine. It thus, lacks the phosphorylation site to interact with PTP SHP-1. Amino acid sequence for Neg is as; MEGLN***F***MVLATKSS*Ter*.

Additionally, Pos and Neg were modified by adding self annealing flanking amino acid sequence (GEFLIVIKSG) on both N and C termini. (Pos and Neg with self annealing flanking amino acid sequence were designated as PC and NC respectively). Flanking sequence is reported to protect peptides from protease degradation and provides a loop like structure to the expressed peptide (Gururaja *et al.,* 2000). In addition to these peptides one more peptide, referred as Non specific peptide (NSP) having no resemblance with the Ros tyrosine phosphorylation domain derived peptides and lacking tyrosine residue was used as a negative control. Peptides were expressed from pLNHX retroviral vector as a part of a bi-cistronic mRNA coding for the peptides and EYFP, both are translated from same mRNA transcript due to the presence of IRES.

Effect on NIH3T3TrkA Ros cell growth by short domain specific peptides

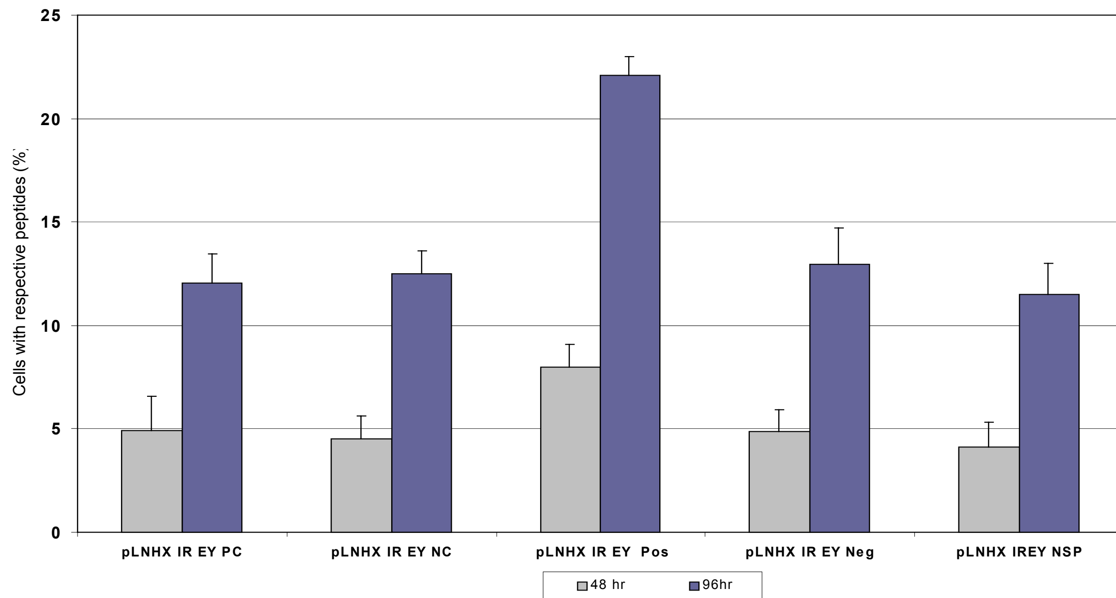


Fig 1; NIH3T3 TrkA Ros cells were infected with viruses from different pLNHX retroviral vector constructs separately. Viruses were collected, filtered after 48h of transfection of Ecopack T[M] 293 packaging cell line. NIH3T3 TrkARos cells were analyzed to determine percentage of cells with the respective peptide after 48h and 96h of viral infection. A significant increase in the number of cells expressing Pos was found compared to cells expressing other peptides, which significantly increased after 96 h of infection. NIH3T3 TrkA cells were grown in absence of anhydrotetracycline (atc) throughout the whole study indicating expression of SHP-1PTP.

Effect of NGF on NIH3T3TrkA Ros cells expressing peptides (cells grown in absence of atc)

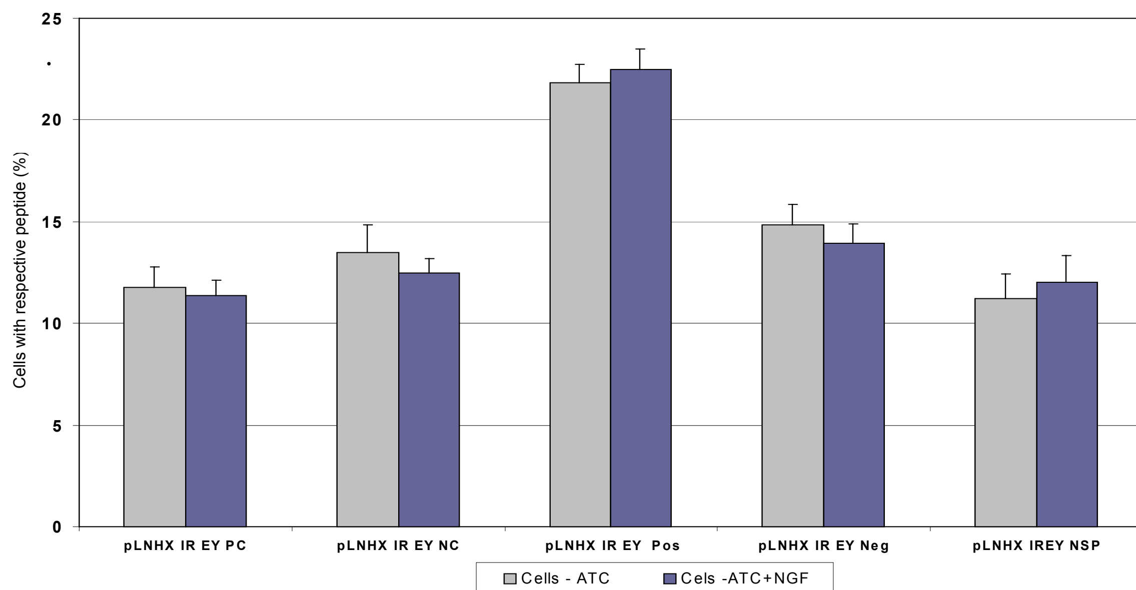


Fig 2; NIH3T3TrkARos cells after being infected with viruses from various retroviral vector constructs separately and grown in absence of atc, were divided after 96h of infection. One group of infected cells were treated with NGF (100ng/mL) for 6 days, while one was grown in absence of NGF as control. Medium with NGF was changed after every two days. Cells were analyzed to determine the number of cells with respective peptide after 6 days of NGF treatment, a relatively small increase in the number of cells expressing Pos was observed.

3.1.2 Effect on NIH3T3TrkA Ros cell growth in absence and presence of PTP SHP-1

NIH3T3TrkARos cells were infected with retroviral vectors expressing different peptides. Cells grown in absence of atc (expressing PTP SHP-1), were analyzed by FACS after 48h and 96h of infection (transduction). As shown in Fig.1 a steep increase in percentage of cells expressing Pos in comparison to cells expressing other peptides was observed. Surprisingly, cells expressing PC (which is similar to Pos except the self annealing flank) showed less proliferation enhancement than Pos expressing cells. Increase in Pos expressing cells, suggest that the expressed peptide might be able to interfere between tyrosine kinase Ros domain and PTP SHP-1 interaction. As Pos sequence has been derived from Ros tyrosine phosphorylation domain it may act as an alternate binding partner for PTP SHP-1 to alter its interaction with Ros tyrosine kinase. Since the peptide and EYFP are translated from the same mRNA transcript increase in number of fluorescent Pos expressing cells directly indicates enhancement in proliferation of cells (Fig 1a). Less proliferative effect observed in PC expressing cells suggests that the peptide is not able to alter the said interaction. Although, PC is similar to Pos except the self annealing flank. PC was expected to enhance more cell proliferation than Pos due to the presence of self annealing clamp sequence that is reported to enhance stability and providing a scaffold like structure to the expressed peptide. The reduced effect of PC may be due to the decrease in flexibility of the peptide as the clamp gives a loop like structure to the expressed peptide making it more structurally constrained and thus possibly less effective for interaction. The loop might also have influence on the frame of the peptide. Neg and NC proved less effective in enhancing cell proliferation as expected due to lack of tyrosine residue in both peptides. Substitution of tyrosine amino acid with phenylalanine devoids the peptide to be an interacting partner for PTP SHP-1. Besides that, NC had clamp sequence which reduces its flexibility. This is consistent with the results as cells expressing Neg and NC did not show increase in proliferation of cells. This may be explained on the basis that these peptides were no longer able to disrupt interaction PTP SHP-1 with tyrosine kinase Ros due to the lack of specific tyrosine residue. Furthermore, expression of a non specific peptide (NSP) with no resemblance to Ros tyrosine phosphorylation domain derived peptides showed no effect on cell proliferation enhancement indicating NSP was unable to interfere the interaction. These results strengthens that Pos expression did play a role in observed cell proliferation enhancement. When cells were grown in presence of atc (no expression of PTP SHP-1) (Fig 3) a slight increase in cell proliferation was observed in Pos expressing cells compared to other cells. This enhancement in cell proliferation seen in Pos expressing cells was comparatively lower than cells grown in the

Effect on NIH3T3TrkA Ros cell growth by short domain specific peptides

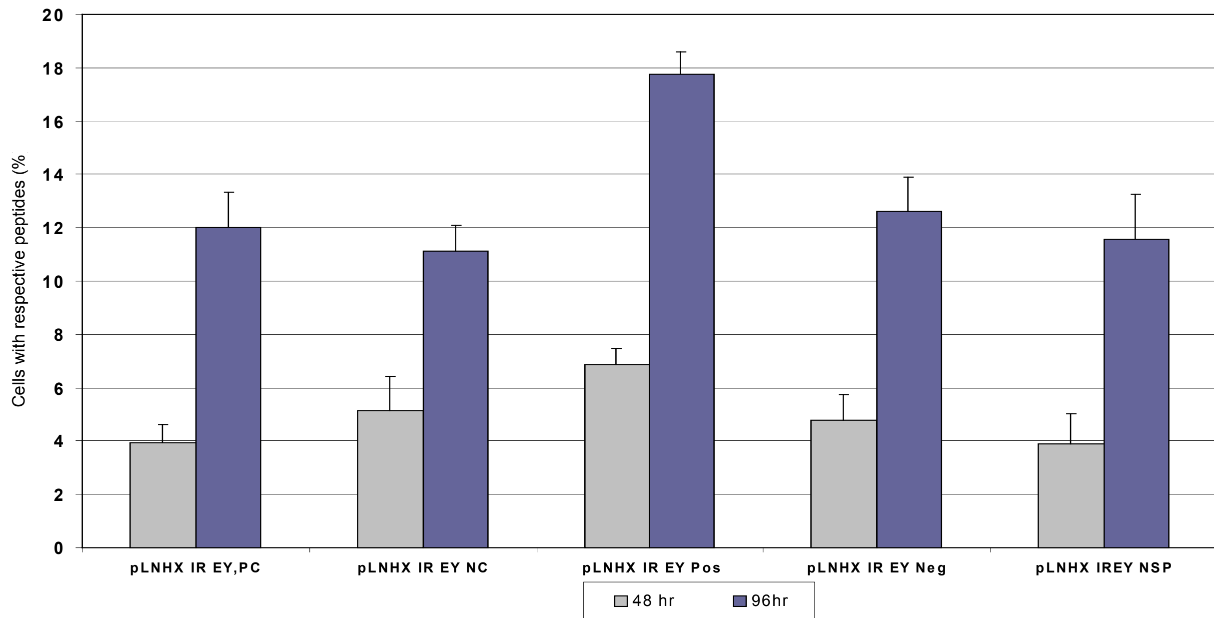


Fig 3; NIH3T3TrkA Ros cells grown in presence of atc were infected with viruses from different pLNHX retroviral vector constructs. Infected cells were analyzed after 48h and 96h of infection to determine the peptide expressing cells. Percentage of Pos expressing cells were found higher than other peptide expressing cells after 48h of infection and a steep increase in Pos expressing cells was observed after 96 of infection compared to other peptide expressing cells. Data is mean of three separate experiments.

Effect of NGF on NIH3T3TrkA Ros cells expressing peptides (cells grown in presence of atc)

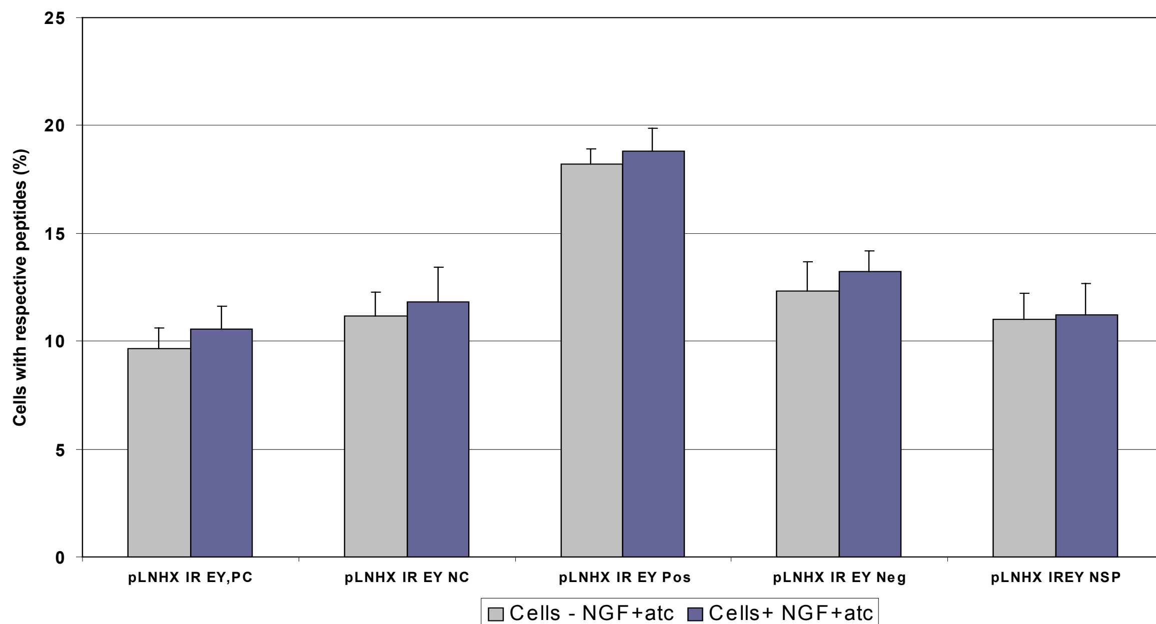


Fig 4; NIH3T3 TrkA Ros cells grown in presence of atc after being infected with viruses from different pLNHX retroviral vector constructs separately were divided after 96h of infection. One group of cells from each virally infected peptide expressing vector were treated with NGF (100ng/mL) for 6 days, while one group was untreated. Medium with NGF was changed after every 2 days. Post NGF treatment cells were analyzed to determine the percentage of the cells expressing the corresponding peptides. NGF has relatively less effect on increased proliferation in peptide expressing cells, except for Pos expressing cells. Data is mean of three separate experiments.

absence of atc, suggesting that in addition to tyrosine kinase Ros and PTP SHP-1 interaction some other factors may also be contributing to the enhanced observed effect.

3.1.2 Stimulation of TrkA Ros by NGF is not influenced by peptides

Modified cells were challenged with NGF (100ng/mL) post 96h of infection to stimulate TrkA Ros receptor. This stimulation had relatively small effect on enhancement in cell proliferation of peptide expressing cells. Increase in number of Pos expressing cells was relatively higher when cells were grown in presence (Fig 4) or in absence of atc (Fig 2) than other peptides. No strong effect on cell proliferation in peptide expressing peptides was observed, indicating NGF did not play any major role in enhancing the effect of peptides. Proliferation assay by counting cells by coulter counter was further done to study the effect on cell proliferation. As seen in Fig 5 and Fig 6 when cells were grown in presence (Fig 5) and absence (Fig 6) of NGF an overall relative increase in cell proliferation was seen. However, slightly enhanced proliferation of Pos expressing cells reconfirmed a possible role of Pos in depicting this enhanced proliferative effect.

Ros tyrosine phosphorylation domain derived peptides were also expressed in normal NIH3T3 cells (Fig 7). In these cells peptides did not induce proliferation and there is no difference in activity between the peptides. Surprisingly, a decrease in PC and NC expressing cells was observed after 48h of infection. These effects clearly indicate that the proliferation seen in NIH3T3TrkA Ros cells is due to the domain specific expressed peptides and is dependent on the expression of the intracellular part of the Ros receptor tyrosine kinase domain from which they were derived. The observed effects clearly showed that the peptides might be able to target specific interactions and could be used to influence a known protein interaction. However, in the present study the enhanced proliferation is not dependent solely on the disruption of Ros tyrosine kinase and PTP SHP-1 interaction.

These results gave us a clue that peptides could be used to interfere a known protein interaction. Based on this method of expressing peptides using efficient expression vector system, peptides derived from specific motifs were used to target interaction between nuclear receptors and coactivators.

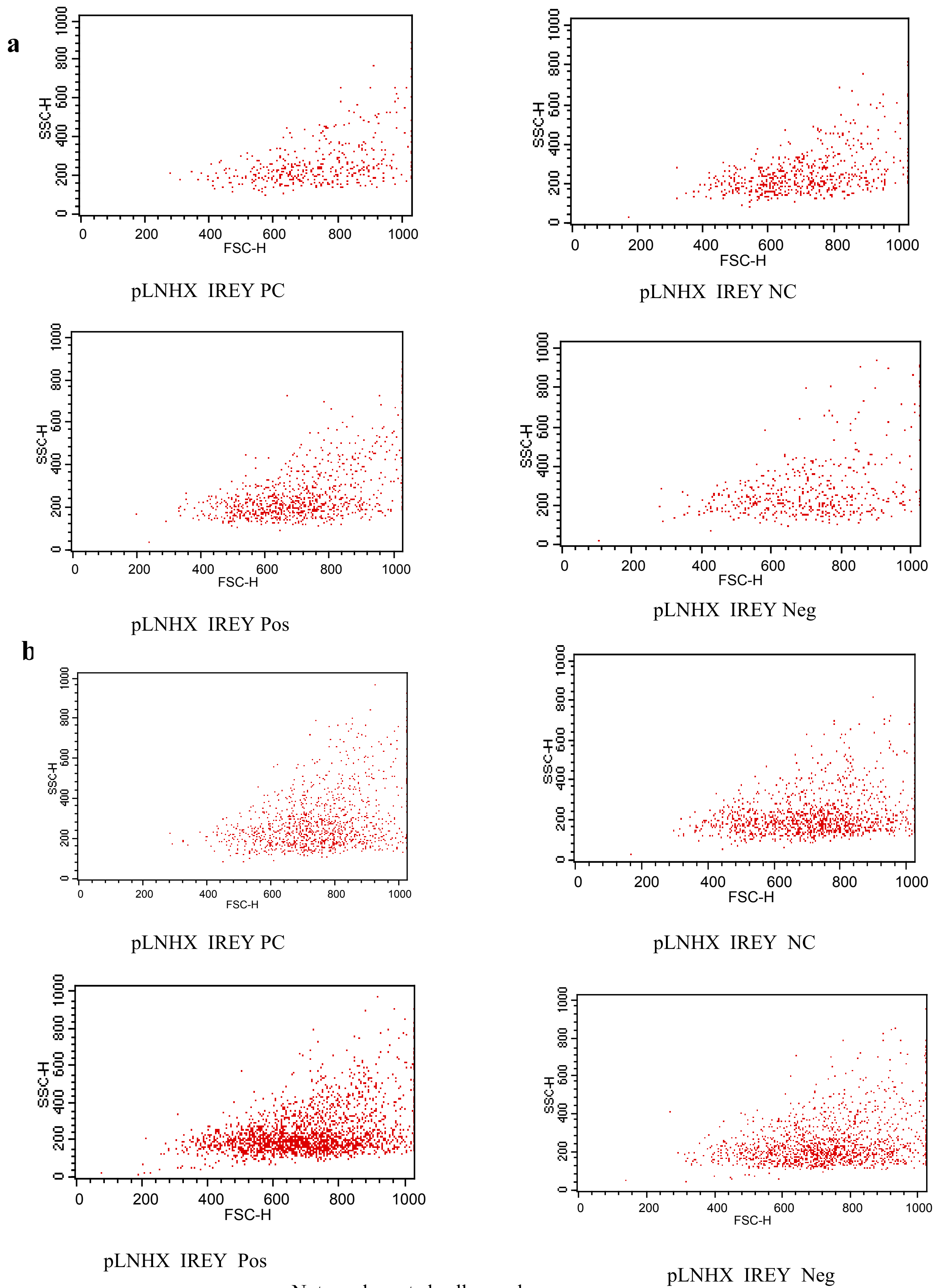


Fig (1a); FACS analysis of NIH3T3TrkA Ros cells infected with viruses from different pLNHX retroviral vectors (a) sorted cells after 48h (b) after 96h of infection. An increase in number of cells can be observed in Pos expressing cells compared to other cells. This enhancement in number of cells reflects increased proliferation in Pos expressing cells due to the peptide. Each experiment was repeated three times.

Proliferation assay of NIH3T3 TrkA Ros cells treated with NGF and grown in presence of atc

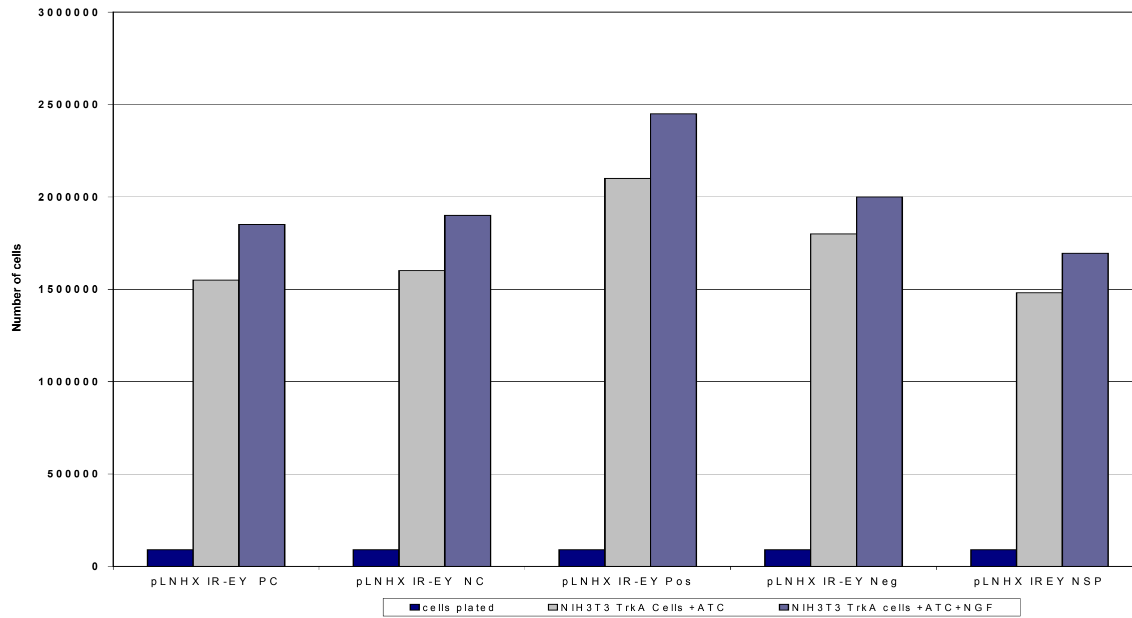


Fig 5; NIH3T3TrkA Ros cells grown in presence of atc, were infected with viruses from different pLNHX retroviral vectors separately. Cells were treated with NGF (100ng/mL) for 6 days before being counted by Coulter counter. One group of virally infected cells was grown in parallel with out NGF. Cell numbers indicate a relatively slight increase in number of NGF treated cells compared to untreated cells. Cell numbers are mean of three separate assays.

Proliferation assay of NIH3T3 TrkA Ros cells treated with NGF and grown in absence of atc

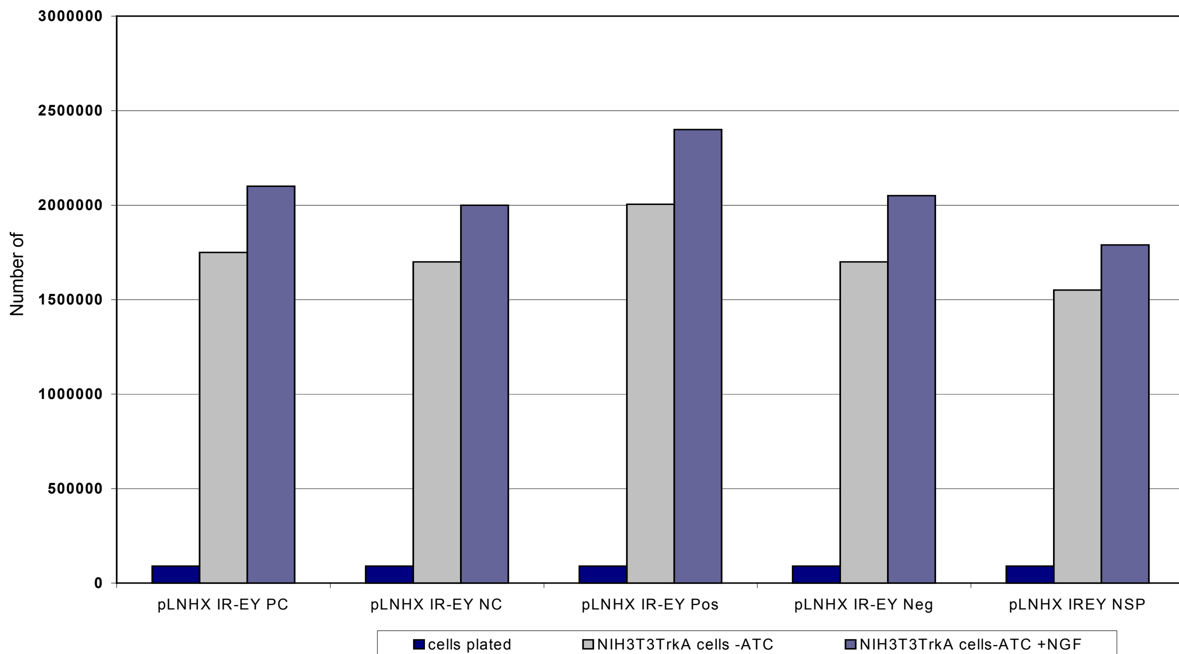


Fig 6; NIH3T3TrkA Ros cells grown in absence of atc were infected with viruses from different pLNHX retroviral vectors separately. Cells were treated with NGF (100ng/mL) for 6 days before being counted by Coulter counter. One group of virally infected cells was grown in parallel with out NGF. Coulter counter counting of cells indicated an increase in number of NGF treated cells as compared to untreated cells . Cell numbers are mean of three separate assays.

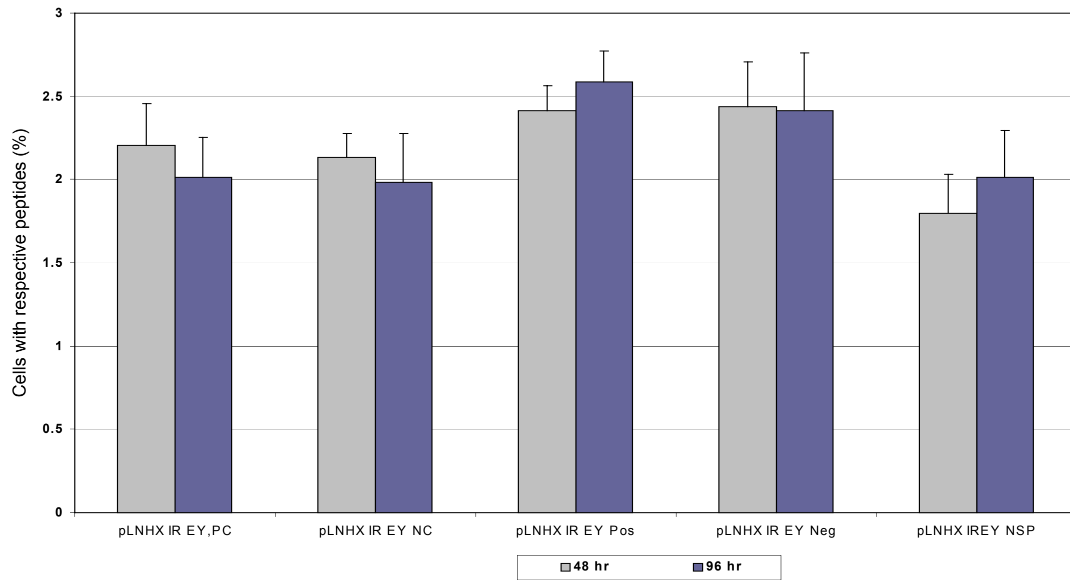
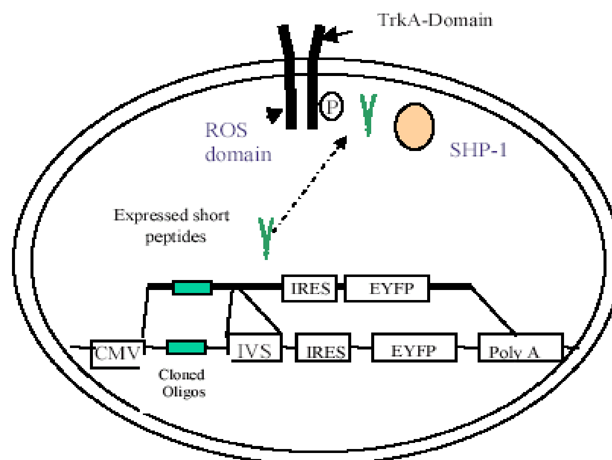


Fig 7; Normal NIH3T3 cells were infected with viruses from different pLNHX retroviral constructs after 24h of plating. Post 48h and 96h of infection cells were analyzed to determine the peptide expressing cells. Cells expressing different peptides did not show any increase in number of cells as seen in NIH3T3TrkA Ros cells, which were modified to study the interaction of peptides with the intended interaction of tyrosine kinase Ros and SHP-1PTP. Since, no effect on NIH3T3 cells was observed it signifies the role of domain specific peptides in enhancing proliferation in NIH3T3TrkA Ros cells. Data is mean of three different experiments



Picture 1;

A Schematic outline of the modified NIH3T3TrkA-Ros cell line highlighting a possible way of the peptides interfering with the tyrosine kinase Ros and SHP-1PTP interaction after their expression in the cells.

3.2 Nuclear receptor coactivator interaction specificity; Effect of short peptides with LXXLL motif on transcription activation

Receptors as transcription factors play an important role in growth, development and disease progression. Receptors are usually present on membranes, cytoplasm and in the nucleus. Binding of ligand to receptor initiates a cascade of signal reactions leading to expression of different genes. Ligands for membrane bound receptors bind directly to them while ligands for nuclear and cytoplasmic receptors had to enter inside the cell to bind their respective receptors. Once a ligand bound to a nuclear receptor it may form a heterodimer or homodimer before binding to hormone response elements (HREs) present on DNA to initiate

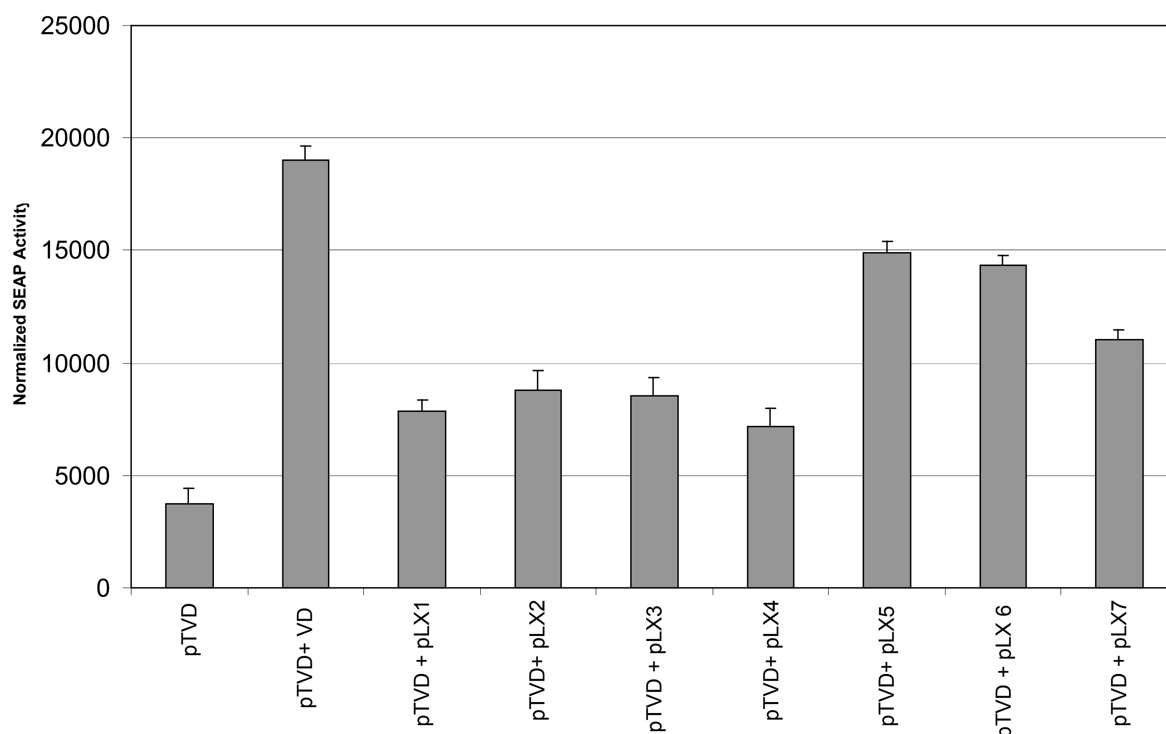


Fig 8; HeLa cells were co-transfected separately with LXXLL peptide expressing vectors and a SEAP reporter vector, which in addition to SEAP reporter gene has Vitamin D response element (VDRE) sequence. Cells were treated with 10^{-6}M vitamin D (Vit. D) post 24 hr of transfection. To determine SEAP activity samples were taken 24h post Vit.D treatment. Vit. D induced expression of reporter gene was significantly suppressed by flanked LXXLL peptides than non flanked peptides indicating their role to antagonize the induced transcription as clear from the SEAP activities. Peptide LX4 showed relatively higher effect in comparison to other flanked LXXLL peptides, while among non flanked ones peptide LX7 has significant effect. Activities are the mean of triplicate assays

transcription. Various coactivators are recruited by receptors to enhance transcription. Coactivators are reported to interact with the AF-2 region of the receptor via specific LXXLL motifs whose consensus sequence and number varies from coactivator to coactivator. In the present study, attempts were carried out to express different LXXLL peptides varying in

number of LXXLL motifs and amino acid residues on N and C termini of the motif, with this aim if they will be able to block receptor and coactivator interaction. Various LXXLL peptides were chosen. In LX1, LX2, LX3 and LX4 pattern of motif and amino acids is as $X_7LX_2LLX_7$ where X is any amino acid and L is Leucine. Peptides with random LXXLL motif and flanking residues around it avoid biasness to any specific amino acid in the expressed peptide. Peptide LX5 contains only one motif (LHRLL) with out any additional amino acid residue around the motif, peptide LX6 has two LXXLL motifs (LHRLL) separated by three amino acid residues and finally peptide LX7 has one motif (LHRLL) with two amino acid on C terminus of motif. In peptides LX5, LX6 and LX7 sequence of the motif is LHRLL. It was chosen after comparing motif sequences from various coactivators and this motif sequence was present in most coactivators that led to its selection. Cells were co-transfected with Peptide-EYFP expressing and SEAP reporter expressing vectors. Each SEAP vectors have varying number of different hormone response element copies. Enhanced yellow fluorescent protein was used to monitor transfection efficiencies.

Effect of LXXLL peptides on 9 cis-retinoic acid induced transcription

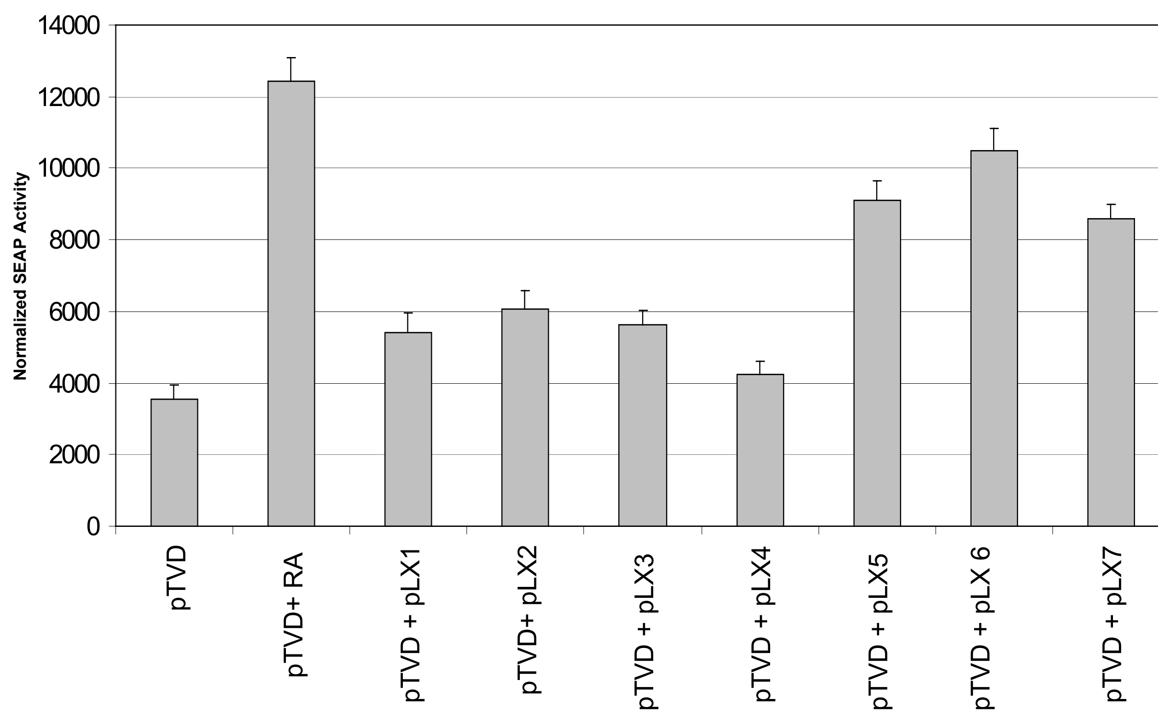


Fig 9; HeLa cells were co-transfected separately with LXXLL peptide expressing vectors and SEAP vector, which in addition to SEAP reporter gene has Vitamin D response element (VDRE) sequence. Cells were treated with 10^{-6} M 9-cis retinoic acid post 24 hr of transfection. To determine SEAP activity samples were taken after 24 h of 9-cis retinoic acid treatment. Cells expressing LXXLL peptides with flanking sequences (LX1-LX4) indicated less SEAP activity signifying the role of peptides in inhibiting the reporter gene expression by acting as antagonists. Non flanked LXXLL peptides were less effective in suppressing the induced effect. Activities are mean of three separate experiments.

All peptides were expressed in two cell lines; HeLa and NIH3T3 by employing bi-cistronic vector expressing EYFP and peptide from the same mRNA transcript. Various drugs like Vitamin D, 9 cis-retinoic acid, Dexamethasone and Forskolin were used separately as inducing agents to study the antagonizing effect of peptides on induced transcription.

3.2.1 Suppression of vitamin D and 9-cis retinoic acid induced transcription.

HeLa cells were co-transfected with Peptide-EYFP expressing vector along with SEAP reporter vector having Vitamin D response element (VDRE) sequence. Post 24h of transfection cells were treated with 10^{-6}M vitamin D (Vit.D) for 24 h. As seen in (Fig 8) Vit. D strongly induced OC promoter driven SEAP reporter gene. With the expression of peptides induced activation got decreased as indicated by the SEAP reporter activity. This decrease may suggest that the peptides have ability to block vitamin D receptor (VDR) and coactivator interaction.

Efficiency of peptide mediated suppression is concentration dependent

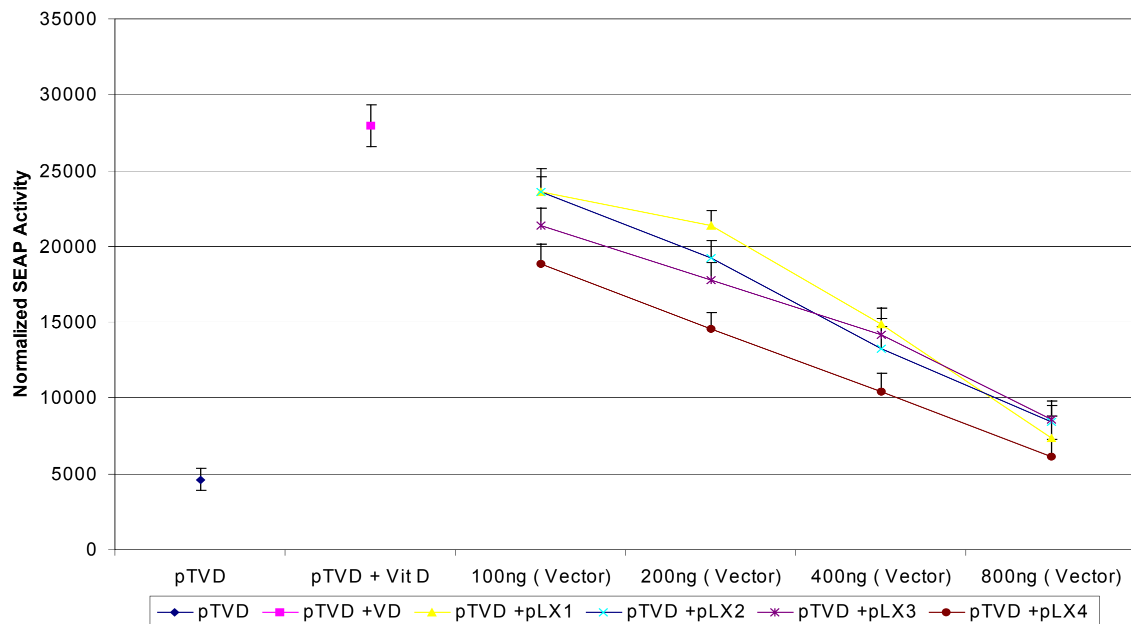


Fig 10; HeLa cells were co-transfected separately with increasing concentration (100ng, 200ng, 400ng and 800ng) of LXXLL peptide expressing vectors as indicated and SEAP vector having Vitamin D response element (VDRE) sequence. Cells were treated with 10^{-6} M Vit D post 24 hr of transfection. SEAP activity was determined after 24 h of Vit D treatment. Vit D induced SEAP gene expression as indicated by SEAP activity. With increase in concentration of peptides SEAP activity showed gradual decrease indicating that with increase in peptide expressing vectors more copies of peptide may be available to antagonize the induced effect . Activities are mean of three separate experiments.

Hall *et al.,* 2000 reported LXXLL peptides interact directly with the AF-2 domain of the nuclear receptor family indicating blockade of this region may prevent transcriptional activation by receptor and supports the prevalent idea that the receptor must recruit an

additional factor(s) through this site to manifest function. Coactivator recruitment is necessary for receptor to carry out transcriptional activation and they interact with ligand bound receptor via LXXLL motif.

Expressed LXXLL peptides in HeLa cells treated with Vit. D showed different extent of antagonizing effect as seen in (Fig 8). With flanked peptides being more potent than non flanked peptides as indicated by SEAP activities, each SEAP activity reported is mean of three separate experiments. Peptides lacking additional sequences were quite ineffective except LX7 which exhibited better suppressive effect than LX5 and LX6. One possible reason for this may be that these peptides are not able to interact effectively with AF-2 domain of receptor to block its interaction with the coactivator due to lack of additional amino acid residues. Observed variation in activities of flanked LXXLL peptides among themselves led to the supposition that these peptides might differ in their affinities to interact

Comparison of LXXLL peptides and non-LXXLL peptides on antagonizing induced dexamethasone transcription

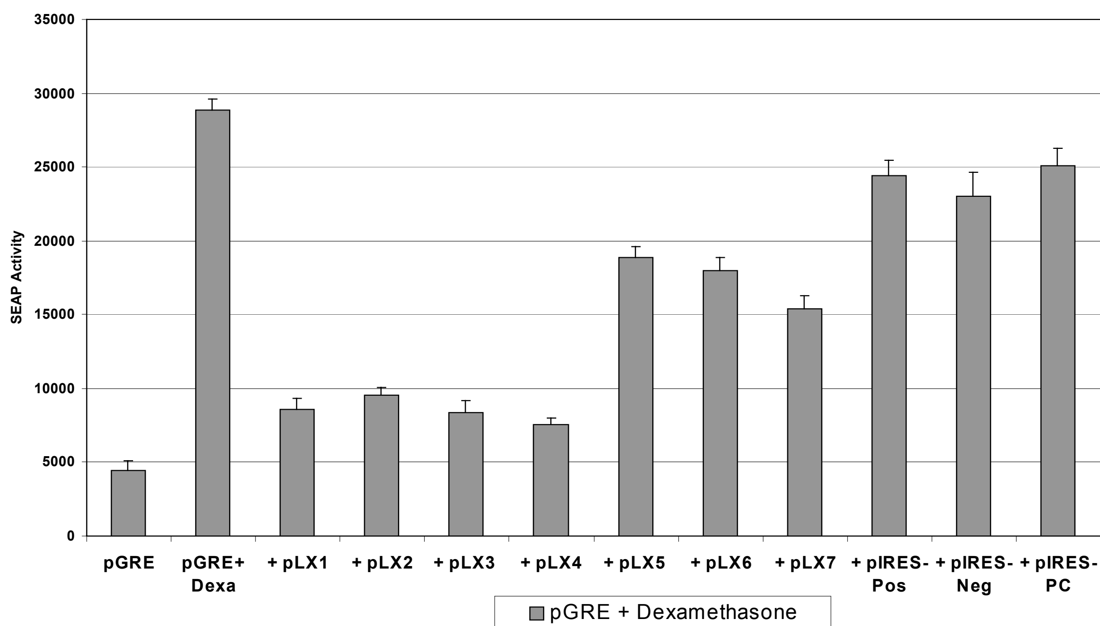


Fig 11; HeLa cells were co-transfected separately with LXXLL peptide expressing vectors and SEAP vector having three tandem copies of glucocorticoid response element (GRE) sequence in one group, while in other group cells where cotransfected with non-LXXLL peptide expressing vectors and SEAP vector. Cells were treated with 10^{-6} M dexamethasone post 24 hr of transfection. SEAP activity was determined after 24h of dexamethasone treatment. Dexamethasone treatment induces SEAP activation which was significantly lowered by expression of peptides to different extent with peptides having long flanking sequences have preferentially better antagonizing effect as indicated by decrese in activities than non flanking LXXLL peptides. Peptides lacking LXXLL motif did not show any considerable effect on suppressing the induced effect which suggest that for a peptide to be an active antagonist LXXLL motif sequence should be present in it.

with ligand bound receptor. Among flanked peptides LX4 and LX1 showed better suppressive effect in Vitamin D induced treatment than LX2 and LX3. 9-cis retinoic acid induced activation (Fig 9) was also suppressed by peptides. The observed pattern of suppression was similar as seen in Vit. D induced activation. However, 9-cis retinoic acid induced SEAP reporter activities were significantly lower than Vit. D. This decrease in activity in case of retinoid treatment may suggest that in VDR-RXR heterodimer, VDR interaction with the coactivator and hormone response element play a significant role.

3.2.2 Efficiency of peptide mediated suppression is concentration dependent

Antagonistic property of peptides was found to be dose dependent (Fig 10). With increase in the concentration of peptide expressing vector a stronger suppressive effect on induction was observed as indicated by decrease in SEAP activity in case of peptides with flanking sequence (LX1-LX4). This indicates that with increase in concentration more copies of peptide may be available to interact with the receptor complex to prevent coactivator recruitment to influence the induced transactivation.

Forskolin induced effect antagonized by expressed peptides

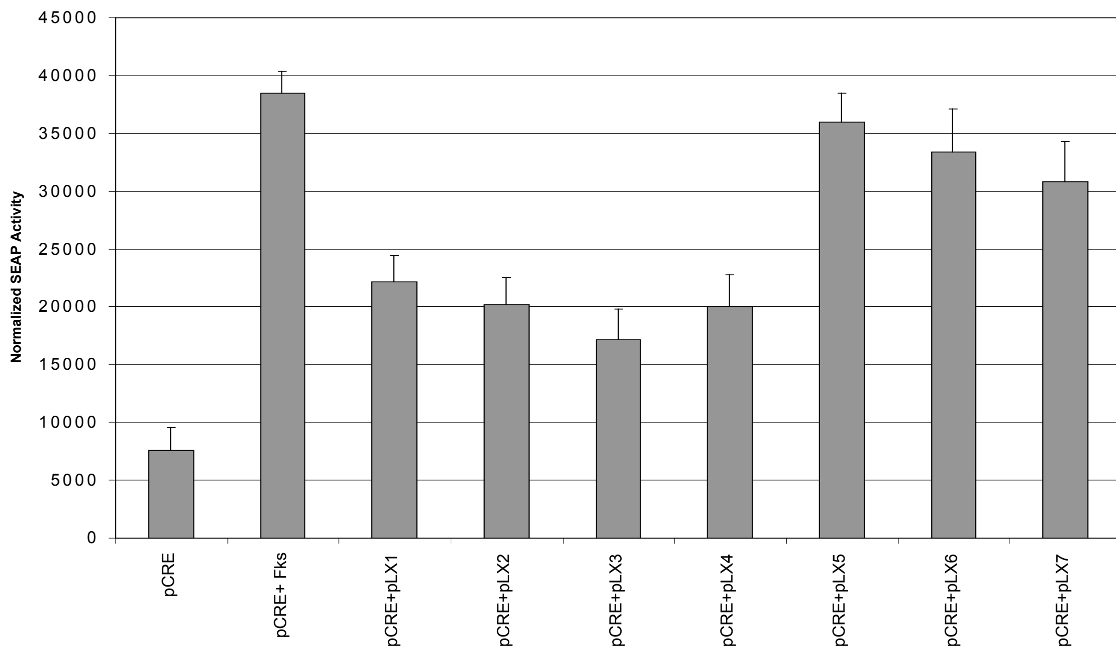


Fig 12; HeLa cells were co-transfected separately with LXXLL peptide expressing vectors and SEAP vector which contains three tandem copies of cAMP response element (CRE) sequences in addition to SEAP reporter gene. Cells were treated with 10µM forskolin post 24 hr of transfection. SEAP activity was determined after 24 h of forskolin. treatment. Forskolin treatment induces SEAP activation which was significantly lowered by expression of peptides to different extent. LXXLL peptides with long flanking sequences have preferentially better antagonizing effect than non flanking LXXLL peptides. Activities are mean of three separate experiments.

Table 1;

SRC1	YSQTSHK	LVKLL	TTTAEQQ	NR1
NcoA1	YSQTSHK	LVQLL	TTTAEQG	NR1
GRIP1	DSKGQTK	LLQLL	TTKSDQM	NR1
SRC1	LTARHKI	LHRLL	QEGSPSD	NR2
NcoA1	LTERHKI	LHRLL	QEGSPSD	NR2
GRIP1	LKEKHKI	LHQLL	QDSSSPV	NR2
SRC1	ESKDHQL	LRYLL	DKDEKDL	NR3
NcoA1	ESKDHQL	LRYLL	DKDEKDL	NR3
GRIP1	KKKENAL	LRYLL	DKDDTKD	NR3
NcoA1	DQCISSQ	LDELL	CPPTTVE	NR4
NcoA1	GVIEKES	LGPLL	LEALDGF	NR5
SRC1	QAQQKSL	LQQLL	TE stop	NR4
NcoA1	QAQQKSL	LQQLL	TE stop	NR6
CBP	AASKHKQ	LSELL	RGGSGSS	NR1
p300	AASKHKQ	LSELL	RGGSGSS	NR1
CBP	RKLIQQQ	LVLLL	HAHKCQR	NR2
p300	RKLIQQQ	LVLLL	HAHKCQR	NR2
CBP	RSISPSA	LQDLL	RTLKSPS	NR3
p300	GTVSQQA	LQNLL	RTLRSPS	NR3
RIP	DSIVLTY	LEGLL	MHQAAGG	NR1
140	GKQDSTL	LASLL	QSFSSRL	NR2
	YGVASSH	LKTLL	KKSKVKD	NR3
	PSVACSQ	LALLL	SSEAHLQ	NR4
	DSIVLTY	LEGLL	MHQAAGG	NR5
	SHQKVTL	LQLLL	GHKNEEN	NR6
LX1	MTIAVVFR	LMCLL	VLGGRVR	
LX2	MLGFFYDL	LWFLL	CVCVLHP	
LX3	MLQTYVVF	LEPLL	FDFSRDR	
LX4	MRVSLLSL	LLRLL	QSIAVYR	
LX5	M	LHRLL		
LX6	M	LHRLL	AAA LSRLL	
LX7	M	LHRLL	QL	

Comparison of LXXLL motifs with amino acid residues around the motif from various coactivators with expressed LXXLL peptides. Expressed peptides share varying number of amino acid residues with coactivators, with peptide LX4 is sharing more number of flanking amino acid residue around LXXLL motif than other peptide. Peptide LX7 shares amino acid Q with other coactivators sequences.

3.2.3 Suppression of dexamethasone and forskolin induced transcription

In order to further confirm role of peptides and their ability to interact and antagonize the induced activation, SEAP vectors with GRE and CRE response elements were used to study the effect of dexamethasone and forskolin on induced transactivation respectively. Dexamethasone binds to glucocorticoid receptor and the receptor ligand complex binds to glucocorticoid response element (GRE) and initiates transcription after coactivator recruitment.

Cross treatment of pCRE-SEAP vector transfected cells with dexamethasone

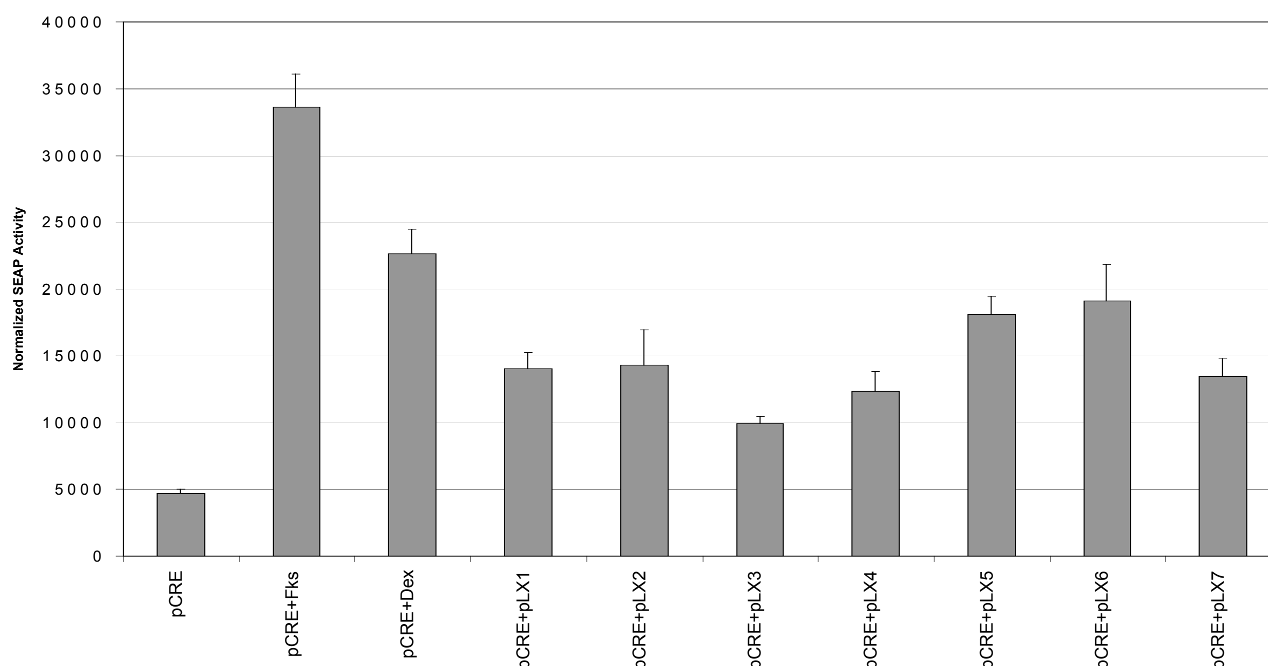


Fig 13; HeLa cells were co-transfected separately with LXXLL peptide expressing vectors and SEAP vector which contains three tandem copies of cAMP response element (CRE) sequence in addition to SEAP reporter gene. Cells were cross treated with dexamethasone post 24 hr of transfection. SEAP activity was determined after 24 h of treatment. Forskolin induced SEAP reporter activity was stronger than cross treatment with dexamethasone. SEAP activity was lowered by LXXLL expressed peptides indicating their role as antagonists for activation. LX3, LX4 and LX7 seem to have better effect than other peptides. In cross treatment reporter activities were comparatively less than normal treatment of drug. Activities are mean of three separate experiments.

Treatment of pGRE-SEAP vector transfected cells with dexamethasone showed a significant increase in SEAP activity as compared reporter activity of untreated transfected cells. Increase in reporter activities indicated the induced effect of dexamethasone on GRE mediated transcription of the reporter gene. On expressing LXXLL peptides a decrease in SEAP activity (Fig 11) was observed in the same pattern as in Vit. D treatment with peptides having flanking sequences being more antagonistic than non flanking ones. Forskolin induced transcription was suppressed by peptides (Fig 12) as in dexamethasone, however peptides vary in their pattern of efficiency.

Dexamethasone mediated transcription of pCRE-SEAP reporter gene indicating a cross talk between glucocorticoids and cAMP. Groul *et al.,* 1993 have reported synergism between glucocorticoids and cAMP. Dexamethasone induced CRE transcription was significantly lower than induced by forskolin (Fig 13). Forskolin was also found to induce transcription of GRE-SEAP reporter gene (Fig 14) indicating a possible cross talks of cAMP and glucocorticoid receptor. Transcription of CRE/GRE SEAP reporter gene by dexamethasone and forskolin respectively was subjected to study the effect of expressed LXXLL peptide to suppress the induced transcription. Peptides were able to suppress the SEAP reporter activity in both cases (Fig 13, 14) with flanking LXXLL peptides being more potent in antagonizing the induced effect compared to non flanked ones. All these results elucidate a potential role for short LXXLL peptides when expressed in vivo to act as antagonists to suppress the induced transcription by different agents.

3.2.4 Adjacent amino acids are major determinants of efficiency

Flanking peptides exhibited variation in reducing the reporter activity that corresponds to their ability to act as antagonistic agents. Among flanking peptides LX4 and in non-flanking peptide LX7 seemed more effective than others. The significant antagonizing effect of peptides with flanking sequences on both N and C termini indicated that presence of amino acid residues around LXXLL play a significant role. On comparing the flanking residues of these peptides with the residues around LXXLL residues in various coactivators (Table 1), LX4 was found to share maximum number of amino acids around LXXLL motifs with high affinity peptides present in various coactivators, followed by LX3, LX1 and LX2. This suggests that residues around the motif may help the peptide in properly interacting with AF-2 domain of activated receptor and block its interaction with the coactivators. However, this blocking seems was also found to dependent on presence of particular amino acid residue present around the motif, as can be argued on the basis of LX4 which shares not only the position but sequence of amino acid residues with coactivators (Table 1). Among other peptides with no or few amino acid residues around the motif peptide LX7 was found to be exerting better effect than LX5 and LX6. On comparing LX7 with coactivators this effect was attributed to the presence of amino acid glutamine (Q) present in the peptide which it shares with high affinity peptides. Thus, even a single residue seems to be having a role in binding to receptor ligand complex and effecting transactivation. Peptides with only one LXXLL motif (LX5) interacted with receptor ligand complex to block recruitment of coactivator and thus acted as antagonists of the induced activation as indicated by reporter gene activities observed

for the first time. A comprehensive comparison of antagonistic effect of flanked peptide and non flanked peptide is elaborated in Table 2.

Table 2: Comparison of expressed LXXLL peptides on antagonizing the induced transcription by different drugs.

	No treatment	Dex	VitD	RA	Dex/cre	Forskolin	Fors/GRE	
Control plasmid	100%	100%	100%	100%	100%	100%	100%	100%
pLX3	100%	34%	45%	45%	51%	45%	44%	44%
pLX4	100%	31%	38%	34%	40%	64%	55%	43%
pLX1	100%	35%	41%	44%	60%	58%	62%	50%
pLX2	100%	39%	46%	49%	69%	53%	70%	54%
pLX7	100%	63%	58%	69%	68%	80%	59%	66%
pLX6	100%	73%	75%	84%	91%	87%	81%	82%
pLX5	100%	77%	78%	73%	84%	94%	73%	80%
	100%	50%	55%	57%	66%	68%	63%	

Comparative analysis of different peptides on treating HeLa cells with vitamin D, retinoic acid, dexamethasone, forskolin and cross treatment of dexamethasone and forskolin after co-transfecting cells with LXXLL peptide expressing vectors and vectors with specific Hormone response elements (HREs) respectively. Peptides with flanking amino acid residues around the LXXLL motif were found to be better in antagonizing the induced transcription by various agents than peptides lacking flanking residues. Among flanked peptides LX4 was having higher suppressive effect in different treatments followed by LX3 except in forskolin treatment where LX3 had better effect on forskolin than LX4. Effect of peptides seems to be related with the number of flanking amino acids a peptide shares with high affinity peptides which was higher in LX4 followed by LX3. Among the non flanked ones LX7 showed better suppressive peptide than LX5 and LX6. LX7 shares amino acid `Q´ with many high affinity peptide explains its role in being more potent than other non flanking peptide. On comparing drug treatments, dexamethasone induced transcription was overall highly suppressed by peptides followed by vitamin D and retinoic acid respectively.

Peptide with two close motifs (LX6) did not act as strong antagonist as thought due to the presence of two motifs. It was believed, if LXXLL motif alone is required for a peptide to act as antagonist then on that basis LX6 should be more effective than other peptides. As per the reporter activity LX6 (Table 2) is the least active peptide in down regulating the induced effect indicating that not only the number of motif are necessary but their specific position is also equally important as seen in most of the coactivators. Peptide LX5 that is devoid of any additional residue resulted merely in suppressing the induced effect, emphasizing the need of additional residues around the motif. To confirm whether the LXXLL peptides do really act as antagonists three different non LXXLL peptides as described earlier Pos, Neg and PC were expressed in combination with pGRE–SEAP vector and treated with dexamethasone. All these peptides lack LXXLL motif, as clear from Fig 11 no significant suppression in induced

activity was observed by these peptides. Ineffectiveness of these peptides confirms role of LXXLL peptides to act as signature motifs in coactivators to recognize and interact with receptor.

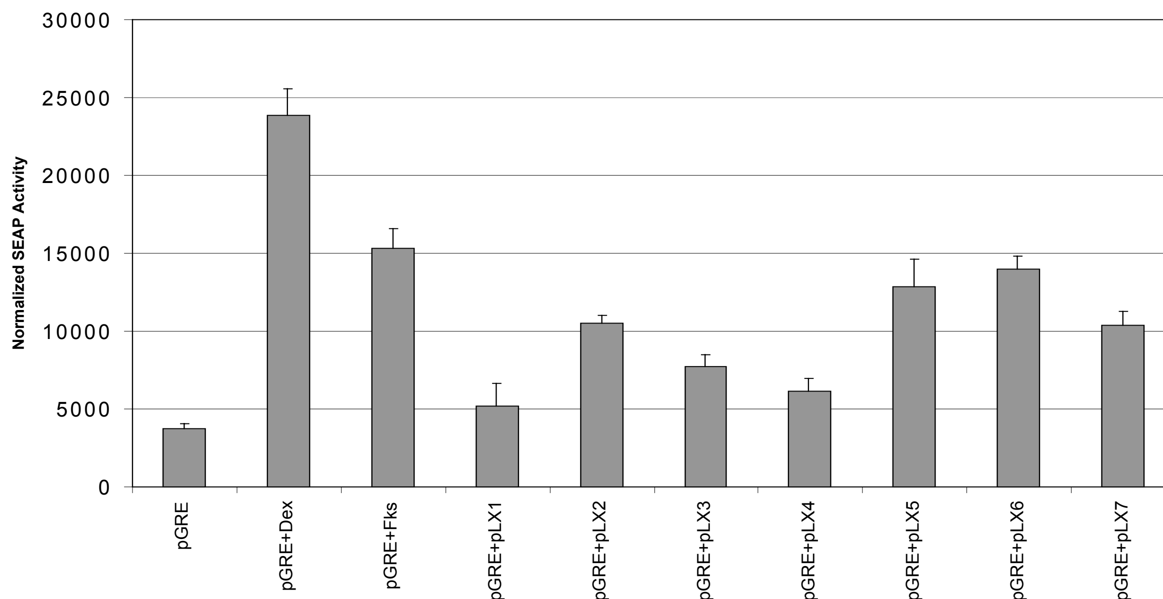


Fig 14; HeLa cells were co-transfected separately with LXXLL peptide expressing vectors and SEAP vector which contains three tandem copies of glucocorticoid response element (GRE) sequence in addition to SEAP reporter gene. Cells were cross treated with 10µM forskolin post 24 hr of transfection. SEAP activity was determined after 24 h of dexamethasone induced SEAP reporter activity was stronger than cross treatment with forskolin. SEAP activity was lowered by LXXLL expressed peptides indicating their role as antagonists for activation. LX1, LX4 and LX3 seem to have better effect than other peptides. Activities are mean of three separate experiments.

3.2.5 Pattern of efficiency is different for nuclear receptor mediated transcription and PKA (forskolin) mediated transcription

Forskolin induces the cAMP pathway by activating adenylate cyclase. cAMP leads to the phosphorylation of CREB which binds to CRE and carries out transcription. Forskolin mediated cAMP response element (CRE) dependent reporter gene expression was significantly lowered by expression of the LXXLL peptides (Fig 12), indicating their role in interacting with CREB complex and thus prevent the coactivator recruitment. Forskolin strongly induced CRE mediated expression of reporter gene than by other agents. Influence of flanked peptides was higher than non flanked ones as seen earlier in dexamethasone and Vit. D treatment. However, change in pattern of peptide efficiency was observed in forskolin treatment with peptide LX3 being more effective as compared to LX4 seen in case of dexamethasone and Vit. D treatment. Although LX3 shares less number of residues with the high affinity peptides present in various coactivators than LX4 peptide. Change in efficiency

may be due to variation in function as PKA mediated phosphorylation of CREB and its interaction with SRC-1 or other proteins occurs in cytoplasm rather than in nucleus.

3.2.6 LXXLL peptides are active in different cell types

Does the effect of expressed peptides on antagonizing the receptor mediated transcription remains constant or varies in different cell type? It was found to remain same in case of NIH3T3 and HeLa cell line. Treatment of NIH3T3 cells with Vit. D after being transfected with VDRE-SEAP vector leads to significant increase in VDRE driven SEAP activity (Fig15) compared to control. Peptides antagonized the Vit. D induced transcription in same way as observed in HeLa cells with LX4 peptide being more effective followed by LX1, LX3 and LX2 among peptides with flanked LXXLL motif, while LX7 was among non flanking peptides. As observed in HeLa cells antagonistic property was dose dependent, same dose dependency was observed in NIH3T3 cells (Fig 16). This emphasis that the role of the peptides is not cell dependent. Similar results as in HeLa on treatment with dexamethasone and Forskolin were observed in NIH3T3 cells (Fig 17). Further confirming role of peptides is not depending on cell type and their ability to interact and suppressive the induced effect remains same.

Vitamin D induced transcription antagonized by short LXXLL peptides expressed in vivo (in NIH3T3 cells)

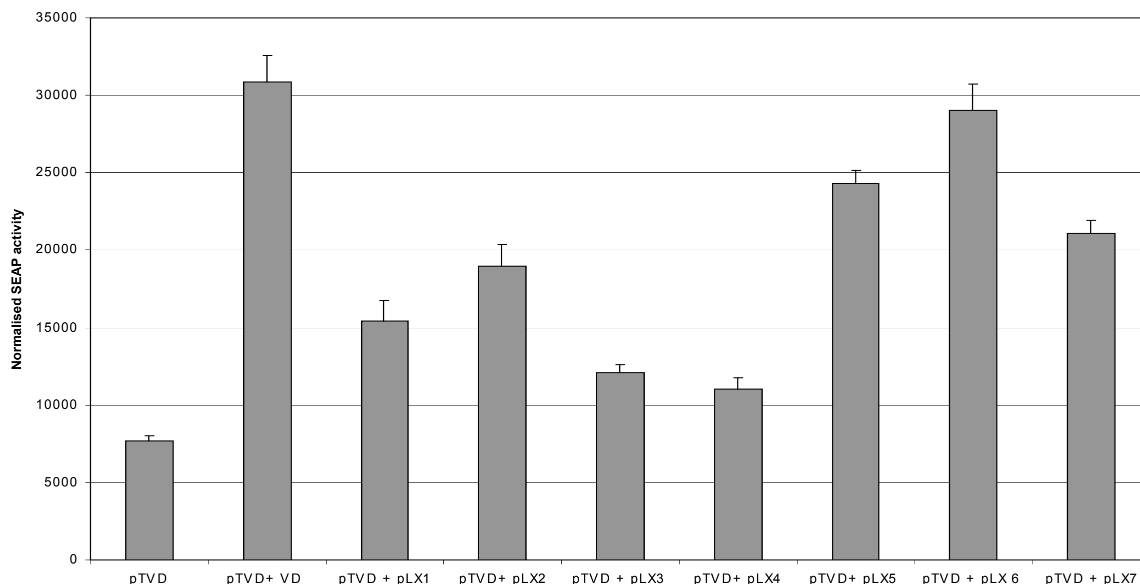


Fig 15 : NIH3T3 cells were co-transfected separately with LXXLL peptide expressing vectors and SEAP vector which contains in addition to SEAP reporter gene vitamin D response element (VDRE) sequence. Cells were treated with 10^{-6} M Vit. D post 24 hr of transfection. For SEAP activity samples were taken after 24 h of Vit D treatment. Lower SEAP activities were observed in case of cells expressing flanked LXXLL peptides; less SEAP reporter induction indicated the role of peptides in antagonizing its expression. Flanked peptides were better suppressing this effect than non flanked ones suggesting the possible role of flanking amino acid residues

3.2.7 Influence of LXXLL peptides on cell proliferation and signal transduction

Dexamethasone is a well known anti-proliferative agent which blocks cell cycle and thus prevents cell growth. While studying the effect of LXXLL peptides on suppressing the dexamethasone induced effect an increase in cell death was observed which was not observed when cells were treated with Vit. D and forskolin. Cell proliferation assay (Fig 18) indicated an increase in cell death in dexamethasone treated cells transfected with LX peptide expressing vectors. Peptides with flanked sequences enhanced anti-proliferative effect more than non flanked ones. To deeper understand this more, protein antibody array analysis was done to determine the various proteins involved in different pathways. Data from array indicated decrease in number of proteins involved in MAPK kinase pathway and increase in some other proteins (Fig 19, 20). Most of the phospho proteins like Phoso Erk2, Erk 5, Erk 4, were decreased. p38 which gets elevated in stress condition was found to be increased. An increase in Phospho Stat3, NF-kß was also observed. Glucocorticoids decrease level of c-myc which plays a role in cell progression, further decrease in its level was observed in cells expressing flanked LXXLL peptides, in addition to it decrease in the akt level was also seen. Increased cell death in presence of LXXLL peptides may be due to decrease in phospho proteins especially that of MAP Kinase which play an important role in cell progression and growth. Enhanced effect on cell death due to LXXLL peptides was not observed in vit D and retinoic acid treatments inferring no down regulation in phospho proteins and other kinases.

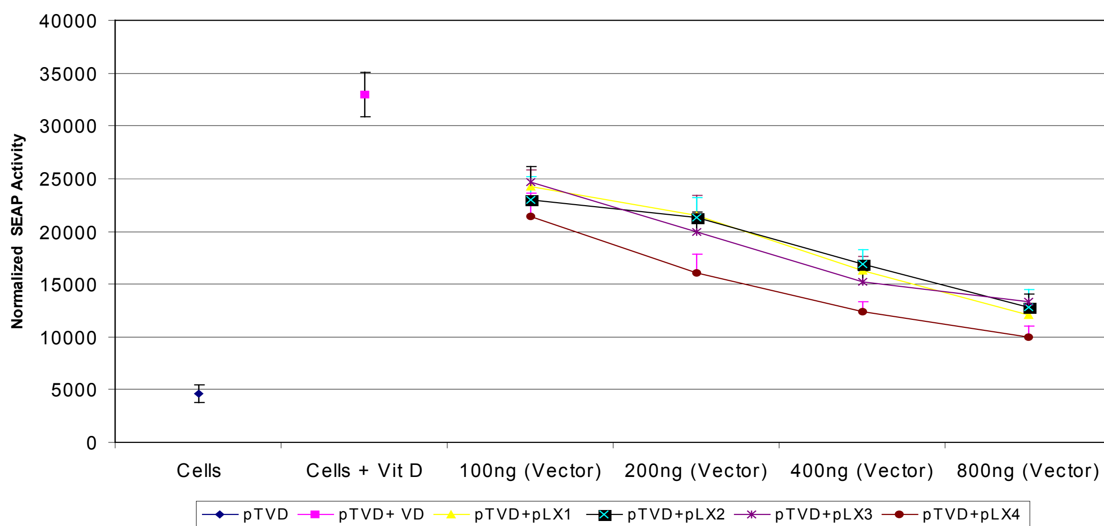
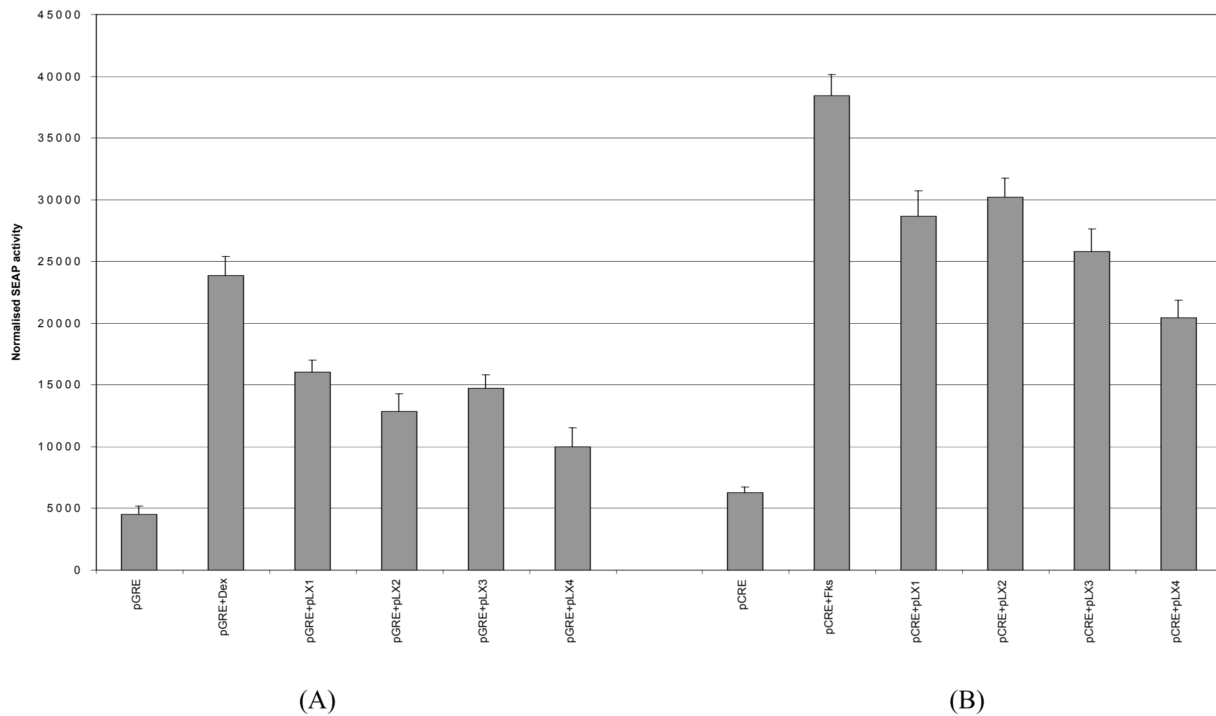


Fig 16; NIH3T3 cells were co-transfected separately with increasing concentration (100ng, 200ng, 400ng and 800ng) of LXXLL peptide expressing vectors and SEAP vector, which in addition to SEAP reporter gene has Vitamin D response element (VDRE) sequence. Cells were treated with 10^{-6} M Vit D post 24h of transfection. SEAP activity was determined after 24h of Vit.D treatment. With increase in concentration of LXXLL peptide expressing vector SEAP activity showed gradual decrease indicating higher concentration of peptides results in more copies of peptide available to antagonize the Vit D effect. Both in HeLa and NIH3T3 cells LX4 exhibited more antagonistic effect



(A) (B)

Fig 17; (A) NIH3T3 cells were co-transfected separately with LXXLL peptide expressing vectors and SEAP vector which contains three tandem copies of glucocorticoid response element (GRE) sequence in addition to SEAP reporter gene. Cells were treated with 10^{-6}M dexamethasone post 24h of transfection

(B) LXXLL expressing vector were co-transfected with pCRE-SEAP vector in NIH3T3 cells and treated with 10µM Forskolin post 24h of transfection. SEAP activity was determined after 24h of treatment. Antagonizing effect of peptides with flanking sequences in NIH3T3 and HeLa cells showed similar order of effectiveness as antagonizing effect of dexamethasone and forskolin.

Cell proliferation of LXXLL peptide expressing cells in presence of dexamethasone

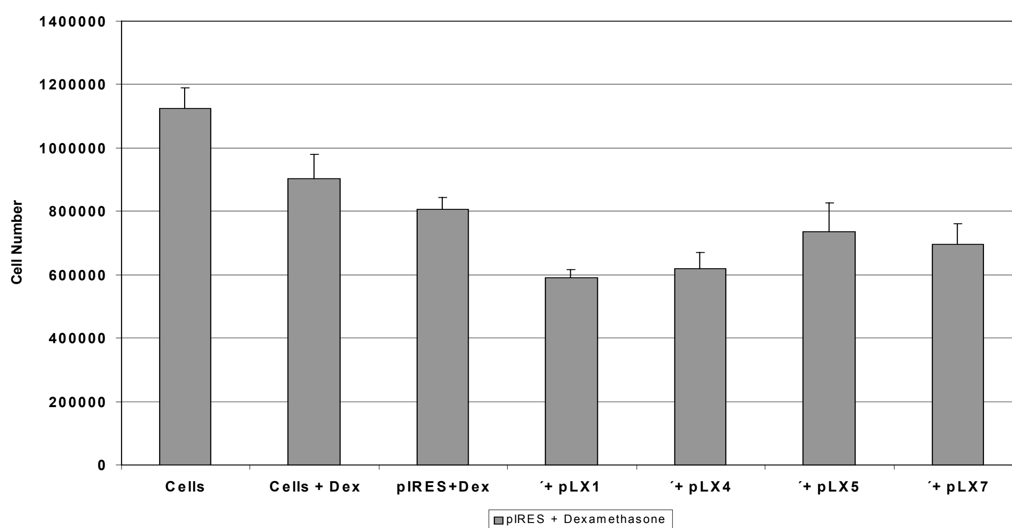


Fig 18; Cell proliferation assay of HeLa cells transfected with various pLX peptide expressing vectors. Post 24h of transfection cells were treated with dexamethasone (10^{-6}M) for 24h, cells transfected with LX1 and LX4 expressing vectors showed more cell death on treatment with dexamethasone than cell transfected with LX5, LX7 and vector alone.

Antibody Array data of cells expressing LXXLL peptides grown in presence of dexamethasone

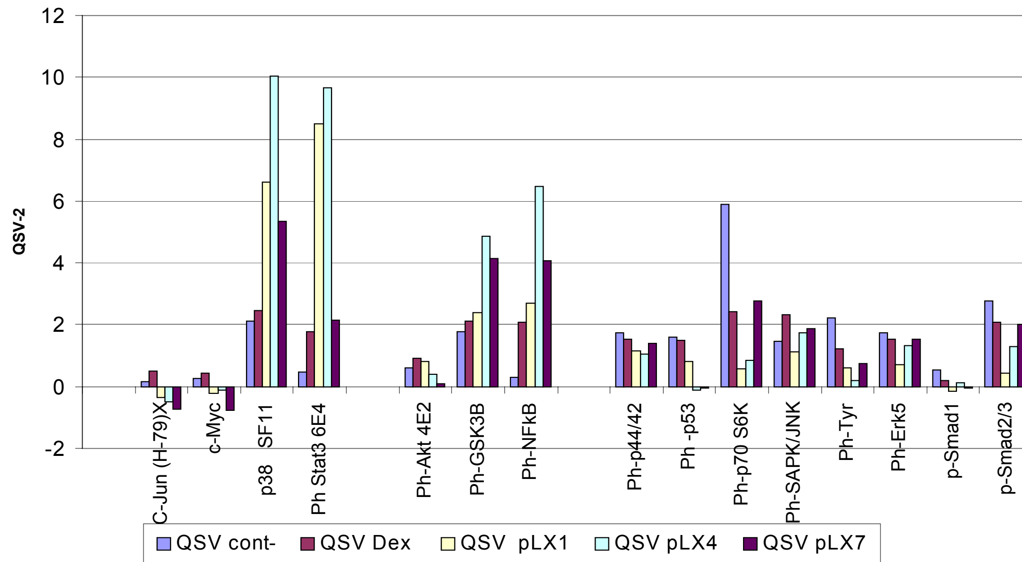


Fig 19; Protein antibody array data of HeLa cells transfected with different peptide expressing vectors. Cells were treated with 10^{-6}M dexamethasone for 24h post 24h of transfection. 50µg of proteins were labelled on the Array Tube. A number of proteins were found to be downregulated and few upregulated on dexamthasone treatment.

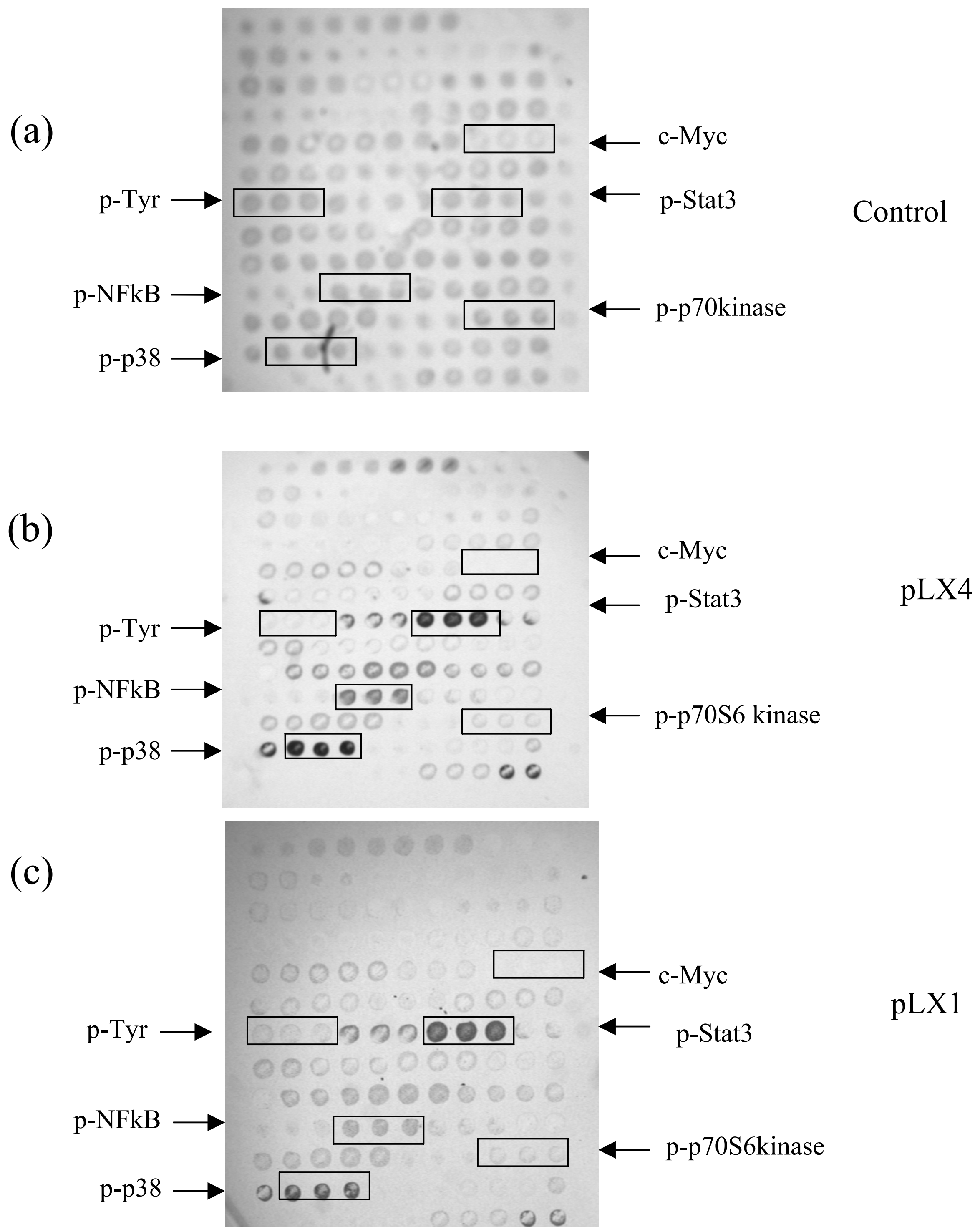


Fig 20; Photomicrographs of Protein antibody array of HeLa cells treated with dexamethasone after being transfected with LXXLL peptide expressing vectors. (a) Control (b) cells with vector pLX4 (c) cells with vector LX1. Protein extracts from transfected cells obtained after 48h of treatment were analyzed by antibody array tubes. Each antibody is spotted three times in the tube. A number of proteins were found to down regulated in cells expressing LX peptides. While an up regulation in stress related proteins was also found.

3.3 Peptide expression library approach to search novel bio active peptide

Peptide library method offers a unique approach to the elucidation of protein interaction networks and signaling pathways. Many enzymes that act on protein-kinases, phosphatases, proteases, deacetylases and acetyltransferases to name a few recognize their substrate on the basis of sequence context surrounding the site of modification. Likewise, protein-protein interactions are often mediated by modular domains, including SH2, PTB, SH3 and FHA domains that interact with short linear stretches of protein sequences, often in the context of post-translational covalent modifications to the binding partner. Peptide libraries offer a rapid means for the elucidation of recognition sequences for these proteins and domains; in essence, a practical alternative to the laborious individual analysis of impossibly large number of peptides or mutant proteins. Developments in bioinformatics enable scanning of protein sequence database with complex databases derived from peptide library studies, providing an avenue for the identification of candidate protein targets.

Peptide library approaches can be broadly grouped into methods employing either synthetic or encoded libraries. Synthetic library methods include spatially addressable positional scanning libraries (PSLs), mixture based oriented peptide libraries (OPLs) and one bead one peptide solid-phase libraries. PSL and OPL approaches have the advantage of allowing exhaustive analysis of the contribution to specificity of each amino acid at each position within the library. Rather than analyzing individual peptides, however, these libraries generate consensus motifs that have the drawback of masking interactions between positions or the existence of multiple binding modes. Encoded libraries, phage display being by far most popular (but also including mRNA display and interact cell expression based systems) and immobilized split-pool synthetic libraries provide a collection of sequences that are positively selected. Although these approaches allow identification of cooperativity between binding subsites, the labor involved with sequencing a large number of clones or beads generally means that weaker selections are overlooked and negative selections cannot be inferred.

Combinatorial peptide libraries have been playing a major role in the search for new drugs, ligands, enzyme substrates, and other specifically interacting molecules, such libraries are composed of millions of peptides. The principal features of these libraries require a versatile repertoire, an easily identifiable tag for each of the library members, a simple method of synthesis, and a compatibility with the biochemical milieu. Two types of combinatorial libraries are in use: synthetic libraries and biological (mainly phage display) ones. An advantage of the biological libraries is due to the ability of each of the library

members to replicate it and to the fact that they carry their own coding sequences. In recent years, there have been a growing number of examples of the successful isolation of peptide ligands for enzymes from phage-displayed combinatorial peptide libraries. These peptides typically bind at or near the active site of enzymes and can inhibit their activity, while the peptide ligand isolated from phage-displayed libraries may not resemble the chemical structure of the normal substrate of enzyme. Peptides can be used as an inhibitor to evaluate the function of the enzyme or for drug discovery efforts (i.e., as a lead compound for peptidomimetic design or as displaceable probe in high-throughput screens of libraries of small molecules). Combinatorial peptide library approach has been applied in identification of biologically active peptides.

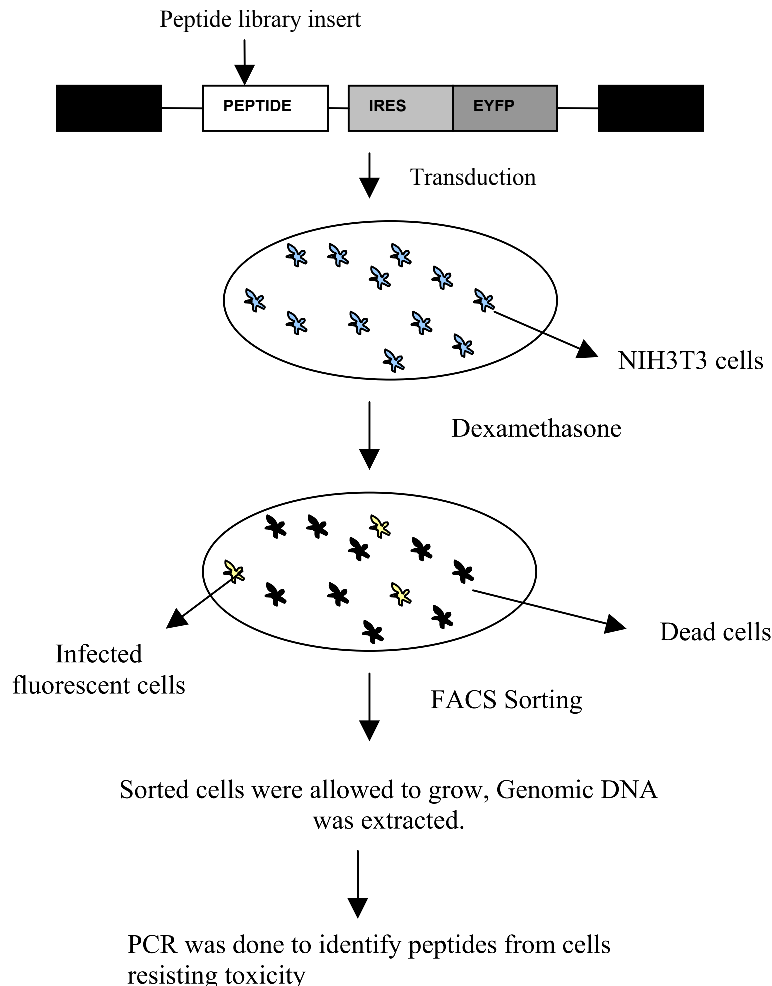
3.3.1 Design and preparation of random peptide libraries

Combinatorial retroviral peptide library approach has been used to screen potential peptides helping in elucidating various mechanisms a cell undergoes. Random peptide library based on nnk codon system, where n denotes all four nucleotides and k denotes g and t was synthesized to screen bioactive peptides. nnk coding system ensures a more equal distribution of the amino acids in the library and two out of three stop codons are omitted. Peptide library has vast diversity due to presence of large number of different peptides which makes it more suitable medium to isolate a bioactive peptide. Peptide libraries are preferred to cDNA libraries due to limited diversity of cDNA library. A random peptide library of 13 amino acids based on pOS IRESGFP vector (pOS) was synthesized. For library synthesis two partly complementary oligonucleotide sequences were used, one having bases for 13 random amino acids and restriction site for Not1 and BamH1 on 5´ and 3` of random bases respectively. Another, short oligonucleotide sequence complementary to first one was used for elongation to make the complementary double stranded, followed by restriction digestion with Not1 and BamH1. Digestion product after cloning in pOS was extensively used for transformation of E.coli to get as many as 10^6 colonies. Each colony represents vector with different peptide of 13 amino acids. All colonies were scrapped and proceeded to get the vector DNA. Peptide library has complexity of 10^6 peptides, theoretically a sufficient number to cover a vast diversity. Peptide libraries have an advantage of comprising a number of different combinations of amino acids thus enhancing chances of getting bioactive peptides.

3.3.2 Identification of peptide conferring resistance to dexamethasone toxicity in fibroblasts

Employing combinatorial peptide approach, an attempt was made to screen peptide library to isolate peptides that could provide resistance to cells against anti-proliferative effect of dexamethasone. Dexamethasone, a known antiproliferative agent blocks cell progression in

different cell lines normally by causing an arrest in the G1-phase of the cell cycle. Random peptide library of 13 amino acids based on pOS was designed with approximately 10^6 peptide combinations, a satisfactory number to cover the theoretical diversity of the library with this aim aim to get a peptide able to overcome the dexamethasone induced toxicity.



Scheme for the peptide library screen. To initiate a screen, a peptide library (complexity 1x 10^6) encoded within the vector was transfected into the retroviral packaging cell line Ecopack Tm 293. The viral particles were collected and then used to transduce NIH3T3 cells. The infected cells were treated with a high dose of dexamethasone. The peptide sequence were rescued from surviving and FACS sorted cells by PCR from genomic DNA, isolated peptide was recloned in the vector to confirm the dexamethasone resistivity.

Retroviral vector approach was employed as it ensures presence of single peptide in a single virus particle, which could be rescued and sequenced. Peptide library was transfected in Eco Pack packaging cell line, viruses were collected after 48h and 72h of infection and used to infect (transduce) target NIH3T3 cells. NIH3T3 cells post 72h of transduction were plated and treated with dexamethasone (10^{-4}M) for 48h. This concentration was chosen after comparing the toxicity on cells by various concentrations (10^{-4}M, 10^{-5}M, and10^{-6}M). 10^{-4}M was found to

be more toxic and causes considerable cell death than other concentrations. On treatment of normal cells with dexamethasone (10^{-4}M) cells die drastically (Fig 23), while cells infected with peptide library on treated with dexamethasone for 48h showed little resistance to dexamethasone induced toxicity. Cells that resisted toxicity were sorted by FACS sorter and allowed to grow in 96-well plate for few days. Genomic DNA was extracted from these cells. PCR was carried out (Fig 22) using vector specific primers to get the specific peptides present in the cells. Sequencing of PCR product resulted in identification of a peptide that could have provided resistance to cells against dexamethasone. Amino acid sequence of the peptide referred as (Dex Pep) was found is as MSRRHRGGLLSVDT. In order to be sure that the peptide provided resistance to dexamethasone toxicity, peptide was recloned in pOS. NIH3T3 cells were transduced with this vector followed by dexamethasone treatment. As seen in (Fig 23) cells infected with screened peptide resisted dexamethasone induced cell death and an enhancement in cell growth was observed compared to dexamethasone treated uninfected cells. To further make sure that this resistive effect is by screened peptide, cells were infected separately with normal pOS and with pOS having a non-specific peptide (a random peptide having no effect on resisting dexamethasone inducing toxicity). Both have no effect on resisting dexamethasone induced toxicity after analyzing cells post 24 and 48 h (Fig 23 and 24) of drug treatment. As seen from figures, with increase in time period of treatment extent of cell death increases. No effect on suppressing induced toxicity by normal vector and vector with non-specific peptide emphasizes the role of screened peptide in resisting toxicity of cells and also indicates that this effect is not vector specific. Proliferation assay (Fig 21) of cells further confirm the role of screened peptide in enhancing the cell growth of dexamethasone treated cells. It overrides dexamethasone induced toxicity to some extent, if not completely. The resistance to dexamethasone induced toxicity by the peptide raises the question whether the peptide after expression is functioning within the cell or it is secreted outside the cell to overcome the dexamethasone induced toxicity shown by the cells. Spent medium from the cells infected with the screened peptide (Dex pep) was added to NIH3T3 cells followed by dexamethasone treatment for 48h. Cells did not resisted dexamethasone induced cell death as was seen in cells expressing the peptide (Dex pep), it may indicate that the peptide is not secreted outside the cell. In case the peptide is secreted outside the cells resistance to the dexamethasone induced cell death should have been observed by adding the spent medium from the infected cells to normal NIH3T3 cells. Lack of resistivity effect on cell toxicity was also observed in cells treated with spent medium from pOS infected cells and pOS IRESGFP vector having non specific peptide. This led us to speculate that the peptide after expression

remains within the cell after expression and functions somehow to overcome the induced toxicity. The mechanism by which this peptide overcomes the dexamethasone toxicity and induces resistance needs to be studied further. Retroviral peptide libraries were used also by other groups (Matza-Porges *et al.,*2003; Xu *et al.,*2001) to search and isolate peptides that influence cellular proliferation and lead to better survival of the cell. So far the targets through which these peptides act have been identified only in a few cases (Xu *et al.,*2001; Tenson *et al.,*1997).

Cell proliferation of NIH3T3 cells; Effect of screened peptide

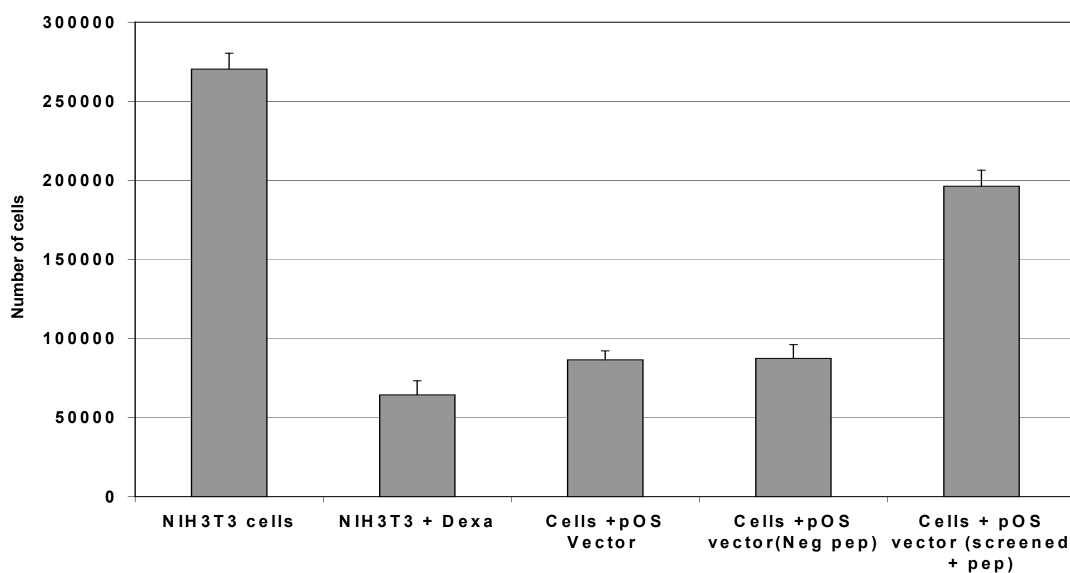


Fig 21; NIH3T3 cells treated with dexamethasone underwent considerable cell death. Cells transduced with pOS control vector and pOS vector with any peptide (referred as Neg peptide) exhibited cell death by dexamethasone treatment for 48h. While cells infected with pOS vector having screened peptide less cell death was observed compared to other infected cells, inferring that resistivity to dexamethasone induced toxicity is due to the screened peptide.

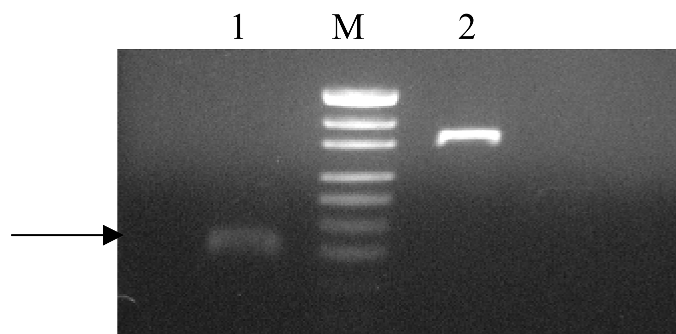
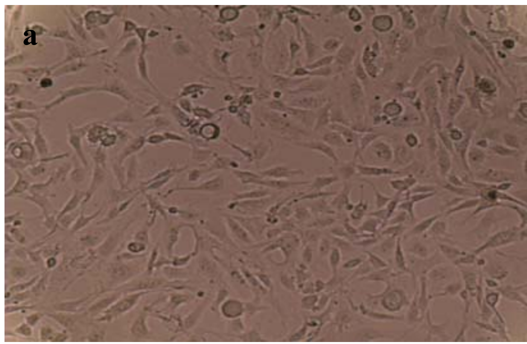
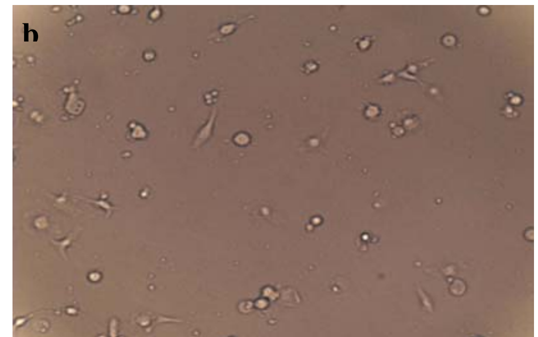


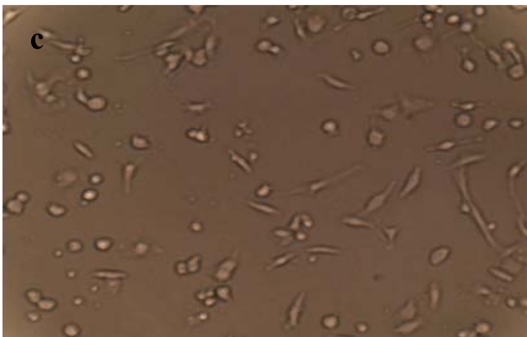
Fig 22; NIH3T3 cells infected with random peptide library, showing resistance to dexamethasone toxicity were sorted by FACS sorter and allowed to grow. Genomic DNA extracted from these cells was used to perform PCR using vector specific primers to isolate provirus sequence, providing resistance to induced toxicity were further recloned to check their response again. Lane 1 and 2 shows PCR products from the extracted DNA using vector specific primers. In case of Lane 1, primers were having Not1 and BamH1 restriction sites, these enzymes were used to digest and recloned PCR product again in the vector.



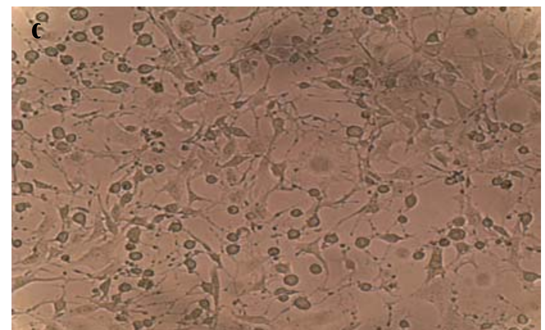
Normal NIH3T3 cells



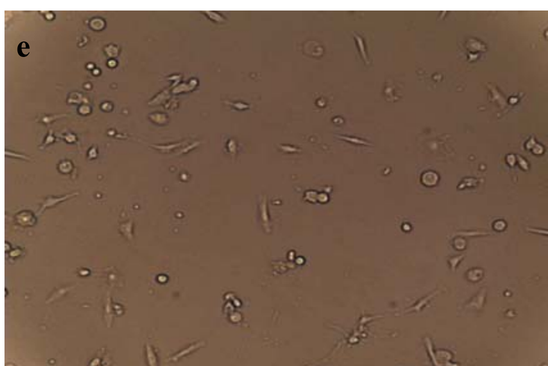
NIH3T3 cells + Dexamethasone



NIH3T3 cells infected with control pOS IRES GFP vector, treated with dexamethasone

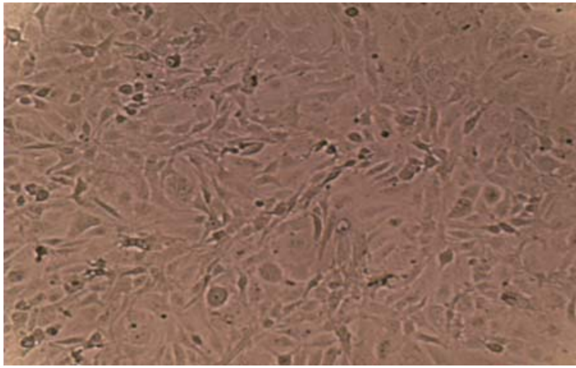


NIH3T3 cells, infected with pOS IRES GFP vector having recloned screened peptide and treated with dexamethasone



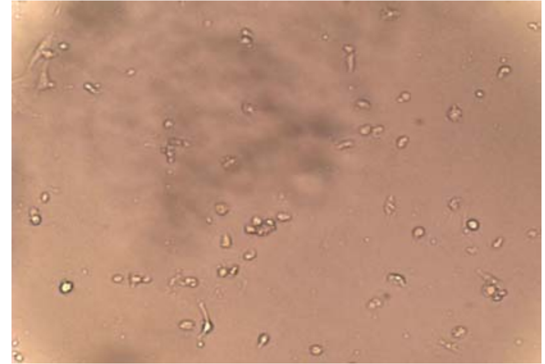
NIH3T3 cells, infected with pOS IRES GFP vector having a non specific peptide and treated with dexamethasone

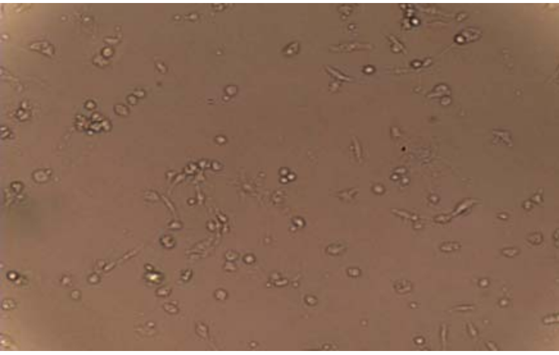
Fig 23; Photomicrographs of (a) control NIH3T3 cell (b) NIH3T3 cells treated with dexamethasone (10^{-4} M) (c) NIH3T3 infected with pOS-IRESGFP vector and treated with dexamethasone (10^{-4}M) (d) NIH3T3 infected with pOS-IRESGFP vector with screened peptide and treated with dexamethasone (e) NIH3T3 infected with pOS-IRESGFP vector with a negative peptide, treated with dexamethasone post 24h of treatment. Dexamethasone (10^{-4} M) caused cell toxicity to a great extent, while cells infected with screened peptide resisted the toxicity, peptide was screened from the cells infected with peptide library post dexamethasone challenge. Recloning of this peptide in pOS IRES GFP vector and infecting the vector in NIH3T3 cells showed a considerable resistance to the dexamethasone induced toxicity. Vector alone and vector with any non-specific peptide did not showed any resistance to the dexamethasone induced toxicity, emphasizing role of the screened peptide.



Normal NIH3T3 cells

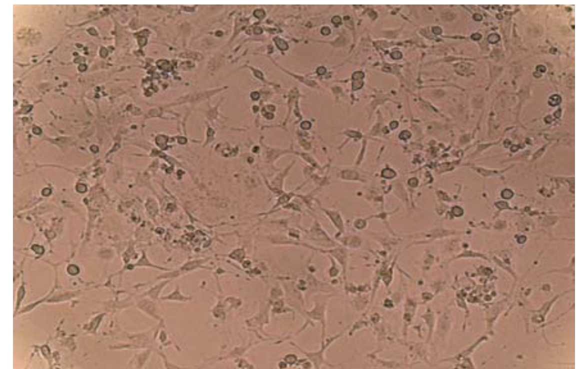
NIH3T3 cells + Dexamethasone

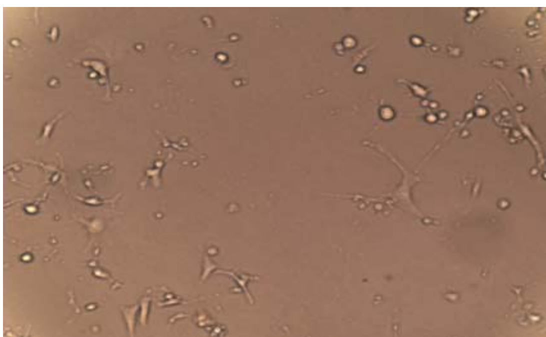




NIH3T3 cells infected with control pOS IRES GFP vector, treated with dexamethasone

NIH3T3 cells, infected with pOS IRES GFP vector having recloned screened peptide and treated with dexamethasone





NIH3T3 cells, infected with pOS IRES GFP vector having a non specific peptide and treated with dexamethasone

Fig 24; Photomicrographs of (a) control NIH3T3 cell (b) NIH3T3 cells treated with Dexamethasone (10^{-4} M) (c) NIH3T3 infected with pOS-IRESGFP vector and treated with dexamethasone (10^{-4} M) (d) NIH3T3 infected with pOS-IRESGFP vector with screened peptide and treated with dexamethasone (e) NIH3T3 infected with pOS-IRESGFP vector with a negative peptide and treated with dexamethasone after 48h of treatment . Dexamethasone (10^{-4} M) caused cell toxicity to a great extent, while cells infected with screened peptide resisted the toxicity; peptide was screened from the cells infected with peptide library post dexamethasone challeng. Recloning of this peptide in pOS IRES GFP vector and infecting the vector in NIH3T3 cells showed a considerable resistance to the dexamethasone induced toxicity. Vector alone and vector with any non specific peptide did not showed any resistance to the dexamethasone induced toxicity, emphasizing role of the screened peptide.

Discussion

Proteins are key players in cellular processes and plays a dominant role in many biological functions within a cell. Many cellular processes and biochemical events are achieved by a group of proteins interacting with each other. Protein interactions also had a pivotal role in the functional selectivity of enzymes participating in cellular signal transduction cascades. Interaction between specific binding proteins control, at least in part, the temporal and spatial orchestration of the functions mediated by these signaling events. The ability to modulate protein-protein interactions provides a means to elucidate the role of specific proteins and enzymes in various signaling cascades in normal and diseased states. Many of the signaling enzymes belong to families of related proteins, each mediating a unique physiologic functions. Little is known, however, about the role of the individual family members, mainly due to the lack of selective modulators. One important strategy to search selective modulators of intracellular signaling enzymes capitalizes on the finding that upon stimulation many enzymes, including protein kinases, translocate from one cell compartment to another, which bring proteins close to their activator and /or specific substrates for their function mediated by selective binding proteins.

Modulation of protein interactions by small molecules (including short peptides) remains a big challenge. *In vivo* expression of short specific peptides derived from domains of one interaction partner could be an approach to alter interaction between two known interacting protein partners. On expressing short peptides derived from Ros tyrosine kinase phosphorylation domain in modified cells, expressing PTP SHP-1 and Ros tyrosine kinase receptor cellular proliferation was successfully modulated. Interaction between tyrosine kinase Ros and PTP SHP-1 is known to inhibit cell proliferation, growth and differentiation (Keilhack *et al.,*2001). Modulation in cell growth was observed only when the receptor from which the peptides are derived is expressed also along with its interaction partner. A significant increase in proliferation was observed with peptide that was exact homologues to the tyrosine phosphorylation domain. Substitution of tyrosine by phenylalanine in the expressed peptide reduced the observed pro-proliferative effect but did not fully abolish the proliferation enhancement. Another modification of peptides, addition of two self annealing flanking clamp sequences on both C and N termini led to reduction of pro-proliferative effect as exhibited by the peptide with out modification. The idea behind the modification was to increase the stability of peptides. Self annealing clamp is reported to give a stem loop like structure (Gururaja *et al.,*2000) to the expressed peptide that reduces chances of proteolytic cleavage and also provides a stable frame to the peptides, but making them less flexible. It

seems that the rigid structure reduced peptide activity significantly which led to the speculation that, for pro-proliferative effect peptides should be in less structurally constrained form and in right frame, to interfere a particular interaction. However, from these observations it is clear that small peptides do exert an effect when expressed *in vivo*. In normal NIH3T3 cells lacking expression of tyrosine kinase Ros and PTP SHP-1, expressed peptides showed no effect on enhanced cellular proliferation. This clearly showed that tyrosine kinase Ros and PTP SHP-1 interaction is influenced by the homologous peptides.

On analyzing effect of peptides in response to stimulation of the chimeric TrkA-Ros receptor with NGF, a slight increase in cell proliferation was observed with Pos. However, relative enhancement by various peptides remained same. This indicates that activation by NGF uses a signaling mechanism that is complementary to the effect mediated by the peptides, which led to the suggestion that expression of domain specific peptides directly influences the activity of the corresponding domain of a target protein. This effect may be mediated by a direct interaction with other proteins that need to interact with the target protein (here Ros) for signal transduction. An alternative explanation might be that expression of homologous peptides interferes with the formation of target protein complexes, which in turn also affect signal transduction. From these observations it was not clear enough to dissect the role of *in vivo* expressed short peptides in Ros-signaling. However, results showed that expression of short peptides can significantly influence cellular proliferation, based on specifically influencing the activity of the protein domain from which the peptides are derived. Addition of the flanking sequence reduced observed enhanced proliferative effect, probably by hindering the interaction of peptide, thus highlighting significance of proper frame required for interaction. From these observations it can be summed up that short peptides can be expressed *in vivo* and are able to exert an influence when presented in right frame.

Peptides (LXXLL) as antagonists of receptor induced transcription

Transcription factors play an important role in expressing various genes to perform different cellular functions. VDR, a transcriptional factor and a member of nuclear receptor (NR) family functions to alter expression of specific genes in response to 1, 25$(OH)_2D_3$ (Evans, 1989). NR recruits additional co-modulators to regulated promoters (Rachez *et al.,* 2000), which involves an activation domain on the receptor and an LXXLL motif within the comodulator; these factors may function to elicit architectural changes in chromatin structure essential for enhanced transcription. In response to Vit D, RXR is believed to be a necessary

partner for VDR to form a heterodimer (Whitfield *et al.,*1995, Cheskis *et al.,*1996). VDR also forms homodimers or heterodimer with RAR.

LXXLL motif are present in coactivators whose number varies from coactivator to coactivator are known to interact with AF-2 domain (Hall *et al.,*2000) of nuclear receptor ligand complex. An attempt to use LXXLL peptides as antagonists to induced effect was carried out. Since different coactivators have specific numbers of consensus LXXLL motifs and are conservely separated from one another, in the present study short peptide with varying number of motifs and number of amino acid residues around the motif were expressed. Pike *et al.,*2003 and Hall *et al.,*2002 reported antagonistic property of LXXLL peptides with different number of motifs, however, for the first time peptides with one LXXLL motif and with or with out additional flanking residues around the motif were employed to study their influence on the induced activation. Peptides with additional amino acid residues on both sides of motif were in format of $X_7LX_2LLX_7$ (where L is leucine and X is any amino acid) which reduces the biasness for any particular amino acid residue. Peptides having additional residues on both N and C termini of LXXLL motif exhibited better suppressing effect on activation than peptides lacking additional residues indicating that these residues have a role in binding and recognizing nuclear receptor to block transcriptional activation induced by Vit D treatment. Comparing amino acid residues around LXXLL motifs in various coactivators (Table1), leads to the result that expressed peptides with additional residues bear residual resemblance with them particularly LX4, it shares residual similarity with the various coactivators and with so called High affinity peptides (Heery *et al.,*2001) present in some strong coactivators which could be the reason for their role in being more potent suppressor peptide than other peptides. Peptides LX1-LX3 with additional residues has relatively lower effect than LX 4 peptide. Comparison of flanking amino acid residues of these peptides with various coactivators showed that they share less residual similarity with them, which could explain their less effectiveness. It may indicate presence of specific amino acid residues are important for the interaction and recognition of the AF2 region of the receptor for the peptides to interact. Peptide LX7 with only two residues on C terminus of motif showed better effect than peptides lacking residues (LX5 and LX6). Peptide LX7 has amino acid glutamine (Q) after LXXLL motif which is shared by few high affinity peptides and also by LX4, implicating presence of a single amino acid residue can have effect in addition to LXXLL motif. (Hall *et al.,*2000) reported presence of tryptophan in a peptide as third amino acid residue before LXXLL motif increases its antagonistic ability.

Is antagonistic effect of peptides dependent only on the flanking residues or also on number and location of LXXLL motifs? Peptide (LX6) was designed with two LXXLL motifs separated by three amino acid residues without additional residues on both C and N termini of motif. If the number of motifs were criteria for a peptide to be better antagonist LX6 was the peptide expected to be more efficient than other peptides. Surprisingly LX6 exhibited less antagonistic property as indicated by the marker (SEAP) activity than other peptides leading to the conclusion that number of motifs is not the most important factor for a peptide to act, especially, if they are close to each other. However, as in most of the coactivator's position of LXXLL motifs have conserved spacing (Heery *et al.,*1997; Torchia *et al.,*1998) and are apart from each other. Observed effects from peptide LX6 reconfirms that number of LXXLL motifs play a role when spaced properly as is case of coactivators. Since, making oligonucleotides for such long peptide is not practically feasible, no attempt was done to express long peptide with varying number of motifs conservely spaced. This may suggest that the AF2 domain where coactivators (LXXLL sequences) interact has a specific binding space and orientation and interacts efficiently with LXXLL motifs conservely spaced. As mentioned earlier peptide with only one LXXLL motif (LX5) and with out additional amino acid residues on C and N termini of motif is employed for the first time for studying effect on induced transcription. Results are indicating that one motif could act as an antagonist to induced effect. However, the effect was considerably less than the peptides with additional residues. This confirms that a single motif can perform the function though not so efficiently.

In addition to the effect of peptides on vitamin D induced transcription, influence on dexamethasone and Forskolin induced effects were studied by expressing peptides. Almost in all treatments peptides with flanking sequences proved to be exerting better effect than non flanked ones. This demonstrates the importance of amino acid residues around the LXXLL motif playing an important role in binding to the AF-2 region of receptor to act as a strong antagonist. Interestingly in case of forskolin treatment peptides exerting antagonistic effect showed considerable variation in their specific effect than observed in dexamethasone and vitamin D treatment. Peptide LX4 was found to have better antagonistic effect in dexamethasone and Vit D, while in forskolin treatment peptide LX3 turned out to be efficient in antagonizing the induced effect. These results indicate peptide affinity varies with treatment and possibly also with the interacting partner (in present case receptor or other proteins activated by ligand binding on receptor). On increasing the concentration of peptide expressing vectors antagonistic effect was found to be concentration dependent, inferring with

higher concentration of peptides possibly more copies of peptides are available to interact with the receptor to block coactivator recruitment.

Is peptide effect cell line specific?

Is antagonistic effect of peptide is subjected to change in case peptides are expressed in different cell lines? To answer this, in addition to HeLa cells peptides were expressed in NIH3T3 cell line. Surprisingly, expressed peptides exert their effects in similar fashion as in case of HeLa cells with peptides having flanking sequence proving to be better antagonists in suppressing the induced transcriptional activity caused by various agents like Vit D, dexamethasone and forskolin. Effect of peptides was also found to be dose dependent as observed in HeLa cells. Thus, indicating a similar antagonistic effect of peptide in HeLa and NIH3T3 cells. Although, being two different cell lines, similar behavior of peptide action may indicate a common mechanism of interaction of ligand binded receptor with coactivators in both cells, which coincides with observed effect of peptide effect on interaction with activated receptor when treated with different transcription inducing agents.

Dexamethasone and forskolin induced cross talk

Glucocorticoids modulate gene expression in mammalian organisms primarily at the level of transcription although there are reports of post transcriptional effects of glucocorticoids (Ulbright *et al.,*1993). Dexamethasone treatment led to CRE mediated transactivation indicating a possible cross talk of dexamethasone with CRE mediating protein CREB, as CREB interacts with CRE to initiate the transcription. Glucocorticoids are reported (Gonzalez *et al.,*1989; Son *et al.,*2001; Whitehead *et al.,*1997) to cause phosphorylation of CREB , a critical step in its activation. In many cases glucocorticoids are reported to have a permissive effect on transcription by enhancing ability of cAMP to induce transcription (Nakamura *et al.,*1987; Noda *et al.,*1988). These studies are in agreement with the observed effect of dexamethasone induced cross talk as confirmed by SEAP activity. However, the transcriptional activity carried out by CREB on stimulation by dexamethasone is considerably lower than that from forskolin induced treatment. CREB is known to bind CRE as homodimer and transcriptional activity is mediated by cAMP dependent protein kinase mediated phosphorylation (Schmid *et al.,*1987) which may suggest that CREB phosphorylation by dexamethasone induction is not sufficient enough to cause the CRE mediated transcription as caused by forskolin. Dexamethasone is also reported to increase mRNA level of CREB (Jungmann *et al.,*1992) in rat C6 glioma cells. Dexamethasone induced transcriptional activity after cross talk with CREB was found to be suppressed by LXXLL peptides, although the transcriptional activity as depicted by SEAP activity was less. However, suppression

pattern of induced cross transactivation by peptides was similar as in case of GRE mediated transcriptional activity of dexamethasone, with LXXLL peptides having flanking amino acid residues proving to be more potent in antagonizing than non flanked peptides. Similar cross transactivation was found in GRE mediated transcriptional activity on treatment with forskolin. This cross treatment was subjected to suppression by LXXLL peptides indicating interruption in normal interaction of proteins was responsible for transcriptional activity. Antagonizing cross talk effect caused by either dexamethasone or forskolin by LXXLL peptides further proved that LXXLL peptides could be strong antagonists of induced effect if LXXLL motif in peptides is having additional amino acid residues and bear residual resemblance with high affinity peptides.

Peptide expression resulted in enhanced anti proliferative effect of dexamethasone, an effect not observed in Vit D and forskolin treatments. Dexamethasone is known to inhibit cell growth by blocking progression at G1 phase and also exerting its effect by inhibiting signal pathways in particular mitogen activated protein kinase (MAPK) pathway (Lasa *et al.,* 2001; Reider *et al.,*1996) There are many reports on the relationship between MAPK and proliferation e.g. follicle-stimulating hormone has growth stimulatory effect on ovarian surface epithelial cells, which may be caused by activation of the MAPK (ERK) cascade (Choi *et al.,*2002). Phorbol esters inhibit fibroblast growth factor-2-stimulated fibroblast proliferation by a p38 MAP kinase dependent pathway (Maher P 2002). Previous reports have suggested the ability of glucocorticoid receptor to suppress transactivation by transcription factors like AP-1, NF-kB or NFAT as hallmark of glucocorticoid action (Cato *et al.,*1996; Barnes, 1998) and p38 kinase as a target for negative regulation by glucocorticoids. However, p38 activation in HO-8910 cells is reported to get enhanced in a time and dose dependent manner on dexamethasone treatment (Xia *et al.,*2003). Antibody array data of MAP Kinases from peptide expressing cells treated with dexamethasone show considerable decrease in various proteins involved in MAP kinase pathway, which is consistent with other known data. Mechanism underlying in down regulating especially ERK1/2 according to (Kassel *et al.,* 2001) might be via increased expression and decreased degradation of the MAP kinase phosphatase-1 (MKP-1). However, antibody array data showed an increase in NF-kB, STATs which are usually down regulated by dexamethasone. An increase in p38 which is negatively regulated in HeLa cells on dexamethasone was also observed. As p38 is also expressed in stress conditions, present enhancement may be correlated with stress induced by expressed peptides, similar can be said of NFkB enhancement. Cross talk between offer an elegant

approach to study cell surface markers, specific proteins, signal transduction p38 and NF-kB (McKay *et al.,*1998) may be also responsible for enhancement in NF-kB expression.

Peptide library screen

A retroviral combinatorial library method based on functional screening of selection of active peptides expressed in mammalian cells was employed in the present study to screen peptide preventing dexamethasone induced toxicity. On functional screening of peptide library in dexamethasone treated cells a peptide was found to provide the cells resistance against the induced toxicity of dexamethasone. Although the size of screened library was not so high, it may be possible to screen a library of higher complexity to get a large number of positive effector peptide. Retroviral vector based transduction ensures stable one cell- one peptide expression as opposed to other library technologies and allows efficient functional screening to isolate the desired peptide. Retroviral library approach based on peptide library has been used by few groups. Xu *et al.,* 2001 reported peptides conferring resistance to taxol employing peptide library of 18 random acids of higher complexity. Tenson *et al.,*1997 screened peptides based on retroviral library of 21 amino acids providing resistance to Erythromycin. Similarly peptides conferring resistance to induced apoptosis were reported by (Matza-Porges *et al.,*2003) on screening 15 amino acid random peptide library. Previously, retroviral library technology has been used in the context of cDNA libraries and in few cases successful functional cloning of cDNA molecules were reported (Rayer *et al.,*1994; Kitamura *et al.,* 1995). Another technology complementary to random peptide library uses libraries of cDNA fragments, termed genetic suppressor elements for functional screening in mammalian cells (Holzmayer *et al.,*1992; Roninson *et al.,*1995). However in the present study instead of cDNA libraries, a synthetic nucleotide based library was preferred as the library components can have any sequence which can be encoded after mixing four nucleotides and not only sequences already existing in nature, which makes it better candidate for drug target discovery too. Results from screening of synthetic or phage display peptide libraries have shown that unnatural higher affinity peptides capable of competing with naturally occurring protein-protein interactions can be pulled out from random libraries (Aramburu *et al.,*1999). Since the size of individual library peptide is in range of 5-20 amino acids which may suggests that the potential effector peptide may act by blocking crucial protein-protein interactions via binding directly to localize active sites on their target proteins. This could be the case with dexamethasone as it blocks cell progression at G1 phase and also down regulates signal pathways especially MAP kinase pathways, it may be argued that the

screened peptide may be able to overcome the dexamethasone mediated effect by acting on some crucial proteins and interfere with its interaction. Peptide action was further confirmed on basis on negative controls and vector alone which exerted no effect on the induced toxic effect by dexamethasone.

Summary

Interaction of proteins is central to various cellular processes like cell growth, proliferation, differentiation and programmed cell death. The interactions can furthermore be attributed to specified biochemical processes like transcription, translation or replication Some proteins are highly specific to interact with certain partners. They also can interact with many other proteins and even form larger multifunctional complexes. To study the specific role of a single protein-protein interaction of a multifunctional protein or protein complex, it is necessary to interrupt each single interaction individually to elucidate their respective role and influence they exert.

There are many ways to interrupt protein interactions. One is expressing specific peptides *in vivo* to target a known protein-protein interaction. In order to accomplish this, an efficient retroviral expression vector system capable of expressing peptides was developed. To target known protein-protein interactions, peptides with sequences homolog to one of the interacting domains were expressed. As one example, peptides derived from the Ros tyrosine phosphorylation domain were expressed to target the interaction between the tyrosine kinase Ros and PTP SHP-1. In a second example peptides based on the LXXLL motif important for the interaction of transcription factors and coactivators were expressed to disrupt this interaction. Furthermore, the peptide expression system was used to establish a peptide library that can be used to screen for new peptides influencing cellular processes.

The dephosphorylation of tyrosine kinase Ros by PTP SHP-1 is an important step to inhibit cellular proliferation and growth. To target this interaction, peptides derived from the Ros tyrosine phosphorylation domain were expressed using retroviral expression system in modified NIH3T3 cells. These cells express an artificial TrkA-Ros fusion receptor tyrosine kinase and under the control of anhydrotetracycline expresses PTP SHP-1. Upon expression of the tyrosine domain homologous peptide an increase in cell proliferation was observed. Surprisingly, this enhancement in cell growth was independent of PTP SHP-1 and of receptor stimulation. To exclude that these peptides stimulate proliferation by an independent mechanism, peptides were also expressed in the unmodified parent NIH3T3 cell line. In this case no significant stimulation was observed. The peptides only enhanced proliferation in the presence of the TrkA-Ros receptor containing the homologous domain. A single point mutation replacing the tyrosine by phenylalanine (negative mutation) significantly reduced the observed enhancement of proliferation. Modification of peptides by addition of self annealing complementary flanking sequences at both N and C termini influenced peptide activity similarly as the replacement of tyrosine. In combination with the negative mutation (phenylalanine) the self annealing clamps had no observable additional effect.

The enhanced proliferation observed with the Ros tyrosine domain peptide was not only the result of a disruption of the Ros SHP-1 interaction. The effect was dependent on the presence of the cytoplasmic Ros domain, the functional homology and a relative flexibility of the peptide. Mutation or structural constrain lead to a strong disruption of the proliferation enhancement.

To address the question if peptides expressed by this system are also active in the nucleus, transcription factor coactivator interactions were targeted. Ligand binding nuclear receptors recruit coactivators for full activation in transcription control. Coactivators interact with AF-2 domains of nuclear receptors via consensus LXXLL motifs present in all coactivators. To interfere with coactivator and receptor interaction short LXXLL peptides with or without additional amino acid residues around the LXXLL motif were expressed in HeLa and NIH3T3 cells. Expressed peptides were found to antagonize transcription activation by various agents like vitamin D, dexamethasone, 9-cis retinoic acid and forskolin. The antagonizing effect of the peptides increased with additional amino acids around the LXXLL motif. The inhibitory effect was found to depend on the number of amino acids that an expressed peptide shares with the high affinity motifs present in strong coactivators when aligned. Presence of two close LXXLL motifs separated by a few amino acids in a peptide were less efficient. The peptide with only one LXXLL motif with out additional amino acids showed the least suppressive effect. These studies demonstrate the importance of neighbouring amino acid residues around the central LXXLL motif for nuclear receptor coactivator interaction selectivity. Peptides lacking the LXXLL motif did not show any antagonistic effect in these experiments.

Comparing transcription induction with the various inducers a pattern of relative specificity for the various flanking amino acids was found. With this, two main groups are identified, one containing Vit D, dexamethasone and 9-cis-retinoic acid and the other for forskolin. This pattern fits well with the known coactivator specificity of the respective transcription factors.

Finally, a combinatorial retroviral peptide library was synthesized to screen novel bioactive peptides. The random peptide library was synthesized on the basis of nnk codon system, where n denotes all four nucleotides and k denotes g and t. This nnk system ensures a more equal distribution of amino acids in the library and of possible three stop codons two are omitted. With this system a random retroviral peptide library encoding for peptides of 13 amino acid residues was prepared. After ligation of the template into the retroviral vector a large number of transformations of E.coli were performed to get at least 10^6 colonies. Each colony represented a single vector with a different peptide. All colonies were scrapped and

used for preparation of vector DNA resulting in a peptide library with about 10^6 independent members, all present in a large number of copies. With this expression library new peptides can be identified in a suitable functional assay. Retroviral infection ensures that in each cell only one peptide is expressed and present over several generations.

The peptide library was employed to screen bioactive peptides conferring resistance to cells against dexamethasone induced toxicity. Higher concentration of dexamethasone (10^{-4} mol/l) caused considerable cell death of NIH3T3 cells. Using this strategy only one successful selection could be carried out. Surprisingly, only a single active peptide was identified. Its activity was reconfirmed after repeated cloning into the retroviral expression system and subsequent infection suppresses the toxic effect of dexamethasone. How the peptide influences cellular regulation is not immediately clear and could not be resolved within this work.

Zusammenfasung

Wechselwirkungen zwischen Proteinen spielen eine zentrale Rolle in verschiedenen regulatorischen Prozessen, die das Zellwachstum, die Vermehrung, Differenzierung und programmierten Zelltod (apoptose) steuern. Auch beim Blick auf biochemischen Prozessen, wie z.B. Transkription und Translation, spielen Protein-Protein-Interaktionen eine entscheidende Rolle. Proteine zeigen eine sehr hohe Spezifität bei der Interaktion mit Partnermolekülen. Sie können darüberhinaus meist mit vielen anderen Proteinen interagieren und multifunktionelle Komplexe bilden. Um die spezifische Rolle einer Protein-Protein-Interaktion innerhalb eines multifunktionellen Proteins oder Komplexes zu untersuchen, ist es erforderlich, jede Interaktion innerhalb des Komplexes individuell beeinflussen zu künnen.

Protein-Wechselwirkungen können durch verschiedene Methoden unterbrochen werden. Eine Möglichkeit bietet die Expression von Peptiden, die gegen spezifische Protein-Protein-Interaktionen gerichtet sind. Dazu wurde im Rahmen der vorliegenden Arbeit ein effizientes Expressionssystem für die kontrollierte Expression kleiner Peptide auf Basis von retroviralen Vektoren entwickelt. In einem ersten Schritt wurde dieses System genutzt, um bekannte Protein-Protein-Interaktionen zu beeinflussen. Dazu wurden Peptide exprimiert, die mit einer der interagierenden Domänen homolog sind und daher die entsprechende Interaktion kompetitiv unterbinden sollten. Zum einen wurde die Tyrosin-Phosphorylierungsdomäne von Ros genutzt, um die Interaktion der Ros-Tyrosin-Kinase mit der Phosphatease SHP-1 zu beeinflussen, zum anderen Peptide mit LXXLL-Motiven, um entsprechender Weise die Interaktion von Transkriptionsfaktoren und Koaktivatoren zu unterbinden. Darüber hinaus wurde ein System zur Expression einer Peptid-Bibliothek entwickelt, die es ermöglicht nach neuen Peptiden zu suchen, die zelluläre Prozesse beeinflussen.

Die Dephosphorylierung von Ros durch SHP-1 Phosphatase ist ein wichtiger Schritt zur Inhibierung zellulärer Proliferation und Wachstum. Um dies zu untersuchen, wurden die Ros-abgeleiteten Peptide mit dem retroviralen Expressionssystem in einer modifizieten NIH3T3 Zelllinie exprimiert. Diese Zellen enthalten einen artifiziellen TrkA-Ros Rezeptor und können auch SHP-1 unter Kontrolle von Tetrazyklin exprimieren. Durch Expression der zur Tyrosin-Domäne homologen Peptide wurde eine signnifikante Zunahme der Proliferation beobachtet. Überraschender Weise war diese unabhängig von SHP-1 und der Aktivierung des Rezeptors. Um auszuschließen, daß die Peptide über einen anderen Mechanismus die Proliferation stimulieren, wurden diese auch in nicht modifizierten NIH3T3 Zellen exprimiert. Dabei konnte keine nennenswerte Stimulierung beobachtet werden. Die Peptide waren nur proliferationsfördernd, wenn der TrkA-Ros Rezpetor mit der homologen Domäne in den

Zellen exprimiert wurde. Das Ersetzen des funktionellen Tyrosins durch Phenylalanin im Peptid (negative Mutation) führte zu einer deutliche Abschwächung der Proliferationsförderung. Eine weitere Veränderung der vom Rezeptor abgeleiteten Peptide durch Hinzufügen von selbst-bindenden („self-annealing“) komplementären flankierenden Sequenzen an N- und C-Terminus führte zu einer vergleichbaren Verminderung der Proliferationsförderung wie das Ersetzen des Tyrosins durch Phenylalanin. In Verbindung mit der negativen Mutation (Phenyalanin) hatten die selbst-bindenden flankierenden Sequenzen jedoch keinen zusätzlichen Effekt.

Die durch das Ros-Tyrosin-Domäne-homolge Peptid erhöhte Zellproliferation konnte nicht auf einer Störung der Wechselwirkung zwischen Ros und der SHP-1 Phosphatase zurückgeführt werden. Der Effekt ist jedoch abhängig von der Anwesenheit der cytoplasmatischen Ros Domäne, der funktionellen Homologie und einer relativen Flexibilität des Peptides. Mutation oder strukturellen Rahmen führten dagegen zu einer starken Abschwächung der Proliferationsförderung.

Mit dem gleichen Vektorsystem wurden auch Peptide mit-einem LXXLL Motiv exprimiert, um zu überprüfen ob mit diesem System auch Interaktionen im Zellkern beeinflußt werden können. Das LXXLL Motiv ist sehr charakteristisch für Koaktivatoren von Transkriptionsfaktoren und spielt eine Rolle bei der Wechselwirkung eines Koaktivators mit der AF-2-Transaktivierungs-Domäne eines aktivierten nukleären Rezeptors. Peptide mit oder ohne zusätzliche Aminosäuren um das LXXLL Motiv, exprimiert in HeLa und NIH3T3 Zellen, wirkten der Trankriptionsinduktion durch verschiedene induzierende Moleküle wie Vitamin D, Dexamethason, 9-cis Retinolsäure und Forskolin entgegen. Der antagonistische Effekt war bei Peptiden mit flankierenden Aminosäuren stärker als bei Peptiden ohne zusätzliche Aminosäuren. Die beobachteten Effekte waren auch von der Anzahl und Lage der Aminosäuren um das Motiv abhängig und um so effektiver, je besser diese mit natürlichen Nachbaraminosäuren in LXXLL Motiven starker Koaktivatoren übereinstimmten. Zwei nah beeinander liegende LXXLL Motive hatten einen geringeren inhibierenden Effekt. Peptide mit nur einem LXXLL Motiv zeigten im Vergleich die geringste Aktivität. Diese Beobachtungen verdeutlichten die Bedeutung zusätzlicher Aminosäuren um das LXXLL Motiv für die Wechselwirkung mit nukleären Rezeptoren und Transkriptionsfaktoren. Peptide ohne LXXLL Motiv hatten keinen antagonistischen Effekt.

In Vergleich der Trankriptionsinduktion durch die verschiedenen Induktoren, konnte darüber hinaus eine Zuordnung der relativen Spezifität für die unterschiedlichen flankierenden Aminosäuren gefunden werden. Dabei entstehen zwei Hauptgruppen, eine mit

Vitamin D und Dexamethason, die andere mit Forskolin, welche gut zur bekannten unterschiedlichen Koaktivatorspezifität der jeweils aktivierten Transkriptionsfaktoren passt.

Neben der Expression bekannter Peptide wurde das retrovirale Expressionssystem auch genutzt um eine retrovirale Peptid-Expressionsbibliothek herzustellen. Die Peptidbibliothek wurde auf der Basis von NNK Kodons generiert, wobei N für alle vier Nukleotide steht während K nur für G und T codiert. Durch das NNK-System wird eine bessere Verteilung von Aminosäuren erreicht und zwei von drei Stopkodons eliminiert. Mit diesem System wurde eine retrovirale randomisierte Peptidbibliothek mit Peptiden von 13 Aminosäuren Länge generiert. Nach Ligation wurden durch wiederholte Transformation mehr als 10^6 Kolonien erhalten, die jeweils einen Vektor mit einem unterschiedlichen Peptid enthalten. Alle Klone wurden in die Präparation von Vektor-DNA eingesetzt und so eine Bibliothek von ungefähr 10^6 unabhängigen Peptiden erhalten. Die sind wiederum in großer Kopienzahl vorhanden. Mit dieser Expressionsbibliothek wird es möglich, in einem geeigneten zellulären Funktionsassay nach neuen Peptiden zu suchen. Durch die retrovirale Infektion wird sichergestellt, daß jede Zelle jeweils nur ein Peptid exprimiert und die Expressionskassetten über mehrere Generationen an Tochterzellen weitergibt.

Als Selektionsstrategie wurde der cytotoxische Effekt von Dexamethason genutzt. Erhöhte Konzentration von Dexamethason (10^{-4} mol/l) führte zu einem signifikantem Absterben von NIH3T3 Zellen. Im Rahmen der Arbeit konnte nur eine erfolgreiche Selektion durchgeführt werden. Diese führte überraschend zur Selektion nur eines einzigen aktiven Peptids. Dessen Aktiviät konnte, auch nach erneuter Klonierung in das retrovirale Expressionssystem und anschließender erneuter Infektion, den cytotoxischen Effekt von Dexamethason unterdrücken. Wie das Peptid in die zelluläre Regulation eingreift, konnte leider bis zum Abschluß dieser Arbeit nicht geklärt werden.

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Agrawal AK, Shukla S, Chaturvedi RK, Seth K, Srivastava N, **Ahmad A***, Seth PK. Olfactory ensheathing cell transplantation restores functional deficits in rat model of Parkinson's disease: a cotransplantation approach with fetal ventral mesencephalic cells. Neurobiol Dis. 2004 Aug;16(3):516-26.

Dar AA and Wolfl S; Expression of short domain specific peptides *in vivo*; A possible approach to influence known protein interaction. (Communicated to J Biochem Biophys Methods)

Dar AA and Wolfl S; Short LXXLL peptides antagonists of induced transcription; Influence of amino acids around LXXLL motif on suppression. (MS under preparation)

Note: Please read *Ahmad A* as *Dar AA*

Selbständigkeitserklärung

Hiermit ich, die vorliegende Arbiet selbständig und unter ausschließlicher Verwendung der angegebenen Quellen- und Hilfsmittel angefertigt zu haben

Jena